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Two New β-Class Milbemycins from *Streptomyces bingchenggensis*: Fermentation, Isolation, Structure Elucidation and Biological Properties

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Abstract Two new β-class milbemycins from *Streptomyces bingchenggensis*, named milbemycin β_{13} and β_{14}, have been isolated and characterized. The producing organism has been deposited at the China General Microbiology Culture Collection Center (Accession No: CGMCC1734). On the basis of detailed spectroscopic analysis and comparison with reported data, their structures were determined to be the hydroxyl derivatives at C-27 of milbemycin β_3 and 25-ethylmilbemycin β_3, respectively. Milbemycins β_{13} and β_{14} possess potent acaricidal and nematocidal activity. The discovery of these two compounds plays an important role in understanding and perfecting the proposed pathways of milbemycins.

Keywords β-class milbemycins, *Streptomyces bingchenggensis*, acaricidal activity, nematocidal activity

Introduction

Since the discovery in 1967 of B-41, a metabolite with outstanding activity against various kinds of mites, more than 30 structurally similar milbemycins have been isolated from fermentation broths of *Streptomyces hygroscopicus* subsp. *aureolacrimosus* [1]. All milbemycins have 16-membered macrolide structures and possess potent anthelmintic activity. Following the discovery of milbemycins, numerous compounds with the same 16-membered macrolide structure were isolated [2~5], including Merck's avermectin with potent anthelmintic activity, Cyanamid's LL-F28249, Glaxo's Factor series compounds, and milbemycins α_{11}~α_{14}.

Many milbemycin compounds have been isolated from other microorganisms, including *Streptomyces cyaneogriseus* subsp. *noncyanogenus* [6], *Streptomyces thermoarchaensis* [7], *Streptomyces hygroscopicus* [8], *Streptomyces* sp. [9, 10], and a hybrid microorganism obtained by protoplast fusion of *Streptomyces avermitilis* and *S. hygroscopicus* [11].

Streptomyces hygroscopicus subsp. *aureolacrimosus* SANK 60286 also produced a new family of milbemycin together with the other milbemycins [3]. There are two kinds of milbemycin compounds, namely β-type and α-type milbemycin. Twelve β-type milbemycin compounds and 27 α-type milbemycin compounds have been reported [12].

In order to discover new milbemycins, we investigated the fermentation broths of a strain of *Streptomyces bingchenggensis*, which has been deposited at the China General Microbiology Culture Collection Center (Accession No: CGMCC1734). We obtained two new β-series milbemycins, named milbemycins β_{13} (**1**) and β_{14} (**2**). In this paper, we describe the fermentation, isolation, structural elucidation and acaricidal activities of the two new milbemycins.

Materials and Methods

Microorganism

The producing organism, *S. bingchenggensis*, was isolated from a soil sample collected in Harbin, China. *S. bingchenggensis* has been deposited at the China General

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Microbiology Culture Collection Center (Accession No: CGMCC1734), and we have determined the 16S rDNA sequence (Accession No: DQ449953 in National Center for Biological Information).

Fermetation

The seed for preculture was spores. The medium for sporulation contained sucrose 4.0 g, yeast extract 2.0 g, malt extract 5.0 g, skim milk 1.0 g in 1 liter water. The pH was adjusted to 7.0 with 1 M NaOH, 20 g of agar added, and this mixture sterilized at 121°C for 30 minutes. The spore suspension was prepared from the agar plates incubated at 28°C for 7～8 days.

A spore suspension of the culture of strain *S. bingchenggensis*, 1.0 ml, was transferred to a 250-ml Erlenmeyer flask that contained 25 ml of the seed medium containing sucrose 0.25 g, polypepton 0.1 g, and K_2HPO_4 1.25 mg. The inoculated flasks were incubated at 28°C for 42 hours on a rotary shaker at 250 rpm. Then 8.0 ml of the culture was transferred into a 1-liter Erlenmeyer flask containing 100 ml of the producing medium consisting of sucrose 8.0%, soybean powder 1.0%, yeast extract 0.2%, meat extract 0.1%, $CaCO_3$ 0.3%, K_2HPO_4 0.03%, $MgSO_4 \cdot 7H_2O$ 0.1%, $FeSO_4 \cdot 7H_2O$ 0.005%, pH 7.2 before sterilization. Fermentation was carried out at 28°C for 8 days on a rotary shaker at 250 rpm.

