STUDIES ON TOMAYMYCIN, A NEW ANTIBIOTIC. I ISOLATION AND PROPERTIES OF TOMAYMYCIN

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Tomaymycin, a new antiphage antibiotic is produced by cultivation of *Streptomyces achromogenes* var. *tomaymyceticus* in a lactose-bouillon medium enriched with yeast extract and phosphate salts. The active substance can be extracted from the culture filtrate by carbon adsorption and further purified by silica column chromatography. Tomaymycin is obtained in crystalline form as a molecular complex with methanol. The complex occurs as colorless platelets, m.p. 145~146°C, $[\alpha]_D^{20}+423°$ (*c* 0.5, pyridine), λ_{max} 224, 237, 260, 320 mμ (in methanol). The molecular formula is $C_{16}H_{20}O_4N_2$. The antiphage activity is strong against *Escherichia coli* T_1, T_3 phages and *Bacillus subtilis* M-2, SP-10 phages. Tomaymycin has also antimicrobial activity against Gram-positive bacteria. The LD_{50} (i.p.) of tomaymycin for mice is estimated at 3.0 mg/kg.

A new antibiotic substance with an antiphage activity was obtained from the culture filtrate of *Streptomyces* sp. No. A-2127, an organism isolated from a soil sample collected at Musashikoganei-city, Japan. This substance possesses strong inhibitory activity against various phages including T-series *Escherichia coli* phages, λ phage and *Bacillus subtilis* phage SP-10.

Although a number of antiphage substances derived from *Streptomyces* species have been described[1~9], the physicochemical and biological properties of this substance indicate it to be a new antibiotic. Consequently we named it tomaymycin.

Strain, A-2127 closely resembles *Streptomyces achromogenes* based on its cultural characteristics.

This paper deals with the characteristics of the producing strain, the fermentation process, the isolation procedure and the properties of tomaymycin.

Materials and Methods

B. subtilis phage SP-10 and *E. coli* phage T 3 were mainly used in the course of screening. Other phages were also used when the antiphage spectrum of the antibiotic was studied.

1. Screening method: A phage plate was prepared as follows: 5 ml of soft agar containing 1×10^8 particles of phages and 5×10^7 host cells were poured on 20 ml of nutrient broth agar in a standard Petri dish. On the plate thus made, paper discs, each soaked in a culture fluid to be tested were placed and the plate was then incubated for 15 hours at

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37°C. When any antiphage substance was present, zone of growth of host bacteria appeared around the paper disc.

2. Assay of the antibiotic against free phage: As tomaymycin was found to inactivate several different phages in free form, a contact method was generally used for assay of the antibiotic. One ml of the standard phage suspension (2×10^5 particles/ml in 0.01 M Tris-HCl buffer, pH 7.2) was added to 1 ml of the sample and allowed to stand for 1 hour at 37°C. Samples were prepared by serial dilution using the 0.01 M Tris-HCl buffer. One tenth ml of the mixture was then assayed for plaque counting. The activity was calculated as the drug concentration (mcg/ml) that inactivated 50 % of phage particles (IAD 50).

3. Antimicrobial activity: The usual dilution method was employed for assaying antibacterial and antifungal activities.

Results

1. Characteristics of the Tomaymycin-producing Strain

Streptomyces A-2127 was isolated from a soil sample collected at Musashikoganei-city, Japan. The cultural characteristics and physiological properties of this strain were observed after 10~14 days incubation at 30°C. The observation are listed in Table 1.

On the basis of these observations, the following characteristics were noticed as distinctive features of this strain.

1) The aerial mycelia were long, usually straight.
2) The color of mature aerial mycelium was gray to dark gray and the vegetative growth were cream or grayish white. No soluble pigment was produced on synthetic agar.
3) Slight chromogenic action was observed on some media.
4) Hydrolytic activity on gelatin, milk or starch was essentially weak.

