THIOTETROMYCIN, A NEW ANTIBIOTIC

TAXONOMY, PRODUCTION, ISOLATION, AND PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES

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A new antibiotic, thiotetromycin, has been isolated from the culture filtrate of *Streptomyces* sp. OM-674 by solvent extraction and silica gel chromatography. The molecular formula of the antibiotic has been determined as $C_{13}H_{18}O_2S$ on the basis of elemental analysis and its high resolution mass spectrometry. The antibiotic contains a thiotetronic acid in the molecule and possesses a selective activity against *Bacteroides fragilis*.

In the course of screening for antibacterial antibiotics of actinomycetes origin, we have found that strain OM-674 produces a new antibiotic which has been designated as thiotetromycin. The antibiotic was found to be selectively active against *Bacteroides fragilis*. The UV, IR and NMR spectra suggest that the antibiotic has a thiotetronic acid in its molecule.

The present paper deals with the taxonomy of strain OM-674 and the production, isolation, and biological and physicochemical properties of thiotetromycin.

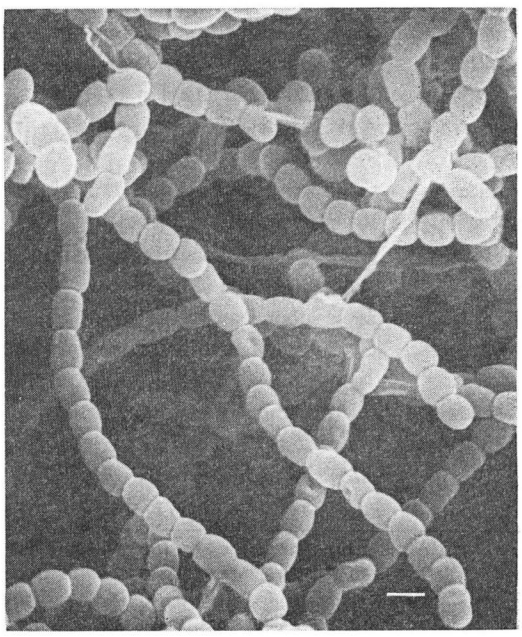
Taxonomy of the Producing Organism

Morphology

The vegetative mycelium grows abundantly on both synthetic and complex agar media, and does not show fragmentation into coccoid and bacillary elements. Though very poor growth of aerial mycelium was observed on glycerol-calcium malate agar, the velvety or powdery aerial mycelium grows moderately or abundantly on other agar media.

The sporophores are of the *Rectus-Flexibilis* type and have more than ten spores per chain (Plate 1). The spores are oval in shape, 0.60×0.88 μm in size, and have a smooth surface (Plate 1). Sclerotic granules, sporangia and flagellated spores were not observed.

Plate 1. Scanning electronmicrograph of aerial mycelia of *Streptomyces* sp. OM-674 on inorganic salts - starch agar. Bar represents 1 μm.



Chemical Compositions

The chemical analyses of sugars in whole cells and diaminopimelic acid (DAP) in cell wall were carried out by the methods of LECHEVALIER

& LECHEVALIER[1]. Strain OM-674 showed the presence of LL-DAP in the cell wall and no characteristic sugar pattern.

Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for two weeks. Color names and hue numbers indicated are those of Color Harmony Manual (4th edition) published by Container Cooperation of America. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% carbon source each. The cultural and physiological characteristics, and the utilization of carbon sources of strain OM-674 are shown in Tables 1, 2 and 3, respectively.

Strain OM-674 exhibits the following properties. Sporophore, *Rectus-Flexibilis*; spore, oval and smooth surface; color of vegetative mycelium, olive or dull gold; color of aerial mycelium, light olive-gray or light brownish gray; melanoid pigment, none; soluble pigment, yellow tint; DAP in cell wall, LL-type.

Table 1. Cultural characteristics of strain OM-674.

