NOTE

A novel macrolide compound from *Streptomyces bingchenggensis*: fermentation, isolation, structure elucidation and biological properties

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Natural products play an important role in drug discovery and have been used for the treatment of diseases for decades. They constitute a leading source of novel molecules for the development of new drug candidates.^{1–3} To search for more bioactive compounds, we have reported earlier the isolation and structure elucidation of milbemycins, β_{13} , β_{14} , α_{28} , α_{29} , and α_{30} , and secomilbemycins A and B from *Streptomyces bingchenggensis*.^{4–6} In this paper, we obtained a novel macrolide compound, ST906 (**1**), from *S. bingchenggensis*, and described the fermentation, isolation, structural elucidation and cytotoxic activity.

MATERIALS AND METHODS

Microorganism

The producing organism, *S. bingchenggensis*, was isolated from a soil sample collected in Harbin, China. *S. bingchenggensis* has been deposited at the China General Microbiology Culture Collection Center (accession no. CGMCC1734; Institute of Microbiology, Chinese Academy of Sciences), and we have determined the 16S rDNA sequence (accession no. DQ449953 in GenBank, National Center for Biological Information).

Fermentation

The seed for preculture was spores. The medium for sporulation contained sucrose (Bei Jing Ao Bo Xing, Beijing, China) 4 g, yeast extract (Bei Jing Ao Bo Xing) 2 g, malt extract (Bei Jing Ao Bo Xing) 5 g and skimmed milk (Nmyili, Huhehaote, China) 1 g in 1 l water. The pH was adjusted to 7.0 with 1 M NaOH to which 20 g of agar was added, and this mixture was sterilized at 121 °C for 30 min. The spore suspension was prepared from the agar plates (20 ml) incubated at 28 °C for 7–8 days.

A spore suspension of the culture of strain *S. bingchenggensis*, 1 ml, was transferred to a 250-ml Erlenmeyer flask that contained 25 ml of the seed medium containing sucrose 0.25 g, polypepton (Bei Jing Ao Bo Xing) 0.1 g and K₂HPO₄ 1.25 mg. The inoculated flasks were incubated at 28 °C for 42 h on a rotary shaker at 250 r.p.m. Then, 8.0 ml of the culture was transferred into a 1-l Erlenmeyer flask containing 100 ml of the producing medium consisting of sucrose (Bei Jing Ao Bo Xing) 8.0%, soybean powder (Comwin, Beijing, China)

1.0%, yeast extract (Bei Jing Ao Bo Xing) 0.2%, meat extract (Bei Jing Ao Bo Xing) 0.1%, CaCO₃ (Bei Jing Hong Xin) 0.3%, K₂HPO₄ 0.03%, MgSO₄·7H₂O 0.1% and FeSO₄·7H₂O 0.005%; pH 7.2 before sterilization. Fermentation was carried out at 28 °C for 8 days on a rotary shaker at 250 r.p.m.

Isolation and purification

The fermentation broth (15 l) was filtered. The resulting cake was washed with water, and both filtrate and wash were discarded. MeOH (3 l) was used to extract the washed cake. The MeOH extract was concentrated to ~1 l under reduced pressure and the resulting concentrate was extracted three times with an equal volume of EtOAc. The combined EtOAc phase was concentrated under reduced pressure to yield 25 g of an oily substance. The residual oily substance was chromatographed on silica gel (Qing Dao Hai Yang Chemical Group Co., Qingdao, China, 100–200 mesh) and eluted with petroleum ether–Me₂CO (95:5–50:50) to give five fractions that were separated by TLC with petroleum ether–acetone (3:1, v/v). Spots were detected on TLC under UV or by heating after spraying with sulfuric acid–ethanol (5:95, v/v). To obtain pure compound ST906 (**1**), the fourth fraction (petroleum ether–acetone 70:30, v/v) of the silica gel chromatography was separated by semipreparative HPLC (Agilent 1100, Santa Clara, CA, USA, Zorbax SB-C18, 5 μ m, 250×9.4 mm i.d.) using a solvent of 96% CH₃OH/H₂O. The eluates were monitored with a photodiode array detector at 220 nm, and the flow rate was 1.5 ml min^{–1} at a room temperature (ST906 (**1**), 17.3 min).