Isolation and Purification

The fermentation broth (10 liters) was filtered. The resulting cake was washed with water, and the both filtrate and wash were discarded. MeOH (10 liters) was used to extract the washed cake. The MeOH extract was concentrated to approximately 2 liters under reduced pressure, and the resulting concentrate was extracted three times with an equal volume of EtOAc. The combined EtOAc phase was concentrated under reduced pressure to yield 25 g of oily substance. The residual oily substance was chromatographed on silica gel, eluting with petroleum ether - Me_2CO (95 : 5～50 : 50). Fractions not containing milbemycin A_3 and A_4 were combined to give 10 g of crude sample upon evaporation of the solvents. The crude sample was applied to a silica gel column and eluted with petroleum ether - Me_2CO (9 : 1～3 : 1) to give five fractions. Fraction 2 was further separated by RP-C_{18} silica gel column chromatography, eluting with MeOH - H_2O (80 : 20～93 : 7), to yield **1** (18 mg) and **2** (50 mg).

General

UV spectra were obtained on a Varian CARY 300 BIO spectrophotometer; IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrometer (ν_{max} in cm^{-1}); ^1H- and ^{13}C-NMR spectra were measured with a Bruker DRX-400 (400 MHz for ^1H and 100 MHz for ^{13}C) spectrometer. Chemical shifts are reported in parts per million (δ), using residual $CHCl_3$ (δ_H 7.26 ppm; δ_C 77.0) as an internal standard, with coupling constants (J) in Hz. ^1H- and ^{13}C-NMR assignments were supported by ^1H-^1H COSY, HMQC and HMBC experiments. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer. Optical rotation was measured on a Perkin-Elmer 341 Polarimeter. Commercial silica gel (Qing Dao Hai Yang Chemical Group Co., 100～200 and 200～300 mesh) and reverse phase C_{18} silica gel were used for column chromatography. Spots were detected on TLC under UV or by heating after spraying with sulfuric acid - ethanol, 5 : 95 (v/v).

Antiparasitic Activity

Antiacaricidal Activity against Adult Mites

MeOH solutions containing 0.1% of individual compounds were diluted 10-fold with water containing 0.01% of detergent to prepare 100 μg/ml solutions. Then appropriate further dilutions were prepared. Two-spotted spider mites, sensitive to organophosphorus insecticides, were inoculated on the primary leaves of cowpea plants. One day after inoculation, leaves of the cowpea plants were soaked in the sample solutions for 1～2 seconds and the leaves were kept at 25°C. After 3 days, survival of the adult insects was determined with a binocular microscope and the mortality (%) was calculated.

Antiacaricidal Activity against Mite Eggs

Sample solutions containing 100, 50, 30, and 10 μg/ml of individual compounds were prepared. Female adult two-spotted spider mites were allowed to lay eggs on the primary leaves of cowpea plants. The adult mites were removed to obtain test leaves each bearing about 40 eggs. In a similar manner to the preceding example, the test leaves were soaked in the sample solutions for 1～2 seconds. After 10 days at 25°C, the number of unhatched eggs was counted, and the unhatched egg rates (%) were calculated.

Antinematocidal Activity

MeOH solutions containing 0.1% of individual compounds were diluted 10-fold with water to prepare solutions containing 100 μg/ml. Then appropriate amounts of the solutions were added to 1-ml portions of an aqueous suspension containing living nematodes, *Caenorhabditis elegans*. The mixtures were left at 25°C for 15 hours after shaking. The number of nematodes that were immobilized

and the total number of the nematodes tested were counted under a stereoscopic microscope. Immobilized rates (%) against the total number of tested nematodes were calculated.

Results and Discussion

Physico-chemical Properties of 1 and 2

Milbemycin β_{13} (**1**, Fig. 1) $C_{31}H_{42}O_6$, white amorphous powder ; $[\alpha]_D^{20}$ +108° (*c* 0.25, Me_2CO); UV λ_{max}^{MeOH} nm (log ε): 246 (3.37); IR (KBr), ν_{max} cm^{-1}: 3600, 3470, 2940, 1673, 1604, 1291, 1174, 994, 965, ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) see Table 1; ESI-MS *m/z* 509 $[M+H]^+$; HRESI-MS *m/z* 509.2904, calcd for $C_{31}H_{41}O_6$ 509.2903.

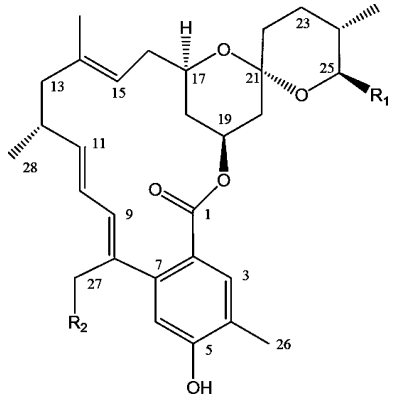
Milbemycin β_{14} (**2**, Fig. 2) $C_{32}H_{44}O_6$, white amorphous powder; $[\alpha]_D^{20}$ +108° (*c* 0.25, Me_2CO); UV λ_{max}^{MeOH} nm (log ε): 246 (3.37); IR (KBr), ν_{max} cm^{-1}: 3600, 3470, 2940, 1670, 1601, 1285, 1170, 1004, 965, ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR ($CDCl_3$, 100 MHz) see Table 1; ESI-MS *m/z* 523 $[M+H]^+$; HRESI-MS *m/z* 523.3059, calcd for $C_{32}H_{43}O_6$ 523.3060.

