The Journal of Antibiotics (2011) 64, 591–594

www.nature.com/ja

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Two new doramectin analogs from *Streptomyces avermitilis* NEAU1069: fermentation, isolation and structure elucidation

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The Journal of Antibiotics (2011) **64,** 591–594; doi:10.1038/ja.2011.48; published online 15 June 2011

Keywords: new doramectin analogs; *Streptomyces avermitilis* NEAU1069; structure elucidation

Microbes have made a phenomenal contribution to the health and well-being of people throughout the world.[1] Nowadays, drug discovery from microbial natural products has become more and more difficult. However, it is not the end of an era but an endless frontier.[2] To explore more bioactive compounds, a strain of *Streptomyces avermitilis* NEAU1069 that produced milbemycins was isolated from a soil sample.[3] Later, two novel macrolide compounds with a cyclohexyl group at the position of C-25, such as doramectin and avermectin B1a, were obtained from the fermentation broth, using bioactivity guided fractionation method, on adding cyclohexanecarboxylic acid to the fermentation medium.[4] The co-existence of milbemycins and avermectin implies that *S. avermitilis* NEAU1069 is an interesting strain and merits further study. Therefore, large amounts of fermentation broth (30 l) were collected and the detailed constituents were investigated; two other new compounds **1** and **2** (Figure 1), similar to doramectin, were subsequently obtained. Here we describe the fermentation, isolation and structure elucidation of these two new doramectin analogs **1** and **2**.

Compound **1** was obtained as a white amorphous powder. Its molecular formula was established to be $C_{44}H_{68}O_{11}$ as deduced from the high-resolution electron spray ionization (HRESI)-MS and ^{13}C NMR data (Table 1). The IR spectrum of **1** showed absorption bands assignable to the hydroxyl group (3447 cm^{-1}) and an ester carbonyl (1705 cm^{-1}). The ^1H NMR (400 MHz, $CDCl_3$) data (Table 1) of **1** displayed three doublet aliphatic methyls at δ 0.90, 1.18, 1.28; three olefinic methyl signals at δ 1.55, 1.72, 1.82; two methoxy signals at δ 3.37, 3.51; one *trans*-double bond at δ 5.59 (dd, J=10.0, 14.8 Hz) and δ 6.00 (dd, J=10.8, 14.8 Hz). Its ^{13}C NMR spectrum revealed 44 carbon resonances, including an ester carbonyl carbon at δ 174.1 (s), a ketal carbon at δ 99.6 (s), 5 sp^2 methines, 3 sp^2 quaternary carbons, 3 secondary methyls, 3 vinylic methyls, 11 methylenes, 14 aliphatic methines (10 oxygenated), 1 oxygenated quaternary carbon and 2 methoxy groups. The above data accounted for 65 protons, indicating the presence of three free hydroxyl groups in **1**. Comparison of the ^1H NMR data of **1** with those of selamectin[5] and doramectin[6] suggested that compound **1** was structurally related to doramectin and selamectin (Figure 1). The differences between **1** and selamectin were in C-5, C-6, C-8 and C-23. The ^1H-^1H COSY and HMBC spectra (Figure 1b) fulfilled the assignment of the structure of **1**. The observed HMBC correlations from δ_H 1.82 (4-Me) and one of methoxy proton signal to C-5 (δ 77.2) demonstrated that the hydroxyimino group in selamectin was replaced by a methoxy group in **1**. The correlation of H-5 and H_2-6 in the ^1H-^1H COSY spectrum showed the C-6 was a methylene. The HMBC correlations between δ_H 1.72 (8-Me) and δ_C 76.2 (s, C-7), δ_C 136.8 (s, C-8), δ_C 124.6 (d, C-9) revealed a methyl group was substituted at C-8. A hydroxyl group attached to C-23 was also supported by the HMBC correlation from the δ_H 0.90 (24-Me) to δ_C 70.0 (d, C-23). As a consequence, the gross structure of **1** was established.

The relative stereochemistry of **1** was assigned on the basis of the concurrence with those of doramectin and 25-cyclohexyl-avermectin B_2.[6]

To confirm the assignment of the sugar moiety of **1**, acid hydrolysis of **1** was performed, and afforded an aglycone and a sugar. The sugar component was identified by TLC co-chromatography with the sugar obtained by acid hydrolysis of selamectin. The identical R_F values of the two resulting sugars indicated that compound **1** and selamectin possessed the same sugar moiety.

