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Bioconversion of Milbemycin-related Compounds: Isolation and Utilization of Non-producer, Strain RNBC-5-51

KOICHI NONAKA*, TAKAHIRO TSUKIYAMA†, KAZUO SATO†, CHIEKO KUMASAKA, FUMIO MARUYAMA and HIROJI YOSHIKAWA

Biomedical Research Laboratories, Sankyo Co., Ltd.,
389-4, Aza-ohtsurugi, Shimokawa, Izumi-machi, Iwaki-shi, Fukushima 971-8183, Japan
†Agroscience Research Laboratories, Sankyo Co., Ltd.,
1041 Yasu, Yasu-cho, Shiga 520-2342, Japan

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A non-producing strain, the so-called strain RNBC-5-51 SANK 60198, was isolated during a screening program of strain improvement in the milbemycin production. Strain RNBC-5-51 indicated almost the same characteristics as those in the parent strain, that is, the abundant spore formation on agar media and the good growth in liquid media. But it does not produce any kind of milbemycins. In addition, strain RNBC-5-51 accumulated precursor-like compounds of milbemycin-polyketide, the production of which were inhibited by the addition of cerulenin.

In the bioconversion experiments, strain RNBC-5-51 converted milbemycin β_6 and A_4 to milbemycin α_{14}, and milbemycin β_7 and A_3 to milbemycin α_{11}, respectively. This strain also converted milbemycin D and avermectin B_{1a}, to 26-(3-methyl-2-butenoyloxy)milbemycin D and 26-(3-methyl-2-butenoyloxy)avermectin B_{1a}, respectively.

These results suggest that milbemycin α_{11} is biosynthesized through the same route as milbemycin α_{14}, and the mutated step in strain RNBC-5-51 might be in the polyketide synthetic pathway of milbemycins. Strain RNBC-5-51 loses the ability for *de novo* synthesis of milbemycins, but it retains the ability to bioconvert the milbemycin skeleton. This strain might be useful for C-26 modification of milbemycin-related compounds.

More than 30 milbemycins, which demonstrate insecticidal and acaricidal activities, produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus* have been isolated and reported by researchers at Sankyo Co., Ltd.[1]. All milbemycins have the same 16-membered macrolide structure, which is biosynthesized *via* a polyketide derived from condensation of several units of acetate, propionate and branched-chain fatty acid, as avermectins have[1]. Among them, milbemycin α_{11} and α_{14} produced by *S. hygroscopicus* subsp. *aureolacrimosus* SANK 60286 have the most effective acaricidal activity[2]. During a screening program of high milbemycin-producing strains, some biosynthetically blocked mutants of *S. hygroscopicus* subsp. *aureolacrimosus* have been isolated and characterized[3,4]. In biosynthetic studies on milbemycins, these mutants were applied to bioconversion experiments with cerulenin, a specific inhibitor of fatty acid and polyketide biosyntheses, to surmise the biosynthetic pathway of milbemycin α_{14}, the final compound of 25-ethylmilbemycins[4]. In the 25-methylmilbemycins, milbemycin A_3 and α_{11}, with insecticidal and acaricidal activity, are important compounds. When the structures between 25-methyl and 25-ethyl milbemycins were compared, it was predicted that 25-methylmilbemycins were biosynthesized by the same route as 25-ethylmilbemycins. However, the same studies as those in the bioconversion experiments with cerulenin could not be performed because intermediates of 25-methylmilbemycins, such as milbemycin β_4, β_5, and α_{27} for 25-ethylmilbemycins, have not been isolated yet from the fermentation broth of milbemycin producers. This problem was overcome by virtue of isolating strains RNBC-5-51, and 57-338, 26-hydroxylmilbemycin A_4-producing strain, which have already been isolated and reported[4]. In this paper we report a proposed biosynthetic pathway of 25-methylmilbemycins, which was determined from

bioconversion experiments using strain RNBC-5-51, and the bioconversion of some milbemycin-related compounds, milbemycin D and avermectin B_{1a} for the modification at C-26 position by virtue of using the same strain, RNBC-5-51.

