Microbial Conversion of Milbemycins: Oxidation of Milbemycin A_4 and Related Compounds at the C-25 Ethyl Group by *Circinella umbellata* and *Absidia cylindrospora*

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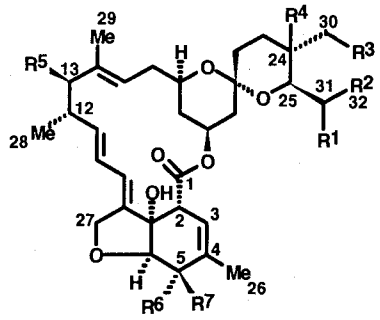
Microbial oxidation of milbemycin A_4 at the C-25 ethyl group was performed. Milbemycin A_4 was converted to 31- and 32-hydroxy derivatives by *Circinella umbellata* SANK 44272 along with 24- and 30-hydroxy derivatives. Related compounds, 5-ketomilbemycin A_4 5-oxime and 13β-fluoromilbemycin A_4 were similarly converted to the hydroxylated compounds by this microorganism. *Absidia cylindrospora* SANK 31472 converted milbemycin A_4 to the corresponding 32-oic acid, 24-hydroxy derivative and a few oxygenated compounds including at the C-25 ethyl group.

Milbemycins are a family of sixteen-membered macrolides produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus*,[1~3] and exhibit potent anti-parasitic and pesticidal activities.

Since the discovery of this unique macrolide, intensive efforts have been directed towards chemical modification mainly at the C-5 and C-13 position of milbemycins in order to improve this biological quality. Some of the 13-alkoxy derivatives of milbemycins, which were synthesized from natural milbemycins *via* 13-hydroxy or 13-iodo derivatives, were found to exhibit excellent anthelmintic properties.[4,5]

As part of our studies on the derivatization of milbemycins, a few years ago we began investigating the microbial conversion of milbemycins, and have consequently demonstrated that the C-13 position and all the methyl groups of milbemycin A_4 were hydroxylated and the 14,15-double bond was epoxidated by zygomycetes or actinomycetes.[6~11] However, we have not found hydroxylated compounds at the C-25 ethyl group of milbemycin A_4 (**1a**) which would be important intermediates to synthesize new milbemycin derivatives. Merck's group recently reported hydroxylation of the *sec*-butyl group at the C-25 position of 22,23-dihydroavermectin B1a aglycone and avermectin by two actinomycetes, *Saccharopolyspora erythrea* ATCC 11635 and *Amycolata autotrophica* ATCC 35203.[12,13] We independently concentrated our screening studies on finding compounds which were modified at the C-25 ethyl group of milbemycin A_4 (**1a**).

Fig. 1. The structures of milbemycin A_4, related compounds and conversion products.



Compound	R^1	R^2	R^3	R^4	R^5	R^6	R^7
1a	H	CH_3	H	H	H	H	OH
1b	H	CH_3	H	H	OH	H	OH
1c	H	CH_3	OH	H	H	H	OH
1d	H	CH_3	H	OH	H	H	OH
1e	OH	CH_3	H	H	H	H	OH
1f	H	CH_2OH	H	H	H	H	OH
1g	H	COOH	H	H	H	H	OH
1h	OH	CH_3	OH	H	H	H	OH
1i	H	CH_2OH	H	H	OH	H	OH
2a	H	CH_3	H	H	H	NOH	
2b	H	CH_3	H	H	OH	NOH	
2c	H	CH_3	OH	H	H	NOH	
2d	H	CH_3	H	OH	H	NOH	
2e	OH	CH_3	H	H	H	NOH	
2f	H	CH_2OH	H	H	H	NOH	
3a	H	CH_3	H	H	F	H	OH
3c	H	CH_3	OH	H	F	H	OH
3d	H	CH_3	H	OH	F	H	OH
3f	H	CH_2OH	H	H	F	H	OH
4a	H	CH_3	H	H	H	O	
5a	H	CH_3	H	H	OH	O	
5f	H	CH_2OH	H	H	OH	O	
6c	H	CH_3	OH	H	OH	OH	H
6d	H	CH_3	H	OH	OH	OH	H
6e	OH	CH_3	H	H	OH	OH	H
6f	H	CH_2OH	H	H	OH	OH	H

The present paper deals with oxidation of the C-25 ethyl group of milbemycin A_4 and related compounds by *Circinella umbellata* SANK 44272 and *Absidia cylindrospota* SANK 31472. The structures of milbemycins and conversion products are shown in Fig. 1.

Materials and Methods

Materials

Milbemycin A_4 (**1a**) was isolated as described previously.[1] 5-Ketomilbemycin A_4 5-oxime (**2a**),[14] 13β-fluoromilbemycin A_4 (**3a**),[15] 5-ketomilbemycin A_4 (**4a**),[14] and 13β-hydroxy-5-ketomilbemycin A_4 (**5a**)[16] were prepared from milbemycin A_4 according to the literature procedures.

Culture Maintenance

Stock cultures of *C. umbellata* SANK 44272 and *A. cylindrospora* SANK 31472 were maintained on potato dextrose agar (Difco) and stored at 4°C. These stock cultures were refreshed every half year.

Culture Medium

MY medium consisted of glucose 1.0%, Polypepton (Nihonseiyaku) 0.5%, yeast extract (Difco) 0.3%, and malt extract (Difco) 0.3%, pH 6.3~6.5.

