ORIGINAL ARTICLE

Isolation, structure elucidation and antibacterial activities of streptothricin acids

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Five streptothricin acids (compounds 1–5) were isolated by ion-exchange resin chromatography and preparative RP-HPLC from the fermentation broth of *Streptomyces qinlingensis*. Their structures were elucidated mainly by analyses of the IR, HR-EIS-MS and NMR spectral data. They were deduced as two known compounds, streptothricin F acid (1) and streptothricin D acid (2), and three new compounds, 12-carbamoylstreptothricin E acid (3), 12-carbamoylstreptothricin D acid (4) and N-acetyl-streptothricin D acid (5), respectively. The antibacterial activities of 1–5 against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* were assayed by micro-broth dilution. Comparison of the MICs with streptothricin F and D showed that the antimicrobial activities of 1–5 were decreased significantly but varied with the structures.

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Keywords: antibacterial activity; streptothricin acid; structural elucidation

Streptothricin antibiotic is a type of broad-spectrum antibiotics. As streptothricin F was isolated from *Streptomyces lavendulae* in 1942,¹ dozens of streptothricins (STs) have been isolated from natural resources. Although they were shown to possess strong antimicrobial activity against many species of fungi and bacteria, their application in the therapeutic area has been hampered because of the nephrotoxicity.^{2–5} Hamano *et al.*⁶ isolated a novel ST-resistance gene (*sttH*) and showed that the hydrolysis of the amide bond of streptolidine lactam could be catalyzed by the enzyme *in vitro*. With the breaking of the amide bond, the antimicrobial activity of streptothricin D (ST-D) was altered from broad-spectrum to bacteria-specific, and its toxicity against eukaryotic cells was also reduced at the same time. These results suggest that ST-D acid is a potential candidate for clinical development or use as a new lead compound for drug discovery. Attempts at preparing ST acids were made from STs chemically, but only ST-F acid, which bears one β -lysine, was readily obtained, whereas others that bear more β -lysine residues were not prepared successfully.^{7,8} In addition, only the bio-activities of ST-F and ST-D acids have been investigated.⁶

In the process of screening for new antibiotics, *S. qinlingensis*, a ST producer, was isolated from soil samples. ST-F, ST-D and two other novel compounds, 12-carbamoyl-ST-F and 12-carbamoyl-ST-D, have been isolated from its fermentation broth in our laboratory.⁹ In subsequent research, a facile LC-MS/MS method based on RP-HPLC coupled with electrospray ionization tandem mass spectrometry was used for the analysis of STs in the fermentation broth of *S. qinlingensis*.¹⁰ A total of 19 ST-like compounds were identified or tentatively characterized on the basis of their mass spectral data; among them, 12 were ST acids. Here, we report the

isolation, structure elucidation and antibacterial activities of five ST acids that were isolated by ion-exchange resin chromatography and preparative RP-HPLC from the fermentation broth of *S. qinlingensis*. These compounds were identified as two known compounds, ST-F acid (1) and ST-D acid (2), and three new compounds, 12-carbamoylstreptothricin E acid (3), 12-carbamoylstreptothricin D acid (4) and N-acetyl-streptothricin D acid (5). The antibacterial activities of 1–5 against *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Bacillus cereus* and *Pseudomonas aeruginosa* were assayed by means of micro-broth dilution. The results indicated that 1–5 exhibited less antibacterial activities when compared with that of ST-D, but the extent of decrease varied with the structures.

RESULTS AND DISCUSSION

Physicochemical properties of 3–5

Compound 3: colorless amorphous powder, m.p. 132–134 °C (dec), $[\alpha]_D^{25}$ –14.0 (c 0.1, MeOH). IR $\nu_{\max}$ cm^{–1}: 3382, 1651, 1555, 1492, 1388, 1070 cm^{–1}. ¹H and ¹³C NMR: see Tables 1 and 2. High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) (*m/z*): 649.3630 [M+H]⁺, calcd for C₂₅H₄₉N₁₀O₁₀, 649.3628.

