SALINOMYCIN, A NEW POLYETHER ANTIBIOTIC

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(Received for publication August 12, 1974)

Salinomycin is a new polyether antibiotic produced by a strain of *Streptomyces albus* (ATCC 21838). The antibiotic is purified by solvent extraction followed by chromatography on alumina or silica gel. It is a weakly acidic compound and has the molecular formula $C_{42}H_{70}O_{11}$. Salinomycin exhibits activity against gram-positive bacteria including mycobacteria and some filamentous fungi, and is effective in the treatment of coccidial infection of poultry.

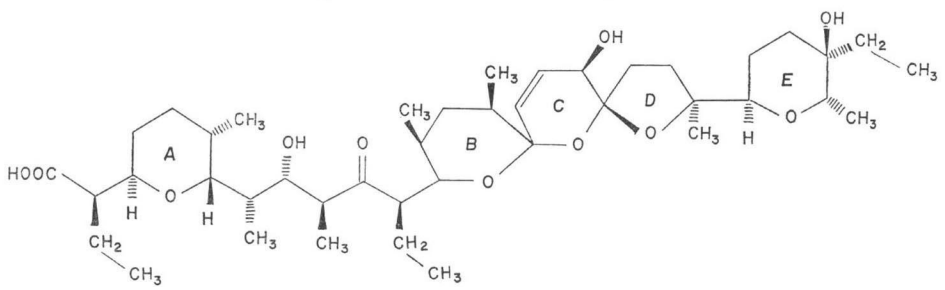
During the course of our screening program for new antibiotics, a new biologically active substance has been isolated from the culture broth of a strain of *Streptomyces* (No. 80614). This substance was designated as salinomycin.

Salinomycin is a member of the polyether antibiotics, which include nigericin[1], K-178[2], K-358[3], X-206[4], lasalocid (X-537A)[5], grisorixin[6], monensin[7], dianemycin[8] and A204A[9], but it can be differentiated clearly from the above compounds on the basis of the chemical and spectral properties.

As a result of taxonomical study, the salinomycin-producing organism was identified as a strain of *Streptomyces albus* (ROSSI-DORIA) WAKSMAN and HENRICI, and the type strain has been deposited in the Fermentation Research Institute, Chiba, Japan and American Type Culture Collection, Rockvill, Marlyland, U.S.A. and accessioned as FERM-P No. 419 and ATCC 21838, respectively.

This paper deals with the taxonomical studies of producing organism, production, isolation and the physico-chemical and biological properties of salinomycin. The structure of salinomycin has been established recently by X-ray crystallographic analysis of its *p*-iodophenacyl ester by KINASHI *et al.*[10] as shown in Fig. 1.

Fig. 1. Structure of salinomycin.



Characteristics of the Salinomycin-Producing Organism

The salinomycin-producing organism is a *Streptomyces* designated as the strain No. 80614, isolated from a soil sample collected at Fuji city, Shizuoka Prefecture, Japan. According to the taxonomical studies described below, this organism was identified with a strain of *Streptomyces albus*. It shows the following properties:

Morphological Characteristics

The morphology of the culture was microscopically observed on inorganic salts-starch agar and oatmeal agar, at 27°C for 10~14 days. The aerial mycelium of the strain No. 80614 branches and produces spirals of two or three volutions in sporophores. Formation of true whorls was not observed on electron microscope, the spores were ellipsoidal and cylindrical, 0.5~1.0 μ×1.0~1.5 μ. The surface of the spores was smooth.

Cultural and Physiological Characteristics

The cultural characteristics and physiological properties of the strain No. 80614 are shown in Tables 1, 2 and 3. All media used in this study were prepared according to the recom-

Table 1. Cultural characteristics of strain No. 80614.

Medium	Growth	Aerial mycelium	Soluble pigment
CZAPEK's agar	Good, raised, white to pale yellow	Poor, white	None
Starch inorganic salts agar	Poor or moderate, white to tan	Moderate, powdery, white to whitish gray	None
Glucose asparagine agar	Poor, thin, white to tan	Moderate to poor, velvety, white to whitish gray	None
Glycerine asparagine agar	Poor to moderate, tan	Poor to moderate, white	None
Calcium malate agar	Good, raised, white to pale tan	None or poor, white	None
Tyrosine agar	Poor, thin, white to pale brown	None	None or faint brown
Nutrient agar	Poor, thin, golden yellow	None or poor, white	None
Yeast malt extract agar	Good, yellowish brown	Good, white to yellowish white	Pale brown
Oatmeal agar	Poor, colorless	Poor to moderate, white to whitish gray	None or pale brown
Glucose nutrient agar	Poor, thin, yellowish white	None or poor, white	None
Glucose peptone agar	Poor, thin, yellowish white	None or poor, white	None or pale brown
Glycerol CZAPEK's agar	Good, raised, white to pale tan	Poor or none, white	None or pale brown
Potato plug	Poor, thin, brown	None or scanty, white to whitish gray	None
Cellulose	Scant, thin, colorless	None	None
Litmus milk	Ring growth in medium, colorless	None	Faint red
Egg	Poor, thin, yellowish white to white	None	None
LOEFFLER's serum medium (27°C 10 days)	Good, raised, brownish yellow	None	None

Table 2. Physiological properties of strain No. 80614.

