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ORIGINAL ARTICLE

Three new milbemycins from a genetically engineered strain *S. avermitilis* MHJ1011

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Three new β-class milbemycins, 13α-hydroxy-4-ethy1 milbemycin β_3 (1), 13α-hydroxy-25-ethy1 milbemycin β_3 (2), 13α-hydroxy milbemycin β_3 (3), were isolated from the broth of the genetically engineered strains *Streptomyces avermitilis* MHJ1011, whose *aveA1* gene was replaced by *milA1* gene seamlessly. Their structures were determined on the basis of extensive spectroscopic analysis and comparison with data from the literature. These three compounds, especially compound 1, exhibited potent acaricidal activity.

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INTRODUCTION

Polyketide synthases have been attractive targets of biosynthetic engineering to make 'unnatural' natural products. By modification or deletion one or more of the genes encoding polyketide domains or modules, the genetically engineered strain was constructed[1] and exploited to make large numbers of new natural products, such as the analogs of erythromycin,[2,3] amphotericin[4,5] and daptomycin.[6] In our previous work, a genetically engineered strain *Streptomyces avermitilis* MHJ1011, whose *aveA1* gene was seamlessly replaced by *milA1* gene from *Streptomyces hygroscopicus*, has been constructed with the aim of obtaining new 16-membered macrolactone compounds. This led to the isolation of tenvermectins A and B (Figure 1) with

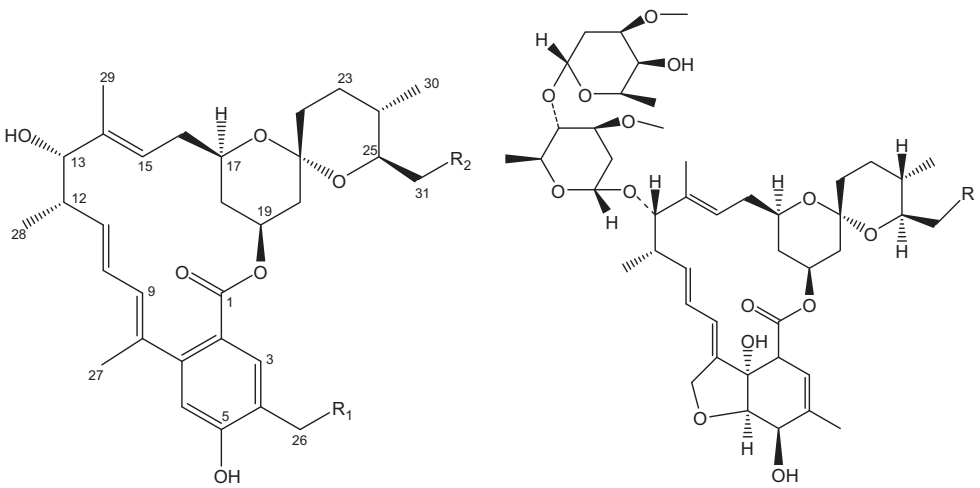

Figure 1 The structures of **1–3**, tenvermectins A and B.

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Table 1 ^{1}H and ^{13}C NMR data of compounds 1–3 (Coupling constants in parenthesis).

