The Journal of Antibiotics (2011) 64, 753–756
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NOTE

# New milbemycins from mutant *Streptomyces bingchenggensis* X-4

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*The Journal of Antibiotics* (2011) **64,** 753–756; doi:10.1038/ja.2011.75; published online 17 August 2011

**Keywords:** milbemycin $\beta_{15}$; seco-milbemycins E and F; *Streptomyces bingchenggensis*

*Streptomyces bingchenggensis* produced milbemycins, including $\alpha_1$ ($A_3$), $\alpha_3$ ($A_4$), $\beta_{13}$, $\beta_{14}$, $\alpha_{28}$, $\alpha_{29}$, $\alpha_{30}$ and ST 906, four seco-milbemycins A, B, C and D, and two cyclic pentapeptides.[1–5] Milbemycins belong to a 16-membered macrolide antibiotic with an outstanding activity against various kinds of mites.[6] During a screening program for high production of $A_3$ and $A_4$, a mutant *S. bingchenggensis* X-4 was obtained by UV treated, *N*-methyl-*N*′-nitroso-*N*-nitroso-guanidine mutation and genetic manipulative techniques. Significant differences of phenotype, such as the morphology of aerial mycelia, and the metabolite HPLC profiles were observed between the wild-type *S. bingchenggensis* and its mutant strain X-4. In the course of investigating the metabolites of this mutant strain, three new interesting compounds milbemycin $\beta_{15}$ (**1**), seco-milbemycins E (**2**) and F (**3**) were isolated from the fermentation broth of *S. bingchenggensis* X-4. The structure of compound **1** was similar with milbemycin D, which is a highly selective and potent nematocide and insecticide.[7] So the bioactivity of compound **1** should be further investigated. Compared with the seco-milbemycins isolated previously,[2,3] the hydroxy groups at C-5 were absent in compounds **2** and **3**. Furthermore, all the milbemycins[3,8] and avermectins[9,10] obtained from microorganisms contain the hydroxyl at C-5, and the 5-dehydroxyl derivatives of milbemycins and avermectins can only be obtained using the synthetic methods.[11] So seco-milbemycins E and F may have an important role in understanding and perfecting the proposed biosynthesis pathways of milbemycins.

The producing strain *S. bingchenggensis* X-4 was maintained on an 1/2YM slant agar consisting of sucrose 0.4%, skim milk 0.1%, yeast extract (OXOID Basingstoke, Hampshire, UK) 0.2%, malt extract (BD Biosciences, San Jose, CA, USA) 0.5%, agar (BD Biosciences) 2.0% at 28 °C for 12 days.[12] A seed 15-l fermentor (FUS-15 L (A), Shanghai Guoqiang Bioengineering Equipment, Shanghai, China) containing 10 l of seed medium (sucrose 1.0%, polypepton 0.2%, $K_2HPO_4$ 0.05%, skim milk 0.05%)[12] was inoculated with 0.5 l of broth cultured in flask with seed medium. The flask with seed medium ($2–6\times10^7$ spores per ml) was cultured for 30 h at 28 °C on a rotary shake at 250 r.p.m. After incubation for 32 h, the seed broth (3 l) in the 15-l fermentor was transferred into the production 50-l fermentor (FUS-50 L (A), Shanghai Guoqiang Bioengineering Equipment) containing 30-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% $MgSO_4$ $7H_2O$, 0.005% $FeSO_4$ $7H_2O$ and 0.3% $CaCO_3$).[12] The culture temperature was 28 °C and the initial pH was 7.40 sterilized by sparging with steam at 121 °C for 30 min. The dissolved oxygen was maintained above 35% by adjusting the agitation speed. The initial aeration and agitation rate in the 15-l reactor was 1 vvm and 180 r.p.m., whereas those in the 50 l were 0.8 vvm and 150 r.p.m., respectively. In the process of fermentation, the pH was not controlled. The fermentation was performed for 10 days at 28 °C.

