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NOTE

Isolation and identification of new macrocyclic lactones from a genetically engineered strain *Streptomyces bingchenggensis* BCJ60

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Avermectins and Milbemycins (AM), the 16-membered macrocyclic lactones with high insecticidal and antihelminthic activity, have been widely used for broad-spectrum parasite control in agricultural, veterinary and medical fields.[1] To date, six avermectins (abamectin, ivermectin, emamectin, doramectin, eprinomectin and selamectin) and five milbemycins (milbemectin, moxidectin, milbemycin oxime, lepimectin and latidectin) have been currently registered as insecticides and veterinary drugs. Among these commercial drugs, abamectin and ivermectin are the best known, because of their long and widespread usage.[2,3] Eprinomectin was the first avermectin drug licensed for treatment of parasitic infections in lactating cows. In the medical field, ivermectin was used to treat onchocerciasis (also known as river blindness) and lymphatic filariasis (also known as elephantiasis).[4] Because the radically lowering the incidence of human parasitic infection, the 2015 Nobel Prize in Physiology or Medicine was awarded to William C. Campbell and Satoshii Omura.[5] Additionally, moxidectin is currently undergoing a phase III clinical trial to compare its efficacy with ivermectin in subjects with *Onchocerca volvulus* infection.[6] Furthermore, AM including ivermectin, milbemycin A4 and milbemycin oxime A4 also demonstrated inhibitory activity against P-glycoprotein, leading to reverse multidrug resistance of tumor cells.[7–9] Given the importance and potential of these compounds, the discovery of AM derivatives has drawn increasing attention.

In our previous work, a genetically engineered strain *Streptomyces bingchenggensis* BCJ60, in which the *milF* gene encoding a C5-ketoreductase responsible for the ketonization of milbemycins was disrupted, has been constructed with the production of 5-oxomilbemycins A3/A4 and the elimination of milbemycins A3, A4, B2 and B3.[10] To further exploit the active constituents produced by this strain, the detailed fractionation of the crude extract was conducted and two new milbemycin derivatives (**1** and **2**, Figure 1) were subsequently obtained. In this paper, we describe the isolation, structural elucidation, acaricidal and nematocidal activities of compounds **1** and **2**.

The strain *S. bingchenggensis* BCJ60 was cultured in 100 l fermentor containing 60 l production medium (16.0% sucrose, 2.0% soybean powder, 0.5% yeast extract, 0.5% meat extract, 0.05% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.005% $FeSO_4{\cdot}7H_2O$ and 0.3% $CaCO_3$) for 10 days at 28 °C. The fermentation broth was filtered. The resulting cake was washed with water, and both filtrate and wash were discarded. Methanol (20 l) was used to extract the washed cake. The MeOH extract was evaporated under reduced pressure to ~3 l at 50 °C and the resulting concentrate was extracted three times using an equal volume of EtOAc. The EtOAc extract (40 g) was subjected to column chromatography over silica gel (100–200 mesh, Qingdao Haiyang Chemical Group Co., Qingdao, China) eluted with a petroleum ether/acetone mixture (100:0–80:20, v/v) to obtain fractions I and II. Fraction II was further fractionated by silica gel eluted with petroleum ether/ethyl acetate (90:10, 85:15 and 80:20, v/v) to give three subfractions. Subfraction II was separated by semi-preparative HPLC (Agilent 1100, Zorbax SB-C3, 5 μm, 250 × 9.4 mm i.d.; Agilent, Palo Alto, CA, USA) eluting with $CH_3OH/CH_3CN/H_2O$ (42:42:16, v/v/v) to afford compound **1** (R_t = 17.5 min, 28.5 mg) and compound **2** (R_t = 16.6 min, 26.1 mg). The ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were measured with a Bruker DRX-400 (Agilent Technologies, Santa Clara, CA, USA) spectrometer. The high resolution electrospray ionization mass spectroscopy was taken on a Q-TOF Micro LC-MS-MS mass spectrometer (Agilent, Boblingen, Germany). Optical rotation was measured on a Perkin-Elmer 341 polarimeter (Anton Paar GmbH, Graz, Austria). UV spectra were recorded on a UV-1800 UV spectrophotometer (Shimadzu, Kyoto,

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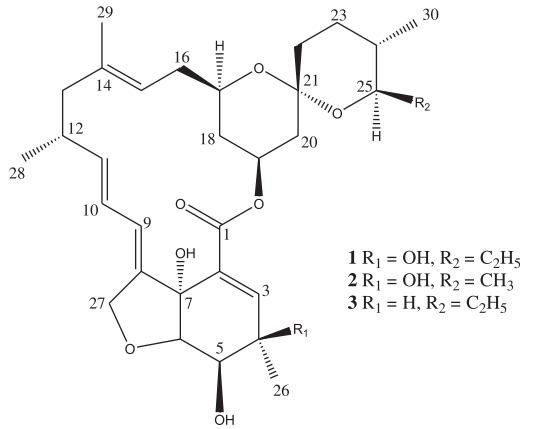

Figure 1 Structures of **1**, **2** and $\Delta^{2,3}$-milbemycin A4 (**3**).

