TETROCARCINS, NOVEL ANTITUMOR ANTIBIOTICS

I. PRODUCING ORGANISM, FERMENTATION AND ANTIMICROBIAL ACTIVITY

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A novel antitumor antibiotic complex has been obtained from the culture broth of Actinomycete strain DO-11 (KY11091) isolated from a soil sample collected in Sendai, Miyagi, Japan. On the taxonomic studies the producing organism is described as *Micromonospora chalcea* KY11091. For the production of the antibiotics, soluble starch served as a good carbon source and yeast extract was the best nitrogen source tested. The antibiotic complex designated as tetrocarcins is active against Gram-positive bacteria, but is not active against Gram-negative bacteria. Tetrocarcin A showed bacteriocidal activity against *Bacillus subtilis*.

In the course of our screening program for new antibiotics, we found a novel antibiotic complex with antibacterial and antitumor activity from Actinomycete strain DO-11. As described in the preceding paper[1] and in the following paper,[2] the complex was isolated and separated into three components designated as tetrocarcins A, B and C.

Tetrocarcins A, B and C contain the novel polycyclic aglycone, tetronolide,[3] that is common to all tetrocarcins. The generic name, tetrocarcin, is proposed on the basis of the unique structure of the aglycone that consists of a derivative of tetronic acid.[4] Details of the structure determination of tetrocarcins will be given in separate papers.[2,3]

In this paper, the taxonomic studies of the producing strain, the fermentation studies and antibacterial activity of tetrocarcins are presented.

Taxonomy

Actinomycete strain DO-11 was isolated from a soli sample collected in Sendai-shi, Miyagi, Japan. The strain has been deposited at Northern Regional Research Laboratories, Peoria, Illinois, U.S.A. and has been assigned accession number NRRL11289.

The taxonomic characterization was carried out according to the methods used in the International Streptomyces Project (ISP).[5] Additional media recommended by WAKSMAN[6] were also used. The various kinds of media were inoculated with the washed mycelia suspended in 0.85% saline obtained from a culture shaken at 28°C for 72 hours in a liquid medium consisted of 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g yeast extract, 5 g peptone per liter of water, pH 7.0 prior to sterilization.

Cultural Characteristics

Strain DO-11 showed good growth on the following medium; yeast extract malt agar (ISP medium 2). On this agar medium colonies were orange in the earlier stages of growth, and gradually turned to black olive. Many spores were found in the black colonies, but formation of true aerial mycelium

was absent. Microscopic observation showed the substrate mycelium to be well developed and branched, but not fragmented. The hyphae were about 0.5 μ in diameter. The spores were borne singly from the substrate mycelia. The spores were spherical to oval and smooth to warty as seen by electron microscopy (Plate 1). Cell wall analysis revealed the presence of meso-diaminopimeric acid.

Appearance on Various Media

The cultural characteristics of strain DO-11 shown in Table 1 were observed after two weeks of incubation at 28°C. No diffusible pigments were observed on all the media tested. The numbers in parentheses corresponds to the hue number used in "Color Harmony Mannual".[7]

Physiological Characteristics

The physiological characteristics of strain DO-11 are shown in Table 2. The temperature range for growth and the pH range for growth were observed after cultivation of 2 days and the action upon milk and decomposition of cellulose was observed after one month. All other observations were made after 20 days.

These characteristics of DO-11 shown in Tables 1 and 2 place the organism in the genus *Micromonosporales* and resemble closely those of *Micromonospora chalcea*.[9] Thus, utilization of carbon sources of *Micromonospora chalcea* ATCC12452 was observed in order to compare with those of DO-11 as shown in Table 3. It is apparent that DO-11 showed the very similar utilization pattern except for D-ribose. It has been reported that spores of *Micromonospora chalcea* ATCC12452 appear to be from smooth to warty.[10,11] Thus we also examined the spores of both DO-11 and *M. chalcea* ATCC12452 under the electron microscope. As shown in Plates 1 and 2, spores from the both strains showed smooth to warty surfaces and the similar shapes indicating the both are closely related, although the spores are of different size. Therefore, we concluded that the strain DO-11 was designated as

Table 1. Cultural characteristics of strain DO-11.