Temperature for growth	21°C~37°C
pH range for growth	5.5~8.5
Tyrosinase reaction	negative
Melanoid pigment	negative
Reduction of nitrate	doubtful
Liquefaction of gelatin	positive
Coagulation of milk	negative
Peptonization of milk	negative
Hydrolysis of starch	positive
Cellulose decomposition	negative
Product	salinomycin

Table 3. Carbon utilization pattern of strain No. 80614.

Carbon source	Response*	Carbon source	Response*
Arabinose	±	Sucrose	±
Glucose	++	Trehalose	+
Galactose	++	Xylose	++
Lactose	+	Salicin	±
Levulose	++	Inulin	++
Mannose	±	Adnit	−
Maltose	±	Dulcit	−
Melezitose	−	*i*-Inosit	−
Melibiose	++	Mannit	++
Raffinose	−	Sorbit	−
Rhamnose	−	Cellobiose	++

*++: strongly positive utilization. +: positive utilization. ±: doubtful utilization. −: negative utilization.

mendation of WAKSMAN's monograph[11] and a report of International Streptomyces Project (ISP)[12].

Cultural and physiological studies were carried out at 27°C and the results were observed at 21st day unless otherwise stated. Utilization of carbon sources was investigated by the method of PRIDHAM and GOTTLIEB[13].

Comparison of Strain No. 80614 with Related *Streptomyces*

As a result of these observations, the following characteristics were noticed as distinctive feature of the strain No. 80614.

1) Spores are born in short spirals and spore surface is smooth.

2) Color of aerial mycelia is white to whitish gray. Color of vegetative growth is non-characteristics, colorless to pale yellow. No soluble pigment is produced on synthetic agar.

3) Melanoid pigment is not formed.

The strain No. 80614 was checked for identity with known species described in BERGEY's Manual[14], WAKSMAN's text book[11] and other literatures. As a result of the above research, the strain No. 80614 was closely related to *Streptomyces albus* (ROSSI-DORIA) WAKSMAN and HENRICI 1943. Therefore, the salinomycin-producing strain No. 80614 was identified as a strain of *Steptomyces albus* (ROSSI-DORIA) WAKSMAN and HENRICI.

Production and Isolation of Salinomycin

The production of salinomycin was carried out by a tank fermentation. *Streptomyces albus* was grown in a 500-ml shake flask containing 100 ml of the following culture medium; 4% glucose, 1% soybean meal, 1% beer yeast and 0.2% $CaCO_3$. The culture was kept for 48 hours on a rotary shaker at 28°C and the culture (1 liter) was seeded into a 200-liter tank fermentor containing 100 liters of the following medium: 2% glucose, 1% starch, 2.5% soybean meal, 0.4% beer yeast, 0.1% meat extract and 0.2% NaCl. The pH was adjusted to 8.5 with 1 N NaOH before sterilization. Incubation was carried out at 28°C for 90 hours under the standard condition of aeration and agitation.

The formation of salinomycin was followed by testing the antibiotic activity at various times during the fermentation. For this procedure, each sample was mixed with an equal volume of methanol and the mixture was shaken for 2 hours and filtered. The filtrate was assayed by paper disk method using *Bacillus subtilis* as a test organism.

A typical time course of fermentation is shown in Fig. 2. The active substance was isolated both from the mycelium as well as from the filtered broth. The fermentation broth was adjusted to pH 9.0 with 5 N NaOH and was filtered after the addition of filter aid. The separated mycelium was extracted with 80% aqueous acetone. The extract was concentrated *in vacuo* and the aqueous residue was adjusted to pH 9.0 with 3 N NaOH and extracted with butyl acetate. The filtered broth was extracted with butyl acetate. The solvent extracts were combined and concentrated to an oily residue under reduced pressure.

Fig. 2. A typical time course of antibiotic production.

