TETROCARCINS E_1, E_2, F AND F-1, NEW ANTIBIOTICS

FERMENTATION, ISOLATION AND CHARACTERIZATION

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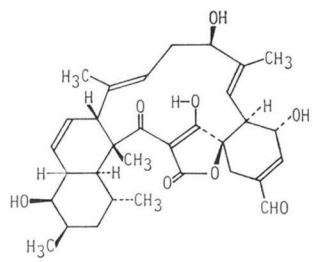
New components of tetrocarcins (E_1, E_2, F and F-1) were found in the culture broth of *Micromonospora chalcea* KY 11091 that was known to produce tetrocarcins A, B and C. Tetrocarcin F-1 consisted of tetronolide and nitro sugar (tetronitrose). Tetrocarcins E_1 and E_2 consisted of F-1 and deoxy sugar (L-digitoxose). Tetrocarcin F consisted of F-1 and two deoxy sugars (their structures were not yet determined). They all showed antibacterial activities against Gram-positive bacteria and the specific activity decreased with decrease in the numbers of deoxy sugars attached to the aglycone.

We have reported that novel antibiotics, tetrocarcins A, B and C were isolated from a culture broth of the newly isolated strain of *Micromonospora chalcea*.[1~3]

They consisted of the common aglycone designated tetronolide[4] (Fig. 1), nitro sugar (tetronitrose) and deoxy sugars (L-amicetose and L-digitoxose).[1,5,6] They showed activity against experimental tumors such as mouse sarcoma 180 and mouse leukemia P388. These findings prompted us to make detailed studies of tetrocarcins fermentation. In the culture broth of tetrocarcins fermentation, we found various new components related to tetrocarcins A, B and C.

Fig. 1. Structure of tetronolide.



In the present paper, fermentation, isolation and properties of tetrocarcins E_1, E_2, F and F-1 are described.

Materials and Methods

Microorganisms

Micromonospora chalcea KY11091 was described previously.[2] *Bacillus subtilis* No. 10707 was from our laboratory stock and used as an indicator for tetrocarcin bioassays. Other bacteria were also from our laboratory stock.

Media and Culture Condition

The seed medium and the culture conditions for the seed culture were the same as reported previously.[2] The fermentation medium consisted of 60 g soluble starch, 10 g soybean meal, 10 g peptone, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$ and 1 g $CaCO_3$ per liter of tap water. The pH of the medium was adjusted to 7.0 prior to sterilization. The fermentation was carried out at 28°C for 72 hours in a 300-liter tank fermentor with aeration (180 liters/minute) and agitation (200 rpm).

Determination of Cell Growth

The growth of cells was expressed as packed cell volume (PCV) that was measured after centrifugation

of culture broths (10 ml) at 1,200×*g* for 10 minutes.

Chromatography

Thin-layer chromatography was carried out on silica gel plates (E. Merck, 0.25 mm, 20×20 cm). Column chromatography was carried out on silica gel (Kanto Chemical Co.), non-ionic porous resin Diaion HP-20 (Mitsubishi Chemical Industry) and charcoal (Wako Co.).

Assay of Tetrocarcins

Total amounts of tetrocarcins were assayed by the paper disc method using *B. subtilis* No. 10707 as an indicator. In some experiments tetrocarcins were measured according to their UV absorbancy at 270 nm, assuming that molar extinction coefficient were the same for all tetrocarcins after developing with the lower layer of a mixture of $CHCl_3$ - MeOH - H_2O (3: 1: 1) in silica gel thin-layer chromatography.

Results and Discussion

Fermentation

Time course of tetrocarcins fermentation was as shown in Fig. 2. After 72-hour cultivation, the fermentation broth contained 200 μg/ml of tetrocarcin A, 10 μg/ml of E_1 and E_2, 20 μg/ml of F and 5 μg/ml of F-1. (Tetrocarcins E_1 and E_2 are interchangeable and cannot be measured separately under this condition.)

Isolation

Tetrocarcins E_1, E_2, F and F-1 were isolated from 180 liters of the broth as shown in Fig. 3. A crude tetrocarcins mixture (about 5 g) obtained by sequential chromatography on Diaion HP-20, charcoal and silica gel was dissolved in a small amount of the lower layer of a mixture of chloroform - methanol - water (3: 1: 1, v/v/v) and applied on a column of silica gel (2 liters) which was equilibrated with the same solvent. The column was developed with 5 liters of the same solvent. Tetrocarcins were eluted in the order of E_1, F, E_2, F-1 and A, but the separation of these components were not complete and further procedures were required

Fig. 2. Time course of the fermentation in a 300-liter tank.

