# New Milbemycins from *Streptomyces hygroscopicus* subsp. *aureolacrimosus*: Fermentation, Isolation and Structure Elucidation

Koichi Nonaka*, Takahiro Tsukiyama†, Yoshihiro Okamoto†, Kazuo Sato†, Chieko Kumasaka, Takahiro Yamamoto, Fumio Maruyama and Hiroji Yoshikawa

Lead Discovery 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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Twelve new milbemycins have been isolated and characterized from some strains derived from *Streptomyces hygroscopicus* subsp. *aureolacrimosus* SANK 60286 and SANK 60526. The metabolites **1**~**4** and **9**~**11** were produced by strain RM28D-688 SANK 60797 as minor products. The metabolites **5**~**8** were obtained from a broth of strain 57-338 SANK 61796. Strain MK-1391 SANK 62896 was used for the production of metabolite **12**. The new metabolites, eight $\alpha$-class and four $\beta$-class compounds, have new structural features. For example, milbemycins $\alpha_{26}$ and $\alpha_{27}$, have the 26-hydroxy moiety, and other derivatives (milbemycins $\alpha_{20\sim23}$) have different side chains at the C-26 position from those of milbemycins $\alpha_{11}$ and $\alpha_{14}$. In addition, 5-hydroxylmilbemycin $\beta_7$ ($\beta_{12}$), involved in the major biosynthetic pathway of 25-methyl and 25-ethyl milbemycins, was discovered.

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]. All milbemycins have 16-membered macrolide structures, which are biosynthesized *via* a polyketide derived from the condensation of several units of acetate, propionate and branched-chain fatty acid[1]. Following the discovery of milbemycins, numerous compounds with the same 16-membered macrolide structure were isolated[2~4], including Merck's avermectin with potent anthelmintic activity, Cyanamid's LL-F28249, Glaxo's Factor series compounds, and milbemycins $\alpha_{11}$ and $\alpha_{14}$.

During a strain improvement program for high milbemycin-producing strains for commercialization, some biosynthetically blocked mutants of *S. hygroscopicus* subsp. *aureolacrimosus* have been isolated and characterized[5~7]. In biosynthetic studies on milbemycins, these mutants were used in bioconversion experiments with cerulenin, a specific inhibitor of fatty acid and polyketide biosyntheses, to delineate the biosynthetic pathway of milbemycin $\alpha_{14}$, the final product of 25-ethylmilbemycins[6]. Furthermore, we successfully obtained a non-producing strain, the so-called strain RNBC-5-51. The bioconversion experiments using this strain were conducted to elucidate the biosynthetic pathway of 25-methylmilbemycins, and to modify some milbemycin-related compounds such as milbemycin D and avermectin $B_{1a}$ at the C-26 position[7].

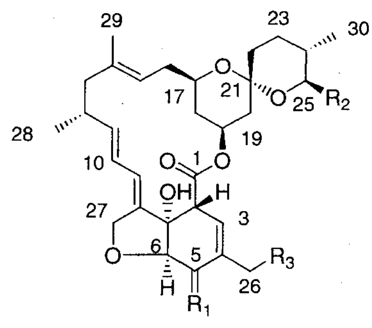
In addition, we investigated the fermentation broth of a few milbemycin-producing strains in more detail to discover new milbemycins, and isolated 12 new metabolites (compounds **1** to **12**, Fig. 1 and 2). In this paper, we report the fermentation conditions and the isolation, and structure elucidation of the new milbemycins.

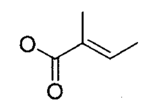
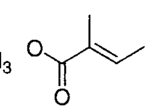
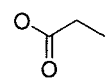
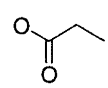
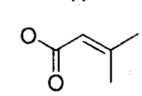
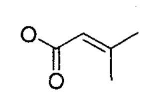
## Results

### Producing Strain

During a screening program for high milbemycin producers, several characteristic mutants were isolated;

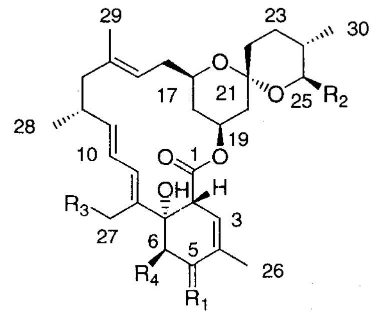
Fig. 1. Structure of milbemycins.




| Milbemycins | $R_1$ | $R_2$ | $R_3$ |
|---|---|---|---|
| $\alpha_{20}$ (**1**) | H, β-OH | $C^{(31)}H_3$ |  |
| $\alpha_{21}$ (**2**) | H, β-OH | $C^{(31)}H_2C^{(32)}H_3$ |  |
| $\alpha_{22}$ (**3**) | H, β-OH | $CH_3$ |  |
| $\alpha_{23}$ (**4**) | H, β-OH | $CH_2CH_3$ |  |
| $\alpha_{24}$ (**5**) | H, β-OMe | $CH_3$ | OH |
| $\alpha_{25}$ (**6**) | H, β-OMe | $CH_2CH_3$ | OH |
| $\alpha_{26}$ (**7**) | H, β-OH | $CH_3$ | OH |
| $\alpha_{27}$ (**8**) | H, β-OH | $CH_2CH_3$ | OH |
| $A_3$ (**13**) | H, β-OH | $CH_3$ | H |
| $A_4$ (**14**) | H, β-OH | $CH_2CH_3$ | H |
| $\alpha_{11}$ (**15**) | H, β-OH | $CH_3$ |  |
| $\alpha_{14}$ (**16**) | H, β-OH | $CH_2CH_3$ |  |

strain RM28D-688 SANK 60797 was isolated as a high producer of milbemycin $\alpha_{11}$ and $\alpha_{14}$, and strains 57-338 SANK 61796 and MK-1391 SANK 62896 were biosynthetically blocked mutants, derived from SANK 60286 and SANK 60526, respectively[6]. Each strain was maintained on a 1/2 YM 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 ($\geqq 10^8$ CFU/ml) in 50% (W/V) glycerol at $-20$°C.

Fig. 2. Structure of milbemycins.




| Milbemycins | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| $\beta_9$ (**9**) | H, β-OMe | $C^{(31)}H_3$ | OH | OH |
| $\beta_{10}$ (**10**) | H, β-OMe | $C^{(31)}H_2C^{(32)}H_3$ | OH | OH |
| $\beta_{11}$ (**11**) | H, β-OH | $CH_3$ | H | OH |
| $\beta_{12}$ (**12**) | H, β-OH | $CH_3$ | H | H |

## Fermentation

To obtain the metabolites **1**~**4** and **9**~**11**, which were minor compounds produced by strain RM28D-688, a large-scale fermentation (30-liter fermentor) had to be conducted. On the other hand, the metabolites **5**~**8** and **12** were produced as major products of strains 57-338 and MK-1391, respectively. Therefore, these metabolites could be isolated from the cultured broth in flasks. The typical cultivation procedures using a fermentor (A) and flasks (B) are described below.

