ORIGINAL ARTICLE

A C G publications

Rec. Nat. Prod. 16:3 (2022) 206-211

records of natural products

Two New 13-oxomilbemycins from a NTG-Induced Mutation Strain of *Streptomyces avermitilis* AVE-H39

Ji-Dong Wang [1], Bing Li [1], Shao-Yong Zhang [1], Yi Wu [1], Wen-Sheng Xiang [2], Li-Qin Zhang [1] and Huan Qi [1*]

[1]*Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province, College of Life Science, Huzhou University, Huzhou 313000, China*

[2]*Life Science and Biotechnology Research Center, School of Life Science, Northeast Agricultural University, Harbin 150030, China*

(Received May 14, 2021; Revised July 06, 2021; Accepted July 08, 2021)

Abstract: Two new 13-oxomilbemycins, 13-oxomilbemycin β_3 (**1**) and 25-ethyl-13-oxomilbemycin β_3 (**2**), were isolated from the broth of a NTG-induced mutation strain of *Streptomyces avermitilis* AVE-H39. The structures of **1** and **2** were determined based on MS and extensive NMR analysis. Compounds **1** and **2** possessed moderate nematocidal activity.

Keywords: *Streptomyces avermitilis* AVE-H39; NTG-induced mutation; 13-oxomilbemycins; nematocidal activity.

1. Introduction

Sixteen-membered macrolides, important members of the polyketides, have been widely used in veterinary and agricultural fields and obtained great success [1-3]. Because of its wide-spread applications, researches on sixteen-membered macrolides are of great interest worldwide. Recently, a new kind of 16-membered macrolide antibiotics (tenvermectins A and B) with better insecticidal property than avermectin and ivermectin have been isolated from the fermentation broth of the two genetically engineered strains *Streptomyces avermitilis* MHJ1011 and *Streptomyces avermitilis* AVE-H39 [4-5]. In the effort to enhance the production of tenvermectins A and B in *S. avermitilis* AVE-H39, a mutant strain AVE-H39C12 was obtained by treating the spores of *S. avermitilis* AVE-H39 with *N*-methyl-*N'*-nitroso-*N*-nitrosoguanidine. Several differences of the HPLC profiles of metabolites were observed between the strain *S. avermitilis* AVE-H39 and its mutant strain AVE-H39C12. As part of an ongoing search for the metabolites of this mutant strain, two new interesting compounds were isolated from the fermentation broth of *S. avermitilis* AVE-H39C12. Here we described the isolation, structural elucidation and nematocidal activity of the two new compounds.

* Corresponding author: E-Mail: lcqhlc@163.com (H. Qi); Phone:+86-572-2321166

The article was published by ACG Publications
http://www.acgpubs.org/journal/records-of-natural-products Month-Month 202x EISSN:1307-6167
DOI: http://doi.org/10.25135/rnp.269.2105.2075
Available online: July 21, 2021

2. Materials and Methods

2.1. General

Optical rotation was measured on Perkin-Elmer 341 Polarimeter (Perkin-Elmer, Suzhou, China). IR spectra in pressed KBr disk were obtained on a Thermo Scientific Nicolet iS20 FTIR spectrometer (Thermo Scientific, Waltham, MA, USA) and UV spectra were recorded on a Thermo Scientific Evolution 201 UV-Visible spectrophotometer (Thermo Scientific, Waltham, MA, USA). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker DRX-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C; Bruker, Rheinstetten, Germany). Chemical shifts are reported in ppm (δ), using $CDCl_3$ (δ_H 7.27; δ_C 77.0) as an internal standard, and coupling constants (J) in Hz. The ESIMS and HRESIMS were taken on an Agilent 6545 Q-TOF LC-MS-MS mass spectrometer (Agilent, Palo Alto, CA, USA). Column chromatography was carried out on silica gel (100–200 mesh; Qingdao Marine Chemical Group Co., Qingdao, Shandong, China) and Sephadex LH-20 (GE Healthcare, Glies, UK). Preparative HPLC (Agilent 1200, Zorbax SB-C18, 5 μm, 250×20 mm inner diameter; 10 mL/min; 220 nm; Agilent, Palo Alto, CA, USA) was further performed to obtain pure compounds. Spots were detected on thin layer chromatography (TLC) under UV or by heating after spraying with sulfuric acid–ethanol (5:95, v/v).

