LAVENDOMYCIN, A NEW ANTIBIOTIC

I. TAXONOMY, ISOLATION AND CHARACTERIZATION

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Lavendomycin, a new basic peptide antibiotic containing novel amino acids, has been isolated from the culture broth of a streptomyces designated as *Streptomyces lavendulae* subsp. *brasilicus*. The antibiotic obtained as colorless crystals ($C_{29}H_{50}N_{10}O_8$, MW 666) is active against Gram-positive bacteria *in vitro* and *in vivo*, however, inactive against Gram-negative bacteria and fungi. An acute toxicity of the antibiotic in mice was LD_{50} >2 g/kg by subcutaneous injection.

In pursuance of our screening program of the search aimed at enhancing antibiotic activity in serum medium, we found a new antibiotic, named lavendomycin†, produced by the strain of *Streptomyces lavendulae* subsp. *brasilicus*. The strain was isolated from the soil sample collected in Yaeyama Islands, Kagoshima Prefecture. The antibiotic exhibited a significant growth inhibition of Gram-positive bacteria. The structure was determined (Fig. 1) by chemical study as discussed in the following paper.

In this paper, the taxonomy of the producing strain, fermentation, isolation, chemical and biological properties of lavendomycin are described.

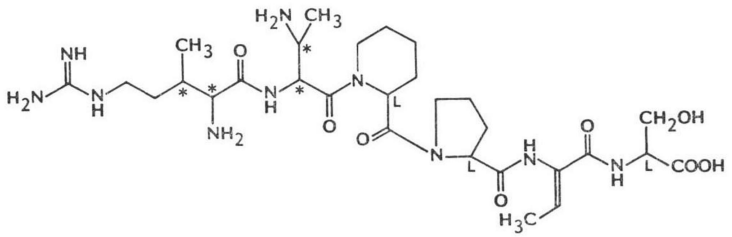
Taxonomical Studies

Morphological observations were made on cultures with light and electron microscopes on cultures grown at 30°C for 14 days on yeast - malt extract agar, oatmeal agar and inorganic salts - starch agar.

Cultural characteristics were observed on nine kinds of media described by SHIRLING and GOTTLIEB[1] and WAKSMAN[2]. The incubation was made at 30°C for 14 days. The color names used in this study were based on Color Standard (Nihon Shikisai Co., Ltd.).

The whole cell analysis was performed by the methods of BECKER *et al.*[3] and YAMAGUCHI[4]. The range of growth temperature and optimum temperature were determined on yeast - malt extract agar using a temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.).

Fig. 1. Structure of lavendomycin.
* Under consideration with absolute configurations.



† Lavendomycin was originally designated as FR-900201.

Table 1. Cultural characteristics of strain No. 1446.

Medium	Aerial mycelium	Reverse side of color	Soluble pigment
Sucrose - nitrate agar	None or very thin	Colorless, small colonies	None
Glucose - asparagine agar	White, thin powdery	Pale yellow, small colonies	Pale yellow
Glycerol - asparagine agar	White, thin powdery	Pale yellow to yellowish brown, small colonies	Pale yellow
Inorganic salts - starch agar	Light brownish gray to brownish gray, powdery	Pale yellow, small colonies	Pale yellow
Nutrient agar	None	Colorless to pale yellow, wrinkled colonies	None
Yeast - malt extract agar	None or very thin	Colorless to pale yellow wrinkled colonies	None or trace
Oatmeal agar	None	Colorless, flat	Pale yellow
Tyrosine agar	White to light brownish gray, powdery	Brown, slightly wrinkled colonies	Brown
Peptone - yeast extract - iron agar	None	Pale yellow, wrinkled colonies	Faint brown

Table 2. Physiological properties of strain No. 1446.

Temperature range for growth	10~37°C
Optimum temperature	26°C
Starch hydrolysis	+
Gelatin liquefaction	−
Milk peptonization	+
Milk coagulation	−
Melanin production	+

+ Positive, − negative.

Table 3. Carbon sources utilization of strain No. 1446.

D-Glucose	+
Sucrose	−
Glycerol	+
D-Xylose	−
D-Fructose	−
Lactose	−
L-Maltose	+
Rhamnose	−
Raffinose	−
D-Galactose	+
L-Arabinose	−
D-Mannose	+
Inositol	−
D-Mannitol	−
Inulin	−
Cellulose	−
Salicin	−
Chitin	−
Sodium citrate	+
Sodium succinate	−
Sodium acetate	−

+ Utilization, − no utilization.

Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[5]. The results were obtained after 14 days at 30°C.

