THIOLACTOMYCIN, A NEW ANTIBIOTIC

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL PROPERTIES

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A strain of actinomycetes, isolated from a soil sample, has produced a novel antibiotic ($C_{11}H_{14}O_2S$) containing a unique thiolactone moiety in its molecule. On the basis of taxonomic studies the producing organism was identified as belonging to the genus *Nocardia*. The antibiotic, named thiolactomycin, exhibits a broad antibacterial spectrum and particularly potent activity against *Salmonella*, *Serratia* and *Bacteroides*. Furthermore, the acute toxicity is weak in experimental animals. These results indicate that thiolactomycin is distinct from other known antibiotics and represents a new type of antibiotic.

In our screening for new antibiotics, we isolated from a soil sample collected in Sayama City, Saitama Prefecture, Japan, a strain of *Nocardia* designated strain No. 2–200, which was found to produce a new antibiotic, thiolactomycin*. The antibiotic was detected in the fermentation broth by the use of the β-lactam antibiotic-sensitive mutant of *Pseudomonas aeruginosa* M-57740 (Table 1). This new antibiotic has proved to be of considerable interest with its unique thiolactone moiety and because of its broad antibacterial spectrum and weak toxicity.

This is a report on the taxonomy of the producing organism and the isolation and biological properties of thiolactomycin.

Taxonomy

Strain No. 2–200 isolated from a soil sample collected in Sayama City, Saitama Prefecture, Japan, has been deposited in the American Type Culture Collection, and in the Fermentation Research Institute, Japan, and has been assigned accession numbers ATCC 31319 and FERM-P 4171, respectively.

Taxonomic characterization was carried out according to the methods of the International Streptomyces Project (ISP).[2] Also other media recommended by WAKSMAN[3] were used. The taxonomy keys in BERGEYS's Manual of Deter-

Table 1. Sensitivity of strains *Pseudomonas aeruginosa* J-272 and M-57740 to various kind of antibiotics.

Antibiotics	MIC (μg/ml)	
	Ps. J-272	*Ps.* M-57740
6-Aminopenicillic acid	1600	6.25
Penicillin G	1600	0.78
Carbenicillin	400	0.39
Cephaloridin	1600	1.56
Ampicillin	1600	0.19
Erythromycin	800	100
Kanamycin	800	100
Chloramphenicol	200	12.5
Thiolactomycin	800	1.56

MIC was determined by the serial agar dilution method with heart infusion medium. Inoculation with 10^6 cells/ml.

* Previous name is 2–200.

Table 2. Cultural characteristics of strain No. 2-200 on various media.

Medium	Appearance of growth		Soluble pigment
	Aerial mycelium	Growth	
Yeast malt extract agar (ISP-2)	White	Good, elevated wrinkled Dull yellow orange	None
Oatmeal agar (ISP-3)	White	Moderate, flat Pale yellow orange	None
Inorganic salts starch agar (ISP-4)	White	Moderate Pale yellowish brown	None
Glycerol asparagine agar (ISP-5)	White, powdery	Poor Dull reddish orange	None
Peptone yeast extract iron agar (ISP-6)	No aerial mycelium	Moderate, elevated wrinkled Pale yellowish brown	None
Tyrosine agar (ISP-7)	White, powdery	Good Pale yellowish brown	None
Glucose asparagine agar	White	Moderate, raised wrinkled Pale yellowish brown	None
Sucrose nitrate agar	White, powdery	Good Dull yellow orange	None
Nutrient agar	Poor, white	Moderate, wrinkled Colorless	None
Glucose peptone gelatin		Wrinkled Colorless	None
Skim milk		Poor	None

Fig. 1. Scanning electron micrograph of *Nocardia* sp. No. 2-200.

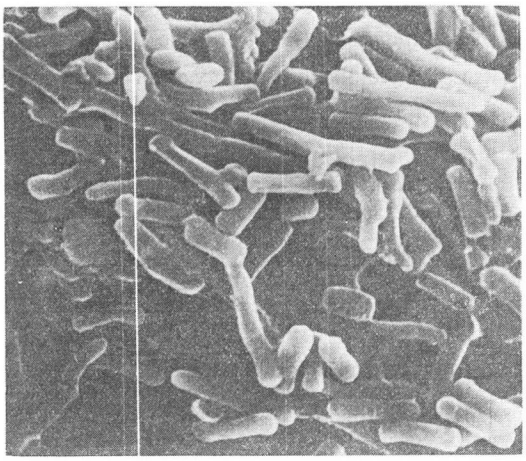


Table 3. Utilization of various carbon compounds by strain No. 2-200.

Carbon compound	Growth
D-Glucose	+
L-Arabinose	±
Sucrose	+
D-Xylose	−
Inositol	−
D-Mannitol	−
D-Fructose	−
Rhamnose	−
Raffinose	−

Symbols: +: positive, ±: doubtful, −: no utilization.

minative Bacteriology (8th *Ed.*) were used for comparison with recognized genera and species of actinomycetes.