2.2. Organisms Material

The parental strain *S*. avermitilis AVE-H39 was grown and maintained on ISP2 agar plate containing malt extract (Becton, Dickinson and Company, Franklin Lake, NJ, USA) 1%, yeast extract (Oxoid Ltd, Basingstoke, UK) 0.4%, glucose (Sinopharm Chemical Reagent Co, Ltd, Shanghai, China) 0.4%, and agar (Sinopharm Chemical Reagent Co, Ltd, Shanghai, China) 2.0% at pH 7.0. To improve the production of tenvermectins A and B, spores of *S. avermitilis* AVE-H39 were treated with *N*-methyl-*N*′-nitroso-*N*-nitrosoguanidine (NTG) using the described method [6-8]. Mutant colonies were obtained by incubation for 7-12 days at 28 °C. Each colony was fermented by shake flask with 30 mL medium consisted of corn starch (Shandong Xiwang Group Ltd, Binzhou, China) 10%, amylase (Sinopharm Chemical Reagent Co, Ltd, Shanghai, China) 0.02%, soybean powder (Ningbo Beilun Jiangnan Grease Co, Ltd, Ningbo, China) 2.0%, yeast extract (Angel Yeast Co., Ltd, Yichang, China) 1.0%, $CaCO_3$ (Sinopharm Chemical Reagent Co, Ltd, Shanghai, China) 0.2%, on a rotary shaker (250 rpm, 28°C) for 7 days. The profiles of the fermentation products were analyzed by HPLC. As a result, several differences on the HPLC profiles were observed between the strain *S. avermitilis* AVE-H39 and its mutant strain AVE-H39C12. Thus, the mutant strain AVE-H39C12 was used for further study.

2.3. Fermentation and Isolation

The mutant strain *S. avermitilis* AVE-H39C12 was incubated on ISP2 agar plates for 8 days at 28 °C, and then the spores were inoculated in the 1L Erlenmeyer flasks with seed medium. Each flask contained 250 mL of seed medium consisted of glucose 0.4%, maltodextrin (Shandong Xiwang Group Ltd, Binzhou, China) 1%, yeast extract 0.4%, $CaCO_3$ 0.2%, pH 7.2, and the medium was sterilized for 20 minutes at 121°C. After incubated on a rotary shaker (250 rpm, 28°C) for 48 h, about 1 L of the seed were inoculated in a 50 L fermentor (Shanghai Baoxing Bioengineering Equipment Co. Ltd., China) which contained 30 L of production medium consisting of corn starch 12%, amylase, 0.02%, soybean powder 3.0%, yeast powder (Angel Yeast Co., Ltd, Yichang, China) 1.0%, mannitol (Qingdao Bright Moon Seaweed Group Co., Ltd., China) 2.0%, $CaCO_3$ 0.3%, defoaming 0.1%, pH 7.2. The fermentation was carried out at 28 °C for 8 days and stirred at 200 rpm with the aeration rate of 1500 L of air per hour, tank pressure control at 0.05 MPa.

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 and the crude extract was subjected to a silica gel column and successively eluted with a stepwise gradient of petroleum ether/EtOAc (90:10–60:40, v/v) to yield six fractions (I–VI) based on the TLC profiles. The fraction II was separated by Sephadex LH-20 column eluting with CH_2Cl_2/ MeOH (1/1, v/v) to afford fraction IIA. Fraction IIA was further purified by preparative HPLC eluting with MeOH/H_2O (85:15, v/v, 10 mL min^{-1}) to give compounds **1** (11 mg, t_R = 17.5 min) and **2** (16 mg, t_R = 19.8 min).

*Compound **1***: Colorless oil; $[\alpha]_D^{25}$ +51 (*c* 0.07, EtOH); UV (EtOH) λ_{max} nm (log ε): 233 (4.50); IR (KBr) ν_{max} cm^{-1}: 3371, 2929, 1672, 1452, 1380, 1278, 1166, 1094, 1003; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data are listed in Table 1; HRESIMS: *m/z* 509.2909 $[M + H]^+$ (calcd for $C_{31}H_{41}O_6$, 509.2898).

