# LEPTOMYCINS A AND B, NEW ANTIFUNGAL ANTIBIOTICS

## I. TAXONOMY OF THE PRODUCING STRAIN AND THEIR FERMENTATION, PURIFICATION AND CHARACTERIZATION

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A strain of Streptomyces was found to produce new antifungal antibiotics. The active compounds were purified and separated into two substances named leptomycin A and B by high performance liquid chromatography.

The molecular formulae of leptomycins A and B are $C_{32}H_{46}O_6$ and $C_{33}H_{48}O_6$ respectively, and physicochemical and biological properties of them are very similar to each other.

Leptomycins A and B exhibit strong inhibitory activity against Schizosaccharomyces and Mucor.

In the course of a screening program for substances which cause abnormal morphology on the growth of various fungi, *Streptomyces* sp. ATS1287 was found to produce substances which caused cell elongation of the fission yeast, *Schizosaccharomyces pombe* and hyphal swelling or curling of *Mucor racemosus* and *Mucor rouxianus*[1].

This strain was found to produce two active substances, named leptomycin A and B (formerly called ATS1287 A and B). This paper deals with the taxonomy of the producing strain and the fermentation, purification and characterization of leptomycins A and B.

### Taxonomic Studies on the Leptomycin-producing Strain

The leptomycin-producing strain was isolated from a soil sample collected in Japan.

The method described by Shirling and Gottlieb[2] was employed for taxonomic identification. Morphological observation were made on the cultures grown at 26.5°C for 14 days on sucrose - nitrate agar, glucose - asparagine agar and oatmeal agar. Mature spore chains have 10 or more spores in the form of *Retinaculum-Apertum* or *Spiral*. The spores are 0.4～0.6 by 1.0～2.0 μm in size with smooth surface (Fig. 1). Cultural characteristics are shown in Table 1. Colonies with the appearance of the gray color series and no soluble pigment were produced. Physiological properties of the producing strain are summarized in Table 2. Melanin production, nitrate reduction, starch hydrolysis, gelatin liquefaction and milk coagulation were negative. Milk pe-

Fig. 1. Electron micrograph of leptomycin-producing strain.

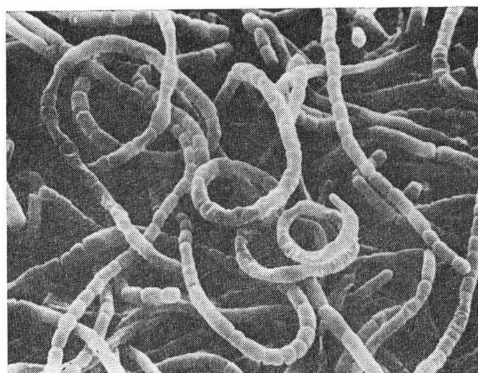


Table 1. Cultural characteristics of the producing strain.

| Medium | Aerial mycelium | Reverse side of colony | Soluble pigment |
|---|---|---|---|
| Sucrose - nitrate agar | Thin, mouse gray | None | None |
| Glucose - asparagine agar | Good, mouse gray | Pale olive buff | None |
| Glycerin - asparagine agar | Good, mouse gray | Pale olive buff | None |
| Nutrient agar | Very thin | Olive buff | None |
| Starch - inorganic salts agar | Very thin | None | None |
| Tyrosine agar | Good, pale olive gray | Olive buff | None |
| Oatmeal agar | Thin, mouse gray | None | None |
| Yeast - malt extract agar | Good, pale mouse gray | Deep olive buff | None |

Table 2. Physiological properties of the producing strain.

| Properties observed | Characteristics |
|---|---|
| Melanine production | None |
| Nitrate reduction | Negative |
| Starch hydrolysis | Negative |
| Gelatin liquefaction | Negative |
| Action on milk | No coagulation<br>Weak peptonization |

Table 3. Utilization of various carbon compounds.

| | | | |
|---|---|---|---|
| L-Arabinose | − | D-Mannitol | − |
| D-Fructose | + | L-Rhamnose | + |
| D-Glucose | + | Raffinose | ± |
| D-Galactose | + | D-Xylose | − |
| *i*-Inositol | − | Salicin | − |
| Sucrose | − | | |

+; Good utilization, ±; doubtful utilization, −; no utilization.

ptonization was weakly positive. Carbon utilization is shown in Table 3. According to these results, the producing strain was judged similar to *Streptomyces carnosus* among the species described in SHIRLING's reports[3,4,5], but its cultural characteristics on yeast - malt extract agar revealed its distinct difference from this species.

