TRIOXACARCINS, NOVEL ANTITUMOR ANTIBIOTICS
II. ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND MODE OF ACTION

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Novel antitumor antibiotics, trioxacarcin complex, were isolated from the fermentation broth of *Streptomyces bottropensis* DO-45 by the use of non-ionic porous resin and silica gel chromatography. The purified components (trioxacarcins A, B and C) were characterized as new antibiotics by their physico-chemical properties and chromatographic behaviors.

The mode of action of trioxacarcin A against *Bacillus subtilis* was found to be through the inhibition of DNA synthesis.

As reported in the preceding paper,[1] novel antibiotics, trioxacarcins A, B and C, have been found in the culture broth of *Streptomyces bottropensis* DO-45. They were active against Gram-positive bacteria *in vitro* and showed antitumor activities against experimental murine tumors.

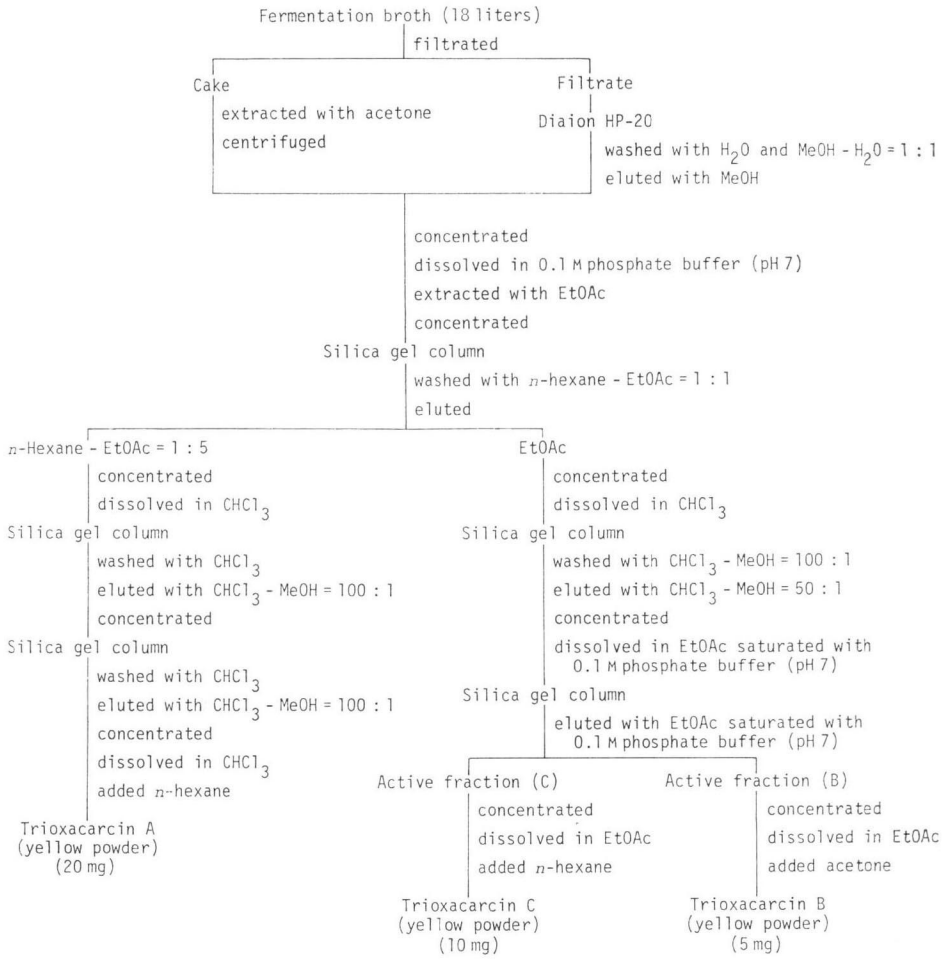
The present paper will describe their isolation, physico-chemical properties and the mode of action of trioxacarcin A.

