MICROBIAL CONVERSION OF MILBEMYCINS: 29-HYDROXYLATION OF MILBEMYCINS BY GENUS *Syncephalastrum*

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Milbemycins are a family of sixteen-membered macrolides produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus*, and demonstrate potent and broad spectrum activity as anthelmintics, acaricides and insecticides.[1~3]

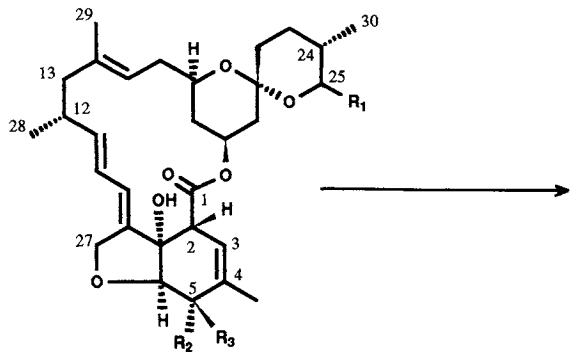
In previous papers, we reported that milbemycins were converted into 30-hydroxy, 13β-hydroxy, and 13β,29-dihydroxy derivatives by using *Amycolata autotrophica* subsp. *amethystina* ATCC 35204, *Cunninghamella echinulata* ATCC 9244, and *Streptomyces cavourensis* SANK 67386, respectively.[4~6] As part of our continuing screening effort for microbial conversion of milbemycins, we observed that *Syncephalastrum racemosum* and *Syncephalastrum nigricans* had conversion abilities to milbemycin A_4 (**1a**).

The present paper describes the isolation and identification of converted products of milbemycin A_4 (**1a**), A_3 (**2a**), D (**3a**) and 5-ketomilbemycin A_4 5-oxime (**4a**) by *S. racemosum* (Fig. 1).

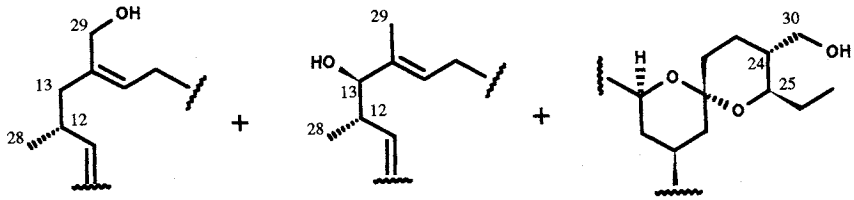
Milbemycins conversion were followed by TLC (Merck Art. No. 5715: EtOAc) and HPLC (column: Waters, Nova pak C_{18} 8 mm × 10 cm; solvent: system 1, acetonitrile - water (75 : 25), with a flow rate of 1.5 ml/minute; system 2, acetonitrile - water (55 : 45), with a flow rate of 1.0 ml/minute; detector: UV 243 nm).

S. racemosum SANK 42672 was cultured in five 500-ml Erlenmeyer flasks containing 100 ml MY medium composed of glucose 1.0%, Polypepton (Daigo Nutritive Chemicals) 0.5%, yeast extract (Difco) 0.3%, and malt extract (Difco) 0.3% (pH 6.3 ~ 6.5) at 26°C on a rotary shaker (200 ~ 220 rpm).

Fig. 1. Microbial conversion of milbemycin A_4 by *Syncephalastrum racemosum*.



1a $R_1 = CH_2CH_3$, $R_2 = H$, $R_3 = OH$
2a $R_1 = CH_3$, $R_2 = H$, $R_3 = OH$
3a $R_1 = CH(CH_3)_2$, $R_2 = H$, $R_3 = OH$
4a $R_1 = CH_2CH_3$, $R_2 = R_3 = NOH$



1b~4b **1c~4c** **1d~4d**

Table 1. Conversion of milbemycins by *Syncephalastrum racemosum* SANK 42672.

