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NOTE

Two new α-class milbemycin metabolites from mutant *Streptomyces avermitilis* NEAU1069-3

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Microbial metabolites attract increasing attention as potential pesticides owing to their potential bioactivity and low toxicity to non-target animals and humans.[1,2] Several microbial metabolites, such as avermectins, milbemycins, have been commercialized and considered to be the most widely used drugs in animal health and agriculture.[3] During the course of the screening program for new natural pesticides and antiparasitic veterinary drugs, two novel macrocyclic lactones, three milbemycins and six new doramectin congeners have been isolated from *Streptomyces avermitilis* NEAU1069.[4–7] To screen for more bioactive compounds, a mutant *S. avermitilis* NEAU1069-3 was obtained by UV treatment and two new doramectin analogs were isolated from its fermentation broth.[8] In further work to explore the chemical diversity of the constituent of *S. avermitilis* NEAU1069-3, two new α-class milbemycins, 23, 24-didehydro-13α-hydroxy milbemycin A_3 (**1**) and 24, 30-didehydro-13α-hydroxy milbemycin A_3 (**2**) were isolated from the broth of *S. avermitilis* NEAU1069-3. Herein, we describe the fermentation, isolation, structural elucidation and insecticidal activity of the two new α-class milbemycins.

The culture and fermentation of *S. avermitilis* NEAU1069-3 were conducted according to the procedure as described previously.[6] Thirty liters of broth from 300×100 ml fermentations 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 2 l at 45 °C and the resulting concentrate was extracted three times using an equal volume of EtOAc. The combined EtOAc phase was concentrated under reduced pressure to yield 26 g of oily substances. The residual oily substance was chromatographed on silica gel (Qingdao Haiyang Chemical Group, Qingdao, China; 100–200 mesh) and eluted with a petroleum ether-acetone mixture (100:0 – 50:50, v/v). The fractions eluted with petroleum-acetone mixture (85:15, v/v) were combined and evaporated to obtain fraction I and the fractions eluted with the petroleum ether-acetone mixture (80:20, v/v) were pooled and concentrated to give fraction II. The fraction I was subjected to Sephadex LH-20 (GE Healthcare, Glies, UK) column eluting with MeOH to give subfraction I. The semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250×9.4 mm i.d.; Agilent, Palo Alto, CA, USA) was applied to obtain pure compounds. The eluates were monitored using a photodiode array detector at 254 nm, and the flow rates were 1.5 ml min^{-1} at room temperature. The subfraction I was further separated by semi-preparative HPLC using a solvent containing a CH_3OH-CH_3CN-H_2O mixture (72:7:21, v/v/v) to obtain compound **1** (t_R 17.3 min, 12 mg) and the fraction II was subjected to Sephadex LH-20 column eluting with MeOH to give subfraction II and the subfraction II was purified by the semi-preparative HPLC using a solvent containing a CH_3OH-CH_3CN-H_2O mixture (69:24:7, v/v/v) to obtain compound **2** (t_R 30.1 min, 8 mg).

Compound **1** was isolated as colorless oil with $[\alpha]_D^{25}$ 43.3 (*c* 0.03, EtOH) and UV (EtOH) λ_{max} nm (log ε): 245 (4.01). The positive HRESIMS showed a pseudo molecular ion at *m/z* 565.2759 $(M+Na)^+$, corresponding to the molecular formula $C_{31}H_{42}O_8$ that required 11 degrees of unsaturation. The IR spectrum of **1** showed absorption bands assignable to the hydroxyl group (3400 cm^{-1}) and carbonyl group (1710 cm^{-1}). The ^1H NMR spectrum of **1** showed two doublet aliphatic methyl signals at δ_H 1.15 (3H, d, $J = 7.0$ Hz), 1.24 (3H, d, $J = 6.7$ Hz), three olefinic methyl signals at δ_H 1.52 (3H, br s), 1.61 (3H, br s), 1.79 (3H, br s) and one trans-double bond at δ_H 5.73 (1H, dd, $J = 14.9$, 9.8 Hz) and 5.93 (1H, dd, $J = 14.9$, 11.2 Hz). Inspection of the ^{13}C NMR (DEPT) and HMQC spectra revealed the existence of an ester carbonyl at δ_C 171.7 (s), a ketal at δ_C 96.7 (s), six oxygenated methines at δ_C 80.7 (d), 76.7 (d), 68.5 (d), 68.1 (d), 67.7 (d) and 67.0 (d), two aliphatic methines at δ_C 45.8 (d), 40.2 (d), one oxygenated quaternary carbon at δ_C 80.4 (s), one oxygenated methylene at δ_C 67.4 (t), five methyls at δ_C 18.9 (q), 18.9 (q), 18.4 (q), 18.3 (q) and 13.7 (q) in addition to four aliphatic methylenes and ten sp^2 carbons. Comparison of the ^1H NMR data (Table 1) of **1** with those of the milbemycin A_3 suggested that **1** was similar to milbemycin A_3[9]. The differences between **1** and milbemycin A_3 were that **1** contained a double bond at C-23 and C-24 and a hydroxy group at C-13. The ^1H-^1H COSY correlation of δ_H 4.19 and δ_H 2.59, and the

