MICROBIAL CONVERSION OF MILBEMYCINS: 30-OXIDATION OF MILBEMYCIN A_4 AND RELATED COMPOUNDS BY *AMYCOLATA AUTOTROPHICA* AND *AMYCOLATOPSIS MEDITERRANEI*

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Microorganisms were screened for their ability to modify milbemycin A_4 (**1a**). Many strains, mostly actinomycetes and zygomycetes, were found to convert milbemycin A_4 (**1a**) to one or more new products. Among these products, M-1, M-2, and M-3 were obtained using *Amycolata autotrophica* subsp. *amethystina* ATCC 35204, and were identified as 30-hydroxymilbemycin A_4 (**1b**), 26,30-dihydroxymilbemycin A_4 (**1c**), and milbemycin A_4 30-oic acid (**1d**), respectively. Other milbemycins and LL-F28249α (**7a**) also underwent 30-hydroxylation by the microorganism. 22,23-Dihydroavermectin B_{1a} (**8a**) was not hydroxylated at any position by *A. autotrophica* subsp. *amethystina* ATCC 35204, but a corresponding hydroxyl product at the C-30 position was obtained using *Amycolatopsis mediterranei* IFO 13415.

Milbemycins are a family of sixteen-membered macrolides produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus*[1~3] and exhibit potent antiparasitic and pesticidal activities. Similar structural features and biological activities have also been reported for avermectin[4] and LL-F28249[5] isolated from the culture broths of *Streptomyces avermitilis* and *Streptomyces cyaneogriseus* subsp. *noncyanogenus*, respectively.

In the course of our investigations of preparing useful new conversion products for further derivatization, and for subsequent use as metabolite reference standards in animal metabolism studies, microbial conversions of milbemycins were examined. RAMOS TOMBO *et al.* have recently demonstrated 13β-hydroxylation and 14,15-epoxidation of milbemycins by *Streptomyces violascens* ATCC 31560[6].

Disclosed herein is microbial modification of milbemycins and related compounds, namely through the oxidation of a C-24 methyl group.

Materials and Methods

Materials

Milbemycins A_3 (**2a**)[1], A_4 (**1a**)[1], D (**3a**)[3], and LL-F28249α (**7a**)[5] were isolated as described previously. 13β-Hydroxymilbemycin A_4 (**4a**)[7], 13β-fluoromilbemycin A_4 (**5a**)[8], and 5-ketomilbemycin A_4 5-oxime (**6a**)[9] were synthesized from milbemycin A_4 (**1a**). 22,23-Dihydroavermectin B_{1a} (**8a**) was isolated from a commercially available mixture of 22,23-dihydroavermectins B_{1a} and B_{1b}.

Microorganisms

Microorganisms were obtained from various culture collections and were also isolated from soil samples.

Culture and Reaction Conditions

For screening, each microorganism was inoculated into 100-ml Erlenmeyer flasks containing 20 ml of MY medium consisting of glucose 1.0%, Polypeptone (Daigo Nutritive Chemicals) 0.5%, yeast extract (Difco) 0.3%, and malt extract (Difco) 0.3%, pH 6.3~6.5. The flasks were incubated at 200~220 rpm on a rotary shaker for a period of 2~3 days, at 26°C for fungi and at 28°C for actinomycetes, bacteria, and yeasts. Then milbemycin A_4 (**1a**) (5% (w/v) in 1,4-dioxane) was added to a final concentration of 500 μg/ml and the cultivation was continued.

Detection and Measurement of Conversion Products

Aliquots of culture broth were withdrawn at different intervals and were extracted with EtOAc. The extract was examined by TLC (Merck Art. No. 5715: EtOAc). Developed chromatograms were detected under 254 nm UV light or by spraying with ammonium molybdate (10% (w/v) in ethanol), followed by warming on a hot plate.

The analytical HPLC was performed using a Nova pak C_{18} (Waters, 8 mm × 10 cm) column. Elution was achieved with one of two solvent systems. System 1 consisted of acetonitrile-water (75:25), with a flow rate of 1.5 ml/minute. System 2 consisted of acetonitrile-water (55:45), with a flow rate of 1.0 ml/minute. UV-detection was performed at 243 nm.