	Proton			*Carbon*		
Number	***1***	***2***	***3***	***1***	***2***	***3***
1				169.6 s	170.0 s	169.4 s
2				123.7 s	121.6 s	123.5 s
3	7.35 s	7.32 s	7.34 s	130.3 d	131.6 d	131.9 d
4				128.7 s	123.0 s	122.8 s
5				155.5 s	157.6 s	156.1 s
6	6.60 s	6.60 s	6.63 s	114.3 d	113.5 d	114.2 d
7				144.0 s	144.5 s	144.1 s
8				134.9 s	134.6 s	134.9 s
9	5.72 d (10.7)	5.60 d (10.7)	5.69 d (10.8)	128.6 d	127.9 d	128.5 d
10	6.16 dd (15.1, 10.8)	6.22 dd (15.1, 10.7)	6.16 dd (15.2, 10.8)	127.4 d	127.0 d	127.3 d
11	5.44 dd (15.1, 9.5)	5.45 dd (15.1, 10.0)	5.43 dd (15.2, 9.5)	134.3 d	134.6 d	134.3 d
12	2.61 m	2.62 m	2.61 m	40.4 d	40.7 d	40.5 d
13	4.02 brs	3.97 brs	4.02 brs	79.1 d	78.6 d	79.0 d
14				137.8 s	138.1 s	137.8 s
15	5.24 (brd, 10.1)	5.30 d (8.6)	5.24 d (10.4)	117.9 d	117.4 d	117.9 d
16	2.38 m	2.33 m	2.38 m	33.4 t	33.0 t	33.4 t
	2.27 m		2.32 m			
17	3.75 m	3.77 m	3.76 m	67.6 d	67.9 d	67.6 d
18	1.98 m	2.00 m	1.97 m	36.7 t	36.6 t	36.7 t
	0.76 q (12.0)	0.79 q (12.2)	0.77 q (11.8)			
19	5.51 m	5.40 m	5.47 m	68.1 d	68.0 d	68.0 d
20	1.97 m	1.93 m	1.97 m	41.2 t	41.2 t	41.2 t
	1.42 t (12.0)	1.37 t (12.0)	1.42 t (12.0)			
21				97.8 s	97.5 s	97.8 s
22	1.68 m	1.66 m	1.67 m	35.8 t	35.3 t	35.8 t
	1.55 m	1.54 m	1.55 m			
23	1.53 m	1.54 m	1.54 m	27.8 t	27.6 t	27.8 t
24	1.26 m	1.32 m	1.26 m	36.6 d	34.3 d	36.6 d
25	3.30 m	3.14 m	3.31 m	71.3 d	76.1 d	71.3 d
26	2.60 q (7.4)	2.17 s	2.22 s	22.5 t	14.3 q	15.4 q
27	2.07 s	2.06 s	2.06 s	18.1 q	17.1 q	18.1 q
28	1.21 d (6.9)	1.18 d (6.6)	1.21 d (6.8)	18.2 q	17.7 q	18.3 q
29	1.64 brs	1.66 brs	1.64 brs	15.1 q	13.9 q	15.1 q
30	0.84 d (6.5)	0.85 d (6.4)	0.84 d (6.5)	17.9 q	16.7 q	17.9 q
31	1.14 d (6.2)	1.74 m	1.14 d (6.2)	19.4 q	25.5 t	19.4 q
		1.35 m				
32	1.23 t (7.5)	1.01 t (7.3)		13.6 q	9.2 q	

excellent insecticidal activity.[7] The in-depth chemical study of fermentation broth of the strain *S. avermitilis* MHJ1011 resulted in identification of three new β-class milbemycins (**1–3**, Figure 1). Herein, we describe the fermentation, isolation, structural elucidation and acaricidal activity of the three new compounds.

RESULTS AND DISCUSSION

Structural elucidation

In the course of fermentation and isolation of tenvermectins A and B from the strain *S. avermitilis* MHJ1011, the by-products were pooled and a crude extract was obtained. The pooling crude extract was isolated by silica gel column chromatography and semi-preparative HPLC to afford three new compounds (**1–3**).

The molecular formula of compound **1** was established to be $C_{32}H_{44}O_6$ as deduced from the HRESI-MS *m/z* 525.3206 $[M+H]^+$ (calcd for $C_{32}H_{45}O_6$ 525.3211) and ^{13}C NMR data (Table 1). The ^{1}H NMR spectrum (Table 1) of **1** displayed three doublet aliphatic methyls at δ_H 0.84 (3H, d, $J=6.5$ Hz), 1.14 (3H, d, $J=6.2$ Hz), 1.21 (3H, d, $J=6.9$ Hz), two olefinic methyl signals at δ_H 1.64 (3H, brs) and 2.07 (3H, s), one trans-double bond at δ_H 5.44 (1H, dd, $J=15.1$, 9.5 Hz), 6.16 (1H, dd, $J=15.1$, 10.8 Hz) and two downfield proton signals at δ_H 7.35 (1H, s) and 6.60 (1H, s). The ^{13}C NMR and HMQC spectra revealed 32 carbon resonances, including an ester carbonyl carbon at δ 169.6 (s), a ketal carbon at δ 97.8 (s), four oxygenated methines at δ_C 79.1 (d), 71.3 (d), 68.1 (d) and 67.6 (d), two aliphatic methines at δ_C 40.4 (d) and 36.6 (d), six methyls at δ_C 19.4 (q), 18.2 (q), 18.1 (q), 17.9 (q), 15.1 (q) and 13.6 (q) in addition to five aliphatic methylenes and 14 sp^2 carbons. By detailed comparison of the NMR data of **1** with those of milbemycin β_3,[8,9] it was revealed that **1** was similar to milbemycin β_3. The differences between **1** and milbemycin β_3 were that a hydroxyl group substituted at C-13 and an ethyl group situated at C-4 in **1**. The ^{1}H-^{1}H COSY correlation of δ_H 2.61/ δ_H 4.02, δ_H 1.23/δ_H 2.60, and the observed HMBC correlations from δ_H 1.21 and 1.64 to δ_C 79.1, from δ_H 1.23 to δ_C 22.5, 128.7

