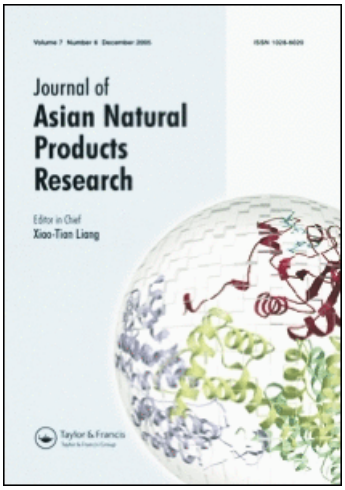

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In the continuing study of the chemical compositions of the strain *Streptomyces bingchenggensis*, three new milbemycin derivatives, milbemycin α_{31} (**1**), secomilbemycins C (**2**), and D (**3**), were isolated. Their structures were established on the basis of extensive spectroscopic analysis.

Keywords: new milbemycin α_{31}; secomilbemycins C and D; *Streptomyces bingchenggensis*

1. Introduction

Since the discovery of B-41, a metabolite with outstanding activity against various kinds of mites, more than 30 kinds of structurally similar milbemycins have been isolated from fermentation broth of *Streptomyces hygroscopicus* subsp. *Aureolacrimosus* [1–4]. All milbemycins have 16-membered macrolide structures. Following the discovery of milbemycins, numerous compounds with the same 16-membered macrolide skeleton were isolated from other microorganisms, including *Streptomyces cyaneogriseus* subsp. *Noncyanogenus* [5], *Streptomyces thermoarchaensis* [6], *S. hygroscopicus* [7], *Streptomyces* sp. E225 (NCIB 12310) [8–10], *Streptomyces* NCIB 11876 [11], *S. hygroscopicus* ATCC 53718 [12], *Streptomyces bingchenggensis* [13–15], and a hybrid microorganism obtained by protoplast fusion of *Streptomyces avermitilis* and *S. hygroscopicus* [16]. Moreover, there are still hundreds of semi-synthetic [17] and bioconversion milbemycins [18].

We have previously reported the isolation and structure elucidation of milbemycins β_{13}, β_{14}, α_{28}, α_{29}, α_{30}, and secomilbemycins A and B from *S. bingchenggensis* [13–15]. In order to search for more bioactive milbemycins, we scaled up the fermentation of the strain *S. bingchenggensis*. In the following investigation of its chemical compositions, three new milbemycin derivatives, milbemycin α_{31} (**1**), secomilbemycins C (**2**), and D (**3**), were isolated. In this paper, we describe the isolation and structural elucidation of the three new milbemycin derivatives.

2. Results and discussion

Compound **1** was obtained as colorless oil with the UV absorption maxima at 356, 301, 257, 227, and 204 nm. In its IR spectrum, the hydroxyl and carbonyl

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absorptions were presented at 3360 and 1708 cm^{-1}, respectively. The molecular formula of **1** was deduced from HR-ESI-MS at *m/z* 521.2980 $[M]^+$ as $C_{32}H_{42}O_6$, which indicated the presence of 12 degrees of unsaturation. The ^1H NMR spectrum (see Table 1) of **1** showed a singlet of sp^2 proton signal at δ 7.04 (s) and an oxygenated methylene proton signal at δ 5.24 (d). Its ^{13}C NMR and DEPT spectral data (see Table 1) displayed 32 carbons, including 12 sp^2 carbons. These data suggested that **1** was the analog of milbemycin β_{13} [15]. Comparison of the ^1H and ^{13}C NMR spectral data of **1** with those of milbemycin β_{13} showed that **1** was very similar to milbemycin β_{13}, except for the obvious difference at positions C-6 and C-27. This further suggested a five-membered ether ring was formed between C-6 and C-27 like other milbemycin α series. The HMBC correlation of H-27 at δ_H 5.24 and C-6 confirmed the above result. So, **1** was elucidated as 5,6-dehydro-2,7-dehydroxy milbemycin A_4. The relative stereochemistry of **1** was assigned by the concurrence with milbemycin A_4 [1,2].

Compounds **2** and **3** were also isolated as colorless oil. Their UV and IR spectra were similar. The HR-ESI-MS spectra gave the same molecular formula as $C_{31}H_{42}O_6$. Comparison of the ^1H and ^{13}C NMR spectral data of **2** and **3** with those of secomilbemycins A and B [15] revealed that **2** and **3** were almost identical to those of secomilbemycins A and B, except for the substituent at C-25, where the ethyl in secomilbemycins A and B was replaced by the methyl in **2** and **3** [15]. In **2**, the NOESY correlations between H-27 and H-10 and between H-6 and H-9 indicated the Δ^8 olefin was *trans*-double bond as in secomilbemycin A. The cross-peaks between H-27 and H-9, H-6, and H-10 in the NOESY experiment of **3** indicated the configuration of Δ^8 olefin was Z as in secomilbemycin B. Thus, the structures of **2** and **3** were established.

