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

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Carriomycin, a new polyether antibiotic, was isolated from culture broth of *Streptomyces hygroscopicus* strain T-42082. It is active against Gram-positive bacteria, several fungi, yeasts and mycoplasma. It is also coccidiostatic. The free acid of carriomycin occurs as colorless prisms having the molecular formula $C_{47}H_{80}O_{15}$ (M.W. 885.15), m.p. 120~122°C and $[\alpha]_D^{25}$ −0.5 in methanol. It has no characteristic absorption maxima in the ultraviolet spectrum. The presence of one carboxyl and three methoxy groups was observed from its infrared, PMR and CMR spectra.

A strain of actinomycete, T-42082, produced an antibiotic of the polyether group in its culture broth. The active principle which we have named carriomycin was extracted from the broth and isolated in crystalline free acid and salts. This paper describes the taxonomic characterization of the strain T-42082, and the production, isolation, and physicochemical and biological characterization of carriomycin.

Taxonomic Characterization of Strain T-42082

Strain T-42082 was isolated from a soil sample collected in Ito City, Shizuoka, Japan. After

Table 1. Cultural characteristics of strain T-42082. Results of 14 day cultivation at 28°C

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose-nitrate agar	Moderate, colorless to ivory	White to light gray	Creamy to ivory	None
Glucose-asparagine agar	Moderate, grayish tan	White to grayish tan with black patches	Grayish tan to yellowish tan	None or pale yellowish tan
Glycerol-asparagine agar	Moderate, grayish tan to yellowish-grayish tan	Olive-gray with black patches	Pale yellowish gray to pale yellow	Pale yellow
Inorganic salts-starch agar	Moderate, pale yellowish tan to grayish-yellowish tan	Grayish tan	Yellowish tan to grayish tan	Pale yellow
Yeast extract-malt extract agar	Abundant, yellowish tan	White to gray or grayish-yellowish tan	Dark brownish tan to dark yellowish tan	Orange-yellowish tan
Oatmeal agar	Abundant, yellowish tan	White to yellowish gray	Greenish-yellowish tan	Yellow
Tyrosine agar	Moderate, yellowish tan	Yellowish gray to grayish tan with black patches	Yellowish tan to dark gray	Yellowish tan
Nutrient agar	Moderate, colorless to creamy	White	Colorless to pale yellow	Colorless to pale yellow

cultivation on glucose-asparagine agar at 28°C for 14 days, it showed the following morphological characteristics.

Vegetative mycelium is well developed and branched. Aerial mycelium, measuring about 1 μm in diameter, branches monopodially and closed spiral spore chains are formed at the end of the side branches. The spore is ellipsoidal to cylindrical ($0.7 \sim 1.0 \times 0.9 \sim 1.4$ μm), with a warty surface. Other special organs such as sporangium, flagellated spore and sclerotium were not observed in the above medium.

Cultural characteristics studied on various media prepared according to the methods of WAKSMAN[1] and SHIRLING and GOTTLIEB[2] are shown in Table 1.

Physiological properties of the strain T-42082 are shown in Table 2.

Table 2. Physiological properties of strain T-42082

Parameter	Reaction
Temperature	growth 15~37°C optimum 24~34°C
Liquefaction of gelatin	positive
Hydrolysis of starch	positive
Peptonization of skimmed milk	positive
Coagulation of skimmed milk	negative
Production of melanoid pigment	negative in tyrosine agar negative in peptone-yeast extract iron agar
Assimilation of carbon sources*	assimilates *i*-inositol, D-mannitol, D-xylose, L-arabinose, D-glucose, D-fructose, rhamnose, sucrose and raffinose

* Examined in PRIDHAM and GOTTLIEB agar[4].

Analysis of above results indicates that strain T-42082 can be identified as *Streptomyces hygroscopicus* WAKSMAN and HENRICI (1948). The strain was therefore named *Streptomyces hygroscopicus* T-42082 and has been deposited at the Institute for Fermentation, Osaka, and American Type Culture Collection under the accession numbers of IFO 13609 and ATCC 31080, respectively.

