A MACROCYCLIC ANTIBIOTIC M-230B PRODUCED BY *MYXOCOCCUS XANTHUS*

ISOLATION AND CHARACTERIZATION

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Myxococcus xanthus strain M516E produced at least three related antibiotics against Gram-positive and Gram-negative bacteria. From physico-chemical properties, a main component was identical to myxovirescin A and a second component, designated M-230B was found to be an antibiotic which is closely related to myxovirescin A. The structure of M-230B was determined from its physico-chemical properties, especially from ^{13}C NMR spectrum as compared with that of myxovirescin A. The addition of alcohol, such as isobutyl alcohol, to the culture medium markedly stimulated production of the antibiotics.

In the course of screening for antibiotics from myxobacteria, a strain M516E was found to produce at least three closely related antibiotics which were active against Gram-positive and Gram-negative bacteria, especially against enterobacteria. Strain M516E was identified as *Myxococcus xanthus* from taxonomic studies. Among the antibiotics produced, the main component was identical to myxovirescin A[1,2] and a second component M-230B was found to be an antibiotic which is closely related to myxovirescin A. This paper describes the properties of the producing organism, the antimicrobial activities of these antibiotics and the isolation and characterization of M-230B.

Materials and Methods

General

Melting points were determined with a Yanaco MPJ-3. UV spectra were examined with a Hitachi 124 spectrophotometer, 1 cm light path, using methanol as a solvent. IR spectra were measured by a Jasco-A202 infrared spectrometer on NaCl. Optical rotation was measured by a Jasco DIP-4 polarimeter, using methanol as a solvent. ^1H NMR and ^{13}C NMR spectra were measured in $CDCl_3$ on a Jeol JNM-FX100 spectrometer using tetramethylsilane (TMS) as the internal reference. Mass spectra were determined with a Jeol JMS-01SG-2 spectrometer equipped with a direct inlet system. For HPLC, a Toyo Soda HLC-803 with a TSK-GEL-LS410KG column was used and the detection was carried out using a Jasco Uvidec-100-IV UV spectrophotometer.

Fermentation

In preliminary fermentation studies, SP medium[3] containing 0.3% Casitone, 0.5% soluble starch, 0.1% raffinose, 0.1% galactose, 0.1% sucrose, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.025% K_2HPO_4, pH 7.0 was used. One hundred milliliters of this medium in a 500-ml shaking flask was inoculated with the organism and incubated on a reciprocal shaker at 30°C for three days. Since we found the stimulation of antibiotic production by the addition of various alcohols, we usually added 0.5% isobutyl alcohol to the fermentation medium.

Assay for Antimicrobial Activity

Antibiotics produced by the organism were assayed using *Escherichia coli* B as a test organism.

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The inhibition of the growth of *E. coli* B was assayed by the paper disk (7 mm in diameter) method for two days at 30°C, using peptone agar containing 0.3% peptone, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% K_2HPO_4 and 1.5% agar, pH 7.0.

Isolation and Purification of Myxovirescin A and M-230B

Fermentation was carried out in a 3-liter volume of shaking flask containing 700 ml of the medium on a reciprocal shaker at 30°C for 6 days at 140 cycles per minute. The medium before autoclaving contained 0.3% peptone, 0.15% soluble starch, 0.1% $MgSO_4 \cdot 7H_2O$, 0.01% K_2HPO_4 and 0.5% isobutyl alcohol, in 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer pH 7.0. The antibiotics produced could be extracted from both the culture supernatant and the cells. Culture broth (20 liters) was centrifuged and the clarified culture supernatant fluid was concentrated *in vacuo* to 1 liter under reduced pressure at 30°C. Then concentrate was extracted with ethyl acetate exhaustively until no activity against *E. coli* B was detected in the spent aqueous phase by the paper disk method. The cells removed by the centrifugation were extracted twice with 500 ml of acetone. After the acetone was evaporated, the residue was extracted with 500 ml of ethyl acetate. Both the extracts from the culture supernatant and from the cells had antibiotic activity and, therefore, these two fractions were combined and concentrated *in vacuo* until the ethyl acetate was removed. The residue was then dissolved in a small amount of chloroform and was applied to silica gel column (2×45 cm) packed with chloroform. Wako gel C-100 was used for silica gel column chromatography, and was activated at 120°C for 60 minutes, cooled, stirred with chloroform and packed as a slurry. The column was eluted with 500 ml of chloroform to remove inactive materials and subsequently eluted with 1 liter of acetone. The active fractions eluted with acetone were concentrated *in vacuo* and were subjected to preparative silica gel thin-layer chromatography (TLC) using chloroform - acetone (3: 2) as the solvent. Preparative TLC (Merck Silica gel $60PF_{254}$) was carried out on 0.75 mm thickness (20×20 cm). The plate was dried overnight at 30°C and activated at 100°C for 60 minutes, cooled and used immediately. The active fractions, located by an UV lamp (254 nm), were scraped off, extracted with 500 ml of methanol and concentrated *in vacuo*. After additional purification by the same TLC twice more, the concentrated materials were further purified by preparative high performance liquid chromatography (HPLC) on a reversed phase silica gel column with a solvent of methanol - water, (4: 1).