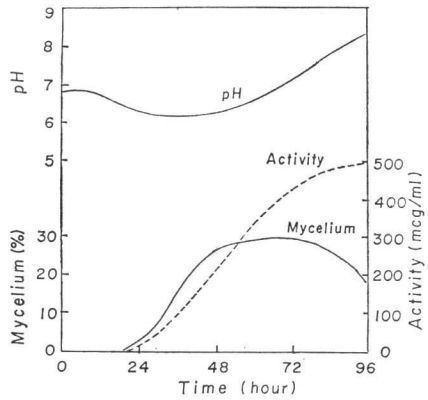


Table 4. Properties of salinomycin and sodium salt.

Property	Acid	Na salt
Melting point (°C)	112.5~113.5	140~142
$[\alpha]_{25}^{D}$ (*c* 1, ethanol)	−63°	−37°
UV absorption	285 nm(ε 108)	285 nm(ε 108)
IR absorption (carbonyl)	5.85	5.85 6.40
Molecular weight	750 (mass)	772 (mass)
Molecular formula	$C_{42}H_{70}O_{11}$	$C_{42}H_{69}O_{11}Na$
Elementary analysis	Calcd. Found	Calcd. Found
C:	67.17 67.18	65.26 65.01
H:	9.39 9.55	9.00 8.95
O:	23.43 23.13	22.76 22.39
Na:		2.97 2.80
pKa′	6.40 (DMF)	

The antibiotic was further purified by column chromatography on alumina and silica gel. The residue was applied onto the top of an alumina column packed with ethyl acetate-hexane mixture (3:1) and the column was washed with the same solvent system, and then developed with ethyl acetate-methanol mixture (3:1). The latter eluate, which contained the antibiotic, was concentrated *in vacuo*. The residue was dissolved in a small amount of chloroform and applied to a column of silica gel, which was developed with chloroform containing 4% methanol. The fractions containing the antibiotic were combined and concentrated to dryness. The dry residue was dissolved in a small amount of acetone - water mixture and chilled in a refrigerator until crystallization was completed. Crude crystals of salinomycin were filtered, washed with water and recrystallized from an acetone-water mixture and dried *in vacuo*. By the above-described isolation procedure, salinomycin was obtained in the form of colorless prism of the sodium salt.

The free acid of salinomycin could be obtained as white amorphous powder by adjusting the fermentation broth to pH 3.0 with 3 N HCl and following the previously described isolation procedure, or by shaking the ethyl acetate solution of salinomycin sodium salt with 0.1 N HCl. Both salinomycin and its sodium salt are readily convertible with each other, and extractable from water into organic solvents.

Properties of Salinomycin

Salinomycin free acid is a white amorphous powder. It is weakly acidic having a pKa′

value of 6.4 (DMF), and melts at 112.5~113.5°C. Salinomycin is levorotatory having optical rotation of −63° in ethanol solution. It is soluble in lower alcohols, acetone, ethyl acetate, benzene, chloroform, carbon tetrachloride, ether, petroleum ether and hexane, and insoluble in water. It is stable as the sodium salt but gradually loses antimicrobial activity under acidic conditions. Some of physico-chemical properties of salinomycin free acid and sodium salt are summarized in Table 4.

Elementary analysis of the salinomycin is as follows:

Calcd. for $C_{42}H_{70}O_{11}$ (M.W. 750):	C 67.17, H 9.39, O 23.43.
Found:	C 67.18, H 9.55, O 23.13.

The molecular weight of salinomycin was determined from the elementary analysis, mass spectrum and its derivatives. The mass spectrum of salinomycin showed the molecular ion peak at *m/e* 750 and a dehydration peak at *m/e* 732, which is good agreement with the molecular formula $C_{42}H_{70}O_{11}$ and the mass spectrum of its sodium salt exihibited the molecular ion peak at *m/e* 772, corresponding to the molecular formula $C_{42}H_{69}O_{11}Na$*.

Esterification of salinomycin with an excess of etherial diazomethane gave a crystalline monomethyl ester (m.p. 99~101°C. Found: C 67.35; H 9.28; O 22.85, Calcd. for $C_{43}H_{72}O_{11}$: C 67.54; H 9.42; O 23.03). Its mass spectrum showed the distinct molecular ion peak at *m/e* 764.

Treatment of salinomycin with acetic anhydride-pyridine gave a crystalline monoacetyl derivative (m.p. 148~150°C. Found: C 66.45; H 9.14, O 24.45; Calcd. for $C_{44}H_{72}O_{12}$: C, 66.66; H 9.09; O 24.24). Its mass spectrum gave 792 for the molecular weight.

The molecular weight and formula of salinomycin methyl ester and acetyl derivative, as determined by elementary analysis and mass spectrometry, are consistent with the formula of salinomycin.

