STUDIES ON THE IONOPHOROUS ANTIBIOTICS. XXIV[1]

LEUSERAMYCIN, A NEW POLYETHER ANTIBIOTIC PRODUCED BY *STREPTOMYCES HYGROSCOPICUS*

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A new antibiotic leuseramycin has been isolated from the cultured mycelium of the strain of *Streptomyces hygroscopicus* TM-531. Physicochemical data, in particular those of ^{1}H- and ^{13}C-NMR spectra, revealed that leuseramycin is closely related to dianemycin in its structure, the former having a methyl group in place of the hydroxymethyl group at C-30. It is active against Gram-positive bacteria, some phytopathogenic fungi and some protozoa.

In the course of screening for antibacterial substances two polyether antibiotics from *Streptomyces* sp. TM-531 were isolated. One of them was identified as dianemycin[2,3] and the other was found to be a new antibiotic, which we designated leuseramycin. Although most polyether antibiotics are not suitable for clinical use, some of them are finding an increasing role in the veterinary field as coccidiostats in poultry breeding and as growth promotors in ruminants such as cattle and sheep[4].

This paper is concerned with the taxonomy and the cultivation of strain TM-531 as well as the isolation, structure and some biological properties of leuseramycin. The antiprotozoal activity of this antibiotic will be reported elsewhere.

Taxonomy

The strain TM-531 was isolated from a soil sample obtained in Omiya City, Saitama Prefecture, Japan. After cultivation on oat meal agar at 28°C for 14 days, it showed the following morphological characteristics.

Vegetative mycelium fully developed with branching. Aerial mycelium branches monopodically with a spiral spore chain formed at the end of the branch (Fig. 1). Spore is oval to short cylindrical ($0.8 \sim 1.0\ \mu \times 1.3 \sim 1.6\ \mu$) and has a rough surface (Fig. 2). Sporangium, flagellated spore and sclerotium are not observed on oat meal agar.

Cultural characteristics studied on various media according to WAKSMAN[5], and GOTTLIEB and SHIRLING[6] are shown in Table 1. Physiological characteristics of the strain TM-531 are summarized in Table 2.

These characteristics indicate that the strain TM-531 belongs to the species of *Streptomyces hygroscopicus* WAKSMAN and HENRICI (1948), and it was named *Streptomyces hygroscopicus* TM-531.

Fig. 1. Photomicrograph of the strain TM-531.
(Oat meal agar, 14 days, 600×)

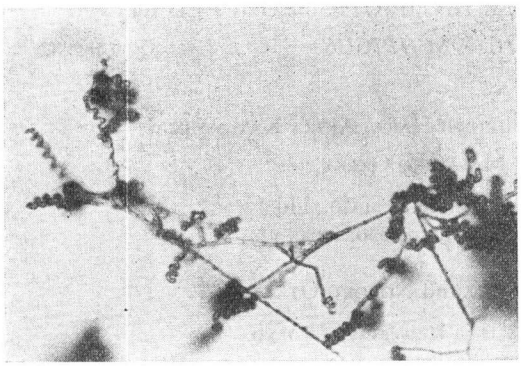


Fig. 2. Electromicrograph of spores of the strain TM-531.
(Oat meal agar, 14 days, 5,000×)

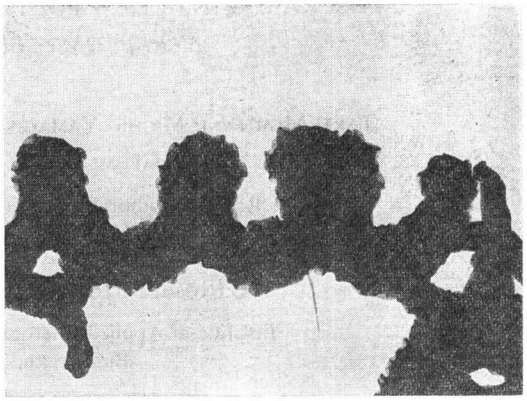


Table 1. Cultural characteristics of the strain TM-531.

