J. Antibiot. 60(12): 739–744, 2007

ORIGINAL ARTICLE

THE JOURNAL OF ANTIBIOTICS

Two New Members of Streptothricin Class Antibiotics from *Streptomyces qinlingensis* sp. nov

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Received: September 12, 2007 / Accepted: November 27, 2007

Abstract Four streptothricin-group antibiotics (**1**~**4**) were isolated from the fermentation broth of *Streptomyces qinlingensis* sp. nov. Along with the known antibiotics streptothricins F (**1**) and D (**3**), two new members of this class (**2**, **4**) were identified as 12-carbamoyl derivatives of **1** and **3**, respectively, mainly by analysis of the IR, HR-MS and NMR spectral data. The antibacterial activities of **1**~**4** against *Escherichia coli* (MICs 3.1, 25.0, 3.1 and 12.5 μg/ml), *Bacillus subtilis* (MICs 6.3, 25.0, 3.1 and 50 μg/ml), *Staphylococcus aureus* (MICs 12.5, >100.0, 6.3, >100.0 μg/ml), *Bacillus cereus* (MICs 25.0, 50.0, 25.0 and 50.0 μg/ml) and *Pseudomonas aeruginosa* (MICs 50.0, >100.0, 50.0, >100.0μg/ml) were assayed by micro-broth dilution. The results based on MIC data indicated that **2** and **4** exhibited significantly less potent antibacterial activities when compared to that of **1** and **3**.

Keywords *Streptomyces qinlingensis*, streptothricin, structural elucidation

Introduction

Streptothricins are a group of *N*-glycoside antibiotics. The first member of this group antibiotics, streptothricin F (**1**), was isolated by Waksman and Woodruff from *Streptomyces lavendulae* in 1942 [1], van Tamelen proposed its structural formula until 1961 [2]. Since then, the probable structures of the other six members of streptothricins (A, B, C, D, E, and X) were also reported [3, 4]. The molecular structures of all these compounds were finally confirmed by a total synthesis of **1** in 1982, and the location of the carbamate moiety was reassigned from C-12 to C-10 [5, 6]. All streptothricins consist of carbamoylated D-gulosamine to which the β-lysine homopolymer (1~7 residues) and the amide form of the unusual amino acid streptolidine (streptolidine lactam) are attached (Fig. 1). Also β-*N*-acetyl derivatives of streptothricins E and D (**3**) were found from *Streptomyces nojiriensis* [7]. Achievements in the study of streptothricin antibiotics were reviewed in 1983 [8].

Streptothricins are broad-spectrum antibiotics, which exhibit the potent activity against wide range of bacteria as well as some pathogenic fungi [1]. Because of their inherent toxicity, none of them has been used clinically [9], but these antibiotics have potential value to exploit as some agricultural fungicides. Zhongshengmycin, which consists of streptothrins A~F, has been registered as

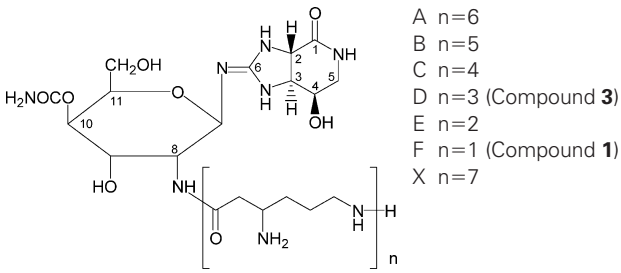

Fig. 1 Structures of streptothricin-group antibiotics A~F and X.

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agricultural antibioctics to control some bacterial diseases in China [10]. Recently, a new streptothricin family antibiotic producing *Streptomyces* spp. SNUS 8810-111 was isolated from a Korea soil sample, and produced **3** and *N*-methylstreptothricin D [11, 12]. Studies were conducted to address the formation and timing of incorporation of the arginine-derived base streptolidine in the biosynthesis of **1** [13]. Although many streptothricin-resistance genes have been identified, only one resistance mechanism has been identified. A novel streptothricin-resistance gene (SttH) was successfully isolated from *S. albulus* [14]. An SttH gene-knockout mutant to better understand the true biologicol role of SttH in *S. albulus* and to clarify whether or not *S. albulus* possesses different streptothricin-resistance gene(s) was also constructed [15].