Compound **2** was obtained as a white amorphous powder. Its molecular formula was established to be $C_{43}H_{62}O_{10}$ on the basis of HRESI-MS at m/z 761.4225 $[M+Na]^+$ (calculated as 761.4235 for $C_{43}H_{62}NaO_{10}$). The ^1H NMR spectrum of **2** displayed a methoxy group at δ 3.52 and two downfield proton signals at δ 6.62, 7.40. Its ^{13}C NMR spectrum showed 43 carbon resonances, including 7 methyls (1 oxygenated), 10 methylenes, 12 aliphatic methines (9 oxygenated), 12 sp^2 carbons, a ketal carbon and an ester carbonyl group. This suggested

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Received 5 October 2010; revised 20 February 2011; accepted 19 May 2011; published online 15 June 2011

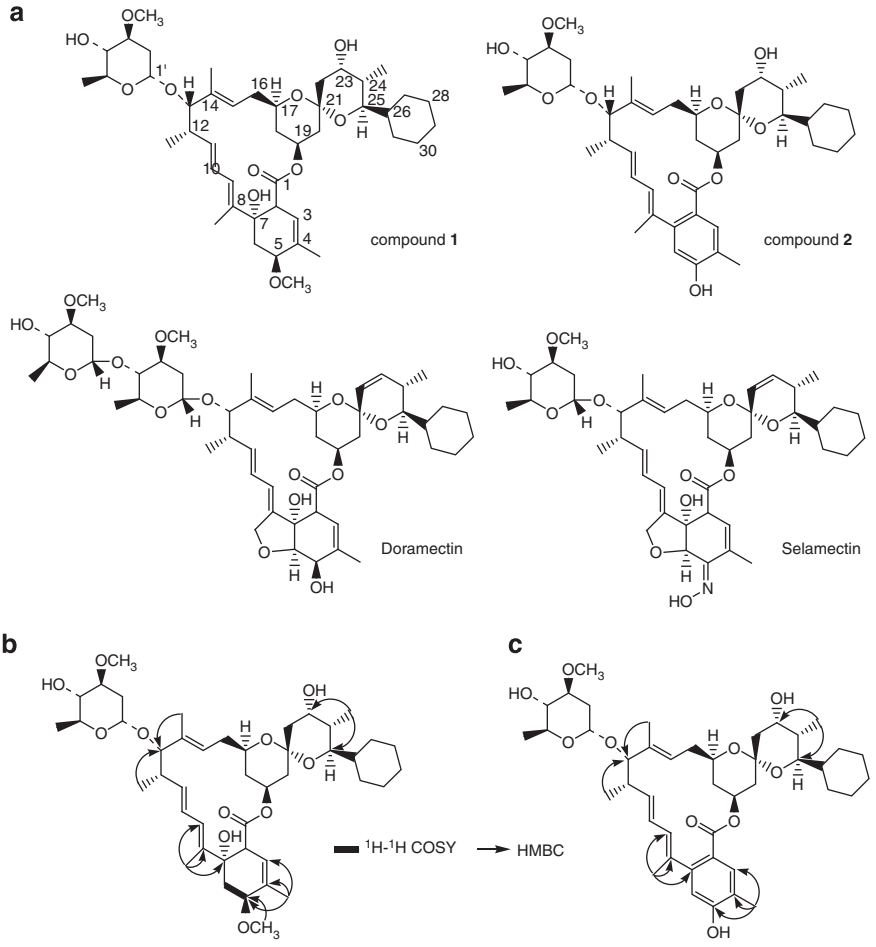

Figure 1 Structures (**a**) and key ^{1}H-^{1}H COSY, HMBC correlations (**b**, **c**) of compounds **1** and **2**.

that **2** was also a doramectin derivative. Comparison of the ^{1}H and ^{13}C NMR data of **2** with those of **1**, the differences between **2** and **1** were in C-2 to C-7 moiety in addition to the absence of a methoxy in **2**. Further, comparing the ^{1}H and ^{13}C NMR data of C-2 to C-7 moiety of **2** with those of the same structural units in milbemycin β_3,[7–9] β_4,[10,11] β_{13}, β_{14},[12] suggested that the C-2 to C-7 moiety in **2** was an aromatic ring as that of milbemycin β_3, β_{13}, β_{14}. The HMBC correlations (Figure 1c) from δ_H 2.23 (4-Me) to δ_C 132.2 (d, C-3), δ_C 122.8 (s, C-4) and δ_C 156.1 (s, C-5), from δ_H 2.07 (8-Me) to δ_C 144.3 (s, C-7), δ_C 134.8 (s, C-8) and δ_C 128.4 (d, C-9) confirmed the presence of a phenyl ring in **2**. As a result, the gross structure of **2** was established as shown in Figure 1. Biogenetically, the relative configuration of **2** was assigned as that of **1**.