Fermentation was carried out in a 300-liter tank using the medium indicated in the text at 30°C with agitation at 200 rpm and aeration of 180 liters per minute.

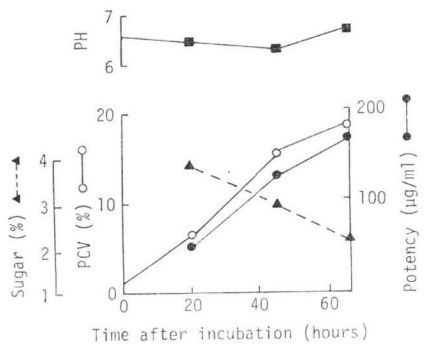
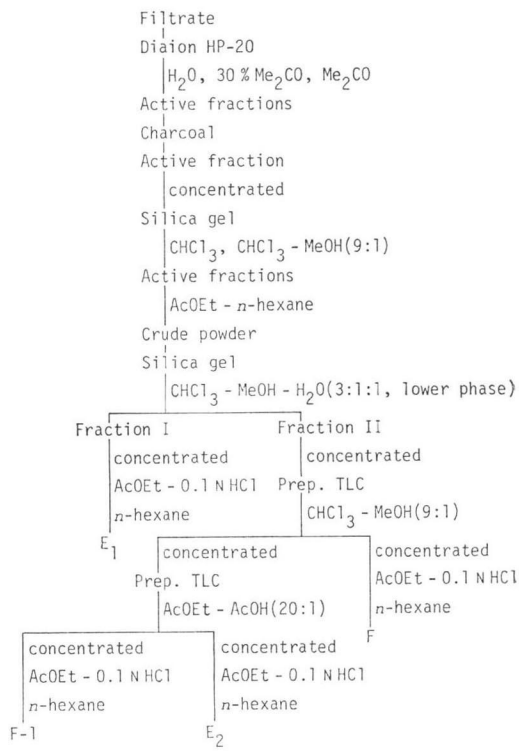


Fig. 3. Isolation procedures for tetrocarcins.



to obtain pure samples. Fractions containing E_1 (Fraction I) were combined, concentrated to dryness and dissolved in ethyl acetate. Then the solution was washed with 0.1 N HCl in order to remove mineral ions. Tetrocarcin E_1 (50 mg) was obtained by precipitation with *n*-hexane. Fractions containing F, E_2 and F-1 were concentrated and subjected to preparative thin-layer chromatography with chloroform - methanol (9: 1, v/v). Tetrocarcin F was separated from tetrocarcins E_2 and F-1, and it was eluted with acetone from silica gel and concentrated to dryness. Tetrocarcin F (70 mg) was obtained by a procedure similar to that for E_1. The mixture of tetrocarcins E_2 and F-1 was also eluted by acetone and concentrated to dryness. Then, these were dissolved in a small amount of acetone and subjected to preparative thin-layer chromatography with ethyl acetate - acetic acid (20: 1, v/v). The zones corresponding to tetrocarcins F-1 and E_2 were separately eluted by acetone and concentrated to dryness. After removal of mineral ions by washing with 0.1 N HCl, tetrocarcin F-1 (1 mg) and E_2 (10 mg) were obtained.

Physico-chemical Properties

The Rf values of tetrocarcins E_1, E_2, F and F-1 on thin-layer chromatography are shown in Table 1. They could be clearly differentiated from each other by this method.

Physico-chemical properties of four components are listed in Table 2. Their UV absorption spectra suggest that they all have the same aglycone, tetronolide. The IR spectra are shown in Fig. 4. PMR spectrum of tetrocarcin F-1 was identical with component f-1 obtained by hydrolysis of tetrocarcin A with 0.2 N HCl in acetone[1,5,6] (Table 3). The molecular ion peak of tetrocarcin F-1 was observed at *m/z* 782 in its FD mass spectrum which was identical with component f-1. Thus, the structure of tetrocarcin F-1 consists of tetronolide and a nitro sugar (tetronitrose) as shown in Fig. 5.[5,6] Tetrocarcin A contains tetrocarcin F-1 and four deoxy sugars (two digitoxoses and two amicetoses).[1,5,6] Tetrocarcin B contains F-1 and three deoxy sugars (two digitoxoses and one amicetose). Their structural elucidation will be reported elsewhere.[6] PMR spectra of tetrocarcins E_1 and E_2 were identical with those of hydrolyzed components 1 and 2[5,6] which were obtained by hydrolysis of tetrocarcin A at pH 2 and consisted of tetrocarcin F-1 and digitoxose (Table 4). Tetrocarcin F is suggested to contain tetrocarcin F-1 and two deoxy sugars according to the PMR spectrum (Fig. 6). It has two ano-

Table 1. Rf values of tetrocarcins on silica gel TLC.

