NOTE

New β -class milbemycin compound from *Streptomyces avermitilis* NEAU1069: fermentation, isolation and structure elucidation

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Microbial metabolites attract increasing attention as potential pesticides. They are expected to overcome the resistance and pollution that have accompanied the use of synthetic pesticides.^{1,2} Our experiments were conducted as part of a program to screen new antibiotics for pesticides and antiparasitic veterinary drugs, or as semisynthetic intermediates. As a result, a new compound **1**, two known milbemycins (compounds **2** and **3**, Figure 1) and avermectin B_{1a} were isolated from the fermentation broth of *Streptomyces avermitilis* NEAU1069 strain, which was newly obtained from a soil sample. The structures of compounds **1**, **2** and **3** were elucidated by UV, IR, electrospray ionization (ESI)-MS, high-resolution ESI (HRESI)-MS, extensive 1D and 2D NMR analyses, and a comparison with data reported in literature. Their structures are similar to milbemycin β 3,^{3–6} β 4,^{7,8} β 13,⁹ β 14⁹ and aromatic S541 analogs,¹⁰ which are generated by the nematode-producing strains *S. thermoarchaensis* NCIB 12015 and NCIB 12212. However, there are differences among them at positions C-23 and C-25. β -Class milbemycins have not been previously reported as being from *S. avermitilis*.^{3–10} This paper describes the fermentation, isolation and identification of compounds **1**, **2** and **3**. Moreover, the spectroscopic data of compounds **2** and **3** are reported for the first time (although the structures of compounds **2** and **3** could be searched in SciFinder Scholar (Chemical Abstract Service, ACS; <http://www.cas.org/products/sfacad/index.html>), no reference was cited. Furthermore, they were not reported). The discovery of these three compounds possibly has an important role in understanding and perfecting the proposed pathways of avermectins and milbemycins. In addition, it is significant that they are useful intermediates in the preparation of other derivatives.¹¹

MATERIALS AND METHODS

Screening, isolation and identification of the producing organism

The agar plate dilution method was used to separate the microorganism reported in this paper. A sample soil of 0.5 kg obtained from Harbin city,

China, was taken in a sterilized test tube and sterilized water was added thereto. After 10 min of setting, supernatant was obtained. The supernatant thus obtained was 100-fold diluted using sterilized water, and 0.1 ml of the diluted solution was distributed onto a yeast–malt extract (yeast extract 4 g l^{–1}, malt extract 10 g l^{–1}, glucose 4 g l^{–1}, agar 20 g l^{–1} and distilled water 1 l, pH 7.0–7.2) taken in a petri dish and cultured at 28 °C for 15 days. Colonies formed on the yeast–malt extract agar, from which a single colony was isolated using a sterilized needle. The obtained colony was transferred to a fresh yeast–malt extract agar at regular intervals for 10 days, followed by submerged fermentation. Metabolites were extracted by adding 4 ml methanol to 2 ml whole broth and mixing intermittently for 3 h. The extracts were examined on two-spotted spider mites to determine whether they have acaricidal activity. Then, the metabolites with acaricidal activity were purified and their structures were determined.

To identify the isolated strain with acaricidal activity in the metabolites, morphological properties of cells, cultural characteristics of cells, morphological characteristics of colonies, physiological characteristics, utilization of a carbon source and 16S rDNA (Accession No: DQ768097 in National Center for Biological Information, Bethesda, MD, USA) were investigated. The strain was named *S. avermitilis* NEAU1069. It has been deposited at the China General Microbiology Culture Collection Center, Beijing, China (Accession No: CGMCC 2943).