Yeast extract - malt extract agar*	G : good, mustard (2 le)		SP : poor, yellow maple (3 le)
	R : dull gold (2 ng)	Tyrosine agar*	G : good, mustard (2 le)
	AM: abundant, velvety, pearl (2 ba)		R : dull gold (2 ng)
	SP : none		AM: abundant, velvety, white (a) and sand (3 cb)
Oatmeal agar*	G : good, mustard (2 le)		SP : none
	R : olive (1½ pi)	Sucrose - nitrate agar**	G : thin, ivory (2 db)
	AM: moderate, velvety, putty (1½ ec)		R : bamboo (2 gc)
	SP : poor, dusty yellow (1½ gc)		AM: moderate, powdery, sand (2 ec)
Inorganic salts - starch agar*	G : good, light mustard tan (2 ie)		SP : poor
	R : light olive (1½ ie)	Glucose - nitrate agar**	G : good, bamboo (2 gc)
	AM: abundant, velvety, parchment (1½ db)		R : mustard (2 le)
	SP : none		AM: moderate, velvety, white (a) and parchment (1½ db)
Glycerol - asparagine agar*	G : good, bamboo (2 gc)		SP : poor
	R : dull gold (2 ng)	Glycerol - calcium malate agar**	G : poor, pastel yellow (1½ hb)
	AM: moderate, velvety, putty (1½ ec)		R : dusty yellow (1½ gc)
	SP : none		AM: poor, powdery, cream (1½ ca)
Glucose - asparagine agar	G : moderate, antique gold (1½ pe)		SP : none
	R : antique gold (1½ ne)	Glucose - peptone agar**	G : good, dull gold (2 ng)
	AM: moderate, velvety, white (a) and pearl (2 ba)		R : dull gold (2 ng)
	SP : poor, bamboo (2 fb)		AM: abundant, velvety, (24½ cb)
Peptone - yeast extract - iron agar*	G : good, penetrant, light ivory (2 ca) and coral rose (6½ ic)		SP : dusty yellow (1½ gc)
	R : bamboo (2 gc), center; tile red (5 ne)	Nutrient agar**	G : moderate, bamboo (2 gc)
	AM: moderate, velvety, white (a) and dawn pink (7 dc)		R : dusty yellow (1½ gc)
			AM: moderate, velvety, sand (2 ec)
			SP : none

* Medium recommended by International Streptomyces Project.
** Medium recommended by S. A. WAKSMAN.
Abbreviation: G, growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of strain OM-674.

Melanin formation	−*
Tyrosinase reaction	−
H_2S production	±
Nitrate reduction	−
Liquefaction of gelatin	+(21°C)
Peptonization of milk	+(37°C)
Coagulation of milk	−(37°C)
Cellulolytic activity	−
Hydrolysis of starch	−
Temperature range for growth	15~40°C

* +, active; ±, weakly active; −, not active.

Table 3. Utilization of carbon sources by strain OM-674.

Carbon source	Utilization*
D-Glucose	+
D-Fructose	±
L-Rhamnose	−
D-Mannitol	+
L-Arabinose	+
i-Inositol	−
Raffinose	±
D-Xylose	+
Sucrose	+
Cellulose	−

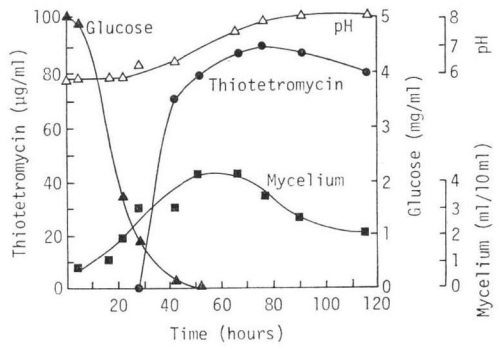
* +, utilized; ±, weakly utilized; −, not utilized.

Based on the taxonomic properties described above, strain OM-674 is considered to belong to the genus *Streptomyces* and to be a strain of the green or gray series of the PRIDHAM and TRESNER grouping[4]. Strain OM-674 has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. OM-674 and the accession No. FERM P-6510.

Production and Isolation

The stock culture of strain OM-674 was inoculated into 100 ml of a seed medium consisting of 1% glucose, 1% starch, 0.5% yeast extract, 0.5% peptone and 0.4% $CaCO_3$ in a 500-ml Sakaguchi flask and incubated at 27°C for 48 hours. One hundred milliliters of the seed culture was transferred to 15 liters of a production medium in a 30-liter jar fermentor and the fermentation carried out at 27°C with aeration of 7 liters/minute and agitation of 300 rpm. The composition of the production medium was 0.5% glucose, 1.0% corn steep liquor, 1.0% oatmeal, 1.0% Pharmamedia, 0.5% K_2HPO_4, 0.5% $MgSO_4 \cdot 7H_2O$ and 1 ml/liter trace salts solution (1 g/liter each; $FeSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$ and $CoCl_2 \cdot 6H_2O$). Fig. 1 shows a typical time course of thiotetromycin production. The antibiotic production started at 30~40 hours after inoculation, then gradually increased reaching a maximum (90 μg/ml) at 75 hours.

Fig. 1. Time course of thiotetromycin production by *Streptomyces* sp. OM-674 in a 30-liter jar fermentor.



