ISOLATION OF LAVENDAMYCIN
A NEW ANTIBIOTIC FROM *STREPTOMYCES LAVENDULAE*

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The isolation of lavendamycin (**1**) an antibiotic closely related to streptonigrin (**2**) is described. Details of the taxonomic description of *S. lavendulae* (strain C-22,030), production of lavendamycin, its isolation and the biological activity are given.

Recently we have reported the structure determination of lavendamycin (**1**), a novel antibiotic closely related to streptonigrin (**2**) in structure (Fig. 1).[1] In this paper we report the characterization of the producing culture, the fermentation conditions, the isolation of, and biological activity of **1**.

In the course of screening actinomycetes for the production of agents inducing lysogenic *Escherichia coli* strain W1709 (λ)[2] strain C-22,030 was isolated from a soil sample collected from Wadington, New York. An antibiotic produced by strain C-22,030 inhibited *Staphylococcus aureus* FDA strain 209P but was less active aginst a derived *S. aureus* Bristol A9626, streptonigrin resistant strain. However, the antibiotic from strain C-22,030 chromatographed differently from streptonigrin on paper chromatography. The level of activity to induce *E. coli* strain W1709 was increased as a result of a media improvement and strain selection program. With further improvement activity against the Walker 256 intramuscular tumor[3] in the rat was observed.

Taxonomic Description

An inoculum for *Streptomyces lavendulae* NRRL B-1230[4] and strain C-22,030 was prepared for comparative diagnostic studies according to a procedure described by GOTTLIEB.[5] On solid media with abundant aerial mycelium both organisms exhibit pink to red sporulation.

The sporophore of strain C-22,030 is usually flexuous and spores are in chains that are predominantly loose spirals with hooks and loops. Spores formed on yeast extract agar[6] were examined with electron microscopy and were found to have smooth surfaces. The dimensions of spores are 1.1×1.42 μ.

Fig. 1. Structure of lavendamycin (**1**) and streptonigrin (**2**).

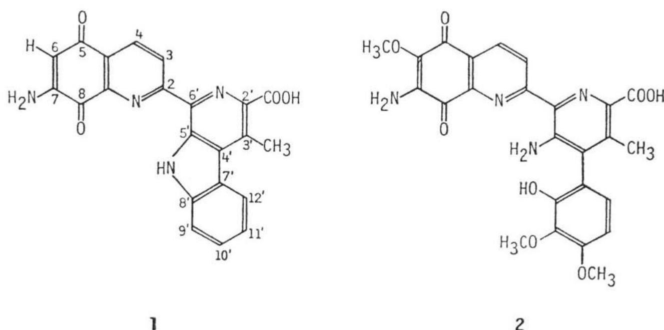

Table 1. Cultural characteristics of strain C-22,030.

Agar medium	Description*
Yeast extract agar[6]	A: Abundant, long sporophores some flexuous with loose spirals or loops, Rosedust 6 B-2 (grayish red #19)
	V: Not visible
	R: Bure 13H-5 (moderate yellowish brown #77)
	S: Light tan
Carvajal's oatmeal agar[6]	A: Abundant rose pink sporulation, long sporophores with open loose spirals, Tonquet 4 B-6 (grayish yellowish pink #32)
	V: Not visible
	R: Sombrero 11 D-4 (grayish yellow #90)
	S: None
Glycerol asparagine agar[7]	A: Sparse white, no sporophores, White 1 A-7
	V: Thin and colorless
	R: Colorless
	S: None
Inorganic salts - starch agar[6]	A: Abundant, short sporophores with compact spirals and long sporophores (some flexuous) with compact to loose spirals, Rosedust 6 B-2 (grayish red #19) and Woodrose 6 B-9 (light grayish reddish brown #45)
	V: Not visible
	R: Flesh 11 A-1 (pale orange yellow #73)
	S: None

* A: Aerial mycelium, V: vegetative mycelium, R: reverse, S: soluble pigment.
Incubation at 28°C for 14 days. Plate numbers refer to color blocks described by MAERZ and PAUL.[8]
ISCC-NBS[9] designation is given in parentheses.

The sporophore of *S. lavendulae* NRRL B-1230 is flexuous and spores are in chains of loose or tight spirals. The spore wall of spores from yeast extract agar is smooth and the dimensions are $0.71\sim1.1\times1.4\sim2.1\ \mu$.