In the process of screening new antibiotics, we found four streptothricin antibiotics (**1**～**4**) from the fermentation broth of *S. qinlingensis* sp. nov, a new *Streptomyces* strain isolated from a soil sample collected in Qinling mountain, Shannxi province, China. In the present paper, the isolation, structure elucidation, and preliminary bioassay of two new streptothricin-group antibiotics from *S. qinlingensis* are presented.

Materials and Methods

Microorganism

The producing strain *S. qinlingensis* sp. nov was isolated from a soil sample collected in Qinling mountain, Shannxi province, China, and identified by testing its morphology, physiology, biochemistry, and 16S rRNA gene sequence as a new strain. The voucher specimen of this actinomycete was deposited at the China General Microbiological Culture Collection Center as CGMCC1381, and the 16S rDNA sequence was registered in Genebank (Accession No: AM167521 in National Center for Biological Information).

Fermentation

The spores of *S. qinlingensis* sp. nov. grown on Gause's No. 1 agar was used to inoculate into a 250-ml flask containing 100 ml of a sterile seed medium consisting of glucose 0.8%, soluble starch 0.8%, beef extract 0.6%, peptone 1.0%, and NaCl 0.5%, pH 7.2. The flask was shaken on a shaker at 210 rpm for 24 hours at 28°C.

Ten milliliters of the seed culture was transferred to 250-ml flasks containing of 100 ml of a sterile producing medium consisting of glucose 3.0%, millet steep liquor 1.0%, peptone 1.5%, NaCl 0.5%, and $CaCO_3$ 0.5%, pH 7.2. Fermentation was carried out at 210 rpm for 96 hours at 28°C on a rotary shaker.

Isolation and Purification

Ten liters of harvest fermentation broth were adjusted to pH 3.5 with oxalic acid and stirred for 30 minutes. The acidic broth was filtered and adjusted to pH 8.0, and it was passed through a column of HD-2 (Na^+, 2 liters). Active principle adsorbed on the column was eluted with 0.5 N HCl (8.0 liters). The eluent was adjusted to pH 7.0 and concentrated *in vacuo* to a small volume (about 300 ml). The concentrated solution was diluted with MeOH (3.0 liters) to precipitate NaCl. MeOH was removed on a rotary evaporator, and the aqueous solution was applied on a column of CM-Sephadex C-25 (0.5 liters, Na^+). The column was eluted with a linear gradient of water and 1.0 M NaCl solution (1.0 liter each). The eluents were collected in 50-ml fractions with two acitive peaks observed at fractions 61～68 and 90～96, which were combined to two fractions. Two active fractions were desalted further by charomatography on active carbon columns and concentrated under reduced pressure to a small volume (about 10 ml), respectively. The concentrated fractions were further separated by ion-pair RP-HPLC on a column of Hypersil ODS-BP (column size 10×250 mm, particle size 10 μm, flow rate 3.5 ml/minute, monitored by RI detector), fractions 61～68 was eluted with 5.0% MeOH containing 2.0% TFA, and fraction 90～96 was eluted with 8.0% MeOH containing 6.0% TFA. Four main fractions were collected according to the peaks in the HPLC profiles, each fraction was lyophilized, redissolved in water and passed through a column packed with 717 resin (Cl^-, 50 ml) to exchange TFA for chloride. Finally, the samples were lyophilized and stored as solids. **1** (152 mg) and **2** (28 mg) were obtained from fraction 61～68, **3** (160 mg) and **4** (80 mg) were from fraction 90～96, respectively.

General Methods

Melting points were measured on an X4 apparatus and uncorrected. IR spectra were recorded on a Nicolet FT-IR 750 spectrometer (KBr plate, ν_{max} in cm^{-1}); all ^1H-, ^{13}C-NMR, DEPT, COSY, HSQC and HMBC spectra were taken on a Bruker Avance 500 MHz (500 MHz for ^1H and 125 MHz for ^{13}C, respectively) spectrometer in D_2O with TMS as an internal standard. The ESI-MS and HRESI-MS spectra were obtained on a Finnigan LCQ LC/MS^n, and a Bruker APEX II mass spectrometer using glycerol as matrix. Optical rotation was measured in MeOH solution on a Perkin-Elmer 341 Polarimeter. Ion-exchange resins HD-2 and 717 were purchased from Shanghai Huazhen Sci. & Tch. Co., Ltd.

