MILBEMYCINS, A NEW FAMILY OF MACROLIDE ANTIBIOTICS

FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOCONVERSION OF MILBEMYCINS J AND K

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Strain Rf-107, a mutant of *Streptomyces hygroscopicus* subsp. *aureolacrimosus*, obtained with ultraviolet irradiation, produced two new macrolide antibiotics, milbemycins J and K without production of any of the other milbemycins described in the previous paper. Fermentation studies on the strain were conducted in shake flasks and 30-liter jar fermentors. Isolation of the antibiotics was performed by adsorption on resinous adsorbent followed by elution with aqueous MeOH. Purification of milbemycins J and K was completed with Lobar Si 60 column chromatography to give colorless crystals. Physico-chemical data, such as UV, IR and NMR spectra are described. Milbemycins J and K were readily converted by the intact cells of the parent strain to milbemycins α_1 and α_3, respectively. Physico-chemical characterization and the bioconversion studies revealed that milbemycins J and K were new antibiotics having the 16-membered macrocyclic lactone with a 6,6-membered spiroketal ring system.

Streptomyces hygroscopicus subsp. *aureolacrimosus* produced 13 milbemycins with insecticidal and acaricidal activity[1]; all the milbemycins have the 16-membered macrolide structure[2,3]. It was also reported that the strain produced five new milbemycins D, E, F, G and H[4].

We isolated some mutants of the organism by means of treatment with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) and ultraviolet (UV) irradiation. One of these mutants, strain Rf-107[4], produced two new milbemycins J and K without production of any other known milbemycins.

In this paper, we describe the fermentation, isolation, physico-chemical properties and bioconversion of milbemycins J and K. Biological properties and structure elucidation of the antibiotics will be reported elsewhere.

Materials and Methods

Microorganism

The strain Rf-107 and the parent strain were maintained on YM slant agar.

Fermentation

Fermentation studies were carried out in 30-liter jar fermentors. The seed culture of strain Rf-107 was prepared by cultivation at 28°C for 2 days on a rotary shaker (220 rpm, 7 cm) in a 2-liter Erlenmeyer flask containing 500 ml of a medium with the following composition: 1% sucrose, 0.35% Polypepton and 0.05% K_2HPO_4. One liter of a good preculture was then inoculated into a 30-liter jar fermentor containing 20 liters of a fermentation medium (BM-2) with the following composition: 6% glucose, 1% soybean meal, 1% skim milk, 0.5% corn starch and 0.05% $CaCO_3$. The pH of the BM-2 medium was adjusted to 7.2 before sterilizing at 120°C for 25 minutes. Fermentation was carried out under the fol-

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lowing condition: temperature 28°C, aeration 10 liters/minute, agitation 150~250 rpm and internal pressure 0.5 kg/cm^2.

Isolation

The extraction, isolation and purification procedures of milbemycins J and K were almost identical to those mentioned in the previous paper[1,5]. Adsorbent resin HP-10 and Lobar column Si 60 for chromatography were purchased from Mitsubishi-Kasei Co., Ltd. and E. Merck Co., Ltd., respectively.

Fermentation broth (30 liters) containing milbemycins J and K was adjusted to pH 3.0 and filtered with Celite. The cake (5 kg) was extracted twice with 10 liters of MeOH; to 20 liters of the extract, 20 liters of water was added. The resulting aqueous MeOH was extracted with 50 liters of *n*-hexane. The *n*-hexane was evaporated under reduced pressure to obtain the residue, which was dissolved in 500 ml of MeOH. To this solution 500 ml of water was added. This solution was applied to 1 liter of Diaion HP-10 column. The adsorbate was washed with 2 liters of 60% aqueous MeOH, and eluted with 1 liter of 95% aqueous MeOH. Fractions containing milbemycins were collected and 800 ml of water was added to 1 liter of the fractions. The approximately 50% aqueous MeOH solution was extracted with 2 liters of *n*-hexane. The extracts were evaporated under reduced pressure to obtain 18 g of oily substance. The residue was dissolved in 20 ml of *n*-hexane and then applied to a Lobar column Si 60. The column was eluted with a mixture of *n*-hexane and ethyl acetate (8: 2). The fractions containing milbemycins J and K were pooled separately on the basis of TLC analysis[1] and the solvent was removed under reduced pressure. The solid thus obtained was recrystallized from a mixture of *n*-hexane and chloroform (5: 1) to yield milbemycin J (1.8 g) as colorless crystals. Colorless crystals (1.1 g) of milbemycin K were obtained after recrystallization from *n*-hexane.