Microbial Conversion of Milbemycins

The spores or mycelia from the slants were used to inoculate into either 20 ml medium/100-ml Erlenmeyer flasks or 100 ml medium/500-ml Erlenmeyer flasks. The flasks were incubated at 200~220 rpm on a rotary shaker for a period of 2 days at 26°C. Then the substrate (5% (w/v) in 1,4-dioxane) was added to a final concentration of 500 μg/ml of milbemycin A_4 (**1a**) or 250 μg/ml of 5-ketomilbemycin A_4 5-oxime (**2a**), 13β-fluoromilbemycin A_4 (**3a**), 5-ketomilbemycin A_4 (**4a**) and 13β-hydroxy-5-ketomilbemycin A_4 (**5a**), and cultivation was continued for 7 additional days.

HPLC Analysis

Analytical HPLC was performed using a Nova pak C_{18} (Waters, 8 × 100 mm) 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.

Preparative HPLC

Preparative HPLC was performed on a Sensyu pak ODS-H-5251 (Sensyu kagaku, 20 × 250 mm, flow rate of 13~15 ml/minute) or Sensyu pak ODS-H-4251 (10 × 250 mm, flow rate of 4 ml/minute) with a mixture of acetonitrile - water (40 : 60~70 : 30). UV-detection was performed at 243 nm.

Isolation of the Conversion Products

The culture broth was filtered and the filtrate was extracted with EtOAc. The mycelium was extracted with 80% MeOH. The MeOH extract was then evaporated and the resulting aqueous solution was extracted with EtOAc. The combined EtOAc extracts were evaporated and chromatographed on silica gel (Wakogel C-100, 20~90% EtOAc in *n*-hexane as eluent). Fractions were collected and analyzed by HPLC. Similar fractions were combined and subsequently chromatographed on reverse phase silica gel (Fuji-gel hanbai ODSQ3, 0~33% MeOH in H_2O) or applied to preparative HPLC.

Separation of 24-Hydroxymilbemycin A_4 and 32-Hydroxymilbemycin A_4

To a solution of a mixture (92.2 mg), obtained from *C. umbellata* incubation broth with milbemycin A_4 (**1a**) after purification of reverse phase silica gel chromatography in dry DMF (2 ml) was added *t*-butyldimethylsilylchloride (30 mg) and imidazole (12 mg). After stirring at room temperature for 1 hour, the reaction mixture was poured into water, and extracted with ethyl acetate. The extract was washed with water and brine, dried ($MgSO_4$), and evaporated under reduced pressure. The residue was purified by preparative TLC (Merck Art. No. 5744: *n*-hexane - EtOAc (5 : 1)) to give 32-hydroxy-32,5-di-*O*-*t*-butyldimethylsilylmilbemycin A_4 and 24-hydroxy-5-*O*-*t*-butyldimethylsilylmilbemycin A_4. Each compound was desilylyzed (*p*-toluenesulphonic acid and methanol).

Spectral Analysis

IR spectra were recorded wih a Nicolet 5S × C FT-IR spectrophotometer. NMR spectra were measured at 270 MHz on a JEOL JNM GX 270 spectrometer. Mass spectra were measured on a JEOL JMS-D 300 spectrometer.

Results and Discussion

Microbial Conversion of Milbemycin A_4 by *C. umbellata* SANK 44272

A few strains of zygomycetes were found to convert milbemycin A_4 (**1a**) to 13β-hydroxymilbemycin A_4 (**1b**), 30-hydroxymilbemycin A_4 (**1c**) and three other unknown compounds (**1d**, **1e**, and **1f**) on the HPLC. *Circinella umbellata* SANK 44272 was chosen to be used in the following preparative-scale study for characterizing new converted products.

Milbemycin A_4 (500 mg) was added to the growing culture (ten 500-ml flasks) of *Circinella umbellata* SANK 44272 and the incubation was continued. The culture broth was extracted and chromatographed on normal and reverse phase silica gel chromatographies as described in Materials and Methods. 30-Hydroxymilbemy-

cin A_4 (**1c**, 73.0 mg), **1e** (3.7 mg), **1f** (10.7 mg) and a mixture of **1d** and **1f** (92.2 mg) were obtained. The mixture was derivatized to *t*-butyldimethylsilylmilbemycins, followed by purification and desilylation to give **1d** (30.0 mg) and **1f** (38.2 mg) as shown in Materials and Methods. From the analyses of MS and ^{1}H NMR spectra, **1d**, **1e**, and **1f** were determined as 24-, 31- and 32-hydroxymilbemycin A_4, respectively.

Other strains of zygomycetes which converted milbemycin A_4 to **1b**, **1c**, **1d**, **1e** and **1f** are shown in Table 1, along with the conversion efficiency determined by HPLC analysis.

Application of *C. umbellata* SANK 44272 or Related Compounds

5-Ketomilbemycin A_4 5-oxime (**2a**), 13β-fluoromilbemycin A_4 (**3a**), 5-ketomilbemycin A_4 (**4a**) and 13β-hydroxy-5-ketomilbemycin A_4 (**5a**) were examined as substrates of *C. umbellata* SANK 44272.

5-Ketomilbemycin A_4 5-oxime (**2a**, 500 mg) in twenty 500-ml flasks was converted to **2c**, **2d**, **2e** and **2f** by *C. umbellata* SANK 44272, which were, after being purified by silica gel column chromatography and preparative HPLC, determined as the corresponding 30-, 24-, 31- and 32-hydroxy compounds (**2c**, 7.5 mg; **2d**, 46.2 mg; **2e**, 1.1 mg; **2f**, 6.9 mg) respectively based on their MS and ^{1}H NMR spectra. The 30-hydroxy compound (**2c**) was previously isolated from the conversion broth of **2a** by *Amycolata autotrophica* ATCC 35204[6]. The other three products were newly obtained in this study.