Antibacterial Assay

MICs of **1**～**4** against *Bacillus subtilis*, *Staphylococcus*

aureus, *Escherichia coli*, *Bacillus cereus*, and *Pseudomonas aeruginosa* were tested by the micro-broth dilution method [16]. The inoculum was prepared by suspending several colonies from an overnight culture of tested bacteria from 5.0% sheep blood agar media in Mueller-Hinton broth, and adjusting to a 0.5 McFarland standard (approximately 1.5×10^8 colony-forming units per ml). A further dilution of 1 : 200 was made by placing 0.25 ml of the adjusted suspension into 49.75 ml of Mueller-Hinton broth. Stock solutions of tested compounds and streptomycin (positive control) in sterile water were prepared at the concentration of 1000 μg/ml and used immediately or stored in working samples at −20°C until used. Two-fold serial dilutions of the tested compounds were prepared in Mueller-Hinton broth. All antimicrobial solutions were prepared in large volumes (50 ml); 0.1 ml samples of the antibiotic solutions and 0.1 ml inoculated suspension of the test microorganism were delivered to wells of a 96-well plate. The final concentration of inoculum in each well was 3.7×10^5 colony-forming units per ml. MIC endpoints were read after 18 hours of incubation at 35°C, and were defined as the lowest concentration of antibiotics that resulted in no bacterial growth as indicated by the ODs at 650 nm. The blank control tube only contained organisms. Three replications were used for each sample.

Results and Discussion

Physico-chemical Properties of 1∼4

1: white amorphous powder, m.p. 220∼222°C (dec.), $[\alpha]_D^{25}$ −45° (*c* 0.1, MeOH). IR (KBr), ν_{max} cm^{-1}: 3271, 3052,

Table 1 ^{1}H-NMR data for streptothricin antibiotics (**1**∼**4**) in D_2O

Position n	**1** (Streptothricin F)		**2** (12-Carbamoylstreptothricin F)		**3** (Streptothricin D)		**4** (12-Carbamoylstreptothricin D)	
	δ	*J* (Hz)	δ	*J* (Hz)	δ	*J* (Hz)	δ	*J* (Hz)
2	4.64 (d)	14	4.64 (d)	14	4.63 (d)	15	4.63 (d)	15
3	4.10 (d)	14	4.10 (d)	14	4.09 (m)	—	4.09 (m)	—
4	4.74 (m)	—	4.75 (m)	—	4.73 (m)	—	4.74 (m)	—
5	3.83 (dd)	6, 15	3.84 (dd)	6, 15	3.81 (dd)	6, 15	3.81 (dd)	5, 15
5	3.42 (d)	15	3.43 (d)	15	3.42 (d)	15	3.42 (d)	15
7	5.11 (d)	10	5.07 (d)	10	5.10 (d)	10	5.05 (d)	10
8	4.28 (dd)	3, 10	4.35 (dd)	3, 8	4.26 (dd)	3, 10	4.32 (m)	—
9	4.18 (t)	3	4.11 (t)	3.5	4.16 (t)	3.5	4.08 (t)	3.5
10	4.79 (m)	—	3.90 (d)	3.5	4.79 (m)	—	3.89 (d)	3.5
11	4.35 (t)	6	4.35 (t)	6	4.34 (t)	6	4.34 (m)	—
12	3.75 (d)	6	4.26 (m)	—	3.73 (d)	6	4.26 (m)	—
12	3.75 (d)	6	4.26 (m)	—	3.73 (d)	6	4.26 (m)	—
15	2.82 (dd)	4, 16	2.83 (dd)	5, 17	2.76 (dd)	5, 16	2.76 (dd)	5, 16
15	2.70 (dd)	4, 16	2.71 (dd)	8, 17	2.68 (dd)	8, 17	2.68 (dd)	8, 17
16	3.71 (m)	—	3.71 (m)	—	3.66 (m)	—	3.65 (m)	—
17	1.81 (m)	—	1.81 (m)	—	1.72 (m)	—	1.71 (m)	—
18	1.81 (m)	—	1.81 (m)	—	1.65 (m)	—	1.64 (m)	—
19	3.07 (m)	—	3.07 (m)	—	3.25 (t)	7	3.24 (m)	—
21					2.73 (dd)	5, 16	2.72 (dd)	5, 16
21					2.62 (dd)	8, 17	2.61 (dd)	8, 17
22					3.66 (m)	—	3.66 (m)	—
23					1.72 (m)	—	1.71 (m)	—
24					1.65 (m)	—	1.64 (m)	—
25					3.22 (t)	7	3.24 (m)	—
27					2.70 (dd)	5, 16	2.69 (dd)	5, 16
27					2.60 (dd)	8, 17	2.59 (dd)	8, 17
28					3.66 (m)	—	3.65 (m)	—
29					1.81 (m)	—	1.78 (m)	—
30					1.81 (m)	—	1.78 (m)	—
31					3.06 (t)	6	3.05 (m)	—