Materials and Methods

Microorganisms

During a screening program of a high milbemycin α_{11} and α_{14}-producer, a biosynthetically blocked mutant, non-producer of milbemycins, strain NBC-5-51 was isolated by means of mutagen treatment of strain NBC-4. Subsequently, a spore suspension, so-called RNBC-5-51 SANK 60198, was prepared from several slants obtained by means of mono spore isolation. In this study we used strains RNBC-5-51 and 57-338[4] for the bioconversion experiments. Each strain was maintained on a 1/2YM agar slant (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 as a spore suspension in 50% (W/V) glycerol at −20°C.

Fermentation and Bioconversion

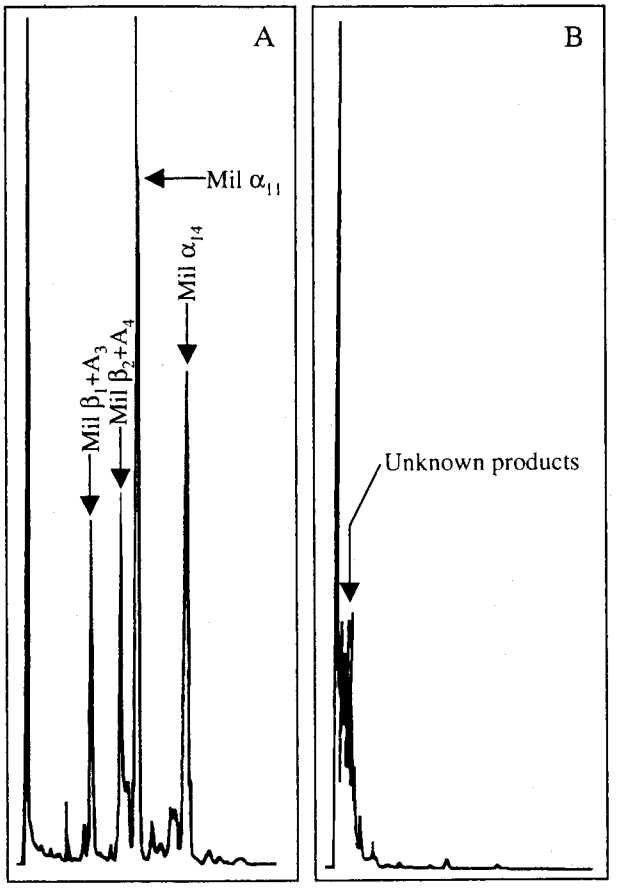
For seed preparation, spore suspension was inoculated into PS medium (sucrose 1.0%, Polypepton® 0.35%, K_2HPO_4 0.05%, pH 7.2) and cultured for 3 days at 28°C on a rotary shaker. Next, 1 ml of seed culture was transferred into a 100 ml Erlenmeyer flask containing 15 ml of production medium designated 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 conducted for a desired period on a rotary shaker at 28°C. For the bioconversion experiment using strain 57-338, cerulenin (final conc. 25 μg/ml) was added to the culture at the beginning and every 24 hours to prevent the formation of milbemycin-polyketide. After 72 hours of cultivation, milbemycin A_3 was added (final conc. 80 μg/ml) and the culture was incubated for another 24 hours. In the case of strain RNBC-5-51, the bioconversion experiments were carried out under the same conditions as described above but without cerulenin.

Detection of Milbemycin-related Compounds by HPLC

To analyze the 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. After filtration, 10 μl of the filtrate was then subjected to HPLC analysis using a NOVA-PAK® C18 column (3.9 mm i.d.×150 mm, Waters). The column was eluted with a mixture of MeCN - MeOH - H_2O (8 : 8 : 5) at a flow rate of 1.5 ml/minute. The chromatography was monitored by absorbance at 242 nm.

Fig. 1. HPLC profile of methanol extract of culture broth.

A and B indicate the HPLC profiles of strains NBC-4 (parent strain) and NBC-5-51, respectively.



