

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

Ji-Dong Wang <sup>1</sup>, Bing Li <sup>1</sup>, Shao-Yong Zhang <sup>1</sup>, Yi Wu <sup>1</sup>,  
Wen-Sheng Xiang <sup>2</sup>, Li-Qin Zhang <sup>1</sup> and Huan Qi <sup>1\*</sup>

<sup>1</sup>Key Laboratory of Vector Biology and Pathogen Control of Zhejiang Province,  
College of Life Science, Huzhou University, Huzhou 313000, China

<sup>2</sup>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  $\beta_3$  (**1**) and 25-ethyl-13-oxomilbemycin  $\beta_3$  (**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. © 2021 ACG Publications. All rights reserved.

### 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](mailto:lcqhlc@163.com) (H. Qi); Phone:+86-572-2321166

## 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).  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on a Bruker DRX-400 spectrometer (400 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$ ; Bruker, Rheinstetten, Germany). Chemical shifts are reported in ppm ( $\delta$ ), using  $\text{CDCl}_3$  ( $\delta_{\text{H}}$  7.27;  $\delta_{\text{C}}$  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  $\mu\text{m}$ , 250 $\times$ 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%,  $\text{CaCO}_3$  (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%,  $\text{CaCO}_3$  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%,  $\text{CaCO}_3$  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

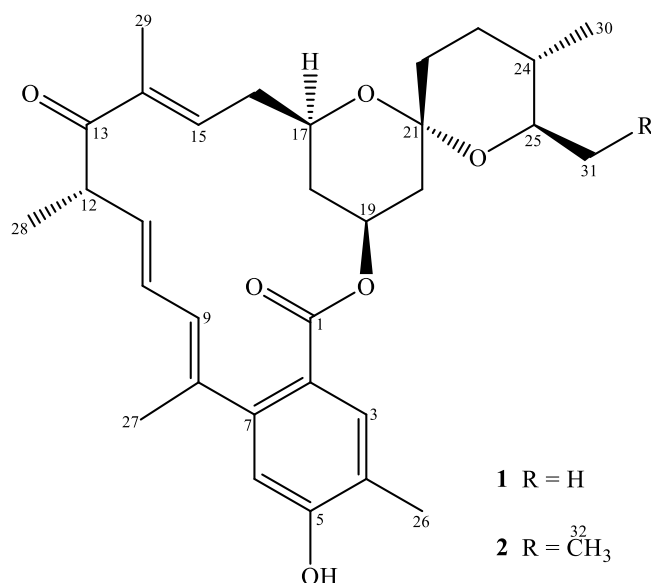
phase was concentrated under reduced pressure and the crude extract was subjected to a silica gel column and successively eluted with a stepwise gradient of petroleum ether/EtOAc (90:10–60:40, v/v) to yield six fractions (I–VI) based on the TLC profiles. The fraction II was separated by Sephadex LH-20 column eluting with CH<sub>2</sub>Cl<sub>2</sub>/ MeOH (1/1, v/v) to afford fraction IIA. Fraction IIA was further purified by preparative HPLC eluting with MeOH/H<sub>2</sub>O (85:15, v/v, 10 mL min<sup>-1</sup>) to give compounds **1** (11 mg, *t*<sub>R</sub> = 17.5 min) and **2** (16 mg, *t*<sub>R</sub> = 19.8 min).

**Compound 1:** Colorless oil;  $[\alpha]_D^{25} +51$  (*c* 0.07, EtOH); UV (EtOH)  $\lambda_{\max}$  nm (log  $\epsilon$ ): 233 (4.50); IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3371, 2929, 1672, 1452, 1380, 1278, 1166, 1094, 1003; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) and <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) spectral data are listed in Table 1; HRESIMS: *m/z* 509.2909 [M + H]<sup>+</sup> (calcd for C<sub>31</sub>H<sub>41</sub>O<sub>6</sub>, 509.2898).

