ANTIBIOTIC NO. 6016, A POLYETHER ANTIBIOTIC

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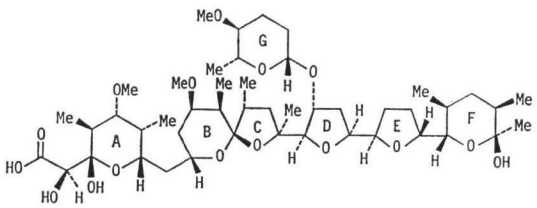
A new polyether antibiotic, No. 6016, was isolated from the culture of *Streptomyces albus* strain No. 6016. The antibiotic was obtained as colorless prisms having a molecular formula of $C_{46}H_{77}O_{16}Na$, m.p. 192~195°C (dec.), and has only end absorption in ultraviolet region. The infrared and NMR spectra of the antibiotic suggest the presence of carbonyl and methoxyl groups. The antibiotic No. 6016 exhibits activity against Gram-positive bacteria including mycobacteria and is effective in the treatment of coccidiosis of fowl.

In the course of our screening program for new antibiotics, the strain No. 6016, which was identified as a strain of *Streptomyces albus*, was found to produce a new antibiotic. The substance was designated as antibiotic No. 6016. The antibiotic was extracted from the fermentation broth with organic solvents, isolated by chromatography on alumina or silica gel, and finally purified as the sodium salt. Antibiotic No. 6016 and its sodium salt are easily soluble in most organic solvents but insoluble in water. From its physico-chemical characteristics, antibiotic No. 6016 was found to be a new member of the polyether group of antibiotics. The entire structure of antibiotic No. 6016 has been established recently with X-ray analysis of its thallium salt by ŌTAKE *et al.*[1] as shown in Fig. 1.

Fig. 1. Structure of antibiotic No. 6016.



This paper deals with the taxonomic studies of the producing organism, production, isolation and the physico-chemical and biological properties of antibiotic No. 6016.

Taxonomy

The strain 6016 was isolated from a soil sample in December of 1975. The organism grows well on many standard media including the ISP media that are recommended by SHIRLING and GOTTLIEB[2] for the description of streptomycete cultures.

The vegetative mycelium does not fragment into coccoid or bacillary elements. The aerial mycelium is simply branched and terminates in long open coils (Fig. 2). Sporangia, flagellated spores, sclerotia, cormia, and true verticils were not observed. Spores were oblong to cylindrical, 0.6~0.7×0.8~0.9 μm, with smooth surface (Fig. 3). LL-Diaminopimelic acid and glycine are detected in its whole-cell hydrolysates by method of LECHEVALIER *et al.*[3].

The cultural characteristics of the strain 6016 on various media are shown in Table 1. The agar plates were incubated at 28°C, and the results were recorded after 21 days of incubation. The physiological reactions of strain 6016 are summarized in Table 2.

Fig. 2. Strain 6016. Spore chains.
Yeast-starch agar, 28°C, 14 days. ×1,200.

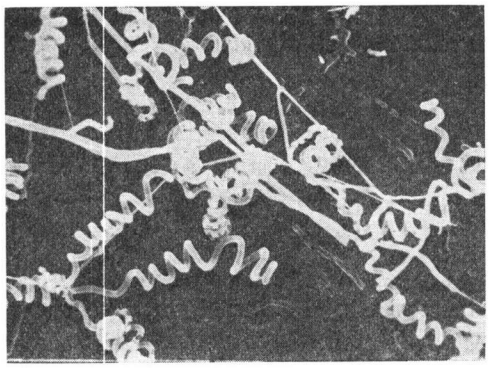


Fig. 3. Strain 6016. Spore chains.
Glucose-asparagin agar, 28°C, 14 days ×9,600.

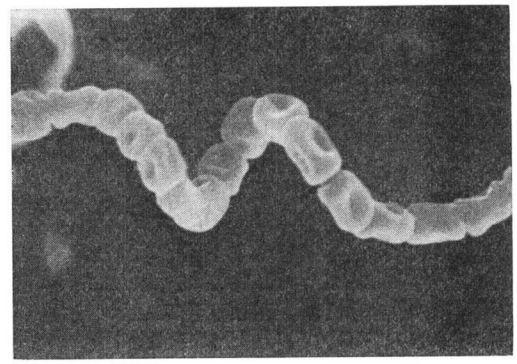


Table 1. Cultural characteristics of strain 6016.

