AN INHIBITOR OF (Na^+, K^+)-ATPase PRODUCED BY *STREPTOMYCES PSEUDOVENEZUELAE* MF722-02; PURIFICATION AND PROPERTIES

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A culture product of *Streptomyces pseudovenezuelae* MF722-02, with a molecular formula of $C_{29}H_{32}N_2O_7$, was isolated as yellow needles from culture broths and mycelia of the organism by means of a series of solvent extraction, column chromatography and crystallization. The antibiotic is active against some Gram-positive bacteria, inhibits growth *in vitro* of cells of mouse leukemia L-1210, prolongs the life span of mice inoculated with the leukemia cells, enhances deoxycholate-induced hemolysis *in vitro* and inhibits (Na^+, K^+)-ATPase *in vitro*.

Some inhibitors of membrane-bound enzymes show antitumor activity and/or immunopotentiator activity[1]. In the course of screening for potential antitumor antibiotics, a culture product of *Streptomyces pseudovenezuelae* MF722-02 (referred to as MF722-02 product) attracted our attention because of its enhancing effect on deoxycholate-induced haemolysis. Haemolytic effect is a characteristic of some polyene antibiotics[2]; however, a crude preparation of MF722-02 product showed no indication of the polyene structure suggesting some unique interaction with the cytoplasmic membrane. MF722-02 product was purified based on its activity against *Micrococcus lysodeikticus*. Physicochemical properties of the purified preparation as well as its antibacterial spectrum resembled those of isemycin[3] indicating a structural similarity between the two antibiotics. MF722-02 product inhibited (Na^+, K^+)-ATPase *in vitro*. The cytotoxicity to leukemia cells seems, at least in part, to be due to inhibition of (Na^+, K^+)-ATPase of the cytoplasmic membrane. These studies are dealt with in this paper.

Materials and Methods

Effect on (Na^+,K^+)-ATPase

The enzyme from porcine cerebral cortex was purchased from Sigma. A reaction mixture contained, in 200 μl, the following components at their indicated concentrations: ATP-2Na, 3 mM; NaCl, 140 mM; KCl, 14 mM; $MgCl_2$, 5 mM; EDTA, 2.5 mM; tris-HCl (pH 7.4), 50 mM; a test sample at a required concentration; and (Na^+,K^+)-ATPase, 10^{-1} unit/ml. The test sample was added as a dimethyl sulfoxide solution whose volume was not over 2 μl. The enzyme was added last, thereby initiating the reaction. The reaction proceeded at 37°C for 30 minutes and was terminated by mixing with 1,400 μl of 100 mM sodium acetate buffer containing 10% sodium dodecyl sulfate. The mixture was then mixed with 200 μl each of 1% ammonium molybdate solution and 1% ascorbic acid solution in this order, left standing at room temperature for 180 minutes and read at 740 nm for its optical absorption.

Effect on Deoxycholate-Induced Haemolysis

The assay method of KUNIMOTO *et al.*[4] was followed except that mouse red blood cells were replaced by horse red blood cells and that Dulbeccos' phosphate buffer (GIBCO No. 310-4080, 1/10 diluted and

pH adjusted to 7.4) was used as the saline solution. Two ml of horse defibrinated blood (Japan Biotest Institute) was centrifuged at 1,500×*g* for 5 minutes. The red blood cells (precipitate) were washed once in 10 ml of the phosphate buffer, suspended in 15 ml of the same buffer and a 100 μl portion was added to an assay mixture.

Results and Discussion

The Producer Strain

The producer strain was isolated from a soil sample collected at Kawasaki City, Kanagawa Prefecture in 1977 and designated MF722-02 in our institute. The morphological and physiological characteristics of the strain indicated that it belongs to *Streptomyces pseudovenezuelae*[5] (data not shown).

