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Purification and Structure Elucidation of the Polyether Antibiotic Alborixin from *Streptomyces pulcher* CRF17

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Authors' contributions

This work was carried out in collaboration between all authors. Author IS isolated and identified the strain Streptomyces pulcher CRF17, performed fermentation, solvent extraction, compounds purification, spectroscopic studies, compound identification and wrote the first draft of the manuscript. Author KAS helped in compounds purification, MS and NMR spectra interpretation, compound identification and manuscript write-up etc. Author SH overall supervised the study and finalized the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To screen *Streptomyces* sp. from saline soil in Pakistan, for its antimicrobial activity and to purify and identify the active metabolites produced by mass spectrometry and NMR spectroscopy. Strain identification by morphological, biochemical, physiological characterization and by 16S rRNA gene sequencing.
Study Design: Cultivation in lab fermenter, solvent extraction and purification of the compounds by column chromatography, identification of the compounds by mass spectrometry and NMR spectroscopy, determination of the antimicrobial activity and cytotoxicity.
Place and Duration of Study: Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan and Institute of Organic and Bio molecular Chemistry, University of Göttingen, Germany, between February, 2007 and April, 2009.
Methodology: The strain was cultivated in a 20 liter fermenter (working volume 10 liters)

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and the culture broth was extracted with ethyl acetate, acetone and methanol. The resultant crude extract was fractionated on silica gel and the components were purified by column chromatography (silica gel, sephadex column and preparative TLC). The pure component was identified by mass spectrometry (ESI and HRESI-MS), NMR analysis (^{1}H and ^{13}C NMR) and by comparison with reference data, the antimicrobial activity and cytotoxicity was determined by disc diffusion assay and by micro well cytotoxicity assay against *Artimia salina*.

Results: The morphological, biochemical and physiological characterization suggested that isolate CRF17 belongs to the genus *Streptomyces*. Further NCBI BLAST of the partial 16S rDNA gene sequence 1420 bp (gene bank accession number: EU294134) from the isolate CRF17 showed 99% identity and 98% query coverage towards *Streptomyces pulcher*. The scale up fermentation of the isolate CRF17 yielded active compound and was identified as alborixin (1).

Conclusion: The isolate *Streptomyces pulcher* CRF17 is a potent producer of the antibiotic alborexin and can be exploited for its commercial production.

Keywords: Alborixin; Polyether Antibiotics; Streptomyces sp. CRF17; Streptomyces pulcher.

1. INTRODUCTION

Polyether ionophores represent a large group of naturally occurring biologically active compounds produced mainly by plants and microorganisms and are able to transport metal cations across cell membranes [1,2]. They are polyketides in origin and the major building blocks are acetate, propionate and butyrate. The major components may possess methyl substituent on each of the cyclic ether units but in addition small amounts of ethyl homologues may also be present. The majority of polyethers are characterized by a linear series of tetrahydrofuran and tetrahydropyran residues, frequently linked by spiroketal systems [3]. These antibiotics show a wide range of activities against Gram-positive organisms, mycobacterium, fungi and yeasts but they have found little applications as human therapeutic agents because of their toxicity. However currently they are widely applied as antibiotics in veterinary medicine due to their effectiveness against coccidiosis and infections caused by Gram-positive microorganisms. The most widely used polyether antibiotics of veterinary importance are monensin [4] alborixin (1) [5,6,7,8], lasalocid [9], X-206 (2) [10], salinomycin [11], maduramicin [12,13] and semduramycin [14], which are produced by various actinomycetes genera. They disturb the metal homeostasis of the microorganism thereby consecutively disrupting series of energy-depending processes which leads to ultimate death of the microbial cell [15].

This family of polyethers mainly derives from different species of actinomycetes of soil microbiota [16]. Actinomycetes have provided many important bioactive compounds of high commercial value and they continue to be routinely screened for new bioactive substances. These searches have been remarkably successful and approximately two thirds of naturally occurring antibiotics have been isolated from actinomycetes [17]. These filamentous bacteria produce over 10,000 bioactive compounds, 7600 derived from *Streptomyces* and 2500 from actinomycetes (rare actino) species, which represent the largest group (45%) of bioactive microbial metabolites [18].