Production and Isolation of Carriomycin

Streptomyces hygroscopicus T-42082 was cultivated at 28°C for 14 days on a slant of glucose-asparagine agar. Approximately 0.25 cm^2 of the grown cell mat with abundant spores on it was cut out and inoculated into 500 ml of seed medium in a 3-liter Sakaguchi shaking flask. The seed medium, with pH adjusted to 7.0 before sterilization, contained (g/liter): glucose, 20; soluble starch, 30; soy-bean flour, 10; corn-steep liquor, 10; Polypepton, 5; NaCl, 3; precipitated $CaCO_3$, 5. Two such seed flasks were incubated at 28°C for 2 days with reciprocal shaking (120 oscillations per min., 10 cm distance). The culture broth was then transferred to 30 liters of main fermentation medium in a 50-liter tank and aerobic, stirred cultivation was carried out at 28°C for 6 days with air-sparging at the rate of 30 liters/min. The main fermentation medium, with pH adjusted to 7.0 before sterilization, contained (g/liter): glucose, 20; soluble starch, 20; glycerol, 10; corn-steep liquor, 10; cotton-seed meal, 10; Polypepton, 5; NaCl, 5; precipitated $CaCO_3$, 5. NaOH was used to adjust pH.

To 25 liters of the fermented broth adjusted to pH 9 with concentrated NaOH, 25 liters of acetone was added. After stirring the mixture for 1 hour at room temperature, mycelia were filtered off and extracted again with 17 liters of acetone. The extracts were combined and concentrated *in vacuo* until no acetone remained. The concentrated aqueous solution was extracted twice with equal volumes of ethylacetate, followed by drying with anhydrous Na_2SO_4. The extracts were concentrated *in vacuo* to 800 ml and passed through a column of activated charcoal (2×33 cm), then the column was washed with 500 ml of ethylacetate. The fractions active against *Staphylococcus*

aureus FDA 209P were combined and the solvent was evaporated. To the oily residue was added 500 ml of *n*-hexane. The resultant solid material was collected by filtration and crystallized from aqueous acetone (5.6 g). On recrystallization from aqueous acetone, crystals (4.5 g) of the mixed sodium and potassium salts of carriomycin were obtained (1.5% Na, 1.9% K). One gram of the mixed salt was dissolved in aqueous acetone and the solution was adjusted to pH 4 with 1 N HCl. After evaporating off acetone, the residual aqueous solution was extracted twice with equal volumes of ethylacetate. The extracts were dried with anhydrous Na_2SO_4 and concentrated to dryness *in vacuo*. The resultant crystalline powder was recrystallized from aqueous acetone. The crystals obtained (0.7 g) contained no ash, showed free acid type infrared absorption spectrum (Fig. 1) and were therefore identified as carriomycin free acid.

The free acid form of carriomycin was dissolved in aqueous acetone and 1 N NaOH was added to pH 9.5. After removing acetone, the antibiotic was extracted with ethylacetate. The extract was concentrated to dryness and the resultant crystalline powder was recrystallized from aqueous acetone. The resulting crystals contained 2.6% Na, showed salt form in the infrared absorption spectrum (Fig. 2), and were identified as sodium salt of carriomycin (C 62.27%, H 8.99%). Potassium salt of carriomycin was obtained by a similar procedure in which KOH was used in place of NaOH.

Fig. 1. Infrared absorption spectrum of free acid of carriomycin (KBr)