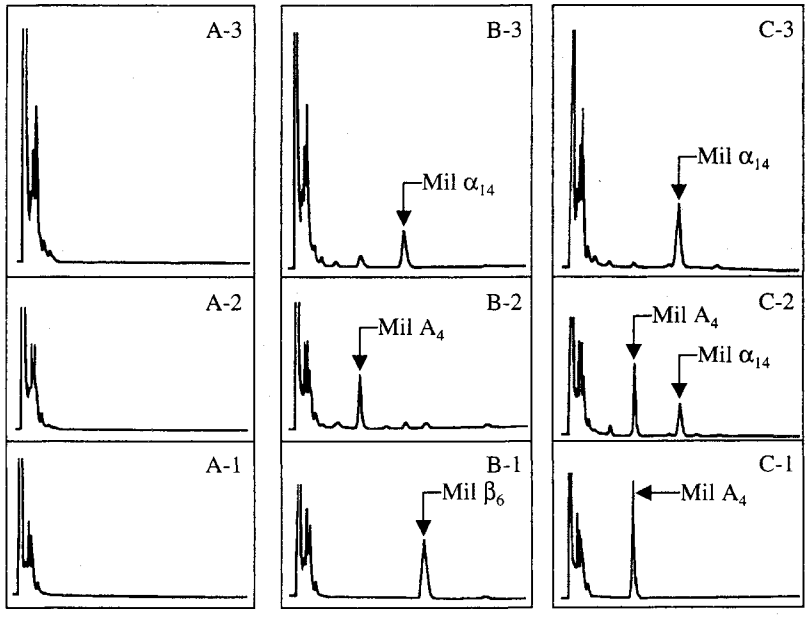
Results

Biosynthetic Characteristics of Strain RNBC-5-51

Strain NBC-5-51, non-producer of milbemycins, which was obtained from milbemycin α_{11} and α_{14}-producing strain, NBC-4, showed morphological features, such as the spore formation on agar media and growth in liquid media, identical to those of milbemycin-producing strains. Strain NBC-5-51 produced no milbemycins, but accumulated unknown products eluted quickly on ODS column (Fig. 1). The formation of these unknown products was inhibited by the addition of cerulenin (data not shown.). Furthermore, milbemycin β_6 (**1b**) and A_4 (**7b**) added to the culture broth of strain RNBC-5-51 were converted to milbemycin α_{14}

Fig. 2. Bioconversion of milbemycin β_6 and A_4 by strain RNBC-5-51.

A: control, B: bioconversion of milbemycin β_6, C: bioconversion of milbemycin A_4.



HPLC traces 1, 2 and 3 recorded 5 minutes after, 5 hours after and 24 hours after the addition of substrates, respectively.

(**9b**) (Fig. 2). It was suggested that the mutated enzyme in the milbemycin biosynthesis of strain RNBC-5-51 could be the milbemycin-polyketide synthase involved in the milbemycin-biosynthetic pathway. Therefore, strain RNBC-5-51 was applied to the bioconversion experiment (without cerulenin) to confirm the biosynthetic pathway of 25-methylmilbemycins. Based on earlier results, we thought that the starter compound for 25-methylmilbemycins was milbemycin β_7, equivalent to milbemycin β_6, and that the intermediate of milbemycin α_{11} was milbemycin A_3, equivalent to milbemycin A_4. Milbemycin β_7 (**1a**) and A_3 (**7a**) added to a culture broth of strain RNBC-5-51 were converted to milbemycin α_{11} (**9a**), as expected (Fig. 3).

Bioconversion of Milbemycin A_3 by Strain 57-338, and Isolation and Structure Elucidation of Its Converted Product

