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Bioconversion of Milbemycin-related Compounds: Biosynthetic Pathway of Milbemycins

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Streptomyces hygroscopicus subsp. *aureolacrimosus* SANK 60286 and SANK 60576 produce many kinds of milbemycins. Among them, milbemycin α_{11}, α_{14}, A_3, and A_4 have the most effective acaricidal activity. In this study, we investigated the terminal biosynthetic pathway to milbemycin α_{14} and A_4 which accumulated as the final products in these strains. Using cerulenin, a specific inhibitor of fatty acid and polyketide biosynthesis, we conducted bioconversion experiments with cultures of several mutants, including milbemycin A_4- and α_{14}-producing strains. The bioconversions of milbemycin β_6 to milbemycin A_4 and milbemycin A_4 to milbemycin α_{14} could be identified. For the biosynthesis of milbemycin A_4 from milbemycin β_6 in the milbemycin A_4-high producing strain, there appeared to be two separate pathways exhibiting different sequences of furan ring formation and C-5 keto reduction steps.

Since the discovery in 1967 of B-41, a metabolite with an outstanding activity against various kinds of mites, more than 30 kinds of structurally similar milbemycins have been isolated from a fermentation broth of the *Streptomyces hygroscopicus* subsp. *aureolacrimosus*[1]. Researchers at Sankyo Co., Ltd. elucidated the structure of milbemycins using X-ray crystallographic analysis of the *p*-bromophenyl-urethane derivative, mass spectrometry, and ^1H and ^{13}C NMR spectroscopy[2]. The structure was basically a 16-membered lactone with a spiroketal ring system consisting of two six-membered rings and cyclohexenediol or phenol. Following the discovery of milbemycins, numerous compounds with the same 16-membered macrolide structure were isolated, including Merck's avermectin with potent anthelmintec activity, Cyanamid's LL-F28249, Glaxo's Factor series compounds, and our new milbemycins α_{11}, α_{14} produced by the newly isolated strain, SANK 60286. Based on many preliminary tests, a mixture of milbemycin A_3 and A_4 was selected as a candidate for an acaricide. After making great efforts to develop the production process, we began to market Milbemectin (a mixture of milbemycin A_3 and A_4) as an acaricide for the control of mites in 1990. Furthermore, in animal health fields, 5-oxime derivatives of milbemycin A_3 and A_4 were found to be highly effective as anthelmintics and were marketed in 1990.

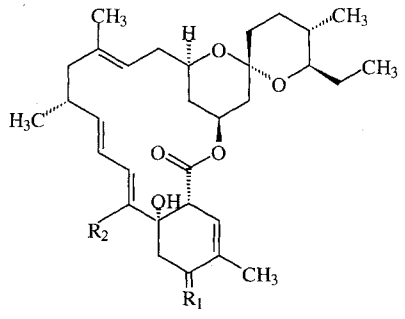
In biosynthetic studies, it was reported that the macrolide ring of milbemycins was biosynthesized *via* a polyketide derived from the condensation of several units of acetate, propionate, and a branched chain fatty acid[3]. For avermectins, the clustered genes encoding the biosynthetic enzymes have been cloned and the biosynthetic order of avermectins after the formation of 16-membered macrocyclic lactones has been studied[4~7]. However, further study of the biosynthetic pathway of milbemycins has been limited to an investigation of the pathway from milbemycin J and K to A_3 and A_4 using an intact-cell and cell-free system of the strain Rf-107[8].

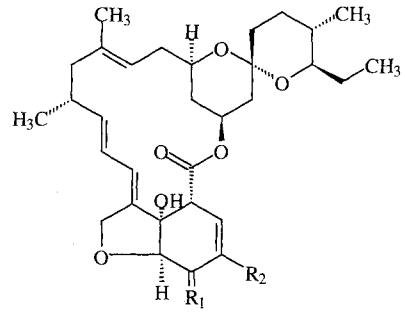
Cerulenin is a specific inhibitor of the condensation reaction in the biosynthesis of fatty acids and polyketides with no effect on the growth of those producing microorganisms[9]. During a screening program for isolating a high-producing strain of milbemycins, we obtained several improved strains and blocked mutants. These strains enabled us to study the biosynthetic sequence of various milbemycins under conditions that allowed cerulenin to inhibit the formation of the

Table 1. Microorganisms and major products.

