MICROBIAL CONVERSION OF MILBEMYCINS: 13β,29-DIHYDROXYLATION OF MILBEMYCINS BY SOIL ISOLATE *Streptomyces cavourensis*

KEIKO NAKAGAWA, KAZUO SATO,† TAKAO OKAZAKI†† and AKIO TORIKATA

Fermentation Research Laboratories, Sankyo Co., Ltd., 1-2-58 Hiromachi, Shinagawa-ku, Tokyo 140, Japan
†Agricultural Chemicals Research Laboratories, Sankyo Co., Ltd., 1041 Yasu, Yasu-cho, Shiga 520-23, Japan
††Tsukuba Research Laboratories, Sankyo Co., Ltd., 33 Miyukigaoka, Tsukuba-shi, Ibaragi 305, Japan

(Received for publication February 1, 1991)

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_3 (**2b**), A_4 (**1b**), and D (**3b**) from milbemycins A_3 (**2a**), A_4 (**1a**), and D (**3a**), respectively, and 13β,29-dihydroxymilbemycin A_4 (**1c**) from 29-hydroxymilbemycin A_4 using *Cunninghamella echinulata* ATCC 9244.[4] During the screening for microbial conversion of milbemycin A_4 (**1a**)[5] we found that a soil isolate actinomycete, strain SANK 67386, converted milbemycin A_4 (**1a**) to 13β,29-dihydroxymilbemycin A_4 (**1c**) besides 13β-hydroxymilbemycin A_4 (**1b**).

The present paper deals with the 13β-hydroxylation and 13β,29-dihydroxylation of milbemycins A_3 (**2a**), A_4 (**1a**), and D (**3a**) by strain SANK 67386 (Fig. 1).

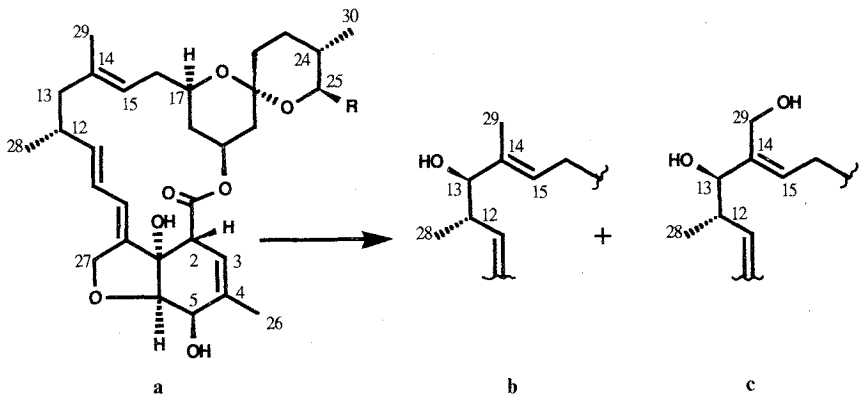
Strain SANK 67386 was isolated from solonized brown Mallee soil collected at Adelaide in Australia. On the descriptive media of SHIRLING and GOTTLIEB,[6] and WAKSMAN,[7] after 14 days at 28°C, the aerial mycelium was abundant and formed yellowish gray mass. The color of the vegetative mycelium was pale yellow to light olive gray. The strain only produced a melanoid pigment.

The substrate hyphae are long, irregularly branched, and do not fragment into short elements or formed spores. Mature spore chains are generally straight to flexuous or hooked and very long, with 10 to more than 50 spores per chain. Spores are elliptical with a smooth surface. Cell walls and whole cells were analyzed by the procedure of BECKER *et al.*,[8] and LECHEVALIER,[9] respectively. The cell wall contained LL-diaminopimelic acid and glycine as major constituents. The whole cell sugar pattern was not characteristic. The strain was considered to be a cell wall type I.

Based on morphology and analyses of the cell wall and whole cell extracts, the strain was identified as *Streptomyces cavourensis*.[10]

The conversion of milbemycins was 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

Fig. 1. Microbial conversion of milbemycins by *Streptomyces cavourensis* SANK 67386.