Isolation of the Conversion Products

Amycolata autotrophica subsp. *amethystina* ATCC 35204 was cultured in six 500-ml Erlenmeyer flasks containing 100 ml of MY medium at 28°C on a rotary shaker (200~220 rpm). After 4 days of cultivation milbemycin A_4 (**1a**) (5% (w/v) in 1,4-dioxane) was added to a final concentration of 500 μg/ml and cultivation was continued for one additional day. The culture broth from the flasks was pooled and extracted with two 600-ml portions of EtOAc. The extract was dried over anhydrous sodium sulfate and concentrated *in vacuo*. The residue was chromatographed on silica gel (Wako gel C-100). Elution with 20~90% EtOAc in *n*-hexane gave three conversion products, M-1 (114.6 mg), M-2 (47.7 mg), and M-3 (3.0 mg).

Results and Discussion

Screening of Microorganisms for Ability to Modify Milbemycin A_4 (1a)

A total of 186 strains of fungi, 345 strains of actinomycetes, 44 strains of bacteria, and 33 strains of yeasts were tested for their ability to convert milbemycin A_4 (**1a**). Many strains of actinomycetes and zygomycetes formed conversion products. Among these, M-1, M-2, and M-3 were isolated from the culture broth of *A. autotrophica* subsp. *amethystina* ATCC 35204 (formerly *Nocardia autotrophica* subsp. *amethystina* ATCC 35204[10]). The chromatograms of the oxidation products are summarized in Table 1. Representative microorganisms that converted milbemycin A_4 (**1a**) to M-1 are shown in Table 2. As a result of the screening, *A. autotrophica* subsp. *amethystina* ATCC 35204 and *Amycolatopsis mediterranei* IFO 13415 (formerly *Streptomyces mediterranei* IFO 13415[10]) carried out the transformation to M-1 more efficiently than the other actinomycetes, and they were used for subsequent work. In some cases certain strains were found to convert milbemycin A_4 (**1a**) to other products. These results will be reported elsewhere.

Identification of M-1, M-2, and M-3

From the analyses of NMR and MS and from the chemical evidence, M-1, M-2, and M-3, obtained as described in Materials and Methods, were identified as 30-hydroxymilbemycin A_4 (**1b**),

Table 1. TLC Rf values and HPLC Rt's of M-1, M-2, and M-3.

Compound	TLC Rf values	HPLC Rt's (minutes)	
		System 1	System 2
1a	0.59	16.07	—
M-1 (**1b**)	0.44	3.08	8.91
M-2 (**1c**)	0.08	2.34	5.01
M-3 (**1d**)	0.10	2.54	5.66

Table 2. Representative microorganisms capable of converting milbemycin A_4 to M-1.

Microorganism	Conversion efficiency[a]
Amycolata autotrophica ATCC 35202	+1
A. autotrophica subsp. *canberrica* ATCC 35203	+1
A. autotrophica subsp. *amethystina* ATCC 35204	+3
Amycolatopsis mediterranei IFO 13415	+3
A. orientalis IFO 12806	+2
Streptomyces flavovirens IFO 12771	+1
S. griseolus IFO 12777	+1
S. roseus IFO 12818	+1
Absidia reflexa IFO 5874	+2
Circinella umbellata IFO 4452	+2
Mortierella isabellina NRRL 1757	+3
Mucor recurvus IFO 8093	+2
Bacillus megaterium IFO 12108	+1

[a] +1: 0.5~10%, +2: 10~30%, +3: more than 30% (HPLC analysis).

Table 3. TLC Rf values and HPLC Rt's of milbemycin A_4 related compounds and their conversion products.

Compound	TLC Rf values	HPLC Rt's (minutes)	
		System 1	System 2
2a	0.59	11.80	—
2b	0.41	2.83	7.12
3a	0.62	24.64	—
3b	0.50	3.64	12.65
4a	0.46	3.50	10.86
4b	0.26	2.00	3.38
5a	0.60	8.84	—
5b	0.45	2.55	6.30
6a	0.69	18.91	—
6b	0.55	3.22	10.65
7a	0.55	11.03	—
7b	0.40	3.20	9.96
8a	0.40	24.19	—
8b	0.30	3.18	9.87

26,30-dihydroxymilbemycin A_4 (**1c**), and milbemycin A_4 30-oic acid (**1d**), respectively. Physico-chemical properties of these compounds are given below. Structures of the conversion products of milbemycin A_4 (**1a**) by *A. autotrophica* subsp. *amethystina* ATCC 35204 as well as a proposed bioconversion pathway are presented in Fig. 1. Milbemycin A_4 (**1a**) was converted predominantly to the 30-hydroxy derivative (**1b**), and subsequently converted to the 26,30-dihydroxy derivative (**1c**). The 30-oic acid might be formed as a minor product from the 30-hydroxy derivative.