Medium	Growth	Aerial mycelium	Color of reverse side colony	Soluble pigment
Sucrose-nitrate agar	Moderate	Moderate, white	Ivory (2 fb)*	None
Glucose-asparagine agar	Moderate	Moderate to good, white	Bamboo, Buff; Straw; Wheat (2 fb)	None
Glycerol-asparagine agar (ISP #5)	Good	Good, white	2 fb	None
Inorganic salt-starch agar (ISP #4)	Good	Good, white	2 fb to Bamboo; Chamois (2 gc)	None
Tyrosine agar (ISP #7)	Moderate	Good, white	2 fb	None
Yeast extract-malt extract agar (ISP #2)	Abundant	Abundant, white	Honey Gold; Lt. Gold (2 ic)	Faint yellow
Oatmeal agar (ISP #3)	Abundant	Abundant, white	Lt. Ivory; Egg Shell (2 ca)	None
Nutrient agar (Difco)	Good	Moderate, white	2 fb	None
Yeast-starch agar	Moderate to good	Good, white	2 fb	None

* Color Harmony Manual, 4th edition, 1958.

Table 2. Physiological characteristics of strain 6016.

Melanoid production on	
Tyrosine agar (ISP #7)	negative
Peptone-yeast extract iron agar (ISP #6)	negative
Tryptone-yeast extract broth (ISP #1)	negative
Melanin formation agar stab (Waksman #42)	negative
Hydrolysis of	
Starch (Inorganic salts-starch agar, ISP #4)	positive (weak)
Gelatin (Glucose-peptone-gelatin)	positive
Skim milk	coagulated and peptonized
Salt tolerance (BENNETT's agar +0, 4, 7, 10 & 13 % NaCl)	≧13 %
Temperature relationship (Yeast-starch agar, thermogradient incubator)	Optimum: 21~38°C. No growth at 10°C and 45°C
Carbon utilization (PRIDHAM and GOTTLIEB basal, ISP #9)	++: D-glucose, L-arabinose, D-fructose, rhamnose, D-mannitol +: D-xylose, sucrose, raffinose ±: *i*-inositol −: no carbon
Whole cell hydrolysate	LL-diaminopimelic acid and glycine, type I cell wall

Based on its characteristics, the strain 6016 is considered to be a strain of *Streptomyces albus* sensu HÜTTER[4]. The taxonomic characters of strain 6016 were compared with *S. albus* from the description of HÜTTER's monograph[4], SHIRLING and GOTTLIEB[5] and BERGEY's Manual, 8th edition[6] (Table 3).

Production and Isolation of Antibiotic No. 6016

To prepare seed culture, ten 500 ml flasks, each containing 100 ml medium, were inoculated with a loopful of culture from agar medium and were incubated with shaking at 30°C for 48 hours. The medium, with pH adjusted to 7.0 before sterilization, contained; glucose 1.5%; soluble starch 1.5%; soybean meal 2.0%; dry yeast 0.5% and sodium chloride 0.2%. One liter, of seed culture was transferred into a 200-liters tank containing 100 liters of the following medium: soluble starch 6.0%, soybean meal 2.0%, dry yeast 0.2%, and sodium chloride 0.2%. The pH was adjusted to 7.0 with 1 N NaOH before sterilization. The fermentation was carried out at 30°C for 90 hours with aeration (100 liters per minute) and agitation (250 r.p.m.). Potency was assayed by the paper disc method using a *Bacillus subtilis* PCI-219 as a test organism. A typical time course of fermentation is shown in Fig. 4. The active substance was isolated both from the mycelium as well as from filtered broth.

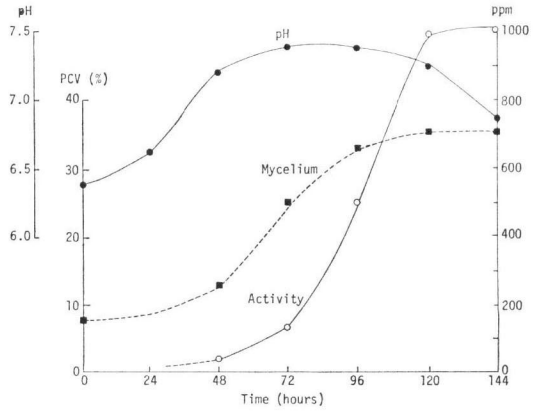
The fermentation broth was filtered after addition of filter aid. The mycelium was extracted with 50% aqueous acetone. The extract was concentrated *in vacuo* and residued aqueous solution was combined with filtered broth. The solution, after adjustment to pH 9.0 with 5 N NaOH, was extracted

Table 3. Comparison of taxonomic characteristics of the strain 6016 and *Streptomyces albus*.