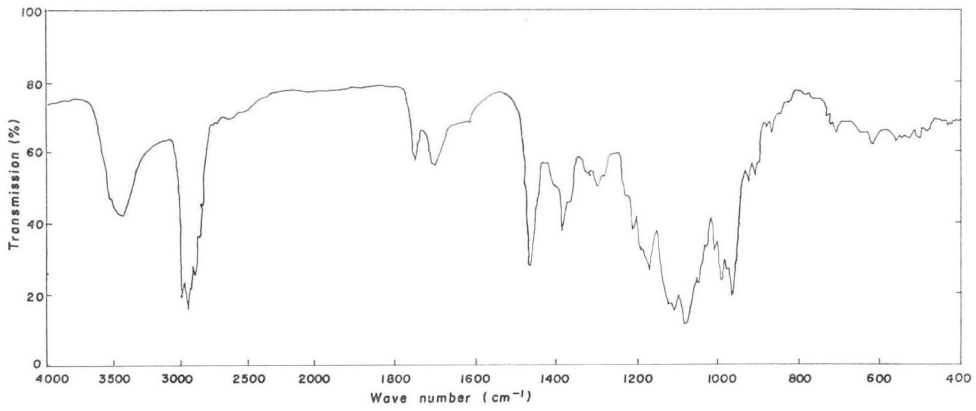
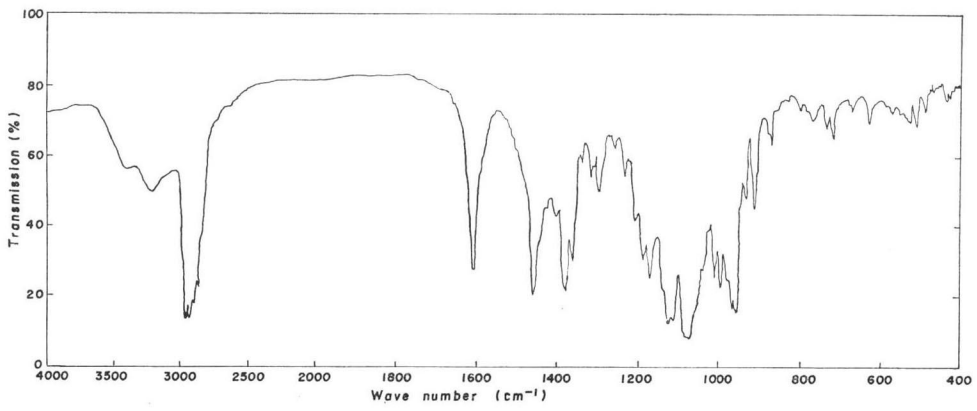


Fig. 2. Infrared absorption spectrum of carriomycin sodium salt (KBr)



Physical and Chemical Properties of Carriomycin

Carriomycin and its salts isolated as described above occur as colorless prisms. Melting points are: free acid, 120~122°C; sodium salt, 178~180°C (with decomposition); potassium salt, 180~182°C (with decomposition); mixture of sodium and potassium salts, 178~180°C (with decomposition). Elemental analysis gave the following values: free acid, C 64.01, H 9.30, O 26.04 (%); sodium salt, C 62.27, H 8.99, Na 2.50 (%); potassium salt, C 61.62, H 8.79, K 4.25 (%). The molecular weight of carriomycin determined by vapor pressure osmometry was 859 (calcd. 885). The number of carbon atoms per molecule determined from CMR (see Fig. 5) was 47. Thus the molecular formula of carriomycin would be $C_{47}H_{80\sim82}O_{14\sim15}$, and that for its potassium salt, $C_{47}H_{79\sim81}O_{14\sim15}K$. Elemental analysis calculated for $C_{47}H_{80}O_{15}$ is C 63.78, H 9.11, O 27.11 (%), and that for $C_{47}H_{79}O_{15}K$ is C 61.15, H 8.62, O 26.00, K 4.24 (%).

Fig. 3. PMR spectrum of carriomycin sodium salt (deuterochloroform)