The mature spores occurred in chains of 10 to 30 spores forming loops, hooks or extended spirals (*retinaculiaperti*). The spores are cylindrical or oval and $0.3\sim0.7\times0.6\sim1.8$ μm in size with smooth surfaces. Aerial mycelium was in the gray color series on inorganic salts - starch agar. Pale yellow soluble pigment was produced. No distinctive reverse side pigment was formed. Cultural characteristics are shown in Table 1.

Whole cell hydrolysates of the producing strain of lavendomycin (strain No. 1446) contained LL-diaminopimelic acid.

Physiological properties of this strain are shown in Table 2. This strain can grow at temperature range from 10°C to 37°C with the growth optimum at 26°C. Melanoid pigments are formed in peptone - yeast extract - iron agar, tyrosine agar and Tryptone - yeast extract broth. Hydrolysis of starch and milk peptonization were positive.

D-Glucose, D-mannose, D-galactose, maltose, glycerol and sodium citrate were utilized for growth

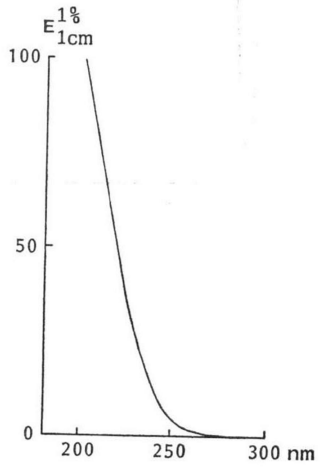
Table 4. Media used for production of lavendomycin.

Seed medium		Production medium	
Corn starch	2 %	Corn starch	2 %
Gluten meal	0.5	Gluten meal	0.5
Corn steep liquor	0.5	Corn steep liquor	0.5
Dried yeast	0.5	Dried yeast	0.5
pH adjusted to 6.8		$CaSO_4 \cdot 2H_2O$	0.5
		pH adjusted to 7.0	

Fig. 2. Purification procedure of lavendomycin.

Fermentation broth (23.4 liters)
|
Diaion HP-20 (6 liters)
| washed with H_2O (12 liters)
| eluted with 50% aq MeOH (30 liters)
| concd *in vacuo* to 1 liter
CM-Sephadex C-25 (H^+, 500 ml)
| washed with H_2O (500 ml)
| eluted with 2.8% aq NH_4OH (1.2 liters)
| concd *in vacuo* to 100 ml
DEAE-Sephadex A-25 (OH^-, 100 ml)
| washed with H_2O (300 ml)
| combined the effluent and wash solution
| concd *in vacuo* to 100 ml
| adjusted to pH 6.0
Diaion HP-20 (50 ml)
| washed with H_2O (100 ml)
| eluted with 65% aq MeOH (150 ml)
| concd to dryness
Crude powder
| recrystallized from MeOH (15 ml)
Colorless crystals (99 mg)

Fig. 3. UV absorption spectrum of lavendomycin (in H_2O).



(Table 3).

Microscopic studies and cell wall composition of the strain No. 1446 indicated that this strain belongs to the genus *Streptomyces*. Accordingly, a comparison of this strain was made with the published descriptions[6~9] of various *Streptomyces* species. The strain No. 1446 is considered to resemble *Streptomyces lavendulae* subsp. *brasilicus*. Thus, taxonomic comparison was carried out using the standard culture of *S. lavendulae* subsp. *brasilicus*. The microbiological characteristics of the strain No. 1446 are in good agreement with those of *S. lavendulae* subsp. *brasilicus*. The strain No. 1446 has, therefore, been identified as *Streptomyces lavendulae* subsp. *brasilicus* (FERM-P 5649)[10].

Fermentation

A loopful of *S. lavendulae* subsp. *brasilicus* on a mature slant was inoculated to each of three 500-ml Erlenmeyer flasks containing 150 ml of sterile seed medium. The flasks were incubated at 30°C for three days on a rotary shaker (220 rpm, 5.1-cm throw). A 30-liter fermentor with 25.5 liters of production medium was inoculated with 2% of the mature seed broth. The compositions of the seed and production media are shown in Table 4.

The fermentation was carried out at 30°C for three days under aeration of 25.5 liters/minute and agitation of 150 rpm. The progress of the fermentation was monitored by *Staphylococcus aureus* 209P as a test organism on serum agar (horse serum 20%, $FeSO_4 \cdot 7H_2O$ 0.01% and agar 1.5%).

Fig. 4. IR spectrum of lavendomycin (in Nujol).