### Fermentation Process A for the Production of Metabolites **1**~**4** and **9**~**11** by Strain RM28D-688

For the first stage preculture, 2 ml of spore suspension was inoculated into 550 ml of PS medium contained in a 2-liter Erlenmeyer flask. The inoculated flask was incubated on a rotary shaker at 28°C for 2 days. Next, the first stage seed was transferred into a 30-liter fermentor fitted with 2 vaned-disc impellers containing 20 liters of the same PS seed medium to give the second stage seed. Sterile air was supplied (10 liter/minute) and the second stage seed was agitated at 180 rpm. The second stage preculture was carried out at 28°C for 24 hours. Subsequently, 600 ml of the second seed culture was transferred into a 30-liter fermentor fitted with 2 vaned-disc impellers, containing 20 liter of the production medium designated as a modified TY-1-3 (sucrose 12%, dextrin 3%, 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 fermentation was carried out at 28.5°C for 12 days, adding extra aliquots of antiforming agent (CB-442, NOF Corp.). Sterile air was supplied (10 liter/minute) and inner pressure was maintained at 0.05 MPa. D.O. level was controlled at 5 ppm by changing the agitation speed.

### Fermentation Process B for the Production of Metabolites **5**~**8** and **12** by Strains 57-338 and MK-1391

A portion of spore suspension or a loopful of mature spores from the slant culture was inoculated into a 500-ml Erlenmeyer flask containing 50 ml of seed medium. After cultivating for 3 days at 28°C on a rotary shaker, 1 ml of the seed culture was transferred into a 100-ml Erlenmeyer flask containing 15 ml of the production medium, 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 11 days at 28°C on a rotary shaker.

Fig. 3. Isolation and purification procedure of **1**~**4** and **9**~**11**.

Fermentation broth (170 L)
- filtration

Filter cake
- extract with MeOH (90 %)
- filtration

Filtrate
- diluted with water
- extracted with *n*-hexane

*n*-Hexane layer
- concentrated *in vacuo*
- dissolved in *n*-hexane

Silica gel column chromatography
- eluted with *n*-hexane-acetone solutions

Preparative reverse phase HPLC
- YMC-Pack ODS-AM R-3105-20AM (YMC Co., Ltd.)
- eluted with 60 % MeCN
- diluted with water
- extracted with *n*-hexane
- concentrated *in vacuo*

colorless amorphous powder
(**1**; 330 mg, **2**; 230 mg, **3**; 80 mg, **4**; 178 mg, **9**; 248 mg, **10**; 560 mg, **11**; 310 mg)

## Isolation and Purification

The isolation and purification procedures for **1**~**12** are summarized in Fig. 3, 4, and 5, respectively. The metabolites **1**~**12** were extracted with aqueous MeOH (90%) from the filtrated mycelial cake or the culture broth of some milbemycin producing strains. The extracts were purified by a combined method of solvent partition, column chromatography, and preparative HPLC. All compounds were finally obtained as a colorless amorphous powder.

## Structure Elucidation

The structures of the new milbemycins were determined by the analysis and the comparison of $^{1}H$ NMR, $^{13}C$ NMR, MS, and IR data with those of milbemycins $A_3$ (**13**) and $A_4$ (**14**)[8], and $\alpha_{11}$ (**15**) and $\alpha_{14}$ (**16**)[4]. The $^{1}H$ and $^{13}C$ NMR data of new milbemycins are summarized in Tables 1, 2, and 3. The molecular formula was established from the HR-MS spectra. In the $^{1}H$ and $^{13}C$ NMR spectra of the new milbemycins, signals corresponding to the 16-membered macrolide structures were found. The structural difference between milbemycins $A_3$ (**13**), $A_4$ (**14**), and the twelve new

Fig. 4. Isolation and purification procedure of **5**~**8**.

Fermentation broth (1.2 L)
- extract with MeOH (90 %)
- filtration

Filtrate
- diluted with water
- extracted with *n*-hexane

*n*-Hexane layer
- concentrated *in vacuo*
- dissolved in *n*-hexane

Preparative reverse phase HPLC
- YMC-Pack ODS-AM SH-364-20AM (YMC Co., Ltd.).
- eluted with 66.5 % MeCN
- diluted with water
- extracted with *n*-hexane
- concentrated *in vacuo*

colorless amorphous powder
(**5**; 64.6 mg, **6**; 43 mg, **7**; 75.5 mg, **8**; 71.8 mg)

Fig. 5. Isolation and purification procedure of **12**.

Fermentation broth (1.2 L)
- extract with MeOH (90 %)
- filtration

Filtrate
- diluted with water
- extracted with *n*-hexane

*n*-Hexane layer
- concentrated *in vacuo*
- dissolved in *n*-hexane

Silica gel column chromatography
- eluted with *n*-hexane-acetone solutions

Preparative reverse phase HPLC
- YMC-Pack ODS-AM SH-364-20AM (YMC Co., Ltd.).
- eluted with 66.5 % MeCN
- diluted with water
- extracted with *n*-hexane
- concentrated *in vacuo*

colorless amorphous powder
(**12**; 120.1 mg)

milbemycins was found in the substitution at 5-, 6-, 26-, and 27-positions.

The $^1$H and $^{13}$C NMR data, and MS data of milbemycins $\alpha_{20}$ (**1**), $\alpha_{21}$ (**2**), $\alpha_{22}$ (**3**), and $\alpha_{23}$ (**4**) showed substitutions of the *trans*-2-methyl-2-butenoyloxy side chain and propionyloxy side chain at the 26-position, respectively. The MS data of milbemycins $\alpha_{24}$ (**5**), $\alpha_{25}$ (**6**), $\alpha_{26}$ (**7**), and $\alpha_{27}$ (**8**) suggested that they contained an extra oxygen atom. Signals for the three protons of the 26-methyl signal disappeared, and the signals for two protons in the substituted allylic region of the NMR spectra appeared. These observations confirmed the existence of a hydroxyl group at 26-position. Similarly, the $^1$H NMR data of milbemycins $\alpha_{24}$ (**5**), and $\alpha_{25}$ (**6**) also indicated the substitution of the hydroxyl group at 5-position by a methoxy group.

The $^1$H NMR spectra of milbemycins $\beta_9$ (**9**) and $\beta_{10}$ (**10**) indicated the substitution of the hydroxyl group at 5-position by a methoxy group. Shift of the conspicuous signals for the two allylic protons of the 27-position were also observed. This observation and the chemical shift of the signals for the proton of 6-position suggested the cleavage of the furan ring to form corresponding diol moiety. In addition, the MS data of milbemycins $\beta_9$ (**9**) and $\beta_{10}$ (**10**) supported these results.

In the $^1$H NMR spectra of milbemycin $\beta_{11}$ (**11**), signals for the two allylic protons of the 27-position disappeared, and the signals for three protons of allylic methyl group appeared. This observation and the chemical shift of the signal for the proton of 6-position suggested the cleavage of the furan ring and the existence of the methyl group at the 8-position. The MS data of milbemycin $\beta_{11}$ (**11**) supported this result.

