TRIOXACARCINS, NOVEL ANTITUMOR ANTIBIOTICS

I. PRODUCING ORGANISM, FERMENTATION AND BIOLOGICAL ACTIVITIES

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A group of antitumor antibiotics with a novel skeleton, trioxacarcins, was discovered in culture broths of actinomycete strain DO-45. The producing organism was subsequently determined to be a new strain and named *Streptomyces bottropensis* DO-45. The antibiotics are active against Gram-positive bacteria and experimental murine tumors such as sarcoma 180 and leukemia P388.

In the course of our screening program for new antitumor antibiotics, we encountered an actinomycete species capable of producing new antitumor antibiotics, trioxacarcins A, B and C, that were previously designated DC-45-A, DC-45-B_1 and DC-45-B_2, respectively.[1]

Trioxacarcins A, B and C contain novel polycyclic chromophores. The generic name trioxacarcin is proposed on the basis of the unique structures of the aglycones that consist of three cyclic ether moieties. Details of the structure determination will appear in the near future.[2]

This report will describe the taxonomic studies of the producing strain, the fermentation studies and the biological activities of trioxacarcins. The isolation, physico-chemical properties and mode of action of trioxacarcin A will be reported in the following paper.[3]

Taxonomy

An actinomycete strain DO-45 was isolated from a soil sample collected in Sapporo-shi, Hokkaido, Japan. The strain has been deposited at Northern Regional Research Laboratories, Peoria, Illinois, U.S.A. and has been assigned accession number NRRL 12051.

The taxonomic characterization was carried out according to the methods used in the International Streptomyces Project (ISP).[4] The various kinds of media were inoculated with washed mycelia suspended in 0.85% saline obtained from a culture shaken at 28°C for 72 hours in a liquid medium consisting 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

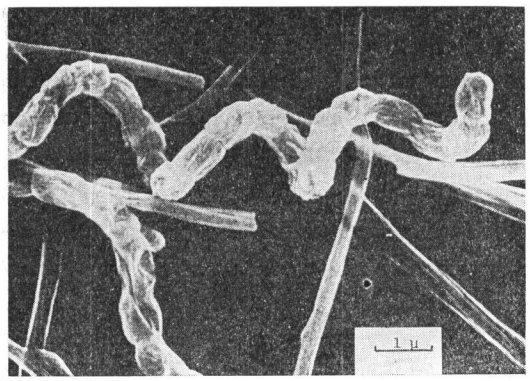
Strain DO-45 grew well on various media as shown in Table 1. Abundant aerial mycelium with simple branching and spiral spore chains were observed by microscope. The mature spore chains were generally long with 10 to 30 or more spores per chain. The spores were oval in shape, with smooth surface as seen by electron microscope (Fig. 1). Cell wall analysis revealed the presence of LL-diamino-

Table 1. Cultural characteristics of strain DO-45.

Medium	Growth	Color of colony*		Growth and color of aerial mycelium	Pigment
		Surface	Reverse		
CZAPECK's agar (Waksman No. 1)	Good flat	Oatmeal (2ec)	Oatmeal (2ec)	Moderate natural string (2de)	None
Glucose - asparagine agar (Waksman No. 2)	Good flat	Light rose beige (4ec)	Mustard (2le)	Poor natural (3dc)	Cinnamon (3lc)
Yeast extract - malt extract agar (ISP No. 2)	Good raised	Rose beige (4gc)	Mustard (2le)	Poor white (a)	Chestnut brown (4ni)
Oatmeal agar (ISP No. 3)	Good raised	Oatmeal (2ec)	Oatmeal (2ec)	Good covert gray (3fe)	None
Inorganic salts - starch agar (ISP No. 4)	Moderate flat	Pussywillow gray (5dc)	Sand (3gc)	Moderate white (a)	Camel (3ie)
Glycerol - asparagine agar (ISP No. 5)	Good raised	Shell (3ca)	Shell (3ca)	None	None
Peptone - yeast extract - iron agar (ISP No. 6)	Good flat	Oatmeal (2ec)	Oatmeal (2ec)	None	Clove brown (3ni)
Tyrosine agar (ISP No. 7)	Good flat	Yellow tint (2cb)	Ivory tint (1ba)	None	None
Nutrient agar	Poor flat	Camel (3ie)	Bamboo (2gc)	None	Camel (3ie)

* Color designation from Color Harmony Manual, 4th Edition, Container Corporation of America, 1958.