Isolation

Activity against *Bacillus subtilis* and thin-layer chromatography were used to monitor trioxacarcins during their isolation from the culture broth of *S. bottropensis*. Since trioxacarcins are lipophilic and weakly acidic substances, they were isolated by the usual methods for such compounds (Fig. 1). Trioxacarcins were found both in mycelia and the culture filtrate. The whole broth (18 liters) was filtered with aid of 10% celite. The solid cake including the mycelia was extracted with 5 liters of acetone and the filtrate was concentrated *in vacuo*. The culture filtrate (16 liters) was applied on a column (1 liter) of non-ionic porous resin, Diaion HP-20 (Mitsubishi Chemical Ind.). After washing with water (2 liters) and then with aqueous methanol (1: 1, v/v, 2 liters) to remove impurities, the column was eluted with 5 liters of methanol. The eluate was concentrated *in vacuo* and combined with the concentrated fraction of cell extract. The combined fraction was concentrated to dryness *in vacuo* and the dried material was dissolved in a small amount of 0.1 M phosphate buffer (pH 7.0), then extracted three times with ethyl acetate. The solvent layer was concentrated *in vacuo* and adsorbed onto celite to obtain a powder. The powder was carefully put on a column (500 ml) packed with silica gel (Wakogel C-200, Wako Junyaku, Japan) which had been suspended in *n*-hexane - ethyl acetate (1: 1, v/v). The column was eluted with a mixture of *n*-hexane - ethyl acetate (1: 5, v/v) to obtain trioxacarcin A and then with ethyl acetate to obtain the mixture of trioxacarcins B and C with some amounts of trioxacarcin A. Each fraction of eluate was tested by silica gel TLC using the solvent systems of chloroform - methanol (9: 1, v/v) and ethyl acetate saturated with 0.1 M phosphate buffer (pH 7.0) (Table 1).

The fractions containing trioxacarcin A were concentrated *in vacuo* and dissolved in a small amount of chloroform, then applied on a column (100 ml) of silica gel (Wakogel C-200) which was previously

Fig. 1. Process for isolation of trioxacarcins.



suspended in chloroform. After washing with 300 ml of chloroform, the column was eluted with a mixture of chloroform - methanol (100: 1, v/v). The active fractions were concentrated and rechromatographed on a column (100 ml) of silicic acid (Mallinckrodt, Inc., U.S.A.). The column was eluted with chloroform - methanol (100: 1, v/v). The active fractions were concentrated to dryness and dissolved in a small amount of chloroform. Trioxacarcin A was obtained by precipitation with *n*-hexane.

Table 1. Chromatographic mobility of trioxacarcins on silica gel TLC.

Solvent system	Rf values of trioxacarcins		
	A	B	C
$CHCl_3$ - MeOH, 9: 1 v/v	0.85	0.50	0.45
EtOAc - Acetic acid, 9: 1 v/v	0.70	0.25	0.35
EtOAc saturated 0.1 M phosphate buffer (pH 7)	0.70	0.30	0.40

The mixture of B, C and a small amount of A obtained by the first silica gel chromatography were concentrated to dryness, dissolved in a small amount of chloroform and applied on a column (100 ml) of silica gel (Wakogel C-200) which was previously suspended and packed in chloroform. After washing with a mixture of chloroform - methanol (100: 1, v/v), the column was eluted with chloroform - methanol (50: 1, v/v) to obtain the mixture of B and C free from the contamination of A. The fractions of B and

C were concentrated to dryness and dissolved in a small amount of the solvent layer of the mixture of 0.1 M phosphate buffer (pH 7.0) and ethyl acetate, which was then transferred to a column (100 ml) packed with silica gel (Wakogel C-200) which had been suspended and packed in the solvent layer of the mixture of phosphate buffer (pH 7.0) and ethyl acetate. The column was eluted with the same solvent, and then C and B were eluted in this order. The fractions containing B and C were collected separately and concentrated to dryness. Each sample was dissolved in a small amount of ethyl acetate. Trioxacarcin B powder was obtained by adding acetone, and C powder was obtained by adding *n*-hexane.