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Table 1 ^{1}H and ^{13}C NMR data of compounds 1 and 2

	δ_H (*J* in Hz)		δ_C	
Position	***1***	***2***	***1***	***2***
1			171.7 (s)	173.6 (s)
2	3.20 br s	3.26 br s	45.8 (d)	45.7 (d)
3	5.37 br s	5.39 br s	118.4 (d)	118.1 (d)
4			136.7 (s)	137.8 (s)
5	4.19 br s	4.29 br s	67.7 (d)	67.7 (d)
6	3.80 br s	3.97 d (6.2)	80.7 (d)	79.1 (d)
7			80.4 (s)	80.2 (s)
8			140.5 (s)	140.0 (s)
9	5.83 br d (11.2)	5.81 d (10.4)	120.2 (d)	120.3 (d)
10	5.93 dd (14.9, 11.2)	5.75 dd (14.8, 10.4)	124.8 (d)	124.8 (d)
11	5.73 dd (14.9, 9.8)	5.70 dd (14.8, 9.8)	137.1 (d)	136.9 (d)
12	2.59 m	2.53 m	40.2 (d)	40.2 (d)
13	4.19 br s	4.02 br s	76.7 (d)	77.6 (d)
14			139.1 (s)	138.7 (s)
15	5.53 br t (8.0)	5.39 m	117.3 (d)	117.2 (d)
16	2.23 m	2.34 m	34.0 (t)	34.2 (t)
17	3.77 m	3.71 m	68.5 (d)	67.9 (d)
18	1.92 m	1.82 m	36.3 (d)	36.7 (t)
	0.85 t (12.2)	0.87 q (12.2)		
19	5.11 m	5.39 m	68.1 (d)	68.3 (d)
20	2.21 m	2.00 m	40.9 (t)	40.6 (t)
	1.29 t (12.0)	1.37 t (11.9)		
21			96.7 (s)	97.9 (s)
22	1.97 m	1.64 m	35.7 (t)	37.2 (t)
	2.17 m			
23	5.29 m	2.59 m	116.2 (d)	28.6 (t)
24		2.21 m	134.6 (s)	147.2 (s)
25	4.11 m	4.14 q (6.4)	67.0 (d)	66.3 (d)
26	1.79 br s	1.87 br s	18.9 (q)	19.9 (q)
27	4.64 dd (14.4, 2.1)	4.66 dd (14.6, 2.2)	67.4 (t)	68.5 (t)
	4.57 dd (14.4, 2.1)	4.70 dd (14.6, 1.8)		
28	1.15 d (7.0)	1.18 d (7.0)	18.9 (q)	19.1 (q)
29	1.52 br s	1.54 br s	13.7 (q)	14.6 (q)
30	1.61 br s	4.79 d (4.6)	18.3 (q)	106.9 (t)
31	1.24 d (6.7)	1.30 d (6.4)	18.4 (q)	17.4 (q)

observed HMBC correlation from H_3-30 to δ_C 116.2 (C-23), δ_C 135.6 (C-24) and δ_C 62.0 (C-25) and from H_3-28 and H_3-29 to δ_C 76.7 (C-13) further confirmed the structural assignment of **1**. As a result, the gross structure of **1** was established as shown in Figure 1. The small coupling constant of H-13 (1H, br s) compared with that of doramectin[10] indicated that the 13-hydroxy was α-oriented. The other relative stereochemistry of **1** was assigned by analogy with milbemycin A_3.