13β-Fluoromilbemycin A_4 (**3a**, 45 mg) in nine 100-ml Erlenmeyer flasks was similarly subjected to conversion by *C. umbellata* SANK 44272 to give 13β-fluoro-30-hydroxymilbemycin A_4 (**3c**, 4.8 mg), and two unknown compounds, **3d** and **3f**. After purification by preparative HPLC, **3d** (4.9 mg) and **3f** (4.6 mg) were revealed as 13β-fluoro-24-hydroxymilbemycin A_4 and 13β-fluoro-32-hydroxymilbemycin A_4.

No conversion product was detected in the broth containing 5-ketomilbemycin A_4 (**4a**).

Finally, conversion of 13β-hydroxy-5-ketomilbemycin A_4 (**5a**) to the corresponding 31- or 32-hydroxylated derivatives by using *C. umbellata* SANK 44272 was investigated. The conversion mixtures showed multi-components and we isolated six new conversion products. However, there were insufficient spectral data to assign definitive structures to these products.

Table 1. Strains converting milbemycin A_4 (**1a**) to 31-hydroxymilbemycin A_4 (**1e**), 32-hydroxymilbemycin A_4 (**1f**), 24-hydroxymilbemycin A_4 (**1d**), 13β-hydroxymilbemycin A_4 (**1b**) and 30-hydroxymilbemycin A_4 (**1c**).

Microorganism	Conversion efficiency[a]				
	1b	**1c**	**1d**	**1e**	**1f**[b]
Circinella umbellata SANK 44272	tr	+1	+3	+2	+2
Cunninghamella echinulata NRRL 3654	+1	+2	+2	+2	+1
Cunninghamella echinulata IFO 4447	tr	+3	+2	+1	+2
Absidia cylindrospora SANK 31472	tr	tr	+1	+1	+1

[a] +1: 0.5~5%, +2: 5~15%, +3: 15%~, tr: trace (HPLC analysis),

[b] **1b**: 13β-hydroxymilbemycin A_4, **1c**: 30-hydroxymilbemycin A_4, **1e**: 31-hydroxymilbemycin A_4, **1f**: 32-hydroxymilbemycin A_4, **1d**: 24-hydroxymilbemycin A_4.

Microbial Conversion of Milbemycin A_4 (**1a**) by *A. cylindrospora* SANK 31472

The HPLC pattern of the *A. cylindrospora* SANK 31472 incubation broth with milbemycin A_4 was different from those of other microorganism conversion broths. In addition to small amounts of 30-, 13β-, 31- and 32-hydroxymilbemycin A_4, 24-hydroxymilbemycin A_4 and three unknown conversion products (**1g**, **1h** and **1i**) were detected by HPLC. Bioconversion of milbemycin A_4 (**1a**) was carried out in nine 500-ml flasks (milbemycin A_4, 450 mg). The conversion products were purified by normal and reverse phase silica gel chromatography, and the structures were characterized. **1g** (15.3 mg) was the major product and was identified as milbemycin A_4 32-oic acid, and **1h** (8.5 mg) and **1i** (5.6 mg) were determined as 30,31-dihydroxymilbemycin A_4 and 13β,32-dihydroxymilbemycin A_4, respectively, in addition to 24-hydroxymilbemycin A_4 (**1d**, 6.3 mg) from the analyses of MS and ^{1}H NMR spectra.

Application of *A. cylindrospora* SANK 31472 to the Conversion of Related Compounds

Microbial conversion of 13β-hydroxy-5-ketomilbemycin A_4 (**5a**, 105 mg) was performed in twenty one 100-ml Erlenmeyer flasks by *A. cylindrospora* SANK 31472. Five conversion products were detected by analytical HPLC. They were isolated from the extracts of culture broth by preparative HPLC and determined as 13β,32-dihydroxy-5-ketomilbemycin A_4 (**5f**, 2.7 mg), 5-*epi*-13β,30-dihydroxymilbemycin A_4 (**6c**, 1.7 mg), 5-*epi*-13β,24-dihydroxymilbemycin A_4 (**6d**, 1.4 mg), 5-*epi*-13β,31-dihydroxymilbemycin A_4 (**6e**, 4.1 mg) and 5-*epi*-13β,32-dihydroxymilbemycin A_4 (**6f**, 8.5 mg) from the analyses of MS and ^{1}H NMR spectra.

The retention times are listed in Table 2.

In conclusion, the microbial conversion of milbemycin A_4 by using *C. umbellata* SANK 44272 has enabled us to obtain 32- and 31-hydroxymilbemycin A_4. These compounds will enable us in turn to synthesize new

Table 2. HPLC retention times of milbemycins and conversion products.