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 ~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 30 g of oily substances. The residual oily substance was chromatographed on silica gel (Qingdao Haiyang Chemical Group, Qingdao, China; 100–200 mesh) and eluted with a petroleum ether–acetone mixture (100:0–50:50, v/v). The fractions eluted with the petroleum ether–acetone mixture (90:10, v/v) were combined and evaporated to obtain fraction I and the fractions eluted with the petroleum ether–acetone mixture (85:15, v/v) were pooled and concentrated to give fraction II. The fraction I was subjected to Sephadex LH-20 (GE Healthcare, Glies, UK) gel column eluting with MeOH to give subfraction I. The semi-preparative HPLC (Agilent 1100, Zorbax SB-C18, 5 μm, 250×9.4 mm i.d.; Agilent, Palo Alto, CA, USA) was applied to obtain pure compounds. The eluates were monitored using a photodiode array detector at 220 nm, and the flow rates were 1.5 ml $min^{-1}$ at room temperature. The subfraction I was further separated by semi-preparative HPLC using a solvent containing a $CH_3OH$–$H_2O$ mixture (95:5, v/v) to obtain compounds

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Received 6 April 2011; revised 18 July 2011; accepted 21 July 2011; published online 17 August 2011

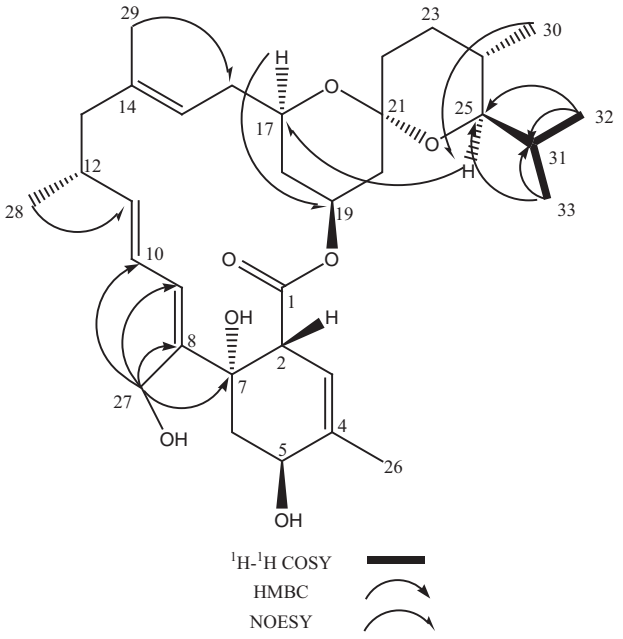


**Figure 1** The structure and key correlations of $^1$H-$^1$H COSY and HMBC of compound **1**.

**2** ($t_R$ 14.5 min, 14 mg) and **3** ($t_R$ 16.2 min, 18 mg). The fraction II was subjected to Sephadex LH-20 gel column eluting with MeOH to give subfraction II. The subfraction II was purified by the semi-preparative HPLC using a solvent containing a $CH_3OH$–$CH_3CN$–$H_2O$ mixture (48:45:7, v/v/v) to obtain compound **1** ($t_R$ 24.5 min, 13 mg).

Compound **1** (Figure 1) was isolated as colorless oil with UV (EtOH) $\lambda_{max}$ (nm) (log ε): 242 (4.21) and $[\alpha]_D^{25}$+87 (c 0.10, EtOH). Its molecular formula was established to be $C_{33}H_{50}O_7$ as deduced from the high-resolution electrospray ionization (HRESI)-MS *m/z* 581.3451 ($(M+Na)^+$, calcd for $C_{33}H_{50}O_7Na$, 581.3449) and $^{13}$C NMR data (Table 1). The IR spectrum of **1** showed absorption bands assignable to the hydroxyl group (3450 $cm^{-1}$) and an ester carbonyl (1715 $cm^{-1}$). The $^1$H NMR (400 MHz, $CDCl_3$) data (Table 1) of compound **1** exhibited one *trans* double bond at $\delta$ 5.50 (dd, *J*=14.6, 9.9 Hz) and $\delta$ 6.26 (dd, *J*=14.6, 11.2 Hz), two olefinic methyls at $\delta$ 1.59 (br s), 1.85 (br s), and four doublet aliphatic methyls at $\delta$ 0.79 (d, *J*=6.1 Hz), 0.84 (d, *J*=6.8 Hz), 1.01 (d, *J*=7.0 Hz) and 1.03 (d, *J*=6.8 Hz). Its $^{13}$C NMR and DEPT data (Table 1) showed 33 carbon resonances, including an ester carbonyl at $\delta$ 173.5 (s), a ketal at $\delta$ 97.4 (s), one oxygenated methylene at 57.8 (t), four oxygenated methines at $\delta$ 78.3 (d), 68.8 (d), 68.3 (d), 67.4 (d), one oxygenated quaternary carbon at $\delta$ 75.9 (s), in addition to five $sp^2$ methine carbons, three $sp^2$ quaternary carbons, seven aliphatic methylenes, four aliphatic methines and six methyls. The above NMR data showed that the compound **1** has the milbe-

