TETROCARCINS, NOVEL ANTITUMOR ANTIBIOTICS

II. ISOLATION, CHARACTERIZATION AND ANTITUMOR ACTIVITY

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(Received for publication June 16, 1980)

Novel antitumor antibiotics, tetrocarcin complex composed of three components (A, B and C) were isolated from the culture broth of *Micromonospora chalcea* KY11091 through various procedures. Tetrocarcins showed marked activity against experimental tumors such as mouse sarcoma 180 and mouse leukemia P388. Multiple injections showed better therapeutic effect against tumors. Mode of action of tetrocarcin A against *Bacillus subtilis* was found to be through the inhibition of RNA synthesis.

As reported in the preceding papers,[1,2] novel antitumor antibiotics, tetrocarcins, have been found in the culture broth of *Micromonospora chalcea* KY11091. They were active against Gram-positive bacteria *in vitro* and showed antitumor activity against mouse tumors. The preliminary report on their physico-chemical characteristics has also appeared.[1]

The present report describes their isolation, physico-chemical characteristics and antitumor activity in detail. Furthermore, mode of action of tetrocarcin A is described.

Isolation

Activity against *Bacillus subtilis*[2] and thin-layer chromatography were used to monitor the isolation of tetrocarcins from the culture broth of *Micromonospora chalcea* KY11091. Since tetrocarcins are lipophilic and weakly acidic substances, they were isolated by the usual methods for such compounds (Fig. 1). The culture broth (16 liters) was filtered with aid of 10% Celite. The filtrate (15 liters) was adsorbed on a column (1 liter) of non-ionic porous resin, Diaion HP-20 (Mitsubishi Chemical Industries). After washing with 2 liters of water and 2 liters of 30% aqueous acetone, the column was eluted with 5 liters of acetone. The eluate was passed through a column (500 ml) of activated carbon

Fig. 1. Isolation procedure for tetrocarcins.

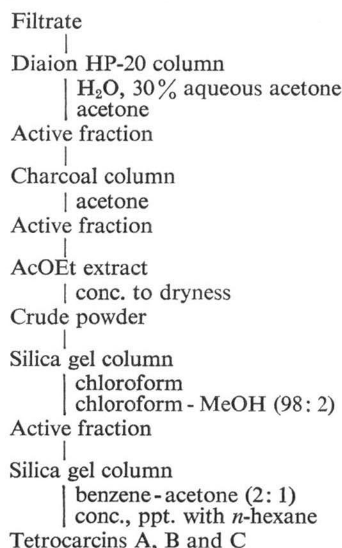

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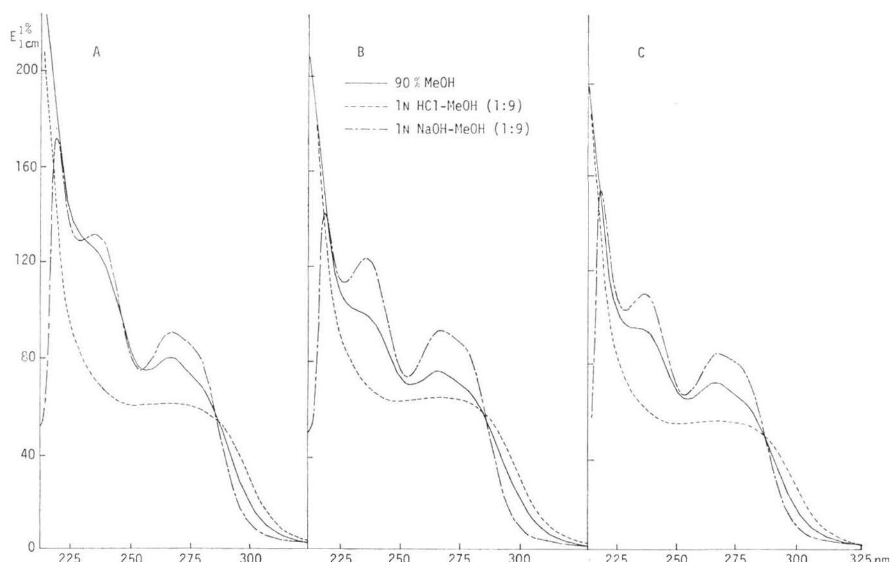
Fig. 2. UV absorption spectra of tetrocarcins A, B and C.