Bioconversion

A spore suspension (1 ml) of *S. hygroscopicus* subsp. *aureolacrimosus* parent strain was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium consisting of 1% sucrose, 0.35% Polypepton and 0.05% K_2HPO_4. The inoculum was incubated at 28°C for 48 hours on a rotary shaker (220 rpm, 7 cm). Five milliliters of the culture broth thus obtained were transferred into a 500-ml Erlenmeyer flask containing 100 ml of the medium consisting of 6% glucose, 0.2% meat extract, 0.2% corn steep liquor, 0.3% Polypepton, 0.5% skim milk, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.05% $CaCO_3$ (pH 7.2 before sterilization). After incubation of the flasks on the rotary shaker at 28°C for 48 hours, cells were harvested from 50 ml of the culture broth by centrifugation at $600 \times g$ for 10 minutes, washed twice with 50 ml of sterile saline, and then employed in the bioconversion as intact cells. For the preparation of the cell free extracts, 20% (wt/vol) suspension of washed cells in 0.1 M phosphate buffer (pH 7.0) were cooled in an ice water bath, and disrupted for 10 minutes with an ultrasonic disintegrator (Branson Sonifier model 200) maintaining the suspension below 4°C. The homogenate was subjected to centrifugation at $18,000 \times g$ for 30 minutes at 4°C. Milbemycins J, K, α_1 and α_3 in the reaction mixture were monitored by TLC and HPLC[5].

Results and Discussion

Fermentation

Fermentation studies on strain Rf-107 were conducted in 30-liter jar fermentors containing 20 liters of the BM-2 medium. Typical tropophase-idiophase kinetics were exhibited (Fig. 1). The packed cell volume in the culture broth increased in the first 3 days and remained constant thereafter. The antibiotics were first detected in the broth after 5 days cultivation and increased at a linear rate for the next 5 days. The yields of milbemycins J and K reached 300 μg/ml and 270 μg/ml at 12 days cultivation, respectively. The HPLC pattern of the culture broth after 12 days cultivation showed that strain Rf-107 produced two new milbemycins J and K as major products and some minor components without production of any other known milbemycins. The results described above suggest that strain Rf-107 was blocked in the biosynthetic pathway of milbemycins which were produced by the parent strain and strain Au-3.

Fig. 1. Time course of milbemycins J and K production by strain Rf-107 in a 30-liter jar fermentor.

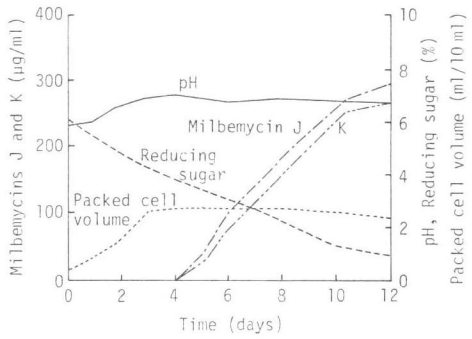


Fig. 2. HPLC pattern of 12-day culture broth of strain Rf-107.

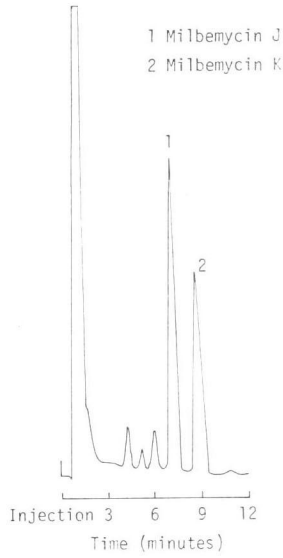


Fig. 3. Isolation and separation of milbemycins J and K.