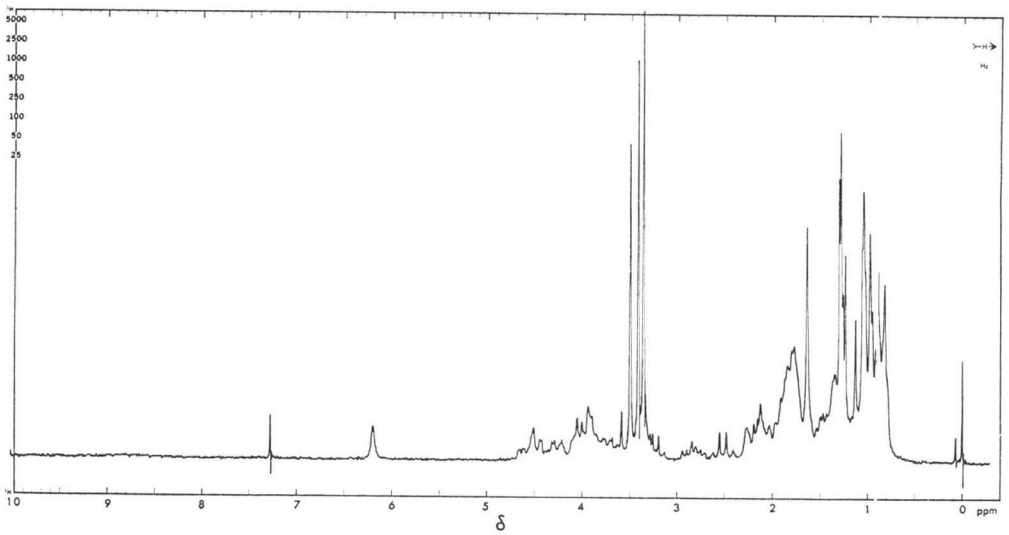


Fig. 4. CMR spectrum of carriomycin sodium salt (deuterochloroform)

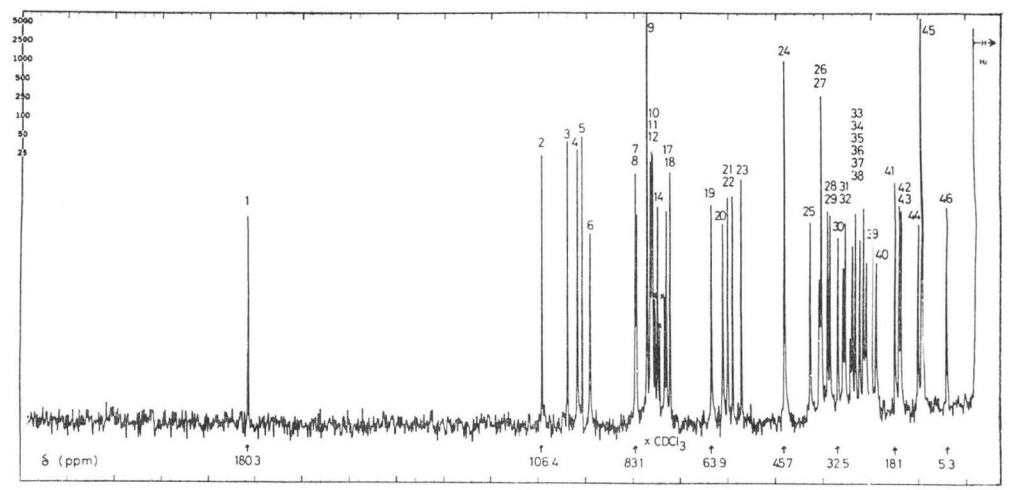


Table 3. ^{13}C Chemical shifts for carriomycin sodium salt and assignment of respective peaks. Carbons (asterisked) in the functional groups are indicated as follows: **I**, C–*C(H)(H)–H; **II**, C–*C(H)–C; **III**, C–*C(C)–C; **IV**, O–*C(H)–H; **V**, C–*C(H)–O; **VI**, C–*C(C)–O; **VII**, O–*C(H)–O; **VIII**, O–*C(C)–O; **IX**, C–*C(=O)–O

Peak in Fig. 4	Chemical shift (ppm)	Functional group assigned	Remarks
1	180.268	**IX**	
2	106.380	**VIII**	
3	100.004	**VIII**	
4	97.513	**VII**	
5	96.368	**VIII**	
6	94.374	**V** (or **VII**)	
7	83.083	**V**	
8	82.801	**VI**	
9	80.111	**V**	two carbons
10	79.325	**V**	
11	78.967	**V**	
12	78.733	**V**	
14	77.521	**V**	
17	75.157	**V**	
18	74.223	**V**	
19	63.890	**V**	
20	61.029	**V**	
21	59.802	**IV**	
22	58.630	**IV**	
23	56.449	**IV**	
24	45.738	**III**	two carbons
25	39.401	**II**	
26	37.117	**II** (or **III**)	
27	36.648	**III** (or **II**)	two carbons
28	35.047	**III**	
29	34.463	**III**	
30	32.463	**II**	
31	31.157	**III**	
32	30.637	**II**	
33	29.381		impurity
34	28.849	**II**	
35	28.118	**II**	
36	27.021	**II**	
37	26.120	**I**	
38	25.377	**II**	
39	23.891	**II**	
40	22.794	**II**	
41	18.071	**I**	
42	17.057	**I**	
43	16.632	**I**	
44	12.338	**I**	
45	11.412	**I**	three carbons
46	5.310	**I**	

Functional groups were assigned from chemical shifts and off resonance.

