

FERENSIMYCINS A AND B, TWO POLYETHER ANTIBIOTICS
TAXONOMY, FERMENTATION, ISOLATION, CHARACTERIZATION
AND STRUCTURAL STUDIES

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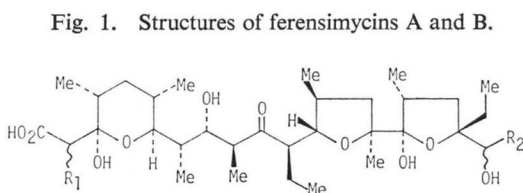
Ferensimycin A* (I), $C_{34}H_{59}O_{10}Na$, mp $133\sim 135^{\circ}C$, and ferensimycin B** (II), $C_{35}H_{61}O_{10}Na$, mp $143\sim 145^{\circ}C$, were isolated as their sodium salts from the fermentation broth of *Streptomyces* sp. No. 5057, a strain similar to *Streptomyces myxogenes* Shōmura *et al.* The physicochemical data of I and II showed that they are both closely related congeners of lysocellin (III). Ferensimycins A and B exhibit activity against Gram-positive bacteria and are effective in the treatment of coccidiosis of fowl.

During the course of a screening program for new antibiotics, a streptomycete, strain No. 5057, was found to produce two new antibiotics which were designated as ferensimycins A (I) and B (II). These antibiotics were purified by alumina or silica gel chromatography of the solvent-extract of the fermentation broth and were obtained as their sodium salts. Ferensimycins A, B and their sodium salts are easily soluble in most organic solvents but insoluble in water. Their physicochemical characteristics showed that ferensimycins A and B are new members of the polyether antibiotics. Their structures (Fig. 1) have been elucidated by mass, 1H and ^{13}C NMR spectra.

This paper deals with the taxonomic studies of the producing organism, fermentation process, isolation, physicochemical and biological properties of ferensimycins A and B and their structural studies.

Taxonomy of Producing Strain

Strain 5057 was isolated from a garden soil collected in Okayama-shi, Okayama Prefecture, Japan, in December of 1974. The strain has been deposited at The Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, and KCC Culture Collection of Actinomycetes, where it has been assigned accession number FERM-P No. 3464 and KCC U-0247, respectively.



Ferensimycin A: $R_1=CH_3$ $R_2=CH_3$
Ferensimycin B: $R_1=CH_3$ $R_2=CH_2CH_3$

* Formerly called antibiotic No. 5057-A. Japan Patent 54-8,641, 1979.

** Formerly called antibiotic No. 5057-B. Presented at the 57th Annual Meeting of Agric. Chem. Soc. Japan, Abstracts p. 166, Tokyo, April 1, 1982.

Procedures recommended in the International Streptomyces Project (ISP)¹⁾ were employed for the taxonomic characterization. Scanning electron micrographs (SEM) were made with an electron microscope (Model MSM-101, Hitachi-Akashi, 30KV, specimen angle 0°). Specimens were coated with gold in an ion-coater (Model IB-3, Eiko). The whole-cell hydrolysate analysis of the strain was performed by the method of LECHEVALIER and LECHEVALIER.²⁾ Paper chromatography showed that LL-diaminopimelic acid and glycine are present. No diagnostic sugars were observed. The strain 5057 is believed to be of cell wall Type I.

The vegetative mycelium does not fragment into coccoid or bacillary elements. The aerial mycelium is alternatively branched and terminates in short coils (Fig. 2). Spores were oblong to cylindrical, about $0.6 \times 0.9 \mu\text{m}$ in size with smooth surface (Fig. 3). The strain abundantly produces slime on glycerol - asparagine agar (ISP #5), tyrosine agar (ISP #7) and BENNETT's agar. On the above media, the growth surface was covered with a lot of slime and aerial mycelia could not be recognized with naked eye. The appearance of the slimy cultures is the same as that of bacteria (Fig. 4). On other media, strain 5057 produced slime in certain amounts and many pseudosporangia were observed as the slime accumulated (Fig. 5).

True sporangia, flagellated spores, sclerotia, synnemata and true verticils were not observed. The cultural characteristics of strain 5057 are shown in Table 1. The agar plates were incubated at 28°C,

Fig. 2. Aerial mycelium of strain 5057.

Coiled terminals on inorganic salts-starch agar, 28°C for 4 days (SEM). Short white bar 5.0 μm .