By the above procedures, the amounts of trioxacarcins A, B and C obtained were 20 mg, 5 mg and 10 mg, respectively.

Physico-chemical Properties

Trioxacarcins are weakly acidic, yellow amorphous powders. They are soluble in organic solvents such as MeOH, $CHCl_3$, acetone *etc.*, but each component exhibited slight differences in solubility (Table 2). Trioxacarcins did not give molecular peaks on EI- and FD-mass spectroscopy and molecular weight could not be determined. However, on the basis of their CMR spectra that showed 42 peaks and elemental analyses, their molecular formulae were estimated (Table 2). The CMR spectra also indicated the presence of anomeric carbons (90~100 ppm) suggesting that trioxacarcins might contain glycosidic linkages. These estimations were substantiated by the structure determination.[3] Other characteristics are summarized in Table 2.

The infrared absorption spectra of trioxacarcins are given in Fig. 2 and each of them showed a hydrogen bonded C=O band at 1620 cm^{-1}.

The antibiotics gave a blue color with ferric chloride-potassium ferricyanide reagent, a reddish brown color with sulfuric acid, a dark green color with ferric chloride, and a reddish purple color with FEHLING reagent. They were positive to EHRLICH reagent, but negative to ninhydrin and anthrone tests.

The ultraviolet absorption spectra exhibited characteristic peaks common to all trioxacarcins (Fig.

Table 2. Physico-chemical characteristics of trioxacarcins.

	Trioxacarcin A	Trioxacarcin B	Trioxacarcin C
M.P.	177~183°C	193~194°C	181~182°C
Anal. Found.	C 55.11 H 5.74	C 54.20 H 6.15	C 54.34 H 6.03
M.W.[2]	877	895	879
Mol. Form.[2]	$C_{42}H_{52}O_{20}$	$C_{42}H_{54}O_{21}$	$C_{42}H_{54}O_{20}$
$[\alpha]_D^{25}$	−15.3° (*c* 1.0, EtOH)	−122.7° (*c* 1.0, $CHCl_3$)	−10° (*c* 0.2, EtOH)
UV: λ_{max} nm (ε) (in 90% MeOH)	233 (29100) 271 (40100) 399 (12200)	233 (30400) 271 (41300) 399 (12300)	233 (27600) 271 (36300) 399 (10700)
Nature	Yellow powder	Yellow powder	Yellow powder
Solubility			
Soluble	MeOH, acetone, EtOAc, $CHCl_3$	MeOH, EtOH, $CHCl_3$	MeOH, acetone, EtOAc, $CHCl_3$
Slightly soluble	Water, EtOEt, benzene	Acetone, EtOAc	Water, EtOEt, benzene
Insoluble	*n*-Hexane	*n*-Hexane, EtOEt	*n*-Hexane

Fig. 2. IR spectra of trioxacarcins A, B and C (KBr pellets).

