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RESEARCH ARTICLE

PURIFICATION AND STRUCTURE DETERMINATION OF THREE BIOACTIVE MOLECULES FROM A NEWLY ISOLATED *Streptomyces sp.* AH 47

[1,*]Abdelhalem A. Hamza, [1]Hiba A. Ali, [2]Benjamin R. Clark, [2]Cormac D. Murphy and [3]Elsheik A. Elobied

[1]National Centre for Research, Commission for Biotechnology and Genetic Engineering, Khartoum, Sudan
[2]School of Biomolecular and Biomedical Science, Centre for Synthesis and Chemical Biology, Ardmore House, University College Dublin, Dublin 4, Ireland
[3]Ahfad University, College of pharmacology, Khartoum, Sudan

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ABSTRACT

A new actinomycete sp. designated as AH 47 was isolated from the soil sample, collected from different geographical area in the Sudan and selected for its antimicrobial activities against bacteria and fungi. These isolate was taxonomically characterized on the basis of morphological, physiological and cultural characteristic studies and strongly suggested that this strain is a member of the genus Streptomyces. Nucleotide sequence of the 16S rRNA gene (1277 pb) of the AH 47 strain exhibited high similarity to the Streptomyces 16S rRNA genes. 16S rRNA sequence of strain has been submitted in the NCBI Gen Bank database (accession number GU013556).
The active antibiotics were isolated from 1 L culture supernatant and from the biomass using reverse phase HPLC-DAD, various separation and purification steps, led to the isolation of three pure bioactive molecules. The chemical structure of these compounds was established on the basis on their ESI-MS and EI-MS, 1H and13C NMR data and by comparison with reference data from literature. These compounds were chemically characterized as actinomycin-D (m/z 1255), actinomycin X_2 (m/z 1269) and actinomycin $X\alpha_B$ (m/z 1271). This new *Streptomyces species* was able to produce a mixture of actinomycins and serves as a promising source of these antibiotics.

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INTRODUCTION

All known natural actinomycins contain the same phenoxasone chromophore and differ only in the amino acid content of the peptide side chains. Chemical and biological properties of many actinomycin complexes have been reviewed (Mauger., 1980). Among the actinomycins, actinomycin D has been studied most extensively and is widely used for treatment of malignant tumors, such as Wilms' tumor (Farber *et al*., 2002) and childhood rhabdomyosarcoma (Womer., 1997). It also has been proposed for treatment of AIDS because of its effectiveness in inhibiting the minus-strand transfer process in HIV-1 (Guo *et al*., 1998). The biological effects of actinomycin D are believed to be the consequence of its ability to intercalate into duplex DNA, which results in the inhibition of DNA-dependent RNA polymerase activities and thus protein synthesis (Wadkins *et al*., 1998). Because of its unique biological properties, actinomycin D has become an important tool in clinical, molecular, and cell biology (Chen *et al*., 2004).. Structural modification of actinomycins to minimize their unwanted side effects and to investigate the structure/function relationship for this class of compounds is an ongoing and active area of research (Bolognese *et al*., 2002). Actinomycin X 2 is structurally related to actinomycin D possesses higher cytotoxicity toward cultured human leukemia (HL-60) cells than does actinomycin D, and it induces cell death via apoptosis (Kurosawa *et al*., 2006). This paper describes the isolation of actinomycete strain. AH 47 from the Sudanese soil samples. The identification of this strain and the isolation, purification, as well as the structure elucidation of the actinomycins has been assessed.