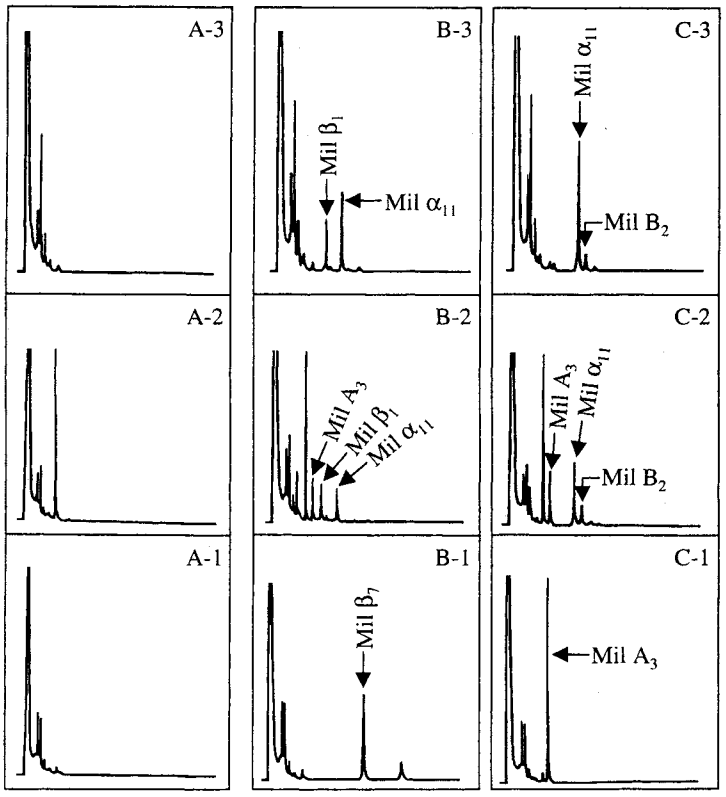
In the above experiments, 26-OH milbemycin A_3, a thought-to-be intermediate between A_3 and α_{11}, was not detected in the culture broth of strain RNBC-5-51. Therefore, in order to find out 26-OH milbemycin A_3, a bioconversion experiment of milbemycin A_3 using strain 57-338, which does accumulate 26-OH milbemycin A_4 (milbemycin α_{27}: **8b**), was conducted under cerulenin-added conditions. The converted unknown product appeared by the addition of milbemycin A_3 (**7a**), as expected. To determine its structure, the isolation of the new compound was performed from the 1,200 ml-culture broth of strain 57-338. To the culture broth, 4,800 ml of MeOH was added, and the resulting solution was stirred at room temperature for 30 minutes. The precipitate was removed by filtration and the filtrate was diluted twice with water. The resulting-aqueous MeOH solution was extracted with equal volume of *n*-hexane and the extract was concentrated *in vacuo* at 37°C. The oily residue was dissolved in 20 ml of MeOH and purified by preparative HPLC. This new milbemycin was obtained as a white amorphous powder (75.6 mg). As shown in Fig. 5, this structure was determined from its physico-chemical properties and spectral data (data not shown), and it was designated as milbemycin α_{26} (**8a**).

Bioconversion of Milbemycin D and Avermectin B_{1a} by Strain RNBC-5-51, and Isolation and Structure Elucidation of their Converted Products

Strain RNBC-5-51 also had the ability to bioconvert other milbemycin analogues, which were not found out in a culture broth of milbemycin α_{11} and α_{14}-producing strain. Milbemycin D (**7c**) and avermectin B_{1a} (**7d**) added to a

Fig. 3. Bioconversion of milbemycin β_7 and A_3 by strain RNBC-5-51.

A: control, B: bioconversion of milbemycin β_7, C: bioconversion of milbemycin A_3.



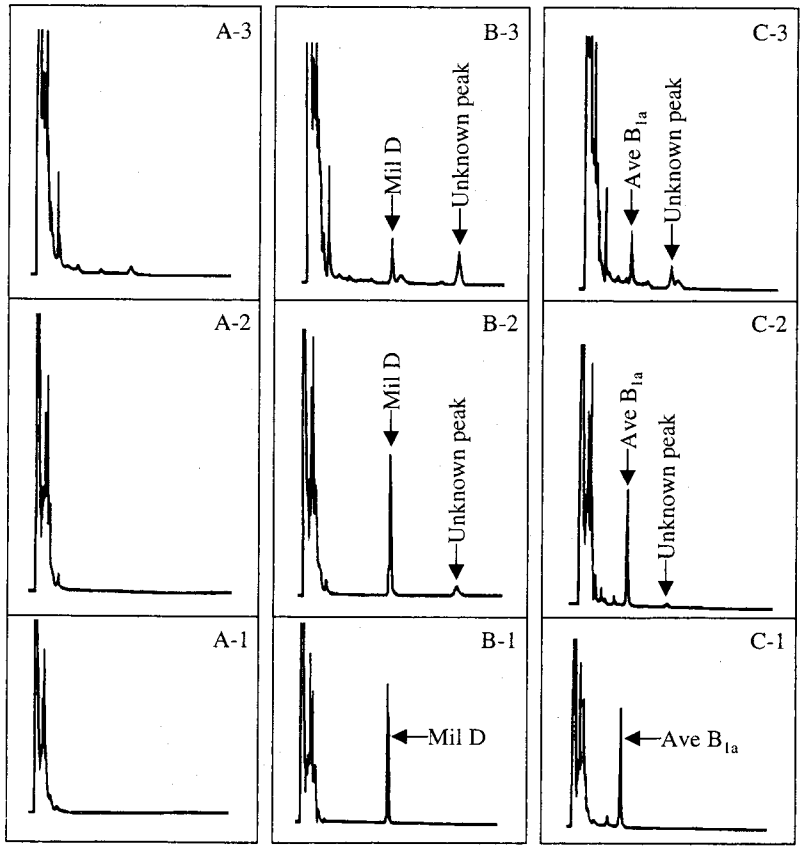
HPLC traces 1, 2 and 3 recorded 5 minutes after, 5 hours after and 24 hours after the addition of substrates, respectively.

