STUDIES ON A NOVEL CYTOCIDAL ANTIBIOTIC, TRIENOMYCIN A

TAXONOMY, FERMENTATION, ISOLATION, AND PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS

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A new antibiotic, trienomycin A, has been isolated from the culture broth of *Streptomyces* sp. No. 83-16. The physico-chemical characteristics of the antibiotic suggested that it belongs to the group of ansamycin antibiotics with a triene moiety in the molecule. The molecular formula of trienomycin A is $C_{36}H_{50}N_2O_7$ (MW 622). The antibiotic possesses potent cytocidal activity against HeLa S_3 and PLC hepatoma cells at concentrations of 0.1 and 0.01 μg/ml (IC_{50} value) respectively. However, trienomycin A showed no antimicrobial activity against the bacteria, fungi and yeasts examined with the exception of weak activity *versus Piricularia oryzae* at a concentration of 1,000 μg/ml.

In the course of a screening program for novel antibiotics showing cytocidal activity, trienomycin A was isolated from the fermentation broth of *Streptomyces* sp. No. 83-16. The present paper deals with taxonomic studies of the producing strain, and the production, isolation and physico-chemical properties of the new antibiotic. The biological activity of trienomycin A against human tumor cells and microorganisms *in vitro* is also described.

Materials and Methods

Taxonomic Studies

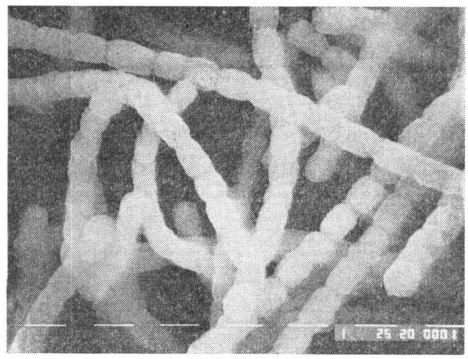
For taxonomic studies, most cultures were grown in accordance with methods adopted by the International Streptomyces Project. For experiments on cultural properties, all cultures were incubated at 27°C and were observed for 15~20 days. The color recorded for mature cultures was described according to the "Color Harmony Manual"[1]. Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB[2,3]. The type of diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[4].

Fermentation

The stock culture of the producing organism was inoculated into a 500-ml Sakaguchi flask containing 100 ml of the seed medium consisting of 2.0% glucose, 0.5% peptone, 0.5% meat extract, 0.3% dry yeast (Fermipan, Gist-Brocades, Holland), 0.5% NaCl and 0.3% $CaCO_3$ (adjusted to pH 7.0 before sterilization). The flask was incubated at 27°C for 72 hours on a reciprocal shaker. The resulting culture (3×100 ml) was transferred to a 30-liter jar fermentor containing 20 liters of the same medium described above and the fermentation was carried out at 28°C for 72 hours with an agitation rate of 160 rpm and an aeration rate of 20 liters per minute. Detection of the antibiotic in the fermentation broth was followed by a cytocidal assay using HeLa S_3 cells described below or by HPLC analysis [column: YMC packed column A-302 (4.6 mm×150 mm); Yamamura Chemical Lab., Japan; solvent: 64% aq MeOH; detection: UV 272 nm].

After 72 hours of fermentation, the amount of trienomycin A in the broth filtrate reached a maximum (2.0 μg/ml).

Fig. 1. Electron micrograph of the strain No. 83-16 (bars in the photo represent 1 μm).



Isolation and Purification

The harvested broth (60 liters) of *Streptomyces* sp. No. 83-16 was centrifuged, and the resulting supernatant was adjusted to pH 5.0 with 3 N HCl and then extracted with EtOAc (30 liters $\times$2). The combined EtOAc layers were concentrated to *ca.* 4 liters *in vacuo* and washed with H_2O (2 liters). The organic layer was dried over Na_2SO_4 and concentrated under reduced pressure to give a brown oil (*ca.* 10 g).

The oliy residue was chromatographed on a silica gel column (Kieselgel 60 F_{254}, Merck, 500 ml) using a toluene - acetone mixture as the developing solvent (49: 1 ~ 0: 50, stepwise). Fractions exhibiting cytocidal activity on HeLa cells were collected and rechromatographed on a silica gel column using a $CHCl_3$ - MeOH gradient to give crude trienomycin A (*ca.* 200 mg).