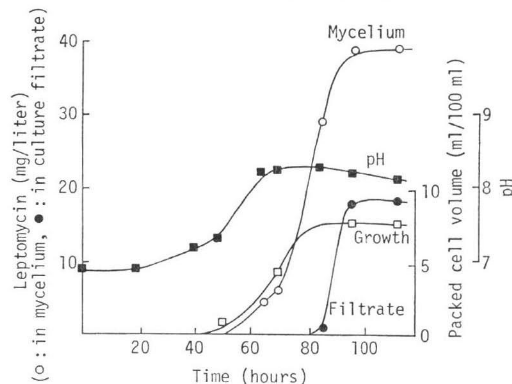
## Fermentation

Erlenmeyer flasks (500 ml) containing 100 ml of the seed medium were inoculated with spores from the slant culture of the producing strain and incubated at 26.5°C on a rotary shaker with 10 cm throw at 100 rpm for 4 days. Five 30 liters fermentors with 15 liters of the production medium were inoculated with 2~5% volume of the seed culture. Composition of the seed and the production media are shown in Table 4. The fermentation was carried out at 26.5°C for 96 hours under aeration of 15 liters/minute and agitation of 200 rpm. The activity appeared 2 days after the inoculation and reached a maximum after 4 days cultivation (Fig. 2). No distinct loss of the activity occurred during prolonged cultivation.

Table 4. Media used for production of leptomycins.

| Seed medium | | Production medium | |
|---|---|---|---|
| Glucose | 0.5% | Soybean flour | 5 % |
| Malt extract | 0.5 | Soybean oil | 3 |
| Yeast extract | 0.2 | Dried yeast | 0.3 |
| KCl | 0.4 | KCl | 0.3 |
| | | $K_2HPO_4$ | 0.02 |
| | | $CaCO_3$ | 0.3 |
| pH was adjusted to 7.3 | | pH was adjusted to 7.2 | |

Fig. 2. Time course of leptomycin production.

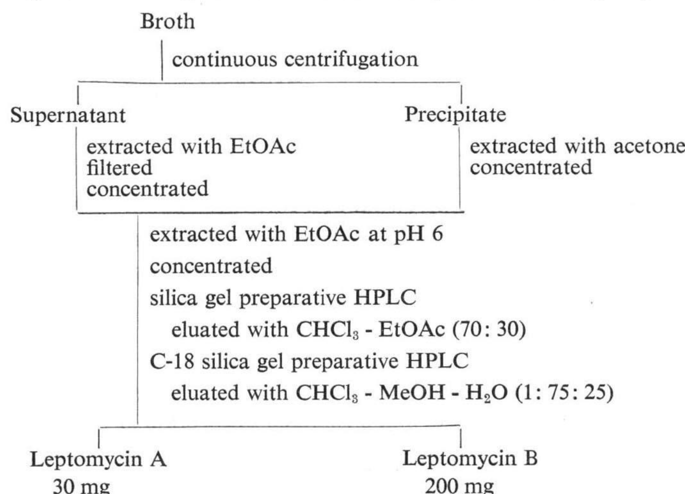


## Isolation and Purification

The flow diagram of the isolation and purification method for the preparation of leptomycin is shown in Fig. 3.