3. Experimental

3.1 General experimental procedures

Optical rotation was measured on a Perkin-Elmer 341 polarimeter. The 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 the internal standard, with coupling constants (J) in Hertz. ^1H and ^{13}C NMR assignments were supported by ^1H–^1H COSY, HMQC, and HMBC experiments. The ESI-MS and HR-ESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer. Commercial silica gel (100–200 and 200–300 mesh, Qingdao Hai Yang Chemical Group Co., Qingdao, China) was used for column chromatography. Spots were detected on TLC under UV or by heating after spraying with sulfuric acid/ethanol, 5:95 (v/v).

The producing organism, *S. bingchenggensis*, was isolated from a soil sample collected in Harbin, China. *S. bingchenggensis* has been deposited at the China General Microbiology Culture Collection Center (Accession No. CGMCC1734), and the 16S rDNA sequence has been determined (Accession No. DQ449953 in National Center for Biological Information).

3.2 Fermentation

The seed for preculture was spores. The medium for sporulation contained sucrose 4 g, yeast extract 2 g, malt extract 5 g, and skim milk 1 g in 1 l water. The pH was adjusted to 7.0 with 1 M NaOH, 20 g of agar was added, and this mixture sterilized at 121°C for 30 min. The spore suspension was prepared from agar plates incubated at 28°C for 7–8 days.

Table 1. ^{1}H and ^{13}C NMR spectral data of milbemycin α_{31} (**1**), secomilbemycins C (**2**), and D (**3**) (in $CDCl_3$).

	Proton			Carbon		
Position	**1**	**2**	**3**	**1**	**2**	**3**
1				167.4 s[a]	165.8 s	165.4 s
2				118.7 s	114.5 s	117.1 s
3	7.04 s	7.76 s	7.89 s	125.7 d	133.2 d	133.6 d
4				125.1 s	126.1 s	125.9 s
5				141.6 s	160.5 s	158.9 s
6		7.01 s	6.92 s	150.3 d	107.5 d	112.3 d
7				123.7 s	138.2 s	137.0 s
8				132.0 s	123.6 s	124.8 s
9	6.23 dt (11.2, 2.6)	6.49 d (11.1)	6.30 d (11.1)	123.4 d	128.0 d	130.8 d
10	5.85 dd (14.9, 11.2)	6.19 dd (14.9, 11.1)	6.54 dd (15.0, 11.1)	124.2 d	121.7 d	123.7 d
11	5.50 dd (14.9, 9.7)	5.97 dd (14.9, 6.6)	5.88 dd (15.0, 7.8)	143.2 d	146.4 d	147.5 d
12	2.51 m	2.53 m	2.48 m	35.6 d	33.8 d	36.3 d
13	2.31 m	2.20 m	2.06 m	48.5 t	48.2 t	46.6 t
14	1.90 br t (12.2)	0.90 dd (12.9, 10.2)		136.9 s	135.4 s	135.7 s
15	5.03 dd (10.4, 4.4)	5.09 t (6.9)	5.26 t (6.8)	122.0 d	122.3 d	121.5 d
16	2.25 m	2.28 m	2.26 m	34.3 t	34.3 t	34.2 t
17	3.68 m	3.60 m	3.58 m	67.6 d	68.1 d	68.2 d
18	2.12 m, 1.02 m	2.11 m, 1.20 m	2.02 m, 1.21 m	36.1 t	40.3 t	40.1 t
19	5.47 m	4.24 m	4.17 m	69.4 d	65.8 d	65.2 d
20	2.22 m, 1.33 m	2.08 m, 1.34 br t (11.9)	2.02 m,1.32 br t (12.0)	41.6 t	44.7 t	44.7 t
21				97.7 s	97.6 s	97.8 s
22	1.65 m, 1.54 m	1.65 m, 1.54 m	1.65 m, 1.53 m	35.6 t	35.8 t	35.5 t
23	1.50 m	1.50 m	1.50 m	27.9 t	27.8 t	27.7 t
24	1.35 m	1.24 m	1.25 m	34.3 d	36.6 d	36.5 d
25	3.10 m	3.26 m	3.26 m	76.0 d	71.3 d	71.3 d
26	2.23 s	2.20 s	2.27 s	15.2 q	15.6 q	15.7 q
27	5.24 d (2.6)	5.05 br s	4.80 d (11.5), 4.73 d (11.5)	74.9 t	66.7 t	73.9 t
28	1.05 d (6.8)	1.09 d (6.1)	1.03 d (6.8)	22.2 q	19.4 q	21.1 q
29	1.52 br s	1.62 br s	1.61 br s	15.3 q	16.1 q	17.5 q
30	0.83 d (6.5)	0.82 d (6.5)	0.81 d (6.5)	17.8 q	17.9 q	17.9 q

