ISOLATION OF A NEW POLYETHER ANTIBIOTIC, LONOMYCIN

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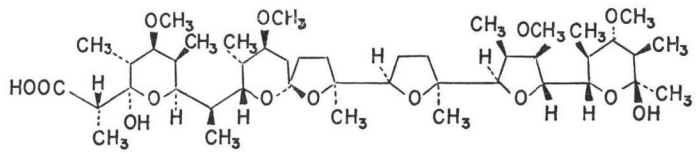
(Received for publication September 29, 1975)

A new polyether antibiotic, lonomycin, was isolated from the culture of *Streptomyces ribosidificus* strain TM-481. The antibiotic obtained as a sodium salt is a colorless prism having a molecular formula of $C_{44}H_{75}O_{14}Na$(M.W. 850), m.p. 188~189°C, and has no absorption maxima in the ultraviolet region. The infrared and NMR spectra of the antibiotic suggest the presence of a carboxyl and four methoxyl groups. Lonomycin shows antimicrobial activity against gram-positive bacteria.

In the course of our search for new antibiotics, the strain TM-481, which was identified as *Streptomyces ribosidificus*, was found to produce a new antibiotic. It was extractable into organic solvents from the filtrate of the cultured broth and showed moderate activity against gram-positive bacteria. The antibiotic and its sodium salt are easily soluble in most of organic solvents but insoluble in water. From its physico-chemical characteristics, the antibiotic, named lonomycin, was found to be a new member of the polyether class of antibiotics. The entire structure of the antibiotic was elucidated by an X-ray analysis as shown in Fig. 1.[1]

Fig. 1. Structure of lonomycin



In this paper, taxonomical studies of the strain TM-481, fermentation, isolation and characterization of the antibiotic are described.

Characteristics of the Strain TM-481

For the study of the growth characteristics, a variety of the standard media were prepared according to GOTTLIEB and SHIRLING,[2] and WAKSMAN.[3]

Morphological Observation

Substrate mycelia develops well and branches on glucose asparagine agar but formation of aerial mycelia is poor. Aerial mycelia on oat meal agar, yeast-malt extract agar and starch agar form abundant spores.

Microscopic examination of the culture grown on yeast-malt extract agar reveals branched aerial mycelia and spiral spore chains. A mature spore-chain generally consists of about 10

spores. An electron micrograph of the spore shows an oval to spherical ($0.7\sim1.0\times1.0\sim1.4\ \mu$) shape with spiny surface.

Cultural Characteristics

The cultural characteristics of the strain TM-481 are listed in Table 1.

Table 1. Cultural characteristics of strain TM-481

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Poor, yellowish cream to yellowish green	Poor, pale yellow	None
Glucose-asparagine agar	Poor, colorless to yellowish cream	Powdery, grayish brown	None, pale yellow after 2 weeks
Glycerol-asparagine agar	Good, yellowish cream	White to light gray or yellowish green	None
Starch agar	Good, yellowish cream to light gray	Abundant, pale yellowish green to dark gray	None
Tyrosine agar	Good, yellowish cream	Abundant, white to light gray or pale yellowish green	None
Nutrient agar	Poor, yellowish cream	None	None
Yeast extract-malt extract agar	Good, grayish yellow to yellowish green	Abundant, pale yellowish green to dark gray	None
Oat meal agar	Good, yellowish cream to light gray	Abundant, yellowish green to dark gray	None

Physiological Properties

Physiological properties of this strain are as follows:
Growth temperature range: 15~45°C on oat meal agar.
Optimum growth temperature: 30~35°C.
Liquefaction of gelatin: slightly positive around the growth of 5~7 days at 20°C.
Hydrolysis of starch: positive.
Coagulation of milk: negative.
Peptonization of milk: negative at 30°C, positive at 37°C.
Melanin production: negative.
Liquefaction of LOEFFLER's coagulated serum: positive.
Reduction of nitrate: positive.

Carbon source utilization test by the method of PRIDHAM and GOTTLIEB shows that this strain is more or less able to utilize D-glucose, L-arabinose, D-fructose, sucrose, inositol, rhamnose, raffinose, and D-mannitol, but not D-xylose at all.

From the above results, the microbiological characteristics of the strain TM-481 may be summarized as follows: Strain TM-481 forms aerial mycelia and spiny-surfaced spores in spiral chains. Growth on synthetic media is poor and the color of vegetative mycelia is yellowish cream to yellowish green. Soluble pigment is not usually observed except for culture on glucose-asparagine agar which produces pale yellow one. Vegetative mycelia on organic media show good growth with yellowish cream to yellowish green color and aerial mycelia show gray to yellowish green color. Spore formation is abundant. Soluble pigment is not produced on organic media.

These characteristics of the strain TM-481 closely relate with those of WAKSMAN's "Flavus-series Streptomyces".[3] Among the known flavus-like species, *Streptomyces ribosidificus* which was reported by SHOMURA *et al.*[4] to produce ribostamycin, an aminoglycoside antibiotic, particularly resembles the strain TM-481 with respect to the form of spore chains having open spirals, to the lack of the productivity of soluble pigment on synthetic or organic agar media, to the ability of growing even at 45°C, a relatively high temperature, as well as to the ability of producing ribostamycin. The slight difference between them is that TM-481 utilizes fructose and liquefies gelatin but *Streptomyces ribosidificus* does not.