Since the activity was present in both culture filtrate and mycelia, it was extracted from each fraction after separation by continuous centrifugation. The supernatant was extracted twice with equal volumes of ethyl acetate, and the extract was concentrated to 500 ml. The mycelia were extracted twice with equal weight volumes of acetone, and the extract was concentrated to 500 ml. After combining both of the concentrated extracts, the pH was adjusted to 6.0 with conc. HCl, and the active substances were extracted three times with equal volumes of ethyl acetate. They were concentrated under reduced pressure to dryness, and the residue applied to silica gel preparative high performance liquid chromatography (column volume 500 ml). After washing with 3 liters of chloroform, the activity was eluted with 2 liters of a mixture of chloroform and ethyl acetate (70: 30, v/v). The active fractions were concentrated under reduced pressure to dryness and subjected to reverse phase preparative liquid chromatography (column volume 500 ml) with the solvent system composed of chloroform, methanol and water (1: 75: 25, v/v/v). The activity was eluted to give fractions A and B. Leptomycins A (30 mg) and B (200 mg) were obtained from each fraction, respectively.

Fig. 3. Flow diagram of the isolation and purification of leptomycins.




## Physicochemical Properties

The physicochemical properties of leptomycins A and B are summarized in Table 5. Both compounds were isolated as yellow sticky oils which were soluble in methanol, ethanol, ethyl acetate and ethyl ether but insoluble in *n*-hexane and water. Their chromatographic behavior on HPLC is presented in Table 6. Color reactions are as follows; positive in iodine vapor, potassium permanganate and rhodamine B tests and negative in ninhydrin test. Field desorption mass spectrometry on leptomycin B gave a peak at $m/z$ 541 $(M + H^+)$ and secondary ion mass spectrometry at $m/z$ 563 $(M + Na^+)$ and 579 $(M + K^+)$. The UV spectrum of leptomycin B showed a maximum absorption at 225 nm and a shoulder absorption at 240 nm, suggesting the presence of dienes and conjugated carbonyl groups. In the IR spectrum (Fig. 4), characteristic absorptions attributable to alcohol and carbonyl groups were observed at 3500~3200 and 1700 $cm^{-1}$ respectively. The $^1H$ NMR spectrum (in $CDCl_3$, 400 MHz) is

Table 5. Physicochemical properties of leptomycins A and B.

| | Leptomycin A | Leptomycin B |
|---|---|---|
| Appearance | Yellow sticky oil | Yellow sticky oil |
| $[\alpha]_D$ (MeOH) | −31.8° (*c* 0.15) | −24.5° (*c* 0.70) |
| Molecular weight | 526 | 540 |
| Molecular formula | $C_{32}H_{46}O_6$ | $C_{33}H_{48}O_6$ |
| UV spectrum in EtOH (ε) | $\lambda_{max}$ 225 (19000), 243 nm (sh 17000) | $\lambda_{max}$ 225 (20000), 240 nm (sh 15000) |
| IR spectrum $\nu_{max}^{film}$ | 3500~3200 (-OH), 1700 $cm^{-1}$ (C=O) | 3500~3200 (-OH), 1700 $cm^{-1}$ (C=O) |

Fig. 4. IR spectrum of leptomycin B (film).

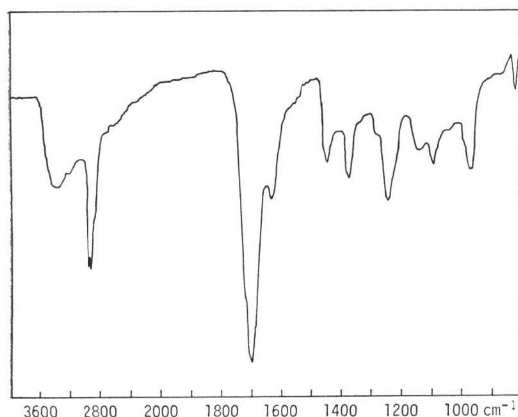



Table 6. Chromatographic behavior on HPLC.

| Solvent system | Retention time (minutes) | |
|---|---|---|
| | Leptomycin A | Leptomycin B |
| $CHCl_3$ - $CH_3OH$ - $H_2O$ (1:75:25) | 23.6 | 31.3 |
| $CHCl_3$ - 0.1 M $CH_3COONH_4$ (76:24) | 9.7 | 11.9 |