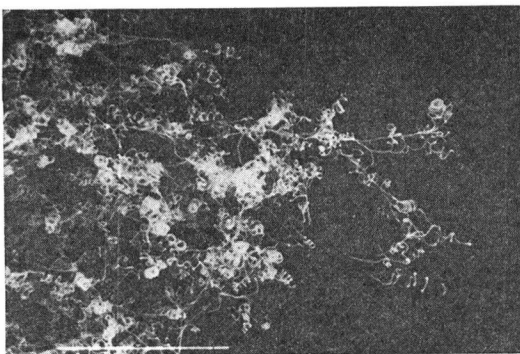


Fig. 3. Smooth spores of strain 5057 on inorganic salts - starch agar, at 27°C, 2 weeks (TEM, bar 1 μm).

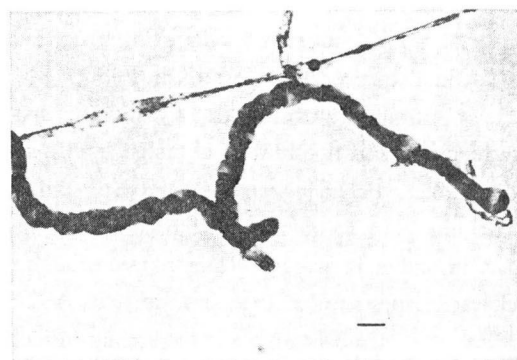


Fig. 4. Slimy appearance of strain 5057. (BENNETT's agar, 28°C, 10 days).

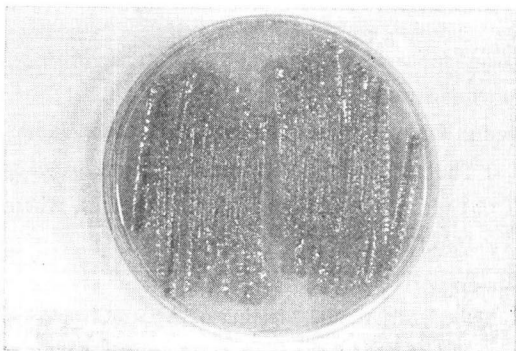


Fig. 5. Pseudosporangium formation of strain 5057 on inorganic salts - starch agar. 28°C, 2 weeks (SEM, short white bar 5.0 μm).

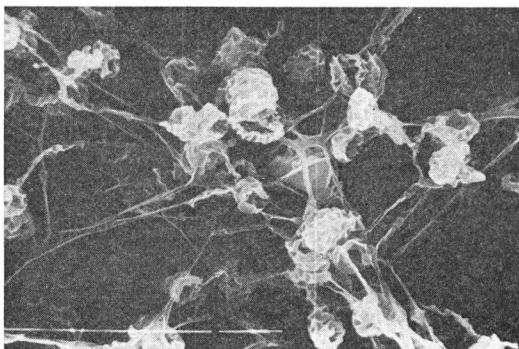


Table 1. Cultural characteristics of strain 5057.

	Growth	Aerial mycelium	Growth color	Soluble pigment	Slime formation
Sucrose nitrate agar	Poor	None	3 ba*	None	
Glucose asparagine agar	Moderate	Poor		Faint cinnamon	
Glycerol asparagine agar (ISP #5)	Good	None (covered with slime)	6½ pi	5 le	Strong
Inorganic salts-starch agar (ISP #4)	Good	Moderate to good, off white to 3 ih	5 pg ~ 5 pl	Light brown	
Tyrosine agar (ISP #7)	Good	Scant, around the growth	6 pl ~ 6 pn	Dark brown	Strong
Nutrient agar (Difco)	Poor	None		Dark brown	
Yeast malt agar (ISP #2)	Moderate	Poor	5 pi	Light brown, 4 ng	Moderate
Oatmeal agar (ISP #8)	Moderate	Good, 3 fe (hygroscopic)	3 ec	None	
BENNETT's agar	Abundant	None (covered with slime)	5 pi	Dark brown	Strong

* The color schema used was that taken the Color Harmony Manual, 4th ed., 1958 (Container Corporation of America, Chicago)

Table 2. Physiological characteristics of strain 5057.

Melanin production on Tyrosine agar (ISP #7)	Positive	Adenine	Positive
Peptone - yeast extract iron agar (ISP #6)	Positive	Salt tolerance (Yeast starch agar +0, 4, 7, 10 & 13% NaCl)	<4%
Tryptone yeast extract broth (ISP #1)	Positive	Temperature relationship (Yeast malt agar, ISP #2, thermogradient incubator, 14 days)	Optimum: 23 ~ 30°C No growth at 13°C and 42°C
Melanin formation agar (Waksman #42)	Positive	Carbon utilization (Pridham and Gottlieb basal, ISP #9)	
Hydrolysis of Starch (ISP #4)	Positive	+ ~ ++ :	D-Glucose, D-xylose, D-fructose, lactose, melibiose, glycerol, <i>i</i> -inositol
Gelatin (glucose peptone gelatin, 23°C, 4 weeks)	Negative		
Skim milk (27°C and 37°C)	Peptonized	— ~ ± :	L-Arabinose, sucrose, rhamnose, melezitose, D-mannitol, dulcitol, sorbitol, inulin, salicin, 0.5% aesculin
Solubility of (basal agar, Difco Nutrient agar)			
Tyrosine	Positive		
Xanthine	Positive		
Hypoxanthine	Positive		