Table 1. $^{1}$H-NMR spectral data of the $\alpha$ series of the new milbemycins ($\delta$ppm, $CDCl_3$).

| Position | $\alpha_{20}$ (1) | $\alpha_{21}$ (2) | $\alpha_{22}$ (3) | $\alpha_{23}$ (4) |
|---|---|---|---|---|
| 2 | 3.20~3.37 (1H of 2H, m) | 3.33 (1H, m) | 3.32 (1H, br) | 3.32 (1H, t, J=2.0 Hz) |
| 3 | 5.80 (1H, br) | 5.74 (1H, br) | 5.65~5.90 (1H of 3H, m) | 5.70~5.90 (1H of 3H, m) |
| 5 | 4.49 (1H, m) | 4.49 (1H, m) | 4.48 (1H, d, J=5.8Hz) | 4.48 (1H, t, J=6.1 Hz) |
| 6 | 4.00 (1H, d, J=6.2 Hz) | 3.99 (1H, d, J=6.2 Hz) | 3.99 (1H, d, J=5.8Hz) | 3.98 (1H, d, J=6.1 Hz) |
| 9 | 5.68~5.85 (1H of 2H, m) | 5.68~5.86 (1H of 2H, m) | 5.65~5.90 (1H of 3H, m) | 5.70~5.90 (1H of 3H, m) |
| 10 | 5.68~5.85 (1H of 2H, m) | 5.68~5.86 (1H of 2H, m) | 5.65~5.90 (1H of 3H, m) | 5.70~5.90 (1H of 3H, m) |
| 11 | 5.33~5.47 (1H of 2H, m) | 5.34~5.46 (1H of 2H, m) | 5.30~5.50 (1H of 2H, m) | 5.30~5.50 (1H of 2H, m) |
| 12 | 2.42 (1H, m) | 2.35~2.50 (1H, m) | 2.45 (1H, m) | 2.40 (1H, m) |
| 13 | 2.07~2.30 (1H of 3H, m) | 2.10~2.30 (1H of 3H, m) | 2.15~2.30 (1H of 3H, m) | 2.05~2.20 (1H of 3H, m) |
| | 0.79~1.90 (1H of 9H, m) | 0.78~1.95 (1H of 11H, m) | 0.80~1.90 (1H of 11H, m) | 0.80~1.92 (1H of 11H, m) |
| 15 | 4.99 (1H, m) | 4.96 (1H, m) | 4.99 (1H, t, J=7.5 Hz) | 4.97 (1H, t, J=7.0 Hz) |
| 16 | 2.07~2.30 (2H of 3H, m) | 2.10~2.30 (2H of 3H, m) | 2.15~2.30 (2H of 3H, m) | 2.05~2.20 (2H of 3H, m) |
| 17 | 3.45~3.65 (1H, m) | 3.50~3.65 (1H, m) | 3.55 (1H, m) | 3.55 (1H, m) |
| 18 | 0.79~1.90 (2H of 9H, m) | 0.78~1.95 (2H of 11H, m) | 0.80~1.90 (2H of 11H, m) | 0.80~1.92 (2H of 11H, m) |
| 19 | 5.33~5.47 (1H of 2H, m) | 5.34~5.46 (1H of 2H, m) | 5.30~5.50 (1H of 2H, m) | 5.30~5.50 (1H of 2H, m) |
| 20 | 2.00 (1H, m) | 2.00 (1H, m) | 2.00 (1H, dd, J=11.8, 3.7 Hz) | 2.00 (1H, dd, J=12.0, 3.4 Hz) |
| | 0.79~1.90 (1H of 9H, m) | 0.78~1.95 (1H of 11H, m) | 0.80~1.90 (1H of 11H, m) | 0.80~1.92 (1H of 11H, m) |
| 22 | 0.79~1.90 (2H of 9H, m) | 0.78~1.95 (2H of 11H, m) | 0.80~1.90 (2H of 11H, m) | 0.80~1.92 (2H of 11H, m) |
| 23 | 0.79~1.90 (2H of 9H, m) | 0.78~1.95 (2H of 11H, m) | 0.80~1.90 (2H of 11H, m) | 0.80~1.92 (2H of 11H, m) |
| 24 | 0.79~1.90 (1H of 9H, m) | 0.78~1.95 (1H of 11H, m) | 0.80~1.90 (1H of 11H, m) | 0.80~1.92 (1H of 11H, m) |
| 25 | 3.20~3.37 (1H of 2H, m) | 3.08 (1H, dt, J=9.2, 2.6 Hz) | 3.26 (1H, dd, J=9.7, 6.3 Hz) | 3.08 (1H, dt, J=9.1, 2.5 Hz) |
| 26 | 4.70 (2H, br) | 4.70 (2H, br) | 4.70 (2H, br) | 4.70 (2H, br) |
| 27 | 4.86 (1H, d, J=13.9 Hz) | 4.86 (1H, d, J=13.6 Hz) | 4.79 (1H, d, J=13.6 Hz) | 4.65~4.80 (2H, m) |
| | 4.66 (1H, d, J=13.9 Hz) | 4.73 (1H, d, J=13.6 Hz) | 4.67 (1H, d, J=13.6 Hz) | |
| 28 | 1.15 (3H, d, J=6.2 Hz) | 1.01 (3H, d, J=6.6 Hz) | 1.00 (3H, d, J=6.7 Hz) | 1.00 (3H, d, J=6.9 Hz) |
| 29 | 1.53 (3H, br) | 1.53 (3H, br) | 1.53 (3H, br) | 1.53 (3H, br) |
| 30 | 0.83 (3H, d, J=6.6 Hz) | 0.82 (3H, d, J=6.2 Hz) | 0.83 (3H, d, J=6.7 Hz) | 0.82 (3H, d, J=6.4 Hz) |
| 31 | 1.01 (3H, d, J=6.6 Hz) | 0.78~1.95 (2H of 11H, m) | 1.14 (3H, d, J=6.3 Hz) | 0.80~1.92 (2H of 11H, m) |
| 32 | | 0.99 (3H, t, J=7.0 Hz) | | 0.98 (3H, t, J=7.7 Hz) |
| C(5)OH | 2.72 (1H, d, J=7.7 Hz) | 2.73 (1H, d, J=7.7 Hz) | 0.80~1.90 (1H of 11H, m) | 2.65 (1H, br) |
| $C(5)OCH_3$ | | | | |
| C(7)OH | 4.13 (1H, s) | 4.12 (1H, s) | 0.80~1.90 (1H of 11H, m) | 4.12 (1H, s) |
| C(26)OH | | | | |
| $C(26)OC(O)C(CH_3)C\underline{H}CH_3$ | 6.90 (1H, m) | 6.90 (1H, m) | | |
| $C(26)OC(O)C(CH_3)CHC\underline{H}_3$ | 1.79 (3H, d, J=6.2 Hz) | 1.79 (3H, dd, J=7.0, 1.1 Hz) | | |
| $C(26)OC(O)C(C\underline{H}_3)CHCH_3$ | 1.84 (3H, d, J=1.5 Hz) | 1.85 (3H, d, J=1.1 Hz) | | |
| $C(26)OC(O)C\underline{H}_2CH_3$ | | | 2.38 (2H, q, J=7.5 Hz) | 2.38 (2H, q, J=7.7 Hz) |
| $C(26)OC(O)CH_2C\underline{H}_3$ | | | 1.15 (3H, t, J=7.5 Hz) | 1.15 (3H, t, J=7.7 Hz) |