Tetrocarcins	Solvent		
	I	II	III
A	0.56	0.37	0.46
E_1	0.73	0.46	0.75
E_2	0.63	0.40	0.69
F	0.67	0.41	0.72
F-1	0.61	0.43	0.80

I: $CHCl_3$ - MeOH (9: 1, v/v)
II: Toluene - Me_2CO (2: 3, v/v)
III: AcOEt - AcOH (20: 1, v/v)

Fig. 4. IR spectra of tetrocarcins (KBr).

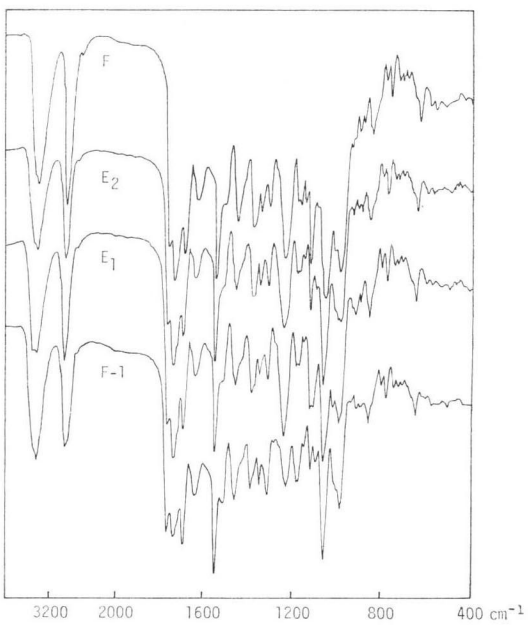


Table 2. Physico-chemical properties of tetrocarcins.

	E_1	E_2	F-1	F
MP	207~210°C	205~208°C	207~210°C	201~204°C
$[\alpha]_D$	−31.6°	−27.4°	+36.2°	−49.4°
(Me_2CO)	(*c* 1.0)	(*c* 1.0)	(*c* 1.0)	(*c* 1.0)
Anal.	C H N	C H N	C H N	C H N
Found	61.3 6.9 3.0	61.4 7.1 2.9	62.9 7.2 3.7	61.8 7.4 2.6
	$C_{49}H_{66}N_2O_{17}$	$C_{49}H_{66}N_2O_{17}$	$C_{41}H_{54}N_2O_{13}$	
Calcd.	C H N	C H N	C H N	
	61.6 7.0 2.9	61.6 7.0 2.9	62.9 7.0 3.6	
UV λ_{max} (ε)	232sh (17000)	232sh (17000)	232sh (17800)	232sh (151)*
in 90% MeOH	268 (10300)	268 (10300)	268 (10100)	268 (92)
	278sh (9100)	278sh (9100)	278sh (8900)	278sh (79)

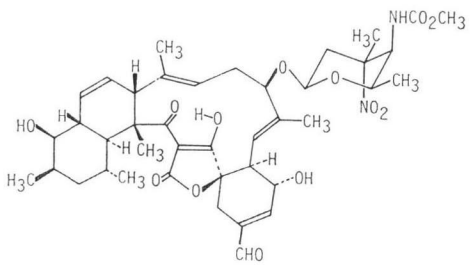
* $E^{1\%}_{1cm}$

Table 3. Chemical shifts of PMR spectra of tetrocarcin F-1 and hydrolyzed component f-1 of tetrocarcin A.

Protons	Component f-1	F-1
$-CH_3$	1.05 d 1.18 d 1.38 s 1.53 s 1.60 s 1.66 s	1.05 d 1.17 d 1.33 s * * 1.66 s
$-CO_2CH_3$	3.72 s	3.73 s
-CHO	9.52 s	9.59 s

* The signals of these two methyl groups were overlapped on the signals of impurities and could not be assigned undoubtedly.

Fig. 5. Structure of tetrocarcin F-1.



meric protons, in contrast to E_1 and E_2 that have one anomeric proton. The structure determination of tetrocarcin F is under progress now. The fact that tetrocarcins having one to four deoxy sugars were in the culture broth may reflect the biosynthetic sequence of tetrocarcin A.