	6016	*Streptomyces albus*		
		Sensu HÜTTER[4]	ISP description[5]	BERGEY's Manual[6]
Spore-chain morphology	spirales	spirales	spirales	spirales
Spore surface	smooth	smooth or slightly warty	smooth	smooth
Shape and size of spores	oblong to cylindrical, 0.6~0.7×0.8~0.9 μm	oblong, 0.8~1.4× 0.4~0.9 μm		
Aerial mass color	white	chalk white; pale yellowish white to pale grayish white	white or yellow color series	white
Color of vegetative mycelium	pale yellowish	yellowish white, light yellow, rarely brownish yellow to light gray	pale yellow	
Melanin	negative	negative	negative	negative
NaCl tolerance	≧13 %			≧13 %, but <15 %
Growth on CZAPEK's agar	moderate			poor
Carbon utilization	Positive: D-glucose, L-arabinose, D-fructose, rhamnose, D-mannitol Weak growth: D-xylose, sucrose, raffinose Trace: *i*-inositol		Positive: D-glucose, D-xylose, D-mannitol, D-fructose No growth or trace: *i*-inositol Variable: L-arabinose, raffinose	Positive: D-glucose, D-xylose, D-mannitol Trace: D-fructose Negative: L-arabinose, L-rhamnose, raffinose, *i*-inositol

with an equal volume of ethyl acetate. The solvent extract was concentrated *in vacuo* to an oily substance. The residue was dissolved in *n*-hexane - ethyl acetate mixture (2 : 1), and then applied onto the top of the alumina column packed with *n*-hexane. The column was washed in *n*-hexane - ethyl acetate mixture (2 : 1) and then eluated with in *n*-hexane - ethyl acetate mixture (1 : 1). The active fractions were collected and evaporated to dryness. The antibiotic was further purified by chromatography on a silica gel column, which was developed with chloroform containing 5% methanol. The fractions containing the antibiotic were combined and concentrated to dryness. The dry residue was dissolved in a small amount of *n*-hexane - ethyl acetate mixture and chilled in a refrigerator until crystallization was completed. The crude crystals of antibiotic No. 6016 were filtered, washed with *n*-hexane and recrystallized from *n*-hexane - ethyl acetate mixture. By the above described isolation procedure, the sodium salt of antibiotic No. 6016 was obtained in the form of colorless prisms.

Fig. 4. Time course of antibiotic No. 6016 production.
PCV: Packed cell volume %.



Properties of Antibiotic No. 6016

The sodium salt of antibiotic No. 6016 decomposed at 192~195°C. Optical rotation of the sodium salt showed, $[\alpha]_D^{25}$ −42.5° (*c* 1.0, MeOH). Elemental analysis gave the following values: C 60.50, H 8.61, O 28.06, Na 2.77% (calcd. for $C_{46}H_{77}O_{16}Na$; C 60.79, H 8.47, O 28.19, Na 2.53%).

Titration in 66% ethanol indicated that antibiotic No. 6016 is a monocarboxylic acid with pKá of

Fig. 5. Infrared absorption spectrum of antibiotic No. 6016 Na salt (KBr).

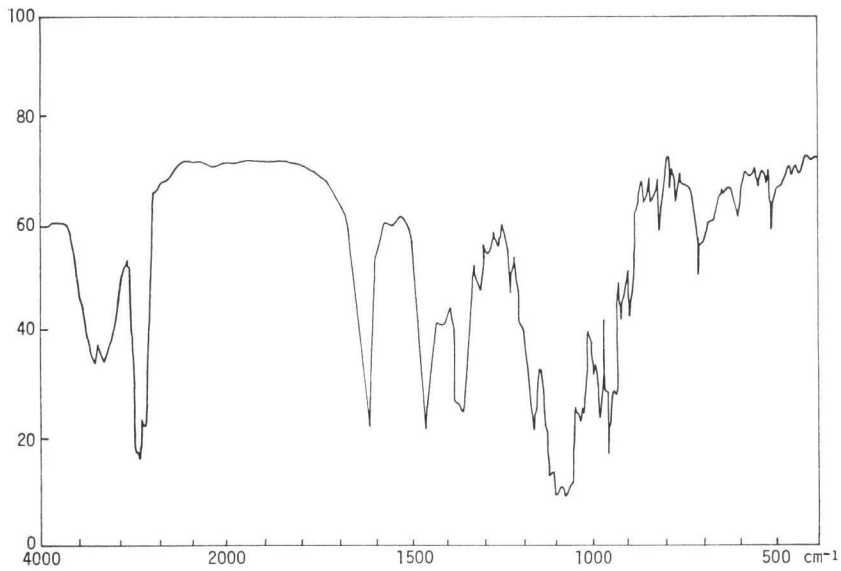
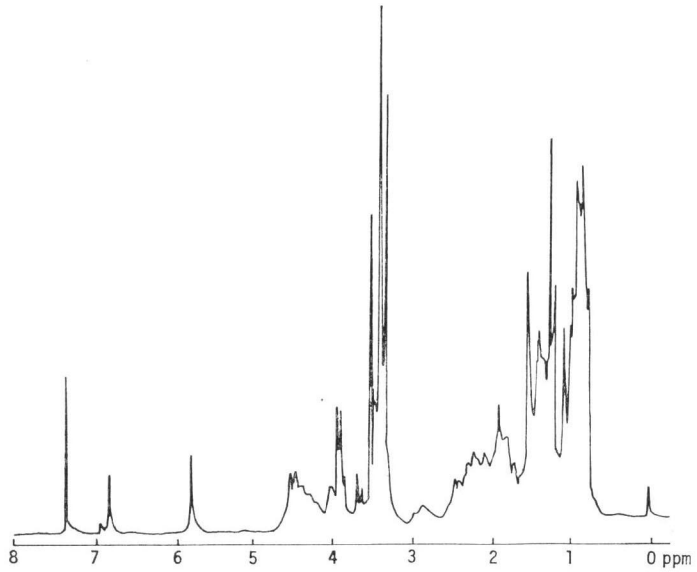