| Position | $\alpha_{24}$ (5) | $\alpha_{25}$ (6) | $\alpha_{26}$ (7) | $\alpha_{27}$ (8) |
|---|---|---|---|---|
| 2 | 3.34 (1H, m) | 3.34 (1H, m) | 3.33 (1H, m) | 3.32 (1H, m) |
| 3 | 5.65~5.85 (1H of 3H, m) | 5.65~5.85 (1H of 3H, m) | 5.70 (1H, br) | 5.71 (1H, br) |
| 5 | 4.10~4.30 (1H of 3H, m) | 4.10~4.30 (1H of 3H, m) | 4.58 (1H, m) | 4.59 (1H, m) |
| 6 | 4.05 (1H, d, J=5.5Hz) | 4.05 (1H, d, J=5.6 Hz) | 3.98 (1H, d, J=6.4 Hz) | 3.98 (1H, d, J=6.1 Hz) |
| 9 | 5.65~5.85 (1H of 3H, m) | 5.65~5.85 (1H of 3H, m) | 5.74~5.84 (1H of 2H, m) | 5.74~5.85 (1H of 2H, m) |
| 10 | 5.65~5.85 (1H of 3H, m) | 5.65~5.85 (1H of 3H, m) | 5.74~5.84 (1H of 2H, m) | 5.74~5.85 (1H of 2H, m) |
| 11 | 5.28~5.50 (1H of 2H, m) | 5.30~5.45 (1H of 2H, m) | 5.30~5.48 (1H of 2H, m) | 5.30~5.47 (1H of 2H, m) |
| 12 | 2.40 (1H, m) | 2.43 (1H, m) | 2.15~2.55 (1H of 4H, m) | 2.35~2.55 (1H, m) |
| 13 | 2.10~2.29 (1H of 3H, m) | 2.12~2.30 (1H of 3H, m) | 2.15~2.55 (1H of 4H, m) | 2.15~2.35 (1H of 3H, m) |
| | 0.80~1.92 (1H of 11H, m) | 0.80~1.91 (1H of 13H, m) | 0.82~1.95 (1H of 10H, m) | 0.82~1.95 (1H of 12H, m) |
| 15 | 5.01 (1H, t, J=7.5 Hz) | 4.96 (1H, t, J=7.3Hz) | 5.00 (1H, m) | 4.97 (1H, t, J=7.4 Hz) |
| 16 | 2.10~2.29 (2H of 3H, m) | 2.12~2.30 (2H of 3H, m) | 2.15~2.55 (2H of 4H, m) | 2.15~2.35 (2H of 3H, m) |
| 17 | 3.55 (1H, m) | 3.55 (1H, m) | 3.55 (1H, m) | 3.57 (1H, m) |
| 18 | 0.80~1.92 (2H of 11H, m) | 0.80~1.91 (2H of 13H, m) | 0.82~1.95 (2H of 10H, m) | 0.82~1.95 (2H of 12H, m) |
| 19 | 5.28~5.50 (1H of 2H, m) | 5.30~5.45 (1H of 2H, m) | 5.30~5.48(1H of 2H, m) | 5.30~5.47 (1H of 2H, m) |
| 20 | 2.02 (1H, m) | 2.01 (1H, dd, J=11.7, 3.4Hz) | 1.99 (1H, m) | 2.01 (1H, m) |
| | 0.80~1.92 (1H of 11H, m) | 0.80~1.91 (1H of 13H, m) | 0.82~1.95 (1H of 10H, m) | 0.82~1.95 (1H of 12H, m) |
| 22 | 0.80~1.92 (2H of 11H, m) | 0.80~1.91 (2H of 13H, m) | 0.82~1.95 (2H of 10H, m) | 0.82~1.95 (2H of 12H, m) |
| 23 | 0.80~1.92 (2H of 11H, m) | 0.80~1.91 (2H of 13H, m) | 0.82~1.95 (2H of 10H, m) | 0.82~1.95 (2H of 12H, m) |
| 24 | 0.80~1.92 (1H of 11H, m) | 0.80~1.91 (1H of 13H, m) | 0.82~1.95 (1H of 10H, m) | 0.82~1.95 (1H of 12H, m) |
| 25 | 3.26 (1H, dd, J=9.7, 6.3 Hz) | 3.08 (1H, m) | 3.27 (1H, dd, J=9.6, 6.4 Hz) | 3.08 (1H, dt, J=9.0, 2.3 Hz) |
| 26 | 4.10~4.30 (2H of 3H, m) | 4.10~4.30 (2H of 3H, m) | 4.19~4.34 (2H, m) | 4.20~4.35 (2H, m) |
| 27 | 4.73 (1H, d, J=14.6 Hz) | 4.72 (1H, d, J=14.0 Hz) | 4.60~4.75 (2H, m) | 4.65~4.80 (2H, m) |
| | 4.63 (1H, d, J=14.6 Hz) | 4.63 (1H, d, J=14.0 Hz) | | |
| 28 | 1.14 (3H, d, J=6.2 Hz) | 1.00 (3H, d, J=6.6 Hz) | 1.15 (3H, d, J=6.1 Hz) | 1.01 (3H, d, J=6.7 Hz) |
| 29 | 1.53 (3H, br) | 1.53 (3H, br) | 1.56 (3H, br) | 1.54 (3H, br) |
| 30 | 0.82 (3H, d, J=6.4 Hz) | 0.81 (3H, d, J=6.4 Hz) | 0.83 (3H, d, J=6.4 Hz) | 0.83 (3H, d, J=6.4 Hz) |
| 31 | 1.00 (3H, d, J=6.3 Hz) | 0.80~1.91 (2H of 13H, m) | 1.01 (3H, d, J=6.4 Hz) | 0.82~1.95 (2H of 12H, m) |
| 32 | | 0.98 (3H, t, J=7.6 Hz) | | 0.98 (3H, t, J=7.8 Hz) |
| C(5)OH | | | 2.74 (1H, d, J=7.6 Hz) | 2.78 (1H, d, J=7.3 Hz) |
| $C(5)OCH_3$ | 3.53 (3H, s) | 3.53 (3H, s) | | |
| C(7)OH | 0.80~1.92 (1H of 11H, m) | 0.80~1.91 (1H of 13H, m) | 4.18 (1H, s) | 4.20 (1H, s) |
| C(26)OH | 0.80~1.92 (1H of 11H, m) | 0.80~1.91 (1H of 13H, m) | 0.82~1.95 (1H of 10H, m) | 0.82~1.95 (1H of 12H, m) |
| $C(26)OC(O)C(CH_3)C\underline{H}CH_3$ | | | | |
| $C(26)OC(O)C(CH_3)CHC\underline{H}_3$ | | | | |
| $C(26)OC(O)C(C\underline{H}_3)CHCH_3$ | | | | |
| $C(26)OC(O)C\underline{H}_2CH_3$ | | | | |
| $C(26)OC(O)CH_2C\underline{H}_3$ | | | | |

$^{1}$H-NMR spectra for milbemycins $\alpha_{20}$ (1), $\alpha_{21}$ (2), and $\alpha_{24}$ (5) were measured at 200 MHz.
$^{1}$H-NMR spectra for milbemycins $\alpha_{22}$ (3), $\alpha_{23}$ (4), $\alpha_{25}$ (6), $\alpha_{26}$ (7), and $\alpha_{27}$ (8) were measured at 270 MHz.