Application of *A. autotrophica* subsp. *amethystina* ATCC 35204 for Conversion of Related Compounds

Conversion of milbemycin A_3 (**2a**), milbemycin D (**3a**), 13β-hydroxymilbemycin A_4 (**4a**), 13β-fluoromilbemycin A_4 (**5a**), 5-ketomilbemycin A_4 5-oxime (**6a**), LL-F28249α (**7a**), and 22,23-dihydroavermectin B_{1a} (**8a**) were examined with *A. autotrophica* subsp. *amethystina* ATCC 35204 using a similar method as for milbemycin A_4 (**1a**). *A. autotrophica* subsp. *amethystina* ATCC 35204 was able to convert these compounds to corresponding 30-hydroxy compounds, except 22,23-dihydroavermectin B_{1a} (**8a**). Chromatograms of the hydroxylation products are summarized in Table 3. Preparation of the conversion products is shown in Table 4. Physico-chemical properties of these compounds are listed below. Structures of the substrates and the products are presented in Fig. 2.

Microbial Conversion of 22,23-Dihydroavermectin B_{1a} (**8a**) to the 30-Hydroxy Compound

22,23-Dihydroavermectin B_{1a} (**8a**) was converted to a more polar product by *A. mediterranei* IFO 13415. The product was extracted and isolated. From the physico-chemical properties the product was

Fig. 1. Proposed pathway for bioconversion of milbemycin A_4 (**1a**) by *Amycolata autotrophica* subsp. *amethystina* ATCC 35204.

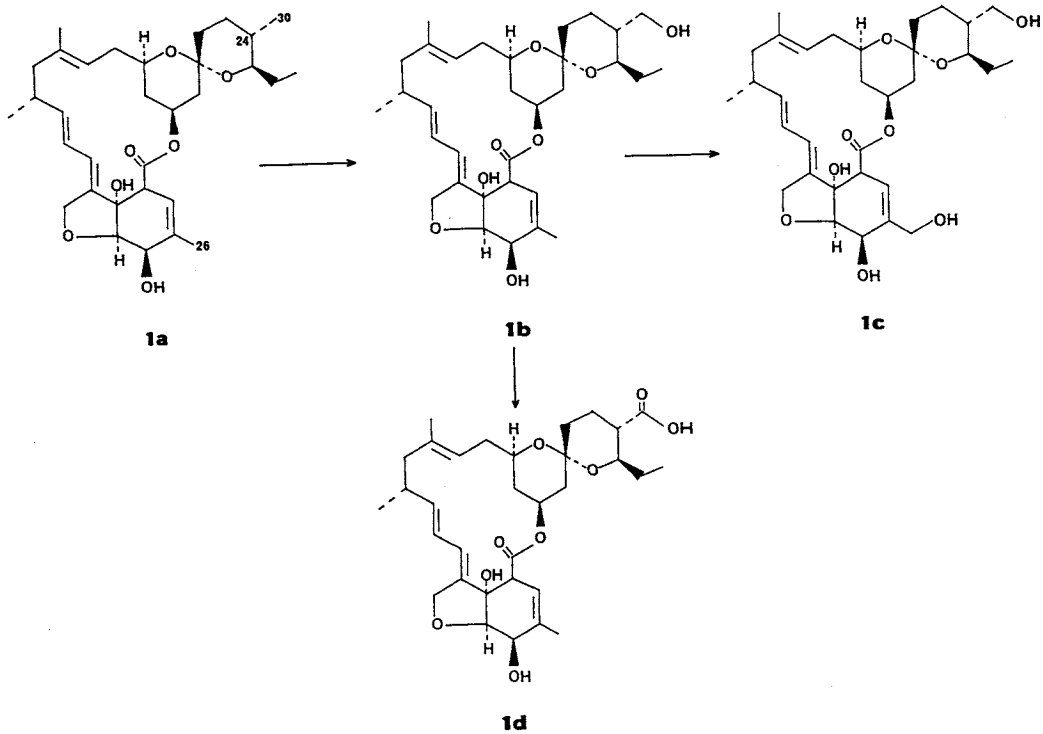


Table 4. Preparation of corresponding 30-hydroxy compounds.