The cultural characteristics of strain No. 2-200 shown in Table 2 were observed after 18 days of incubation at 28°C. The substrate mycelia divided into short rods with age; for instance, most of the aerial mycelia incubated on a sucrose-nitrate medium at 28°C for 21 days fragmented into bacillary elements. No sporophores or spore chains like those found in the genus *Streptomyces* were observed. Almost all substrate mycelia were pleomorphic and fragmented into rods. Scanning electron microscopy (Hitachi SEM-700) clearly depicts the fragmentation of the vegetative mycelia (Fig. 1).

The physiological characteristics of strain No. 2–200 were determined as follows: Hydrolysis of starch and liquefaction of gelatin were positive, while formation of tyrosinase and H_2S, and coagulation and peptonization of milk were negative. Utilization of carbon sources was examined on ISP medium 9 (Table 3). D-Glucose and sucrose were well utilized for growth. Analysis of whole cell hydrolysates by the methods of BOONE[4] demonstrated the presence of meso-diaminopimelic acid.

These morphological and physiological characteristics indicate the classification of strain No. 2–200 as a member of morphological group III of the genus *Nocardia*.

Screening Method

Actinomycetes strains, isolated from several soil samples, were grown on 8 ml of BENNET's agar slant at 28°C for 2 weeks and the mature slants were extracted with 2 ml of pH 7.0, 0.05 M phosphate buffer at 28°C for 2 hours shaken on a tube shaker. The antibacterial activity of the extracts was examined by the paper disk agar diffusion method. *Pseudomonas aeruginosa* M-57740, which is an ampicillin sensitive mutant of *P. aeruginosa* J-272, was used as the test organism. *Nocardia* sp. No. 2–200 was detected as an active strain against this mutant organism, but not against *P. aeruginosa* J-272 or *Bacillus subtilis* PCI-219 by the agar diffusion method.

Fermentation

Nocardia sp. No. 2–200 grown on mature slant cultures of BENNETT's agar was used to inoculate 500 ml flasks containing 100 ml each of sterile growth medium. The seed flasks were shaken on a rotary shaker for 48~72 hours at 28°C. The seed medium consisted of 30 g glucose, 5 g peptone, 5 g Bouillon, 3 g NaCl, 2 g yeast extracts and 2 g $CaCO_3$ per liter of tap water. A 2% vegetative seed was used to inoculate the fermentation medium which consisted of 40 g glycerol, 30 g corn steep liquor, 4 g $NaNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$ and 2 g $CaCO_3$ per liter of tap water. The medium was adjusted to pH 5.5 with KOH prior to sterilization. A typical fermentation time course is shown in Fig. 2. The amount of the antibiotic produced by the fermentation was measured by high performance liquid chromatography (HPLC) under the following conditions: Hitachi 635, UV wave-length, 238 nm; Flow rate, 1.0 ml/

Fig. 2. Time course of the production of thiolactomycin.

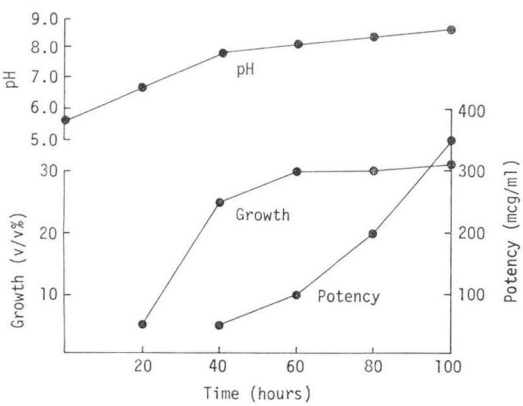
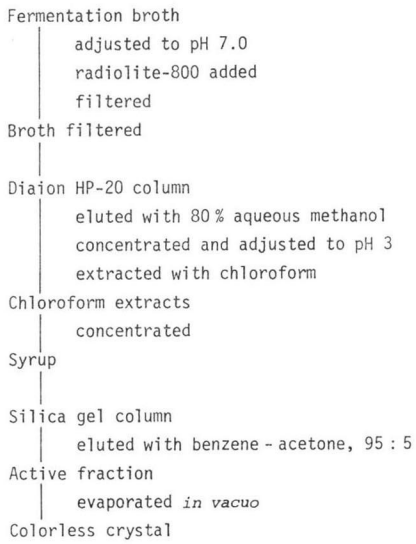


Fig. 3. Isolation procedure for thiolactomycin.



minute; Column size, 4.0×150 mm; Packing, LiChrosorb RP-18 5 μ; Solvent, CH_3CN - H_2O - H_3PO_4 (550: 450: 1); Retention time, 4.5 minutes. Antibiotic activity was first detected in the culture broth at about 24 hours and reached a maximum at about 80 hours. As the strain grew the initial pH 6.0 gradually rose to pH 8.6.