*Compound **2***: Colorless oil; $[\alpha]_D^{25}$ +48 (*c* 0.15, EtOH); UV (EtOH) λ_{max} nm (log ε)： 227 (4.56); IR (KBr) ν_{max} cm^{-1}： 3384, 2929, 1707, 1455, 1380, 1278, 1165, 1100, 988; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) spectral data are listed in Table 1; HRESIMS: *m/z* 523.3060 $[M + H]^+$ (calcd for $C_{32}H_{43}O_6$, 523.3054).

2.4. Nematicidal Activity

The nematicidal activities of compounds **1-2** against *Bursaphelenchus xylophilus* were tested according to the described method using the commercial milbemycins A3/A4 as a positive control [4].

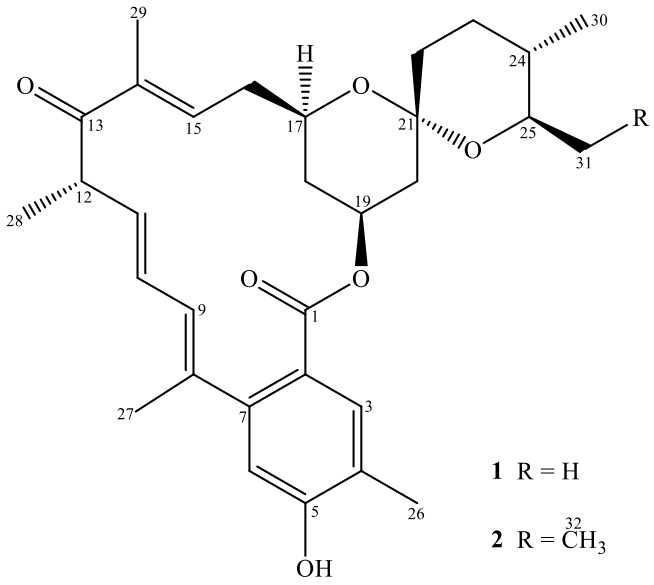

Figure 1. Structures of compounds **1** and **2**

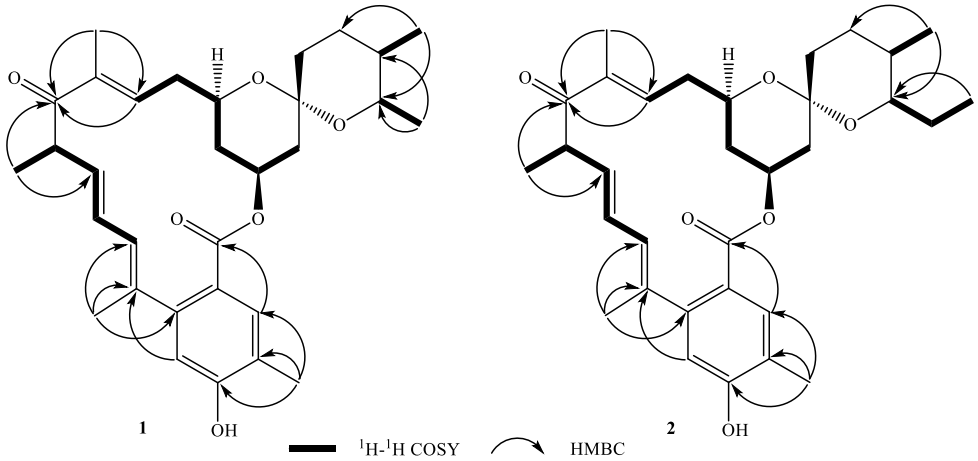

Figure 2. Key ^{1}H-^{1}H COSY and HMBC correlations of compounds **1** and **2**.