Results and Discussion

Organism

A strain, M516E, of myxobacteria, was isolated from a soil sample collected in Sendai, Miyagi, Japan by the method of SINGH[4]. Vegetative cells of strain M516E were rods with rounded ends, 0.5×5~8 μm in size. This strain is strictly aerobic and possesses bacteriolytic activities. Fruiting bodies formed on ECM agar[3] contained refractile spherical microcysts. On the basis of morphological examination, it was concluded that the strain M516E belonged to the genus *Myxococcus*. Further taxonomic characteristics of strain M516E which were examined according to BERGEY's Manual of Determinative Bacteriology 8th edition[6] are as follows: 1) The isolated strain has deliquescent fruiting bodies but they are not raised on a well defined persistent stalk. 2) Myxospore is 1.5 μm or more in diameter. 3) Color of vegetative cell mass is yellow or orange. 4) No diffusible pigment was observed on agar. From these taxonomic characteristics, the strain M516E was identified as *Myxococcus xanthus*.

Effect of Alcohol on the Production of Antibiotics

The amounts of antibiotics produced in the culture supernatant and those extracted from the cells with acetone were very low (approximately 1 μg/ml in the culture supernatant) and, therefore, the culture supernatant did not show any detectable antibacterial activity against *E. coli* B by the paper disk method. In order to enhance the production of these antibiotics, various organic compounds were

added to the culture medium. As shown in Table 1, some alcohols such as isopropyl alcohol, some butyl alcohols, or isoamyl alcohol had a remarkable stimulating activity for the production of the antibiotics. When strain M516E was repeatedly cultured in the SP medium containing isobutyl alcohol, the production of these antibiotics increased progressively. Strain M516E, which was cultured in this way, was designated strain M516E type R. Although this strain was still slightly sensitive to isobutyl alcohol, it produced these antibiotics even without the addition of isobutyl alcohol to the culture medium. The mechanism of this stimulative effect has not yet been clarified. It is reasonable to consider that alcohol, especially isobutyl alcohol is a precursor of these antibiotics after oxidation and conversion to propionyl CoA since myxovirescins are macrolides which are formed partially as polyketides of propionate. It has been reported that addition of some alcohols, such as isopropyl alcohol or *n*-butyl alcohol to the growing myxobacterial cell suspension, resulted in transformation of vegetative cells to myxospore[5]. Although no myxospore could be seen in the culture broth under the present conditions, it is possible to consider that enhanced production of the antibiotics has some relation to cellular morphogenesis. It may also be presumed that the membranes of the cells were damaged by these alcohols and consequently antibiotics would be leaked out of the cells easily.

Strain M516E type R seems to be a spontaneous mutant of the strain M516E and it is a suitable strain for antibiotic production. Interestingly, this strain can form fruiting bodies on SP agar, while the wild type strain can not. From these observations, strain M516E type R is a useful strain for studies on cellular morphogenesis also.

Isolation

The separation of myxovirescin A and M-230B was achieved by preparative HPLC on a reversed phase silica gel column with a solvent of methanol - water (4: 1) as shown in Fig. 1. The eluate was concentrated, freeze dried and the residue crystallized from methanol - water. Sixty mg of crystalline myxovirescin A and 10 mg of crystalline M-230B were obtained from this fermentation. The procedure for the isolation of these antibiotics is shown in Fig. 2.

Table 1. Effect of organic compounds on the production of the antibiotics.

Organic compounds	Addition of organic compounds to the medium (%)	Antibiotics production (clear zone diameter, 7 mm disk) (mm)
None		0
Ethyl alcohol	1.0	0
Propyl alcohol	0.5	12
Isopropyl alcohol	0.5	18
n-Butyl alcohol	0.5	17
Isobutyl alcohol	0.5	20
tert-Butyl alcohol	0.5	20
Amyl alcohol	0.2	9
Isoamyl alcohol	0.2	20
D-Sorbitol	1.0	0
D-Mannitol	1.0	0
L-Threonine	1.0	0

Cultured in SP medium.
Organic compounds were added before autoclaving (120°C, 15 minutes).