Table 2. [1]H-NMR spectral data of the $\beta$ series of the new milbemycins ($\delta$ppm, $CDCl_3$).

| Position | $\beta_9$ (**9**) | $\beta_{10}$ (**10**) | $\beta_{11}$ (**11**) | $\beta_{12}$ (**12**) |
|---|---|---|---|---|
| 2 | 3.35 (1H, br) | 3.33 (1H, br) | 3.74 (1H, dd, $J$=4.7, 2.3 Hz) | 3.40 (1H, m) |
| 3 | 5.27 (1H, br) | 5.29 (1H, br) | 5.27 (1H, m) | 5.25 (1H, t, $J$=1.5 Hz) |
| 5 | 3.75 (1H, m) | 3.75 (1H, m) | 4.46 (1H, br) | 4.47 (1H, br) |
| 6 | 4.02 (1H of 2H, br) | 4.02 (1H of 2H, br) | 3.86 (1H, d, $J$=3.9 Hz) | 1.15~2.00 (2H of 11H, m) |
| 9 | 6.46 (1H, d, $J$=10.9 Hz) | 6.47 (1H, d, $J$=10.9 Hz) | 6.05~6.20 (1H of 2H, m) | 6.22 (1H, d, $J$=11.6 Hz) |
| 10 | 6.30 (1H, dd, $J$=14.6, 10.9 Hz) | 6.30 (1H, dd, $J$=14.5, 10.9 Hz) | 6.05~6.20 (1H of 2H, m) | 6.04 (1H, dd, $J$=14.3, 11.6 Hz) |
| 11 | 5.42 (1H, dd, $J$=14.6, 9.7 Hz) | 5.45 (1H, dd, $J$=14.5, 9.8 Hz) | 5.35~5.55 (1H of 2H, m) | 5.25~5.50 (1H of 2H, m) |
| 12 | 2.50 (1H, m) | 2.45 (1H, m) | 2.40~2.58 (1H of 2H, m) | 2.42 (1H, m) |
| 13 | 2.10~2.30 (1H of 3H, m) | 2.10~2.35 (1H of 3H, m) | 2.12~2.38 (1H of 4H, m) | 2.00~2.42 (1H of 4H, m) |
| | 1.15~1.90 (1H of 10H, m) | 1.20~1.85 (1H of 11H, m) | 0.70~1.95 (1H of 10H, m) | 1.15~2.00 (1H of 11H, m) |
| 15 | 4.82 (1H, dd, $J$=9.8, 4.5 Hz) | 4.80 (1H, m) | 4.85 (1H, d, $J$=8.3 Hz) | 4.85 (1H, dd, $J$=9.4, 4.2 Hz) |
| 16 | 2.10~2.30 (2H of 3H, m) | 2.10~2.35 (2H of 3H, m) | 2.12~2.38 (2H of 4H, m) | 2.00~2.42 (2H of 4H, m) |
| 17 | 3.55 (1H, m) | 3.55 (1H, m) | 3.62 (1H, m) | 3.54 (1H, m) |
| 18 | 0.70 (1H, m) | 0.78 (1H, m) | 0.70~1.95 (2H of 10H, m) | 0.75 (1H, m) |
| | 1.15~1.90 (1H of 10H, m) | 1.20~1.85 (1H of 11H, m) | | 1.15~2.00 (1H of 11H, m) |
| 19 | 5.32 (1H, m) | 5.35 (1H, m) | 5.35~5.55 (1H of 2H, m) | 5.25~5.50 (1H of 2H, m) |
| 20 | 1.15~1.90 (2H of 10H, m) | 1.90 (1H, m) | 0.70~1.95 (2H of 10H, m) | 1.15~2.00 (2H of 11H, m) |
| | | 1.20~1.85 (1H of 11H, m) | | |
| 22 | 1.15~1.90 (2H of 10H, m) | 1.20~1.85 (2H of 11H, m) | 0.70~1.95 (2H of 10H, m) | 1.15~2.00 (2H of 11H, m) |
| 23 | 1.15~1.90 (2H of 10H, m) | 1.20~1.85 (2H of 11H, m) | 0.70~1.95 (2H of 10H, m) | 1.15~2.00 (2H of 11H, m) |
| 24 | 1.15~1.90 (1H of 10H, m) | 1.20~1.85 (1H of 11H, m) | 0.70~1.95 (1H of 10H, m) | 1.15~2.00 (1H of 11H, m) |
| 25 | 3.24 (1H, dd, $J$=9.8, 6.4 Hz) | 3.04 (1H, dt, $J$=9.5, 2.5 Hz) | 3.26 (1H, dd, $J$=9.7, 6.3 Hz) | 3.25 (1H, dd, $J$=6.2, 2.7 Hz) |
| 26 | 1.78 (3H, br) | 1.78 (3H, br) | 1.82 (3H, br) | 1.84 (3H, br) |
| 27 | 4.30 (1H, m) | 4.30 (1H, m) | 1.92 (3H, br) | 1.71 (3H, d, $J$=1.0 Hz) |
| | 4.17 (1H, d, $J$=13.0 Hz) | 4.18 (1H, m) | | |
| 28 | 1.02 (3H, d, $J$=6.6 Hz) | 1.03 (3H, d, $J$=6.4 Hz) | 1.05 (3H, d, $J$=6.6 Hz) | 1.01 (3H, d, $J$=6.9 Hz) |
| 29 | 1.60 (3H, br) | 1.60 (3H, br) | 1.63 (3H, br) | 1.59 (3H, br) |
| 30 | 0.82 (3H, d, $J$=6.6 Hz) | 0.81 (3H, d, $J$=6.7 Hz) | 0.82 (3H, d, $J$=6.6 Hz) | 0.82 (3H, d, $J$=6.4 Hz) |
| 31 | 1.10 (3H, d, $J$=6.4 Hz) | 1.20~1.85 (2H of 11H, m) | 1.10 (3H, d, $J$=6.3 Hz) | 1.11 (3H, d, $J$=6.2 Hz) |
| 32 | | 0.95 (3H, t, $J$=7.3 Hz) | | |
| C(5)OH | | | 2.40~2.58 (1H of 2H, m) | 2.00~2.42 (1H of 4H, m) |
| C(5)OC<u>H</u>$_3$ | 3.45 (3H, s) | 3.45 (3H, s) | | |
| C(6)OH | 3.84 (1H, s) | 3.83 (1H, s) | 2.12~2.38 (1H of 4H, m) | |
| C(7)OH | 4.02 (1H of 2H, br) | 4.02 (1H of 2H, br) | 4.05 (1H, s) | 3.91 (1H, br) |
| C(27)OH | 1.15~1.90 (1H of 10H, m) | 1.20~1.85 (1H of 11H, m) | | |

[1]H-NMR spectra for milbemycins $\beta_9$ (**9**), $\beta_{10}$ (**10**), $\beta_{11}$ (**11**), and $\beta_{12}$ (**12**) were measured at 270 MHz.

The comparison of the NMR and MS data of milbemycin $\beta_{12}$ (**12**) with those of milbemycin $\beta_{11}$ (**11**) indicated the lack of a hydroxyl group at 6-position.

From the above data and the 2D NMR studies, the structures of the twelve new milbemycins were consequently determined as shown in Fig. 1 and 2.

## Discussion

In this paper, we report that twelve new milbemycins, milbemycin $\alpha_{20}$~$\alpha_{27}$ (**1~8**) and $\beta_9$~$\beta_{12}$ (**9~12**) were isolated from milbemycin-producing strains, *Streptomycs hygroscopicus* subsp. *aureolacrimosus* SANK 60797, SANK 61796 and SANK 62896, respectively.