***Corresponding author:** Abdelhalem A. Hamza
National Centre for Research, Commission for Biotechnology and Genetic Engineering, Khartoum, Sudan

MATERIALS AND METHODS

Isolation, characterization and identification of Streptomyces sp. AH47

Streptomyces strains AH 47 was isolated from Sudanese soil samples, collected from different locations in the Sudan. Isolation of the strains 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 lyophils and as spore suspensions at -80 °C as described by (Waksman and Hopwood, 1940). The bacteria 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). The isolate was grown in nutrient broth for the preparation of genomic DNA which was extracted according to methods described by (Nikodinovite *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 isolation of actinomycins

The production of antibiotic by the isolate was examined in different media, and tryptone soya broth (TSB) gave the maximum growth and antibiotic activity. Baffled Erlenmeyer flasks (250 ml), containing 50 ml of medium, were inoculated from a spore suspension (10^5 ml^{-1}) and incubated on a rotary shaker (200 rpm) at 30 °C for 48 h. The cultured broth (1 L) was centrifuged at 6000 rpm for 15 minutes to remove the biomass. Activities against test organisms (*Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Aspergillus niger, A. flavus)* were monitored during the isolation, using the antibiotic disk method. Mycelium and supernatant were extracted with ethyl acetate and the antimicrobial activities were observed in the organic phase. Extracts from both supernatant and mycelium were combined and concentrated under vacuum to give a red-orange powder. The crude organic extract having antimicrobial activities, was subjected to various separation steps (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 StableBond column. Peak purity was assessed by analytical HPLC with a gradient elution of acetonitile over 20 minutes, flow rate of 0.8 ml/min, using a Thermo Hypersil C18 column (4.6 x 150 mm 5 µm) and five peaks were obtained, collected separately, concentrated and the bioactivity was assessed. ^{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 wasrecorded on a Quattro Triple Quadrupole Mass Spectrometer, Finnigen TSQ 7000with nano-ESI-API-ion source. ESI-HRMS was measured on Micromass LCTmass spectrometer coupled with a HP 1100 HPLC with a 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).

RESULTS AND DISCUSSION

Cultural and physiological characteristics and identification of Streptomyces sp.AH47

During screening of bacteria from Sudanese soil for bioactive natural products, different streptomyces bacteria were isolated and tested for their antibiotic production against a range of target organisms. A new aerobic bacterium designated AH 47, which appeared to be actinomycetes Figure (1), displayed a broad antimicrobial spectrum and were selected for further analysis. The cultural and physiological characteristics of the isolates on variou media are listed in Tables 1 and 2, respectively, when cultured on International Streptomyces Project (ISP) media and various other media. As shown in Table 1, strain 47 produced well-developed grey spores on most media tested. They showed good growth on most media except ISP2 media and Bennett's Agar. ISP medium 3 and ISP medium 4 were the best media for growth, spore formation and soluble pigment production. Permissive temperature ranges for the growth and sporulation of the strain 47 was 25-45 °C with an optimum at 37°C; pH ranges for growth ranged from 6.0-8.0 with an optimum at 7. According to culture characteristics (Table 2) strain 47 grew well and colonies were detachable on the ISP 3 and 4 media. The spore chains were white grey to dark grey. The comparison of these observed cultural characteristics with those of the known actinomycete species described in Bergey's manual of Systematic bacteriology, strongly suggested that strain AH 47 belong to the genus Streptomyces. Light and scan electron microscopy revealed that Streptomyces AH 47 isolate is Gram positive, filamentous and spore forming. The spore chain is with open loops, hooks and primitive spirals Figure (2). 16S rRNA sequence data have proved invaluable in streptomycetes systematic, in which they have been used to identify several newly isolated *Streptomyces species* [Mellouli *et al*, 2003; Fourati *et al*., 2005). In order to confirm the identified isolates, 16S rRNA sequence of the strain AH47 was compared to those in Gene Bank by carrying out a BLAST search. The 16S rDNA of the strain AH 47 had 94% sequence homology to a range of *Streptomyces* spp. A selection of these sequences was chosen to create a phylogenetic tree. It was apparent from the GeneBank database that *Streptomyces sp.* AH47 (1277 bp) was closely related to *Streptomyces themocarbodoxydus. Streptomyces* AD 16, *Streptomyces* GB 12 and *Streptomyces xylophagus,* the highest sequence similarity (94 %) Figure (3). However, the literature survey and phylogenetic analysis indicated that strain 47 is quite different from recognized *Streptomyces* species.