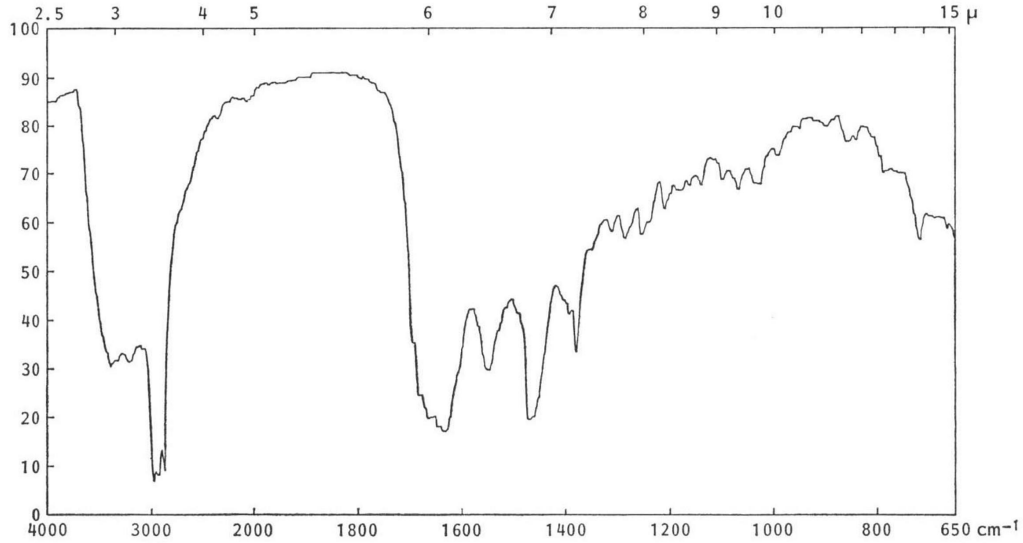


Fig. 5. ^1H NMR spectrum of lavendomycin (in D_2O).

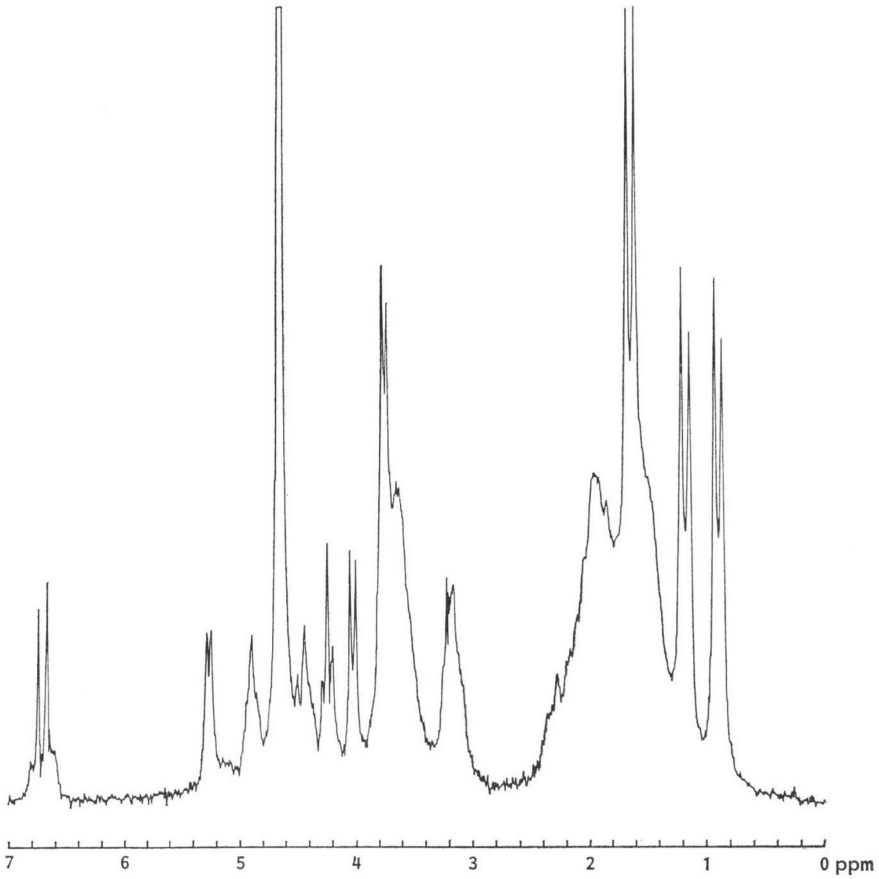
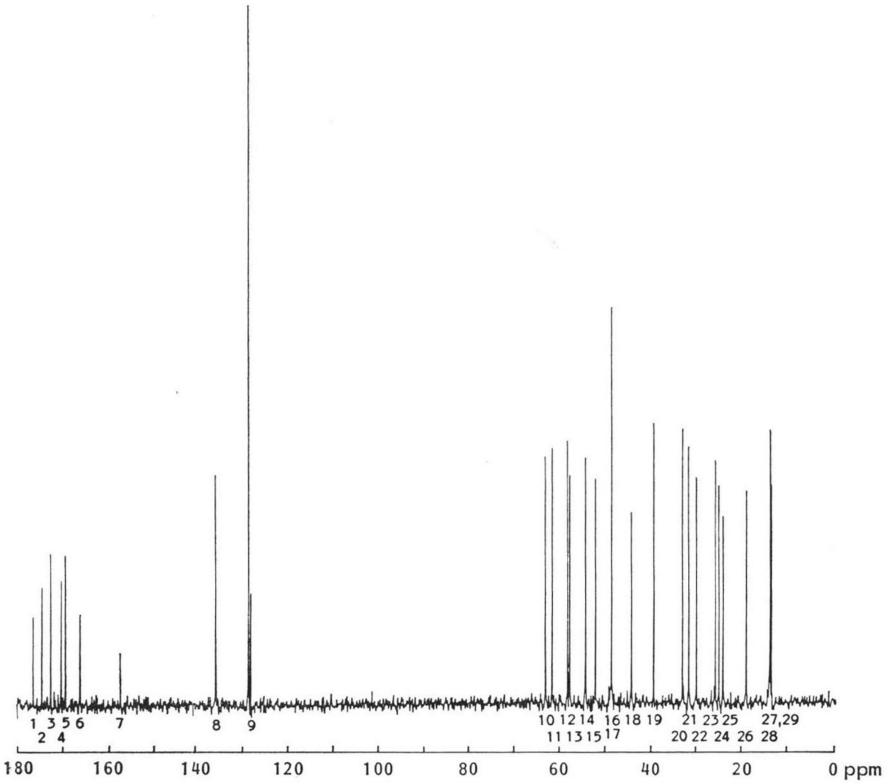


