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Piericidins C_7 and C_8, New Cytotoxic Antibiotics Produced by a Marine *Streptomyces* sp.

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Abstract Piericidins C_7 and C_8, two new members of the piericidin family, were isolated from a marine actinomycete. The producing organism was identified as *Streptomyces* sp. based on the cultural, biochemical and chemotaxonomical characteristics as well as the 16S rRNA sequence. Piericidins C_7 and C_8 showed selective cytotoxicity against rat glia cells transformed with the adenovirus E1A gene (IC_{50} C_7: 1.5 nM, C_8: 0.45 nM) and Neuro-2a mouse neuroblastoma cells (IC_{50} C_7: 0.83 nM, C_8: 0.21 nM).

Keywords piericidin, marine actinomycete, *Streptomyces*, cytotoxic antibiotic

Introduction

The retinoblastoma tumor suppressor protein (pRB) plays an important role in cell-cycle and apoptosis control in mammalian cells and is inactivated during the development of a wide variety of human cancers [1, 2]. The adenovirus E1A gene product inactivates pRB thereby stimulating host cell DNA synthesis. Although this function of E1A induces the host cells to undergo apoptosis, stable transformants expressing E1A seem to exhibit an attenuated apoptotic response to E1A [3]. Then E1A-transformed cells might be an appropriate model for pRB-inactivated tumor cells. In the course of screening for antitumor antibiotics against E1A-transformed cells from marine microorganisms [4], an actinomycete was found to produce two new members of the piericidin family, piericidins C_7 and C_8 (Fig. 1). Both compounds showed selective cytotoxicity against rat glia cells transformed with E1A [5].

This paper describes the taxonomy of the producing organism, and the fermentation, isolation, physicochemical properties and biological activities of piericidins C_7 and C_8. The structure elucidation of the piericidins is described in the accompanying paper [6].

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Materials and Methods

Taxonomy

The characterization of the culture was carried out by the methods of the International Streptomyces Project (ISP) [7, 8]. Cell wall amino acids were analyzed by the methods of Becker [9]. Cellular fatty acid and quinone compositions were analyzed by GC-MS and LC-MS, respectively. The DNA G+C content (mol%) of the strain was determined using HPLC analysis of hydrolyzed genome DNA according to Tamaoka and Komagata [10]. Genomic DNA was purified by using Genomic-tip and buffer set (Qiagen). The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions 8～27 as a forward primer and 1492～1510 as a reverse primer (*Escherichia coli* numbering system) [11]. Sequencing of the 16S rRNA gene was done by Takara Bio Inc. The 16S rRNA gene sequence was compared with the bacterial sequence data stored in DDBJ database by using BLAST algorithm [12].

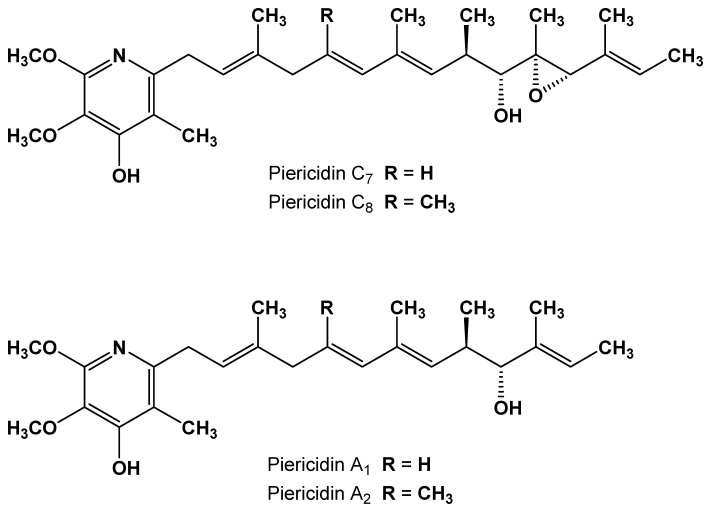

Fig. 1 Structures of piericidins C_7, C_8, A_1 and A_2.

Table 1 Culture characteristics of strain YM14-060

Agar medium	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract - malt extract	White	Yellow	None
Oatmeal	Gray-color series	Yellow	None
Inorganic salts - starch	White	White	None
Glycerol - asparagine	Yellow	Yellow	None

Spectroscopic Measurement

UV and IR spectra were measured on Shimadzu UV-1700 and JASCO FT/IR-410 spectrometers, respectively. Mass spectra were obtained on a JEOL SX-102A spectrometer in the FAB mode using *m*-nitrobenzyl alcohol as matrix and polyethylene glycol as internal standard. Optical rotations were recorded on a JASCO P-1030 spectropolarimeter.