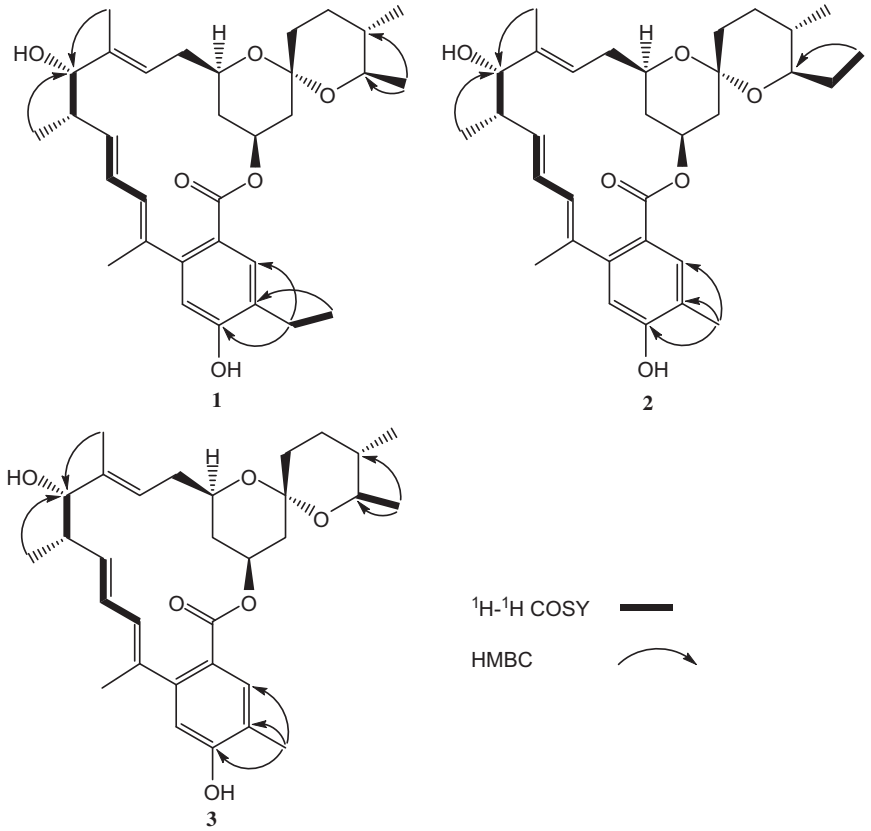

Figure 2 Key ^{1}H-^{1}H COSY and HMBC correlations of **1–3**.

Table 2 Acaricidal activity of 1-3 against *Tetranychus cinnabarinus.*

Compounds	*Virulence regression equation (Y)*	*LC_{50} (mg l^{-1})*	*Coefficient correlation*	*95% confidence limits*
1	6.7175+1.0239*X*	0.0210	0.9569	0.01–0.04
2	6.0897+1.1006*X*	0.1023	0.8985	0.02–0.42
3	5.9488+0.9855*X*	0.1090	0.9568	0.02–0.55
[a]Milbemycins A3/A4	6.6414+1.1021*X*	0.0324	0.9478	0.01–0.08
[b]Tenvermectins A/B	8.7534+1.6327X	0.0050	0.9514	0.00–0.01

[a]Milbemycins A3 and A4 mixture, 30: 70 (in weight).
[b]Tenvermectins A and B mixture, 75: 25 (in weight).