Japan). IR spectra were recorded on a Thermo Nicolet Avatar FT-IR spectrophotometer (Thermo, Tokyo, Japan) using KBr discs.

Compound **1** was isolated as a white amorphous powder with UV (EtOH) λ_{max} (nm) (log ε): 251 (4.44) and $[\alpha]_D^{25} + 170$ (c 0.25, EtOH). Its molecular formula was determined to be $C_{32}H_{46}O_8$ on the basis of high resolution electrospray ionization mass spectroscopy at *m/z* 557.3122 $[M\text{-}H]^-$ (calcd for $C_{32}H_{45}O_8$, 557.3120). Analysis of the IR spectrum indicated the presence of hydroxyl group at 3461 cm^{-1} and carbonyl group at 1717 cm^{-1}, respectively. The ^1H NMR spectrum of compound **1** (Table 1) clearly indicated the presence of five methyls at δ_H 0.84 (d, J=6.5 Hz), 1.00 (t, J=7.4 Hz), 1.01 (d, J=7.4 Hz), 1.39 (s) and 1.50 (br s), one trans-double bond at δ_H 5.70 (dd, J=14.8 Hz, 11.2 Hz) and 5.47 (dd, J=14.8 Hz, 10.0 Hz). The ^{13}C NMR and DEPT spectra (Table 1) of **1** displayed 32 carbon signals, including an ester carbonyl, eight sp^2 carbons, five methyls, eight methylenes, seven aliphatic methines (including five oxygenated ones), two oxygenated quaternary carbons and one ketal carbon. The ^1H-^1H COSY spectrum (Figure 2) of **1** revealed four isolated spin systems. The correlations of H-5/H-6, H-9/H-10/H-11/H-12/H_2-13, H-15/H_2-16/H-17/H_2-18/H-19/H_2-20 and H_2-22/H_2-23/H-24/H-25/H_2-31/H_3-32 protons in the ^1H-^1H COSY spectrum indicated the four structural units of C-5-C-6, C-9-C-13, C-15-C-20 and C-22-C-32. The observed HMBC correlations (Figure 2) of H_3-28/C-11, C-12; H_3-29/C-15; H_3-30/C-24; and H-19/C-1 established the linkage of C-28-C-11, C-29-C-15, C-30-C-24 and C-19-O-C-1. On the basis of these observations and a comparison of its spectroscopic data with those of $\Delta^{2,3}$-milbemycin A4[11,12] (**3**, Figure 1) suggested that compound **1** was similar to $\Delta^{2,3}$-milbemycin A4 except that a methine group in $\Delta^{2,3}$-milbemycin A4 was replaced by an oxygenated quaternary carbon in **1**. In the HMBC spectrum, the observed correlations from H_3-26 (δ_H 1.39) to C-3 (δ_C 136.3), C-4 (δ_C 68.8) and C-5 (δ_C 70.8) indicated that a hydroxyl group was connected to C-4. Therefore, the planar structure of compound **1** was established and named 4-hydroxy-$\Delta^{2,3}$-milbemycin A4.

The NOESY correlation (Figure 3) observed between H-5 and H_3-26 showed the H-5 and H_3-26 having the same orientation. The large coupling constant (14.8 Hz) of H-10 and H-11 and the NOESY crossing peaks between H_3-27 and H-10 and H_3-29 and H_2-16 indicated that the geometry of the three double bonds at C-8 and C-9, C-10 and C-11 and C-14 and C-15 were *E*. The NOESY correlations of H-17 and H-25, H_3-30 and H-25 and H-17 and H-19 suggested that these protons having the same orientations.