culture broth of strain RNBC-5-51 were converted to unknown products, as expected (Fig. 4). To elucidate the structure of the converted products, approximately 1,200 ml of the broth was recovered after the bioconversion experiments using strain RNBC-5-51. Each broth was extracted with 4,800 ml of MeOH. Subsequently, each filtrated extracts diluted by the addition of an equal volume of water were re-extracted with *n*-hexane. The *n*-hexane layers were concentrated, redissolved in *n*-hexane, and applied to the silica gel column, which was successively eluted with *n*-hexane - acetone; 97.5 : 2.5 (I), 95 : 5 (II), 90 : 10 (III), 80 : 20 (IV), 70 : 30 (V). The converted products from milbemycin D and avermectin B_{1a} were eluted in Fr. III and V, respectively. These fractions, concentrated under reduced pressure, were dissolved in MeOH and further purified by preparative HPLC. The converted products of milbemycin D and avermectin B_{1a} were obtained in 37.9 mg and 5.7 mg yield as white amorphous powder, respectively. The converted products were identified as 26-(3-methyl-2-butenoyloxy)milbemycin D (**9c**) and 26-(3-methyl-2-butenoyloxy)avermectin B_{1a} (**9d**), respectively. The spectral data are as follows:

26-(3-methyl-2-butenoyloxy)milbemycin D (**9c**): IR ν_{max} cm^{-1} (NaCl film) 3455, 2955, 2915, 2855, 1715, 1650, 1450, 1380, 1340, 1225, 1180, 1150, 1140, 1120, 1065, 1055, 1010, 990, 960; ^1H NMR (270 MHz, $CDCl_3$, δ) 5.70~5.86 (4H, m), 5.30~5.45 (2H, m), 4.96 (1H, t, J=6.9 Hz), 4.65~4.84 (4H, m), 4.49 (1H, br s), 4.09 (1H, s), 3.99 (1H, d, J=5.9 Hz), 3.59 (1H, m), 3.33 (1H, d, J=2.0 Hz), 3.08 (1H, d, J=7.4 Hz), 2.74 (1H, br s), 2.41~2.45 (1H, m), 2.17 (3H, d, J=1.0 Hz), 2.11~2.31 (3H, m), 2.03 (1H, m), 1.90 (3H, d, J=1.0 Hz), 1.82 (1H, m), 1.53 (3H, br s), 1.04 (3H, d, J=6.9 Hz), 1.00 (3H, d, J=6.4 Hz), 0.85 (3H, d, J=6.4 Hz), 0.80 (3H, d, J=5.9 Hz), 0.80~2.45 (9H, m); ^{13}C NMR (67.5 MHz, $CDCl_3$, δ) 173.1, 166.3, 157.7, 143.1, 139.2, 136.8, 123.4, 121.6, 120.9, 120.6, 115.6, 97.5, 80.4, 79.2, 78.4, 69.0, 68.6, 67.4, 64.8, 63.5, 48.5, 45.6, 41.4, 36.7, 35.9, 35.7, 34.7, 31.6, 28.3, 28.0, 27.4, 22.3, 20.9, 20.3, 17.3, 15.5, 14.2; EI-MS (*m/z*): 654 (M^+), 554; HREI-MS (*m/z*): [M^+]: Calcd for $C_{38}H_{54}O_9$,

Fig. 4. Bioconversion of milbemycin D and avermectin B_{1a} by strain RNBC-5-51.