Medium	Growth	Color*
CZAPEK's agar (WAKSMAN No. 1)	good, flat	dusty orange (41c) to black olive (1po)
Glucose-asparagine agar (WAKSMAN No. 2)	poor to moderate, flat	russet orange (4nc) to dark olive (1pn)
Yeast-malt extract agar (ISP No. 2)	good, raised	terra cotta (5pe) to deep brown (5nl)
Oatmeal agar (ISP No. 3)	moderate to good, flat	apricot (4ia) to clove brown (3pl)
Inorganic salts-starch agar (ISP No. 4)	good, flat	russet orange (4nc) to black olive (1po)
Glycerol-asparagine agar (ISP No. 5)	poor to moderate, flat	light melon yellow (3ea)
Peptone-yeast extract-iron agar (ISP No. 6)	moderate, flat	orange (41a) to luggage tan (4ne)
Tyrosine agar (ISP No. 7)	moderate, flat	black olive (1po)
Tyrosine agar[8]	good, flat	orange rust (4pe) to terra cotta (5pe)
Nutrient agar	moderate, flat	apricot (4ia)
EMERSON's agar	good, raised	orange rust (4pe) to chocolate brown (5po)
BENNET's agar	good, raised	ebony brown (8pn)
$CaCO_3$-neutralized potato plugs	good, raised	russet orange (4nc)

* Color designation from Color Harmony Manual.

Table 2. Physiological properties of strain DO-11.

Liquefaction of gelatin	weakly positive
Peptonization of milk	weakly positive
Coagulation of milk	positive
Proteolytic action on milk agar	strong
Decomposition of cellulose	weakly positive
Hydrolysis of starch	positive
Formation of tyrosinase	negative
Formation of melanoid pigment	negative
pH range for growth*	5.5~9.0 (opt. pH 6.0~8.0)
Temperature range*	21~45°C (opt. temp. 28~40°C)

* The medium composed of 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g yeast extract, 5 g peptone per liter of water, pH 7.0 (prior to sterilization).

Table 3. Comparison of utilization of carbohydrates by strain DO-11 and *Micromonospora chalcea* ATCC12452.

Carbohydrate	DO-11	ATCC12452
L-Arabinose	+	+
D-Arabinose	−	−
D-Xylose	+	+
D-Glucose	+	+
D-Fructose	+	+
Sucrose	+	+
Inositol	−	−
L-Rhamnose	−	−
D-Mannitol	−	−
Raffinose	+	+
Melibiose	+	+
D-Ribose	+	−
Salicin	+	+
Glycerol	±	±

Plate 1. Electron micrograph of spores of the strain DO-11. (×25,000)

The organism was grown in the liquid medium as indicated in Table 2 for 3 days and harvested by centrifuging at 3,000 rpm for 10 minutes. The pellet was fixed with 2% (v/v) glutaraldehyde for 2 hours in ice and then washed with veronal acetate buffer (pH 6.1). The fixed pellet was dehydrated in a graded acetone series from 50% to 100%, followed by iso-amylacetate and examined in JEM 100C scanning electron microscope type ASID-4D which was operated at 40 KV.