Isolation Procedures

The isolation procedure for thiolactomycin is shown in Fig. 3. Activity against *P. aeruginosa* M-57740 and thin-layer chromatography were used to monitor the isolation of the antibiotic from the culture broth. The culture broth (18 liters) was adjusted to pH 7.0 with dilute H_2SO_4 and filtered with the aid of 10% Radiolite-800. The filtrate (16 liters) was adsorbed on a column of non-ionic porous resin, Diaion HP-20 (1 liter). After washing with 2 liters of 10% aqueous methanol, the column was eluted with 3 liters of 80% aqueous methanol. The eluate was adjusted to pH 3 with dilute HCl and extracted with an equal amount of chloroform. The upper water layer was discarded, and the lower chloroform layer was dried with sodium sulfate and concentrated *in vacuo* to obtain an oily substance containing the antibiotic. The oily substance was applied to a silica gel column, then, after washing with 2 liters of benzene, the active fraction was eluted with a mixture of benzene - acetone (95: 5) and concentrated *in vacuo*. Colorless needles of thiolactomycin (2.4 g) were obtained by crystallization with *n*-hexane - acetone.

Biological Properties

Thiolactomycin exhibited an antibacterial spectrum as shown in Table 4. One loopful of inoculum of approximately 10^8 cells in 1 ml of medium was streaked on agar plates containing two-fold decremental dilutions of the antibiotic. The inoculated plates were incubated at 37°C for 24 hours before reading. Then, the antibacterial activity of thiolactomycin against anaerobic bacteria is compared with that of ceftezole in Table 5. Thiolactomycin had antibacterial activity against a wide range of bacterial species, especially against *Salmonella, Serratia* and *Bacteroides*.

Table 4. Antibacterial activity of thiolactomycin.

Microorganism		MIC (μg/ml)	
		NA	ANTI-3
B. subtilis	PCI-219	50	50
B. cereus	T-1	100	100
M. luteus	B	12.5	12.5
S. epidermidis	T-3	100	100
S. aureus	209-P	50	25
S. aureus	JU-5	400	100
E. coli	NIHJ	200	200
E. coli	No-9	100	100
S. enteritidis	T-1	12.5	6.25
S. paratyphi A	T-1	50	50
K. pneumoniae	3K-25	200	200
K. pneumoniae	15 C	200	100
Sh. flexneri 2b	TO-1	12.5	12.5
Pr. mirabilis	1287	100	100
S. marcescens	FU-111	100	50
Ps. aeruginosa	J-272	800	800
Ps. aeruginosa	GMB-75	6.25	6.25

Plate agar dilution method.
NA: Nutrient agar (Eiken).
ANTI-3: Antibiotic agar No. 3 (Difco).
Inoculum size: 1 loopful of 10^6 cells/ml.

Table 5. Minimum inhibitory concentration (MIC) for anaerobic bacteria.

Microorganism	MIC (μg/ml)	
	Thiolactomycin	Ceftezole
Bacteroides fragilis V-6	3.12	100
Bacteroides fragilis V-7	12.5	25
Bacteroides fragilis V-8	6.25	100
Fusobacterium glutinosum 1006	100	6.25
Fusobacterium necroforum S-45	3.25	—

Incubation at 37°C for 40 hours on Gam agar medium using anaerobic incubator. Inoculation with 10^6 cells/ml.

Thiolactomycin sodium salt was weakly toxic. The median lethal dose (LD_{50}) upon single administration in *dd*Y male mice was 455 mg/kg for intravenous injection, 520 mg/kg for intraperitoneal injection and 1,689 mg/kg for oral administration. During 14 days of observation after intravenous injection, no delayed toxicity was observed.

Discussion

The new antibiotic thiolactomycin was discovered using our screening system which combines the use of a β-lactam antibiotic-sensitive mutant with extraction with neutral phosphate buffer from mature slants of soil microorganisms. In order to detect very small amount of compounds, the screening system with the β-lactam sensitive mutant has been useful and a large number of the novel antibiotics have been detected by the use of these systems.[5,6] On the other hand, antibiotics produced only on an agar plate have been known. Fumaramidomycin, a typical antibiotic, is produced only on an agar plate, not in submerged culture.[7]

On the basis of taxonomic studies the producing microorganism was identified as belonging to the morphological group III of the genus *Nocardia*. This genus has recently been noticed to produce other novel antibiotics.[6,8]

Selective inhibitory effect of thiolactomycin on β-lactam antibiotic-sensitive cells led us to think that it was a β-lactam like antibiotic, however its antibacterial spectrum and chemical properties made us modify that idea. As its structure will be represented in the following paper,[1] thiolactomycin has a 5-membered thiolactone ring, but does not contain nitrogen in the ring like β-lactam antibiotics.

The broad antibacterial spectrum, relatively good therapeutic effect *in vivo* and weak toxicity in experimental animals suggest that thiolactomycin has potential as an effective chemotherapeutic agent.

Descriptions of the antibiotic's structure,[1] and its biological activity *in vitro*[9] and *in vivo*[10] are reported in detail in following papers.

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