	Compound[a]	HPLC Rt's[b] (minutes)			Compound[a]	HPLC Rt's[b] (minutes)	
		System 1	System 2			System 1	System 2
1a	Milbemycin A_4	16.07	–	**2e**	31-Hydroxy-5-ketomilbemycin A_4 5-oxime	5.63	26.57
1b	13β-Hydroxymilbemycin A_4	3.50	10.86	**2f**	32-Hydroxy-5-ketomilbemycin A_4 5-oxime	5.27	24.78
1c	30-Hydroxymilbemycin A_4	3.08	8.91	**3a**	13β-Fluoromilbemycin A_4	8.84	–
1d	24-Hydroxymilbemycin A_4	4.76	16.52	**3c**	13β-Fluoro-30-hydroxymilbemycin A_4	2.55	6.30
1e	31-Hydroxymilbemycin A_4	5.17	19.19	**3d**	13β-Fluoro-24-hydroxymilbemycin A_4	3.23	9.71
1f	32-Hydroxymilbemycin A_4	4.85	17.88	**3f**	13β-Fluoro-32-hydroxymilbemycin A_4	3.55	11.75
1g	Milbemycin A_4 32-oic acid	4.20	16.76	**4a**	5-Ketomilbemycin A_4	25.61	–
1h	30,31-Dihydroxymilbemycin A_4	3.33	9.18	**5a**	13β-Hydroxy-5-ketomilbemycin A_4	22.44	4.88
1i	13β,32-Dihydroxymilbemycin A_4	2.22	4.27	**5f**	13β,32-Dihydroxy-5-ketomilbemycin A_4	7.15	2.72
2a	5-Ketomilbemycin A_4 5-oxime	18.91	–	**6c**	5-*epi*-13β,30-Dihydroxymilbemycin A_4	2.03	3.60
2b	13β-Hydroxy-5-ketomilbemycin A_4 5-oxime	3.84	14.75	**6d**	5-*epi*-13β,24-Dihydroxymilbemycin A_4	2.13	3.97
2c	30-Hydroxy-5-ketomilbemycin A_4 5-oxime	3.22	10.65	**6e**	5-*epi*-13β,31-Dihydroxymilbemycin A_4	2.28	4.54
2d	24-Hydroxy-5-ketomilbemycin A_4 5-oxime	5.14	22.17	**6f**	5-*epi*-13β,32-Dihydroxymilbemycin A_4	2.44	5.17

[a] **a**: Substrate, **b~i**: porducts, [b] Rt's relative to **1b**.

milbemycin derivatives with long side chains at C-25.

Milbemycins A_4, 5-ketomilbemycin A_4 5-oxime and 13β-fluoromilbemycin A_4 were similarly converted to the corresponding 32- or 31-hydroxylated products by *C. umbellata*.

A. cylindrospora was also determined to be able to oxidize the C-31 and C-32 positions of milbemycin A_4 and related compounds.

Finally, compared with milbemycins, the oxygenated compounds newly obtained in this study did not improve their biological properties.

Physico-chemical Properties

24-Hydroxymilbemycin A_4 (**1d**): MS *m/z*: 558 (M, $C_{32}H_{46}O_8$), 522, 430, 412, 314, 280, 261 211, 183, 151; HREI-MS found: 558.3203, calcd for $C_{32}H_{46}O_8$: 558.3193; IR (KBr): 3650~3200, 2967, 2926, 2876, 1715 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.81~0.94 (1H, m, 18-H), 1.00 (3H, d, J=6.6 Hz, 28-H_3), 1.02 (3H, t, J=6.6 Hz, 32-H_3), 1.12 (3H, s, 30-H_3), 1.22~1.95 (9H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 31-H_2), 1.53 (3H, s, 29-H_3), 1.88 (3H, s, 26-H_3), 1.99~2.14 (1H, m, 20-H), 2.14~2.31 (3H, m, 13-H, 16-H_2), 2.31~2.49 (2H, m, 5-OH, 12-H), 3.27 (1H, m, 2-H), 3.33 (1H, dd, J=2.9, 9.3 Hz, 25-H), 3.58 (1H, m, 17-H), 3.95 (1H, d, J=5.9 Hz, 6-H), 3.99 (1H, br s, 7-OH), 4.29 (1H, br s, 5-H), 4.60~4.78 (2H, m, 27-H_2), 4.95 (1H, t, J=5.1 Hz, 15-H), 5.27~5.44 (3H, m, 3-H, 11-H, 19-H), 5.68~5.91 (2H, m, 9-H, 10-H).

31-Hydroxymilbemycin A_4 (**1e**): MS *m/z*: 558 (M, $C_{32}H_{46}O_8$), 522, 430, 412, 280, 211, 183, 151; HREI-MS found: 555.3189, calcd for $C_{32}H_{46}O_8$: 558.3193; IR (KBr): 3650~3100, 2967, 2927, 2868, 1717 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.82~0.96 (1H, m, 18-H), 0.89 (3H, d, J=6.1 Hz, 30-H_3), 1.01 (3H, d, J=6.4 Hz, 28-H_3), 1.31~1.91 (8H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H), 1.32 (3H, d, J=6.4 Hz, 32-H_3), 1.53 (3H, s, 29-H_3), 1.87 (3H, s, 26-H_3), 2.03~2.09 (1H, m, 20-H), 2.17~2.47 (5H, m, 5-OH, 12-H, 13-H, 16-H_2), 3.04 (1H, d, J=10.0 Hz, 25-H), 3.27 (1H, q, J=2.4 Hz, 2-H), 3.49~3.60 (1H, m, 17-H), 3.90~4.00 (3H, m, 6-H, 7-OH, 31-H), 4.30 (1H, t, J=6.5 Hz, 5-H), 4.62~4.74 (2H, m, 27-H_2), 4.95 (1H, dd, J=6.9, 8.1 Hz, 15-H), 5.26~5.44 (3H, m, 3-H, 11-H, 19-H), 5.70~5.83 (2H, m, 9-H, 10-H).

