MALIOXAMYCIN, A NEW ANTIBIOTIC WITH SPHEROPLAST-FORMING ACTIVITY

I. PRODUCING ORGANISM, FERMENTATION, ISOLATION AND CHARACTERIZATION

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(Received for publication August 22, 1980)

Strain No. 15748, identified as *Streptomyces lydicus*, produced a new antibiotic malioxamycin. Malioxamycin is a water-soluble, amphoteric antibiotic with low molecular weight, having a valine and a malic acid moieties in its structure. It gave a positive ninhydrin and ferric chloride reactions. Malioxamycin inhibited the growth of some Gram-negative bacteria by acting on peptidoglycan synthesis in the cell wall and produced spheroplasts from those sensitive bacteria.

In the course of our screening program directed toward discovery of new antibiotics with spheroplast-forming activity, we have already reported globomycin,[1] pentalenolactone and iso-U-22956.[2] In this screening program, it has been found that strain No. 15748, isolated from a soil sample collected at the lakeside of Biwa, Shiga Prefecture, Japan, produced a new antibiotic, malioxamycin. Using ion exchange chromatography, the antibiotic was isolated in a crystalline form and determined to be a new antibiotic having a unique structure and biological properties. In this paper, we report the production, isolation, physico-chemical and biological properties of malioxamycin.

Identification of Strain No. 15748

Taxonomic studies of strain No. 15748 were carried out by the method described by SHIRLING and GOTTLIEB. When this strain was examined microscopically, it was found to have well-developed vegetative mycelia with monopodial branching, which did not fragment readily, and aerial mycelia which formed incomplete spirals, loops or hooks. The mature spore chains were generally long with 10 to 50 or more spores per chain. The spore surfaces were smooth. On most of the media tested, growth was good and the aerial mycelium was white to gray mass color (Table 1). Physiological properties and the results of carbon utilization tests of strain No. 15748 are shown in Tables 2 and 3, respectively. As shown in Table 2, no detectable melanin formation was observed. The cell wall composition was determined by the methods of BECKER[3] and BOON.[4] LL-Diaminopimelic acid and glycine were found to be the major constituents. This is in accordance with cell wall type II.

From these characteristics, strain No. 15748 was classified as a member of the genus *Streptomyces* and *S. lydicus* NRRL 2433 was selected as the most closely related species. Simultaneous comparison of these two strains resulted in good agreement of their morphological and physiological properties except for the following differences. Strain No. 15748 formed no aerial mycelium on nutrient agar and *S. lydicus* developed abundant white aerial mycelia. Strain No. 15748 utilized D-cellobiose and inulin but

Table 1. Cultural characteristics of strain No. 15748 and *Streptomyces lydicus* NRRL 2433.

	No. 15748	*S. lydicus* NRRL 2433
Sucrose-nitrate agar	G: Good AM: White to brownish white R: Pale yellowish brown SP: None	G: Good AM: White R: Pale yellow SP: None
Glucose-asparagine agar	G: Poor AM: Brownish white R: Yellowish gray SP: None	G: Good AM: White to brownish gray R: Clear brownish gray SP: None
Glycerol-asparagine agar (ISP 5)	G: Poor AM: White R: Yellowish gray SP: None	G: Very poor AM: White R: Yellowish gray SP: None
Inorganic salts-starch agar (ISP 4)	G: Good AM: Clear brownish white R: Pale brown to yellowish brown SP: None	G: Good AM: White to gray or brownish gray R: Pale yellowish brown SP: None
Tyrosine agar (ISP 7)	G: Good AM: White to pale brown R: Pale yellowish brown SP: None	G: Abundant AM: White to pale brown R: Yellowish brown SP: None
Nutrient agar (Difco)	G: Good AM: None R: Pale yellow SP: None	G: Good AM: White R: Pale yellow SP: None
Yeast extract-malt extract agar (ISP 2)	G: Abundant AM: White to brownish white R: Yellowish brown SP: None	G: Good AM: Pale brown R: Yellowish brown SP: None
Oatmeal agar (ISP 3)	G: Good AM: Clear brownish white R: Pale yellowish brown SP: None	G: Good AM: Grayish brown R: Pale yellowish brown SP: None

G: Growth AM: Aerial mycelium R: Reverse SP: Soluble pigment

S. lydicus did not. Conversely, *S. lydicus* utilized β-lactose and *i*-inositol but strain No. 15748 did not. These differences, however, are not sufficient to differentiate strain No. 15748 from *S. lydicus*, hence the former was named *S. lydicus* No. 15748.