Streptomyces A-2127 was checked for identity with known species described in BERGEY's Manual[10] and WAKSMAN's text book[11] and other published literatures. *Streptomyces achromogenes* and the other 7 species cited below were found to resemble closely strain A-2127. However they had some different characters described as follows:

Streptomyces achromogenes[12]: On glucose asparagine agar, a slightly brown soluble pigment is sometimes produced. On bouillon agar no soluble pigment is observed. On milk media, coagulation occurs.

Streptomyces alboflavus[13]: On starch media, growth is thin, spreading, and no aerial mycelium is produced. On glucose asparagine media, growth is restricted, much folded, with sulfur yellow surface. Nitrate reduction is positive.

Streptomyces althioticus[14]: On CZAPEK's agar, the color of aerial mycelium turns white to gray. On glucose asparagine agar or gelatin stab, brownish soluble pigment is produced. On milk medium, there is a yellowish orange soluble pigment and peptonization occurs.

Streptomyces antimycoticus[15]: Sporophores with spirals typically in dense clusters. On CZAPEK's agar, aerial mycelium is abundant and light neutral gray. On bouillon agar, aerial mycelium is white.

Streptomyces aureofaciens[16]: Sporophores are flexous, producing open spirals. On CZAPEK's agar, faint brown soluble pigment is produced. On bouillon agar or potato plug, no soluble pigment is observed.

Streptomyces fungicidicus[17]: Aerial mycelium produces numerous spirals. Hydrolysis on starch or gelatin is strong.

Streptomyces griseolus[18]: Sporophores are short. On CZAPEK's agar, aerial mycelium

Table 1. Cultural characteristics of strain A 2127

Medium	Growth	Aerial mycelium	Soluble pigment	Remarks
CZAPEK's agar	White-pale yellowish, colony-like growth	Thin, white, powdery	None	
Starch ammonium agar	Pale grayish	Dark gray, powdery	None	Diastatic action ; weak
Glucose asparagine agar	White~light ivory, colony-like growth	None or thin, white powdery	None	
Ca-malate agar	Cream color	White to dark gray, powdery	None	Solubilized calcium malate
Tyrosin agar	Colorless or light brownish thin growth	None	None	Tyrosinase ; negative
Bouillon agar	Creamy, colony-like growth	None	Slightly brownish	
BENNETT's agar	Light creamy, colony-like growth	None	None	
BENNETT's agar (37°C)	Brownish, colony-like growth	Dark grayish powdery	Brown	
Glucose bouillon	Creamy, colony-like growth	None	Light brown	
Glucose CZAPEK's solution	Colorless, little colony-like growth in liquid medium	Thin, white, powdery	None	Nitrite formation ; negative, occasionally positive
Milk	Creamy, surface ring growth	None	Faint grayish brown	Slightly peptonized Coagulation ; negative
Gelatin stab (15~20 days)	Creamy growth	None	None	Liquefaction weak
Potato plug	Grayish cream wrinkled growth	Thin, white, powdery	Dark brown	
Cellulose	No growth			
Morphology	Aerial hyphae	Thick, long and straight		
Carbon utilization	Readily utilized	glucose, xylose, mannose, fructose, mannit		
	Moderately utilized	arabinose, rhamnose, sucrose, lactose, trehalose, raffinose, inosit		
	Not utilized	salicin		

is gray, later becoming pallid neutral gray with a yellowish tone. Faint brown soluble pigment is produced on either CZAPEK's agar or calcium malate agar. On bouillon agar, aerial mycelium of white with a grayish tinge is produced. Milk peptonization and gelatin liquefaction are positive. Optimal temperature for growth is 25°C.

Streptomyces lipmanii[13]: On starch agar, no aerial mycelium is observed and hydrolytic action is positive. The color of growth on calcium malate agar is dark brown. Coagulation of milk, proteolytic action on milk and gelatin are rather strong. Soluble pigment on potato plug is purplish. Optimal temperature is 25°C.

In view of the comparison with the above species, *Streptomyces* A-2127 appears to be most closely related to *Streptomyces achromogenes*. However strain A-2127 produces a new antibiotic tomaymycin. Thus *Streptomyces* A-2127 is considered to be a variant of *S. achromogenes* and is designated as *Streptomyces achromogenes* var. *tomaymyceticus*.