Substrate	Concentration (μg/ml)	Conversion time (days)	Product[a] yield (%)		
			b	**c**	**d**
1a	500	7	5.7	1.5	2.8
2a	250	7	1.6	2.1	1.2
3a	500	7	1.0	1.1	1.0
4a	500	7	2.1	3.5	1.9

[a] **b**: 29-Hydroxy derivative; **c**: 13β-hydroxy derivative; **d**: 30-hydroxy derivative.

After the cultivation for 2 days, milbemycin A_4 (5% (w/v) in 1,4-dioxane) was added to a final concentration of 500 μg/ml and cultivation was additionally continued for 7 days. Then the culture broth was filtered and the filtrate was extracted with three 300-ml portions of EtOAc. EtOAc extract was dried over anhydrous sodium sulfate and evaporated. The mycelium was extracted with 80% MeOH. MeOH was then removed from the extract by evaporation and resulting aqueous solution was extracted and evaporated in a similar manner as the filtrate. These extracts were combined and evaporated *in vacuo*. The residue was subjected to silica gel chromatography (20~90% EtOAc in *n*-hexane as an eluent) and three products were isolated and characterized as 29-hydroxymilbemycin A_4 (**1b**), 13β-hydroxymilbemycin A_4 (**1c**) and 30-hydroxymilbemycin A_4 (**1d**). These hydroxylated products were identical to the authentic compounds on the basis of the comparison of NMR and mass spectrum.[4,5,7] According to the patent procedures,[7] which was not easily accessible (multi steps and low yield), authentic 29-hydroxymilbemycin A_4 was obtained with synthetic methods. In contrast to the synthetic procedures, preparation of 29-hydroxymilbemycin A_4 by a single microbial conversion step offers interesting and variable prospects.

Milbemycins A_3 (**2a**), D (**3a**) and 5-ketomilbemycin A_4 5-oxime (**4a**)[8] were converted using similar method as for milbemycin A_4 (**1a**). The corresponding 29-hydroxy derivatives, 30- and 13β-hydroxy derivatives were also obtained. Yields of each conversion are summarized in Table 1. The Rf values on TLC and HPLC retention times of 29-hydroxy derivatives are listed in Table 2. The physicochemical properties of 29-hydroxymilbemycins A_4 (**1b**), A_3 (**2b**), D (**3b**), and 29-hydroxy-5-ketomilbemycin A_4 (**4b**) were as follows:

29-Hydroxymilbemycin A_4 (**1b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2960 (s), 2928 (s), 2873 (s), 1715 (s); ^1H NMR (270 MHz, $CDCl_3$) δ 5.70~5.85 (2H, m, 9-H, 10-H), 5.31~5.48 (3H, m, 3-H, 11-H, 19-H),

Table 2. TLC Rf values and HPLC retention times of milbemycins and conversion products.

Compound[a]	TLC Rf[b] values	HPLC Rt's[b] (minutes)	
		System 1	System 2
1a	0.59	16.07	—
1b	0.38	3.54	11.50
1c	0.46	3.50	10.86
1d	0.44	3.08	8.91
2a	0.59	11.80	—
2b	0.38	3.02	8.27
2c	0.46	3.02	8.04
2d	0.41	2.83	7.12
3a	0.62	24.64	—
3b	0.39	4.74	19.58
3c	0.48	4.59	18.26
3d	0.50	3.64	12.65
4a	0.69	18.91	—
4b	0.60	3.86	15.32
4c	0.60	3.84	14.75
4d	0.55	3.22	10.65

[a] **a**: Substrate; **b**~**d**: products.
[b] Rf values and retention times relative to **1c**.

5.12 (1H, t, J=7.9 Hz, 15-H), 4.70, 4.64 (2H, ABq, J=15.3 Hz, 27-H_2), 4.29 (1H, br s, 5-H), 4.27, 3.94 (2H, ABq, J=12.1 Hz, 29-H_2), 4.11 (1H, s, 7-OH), 3.96 (1H, d, J=6.0 Hz, 6-H), 3.60 (1H, m, 17-H), 3.25 (1H, m, 2-H), 3.07 (1H, dt, J_d=2.6 Hz, J_t=9.4 Hz, 25-H), 2.49~2.63 (2H, m, 13-H, 16-H), 2.17~2.40 (3H, m, 5-OH, 12-H, 16-H), 2.00 (1H, ddd, J=1.7, 4.9, 12.1 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.20~1.85 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.03 (3H, d, J=6.9 Hz, 28-H_3), 0.99 (3H, t, J=6.9 Hz, 32-H_3), 0.80~0.95 (1H, m, 18-H), 0.83 (3H, d, J=6.5 Hz, 30-H_3); MS *m/z* 558 (M^+, $C_{32}H_{46}O_8$), 540, 430, 412, 394, 369, 330, 279, 261, 246, 195, 181, 167, 151; HREI-MS calcd for $C_{32}H_{46}O_8$: 558.3193, found: 558.3193.