Culture Conditions of the Producer Strain and Isolation of the Product

Streptomyces pseudovenezuelae MF722-02 was shake-cultured at 27°C in a medium containing 2.0% glycerol, 4.0% soybean meal, 1.0% Sunmaruto (a crude preparation of maltose; Hayashibara Bio. Chem. Inc., Okayama, Japan), 0.1% Polypeptone, 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% $CaCl_2$ and 0.3% NaCl, pH being adjusted to 7.4. Small pieces of mycelial mat were withdrawn from a slant culture, inoculated into 4 conical flasks each containing 110 ml of the above medium and incubated at 27°C with rotary shaking for 39 hours (seed culture). About 2.5 ml portions of the seed culture were transferred to 180 flasks each containing 110 ml of the same medium and incubated likewise. Production of MF722-02 product was followed by detection of a UV-absorbing spot with Rf 0.70 on silica-gel thin-layer chromatograms developed with $CHCl_3$ - methanol (9: 1, v/v). Incubation was terminated after 53 hours, and the culture broth (20 liters) was filtered with a Teflon net to separate mycelia from filtrate (Filtrate I). The mycelia were extracted twice with 5 liters of methanol. The methanol fractions were combined and evaporated to dryness *in vacuo* below 40°C. The residue was dissolved in 2 liters of water and extracted twice with 5 liters of butyl acetate. The butyl acetate fractions were combined and evaporated *in vacuo* to dryness leaving 2,388 mg of yellowish-brown powder (Crude powder I). On the other hand, Filtrate I was mixed with 300 g of silanised silica gel (Merck) for 4 hours and the mixture was left standing overnight in a cold room. The gel was collected by filtration, placed in a column and eluted with 1.5 liters of methanol. The eluate was dried *in vacuo* leaving 2,089 mg of yellowish-brown powder (Crude powder II). Crude powder I and II were combined, mixed with 2 liters of petroleum ether and filtered leaving 3,568 mg of petroleum ether-insoluble powder (Crude powder III). The powder was mixed with 200 ml of methanol and filtered and the methanol soluble fraction was concentrated to about 10 ml by evaporation *in vacuo*. The solution was adsorbed on 7.2 g of silica gel (Wakogel C-200) and the powder was dried *in vacuo*, suspended in 10 ml of $CHCl_3$, and layered on a column (4×30 cm) of Wakogel previously saturated with $CHCl_3$. A stepwise elution was conducted with 500 ml of $CHCl_3$ and with about 300 ml of a mixture of $CHCl_3$ - methanol (100: 1, v/v). Fractions showing antibacterial activity against *M. lysodeikticus*, being eluted with the $CHCl_3$ - methanol mixture, were combined (220 ml) and dried *in vacuo* leaving 509 mg of yellow powder (Crude powder IV). Serial crystallization from methanol and from $CHCl_3$ - methanol gave 95 mg of yellow needle crystals. Overall recovery based on the antibiotic titer with *M. lysodeikticus* was 10.2% in MF722-02 product. True recovery of MF722-02 product must be somewhat higher because thin-layer chromatograms of crude preparations showed presence of two other antibacterial substances in minor quantities.

Physicochemical Properties

Some physicochemical properties of MF722-02 product are summarized in Table 1. As shown in Fig. 1, this compound has UV absorption maxima at 278 nm (log ε 4.56), 318 nm (log ε 4.44) and a shoulder at 264 nm (log ε 4.51) indicating the presence of a conjugated chromophore. The IR spectrum of this compound (Fig. 2) shows olefinic absorptions at 982 and 1600 cm^{-1}. FD-Mass spectrometry gave a molecular ion of m/z 520 consistent with the molecular formula of $C_{29}H_{32}N_2O_7$. The ^1H NMR spectrum of this compound in $CDCl_3$ - CD_3OD (7: 3) revealed the presence of eleven olefinic protons in the

Table 1. Physicochemical properties of the culture product of *Streptomyces pseudovenezuelae* MF722-02.