and results were recorded after 21 days of incubation. The physiological reactions of strain 5057 are summarized in Table 2. Slime production on several agar media showed that the strain is an unusual myxogenic streptomycete.

Based on its characteristics, strain 5057 is considered to be a myxogenic strain of the genus *Streptomyces* Waksman and Henrici 1943 nom. approb. The data in this study indicate that strain 5057 closely resembles *Streptomyces myxogenes* Shōmura and Niida non nom. approb.³⁾ Some differences between characteristics of strain 5057 and *S. myxogenes* are shown in Table 3. However, differences are not enough to conclude that these two strains are distinct species and strain 5057 is considered to represent a new subspecies of *S. myxogenes*.

Table 3. Comparison of taxonomic characteristics of strain 5057 and *Streptomyces myxogenes* non nom. approb.

	5057	<i>S. myxogenes</i> SF-1130 ³⁾
Spore-chain morphology	<i>Spirals</i>	<i>Spirals</i>
Spore surface	Smooth	Smooth
Shape and size of spores	Oblong to cylindrical 0.6×0.9 μm	Oblong to cylindrical 0.6~0.7×1.0 μm
Aerial mass color	White to brownish gray	White to gray
Color of vegetative growth	Colorless to dark brown	Colorless to dark grayish brown
Presence of aerial mycelium on		
Sucrose nitrate agar	—	+
Glucose asparagine agar	+	—
Tyrosine agar	+	—
Melanine formation	Positive	Positive
Hydrolysis of		
Gelatin	Negative	Positive
Skim milk (peptonization)	Positive	Negative
Slime formation		
Glucose asparagine agar	—	+
Glycerol asparagine agar	++	—
Starch agar	—	+
Oatmeal agar	—	+
BENNETT's agar	++	
Carbon utilization		
Positive:	D-Glucose, D-fructose, D-xylose, raffinose, <i>i</i> -inositol	D-Glucose, D-xylose, <i>i</i> -inositol, raffinose
Doubtful or negative:	L-Arabinose, rhamnase, D-mannitol	L-Arabinose, D-fructose, D-mannitol, rhamnase
Antibiotic	Polyether	Oligosaccharide

Table 4. Preliminary taxonomic comparison between strain 5057 and three strains studied.

	5057	79-M-2-53	T-142	C37-87
Spore-chain morphology	<i>Spirals</i>	<i>Spirals</i>	<i>Rectiflexibilis</i>	<i>Rectiflexibilis</i>
Spore surface	Smooth	Spiny	Smooth	Smooth
Length of spore chain	Moderate	Moderate to long	Short	Short
Presence of pseudosporangium	Abundant	None	None	None
Slime	Abundant	Usually none	Usually none	Usually none
Isomer of A ₂ pm	LL	LL	LL	LL

Since the name *S. myxogenes*, however, has not been found in the Approved Lists of Bacterial Names and its supplements, strain 5057 is tentatively identified as *Streptomyces* sp. No. 5057.

Three additional streptomycetes, strain 79-M-2-53, T-142 and C37-87 have been independently isolated from soil samples collected in Japan, and they were found to be new producers of antibiotic 5057. Some taxonomic characteristics of the three strains are shown in Table 4 in comparison with strain 5057. It seems that strains T-142 and C37-87 are taxonomically identical, however, these strains are distinguished from *Streptomyces* sp. No. 5057 in many aspects.

Production and Isolation

Ten 500-ml flasks each containing 100 ml of the following medium, were inoculated with a loopful of the culture from an agar slope, and were incubated with shaking at 30°C for 48 hours. The medium, whose pH was adjusted to 6.0 before sterilization, contained: 6.0% soluble starch, 2.5% soybean meal, 0.5% dry yeast, 0.3% ammonium sulfate and 0.3% calcium carbonate. One liter of the above seed culture was transferred into a 200-liter tank containing 100 liters of the same medium. The fermentation was carried out at 30°C for 96 hours with aeration (100 liters/minute) and agitation (250 r.p.m.). Potency was assayed by the paper disc method using *Staphylococcus aureus* FDA 209P JC-1 as the test organism. A typical time course of the fermentation is shown in Fig. 6.