From these features it is reasonable to conclude that the strain TM-481 belongs to the species of *Streptomyces ribosidificus*.

To prepare seed, four 500-ml SAKAGUCHI flasks, each containing 100 ml of a liquid medium, were inoculated with a loopful of culture from agar and were incubated with shaking at 30°C for 48 hours. The medium consisted of 1.0% glucose, 2.0% oat meal, 0.3% meat extract, 0.3% sodium chloride and 0.2% calcium carbonate. The seed, 400 ml, was transferred into a 30-liter glass jar containing 20 liters of the same medium and fermentation was carried out at 30°C for 48 hours with aeration (20 liters/minute) and agitation (200 r.p.m.).

A maximum titer corresponding to 170 mcg/ml of lonomycin appeared in the culture fluid after 48 hours of fermentation. Potency was assayed by the paper-disc agar-diffusion method using a ribostamycin-resistant strain, *Staphylococcus epidermidis* TPR-25.

Isolation and Purification

The fermentation broth was centrifuged to remove mycelia and 5 liters of ethyl acetate was added for extraction to 15 liters of the supernatant followed by agitation for 30 minutes. The ethyl acetate extract was separated from the aqueous layer and concentrated *in vacuo* below 50°C giving a brown syrup. The syrup was extracted three times with 15-ml portions of benzene and an insoluble part was removed by filtration. After the collected filtrate was evaporated to dryness, the residue was extracted three times with 50-ml portions of methanol, and then the extracts were collected and concentrated *in vacuo* to give 5 g of a yellow syrup.

Two grams of partially purified antibiotic thus obtained were dissolved in 5 ml of benzene and the solution was charged on a silica gel column packed with *ca.* 100 g of Kiesel gel 60 (Merck Co., Ltd.). Subsequently, the column was washed with benzene and was developed with a mixture of benzene and acetone (4:1). The active fractions were collected and evaporated to dryness. The residue was further purified by gel filtration on Sephadex LH 20 using methanol. Evaporation of the collected active fractions gave 600 mg of a white powder. The powder was recrystallized from *n*-hexane to obtain 480 mg of lonomycin.

The sodium salt of lonomycin was isolated as colorless prisms which melted at 188~189°C. The molecular weight determined by vapor pressure osmometry is 846. Optical rotation of lonomycin sodium salt shows $[\alpha]_D^{25}+47°$ (*c* 1.0, methanol). Elemental analysis gives the following values: C 61.93, H 8.64, O 26.75, Na 2.68 (%). These values imply the calculated formula to be $C_{44}H_{75}O_{14}Na$ (M.W. 850; C 62.10, H 8.88, O 26.32, Na 2.70 (%)). Titration in 66% dimethylformamide indicates that lonomycin is a monocarboxylic acid with pKá of 6.5. It is soluble in alcohols, acetone, ethyl acetate, chloroform, ethyl ether, benzene and *n*-hexane but

insoluble in water. It is hardly extractable to an aqueous layer from an organic solvent layer in the range of pH 3.0 ~ 9.0. Ultraviolet spectrum in methanol shows only end absorption. Infrared absorption spectrum in KBr pellet exhibits characteristic bands at 3400, 3180, 2980, 2940, 2880, 2810, 1590, 1460~1450, 1400, 1385, 1375, 1300, 1240, 1210~1190, 1170, 1140, 1120, 1095, 1075, 1040, 1020, 1000, 985, 965, 940, 915, 890, 870, 865 and 830 cm^{-1} (Fig. 2). Nuclear magnetic resonance at 60 MHz in deuterochloroform gives four singlets at 3.25~3.45 ppm suggesting the presence of four methoxyl groups (Fig. 3). The presence of a carboxyl group is indicated at 11.03 ppm in the nuclear magnetic resonance spectrum of the free acid which was prepared from the sodium salt by treating its benzene solution with diluted hydrochloric acid. Further, the shift of infrared absorption band of the sodium salt (1590 cm^{-1}) to that of the free acid (1740 cm^{-1}) also suggests the presence of a carboxyl group. Lonomycin gives a characteristic red color on thin-layer plates by spraying with 3% vanillin in 1.5% ethanolic sulfuric acid followed by heating at 100°C for 5 minutes.[5] Ninhydrin, MOLISCH and ferric chloride reactions are negative.