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose-nitrate agar	Good	None	Pale yellowish-brown	None
Glucose-asparagine agar	Moderate	None or poor	Pale yellow	None
Inorganic salts-starch agar	Good	Good; grayish-brown; becomes moist and exhibits dark brown patches	White to pale gray	None
Glycerol-asparagine agar	Good	Good; pale yellow to grayish-brown	Pale yellow to gray	None
Tyrosine agar	Good	Good; pale yellow to grayish-brown	Pale yellow to pale gray	Pale brown
Peptone-yeast extract iron agar	Moderate	None or poor	Pale yellowish-brown	None
Nutrient agar	Poor	None	Pale yellow	None
Yeast extract-malt extract agar	Good	Good; pale yellow to grayish-brown; becomes moist and exhibits dark brown patches	Pale yellow to pale gray	None
Oat meal agar	Good	Good; grayish-brown; becomes moist and exhibits dark brown patches	White to pale gray	None

Cultivation

Stock slants of the strain TM-531 with heavy sporulation were prepared by cultivating the organism on oat meal agar at 30°C for 7 days. A liquid seed-medium (pH 7.0) containing 2% of oat meal and glucose, 0.3% of meat extract, NaCl and $CaCO_3$, and 0.04% of $Fe_2(SO_4)_3$ and $MnCl_2$ was inoculated with spores of the stock culture and incubated on a reciprocal shaker at 30°C for 72 hours. The seed was inoculated into the main-cultivation medium of the same composition as mentioned above at a rate of 2% (v/v).

Fermentation was carried out in a 250-liter fermentor containing 200 liters of the medium at 30°C for 72 hours with aeration at 200 liters per minute and agitation at 150 r.p.m. The antibiotics

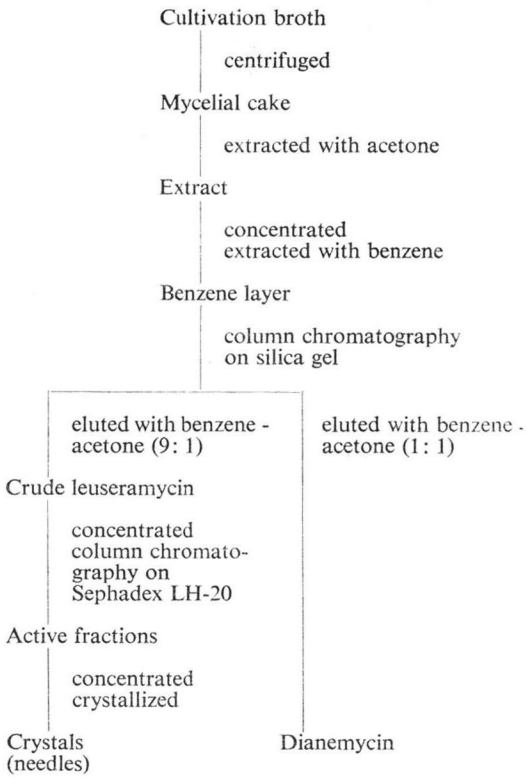
Table 2. Physiological characteristics of the strain TM-531.

Property observed	Characteristics
Temperature (growth)	18~45°C
(optimum)	28~35°C
Liquefaction of gelatin	Positive
Hydrolysis of starch	Positive
Peptonization of milk	Positive
Coagulation of milk	Negative
Production of melanoid pigment	Negative
Reduction of nitrate	Positive
Liquefaction of LOEFFLER's coagulated serum	Positive
Assimilation of carbon sources*	(Assimilates) L-arabinose, D-xylose, D-glucose, D-mannose, D-fructose, L-rhamnose, raffinose, D-mannitol, maltose, galactose, starch, inulin, lactose, sucrose, inositol and trehalose.

* Examined on PRIDHAM-GOTTLIEB agar.

were accumulated mainly on the mycelium.

Fig. 3. Isolation and purification of leuseramycin.