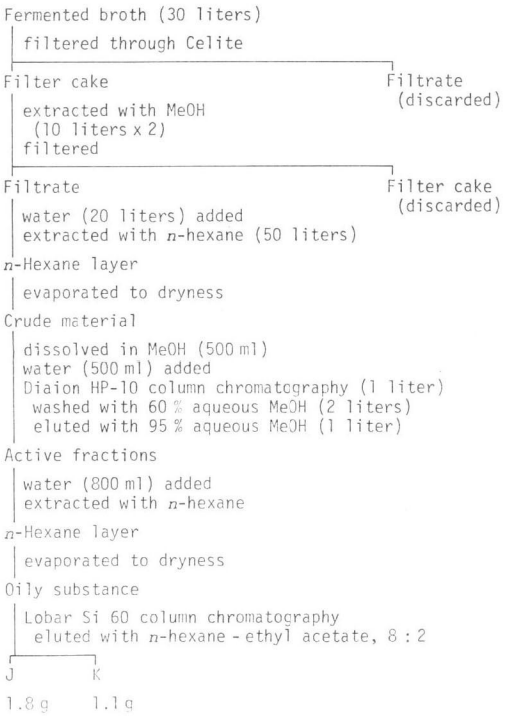
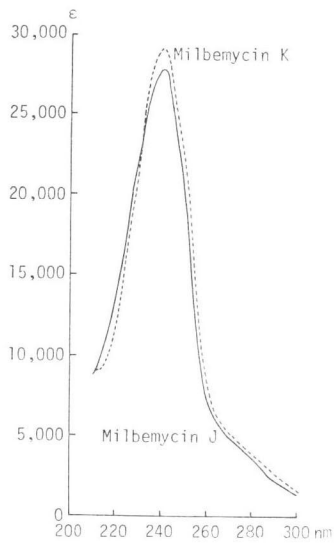


Fig. 4. UV absorption spectra of milbemycins J and K in ethanol.



Isolation

The isolation and separation procedure of milbemycins J and K is outlined in Fig. 3. Milbemycins J and K were obtained as colorless crystals after recrystallization from a mixture of *n*-hexane - chloroform (5: 1) and *n*-hexane, respectively.

Physico-chemical Properties

Physico-chemical data of milbemycins J and K are listed in Table 1. Milbemycins J and K are easily soluble in *n*-hexane, benzene, chloroform and acetone, soluble in MeOH and EtOH but insoluble

Table 1. Physico-chemical properties of milbemycins J and K.

Milbemycin	J	K
Nature	Colorless crystals	Colorless crystals
mp (°C)	213~215	215~218
Mol. Form.	$C_{31}H_{42}O_7$	$C_{32}H_{44}O_7$
M.W. (mass)	526	540
UV λ_{max}^{EtOH} nm (ε)	240 (28000)	240 (29200)
$[\alpha]_D^{27}$*	+40	+42
Rf**	0.67	0.74

* *c* 0.25, acetone.
** Silica gel 60 F-254 plates (Merck), developed in dioxane - CCl_4, 15: 85.

in water. The UV absorption spectra of milbemycins J and K showed a single maximum at 240 nm (ε 28000 and 29200), wihch is ascribed to a conjugated diene system and/or an α,β-unsaturated ketone (Fig. 4). In IR spectra of milbemycins J and K, absorption bands at 3510, 1730 and 1680 cm^{-1} were assigned to tertiary hydroxyl, 16-membered lactone and α,β-unsaturated carbonyl groups, respectively (Fig. 5). An olefinic proton signal at 5.41 ppm in the ^1H NMR spectra of milbemycin α_1 or α_3 at C-3 position shifted to a low field at 6.54 ppm in those

Fig. 5-a. IR spectrum of milbemycin J (Nujol).