The antibiotic was further purified by preparative HPLC [column: YMC packed column A-324 (10 mm $\times$ 300 mm); Yamamura Chemical Lab., Japan; solvent: 64% aq MeOH; detection: UV 254 nm] or by preparative TLC (Kieselgel 60 F_{254}, Merck) developed with $CHCl_3$ - MeOH (19: 1) to give a colorless powder [mp 128 ~ 132°C, $[\alpha]_D^{20}$ +174° (*c* 0.1, MeOH), Rf value on silica gel TLC 0.47 ($CHCl_3$ - MeOH, 9: 1)].

Antimicrobial Activity

The antimicrobial spectrum of trienomycin A was determined using 8 mm paper discs (Toyo Seisakusho Co., Ltd.) and Mueller Hinton agar medium (Difco) for bacteria and potato broth agar medium for fungi or yeasts.

Antimicrobial activity was observed after 24 hours incubation at 37°C for bacteria or longer incubation at 27°C for fungi or yeasts.

Effect of Trienomycin A on Human Tumor Cells *In Vitro*

HeLa S_3 or PLC human hepatoma cells were maintained in monolayers in EAGLE's minimum essential medium (MEM) or WILLIAM's medium supplemented with 10% bovine serum and kanamycin (60 μg/ml) respectively at 37°C.

To determine the effects of the antibiotic on the growth of mammalian cells, HeLa S_3 cells (4×10^4) or PLC cells (5×10^4) in 2 ml of the medium were placed in 2 cm^2-culture plates (Falcon 3047) and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture dish was filled with a fresh medium containing a different concentration of trienomycin A and incubated for 72 hours. The cells were then trypsinized to form a single cell suspension, and counted using a hemocytometer.

The other assay system, a colony formation method, was performed as follows. A single cell suspension of HeLa S_3 cells (200 cells per 5 ml of MEM) was placed in a 5 cm-Petri dish (NUNCLON, Nunc Co.). After preincubation for 24 hours, trienomycin A dissolved in MeOH was added to the medium (final concentration of MeOH was 1.0%) and incubated under the same conditions as described above for 8 days. The colonies were then fixed with MeOH, stained with GIEMSA's solution, and counted.

Results

Taxonomic Studies

An electron micrograph of strain No. 83-16 is shown in Fig. 1. The aerial mycelia were well developed on yeast extract - malt extract agar and were long, with no verticil formation. The chains of spores consisted of more than 20 spores and were almost straight. Most of the spores were short

Table 1. Cultural properties of strain No. 83-16.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Tyrosin agar	Good	Brownish gray	Dark brown	Olive gray
Yeast extract - malt extract agar	Good	Shadow gray	Grayish yellow brown	Pale yellow brown
Nutrient agar	Good	Shadow gray	Beige	Chocolate brown
Glycerol - asparagine agar	Moderate	Beige gray	Pale yellow brown	Grayish yellow brown
Inorganic salts - starch agar	Moderate	Dark covert gray	Brownish gray	Grayish yellow brown
Oatmeal agar	Poor	Light brownish gray	Light brownish gray	Light brownish gray
Peptone - yeast extract - iron agar	Poor	Parchment	Light brownish gray	Chocolate brown

Table 2. Physiological characteristics of strain No. 83-16.

Nitrate reaction	Negative
Liquefaction of gelatin	Doubtful
Starch hydrolysis	Negative
Coagulation of milk	Negative
Peptonization of milk	Doubtful
Melanin formation	Positive
Tyrosinase reaction	Positive
Production of H_2S	Negative

Table 3. Utilization of carbon sources by strain No. 83-16.

Responses	Carbon source
Positive	L-Arabinose, D-xylose, D-glucose, D-fructose, inositol, L-rhamnose, D-raffinose, D-mannitol

Table 4. Physico-chemical properties of trienomycin A and mycotrienins I and II.