Column; Nucleosil 5C-18, 8 mm×300 mm.
Detection; UV absorption at 230 nm.
Flow rate; 1 ml/minute.

shown in Fig. 5. Forty seven proton atoms (without a carboxylate ion proton) were resolved. In the $^{13}C$ NMR spectrum (in $CDCl_3$, 25 MHz), the signals of thirty three carbon atoms were resolved and assigned to eight methyls, three methylenes, seven methin groups (included two CH–O–), twelve $sp^2$ and three carbonyl carbon atoms (Fig. 6). Thus, the molecular formula of leptomycin B was determined as $C_{33}H_{48}O_6$.

The UV and IR spectra of leptomycin A were very similar to those of leptomycin B. In the UV spectrum, a maximum absorption at 225 nm and a shoulder absorption at 243 nm were observed. The IR spectrum of leptomycin A is shown in Fig. 7. Field desorption mass spectrometry on leptomycin

Fig. 5. $^1H$ NMR spectrum of leptomycin B (in $CDCl_3$, 400 MHz).

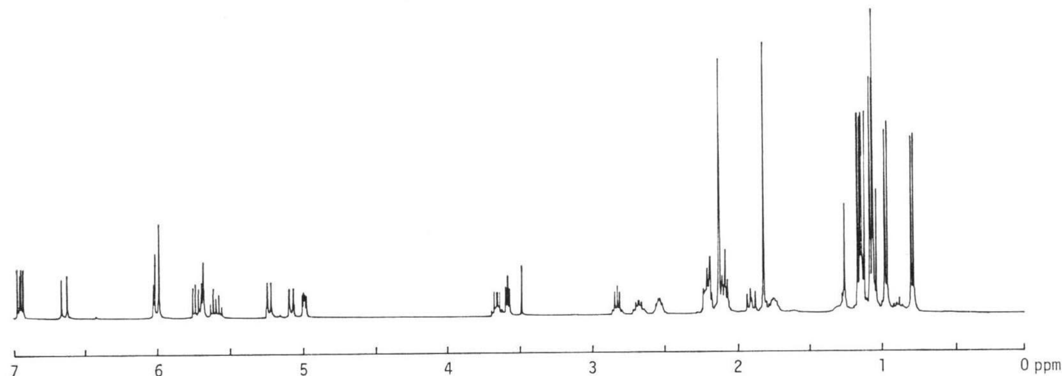


Fig. 6. $^{13}C$ NMR spectrum of leptomycin B (in $CDCl_3$, 25.05 MHz).

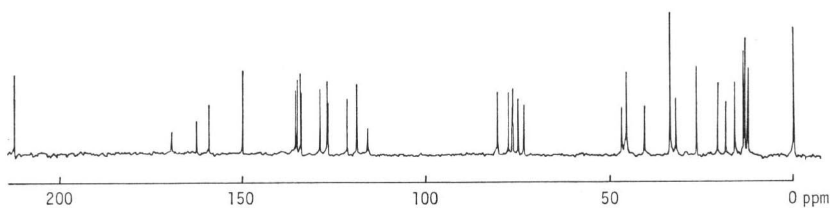



Fig. 7. IR spectrum of leptomycin A (film).