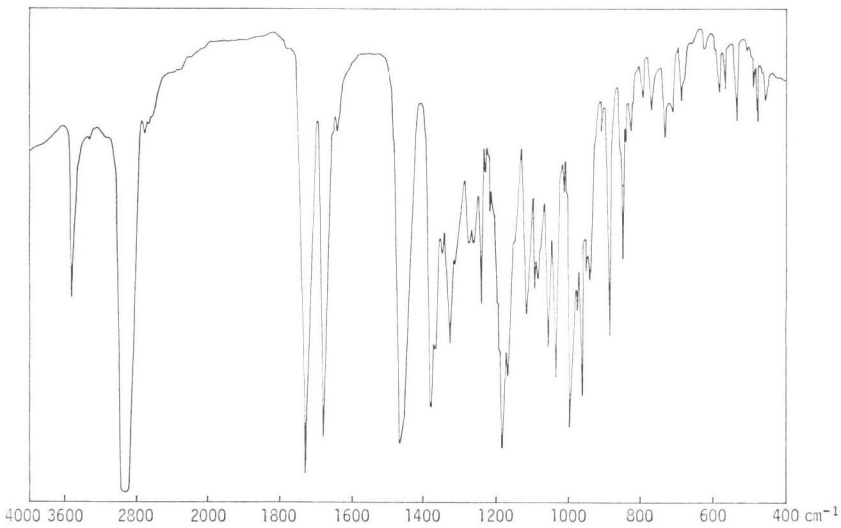


Fig. 5-b. IR spectrum of milbemycin K (Nujol).

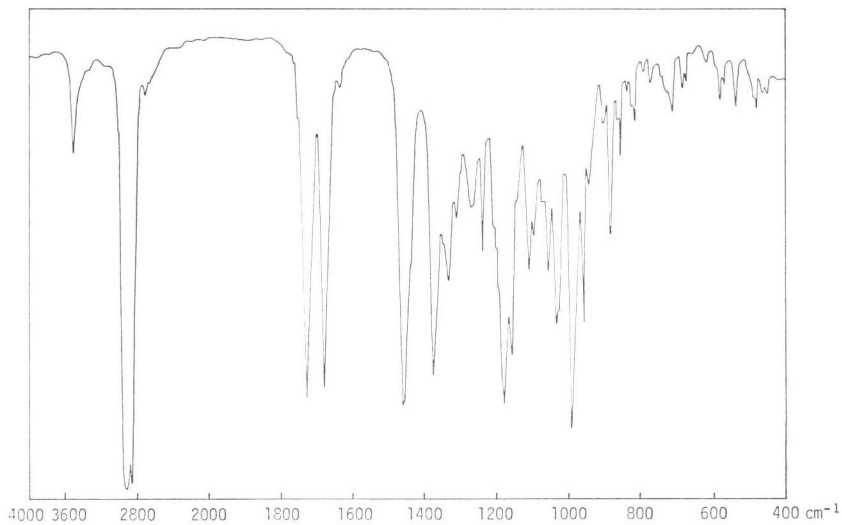


Fig. 6-a. ^1H NMR spectrum of milbemycin J ($CDCl_3$).

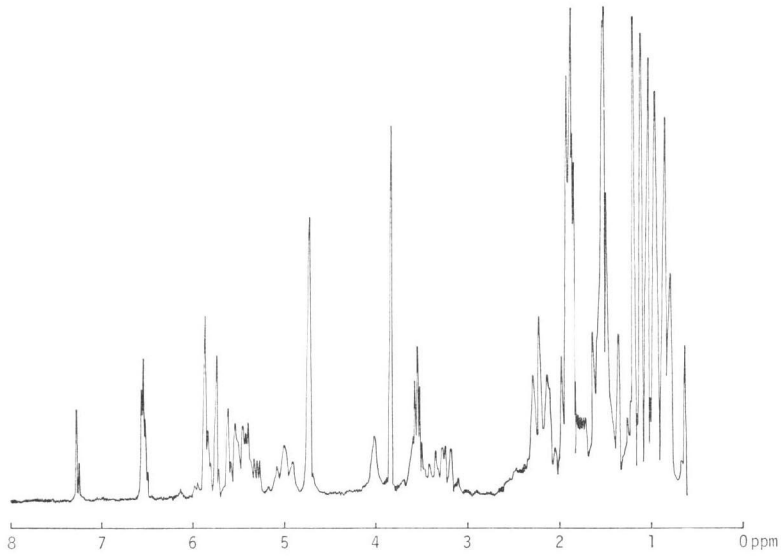
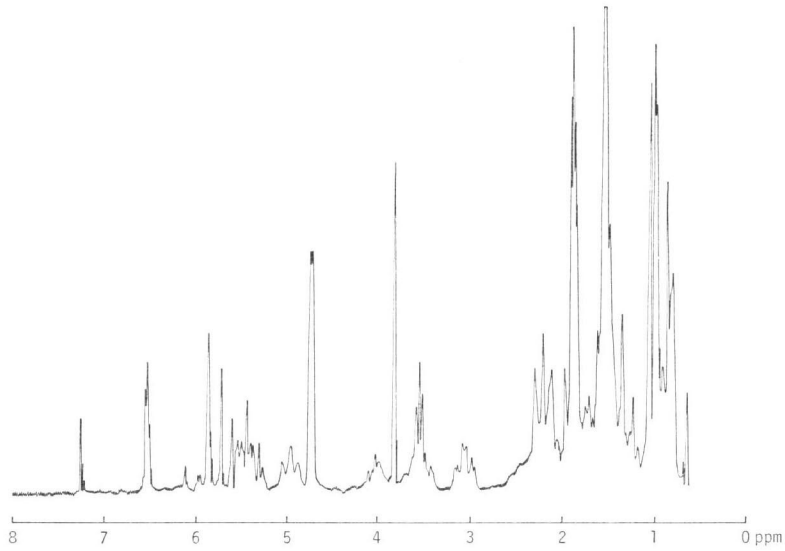