2955, 1708, 1653, 1552, 1493, 1394, 1354, 1302, 1189, 1134, 1078, 949, 914, 851 cm^{-1}; ^1H- and ^{13}C-NMR: see Tables 1 and 2; ESI-MS (*m/z*): 503 $[M+H]^+$, 252 $[M+2H]^{2+}$; HR-ESI-MS (*m/z*): 503.2566 $[M+H]^+$, Calcd. for $C_{19}H_{35}N_8O_8$, 503.2572.

2: white amorphous powder, m.p. 184～186°C (dec), $[\alpha]_D^{25}$ −29° (*c* 0.1, MeOH). IR ν_{max} cm^{-1}: 3332, 2924, 1707, 1652, 1611, 1558, 1481, 1391, 1312, 1280, 1078, 957, 925, 888 cm^{-1}. ^1H- and ^{13}C-NMR: see Tables 1 and 2. ESI-MS (*m/z*): 503 $[M+H]^+$, 252 $[M+2H]^{2+}$; HR-ESI-MS (*m/z*): 503.2569 $[M+H]^+$, Calcd. for $C_{19}H_{35}N_8O_8$, 503.2572.

3: white amorphous powder, m.p. 120～122°C (dec), $[\alpha]_D^{25}$ −3.5° (*c* 0.1, MeOH). IR (KBr), ν_{max} cm^{-1}: 3382, 3274, 3081, 2936, 1718, 1651, 1555, 1448, 1388, 1309, 1258, 1188, 1129, 1071, 974, 953, 926, 849 cm^{-1}; ^1H- and ^{13}C-NMR: see Tables 1 and 2; ESI-MS (*m/z*): 759 $[M+H]^+$, 380 $[M+2H]^{2+}$; HR-ESI-MS (*m/z*): 759.4473 $[M+H]^+$, Calcd. for $C_{31}H_{59}N_{12}O_{10}$, 759.4477.

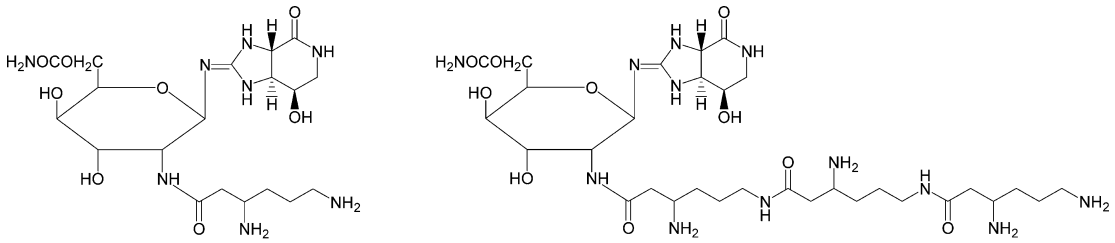
4: white amorphous powder, m.p. 116～118°C (dec), $[\alpha]_D^{25}$ −6.5° (*c* 0.1, MeOH). IR ν_{max} cm^{-1}: 3383, 3067, 2930, 1718, 1652, 1558, 1491, 1390, 1309, 1253, 1069, 951, 849 cm^{-1}; ^1H- and ^{13}C-NMR: see Tables 1 and 2; ESI-MS (*m/z*): 759 $[M+H]^+$, 380 $[M+2H]^{2+}$; HR-ESI-MS (*m/z*): 759.4478 $[M+H]^+$, Calcd. for $C_{31}H_{59}N_{12}O_{10}$, 759.4477.