Table 1 The NMR spectroscopic data for compounds 1 and 2 (in $CDCl_3$)

	δ_H (mult., J in Hz)		δ_C (p.p.m.)	
Position	***1***	***2***	***1***	***2***
1			168.6	168.9
2			129.4	128.9
3	6.23 (s)	6.15 (s)	136.3	137.6
4			68.8	69.1
5	4.01 (d, 3.1)	3.97 (br s)	70.8	71.4
6	4.25 (d, 3.8)	4.24 (d, 3.7)	80.8	80.1
7			74.8	74.9
8			136.8	137.4
9	6.15 (d, 11.2)	6.14 (d, 11.2)	124.1	123.9
10	5.70 (dd, 14.8, 11.2)	5.72 (dd, 14.7, 11.2)	123.7	123.8
11	5.47 (dd, 14.8, 10.0)	5.47 (dd, 14.7, 9.0)	144.2	143.9
12	2.42 (m)	2.41 (m)	35.9	35.8
13	2.22 (m)	2.25 (m)	48.4	48.5
	1.91 (m)	1.88 (m)		
14			136.5	136.5
15	4.95 (br d, 5.9)	4.99 (br d, 5.8)	120.8	120.8
16	2.25 (m)	2.25 (m)	34.9	35.0
17	3.61 (m)	3.62 (m)	67.3	67.3
18	0.76 (q, 11.7)	0.80 (q, 11.6)	36.7	36.6
	1.88 (m)	1.90 (m)		
19	5.39 (m)	5.41 (m)	69.2	69.2
20	1.55 (t, 12.0)	1.57 (t, 11.8)	40.4	40.2
	1.97 (m)	1.96 (m)		
21			97.3	97.5
22	1.53 (m)	1.59 (m)	35.7	35.7
	1.68 (m)	1.71 (m)		
23	1.56 (m)	1.58 (m)	27.8	27.7
24	1.33 (m)	1.27 (m)	34.3	36.6
25	3.09 (m)	3.29 (m)	76.1	71.4
26	1.39 (s)	1.40 (s)	24.2	24.9
27	4.60 (d, 14.0)	4.52 (dd, 14.0, 2.2)	68.7	68.2
		4.59 (dd, 14.0, 2.2)		
28	1.01 (d, 7.4)	1.02 (d, 6.6)	22.1	22.1
29	1.50 (br s)	1.53 (br s)	15.4	15.4
30	0.84 (d, 6.5)	0.86 (d, 6.6)	17.7	17.8
31	1.35 (m)	1.17 (d, 6.2)	25.7	19.4
	1.68 (m)			
32	1.00 (t, 7.4)		10.1	

The other chiral centers of **1** were assigned as occurring with 5-oxomilbemycins A3/A4 and milbemycin A4.[10,13]

The Modified Mosher's method[14] was applied to determine the absolute configuration of C-5. The 5-OH in **1** was derivatized with (*R*)- and (*S*)-α-methoxy-α-(trifluoromethyl)-phenylacetyl chloride (MTPA-Cl) to yield (*S*)- and (*R*)-MTPA esters, respectively. Unfortunately, the $\Delta\delta_{SR}$ ($\Delta\delta_{SR}=\delta_S—\delta_R$ in p.p.m.) values between (*S*)- and (*R*)-MTPA esters at H-3, H-6 and H-26 were all negative and it showed that the chirality of C-5 in **1** could not be determined by application of the Modified Mosher's method. The configurations of C-4 and C-5 atoms presented in the Figures 1, 2 and 3 are the most probable because they have been concluded logically based on the comparing with structure of the closely relative described earlier $\Delta^{2,3}$-milbemycin A4 as a component of the similar milbemycin-producing strain.