In conclusion, we obtained two new doramectin analogs from the culture broth of *S. avermitilis* NEAU1069. Along with previously obtained analogs of doramectin, avermectin and milbemycin from *S. avermitilis* NEAU1069,[3,4] we believe that these two new compounds possibly have important roles in understanding and perfecting the proposed biosynthetic pathways of avermectins and milbemycins.

EXPERIMENTAL PROCEDURE

General procedures and reagents

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); ^{1}H- and ^{13}C-NMR spectra were measured using 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 the residual $CHCl_3$ (δ_H 7.26; δ_C 77.0) as an internal standard, and coupling constant (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 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). Column chromatography was carried out on silica gel (100–200 mesh, Qing Dao Hai Yang Chemical Group, Qingdao, China). Semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 µm, 250×9.4 mm i.d.; Agilent, Palo Alto, CA, USA) was further performed to obtain pure compounds. All chemicals used in the study, such as methanol (MeOH), ethyl acetate (EtOAc), petroleum ether (60–90 °C) and acetone, were of analytical grade.

Strain fermentation, isolation and purification of compounds

Details of the producing strain, *S. avermitilis* NEAU1069, were described in a previous paper.[3,4] The strain was maintained on a YMS medium containing soluble starch (Bei Jing Ao Bo Xing, Beijing, China) 10 g, yeast extract (Bei Jing Ao Bo Xing) 2 g, KNO_3 1 g and agar 20 g in 1.0 l tap water, pH 7.0. The seed medium consisted of glucose (Bei Jing Ao Bo Xing) 20 g, soybean flour (Cormwin, Beijing, China) 15 g and yeast autolysate (Bei Jing Ao Bo Xing) 5.0 g in 1.0 l tap 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

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

	Proton		Carbon	
Position	***1***	***2***	***1***	***2***
1			174.1 s	169.6 s
2	3.45 m		47.9 d	123.3 s
3	5.33 m	7.40 s	118.4 d	132.2 d
4			138.6 s	122.8 s
5	4.05 m		77.2 d	156.1 s
6	1.79 m	6.62 s	37.1 t	114.2 d
	2.22 m			
7			76.2 s	144.3 s
8			136.8 s	134.8 s
9	6.27 d (10.8)	5.71 br d (11.2)	124.6 d	128.4 d
10	6.00 dd (10.8, 14.8)	6.08 dd (11.2, 15.0)	126.2 d	126.9 d
11	5.59 dd (10.0, 14.8)	5.48 dd (10.0, 15.0)	136.2 d	135.7 d
12	2.45 m	2.52 m	40.7 d	40.9 d
13	3.97 br s	4.01 br s	83.0 d	82.9 d
14			135.0 s	134.8 s
15	4.82 m	4.09 br d (10.2)	117.5 d	118.0 d
16	2.29 m	2.25 m	34.2 t	33.3 t
17	3.75 m	3.83 m	68.4 d	68.5 d
18	0.74 q (12.4)	0.78 q (11.8)	36.3 t	36.4 t
	1.79 m	1.96 m		
19	5.31 m	5.48 m	67.2 d	67.2 d
20	1.47 t (11.6)	1.44 t (12.0)	40.6 t	40.8 t
	1.90 dd (4.8, 12.4)	1.96 m		
21			99.6 s	99.9 s
22	1.65 m	1.66 m	41.2 t	41.2 t
	1.96 dd (2.8, 14.0)	1.96 m		
23	3.68 br s	3.77 br s	70.0 d	70.2 d
24	1.63 m	1.65 m	35.1 d	35.1 d
25	3.38 d (10.0)	3.41 d (11.6)	72.5 d	72.5 d
26	1.50 m	1.52 m	38.1 d	38.1 d
27	1.18 m	1.23 m	26.9 t	26.9 t
	1.77 m	1.77 m		
28	1.18 m	1.23 m	26.4 t	26.4 t
	1.77 m	1.65 m		
29	1.46 m	1.52 m	31.2 t	31.1 t
	1.56 m	1.65 m		
30	1.18 m	1.23 m	24.5 t	24.5 t
	1.58 m	1.60 m		
31	1.18 m	1.23 m	26.5 t	26.5 t
	1.65 m	1.77 m		
4-Me	1.82 s	2.23 s	19.4 q	15.4 q
5-OCH_3	3.37 s		56.8 q	
8-Me	1.72 s	2.07 s	13.3 q	18.3 q
12-Me	1.18 d (6.8)	1.16 d (6.8)	20.1 q	19.6 q
14-Me	1.55 br s	1.58 br s	15.7 q	15.6 q
24-Me	0.90 d (6.8)	0.91 d (6.8)	13.8 q	13.8 q
1′	4.78 d (3.6)	4.81 d (3.6)	95.0 d	94.9 d
2′	1.51 m	1.53 m	34.0 t	33.9 t
	2.23 m	2.35 m		
3′	3.60 m	3.60 m	78.3 d	78.3 d
3′-OCH_3	3.51 s	3.52 s	56.8 q	56.8 q
4′	3.17 t (9.2)	3.18 t (9.1)	76.2 d	76.2 d
5′	3.90 dd (6.4, 9.6)	3.89 dd (6.3, 9.5)	68.0 d	67.9 d
5′-Me	1.28 d (6.8)	1.28 (6.8)	17.7 q	17.7 q