Substrate	Microorganism	Concentration (μg/ml)	Conversion time (days)	Product	Yield (%)
2a	*Amycolata autotrophica*[a]	500	4	**2b**	22
1a	*A. autotrophica*	500	1	**1b**	37
3a	*A. autotrophica*	500	7	**3b**	9
4a	*A. autotrophica*	500	1	**4b**	50
5a	*A. autotrophica*	500	2	**5b**	19
6a	*A. autotrophica*	500	7	**6b**	10
7a	*A. autotrophica*	300	4	**7b**	5
8a	*Amycolatopsis mediterranei*	250	7	**8b**	26

[a] *A. autotrophica* subsp. *amethystina*.

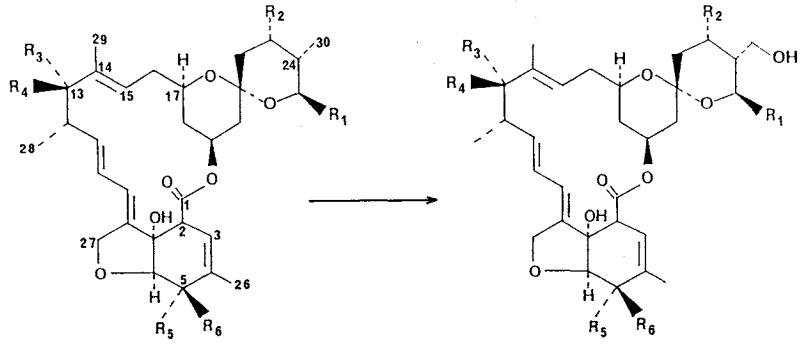
identified as 22,23-dihydro-30-hydroxyavermectin B_{1a} (**8b**).

MIWA *et al.* reported the metabolism of avermectins by liver microsomes, and they isolated a very small amount of hydroxy compounds of avermectins at the C-30 position[11]. But chemical and microbial hydroxylation of milbemycins or avermectins at the C-30 position has not yet been reported. Compared with milbemycins, the hydroxylated compounds newly obtained in this study did not improve acaricidal activities. Success of microbial hydroxylation of milbemycins and related compounds at the C-30 position will lead us to the synthesis of new useful milbemycin derivatives.

Physico-chemical Properties

The ^1H NMR spectral data are listed in Table 5.

Fig. 2. Structures of milbemycins and related compounds, and corresponding 30-hydroxy compounds.



a b

	R_1	R_2	R_3	R_4	R_5	R_6
2	CH_3	H	H	H	H	OH
3	$CH(CH_3)_2$	H	H	H	H	OH
4	CH_2CH_3	H	H	OH	H	OH
5	CH_2CH_3	H	H	F	H	OH
6	CH_2CH_3	H	H	H	NOH	
7	$\overset{31}{C}H(CH_3)=\overset{32}{C}H\overset{33}{C}H(CH_3)_2$	OH	H	H	H	OH
8	$\overset{31}{C}H(CH_3)\overset{32}{C}H_2\overset{33}{C}H_3$	H	(L-oleandrosyl)$_2$	H	H	OH

30-Hydroxymilbemycin A_4 (**1b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2959 (s), 2924 (s), 2874 (s), 1713 (s); MS *m/z* 558 (M^+, $C_{32}H_{46}O_8$), 540, 430, 412, 372, 314, 280, 261, 248, 211, 183, 151; HREI-MS Calcd for $C_{32}H_{46}O_8$: 558.3193, Found: 558.3194.

26,30-Dihydroxymilbemycin A_4 (**1c**): IR (KBr) cm^{-1} 3600~3200 (br s), 2960 (s), 2925 (s), 2874 (s), 1720 (s), 1672 (m); MS *m/z* 574 (M^+, $C_{32}H_{46}O_9$), 556, 538, 430, 280, 261, 211, 183; HREI-MS Calcd for $C_{32}H_{46}O_9$: 574.3142, Found: 574.3139.