A: control, B: bioconversion of milbemycin D, C: bioconversion of avermectin B_{1a}.



HPLC traces 1, 2 and 3 recorded 5 minutes after, 5 hours after and 24 hours after the addition of substrates, respectively.

654.3765; found, 654.3768.

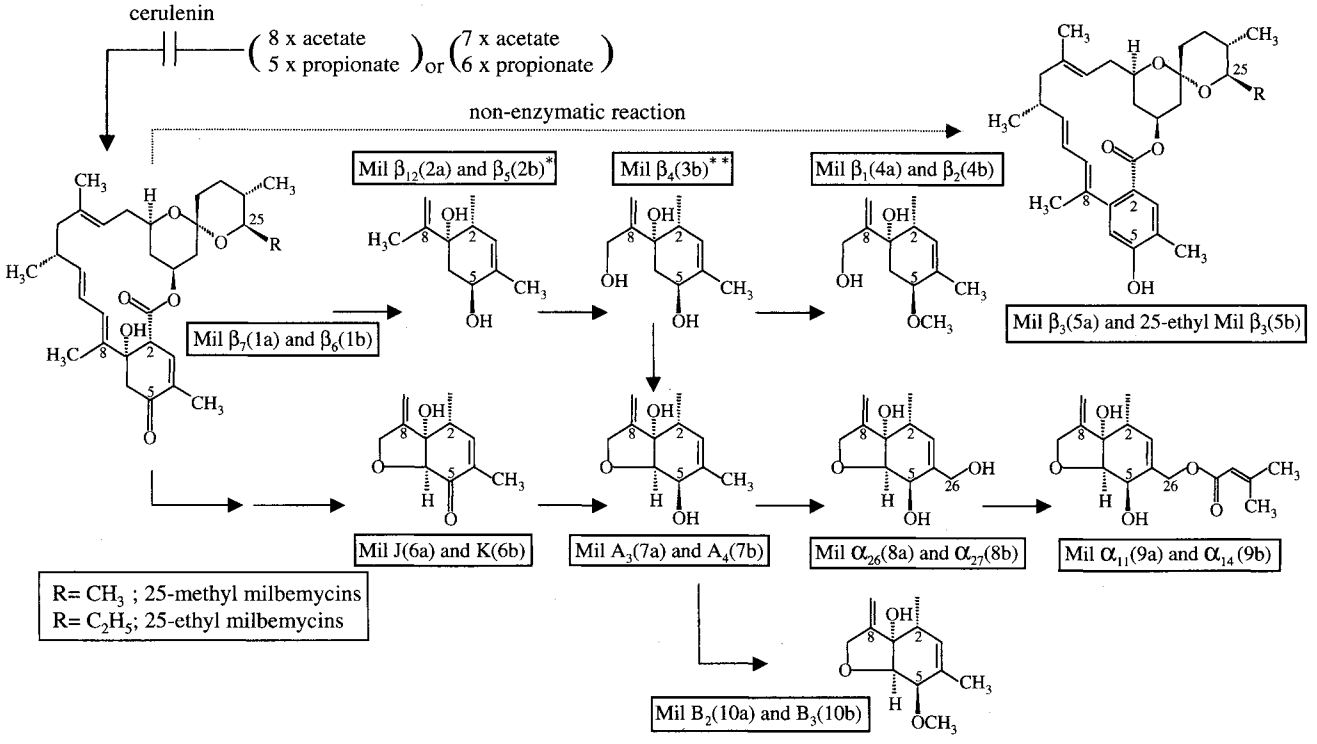
26-(3-methyl-2-butenoyloxy)avermectin B_{1a} (**9d**); IR ν_{max} cm^{-1} (NaCl film) 3465, 2960, 2925, 2870, 1716, 1655, 1650, 1450, 1380, 1340, 1295, 1270, 1225, 1190, 1180, 1140, 1115, 1070, 1050, 995; ^1H NMR (270 MHz, $CDCl_3$, δ) 5.71~5.90 (6H, m), 5.55 (1H, dd, J=9.9, 2.5 Hz), 5.40~5.46 (2H, m), 4.98 (1H, m), 4.66~4.84 (5H, m), 4.48 (1H, m), 3.44 (3H, s), 3.43 (3H, s), 3.13~4.11 (12H, m), 2.72 (1H, d, J=7.4 Hz), 2.42~2.62 (2H, m), 2.16 (3H, d, J=1.0 Hz), 1.90 (3H, d, J=1.0 Hz), 1.50 (3H, br s), 1.29 (3H, d, J=6.4 Hz), 1.25 (3H, d, J=6.0 Hz), 1.17 (3H, d, J=6.9 Hz), 0.82~2.37 (23H, m); ^{13}C NMR (67.5 MHz, $CDCl_3$, δ) 173.3, 166.3, 157.8, 139.4, 138.3, 137.0, 136.3, 135.2, 127.8, 124.7, 121.3, 120.7, 118.3, 115.6, 98.5, 95.8, 95.0, 81.9, 80.6, 80.4, 79.4, 79.1, 78.2, 77.2, 76.1, 75.0, 68.6, 68.4, 68.1, 67.3, 64.7, 63.5, 56.5, 56.4, 45.6, 40.5, 39.8, 36.6, 35.2, 34.5, 34.2, 30.6, 29.7, 27.5, 27.4, 20.3, 20.2, 18.4, 17.7, 16.4, 15.1, 13.0, 12.0; FAB-MS (m/z): $(M+H+(EtO)_3N)^+$: 1120 ($C_{53}H_{78}O_{16}+H+(EtO)_3N)^+$, 1102, 1020.