29-Hydroxymilbemycin A_3 (**2b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2967 (s), 2928 (s), 2874 (s), 1716 (s); ^1H NMR (270 MHz, $CDCl_3$) δ 5.70~5.82 (2H, m, 9-H, 10-H), 5.31~5.47 (3H, m, 3-H, 11-H, 19-H),

5.15 (1H, t, J=7.8 Hz, 15-H), 4.70, 4.64 (2H, ABq, J=15.3 Hz, 27-H_2), 4.29 (1H, m, 5-H), 4.27, 3.94 (2H, ABq, J=12.3 Hz, 29-H_2), 4.10 (1H, s, 7-OH), 3.96 (1H, d, J=6.4 Hz, 6-H), 3.58 (1H, m, 17-H), 3.20~3.31 (2H, m, 2-H, 25-H), 2.50~2.69 (2H, m, 13-H, 16-H), 2.22~2.40 (3H, m, 5-OH, 12-H, 16-H), 2.00 (1H, ddd, J=1.6, 4.9, 12.1 Hz, 20-H), 1.87 (3H, d, J=1.6 Hz, 26-H_3), 1.15~1.80 (8H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H), 1.15 (3H, d, J=6.1 Hz, 31-H_3), 1.03 (3H, d, J=6.5 Hz, 28-H_3), 0.80~0.95 (1H, m, 18-H), 0.83 (3H, d, J=6.5 Hz, 30-H_3); MS m/z 544 (M^+, $C_{31}H_{44}O_8$), 508, 458, 416, 398, 351, 319, 285, 266, 247, 215, 181, 163, 153; HREI-MS calcd for $C_{31}H_{44}O_8$: 544.3036, found: 544.3023.

29-Hydroxymilbemycin D (**3b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2962 (s), 2928 (s), 2872 (s), 1714 (s); ^1H NMR (270 MHz, $CDCl_3$) δ 5.70~5.85 (2H, m, 9-H, 10-H), 5.27~5.46 (3H, m, 3-H, 11-H, 19-H), 5.11 (1H, dd, J=6.3, 9.7 Hz, 15-H), 4.67 (2H, br s, 27-H_2), 4.29 (1H, d, J=6.4 Hz, 5-H), 4.26, 3.94 (2H, ABq, J=12.0 Hz, 29-H_2), 4.05 (1H, br s, 7-OH), 3.96 (1H, d, J=6.4 Hz, 6-H), 3.65 (1H, m, 17-H), 3.26 (1H, m, 2-H), 3.07 (1H, dd, J=2.0, 9.3 Hz, 25-H), 2.48~2.64 (2H, m, 12-H, 16-H), 2.25~2.38 (2H, m, 16-H, 13-H), 2.00 (1H, ddd, J=1.5, 4.9, 11.7 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.25~1.95 (9H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H), 1.05 (3H, d, J=6.8 Hz, 32-H_3), 1.03 (3H, d, J=6.4 Hz, 28-H_3), 0.75~0.90 (1H, m, 18-H), 0.86 (3H, d, J=6.8 Hz, 33-H_3), 0.80 (3H, d, J=6.9 Hz, 30-H_3); MS m/z 572 (M^+, $C_{33}H_{48}O_8$) 474, 444, 408, 330, 275, 209, 151; HREI-MS calcd for $C_{33}H_{48}O_8$: 572.3349, found: 572.3329.