Fig. 6-b. ^1H NMR spectrum of milbemycin K ($CDCl_3$).



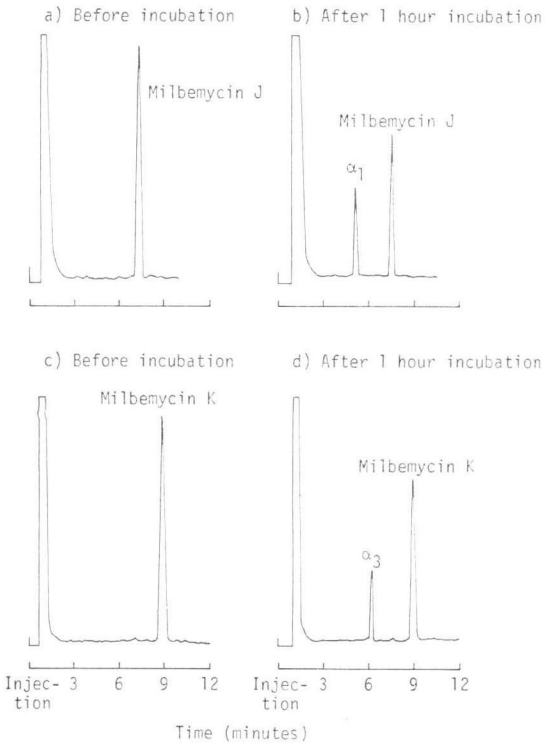
of milbemycins J and K (Fig. 6). The mass spectra of milbemycins J and K showed a molecular ion peak (*m/z* M^+: 526) and (*m/z* M^+: 540), respectively. The physico-chemical properties described above revealed that milbemycins J and K are two new macrolides possessing 16-membered macrocyclic lactone structure with cyclohexenone as shown in following paper.

Bioconversion of Milbemycins J and K

To study the structure relationship between milbemycins J and K, and other milbemycins, the bioconversion studies were carried out using intact cells and cell free extracts of the parent strain. It was confirmed that milbemycins J and K were converted by the intact cells of the parent strain to milbemycins

Fig. 7. HPLC pattern of the reaction mixture for the bioconversion of milbemycins J and K by the intact cells.

Milbemycins J and K were dissolved in $(CH_3)_2$-SO and added to the reaction mixture, which contained 0.64 mmole of milbemycin J or 0.22 mmole of milbemycin K and 11 mg (dry weight) of washed cells in a total volume of 1 ml of 0.1 M phosphate buffer (pH 7.0). Incubation was carried out at 28°C with shaking. The reaction mixture was mixed with 3 ml of acetone and shaken for about 2 minutes. The mixture was centrifuged at 3,000 rpm for 5 minutes and 10 μl of the supernatant solution was injected into the HPLC.