Table 1 – *continued*

Position	Proton			Carbon		
	1	**2**	**3**	**1**	**2**	**3**
31	1.70 m, 1.41 m	1.10 d (5.8)	1.10 d (6.2)	25.7 t	19.4 q	19.3 q
32	1.02 t (7.4)			10.2 q		

Note: Coupling constants are in parenthesis.
[a]By DEPT sequence.

A spore suspension of the culture of strain *S. bingchenggensis*, 5 ml, was transferred to a 1000-ml Erlenmeyer flask that contained 100 ml of the seed medium containing sucrose 0.25 g, polypeptone 0.1 g, and K_2HPO_4 1.25 mg. The inoculated flasks were incubated at 28°C for 42 h on a rotary shaker at 250 rpm. Then 1 l of the culture was transferred into 50 l fermenter containing 30 l 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%, and $FeSO_4{\cdot}7H_2O$ 0.005%, pH 7.2 before sterilization. Fermentation was carried out at 28°C for 8 days with the internal pressure and air flow rate fixed at 150 kPa and 0.5 vvm, respectively.

3.3 Extraction and isolation

The fermentation broth (30 l) was filtered. The resulting cake was washed with water and both filtrate and wash were discarded. Methanol (10 l) was used to extract the washed cake. The MeOH extract was evaporated under reduced pressure to approximately 2 l at 28°C 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 60 g of oily substances. The residual oily substance was chromatographed on silica gel and eluted with petroleum ether/acetone (95:5–50:50) to give five fractions. The semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250 × 9.4 mm i.d.) was further applied to obtain pure compounds. The eluates were monitored with a photodiode array detected at 220 nm and the flow rate was 1.5 ml/min at room temperature. The second fraction (petroleum ether/acetone 9:1) was separated by semi-preparative HPLC using a solvent of 96% CH_3CN/H_2O to afford **1** (t_R 30 min, 12 mg). Fraction **3** (petroleum ether/acetone 7:3) was also

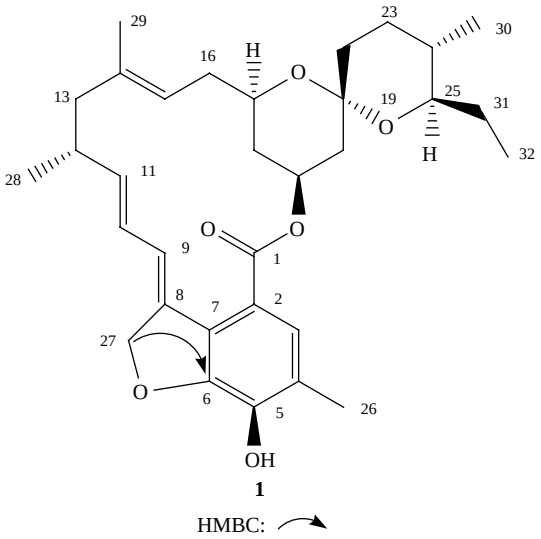

Figure 1. The structure and key HMBC correlations of **1**.

separated by semi-preparative HPLC (CH_3CN/H_2O 7:3) to give **2** (t_R 27.3 min, 25 mg) and **3** (t_R 26.1 min, 9 mg).

*3.3.1 Compound **1***

Compound **1** (Figure 1) was obtained as $C_{32}H_{42}O_6$, colorless oil; $[\alpha]_D^{20}$ +67.9 ($c = 0.14$, EtOH); UV (EtOH) λ_{max} nm (log ε): 356 (3.86), 301 (3.89), 257 (4.18), 227 (4.14), 204 (4.14); IR (KBr) ν_{max} cm^{-1}: 3360, 2922, 1708, 1616, 1509, 1455, 1376, 1277, 1204, 1103, 1031, 986; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR spectral data ($CDCl_3$, 100 MHz) are given in Table 1; ESI-MS *m/z* 521 $[M-H]^+$; HR-ESI-MS *m/z* 521.2980 $[M]^+$ (calcd for $C_{32}H_{42}O_6$, 521.2903).

