MICROBIAL CONVERSION OF MILBEMYCINS: 28-HYDROXYLATION OF MILBEMYCINS BY *Amycolata autotrophica*

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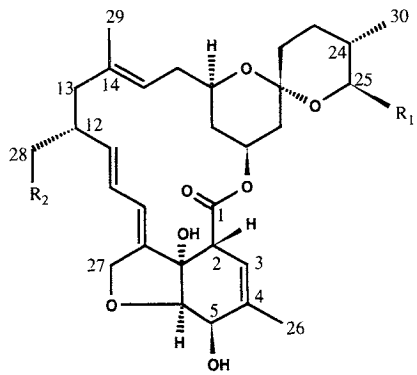
Milbemycins are a family of sixteen-membered macrolides produced by *Streptomyces hygroscopicus* subsp. *aureolacrimosus*. They exhibit broad-spectrum insecticidal and acaricidal activity.[1~3]

In the course of our studies on the microbial conversion of milbemycins, we obtained 13β-hydroxymilbemycins A_4, A_3, D, 13β-hydroxy-LL-F28249α, 28-hydroxymilbemycin D, and 28-hydroxy-LL-F28249α by using *Cunninghamella echinulata*.[4] In the former effort, some of the microorganisms were estimated to convert milbemycin A_4 (**1a**) into 28-hydroxy derivative (**1b**) which was anticipated as a useful compound for synthesizing new milbemycin derivatives. However, the low conversion yield prevented isolation and structure-determination of the fermentation mixtures. We continued the screening to find a microorganism which possessed higher hydroxylation activity. Consequently *Amycolata autotrophica* subsp. *canberrica* ATCC 35203 was found to convert milbemycin A_4 (**1a**) into 28-hydroxymilbemycin A_4 (**1b**) efficiently.



1a $R_1=CH_2CH_3$ $R_2=H$
1b $R_1=CH_2CH_3$ $R_2=OH$
2a $R_1=CH_3$ $R_2=H$
2b $R_1=CH_3$ $R_2=OH$

The present paper deals with the 28-hydroxylation of milbemycin A_4 (**1a**) and A_3 (**2a**) by *A. autotrophica* ATCC 35203.

Converted milbemycins were detected by TLC (Merck Art. 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).

A. autotrophica ATCC 35203 was cultured in twenty 500-ml Erlenmyer flasks containing 100 ml MY medium composed of 1.0% of glucose, 0.5% of Polypepton (Daigo Nutritive Chemicals), 0.3% of yeast extract (Difco), and 0.3% of malt extract (Difco) (pH 6.3~6.5), at 28°C on a rotary shaker (200~220 rpm). After 2 days cultivation, milbemycin A_4 (5% [w/v] in 1,4-dioxane) was added to a final concentration of 250 μg/ml and cultivation was continued for seven additional days. Then the culture broth was extracted with three 1,000-ml portions of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and evaporated. This extract was then purified by silica gel chromatography (20~90% EtOAc in *n*-hexane as an eluent) to give 32 mg (6.2%) of 28-hydroxymilbemycin A_4 (**1b**).

Milbemycin A_3 (**2a**) (500 mg) which is a congener of milbemycin A_4 (**1a**), was subjected to the similar conversion conditions as used for milbemycin A_4, and 11 mg (2.1%) of the corresponding 28-hydroxy derivative was obtained. The Rf values on TLC and HPLC retention times of 28-hydroxy derivatives are listed in Table 1. The physico-chemical properties of 28-hydroxymilbemycin A_4 (**1b**) and A_3 (**2b**) were as follows:

Table 1. 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	0.18	4.97	18.26
2a	0.59	11.80	—
2b	0.18	3.98	12.29

[a] **a**: Substrate; **b**: product.
[b] Rf values and retention times relative to 13β-hydroxymilbemycin A_4.[4]