32-Hydroxymilbemycin A_4 (**1f**): MS *m/z*: 558 (M, $C_{32}H_{46}O_8$), 522, 430, 412, 314, 280, 211, 183, 151; HREI-MS found: 558.3205, calcd for $C_{32}H_{46}O_8$: 558.3193; IR (KBr): 3650~3100, 2953, 2925, 2875, 1715 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.80~0.96 (1H, m, 18-H), 0.85 (3H, d, J=6.5 Hz, 30-H_3), 1.01 (3H, d, J=6.9 Hz, 28-H_3), 1.33~2.05 (11H, m, 13-H, 18-H, 20-H_2, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.87 (3H, s, 26-H_3), 1.53 (3H, s, 29-H_3), 2.17~2.51 (5H, m, 5-OH, 12-H, 13-H, 16-H_2), 3.26 (1H, q, J=2.4 Hz, 2-H), 3.44 (1H, dt, J_d=2.8, J_t=9.3 Hz, 25-H), 3.53~3.64 (1H, m, 17-H), 3.83 (2H, t, J=4.8 Hz, 32-H_2), 3.96 (1H, d, J=6.1 Hz, 6-H), 3.97 (1H, br s, 7-OH), 4.29 (1H, br s, 5-H), 4.62~4.74 (2H, m, 27-H_2), 4.96 (1H, t, J=7.7 Hz, 15-H), 5.22~5.44 (3H, m, 3-H, 11-H, 19-H), 5.69~5.84 (2H, m, 9-H, 10-H).

24-Hydroxy-5-ketomilbemycin A_4 5-oxime (**2d**): MS *m/z*: 571 (M, $C_{32}H_{45}NO_8$), 553, 292, 211, 183, 151; HREI-MS: found: 571.3143, calcd for $C_{32}H_{45}NO_8$: 571.3145; IR (KBr): 3650~3100, 2967, 2927 2876, 1716 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.91 (1H, q,

J=12.1 Hz, 18-H), 1.01 (3H, d, J=6.5 Hz, 28-H_3), 1.05 (3H, t, J=7.3 Hz, 32-H_3), 1.13 (3H, s, 30-H_3), 1.41 (1H, t, J=11.7 Hz, 20-H), 1.49~1.94 (8H, m, 13-H, 18-H, 22-H_2, 23-H_2, 31-H_2), 1.54 (3H, s, 29-H_3), 1.94 (3H, q, J=0.8 Hz, 26-H_3), 2.05~2.12 (1H, m, 20-H), 2.17~2.27 (3H, m, 13-H, 16-H_2), 2.37~2.50 (1H, m, 12-H), 3.35 (1H, dd, J=3.2, 10.1 Hz, 25-H), 3.37~3.41 (1H, m, 2-H), 3.55~3.65 (1H, m, 17-H), 4.65~4.81 (2H, m, 27-H_2), 4.67 (1H, s, 6-H), 4.92~4.98 (1H, m, 15-H), 5.34~5.45 (2H, m, 11-H, 19-H), 5.72~5.81 (2H, m, 3-H, 10-H), 5.87 (1H, dt, J_d=11.3, J_t=2.0 Hz, 9-H).

31-Hydroxy-5-ketomilbemycin A_4 5-oxime (**2e**): MS m/z: 571 (M, $C_{32}H_{45}NO_8$), 553, 292, 211, 183, 151; HREI-MS found: 571.3160, calcd for $C_{32}H_{45}NO_8$: 571.3145; IR (KBr): 3650~3100, 2954, 2925, 2855, 1716 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.84~0.97 (1H, m, 18-H), 0.89 (3H, d, J=6.5 Hz, 30-H_3), 1.01 (3H, d, J=6.4 Hz, 28-H_3), 1.31~2.10 (9H, m, 13-H, 18-H, 20-H_2, 22-H_2, 23-H_2, 24-H), 1.33 (3H, d, J=6.5 Hz, 32-H_3), 1.54 (3H, s, 29-H_3), 1.95 (3H, d, J=1.2 Hz, 26-H_3), 2.18~2.27 (3H, m, 13-H, 16-H_2), 2.38~2.52 (1H, m, 12-H), 3.05 (1H, d, J=10.1 Hz, 25-H), 3.37~3.41 (1H, m, 2-H), 3.50~3.61 (1H, m, 17-H), 3.90~3.97 (1H, m, 31-H), 4.65~4.81 (2H, m, 27-H_2), 4.67 (1H, s, 6-H), 4.92~4.97 (1H, m, 15-H), 5.33~5.46 (2H, m, 11-H, 19-H), 5.72~5.91 (3H, m, 3-H, 9-H, 10-H).

32-Hydroxy-5-ketomilbemycin A_4 5-oxime (**2f**): MS m/z: 571 (M, $C_{32}H_{45}NO_8$), 553, 537, 292, 211, 183, 151; HREI-MS: found: 571.3134, calcd for $C_{32}H_{45}NO_8$: 571.3145; IR (KBr): 3650~3100, 2955, 2926, 2875, 1715 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.84~0.98 (1H, m, 18-H), 0.85 (3H, d, J=6.2 Hz, 30-H_3), 1.02 (3H, d, J=6.6 Hz, 28-H_3), 1.35~2.05 (11H, m, 13-H, 18-H, 20-H_2, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.54 (3H, s, 29-H_3), 1.94 (3H, s, 26-H_3), 2.15~2.52 (4H, m, 12-H, 13-H, 16-H_2), 3.35~3.39 (1H, m, 2-H), 3.44 (1H, dt, J_d=2.4, J_t=9.5 Hz, 25-H), 3.56~3.63 (1H, m, 17-H), 3.83~3.85 (3H, m, 7-OH, 32-H_2), 4.66 (1H, s, 6-H), 4.66~4.80 (2H, m, 27-H_2), 4.93~5.00 (1H, m, 15-H), 5.28~5.46 (2H, m, 11-H, 19-H), 5.71~5.90 (3H, m, 3-H, 9-H, 10-H), 7.68 (1H, s, 5=NOH).