The present study report the selective isolation, taxonomy and preparative screening of the saline isolate CRF17. The lab scale cultivation of the isolate and subsequent isolation and

purification of the bioactive substances by manual column chromatography yielded active compound, which has been identified as alborixin (1) by mass spectrometry, NMR spectroscopy and by comparison with literature data. The isolate exhibits distinctive morphological, biochemical and physiological characteristics and share 98% similarity of the 16S rRNA gene with *Streptomyces pulture*.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of *Streptomyces* sp. CRF17

The isolate CRF17 was isolated from the soil of a saline agricultural land in district Jhang, Punjab, Pakistan. The soil sample was serially diluted and was plated on the selective media: Casein-KNO_3 agar (glycerol 10 g, KNO_3 2 g, casein 0.3 g, $NaCl_2$ g, K_2HPO_4 2 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, $CaCO_3$ 0.02 g, $FeSO_4 \cdot 7\ H_2O$ 0.01 g, agar 18 g in one liter, cycloheximide 50 µg/ml, as an antifungal agent). The presumptive streptomycetes colonies were selected from the crowding and purified by repeated sub culturing on GYM agar (Malt extract 10g, Yeast extract 4g, Glucose 4g, Agar 15g, in 1 L distilled water). The purified culture of the isolate was characterized at molecular level. Cultural characteristics were observed during the incubation at 28ºC for 21 days on GYM agar. The biochemical and physiological characterization includes determination of optimal growth temperature, formation of melanin, utilization of nine different sugars as carbon source, utilization of organic acids, utilization of oxalates, hydrolysis of urea and hemolysis. For 16S rRNA gene sequencing high-molecular weight chromosomal DNA of the isolate was prepared from GYM medium grown mycelia following the methods described by Kieser et al. [19] and Felnagle et al. [20]. PCR amplification of the 16S rRNA gene of strains was performed using two primers P1: 5′-AGAGTTTGATCATGGC-3′ and P2: 5′-TACCTTGTTACGACTT-3′. Approximately 300 ng genomic template DNA was used with 150 pmol of each primer per 50 µl of reaction volume. Amplification was performed in an automated thermocycler (Eppendorf Mastercycler) using 1U Pfu DNA polymerase (Fermentas) and the recommended buffer system according to the following amplification profile: 1, 94ºC 5 min; 2, 94ºC 1 min; 3, 55ºC 1 min; 4, 72ºC 2 min; 5, 72ºC 5 min; 30 cycles. The PCR product was analyzed by agarose gel electrophoresis and 1.5kb DNA fragment was purified by QIAquick Gel Extraction column (Cat. # 28704, Qiagen, Inc), the sequencing was done using dye-terminator chemistry with Mega-BACE 1000/4000 DNA automated sequencers (Amersham Bioscience). The obtained 16S rRNA gene sequence data was analyzed using advance BLAST search program at the NCBI website: http://www.ncbi.nlm.nih.gov/BLAST/. The nucleotide sequence data was deposited to the gene bank, and gene bank accession number was obtained. The isolate was deposited to the departmental microbial culture collection (Bacterial culture collection at the department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan), under the preservation code: MMG-Actinos 34.

2.2 Determination of Antimicrobial Activity and Cytotoxicity

The isolate CRF17 was cultivated on small scale as shaking culture and was extracted on celite bed by ethyl acetate to obtain crude extract. The antimicrobial activity of crude extract as well as pure compounds was determined by disc diffusion bioassays, against a panel of nine test organisms including *Staphylococcus aureus*, *Bacillus subtilis*, *Streptomyces viridochromogenes* (Tü57), *Escherichia coli*, *Candida albicans* and *Mucor miehemi*, *Chlorella* sorokiniana, *Chlorella vulgaris* and *Scenedesmus subspicatus*. Paper disks with diameter of 9 mm were impregnated with 40 µl of crude extract solution (crude extract 1 µg/µl dissolved

in CH_2Cl_2/MeOH 1:1); the disks were dried under sterile conditions and were placed on the surface of test plates. The cytotoxicity of the crude extract and pure compound was determined against larvae of brine shrimp (*Artimia salina*) using a microwell cytotoxicity assay as described by Solis et al. [21]. In preliminary screening the isolate CRF17 exhibited promising antimicrobial activity against the test organisms, so it was selected as a competent strain to be studied further in preparative screening.