Fig. 1. Spores of *Streptomyces bottropensis* DO-45 grown on oatmeal agar: electron micrograph.



pimelic acid.

The cultural characteristics of strain DO-45 shown in Table 1 were observed after two weeks of incubation at 28°C. The aerial mycelia were white (colorless) to gray on agar media and gray colored on oatmeal agar. Melanoid pigments were produced on peptone - yeast extract - iron agar but not on tyrosine agar.

Table 2. Physiological properties of strain DO-45.

Liquefaction of gelatin	Negative
Liquefaction of milk	Weakly positive
Peptonization of milk	Negative
Decomposition of cellulose	Weakly positive
Hydrolysis of starch	Positive
Formation of tyrosinase	Negative
Formation of melanoid pigments	Positive
Optimum growth temperature*	28~38°C
Optimum growth pH*	6.8~7.5

* The medium contained 10 g glucose, 24 g soluble starch, 3 g beef extract, 5 g peptone per liter of water; pH 7.0.

Table 3. Utilization of carbohydrates by strain DO-45.

D-Arabinose	+	Sucrose	++
D-Xylose	++	*m*-Inositol	++
D-Glucose	++	Raffinose	++
D-Fructose	++	L-Rhamnose	+
D-Mannitol	++		

Physiological Characteristics

The physiological characteristics of strain DO-45 are shown in Table 2. The temperature range for growth and the pH range for growth was 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. Utilization of carbohydrate by strain DO-45 is shown in Table 3.

These characteristics of DO-45 shown in Tables 1, 2 and 3 place the organism in the genus *Streptomyces* and resemble those of *Streptomyces collinus, Streptomyces bottropensis* and *Streptomyces parvullus.*[5]

In literature descriptions,[6,7,8] *S. collinus* forms red vegetative mycelium on salts-starch agar and forms very short spore chains, *S. parvullus* forms no melanoid pigments on peptone-yeast extract-iron agar and tyrosine agar and utilizes raffinose poorly, and *S. bottropensis* produces melanoid pigments on peptone yeast extract-iron agar and tyrosine agar and utilizes raffinose poorly. These characteristics indicates that strain DO-45 resembles closely *S. bottropensis*, although strain DO-45 does not produce melanoid pigments on tyrosine agar, utilizes raffinose very well and forms very light gray colored aerial mycelia compared to dark gray colored aerial mycelia of *S. bottropensis*. Therefore, we concluded that strain DO-45 could be designated as *Streptomyces bottropensis* DO-45.

It is important to note that no other member of the *Actinomycetales* is reported to produce trioxacarcins or related antibiotics.

Fermentation

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, 5 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.

Basal inorganic salts of the fermentation media were 1 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 3 g NaCl, 70 mg $CuSO_4 \cdot 5H_2O$, 10 mg $FeSO_4 \cdot 7H_2O$, 8 mg $MnCl_2 \cdot 4H_2O$, 2 mg $ZnSO_4 \cdot 7H_2O$ and 0.006 mg $CoCl_2 \cdot 7H_2O$ per liter of tap water. The pH of media was adjusted to 7.0 prior to sterilization. Using the above basal medium, the effect of carbon and nitrogen sources in the fermentation medium was investigated in test tubes at 28°C for 5 days.

As trioxacarcins were present in both mycelia and culture filtrates, their amounts in whole broth were determined as follows. To 4 ml of the culture broth in a test tube, 2 ml of acetone and 2 ml of ethyl acetate were added, and mixed vigorously. After centrifugation at 1200×*g* for 10 minutes, a portion (usually 10 μl) of upper layer was applied on a silica gel plate and developed with ethyl acetate saturated with 0.1 M phosphate buffer (pH 7.0). The amount of each component of trioxacarcins was determined by measuring the intensity of fluorescence at 475 nm with excitation at 400 nm using a Shimadzu CS-900 chromatoscanner.