Fermentation

The strain was maintained on a YMS (yeast extract–malt extract–soluble starch) medium containing soluble starch (Beijing Ao Bo Xing, Beijing, China) 10 g, yeast extract (Beijing Ao Bo Xing) 2 g, KNO₃ 1 g and agar 20 g in 1.0 l tap water, pH 7.0. The seed medium consisted of glucose (Beijing Ao Bo Xing) 20 g, soybean flour (Comwin, Beijing, China) 15 g and yeast autolysate (Beijing Ao Bo Xing) 5.0 g in 1.0 l water, pH 7.0. Both the media were sterilized at 121 °C for 20 min. Slant cultures were incubated for 6–8 days at 28 °C.

A total of 10 ml of sterile water was added to the slant of the YMS medium. The spores were scraped and transferred onto a sterile tube containing glass beads; the spore suspension was then filtered through six layers of a sterile filter cheesecloth and adjusted to 10⁷–10⁸ c.f.u ml^{–1}. A 2.0 ml of the spore suspension was inoculated into a 250-ml flask containing 25 ml of seed medium and incubated at 28 °C for 24 h, shaken at 250 r.p.m. Then, 8 ml of the culture was

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Figure 1 The structures of compounds **1**, **2** and **3**.

transferred into a 1-l Erlenmeyer flask containing 100 ml of the producing medium consisting of corn starch (Comwin) 10%, soybean powder 1%, cotton flour (Comwin) 1%, α -Amylase (Beijing Ao Bo Xing) 0.02%, NaCl 0.1%, K_2HPO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCO_3$ 0.7% and pH 7.0, before sterilization. Fermentation was carried out at 28 °C for 12–13 days on a rotary shaker at 250 r.p.m.

Isolation and purification

A total of 3 l of broth from 40 producing fermentations was filtered. The resulting cake was washed with water (3 l), and both filtrate and wash were discarded. Methanol (1 l) was used to extract the washed cake. The MeOH extract was evaporated under reduced pressure to approximately 0.2 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 5 g of oily substances. The residual oily substance was chromatographed on silica gel (Qingdao Haiyang Chemical Group, Qingdao, Shandong, China; 100–200 mesh) and eluted with a petroleum (60 °C –90 °C)–acetone mixture (95:5, 75:25 and 50:50, v/v). The fractions eluted with the petroleum–acetone mixture (95:5–75:25, v/v) were combined and evaporated to obtain a crude mixture. The crude mixture was applied to a silica gel column and eluted with a petroleum–EtOAc mixture (95:5, 85:25, 75:25, 65:35 and 50:50, v/v) to give five fractions.

Semipreparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μ m, 250 $\times$ 9.4 mm i.d.; Agilent, Palo Alto, CA, USA) and preparative HPLC (Shimadzu LC-8A, Shimadzu-C18, 5 μ m, 250 $\times$ 20 mm i.d.; Shimadzu, Kyoto, Japan) were further

performed to obtain pure compounds. The eluates were monitored using a photodiode array detected at 220 nm, and the flow rates were 1.5 ml min⁻¹ for the semipreparative HPLC and 20 ml min⁻¹ for the preparative HPLC at room temperature. Fraction one was purified by semipreparative HPLC using a solvent containing a CH₃OH–H₂O mixture (97:3, v/v) to obtain **2** (t_R 10.6 min, 13 mg). Fraction two was also subjected to silica gel elution using petroleum–EtOAc mixture (85:15 and 75:25, v/v) to obtain two subfractions. Subfraction one was further separated by semipreparative HPLC elution using CH₃OH–H₂O mixture (92:8, v/v) to afford compound **1** (t_R 12.3 min, 11 mg). Subfraction two was purified by preparative HPLC using CH₃OH–H₂O mixture (8:2, v/v) to obtain **3** (t_R 17.1 min, 24 mg).