General

UV spectra were obtained on a CARY 300 BIO spectrophotometer (Varian, Palo Alto, CA, USA); IR spectra were recorded on a Nicolet Magna FT-IR 750 spectrometer (Thermo Scientific, Waltham, MA, USA); ¹H and ¹³C NMR spectra were measured with a DRX-400 (400 MHz for ¹H and 100 MHz for ¹³C) spectrometer (Bruker, Bremen, Germany). Chemical shifts are reported on parts per million (δ), using the residual CHCl₃ (δ_{H} 7.26; δ_{C} 77.0) as an internal standard, and coupling constant (*J*) in Hertz. ¹H and ¹³C NMR assignments were supported by ¹H–¹H COSY, heteronuclear multiple quantum coherence and heteronuclear multiple bond coherence (HMBC) experiments. The electrospray ionization-MS (ESI-MS) and high-resolution electrospray ionization-MS (HRESI-MS) spectra were taken on a Q-TOF Micro LC–MS–MS mass

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spectrometer. Optical rotation was measured on a 341 Polarimeter (PerkinElmer, Shanghai, China).

Biological assays

The cytotoxicity of compound on tumor cells was assayed according to published procedures.⁷ Human colon carcinoma cell line, HCT-116, was routinely cultured in Dulbecco's modified Eagle's medium containing 10% calf serum at 37 °C for 4 h in a humidified atmosphere of 5% CO₂ incubator. The adherent cells at their logarithmic growth stage were digested, and were inoculated onto a 96-well culture plate at a density of 1.0×10^4 /well for the determination of proliferation. Test samples were added to the medium, and incubation was continued for 72 h. Coloration substrate, cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan), was added to the medium followed by further incubation for 3 h. Absorbance at 450 nm with a 600 nm reference was measured thereafter. Media and DMSO control wells, in which compound was absent, were included in all the experiments to eliminate the influence of DMSO. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition (\%)} = [\text{OD}_{\text{control}} - \text{OD}_{\text{treated}}] / \text{OD}_{\text{control}} \times 100$$

The cytotoxicity of the compound on tumor cells was expressed as IC₅₀ values (the drug concentration reducing the absorbance in treated cells by 50%, with respect to untreated cells) and was calculated by the LOGIT method.

Physicochemical properties of compound ST906 (1)

ST906 (1, Figure 1) C₃₂H₄₂O₇, colorless oil; $[\alpha]_D^{20} +25.7^\circ$ (*c* 0.07, EtOH); UV (EtOH) λ_{max} nm (log ϵ): 206 (4.18), 228 (4.34) and 290 (3.84); IR (KBr), ν_{max} cm⁻¹: 3360, 2924, 1704, 1619, 1510, 1457, 1376, 1263, 1207, 1101, 1027 and 985; ¹H NMR (CDCl₃, 400 MHz) and ¹³C NMR (CDCl₃, 100 MHz) see Table 1; ESI-MS *m/z* 537 [M-H]⁺; HRESI-MS *m/z* 537.2843 [(M-H)⁺, calcd for C₃₂H₄₁O₇, 537.2846].

Structural elucidation

Compound ST906 (1) was obtained as a colorless oil with the UV absorptions at λ_{max} nm: 206, 228, 257 and 290. In its IR spectrum, the hydroxyl and carbonyl absorptions were presented at 3360 cm⁻¹ and 1704 cm⁻¹, respectively. The molecular formula of 1 was established as C₃₂H₄₂O₇ on the basis of HRESI-MS and NMR analyses, which indicated the presence of 12° of unsaturation. The ¹H NMR spectrum (see Table 1) of ST906 (1) showed two downfield singlet signals at δ 7.83 (s) and 7.59 (s), two doublet methyls at δ 1.03 (d) and δ 0.83 (d), a methyl triplet at δ 1.01 (t) and two singlet olefinic methyls at δ 2.35 (s) and δ 1.74 (br s). A *trans*-double bond was also presented by the signals at δ 6.94 (d, *J*=15.8 Hz) and δ 6.15 (dd, *J*=15.8, 9.4 Hz) in the ¹H NMR spectrum. The ¹³C NMR and DEPT