The ultraviolet absorption spectrum of salinomycin showed a maximum of low intensity at 285 nm (ε 108), corresponding to a carbonyl group, in methanol solution, and at 285 nm (ε 218) in alkaline methanol solution, respectively. As shown in Fig. 3, the infrared absorption

Fig. 3. Infrared absorption spectrum of salinomycin and its sodium salt (KBr).

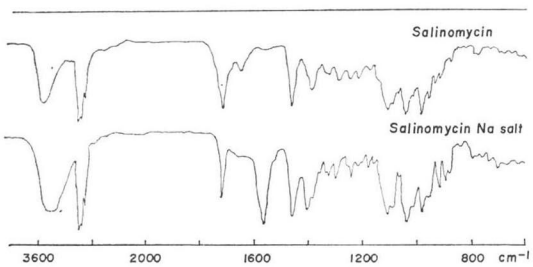
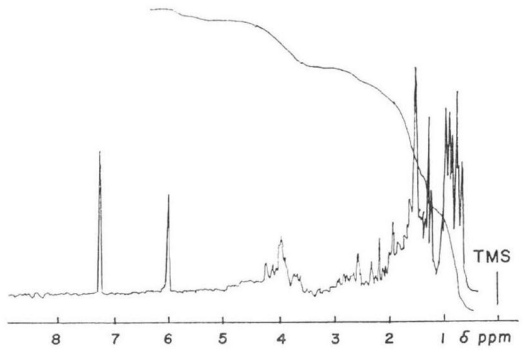


Fig. 4. NMR spectrum of salinomycin in $CDCl_3$ at 100 MHz.



* The previously reported molecular formula $C_{39}H_{65}O_{11}Na$[15] has been revised by H. KINASHI, N. ŌTAKE and H. YONEHARA[10].

spectra of salinomycin and its sodium salt showed bands at 3300~3500 cm^{-1}, 1710 cm^{-1} and 1560 cm^{-1} (in sodium salt), indicating the presence of hydroxyl, ketone and carboxyl groups.

The n.m.r. spectrum of salinomycin taken in deuterochloroform at 100 MHz is illustrated in Fig. 4. The spectrum suggested the presence of many C-methyl groups at δ 0.7~1.5, two unsplit vinyl protons at δ 6.0. In contrast to other polyether antibiotics, the absence of methoxyl signal was characteristic to salinomycin.

Biological Properties of Salinomycin

The antimicrobial activity of salinomycin was determined by the agar dilution method. The results are presented in Table 5. Salinomycin is active against gram-positive bacteria including mycobacteria and some filamentous fungi. No activity was observed against gram-

Table 5. Antimicrobial activity of salinomycin free acid.

Test organisms	Medium	M.I.C. (mcg/ml)
Bacillus subtilis PCI 219	N	3.12
cereus IFO 3466	N	0.78
circulans IFO 3329	N	3.12
megaterium IFO 3003	N	>100
Staphylococcus aureus FDA 209P	N	1.56
*aureus**	N	3.12
epidermidis IFO 3762	N	3.12
Sarcina lutea NIHJ	N	3.12
Micrococcus flavus IFO 3242	N	3.12
luteus IFO 2763	N	1.56
Mycobacterium smegmatis ATCC 607	GN	25.0
phlei IPCR	GN	12.5
avium IFO 3153	GN	12.5
Escherichia coli NIHJ, P-17	N	>100
Klebsiella pneumoniae PCI 602	N	>100
Proteus vulgaris OX-19	N	>100
morganii CCM 680	N	>100
Xanthomonas oryzae	SPP	50.0
citri NIAS	SPP	>100
Pseudomonas aeruginosa IFO 3445	N	>100
aureofaciens IFO 3756	N	>100
Candida albicans YU 1200	PS	>100
Piricularia oryzae	PS	25.0
Alternaria kikuchiana NIAS, A-14	PS	50.0
tenuis IFO 4026	PS	>100
Ophiobolus miyabeanus	PS	>100
Diaphorthe citri	PS	>100
Pellicularia filamentosa NIAS, C-37	PS	>100
Penicillium chrysogenum	PS	>100
Aspergillus niger IFO 6341	PS	>100
fumigatus IMA	PS	>100

* A resistant strain isolated from patient (resistant to streptomycin, erythromycin, chloramphenicol and tetracycline).

N: nutrient agar

GN: 4% glycerol nutrient agar

SPP: sucrose 20, potato 50, peptone 5, Na_2HPO_3 2, $NaNO_3$ 0.5 (g/liter).