General

Melting points were measured using a Fisher–Johns micro-melting point apparatus (corrected; Fisher–Johns, Pittsburgh, PA, USA); 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); ¹H and ¹³C-NMR spectra were measured using a Bruker DRX-400 (400 MHz for ¹H and 100 MHz for ¹³C) spectrometer (Bruker, Rheinstetten, Germany); chemical shifts are reported in p.p.m (δ) using the residual CHCl₃ (δ_H 7.26; δ_C 77.0) as an internal standard, and coupling constant (*J*) in Hz. ¹H and ¹³C-NMR assignments were supported by ¹H–¹H COSY, heteronuclear multiple quantum coherence and heteronuclear multiple bond correlation (HMBC) experiments. The ESI-MS and HRESI-MS spectra were taken on a Q-TOF Micro LC-MS-MS mass spectrometer (Waters,

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

Position	Proton			Carbon		
	1	2	3	1	2	3
1				168.9 s ^a	169.1 s	169.0 s
2				123.6 s	123.8 s	123.3 s
3	7.41 s	7.39 s	7.39 s	132.3 d	132.1 d	132.2 d
4				122.5 s	122.4 s	122.7 s
5				155.8 s	155.6 s	156.1 s
6	6.61 s	6.61 s	6.61 s	114.3 d	114.2 d	114.2 d
7				144.5 s	144.3 s	144.3 s
8				135.0 s	135.0 s	135.1 s
9	5.70 d (10.8)	5.73 d (10.8)	5.68 dd (1.1, 10.9)	128.5 d	128.5 d	128.4 d
10	6.16 dd (10.8, 15.0)	6.17 dd (10.9, 15.1)	6.16 dd (10.9, 15.1)	127.3 d	127.4 d	127.3 d
11	5.45 dd (9.6, 15.2)	5.45 dd (9.6, 14.5)	5.45 dd (9.6, 15.1)	134.5 d	134.3 d	134.3 d
12	2.64 m	2.62 m	2.61 m	40.7 d	40.6 d	40.6 d
13	4.02 br s	4.02 br s	4.02 br s	79.1 d	79.1 d	79.1 d
14				138.3 s	137.8 s	138.3 s
15	5.17 br d (8.6)	5.21 br d (8.7)	5.20 d (8.9)	117.4 d	117.7 d	117.2 d
16	2.28 m	2.32 m	2.34 m	33.1 t	33.7 t	33.4 t
		2.39 m				
17	3.76 m	3.98 m	3.84 m	68.6 d	68.6 d	68.7 d
18	0.78 q (12.0)	0.79 q (12.1)	0.79 q (12.4)	36.1 t	36.6 t	36.3 t
	1.97 m	1.97 m	1.97 m			
19	5.43 m	5.43 m	5.37 m	67.4 d	67.9 d	67.2 d
20	1.47 t (12.0)	1.54 m	1.47 t (6.9)	40.8 t	40.7 t	41.0 t
	2.20 m	1.98 m	1.98 m			
21				100.8 s	96.0 s	99.9 s
22	2.47 br s	5.55 dd (2.6, 9.8)	1.67 dd (3.0, 14.1)	51.6 t	128.1 d	41.3 t
			1.96 m			
23		5.74 dd (1.7, 9.8)	3.83 br s	207.4 s	136.1 d	70.2 d
24	2.45 m	2.27 m	1.64 m	46.6 d	30.6 d	35.8 d
25	3.51 d (10.4)	3.48 dd (1.7, 10.0)	3.55 dd (1.2, 10.7)	76.8 d	75.2 d	71.3 d
26	1.58 m	1.58 m	1.53 m	36.1 d	35.3 d	35.2 d
27	1.52 m	1.50 m	1.49 m	27.4 t	27.6 t	27.4 t
28	0.96 t (7.6)	0.93 t (7.4)	0.95 t (7.3)	12.4 q	12.1 q	12.5 q
29	2.23 br s	2.24 br s	2.21 br s	15.3 q	15.3 q	15.3 q
30	2.07 br s	2.08 br s	2.06 d (1.1)	18.3 q	18.2 q	18.2 q
31	1.21 d (6.9)	1.21 d (6.9)	1.21 d (6.9)	18.4 q	18.3 q	18.4 q
32	1.62 br s	1.63 br s	1.63 br s	15.1 q	15.1 q	15.1 q
33	0.97 d (6.7)	0.91 d (7.1)	0.91 d (6.9)	8.8 q	16.4 q	13.8 q
34	0.97 d (6.7)	0.92 d (6.6)	0.86 d (6.4)	11.4 q	12.8 q	11.5 q

^aBy DEPT sequence.