Growth characteristics of strain C-22,030 (Table 1) and *S. lavendulae* NRRL B-1230 (Table 2) were obtained from cultures incubated at 28°C for fourteen days on agar media in a cross-hatched pattern. Color names are listed according to color blocks of MAERZ and PAUL.[8] These names are converted to the ISCC-NBC color name and number.[9]

Strain C-22,030 utilizes D-glucose, D-fructose, L-arabinose, *i*-inositol and D-mannitol but does not utilize sucrose, raffinose, rhamnose or xylose when tested by the procedure of PRIDHAM and GOTTLIEB.[10] *S. lavendulae* NRRL B-1230 utilizes D-glucose and D-fructose but does not utilize sucrose, L-arabinose, *i*-inositol, D-mannitol, D-xylose, rhamnose or D-xylose. Both strains hydrolyze starch in inorganic salts-starch agar.[6]

Hydrogen sulfide is produced by both strains in peptone iron agar (Difco Laboratories, Detroit, Michigan) plus 0.1% yeast extract (Baltimore Biological Laboratory, Baltimore, Maryland). Both strains produce melanin-like pigment in that medium as well as in Tryptone yeast extract broth[5] and tyrosine agar.[11] Neither strain grows at 50°C on tomato paste oatmeal agar[6] but good growth occurs at 28°C.

Strain C-22,030 utilizes L-arabinose, *i*-inositol and D-mannitol while *S. lavendulae* NRRL B-1230 does not. Spores of both strains differ in size. However, several similarities justify placing strain C-22,030 in the species *S. lavendulae*.

Table 2. Cultural characteristics of *Streptomyces lavendulae* NRRL B-1230 IMRU 3440.

Agar medium	Description*
Yeast extract agar[6]	A: Abundant rose gray sporulation, flexuous sporophores ending in loops and spirals of 1~2 turns, between Mist plate 53 B-1 and Mauveglow plate 53 B-2 (reddish gray #22 and light grayish red #18) V: Not visible R: Stag plate 14 E-7 (moderate yellowish brown #77) S: Tan
Carvajal's oatmeal agar[6]	A: Abundant pink to rose sporulation flexuous sporophores ending in loops, hooks and loose spirals, edge of growth: Zephyr plate 42 B-1 (pale pink #7 and grayish pink #8), center of growth: Sappho plate 44 C-1 (light grayish red #18) V: Not visible R: Parchment plate 12 B-3 (grayish yellow #90) S: Tan
Glycerol asparagine agar[7]	A: Moderate grayish white to blue gray sporulation, flexuous sporophores with a few hooks, loops and loose spirals, Grayish white 34 A-1 and Irisglow 44 B-3 (purplish gray #233) V: Light tan R: Oyster white 10 B-1 (pale yellow green #121) to Ecru beige 11 C-2 (grayish yellow #90) S: None
Inorganic salts-starch agar[6]	A: Moderate to abundant, flexuous sporophores with loops, hooks, many loose spirals and some tightly wound spirals, Yellowish pink to pinkish gray 10 A-1 (yellowish white and grayish yellowish pink #32) V: Light tan R: Polar bear 9B-2 (orange yellow #73) and Ivory 10 B-2 (pale yellow #89) S: None

* A: Aerial mycelium, V: vegetative mycelium, R: reverse, S: soluble pigment.
Incubation at 28°C for 14 days. Plate numbers refer to color blocks described by MAERZ and PAUL.[8] ISCC-NBS[9] designation is given in parentheses.

Production of Lavendamycin

Strain C-22,030 was maintained on agar slants consisting of glucose 4 g, yeast extract 4 g, malt extract 10 g and agar 20 g made up to one liter with deionized water. To prepare an inoculum for production of lavendamycin spores from an agar slant culture of the strain were transferred to a 500-ml Erlenmeyer flask. This flask contained 100 ml of medium consisting of glucose 20 g, ammonium sulfate 3 g, $ZnSO_4 \cdot 7H_2O$ 0.03 g, cornsteep liquor 10 g, Pharmamedia (cottonseed embryo meal from Traders Oil Mill Co., Fort Worth, Texas) 10 g and $CaCO_3$ 4 g made up to one liter with deionized water. After 48 hours of incubation at 27°C on a Gyrotory tier shaker (Model G53, New Brunswick Scientific Co., New Brunswick, New Jersey) set at 210 rev/minute and describing a 5.1 cm circle, 4 ml was transferred to 500-ml Erlenmeyer flasks containing a production medium selected from studies for improved lavendamycin yield. This medium consisted of corn starch 40 g, peanut meal 10 g, linseed meal 10 g and $CaCO_3$ 5 g made up to one liter with deionized water. The production culture was incubated at 27°C for seven days on the previously described shaker.