Fig. 6. ^{13}C NMR spectrum of lavendomycin (in D_2O).



No.	δ (ppm)	No.	δ (ppm)	No.	δ (ppm)	No.	δ (ppm)
1	176.35 (s)	8	136.19 (d)	15	51.96 (d)	23	25.73 (t)
2	174.68 (s)	9	128.10 (s)	16,17	48.60 (t,t)	24	24.96 (t)
3	172.61 (s)	10	62.94 (t)	18	44.22 (t)	25	24.01 (t)
4	170.22 (s)	11	61.44 (d)	19	39.30 (t)	26	18.97 (t)
5	169.30 (s)	12	58.18 (d)	20	32.87 (d)	27	13.96 (q)
6	166.16 (s)	13	57.67 (d)	21	31.58 (d)	28	13.73 (q)
7	157.29 (s)	14	54.15 (d)	22	29.88 (t)	29	13.44 (q)

Isolation Procedure

The flow diagram of the isolation procedure described below is shown in Fig. 2. Most of the activity was present in the broth filtrate. The cultured broth was filtered with a filter aid (Radio-lite). The filtrate (23.4 liters) was run over a column of high-porous adsorption resin Diaion HP-20 (6 liters). The column was washed successively with water (12 liters) and eluted with 50% aqueous methanol (30 liters). The active fractions were concentrated *in vacuo* to 1 liter. The concentrate was adsorbed on a column of CM-Sephadex C-25 (H^+ form, 500 ml). After washing with 500 ml of water, the column was eluted with 2.8% aqueous ammonia (1.2 liters). Active fractions were combined and concentrated *in vacuo* to 100 ml. The concentrate was passed through a column of DEAE-Sephadex A-25 (OH^- form, 100 ml). The column was washed with water (300 ml). The effluent and wash solution were pooled, and then concentrated *in vacuo* to 100 ml. After adjusting to pH 6.0 with 1 N HCl, the solution was subjected to a column of Diaion HP-20 (50 ml) to remove the salts. After washing with

Table 5. Antimicrobial activity of lavendomycin.

Test organisms	MIC (μg/ml)	
	Medium 1[a]	Medium 2[b]
Staphylococcus aureus 209P JC-1	<0.1	0.39
〃 33	<0.1	0.78
〃 47	0.1	1.56
Staphylococcus epidermidis 59	1.56	>400
〃 87	<0.1	0.39
Streptococcus faecalis 50	12.5	12.5
Escherichia coli NIHJ JC-2	>400	>400
Klebsiella pneumoniae NCTC-418	>400	>400
Candida albicans	>400	ND
Aspergillus niger	>400	ND

[a] Serum agar.
[b] Mueller Hinton agar.