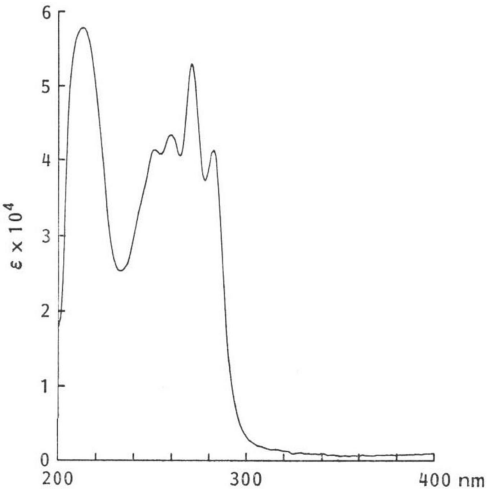
	Trienomycin A	Mycotrienin I[6]	Mycotrienin II[6]
Producing organism	*Streptomyces* sp. No. 83-16	*Streptomyces rishiriensis* T-23	*Streptomyces rishiriensis* T-23
Appearance	Colorless powder	Yellow powder	Colorless powder
Molecular formula	$C_{36}H_{50}N_2O_7$	$C_{36}H_{48}N_2O_8$	$C_{36}H_{50}N_2O_8$
MS m/z (M^+)	622.3607	636.3439	638.3549
UV λ_{max}^{MeOH} nm (ε)	252 (40,900), 260 (42,900), 271 (55,300), 282 (40,700)	262 (38,500), 272 (49,600), 282 (38,800), 383 (3,400)	260 (40,800), 270 (52,300). 280 (40,500), 310 (5,900)
IR ν_{max} cm^{-1}	3400 (NH, OH), 1730, 1205 (ester), 1650, 1540 (amide), 1000 (triene) in KBr	3340 (NH, OH), 1730, 1202 (ester), 1650, 1535 (amide), 1000 (triene) in $CHCl_3$	3340 (NH, OH), 1730, 1200 (ester), 1650, 1535 (amide), 1003 (triene) in Nujol

and cylindrical and measured $0.5 \sim 0.7 \times 0.8 \sim 1.0$ μm in size, and the surfaces were warty.

Culture characteristics, physiological properties and utilization of carbon sources of strain No. 83-16 are shown in Tables 1, 2 and 3, respectively. Cell wall analysis showed the presence of LL-diaminopimelic acid.

Microscopic studies and the cell wall type indicated that strain No. 83-16 belongs to the genus *Streptomyces*.

Fig. 2. UV spectrum of trienomycin A (in MeOH).

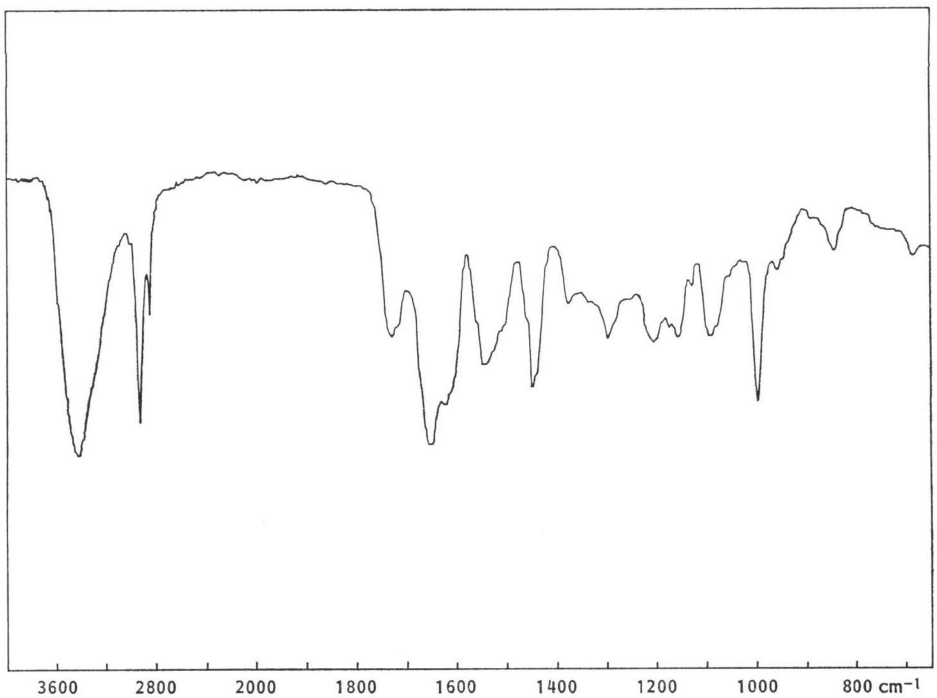


Physico-chemical Properties of Trienomycin A

The preliminary physico-chemical properties of trienomycin A are summarized in Table 4. This antibiotic is soluble in MeOH, EtOH, acetone, EtOAc, BuOAc, $CHCl_3$, CH_2Cl_2 and THF, but practically insoluble in H_2O and *n*-hexane. The UV spectrum of trienomycin A (Fig. 2) showed the presence of a triene moiety (λ_{max}^{MeOH} 260, 271 and 282 nm)[5~10]. The IR spectrum of the antibiotic suggested the presence of an amide group (ν_{max}^{KBr} 1650 and 1540 cm^{-1}), ester group (1730 and 1205 cm^{-1}) and triene group (1000 cm^{-1}) in the molecule (Fig. 3). In the mass spectra (FD-MS and EI-MS), a molecular ion peak was observed at *m/z* 622.

