A NEW ANTIBIOTIC, CYPEMYCIN

TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL CHARACTERISTICS

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A new peptide antibiotic, cypemycin, with a molecular weight of 2,097 (M+H), was isolated from the culture broth of *Streptomyces* sp. OH-4156. The antibiotic possesses cytocidal activity against P388 leukemia cells *in vitro* at a concentration of 1.3 μg/ml (IC_{50} values), and the antibiotic showed antimicrobial activities against *Micrococcus luteus* (MIC, 0.2 μg/ml).

In the course of a screening program for novel antibiotics showing cytocidal activity, cypemycin was isolated from the culture broth of *Streptomyces* sp. OH-4156 which had been isolated from a soil sample collected in Tokyo, Japan. The antibiotic exhibited cytocidal activity against mammalian tumor cells *in vitro*, and antimicrobial activity against *Micrococcus luteus*.

The present paper deals with taxonomic studies of the producing strain, and the production, isolation and physico-chemical properties of the new antibiotic. The preliminary biological activities of cypemycin are also described.

Materials and Methods

General Experimental Procedures

Silica gel 60 (Merck) and Sephadex LH-20 (Pharmacia Fine Chemicals) were used for column chromatography and pre-coated TLC plates of Silica gel 60 (Merck) was used for TLC analysis.

Taxonomic Studies

The type diaminopimelic acid (DAP) was determined by the method of TAKAHASHI *et al.*[1]. To investigate the cultural and physiological characteristics, the International Streptomyces Project (ISP) media recommended by SHIRILING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for 2 weeks. Color names and hue numbers indicated in Table 1 are those of the Color Harmony Manual (4th Ed.)[4]. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[5] containing a 1% carbon source at 27°C.

Cytotoxic Activity Tests

Seven strains of established mammalian cells were maintained in monolayers or in suspension in EAGLE's minimum essential medium (MEM) supplemented with 10% bovine serum and kanamycin (60 μg/ml). To determine the cytotoxicity of cypemycin, cells suspended in 200 μl of the medium were plated in a 96-well culture plate (Falcon) and incubated for 24 hours at 37°C in a 5% CO_2-95% air atmosphere. To each well was added 5 μl of medium containing a different concentration of cypemycin. After 72 hours of incubation, the cell growth was evaluated by the method of MIRAVELLI *et al.*[6].

Antimicrobial Activity Test

The antimicrobial spectrum of cypemycin was determined using 6 mm paper disks (Toyo Seisakusho Co., Ltd.). Bacteria were grown on Mueller-Hinton agar medium (Difco) and fungi or yeast on potato-broth agar medium. Antimicrobial activity was observed after 24 hours of incubation at 37°C for bacteria or longer incubation at 27°C for fungi and yeasts.

Results and Discussion

Taxonomy of the Producing Strain OH-4156

The vegetative mycelia grow abundantly on both synthetic and complex agar media, and do not show fragmentation into coccoid or bacillary elements. The aerial mycelia grow abundantly on yeast extract-malt extract agar, but poorly on other media. The mature sporophores formed spiral spore chains and had more than 20 spores per chain. The

Fig. 1. Electron micrograph of spore chains of *Streptomyces* sp. OH-4156 grown on agar plate for 10 days.

Bar represents 1.0 μm.

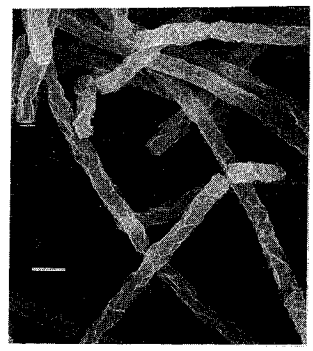


Table 1. Cultural characteristics of strain OH-4156.