2.3 Fermentation, Isolation and Purification

Working isolate was cultivated in a 20 litre lab fermenter (Biostat E, working volume 10 litres) using GYM medium. The fermenter was filled with 9 litre water along with appropriate quantity of GYM medium and was then closed. The inlet and outlet openings of the tubes were closed with stoppers and clamps. The pH electrode port was closed with the metal stopper. The fermenter was autoclaved for 30 minutes at 121ºC. Afterwards the air supply, stirring motor and water circulation pumps were switched on. The acid (2N HCl), base (2N NaOH) and antifoam (1% Niax/70% ethanol) were filled in their respective jars and were connected to the system. The pH electrode was sterilized with 70% ethanol and adjusted in the pH electrode port in the lid. The pre-culture was prepared by inoculating the isolate CRF17 from well grown plates into four 1 litre Erlenmeyer flasks each containing 250 ml of GYM medium. The flasks were incubated at 28ºC on a linear shaker for four days. Pre-culture with the ratio of 10% was used to inoculate the fermenter. The fermentation was carried out for five days at 28ºC. After harvesting the dark brown culture broth, it was filtered over celite by filter press (Schenk Niro 212 B40) to separate the mycelium from water phase. The water phase was adsorbed on Amberlite XAD-16 resin in a large size glass column (100 × 5 cm) and the Amberlite XAD-16 resin was eluted with methanol (2 L) followed by evaporation and the water residue was extracted with ethyl acetate (3 times, 1 L each). On the other hand, the mycelia cake was extracted with ethyl acetate (3 times, 1 L each) and acetone (1L, 1 time). The solutions (ethyl acetate 3 L (from XAD-16 extract), ethyl acetate 3 L (from mycelium extract), and acetone 1 L) containing the active metabolite were evaporated by a rotary evaporator (*Rotavapor* R152). The TLC of all the extracts showed identity which was then collected giving 0.71 g crude extract. The crude extract was subjected to column chromatography on silica gel column (30 g, 1.5 × 50 cm) using a CH_2Cl_2: MeOH gradient, which resulted into two main fractions. The first fraction was identified as fats based on TLC and spraying reagents, while the second fraction was subjected to further purification using PTLC (CH_2Cl_2/10%MeOH) and sephadex LH-20 (CH_2Cl_2/40% MeOH) affording alborixin (1; 17.1 mg) as a white amorphous solid (Figs. 1 and 2).

Alborixin (1): $C_{48}H_{84}O_{14}$ (885); white amorphous solid; UV non-absorbing (254 nm); dark-green, turned 2 hours later to reddish-brown with anisaldehyde/H_2SO_4 spraying reagent; Rf 0.35 (silica gel, 10% MeOH-CH_2Cl_2); ^{1}H NMR ($CDCl_3$, 500 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) (Supplementary Figs S3 and S4); (–)-ESI-MS: m/z 884 $[M - H]^-$, 1767 $[2M - H]^-$; (+)-ESI-MS: m/z 908 $[M + Na]^+$; (+)-HRESI-MS: m/z 902.6192130 $[M + NH4]^+$ (calcd for $C_{48}H_{88}O_{14}N$, 902.6199330)(Supplementary Figs. S1 and S2) Fig 2.

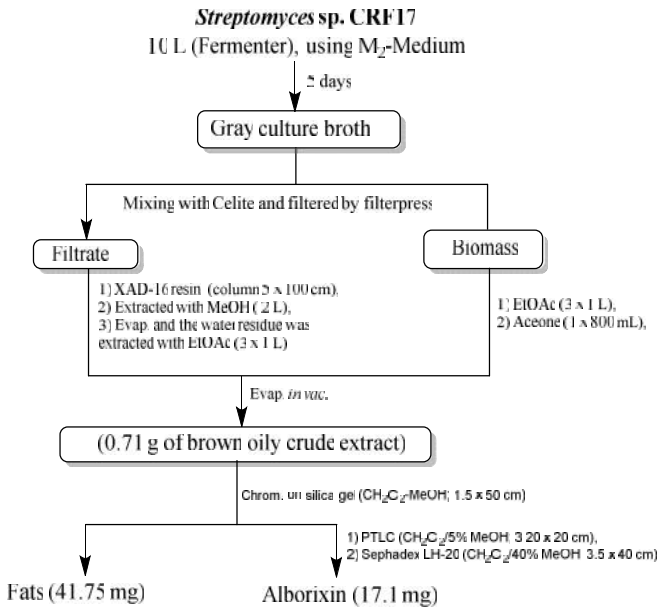

Fig. 1. Working up scheme of the *Streptomyces* sp. CRF 17

2.4 Mass Spectrometry and NMR Analysis

Mass spectra: EI MS of the purified fractions were recorded on a Finnigan MAT 95 spectrometer (70 eV). ESI-MS with Quattro Triple Quadruple mass spectrometer Finigan MAT-Incos 50, ESI-MS LCQ (Finnigan). HRESI-MS was recorded by ESI MS on an Apex IV 7 Tesla FT Ion Cyclotron Resonance MS (Bruker Daltonics, Billerica, MA, USA) with perflurokerosine as standard.