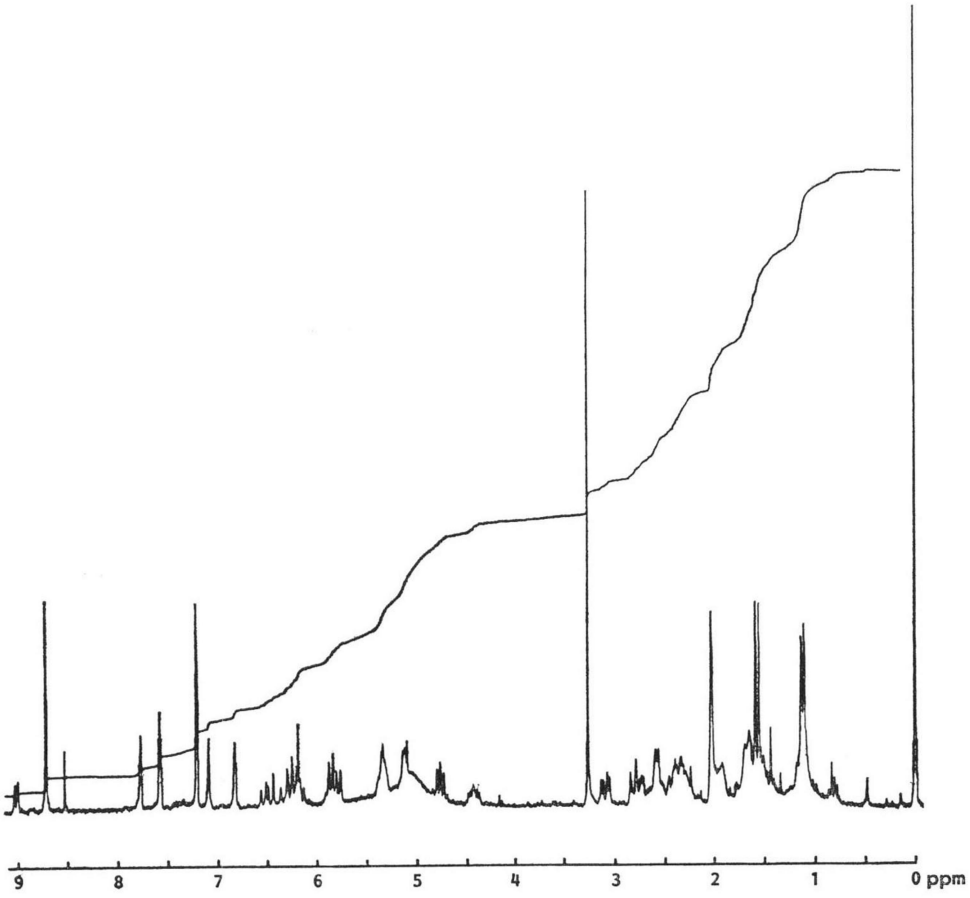
The molecular formula of trienomycin A was elucidated by high resolution mass spectrometry; observed: *m/z* 622.3607; calcd for $C_{36}H_{50}N_2O_7$: 622.3615. The ^1H NMR spectrum of trienomycin A is shown in Fig. 4. These characteristics suggested that this antibiotic is similar to mycotrienins I and II, and ansatrienins A and B* (Table 4).

Fig. 3. IR spectrum of trienomycin A (KBr).



* ZEECK and his associates reported the structures of ansatrienins A and B which have the same structures as mycotrienins I and II, respectively, except for the stereochemistry of alanine[7,9].

Fig. 4. ^1H NMR spectrum of trienomycin A (200 MHz, in pyridine-d_5, TMS).