None of the nitrogen sources added singly to the basal carbon source medium containing 60 g soluble starch and 10 g glucose per liter of the medium showed substantially higher total amounts of trioxacarcins than the control (10 g corn steep liquor). However the amounts of each antibiotic component produced varied greatly. The control (10 g corn steep liquor) was chosen as the nitrogen source, for it gave good production of all three components and we wanted to obtain all three components. The effect of carbon sources was examined using corn steep liquor as the nitrogen source and soluble starch was selected as the carbon source for the production of all the components. Data of fermentation experiments are summarized in Tables 4 and 5.

Using the fermentation medium designed from data of the above experiments, a large scale fermentation (30-liter jar fermentor) was carried out and the time course of a typical fermentation is shown in

Table 4. Effect of nitrogen sources on production of trioxacarcins.

Nitrogen source (10 g/liter)	Trioxacarcins (μg/ml)			
	A	B	C	Total
Yeast extract	66	14	12	92
Peptone	70	16	6	92
Soybean meal	72	—	5	77
Soy casein	71	—	7	78
Pharmamedia (cottonseed flour)	63	6	20	89
Corn steep liquor	40	12	24	76

Carbon sources were 60 g soluble starch and 10 g glucose per liter of medium.

Table 5. Effect of carbon sources on production of trioxacarcins.

Carbon source (60 g/liter)	Trioxacarcins (μg/ml)			
	A	B	C	Total
Soluble starch	47	27	16	90
Dextrin	49	13	8	70
Lactose	5	—	—	5
Fructose	54	8	22	84
Maltose	20	6	9	35
Glucose	4	—	—	4
Glycerol	—	—	—	—
Sucrose	28	5	5	38
Sorbitol	5	—	—	5
Soluble starch (60 g/liter)+ glucose (10 g/liter)	32	10	20	62

Nitrogen source was 10 g corn steep liquor per liter of medium.

Table 6. Antibacterial activity (MIC) of trioxacarcins.

Test organism	Trioxacarcin (μg/ml)		
	A	B	C
Staphylococcus aureus ATCC 6538P	0.2	50	0.4
Bacillus subtilis No. 10707	0.01	12	0.01
Klebsiella pneumoniae ATCC 10031	1.5	>100	1.5
Salmonella typhosa ATCC 9992	50	>100	>100
Escherichia coli ATCC 26	12	>100	50

Medium: Nutrient agar (Eiken Chemical Co. Ltd.)

Fig. 2. Time course of trioxacarcins production.

Fermentation was carried out in a 30-liter jar fermentor using the medium indicated in the text at 30°C, agitation 250 rpm, aeration at 15 liters per minute. Total activity was determined by disc method on nutrient agar, using *B. subtilis* as the test organism and was expressed by diameters of antibacterial zones.

Packed cell volume was determined by centrifugation at 1200×*g* for 10 minutes.

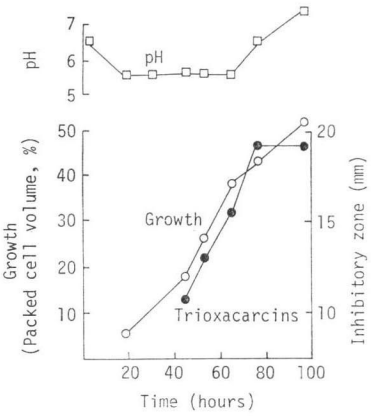


Fig. 3. Effect of trioxacarcin A on the growth of *Bacillus subtilis*.

Trioxacarcin A was added at the time of the arrow indicated and the numbers in the Fig. 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 acids, 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 Biophotorecorder (Toyo Kagaku Sangyo, Japan) at 37°C.