in order to remove the pigments and the impurities. The column was then eluted with acetone and the active fractions were pooled. After the removal of acetone *in vacuo*, the residue was extracted with ethyl acetate and the extract was concentrated to obtain 500 mg of crude powder. It was dissolved in small amounts of chloroform and applied on a column (500 ml) of silica gel (Kanto Chemical Co.). After washing with 1.5 liters of chloroform, the column was eluted with a mixture of chloroform - methanol (98: 2). The active fractions were concentrated to dryness, yielding a mixture (300 mg) of tetrocarcins A, B and C. The mixture was dissolved in about 10 ml of a mixture of benzene - acetone (2: 1) and chromatographed on a column (200 ml) of a silica gel with the same solvent. Each fraction containing tetrocarcins A, B and C was rechromatographed on a silica gel column respectively, because their separation was incomplete. The fractions corresponding to tetrocarcins A, B and C were concentrated to dryness and dissolved in a small volume of ethyl acetate. Tetrocarcins A (150 mg), B (15 mg) and C (5 mg) were obtained by precipitation with *n*-hexane.

Physico-chemical Characteristics

Tetrocarcins A, B and C were obtained as amorphous white powders. They were freely soluble in lower alcohols, acetone, ethyl acetate and chloroform, slightly soluble in benzene and water, but insoluble in *n*-hexane. They gave a violet color in concentrated sulfuric acid. They were positive in $KMnO_4$, 2,4-dinitrophenylhydrazine, $FeCl_3$ and *p*-anisidine HCl reaction, but negative to ninhydrin, anthrone and MOLISCH reagents.

Tetrocarcins A, B and C showed a UV maximal absorption at 268 nm with shoulders at 232 ~ 234 and 278 nm in 90% MeOH as illustrated in Fig. 2. These data showed that all three components contain a very similar chromophore and this assumption was confirmed by the structure determination of the antibiotics, which will be published elsewhere.

The PMR spectra are shown in Fig. 3 and indicate the presence of a formyl and an acetyl group. The IR spectra are illustrated in Fig. 4. Other physico-chemical properties have been described in

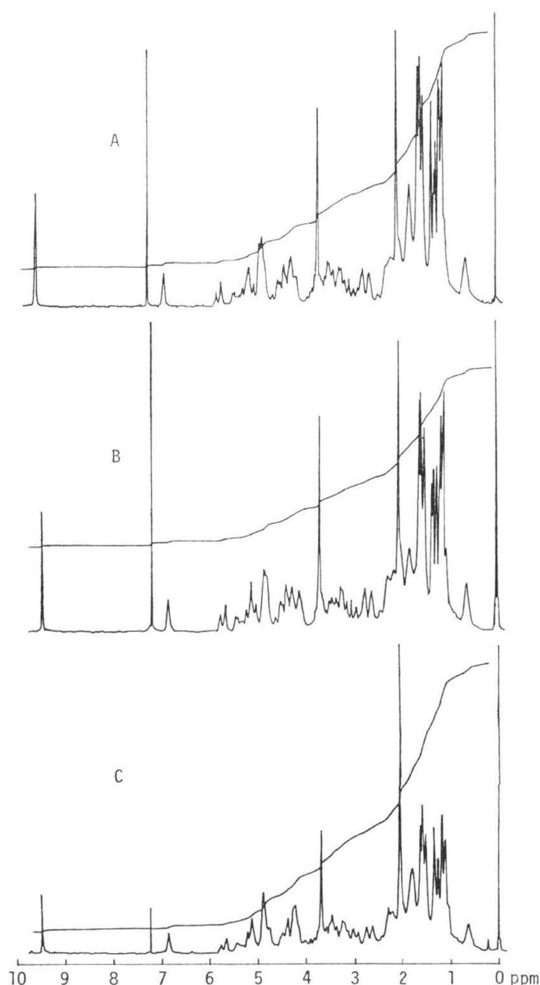
the previous paper.[1)]

Antitumor Activity

The acute toxicities (LD_{50}) of tetrocarcins were calculated from the number of survivors at 14 days after a single intraperitoneal injection into *ddY* mice. The LD_{50} values of tetrocaricns A, B and C in mice were 60~80 mg/kg of body weight by intraperitoneal injection.