Compound **2** was isolated as colorless oil with $[\alpha]_D^{25}$73.3 (*c* 0.03, EtOH) and UV (EtOH) λ_{max} nm (log ε): 245 (4.29). It's molecular formula was determined to be $C_{31}H_{42}O_8$ on the basis of HRESIMS at *m/z* 565.2743 $[M+Na]^+$ (cald 565.2772 for $C_{31}H_{42}NaO_8$), indicating 11 degrees of unsaturation. The IR spectrum of **2** showed absorption bands assignable to the hydroxyl group (3400 cm^{-1}) and carbonyl group (1730 cm^{-1}). The ^1H NMR spectrum of **2** showed one trans-double bond at δ_H 5.75 (1H, dd, $J=14.8$, 10.4 Hz) and 5.70 (1H, dd, $J=14.8$, 9.8 Hz), two olefinic methyls at δ_H 1.54 (3H, br s), 1.87 (3H, br s), two aliphatic doublet methyls at δ_H 1.18 (3H, d, $J=7.0$ Hz) and 1.30 (3H, d, $J=6.4$ Hz). The ^{13}C NMR and DEPT spectra of **2** displayed an ester carbonyl at δ_C 173.6 (s), a ketal carbon at δ_C 97.9 (s), four sp^2 quaternary carbons, five sp^2 methines, one sp^2 methylene, one oxygenated quaternary carbon, six oxygenated methines, one oxygenated methylene in addition to two aliphatic methines, five aliphatic methylenes and four methyls. Detailed analysis of the ^1H and ^{13}C NMR data of **2** (Table 1) suggested that **2** has a same scaffold as **1**, except the $\Delta^{23,24}$ olefin in **1** was disappeared and a $\Delta^{24,30}$ olefin was present in **2**. The downfield ^{13}C NMR chemical shift at C-30 (δ_C 106.9) and the HMBC correlations (Figure 1) from δ_H 4.79 to δ_C 28.6 (t, C-23), 147.2 (s, C-24) and 66.3 (d, C-25) and from δ_H 1.30 (3H, d) to 147.2 (s, C-24) and 66.3 (d, C-25) further confirmed the structural assignment of **2** as shown in Figure 1. The relative stereochemistry of **2** was assigned by analogy with **1**.

The insecticidal activities of compounds **1** and **2** against *Brevicoryne brassicae* (L.) were tested by the leaf dip method and the insecticidal capacities of the two compounds were compared with milbemycin A_3/A_4. In this technique, the aphids were collected from insecticide-

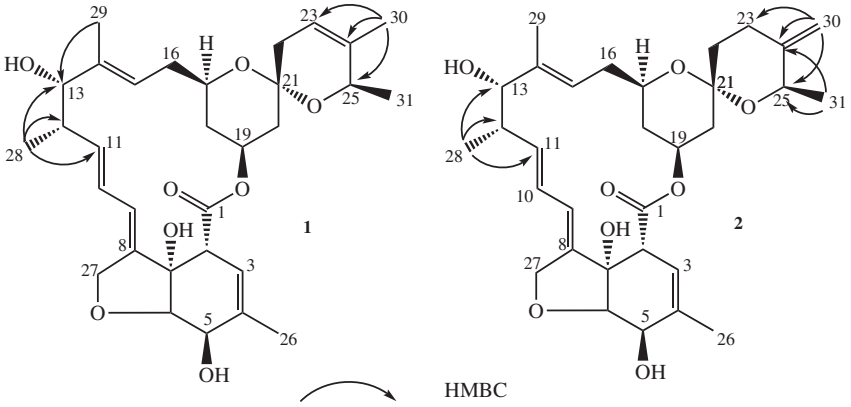

Figure 1 Structures and key HMBC correlations of compounds **1** and **2.**

Table 2 Insecticidal activities of compounds 1 and 2 against *Brevicoryne brassicae*

Compound	*Y*	*IC_{50} (mg l^{-1})*	*Coefficient correlation*	*95% Confidence interval*
1	5.4063+1.3447X	0.4987	0.9928	0.41–0.61
2	5.3108+1.2708X	0.5694	0.9972	0.46–0.71
Milbemycins A_3/A_4[a]	5.0878+1.4836X	0.8726	0.9945	0.7–1.09

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

free flowering-Chinese cabbage and reared at room temperature. Serial dilutions (five concentrations) of each compound were prepared in distilled water for use in the bioassays. Leaf discs with a diameter of ~35 mm were prepared from cabbage leaves and dipped for 30s in various concentrations of each test material. Leaf discs dipped only in distilled water served as controls. The dried leaf discs were then transferred to Petri dishes and 80–90 aphids were released in each dish. The mortality was recorded after 24 h. The mortality of the treated compound was corrected using the control mortality and the corrected data were used to calculate LC_{50} values. The bioassays were performed simultaneously on three replicates for each concentration. The bioassay results (Table 2) demonstrated that compounds **1** and **2** have good aphidicidial activities and would be a potential insecticide

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