Specific rotations of carriomycin and its sodium salt as 1% solution in methanol showed $[\alpha]_D^{25}$ $-0.5°$ and $-0.1°$, respectively.

Both carriomycin and its salts were easily soluble in methanol, ethanol, acetone, ethylacetate, $CHCl_3$, benzene, toluene and CCl_4, slightly soluble in petroleum ether and cyclohexane, but were almost insoluble in water. Carriomycin gave a purple color on silica gel plates with vanillin-H_2SO_4 reagent[4]. It gave an indigo-blue color with aniline-phthalic acid and pale orange color with DRAGENDORFF's reagent, but was negative to MOLISCH, BARTON, molybdate, benzidine, ninhydrin and potassium permanganate reagents.

Carriomycin shows no characteristic absorption maxima in the ultraviolet absorption spectrum. Infrared absorption spectra of carriomycin and its sodium salt are illustrated in Figs. 1 and 2. The dominant absorptions (wave numbers) are as follows: free acid, 2940, 1700, 1462, 1383, 1085, 972 cm^{-1}; sodium salt, 2935, 1610, 1461, 1382, 1080, 972 cm^{-1}. Peaks at 1700 cm^{-1} and 1610 cm^{-1} correspond to COOH and COO^-, respectively.

In the PMR spectrum of carriomycin sodium salt at 90 MHz in deuterochloroform, three methoxy groups at 3.38, 3.42 and 3.51 ppm were indicated (Fig. 3).

CMR spectrum of carriomycin sodium salt in deuterochloroform (Fig. 4) shows the presence of various functional groups indicated in Table 3. Carriomycin contains one carboxyl and three methoxy groups.

Thin-layer chromatography of carriomycin sodium salt in various solvent systems on silica gel plates (Kieselgel 60, Merck) gave the following Rf values: ethylacetate, 0.73; benzene - ethylacetate (1: 1), 0.51; benzene - acetone (1: 1), 0.89; benzene - acetone (9: 1), 0.18; methanol - $CHCl_3$ (1: 19), 0.87; $CHCl_3$ - ethylacetate (2: 3), 0.62.

Biological Properties of Carriomycin

Carriomycin was very active against Gram-positive bacteria, and *Mycobacterium smegmatis*. It was moderately active against *Mycobacterium* sp. ATCC 607, *Candida albicans* and *Pyricularia oryzae*, but not active against Gram-negative bacteria, *Aspergillus niger* and *Penicillium chrysogenum*. Several mycoplasma were moderately sensitive to carriomycin (Table 4).

Anticoccidial activity against *Eimeria tenella, E. acervulina* and *E. maxima* was also demonstrated[5]; addition of 120 ppm carriomycin sodium salt in feed completely protected chicks from coccidiosis caused by the three *Eimeria* species.

The acute toxicity [LD_{50}] of carriomycin sodium salt in a preliminary examination in mice was about 2,000 mg/kg by oral administration and 125~250 mg/kg by intraperitoneal route. The oral toxicity of carriomycin sodium salt in chicks was 1,100 mg/kg.