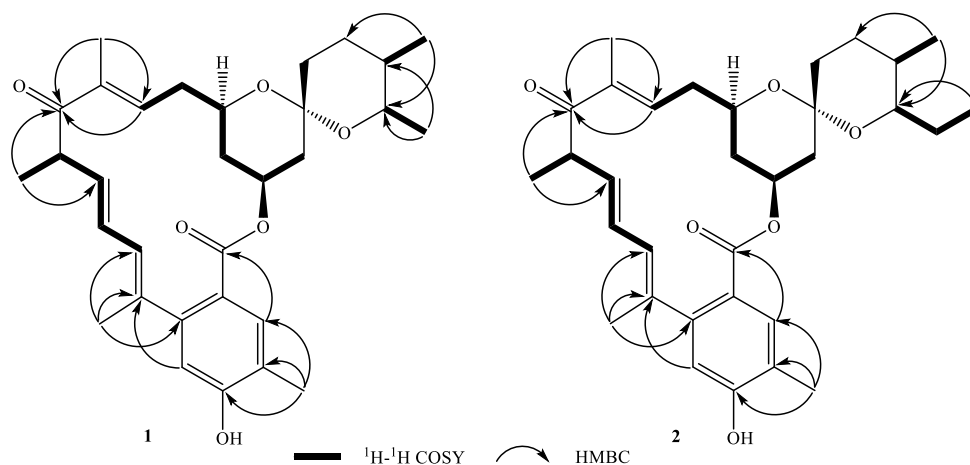
**Compound 2:** Colorless oil;  $[\alpha]_D^{25} +48$  (*c* 0.15, EtOH); UV (EtOH)  $\lambda_{\max}$  nm (log  $\epsilon$ ): 227 (4.56); IR (KBr)  $\nu_{\max}$  cm<sup>-1</sup>: 3384, 2929, 1707, 1455, 1380, 1278, 1165, 1100, 988; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>) and <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) spectral data are listed in Table 1; HRESIMS: *m/z* 523.3060 [M + H]<sup>+</sup> (calcd for C<sub>32</sub>H<sub>43</sub>O<sub>6</sub>, 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**