For production in a tank fermentor 200 ml of inoculum culture was transferred to a tank with 38 liters of medium consisting of glucose 30 g, safflower meal 30 g, peanut meal 10 g and $CaCO_3$ 5 g made up to one liter with tap water. The tank was agitated at 375 rev/minute, the air flow was 91 liters/

minute, the back pressure was 1 atm and the tank was incubated at 27°C for 170 hours. The same production medium was used for a tank fermentor (D-fermentor) with 3,000 liters of medium, which was inoculated with 23 liters of culture. The tank was agitated at 155 rev/minute, the air flow was 2,100 liters/minute the back pressure was 1 atm and the tank was incubated at 27°C for 170 hours.

Isolation of Lavendamycin

The whole broth from a D-fermentor, approximately 1,700 liters, was extracted at broth pH (7.5 ~ 8.0) with an equal volume of *n*-butanol. After filtration, the organic solvent phase was separated and concentrated *in vacuo* to a minimal volume. The reside was diluted with excess petroleum ether (heptane fraction, 20 ~ 30 volumes). This afforded 8.8 kg of tacky solids. These were slurried successively with diethyl ether, methanol, water, methanol, diethyl ether, and methanol to give a final crude material, 135 g.

The crude material from the extraction was only slightly soluble in solvents typically useful for normal and reverse phase chromatography. Normal and reverse phase HPLC systems were developed and found to serve as useful analytical tools to monitor the purification of lavendamycin. Translation of the analytical separation to preparative HPLC was unsuccessful, due to the relative insolubility of lavendamycin and co-produced substances in the mobile phases employed.

The fact that the crude materials demonstrated high solubility and stability in trifluoroacetic acid proved to remedy the problem of preparative purification. Silica gel TLC solvent systems which contained 10 ~ 20 % (v/v) trifluoroacetic acid were developed. Application of these systems to classical open column chromatography afforded purified lavendamycin.

Table 3. Minimum inhibitory concentration (μg/ml).

Organisms	Strain designation	Lavendamycin	Streptonigrin
Streptococcus pneumoniae	A- 9585	2	0.13
Streptococcus pyogenes	A- 9604	4	0.25
Staphylococcus aureus	A- 9537	0.16	0.016
〃 〃 (Pen Res.)	A- 9606	125	125
Streptococcus faecalis	A-20688	1	1
Escherichia coli	A-15119	1	1
〃 〃	A-20341-1	4	1
Klebsiella pneumoniae	A-15130	>125	8
Proteus mirabilis	A- 9900	2	1
Proteus vulgaris	A-21559	0.13	0.016
Serratia marcescens	A-20019	32	1
Enterobacter cloacae	A- 9659	125	32
Pseudomonas aeruginosa	A- 9843A	16	8
Candida albicans	A- 9540	16	0.5
〃 〃	A-15049	>16	2
〃 〃	A-15050	>16	2
Candida tropicalis	A-15051	>16	8
Candida krusei	A-15052	>16	8
Trichophyton rubrum	WW	0.5	8
Trichophyton mentagrophytes	A- 9870	0.5	4
Microsporum canis	A- 9872	0.5	16
〃 〃	A-22494	0.25	4

Biological Effects

Antimicrobial Activity

The minimum inhibitory concentration (MIC) of lavendamycin was determined with a standard 2-fold tube dilution procedure.[2] The results are shown in Table 3 along with testing of streptonigrin. In general the inhibition pattern is similar with lavendamycin being less potent. The only exception is with the fungi *Trichophyton rubrum, T. mentagrophytes* and *Microsporum canis* where lavendamycin is more potent.

Induction of Lysogenic Bacteria

Lavendamycin was tested for its ability to induce bacteriophage production in the lysogenic strain of *Escherichia coli* W1709 (λ) using the methods of PRICE, *et al.*[12] The minimum inducing concentration of 0.003 μg/ml is comparable to that of streptonigrin: 0.008 μg/ml, thus indicating a dissociation between inducing activity and other biological properties in comparing the two antibiotics.

Antitumor Effects

Tests for inhibition of P-388 leukemia in mice were performed using procedures previously reported.[13] The line of P-388 used was received from Bristol Banyu Research Institute through the courtesy of Dr. H. KAWAGUCHI. This line is sensitive to bleomycin products and is designated P-388-J. Lavendamycin was slightly active both on a daily, qd 1→9, schedule and given as a single dose (Table 4). In other tests, not shown, lavendamycin had no inhibitory effect on standard P-388 or L-1210 leukemias.

Table 4. Effect of lavendamycin of P-388-J leukemia (Exp. #6450).