**Table 1 $^1$H- and $^{13}$C NMR data of milbemycin $\beta_{15}$ (1), secomilbemycins E (2) and F (3) (coupling constants in parenthesis)**

| | *Proton* | | | *Carbon* | | |
|---|---|---|---|---|---|---|
| *Number* | ***(1)*** | ***(2)*** | ***(3)*** | ***(1)*** | ***(2)*** | ***(3)*** |
| 1 | | | | 173.5 s* | 165.1 s | 165.1 s |
| 2 | 3.47 d (2.0) | | | 49.2 d | 123.0 s | 123.0 s |
| 3 | 5.29 br s | 7.92 br s | 7.92 br s | 118.0 d | 130.6 d | 130.6 d |
| 4 | | | | 139.2 s | 138.5 s | 138.5 s |
| 5 | 4.45 m | 7.38 dd (8.4, 1.6) | 7.38 dd (8.4, 1.6) | 68.3 d | 134.9 d | 134.9 d |
| 6 | 2.20 m | 7.47 d (8.4) | 7.49 d (8.4) | 41.6 t | 122.6 d | 122.7 d |
| | 1.90 m | | | | | |
| 7 | | | | 75.9 s | 135.9 s | 135.9 s |
| 8 | | | | 139.5 s | 124.1 s | 124.1 s |
| 9 | 6.41 d (11.2) | 6.65 d (11.2) | 6.65 d (11.2) | 129.8 d | 127.6 d | 127.6 d |
| 10 | 6.26 dd (14.6, 11.2) | 6.32 dd (14.9, 11.2) | 6.32 dd (14.9, 11.2) | 123.9 d | 122.5 d | 122.5 d |
| 11 | 5.50 dd (14.6, 9.9) | 5.94 dd (14.9, 7.6) | 5.95 dd (14.9, 7.6) | 144.1 d | 146.6 d | 146.6 d |
| 12 | 2.46 m | 2.51 m | 2.51 m | 36.3 d | 35.4 d | 35.4 d |
| 13 | 2.18 m | 2.11 dd (13.4, 7.0) | 2.11 dd (13.4, 7.0) | 48.4 t | 47.3 t | 47.3 t |
| | 1.85 m | 2.00 m | 2.00 m | | | |
| 14 | | | | 136.4 s | 134.9 s | 134.8 s |
| 15 | 4.85 br d (6.8) | 5.22 t (6.7) | 5.22 t (6.9) | 120.8 d | 122.5 d | 122.5 d |
| 16 | 2.23 m | 2.23 m | 2.23 m | 34.5 t | 34.4 t | 34.4 t |
| 17 | 3.59 m | 3.52 m | 3.53 m | 67.4 d | 68.0 d | 68.1 d |
| 18 | 1.75 m | 1.97 m | 1.97 m | 36.8 t | 40.3 t | 40.4 t |
| | 0.77 m | 1.13 t (11.8) | 1.14 t (11.8) | | | |
| 19 | 5.33 m | 4.09 m | 4.10 m | 68.8 d | 64.8 d | 65.0 d |
| 20 | 1.93 m | 1.97 m | 2.00 m | 41.3 t | 44.8 t | 45.0 t |
| | 1.37 t (11.8), | 1.28 m | 1.28 m | | | |
| 21 | | | | 97.4 s | 97.4 s | 97.2 s |
| 22 | 1.64 m | 1.65 m | 1.65 m | 35.7 t | 35.8 t | 35.7 t |
| | 1.49 m | 1.52 m | 1.52 m | | | |
| 23 | 1.49 m | 1.52 m | 1.52 m | 28.0 t | 27.8 t | 28.0 t |
| 24 | 1.43 m | 1.25 m | 1.31 m | 31.6 d | 36.6 d | 34.6 d |
| 25 | 3.06 dd (9.6, 1.8) | 3.25 m | 3.03 m | 78.3 d | 71.1 d | 75.9 d |