(Figure 2) supported the assignment. Compared with milbemycin β_3, the 30 mass unit enhancement of **1** further confirmed the structure of **1**. In the ^{1}H NMR spectrum, the signal of H-13 displayed a broad singlet, thus the 13-hydroxy group was determined to be α.[10] The other relative stereochemistry of **1** was assigned as occurring with that of tenvermectins A and B.[7] So, the structure of **1** was assigned as 13α-hydroxy-4-ethy1 milbemycin β_3.

Compounds **2** and **3** were also obtained as colorless oil. The ^{1}H and ^{13}C NMR data (Table 1) of **1** and **2** were very similar to those of **1**, which suggested that the three compounds possessed the same skeleton. The HMBC correlations from H_3-32 to C-25, from H_3-26 to C-3, C-4, C-5 and the cross peak of H_3-32/H_2-31 in the ^{1}H-^{1}H COSY spectrum (Figure 2) revealed that **2** is 13-hydroxy-25-ethy1 milbemycin β_3. For **3**, the HMBC correlations between H_3-26 and C-3, C-4, C-5, between H-31 and C-24, C-25 and the ^{1}H-^{1}H COSY correlation of H_3-31/H-25 (Figure 2) established the structure of **3** to be 13-hydroxy milbemycin β_3. The relative configurations of **2** and **3** were assigned by analogy to **1**.

Biological activity

The three new β-class milbemycins **1–3** possess potent acaricidal activity (Table 2). Especially, **1** was more active than **2**, **3** and milbemycins A3/A4 mixture against larval mites. This result further demonstrated C-26 substituted milbemycins possessing high activity.[11]

MATERIALS AND METHODS

General

UV spectra were obtained on a Varian CARY 300 BIO spectrophotometer (Varian, Palo Alto, CA, USA); IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrometer (Nicolet Magna, Madison, WI, USA); ^{1}H and ^{13}C NMR spectra were measured with a Bruker DRX-400 (400 MHz for ^{1}H and 100 MHz for ^{13}C) spectrometer (Bruker, Rheinstetten, Germany). Chemical shifts are reported in p.p.m. (δ), using residual $CHCl_3$ (δ_H 7.26 ppm; δ_C 77.0) as an internal standard, with coupling constants (J) in Hz. ^{1}H and ^{13}C NMR assignments were supported by ^{1}H-^{1}H COSY, HMQC and HMBC experiments. The ESI-MS and high resolution electrospray ionization (HRESI-MS) spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer (Waters, Milford, MA, USA). Optical rotation was measured on a Perkin-Elmer

341 polarimeter (Perkin-Elmer, Fremont, CA, USA). Commercial silica gel (Qing Dao Hai Yang Chemical Group Co., 100–200 mesh). Spots were detected on TLC under UV or by heating after spraying with sulfuric acid-ethanol, 5:95 (v/v).

Microorganism

Avermectin is produced by *S. avermitilis* and milbemycin can be produced by *Streptomyces hygroscopicus*. Both avermectin and milbemycin are biosynthesized by polyketide synthases. The C22–25 substructures of avermectin and milbemycin are determined by *aveA1* and *milA1*, respectively. So plasmid used for replacing *aveA1* gene with *milA1* gene was constructed and it was transformed into *S avermitilis* G8–17, an avermectin B industrial strain. After subculture of a transformant, the genetically engineered strain MHJ1011, whose *aveA1* gene was replaced by *milA1* gene seamlessly, was screened.[7]

Fermentation

The strain *S. avermitilis* MHJ1011 was maintained on the medium containing glucose 10 g, malt extract (Bei Jing Ao Bo Xing, Beijing, China) 3 g, yeast extract (Bei Jing Ao Bo Xing) 3 g, $K_2HPO_4{\cdot}3H_2O$ 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, NaCl 0.5 g, KNO_3 1 g and agar 20 g in 1.0 l of tap water, pH 7.0. The seed medium consisted of corn starch (Shanghai Guoqiang Bioengineering Equipment, Shanghai, China) 25.0 g, soybean meal (Ningbo Beilun Jiangnan Grease Co., Ltd, Ningbo, China) 8.0 g, peanut meal (Bei Jing Ao Bo Xing) 10.0 g, yeast extract 9.5 g, $CoCl_2{\cdot}6H_2O$ 0.03 g in 1-liter water and pH 7.4. All the media were sterilized at 121 °C for 20 min. Slant culture was incubated for 6–7 days at 28 °C. Fermentation was carried out in 50 l of fermentor (containing 30 l of production medium). The producing medium was composed of corn starch 14%, soybean meal 0.2%,α-amylase (Bei Jing Ao Bo Xing) 0.003%, yeast extract 1.0%, zeolite powder (Kaixi, Jiande City, Zhejiang, China) 0.2%, $CoCl_2{\cdot}6H_2O$ 0.002%, $MnSO_4$ 0.0024%, $NaMoO_4$ 0.0024% and pH 7.4. The fermentation was conducted at 28 °C for 10 days stirred at 100 r.p.m with an aeration rate of 900 l of air per hour.