Fig. 1. HPLC chromatogram of the antibiotics.
About 40 mg of the crude antibiotics was loaded on the column (20 mm × 30 cm) and eluted with solvent at flow rate of 3.0 ml/minute.

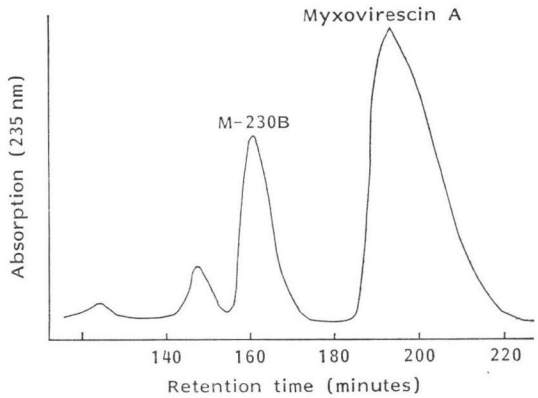


Fig. 2. Isolation procedures for the antibiotics.

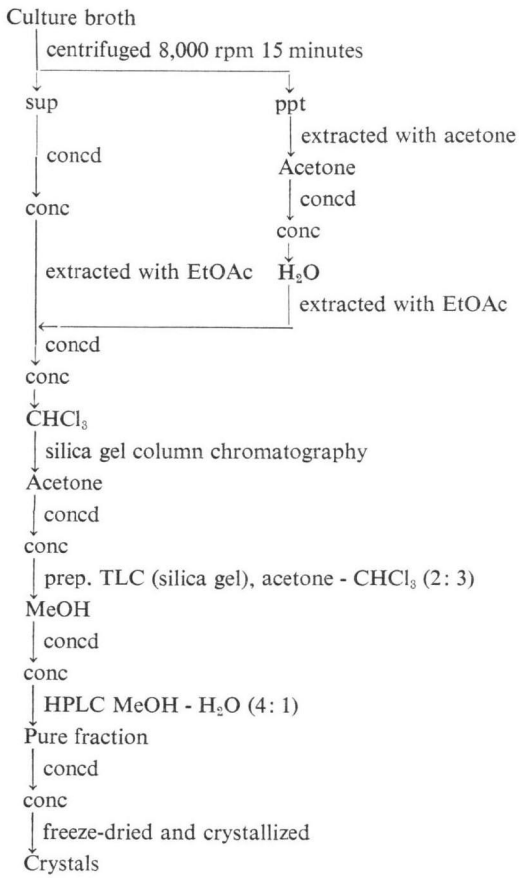


Table 2. Minimal inhibitory concentration of the antibiotics (agar dilution method).

Test organism	MIC (μg/ml)	
	M-230B	Myxovirescin A*
Escherichia coli B	0.4	0.4
Escherichia coli K-12	0.8	1.6
Salmonella typhimurium	3.2	3.2
Pseudomonas aeruginosa	>100	>100
Pseudomonas fluorescens	>100	>100
Pseudomonas marginalis	>100	>100
Erwinia carotovora	50	50
Serratia marcescens	100	50
Micrococcus luteus	>100	>100
Bacillus subtilis	50	>100
Bacillus megaterium	50	100
Staphylococcus aureus	50	50

Assayed on nutrient agar containing 0.5% beef extract, 0.5% Peptone, 0.25% NaCl and 1.5% agar, pH 7.0.

* Myxovirescin A was prepared from the culture of strain M516E.

Antimicrobial Activities

The antibacterial activities of the purified materials thus obtained were assayed by the agar dilution method. Bacteria were grown for 48 hours at 30°C. As shown in Table 2, myxovirescin A and M-230B showed strong antibacterial activities against enterobacteria such as *E. coli* and *Salmonella typhimurium*, but they showed less activities against other Gram-positive and Gram-negative bacteria. These compounds

Fig. 3. UV spectrum of M-230B.
Concentration was 20 μg/ml (in methanol).

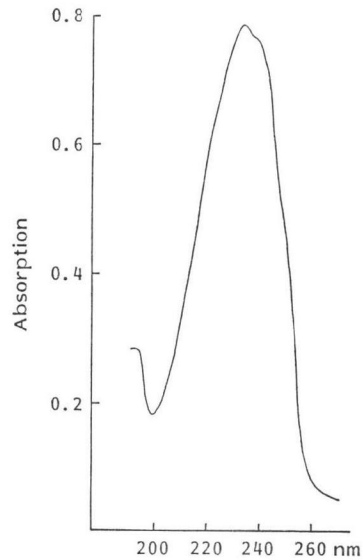


Table 3. Physico-chemical properties of M-230B and myxovirescin A.