The fermentation broth was filtered after addition of filter aid. The acetone extract of the mycelium was concentrated *in vacuo* and the aqueous residue was combined with the filtered broth. The mixture was extracted with an equal volume of ethyl acetate after adjustment to pH 9.0 with 5 N NaOH, and the extract was concentrated *in vacuo* to leave an oily substance. The residue was dissolved in a mixture of *n*-hexane - ethyl acetate (2: 1) and was applied to an alumina column packed with *n*-hexane. The column was washed with a *n*-hexane - ethyl acetate mixture (2: 1) and then was eluted with *n*-hexane - ethyl acetate (1: 1). Active fractions were collected and evaporated to dryness. The antibiotics were purified by chromatography on a silica gel column which was developed with chloroform containing 5% methanol. The rich fractions were combined and concentrated to dryness. The dry residue was crystallized from a small amount of a mixture of chloroform and ethyl acetate. The mixture of the two components, I and II, was further separated by chromatography on Lobar column (E. Merck): the column was developed with *n*-hexane - ethyl acetate (1: 2), to elute first II and then I. The active fractions were respectively concentrated *in vacuo*, and the dry residues were crystallized from chloroform - ethyl acetate mixtures. Ferensimycins A and B sodium salts were obtained by the procedure described above.

Physicochemical and Biological Properties

Antibiotics I and II showed very similar chemical and physicochemical properties. They were soluble in lower alcohols, acetone, ethyl acetate, benzene, chloroform and ethyl ether but insoluble in water. They gave positive color reactions with 2,4-dinitrophenylhydrazine and DRAGENDORFF reagents (even though they do not contain nitrogen atoms) but gave negative reaction to vanillin - H₂SO₄ or KMnO₄. The physicochemical properties of the sodium salts of I and II are summarized in Table 5. The ultraviolet and infrared absorption spectra of I and II sodium salts are shown in Figs. 7 and 8, respectively.

The minimum inhibitory concentrations (MIC) of I and II against a variety of microorganisms

Fig. 6. Time course of the production of ferensimycins A and B.

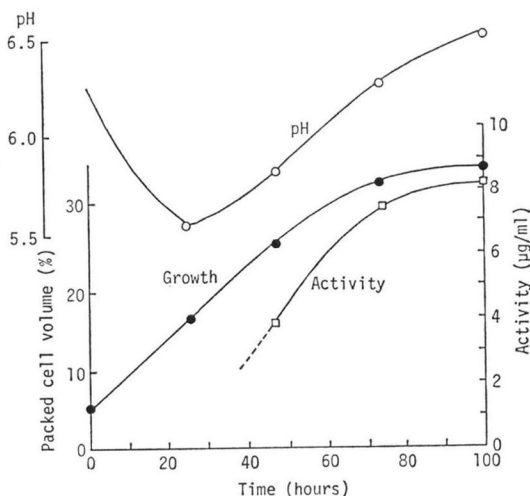


Table 5. Properties of ferensimycins A and B (Na).

	Ferensimycin A (I)	Ferensimycin B (II)
Appearance	Colorless needles	Colorless needles
Melting point	133~135°C	143~145°C
Specific rotation $[\alpha]_D^{25}$	+1.0° (c 1, MeOH)	+5.5° (c 1, CHCl ₃)
Elemental analysis: Found	C 62.74, H 9.45, O 25.14, Na 3.64	C 59.96, H 9.05, O 27.60, Na 3.41
Calcd. for	C ₃₄ H ₅₉ O ₁₀ Na C 62.77, H 9.08, O 24.62, Na 3.54	C ₃₈ H ₆₁ O ₁₀ Na·2H ₂ O C 60.00, H 9.29, O 27.43, Na 3.29
Silica gel TLC: Rf value		
<i>n</i> -Hexane - EtOAc (1:2)	0.56	0.62
Benzene - EtOAc (1:1)	0.28	0.37
UV absorption: λ_{max} (E _{1cm} ^{1%})	292 nm (0.76) in MeOH	291 nm (0.66) in CHCl ₃
Color reaction: Positive	2,4-Dinitrophenylhydrazine, DRAGENDORFF, I ₂ vapor	
Negative	KMnO ₄ , Vanillin-H ₂ SO ₄	

Table 6. Antimicrobial activities of ferensimycins A and B (Na).