Structure Elucidation

The IR spectra of **1**～**4** showed the characteristic absorption bands at 1652～1653 and 1558～1560 cm^{-1} which suggested that they are all peptides. The molecular formula of **1** and **2** were determined to be $C_{19}H_{34}N_8O_8$, **3** and **4** were

Table 2 ^{13}C-NMR data for **1**～**4** in D_2O

Position	**1**	**2**	**3**	**4**
1	172.5	172.4	172.5	172.4
2	56.9	56.9	57.0	56.9
3	63.4	63.4	63.4	63.4
4	63.4	63.4	63.4	63.4
5	51.8	51.8	51.8	51.9
6	165.3	164.9	165.3	164.9
7	81.3	81.5	81.4	81.6
8	51.4	51.8	51.4	51.9
9	69.0	70.4	69.1	70.4
10	72.6	71.6	72.6	71.7
11	76.1	75.0	76.1	75.0
12	62.9	66.5	62.9	66.5
13	160.4	161.5	160.5	161.6
14	174.6	174.6	174.8	174.7
15	38.8	38.8	39.0	39.1
16	50.8	50.8	51.1	51.0
17	31.6	31.6	32.1	32.1
18	25.5	25.5	26.9	26.9
19	41.5	41.5	41.4	41.4
20			174.4	174.4
21			39.2	39.2
22			51.1	51.0
23			32.1	32.1
24			26.9	26.9
25			41.4	41.5
26			174.2	174.2
27			39.3	39.4
28			51.3	51.3
29			31.7	31.7
30			25.5	25.2
31			41.5	41.5



2 (12-Carbamoylstreptothricin F) **4** (12-Carbamoylstreptothricin D)

Fig. 2 Structures of two novel streptothricin antibiotics (**2** and **4**).

Table 3 Antibacterial *in vitro* activity of **1**∼**4** by micro-broth dilution

Compounds	MIC (μg/ml)				
	Bacillus subtilis (No. 1.0088)	*Staphylococcus aureus* (No. 1.0089)	*Escherichia coli* (No. 1.1636)	*Bacillus cereus* (No. 1.1846)	*Pseudomonas aeruginosa* (No. 1.2031)
1 (Streptothricin F)	6.3	12.5	3.1	25.0	50.0
2	25.0	>100.0	25.0	50.0	>100.0
3 (Streptothricin D)	3.1	6.3	3.1	25.0	50.0
4	50.0	>100.0	12.5	50.0	>100.0
Streptomycin	3.1	50.0	32.0	6.3	50.0

determined to be $C_{31}H_{58}N_{12}O_{10}$, on the basis of ^{13}C-NMR and HR-ESI-MS. Based on the spectroscopic analysis, and comparison the ^{1}H- and ^{13}C-NMR data with that reported in reference [17, 18], **1** and **3** were identified as streptothricin F and D, respectively. Comparison the ^{1}H- and ^{13}C-NMR data of **2** and **4** with that of **1** and **3**, we found that the chemical shift of C-9 upfield 1.4 δ, C-10 downfield 1.0 δ, C-11 downfield 1.1 δ and C-12 upfield 3.6 δ in the ^{13}C-NMR spectra, and the chemical shift of H-10 upfield 0.9 δ and H-12 downfield 0.5 δ in the ^{1}H-NMR spectra. These differences maybe attribute to the variety of the substitution position of the carbamoyl group attached to the D-gulosamine in which the substitution position of carbamoyl was transfered from C-10 to C-12 [18]. This presumption was confirmed by the HMBC experiment. In the HMBC spectra of **1** and **3**, the protons of H-9, H-10 and H-11 were correlated with the carbonyl of carbamoyl group. However, the carbonyl of carbamoyl group was correlated with the protons of H-10, H-11 and H-12 in the HMBC spectra of **2** and **4**. Thus the structures of **2** and **4** were finally elucidated as carbamoyl derivatives at C-12 of **1** and **3**, respectively. To our best knowledge, there are only two similar structure compounds were reported before, which were found in the course of screening *N*-type calcium channel blockers in 1998, and they were finally identified as carbamoyl derivatives at C-12 of streptothricin C and B [19, 20], respectively. So **2** and **4** are two novel streptothricin antibiotics (Fig. 2).

Biological Activity

Antimicrobial activities of **1**∼**4** against *B. subtilis*, *S. aureus*, *E. coli*, *B. cereus* and *P. aeruginosa* were determined by the micro-broth dilution method, which were shown in Table 3. Comparing the MICs of **2** and **4** to those of **1** and **3**, the antimicrobial activities of the former two compounds were significantly lower, indicating that the position of the carbamoyl group in the glucosamine moiety plays important role for the antimicrobial properties of this class of streptothricin antibiotic.

Acknowledgements This work was supported by the National Key Project for Basic Research of China (No. 2003CB114404) and the Hi-Tech Research and Development Project of China (No. 2002AA2455121). We wish to thank Xi'an Modern Chemistry Research Institute for NMR analysis.

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