1 R = CH_2CH_3
2 R = CH_3
3 R = $CH(CH_3)_2$

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. cavourensis SANK 67386 was cultured in five 500-ml Erlenmyer 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 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 500 μg/ml and cultivation was continued for seven more days. The cultured broth was then filtered and the filtrate was extracted with three 300-ml portions of EtOAc. The EtOAc extract was dried over anhydrous sodium sulfate and evaporated. The mycelium was extracted with 80% MeOH and the MeOH was evaporated, the resulting aqueous solution was extracted and evaporated in a similar manner as the filtrate. The extracts were combined and purified by silica gel chromatography (20~90% EtOAc in *n*-hexane as an eluent) to give 13β-hydroxymilbemycin A_4 (**1b**) and 13β,29-dihydroxymilbemycin A_4 (**1c**). The configurational assignment of the hydroxy group at the C-13 position was based on the comparison of the coupling constants of 13α- and 13β-hydroxy compounds in ^{1}H NMR spectra.[11,12]

Milbemycins A_3 (**2a**) and D (**3a**) were similarly subjected to the same microbial conversion by *S. cavourensis* to produce the corresponding 13β-hydroxy and 13β,29-dihydroxy derivatives. The yields are shown in Table 1. The Rf values on TLC and HPLC Rt's are listed in Table 2. The physicochemical properties of 13β,29-dihydroxymilbemycins A_3 (**2c**) and D (**3c**) were as follows:

13β,29-Dihydroxymilbemycin A_3 (**2c**): IR (KBr) cm^{-1} 3600~3150 (br s), 2967 (s), 2929 (s), 2873 (s), 1720 (s); ^{1}H NMR (270 MHz, $CDCl_3$) δ 5.72~5.90 (2H, m, 9-H and 10-H), 5.25~5.45 (4H, m, 3-H, 11-H, 15-H and 19-H), 4.66, 4.71 (2H, ABq, J = 15.0 Hz, 27-H_2), 4.13, 4.48 (2H, ABq, J = 12.2 Hz, 29-H_2), 4.29 (1H, d, J = 5.9 Hz, 5-H), 4.02 (1H, s, 7-OH), 3.96 (1H, d, J = 6.3 Hz, 6-H), 3.78 (1H, d, J = 10.3 Hz, 13-H), 3.58 (1H, m, 17-H), 3.25 (2H, m, 2-H and 25-H), 2.57 (1H, m, 12-H), 2.25~2.45 (2H, m, 16-H_2), 2.01 (1H, ddd, J = 1.4, 3.4 and 12.2 Hz, 20-H), 1.87 (3H, d, J = 2.0 Hz, 26-H_3), 1.20~1.80 (7H, m, 18-H, 20-H, 22-H_2, 23-H_2 and 24-H), 1.18 (3H, d, J = 6.3 Hz, 31-H_3), 1.15 (3H, d, J = 6.3 Hz, 28-H_3), 0.84 (3H, d, J = 6.8 Hz, 30-H_3), 0.75~0.90 (1H, m, 18-H); MS *m/z* 542 ($M^+ - H_2O$, $C_{31}H_{42}O_8$), 281, 263, 237, 199, 181, 153, 129; HREI-MS Calcd for $C_{31}H_{42}O_8$: 542.2879, Found: 542.2879.

Table 1. Conversion of milbemycins by *Streptomyces cavourensis*.

Substrate	Concentration (μg/ml)	Conversion time (days)	Products[a] yield (%)	
			b	**c**
1a	500	7	36.1	16.4
2a	500	8	17.5	1.5
3a	500	7	4.3	3.3

[a] **b**: 13β-Dihydroxy derivative, **c**: 13β,29-dihydroxy derivative.

Table 2. TLC Rf values and HPLC Rt's 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	0.46	3.50	10.86
1c	0.18	2.52	5.49
2a	0.59	11.80	—
2b	0.46	3.02	8.04
2c	0.18	2.27	4.52
3a	0.62	24.64	—
3b	0.48	4.59	18.26
3c	0.19	3.00	8.03

[a] **a**: Substrate, **b** and **c**: products.
[b] Rf values and Rt's relative to **1b**.