In previous biosynthetic studies on milbemycins, we have reported the major biosynthetic pathway of milbemycins, which was determined by using an intact-cell and cell-free system of the strain Rf-107[5] or a bioconversion system of blocked mutants[6,7]. In this biosynthetic pathway, milbemycin $\alpha_{26}$ and milbemycin $\alpha_{27}$ would have been the precursors of C-26 derivatives of milbemycins, such as milbemycin $\alpha_9$~$\alpha_{15}$[1] and $\alpha_{20}$~$\alpha_{23}$, respectively. We confirmed that milbemycin $\alpha_{27}$ (or $\alpha_{26}$) was determined

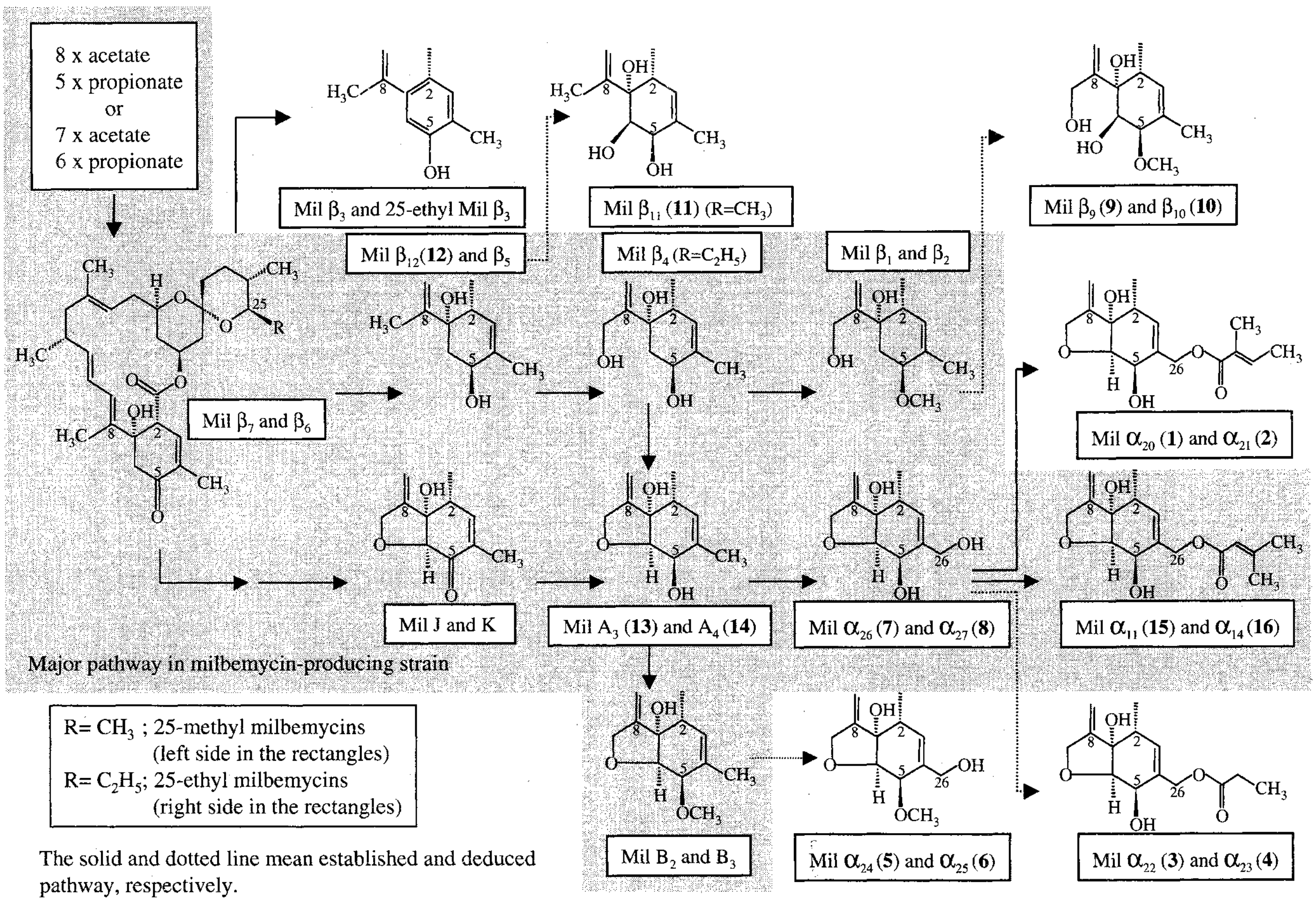
Table 3. $^{13}$C-NMR spectral data of the new milbemycins ($\delta$ppm, $CDCl_3$).