For anti-leukemia P388 tumor experiment, five male CDF_1 mice weighing 18~22 g were used for each group as test animals and 1×10^6 cells of leukemia P388 tumor, which was donated by the Cancer Chemotherapy Center, were implanted intraperitoneally to the mice. After 24 hours following implantation, tetrocarcins suspended in 0.85% NaCl solution containing 0.2% Tween 80 were administered singly intraperitoneally. For comparison, mitomycin C was administered to a group of test animals intraperitoneally as in the case of tetrocarcins. The average survival period (ASP; days) and T/C (the ratio of the median survival time of the treated group divided by that of the control group) after implantation were determined. When 75 mg/kg of tetrocarcin A was injected, T/C was 1.50 as shown in Table 1.

Fig. 3. 100 MHz PMR spectra of tetrocarcins A, B and C in $CDCl_3$.



For anti-sarcoma 180 ascites tumor experiments, six male *ddY* mice weighing 18~22 g were used for each group as test animals and 5×10^6 cells of sarcoma 180, which was obtained from the National Cancer Center, were implanted subcutaneously at the axillary region of mice. After 24 hours following implantations, tetrocarcins were injected intraperitoneally as in the case of leukemia P388. Seven days after implantation the average tumor volume (mm^3) and T/C (the ratio of the median tumor volume of the treated group divided by that of the control group) were determined. When 75 mg/kg of tetrocarcin A was injected, T/C was 0.42, and when 10 mg/kg/day was injected once daily for 6 days, T/C was 0.13 as shown in Table 2. The therapeutic effectiveness was higher for multiple dose treatment than for single dose treatment.

Effect of Tetrocarcin A on the Synthesis of Cellular Macromolecules in *Bacillus subtilis*

Tetrocarcins exhibited rather narrow weak antibacterial spectra. However, their activities against experimental tumors are marked. Thus it is of interest to study the effect of tetrocarcins on the synthesis of macromolecules. The effect of tetrocarcin A on the synthesis of DNA, RNA and protein in

Fig. 4. IR spectra of tetrocarcins A, B and C. (KBr pellets)

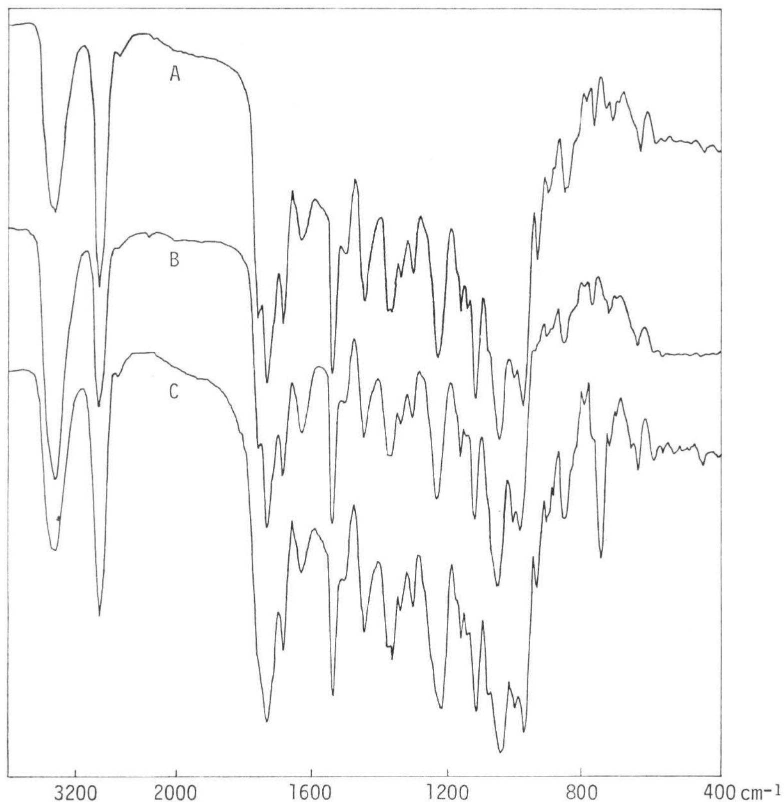


Table 1. Antitumor activity of tetrocarcins against mouse leukemia P388 (i.p.-i.p.).

Compounds	Dose (mg/kg)	ASP (days)	T/C*
Control	None	9.4	
Tetrocarcin A	25	11.2	1.20
	50	13.4	1.40
	75	14.2	1.50
Control	None	12.0	
Tetrocarcin B	12.5	14.4	1.20
	25	16.7	1.39
	50	18.0	1.50
Mitomycin C	4.2	17.0	1.80

* T/C: the ratio of median survival time of the treated group divided by that of the control group.

Table 2. Antitumor activity of tetrocarcins against mouse sarcoma 180 (s.c.-i.p.).