Structural Elucidation

The structures of the new milbemycins were determined by the analysis and the comparison of ^{1}H-NMR, ^{13}C-NMR, MS, and IR data with those of milbemycin β_3 (**3**), 25-ethylmilbemycin β_3 (**4**), and β_4 [5, 10, 13～15]. The ^{1}H- and ^{13}C-NMR data of new milbemycins are summarized in Table 1. The molecular formula was established from the HR-ESI-MS spectra. In the ^{1}H- and ^{13}C-NMR spectra of the new milbemycins, signals corresponding to the 16-membered macrolide structures were found. The structural difference between **1** and **3**, and between **2** and **4** was found only in the substitution at position 27.

By detailed comparison the ^{1}H- and ^{13}C-NMR data of **1** with those of **3**, it was shown that **1** was a derivative of β_3, in which a methyl at position 27 was replaced by a hydroxymethyl group. The HMBC correlation signals observed between δ_H 4.53, 4.56 and δ_C 131.8 (d, C-9), 140.8 (s, C-7), 136.0 (s, C-8) also indicated the presence of a hydroxymethyl group at position 27 in **1**. The 16 mass unit molecular weight enhancement of **1**, compared with that of **3**, further confirmed the structure of **1**. So the structure of **1** was elucidated and named milbemycin β_{13}.

The ^{1}H- and ^{13}C-NMR data of **2** were very similar to those of **1**, except for the methene signal (δ_H 1.70, 1.36, δ_C 25.8). Thus, **2** was assigned to be the 27-hydroxyl derivative of **4**. The HMBC correlations of δ_H 4.51, 4.54 and δ_C 131.6 (d, C-9), 136.4 (s, C-8), 140.9 (s, C-7) further confirmed the presence of a hydroxymethyl group at position 27 in **2**. So the structure of **2** was established and named milbemycin β_{14}.



milbemycin β_{13} (**1**): R_1=CH_3 R_2=OH
milbemycin β_{14} (**2**): R_1=CH_2CH_3 R_2=OH
milbemycin β_3 (**3**): R_1=CH_3 R_2=H
25-ethylmilbemycin β_3 (**4**): R_1=CH_2CH_3 R_2=OH

Fig. 1 The structures of **1**, **2**, milbemycin β_3 and 25-ethylmilbemycin β_3.

The structures of two new β-class milbemycin β_{13} and β_{14} are the hydroxyl derivatives at C-27 of **3** and **4**, respectively. Milbemycin β_3 and 25-ethyl milbemycin β_3 were deduced [5, 12] to derive from a non-enzymatic reaction of milbemycin β_7 and milbemycin β_6 (Fig. 2). If the above deduction is true, **1** and **2** are derived from a non-enzymatic reaction of 27-hydroxymilbemycin β_7 and milbemycin β_6, respectively. However we have not yet isolated 27-hydroxymilbemycin β_7 and milbemycin β_6 from *S. bingchenggensis*. These questions, for example, whether there is difference in biosynthetic pathway of milbemycins between *S. bingchenggensis* and *S. hygroscopicus* subsp., supply many new subjects for us. However, the discovery of two compounds plays an important role in understanding and perfecting the proposed pathways of milbemycins.

Biological Activity

Two new β-class milbemycins β_{13} and β_{14} possess potent acaricidal and nematocidal activity (Tables 2 and 3). Especially they were more active than known milbemycins against unhatched mite eggs. Generally, biological activities of α-type milbemycins are higher than that of β-class milbemycins, but semi-synthesis of higher-activity milbemycins has been researched extensively [16～23].

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Table 1 ^{1}H- and ^{13}C-NMR data of milbemycin β_{13} (**1**) and β_{14} (**2**) (Coupling constants in parenthesis)