Production of Malioxamycin

The medium used both for the seed culture and fermentation of malioxamycin was composed of 1.5% soluble starch, 1.5% glycerol, 1.0% soybean meal, 1.0% fish meal, 0.5% corn steep liquor, 1.0%

K_2HPO_4, 0.3% $CaCO_3$ and 0.01% Disfoam CB-442 (anti-foaming agent manufactured by Nippon Yushi Co.), pH 7.2 before sterilization. The spores of *S. lydicus* No. 15748 grown on a slant were inoculated into a 500-ml Erlenmeyer flask containing 80 ml of the medium and incubation was carried out at 28°C for 3 days on a rotary shaker. The resulting seed culture (35 ml) was then transferred into a 2-liter Erlenmeyer flask containing 700 ml of the medium and the flask was shaken under the conditions described above. After inoculation of 3 liters of the secondary seed culture into a 600-liter tank containing 300 liters of the medium, the fermentation was conducted at 28°C for 191 hours with aeration of 300 liters/minute and agitation of 300 rpm.

Table 2. Physiological properties of strain No. 15748 and *Streptomyces lydicus* NRRL 2433.

		No. 15748	*S. lydicus* NRRL 2433
Tyrosinase reaction		negative	negative
Nitrate reduction		positive	positive
Starch hydrolysis		positive (strong)	positive (strong)
Gelatin liquefaction		positive	positive
Milk coagulation	26°C	negative	negative
	37°C	negative	negative
Milk peptonization	26°C	positive (pH 6.2)	positive (pH 6.2)
	37°C	positive (pH 6.0)	positive (pH 6.2)
Melanin formation			
Tryptone-yeast extract broth (ISP 1)		negative	negative
Peptone-yeast extract iron agar (ISP 6)		negative	negative

Table 3. Carbon utilization pattern of strain No. 15748 and *Streptomyces lydicus* NRRL 2433.

	No. 15748	*S. lydicus* NRRL 2433		No. 15748	*S. lydicus* NRRL 2433
D-Glucose	+	+	Raffinose	+	+
L-Arabinose	+	+	*i*-Inositol	−	+
D-Xylose	±	±	D-Mannitol	+	+
D-Fructose	+	+	Dulcitol	−	−
L-Rhamnose	−	−	Inulin	+	−
D-Galactose	+	+	Dextrin	+	+
D-Mannose	+	+	Soluble starch	+	+
Sucrose	+	+	Salicin	−	±
D-Cellobiose	+	−	Na-acetate	−	−
Melibiose	+	+	Na-succinate	+	+
β-Lactose	−	+	Glycerol	+	+
Maltose	+	+	Cellulose	−	−
Trehalose	±	+	Negative control	−	−

+, positive ±, weakly positive −, negative

Isolation and Purification of Malioxamycin

A 300-liter aliquot of the fermentation broth was adjusted to pH 7.0 with 20% H_2SO_4 and filtered with the aid of diatomaceous earth (1%). The filtrate thus obtained (260 liters) was passed through a column packed with 50 liters of Diaion HP-20 (Mitsubishi Kasei Co.), which was then washed with distilled water to give a combined effluent of 410 liters. The effluent was adsorbed on a column of Diaion SK-1B (H^+ form, 50 liters), washed with water (310 liters) and eluted with 310 liters of 0.5 N NH_4OH. The eluate was concentrated *in vacuo* to 7 liters. Twenty liters of methanol was then added to the concentrate and the mixture was kept overnight at 4°C. After removal of the resulting precipitate, the

supernatant was concentrated and lyophilized. Of 160 g of the crude powder of malioxamycin thus obtained 55 g was dissolved in 5 liters of water and applied on a column of 1.5 liters of SP-Sephadex (H^+ form, Pharmacia Co.) and developed with water. The active fraction (2.8 liters) was then evaporated to about 50 ml *in vacuo* and lyophilized to yield a partially purified powder (410 mg). This powder was chromatographed on a column of Sephadex G-15 (Pharmacia Co.) equilibrated with