α_1 and α_3, respectively (Fig. 7). The bioconversion of milbemycins J and K to milbemycins α_1 and α_3 increased at a linear rate for the first hour (Fig. 8). The optimum pH of the bioconversion of milbemycins J and K to milbemycins α_1 and α_3 was 7.0. The effect of cell age on the conversion activities was examined with the intact cells which were harvested during 24~190 hours cultivation. Maximal apparent specific activity was observed in 72-hour cultured cells, and relatively high con-

Fig. 8. Time course of the bioconversion of milbemycins J and K by the intact cells.

The experimental methods are the same as described in Fig. 7.

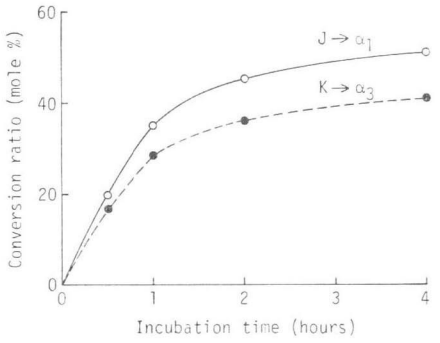
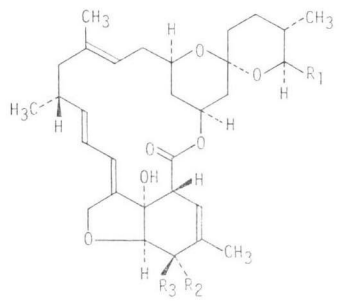


Fig. 9. Structure of milbemycins α_1, α_3, J and K.



Milbemycin	R_1	R_2	R_3
α_1	CH_3	H	OH
α_3	C_2H_5	H	OH
J	CH_3	=O	
K	C_2H_5	=O	

Table 2. Conversion of milbemycins J and K to milbemycins α_1 and α_3 by crude extracts.

The reaction mixture contained milbemycin J (0.65 mmole) or milbemycin K (0.23 mmole) and 0.8 ml of cell free extracts (4.1 mg protein/ml) in a total volume of 1 ml of 0.1 M phosphate buffer (pH 7.0), and was incubated at 28°C for 20 hours with shaking.

	Conversion ratio (mole%)	
	J→α_1	K→α_3
None	2.7	Trace
+NADH 1 mmole	37.5	33.7
+NADPH 1 mmole	13.9	12.8

Milbemycins J and K were dissolved in $(CH_3)_2$-SO.

version activities were maintained during 48~190 hours. It was found in the experiments described above that both milbemycin production and milbemycins J and K conversion occurred simultaneously. However, the intact cells of strain Rf-107 could not convert milbemycins J and K to milbemycins α_1 and α_3, but accumulated milbemycins J and K because of a deficiency of the enzyme related to the conversion.

Milbemycins J and K were also converted by the cell free extracts to milbemycins α_1 and α_3, respectively (Table 2). In the presence of NADH about 37% of the added milbemycin J and 33% of the added milbemycin K were converted to milbemycins α_1 and α_3, respectively. In the presence of NADPH about 14% of the added milbemycin J and 13% of the added milbemycin K were converted to milbemycins α_1 and α_3, respectively. However, in the absence of both NADH and NADPH only a trace of milbemycins J and K were converted to milbemycins α_1 and α_3, respectively. The above results suggest that milbemycins J and K are precursors of milbemycins α_1 and α_3, respectively (Fig. 9).

FURUMAI *et al.*[6] reported the bioconversion pathway of platenomycins, a family of 16-membered macrolide antibiotics, in which platenolide I (C-9, O=C<) was converted to platenolide II (C-9, HO–CH<) by the washed mycelium of the blocked mutants of *Streptomyces platensis* subsp. *malvinus* MCRL 0388. A similar reaction was observed between carbomycin A and maridomycin II or carbomycin B and leucomycin A_3 using intact cells or growing cultures of *Streptomyces hygroscopicus* No. B-5050-HA and its mutants[7]. MATSUHASHI *et al.* also reported the interconversion between midecamycin A_1 and A_3 by the enzyme system extracted from *Streptomyces mycarofaciens* No. 510-19[8]. Our experimental results agree with the above reports.

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