**Table 1 (Continued)**

| | *Proton* | | | *Carbon* | | |
|---|---|---|---|---|---|---|
| *Number* | ***(1)*** | ***(2)*** | ***(3)*** | ***(1)*** | ***(2)*** | ***(3)*** |
| 26 | 1.85 br s | 2.39 s | 2.40 s | 19.0 q | 21.1 q | 21.1 q |
| 27 | 4.23 d (12.2)<br>4.18 d (12.2) | 5.15 br s | 5.16 br s | 57.8 t | 66.5 t | 66.5 t |
| 28 | 1.03 d (6.8) | 1.03 d (6.7) | 1.03 d (6.7) | 21.7 q | 19.8 q | 19.8 q |
| 29 | 1.59 br s | 1.62 br s | 1.63 br s | 15.9 q | 16.4 q | 16.4 q |
| 30 | 0.79 d (6.1) | 0.82 d (6.5) | 0.81 d (6.5) | 17.3 q | 17.9 q | 17.9 q |
| 31 | 1.85 m | 1.09 d (6.4) | 1.62 m<br>1.26 m | 28.3 d | 19.4 q | 25.8 t |
| 32 | 0.84 d (6.8) | | 0.89 t (7.1) | 14.1 q | | 10.2 q |
| 33 | 1.01 d (7.0) | | | 20.9 q | | |
| 7-OH | 3.86 s | | | | | |

By DEPT sequence.

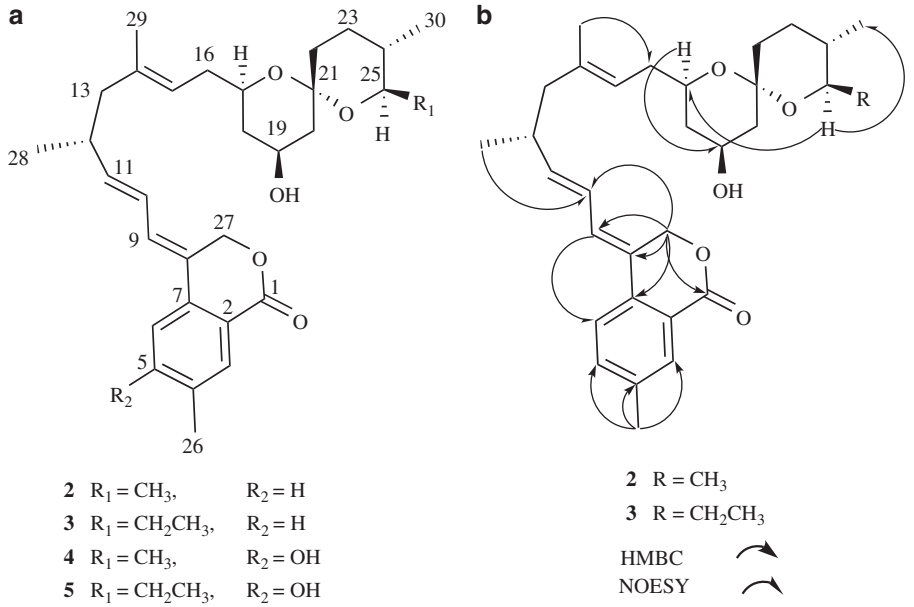


**Figure 2** The structures (**a**) of compounds **2**, **3**, **4** and **5**, as well as the key correlations of HMBC and NOESY (**b**) compounds **2** and **3**.