spectra of ST906 (1) indicated 32 carbons, including one ester carbonyl carbon, 12 *sp*² carbons, one ketal carbon, 5 methyls, 7 aliphatic methylenes and 6 *sp*³ methines (including four oxygenated ones). These data suggested that ST906 (1) was the analog of milbemycin β_{14} .⁴ Besides the six olefins, the presence of five rings was required to satisfy the degrees of unsaturation, so a five-membered ether ring was formed between C-6 and C-27 similar to other milbemycin α -series. The observed HMBC correlations (Figure 2) from δ_{H} 1.03 to δ_{C} 40.1, 43.3 and 86.6, and a ¹H-¹H COSY correlation between δ_{H} 4.56 and δ_{H} 6.15 suggested that a hydroxyl was presented at C-11 and the *trans*-double bond was in C-9 and C-10. The long-range ¹H-¹³C correlation between δ_{H} 7.83 and δ_{C} 120.0, 123.6 and 142.4, and between δ_{H} 6.94 and δ_{C} 144.4 showed that a double bond was present in C-8 and C-27. Thus, the gross planar structure of ST906 (1) was established.

The relative stereochemistry of ST906 (1) was established on the basis of that of other milbemycins, except that of the new chiral center of C-11. In the NOESY experiment, the absent correlated signal

Table 1 ¹H and ¹³C NMR data of ST906 (1) (coupling constants in parenthesis)

Number	Proton	Carbon	Number	Proton	Carbon
1		167.2 s ^a	19	5.63 m	70.0 d
2		117.1 s	20	2.16 m	41.3 t
3	7.59 s	129.7 d		1.33 m	
4		119.6 s	21		97.5 s
5		142.4 s	22	1.65 m	35.6 t
6		142.4 s		1.53 m	
7		123.6 s	23	1.50 m	27.9 t
8		120.0 s	24	1.33 m	34.3 d
9	6.94 d (15.8)	126.7 d	25	3.11 m	76.0 d
10	6.15 dd (15.8, 9.4)	126.4 d	26	2.35 s	14.8 q
11	4.56 br d (9.4)	86.6 d	27	7.83 br s	144.4 d
12	1.86 m	40.1 d	28	1.03 d (7.1)	14.0 q
13	2.14 m	43.3 t	29	1.74 br s	18.3 q
14		137.2 s	30	0.83 d (6.5)	17.8 q
15	5.35 br t (7.3)	122.2 d	31	1.67 m	25.7 t
16	2.46 m	33.3 t		1.35 m	
	2.32 m		32	1.01 t (6.2)	10.1 q
17	3.93 m	67.0 d			
18	2.14 m	35.6 t			
	1.42 m				

^aBy DEPT sequence.

Figure 1 The structures of ST906 (1), milbemycin α -class and LL-F 28249v.

Figure 2 The important HMBC correlations of ST906 (1).

between H-11 and H₃-28 attempted to assign the hydroxyl of C-11 in the same orientation as the methyl of C-28.

The structure of ST906 (1) is similar to milbemycin α -class; however, there are significant differences between them. Milbemycins have a double bond between C-3 and C-4, whereas ST906 (1) possesses a benzene structure. Although a benzene structure was found in the milbemycin β -class,⁴ only compound LL-F 28249v presents a benzene structure in milbemycin α -class (Figure 1).^{8,9} ST906 (1) possesses both double bond between C-8 and C-27, and between C-9 and C-10. For milbemycin α - and β -classes, the position of two double bonds is presented at C-8 and C-9, and at C-10 and C-11, respectively. Moreover, ST906 (1) got a hydroxyl at C-11, whereas for milbemycins there is no such evidence. It is concluded that ST906 (1) is a novel macrolide compound.

Biological activity

We examined the inhibitory activity of compound ST906 (1) against the growth of human colon carcinoma cell line, HCT-116, using the CCK-8 colorimetric method as described in the Materials and methods section. Compound ST906 (1) dose-dependently inhibited the growth of HCT-116 cells with an IC₅₀ value of 4.8 $\mu\text{g ml}^{-1}$. However, the IC₅₀ value for control doxorubicin was 4.2 $\mu\text{g ml}^{-1}$. Bioassay results showed that ST906 (1) had strong cytotoxic activity.

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