Nuclear Magnetic Resonance (NMR) Spectroscopy: ^{1}H NMR spectra were measured by: Varian Unity 300 (300 MHz), Bruker AMX 300 (300 MHz), and Varian Inova 500 (499.8 MHz). Coupling constants (*J*) in Hz. ^{13}C NMR spectra were measured by: Varian Unity 300 (75.5 MHz), Varian Inova 500 (125.7 MHz). Chemical shifts (δ) were measured relative to tetramethylsilane (TMS) as internal standard.

Flash chromatography was carried out on silica gel (230-400 mesh). R_f value was measured on Polygram SIL G/UV$_{254}$ TLC cards (Macherey-Nagel & Co.). Size exclusion chromatography was done on sephadex LH-20 (Lipophilic Sephadex, Amersham Biosciences Ltd; purchased from Sigma-Aldrich Chemie, Steinheim, Germany).

3. RESULTS AND DISCUSSION

3.1 Taxonomy of the Producing Strain

The isolate CRF17 produced yellowish substrate mycelium and off white aerial mycelium which turns to gray on prolonged incubation, along with significantly visible soluble pigments that diffuse into the surrounding culture medium. It produced round colonies which were hard and embedded into the agar medium with a diameter of 5 mm. In microscopic analysis the

isolate CRF17 exhibited characteristic streptomycetes-like filamentous structures. In biochemical and physiological characterization the strain exhibited growth at the temperatures 28ºC and 37ºC. The production of melanin was indicated by the appearance of a dark blue to black color in the agar medium. Among different sugars tested to be utilized as carbon sources, the strain CRF17 exhibited positive growth on seven different compounds; however it could not grow on D-glactose, raffinose and sucrose. Additionally, the isolate exhibited positive results in utilization of organic acids, utilization of oxalates, and negative urea hydrolysis and hemolysis tests (Table 1). The comparison of these characteristics with those of actinomycete species described in Bergey's Manual of Systematic Bacteriology [22] strongly suggested that the isolate CRF17 belongs to the genus *Streptomyces*. The nucleotide sequence of 1420 bp (Accession number EU294134) of the 16S rRNA gene of the *Streptomyces* sp. CRF17 was determined in both strands. The BLAST analysis of this sequence through matching with reported 16S rRNA gene sequences in the gene bank showed high similarity (99%) to *Streptomyces pulcher*.

Table 1. Morphological, biochemical and physiological characteristics of *Streptomyces* sp. CRF17

Growth pattern	well grown ~ rough
Substrate mycelium	yellowish
Ariel mycelium	off white to grayish
Growth temperature range	28~37°C
Production of melanin	+
Utilization of organic acids	+
Utilization of oxalates	+
Hydrolysis of urea	-
Hemolysis	-
Carbon source utilization	
D-Glucose	+
D-Fructose	+
L-Arabinose	+
Sucrose	-
D-Fructose	+
D-Mannitol	+
Raffinose	-
D-Galactose	-
Soluble starch	+
Glycerol	+

(+)= Positive test results or growth on the carbon source, (-) = Negative test result or no growth on the carbon source

3.2 Structure Elucidation of the Purified Compounds

The *Streptomyces* sp. CRF17 was scaled up cultivation as 20 L lab fermenter (Biostat E) with 10 liter working volume which grown for 5 days and yielded 0.71 g crude extract. Fractionation and purification of the obtained crude extract (0.71 g) using various chromatographic techniques afforded compound 1 (17.1 mg) in pure form as sole product of the crude extract (Fig 1).

Compound 1 was isolated as white amorphous solid, which was UV non-absorbent (analyzed under UV at 254 nm), it showed a dark-green coloration on TLC, which turned to

reddish-brown 2 hours later, after spraying with anisaldehyde/sulphuric acid reagent. The molecular weight of compound 1 was confirmed as 885 Dalton, based on the (+) and (–) ESI-MS (Supplementary Fig. S1). The molecular formula of 1 was deduced as $C_{48}H_{84}O_{14}$ (*m/z* 902.6192130 $[M + NH4]^+$; Calcd 902.6199330 for $C_{48}H_{88}O_{14}N$) on the basis of HRESI-MS (Supplementary Fig S2) and of ^1H and ^{13}C NMR data.