Fig. 6. NMR Spectrum of antibiotic No. 6016 sodium salt in $CDCl_3$ (100 MHz).



3.8. It is soluble in alcohols, acetone, ethyl ether, chloroform and ethyl acetate, but insoluble in water. It is stable as the sodium salt but unstable under acidic conditions.

Ultraviolet spectrum in methanol showed only end absorption. Infrared absorption spectrum in KBr disc is shown in Fig. 5. Nuclear magnetic resonance at 100 MHz in deuterochloroform exhibited three methoxyl groups (Fig. 6). On the thin-layer chromatograms of silica gel, the following Rf values were observed: 0.40 with benzene - ethyl acetate (1 : 1), 0.43 with chloroform - methanol (20 : 1), 0.46 with chloroform - acetone (5: 1) and 0.28 with benzene - acetone (5: 1).

The minimum inhibitory concentration (MIC) of antibiotic No. 6016 for a variety of microorganisms is given in Table 4. Determination of MIC was carried out using the serial agar dilution method. Antibiotic No. 6016 is active against Gram-positive bacteria including mycobacteria. No activity was observed against Gram-negative bacteria and fungi.

The acute toxicity of antibiotic No. 6016 in mice was examined. The LD_{50} was 23 mg/kg intraperitoneally and 63 mg/kg orally.

Table 4. Antimicrobial activity of antibiotic No. 6016 Na salt.

Test organisms	MIC (mcg/ml)	Medium*
Bacillus subtilis PCI-219	1.56	N
Bacillus cereus IAM-1729Y	0.39	N
Staphylococcus aureus FDA-209P-JC-1	1.56	N
Staphylococcus aureus (resistant)**	1.56	N
Micrococcus flavus IFO-3242	0.78	N
Escherichia coli NIHJ-JC-2	100	N
Klebsiella pneumoniae	100	N
Proteus vulgaris OX-19	100	N
Mycobacterium avium IFO-3153	0.78	GN
Mycobacterium smegmatis ATCC-607	0.78	GN
Alternaria kikuchiana	100	P
Diaporthe citri	12.5	P
Pyricularia oryzae	100	P

* N: nutrient agar
GN: glycerin nutrient agar
P: potato sucrose agar

** Resistant to streptomycin, erythromycin, leucomycin and penicillin.

The anticoccidial evaluation of antibiotic No. 6016 was carried out with 9-days-old chickens in-

fected with *Eimeria tenella* oocyst. The test was continued for 8 days. Antibiotic No. 6016 was effective in reducing mortality of chickens and increasing average body weight of treated infected chickens compared to untreated infected controls. The anticoccidial evaluation of No. 6016 is under investigation and will be published later in a separate paper.

Discussion

A strain of *Streptomyces albus* produces an antibiotic, designated as No. 6016, which is suggested to have three methoxyl groups in the molecule and only end absorption in ultraviolet region. Antibiotic No. 6016 was compared with other polyether antibiotics which have no characteristic ultraviolet absorption maxima and three methoxy groups in the molecule, such as A-28695B[7], A-204B[8], CP-38295[9], carriomycin[10]. These, however, are differentiated from each other in physico-chemical and biological properties.

Acknowledgment

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