Medium	Cultural characteristics	Medium	Cultural characteristics
Yeast extract-malt extract agar[a]	G: Good, colorless R: Mustard gold (2ne) AM: Abundant, charcoal gray (h) SP: None	Tyrosine agar[a]	G: Moderate, covert tan (2ge) R: Covert tan (2ge) AM: Moderate, silver gray (3fe) SP: Deep brown (4pl)
Oatmeal agar[a]	G: Moderate, colorless R: Dark covert gray (2ih) AM: Moderate, beige gray (3ih) SP: None	Sucrose-nitrate agar[b]	G: Poor, colorless R: Pearl (3ba) AM: Poor, pearl (3ba) SP: None
Inorganic salts-starch agar[a]	G: Good, Lt. ivory (2ca) R: Mustard (2le) AM: Abundant, beige gray (3ih) SP: None	Glucose-nitrate agar[b]	G: Moderate, bamboo (2gc) R: Bamboo (2gc) AM: None SP: Lt. ivory (2ca)
Glycerol-asparagine agar	G: Good, Lt. ivory (2ca) R: Lt. yellow (1½ea) AM: Abundant, silver gray (3fe) SP: None	Glycerol-calcium malate agar[b]	G: Moderate, cream (1½ca) R: Pearl (2ba) AM: None SP: None
Peptone-yeast extract-iron agar[a]	G: Moderate, bamboo (2gc) R: Bamboo (2gc) AM: None SP: Mustard brown (2ni)	Glucose-peptone agar[b]	G: Good, mustard (2le) R: Gold (2le) AM: Moderate white (a) SP: Mustard (2le)
Glucose-asparagine agar	G: Good, Lt. ivory (2ca) R: Bamboo (2gc) AM: Abundant, beige gray (3ih) SP: None	Nutrient agar[b]	G: Good, bamboo (2gc) R: Gold (2lc) AM: Abundant, silver gray (3fe) SP: None

[a] Medium recommended by international streptomyces project.

[b] Medium recommended by S. A. WAKSMAN.

Abbreviations: G; growth of vegetative mycelium, R; reverse, AM; aerial mycelium, SP; soluble pigment.

Table 2. Physiological properties of strain OH-4156.

Melanin formation	+
Tyrosinase reaction	+
H_2S production	+
Liquefaction of gelatin (20°C)	+
Peptonization of milk (37°C)	+
Coagulation of milk (37°C)	−
Nitrate reduction	−
Hydrolysis of starch	+
Cellulolytic activity	−
Temperature range for growth	10~37°C

+: Positive, −: negative.

Table 3. Utilization of carbon sources by strain OH-4156.

Utilized:	D-Glucose, L-arabinose, D-xylose
Partially:	D-Fructose, sucrose
Nonutilized:	Raffinose, melibiose, D-mannitol, L-rhamnose, *i*-inositol

Table 4. Physico-chemical properties of cypemycin.

Appearance	White powder
MP (°C)	188~193
Optical rotation	$[\alpha]_D^{25}$ −70.3° (*c* 1.0, MeOH)
FAB-MS (*m/z*)	2,097 $(M+H)^+$, 2,119 $(M+Na)^+$, 2,135 $(M+K)^+$
UV λ_{max}^{MeOH} nm (log ε)	203 (4.78), 225 (sh, 4.65), 265 (sh, 4.02)
UV $\lambda_{max}^{MeOH-HCl}$ nm (log ε)	203 (4.79), 225 (sh, 4.66), 265 (sh, 4.10)
UV $\lambda_{max}^{MeOH-NaOH}$ nm (log ε)	205 (5.23), 230 (sh, 4.55), 265 (sh, 3.92)
IR ν_{max} (KBr) cm^{-1}	3450, 3300, 3000, 1660, 1530, 1340, 1260
Color reaction (Positive)	H_2SO_4, iodine
Solubility:	
Soluble	MeOH, EtOH, $CHCl_3$, benzene
Insoluble	H_2O, acetone, EtOAc, hexane

spores were oval in shape, 0.7 × 0.4 μm in size and had a smooth surface (Fig. 1). Sclerotic granules, sporangia and flagellated spores were not observed.

DAP in the cell wall of strain OH-4156 was determined to be the LL-type. The cultural characteristics and the utilization of carbon sources are shown in Tables 1, 2, and 3.