13β,29-Dihydroxymilbemycin D (**3c**): IR (KBr) cm^{-1} 3600~3200 (br s), 2963 (s), 2930 (s), 2872 (s), 1716 (s); ^{1}H NMR (270 MHz $CDCl_3$) δ 5.72~5.90 (2H, m, 9-H and 10-H), 5.25~5.45 (4H, m, 3-H, 11-H, 15-H and 19-H), 4.68 (2H, br s, 27-H_2), 4.12, 4.47 (2H, ABq, J = 12.2 Hz, 29-H_2), 4.30 (1H, t, J = 6.1 Hz, 5-H), 4.01 (1H, s, 7-OH), 3.96 (1H, d, J = 6.1 Hz, 6-H), 3.79 (1H, dd, J = 4.9 and 10.3 Hz, 13-H), 3.61 (1H, m, 17-H), 3.25 (1H, m, 2-H), 3.07 (1H, dd, J = 2.0 and 9.3 Hz, 25-H), 2.60 (1H, m, 12-H), 2.30~2.45 (2H, m, 16-H_2), 2.16 (1H, br s, 5-OH), 2.02 (1H, ddd, J = 1.7, 4.9 and 12.2 Hz, 20-H), 1.87 (3H, s, 26-H_3), 1.25~1.70 (8H, m, 18-H, 20-H, 22-H_2, 23-H_2, 24-H and 31-H), 1.18 (3H, d, J = 6.8 Hz, 28-H_3), 1.04 (3H, d, J = 6.8 Hz, 32-H_3), 0.86 (3H, d, J = 6.8 Hz, 33-H_3), 0.81 (3H, d, J = 5.9 Hz, 30-H_3), 0.74~0.90 (1H, m, 18-H); MS *m/z* 570 ($M^+ - H_2O$, $C_{33}H_{46}O_8$) 552, 309, 291, 262, 237, 226, 209, 181, 157, 139; HREI-MS Calcd for $C_{33}H_{46}O_8$: 570.3193, Found: 570.3188.

In our previous studies on the microbial conversion of milbemycins, 13β,24- and 13β,30-di-

hydroxymilbemycins had been obtained as minor products along with 13β-hydroxy derivatives using *C. echinulata* ATCC 9244.[4] The C-24 and C-30 which were far from the C-13 were preferentially hydroxylated by this microorganism. In contrast, *S. cavourensis* produced 13β,29-dihydroxymilbemycin A_4 along with 13β-hydroxy derivatives. It is of interest that the C-29 near to the C-13 was preferentially hydroxylated.

This study gives a new convenient method to produce 13β,29-dihydroxymilbemycins which will be used as metabolite reference standards in animal metabolism studies of milbemycins.

References

1) TAKIGUCHI, Y.; H. MISHIMA, M. OKUDA, M. TERAO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Fermentation, isolation and physico-chemical properties. J. Antibiotics 33: 1120~1127, 1980
2) OKAZAKI, T.; M. ONO, A. AOKI & R. FUKUDA: Milbemycins, a new family of macrolide antibiotics: Producing organism and its mutants. J. Antibiotics 36: 438~441, 1983
3) MISHIMA, H.; J. IDE, S. MURAMATSU & M. ONO: Milbemycins, a new family of macrolide antibiotics. Structure determination of milbemycins D, E, F, G, H, J and K. J. Antibiotics 36: 980~990, 1983
4) NAKAGAWA, K.; S. MIYAKOSHI, A. TORIKATA, K. SATO & Y. TSUKAMOTO: Microbial conversion of milbemycins: Hydroxylation of milbemycin A_4 and related compounds by *Cunninghamella echinulata* ATCC 9244. J. Antibiotics 44: 232~240, 1991
5) NAKAGAWA, K.; A. TORIKATA, K. SATO & Y. TSUKAMOTO: Microbial conversion of milbemycins: 30-Oxidation of milbemycin A_4 and related compounds by *Amycolata autotrophica* and *Amycolatopsis mediterranei*. J. Antibiotics 43: 1321~1328, 1990
6) SHIRLING, E. B. & D. GOTTLIEB: Methods for the characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
7) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Vol. 2. Classification, Identification and Description of Genera and Species. Williams & Wilkins Co., 1961
8) BECKER, B.; M. P. LECHEVALIER & H. A. LECHEVALIER: Chemical composition of cell-wall preparations from strains of various form-genera of aerobic actinomycetes. Appl. Microbiol. 13: 236~243, 1965
9) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. & Clin. Med. 71: 934~944, 1968
10) SKARBEK, J. D. & L. R. BRADY: *Streptomyces cavourensis* sp. nov. (nom. rev.) and *Streptomyces cavourensis* subsp. *washingtonensis* subsp. nov., a chromomycin-producing subspecies. Int. J. Syst. Bacteriol. 28: 45~53, 1978
11) MROZIK, H.; J. C. CHABALA, P. ESKOLA, A. MATZUK, F. WAKSMUNSKI, M. WOODS & M. H. FISHER: Synthesis of milbemycins from avermectins. Tetrahedron Lett. 24: 5333~5336, 1983
12) MROZIK, H.; B. O. LINN, P. ESKOLA, A. LUSI, A. MATZUK, F. A. PREISER, D. A. OSTLIND, J. M. SHAEFFER & M. H. FISHER: Syntheses and biological activities of 13-substituted avermectin aglycons. J. Med. Chem. 32: 375~381, 1989