The proton NMR spectrum of 1 in $CDCl_3$ displayed two singlet protons at δ 6.46 and 6.01 of hydroxyl functions, which disappeared when D_2O was added. Furthermore, the proton NMR spectrum of 1 was rich in aliphatic proton signals with several oxygenated protons in the range of δ 4.60~3.00 ppm. In addition to the other signals, one triplet, eight doublet and three singlet methyls were observed in the range between δ 1.40~0.78 ppm (Supplementary Fig. S3). The ^{13}C NMR spectrum of compound 1 confirms the presence of 48 carbon atoms. The signal at δ 180.7 is due to carboxyl carbon (-COOH). The 15 carbon signals observed between δ 108.4~67.7 are corresponding to the carbons bounded to at least one oxygen atom. The carbon signals observed at δ 108.3, 99.5 and 97.8 probably corresponding to three hemiacetals. In the up-field region (46.4~8.5 ppm), it showed 32 carbon signals corresponding to twelve methylenes, eight methins and twelve methyls (Supplementary Fig. S4).

A substructure search in AntiBase [1] supported by the NMR data and molecular weight resulted in alborixin (1) (Fig. 2) which had previously isolated from *Streptomyces albus* [5,6,7] with a wrong reported stereochemistry at C-22 position. The revised structure of alborixin (1) which was previously reported with a wrong stereochemistry at C-22 was later corrected by Seto et al. [8] in comparison with the closely related polyether antibiotic X-206 [9]. Alborixin (1) is an ionophore antibiotic of the nigericin group, it is active against Gram positive bacteria and is coccidiostatic but is very toxic [5,6,7,8].

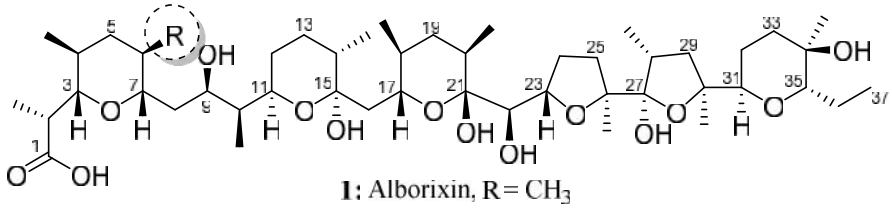

Fig. 2. Structure of Alborixin (1) and its closely related antibiotic X-206 (2)

3.3 Antimicrobial Activities and Cytotoxicity

The crude extract obtained from the culture broth of the isolate *Streptomyces* sp. CRF17 exhibited strong antimicrobial activity against the Gram-positive bacteria, *Staphylococcus aureus*, *Bacillus subtilus* and *Streptomyces viridochromogens* Tü57 with an inhibition zone of 23 mm, 15 mm and 11 mm, respectively. In case of Gram-negative bacteria and fungal pathogens a zone of inhibition of 15 mm against *Escherichia coli*, minor inhibition of *Candida albicans* and about 18 mm zone of inhibition against *Mucor mehei* was observed (Table 2). Additionally the crude extract exhibited 69.3% mortality of the larvae of *Artimia salina* in microwell cytotoxicity assay (Table 2). The pure alborixin (1) recovered from the culture broth of the isolate CRF17, exhibited antimicrobial activity against *Staphylococcus aureus*, *Bacillus subtilus* and *Streptomyces viridochromogens* Tü57 with the zone of inhibition of 16

mm, 12 mm and 12 mm respectively. In addition, compound 1 exhibited 28.9% mortality of the larvae of *Artimia salina* in microwell cytotoxicity assay (Table 2).

Table 2. Antimicrobial activity and cytotoxicity of the crude extract and pure compound (Alborexin) isolated from *Streptomyces* sp. CRF17

Test organism	Zone of inhibition (mm)	
	Crude Extract	Pure compound
Staphylococcus aureus	23	16
Bacillus subtilis	15	12
Streptomyces viridochromogens (Tü 57)	11	12
Escherichia coli	15	0
Candida albicans	10	0
Mucor mehei	18	0
Chlorella vulgaris	0	0
Chlorella sorokiana	0	0
Scendesmus subspicatus	0	0
Cytotoxicity: % mortality (*Artimia salina*)	69.3	28.9

4. CONCLUSION

In this study we investigated a saline isolate *Streptomyces* sp. CRF17 for the production of active antimicrobial agents. The lab scale fermentation and subsequent isolation, purification and structure elucidation of the metabolites reveal that the isolate is a prolific producer of the antibiotic alborexin and can be exploited for its commercial production. The taxonomic studies including genetic identification confirmed that the isolate CRF17 has maximum similarity (98%) with *Streptomyces pulcher*.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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