Table 1. Cultural characteristics of strains AH 47 on different media

Medium	Growth	Spore colour	Vegetative Mycelia	Soluble pigment
ISP2	Poor	Grey, white edges	Cream	None
ISP3	Abundant	Grey	Cream	Green-yellow
ISP4	Abundant	Dark grey	Yellow	Yellow
ISP5	Poor	None	None	None
ISP6	Abundant	None	None	None
ISP7	Abundant	None	None	None
Nutrient agar	Poor	None	None	None
Bennett agar	Poor	None	None	None

Table 2. Physiological and biochemical properties of strains AH 47

Property	Reaction
Melanin formation (ISP6 and ISP7 media	+
Starch hydrolysis (tryptone soya agar medium)	+
Casein hydrolysis (Casein Agar medium)	+
Unease production (Nitrate Peptone Broth medium)	+
Gelatin hydrolysis (Nutrient Gelatine medium)	-
Soluble pigment production (ISP media)	-
H_2S production (Triple sugar iron Agar medium)	-
pH range of growth (ISP medium 4)	6 – 8
Temperature range of growth (ISP medium 4)	25 – 45
Antibiotic resistance	
Ampicillin (20 mg/l)	+
Kanamycin (25 mg/l)	-
Vancomycin (5 mg/l)	-
Steptomycin (10 mg/l)	-
NaCl tolerance	
NaCl 2% (ISP medium 4)	+
NaCl 4% (ISP medium 4)	+
NaCl 7% (ISP medium 4)	-
Growth on inhibitory compounds	
Phenol 0.1% (ISP medium 4)	+
Lysozyme 0.005% (ISP medium 4)	+
Sodium azide 0.01% (ISP medium 4)	-
Crystal violet 0.05% (ISP medium 4)	-

Table 3. Peak No HPLC data (Rt), molecular weight, (m/z), and assigned structures of compounds in the ethyl acetate extract of *Streptomyces* sp. AH 47

Peak No	R_t (min)	M+ (*m/z*)	UV nm	Metabolites
1	15.36	1269	220	Actinomycin X_2
2	16.3	1255	441	Actinomycin-D
3	16.53	1271	242	Actinomycin $X_{αB}$

It is clear from the combination of genotypic and phenotypic data that strain AH47 should be classified as a novel genomic species of the genus *Streptomyces*. The 16S rDNA sequence of the isolate was submitted to GenBank (accession numbers GU013556). Interestingly, neither of the strains that are phylogenetically most closely related to strain AH47 are known to produce actinomycins, thus, we have identified new actinomycin-producing isolate.

Figure 1. Morphological type of Colonies of *Streptomyces sp.* AH 47 in ISP4 medium after 10 days incubation at 30°C

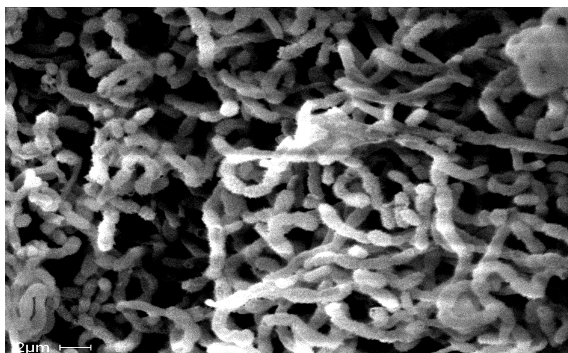

Figure 2. Scanning electron micrographs of the spore chains of *Streptomyces* sp. AH 47.

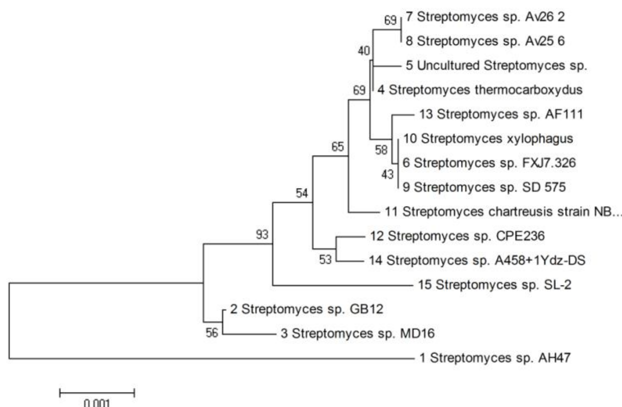

Figure 3. Phylogenetic tree showing the relationship between strain AH 47 and representative species of the genus *Streptomyces* and other taxa based on nearly complete 16S rRNA gene sequences

