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Aureolic acids from a marine-derived *Streptomyces* sp. WBF16

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ABSTRACT

A marine-derived actinomycete (*Streptomyces* sp. WBF16) exhibiting antitumor activities was investigated. The strain was identified using morphological, biochemical and genetic techniques. 16S rDNA sequence of the isolate indicated that it was most closely related to *Streptomyces coelicolor A3* (**2**). Furthermore, a new aureolic acid (Chromomycin B, **1**), along with Chromomycin A_2 (**2**) and Chromomycin A_3 (**3**) were isolated from its secondary metabolites. Their structures were determined by chemical and spectroscopic methods including 1D, 2D NMR and HRMS. Compounds **1**–**3** showed strong cytotoxicity against SGC7901, HepG2, A549, HCT116 and COC1 and HUVEC.

1. Introduction

The aureolic acids are a family of related antitumor antibiotics that include olivomycin A, Chromomycin A_2 or A_3, mithramycin, chromocyclomycin and so on (Remers 1979a; Skarbek and Speedie 1981; Remers and Iyengar 1995; Wohlert et al. 2001; Ogawa et al. 1998; Katahira et al. 1998; Lombó et al. 2006). All aureolic acid type compounds (with the exception of chromocyclomycin) contain a tricyclic aglycon with two aliphatic side chains attached at C-3 and C-7.

Now, Chromomycin have been used clinically in the treatment of some tumor diseases (Jia et al. 2007; Remers 1979b) such as disseminated embryonal cell carcinoma (Skarbek and Speedie 1981; Kennedy et al. 1968; Elias and Evans 1972), hyperglycaemia (Dewick 1997), β-thalassemia and sickle cell anemia (Fibach et al. 2003). As suggested mechanism of action, MTM binds to the DNA, as a Mg^{2+} dimer, which cross-links the two strands (Sastry and Patel 1993; Sastry et al. 1995; Huang et al. 1995), so this action block RNA synthesis, rendering the drug potent anti-tumor activity.

However, clinical use of Chromomycin was limited by the side effects (Calabresi and Chabner 1991). Therefore, we paid more attention to investigate new aureolic acids from marine-derived *Streptomyces* sp.

In this paper, a marine-derived actinomycete was identified as *Streptomyces* sp. WBF16 by morphological, biochemical, genetic techniques and 16S rRNA analysis. And its secondary metabolites including one new Chromomycin derivatives (**1**) (Fig. 3), together with the known Chromomycin A_2 (**2**) and A_3 (**3**) were elucidated by chemical and spectroscopic methods including 1D, 2D NMR and HRMS.

2. Material and methods

2.1. Experiment materials

NMR, Bruker DRX-500; HREIMS, Mariner ESI spectrometer. Sephadex LH-20 (Amersham Pharmacia Biotech Co., Ltd, America). HPLC was performed using a JASCO2000 series instrument equipped with an UV-2075 detector and an YMC-Pack ODS-A (250 mm × 20 mm) column.

2.2. Sample collection

Samples were collected from sea sediments located in Bijiatuan (37°32′N, 122°03′E), in the city of Weihai, China. They were collected and kept in the box with ice until transported to laboratory for further isolation.

2.3. Biochemical and physiological characterization

The morphological characteristics of the isolate were examined according to the methods reported by Shirling for the International *Streptomycete* Project (Shirling and Gottlieb 1966). Cultural

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characters of the strain were studied on International *Streptomyces* Project (ISP) media including YMD agar (ISP-2), oat meal agar (ISP-3), starch inorganic salt agar (ISP-4), glycerol aspar agine salts agar (ISP-5), peptone yeast extract iron agar (ISP-6), tyrosine agar (ISP-7) and non-ISP media like Gause-agar and Waksman No. 2 (Waksman 1961).

The utilization of carbon sources was determined by referring the method of Gottlieb (1961). The strain's tolerance of NaCl, the ability to produce H_2S and different enzymes were obtained by standard protocols (Holding and Collee 1971).

Cell wall composition was analyzed by the method of Lechevalier and Lechevalier (1970), using thin layer chromatography plates as described by Staneck and Roberts (1974).

2.4. Phylogenetic analysis of the strain

Genomic DNA of the strain was extracted by the method according to Weisburg et al. (1991) and Chun and Goodfellow (1995). 16S rRNA gene was amplified with primers forward (5′-CGGAGAGTTTGATCCTGGCTCAG-3′) and reverse (5′-AAAGGAGGTGATCCA GCCGCA-3′). The conditions for thermal cycling were as follows: denaturation of the target DNA at 94 °C for 5 min followed by 30 cycles at 94 °C for 45 s, primer annealing at 55 °C for 45 s, and primer extension at 72 °C for 10 min. The fragments were separated on 1% agarose gel, eluted and purified by the Clean-up gene kit (U-gene, China). The obtained PCR product was sequenced by the ABI 3730XL Genetic Analyzer (Applied Biosystems, USA). The sequence was compared for similarity with the reference species of bacteria contained in genomic database banks, using the NCBI BLAST program. The sequences were aligned *via* Clustal X 2.1 software (Thompson et al. 1997). The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The statistical significance of the tree was obtained by bootstrap analysis of sequence data with help of MEGA version 4.1 (Kumar et al. 2001).