Test organisms	MIC (μ g/ml)		Medium
	Ferensimycin A	Ferensimycin B	
<i>Bacillus subtilis</i> PCI-219	0.39	0.5	N*
<i>Staphylococcus aureus</i> FDA-209P JC-1	0.78	0.5	N
<i>Staphylococcus aureus</i> (resistant)**	0.78	0.5	N
<i>Micrococcus flavus</i> IFO-3242	0.78	—	N
<i>Mycobacterium smegmatis</i> ATCC 607	6.25	5.0	GN
<i>Escherichia coli</i> NIHJ-J2	50	100	N
<i>Proteus vulgaris</i>	50	100	N
<i>Piricularia oryzae</i>	12.5	50	P
<i>Cochliobolus miyabianus</i>	25	50	P

* N: Nutrient agar. GN: Glycerin nutrient agar. P: Potato sucrose agar.

** Resistant to penicillin, streptomycin, chloramphenicol, tetracycline, erythromycin, kanamycin, sulfonamide.

are given in Table 6. MIC values were determined by using the serial agar dilution method. These antibiotics were found to be active against Gram-positive bacteria but inactive against Gram-negative bacteria and fungi.

The acute toxicity of I and II in mice was also examined. The LD₅₀ (i.p.) values of I and II were 30~50 mg/kg and 50 mg/kg, respectively. Oral toxicity of II was 500 mg/kg (LD₅₀).

Structure of I and II

The structural elucidation of I and II was performed by comparing their mass, ¹H and ¹³C

Fig. 7. UV Spectra of ferensimycins A and B.

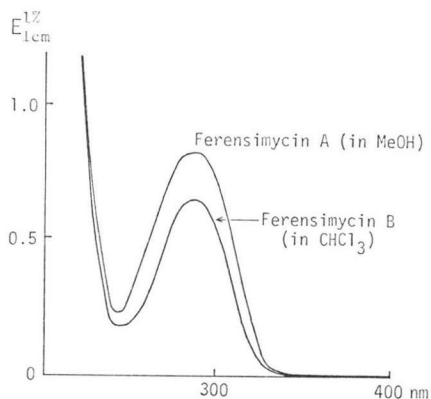


Fig. 8. IR Spectra of ferensimycins A(I) and B(II) Na salt (KBr).

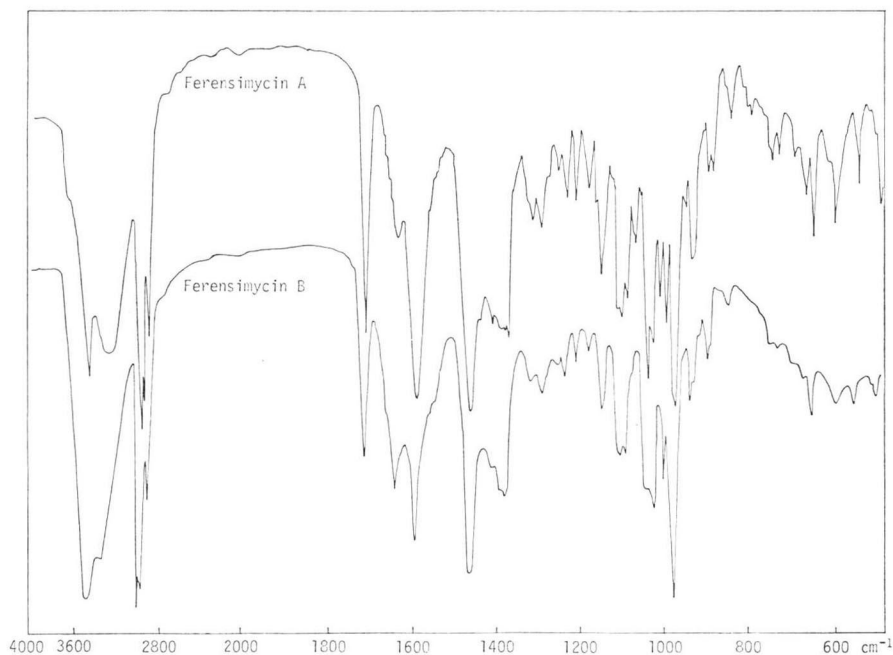
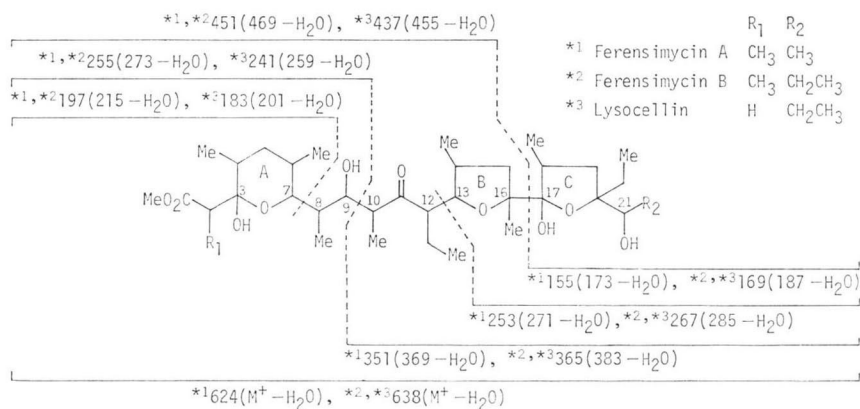


Fig. 9. Diagnostic fragmentation of ferensimycins A, B and lysocellin (methyl ester).