mycin skeleton. In the $^1$H-$^1$H COSY spectrum (Figure 1), the correlations from $\delta$ 0.84 ($H_3$-32), 1.01 ($H_3$-33) and 1.85 (H-31) and the HMBC crossing signals (Figure 1) between $\delta$ 0.84, 1.01 and $\delta$ 28.3 (C-31) indicated the presence of an isopropyl moiety. Comparison of the $^1$H and $^{13}$C NMR spectral data of compound **1** with those of milbemycin $\beta_{12}$[13] revealed that compound **1** was similar to those of milbemycin $\beta_{12}$, except for the substituents at C-8 and C-25, where the methyl at C-8 and the ethyl group at C-25 in milbemycin $\beta_{12}$ were replaced by a hydroxymethyl group and isopropyl group in compound **1**, respectively. The observed HMBC correlations (Figure 1) from $H_3$-32 ($\delta_H$ 0.84)/$H_3$-33 ($\delta_H$ 1.01) to C-25 ($\delta_C$ 78.3) and from $H_2$-27 ($\delta_H$ 4.18, 4.23) to C-7 ($\delta_C$ 75.9), C-9 ($\delta_C$ 129.8), C-8 ($\delta_C$ 139.5) confirmed the structural assignment of compound **1**. Thus, the gross structure of compound **1** was established.

In compound **1**, the large *J* (14.6 Hz) of H-10 and H-11 and the NOESY crossing peaks (Figure 1) between $H_2$-27 and H-10, $H_3$-29 and $H_2$-16 indicated that the three double bonds at C-8 and C-9, C-10 and C-11, C-14 and C-15 were *E*. The NOESY correlations of H-17, $H_3$-30 and H-25, H-17 and H-19, H-11 and $H_3$-28 suggested that these protons having the same orientations as in milbemycin $\beta_{12}$.

From a biogenetic point of view, the relative stereochemistry of the chiral centers in compound **1** was assigned based on that of milbemycin E[14] and milbemycin $\beta_{12}$. The assumption was supported by the similar optical rotation values of **1** and milbemycin E ($[\alpha]_D^{27}$+157 (c 0.25, acetone)).

Compound **2** (Figure 2a) was obtained as colorless oil with UV (EtOH) $\lambda_{max}$ (nm) (log ε): 288 (4.08) and $[\alpha]_D^{25}$+29.6 (c 0.27, EtOH). Its molecular formula was established to be $C_{31}H_{42}O_5$ as deduced from the HRESI-MS *m/z* 517.3027 $[M+Na]^+$ (calcd for $C_{31}H_{42}O_5Na$ 517.3006) and $^{13}$C NMR data (Table 1). The IR spectrum of compound **2** showed absorption bands assignable to the hydroxyl group (3454 cm$^{-1}$) and an ester carbonyl (1724 cm$^{-1}$). The $^1$H NMR (400 MHz, $CDCl_3$) data (Table 1) of compound **2** displayed one *trans* double bond at $\delta$ 5.90 (dd, *J*=14.9, 7.6 Hz) and $\delta$ 6.32 (dd, *J*=14.9,