Discussion

Strain RNBC-5-51, which accumulated no milbemycins, was successfully isolated by virtue of mutagen treatment during a screening program for high producers of milbemycins. Although many kinds of non-producers for milbemycins, such as strain Nt-15[5], and some kinds of morphological mutants[6] have already been isolated and reported, these isolated strains except for morphological mutants, could not be cultured normally in a production medium. Although the morphological mutants were able to be cultured as well as milbemycin-producing strains, they could not convert milbemycins[6].

On the other hand, the bioconversion experiments using

Fig. 5. Proposed pathways of milbemycin α_{11} and α_{14} biosyntheses.



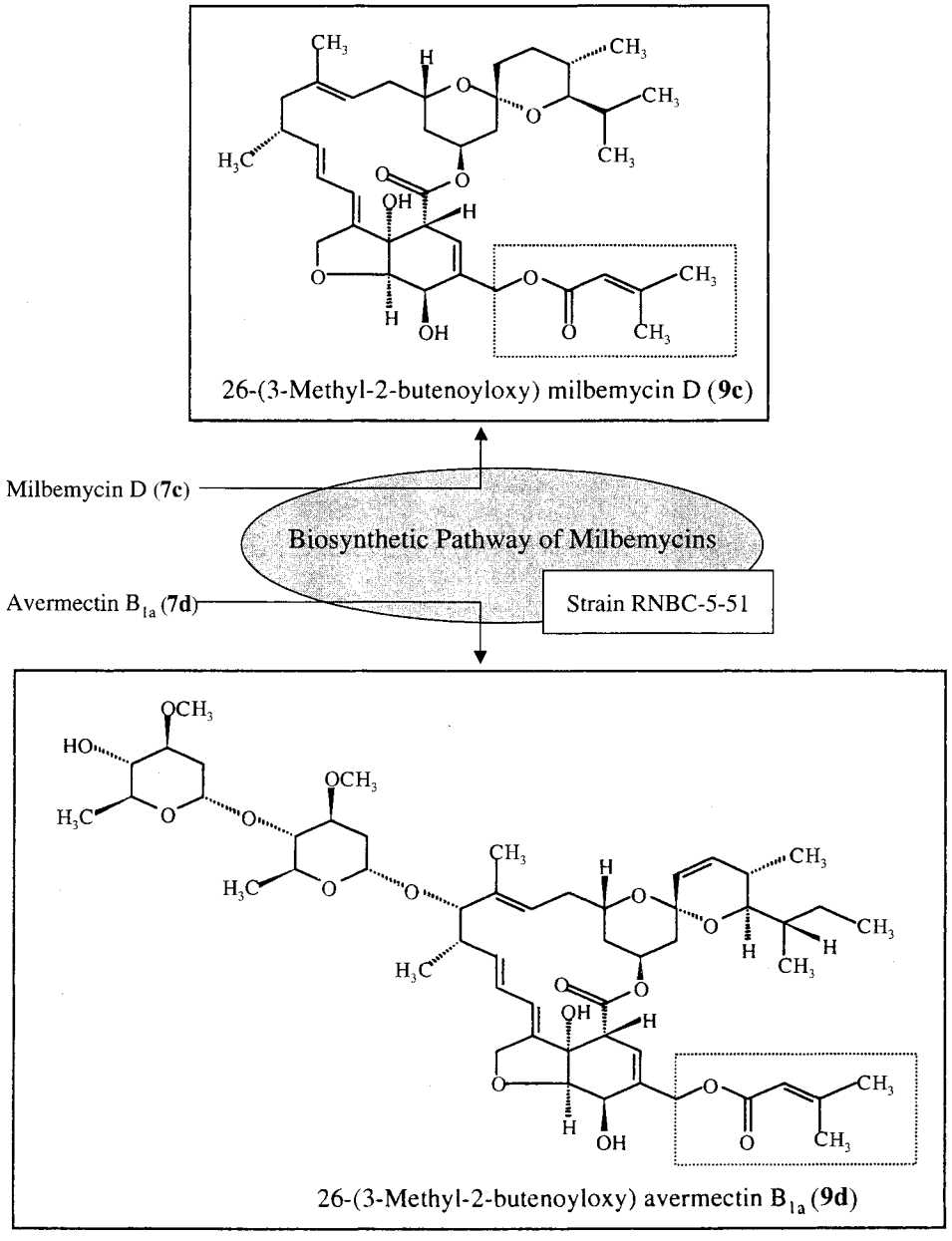
* In naming the different milbemycins in the rectangles, all a and bs in parentheses indicate 25-methylmilbemycin and 25-ethylmilbemycin, respectively.