Isolation

The procedure for the isolation of leuseramycin is shown in Fig. 3. After separation of the supernatant from 200 liters of the cultured broth by centrifugation, 5.0 kg of mycelial cake were extracted twice with 20 liters of acetone. The extracts were combined and concentrated *in vacuo* until the acetone was removed. The residue was extracted twice with 2 liters of benzene, followed by drying of the benzene extract over anhydrous Na_2SO_4. The combined extracts were concentrated to 500 ml and applied to a silica gel column (2,000 ml) packed with benzene. The column was developed with benzene to remove inactive oils and was subsequently developed with a mixture of benzene and acetone (9: 1). The active fractions, monitored by T.L.C. using vanillin-H_2SO_4 spray, were combined and concentrated to give a yellowish powder. One tenth of the powder was dissolved in 10 ml of acetone and applied to a Sephadex LH-20 column (500 ml) equilibrated with acetone. The fractions containing leuseramycin were pooled and concentrated to *ca.* 25 ml. Addition of an equal volume of diluted HCl (10^{-3} M) produced crystals of leuseramycin. The crystals were dissolved and recrystallized from acetone - water (2: 1) to give 1.0 g of needle crystals.

Physicochemical Properties

Leuseramycin is soluble in benzene, chloroform and acetone, slightly soluble in *n*-hexane, methanol and ethanol, and practically insoluble in water. Some physicochemical properties of leuseramycin are listed in Table 3. The UV and IR spectra of leuseramycin, shown in Fig. 4 and Fig. 5, respectively,

Table 3. Physicochemical properties of leuseramycin (free acid).

Nature	white needle
Melting point	88~91°C
Specific rotation	+81.2° (*c* 1, $CHCl_3$)
$[\alpha]_D^{26}$	+43.6° (*c* 0.5, MeOH)
Molecular weight	843±10*
Formula	$C_{47}H_{78}O_{13}$ (851.1)
Elemental analysis	
Found	C: 66.40, H: 9.49, O: 24.25
Calcd.	C: 66.32, H: 9.24, O: 24.44
UV absorption	λ_{max} 232 nm, $E_{1cm}^{1\%}$ 177.6 (EtOH)
Rf values on silica-gel TLC	0.22 (ethyl acetate)
	0.24 (benzene - acetone, 2: 1)
	0.35 (*n*-hexane - acetone, 2: 1)

* Vapor pressure osmometry

Fig. 4. UV spectrum of free acid of leuseramycin (EtOH).

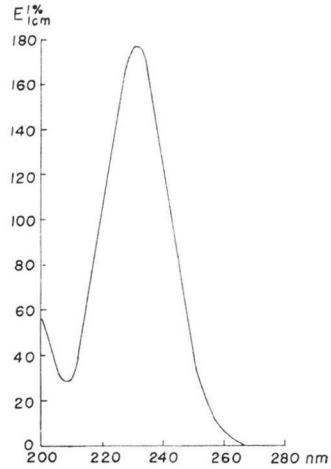


Fig. 5. IR spectrum of free acid of leuseramycin (KBr).

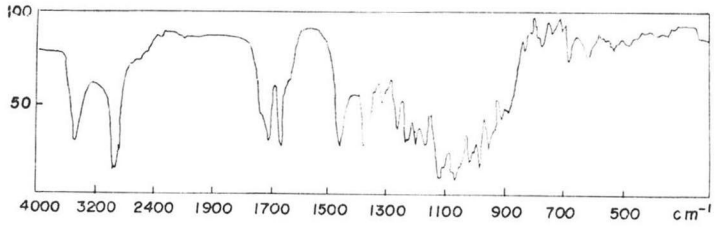
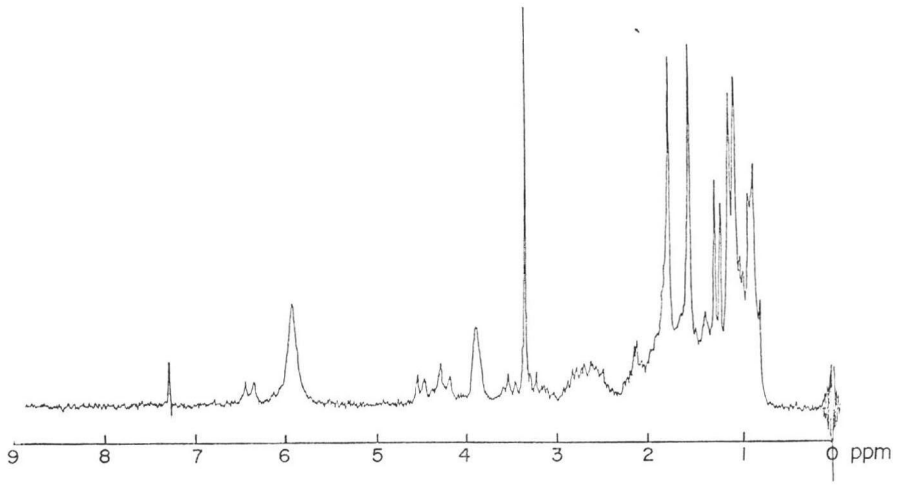