A 75-hour culture (15 liters) was clarified by centrifugation to obtain about 12 liters of supernatant fluid. After the supernatant fluid was adjusted to pH 4.5 with 6 N HCl, the antibiotic was extracted twice with 6 liters of ethyl acetate. The solvent layer was concentrated *in vacuo* to dryness yielding 4 g of a brown paste. The paste was dissolved in 10 ml of benzene and then chromatographed on silica gel (Merck, Kieselgel 60, 120 g) eluting with a mixed solvent of benzene and acetone (20: 1, v/v). The active fractions were combined and concentrated *in vacuo* to give a pale yellowish powder. The powder was crystallized from *n*-hexane and acetone to give 180 mg of colorless needles.

The antibiotic activity was assayed by paper disc method against *Bacteroides fragilis* ATCC 23745

on agar plates. The antibiotic was also detected by thin-layer chromatography on silica gel (Merck, GF_{254}), developing with a mixed solvent of benzene and acetone (7: 3, v/v): its Rf value was 0.48.

Physicochemical Properties

Thiotetromycin is an acidic compound: mp 92°C, $[\alpha]_D^{25}$ +124° (*c* 1.0, methanol). Elemental analysis gave the following values: C 65.30, H 7.57, N 0, S 13.21%. The calculated values for $C_{13}H_{18}O_2S$ are C 65.51, H 7.61, S 13.45%. The molecular formula determined from the analysis is supported by the following molecular ions of the free acid and its monoacetate in the high resolution mass spectrometry: free acid (found, 238.102; calcd. for $C_{13}H_{18}O_2S$, 238.102) and the monoacetate (found, 280.111; calcd. for $C_{15}H_{20}O_3S$, 280.113). The UV spectrum in ethanol (Fig. 2) showed maxima at 238 nm (ε 30,100) and 300 nm (ε 4,700). The IR and ^1H NMR spectra of thiotetromycin are shown in Figs. 3 and 4, respectively.

Fig. 2. UV spectrum of thiotetromycin (EtOH).

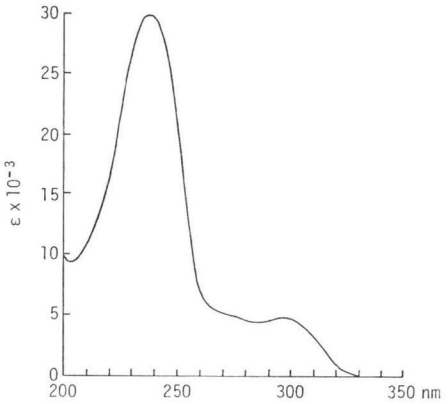
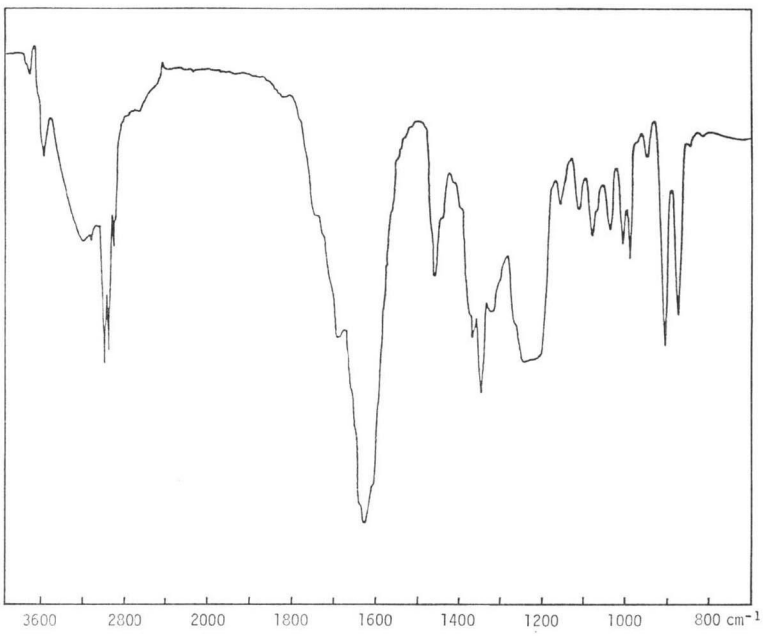


Fig. 3. IR spectrum of thiotetromycin ($CHCl_3$).



Biological Properties

Antimicrobial activities were assayed by conventional agar dilution method using heart infusion agar for aerobic bacteria and GAM (Nissui Seiyaku Co. Ltd.) agar for anaerobic bacteria. Thiotetromycin exhibits a selective activity against *Bacteroides fragilis* (Table 4). Intraperitoneal injection to mice at 400 mg/kg exhibited no overt toxicity.