Isolation and purification

The final 30 l of fermentation broth was filtered and the resulting cake was extracted with ethanol (10 l). The ethanol extract was evaporated under reduced pressure to 1 l at 45 °C and subsequently extracted three times using an equal volume of ethyl acetate. The combined ethyl acetate phase was concentrated under reduced pressure to yield 260 g of oily substances. A amount of 5 g of the residual oily substance was subjected to a silica gel column and successively eluted with a stepwise gradient of petroleum ether/acetone (90:10–60:40, v/v) to afford four fractions (I–IV) based on the (TLC profiles. The fraction I eluted with petroleum ether/acetone (90:10–80:20, v/v) was chromatographed on silica gel and eluted with petroleum ether-acetone (100:0–70:30, v/v) to give eight fractions. The fourth fraction was separated by the semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250 × 9.4 mm inner diameter; Agilent, Palo Alto, CA, USA) using a solvent containing a CH_3CN: H_2O mixture (95: 5, v/v) to obtain compounds **1** (t_R 15.1 min, 80 mg), **2** (t_R 14.2 min, 40 mg) and **3** (t_R 13.5 min, 35 mg).

13α-hydroxy-4-ethy1 milbemycin β_3 (**1**): colorless oil; $[\alpha]_D^{25} + 25.7$ (*c* 0.13, EtOH); UV (EtOH) λ_{max} nm (log ε): 247 (4.17); IR (KBr), ν_{max} cm^{-1}: 3396, 1703, 2929, 1382, 1281, 1162, 994; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data see Table 1; ESI-MS *m/z* 525 $[M+H]^+$; HRESI-MS *m/z* 525.3206 $[M+H]^+$ (calcd for $C_{32}H_{45}O_6$ 525.3211).

13α-hydroxy-25-ethy1 milbemycin β_3 (**2**): colorless oil; $[\alpha]_D^{25} + 25.7$ (*c* 0.07, EtOH); UV (EtOH) λ_{max} nm (log ε): 247 (4.33); IR (KBr), ν_{max} cm^{-1}: 3395, 1714, 2928, 1455, 1163, 996; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data see Table 1; ESI-MS *m/z* 525 $[M+H]^+$; HRESI-MS *m/z* 525.3205 $[M+H]^+$ (calcd for $C_{32}H_{45}O_6$ 525.3211).

13α-hydroxy milbemycin β_3 (**3**): colorless oil, $[\alpha]_D^{25} + 36.6$ (*c* 0.17, EtOH); UV (EtOH) λ_{max} nm (log ε): 245 (3.97); IR (KBr), ν_{max} cm^{-1}: 3409, 1702, 2928, 1382, 1279, 1163, 997; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data see Table 1; ESI-MS *m/z* 509 $[M-H]^-$; HRESI-MS *m/z* 509.2908 $[M-H]^-$ (calcd for $C_{31}H_{41}O_6$ 509.2909).

Acaricidal activity test

The larvicidal activities of test compounds against *Tetranychus cinnabarinus* reared in the laboratory were tested according to the reported procedure.[12] Each test sample was prepared in acetone at a concentration of 1000 mg l^{-1} and diluted to the required concentration of 0.01, 0.005, 0.0025, 0.001 and 0.0005 mg l^{-1} with distilled water containing alkylphenol ethoxylates (1/1000). The primary leaves of *Vicia faba* L. species were infected with carmine spider mites. At 2 h after infection, 10 fourth-instar mite larvae were dipped in the diluted solutions of related chemicals for 5 s before the superfluous liquid was removed and larvae were kept in a conditioned room. The experiments were repeated three times and blank controls. The mortality was evaluated 24 h after treatment by examining the adult mites under a binocular microscope to determine the living and dead individuals.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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