2.5. Fermentation, extraction, purification and structural elucidation of bioactive metabolites

The marine actinomycete WBF16 was cultivated in 0.5 L conical flasks containing 0.3 L of soy medium (20 g soluble starch, 20 g soybean powder, 1.0 g KNO_3, 25 g sea salt) for 7 days and shaken at 200 rpm at 28 °C. After cultivation, fermentation broth was concentrated and added to macroporous resin to adsorb the organic products. The resin was eluted with deionized water–30% ethanol/water–100% ethanol/water in turn. The ethanol soluble fraction was dried *in vacuo* to yield 10 g of extract.

The crude extract was dissolved in methanol and chromatographed on a silica gel column using $CHCl_3$/MeOH (gradient 20:1, 10:1, and 2:1) to deliver 3 fractions Fr. I–Fr. III. Fr. II was purified on Sephadex LH-20 using MeOH to afford two sub-fractions (Fr. IIa and Fr. IIb). Fr. IIa was purified by reversed phase HPLC (250 mm × 20.0 mm, 8 mL/min) using 48% CH_3CN (0.02% Formic acid) to afford Chromomycin A_2 (**2**, 200 mg, t_R = 47 min) and Chromomycin A_3 (**3**, 90 mg, t_R = 24 min). Fr. IIb was purified with the HPLC using 90% methanol (0.02% Formic acid) to afford compound **1** (12 mg, 40 min).

2.6. Chromomycin B (*1*)

Yellow amorphous powder; ^{1}H NMR (500 MHz, $CDCl_3$) see Table 3, ^{13}C NMR (125 MHz, $CDCl_3$) see Table 3; ESI-MS $[M-H]^-$ *m/z* 1152, HRESIMS $[M-H]^-$ m/z 1151.5239 (calcd for $[C_{57}H_{84}O_{24}-H]^-$, 1151.5274)

Table 1
Cultural characteristics of actinobaterium strain WBF-16.

Culture media	Growth	Pigment production
[a]ISP-2	Good	–[b]
ISP-3	Good	–
ISP-4	Good	–
ISP-5	Good	–
ISP-6	Good	Light yellow
ISP-7	Good	Dark yellow
Gause-agar	Good	Dark yellow
Waksman No. 2	Good	–

[a] International *Streptomyces* Project Media.
[b] No production.

2.7. Cytotoxicity assays

Human gastric cancer cell line (SGC7901), human liver hepatocellular carcinoma cell line (HepG2), human lung adenocarcinoma epithelial cell line (A549), human colon cancer cell line (HCT116), Human ovarian cancer cell line (COC1) and Human Umbilical Vein Endothelial Cells (HUVEC) were maintained in RPMI-1640 medium with 5% (v/v) fetal bovine serum and cultured in 96 well microtiter plates. Appropriate dilutions of **1** were added into wells and the cells cultured in a humidified environment in the presence of 5% CO_2 at 37 °C for 72 h. The CCK-8 method was used to evaluate the survival rates of the cancer cell (Ma et al. 2007). The final concentration of compounds **1**–**3** that gave 50% inhibition of cell growth was expressed as the IC_{50} values. Results were expressed as the mean value of triplicate determinations.

3. Results

3.1. Taxonomy of the strain

Cultural, morphological and physiological characteristics of WBF16 from marine sediments were studied according to the standard procedures. Table 1 depicts the cultural characteristics of strain WBF16. It exhibited good growth on ISP-2, ISP-3, ISP-4, ISP-5, ISP-6, ISP-7, Gause-agar and Waksman No. 2 media. The color of the aerial mycelium appeared light yellow (see Supplementary Data File I), while that of substrate mycelium went from light yellow to dark gray (see Supplementary Data File II). The strain produced reddish dark yellow pigment on ISP-7 and Gause-agar and exhibited light yellow pigmentation on ISP-6. Fig. 1 shows the sporophor morphology of the strain was a spiral type, and it may

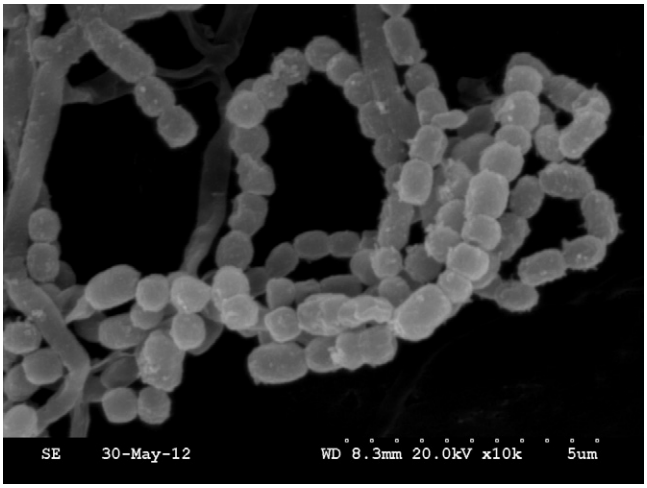

Fig. 1. Scanning electron micrograph of sporotrichial and spore of *Streptomyces* sp. WBF16.