Strain		Major accumulated products	Origin
Streptomyces hygroscopicus subsp. *aureolacrimosus*			
	SANK 60576	β_1, β_2, β_3, A_3 A_4, B_2, B_3, $\alpha_{5\sim10}$, D, E, F, G, H	Parent strain
	SANK 60286	β_1, β_2, A_3, A_4, $\alpha_{11\sim15}$	Parent strain
RDGr	SANK 66893	A_3, A_4	SANK 60576
Rf-107	SANK 62996	J, K, β_6	SANK 60576
MK-1391	SANK 62896	β_5, β_6, β_7, β_{12}	SANK 60576
RM28D-688	SANK 60797	β_1, β_2, α_{11}, α_{14}	SANK 60286
M28D-10/82	SANK 62296	β_1, β_2, B_2, B_3	SANK 60286
57-338	SANK 62196	B_2, B_3, α_{27}	SANK 60286
BC-5-55	SANK 62396	β_6, β_7	SANK 60286

Fig. 1. Structure of 25-ethyl milbemycins.





β-type milbemycin	R_1	R_2
β_2	H, β-OCH_3	CH_2OH
β_4	H, β-OH	CH_2OH
β_5	H, β-OH	CH_3
β_6	O	CH_3

α-type milbemycin	R_1	R_2
A_4	H, β-OH	CH_3
B_3	H, β-OCH_3	CH_3
K	O	CH_3
α_{14}	H, β-OH	$CH_2OCOCH=C(CH_3)_2$
α_{27}	H, β-OH	CH_2OH

macrolide ring. In this paper, we report a bioconversion study conducted with cerulenin to deduce the terminal biosynthetic pathway of milbemycins including milbemycin A_4 and α_{14}.

Materials and Methods

Microorganisms

During a screening program of high producing milbemycin strain, some blocked mutants were isolated by means of mutagen treatment and mono spore isolation (Table 1). Each strain was maintained on 1/2YM slant agar (sucrose 0.4%, skim milk 0.1%, yeast extract 0.2%, malt extract 0.5%, agar 2.0%, pH 7.2) at 28°C or in 50% (W/V) glycerol solution at −20°C.

Preparation of Milbemycin-related Compounds

The structure of the compounds used for bioconversion is shown in Fig. 1. All the compounds except 26-OH milbemycin A4, the so-called milbemycin α_{27}, have already been isolated and reported[1]. Milbemycin α_{27} was isolated and purified by silica gel chromatography and preparative HPLC. This structure was determined in comparison with other milbemycin structures after some analyses (data not shown).

Fermentation and Bioconversion

Spore suspension was inoculated into PS medium (sucrose 1.0%, Polypepton 0.35%, K_2HPO_4 0.05%, pH 7.2) and cultured for 3 days at 28°C on a rotary shaker. Next, 1 ml of seed culture was transferred into a 100 ml

Erlenmeyer flask containing 15 ml of production medium designated as TY-1-3 (sucrose 12%, Pharmamedia® 1.1%, soybean meal 1.1%, skim milk 1.1%, K_2HPO_4 0.1%, $FeSO_4 \cdot 7H_2O$ 0.01%, $CaCO_3$ 0.25%, pH 7.2). The cultivation was continued for the desired period on a rotary shaker at 28°C. For the bioconversion experiment, cerulenin (final conc. 25 μg/ml) was added to the culture at the beginning and every 24 hours to prevent the formation of aglycon. After 72 hours of cultivation, each milbemycin-related compound was added (final conc. 80 μg/ml) and the culture was incubated for a further 24 hours. Under these conditions, the bioconversion experiments were carried out with a blocked mutant and a deduced intermediate which was converted to a major product by the tested mutant.

Detection of Milbemycin-related Compounds by HPLC

To analyze converted product in the culture broth, 0.5 ml of the broth was mixed with 4.5 ml of MeOH and sonicated for 20 minutes. Ten μl of the filtered solution

Fig. 2. Time course of milbemycin $\alpha_{11}+\alpha_{14}$ production.