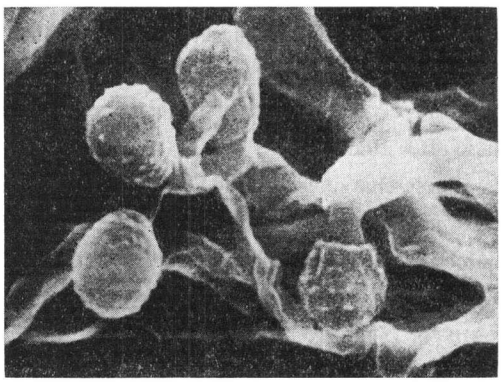
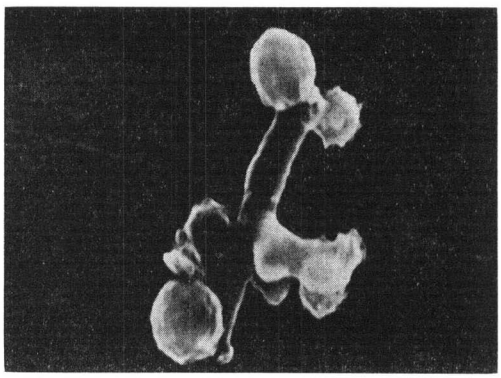


Plate 2. Electron micrograph of spores of *Micromonospora chalcea* ATCC12452. (×25,000)

Preparation of the sample was according to the same method as described in Plate 1.



Micromonospora chalcea KY11091. It is important to note that no other *Micromonosporales* and other Actinomycetales are reported to produce tetrocarcins or related antibiotics.

Fermentation

The seed flasks were inoculated with stock cultures maintained in a deep freezer (−70°C) and grown for 48~72 hours at 28°C. The seed medium consisted of 4 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 1.5 g KH_2PO_4, 4 g $(NH_4)_2SO_4$, 20 g sucrose, 10 g fructose, 10 g glucose, 5 g corn steep liquor and 20 g $CaCO_3$ per liter of tap water. A 5% vegetative seed was used to inoculate into the fermentation medium.

Table 4. Effect of carbon sources on the production of tetrocarcins.

Carbon source	(g/liter)	Growth PCV (%)	Tetrocarcins (μg/ml)
Soluble starch	70	22	100
	40	18	130
Sucrose	70	20	95
	40	17	40
Glucose	70	15	trace
	40	10	trace
Fructose	70	10	trace
	40	10	trace
Lactose	70	6	70
	40	7	trace
Sorbitol	70	2	trace
	40	3	trace
Sucrose +glucose	30 ea.	20	70

Table 5. Effect of nitrogen sources on the production of tetrocarcins.

Nitrogen source	(g/liter)	Growth PCV (%)	Tetrocarcins (μg/ml)
Yeast extract	2	15	80
	4	18	90
	8	25	120
Corn steep liquor	2	10	60
	4	15	70
	8	20	70
Meat extract	2~8	5~8	trace
Peptone	2~8	3~5	trace

Fig. 1. Time course of tetrocarcins production.
Fermentation was carried out in a 30-liter jar fermentor using the medium indicated in the text at 30°C with agitation of 250 rpm and aeration of 15 liters per minute.

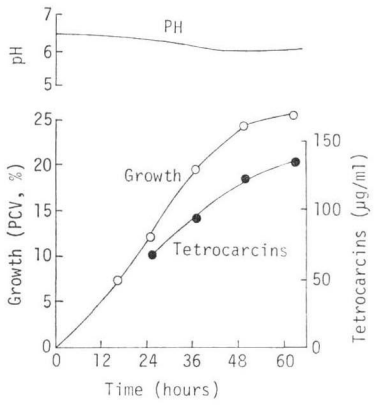
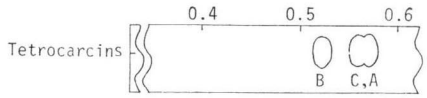
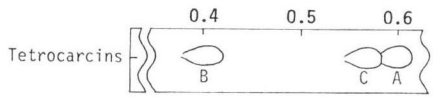


Fig. 2. Comparison of Rf values of tetrocarcins.
(1) Silica gel: $CHCl_3$-MeOH=9:1