2. Fermentation Process

The most suitable medium for production of the antibiotic contains lactose 3 %, Polypeptone 1 %, meat extract 1 %, yeast extract 1 %, NaCl 0.25 %, potassium phosphate

monobasic 1.5 % and sodium phosphate dibasic 0.4 % (initial pH 6.2). This medium was used for all stages of growth both seed and main cultures. After 72 hours of incubation at 30°C on a reciprocating shaker, precultures in flasks were used to a seed fermentor (inoculum size 5 %). After 48 hours of cultivation at 30°C, the seed tank broth was transferred to the main fermentor (inoculum size 10 %). Cultivation in main tank was continued at 30°C for 48 hours with adequate aeration and agitation. Throughout the culture, the pH of cultural broth was maintained between 6.2 and 6.4. The potency of tomaymycin reached a maximum at 48 hours after inoculation. The control of pH with phosphate buffer appears to be indispensable to this fermentation.

3. Isolation Procedure

The culture filtrate was added with activated carbon (0.3 %), and the mixture stirred for about 30 minutes. The active substance was completely adsorbed on the carbon and gradually eluted with the following solvent system: methanol-pyridine-ammonium water-water (86 : 3 : 1 : 10).

The oily matter obtained by concentrating the eluate from the carbon was washed with petroleum ether and *n*-hexane. The residues were dissolved in water and this aqueous solution was extracted with chloroform after acidification to pH 3.0 with hydrochloric acid. The extract was concentrated and chromatographed on a column of silicic acid that was developed with ethyl-acetate. Active fractions were collected and concentrated to dryness *in vacuo*. Residual yellowish powder was dissolved in a small amount of methanol. Crude crystals of tomaymycin were obtained by keeping the solution in a refrigerator for 2 or 3 days. Crystallized tomaymycin was dissolved in a large amount of chloroform and refluxed for one hour. After concentration *in vacuo* *n*-hexane was added thereto whereby des-methanol tomaymycin was precipitated. The precipitate was collected by filtration and subjected again to the above treatment. The finally obtained material was washed with ether at a cold place to give des-methanol tomaymycin as a powder.

4. Physical and Chemical Properties of Tomaymycin

Des-methanol tomaymycin and its derivatives are very labile substances that are difficult to characterize. In contrast, tomaymycin, the molecular complex of the antibiotic and methanol, possesses comparatively high stability. It was obtained as colorless platelets that melted at 145~146°C. It was optically active, $[\alpha]_D^{20}+423$ (*c* 0.5, pyridine). The results of the elementary analysis were as follows:

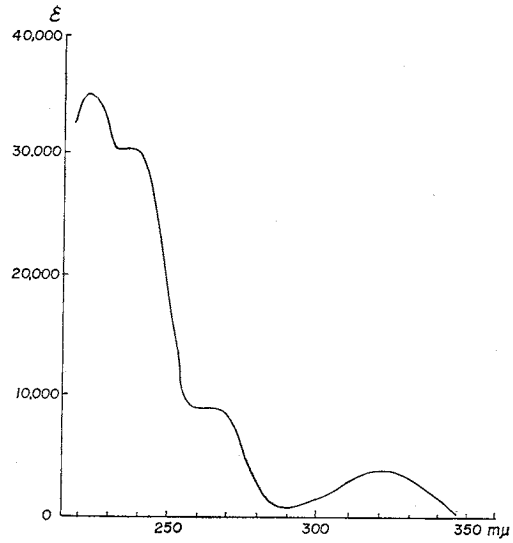
Anal. Found: C 62.95, H 6.66, O 21.25, N 9.05.
Calcd. for $C_{16}H_{20}O_4N_2$: C 63.16, H 6.58, O 21.05, N 9.21.

In the mass spectrum of tomaymycin, the molecular ion peak (M, *m/e* 304) was not recognized. The fragment peak (M-32, *m/e* 272) at the high mass end was due to the loss of methanol from tomaymycin.