Compounds	Dose (mg/kg)	Tumor volume (mm^3)	T/C
Control	None	1405	
Tetrocarcin A	25	1096	0.78
	50	730	0.52
	75	590	0.42
	2.5×6 (days 1~6)	1082	0.77
	5.0×6 (days 1~6)	688	0.49
	10×6 (days 1~6)	183	0.13
Control	None	1467	
Tetrocarcin B	12.5	1027	0.70
	25	734	0.50
	50	542	0.37
	10×5	836	0.57
	20×5	440	0.30
Mitomycin C	4.2	450	0.32

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

Fig. 5. Effects of tetrocarcin A on macromolecular synthesis in *Bacillus subtilis*.

(A) Incorporation of leucine-^{3}H into the acid-insoluble fraction.

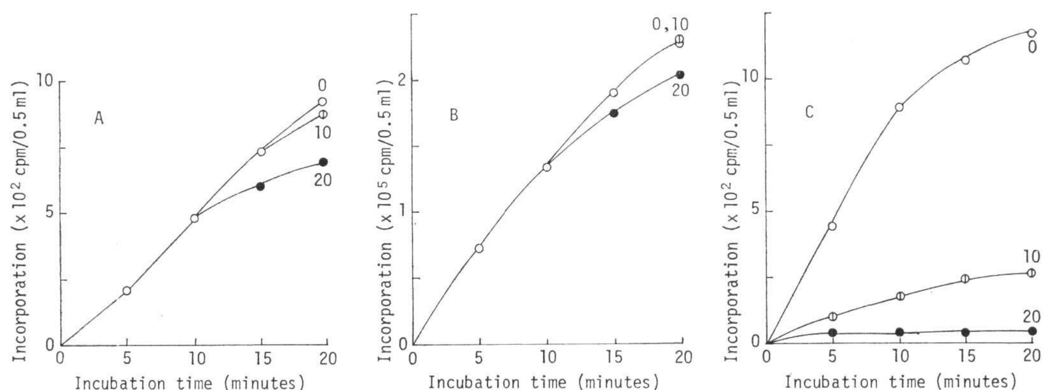
When the cell density of *B. subtilis* in the medium consisted of 0.2 g $MgSO_4 \cdot 7H_2O$, 2 g citric acid, 10 g K_2HPO_4, 3.5 g $NaNH_4HPO_4 \cdot 4H_2O$, 5 g glucose, 1 g Casamino acid, 2 g yeast extract, 50 mg tryptophan and 50 mg arginine per liter of tap water (pH 7.0 prior to sterilization) reached $OD_{660nm}=0.1$, L-leucine-4,5-^{3}H (0.2 μCi/ml) was added. After the addition of the radioactive precursor, 0.5 ml samples were removed and treated as described in the text. All the incubation were carried out at 37°C with shaking. Numbers in Figures indicate amounts of the drug added.

(B) Incorporation of thymidine-^{3}H into the acid-insoluble fraction.

Experimental procedures were the same as those described in (A) except that thymidine-methyl-^{3}H (1 μCi/ml) was added.

(C) 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.



growing cells of *B. subtilis* are presented in Fig. 5. The synthesis of DNA, RNA and protein were followed by measuring the incorporation of labeled thymidine (methyl-^{3}H), uracil-2-^{14}C and L-leucine 4, 5-^{3}H into acid-insoluble precipitates. After the addition of radioactive precursors, 0.5-ml samples were removed at intervals and poured into 2.5 ml of ice-cold 5% trichloroacetic acid and placed for one hour in the ice bath. They were filtered through HA Millipore filters (0.45 μ) and washed with 15 ml of cold 5% trichloroacetic acid. The filters were dried and counted in vials containing toluene scintillation fluid consisted of 4 g 2,5-diphenyloxazole and 0.1 g 2,2′-*p*-phenylene-bis-(5-phenyloxazole) per liter of toluene.

Inhibition by tetrocarcin A of DNA synthesis was only slight and detected only after 20 minutes at the concentration of 20 μg/ml. However, protein synthesis was inhibited in 10~15 minutes, although the inhibition was slight. RNA synthesis was blocked completely at 5 minutes and even at the concentration of 10 μg/ml where inhibition of the growth was very slight (Fig. 5) the synthesis was severely inhibited. These results indicated that tetrocarcin A primarily inhibits RNA synthesis and has some effect on protein synthesis, without affecting DNA synthesis.

References

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