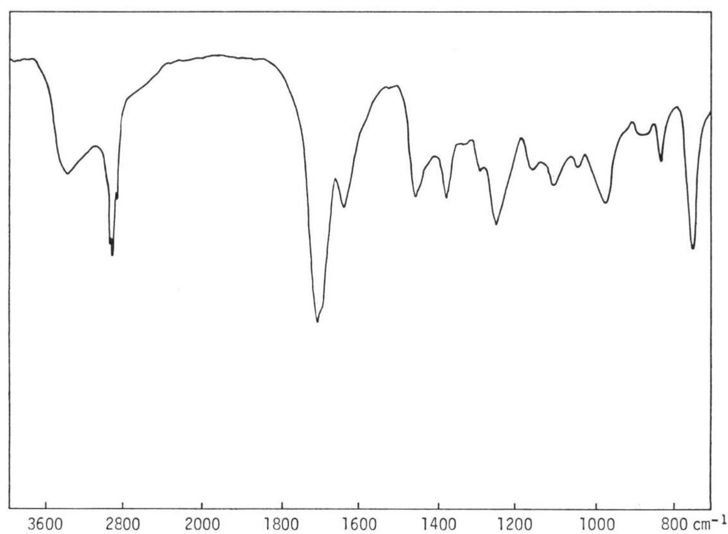



Fig. 8. $^1H$ NMR spectrum of leptomycin A (in $CDCl_3$, 400 MHz).

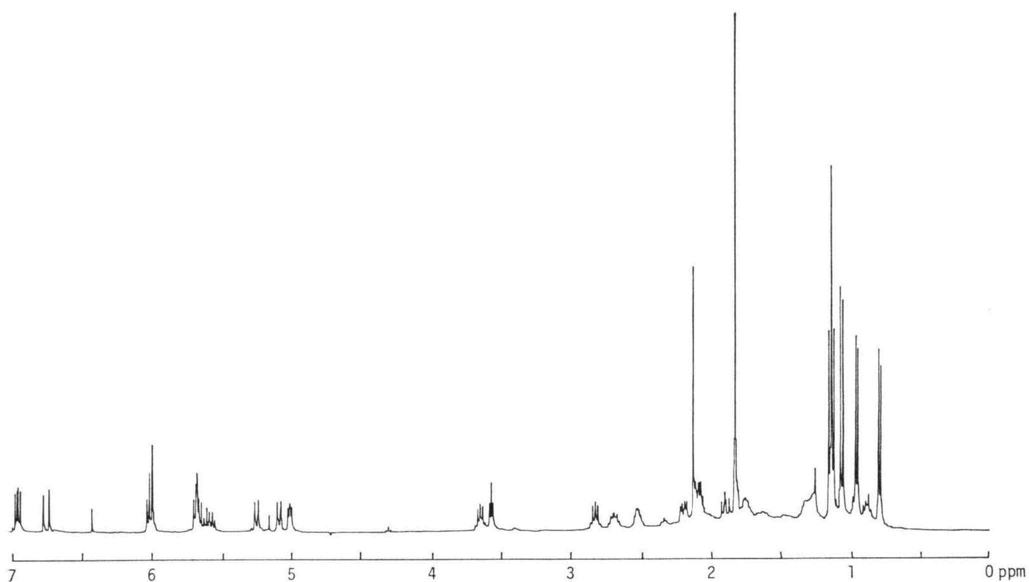


Table 7. Antimicrobial activity of leptomycins.

| Organisms | MIC (μg/ml) | |
|---|---|---|
| | Leptomycin A | Leptomycin B |
| *Aerobacter aerogenes* ATCC 8724 | NT | >100 |
| *Bacillus subtilis* PCI 219 | 100 | 100 |
| *Corynebacterium equi* IFO 3730 | NT | >100 |
| *Mycobacterium smegmatis* IFO 3038 | >100 | >100 |
| *Staphylococcus aureus* FDA 209P | >100 | 100 |
| *Pseudomonas aeruginosa* IAM 1180 | NT | >100 |
| *Aspergillus nidulans* IAM 2155 | >100 | >100 |
| *Penicillium chrysogenum* FAT 917 | >100 | >100 |
| *Paecilomyces varioti* IAM 5001 | NT | >100 |
| *Candida albicans* IAM 4888 | >100 | >100 |
| *Saccharomyces cerevisiae* IAM 4020 | >100 | >100 |
| *Schizosaccharomyces pombe* IAM 4863 | 0.1 | 0.012 |
| *Rhodotorula minuta* IFO 0387 | NT | 0.25 |
| *Mucor rouxianus* IFO 5775 | 0.4 | 0.031 |
| *M. rouxianus* ATCC 8099 | NT | 0.062 |
| *M. racemosus* IFO 5403 | NT | 0.125 |
| *M. hiemalis* IAM 6095 | NT | 0.25 |
| *M. rouxii* NI 1066 | NT | 0.031 |
| *M. pusillus* IAM 6122 | NT | 0.5 |
| *M. javanicus* IAM 6087 | NT | 1 |
| *Rhizopus javanicus* IAM 6241 | NT | 4 |
| *R. niveus* Rh-2-1 | NT | 4 |

NT; Not tested.