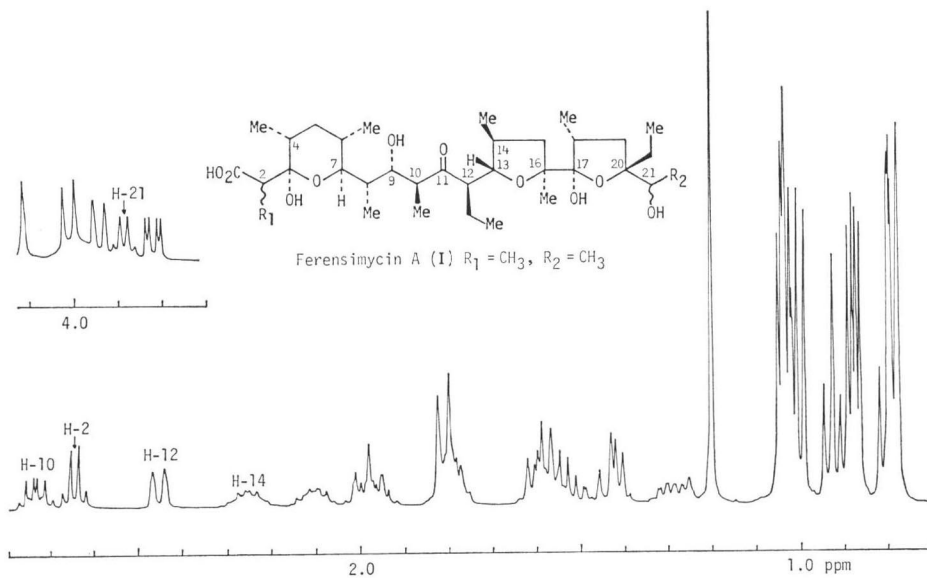
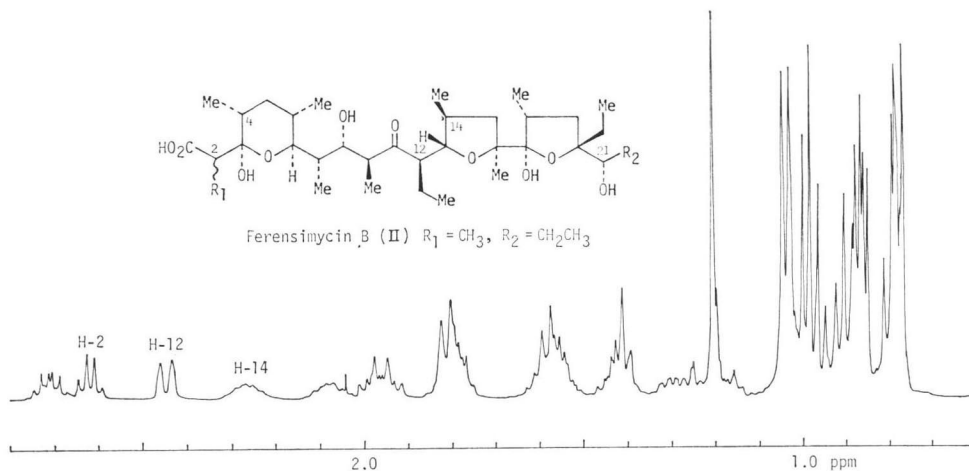
NMR spectra to those of lysocellin (**III**),^{4,5)} which is an antibiotic structurally very similar to **I** and **II**.

1) Mass Spectra (EI)

Instead of the M^+ ion of the methyl esters of **I**, **II** and **III** dehydration peaks were observed in their mass spectra, with m/z values of 624, 638 and 624, respectively. The fragmentation patterns are shown in Fig. 9. The fragment containing the ring A, which occurs by the cleavage of the C7-C8 bond, is larger for both **I** and **II** than **III** by 14 mass units. Scission at the C16-C17 bond produces fragments containing the C ring which are 14 mass units larger for both **II** and **III**, than for **I**.