△ PCV of cerulenin-added culture, ▲ PCV of control culture, ◇ productivity of cerulenin-added culture, ◆ productivity of control culture.

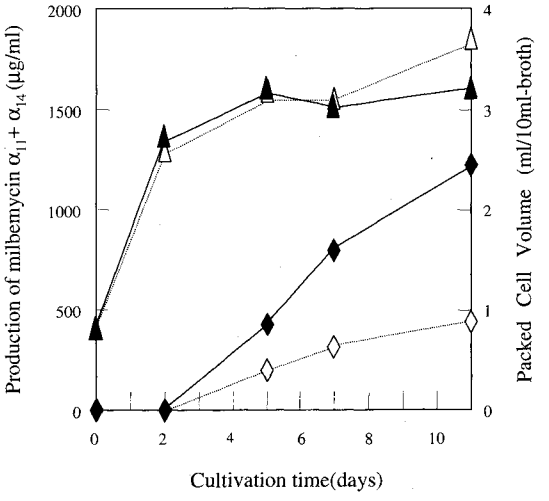
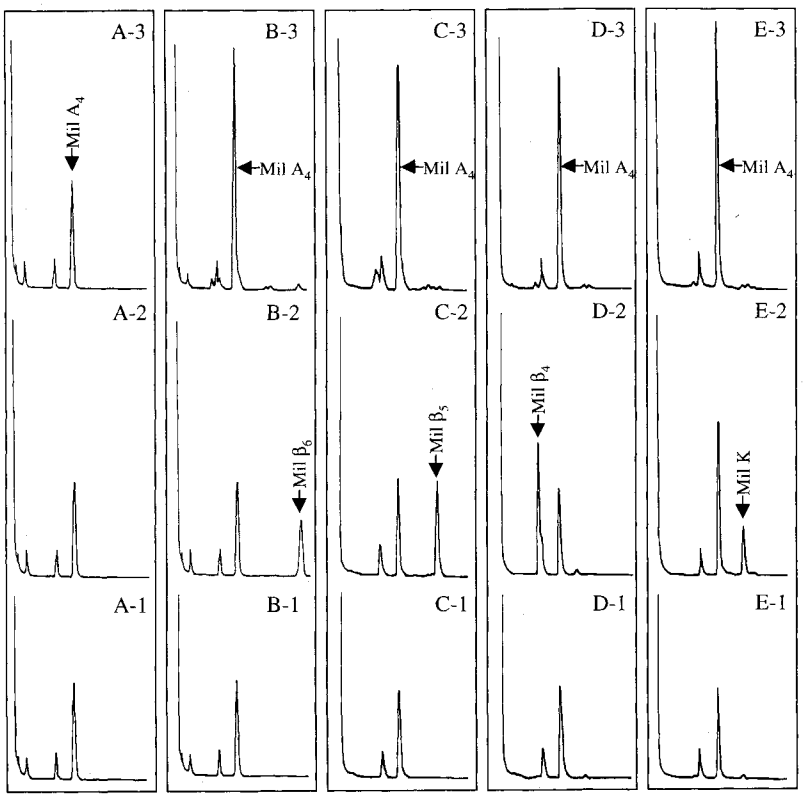


Fig. 3. Bioconversion of milbemycin-related compounds by strain RDGr.

A: Control (with cerulenin), B: bioconversion of milbemycin β_6, C: bioconversion of milbemycin β_5, D: bioconversion of milbemycin β_4, E: bioconversion of milbemycin K.



HPLC traces 1, 2, and 3 recorded before, 0 hour after, and 24 hours after the addition of milbemycin-related compounds, respectively.

was then injected into the column. HPLC analysis was performed on a NOVA-PAK® C18 (3.9 mm i.d. × 150 mm, Waters) or a J'-sphere ODS-L80 (4.6 mm i.d. × 150 mm, YMC Co., Ltd.). The column was eluted with a mixture of MeCN-MeOH-H_2O (8:8:5) or 66.5% MeCN at a flow rate of 1.5 ml per minute. Detection was done by UV absorbance at 242 nm.