Table 4. Chemical shifts of PMR spectra ($CDCl_3$).

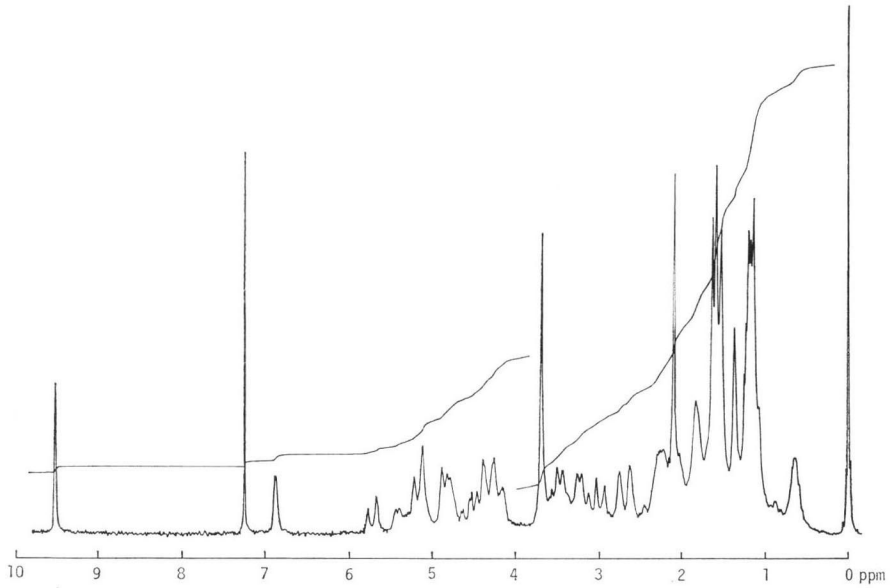
Protons	Component 1	E_1	Component 2	E_2
$-CH_3$	1.13 d	1.13 d	1.14 d	1.14 d
	1.17 d	1.16 d	1.17 d	1.17 d
	1.22 d	1.22 d	1.30 d	1.30 d
	1.37 s	1.37 s	1.36 s	1.36 s
	1.54 s	1.54 s	1.54 s	1.54 s
	1.60 s	1.60 s	1.60 s	1.60 s
	1.64 s	1.64 s	1.64 s	1.63 s
$-OCOCH_3$	2.14 s	2.14 s	2.13 s	2.13 s
$-CO_2CH_3$	3.72 s	3.72 s	3.72 s	3.72 s
-CHO	9.58 s	9.59 s	9.58 s	9.58 s

Table 5. Antibacterial activity of tetrocarcins.

Test organisms	MIC (μg/ml)			
	E_1 & E_2	F	F-1	A
Staphylococcus aureus ATCC 6538P	50	25	>200	20
Bacillus subtilis No. 10707	20	3.0	150	0.05
Klebsiella pneumoniae ATCC 10031	>200	>100	>200	>200
Escherichia coli ATCC 26	>200	>100	>200	>100
Shigella sonnei ATCC 9290	>200	>100	>200	>100
Serratia marcescens ATCC 4003	>200	>100	>200	100

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

Fig. 6. ^1H NMR spectrum of tetrocarcin F (100 MHz, $CDCl_3$).



Antibacterial Properties

Minimum inhibitory concentrations (MIC) of tetrocarcins E_1, E_2, F and F-1 against different groups of bacteria are shown in Table 5. Tetrocarcins E_1, E_2, F and F-1 were moderately active against *Bacillus subtilis*, rather weakly active against *Staphylococcus aureus*, and not active against the Gram-negative bacteria tested.

Table 6. MIC against *B. subtilis* and the number of deoxy sugars attached to tetrocarcins.

Tetrocarcins	MIC (μg/ml)	Numbers of deoxysugars
A	0.05	4
B	0.1	3
F	3.0	2
E_1	20	1
E_2	20	1
F-1	150	0

As shown in Table 6 antibacterial activity is proportional to the numbers of deoxy sugars in tetrocarcins, that is, the activity decreased as the following sequence: tetrocarcin A (containing four deoxy sugars, two L-digitoxoses and two L-amicetoses), B (containing three deoxy sugars, two L-digitoxoses and one L-amicetose), F (containing two deoxy sugars), E_1 and E_2 (containing one deoxy sugar, L-digitoxose) and F-1 (containing no deoxy sugar).

Acknowledgements

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