| Position | $\alpha_{20}$ (1) | $\alpha_{21}$ (2) | $\alpha_{22}$ (3) | $\alpha_{23}$ (4) | $\alpha_{24}$ (5) | $\alpha_{25}$ (6) | $\alpha_{26}$ (7) | $\alpha_{27}$ (8) | $\beta_9$ (9) | $\beta_{10}$ (10) | $\beta_{11}$ (11) | $\beta_{12}$ (12) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 173.6 | 173.4 | 173.1 | 173.0 | 173.4 | 173.3 | 173.2 | 173.2 | 174.1 | 174.1 | 174.6 | 174.0 |
| 2 | 46.0 | 46.0 | 45.6 | 45.6 | 45.5 | 45.5 | 45.6 | 45.6 | 44.7 | 44.7 | 44.4 | 47.9 |
| 3 | 121.0 | 121.0 | 120.6 | 120.7 | 119.7 | 119.8 | 120.0 | 120.0 | 118.6 | 118.6 | 118.2 | 118.2 |
| 4 | 139.7 | 139.6 | 139.1 | 139.1 | 139.4 | 139.3 | 140.0 | 140.0 | 136.0 | 136.0 | 136.1 | 136.5 |
| 5 | 69.2 | 69.2 | 68.9 | 68.8 | 69.0 | 69.0 | 68.9 | 68.9 | 69.5 | 69.5 | 69.9 | 68.6 |
| 6 | 79.6 | 79.7 | 79.1 | 79.1 | 75.7 | 76.0 | 79.1 | 79.0 | 79.3 | 79.3 | 71.3 | 41.0 |
| 7 | 80.8 | 80.8 | 80.3 | 80.4 | 80.4 | 80.4 | 80.4 | 80.4 | 76.8 | 76.8 | 77.8 | 76.6 |
| 8 | 137.1 | 137.1 | 136.9 | 136.9 | 138.1 | 138.1 | 139.2 | 139.1 | 139.1 | 139.1 | 136.7 | 139.0 |
| 9 | 121.7 | 121.6 | 121.7 | 121.8 | 120.9 | 120.9 | 120.9 | 120.9 | 124.1 | 124.1 | 124.1 | 124.6 |
| 10 | 123.8 | 123.8 | 123.3 | 123.3 | 123.4 | 123.4 | 123.4 | 123.3 | 130.6 | 130.6 | 126.3 | 124.8 |
| 11 | 143.5 | 143.5 | 143.1 | 143.2 | 142.8 | 142.8 | 143.0 | 143.1 | 143.7 | 143.8 | 140.7 | 140.8 |
| 12 | 37.0 | 36.4 | 36.5 | 35.9 | 36.5 | 35.9 | 36.5 | 36.0 | 36.5 | 36.0 | 36.6 | 36.5 |
| 13 | 49.0 | 48.9 | 48.5 | 48.5 | 48.6 | 48.5 | 48.5 | 48.5 | 48.5 | 48.4 | 48.8 | 48.6 |
| 14 | 137.4 | 137.4 | 136.5 | 136.5 | 137.0 | 137.0 | 136.9 | 136.9 | 134.5 | 134.5 | 135.7 | 136.2 |
| 15 | 121.4 | 121.3 | 120.9 | 120.9 | 120.4 | 120.5 | 120.5 | 120.6 | 120.9 | 120.8 | 121.2 | 120.7 |
| 16 | 35.1 | 35.1 | 34.7 | 34.7 | 34.7 | 34.7 | 34.7 | 34.7 | 34.6 | 34.6 | 35.7 | 34.6 |
| 17 | 65.2 | 65.3 | 64.7 | 64.8 | 64.8 | 64.7 | 65.8 | 65.8 | 67.5 | 67.5 | 67.5 | 67.6 |
| 18 | 37.0 | 37.1 | 36.6 | 36.7 | 36.6 | 36.6 | 36.5 | 36.6 | 36.4 | 36.5 | 36.6 | 36.4 |
| 19 | 68.0 | 67.9 | 67.5 | 67.4 | 67.5 | 67.4 | 67.5 | 67.4 | 68.3 | 68.3 | 68.1 | 68.0 |
| 20 | 41.6 | 41.7 | 41.1 | 41.3 | 41.1 | 41.2 | 41.1 | 41.3 | 40.9 | 41.1 | 40.8 | 41.1 |
| 21 | 98.0 | 97.8 | 97.5 | 97.4 | 97.5 | 97.4 | 97.6 | 97.4 | 97.5 | 97.3 | 97.6 | 97.5 |
| 22 | 36.1 | 36.0 | 35.6 | 35.6 | 35.7 | 35.6 | 35.6 | 35.7 | 35.7 | 35.6 | 34.4 | 35.7 |
| 23 | 28.1 | 28.3 | 27.7 | 27.8 | 27.7 | 27.9 | 27.7 | 27.9 | 27.7 | 27.8 | 27.7 | 27.7 |
| 24 | 36.4 | 34.7 | 36.0 | 34.2 | 35.9 | 34.2 | 36.0 | 34.2 | 36.0 | 34.4 | 35.6 | 36.3 |
| 25 | 71.8 | 76.4 | 71.3 | 76.0 | 71.5 | 75.7 | 71.3 | 76.0 | 71.2 | 75.9 | 72.6 | 71.2 |
| 26 | 64.9 | 64.9 | 64.3 | 64.3 | 64.8 | 64.7 | 64.6 | 64.7 | 19.2 | 19.2 | 19.2 | 19.0 |
| 27 | 69.0 | 69.0 | 68.6 | 68.6 | 68.3 | 68.3 | 68.5 | 68.6 | 57.3 | 57.3 | 13.5 | 13.2 |
| 28 | 22.7 | 22.8 | 22.3 | 22.2 | 22.3 | 22.3 | 22.3 | 22.3 | 21.5 | 21.5 | 20.5 | 22.0 |
| 29 | 16.0 | 16.0 | 15.5 | 15.5 | 15.5 | 15.5 | 15.5 | 15.5 | 16.0 | 16.0 | 15.9 | 15.9 |
| 30 | 18.3 | 18.2 | 17.8 | 17.7 | 17.9 | 17.7 | 17.8 | 17.7 | 17.8 | 17.7 | 17.8 | 17.8 |
| 31 | 19.8 | 26.1 | 19.3 | 25.7 | 19.3 | 25.7 | 19.3 | 25.7 | 19.3 | 25.7 | 19.3 | 19.3 |
| 32 | | 10.6 | | 10.1 | | 10.1 | | 10.1 | | 10.1 | | |
| C(5)O<u>C</u>H$_3$ | | | | | 57.6 | 57.6 | | | 57.7 | 57.7 | | |
| C(26)O<u>C</u>(O)C(CH$_3$)CHCH$_3$ | 168.2 | 168.2 | | | | | | | | | | |
| C(26)OC(O)<u>C</u>(CH$_3$)CHCH$_3$ | 128.7 | 128.7 | | | | | | | | | | |
| C(26)OC(O)C(CH$_3$)<u>C</u>HCH$_3$ | 138.5 | 138.4 | | | | | | | | | | |
| C(26)OC(O)C(CH$_3$)CH<u>C</u>H$_3$ | 14.9 | 14.9 | | | | | | | | | | |
| C(26)OC(O)C(<u>C</u>H$_3$)CHCH$_3$ | 12.6 | 12.6 | | | | | | | | | | |
| C(26)O<u>C</u>(O)CH$_2$CH$_3$ | | | 174.2 | 174.2 | | | | | | | | |
| C(26)OC(O)<u>C</u>H$_2$CH$_3$ | | | 27.5 | 27.5 | | | | | | | | |
| C(26)OC(O)CH$_2$<u>C</u>H$_3$ | | | 9.1 | 9.1 | | | | | | | | |

$^{13}$C-NMR spectra for milbemycins $\alpha_{20}$ (**1**) and $\alpha_{21}$ (**2**) were measured at 50 MHz.
$^{13}$C-NMR spectra for milbemycins $\alpha_{22}$ (**3**), $\alpha_{23}$ (**4**), $\alpha_{24}$ (**5**), $\alpha_{25}$ (**6**), $\alpha_{26}$ (**7**), $\alpha_{27}$ (**8**), $\beta_9$ (**9**), $\beta_{10}$ (**10**), $\beta_{11}$ (**11**), and $\beta_{12}$ (**12**) were measured at 67.5 MHz.

to be a precursor of milbemycin $\alpha_{21}$ (or $\alpha_{20}$) by the bioconversion experiments using strain BCW-4-3 SANK 62099, which accumulated milbemycin $\alpha_{20}$ and $\alpha_{21}$ (data not shown). Furthermore, in another bioconversion experiment using strain RDGr, milbemycin $A_3$ and $A_4$-high producers[6)], milbemycin $\beta_{12}$ was converted to milbemycin $A_3$, as expected (data not shown). Milbemycin $\beta_{12}$ would have been the precursor of milbemycin $A_3$ or $\beta_1$ *via* a hypothetical C-27 hydroxylmilbemycin $\beta_{12}$, which has not been isolated yet. These new milbemycins may be part of the proposed milbemycin biosynthetic pathway as shown in Fig. 6.

Fig. 6. Proposed pathway of milbemycins.




## Experimental

General

HPLC analysis was basically 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.). These columns were 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, respectively. However, milbemycin $\alpha_{20}$ (or $\alpha_{21}$) could not be separated from milbemycin $\alpha_{11}$ (or $\alpha_{14}$) by using these systems. In the case of the HPLC analysis for milbemycin $\alpha_{20}$ and $\alpha_{21}$, J′-sphere ODS-M80 (4.6 mm i.d.×150 mm, YMC Co., Ltd.), which was eluted with 66.5% MeCN at a flow rate of 1.0 ml per minute, was used. All chromatograms were monitored with an absorbance at 242 nm.

$^1$H NMR (200 MHz) and $^{13}$C NMR (50 MHz) spectra were measured on a Varian Gemini-200 FT NMR Spectrometer. $^1$H NMR (270 MHz) and $^{13}$C NMR (67.5 MHz) spectra were measured on a JEOL JNM-GX 270 FT NMR Spectrometer. Mass spectra were measured on a Fisions Instruments VG Autospec. IR spectra were measured on a Perkin Elmer 1600 series FT IR.