Results

Effect of Cerulenin on Milbemycin Production

Milbemycin production of strain RM28D-688, milbemycin α_{11}/α_{14}-producing strain, was not affected when cerulenin was added at the beginning of cultivation, but it was inhibited strongly when cerulenin was added to the culture broth every 24 hours. Figure 2 shows the time course of milbemycin α_{11} and α_{14} production with or without cerulenin. In the cultivation with addition of 25 μg/ml of cerulenin every 24 hours for 11 days, small amounts of milbemycin α_{11} and α_{14} were detected by HPLC analysis from day 3, and its production was inhibited to 40% of the control culture. In case over 25 μg/ml of cerulenin was added to the culture broth, the growth of tested strain was unstable and no bioconversions were occurred. We also confirmed that the successive addition of 25 μg/ml of cerulenin inhibited milbemycins production of other strains used for the following bioconversion tests (data not shown). Therefore, we set up the experimental conditions for bioconversion as indicated in Materials and Methods. The bioconverted product was determined by comparing with the control culture (with cerulenin, without milbemycins) as shown in Fig. 3.

Bioconversion of 25-Ethyl Milbemycin-related Compounds by Strain RDGr

Strain RDGr produced milbemycin A_3 and A_4 as major products. Milbemycin β_6 added into the cerulenin-supplemented culture of strain RDGr was converted to milbemycin A_4. Milbemycin K, β_5, and β_4 were also converted to milbemycin A_4 under the same conditions, but milbemycin α_{27} was not converted to milbemycin α_{14} (Fig. 3. and Table 2).

Bioconversion of 25-Ethyl Milbemycin-related Compounds by Strain MK-1391

Strain MK-1391 derived from milbemycin A_4-producing strain produced milbemycin β_5, β_6, β_7, and β_{12} as major products. Milbemycin β_6 and K were converted to milbemycin β_5 and A_4, respectively. Milbemycin β_4 was converted to milbemycin β_2 and unknown products under the same conditions, but milbemycin β_5 was not converted (Table 2).

Table 2. Bioconversion of milbemycin-related compounds.

Strain	Substrate	Product
RDGr	β_4	A_4
	β_5	A_4
	β_6	A_4
	K	A_4
	α_{27}	Not converted
Rf-107	K	Not converted
	β_4	β_2
	β_5	β_2
	β_6	K
	A_4	B_3
MK-1391	β_4	β_2, unknown product
	β_5	Not converted
	β_6	β_5
	K	A_4
RM28D-688	β_4	β_2
	β_5	β_2
	β_6	β_2
	α_{27}	α_{14}
M28D-10/82	β_4	β_2
	β_5	β_4, β_2
	K	A_4
	A_4	B_3
	α_{27}	α_{14}
57-338	A_4	α_{27}, B_3
BC-5-55	β_4	β_2
	β_5	Not converted
	K	A_4, α_{27}
	A_4	α_{27}, α_{14}
	α_{27}	α_{24}

Bioconversion of 25-Ethyl Milbemycin-related Compounds by Strain Rf-107

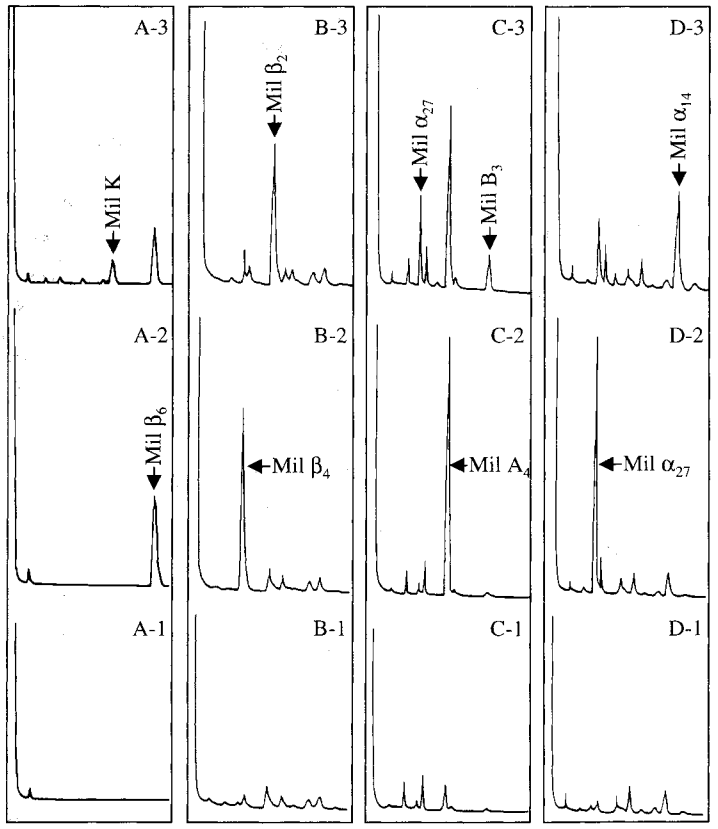
Strain Rf-107 derived from milbemycin A_4-producing strain produced milbemycin J, K, and β_6 as major products. Milbemycin β_6 was converted to milbemycin K. Milbemycin β_5 and β_4 were both converted to milbemycin β_2. Milbemycin A_4 was converted to milbemycin B_3 under the same conditions, but milbemycin K was not converted (Fig. 4. and Table 2).