29-Hydroxy-5-ketomilbemycin A_4 5-oxime (**4b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2959 (s), 2928 (s), 2872 (s), 1715 (s); ^1H NMR (270 MHz, $CDCl_3$) δ 5.73~5.91 (3H, m, 3-H, 9-H, 10-H), 5.31~5.51 (2H, m, 11-H, 19-H), 5.13 (1H, dd, J=7.2, 15.1 Hz, 15-H), 4.77, 4.69 (2H, dABq, J_d=2.2 Hz, J_{AB_q}=14.7 Hz, 27-H_2), 4.67 (1H, s, 6-H), 4.27, 3.94 (2H, AB_q, J=11.7 Hz, 29-H_2), 4.00 (1H, s, 7-OH), 3.60 (1H, m, 17-H), 3.38 (1H, m, 2-H), 3.07 (1H, dt, J_d=1.7 Hz, J_t=8.5 Hz, 25-H), 2.49~2.63 (2H, m, 12-H, 16-H), 2.15~2.36 (2H, m, 13-H, 16-H), 2.00 (1H, ddd, J=2.0, 5.3, 12.1 Hz, 20-H), 1.94 (3H, d, J=2.0 Hz, 26-H_3), 1.20~1.85 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 1.03 (3H, d, J=6.4 Hz, 28-H_3), 1.00 (3H, t, J=7.3 Hz, 32-H_3), 0.80~0.95 (1H, m, 18-H), 0.82 (3H, d, J=6.3 Hz, 30-H_3); MS m/z 571 (M^+, $C_{32}H_{45}NO_8$), 537, 429, 397, 370, 336, 292, 279, 240, 211, 195, 181, 167, 149; HREI-MS calcd for $C_{32}H_{45}NO_8$: 571.3145, found: 571.3134.

Table 3. 29-Hydroxymilbemycin A_4-producing strains from milbemycin A_4.

Microorganism	Conversion efficiency[a]		
	1b	**1c**	**1d**[b]
Syncephalastrum racemosum SANK 41872	+2	+1	+1
S. racemosum SANK 41972	+1	+1	tr
S. racemosum SANK 42672	+2	+1	tr
S. nigricans SANK 42172	+2	+1	+1
S. nigricans SANK 42272	+1	+1	tr
S. nigricans SANK 42372	+1	+1	+1
Rhizopus circinans SANK 16480	+1	+2	tr

[a] +1: 0.5~10%, +2: 10~30%, tr: trace (HPLC analysis).

[b] **1b**: 29-Hydroxymilbemycin A_4, **1c**: 13β-hydroxymilbemycin A_4, **1d**: 30-hydroxymilbemycin A_4.

Other cultures which were found to convert milbemycin A_4 (**1a**) to 29-hydroxymilbemycin A_4 (**1b**) and their conversion efficiency are given in Table 3. When milbemycin A_4 (**1a**) was hydroxylated to 13β,29-dihydroxymilbemycin A_4 by *Streptomyces cavourensis* SANK 67386 (unpublished data), trace amounts of 29-hydroxymilbemycin A_4 (**1b**) was found in the fermentation mixtures by HPLC detection. In the current study, *Syncephalastrum* spp. afforded 29-hydroxymilbemycin A_4 (**1b**) as a major product from milbemycin A_4 (**1a**). In the previous report, only *Syncephalastrum* spp. could convert ML-236B,[9] a competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, predominantly to 6α-hydroxy-ML-236B while a number of organisms hydroxylated at 6β-position.[10,11] It is of interest to note that the difference of stereoselectivity in the hydroxylation of ML-236B between *Syncephalastrum* spp. and other microorganisms was one of site selectivity in the hydroxylation of milbemycins. The characterization of the oxygenase in *Syncephalastrum* spp. is now under investigation. It was also found that *Rhizopus circinans*, which similar to *Syncephalastrum* belongs to the *Mucorales*, possessed 29-hydroxylation activity of milbemycin A_4 (**1a**) but with lower activity.

In conclusion, the microbial conversion of milbemycins by *Syncephalastrum* spp. to effect the preferential hydroxylation at the 29-position was apparently indicated.

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