Biological Properties of Trienomycin A

Antimicrobial activity of trienomycin A was determined by the paper disc method. The antibiotic at a concentration of 1,000 μg/ml produced an inhibitory zone (15 mm in diameter) against *Piricularia oryzae* but was inactive against Gram-positive and Gram-negative bacteria, fungi and yeasts (Table 5). The cytocidal activity of trienomycin A was investigated using two strains of human tumor cells *in vitro*. When the cells were exposed to the antibiotic for 3 days, the growths of HeLa S_3 and PLC hepatoma cells were inhibited at concentrations of 0.1 and 0.01 μg/ml, respectively (Table 6). In the colony formation assay, the IC_{50} value of trienomycin A on HeLa S_3 cells was about 0.01 μg/ml (Fig. 5).

Discussion

A new antibiotic, trienomycin A, was isolated from the culture filtrate *Streptomyces* sp. No. 83-16. The physiological morphological and culture properties of *Streptomyces* sp. No. 83-16 are compared with those of *S. rishiriensis* T-23[6], which produces mycotrienins, and it was found that no similarities were found between these two strains except the carbon utilization pattern.

The physico-chemical characteristics of trienomycin A suggest that this antibiotic belongs to the group of ansamycin antibiotics with a triene moiety in the molecule. Several ansamycin antibiotics such as naphthomycin ($C_{40}H_{46}ClNO_9$)[11], actamycin ($C_{39}H_{45}NO_{10}$)[12], mycotrienins I ($C_{36}H_{48}N_2O_8$) and

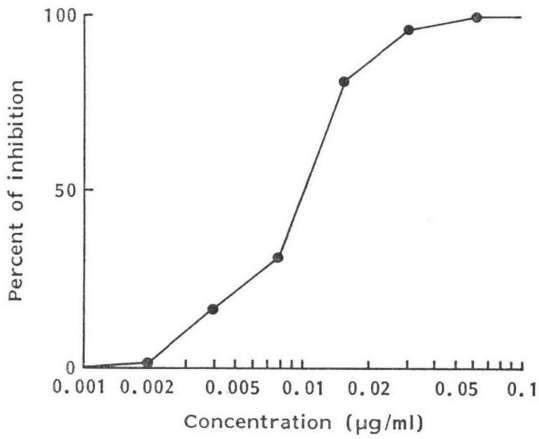
Table 5. Antimicrobial spectrum of trienomycin A.

Organism	Inhibitory zone (mm) at 1 mg/ml
Bacillus subtilis PCI 219	—
B. cereus IFO 3001	—
Micrococcus luteus ATCC 9341	—
Staphylococcus aureus FDA 209P	—
Salmonella typhimurium KB 20	—
Shigella flexneri E 20	—
S. sonnei E 33	—
Escherichia coli NIHJ	—
Klebsiella pneumoniae PCI 602	—
Enterobacter aerogenes IAM 1183	—
Proteus vulgaris IFO 3167	—
Candida albicans KF 1	—
Saccharomyces sake KF 26	—
Schizosaccharomyces pombe IAM 4863	—
Rhizopus javanicus IAM 6241	—
Aspergillus niger ATCC 6275	—
Alternaria kikuchiana KF 185	—
Mucor racemosus IFO 5403	—
Piricularia oryzae KF 180	15 (29)

—: No inhibition.

Number in parenthesis indicates an incomplete inhibitory zone.

Fig. 5. Effect of trienomycin A on colony formation of HeLa S_3 cells.



II ($C_{36}H_{50}N_2O_8$)[5~8], and ansatrienins A ($C_{36}H_{48}N_2O_8$), B ($C_{36}H_{50}N_2O_8$), A_2 ($C_{34}H_{46}N_2O_8$) and A_3 ($C_{34}H_{46}N_2O_8$)[9,10] are known to possess a triene moiety in their structures. Among them, mycotrienins I and II especially are similar to trienomycin A. However, trienomycin A differs from these antibiotics in physico-chemical properties as shown in Table 4.

The structural elucidation and other biological properties including antitumor effects and acute toxicity of trienomycin A will be reported elsewhere.

Table 6. Cytocidal activities of trienomycin A vs. HeLa S_3 and PLC hepatoma cells *in vitro*.

HeLa S_3		PLC	
Concentration (μg/ml)	Inhibition (%)	Concentration (μg/ml)	Inhibition (%)
6.4	92.5	4.0	96.9
1.6	87.6	1.0	94.0
0.4	82.2	0.25	87.9
0.1	69.2	0.063	75.5
0.025	27.2	0.016	59.7
0.006	−2.1	0.004	30.0
Control	0	Control	0

See text for experimental details.

Acknowledgments

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