Milbemycin A_4 30-Oic Acid (**1d**): IR (KBr) cm^{-1} 3700~3100 (br s), 2966 (s), 2927 (s), 2879 (s), 1716 (s); ^{13}C NMR (67.8 MHz, $CDCl_3$) δ 178.8 (COOH), 173.5 (C-1), 142.9 (C-11), 139.4, 137.8 and 137.1 (C-4, C-8, C-14), 123.4 (C-10), 120.8 (C-15), 120.4 (C-9), 118.1 (C-3), 97.2 (C-21), 80.2 (C-7), 79.5 (C-6); MS *m/z* 572 (M^+, $C_{32}H_{44}O_9$), 444, 426, 294, 275, 248, 225, 197, 179, 151; HREI-MS Calcd for $C_{32}H_{44}O_9$: 572.2985, Found: 572.2970.

30-Hydroxymilbemycin A_3 (**2b**): IR (KBr) cm^{-1} 3600~3200 (br s), 2967 (s), 2951 (s), 2925 (s), 2870 (s), 1716 (s); MS *m/z* 544 (M^+, $C_{31}H_{44}O_8$), 416, 266, 247, 197, 169, 151; HREI-MS Calcd for $C_{31}H_{44}O_8$: 544.3036, Found: 544.3043.

30-Hydroxymilbemycin D (**3b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2963 (s), 2927 (s), 2871 (s), 1716 (s); MS *m/z* 572 (M^+, $C_{33}H_{48}O_8$), 444, 426, 372, 314, 294, 275, 248, 225, 197, 179, 151; HREI-MS Calcd for $C_{33}H_{48}O_8$: 572.3349, Found: 572.3349.

13β,30-Dihydroxymilbemycin A_4 (**4b**): IR (KBr) cm^{-1} 3600~3200 (br s), 2962 (s), 2928 (s), 2873 (s), 1720 (s), 1620 (w); MS *m/z* 574 (M^+, $C_{32}H_{46}O_9$), 556, 446, 295, 277, 211, 183, 151; HREI-MS Calcd for $C_{32}H_{46}O_9$: 574.3142, Found: 574.3147.

13β-Fluoro-30-hydroxymilbemycin A_4 (**5b**): IR (KBr) cm^{-1} 3600~3200 (br s), 2968 (s), 2928 (s), 2874 (s), 1717 (s); MS *m/z* 576 (M^+, $C_{32}H_{45}FO_8$), 448, 332, 266, 211, 183, 151; HREI-MS Calcd for $C_{32}H_{45}FO_8$:

Table 5. ^1H NMR spectral data of conversion products in $CDCl_3$ (270 MHz).