The strain exhibited the following properties. Sporophores were spirals; spores were oval and had smooth surfaces; the vegetative mycelia were brown or beige and the aerial mycelia were gray or white; melanoid pigment was produced.

Based on the taxonomic properties described above, strain OH-4156 was considered to belong to the genus *Streptomyces*[7]. The strain was deposited in the National Institute of Bioscience and Human-Technology, (formerly the Fermentation Research Institute), Agency of Industrial Science and Technology, Japan, under the name of *Streptomyces* sp. OH-4156. The accession No. is FERM P-12947.

Fermentation and Isolation of the Active Components

A stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 100 ml of seed medium consisting of 0.1% glucose, 2.4% starch, 0.3% peptone, 0.3% meat extract, 0.5% yeast extract, and 0.4% $CaCO_3$ (pH 7.0 before sterilization). The fiasks were inoculated at 27°C for 72 hours on a reciprocal shaker. Then 400 ml of the resulting culture were transferred to a 30-liter fermentor containing 20 liters of medium consisting of 0.5% maltose, 1.5% dry yeast, 2.5% Ebios (dry brewery's yeast), 1% KBr, 0.05% KH_2PO_4 and 0.05% $MgSO_4$ (pH 7.0). The fermentation was carried out at 27°C for 72 hours using an agitation rate of 250 rpm and an aeration rate of 5 liters/minute.

The mycelia collected from fermentation broth were mixed with methanol, and the crude substance was extracted. The extract was concentrated *in vacuo* to give a brown syrup, and was subjected to silica gel column chromatography (i.d. 5 × 25 cm) using $CHCl_3$-methanol (5 : 1) as solvent. Fractions exhibiting cytocidal activity against B16 melanoma cells were collected. Further separation of the active fractions (142 mg) using Sephadex LH-20 column chromatography (15 × 700 mm) eluted with methanol gave a crude

fraction containing cypemycin (93 mg). The crude was dissolved in a small amount of MeOH, cooled to 5°C for one day and centrifuged, and the precipitate was collected. The precipitate was washed with a small volume of MeOH to yield a white powder (48.5 mg). Final purification with HPLC (Capcell Pak C_{18} Shiseido, 20 × 250 mm) eluted with 80% methanol gave a white powder (42 mg).

Fig. 2. UV spectrum of cypemycin.

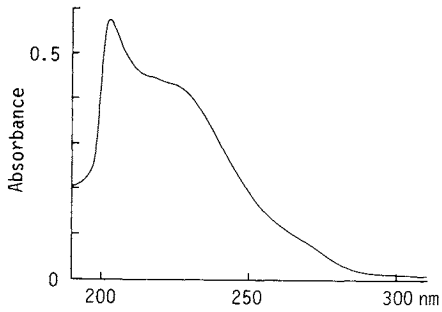


Fig. 3. IR spectrum of cypemycin.

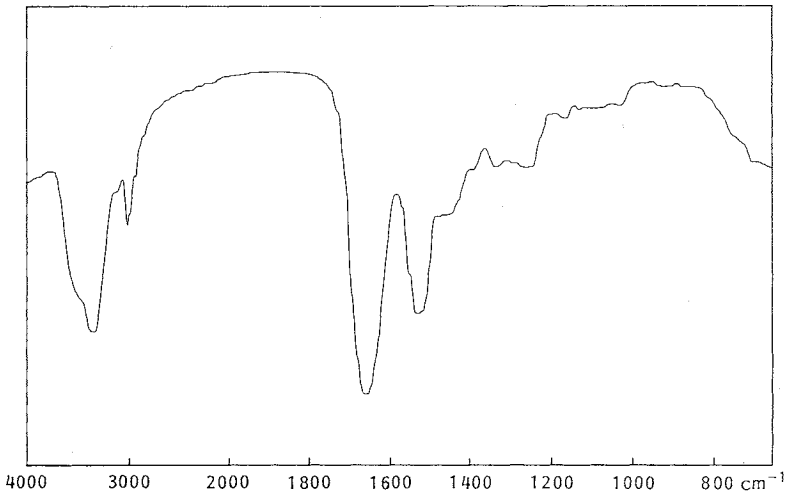


Fig. 4. ^{1}H NMR spectrum of cypemycin.