2) ¹H NMR Spectra

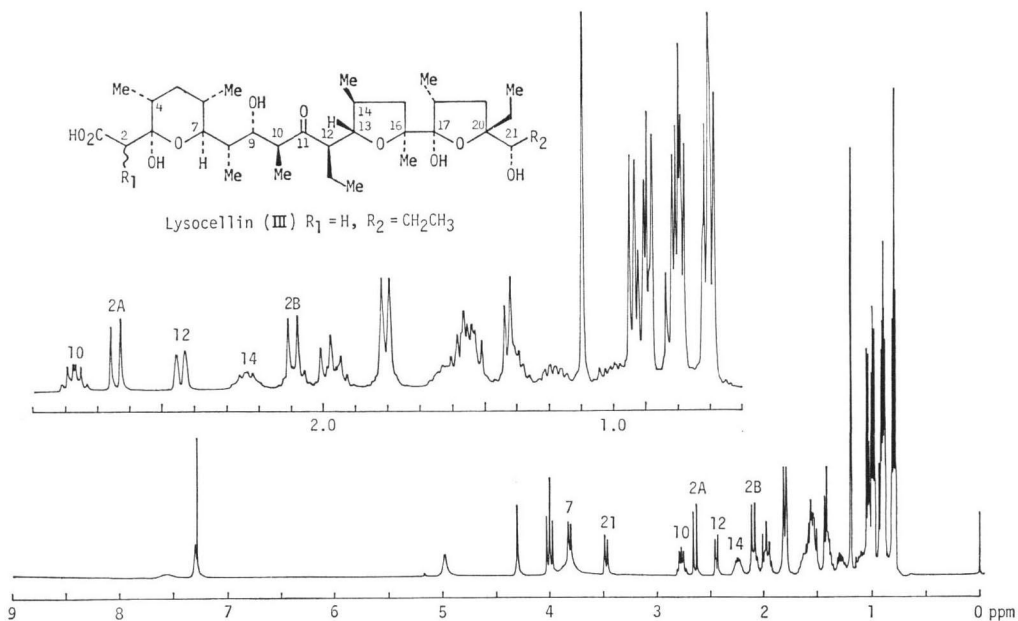
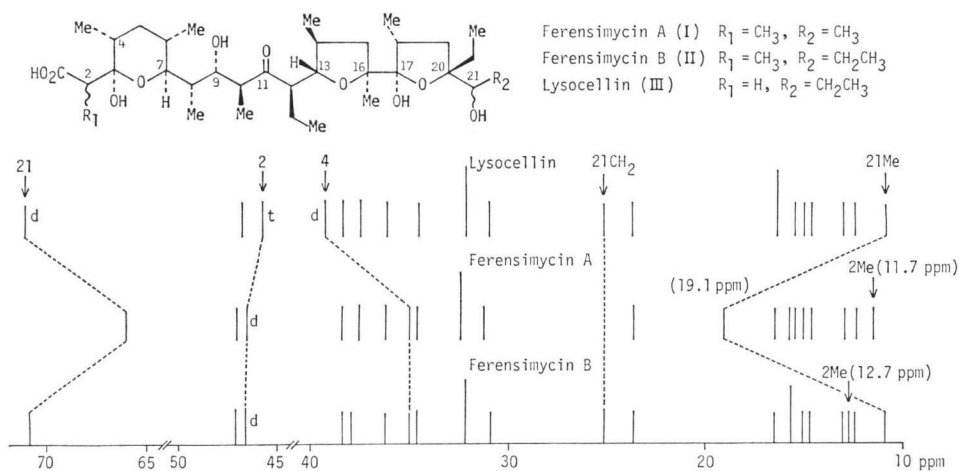
Figs. 10, 11 and 12 show the 400 MNz ¹H NMR spectra of the Na salts of **I**, **II** and **III** in CDCl₃,

Fig. 10. ^1H NMR spectrum of ferensimycin A (I) Na salt (400 MHz in CDCl_3).Fig. 11. ^1H NMR spectrum of ferensimycin B (II) Na salt (400 MHz in CDCl_3).

respectively. Comparison of these shows that the two H-2 and H-21 doublets of **III** are replaced by two quartets centered at 2.64 ppm (H-2) and 3.88 ppm (H-21) in **I**. Spin decoupling experiments proved these peaks to be connected to doublets at 1.0264 ppm (2-Me proton) and 1.0325 ppm (21-Me proton), respectively. Similarly, the quartet centered at 2.62 ppm for H-2 in **II** becomes a singlet upon irradiation of a doublet at 1.04 ppm (2-Me proton).