As shown in Fig. 1, the ultraviolet absorption spectrum of tomaymycin exhibits maxima in methanol at 224 mμ (ε 36,000), 237 mμ (ε 30,000), 260 mμ (ε 9,000) and 320 mμ (ε 3,600). The infrared spectra of tomaymycin and des-methanol tomaymycin are shown in Fig. 2-a and 2-b.

Tomaymycin gradually changed to dark brown on exposure to sun light and lost its antiphage activity. It also lost its activity under strongly acidic conditions but was stable at pH 4~9 when heating at 70°C for 1 hour. Tomaymycin is soluble in various organic solvents such as methanol, butanol, acetone, ethyl acetate, chloroform, pyridine and alkaline water. It is slightly soluble in ether and benzene and almost insoluble in petroleum ether, *n*-hexane and acidic water. It seemed to be a weak acidic substance.

Fig. 1. UV spectrum of tomaymycin (methanol).



Tomaymycin gave positive reaction to FEHLING, TOLLENS, ferric chloride tests, but negative reaction to PAULI, EHRLICH, SAKAGUCHI and anthrone tests.

5. Biological Properties

The antibiotic spectrum was obtained by dilution methods using glucose bouillon

Fig. 2-a. IR spectrum of tomaymycin (Nujol).

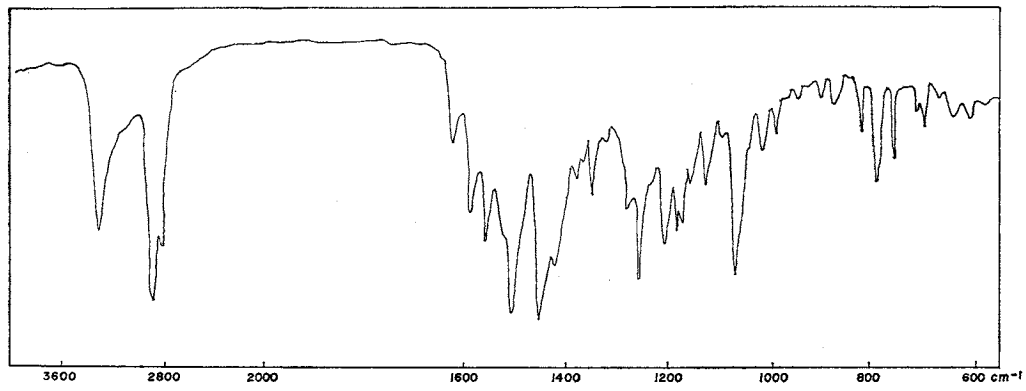


Fig. 2-b. IR spectrum of des-methanol tomaymycin (Nujol).