Analysis		Calcd. for $C_{29}H_{32}N_2O_7$ (molecular weight 520) C 66.91, H 6.20, N 5.38, O 21.52 Found C 66.78, H 6.35, N 5.37, O 20.82
Melting point		194°C (decomp.)
Rf (silica gel thin-layer chromatography)		0.56 (benzene - methanol, 9: 1) 0.70 (chloroform - methanol, 9: 1)
$[\alpha]_D^{23}$		−81.0° (*c* 0.667, chloroform)
Color reaction	Positive	EHRLICH, RYDON-SMITH, TOLLENS, FEHLING ferric chloride
	Negative	SAKAGUCHI, ninhydrin
Solubility	Soluble	Dimethyl sulfoxide, ethyl acetate, chloroform, acetone, methanol
	Insoluble	Water, *n*-hexane

Fig. 1. UV spectrum of the culture product of *Streptomyces pseudovenezuelae* MF722-02.
Recorded on a Hitachi EPS-3T UV spectrometer.

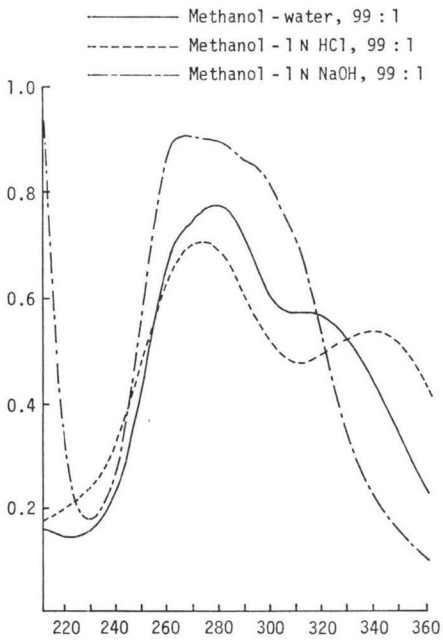
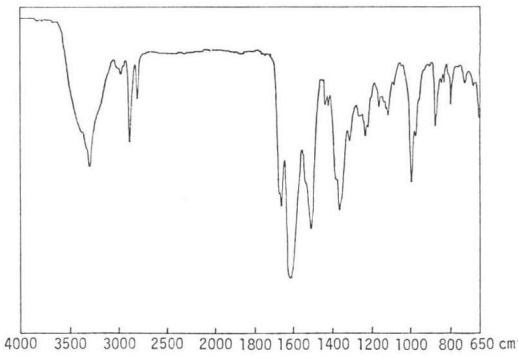


Fig. 2. IR spectrum of the culture product of *Streptomyces pseudovenezuelae* MF722-02.
Recorded on a Hitachi EPI-S2 IR spectrometer.



molecule at δ 5.7 to 7.5, two methine protons at δ 3.63 and 3.70, two methylene protons at δ 2.58, and multiplet signals of eleven protons attributed to five methylene and one methine at δ 0.8 to 2.4 (Fig. 3). The ^{13}C NMR spectrum of this compound in $CDCl_3$ - CD_3OD showed twenty-eight signals (Fig. 4, Table 2). Since another broad signal was observed at δ 185 in deuteriodimethylsulfoxide solution (data not shown), all the twenty-nine carbons of the molecule were identified by ^{13}C NMR spectrometry.

The physicochemical properties of MF722-02 product resembled those of isemycin[3] but were not

Fig. 3. ^{1}H NMR spectrum of the fermentation product of *Streptomyces pseudovenezuelae* MF722-02. Recorded on a Varian XL-100 NMR spectrometer (100 MHz) using $CDCl_3$ - CD_3OD (7: 3) as the solvent system.