Bioconversion of 25-Ethyl Milbemycin-related Compounds by Strain RM28D-688

Strain RM28D-688 produced four milbemycin-related

Fig. 4. Bioconversion of milbemycin-related compounds.

A: Bioconversion of milbemycin β_6 by strain Rf-107, B: bioconversion of milbemycin β_2 by strain RM28D-688, C: bioconversion of milbemycin A_4 by strain 57-338, D: bioconversion of milbemycin α_{27} by strain RM28D-688.



HPLC traces 1, 2, and 3 recorded before, 0 hour after, and 24 hours after the addition of milbemycin-related compounds, respectively.

compounds, β_1, β_2, α_{11}, and α_{14}, as major products. Milbemycin β_6 was converted to milbemycin β_2. Milbemycin β_5 and β_4 were both converted to milbemycin β_2 under the same conditions. Milbemycin α_{27} was converted to milbemycin α_{14} as a final product in the biosynthetic pathway of 25-ethyl milbemycins (Fig. 4. and Table 2).

Bioconversion of 25-Ethyl Milbemycin-related Compounds by Strain M28D-10/82

Strain M28D-10/82 derived from milbemycin α_{14}-producing strain produced four milbemycin-related compounds, β_1, β_2, B_2, and B_3, as major products. Milbemycin K, A_4, and α_{27} were converted to milbemycin A_4, B_3, and α_{14}, respectively. Milbemycin β_5 was converted to milbemycin β_2 *via* β_4 under the same conditions (Table 2).

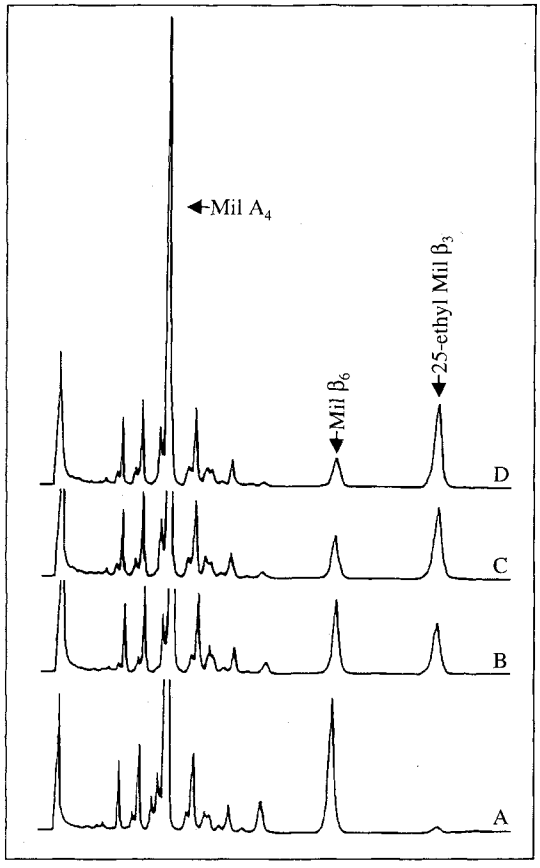
Bioconversion of Milbemycin A_4 by Strain 57-338

Strain 57-338 derived from milbemycin α_{14}-producing strain produced milbemycin B_2, B_3, and α_{27} as major products. Milbemycin A_4 was converted to milbemycin α_{27} and B_3 by strain 57-338 (Fig. 4. and Table 2).