Isolation and Physico-chemical Properties of **1~4** and **9~11**

The metabolites **1~4** and **9~11** were isolated from a 170-liter culture broth of strain RM28D-688. Mycelia were recovered from the harvested broth to which was added 5% (W/W) Celite® (Celite Corp.), by filtration with a Buchner funnel. The resulting mycelial cake was extracted with 100 liters of aqueous MeOH (90%). Subsequently, the filtrate diluted with an equal volume of water was re-extracted with *n*-hexane. The *n*-hexane layers were concentrated *in vacuo* at 37°C, redissolved in *n*-hexane, and applied to a silica gel column (4 kg) equilibrated with *n*-hexane. The column was successively eluted with *n*-hexane - acetone solutions (97.5 :

2.5, 95 : 5, 90 : 10). The desired fractions detected by HPLC were concentrated under reduced pressure and the concentrates were dissolved in MeOH and further purified by preparative HPLC using YMC-Pack ODS-AM R-3105-20AM (100 mm i.d.×500 mm, YMC Co., Ltd.). The column was eluted with 60% MeCN at a flow rate of 200 ml per minute. Each fraction in which the desired metabolite was detected by HPLC analysis, was diluted with an equal volume of water. The resulting solutions were extracted with an equal volume of *n*-hexane - EtOAc solution (1 : 1, V/V) and the extracts were concentrated *in vacuo* at 37°C to dryness to give pure milbemycins. Each metabolite was obtained as a colorless amorphous powder, respectively (**1**; 330 mg, **2**; 230 mg, **3**; 80 mg, **4**; 178 mg, **9**; 248 mg, **10**; 560 mg, **11**; 310 mg).

Milbemycin $\alpha_{20}$ (**1**): IR $\nu_{max}$ (film) cm$^{-1}$ 3455, 1710; MS *m/z* 626 ($M^+$, $C_{36}H_{50}O_9$), 526; HREI-MS calcd for $C_{36}H_{50}O_9$: 626.3455, found: 626.3453.

Milbemycin $\alpha_{21}$ (**2**): IR $\nu_{max}$ (film) cm$^{-1}$ 3465, 1710; MS *m/z* 640 ($M^+$, $C_{37}H_{52}O_9$), 540; HREI-MS calcd for $C_{37}H_{52}O_9$: 640.3611, found: 640.3613.

Milbemycin $\alpha_{22}$ (**3**): IR $\nu_{max}$ (film) cm$^{-1}$ 3465, 1730, 1725; MS *m/z* 600 ($M^+$, $C_{34}H_{48}O_9$), 582, 564, 526; HREI-MS calcd for $C_{34}H_{48}O_9$: 600.3298, found: 640.3298.

Milbemycin $\alpha_{23}$ (**4**): IR $\nu_{max}$ (film) cm$^{-1}$ 3465, 1735; MS *m/z* 614 ($M^+$, $C_{35}H_{50}O_9$), 540; HREI-MS calcd for $C_{35}H_{50}O_9$: 614.3455, found: 614.3456.

Milbemycin $\beta_9$ (**9**): IR $\nu_{max}$ (film) cm$^{-1}$ 3455, 1705; MS *m/z* 560 ($M^+$, $C_{32}H_{48}O_8$), 542; HREI-MS calcd for $C_{32}H_{48}O_8$: 560.3349, found: 560.3350.

Milbemycin $\beta_{10}$ (**10**): IR $\nu_{max}$ (film) cm$^{-1}$ 3465, 1705; MS *m/z* 574 ($M^+$, $C_{33}H_{50}O_8$), 556, 538; HREI-MS calcd for $C_{33}H_{50}O_8$: 574.3506, found: 574.3507.

Milbemycin $\beta_{11}$ (**11**): IR $\nu_{max}$ (film) cm$^{-1}$ 3465, 1705; MS *m/z* 530 ($M^+$, $C_{31}H_{46}O_7$), 512, 494; HREI-MS calcd for $C_{31}H_{46}O_7$: 530.3244, found: 530.3244.

## Isolation and Physico-chemical Properties of 5~8

To the 1,200 ml culture broth of strain 57-338, 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 an equal volume of *n*-hexane and the extract was concentrated *in vacuo* at 37°C. The oily residue was dissolved in MeOH and purified by preparative HPLC using YMC-Pack ODS-AM SH-364-20AM (30 mm i.d.× 300 mm, YMC Co., Ltd.). The column was eluted with 66.5% MeCN at a flow rate of 10 ml per minute. Each new milbemycin, **5~8**, was obtained as a colorless amorphous powder, respectively (**5**; 64.6 mg, **6**; 43 mg, **7**; 75.5 mg, **8**; 71.8 mg).

Milbemycin $\alpha_{24}$ (**5**): IR $\nu_{max}$ (film) cm$^{-1}$ 3425, 1715; MS *m/z* 558 ($M^+$, $C_{32}H_{46}O_8$), 540, 508; HREI-MS calcd for $C_{32}H_{46}O_8$: 558.3193, found: 558.3194.

Milbemycin $\alpha_{25}$ (**6**): IR $\nu_{max}$ (film) cm$^{-1}$ 3445, 1725; MS *m/z* 572 ($M^+$, $C_{33}H_{48}O_8$), 554, 522; HREI-MS calcd for $C_{33}H_{48}O_8$: 572.2985, found: 572.2987.

Milbemycin $\alpha_{26}$ (**7**): IR $\nu_{max}$ (film) cm$^{-1}$ 3415, 1725; MS *m/z* 544 ($M^+$, $C_{31}H_{44}O_8$), 526; HREI-MS calcd for $C_{31}H_{44}O_8$: 544.3036, found: 544.3035.

Milbemycin $\alpha_{27}$ (**8**): IR $\nu_{max}$ (film) cm$^{-1}$ 3425, 1720; MS *m/z* 558 ($M^+$, $C_{32}H_{46}O_8$), 540; HREI-MS calcd for $C_{32}H_{46}O_8$: 558.3193, found: 558.3194.

## Isolation and Physico-chemical Properties of 12

For metabolite **12**, the culture broth of strain MK-1391 was extracted with MeOH, re-extracted with *n*-hexane and concentrated *in vacuo* at 37°C. The oily residue dissolved in *n*-hexane was applied to a silica gel column (30 g) previously equilibrated with *n*-hexane, which was successively eluted with *n*-hexane - acetone solutions (97.5 : 2.5, 95 : 5, 90 : 10, 80 : 20). The desired fraction detected by HPLC analysis was concentrated under reduced pressure; the concentrates were dissolved in MeOH and further purified by preparative HPLC using YMC-Pack ODS-AM SH-364-20AM (30 mm i.d.×300 mm, YMC Co., Ltd.). The column was eluted with 66.5% MeCN at a flow rate of 10 ml per minute. The fraction containing a metabolite detected by HPLC analysis, was diluted by the addition of water, extracted with an equal volume of *n*-hexane - EtOAc solution (1 : 1, V/V), and concentrated *in vacuo* at 37°C to dryness to give a colorless amorphous powder (120.1 mg).

Milbemycin $\beta_{12}$ (**12**): IR $\nu_{max}$ (film) cm$^{-1}$ 3453, 1710; MS *m/z* 514 ($M^+$, $C_{31}H_{46}O_6$), 496, 478; HREI-MS calcd for $C_{31}H_{46}O_6$: 514.3296, found: 514.3296.

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