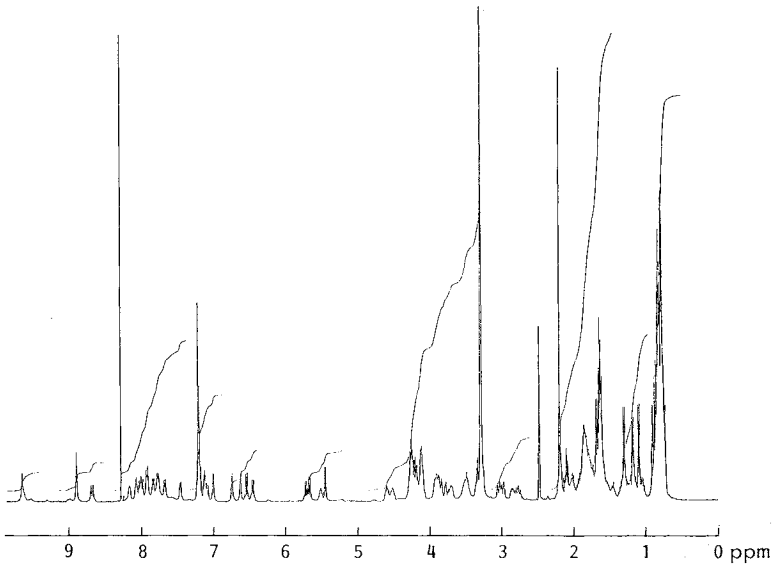
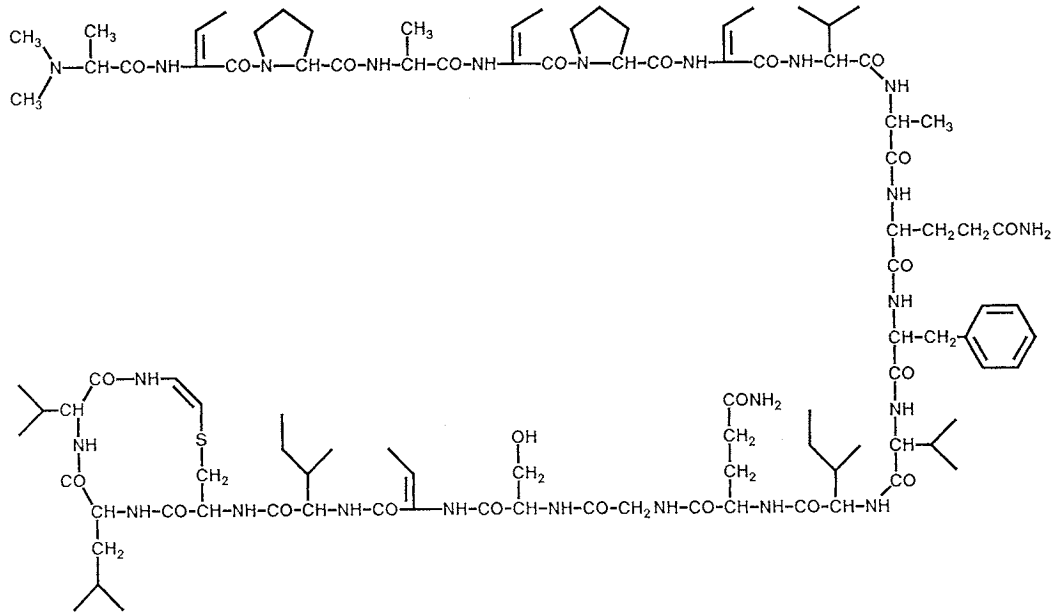


Fig. 5. Structure of cypemycin (**1**).