Table 4. Antimicrobial activity of carriomycin sodium salt

	Organism	Minimum inhibitory concentration (μg/ml)
Gram-positive bacteria*	*Bacillus subtilis* PCI 219	0.31
	Bacillus subtilis ATCC 6633	0.31
	Bacillus cereus	<0.1
	Bacillus megaterium	0.2~0.31
	Bacillus pumilus	0.2
	Staphylococcus aureus FDA 209P	0.2
Gram-negative bacteria*	*Escherichia coli* NIHJ	>100
	Proteus vulgaris	>100
	Pseudomonas aeruginosa	>100
Mycobacterium*	*Mycobacterium* sp. ATCC 607	10
	Mycobacterium smegmatis	0.4
Yeast and fungi*	*Candida albicans*	10
	Aspergillus niger	>50
	Penicillium chrysogenum	>50
	Pyricularia oryzae P-18	10
Mycoplasma**	*Acholeplasma laidlawii*	3.9
	Mycoplasma mycoides	2.0
	Mycoplasma gallisepticum	7.8
	Mycoplasma pulmonis	7.8

* Examined by agar dilution method in Trypticase-soy agar (BBL).

** Examined by broth dilution method in PPLO-broth (Difco) supplemented with 20% horse serum and 2.5% fresh-yeast extract.

Comparison of Carriomycin with Other Antibiotics

Carriomycin is a monocarboxylic acid composed of carbon, hydrogen and oxygen. Many ether bonds are shown by the CMR spectrum. Overall profiles of infrared absorption spectra of carriomycin and its salts are most similar to those of polyether group antibiotics. Like most polyether antibiotics, both free acid and salt form of carriomycin are readily soluble in organic solvents with a wide range of polarity. Color reactions of carriomycin to various reagents are also similar to those of other polyether antibiotics. It is active against Gram-positive bacteria, but not active against Gram-negative bacteria. It also inhibits the growth of mycoplasma and *Eimeria* species, consistent with a number of known polyethers.

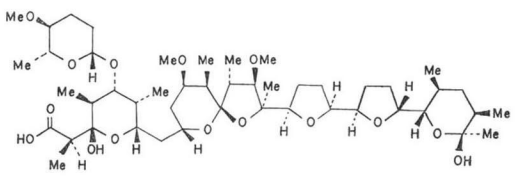
Many polyether antibiotics have been found, especially in recent years. Some of them, *e.g.* dianemycin[6] and X-537A (lasalocid)[7], have characteristic absorption maxima in the ultraviolet spectrum. On the contrary, carriomycin has no characteristic absorption maxima in the ultraviolet spectrum and can be distinguished from them. Polyether antibiotics without ultraviolet absorption maxima can be grouped according to the number of methoxy groups contained in their structures. Carriomycin has three methoxy groups as evidenced by PMR and CMR spectra. Antibiotic A-28695B[8], and antibiotic T-40517[9] which is identical with later found antibiotics CP-38295[10] or C20–12[11], are the presently known examples of polyether antibiotics possessing three methoxy groups. We directly compared carriomycin with antibiotic T-40517. In thin-layer chromatography with ethylacetate as the solvent, carriomycin showed an Rf value of 0.73, while antibiotic T-40517 showed an Rf value of 0.59. Moreover the color reaction of carriomycin with vanillin-H_2SO_4 reagent[4] was purple, but that of antibiotic T-40517 was red. These two antibiotics differed also in other physical and chemical properties such as melting point, infrared absorption spectrum and specific rotation. Carriomycin is different from antibiotic A-28695B in melting point, specific rotation and infrared absorption spectrum. Melting point of antibiotic A-28695B sodium salt is 161~162°C without decomposition while that of carriomycin sodium salt is 178~180°C with decomposition.

There are additional polyether antibiotics which have no characteristic ultraviolet absorption maxima, but the number of methoxy groups has not been determined: M-4164-A[12] and SF-1195.[13] Antibiotics SF-1195 and M-4164-A differ from carriomycin in melting points and infrared absorption spectra.

The most conspicuous characteristic of carriomycin is its extreme low toxicity among polyether antibiotics. This fact together with the above-described physical and chemical evidences discriminate between carriomycin and other known polyether antibiotics.

The entire structure of carriomycin was elucidated by ŌTAKE *et al.*[14] as shown in Fig. 5. The structure differs considerably from the known polyether antibiotics[15] with exception of antibiotic A-204A. The only difference between carriomycin and A-204A is that the former antibiotic lacks two of the five methoxyls present in A-204A, at carbon C-6 and C-27.[16]

Fig. 5. Structure of carriomycin[1]



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