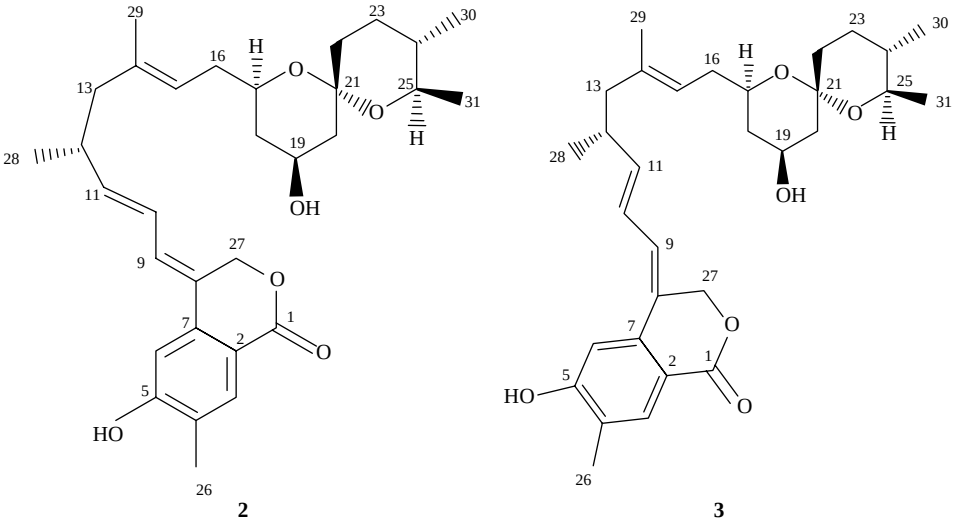

Figure 2. The structures of secomilbemycin C (**2**) and D (**3**).

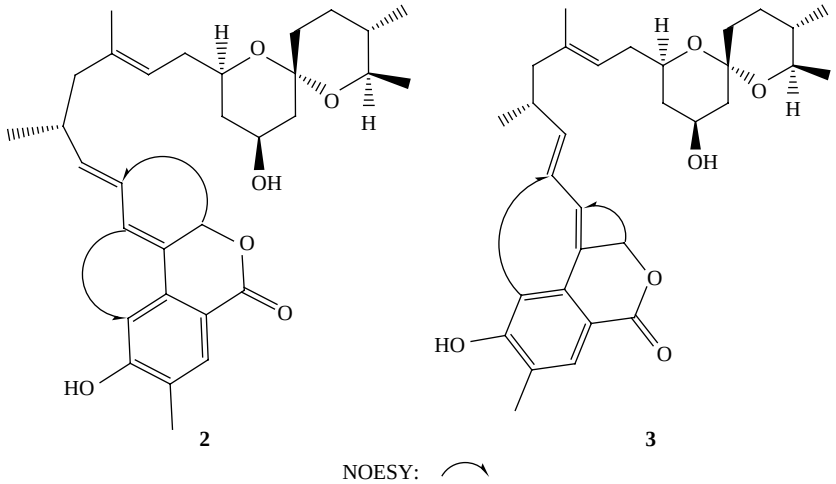

Figure 3. The key NOESY correlations of **2** and **3**.

*3.3.2 Compound **2***

Compound **2** (Figure 2) was obtained as $C_{31}H_{42}O_6$, colorless oil; $[\alpha]_D^{20}$ +25.0 ($c = 0.2$, EtOH); UV (EtOH) λ_{max} nm (log ε): 305 (4.30), 271 (4.38), 204 (4.29); IR (KBr) ν_{max} cm^{-1}: 3250, 2923, 1686, 1606, 1454, 1383, 1278, 1162, 1096, 986; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data are given in Table 1; ESI-MS *m/z* 509 $[M-H]^+$; HR-ESI-MS *m/z* 509.2980 $[M]^+$ (calcd for $C_{31}H_{42}O_6$, 509.2903).

*3.3.3 Compound **3***

Compound **3** (Figure 2) was obtained as $C_{31}H_{42}O_6$, colorless oil; $[\alpha]_D^{20}$ +27.5 ($c = 0.04$, EtOH); UV (EtOH) λ_{max} nm (log ε): 296 (4.23), 270 (4.31), 206 (4.43); IR (KBr) ν_{max} cm^{-1}: 3230, 2922, 1686, 1607, 1455, 1383, 1280, 1164, 1025, 982; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) spectral data are given in Table 1; ESI-MS *m/z* 509 $[M-H]^+$; HR-ESI-MS *m/z* 509.2980 $[M]^+$ (calcd for $C_{31}H_{42}O_6$, 509.2903).

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