(2) Silanized silica gel: $CHCl_3$-Dioxane=95:5



Basal components of the fermentation media were 0.09 g $MgSO_4 \cdot 7H_2O$, 0.15 g KH_2PO_4, 0.21 g K_2HPO_4 and 30 mg $ZnSO_4 \cdot 7H_2O$ per liter of tap water. The pH of media was adjusted to 7.0 prior to sterilization. Using the above basal medium, a number of carbohydrate and nitrogen sources were investigated for their effect on growth of *Micromonospora chalcea* KY11091 and on the production of the antibiotics, tetrocarcins. Among the carbohydrates tested, soluble starch gave the best, while glucose gave the poorest tetrocarcins production, although the growth of the mycelium were of similar extent (Table 4). Yeast extract was the best nitrogen source for the production of tetrocarcins (Table 5). Thus the fermentation medium was determined as follows: 40 g soluble starch, 8 g yeast extract, 0.09 g $MgSO_4 \cdot 7H_2O$, 0.15 g KH_2PO_4, 0.21 g K_2HPO_4 and 30 mg $ZnSO_4 \cdot 7H_2O$ per liter of tap water.

The time course of a typical fermentation is shown in Fig. 1. Antibiotic activity appeared in the culture supernatant at about 24 hours and reached maximum at about 64 hours. The active materials were isolated by sequential chromatography on non-ionic porous resin HP-20 and silica gel. Details of the isolation and their physico-chemical characteristics are given in the following paper.[2]

Tetrocarcins A, B and C were detected by thin-layer chromatography on silica gel or silanized

Table 6. Antimicrobial activity of tetrocarcins.

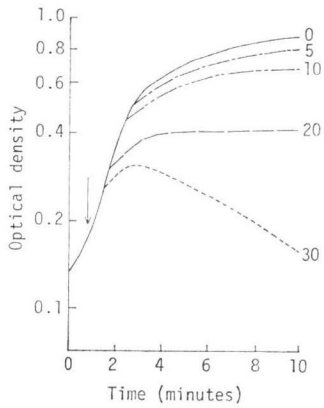
Test organisms	MIC (μg/ml)		
	A	B	C
Staphylococcus aureus ATCC6538P	20	20	30
Bacillus subtilis No. 10707	0.1	0.1	0.1
Klebsiella pneumoniae ATCC10031	>100	>100	>100
Escherichia coli ATCC26	>100	>100	>100
Shigella sonnei ATCC9290	>100	>100	>100

The medium consisted of 3 g tryptone, 3 g meat extract, 1 g yeast extract, 1 g glucose and 16 g agar per liter of tap water. The pH of the medium was adjusted to 7.0 prior to sterilization.

silica gel as shown in Fig. 2. They were visualized by exposing the plates to UV light or by bioautography using *Bacillus subtilis* as an indicator strain.

Fig. 3. Effect of tetrocarcin A on the growth of *Bacillus subtilis*.

Tetrocarcin A was added at the time indicated by the arrow and the numbers in the figure indicate amounts of the drug added (μg/ml). The medium consisted of 0.2 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid, 10 g K_2HPO_4, 3.5 g $NaNH_4HPO_4 \cdot 4H_2O$, 5 g glucose, 1 g Casamino acid, 2 g yeast extract, 50 mg tryptophan and 50 mg arginine per liter of tap water (pH 7.0 prior to sterilization). Growth was automatically recorded with the Bio-photorecorder (Toyo Kagaku Sangyo, Japan) at 37°C.



Antibacterial Activity

Antibacterial activity was determined by the agar dilution method at pH 7.0. As shown in Table 6, tetrocarcins A, B and C had moderate activity against *Staphylococcus aureus*, strong activity against *Bacillus subtilis*, while no activity was observed against the Gram-negative bacteria tested.

The effect of tetrocarcin A on the growth of *Bacillus subtilis* is shown in Fig. 3. The growth was inhibited at the concentration of 5 μg per ml and increase of the antibiotics showed more suppressive effect on the growth. At the concentration of 30 μg/ml, lysis of cells was observed indicating that tetrocarcin A acts as a bacteriocidal antibiotic.

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