13β-Fluoro-24-hydroxymilbemycin A_4 (**3d**): MS m/z: 576 (M, $C_{32}H_{45}FO_8$), 558, 448, 428, 332, 279, 266, 211, 183, 151; HREI-MS found: 576.3097, calcd for $C_{32}H_{45}FO_8$: 576.3098; IR (KBr): 3650~3100, 2971, 2932, 2875, 1716 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.92 (1H, q, J=12.1 Hz, 18-H), 1.04 (3H, t, J=7.3 Hz, 32-H_3), 1.13 (3H, s, 30-H_3), 1.16 (3H, d, J=6.4 Hz, 28-H_3), 1.39 (1H, t, J=11.7 Hz, 20-H), 1.50~1.92 (7H, m, 18-H, 22-H_2, 23-H_2, 31-H_2), 1.62 (3H, s, 29-H_3), 1.88 (3H, s, 26-H_3), 2.07~2.11 (1H, m, 20-H), 2.30~2.36 (2H, m, 16-H_2), 2.57~2.67 (1H, m, 12-H), 3.26~3.28 (1H, m, 2-H), 3.33 (1H, dd, J=2.5, 10.3 Hz, 25-H), 3.56~3.64 (1H, m, 17-H), 3.94 (1H, br s, 7-OH), 3.97 (1H, d, J=6.4 Hz, 6-H), 4.29 (1H, d, J=6.4 Hz, 5-H), 4.40 (1H, dd, J=10.3, 47.9 Hz, 13-H), 4.68 (1H, dd, J=2.0, 14.7 Hz, 27-H), 4.70 (1H, dd, J=2.0, 14.7 Hz, 27-H), 5.26~5.38 (3H, m, 11-H, 15-H, 19-H), 5.40 (1H, s, 3-H), 5.76~5.88 (2H, m, 9-H, 10-H).

13β-Fluoro-32-hydroxymilbemycin A_4 (**3f**): MS m/z: 576 (M, $C_{32}H_{45}FO_8$), 558, 448, 428, 332, 279, 266, 211, 183; HREI-MS found: 576.3083, calcd for $C_{32}H_{45}FO_8$: 576.3098; IR (KBr): 3650~3100, 2966, 2928, 2873, 1719 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.86 (3H, d, J=6.4 Hz, 30-H_3), 0.84~0.97 (1H, m, 18-H), 1.16 (3H, d, J=6.8 Hz, 28-H_3), 1.25~1.80 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.61 (3H, s, 29-H_3), 1.87 (3H, s, 26-H_3), 1.99~2.10 (1H, m, 20-H), 2.26~2.37 (2H, m, 16-H_2), 2.55~2.67 (1H, m, 12-H), 3.25~3.28 (1H, m, 2-H), 3.44 (1H, dt, J_d=2.9, J_t=9.31 Hz, 25-H), 3.50~3.70 (1H, m, 17-H), 3.81~3.85 (2H, m, 32-H_2), 3.91 (1H, br s, 7-OH), 3.96 (1H, d, J=6.4 Hz, 6-H), 4.25~4.32 (1H, br s, 5-H), 4.40 (1H, dd, J=10.3, 47.9 Hz, 13-H), 4.62~4.76 (2H, m, 27-H_2), 5.20~5.33 (3H, m, 11-H, 15-H, 19-H), 5.40 (1H, s, 3-H), 5.76~5.90 (2H, m, 9-H, 10-H).

Milbemycin A_4 32-oic acid (**1g**): MS m/z: 572 (M, $C_{32}H_{46}O_9$), 444, 426, 294, 275 225, 197, 151; HREI-MS found 572.2991, calcd for $C_{32}H_{46}O_9$: 572.3134; IR (KBr): 3650~3100, 2958, 2927, 2866, 1723 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.75~0.92 (1H, m, 18-H), 0.86 (3H, d, J=6.5 Hz, 30-H_3), 0.91 (3H, d, J=6.5 Hz, 28-H_3), 1.17~1.70 (6H, m, 20-H, 22-H_2, 23-H_2, 24-H), 1.50 (3H, s, 29-H_3), 1.75~1.95 (2H, m, 13-H, 18-H), 1.83 (3H, s, 26-H_3), 2.10~2.42 (5H, m, 12-H, 13-H, 16-H_2, 20-H), 2.37 (1H, dd, J=10.9, 14.9 Hz, 31-H), 2.63 (1H, dd, J=3.2, 14.9 Hz, 31-H), 3.24~3.27 (1H, m, 2-H), 3.65 (1H, dt, J_d=3.2, J_t=10.9 Hz, 25-H), 3.79~3.88 (1H, m, 17-H), 3.97 (1H, d, J=5.6 Hz, 6-H), 4.29~4.35 (1H, m, 5-H), 4.61 (1H, dd, J=2.0, 14.1 Hz, 27-H), 4.69 (1H, dd, J=2.0, 14.1 Hz, 27-H), 4.93~5.02 (2H, m, 15-H, 19-H), 5.38 (1H, d, J=1.2 Hz, 3-H), 5.49 (1H, dd, J=9.3, 14.5 Hz, 11-H), 5.63 (1H, dd, J=10.9, 14.5 Hz, 10-H), 5.90 (1H, d, J=10.9 Hz, 9-H).