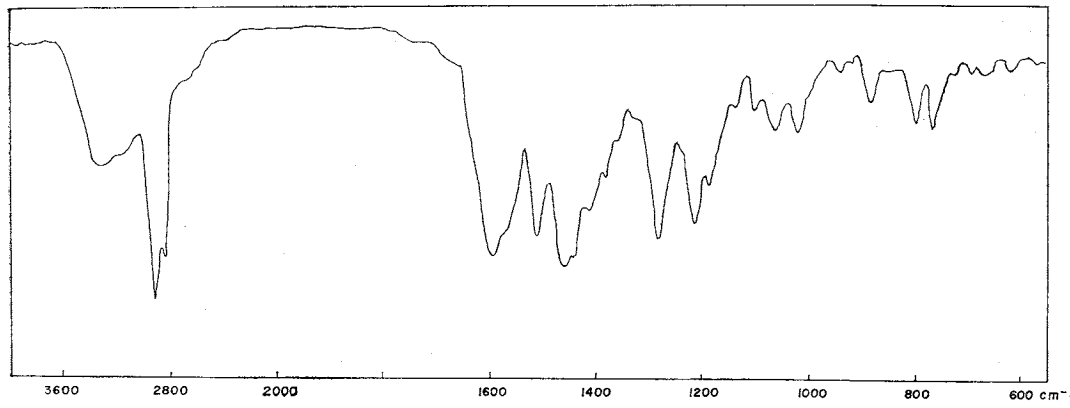


Table 2. Antimicrobial spectrum of tomaymycin

Test organisms	Minimum inhibitory concentration (mcg/ml)
Staphylococcus aureus FDA 209 P	6.2
Bacillus subtilis ATCC-6633	12.5
Corynebacterium xerosis	25.0
Sarcina lutea	25.0
Escherichia coli	100
Pseudomonas aeruginosa	100
Proteus vulgaris	100
Aspergillus niger	50
Penicillium chrysogenum Q-176	25
Saccharomyces cerevisiae	50
Torula utilis	50
Candida albicans	50

Table 3. Antiphage spectrum of tomaymycin

Test phage	IAD_{50} (mcg/ml)
Escherichia coli T_1	0.1
T_2	3.2
T_3	0.1
T_4	3.2
λ	1.0
Bacillus subtilis M-2	0.2
SP-10	0.2
Lactobacillus acidophilus J_1	>100
Pseudomonas aeruginosa P_1	>100

Table 4. Comparison of tomaymycin, anthramycin and dextrochrysin

	Tomaymycin	Anthramycin methyl ether (monohydrate)	Dextrochrysin
Melting point	145~146°C (dp)	above 120°C (dp)	250~255°C (dp)
Molecular formula	$C_{16}H_{20}N_2O_4$	$C_{17}H_{19}N_3O_4 \cdot H_2O$	
Elemental analysis	C 62.95 H 6.66 N 9.05 O 21.25	C 58.99 H 6.27 N 12.10 O 22.96	C 61.47 H 6.82 N 10.25 O 21.46
UV spectrum	λ_{max}^{MeOH} (ε) : 224 (36,000) 237s (30,000) 260s (9,000) 320 (3,600)	$\lambda_{max}^{CH_3CN}$ (ε) : 231 (22,100) 334 (36,800) 360s (22,500)	λ_{max}^{MeOH} ($E_{cm}^{1\%}$) : 240s (410) 335 (850)
IR spectrum	ν_{max}^{nujol} (cm^{-1}) 3,340 1,640 1,600 1,570 1,510	ν_{max}^{KBr} (cm^{-1}) 3,400 3,340 3,220 1,665 1,615 1,600 1,590 1,560 1,520	ν_{max}^{nujol} (cm^{-1}) 3,370 3,330 1,650 1,600 1,560 1,520
Rf {Silica gel / EtOAc - MeOH 4 : 1}	0.62	0.50	0.46

media for bacteria and SABOURAUD media for fungi and yeasts. Minimal concentrations at which complete inhibition were observed against a series of organisms are shown in Table 2. Tomaymycin was primarily active against Gram-positive bacteria and weakly active against yeasts and fungi.

The antiphage activity is shown in Table 3. It was found to be very active against some bacteriophages by the contact method previously described. T1 and T3 phages were most sensitive to this antibiotic. In contrast, *Lactobacillus acidophilus* phage and *Pseudomonas aeruginosa* phage were not inactivated even at an antibiotic concentration of 100 mcg/ml.

When tomaymycin was suspended with 5% carboxymethyl cellulose and injected intraperitoneally to mice, the LD_{50} was calculated at 3 mg/kg.

Discussion

The antibiotic was obtained as crystals from methanol solution and as a pale yellowish powder from a non-alcoholic solvent. The infrared data (Fig. 2-a and 2-b) indicated that the crystalline product and the powder were chemically different, although a comparison showed no significant differences in biological properties. Structural studies established that crystalline tomaymycin differed from des-methanol tomaymycin by one methoxy group. It was also found that tomaymycin is related structurally to anthramycin[19,20], an antibiotic with a pyrrolobenzodiazepine nucleus. However, clear differences were found between physicochemical properties of the two as seen in Table 4.

In addition, dextrochrysin[21], a second antibiotic related to anthramycin was compared with tomaymycin. Once again apparent differences were found between their physicochemical properties as shown in Table 4.

On the basis of their data it is concluded that tomaymycin is a new antibiotic.

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