Fig. 1. IR spectrum of malioxamycin. (KBr)

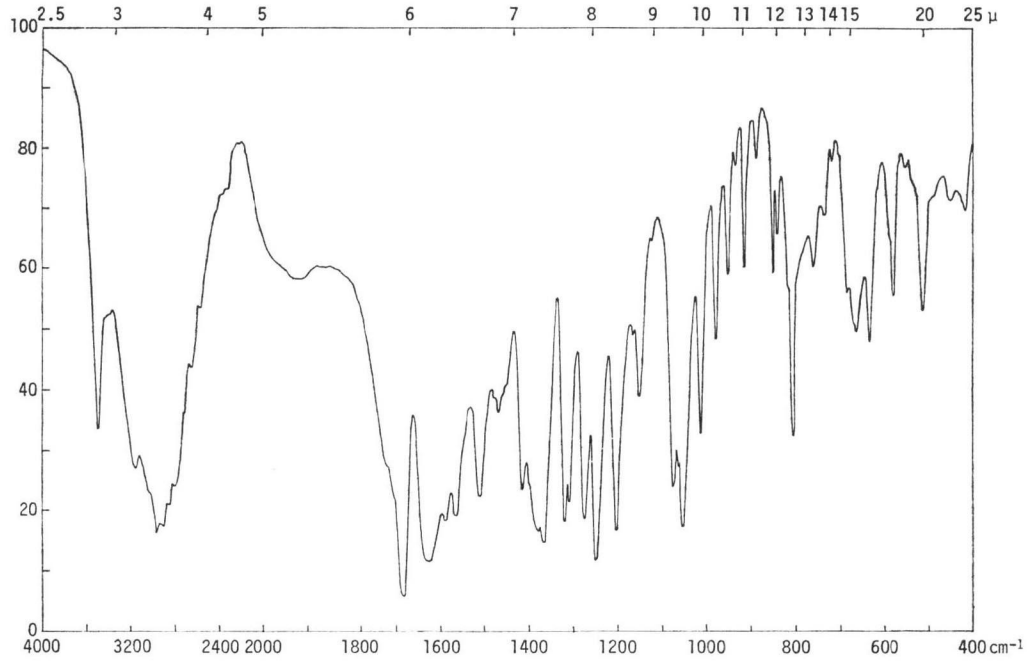
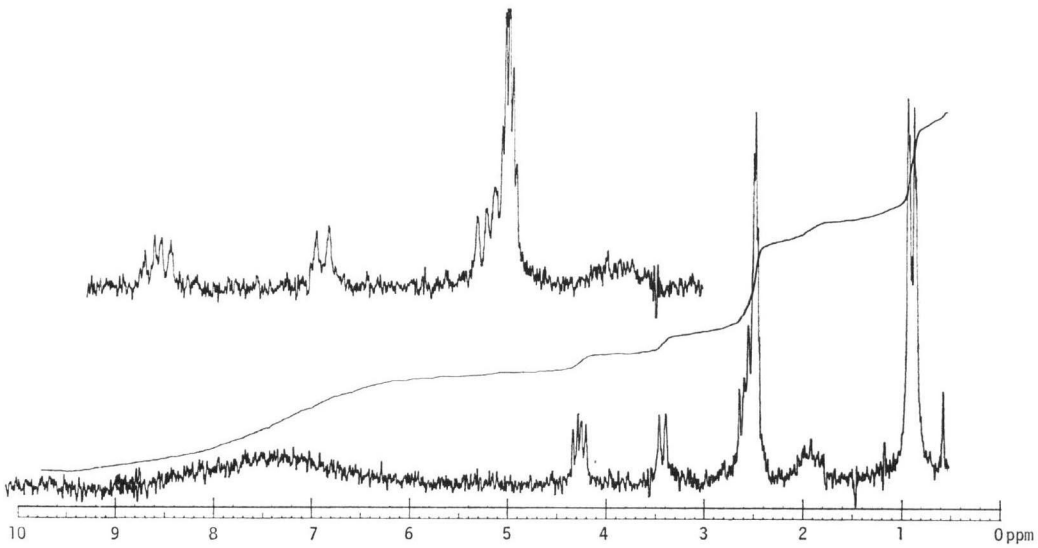