Table 6. Protective efficacy in experimental infection in mice of lavendomycin.

Test organisms	Cell number (cells/mouse)	ED_{50} value (mg/kg)
Staphylococcus aureus 47	1.5×10^7	2.33
Staphylococcus epidermidis 87	2.6×10^8	1.68
Streptococcus faecalis 50	2.6×10^5	13.2

water (100 ml), the active principle was eluted with 65% aqueous methanol, and then concentrated *in vacuo* to dryness. Final purification was achieved by dissolving the above residue in hot methanol (15 ml). Dihydrochloride of lavendomycin was obtained as a colorless crystal (99 mg).

Physico-chemical Properties

Lavendomycin is a basic (pK_1 2.86, pK_2 6.39 and pK_3 8.04) colorless crystal which decomposes at more than 205°C. It is soluble in water and methanol, and slightly soluble in ethanol, but hardly soluble in acetone, chloroform and ethyl acetate. The optical rotation is $[\alpha]_D^{21}$ −47.4° (*c* 0.4, H_2O). The molecular formula is established as $C_{29}H_{50}N_{10}O_8$ (MW 666.73) by elemental analysis and FD-MS (*m/z* 667 ($M^+ + 1$)).

Anal Calcd for $C_{29}H_{50}N_{10}O_8 \cdot 2H_2O$: C 49.56, H 7.74, N 19.93.
Found: C 49.27, H 7.51, N 19.81.

As shown in Fig. 3, the UV absorption spectrum showed only end absorption in aqueous solution. The IR spectrum shown in Fig. 4 suggests the presence of peptide bonds (1630 and 1550 cm^{-1}) in Nujol. ^1H NMR and ^{13}C NMR spectra in D_2O were shown in Figs. 5 and 6, respectively. Lavendomycin gives positive reactions with ninhydrin and iodine tests, but negative with Tollens and Dragendorff reactions. Thin-layer chromatography was carried out on a silica gel sheet (Merck chromatogram sheet 60 F254) using the solvent system of butanol - acetic acid - water (4: 1: 2). Rf value shows 0.04 in this system.

Acid hydrolysis of lavendomycin with 6 N HCl at 110°C for 24 hours in a sealed tube yielded one mol of serine, two mol of proline (in fact, mixtures of one mol of proline and homoproline respectively) and three mol of unknown amino acid. It shows that lavendomycin is a new peptide antibiotic containing novel amino acids.

Biological Properties

The antimicrobial spectrum of lavendomycin is shown in Table 5. This test was conducted by the agar dilution streak method, using the serum agar medium mentioned above and Mueller Hinton agar for bacteria, which were incubated at 37°C for 20 hours, and malt extract medium for fungi. Lavendomycin showed antibacterial activity against Gram-positive bacteria, but it has no activity against Gram-negative bacteria and fungi. The *in vivo* activity of lavendomycin against experimental infections due to *Staphylococcus aureus* 47, *Staphylococcus epidermidis* 87 and *Streptococcus faecalis* 50 was examined. One hour after the intraperitoneal challenge with a lethal dose of the pathogen given in a 5% suspension of mucin the drug solutions were administered subcutaneously. A group of the mice (ICR mouse, 4 weeks, male) was used for each dosage level with animal being observed seven days to determine the ED_{50} values. As shown in Table 6, lavendomycin gave fairly good protection against *Staphylococcus*, but gave less effective against *Streptococcus*.

The acute toxicity (LD_{50}) by intraperitoneal injection into mice (ICR) was greater than 2 g/kg.

Discussion

It is well known that there are numerous antibiotics greatly diminishing their biological activity because of binding with proteins in serum. Although these antibiotics, in this case, are excellent *in vitro*, the *in vivo* activity is not good. As expected the reverse phenomena mentioned above, we adopted serum agar on the first step of screening for searching an effective antibiotic *in vivo*. In result, this screening system led to two novel peptide antibiotics, named FR-900129* and lavendomycin.

These antibiotics possess several unique features; (1) from viewpoint of chemistry, they contain a linear hexa or heptapeptide including novel amino acids, (2) of which the *C*-terminus is serine, (3) from biological point, they are active against Gram-positive bacteria *in vitro* and *in vivo* (Tables 5 and 6). Although the exact mechanism accounting for these activities has not yet been elucidated, the simplest possibility is that a certain factor in serum cooperate to make its *in vivo* activity.

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* Cirratiomycin B[12,13] and antrimycin A[14~16] were identified to be FR-900129[11].

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