Number	Proton		Carbon	
	β_{13} (**1**)	β_{14} (**2**)	β_{13} (**1**)	β_{14} (**2**)
1			169.0 s[a]	168.9 s
2			123.9 s	124.0 s
3	7.39 s	7.41 s	132.1 d	132.2 d
4			123.5 s	123.6 s
5			156.3 s	156.6 s
6	6.70 s	6.72 s	114.4 d	114.5 d
7			140.8 s	140.9 s
8			136.0 s	136.4 s
9	5.82 d (11.0)	5.81 d (11.0)	131.8 d	131.6 d
10	6.25 dd (14.9, 11.0)	6.25 dd (15.0, 11.0)	123.9 d	124.0 d
11	5.43 dd (14.9, 8.8)	5.44 dd (15.0, 9.0)	143.2 d	142.9 d
12	2.53 m	2.52 m	35.6 d	35.6 d
13	2.25 m	2.25 m	48.7 t	48.6 t
	1.86 br t (12.4)	1.85 br t (12.5)		
14			135.6 s	135.7 s
15	4.91 br d (9.8)	4.92 br d (9.8)	121.6 d	121.6 d
16	2.34 m	2.35 m	33.8 t	33.8 t
	2.25 m	2.24 m		
17	3.71 m	3.72 m	67.6 d	67.6 d
18	2.04 m	2.04 m	36.7 t	36.8 t
	0.87 m	0.88 m		
19	5.50 m	5.46 m	68.8 d	68.7 d
20	2.01 m	2.03 m	41.2 t	41.4 t
	1.44 br t (12.0)	1.44 br t (12.0)		
21			97.8 s	97.6 s
22	1.67 m	1.67 m	35.7 t	35.7 t
	1.55 m	1.55 m		
23	1.55 m	1.54 m	27.8 t	27.9 t
24	1.25 m	1.35 m	36.6 d	34.4 d
25	3.30 m	3.09 m	71.3 d	76.0 d
26	2.22 s	2.21 s	15.4 q	15.5 q
27	4.56 d (10.0)	4.54 d (10.0)	61.5 t	61.5 t
	4.53 d (10.0)	4.51 d (10.0)		
28	1.06 d (6.6)	1.05 d (6.6)	20.9 q	21.0 q
29	1.65 br s	1.65 br s	16.1 q	16.1 q
30	0.85 d (6.4)	0.83 d (6.4)	17.9 q	17.8 q
31	1.15 d (6.2)	1.70 m	19.4 q	25.8 t
		1.36 m		
32		1.00 t (7.5)		10.1 q

[a] By DEPT sequence.

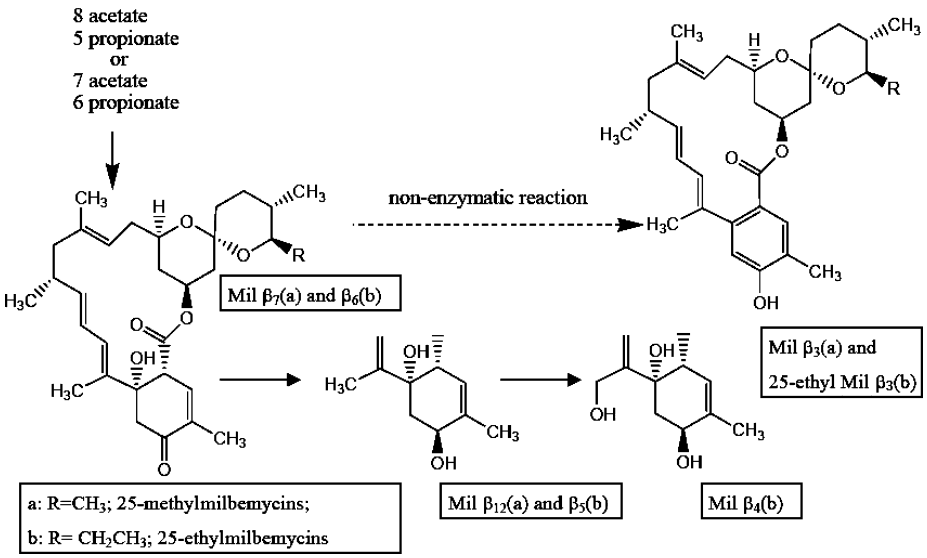

Fig. 2 Proposed pathways of milbemycins by Nonaka *et al.* [5, 12].

Major pathway in milbemycin-producing strain. The solid and dotted line mean established and deduced pathway, respectively.

Table 2 Acaricidal activity of milbemycins against adult mites and mite eggs

Concentration (μg/ml)	Adult mites mortality (%)			Mite eggs unhatched (%)		
	β_{13}	β_{14}	A_3/A_4[a]	β_{13}	β_{14}	A_3/A_4[a]
100	91.2	96.5	100	73.4	87.9	43.5
50	58.7	69.3	100	43.5	67.1	17.3
30	17.3	37.4	93.0	13.7	12.4	4.1
10	5.4	11.2	71.4	0	3.4	

[a] Milbemycins A_3 and A_4 mixtures, 30 : 70 (in volume).

Table 3. Nematocidal activity of milbemycins against *Caenorhabditis elegans*

Concentration (μg/ml)	Immobility (%)		
	β_{13}	β_{14}	A_3/A_4[a]
100	100	100	100
50	76	87	100
30	53	54	91
10	21	31	74

[a] Milbemycins A_3 and A_4 mixtures, 30 : 70 (in volume).

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