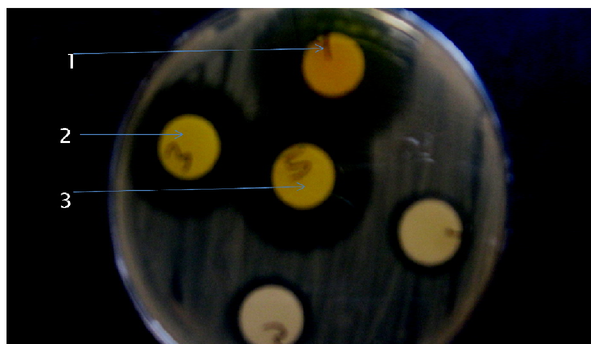

Figure 4. Activity of different actinomycins produced by *Streptomyces* sp. AH 47 against *Bacillus subtilus.*

1- actinomycin D, 2- actinomycin X_2, 3-actinomycin $X_{0\beta}$.

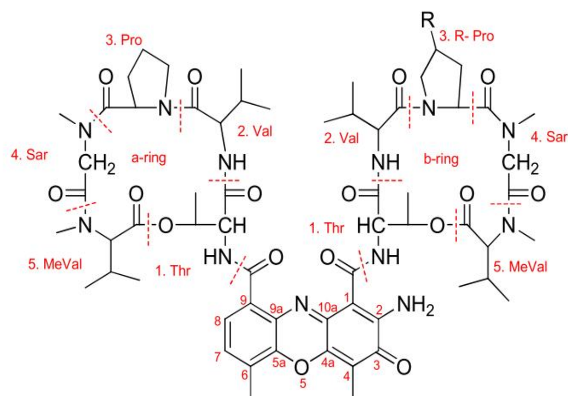

Figure 5. The structures of isolated compounds

1. **Actinomycin D ……… R = H,**
2. **Actinomycin X_2 ………… R = O**
3. **Actinomycin $X_{0\beta}$ ………. R = OH**

Extraction, purification and structure elucidation of the active compounds

Various separation steps were applied to (1 L) culture broth of the *Streptomyces sp.* AH 47. *Streptomyces sp.* AH 47 produced a red/orange coloured active complex in TSB medium. The filtrate and mycelial cake were extracted with ethyl acetate to give a combined organic extract. Preliminary HPLC-DAD analysis of this extract identified a characteristic absorbance spectrum characteristic of actinomycins, which is physiologically eluted from the HPLC column at retention times very close (15.36, 16.3 and 16.53 min) and had similar absorbance maxima (220, 441 and 242 nm). This tentative identification was supported by the observation of strong ions in the ESI (+) MS spectra of the extracts corresponding to the presence of actinomycin D (*m/z* 1255, $[M+H]^+$), actinomycin X_2 (*m/z* 1269) and $X_{0\square}$ (*m/z* 1271) table 3. The identity of the three actinomycins was confirmed by comparison of ^1H and ^{13}C NMR and MS data with those reported in the literature. Interestingly, this combination of actinomycins is also produced by *Streptomyces. padanus* MITKK-103, (Kurosawa, *et al.,* 2006), which is physiologically and phylogenetically distinct from *Streptomyces sp.* AH 47. Actinomycins are an important class of natural products that, despite their first discovery more than 80 years ago, continue to be a focus of many research areas, especially in the biological and medicinal sciences. Thanks to such sustained efforts, today there is a wealth of knowledge for this class of compounds and their microbial producers. To the best of our knowledge, there are at least 19 species of Streptomyces capable of producing actinomycins.