Fig. 6. ^{1}H-NMR spectrum of free acid of leuseramycin ($CDCl_3$).



suggest the presence of an α,β-unsaturated carbonyl system. The 100 MHz ^{1}H-NMR spectrum of leuseramycin, shown in Fig. 6, suggests the presence of one $-OCH_3$ (δ 3.35, s), $-CH-CH=C$ (δ 6.40, d) and $-CH=C-CH_3$ (δ 1.78, s).

Structure

Both physicochemical properties and ^{1}H-NMR spectrum of leuseramycin suggest that this antibiotic is very similar to dianemycin whose structure has been studied by HAMILL *et al.*[2] and CZERWINSKI *et al.*[3] The structural elucidation was performed by detailed comparison of the ^{13}C-NMR spectra of leuseramycin and dianemycin (Table 4). The assignment of ^{13}C-signals of dianemycin has been already elucidated by ŌTAKE *et al.*[7] Two of the forty seven signals observed in the spectrum of leuseramycin are apparently different from those of dianemycin in their chemical shifts. The methylene signal at 65.9 ppm due to C-30 of dianemycin disappears and a new methyl signal is observed at 28.3 ppm in the spectrum of leuseramycin. This change indicates that the hydroxymethyl group at C-30 of the former is replaced by a methyl group in the latter.

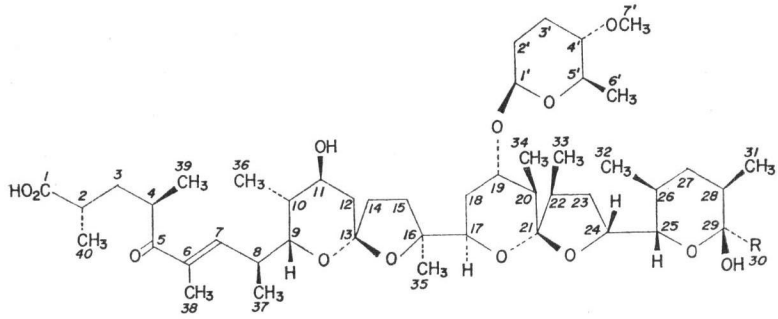
The same substitution can also explain that the methine signal at 34.8 ppm due to C-28 of dianemycin is shifted to 39.0 ppm in leuseramycin. This conclusion agrees with the elemental analysis and is supported by the empirical rules in ^{13}C-NMR spectroscopy for the structural elucidation of polyether antibiotics[8]. Thus, the structure of leuseramycin is illustrated as shown in Fig. 7. The structural relationship between leuseramycin and dianemycin is compared with that between grisorixin[9] and nigericin[10].

Table 4. ^{13}C-NMR spectra of leuseramycin and dianemycin (free acid).