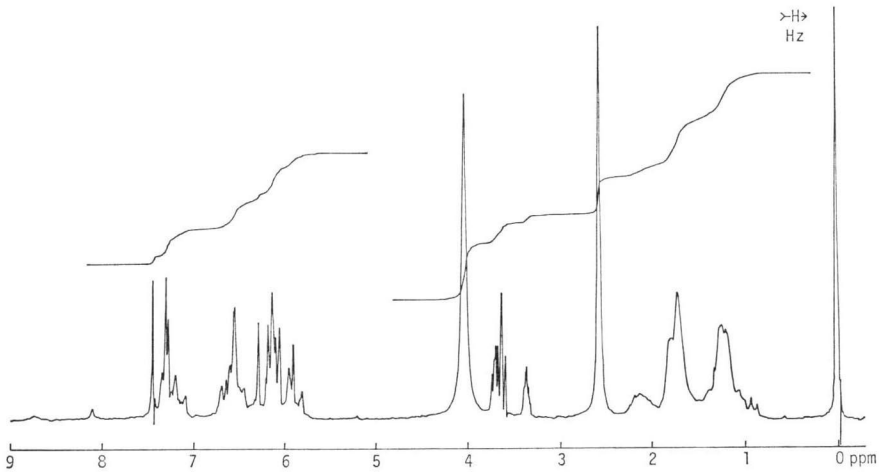
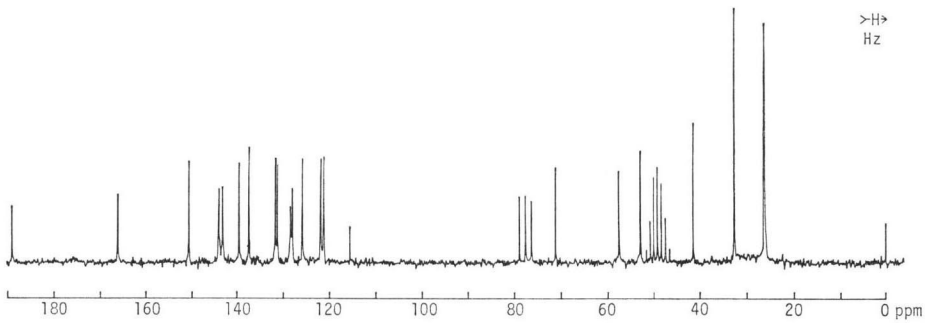


Fig. 4. ^{13}C NMR spectrum of the fermentation product of *Streptomyces pseudovenezuelae* MF722-02. Recorded on a Varian XL-100 NMR spectrometer (25.16 MHz) using $CDCl_3$ - CD_3OD (7: 3) as the solvent system.



identical. Inconsistent points were as follows. (1) $C_{28}H_{30}N_2O_7$ (molecular weight 506) was proposed for isemycin while $C_{29}H_{32}N_2O_7$ (molecular weight 520) was found for MF722-02 product. (2) The UV spectrum of a methanol solution of isemycin had no shoulder at 264 nm. (3) The IR spectrum of isemycin showed weak absorption at 1290 cm^{-1} and 1280 cm^{-1}.

Biological and Biochemical Properties

MF722-02 product was active against various Gram-positive bacteria. The MIC against *M. lysodeikticus* determined by the agar dilution method was 1.56 μg/ml, for example. The antibacterial spectrum (data not shown) was similar to that reported for isemycin. The two antibiotics must belong to the same group in terms of structure and activity. MF722-02 product inhibited the growth *in vitro* of cells of mouse leukemia L-1210 by 50% at a concentration of 0.7 μg/ml. MF722-02 product showed about 30% increase of the life span of mice inoculated with the same leukemia cells when the antibiotic was administered intraperitoneally at doses ranging from 1.25 to 10 mg/kg on days 1 through 10. A test for acute toxicity, determined by intraperitoneal injection, showed that mice died at a dose of 60

Table 2. ^{13}C NMR chemical shifts of the culture product of *Streptomyces pseudovenezuelae* MF-722-02.