Milford, MA, USA). Optical rotation was measured on a Perkin-Elmer 341 Polarimeter (Perkin-Elmer, Fremont, CA, USA).

Compound (1, Figure 1) $\text{C}_{34}\text{H}_{46}\text{O}_7$, white amorphous powder; mp. 98–100 °C; $[\alpha]_{\text{D}}^{25} +74^\circ$ (c 0.031, EtOH); UV (EtOH) λ_{max} nm (log ϵ): 200 (4.57), 243 (4.25); IR (KBr), ν_{max} cm^{-1} : 3448, 1701, 1457, 1379, 1277, 1235, 1151, 989; ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (100 MHz, CDCl_3), for data see Table 1; ESI-MS m/z 565 $[\text{M}-\text{H}]^-$; HRESI-MS m/z 589.3093 $[\text{M}+\text{Na}]^+$; calculated for $\text{C}_{36}\text{H}_{48}\text{O}_7\text{Na}$ 589.3136.

Compound (2, Figure 1) $\text{C}_{34}\text{H}_{46}\text{O}_6$, white amorphous powder; mp. 124–126 °C; $[\alpha]_{\text{D}}^{25} +166^\circ$ (c 0.060, EtOH); UV (EtOH) λ_{max} nm (log ϵ): 200 (4.88), 246 (4.57); IR (KBr), ν_{max} cm^{-1} : 3448, 1700, 2926, 1457, 1378, 1280, 1159, 997; ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (100 MHz, CDCl_3) for data see Table 1; ESI-MS m/z 551 $[\text{M}+\text{H}]^+$; HRESI-MS m/z 573.3180 $[\text{M}+\text{Na}]^+$; calculated for $\text{C}_{36}\text{H}_{48}\text{O}_6\text{Na}$ 573.3187.

Compound (3, Figure 1) $\text{C}_{34}\text{H}_{48}\text{O}_7$, white amorphous powder; mp. 150–153 °C; $[\alpha]_{\text{D}}^{25} +113^\circ$ (c 0.135, EtOH); UV (EtOH) λ_{max} nm (log ϵ): 201 (4.66), 247 (4.40); IR (KBr), ν_{max} cm^{-1} : 3446, 1701, 2928, 1456, 1381, 1280, 1164, 997; ^1H -NMR (400 MHz, CDCl_3) and ^{13}C -NMR (100 MHz, CDCl_3), for data see Table 1; ESI-MS m/z 567 $[\text{M}-\text{H}]^-$; HRESI-MS m/z 591.3315 $[\text{M}+\text{Na}]^+$; calculated for $\text{C}_{36}\text{H}_{50}\text{O}_7\text{Na}$ 591.3292.

Compound 1 was isolated as a white amorphous powder. Its molecular formula was determined to be $\text{C}_{34}\text{H}_{46}\text{O}_7$ on the basis of HRESI-MS at m/z 589.3093 $[\text{M}+\text{Na}]^+$ (calculated as 589.3136 for $\text{C}_{34}\text{H}_{46}\text{NaO}_7$) and ^{13}C -NMR data (Table 1). The IR spectrum of 1 showed hydroxyl absorption at 3448 cm^{-1} . ^1H -NMR data of 1 indicated one triplet aliphatic methyl signal at δ 0.96; three doublet aliphatic methyl signals at δ 0.97, 0.97, 1.21; three olefinic or aromatic methyl signals at δ 1.62, 2.07, 2.23; and two downfield singlet proton signals at δ 7.41 and 6.61. Its ^{13}C -NMR spectrum displayed 34 carbon