3. Results and Discussion

3.1. Structure Elucidation

Compound **1** was obtained as colorless oil with a specific rotation of $[\alpha]_D^{25}$ +51 (*c* 0.07, EtOH) and UV(EtOH) λ_{max} nm (log ε): 233 (4.50). Its molecular formula $C_{31}H_{40}O_6$ was established by the positive HRESIMS ion at *m/z* 509.2909 $[M+H]^+$ (calcd 509.2898), indicating 12 indices of hydrogen deficiency. Absorptions at 3371 and 1672 cm^{-1} in the IR spectrum of **1** revealed the presence of hydroxyl and carbonyl functionalities, respectively. The ^{1}H NMR spectrum of **1** (Table 1) showed the presence of two downfield singlet signals [δ_H 7.50 (1H, s) and 6.59 (1H, s)], one *trans*-double bond [δ_H 6.46 (1H, dd, *J* = 15.0, 10.9 Hz) and 5.41 (1H, dd, *J* = 15.0, 9.5 Hz)], an aromatic methyl [δ_H 2.25 (3H, s)], two olefinic methyls [δ_H 2.12 (3H, d, *J* = 0.9 Hz) and 1.81 (3H, brs)] and three aliphatic doublet methyls [δ_H 1.22 (3H, d, *J* = 6.7 Hz), 1.14 (3H, d, *J* = 6.2 Hz) and 0.85 (3H, d, *J* = 6.5 Hz)]. Its ^{13}C NMR spectrum, complemented by DEPT experiment (Table 1) only exhibited 30 carbon resonances including two carbonyls [δ_C 202.1 and 168.4], five sp^2 quaternary carbons, six protonated sp^2 carbons, one ketal carbon [δ_C 97.7], five sp^3 methines (three of which contained oxygen), five sp^3 methylenes and six methyls. The HMBC correlations (Figure 2) from the two olefinic methyls to the carbon signal (δ_C 137.7) suggested that two sp^2 quaternary carbons were overlapped at δ_C 137.7. The ^{1}H and ^{13}C NMR data of **1** revealed close similarities to those of milbemycin β_3 [9-10] except that a methylene at C-13 in milbemycin β_3 was replaced by a carbonyl group in **1**. The observed HMBC correlation from H_3-28 and H_3-29 to C-13 (δ_C 202.1) established the structure of **1** as 13-oxomilbemycin β_3. The downfield chemical shift of C-15 (δ_H 6.71; δ_C 139.1) further confirmed the presence of a carbonyl group in C-13. From a biosynthetic point of view, the relative configuration of **1** was assigned as that of 25-methyl ivermectin [5].

Compound **2** was isolated as colorless oil with a positive optical rotation of of $[\alpha]_D^{25}$ +48 (*c* 0.15, EtOH) and UV (EtOH) λ_{max} nm (log ε): 227 (4.56). The molecular formula of **2** was established as $C_{32}H_{42}O_6$ based on the HRESIMS ion at *m/z* 523.3060 $[M+H]^+$, implying 12 degrees of unsaturation. The IR spectrum showed absorption bands assignable to the carbonyl group (1707 cm^{-1}) and the hydroxy group (3384 cm^{-1}). A detailed analysis of the ^{1}H and ^{13}C NMR data of **2** (Table 1) revealed that it has the same skeleton as **1**. The only difference between **2** and **1** was in the substituent of C-25, where the methyl group in **1** was replaced by an ethyl group in **2**. The HMBC correlations (Figure 2) from H_3-32 (δ_H 0.96) to C-25 (δ_C 76.3) in conjunction with the crossing peak of H_3-32/H_2-31 in the ^{1}H-^{1}H COSY spectrum (Figure 2) established the structure of **2** as 25-ethyl-13-oxomilbemycin β_3. The relative stereochemistry of **2** was assigned as that of **1**.

Table 1. ^{1}H and ^{13}C NMR spectral data for **1** and **2** in $CDCl_3$

Position	δ_H (mult., *J* in Hz)		δ_C (ppm)	
	1	**2**	**1**	**2**
1			168.4	168.4
2			122.8	122.8
3	7.50 (s)	7.49 (s)	132.8	132.9
4			123.3	123.2
5			156.2	156.2
6	6.59 (s)	6.60 (s)	114.4	114.5
7			144.8	144.8
8			137.7	137.7
9	5.68 (d, 10.9)	5.67 (d, 10.9)	126.9	126.8
10	6.46 (dd, 15.0, 10.9)	6.45 (dd, 15.0, 10.9)	128.9	129.0
11	5.41 (dd, 15.0, 9.5)	5.41 (dd, 15.0, 9.8)	135.5	135.5
12	3.93 (m)	3.94 (m)	46.0	46.0
13			202.1	202.1
14			137.7	137.7
15	6.71 (t, 7.5)	6.73 (t, 7.2)	139.1	139.1
16	2.38 (m) 2.64 (m)	2.37 (m) 2.65 (m)	33.6	33.6
17	3.83 (m)	3.84 (m)	65.7	65.8
18	1.18 (m) 1.96 (m)	1.19 (m) 1.95 (m)	35.3	35.5
19	5.38 (m)	5.38 (m)	68.4	68.5
20	1.41 (t, 11.9) 2.03 (m)	1.41 (t, 11.9) 2.04 (m)	40.8	41.0
21			97.7	97.6
22	1.55 (m) 1.70 (m)	1.53 (m) 1.69 (m)	35.7	35.6
23	1.55 (m)	1.53 (m)	27.7	27.9
24	1.27 (m)	1.33 (m)	36.5	34.3
25	3.27 (m)	3.06 (m)	71.5	76.3
26	2.25 (s)	2.24 (s)	15.3	15.3
27	2.12 (d, 0.9)	2.11 (br s)	19.3	19.2
28	1.22 (d, 6.7)	1.22 (d, 6.6)	16.5	16.5
29	1.81 (br s)	1.80 (br s)	12.2	12.1
30	0.85 (d, 6.5)	0.84 (d, 6.5)	17.9	17.8
31	1.14 (d, 6.2)	1.33 (m) 1.69 (m)	19.3	25.7
32		0.96 (t, 7.3)		10.2