AH 47 and strain may be useful for metabolic engineering of high-value biologically active metabolites such as actinomycin D, actinomycin X_2 and as actinomycin $X\alpha_B$. Utilization of actinomycin X 2 for medicinal purposes has not been reported to any extent in the literature. Whereas actinomycin D has been shown to have higher activity toward human leukemia cell lines such as HL-60 cells than did several structural actinomycin analogues (Takusagawa *et al.*, 2001). This result encourages further research on all actinomycins. It is plausible that AH 47can be used to produce enough of these antibiotics to enable studies of medicinal applications.

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REFERENCE

Bolognese, A., Correale, G., Manfra, M., Lavecchia, A, Mazzoni, O., Novellino, E, Barone, V., Pani, A., Tramontano, E, La Colla, P, Murgioni, C., Serra, I., Setzu, G., Loddo, R (2002). Antitumor agents. 1. Synthesis, biological evaluation, and molecular modeling of 5H-pyrido [3,2-a]phenoxazin-5-one, a compound with potent antiproliferative activity.*J Med Chem*45:5205– 5216.

Chen, F. M, Sha F, Chin KH, Chou SH (2004) The nature of actinomycin D binding to d(AACCAXYG) sequence motifs. Nucleic Acids Res 32:271 – 277.

Farber, S., DAngio, G., Evans, A, Mitus A (2002) Clinical studies ofactinomycin D with special reference to Wilms' tumor in children. 1960. J Urol 168:2560 – 2562; discussion 2563.

Fourati, B., Fguira, L., Fotso, S., Ben Ameur-Mehdi R, Mellouli L, and Laatsch H. (2005). Purification and structure elucidation of antifungal and antibacterial activities from a newly isolated *Streptomyces sp* US80 strain. Res Micro- biol 156:341–347.

Guo J, Wu T, Bess J, Henderson LE, Levin JG (1998) Actinomycin D inhibits human immunodeficiency virus type 1 minus-strand transfer in in vitro and endogenous reverse transcriptase assays. J Virol 72:6716 – 6724.

Kurosawa, K, B., V. P., VanEssendelft, J. L., Willis, L. B., Lessard, P. A., Ghiviriga, I., Sambandan, T. G., Rha, C. K., Sinskey, A. J. (2006). Characterization of Streptomyces MITKK-103, a newly isolated actinomycin X2-producer. Appl Microbiol Biotechnol 72:145–154.

Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, J. D. Thompson, T. J. Gibson, and D. G. Higgins. 2007. Clustal W and clustal X version 2.0. Bioinformatics 23:2947-2948.

Mauger, A. B (1980). The actinomycins. Ellis Horwood, England Men'shikov GP, Denisova SI (1962) Isolation of actinomycins from the group *Actinomyces fluorescens.* Antibiotiki 7:31–32

Mellouli, L., Ben Ameur, R., Sioud, S., Salem, M., and Bejar S. (2003). Isolation, purification and partial characterization of antibacterial activities produced by a new isolated *Streptomyces sp* US24 strain. Res Microbiol 154:345–52.

Nikodinovic, J, Barrow, K. D, and Chuck, J. A. (2003). High yield preparation of genomic DNA from Streptomyces. Biotechniques 35 (5): 932-934.

Shirling, E. B. and Gottlieb. D .(1966). Methods for characterization of *Streptomyces species*. International Journal of Systematic Bacteriology 16:313-340.

Stackebrandt, E., F. A. Rainey, and N. L. WardRainey. 1997. Proposal for a new hierarchic classification system, Actinobacteria classis nov. International Journal of Systematic Bacteriology 47:479-491.

Takusagawa F, Carlson RG, Weaver RF (2001) Anti-leukemia selectivity in actinomycin analogues. Bioorg Med Chem 9:719 –725.

Wadkins RM, Vladu B, Tung CS (1998) Actinomycin D binds to metastable hairpins in single-stranded DNA. Biochemistry 37:11915 – 11923.

Waksman, S. A., Woodruff, H.B. 1940. Bacteriostatic and bacteriocidal substances produced by soil actinomycetes. Proceedings of the Soceity for Experimental Biology in Medicine 45:609-614.

Womer RB (1997) Soft tissue sarcomas. Eur J Cancer 33:2230 –2234; discussion 2234 – 2236

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