Fig. 2. NMR spectrum of malioxamycin. (DMSO)



the lower layer of the solvent system of 1-butanol - acetic acid - water (4: 1: 5) and developed with the upper layer of that system. The fractions with a single spot on thin-layer chromatograms were collected and concentrated *in vacuo*. The concentrate was recrystallized from water and 10 mg of malioxamycin was obtained as colorless needles.

Physico-chemical Properties of Malioxamycin

Malioxamycin obtained as colorless needles showed the following properties. It melted in the range of 158~160°C with decomposition and possessed $[\alpha]_D^{21}$ +37.4° (*c* 1.21, H_2O) and no UV absorption. The IR absorption spectrum (Fig. 1) showed a band at 1625 cm^{-1} corresponding to the amide bond. The NMR spectrum is shown in Fig. 2. The results of elementary analysis were as follows:

Found: C, 40.36; H, 6.79; N, 10.41.
Calcd. for $C_6H_{10}N_2O_6$: C, 40.60; H, 6.81; N, 10.52.

Malioxamycin is soluble in water, ethanol, methanol and acetone, but insoluble in ethyl acetate, chloroform and benzene. It gave a positive reaction with ninhydrin and with ferric chloride. On thin-layer chromatography using cellulose (Eastman chromagram sheet No. 6065, Kodak Co.), malioxamycin gave a single spot of Rf 0.46 with the developing solvent 1-butanol - pyridine - acetic acid - water (15: 10: 3: 10), at 0.47 with 1-butanol - acetic acid - water (3: 1: 1) and at 0.24 with acetonitrile - water (7: 3).

Biological Properties of Malioxamycin

The minimal inhibitory concentrations of malioxamycin against various microorganisms were determined by the serial two-fold agar dilution method. Malioxamycin scarcely inhibited bacterial growth at a concentration of 100 μg/ml, except certain Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae* (Table 4). As will be described in the subsequent paper, malioxamycin has a unique fused hydroxamic acid moiety in its structure. It has been reported that aminooxyacetic acid with a free hydroxamic acid moiety inhibited enzymes such as aminotransferase, racemase and decarboxylase.[5] These enzymes require pyridoxal phosphate (vitamin B_6) as a coenzyme for their activities. In fact, aminooxyacetic acid or aminooxysuccinic acid demonstrated fairly strong antimicro-

Table 4. Minimal inhibitory concentrations of malioxamycin.

Organism	MIC (μg/ml)
Staphylococcus aureus FDA 209P JC-1	>100
Bacillus subtilis PCI 219	>100
Escherichia coli NIHJ JC-2	100
E. coli B	>100
E. coli 665	100
E. coli SANK 72375	50
Klebsiella pneumoniae 846	100
Serratia marcescens SANK 73060	>100
Pseudomonas aeruginosa 1046	>100

Medium; Antibiotic medium No. 3 (Difco).

Fig. 3. Spheroplast formation of *Escherichia coli* B in the presence of malioxamycin.

Scanning electron micrograph of the cell of *E. coli* B after treatment with 100 μg/ml of malioxamycin at 37°C for 5 hours.