11.2 Hz), one aromatic methyl at $\delta$ 2.39 (s), one vinylic methyl at $\delta$ 1.62 (br s), and three aliphatic methyls at $\delta$ 0.82 (d, $J$=6.5 Hz), 1.03 (d, $J$=6.7 Hz) and 1.09 (d, $J$=6.4 Hz). The signals at $\delta$ 7.38 (1H, dd, $J$=8.4, 1.6 Hz), 7.47 (1H, d, $J$=8.4 Hz) and 7.92 (1H, br s) in the $^1$H NMR spectrum of compound **2** showed the presence of a 1, 2, 4-trisubstituted benzene ring moiety. Its $^{13}$C NMR and DEPT data (Table 1) revealed an ester carbonyl at $\delta$ 165.1 (s), a ketal at $\delta$ 97.4 (s), three oxygenated methines, one oxygenated methylene, in addition to 12 $sp^2$ carbons, 6 aliphatic methylene, 2 aliphatic methines and 5 methyls. Comparison of the $^1$H and $^{13}$C NMR spectral data of compound **2** with those of seco-milbemycin C (compound **4**, Figure 2a)[3] revealed that compound **2** was almost identical to those of seco-milbemycin C, except for the substituent at C-5, where the hydroxy in seco-milbemycin C was replaced by a hydrogen atom in compound **2**. The observed HMBC correlations (Figure 2b) from H-27 ($\delta$ 5.15) to C-1 ($\delta$ 165.1) and from $H_3$-26 ($\delta$ 2.39) to C-3 ($\delta$ 130.6), and from C-4 ($\delta$ 138.5) and C-5 ($\delta$ 134.9) further confirmed the structural assignment of compound **2**. Thus, the gross structure of compound **2** was established.

In compound **2**, the NOESY correlations (Figure 2b) between H-27 and H-10 and between H-6 and H-9 indicated the $\Delta^8$ olefin was *trans*-double bond as in seco-milbemycin C. The *E* configuration of the $\Delta^{10}$ olefin of compound **2** was assigned by the large $J$ (14.9 Hz) between H-10 and H-11. The NOESY correlation signal between H-29 and H-16 revealed that the double bond of C-14 and C-15 was *E*. Furthermore, the NOESY correlations (Figure 2a) of H-17, $H_3$-30 and H-25, H-17 and H-19, H-10 and $H_3$-28 showed the orientation of these protons. Thus, the structure of compound **2** was established. Biogenetically, the relative stereochemistry of the chiral centers in compound **2** was assigned based on that of seco-milbemycin C. The assumption was supported by the similarity of optical rotation values between compound **2** and secomilbemycin C ($[\alpha]_D^{20}$+25 (c 0.20, EtOH)).

Compound **3** (Figure 2a) was also isolated as colorless oil with $[\alpha]_D^{25}$+32.9 (c 0.28, EtOH) and UV (EtOH) $\lambda_{max}$ (nm) (log ε): 287 (4.11). The HRESI-MS gave the molecular formula $C_{32}H_{44}O_5$ (found: 531.3133 $[M+Na]^+$, calcd: 531.3149). Its $^1$H and $^{13}$C NMR data (Table 1) were very similar to those of compound **2**. Comparison of the $^1$H and $^{13}$C NMR data of compound **3** with those of compound **2**, suggested that compound **3** was structurally related to compound **2**. The difference between compounds **3** and **2** could be explained by the replacement of a methyl substituent in C-25 in compound **2** by an ethyl group in compound **3**. The stereostructure of compound **3** was assigned as that of compound **2** by the analysis of the NMR data and NOESY correlation signals (Figure 2b). Further comparison of the NMR data with those of seco-milbemycin A (compound **5**, Figure 2a)[2] confirmed the structure assignment of compound **3**.

The acaricidal activity against adult two-spotted spider mites (*Tetranychus urticae* Koch) and mite eggs as well as nematocidal activity against *Caenorhabditis elegans* of compounds **1**, **2** and **3** were evaluated according to the methods described previously.[1,4,5] Although these compounds showed potent acaricidal and nematocidal activities (data not shown), the bioactivities were weaker than those of the commercial acaricide and nematocide milbemycins $A_3$/$A_4$. However, the discovery of compounds **1**, **2** and **3** in the mutant *S. bingchenggensis* X-4 may shed new insight into the biosynthesis of milbemycin.

## ACKNOWLEDGEMENTS

This research work was financially supported by the National Natural Science Foundation of China (30971937), the Program for New Century Excellent Talents in University (NCET-08-0668, 1154-NCET-002), the Outstanding Youth Foundation of Heilongjiang Province (JC200706), the National Key Project for Basic Research (2010CB126102) and the Program for New Teachers in University (20092325120007).

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