30,31-Dihydroxymilbemycin A_4 (**1h**): MS m/z: 574 (M, $C_{32}H_{46}O_9$), 556, 446, 314, 296, 277 248, 227, 199, 151; HREI-MS found: 574.3120, calcd $C_{32}H_{46}O_9$: 574.3134; IR (KBr): 3650~3100, 2967, 2926, 2871, 1719 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.80~1.00 (1H, m, 18-H), 1.01 (3H, d, J=6.4 Hz, 28-H_3), 1.20~

2.10 (9H, m, 13-H, 18-H, 20-H_2, 22-H_2, 23-H_2, 24-H), 1.37 (3H, d, J=6.4 Hz, 32-H_3), 1.54 (3H, s, 29-H_3), 1.88 (3H, d, J=1.6 Hz, 26-H_3), 2.15~2.50 (5H, m, 5-OH, 12-H, 13-H, 16-H_2), 3.21 (1H, d, J=0.8 Hz, 25-H), 3.27~3.30 (1H, m, 2-H), 3.44~3.68 (3H, m, 17-H, 30-H_2), 3.96 (1H, d, J=6.4 Hz, 6-H), 4.27~4.34 (2H, m, 5-H, 31-H), 4.63~4.74 (2H, m, 27-H_2), 4.92~4.97 (1H, m, 15-H), 5.30~5.43 (3H, m, 3-H, 11-H, 19-H), 5.70~5.84 (2H, m, 9-H, 10-H).

13β,32-Dihydroxymilbemycin A_4 (**1i**): MS m/z: 574 (M, $C_{32}H_{46}O_9$), 556, 428, 295, 211, 183, 151; HREI-MS found: 574.3158, calcd, $C_{32}H_{46}O_9$: 574.3134; IR (KBr): 3650~3100, 2956, 2927, 2871, 1717 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.85 (3H, d, J=6.5 Hz, 30-H_3), 0.80~1.00 (1H, m, 18-H), 1.14 (3H, d, J=6.9 Hz, 28-H_3), 1.59 (3H, s, 29-H_3), 1.87 (3H, s, 26-H_3), 1.20~2.15 (10H, m, 18-H, 20-H_2, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.87 (3H, s, 26-H_3), 2.20~2.45 (4H, m, 5-OH, 12-H, 16-H_2), 3.25~3.29 (1H, m, 2-H), 3.44 (1H, dt, J_d=2.8, J_t=9.3 Hz, 25-H), 3.55~3.65 (1H, m, 17-H), 3.72 (1H, d, J=9.7 Hz, 13-H), 3.83 (2H, d, J=5.2 Hz, 32-H_2), 3.93 (1H, s, 7-OH), 3.96 (1H, d, J=6.5 Hz, 6-H), 4.27~4.32 (1H, m, 5-H), 4.64~4.73 (2H, m, 27-H_2), 5.21~5.41 (3H, m, 11-H, 15-H, 19-H), 5.40 (1H, s, 3-H), 5.74~5.85 (2H, m, 9-H, 10-H).

13β,32-Dihydroxy-5-ketomilbemycin A_4 (**5f**): MS m/z: 572 (M, $C_{32}H_{44}O_9$), 554, 295, 277, 259, 241, 211, 183; HREI-MS found: 554.2881, calcd for $C_{32}H_{42}O_8$ (M−H_2O): 554.2881; IR (KBr): 3650~3100, 2958, 2928, 2871, 1724 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.86 (3H, d, J=6.4 Hz, 30-H_3), 0.88~1.02 (1H, m, 18-H), 1.15 (3H, d, J=6.8 Hz, 28-H_3), 1.30~2.10 (10H, m, 18-H, 20-H_2, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.59 (3H, s, 29-H_3), 1.89~1.90 (3H, s, 26-H_3), 2.25~2.45 (3H, m, 12-H, 16-H_2), 3.45 (1H, dt, J_d=2.9, J_t=9.3 Hz, 25-H), 3.49 (1H, s, 7-OH), 3.54~3.70 (2H, m, 2-H, 17-H), 3.73 (1H, d, J=9.8 Hz, 13-H), 3.80~3.90 (3H, m, 6-H, 32-H_2), 4.65~4.81 (2H, m, 27-H_2), 5.24~5.46 (3H, m, 11-H, 15-H, 19-H), 5.74~5.88 (2H, m, 9-H, 10-H), 6.54~6.56 (1H, m, 3-H).