1b	5.68~5.83 (2H, m, 9-H, 10-H), 5.30~5.42 (3H, m, 3-H, 11-H, 19-H), 4.94 (1H, t, J=7.7 Hz, 15-H), 4.70, 4.64 (2H, ABq, J=14.9 Hz, 27-H_2), 4.28 (1H, br s, 5-H), 4.05 (1H, s, 7-OH), 3.95 (1H, d, J=6.2 Hz, 6-H), 3.63 (1H, dd, J=4.4, 11.0 Hz, 30-H), 3.55 (1H, m, 17-H), 3.49 (1H, dd, J=6.0, 11.0 Hz, 30-H), 3.33 (1H, td, J_t=9.3 Hz, J_d=2.2 Hz, 25-H), 3.26 (1H, m, 2-H), 2.30~2.50 (2H, m, 5-OH, 12-H), 2.15~2.28 (3H, m, 13-H, 16-H_2), 2.01 (1H, dd, J=3.3, 11.5 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.52 (3H, s, 29-H_3), 1.20~1.90 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.01 (3H, t, J=7.0 Hz, 32-H_3), 0.99 (3H, d, J=7.0 Hz, 28-H_3), 0.80~0.95 (1H, m, 18-H)
1c	5.70~5.87 (3H, m, 3-H, 9-H, 10-H), 5.32~5.45 (2H, m, 11-H, 19-H), 4.96 (1H, m, 15-H), 4.71 (1H, dd, J=14.7, 2.2 Hz, 27-H), 4.70 (1H, dd, J=14.7, 2.2 Hz, 27-H), 4.58 (1H, m, 5-H), 4.31 (1H, d, J=13.2 Hz, 26-H), 4.26 (1H, d, J=13.2 Hz, 26-H), 3.98 (1H, d, J=6.2 Hz, 6-H), 3.63 (1H, dd, J=11.0, 4.4 Hz, 30-H), 3.50 (1H, dd, J=11.0, 6.2 Hz, 30-H), 3.47~3.66 (1H, m, 17-H), 3.30~3.38 (2H, m, 2-H, 25-H), 2.10~2.50 (5H, m, 5-OH, 12-H, 13-H, 16-H_2), 2.03 (1H, m, 20-H), 1.53 (3H, s, 29-H_3), 1.25~1.94 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 0.82~1.08 (7H, m, 18-H, 28-H_3, 32-H_3)
1d	5.69~5.83 (2H, m, 9-H, 10-H), 5.30~5.44 (3H, m, 3-H, 11-H, 19-H), 4.96 (1H, dd, J=4.5, 10.5 Hz, 15-H), 4.66, 4.71 (2H, ABq, J=15.0 Hz, 27-H_2), 4.29 (1H, d, J=7.0 Hz, 5-H), 4.08 (1H, s, 7-OH), 3.96 (1H, d, J=6.4 Hz, 6-H), 3.51~3.68 (2H, m, 17-H, 25-H), 3.27 (1H, dd, J=2.0, 4.4 Hz, 2-H), 2.40 (1H, m, 12-H), 2.15~2.30 (3H, m, 13-H, 16-H_2), 2.03 (1H, dd, J=3.6, 12.0 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.53 (3H, s, 29-H_3), 1.20~1.95 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.02 (3H, t, J=7.3 Hz, 32-H_3), 1.00 (3H, d, J=6.4 Hz, 28-H_3), 0.81~0.95 (1H, m, 18-H)
2b	5.69~5.83 (2H, m, 9-H, 10-H), 5.33~5.46 (3H, m, 3-H, 11-H, 19-H), 4.98 (1H, t, J=5.6 Hz, 15-H), 4.69 (1H, dd, J=14.1, 2.4 Hz, 27-H), 4.67 (1H, dd, J=14.1, 2.4 Hz, 27-H), 4.29 (1H, m, 5.-H), 4.07 (1H, s, 7-OH), 3.96 (1H, d, J=6.4 Hz, 6-H), 3.63 (1H, dd, J=11.3, 4.4 Hz, 30-H), 3.47~3.58 (3H, m, 17-H, 25-H, 30-H), 3.27 (1H, q, J=2.4 Hz, 2-H), 2.15~2.50 (5H, m, 5-OH, 12-H, 13-H, 16-H_2), 2.01 (1H, ddd, J=12.1, 4.8, 1.6 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.53 (3H, s, 29-H_3), 1.22 (3H, d, J=6.4 Hz, 31-H_3), 1.20~1.91 (8H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H), 1.00 (3H, d, J=6.4 Hz, 28-H_3), 0.81~0.95 (1H, m, 18-H)