Material	Treatment regimen	Dose mg/kg/inj.	MST days	Effect MST % T/C	Average weight change, g	Survivors Day 5
Bleomycin	qd 1→9	16 U	13.0	130	−0.9	6 / 6
		8 U	10.0	100	−0.3	6 / 6
Lavendamycin	qd 1→9	3.2	10.5	105	−1.3	6 / 6
		1.6	13.0	130	−0.3	6 / 6
		0.8	13.5	135	−0.3	6 / 6
		0.4	12.0	120	+0.3	6 / 6
		0.2	13.0	130	+1.0	6 / 6
		0.1	12.0	120	+1.2	6 / 6
Lavendamycin	Once, Day 1	12.8	9.0	90	−2.9	5 / 6
		6.4	12.5	125	−0.6	6 / 6
		3.2	12.0	120	−1.2	6 / 6
		1.6	12.0	120	−0.7	6 / 6
		0.8	11.0	110	−0.1	6 / 6
		0.4	11.5	115	+0.2	6 / 6
Control		Saline	10.0	—	+0.7	10 / 10

Tumor inoculum: 10^6 ascites cells implanted i.p.
Host : BDF_1 ♀ mice.
Tox : Toxicity $<$4/6 survivors, Day 5.
Evaluation : MST = median survival time.
Effect : % T/C = MST treated/MST control $\times$100.
Criteria : T/C $\geqq$ 125 considered significant antitumor effect.

Experimental

Procedure for Bioautographs for C-22,030 Culture Samples and Lavendamycin

Schleicher and Schuell 589 Blue Ribbon Filter paper strips 1.3×58 cm were developed with solvent system butanol - glacial acetic acid - water (2: 1: 1). After development and drying, the strips were placed on a layer of Streptomycin Assay Agar with Yeast Extract (Baltimore Biological Laboratory, Baltimore, MD) seeded with *Bacillus subtilis* ATCC 6633. The plate was incubated at 27°C for 18 hours. Streptonigrin gave a zone with Rf 0.9. Broth samples of strain C-22,030 and lavendamycin gave a zone with Rf 0.4.

HPLC Analysis Systems

The HPLC apparatus consisted of a Waters Assoc. Model M-6000A Solvent Delivery System, a Waters Assoc. Model U6K injector with a 2 ml loop, a μ-Porasil prepacked column (Waters Assoc., 3.9 mm ID×30 cm, 10 μ particle size), a Schoeffel Model SF770 spectroflow monitor UV detector, and a Heath/Schlumberger strip chart recorder. The normal phase HPLC solvent system employed was dichloromethane - methanol - water - 30% aqueous ammonia, 80: 20: 1: 1 (v/v), and resulted in the following separation factor: $K'_{lavendamycin}=1.1$.

The reverse phase HPLC solvent system employed was pH 8 0.01 M KH_2PO_4 - acetonitrile, 80: 20 (v/v). The observed separation factor was: $K'_{lavendamycin}=5.3$.

TLC System and Classical Column Chromatography

Lavendamycin exhibited an Rf 0.3 (brick-red spot) upon development with toluene - acetonitrile - trifluoroacetic acid, 70: 20: 10 (v/v), using 250 μ silica gel GHLF Uniplates (Analtech, Inc.). For preparative column chromatography, solubility was maximized while maintaining adequate resolution through the use of higher proportions of acetonitrile and trifluoroacetic acid. For example, crude material (5.3 g) was chromatographed using a 5 cm ID×45 cm glass column (Fischer and Porter Co.) slurry packed with Woelm Act III silica gel (70~230 mesh) in toluene - acetonitrile - trifluoroacetic acid, 40: 40: 20 (v/v), at a flow rate of 10 ml/minute. This yielded 0.4 g enriched lavendamycin. Accumulated enriched lavendamycin fractions (0.7 g) were reprocessed through a freshly packed column (50 cm ID×45 cm) using a toluene - acetonitrile - trifluoroacetic acid, 50: 30: 20 (v/v), solvent system to yield 453 mg of lavendamycin (**1**); m.p. >300°C (dec.); M.S. *m/z* 398; UV λ_{max}^{MeOH} (a) 239 (49.2), 246 (49.8), 391 (21.1) nm, $\lambda_{max}^{MeOH-HCl}$ (a) 252 (47.4), 277 (36.0), 385 (19.0) nm, $\lambda_{max}^{MeOH-NaOH}$ (a) 245 (94.1), 309 (42.3), 390 (39.6) nm; IR ν_{max}^{KBr} 2800~3800, 1740, 1692, 1610 (strong), 1590 cm^{-1}.

Anal. Calcd. for $C_{22}H_{14}N_4O_4 \cdot H_2O$: C 63.46, H 3.87, N 13.45
Found: C 63.14, H 3.74, N 13.27

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