Two new 13-oxomilbemycins from *Streptomyces avermitilis*

**Figure 2.** Key  $^1\text{H}$ - $^1\text{H}$  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  $[\alpha]_{\text{D}}^{25} +51$  ( $c$  0.07, EtOH) and UV(EtOH)  $\lambda_{\text{max}}$  nm ( $\log \epsilon$ ): 233 (4.50). Its molecular formula  $\text{C}_{31}\text{H}_{40}\text{O}_6$  was established by the positive HRESIMS ion at  $m/z$  509.2909  $[\text{M}+\text{H}]^+$  (calcd 509.2898), indicating 12 indices of hydrogen deficiency. Absorptions at 3371 and 1672  $\text{cm}^{-1}$  in the IR spectrum of **1** revealed the presence of hydroxyl and carbonyl functionalities, respectively. The  $^1\text{H}$  NMR spectrum of **1** (Table 1) showed the presence of two downfield singlet signals [ $\delta_{\text{H}}$  7.50 (1H, s) and 6.59 (1H, s)], one *trans*-double bond [ $\delta_{\text{H}}$  6.46 (1H, dd,  $J = 15.0, 10.9$  Hz) and 5.41 (1H, dd,  $J = 15.0, 9.5$  Hz)], an aromatic methyl [ $\delta_{\text{H}}$  2.25 (3H, s)], two olefinic methyls [ $\delta_{\text{H}}$  2.12 (3H, d,  $J = 0.9$  Hz) and 1.81 (3H, brs)] and three aliphatic doublet methyls [ $\delta_{\text{H}}$  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  $^{13}\text{C}$  NMR spectrum, complemented by DEPT experiment (Table 1) only exhibited 30 carbon resonances including two carbonyls [ $\delta_{\text{C}}$  202.1 and 168.4], five  $sp^2$  quaternary carbons, six protonated  $sp^2$  carbons, one ketal carbon [ $\delta_{\text{C}}$  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 ( $\delta_{\text{C}}$  137.7) suggested that two  $sp^2$  quaternary carbons were overlapped at  $\delta_{\text{C}}$  137.7. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **1** revealed close similarities to those of milbemycin  $\beta_3$  [9–10] except that a methylene at C-13 in milbemycin  $\beta_3$  was replaced by a carbonyl group in **1**. The observed HMBC correlation from H<sub>3</sub>-28 and H<sub>3</sub>-29 to C-13 ( $\delta_{\text{C}}$  202.1) established the structure of **1** as 13-oxomilbemycin  $\beta_3$ . The downfield chemical shift of C-15 ( $\delta_{\text{H}}$  6.71;  $\delta_{\text{C}}$  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  $[\alpha]_{\text{D}}^{25} +48$  ( $c$  0.15, EtOH) and UV (EtOH)  $\lambda_{\text{max}}$  nm ( $\log \epsilon$ ): 227 (4.56). The molecular formula of **2** was established as  $\text{C}_{32}\text{H}_{42}\text{O}_6$  based on the HRESIMS ion at  $m/z$  523.3060  $[\text{M}+\text{H}]^+$ , implying 12 degrees of unsaturation. The IR spectrum showed absorption bands assignable to the carbonyl group (1707  $\text{cm}^{-1}$ ) and the hydroxy group (3384  $\text{cm}^{-1}$ ). A detailed analysis of the  $^1\text{H}$  and  $^{13}\text{C}$  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<sub>3</sub>-32 ( $\delta_{\text{H}}$  0.96) to C-25 ( $\delta_{\text{C}}$  76.3) in conjunction with the crossing peak of H<sub>3</sub>-32/H<sub>2</sub>-31 in the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum (Figure 2) established the structure of **2** as 25-ethyl-13-oxomilbemycin  $\beta_3$ . The relative stereochemistry of **2** was assigned as that of **1**.

**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data for **1** and **2** in  $\text{CDCl}_3$ 

Position	$\delta_{\text{H}}$ (mult., $J$ in Hz)		$\delta_{\text{C}}$ (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)	2.37 (m)	33.6	33.6
	2.64 (m)	2.65 (m)		
17	3.83 (m)	3.84 (m)	65.7	65.8
18	1.18 (m)	1.19 (m)	35.3	35.5
	1.96 (m)	1.95 (m)		
19	5.38 (m)	5.38 (m)	68.4	68.5
20	1.41 (t, 11.9)	1.41 (t, 11.9)	40.8	41.0
	2.03 (m)	2.04 (m)		
21			97.7	97.6
22	1.55 (m)	1.53 (m)	35.7	35.6
	1.70 (m)	1.69 (m)		
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)	19.3	25.7
		1.69 (m)		
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  $\mu\text{g/mL}$ ; **2**, 127.37  $\mu\text{g/mL}$ ; milbemycins A3/A4, 14.26  $\mu\text{g/mL}$ ).

## 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](https://orcid.org/0000-0003-4098-6209)

Bing Li: [0000-0001-5465-5414](https://orcid.org/0000-0001-5465-5414)

Shao-Yong Zhang: [0000-0003-3571-5797](https://orcid.org/0000-0003-3571-5797)

Yi Wu: [0000-0002-2981-7589](https://orcid.org/0000-0002-2981-7589)

Wen-Sheng Xiang: [0000-0002-1826-5985](https://orcid.org/0000-0002-1826-5985)

Li-Qin Zhang: [0000-0002-3859-5412](https://orcid.org/0000-0002-3859-5412)

Huan Qi: [0000-0003-4313-5055](https://orcid.org/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 nematocidal 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  $\beta_3$ , *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-ivermectin studies. 5. Total synthesis of milbemycin  $\beta_3$  and its C(12) epimer, *J. Am. Chem. Soc.* **108**, 2662-2674.

**A C G**  
publications

© 2021 ACG Publications