The minimum inhibitory concentration (MIC) of lonomycin for a variety of microorganisms is given in Table 2. Determination of the MIC was carried out using the serial agar dilution

Fig. 2. Infrared absorption spectrum of lonomycin sodium salt (KBr)

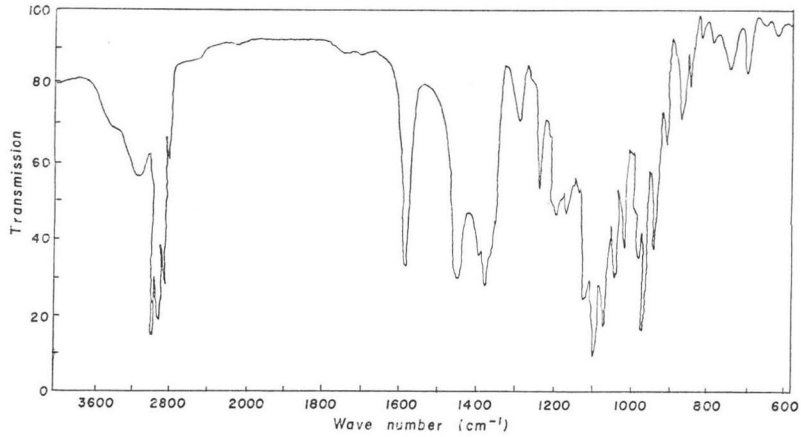


Fig. 3. NMR spectrum of lonomycin sodium salt ($CDCl_3$)

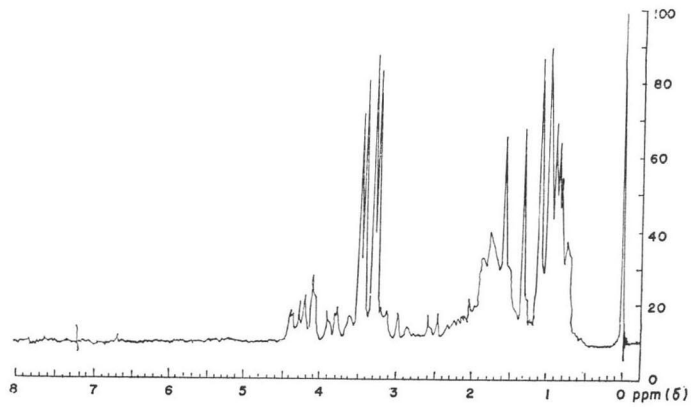


Table 2. Antimicrobial spectra of lonomycin

Microorganisms	MIC(mcg/ml)	Medium
Staphylococcus aureus FDA 209P	3.13	1
Staphylococcus aureus Smith	3.13	1
Staphylococcus aureus TPR-18 (SA-, PC-, TC-, KM-, CP- and Mac-R)	6.25	1
Staphylococcus aureus TPR-23 (SA-, PC-, TC-, SM-, KM-, CP- and Mac-R)	6.25	1
Staphylococcus aureus TPR-26 (SA-, PC-, TC-, SM-, CP- and Mac-R)	6.25	1
Staphylococcus aureus TPR-27 (SA-, PC-, TC-, SM-, KM-, CP- and Mac-R)	6.25	1
Staphylococcus epidermidis TPR-13 (SA-, PC-, CP-, EM- and OM-R)	6.25	1
Staphylococcus epidermidis TPR-14 (PC- and CP-R)	6.25	1
Staphylococcus epidermidis TPR-16 (SA-, PC-, TC- and CP-R)	3.13	1
Staphylococcus epidermidis TPR-25 (SA-, PC-, TC-, SM-, KM-, CP- and Mac-R)	6.25	1
Staphylococcus epidermidis TPR-28 (SA-, PC-, TC-, SM-, KM-, CP- and Mac-R)	3.13	1
Bacillus subtilis PCI 219	3.13	1
Sarcina lutea NIHJ	6.25	1
Escherichia coli B	>50	1
Proteus vulgaris HX 19	>50	1
Aspergillus niger	>50	2
Trichophyton asteroides	>50	2
Candida albicans	>50	2
Saccharomyces cerevisiae	>50	2

Medium 1: heart infusion agar Medium 2: SABOURAUD agar
Abbreviations: Mac: macrolide, R: resistant strain

method. Loopful suspension of the test microorganism (*ca.* 10^8 cells/ml) was inoculated on each of the plates containing the antibiotic at stepwise concentrations and the plates were incubated at 30°C for 24 or 48 hours. The highest concentration of the antibiotic employed in this test was 50 mcg/ml.

The acute toxicity of lonomycin was examined in male *ddY* mice. The LD_{50} doses were 4.86 mg/kg intravenously and 8.28 mg/kg intraperitoneally.

Discussion

HARNED *et al.*[6] and BERGER *et al.*[7] originally reported the isolation of some polyether antibiotics in 1951. Since then a number of novel antibiotics of this group such as nigericin,[6] X-537A,[7] monensin,[5] grisorixin,[8] dianemycin,[9] salinomycin,[10] A-28695A,[11] A-28695B,[11] A-204A,[12] lysocellin,[13] SF-1195,[14] septamycin,[15] and laidlomycin[16] were isolated from the cultures of *Streptomyces* species.

Lonomycin is suggested to have "four" methoxyl groups in the molecule and the number is identical with those of A-28695A and septamycin. These, however, are different each other in physico-chemical and biological properties.

Acknowledgement

The authors are grateful to President SHOJI UEHARA, Executive Director BUN-ICHIRO TERAJIMA and Dr. I. TANAKA, Manager of Research Department, of Taisho Pharmaceutical Co., for their approval to publish this work.

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