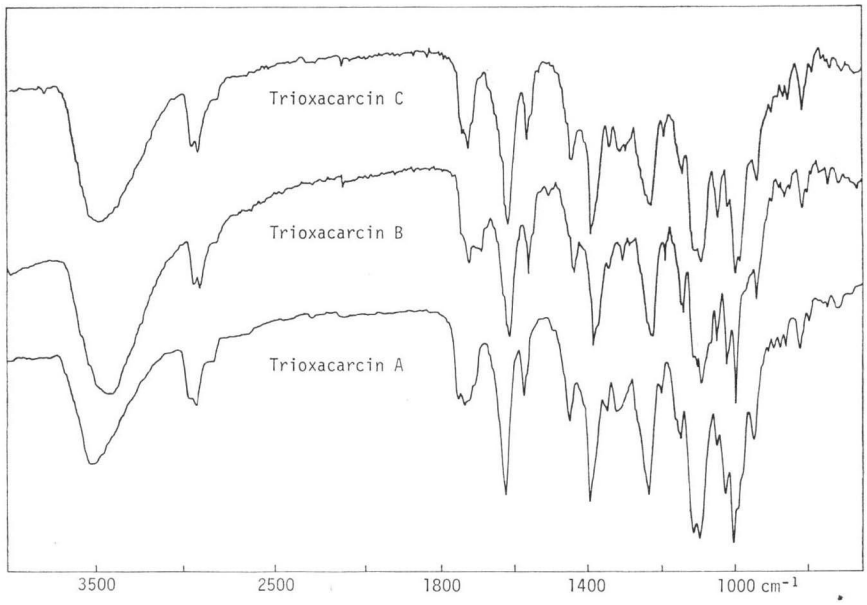
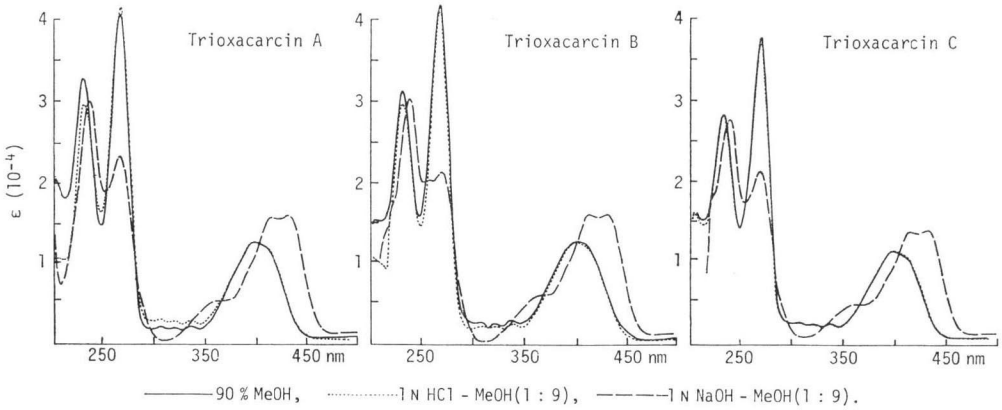


Fig. 3. UV absorption spectra of trioxacarcins A, B and C.



3) suggesting that their chromophores would be the same or very closely related to one another. Their ultraviolet absorption spectra are similar to those of chartreusin[4] and aureolic acid group antibiotics,[5] especially to the latter. However, their fluorescence spectra are clearly different and can be differentiated easily one from the other (Table 3). Moreover, trioxacarcins differ from chartreusin and olivomycin A in melting point, optical rotation, molecular weight, elemental analysis, and ultraviolet absorption spectrum as shown in Table 3.

On the basis of these results, trioxacarcins were determined to be novel antibiotics.

Effect of Trioxacarcin A on the Syntheses of Cellular Macromolecules in *Bacillus subtilis*

Trioxacarcins exhibit rather narrow antibacterial spectra, but their activities against experimental tumors are marked. Thus it is of interest to study the effect of trioxacarcins on the syntheses of macro-

Table 3. Comparison of trioxacarcin A with chartreusin and olivomycin A.

	Trioxacarcin A	Chartreusin	Olivomycin A
M.P.	177~183°C	197.5°C	164~166°C
$[\alpha]_D$	−15.3° (EtOH)	−33° (AcOH)	−36° (EtOH)
UV: λ_{max} nm	233, 271, 399	236, 264, 403	227, 277, 405
Elemental *Anal.*	C: 55.11 H: 5.74	59.89 5.19	57.78 7.69
M.W.	877	642	1200
Mol. Form.	$C_{42}H_{52}O_{20}$	$C_{32}H_{34}O_{14}$	$C_{58}H_{84}O_{26}$
Fluorescence λ_{exc} λ_{emm}	 400 475	 — —	 415 485