A gave a peak at $m/z$ 527 ($M + H^+$) and 549 ($M + Na^+$). The $^1H$ NMR spectrum of leptomycin A (Fig. 8) is also very similar to that of leptomycin B and forty five proton atoms (without a carboxylate proton) were resolved. Thus, the molecular formula of leptomycin A was determined as $C_{32}H_{46}O_6$. The chemical structures of leptomycins will be described in the succeeding paper.

## Biological Properties

The minimum inhibitory concentrations (MIC) of the leptomycins are given in Table 7. Leptomycins A and B are both active against Schizosaccharomyces and Mucor and exhibit weak activity against Rhizopus and Rhodotorula. They showed no effect on other microbes as follows; Aerobacter, Bacillus, Mycobacterium, Corynebacterium, Staphylococcus, Pseudomonas, Aspergillus, Penicillium, Paecilomyces and Candida at the concentration of 50 μg/ml. Acute toxicity of leptomycin (mixture) was examined with mice. When 12.5 mg/kg of the antibiotic was given by i.v. injection, 2 of 5 mice were killed. All the mice survived with dosage below 6.25 mg/kg. The cytotoxicity of leptomycin (mixture) was examined in tissue culture with SV-40 transformed C3H-2K cells. Morphological changes of these cells were observed under a light microscope after 1 to 3 days cultivation with the addition of leptomycin. The results are shown in Table 8. Only 1~10 ng of leptomycin caused a distinct morphological change

Table 8. Cytotoxic activity on tissue culture cells.

| Concentration (ng/ml) | Effect |
|---|---|
| 1,000 | + |
| 100 | + |
| 10 | ± |
| 1 | ± |
| 0.1 | — |
| 0.01 | — |

+; Destruction of cells, ±; rounding of cells, —; no effect.

with rounding of the cells.

## Experimental

General

Preparative HPLC was carried out on a Waters System 500A apparatus with a silica gel column (prep pak silica) and a C-18 bonded silica gel column (prep pak C18). Field desorption mass spectra were measured on a Hitachi M-80A spectrometer. The $^{1}H$ NMR spectra were measured on a JEOL FX-400 spectrometer and $^{13}C$ NMR spectra were measured on a JEOL FX-100 spectrometer using TMS as an internal standard. The IR spectra were measured on a JASCO A-202 and UV spectra on a Hitachi 200 spectrophotometer.

Antimicrobial Assay Procedure of Leptomycin

The antimicrobial activity of leptomycin was assayed using *S. pombe* as an indicator strain.

The microorganism, *S. pombe* IAM4863, was cultured in 10 ml of malt medium (malt extract 1%, yeast extract 0.4%, glucose 2%, pH 6.0) in a test tube with shaking for 18 hours at 30°C. One ml of the culture was mixed with 20 ml of melted malt soft agar medium (agar 0.5%) at 40°C which was then transferred to a Petri dish (8 cm × 23 cm). A paper disc (Toyo, 8 mm, thick) containing 50 μl of solution with an appropriate concentration of leptomycin was dried and put on the plate. After the incubation for 1 day at 30°C, the diameter of the inhibitory zone around the paper disc was measured.

Medium and Assay Method for Cytotoxicity of Leptomycin

A stock of SV-40 transformed C3H-2K cells was grown in Eagle's minimum essential medium supplemented with 10% calf serum. After 24 hours cultivation of these cells ($1 \times 10^{5}$/ml) divided into test plates (5 ml), the growth medium was exchanged with fresh medium containing leptomycin (ethanol solution).

## Acknowledgments

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## References

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