Physico-chemical Properties of Cypemycin

Physico-chemical properties of cypemycin are summarized in Table 4. UV and IR absorption spectra of cypemycin are shown in Figs. 2 and 3, respectively. The IR spectrum showed strong amide absorbance at 1660 and 1530 cm^{-1}, suggesting that cypemycin may be a peptide antibiotic. Fig. 4 show ^1H NMR spectrum of cypemycin, and the structure was determined to be **1** (Fig. 5), a new member of the peptide family of antibiotic. The structure elucidation of cypemycin will be described elsewhere.

Table 5. Cytotoxicities of cypemycin against mammalian cells.

Cell line	Origin	IC_{50} (μg/ml)
HeLa S3	Human cervix carcinoma	>25
B16 melanoma	Mouse melanoma	>25
P388 leukemia	Mouse leukemia	1.3
L929	Mouse fibroblast	>25
HCC-1	Human liver tumor	>25
HCC-M	Human liver tumor	>25
Alex	Human liver tumor	>25

Biological Activity Tests of Cypemycin

Cypemycin showed antimicrobial activity against only *Micrococcus luteus* (MIC = 0.2 μg/ml) and no activity against other Gram-positive and-negative bacteria, fungi, yeast etc. Since known high molecular antibiotics of the peptide group such as bacitracin[8], triculamin[9], siomycin[10] and stenothricin[11] show narrow antibacterial spectra, it was evident that cypemycin showed activity similar to that of such antibiotics. Cytocidal activity of cypemycin was examined against mammalian tumor cells *in vitro*. When the cells were exposed to the antibiotic for 3 days, the IC_{50} values were 1.3 μg/ml against P388 leukemic cells which were the most sensitive among the cell lines as shown in Table 5.

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References

1) TAKAHASHI, Y.; Y. IWAI, H. TOMODA, N. NIMURA, T. KINOSHITA & S. ŌMURA: Optical resolution of 2,6-diaminopimelic acid stereoisomer by high performance liquid chromatography for the chemotaxonomy of actinomycete strains. J. Gen. Appl. Microbiol. 35: 27~32, 1989
2) SHRIRLING, E. B. & D. GOTTLIEB: Methods for characterization of Streptomyces species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Description of Genera and Species. Williams and Wilkins Co., 1961
4) Container Corporation of America: Color Harmony Manual. 4th Ed. Container Corporation of America, Chicago, 1958
5) PRIDHAM, T. G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bacteriol. 56: 107~114, 1948
6) MIRABELLI, C. K.; H. BARTUS, J. O. L. BARTUS, R. JOHNSON, S. M. MONG, C. P. SUNG & S. T. CROOKE: Application of a tissue culture microtiter test for the detection of cytotoxic agents from natural products. J. Antibiotics 38: 758~766, 1985
7) WILLIAMS, S. T.; M. GOODFELLOW & G. ALDERSON: Genus Streptomyces Waksman and Henrici 1943. *In* BERGEY's Manual of Systematic Bacteriology. Volume 4. *Ed.*, S. T. WILLIAMS *et al.*, pp. 2452~2492, Williams and Wilkins Co., 1989
8) LOCKHART, I. M.; E. P. ABRAHAM & G. G. F. NEWTON: *N*-Terminal and sulfur containing residues of bacitracin A. Biochem. J. 61: 534~544, 1955
9) ANZAI, K. & S. SUZUKI: Amino acid sequence of triculamin. Agr. Biol. Chem. 33: 1737~1744, 1969
10) EBATA, M.; K. MIYAZAKI & H. OTSUKA: Studies on siomycin. III. Structural features of siomycin A. J. Antibiotics 22: 434~441, 1969
11) HASENBOHLER, A.; H. KNEIFEL, W. A. KONIG, H. ZAHNER & H. J. ZEILER: Metabolic products of microorganisms, 134. Stenothricin, a new inhibitor of the bacterial cell wall synthesis. Arch. Microbiol. 99: 307~321, 1974