Cells and Cell Culture

RG-E1A-7 cells were established by transfection of primary rat glia cells with pSV2neo containing the adenovirus E1A gene [5]. Other cell lines were obtained from the Japanese Cancer Research Resources Bank (JCRB). All the cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum and 0.1% glucose.

MTT Assay

RG-E1A-7 (2×10^4 cells/ml), Neuro-2a (5×10^4 cells/ml), C6 (1×10^5 cells/ml) and 3Y1 cells (1×10^4 cells/ml) were plated and incubated for 3 days with various concentrations of samples. After the cells were treated with 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hours at 37°C, the relative cell number was measured as absorbance at 570 nm. IC_{50} values were calculated by linear interpolation between the two drug concentrations above and below the 50% inhibition line.

Results and Discussion

Identification of the Producing Organism

The producing organism designated YM14-060 was isolated from unidentified greenish ascidians collected at Iwayama Bay, Palau (depth: 0.5 m, GPS location: N7°20′4.5″, E134°29′47.5″). The aerial mycelium of culture YM14-060 irregularly branched on the main hypha. However, no spore chain was observed on tested media. The culture characteristics and biochemical properties of YM14-060 are summarized in Tables 1 and 2, respectively. Whole-cell analysis showed that the strain contained L,L-diaminopimelic acid, indicating cell-wall type I. The major menaquinones were MK-9(H_4) and MK-9(H_6), and the predominant cellular fatty acid was iso-15 : 0. The DNA G+C content was 73.6 mol%. The 16S rRNA

sequencing of this strain revealed high sequence identity with *Streptomyces* sp. NBRC 13801 (99.7%), *Streptomyces cinnamoneus* LMG 20324 (98.3%) and *Streptomyces griseocarneus* DSM40004T (98.0%). From these characteristics, strain YM14-060 was identified as a member of the genus *Streptomyces* and named *Streptomyces* sp. YM14-060.

Fermentation

The seed medium consisted of Bacto Peptone 5.0 g, yeast extract 1.0 g, iron(III) citrate 0.1 g, distilled water 250 ml and sea water 750 ml (pH 7.6). A 100-ml Erlenmeyer flask containing 40 ml of the medium was inoculated with a stock culture of YM14-060 and was incubated on a rotary shaker at 30°C for 3 days. The seed culture at 2% was transferred to 1-liter Erlenmeyer flasks containing 500 ml of a production medium consisting of Bacto Peptone 5.0 g, yeast extract 1.0 g, iron(III) citrate 0.1 g, sucrose 1.0 g, distilled water 250 ml and sea water 750 ml (pH 7.6). The fermentation was carried out on a rotary shaker at 30°C for 7 days.

Table 2 Physiological and biochemical properties of strain YM14-060

Gelatin liquefaction	−
Hydrolysis of starch	+
Degradation of cellulose	−
Nitrate reduction	−
Skim milk	
Coagulation	+
Peptonization	+
Melanoid pigment production	
Tryptone - yeast extract - iron agar	+
Tyrosine agar	−
Temperature range for growth	20~30°C
Carbon utilization on Pridham & Gottlieb basal agar	
D-Glucose	+
L-Rhamnose	−
D-Mannitol	−
D-Fructose	−
L-Arabinose	−
Raffinose	+
Sucrose	−
D-Xylose	−
Inositol	+

Isolation

The fermentation broth (2.0 liters) was centrifuged and the mycelium was extracted with acetone. After evaporation, the aqueous concentrate was extracted with ethyl acetate. The extract was chromatographed on a silica gel column with chloroform. The active eluate was subjected to HPLC (YMC Pack D-ODS-7, 20×250 mm) with 85% methanol. The two main fractions gave piericidins A_1 (16.9 mg) and A_2 (18.6 mg) [13~15]. The remaining active fraction was further purified by HPLC (YMC Pack D-ODS-7, 20×250 mm) with 78% methanol. Two active fractions thus obtained were separately concentrated to dryness to yield two new substances designated piericidins C_7 (2.7 mg) and C_8 (7.3 mg). The structures of piericidins C_7, C_8, A_1 and A_2 are shown in Fig. 1.

Physico-chemical Properties

The physico-chemical properties of piericidins C_7 and C_8 are summarized in Table 3. The molecular formulae were established as $C_{28}H_{41}NO_5$ for piericidin C_7 and $C_{29}H_{43}NO_5$ for piericidin C_8 by high-resolution FAB-MS. The IR spectrum of the piericidins indicates the presence of hydroxyl groups (~3400 cm^{-1}).