3) ^{13}C NMR Spectra

The assignment of signals in the ^{13}C NMR spectra is usually facilitated by the selective proton decoupling technique which necessitates unequivocal assignments of proton peaks to be irradiated in the ^1H NMR spectra. The chemical shifts of the ^{13}C NMR spectra of the Na salts of **I**, **II** and **III** in CDCl_3 are shown in Table 7 and their graphical comparison in Fig. 13. Upon going from **III** to **I**,

Fig. 12. ^1H NMR spectrum of lysocellin (III) Na salt (400 MHz in CDCl_3).Fig. 13. Pertinent region of ^{13}C NMR spectra of ferensimycins A(I), B(II) and lysocellin (III).

the following changes are observed: a) two new methyl signals are produced at 11.7 and 19.1 ppm, b) the C-21 methylene peak is lost while the C-21 methyne resonance is shifted upfield, c) the C-2 methylene peak for **III** is replaced by a methyne peak at 46.7 ppm and d) the C-4 methyne peak shifts upfield.

Similarly, going from **III** to **II** a) produces a new methyl peak at 12.7 ppm, b) C-2 methylene peak becomes a methyne group, and c) the C-4 methyne peak shift upfield.

The position of the new methyl groups in molecules **I** and **II** were revealed by selective proton decoupling. Irradiation of the 2-Me protons (1.04 ppm) of **II** gives a sharp singlet at 12.7 ppm and, therefore, the peak can be attributed unambiguously to 2-Me. Because of the extreme proximity of

Table 7. ^{13}C NMR chemical shifts of ferensimycins A, B and lysocellin (Na).

Carbon	Functionality	Ferensimycin A (ppm)	Ferensimycin B (ppm)	Lysocellin (ppm)
1	COOH	181.5	181.5	178.1
2	CH ₂	—	—	45.9
2	CH	46.7	46.7	—
3	O-C-O	100.1	100.4	98.0
4	CH	35.0	34.9	39.2
5	CH ₂	36.2	36.3	36.3
6	CH	32.4	32.3	32.2
7	CH-O	80.3	80.4	79.5
8	CH	32.4	32.3	32.2
9	CH-O	76.4	76.4	76.0
10	CH	47.1	47.2	47.1
11	C=O	215.0	214.6	214.7
12	CH	54.9	54.8	54.9
13	CH-O	83.9	83.9	83.3
14	CH	34.9	34.7	34.8
15	CH ₂	42.7	42.7	42.5
16	C-O	86.3	86.4	85.9
17	O-C-O	108.5	108.4	107.8
18	CH	38.3	38.2	38.4
19	CH ₂	37.5	38.0	37.5
20	C-O	87.4	87.5	86.9
21	CH-OH	66.1	71.0	71.4
2-Me		11.6	12.7	—
4-Me		15.7	15.7	16.6
6-Me		16.6	16.6	16.6
8-Me		12.5	12.5	12.6
10-Me		13.0	12.9	12.8
12-Me		5.1	4.8	5.4
14-Me		15.6	15.7	15.5
16-Me		23.9	24.0	23.9
18-Me		14.8	14.8	14.7
20-Me		7.1	7.0	7.2
21-Me		19.1	10.9	11.3
12-CH ₂		15.1	15.2	14.9
20-CH ₂		31.3	30.8	31.0
21-CH ₂		—	25.6	25.5

the two methyl groups (2-Me and 21-Me, 1.0264 and 1.0325 ppm, respectively) in **I** selective irradiation is unsuccessful, but comparison with **II** indicate that the methyl peak at 11.7 ppm, is to be ascribed to the 2-methyl and the 19.1 ppm methyl peak to the 21-methyl. The upfield shift of the C-4 methyne upon going from **III** to **I** or **II** is due to the γ -effect produced by the substitution of the C-2 proton by the methyl group.

The structures of **I** and **II** have been thus assigned as shown in Fig. 1, in accord with the results of analysis of their mass spectra. The stereochemistry of C-2 and C-21 in **I**, and C-2 in **II** including the absolute configuration of **I** and **II** is now under investigation.

Note Added in Proof

After submission of this paper, we were informed from Dr. JOHN W. WESTLEY of Hoffmann-La Roche Inc. that he independently isolated **I** and that the configurations of C-2 and C-21 were determined to be both (*S*) by X-ray analysis. Taking into accounts of the almost completely identical chemical shifts of carbon signals around C-2, the stereochemistry of C-2 of **II** should be identical with that of **I**.

Acknowledgment

The authors are indebted to Dr. J. W. WESTLEY for information on the structure of the polyether antibiotic identical with ferensimycin A prior to publication.

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