	M-230B	Myxovirescin A*
Appearance	Colorless needles	Colorless needles
Mp (°C)	113	37~42
$[\alpha]_D$ in MeOH	+13° (*c* 0.2)	+20° (*c* 0.2)
Molecular formula	$C_{35}H_{59}NO_8$	$C_{35}H_{61}NO_8$
Mass (EI-HI)	621.4240	623.4407
UV λ_{max} nm (ε) in MeOH	230 (25,000), 238 (23,500)	238 (24,000)
IR ν_{max} cm^{-1}	3300~3500, 1710, 1665	3300~3500, 1740, 1710, 1665

* Myxovirescin A was prepared by the strain M516E.

Fig. 4. IR spectrum of M-230B.

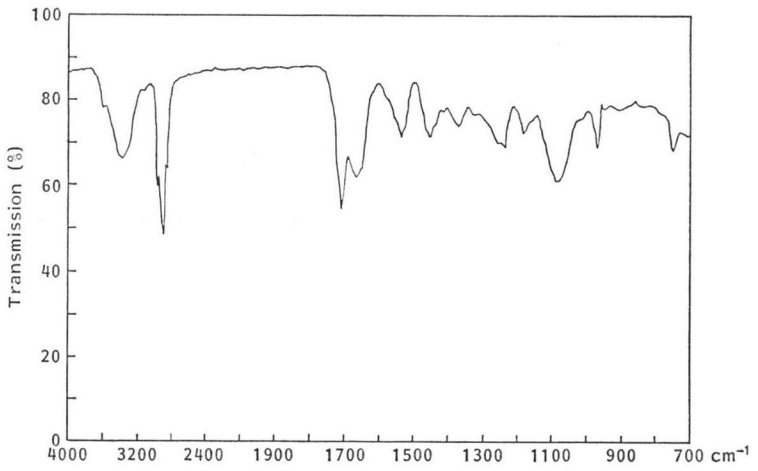
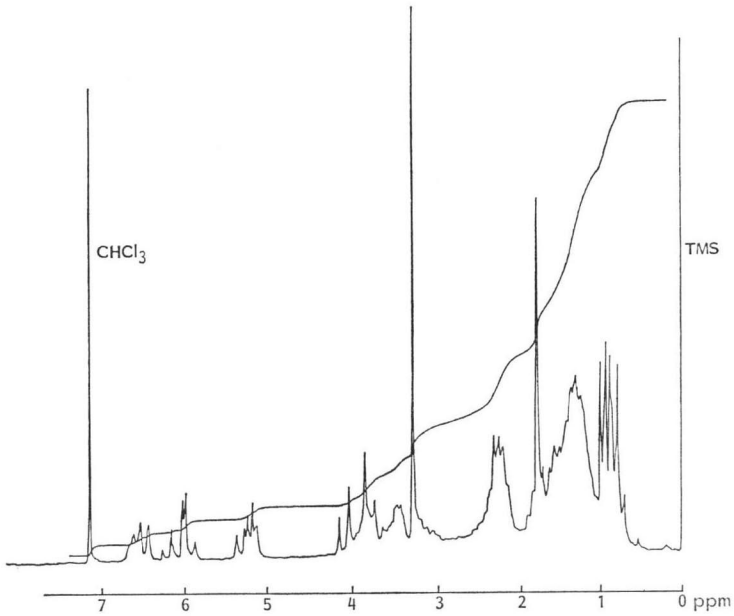


Fig. 5. ^1H NMR spectrum of M-230B in $CDCl_3$ at 100 MHz.



showed no activity against fungi and yeast (*Neurospora crassa* and *Saccharomyces cerevisiae*, respectively).