3.2 Nematicidal Activity

Compounds **1** and **2** displayed moderate nematocidal activities against *Bursaphelenchus xylophilus* (LC50: **1**, 62.24 μg/mL; **2**, 127.37 μg/mL; milbemycins A3/A4, 14.26 μg/mL).

Acknowledgments

This research was financially supported by the Key Research and Development Programs of Zhejiang Province (Grant No: 2020C02028).

Supporting Information

Supporting information accompanies this paper on http://www.acgpubs.org/journal/records-of-natural-products

ORCID
Ji-Dong Wang: 0000-0003-4098-6209
Bing Li: 0000-0001-5465-5414
Shao-Yong Zhang: 0000-0003-3571-5797
Yi Wu: 0000-0002-2981-7589
Wen-Sheng Xiang: 0000-0002-1826-5985
Li-Qin Zhang: 0000-0002-3859-5412
Huan Qi: 0000-0003-4313-5055

References

[1] P. Przybylski (2011). Modifications and biological activity of natural and semisynthetic 16-membered macrolide antibiotics, *Curr. Org. Chem*. **15**, 328-374.

[2] L. K. Bekele and G. G. Gebeyehu (2012). Application of different analytical techniques and microbiological assays for the analysis of macrolide antibiotics from pharmaceutical dosage forms and biological matrices, *ISRN Anal. Chem.* **12**, 546.

[3] H. A. Kirst (1998). Recent developments with macrolide antibiotics, *Exp. Opin. Ther. Patents* **8**, 111-120.

[4] J. Huang, A. L. Chen, H. Zhang, Z. Yu, M. H. Li, N. Li, J. T. Lin, H. Bai, J. D. Wang and Y. G. Zheng (2015). Gene replacement for the generation of designed novel avermectin derivatives with enhanced acaricidal and nematicidal activities, *Appl. Environ. Microbiol.* **81**, 5326-5334.

[5] J. Zhang, Y. J. Yan, J. An, S. X. Huang, X. J. Wang and W. S. Xiang (2015). Designed biosynthesis of 25-methyl and 25-ethyl ivermectin with enhanced insecticidal activity by domain swap of avermectin polyketide synthase, *Microb. Cell. Fact.* **14**, 152 (12 pages).

[6] S. Srikrai and J. E. Robbers (1983). Methods for mutation and selection of the ergot fungus, *Appl. Environ. Microbiol.* **45**, 1165-1169.

[7] V. Delić, D. A. Hopwood and E. J. Friend (1970). Mutagenesis by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) in *Streptomyces coelicolor, Mutat. Res.* **9**, 167-182.

[8] B. B. Chattoo and U. Sinha (1974). Mutagenic activity of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) and *N*-methyl-*N*-nitrosourea (NMU) in *Aspergillus nidulans, Mutat. Res.* **23**, 41-49.

[9] D. R. Williams, B. A. Barner, K. Nishitani and J. G. Phillips (1982). Total synthesis of milbemycin β_3, *J. Am. Chem. Soc*. **104**, 4708-4710.

[10] S. R. Schow, J. D. Bloom, A. S. Thompson, K. N. Winzenberg and A. B. Smith (1986). Milbemycin-avermectin studies. 5. Total synthesis of milbemycin β_3 and its C(12) epimer, *J. Am. Chem. Soc*. **108**, 2662-2674.