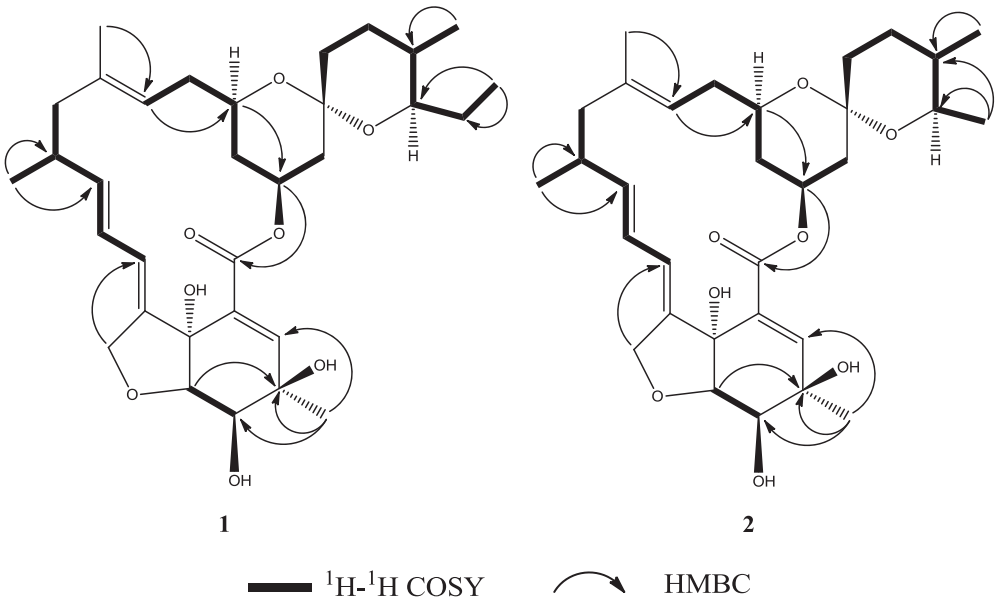

Figure 2 Key ^{1}H-^{1}H COSY and HMBC correlations of **1** and **2**.

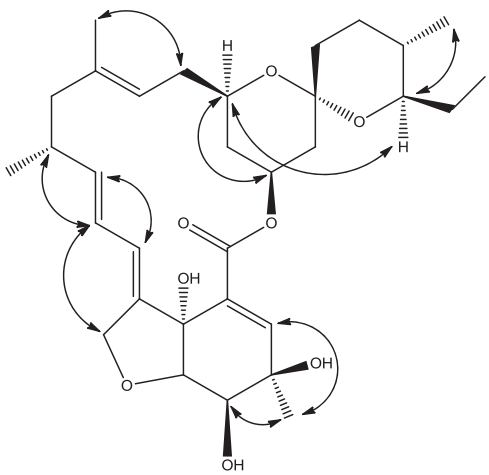

Figure 3 NOESY correlations of **1**.

Compound **2** was obtained as a white amorphous powder with $[\alpha]_D^{25}$ + 162 (*c* 0.30, EtOH) and UV (EtOH) λ_{max} nm (log ε): 251 (4.43). The molecular formula of **2**, $C_{31}H_{44}O_8$, was established on the basis of the high resolution electrospray ionization mass spectroscopy (*m/z* 543.2972 [M-H]$^-$, calcd for $C_{31}H_{43}O_8$, 543.2963). The IR absorption bands revealed the presence of hydroxyl (3451 cm^{-1}) and carbonyl (1699 cm^{-1}) functionalities. Detail analysis of the NMR spectrum suggested the ^{1}H and ^{13}C NMR data (Table 1) of **2** were very similar to those of **1**, except for the absence of a methylene. The HMBC correlations (Figure 2) from H_3-31 (δ_H 1.17) to C-25 (δ_C 71.4) and C-24 (δ_C 36.6) indicated that an ethyl group at C-25 in **1** was replaced by a methyl group in **2**. Thus, the structure of compound **2** was established and named 4-hydroxy-$\Delta^{2,3}$-milbemycin A3. The relative configuration of **2** was assigned as occurring with **1**.

The acaricidal and nematocidal capacities of compounds **1** and **2** against *Tetranychus cinnabarinus* and *Bursaphelenchus xylophilus*, respectively, reared in the laboratory were evaluated according to our reported paper.[15,16] The commercial acaricide and nematocide milbemycins A3/A4 was used as a positive control. As shown in Table 2, the two new macrocyclic lactones **1** and **2** possessed high acaricidal activity against *Tetranychus cinnabarinus* (LC_{50} = 0.129 and 0.111 mg l^{-1}), and nematocidal activity against *Bursaphelenchus xylophilus* (LC_{50} = 5.145 and 5.288 mg l^{-1}), which were comparable to those of commercial pesticide milbemycins A3/A4.

Table 2 Acaricidal and nematocidal activities of compounds 1 and 2

	LC_{50} (mg l^{-1})[a]	
Compounds	*Tetranychus cinnabarinus*	*Bursaphelenchus xylophilus*
1	0.129 ± 0.005	5.145 ± 0.248
2	0.111 ± 0.015	5.288 ± 0.478
Milbemycins A3/A4[b]	0.107 ± 0.007	4.900 ± 0.068

[a]Values are the means ± s.d.s of three independent experiments.
[b]Milbemycins A3 and A4 mixtures, 30:70 (in volume).

In summary, compounds **1** and **2** produced by a genetically engineered strain *Streptomyces bingchenggensis* BCJ60 possess potent acaricidal and nematocidal activities and they not only have potential as biologically based pesticides, but also provide new insight into the biosynthesis of milbemycins.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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