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

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**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. © 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.

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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 ($\delta$), using CDCl$_3$ ($\delta$$^1$H 7.27; $\delta$$^13$C 77.0) as an internal standard, and coupling constants ($J$) in Hz. The ESIMS and HRESIMS were taken on a 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
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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$_2$Cl$_2$/ MeOH (1/1, v/v) to afford fraction IIA. Fraction IIA was further purified by preparative HPLC eluting with MeOH/H$_2$O (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; [α]$^D_{25}$ +51 (c 0.07, EtOH); UV (EtOH) $\lambda_{\text{max}}$ nm (log ε): 233 (4.50); IR (KBr) $v_{\text{max}}$ cm$^{-1}$: 3371, 2929, 1672, 1452, 1380, 1278, 1166, 1094, 1003; $^1$H 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; [α]$^D_{25}$ +48 (c 0.15, EtOH); UV (EtOH) $\lambda_{\text{max}}$ nm (log ε): 227 (4.56); IR (KBr) $v_{\text{max}}$ cm$^{-1}$: 3384, 2929, 1707, 1455, 1380, 1278, 1165, 1100, 988; $^1$H 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
Two new 13-oxomilbemycins from Streptomyces avermitilis

![Figure 2. Key 1H-1H COSY and HMBC correlations of compounds 1 and 2.](image)

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) \( \lambda_{max} \) nm (log \( \varepsilon \)): 233 (4.50). Its molecular formula \( \text{C}_{31}\text{H}_{46}\text{O}_6 \) was established by the positive HRESIMS ion at \( m/z \) 509.2909 [M+H]+ (caled 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 [\( \delta_H \) 7.50 (1H, s) and 6.59 (1H, s)], one trans-double bond [\( \delta_H \) 6.46 (1H, dd, \( J = 15.0, 10.9 \text{ Hz} \)) and 5.41 (1H, dd, \( J = 15.0, 9.5 \text{ Hz} \))], an aromatic methyl [\( \delta_H 2.25 \) (3H, s)], two olefinic methyls [\( \delta_H 2.12 \) (3H, d, \( J = 0.9 \text{ Hz} \)) and 1.81 (3H, brs)] and three aliphatic doublet methyls [\( \delta_H 1.22 \) (3H, d, \( J = 6.7 \text{ Hz} \)), 1.14 (3H, d, \( J = 6.2 \text{ Hz} \)) and 0.85 (3H, d, \( J = 6.5 \text{ Hz} \))]. Its \( ^{13}\)C NMR spectrum, complemented by DEPT experiment (Table 1) only exhibited 30 carbon resonances including two carbonyls [\( \delta_C 202.1 \) and 168.4], five \( sp^2 \) quaternary carbons, six protonated \( sp^2 \) carbons, one ketal carbon [\( \delta_C 97.7 \)], 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 (\( \delta_C 137.7 \)) suggested that two \( sp^2 \) quaternary carbons were overlapped at \( \delta_C 137.7 \). The \( ^1\)H and \( ^{13}\)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-28 and H-29 to C-13 (\( \delta_C 202.1 \)) established the structure of 1 as 13-oxomilbemycin \( \beta_3 \). The downfield chemical shift of C-15 (\( \delta_C 6.71 \); \( \delta_C 139.1 \)) 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 \( [\alpha]_D^{25} +48 \) (c 0.15, EtOH) and UV (EtOH) \( \lambda_{max} \) nm (log \( \varepsilon \)): 227 (4.56). The molecular formula of 2 was established as \( \text{C}_{32}\text{H}_{45}\text{O}_6 \) 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-32 (\( \delta_H \) 0.96) to C-25 (\( \delta_C \) 76.3) in conjunction with the crossing peak of H-32/H-31 in the \( ^1\)H-\( ^1\)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. $^1$H and $^{13}$C NMR spectral data for 1 and 2 in CDCl$_3$

| Position | $\delta_H$ (mult., $J$ in Hz) | $\delta_C$ (ppm) |
|----------|-------------------------------|------------------|
|          | 1                             | 2                | 1                | 2                |
| 1        | 168.4                         | 168.4            | 122.8            | 122.8            |
| 2        | 6.59 (s)                      | 6.60 (s)         | 114.4            | 114.5            |
| 3        | 7.50 (s)                      | 7.49 (s)         | 132.8            | 132.9            |
| 4        | 123.3                         | 123.2            | 156.2            | 156.2            |
| 5        | 6.46 (dd, 15.0, 10.9)         | 6.45 (dd, 15.0, 10.9) | 128.9            | 129.0            |
| 6        | 5.41 (dd, 15.0, 9.5)          | 5.41 (dd, 15.0, 9.8) | 135.5            | 135.5            |
| 7        | 3.93 (m)                      | 3.94 (m)         | 202.1            | 202.1            |
| 8        | 137.7                         | 137.7            | 137.7            | 137.7            |
| 9        | 5.68 (d, 10.9)                | 5.67 (d, 10.9)   | 126.9            | 126.8            |
| 10       | 6.71 (t, 7.5)                 | 6.73 (t, 7.2)    | 139.1            | 139.1            |
| 11       | 2.38 (m)                      | 2.37 (m)         | 33.6             | 33.6             |
| 12       | 2.64 (m)                      | 2.65 (m)         | 65.7             | 65.8             |
| 13       | 1.18 (m)                      | 1.19 (m)         | 35.3             | 35.5             |
| 14       | 1.96 (m)                      | 1.95 (m)         | 68.4             | 68.5             |
| 15       | 5.38 (m)                      | 5.38 (m)         | 40.8             | 41.0             |
| 16       | 1.41 (t, 11.9)                | 1.41 (t, 11.9)   | 97.7             | 97.6             |
| 17       | 2.03 (m)                      | 2.04 (m)         | 97.7             | 97.6             |
| 18       | 1.55 (m)                      | 1.53 (m)         | 35.7             | 35.6             |
| 19       | 1.70 (m)                      | 1.69 (m)         | 27.7             | 27.9             |
| 20       | 1.55 (m)                      | 1.53 (m)         | 36.5             | 34.3             |
| 21       | 1.27 (m)                      | 1.33 (m)         | 71.5             | 76.3             |
| 22       | 3.27 (m)                      | 3.06 (m)         | 15.3             | 15.3             |
| 23       | 2.25 (s)                      | 2.22 (s)         | 19.3             | 19.2             |
| 24       | 2.12 (d, 0.9)                 | 2.11 (br s)      | 16.5             | 16.5             |
| 25       | 1.81 (br s)                   | 1.80 (br s)      | 12.2             | 12.1             |
| 26       | 0.85 (d, 6.5)                 | 0.84 (d, 6.5)    | 17.9             | 17.8             |
| 27       | 1.14 (d, 6.2)                 | 1.33 (m)         | 19.3             | 25.7             |
| 28       | 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).
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Supporting Information

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

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