The key HMBC correlations of compound 1, 2 and 3

Figure 2 The key heteronuclear multiple bond correlations (HMBC) of compounds 1, 2 and 3.

signals, including 1 carbonyl, 1 ester carbonyl, 12 sp^2 carbons, 7 methyls, 5 methylenes and 7 aliphatic methines (including 4 oxygenated and 1 ketal carbon). Comparing the ^1H and ^{13}C -NMR data with those of avermectin¹² suggested that compound 1 may be a derivative of avermectin aglycone. By a detailed comparison of the NMR data of 1 with those of milbemycin β ^{3,3-6} β ^{4,7,8} β ¹³ and β ¹⁴⁹ isolated from the milbemycin-producing strain, it was revealed that compound 1 was similar to milbemycin β 3 except for the differences observed in C-13, C-23 and C-25 positions. In the HMBC spectrum, the observed correlation between δ_{H} 1.21 and δ_{C} 134.5, 40.7, 79.1 showed that C-13 was substituted by a hydroxyl group (Figure 2). Both the HMBC correlated signals of δ_{H} 0.97, δ_{C} 76.8, 46.6, 207.4 and the HMBC correlated signals of δ_{H} 2.47, δ_{C} 100.8, 207.4 indicated that the carbonyl group was in C-23 (Figure 2). The remaining one aliphatic methylene, one aliphatic methine, one doublet aliphatic methyl and one triplet aliphatic methyl showed the presence of an isopropyl group. The three bonds' correlation signals between δ_{H} 3.51 and δ_{C} 11.4, 27.4 assigned the second butyl group at C-25 similar to that of avermectin B_{1a}. The relative stereochemistry of 1 was assigned by concurring with that of avermectin B.¹²

Compound 2 was obtained as a white amorphous powder. Its ^1H and ^{13}C -NMR data were very similar to those of compound 1, except for the presence of another double bond and the absence of one carbonyl and one methylene group. The ^1H - ^1H COSY correlation of δ_{H} 5.55 and δ_{H} 5.74, and the HMBC correlations between δ_{H} 0.91 and δ_{C} 136.1, indicated that the C-23 carbonyl and C-22 methylene (Figure 2) in 1 were replaced by one double bond in compound 2. Thus, the structure of compound 2 was established.

Compound 3 was also isolated as a white amorphous powder. Comparison of the ^1H and ^{13}C -NMR data with those of compound 1

exhibited that the difference between compound 3 and compound 1 was only in the C-23 position, in which one carbonyl in compound 1 was replaced by a hydroxyl in compound 3. The crossing peaks of δ_{H} 0.91 and δ_{C} 70.2 in the HMBC spectrum confirmed the hydroxyl group substituted at C-23 in compound 3 (Figure 2). Consequently, the structure of compound 3 was elucidated.

We have determined the 16S rDNA sequence of the strain (DQ768097), which produces compounds 1, 2 and 3. It shared 99.05, 99.93 and 99.93% identity with the 16S rDNA sequences of *S. avermitilis* strains NBRC 14893 (AB184632), MA-4680 (AB078897) and AF145223, respectively, thereby characterizing strain NEAU1069 as *S. avermitilis*. We also isolated avermectin B_{1a} from the *S. avermitilis* NEAU1069 fermentation broth. Furthermore, compounds 1, 2 and 3 produced by *S. avermitilis* NEAU1069 had the same isopropyl group at C-25 as avermectin. Although compounds 1, 2 and 3 are analogs of milbemycin β ¹³ and β ¹⁴⁹ produced by *S. bingchenggensis*, the 16S rDNA sequence of *S. avermitilis* NEAU1069 only shared 94.84% identity compared with that of *S. bingchenggensis* (DQ449953). β -Class milbemycin compounds isolated from *S. avermitilis* were not reported.

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