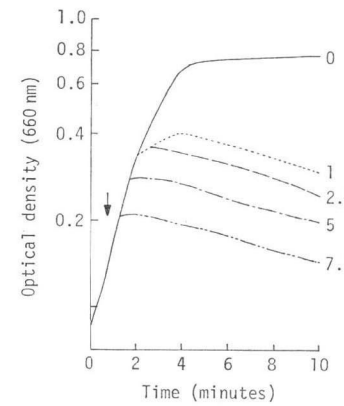


Fig. 2. Antibiotic activity appeared in whole broth extracts at about 40 hours and reached maximum at about 80 hours. The active materials were isolated and purified by sequential chromatography on non-ionic porous resin and silica gel. Details of their isolation and physico-chemical characteristics are given in the following paper.[3]

Biological Activities

Each component of the trioxacarcins was tested for its biological activities. The *in vitro* activities against various bacteria are shown in Table 6. Trioxacarcin A showed strong activity against Gram-positive bacteria, while weak activity was observed against Gram-negative bacteria. Trioxacarcin C showed a similar antibacterial spectrum, but its activities were slightly weaker than trioxacarcin A. Trioxacarcin B showed only weak activity against Gram-positive bacteria and no activity against Gram-negative bacteria.

The effect of trioxacarcin A on the growth of *Bacillus subtilis* is shown in Fig. 3. Growth was inhibited by 1 μg per ml and increasing concentrations showed more extensive effect on growth. Thus 5 μg per ml produced cell lysis indicating bactericidal activity.

The acute toxicities (LD_{50}) of trioxacarcins were calculated from the number of survivors at 14 days after a single intraperitoneal injection into *dd*Y mice. The LD_{50} of trioxacarcins A, B and C were 1 mg/kg, 100 mg/kg and 2 mg/kg, respectively.

Table 7. Antitumor activity of trioxacarcins against murine sarcoma 180 (s.c. - i.p.)

Compounds	Dose (mg/kg)	Tumor volume (mm^3)	T/C*
Control	—	1410	
Trioxacarcin A	0.2	775	0.55
	0.1	930	0.66
	0.2×7	592	0.42
	0.1×7	846	0.60
Trioxacarcin B	75	775	0.55
	50	846	0.60
	20	1057	0.75
Trioxacarcin C	1.0	493	0.35
	0.5	1170	0.83
Mitomycin C	5.6	282	0.20

Drugs were injected intraperitoneally 24 hours after tumor implantation.

* T/C represents the ratio of the median tumor volume of the treated group divided by that of the control group.

Criteria: T/C≤0.5 considered significant antitumor activity.

Table 8. Antitumor activity of trioxacarcins against murine leukemia P388 (i.p. - i.p.).

Compounds	Dose (mg/kg)	Survival days	Increase of life span(%)
Control		10.5	—
Trioxacarcin A	0.4	12.4	18
	0.3	13.4	28
	0.2	12.6	20
	0.1	12.9	23
	0.15×5	12.6	20
	0.10×5	14.8	35
	0.05×5	13.4	28
Trioxacarcin B	50	17.0	62
	25	15.5	48
	12.5	16.4	56
	6.25	14.5	38
Trioxacarcin C	1.0	13.1	25
	0.5	14.0	33
	0.25	13.3	27
Mitomycin C	4.2	16.4	56

Drugs were injected intraperitoneally 24 hours after tumor implantation. Increase of life span (%) was calculated from the average life span of the treated mice and that of the control mice.

Criteria: Increase of life span (%)≥20 considered significant antitumor activity.

Antitumor activities were examined as described in the previous paper.[9] For comparison mitomycin C was similarly administered intraperitoneally to a group of test animals. As shown in Tables 7 and 8, trioxacarcins A, B and C exhibited antitumor activities against murine tumors. The therapeutic effectiveness was higher for multiple dose treatment than for single dose treatment. The effectiveness of trioxacarcins was not as strong as mitomycin C except that trioxacarcin B showed almost the same activity against leukemia P388 as mitomycin C.

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