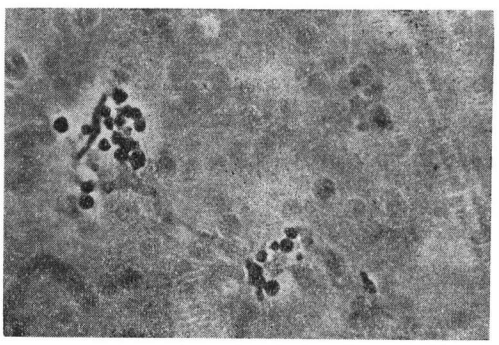
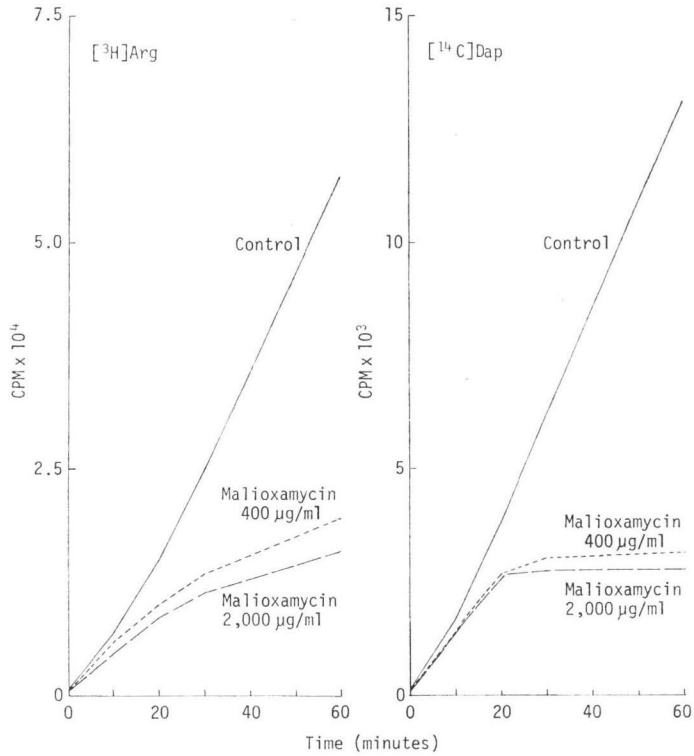


Fig. 4. Incorporation of radioactive precursors into cold 5% TCA insoluble fraction of *E. coli* H2143 ($Dap^- \cdot Lys^-$).



bial activities and they were antagonized by pyridoxal or pyridoxine. Antimicrobial activity of malioxamycin, however, was not antagonized by pyridoxal or pyridoxine indicating that the mode of action of malioxamycin is different from that of the aminooxy acids. To confirm this fact, the effect of malioxamycin on glutamate pyruvate transaminase (GPTase) was examined. All aminooxy acids tested inhibited GPTase strongly ($Ki=5\times10^{-6}$ M) but malioxamycin had only a weak ($Ki=1.3\times10^{-3}$ M) effect.

Malioxamycin induced spheroplast formation in bacteria such as *E. coli* B, sensitive to this antibiotic. Cells of *E. coli* B grown in the presence of 100 μg/ml of malioxamycin at 37°C for 5 hours in trypto-soy broth supplemented with 12% sucrose and 0.1% $MgSO_4 \cdot 7H_2O$, first swelled and finally formed spheroplasts as shown in Fig. 3. This spheroplast formation might be ascribed to the result of inhibition of cell-wall peptidoglycan synthesis. As shown in Fig. 4, incorporation of [^{14}C]-diaminopimelic acid into the cold 5% trichloroacetic acid insoluble fraction of *E. coli* H2143 ($Dap^- \cdot Lys^-$) was strongly inhibited. Malioxamycin also inhibited the incorporation of [^{3}H]-arginine, though to a lesser extent than that of [^{14}C]-Dap. Malioxamycin showed relatively low toxicity. Mice were tolerant to intravenous administration of 100 mg/kg of the antibiotic.

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