Fig. 4. ^1H NMR spectrum of thiotetromycin (90 MHz, $CDCl_3$).

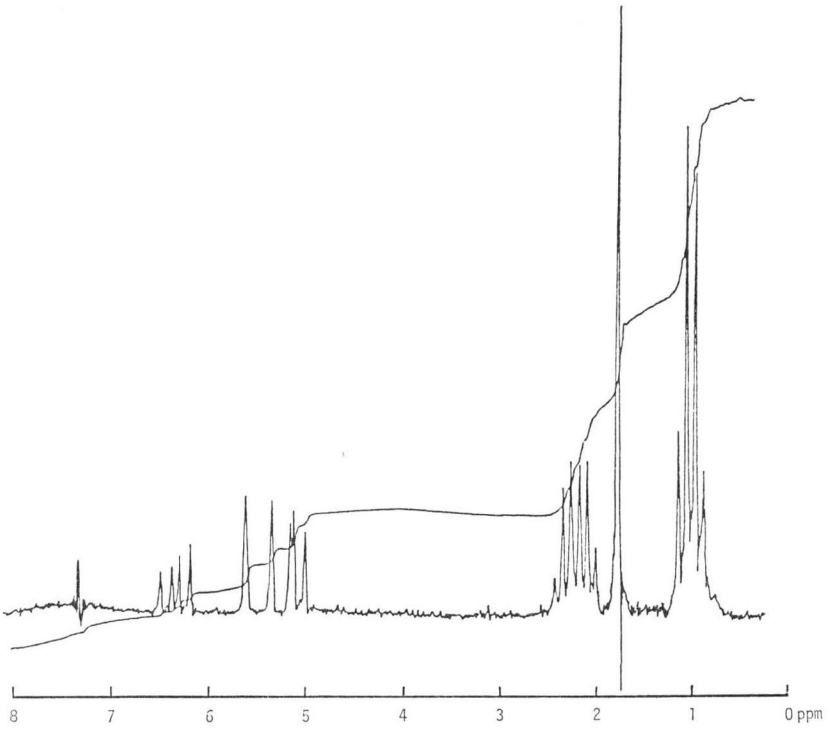


Table 4. Antimicrobial spectrum of thiotetromycin.

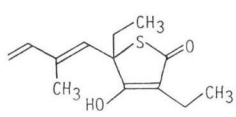
Test organism	MIC (μg/ml)
Staphylococcus aureus ATCC 65389	100
Bacillus subtilis PCI 219	>100
Mycobacterium smegmatis ATCC 607	>100
Escherichia coli NIHJ JC-2	>100
Pseudomonas aeruginosa IFO 3080	>100
Salmonella pullorum	100
Salmonella typhimurium 1	>100
Clostridium perfringens ATCC 13124	>100
Eubacterium lentum ATCC 2559	>100
Eubacterium limosum ATCC 8486	>100
Bifidobacterium bifidum ATCC 11146	>100
Peptococcus prevotii ATCC 9321	>100
Peptococcus variabilis ATCC 14955	>100
Lactobacillus acidophilus IFO 3205	>100
Bacteroides fragilis ATCC 23745	6.25
Fusobacterium necrophorum NIAH 1	>100
Fusobacterium varium ATCC 8501	>100
Veillonella alcalescens subsp. *alcalescens* ATCC 17745	100

Discussion

From the physicochemical properties described above, thiotetromycin was found to resemble thiolactomycin[5,6] which is the first antibiotic containing a thiotetronic acid, a five-membered thiolactone. However, because the molecular formula of the antibiotic ($C_{13}H_{18}O_2S$) is differentiated from that of thiolactomycin ($C_{11}H_{14}O_2S$), it can be reasonable to conclude that thiotetromycin is new. It is of interest that thiolactomycin is an antibiotic of *Nocardia* origin[5], while thiotetromycin is produced by a strain of genus *Streptomyces*.

The antibiotic is selectively active against *Bacteroides fragilis* (Table 4). In the course of screening program using *B. fragilis* as a test organism, TSUKIURA *et al.*[7,8] found new antibiotics,

Fig. 5. Structure of thiotetromycin.



Bu-2313 A and B, which are tetramic acid-containing antibiotics structurally related to streptolydigin[9] and tirandamycin[10]. As we have also observed that tetramic acid-group antibiotics, such as streptolydigins and tirandamycins, possess selectively inhibitory activity against *B. fragilis*[11], the compounds containing tetramic acid, thiotetronic acid or a related moiety may be considered to exhibit selective activity against *B. fragilis*.

Fig. 5 shows the structure of thiotetromycin. A paper describing structure elucidation is now in preparation.

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