**C-8a hydroxylmilbemycin β_{12}, equivalent to structure of milbemycin β_4, among 25-methylmilbemycins has not been isolated yet.

strain RNBC-5-51 enabled us to elucidate the biosynthetic pathway of 25-methyl milbemycins. Strain RNBC-5-51 accumulated unknown products in the culture broth. These products, of which production was inhibited by the addition of cerulenin, have not yet been purified, because they were unstable during the purification process. They probably might be precursors of milbemycins, such as a hypothetical linear polyketide for milbemycin skeleton, or fatty acids which could be of relation to milbemycin biosynthesis. Proposed biosynthetic pathways of 25-methyl and 25-ethyl milbemycins based on our results are summarized in Fig. 5.

From the results of the bioconversion study of milbemycin analogues, such as milbemycin D and avermectin B_{1a}, it was shown that the terminal biosynthetic pathway of milbemycins could introduce a 3-methyl-2-butenoyloxy substructure derived from leucine onto milbemycin analogues at C-26 position (Fig. 6). According to some reports[7,8], hybrid compounds were biosynthesized by the addition of a foreign macrolide ring in the culture broth of a macrolide-producing microorganism under conditions that allowed cerulenin to inhibit the formation of the original macrolide rings. However, in the case of strain RNBC-5-51, cerulenin was unnecessary in the biosynthesis of the hybrid compounds. In a biosynthetic study on avermectins, the technique of mutational biosynthesis of avermectin with a novel C-25 substituent was reported by researchers at Pfizer Ltd.[9,10]. Recently, a genetically engineered erythromycin-producing polyketide synthase which has a loading module for the avermectin-producing polyketide synthase (avermectin-PKS) was constructed[11]. The expression of this hybrid enzyme in erythromycin producers, produced various novel erythromycins which had various starter units because of the wide specificity in the loading module for the avermectin-PKS. These results indicate that the various milbemycin-related compounds which possess various polyketide-chain initiators at C-25 position, are able to take part in a late or terminal biosynthetic step, such as in our proposed milbemycin biosynthetic pathway. Therefore, strain RNBC-5-51 would be able to bioconvert milbemycin-related compounds, such as milbemycin D and avermectin B_{1a},

Fig. 6. Novel milbemycin-related compounds obtained by bioconversion.



which have different starter units. This mutant would give us a way to produce novel milbemycin-related compounds which have the 3-methyl-2-butenoyloxy substructure at C-26 position.

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