28-Hydroxymilbemycin A_4 (**1b**): IR (KBr) cm^{-1} 3650~3150 (br s), 2957 (s), 2928 (s), 2873 (s), 1715 (s), cm^{-1}; ^1H NMR (270 MHz, $CDCl_3$) δ 5.67~5.94 (2H, m, 9-H, 10-H), 5.25~5.44 (3H, m, 3-H, 11-H, 19-H), 5.01 (1H, t, J=7.7 Hz, 15-H), 4.67 and 4.74 (2H, ABq, J=14.3 Hz, 27-H_2), 4.29 (1H, br s, 5-H), 4.16 (1H, s, 7-OH), 3.96 (1H, d, J=6.1 Hz, 6-H), 3.51~3.72 (2H, m, 17-H, 28-H), 3.39 (1H, dd, J=8.1, 10.9 Hz, 28-H), 3.27 (1H, dd, J=2.2, 4.6 Hz, 2-H), 3.08 (1H, dt, J_d=2.8 Hz, J_t=9.2 Hz, 25-H), 2.46~2.59 (1H, m, 12-H), 2.16~2.38 (4H, m, 5-OH, 13-H, 16-H_2), 2.01 (1H, ddd, J=1.6, 5.0, 12.1 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.55 (3H, s, 29-H_3), 1.22~1.94 (10H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H, 31-H_2), 0.99 (3H, t, J=7.3 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^+, $C_{32}H_{46}O_8$), 430, 412, 372, 330, 288, 264, 245,195, 167; HREI-MS calcd for $C_{32}H_{46}O_8$: 558.3193, found: 558.3183.

28-Hydroxymilbemycin A_3 (**2b**): IR (KBr) cm^{-1} 3650~3100 (br s), 2968 (s), 2927 (s), 2873 (s), 1719 (s); ^1H NMR (270 MHz, $CDCl_3$) δ 5.75~5.96 (2H, m, 9-H, 10-H), 5.26~5.43 (3H, m, 3-H, 11-H, 19-H), 5.04 (1H, t, J=7.8 Hz, 15-H), 4.67 and 4.73 (2H, dABq, J_d=2.0 Hz, J_{ABq}=15.5 Hz, 27-H_2), 4.29 (1H, d, J=5.6 Hz, 5-H), 4.10~4.25 (1H, br s, 7-OH), 3.95 (1H, d, J=5.6 Hz, 6-H), 3.50~3.61 (1H, m, 17-H), 3.55 (1H, dd, J=5.4, 10.4 Hz, 28-H), 3.39 (1H, dd, J=8.2, 10.4 Hz, 28-H), 3.21~3.30 (2H, m, 2-H, 25-H), 2.42~2.61 (1H, m, 12-H), 2.16~2.28 (3H, m, 13-H, 16-H_2), 2.02 (1H, dd, J=4.2, 11.8 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.55 (3H, s, 29-H_3), 1.20~1.92 (8H, m, 13-H, 18-H, 20-H, 22-H_2, 23-H_2, 24-H), 1.15 (3H, d, J=6.0 Hz, 31-H_3), 0.85~1.02 (1H, m, 18-H), 0.83 (3H, d, J=6.4 Hz, 30-H_3); MS m/z 544 (M^+, $C_{31}H_{44}O_8$); 416, 398, 372, 330, 288, 264, 250, 231, 181, 167, 153; HREI-MS calcd for $C_{31}H_{44}O_8$: 544.3036, found: 544.3049.

We have reported that C-30, C-26, and C-29 methyl groups of milbemycin A_4 (**1a**) were hydroxylated by microorganisms.[5~7] In this report the 28-hydroxylation of milbemycin A_4 (**1a**) was confirmed. Thus, all the methyl groups of milbemycin A_4 can be selectively hydroxylated by microbial conversion. Further studies on microbial conversion of milbemycin A_4 (**1a**) involving the side chain at C-25 are in progress.

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