5-*epi*-13β,30-Dihydroxymilbemycin A_4 (**6c**): MS m/z: 574 (M, $C_{32}H_{46}O_9$), 556, 538, 446, 428, 295, 277, 211, 183; HREI-MS found: 574.3155, calcd for $C_{32}H_{46}O_9$: 574.3134; IR (KBr): 3650~3100, 2960, 2927, 2873, 1719 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.84~0.98 (1H, m, 18-H), 1.02 (3H, t, J=7.3 Hz, 32-H_3), 1.13 (3H, d, J=6.4 Hz, 28-H_3), 1.34~1.82 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.58 (3H, s, 29-H_3), 1.91 (3H, d, J=1.0 Hz, 26-H_3), 1.99~2.09 (1H, m, 20-H), 2.25~2.43 (3H, m, 12-H, 16-H_2), 3.03~3.07 (1H, m, 2-H), 3.35 (1H, dt, J_d=2.4, J_t=9.3 Hz, 25-H), 3.51~3.66 (3H, m, 17-H, 30-H_2), 3.71 (1H, d, J=9.8 Hz, 13-H), 3.84 (1H, d, J=1.5 Hz, 6-H), 4.02 (1H, s, 5-H), 4.55~4.67 (2H, m, 27-H_2), 5.19~5.47 (4H, m, 3-H, 11-H, 15-H, 19-H), 5.74~5.85 (2H, m, 9-H, 10-H).

5-*epi*-13β,24-Dihydroxymilbemycin A_4 (**6d**): MS m/z: 574 (M, $C_{32}H_{46}O_9$), 556, 538 446, 428, 330, 295, 277, 261, 211, 183; HREI-MS found: 574.3145, calcd for $C_{32}H_{46}O_9$: 574.3134. IR (KBr): 3650~3100, 2968, 2930, 2874, 1721 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.86~0.99 (1H, m, 18-H), 1.04 (3H, t, J=7.3 Hz, 32-H_3), 1.13 (3H, s, 30-H_3), 1.13 (3H, d, J=6.4 Hz, 28-H_3), 1.40 (1H, t, J=11.7 Hz, 20-H), 1.48~1.93 (7H, m, 18-H, 22-H_2, 23-H_2, 31-H_2), 1.59 (3H, s, 29-H_3), 1.91 (3H, s, 26-H_3), 2.05~2.12 (1H, m, 20-H), 2.25~2.41 (3H, m, 12-H, 16-H_2), 3.04~3.07 (1H, m, 2-H), 3.34 (1H, dd, J=3.4, 10.3 Hz, 25-H), 3.54~3.64 (1H, m, 17-H), 3.72 (1H, d, J=9.8 Hz, 13-H), 3.84 (1H, d, J=1.5 Hz, 6-H), 4.04 (1H, s, 5-H), 4.55~4.67 (2H, m, 27-H_2), 5.19~5.24 (1H, m, 15-H), 5.28~5.45 (3H, m, 3-H, 11-H, 19-H), 5.74~5.85 (2H, m, 9-H, 10-H).

5-*epi*-13β,31-Dihydroxymilbemycin A_4 (**6e**): MS m/z: 574 (M, $C_{32}H_{46}O_9$), 556, 428, 295, 277 211 179; HREI-MS found: 574.3166, calcd for $C_{32}H_{46}O_9$: 574.3134; IR (KBr): 3650~3100, 2968, 2929, 2870, 1721 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.86~0.99 (1H, m, 18-H), 0.90 (3H, d, J=6.4 Hz, 30-H_3), 1.13 (3H, d, J=6.4 Hz, 28-H_3), 1.32 (3H, d, J=6.4 Hz, 32-H_3), 1.40 (1H, t, J=11.7 Hz, 20-H), 1.58 (3H, s, 29-H_3), 1.50~1.75 (6H, m, 18-H, 22-H_2, 23-H_2, 24-H), 1.91 (3H, s, 26-H_3), 2.03~2.10 (1H, m, 20-H), 2.22~2.41 (3H, m, 12-H, 16-H_2), 3.02~3.07 (2H, m, 2-H, 25-H), 3.49~3.61 (1H, m, 17-H), 3.72 (1H, d, J=9.8 Hz, 13-H), 3.84 (1H, d, J=1.5 Hz, 6-H), 3.88~3.97 (1H, m, 31-H), 4.04 (1H, s, 5-H), 4.55~4.67 (2H, m, 27-H_2), 5.18~5.24 (1H, m, 15-H), 5.26~5.40 (2H, m, 11-H, 19-H), 5.41~5.43 (1H, m, 3-H), 5.74~5.85 (2H, m, 9-H, 10-H).

5-*epi*-13β,32-Dihydroxymilbemycin A_4 (**6f**): MS m/z: 574 (M, $C_{32}H_{46}O_9$), 538, 428, 348, 295, 277 211, 183; HREI-MS found 574.3136, calcd for $C_{32}H_{46}O_9$: 574.3134; IR (KBr): 3650~3100, 2956, 2927, 2871, 1720 cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 0.84~1.00 (1H, m, 18-H), 0.85 (3H, d, J=6.4 Hz, 30-H_3), 1.14 (3H, d, J=6.4 Hz, 28-H_3), 1.34~1.80 (9H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.58 (3H, s, 29-H_3), 1.90 (3H, s, 26-H_3), 1.98~2.05 (1H, m, 20-H), 2.20~2.44 (4H, m, 12-H, 16-H_2, OH), 3.03~3.07 (1H, m, 2-H), 3.44 (1H, dt, J_d=2.9, J_t=9.3 Hz, 25-H), 3.55~3.67 (1H, m, 17-H), 3.71 (1H, dd, J=2.9, 9.8 Hz, 13-H), 3.75~4.05 (1H, br s, OH), 3.78~3.87 (3H, m, 6-H, 32-H_2), 4.02

(1H, s, 5-H), 4.54~4.66 (2H, m, 27-H_2), 5.20~5.42 (4H, m, 3-H, 11-H, 15-H, 19-H), 5.73~5.84 (2H, m, 9-H, 10-H).

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