Bioconversion of C-25 Ethyl Milbemycin-related Compounds by Strain BC-5-55

Strain BC-5-55 derived from milbemycin α_{14}-producing strain produced milbemycin β_6 and β_7 as major products. The bioconversion of milbemycin K to α_{27} *via* A_4, milbemycin A_4 to α_{14} *via* α_{27}, milbemycin α_{27} to α_{14}, and milbemycin β_4 to β_2 were respectively confirmed, but milbemycin β_5 was not converted (Table 2).

Fig. 5. Conversion of milbemycin β_6 in MeOH extract.



HPLC traces A, B, C, and D recorded 0 hour, 1 week, 2 weeks, and 3 weeks after the extraction of milbemycin-related compounds from culture broth.

Conversion of Milbemycin β_6 in MeOH

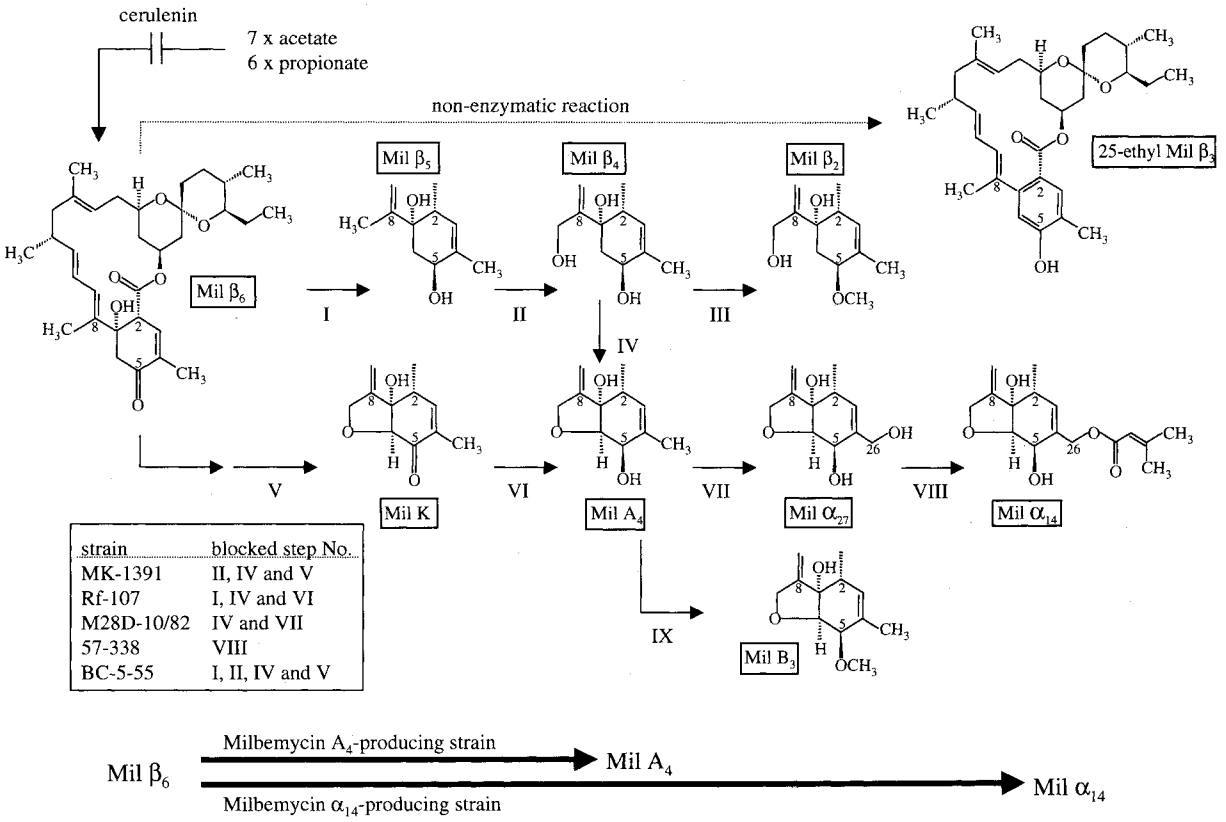
Milbemycin β_6 was ordinarily converted to milbemycin A_4 *via* β_5 and β_4, or *via* milbemycin K by milbemycin-producing strain. When MeOH extract containing milbemycin β_6 was kept at room temperature, milbemycin β_6 was converted to a new compound, 25-ethyl milbemycin β_3 (Fig. 5).