Physico-chemical Properties and Structure of M-230B

The UV and IR spectra of M-230B shown in Figs. 3 and 4, respectively, suggest the presence of an α,β-unsaturated carbonyl system. The 100 MHz ^1H NMR spectrum of M-230B, shown in Fig. 5 suggests the presence of one each $-OCH_3$ (3.36 ppm s) and $-C{=}C-CH_3$ (1.85 ppm s). Some physico-chemical properties of M230B and myxovirescin A are listed in Table 3. Both physico-chemical properties and ^1H NMR spectrum suggest that M-230B is very close in chemical structure to myxovirescin A which has been studied by TROWITZSCH *et al.*[2]. The elucidation of its structure was performed by the comparison of the ^{13}C NMR signals between myxovirescin A and M-230B (Table 4). Six out of thirty

five signals were different. The carbonyl signal at 175.9 ppm due to C-1 in myxovirescin A disappears and a new carbonyl signal appears at 166.7 ppm, and two signals of olefinic carbon appear at 126.1 ppm and 149.1 ppm in M-230B. From the results of UV, IR and ^{1}H NMR spectra which suggest the presence of α,β-unsaturated carbonyl system and allylic protons (1.85 ppm s, not shown in myxovirescin A), we

Table 4. ^{13}C NMR spectral data of myxovirescin A and M-230B.

Carbon No.	Myxovirescin A* (ppm)	M-230B (ppm)	Carbon No.	Myxovirescin A* (ppm)	M-230B (ppm)
C-1	175.9 s	166.7	C-19	30.5 t	30.5
C-2	37.2 d	126.1	C-20	73.5 d	73.5
C-3	40.9 t	149.1	C-21	71.5 d	71.5
C-4	30.4 d	33.8	C-22	36.0 t	36.4
C-5	36.5 t	37.0	C-23	68.4 d	69.0
C-6	26.4 t	27.5	C-24	45.4 t	45.3
C-7	23.8 t	23.7	C-26	171.0 s	171.5
C-8	42.4 t	42.5	C-27	73.6 d	73.9
C-9	212.2 s	212.3	C-29	17.3 q	12.7
C-10	43.1 t	43.0	C-30	19.6 q	20.3
C-11	22.1 t	22.2	C-31	28.3 t	28.3
C-12	34.6 t	34.7	C-32	11.8 q	11.9
C-13	45.1 d	45.1	C-33	70.9 t	71.1
C-14	139.8 d	139.5	C-34	58.1 q	58.3
C-15	125.8 d	125.9	C-35	34.0 t	34.0
C-16	129.7 d	130.1	C-36	18.2 t	18.3
C-17	134.7 s	134.7	C-37	13.7 q	13.7
C-18	30.4 t	30.3			

* Data from W. TROWITZSCH *et al.*[2]

Fig. 6. Assignment of ^{13}C NMR signals.
Number of carbon corresponds to that shown in Table 4.

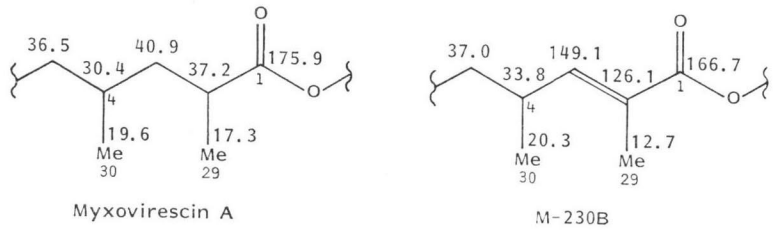
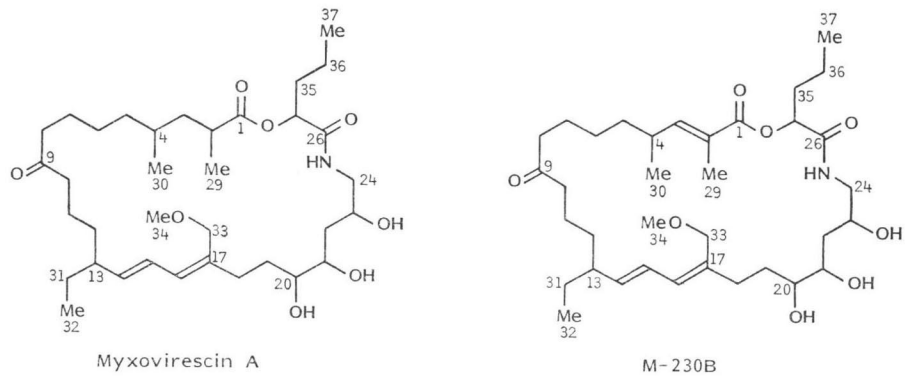


Fig. 7. Structure of myxovirescin A and M-230B.
Number of carbon corresponds to that shown in Table 4.



assumed that double bond exists between C-2 and C-3 in M-230B. Assignments of the different signals are shown in Fig. 6, and the proposed structure of M-230B is illustrated in Fig. 7. M-230B seems to be the same as myxovirescin B described without structure in the patent literature[7].

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