Table 3 Physico-chemical properties of piericidins C_7 and C_8

	Piericidin C_7	Piericidin C_8
Appearance	Colorless oil	Colorless oil
$[\alpha]_D^{23}$	+7.0° (*c* 0.21, MeOH)	+13° (*c* 0.37, MeOH)
Molecular formula	$C_{28}H_{41}NO_5$	$C_{29}H_{43}NO_5$
HR-FAB-MS (*m/z*)		
Found	472.3062 $(M+H)^+$	486.3223 $(M+H)^+$
Calcd.	472.3063	486.3219
UV λ_{max}^{MeOH} nm (ε)	231 (30,000), 237 (30,400) 269 (3,800)	225 (21,600), 267 (6,000)
IR ν_{max} (KBr) cm^{-1}	3420, 1590, 1470	3400, 1590, 1470

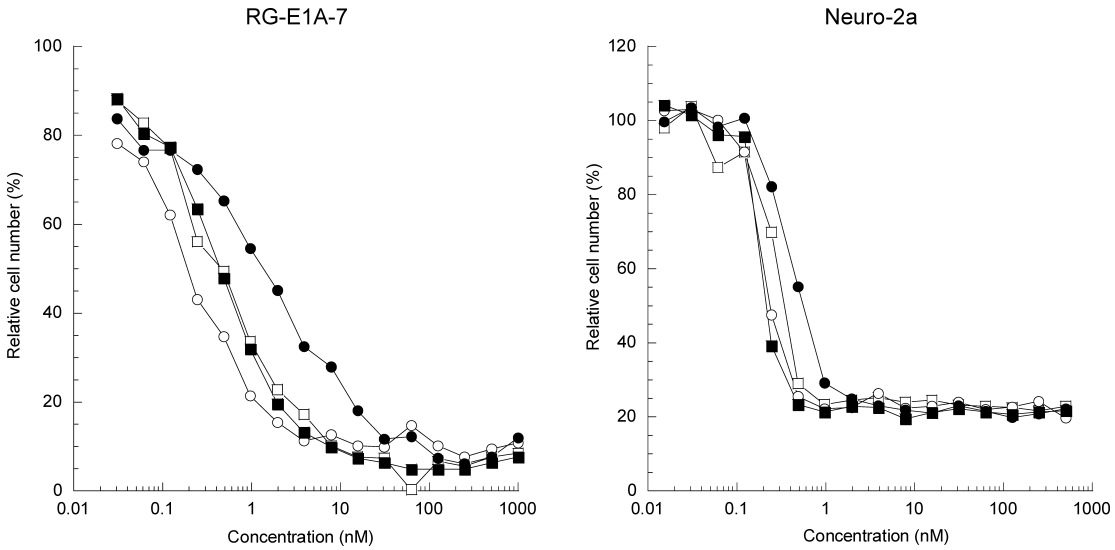

Fig. 2 Effects of piericidins C_7, C_8, A_1 and A_2 on the growth of RG-E1A-7 cells and Neuro-2a cells.

Cells were cultured with various concentrations of piericidin C_7 (●), C_8 (■), A_1 (○) or A_2 (□) for 72 hours and then the relative cell numbers were measured by the MTT method.

Table 4 IC_{50} values (nM) of piericidins against transformed or normal cells

Cell line	Piericidin C_7	Piericidin C_8	Piericidin A_1	Piericidin A_2
RG-E1A-7	1.5	0.45	0.20	0.47
Neuro-2a	0.83	0.21	0.21	0.22
C6	>1,000	>1,000	>1,000	>1,000
3Y1	>1,000	>1,000	>1,000	>1,000

Biological Activity

The biological activities of piericidins were examined by using rat glial cells transformed with the adenovirus E1A gene (RG-E1A-7), Neuro-2a mouse neuroblastoma cells, C6 rat glioma cells and 3Y1 rat normal fibroblasts. Piericidins C_7 and C_8 showed cytotoxicity against RG-E1A-7 cells (IC_{50} C_7: 1.5 nM, C_8: 0.45 nM) and inhibited the growth of Neuro-2a cells (IC_{50} C_7: 0.83 nM, C_8: 0.21 nM) without cytotoxic cell death (Fig. 2). Interestingly, the piericidins at less than 1 μM showed neither cytotoxic nor cytostatic effect on C6 cells and 3Y1 cells (Table 4). Piericidins A_1 and A_2 exhibited similar activity when compared with piericidins C_7 and C_8 (Fig. 2 and Table 4). Piericidins have been reported to inhibit mitochondrial NADH-ubiquinone oxidoreductase [16]. Since mitochondrial F_0F_1-ATPase inhibitors show selective cytotoxicity against RG-E1A-7 cells [17, 18], mitochondrial dysfunction might cause cell death or growth arrest in certain types of tumors. Further studies on the biological activities of piericidins are in progress.

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