3b	5.68~5.85 (2H, m, 9-H, 10-H), 5.29~5.43 (3H, m, 3-H, 11-H, 19-H), 4.94 (1H, t, J=7.7 Hz, 15-H), 4.65, 4.71 (2H, ABq, J=14.9 Hz, 27-H_2), 4.30 (1H, d, J=6.0 Hz, 5-H), 4.05 (1H, s, 7-OH), 3.96 (1H, d, J=6.0 Hz, 6-H), 3.63 (1H, dd, J=3.8, 10.9 Hz, 30-H), 3.59 (1H, m, 17-H), 3.47 (1H, dd, J=6.0, 10.9 Hz, 30-H), 2.30~2.50 (2H, m, 5-OH, 12-H), 2.17~2.28 (3H, m, 13-H, 16-H_2), 2.03 (1H, dd, J=4.0, 11.5 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.53 (3H, s, 29-H_3), 1.20~1.90 (9H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H), 1.06 (3H, d, J=6.8 Hz, 32-H_3), 1.00 (3H, d, J=6.4 Hz, 28-H_3), 0.91 (3H, d, J=6.8 Hz, 33-H_3), 0.79~0.85 (1H, m, 18-H)
4b	5.73~5.85 (2H, m, 9-H, 10-H), 5.40 (1H, s, 3-H), 5.27~5.42 (2H, m, 11-H, 19-H), 5.23 (1H, t, J=8.1 Hz, 15-H), 4.70 (1H, d, J=15.0 Hz, 27-H), 4.69 (1H, d, J=15.0 Hz, 27-H), 4.29 (1H, m, 5-H), 3.99 (1H, s, 7-OH), 3.96 (1H, d, J=6.0 Hz, 6-H), 3.72 (1H, d, J=10.1 Hz, 13-H), 3.63 (1H, dd, J=10.9, 4.4 Hz, 30-H), 3.51 (1H, dd, J=10.9, 6.0 Hz, 30-H), 3.50~3.67 (1H, m, 17-H), 3.35 (1H, td, J_t=9.4 Hz, J_d=2.5 Hz, 25-H), 3.27 (1H, q, J=2.5 Hz, 2-H), 2.25~2.42 (4H, m, 5-OH, 12-H, 16-H_2), 2.03 (1H, m, 20-H), 1.88 (3H, s, 26-H_3), 1.58 (3H, s, 29-H_3), 1.23~1.77 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.13 (3H, d, J=6.4 Hz, 28-H_3), 1.01 (3H, t, J=7.3 Hz, 32-H_3), 0.85~0.99 (1H, m, 18-H)
5b	5.75~5.89 (2H, m, 9-H, 10-H), 5.40 (1H, s, 3-H), 5.24~5.37 (3H, m, 11-H, 15-H, 19-H), 4.70 (1H, dd, J=14.1, 2.8 Hz, 27-H), 4.69 (1H, dd, J=14.1, 2.8 Hz, 27-H), 4.40 (1H, dd, J=47.5, 9.7 Hz, 13-H), 4.30 (1H, m, 5-H), 4.04 (1H, s, 7-OH), 3.96 (1H, d, J=6.5 Hz, 6-H), 3.63 (1H, dd, J=10.9, 4.4 Hz, 30-H), 3.50 (1H, dd, J=10.9, 6.1 Hz, 30-H), 3.50~3.70 (1H, m, 17-H), 3.34 (1H, td, J_t=9.7 Hz, J_d=2.4 Hz, 25-H), 3.27 (1H, q, J=2.4 Hz, 2-H), 2.51~2.67 (1H, m, 12-H), 2.27~2.40 (3H, m, 5-OH, 16-H_2), 2.03 (1H, m, 20-H), 1.88 (3H, s, 26-H_3), 1.61 (3H, s, 29-H_3), 1.20~1.77 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.16 (3H, d, J=6.0 Hz, 28-H_3), 1.01 (3H, t, J=7.3 Hz, 32-H_3), 0.83~0.98 (1H, m, 18-H)
6b	8.45 (1H, br s, 5=NOH), 5.70~5.93 (3H, m, 3-H, 9-H, 10-H), 5.33~5.46 (2H, m, 11-H, 19-H), 4.95 (1H, t, J=7.7 Hz, 15-H), 4.77 (1H, dd, J=2.2, 14.3 Hz, 27-H), 4.69 (1H, dd, J=2.2, 14.3 Hz, 27-H), 4.67 (1H, s, 6-H), 3.96 (1H, s, 7-OH), 3.63 (1H, dd, J=4.0, 11.0 Hz, 30-H), 3.55 (1H, m, 17-H), 3.49 (1H, dd, J=6.2, 11.0 Hz, 30-H), 3.30~3.40 (2H, m, 2-H, 25-H), 2.43 (1H, m, 12-H), 2.12~2.28 (3H, m, 13-H, 16-H_2), 2.01 (1H, dd, J=3.0, 11.5 Hz, 20-H), 1.93 (1H, m, 26-H_3), 1.53 (1H, m, 29-H_3), 1.35~1.90 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.02 (3H, t, J=7.1 Hz, 32-H_3), 1.00 (3H, d, J=7.0 Hz, 28-H_3), 0.81~0.95 (1H, m, 18-H)