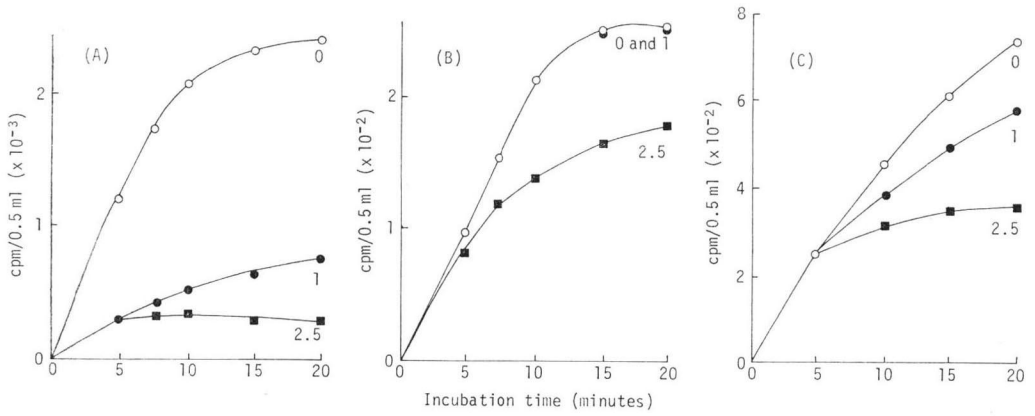
λ_{exc}: Wave length (nm) to give the maximum intensity of fluorescence.
λ_{emm}: Wave length (nm) of the maximum intensity given by λ_{exc}.

Fig. 4. Effects of trioxacarcin A on macromolecular syntheses in *Bacillus subtilis*.

(A) Incorporation of thymidine-^{3}H into the acid-insoluble fraction.
When the cell density of *B. subtilis* in the medium consisting 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 prior to sterilization) reached $OD_{660}=0.1$, thymidine-Me-^{3}H (1 μCi/ml) was added. Their 0.5 ml samples were removed and treated as described in the text. All incubations were carried out at 37°C with shaking. Numbers in Figures indicate amounts of the drug added (μg/ml).

(B) Incorporation of uracil-^{14}C into the acid-insoluble fraction.
Experimental procedures were the same as those described in (A) except that uracil-2-^{14}C (0.05 μCi/ml) was added.

(C) Incorporation of leucine-^{3}H into the acid-insoluble fraction.
Experimental procedures were the same as those described in (A) except that L-leucine-4,5-^{3}H (0.2 μCi/ml) was added.



molecules. The effects of trioxacarcin A on the syntheses of DNA, RNA and protein in growing cells of *B. subtilis* are presented in Fig. 4. The experiments were carried out according to the previous paper.[3]

Inhibition by trioxacarcin A of RNA was only slight and detected only after 10 minutes at the concentration of 2.5 μg/ml. Inhibition of protein synthesis was observed only after 10 minutes and was

not extensive. However, DNA synthesis was blocked completely at 5 minutes even at the concentration of 1 μg/ml where the growth was slightly inhibited. These results indicated that trioxacarcin A primarily inhibits DNA synthesis without gross effects on RNA and protein syntheses.

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References

1) TOMITA, F.; T. TAMAOKI, M. MORIMOTO & K. FUJIMOTO: Trioxacarcins, novel antitumor antibiotics. I. Producing organisms, fermentation and biological activities. J. Antibiotics 34: 1519~1524, 1981
2) SHIRAHATA, K.; T. IIDA & N. HIRAYAMA: The structures of trioxacarcin A, a new antitumor antibiotic, and its related compounds. Abstracts Papers of 24th Symposium on The Chemistry of Natural Products, No. 26, Osaka, October, 1981
3) TAMAOKI, T.; M. KASAI, K. SHIRAHATA, S. OHKUBO, M. MORIMOTO, K. MINEURA, S. ISHII & F. TOMITA: Tetrocarcins, Novel antitumor antibiotics. II. Isolation, characterization and antitumor activity. J. Antibiotics 33: 946~950, 1980
4) BERDY, J.: CRC Handbook of Antibiotic Compounds. VI. Alycyclic, Aromatic and Aliphatic Antibiotics. CRC Press, Inc., Cleveland, Ohio, 1981
5) REMERS, W. A.: The Chemistry of Antitumor Antibiotics. 1, p. 133~175, John Wiley & Sons, New York, 1979