PS: potato-sucrose agar.

negative bacteria or yeast.

The acute toxicity of salinomycin in mice was examined. The LD_{50} of salinomycin in mice was 18 mg/kg intraperitoneally and 50 mg/kg orally.

The anticoccidial estimation of salinomycin was carried out with 14 days-old chikens infected with *Eimeria tenella* oosyst. The test was continued for 9 days period. Salinomycin was effective in reducing mortality of chickens and increasing average body weight of treated infected chickens compared to those of untreated infected controls. The anticoccidial estimation of salinomycin is under progress and will be published later in a separate paper.

Discussion

A strain of *Streptomyces albus* produces an antibiotic, designated as salinomycin which is a new member of the polyether antibiotics. Salinomycin was compared with other polyether antibiotics, such as nigericin[1], K-178[2], K-358[3], lasalocid (X-537A)[5], grisorixin[6], monensin[7], dianemycin[8] and A204A[9]. It could be differentiated well on the basis of the melting point, optical rotation, molecular formula and UV, IR, NMR spectral properties.

Modifications of the fermentation conditions, nutrients balance in the medium, fermentation period, temperature, aeration and agitation were carried out to increase the salinomycin titer. During these experiments, two unknown biologically active substances were detected on the conditions of the high level of glucose (10 %) and soybean meal (5 %) in the early fermentation. These compounds are under study and will be published later.

As shown in Fig. 1, the structure of salinomycin has been established by X-ray crystallographic analysis of the *p*-iodophenacyl ester[10]. The molecule is pentacyclic; the *B*, *C* and *D* ring form a spiroketal moiety, a double bond is present in the C ring. Therefore, salinomycin is a new type of the polyether antibiotics and has the anticoccidial activity in chicken as same as other polyether antibiotics.

References

1) Harned, R.L.; P. Harter, C.L. Corum & K.L. Jones: Nigericin, a new crystalline antibiotic from unidentified *Streptomyces*. Antibiot. & Chemoth. 1:594~596, 1951
2) Magyar, K. & J. Stverteczky: Antibiotics produced by *Streptomyces*. IV. A new antibiotic K-178; chemical studies. Ztschr. f. Allg. Mikrobiologie 4: 265~268, 1964
3) Gyimesi, J.; I. Horvath & A. Szentirmai: Antibiotics produced by *Streptomyces*. V. A new antibiotic K-358. Ztschr. f. Allg. Mikrobiologie 4: 269~272, 1964
4) Berger, J.; A.I. Rachlin, W.E. Scott, L.H. Sterbach & M.W. Goldberg: The isolation of three crystalline antibiotic from *Streptomyces*. J. Am. Chem. Soc. 73:5295~5298, 1951
5) Westley, J.W.; R.H. Evans, J.T. Williams & A. Stempel: Structure of antibiotic X-537A. Chem. Comm. 1970: 71~72, 1970
6) Gachon, P.; A. Kergomard & H. Veschambre: Grisorixin, a new antibiotic related to nigericin. Chem. Comm. 1970: 1421~1422, 1970
7) Haney, M.E. & M.M. Hoehn: Monensin, a new biologically active compound. I. Discovery and isolation. Antimicr. Agents & Chemoth. -1967: 349~352, 1968
8) Hamill, R.L.; M.M. Hoehn, G.E. Pittenger, J. Chamberlin & M. Gorman: Dianemycin, an antibiotic of the group affecting ion transport. J. Antibiotics 22: 161~164, 1969
9) Jone, N.D.; M.O. Cheney, J.W. Chamberlin, R.L. Hamill & Sue Chen: Structure of A204A, a new polyether antibiotic. J. Am. Chem. Soc. 95: 3399~3400, 1973
10) Kinashi, H.; N. Ōtake & H. Yonehara: The structure of salinomycin, a new member of the

polyether antibiotics. Tetrahedron Letters 1973: 4955~4958, 1973
11) WAKSMAN, S.A.: The actinomycetes. Vol. 2. Classification, identification and descriptions of genera and species. The Williams and Wilkins Co., Baltimore, 1961
12) SHIRLING, E.B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Intern. J. Syst. Bact. 16: 313~340, 1966
13) PRIDHAM, T.G. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid for species determination. J. Bact. 56: 107~114, 1948
14) BREED, R.S.; E.G.D. MURRAY & N.R. SMITH: BERGEY's Mannual of Determinative Bacteriology. 7 th Edition. The Williams and Wilkins Co., 1957
15) MIYAZAKI, Y.; J. NAGATSU, H. SUGAWARA & M. SHIBUYA: Japanese Patent Preliminary Publication No. 25392, 1972