Table 5. (Continued)

7b	5.69~5.82 (2H, m, 9-H, 10-H), 5.41 (1H, s, 3-H), 5.28~5.41 (2H, m, 11-H, 19-H), 5.17 (1H, dd, J=9.3, 1.2 Hz, 32-H), 4.95 (1H, m, 15-H), 4.69, 4.68 (2H, ABq, J=15.0 Hz, 27-H_2), 4.29 (1H, t, J=6.0 Hz, 5-H), 3.96 (1H, d, J=6.0 Hz, 6-H), 3.75~3.87 (3H, m, 7-OH, 23-H, 25-H), 3.38~3.75 (4H, m, 17-H, 23-OH, 30-H_2), 3.27 (1H, q, J=2.4 Hz, 2-H), 2.69 (1H, m, 33-H), 2.17~2.65 (5H, m, 5-OH, 12-H, 13-H, 16-H_2), 1.98~2.07 (2H, m, 20-H, 22-H), 1.87 (3H, d, J=1.6 Hz, 26-H_3), 1.67 (3H, d, J=1.2 Hz, 31-CH_3), 1.53 (3H, s, 29-H_3), 1.20~1.91 (5H, m, 13-H, 18-H, 20-H, 22-H, 24-H), 1.08 (3H, d, J=6.9 Hz, 33-CH_3), 1.00 (3H, d, J=6.5 Hz, 28-H_3), 0.82 (3H, d, J=6.9 Hz, 33-CH_3), 0.80~1.00 (1H, m, 18-H)
8b	5.82~5.90 (1H, m, 11-H), 5.65~5.80 (2H, m, 9-H, 10-H), 5.27~5.43 (3H, m, 3-H, 19-H, 1″-H), 4.97 (1H, d, J=8.0 Hz, 15-H), 4.77 (1H, m, 1′-H), 4.71 (1H, dd, J=2.4, 16.2 Hz, 27-H), 4.64 (1H, dd, J=2.4, 16.2 Hz, 27-H), 4.28 (1H, d, J=5.7 Hz, 5-H), 4.09 (1H, br s, 7-OH), 3.95 (1H, d, J=6.4 Hz, 6-H), 3.94 (1H, br s, 13-H), 3.53~3.92 (7H, m, 17-H, 30-H_2, 3′-H, 5′-H, 3″-H, 5″-H), 3.35~3.40 (1H, m, 25-H), 3.41 (s), 3.42 (s) (6H, 3′-OCH_3, 3″-OCH_3), 3.28 (1H, d, J=2.0 Hz, 2-H), 3.23 (1H, t, J=9.2 Hz, 4′-H), 3.15 (1H, t, J=9.2 Hz, 4″-H), 2.52 (1H, m, 12-H), 2.16~2.35 (2H, m, 16-H_2), 1.99 (1H, dd, J=4.6, 12.0 Hz, 20-H), 1.86 (3H, s, 26-H_3), 1.50 (3H, s, 29-H_3), 1.26 (3H, d, J=6.0 Hz) and 1.24 (3H, d, J=5.6 Hz) (5′-CH_3 and 5″-CH_3), 1.10~1.80 (14H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H, 32-H_2, 2′-H_2, 2″-H_2), 1.15 (3H, d, J=6.8 Hz, 28-H_3), 0.91 (3H, t, J=7.2 Hz, 32-CH_3), 0.89 (3H, d, J=6.4 Hz, 31-CH_3), 0.78~0.90 (1H, m, 18-H)

δ ppm downfield from internal TMS.

576.3098, Found: 576.3104.

30-Hydroxy-5-ketomilbemycin A_4 5-Oxime (**6b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2958 (s), 2925 (s), 2873 (s), 1714 (s); MS m/z 571 (M^+, $C_{32}H_{45}NO_8$), 553, 537, 211, 193, 183, 165, 151; HREI-MS Calcd for $C_{32}H_{45}NO_8$: 571.3145, Found: 571.3147.

30-Hydroxy LL-F28249α (**7b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2964 (s), 2925 (s), 2875 (s), 1717 (s); MS m/z 628 (M^+, $C_{36}H_{52}O_9$), 610, 592, 482, 314, 248, 151; HREI-MS Calcd for $C_{36}H_{52}O_9$: 628.3611, Found: 628.3610.

22,23-Dihydro-30-hydroxyavermectin B_{1a} (**8b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2967 (s), 2932 (s), 2877 (s), 1716 (s); MS m/z 728 ($M^+ - C_7H_{13}O_4 + 1$, $C_{41}H_{61}O_{11} + 1$), 666, 602, 584, 568, 323, 305, 145; FAB-MS 913 ($M^+ + 23$, $C_{48}H_{74}O_{15} + Na$).

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