Carbon number	Functional group	Leusera-mycin (ppm)	Dianemycin (ppm)	Carbon number	Functional group	Leusera-mycin (ppm)	Dianemycin (ppm)
1	$-CO_2H$	179.2	179.3	25	–CH(O)	74.5	73.9
2	$-CH(CH_3)$	37.1	37.5	26	$-CH(CH_3)$	32.5	32.9
3	$-CH_2$	40.1	40.4	27	$-CH_2$	38.1	37.3
4	$-CH(CH_3)$	36.8	36.6	28	$-CH(CH_3)$	39.0	34.8
5	–C=O	204.4	204.8	29	–O–C–OH	98.7	98.2
6	$=C(CH_3)$	134.2	134.2	30		28.3 ($-CH_3$)	65.9 ($-CH_2OH$)
7	CH	144.9	144.9	31	$-CH_3$	17.2	17.3
8	$-CH(CH_3)$	38.1	37.8	32	$-CH_3$	17.8	17.8
9	–CH(O)	69.6	69.8	33	$-CH_3$	16.5	16.3
10	$-CH(CH_3)$	36.8	36.6	34	$-CH_3$	13.6	13.4
11	–CH(OH)	70.6	70.6	35	$-CH_3$	27.1	27.0
12	$-CH_2$	33.9	33.7	36	$-CH_3$	10.4	10.4
13	–O–C–O	107.0	107.1	37	$-CH_3$	14.5	14.7
14	$-CH_2$	39.7	39.5	38	$-CH_3$	11.3	11.3
15	$-CH_2$	32.8	32.7	39	$-CH_3$	16.5	16.3
16	$-CO(CH_3)$	87.0	86.9	40	$-CH_3$	18.6	18.4
17	–CH(O)	75.4	75.7	1′	–O–CH–O	101.9	102.2
18	$-CH_2$	25.2	25.2	2′	$-CH_2$	30.7	30.8
19	–CH(O)	80.1	80.1	3′	$-CH_2$	27.7	27.1
20	$-CH(CH_3)$	34.4	34.0	4′	–CH(O)	80.1	80.2
21	–O–C–O	109.0	109.6	5′	–CH(O)	74.6	74.5
22	$-CH(CH_3)$	35.8	36.1	6′	$-CH_3$	18.4	18.4
23	$-CH_2$	29.4	29.9	7′	$-OCH_3$	56.8	56.8
24	–CH(O)	76.9	77.2				

^{13}C-NMR spectra were taken on a JEOL FX-100 spectrometer operating at 25.05 MHz in $CDCl_3$ solution. Chemical shifts are expressed in ppm from internal TMS.

Fig. 7. Structures of leuseramycin and dianemycin.



Leuseramycin: R=CH_3

Dianemycin : R=CH_2OH

Table 5. Antibacterial spectrum of leuseramycin.

Organisms	MIC (mcg/ml)
Staphylococcus aureus FDA 209P	1.56
Staphylococcus aureus Smith	6.25
Staphylococcus aureus TPR-23 (SA-, PC-, TC-, SM-, KM-, CP- and Mac-R)	3.13
Staphylococcus epidermidis TPR-25 (SA-, PC-, TC-, SM-, KM-, CP- and Mac-R)	3.13
Bacillus subtilis ATCC 6633	1.56
Bacillus licheniformis	1.56
Micrococcus luteus NIHJ	3.13
Corynebacterium xerosis NCTC 9755	1.56
Streptococcus faecalis ATCC 8043	6.25
Escherichia coli NIHJ	>100
Pseudomonas aeruginosa P-32	>100
Proteus vulgaris HX-19	>100

Abbreviations: Mac: macrolide, R: resistant strain.
Medium: heart-infusion agar.
Incubation: 24 hours at 37°C.

Table 6. Antifungal spectrum of leuseramycin.

Organisms	Mycelial development (Diameter of colony, mm)	
	Control	Leuseramycin
Glomerella cingulata	27.3	13.4
Pyricularia sasakii	63.3	63.0
Gibberella fujikuroi	27.6	20.2
Botrytis cinerea	47.7	46.7
Helminthosporium sativum	22.8	15.4

Medium: potato-dextrose agar.
Antibiotic concentration: 100 mcg/ml.
Inoculation was performed as follows: The potato-dextrose agar plate, on which the test fungus grew enough, was cut off circularly, 8 mm by diameter, and this agar block was put on the test plate.
Incubation: 7 days at 30°C.

Biological Properties

Leuseramycin has activity against a wide range of Gram-positive bacteria and certain phytopathogenic fungi but is inactive to Gram-negative bacteria. No cross-resistance was observed between leuseramycin and any known antibiotics tested which belong to the groups of macrolide, aminoglycoside, tetracycline and β-lactam. The antibacterial spectrum *in vitro*, assayed by the agar dilution method, is given in Table 5. The antifungal activity *in vitro*, determined by the inhibition of mycelial development, is given in Table 6. The acute toxicity of leuseramycin administered intraperitoneally to *ddY* mice is 37.2 mg/kg (LD_{50}).

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