	δ, ppm		δ, ppm
$-CH_2-$ ×3	26.0 t	-CH=	128.6 d
$-CH_2-$	26.2 t	-CH=	131.5 d
$-CH_2-$ ×3	32.5 t	-CH=	132.0 d
>CH-	41.4 d	-CH=	137.7 d
>CH-	52.9 d	>C=	137.7 s
>CH-	57.5 d	-CH=	139.8 d
>C<	71.2 s	-CH=	143.5 d
>C=	115.6 s	-CH=	144.2 d
-CH=	121.0 d	-CH=	150.8 d
-CH=	121.4 d	>C=	166.3 s
-CH=	126.0 d	>C=	166.4 s
>C=	128.2 s	>C=O	189.6 s

Chemical shifts were measured in $CDCl_3$-CD_3OD (7: 3) at 25.16 MHz using trimethyl silane as the internal reference. Multiplicities on off-resonance experiment are expressed as s, d and t for singlet, doublet and triplet, respectively.

Fig. 5. Effect on deoxycholate-induced haemolysis. ●: MF722-02 product. △: Diketocoriolin B. ○: No drug.

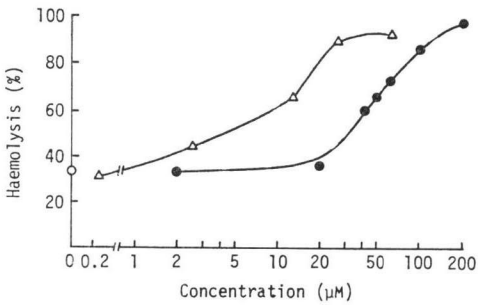
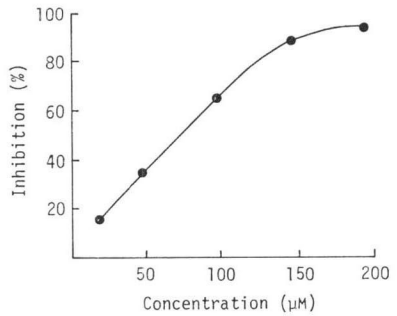


Fig. 6. Inhibition of (Na^+, K^+)-ATPase by the culture product of *Streptomyces pseudovenezuelae* MF722-02.



mg/kg but survived at 30 mg/kg. Some interaction of MF722-02 product with the cytoplasmic membrane of mammalian cells was first suggested by the finding that this compound stimulated deoxycholate-induced haemolysis. Since the same effect was reported with diketocoriolin B (a derivative of coriolin B, a fungus product) having antitumor and immunopotentiator activities, a parallel experiment with diketocoriolin B was conducted for comparison. On the basis of activity/molal concentration, MF 722-02 product was about 1/5 to 1/8 as active as diketocoriolin B, as shown in Fig. 5. Diketocoriolin B was reported to be a specific inhibitor of (Na^+, K^+)-ATPase of various tissues[4], therefore, MF722-02 product was also tested for possible inhibition of this enzyme. As Fig. 6 shows, MF722-02 product inhibited the enzyme, the extent of inhibition being a linear function of the concentration of the product up to 150 μM. The dose required for 50% inhibition (ID_{50}) was estimated to be about 70 μM, indicating that MF722-02 product was a several-fold weaker inhibitor of this enzyme than diketocoriolin B, as the ID_{50} of the latter was reported to range from 5 to 10 μM depending on the source of the enzyme. Although MF722-02 product and diketocoriolin B share a common mechanism of action, it is unlikely that they have any structural similarity. It has been reported that intraperitoneal injection of diketocoriolin B into mice increased the number of spleen cells producing antibody to sheep red blood cells[6]. No such effect was observed with MF722-02 product (data not shown) suggesting that there is no direct correlation between inhibition of (Na^+, K^+)-ATPase of the cytoplasmic membrane and blastogenesis of the lymphocyte. Inhibition by MF722-02 product of (Na^+, K^+)-ATPase should, at least in part, underlie its cytotoxic effect on mammalian cells. The mechanism of antibacterial activity of this compound has not yet been studied. Insensitivity of Gram-negative bacteria to this compound may be ascribed to permeability barrier.

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