Discussion

Cerulenin is a powerful inhibitor useful in analyzing the biosynthetic sequence after the formation of polyketide or macrolide compounds. To elucidate the biosynthetic pathway of milbemycin α_{14} from milbemycin β_6 and the mutated steps in several blocked mutants, we examined bioconversions with cerulenin added to the culture broth. The results of bioconversion by using strains Rf-107 and RDGr, suggest that there are two routes for furan ring formation leading to milbemycin A_4, one is milbemycin $\beta_6 \rightarrow \beta_5 \rightarrow \beta_4 \rightarrow A_4$ and the other is $\beta_6 \rightarrow (X) \rightarrow K \rightarrow A_4$. In strain Rf-107, one of furan ring formation (milbemycin $\beta_6 \rightarrow (X) \rightarrow K$) was working but the other (milbemycin $\beta_4 \rightarrow A_4$) was not. We think there would be two enzymes, which had different substrate specificities, and an intermediate X, C-8a hydroxyl milbemycin β_6, which has not yet been isolated in the culture broth, existed in the pathway of milbemycin β_6 to K. This compound might be unstable and quickly converted to milbemycin K in the culture broth of our milbemycin producer. Although the producing organism is different from ours, the corresponding compound to intermediate X, VM44868, has been already isolated and reported by SmithKline Beecham group[10]. VM44868 would be bioconverted to α-type milbemycin, VM44857, in the culture broth by the similar reaction, equivalent to the steps V and VI in Fig. 6. On the other hand, milbemycin β_6 is non-enzymatically converted to 25-ethyl milbemycin β_3 in MeOH as shown in Fig. 5. The corresponding compound to 25-ethyl milbemycin β_3, VM54339, would be also non-enzymatically converted from VM44868 in MeOH. According to the report by H. IKEDA and S. ŌMURA[11], it has shown that the oxygen in the benzofuran between C-6 and C-8a is derived from molecular oxygen, and this oxdation would be performed by cytochrome P450 type oxidase. By isolating BC-5-55, a strain which accumulated milbemycin β_6 and β_7, we determined that the first formed of the 25-ethyl milbemycins was milbemycin β_6. In addition, the results of bioconversion by using strain BC-5-55, suggest that there would be two enzymes for C-5 keto reduction in the two routes from milbemycin β_6 to A_4. In strain BC-5-55, one of deduced C-5 keto reductase (milbemycin $K \rightarrow A_4$) was working but the other ($\beta_6 \rightarrow \beta_5$) was not. From these bioconversion tests, the plausible biosynthetic pathway from milbemycin β_6, the first formed product, *via* a hypothetical polyketide derived from 7 acetate and 6 propionate units, is summarized in Fig. 6. The blocked steps in the tested strains are also indicated in the Figure.

These two biosynthetic pathways exclusively demonstrated by the milbemycin A_4-high-producing strain, RDGr, have not been reported for avermectin-producing strains. According to some reports[4~6], avermectin-producing strains only have the biosynthetic pathway equivalent to milbemycin $\beta_6 \rightarrow K \rightarrow A_4$. In the strain improvement program, a lot of the strains isolated and cultivated had a tendency to accumulate milbemycin β_6. For strain RDGr, two biosynthetic pathways from

Fig. 6. Proposed pathways of milbemycin α_{14} biosynthesis.



milbemycin β_6 to A_4 might be necessary for high production of milbemycin A_4. Furthermore, the comparison of bioconversion tests between milbemycin A_4- and α_{14}-producing strains indicated that the milbemycin A_4-producing strain had no enzyme for steps VII and VIII in Fig. 6.

Acknowledgments

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