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 10^7–10^8 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, 8.0 ml of the culture was transferred into 1–l Erlenmeyer flask containing 100 ml of the producing medium consisting of corn starch (Cormwin) 10%, soybean powder (Bei Jing Ao Bo Xing) 1%, cotton flour (Cormwin) 1%, α-amylase (Bei Jing Ao Bo Xing) 0.02%, NaCl 0.1%, K_2HPO_4 0.2%, $MgSO_4$ $7H_2O$ 0.1%, $CaCO_3$ 0.7%, cyclohexanecarboxylic acid 0.1%, 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.

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 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 25 g of oily substances. The residual oily substance was chromatographed on silica gel and eluted with a petroleum ether–acetone mixture (95:5–50:50, v/v). The fractions eluted with the petroleum ether–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 ether–EtOAc mixture (95:5–50:50, v/v) to give five fractions (A1–A5). Fraction A3 eluted with a mixture of petroleum ether–EtOAc (75:25, v/v) was also subjected to silica gel eluting with a petroleum ether–EtOAc mixture (85:5–75:25, v/v) to give two subfractions B1 and B2.

Semipreparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250×9.4 mm i.d.) was used to obtain pure compounds. The eluate was monitored using a photodiode array detected at 220 nm, and the flow rate was 1.5 ml min^{-1} at room temperature. Subfraction B1 eluted with a mixture of petroleum ether–EtOAc (75:25, v/v) was further separated by semipreparative HPLC using a solvent containing a CH_3OH–H_2O mixture (92:8, v/v) to obtain compound **1** (t_R 24.2 min, 40 mg) and compound **2** (t_R 21.5 min, 11 mg).

Physicochemical properties of 1 and 2

Compound **1** (Figure 1a) $C_{44}H_{68}O_{11}$, white amorphous powder; melting point 153–155 °C; $[\alpha]_D^{25}$+51 (c 0.2, EtOH); UV (EtOH) λ_{max} nm (log ε): 200 (4.57), 250 (4.18); IR (KBr), ν_{max} cm^{-1}: 3454, 2929, 2855, 1714, 1637, 1452, 1380, 1341, 1167, 1099, 990; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data see Table 1; ESI-MS *m/z* 795 $[M+Na]^+$; HRESI-MS *m/z* 795.4649 $[M+Na]^+$, calculated for $C_{44}H_{68}O_{11}Na$ 795.4654.

Compound **2** (Figure 1a) $C_{43}H_{62}O_{10}$, white amorphous powder; melting point 146–148 °C; $[\alpha]_D^{25}$+124 (c 0.09, EtOH); UV (EtOH) λ_{max} nm (log ε): 200 (4.81), 245 (4.41); IR (KBr), ν_{max} cm^{-1}: 3451, 2928, 2856, 1701, 1614, 1503, 1451, 1378, 1279, 1162, 997; ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data see Table 1; ESI-MS *m/z* 761 $[M+Na]^+$; HRESI-MS *m/z* 761.4225 $[M+Na]^+$, calculated for $C_{43}H_{62}O_{10}Na$ 761.4235.

Acid hydrolysis of ***1***. Compound **1** (5.0 mg) was dissolved in 1 M HCl (1 ml) and then heated at 80 °C for 4 h. Aglycone was extracted with $CHCl_3$ three times, and the aqueous residue was evaporated under reduced pressure. The residue and the sugar obtained from selamectin, by the above procedure, were analyzed by TLC using chloroform–methanol (9:1, v/v) as a developing solvent. The spots were detected by spraying with anisaldehyde–H_2SO_4 reagent followed by heating, and the result showed that the two sugars have the identical R_F values (R_F=0.6).

ACKNOWLEDGEMENTS

This study was supported by the National Natural Science Foundation of China (no. 309771427, 310717150), the National Key Project for Basic Research (no. 2010CB126102) and the Key Science and Technology Program of Heilongjiang province (GA08B101-2).

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