Compound 4: colorless amorphous powder, m.p. 150–152 °C (dec), $[\alpha]_D^{25}$ +8 (c 0.1, MeOH). IR $\nu_{\max}$ cm^{–1}: 3396, 1655, 1558, 1492, 1390, 1067 cm^{–1}; ¹H and ¹³C NMR: see Tables 1 and 2. HR-ESI-MS (*m/z*): 777.4576 [M+H]⁺, calcd for C₃₁H₆₂N₁₂O₁₁, 777.4577.

Compound 5: colorless amorphous powder, m.p. 172–174 °C (dec), $[\alpha]_D^{25}$ +4.2 (c 0.1, MeOH). IR $\nu_{\max}$ cm^{–1}: 3418, 1652, 1554, 1445, 1388, 1071 cm^{–1}; ¹H and ¹³C NMR: see Tables 1 and 2. HR-ESI-MS (*m/z*): 819.4683 [M+H]⁺, calcd for C₃₃H₆₂N₁₂O₁₂, 819.4683.

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Table 1 ^1H NMR data of compounds 1–5 (500 MHz, in D_2O)

Position	1	2	3	4	5
2	4.58 (d, $J=5$)	4.69 (d, $J=5$)	4.63 (d, $J=5$)	4.61 (d, $J=5$)	4.71 (d, $J=5$)
3	4.27 (m)	4.34 (m)	4.30 (m)	4.30 (m)	4.36 (m)
4	4.14 (m)	4.14 (m)	4.15 (m)	4.14 (m)	4.15 (m)
5	3.28 (dd, $J=5, 14$)	3.25 (dd, $J=5, 14$)	3.28 (m)	3.29 (dd, $J=3, 14$)	3.29 (m)
5	3.12 (dd, $J=10, 14$)	3.13 (dd, $J=11, 14$)	3.13 (m)	3.13 (dd, $J=11, 14$)	3.13 (m)
7	5.14 (d, $J=10$)	5.14 (d, $J=10$)	5.09 (d, $J=10$)	5.14 (d, $J=10$)	5.12 (d, $J=10$)
8	4.25 (dd, $J=3, 10$)	4.26 (dd, $J=3, 10$)	4.31 (dd, $J=3, 10$)	4.26 (dd, $J=3, 10$)	4.26 (dd, $J=3, 10$)
9	4.18 (t, $J=3$)	4.15 (t, $J=3$)	4.10 (t, $J=3$)	4.18 (t, $J=3$)	4.08 (t, $J=3$)
10	4.79	4.79	3.88 (d, $J=4$)	3.88 (d, $J=4$)	4.79
11	4.33 (t, $J=6$)	4.33 (t, $J=6$)	4.33 (t, $J=6$)	4.33 (t, $J=6$)	4.32 (t, $J=6$)
12	3.74 (d, $J=6$)	3.74 (d, $J=6$)	4.23 (d, $J=5$)	4.23 (d, $J=5$)	3.72 (d, $J=6$)
12	3.74 (d, $J=6$)	3.74 (d, $J=6$)	4.26 (d, $J=7$)	4.26 (d, $J=7$)	3.72 (d, $J=6$)
15	2.81 (dd, $J=5, 17$)	2.79 (dd, $J=5, 17$)	2.78 (dd, $J=5, 17$)	2.80 (dd, $J=5, 17$)	2.54 (dd, $J=5, 17$)
15	2.71 (dd, $J=8, 17$)	2.65 (dd, $J=8, 17$)	2.65 (dd, $J=8, 17$)	2.71 (dd, $J=8, 17$)	2.43 (dd, $J=8, 17$)
16	3.71 (m)	3.66 (m)	3.68 (m)	3.68 (m)	4.14 (m)
17	1.79 (m)	1.81 (m)	1.74 (m)	1.72 (m)	1.54 (m)
18	1.79 (m)	1.70 (m)	1.68 (m)	1.70 (m)	1.54 (m)
19	3.05 (m)	3.12 (m)	3.24 (m)	3.07 (m)	3.19 (m)
21		2.72 (dd, $J=5, 17$)	2.75 (dd, $J=5, 17$)	2.75 (dd, $J=5, 17$)	2.75 (dd, $J=5, 17$)
21		2.63 (dd, $J=8, 17$)	2.62 (dd, $J=8, 17$)	2.62 (dd, $J=8, 17$)	2.65 (dd, $J=8, 17$)
22		3.66 (m)	3.68 (m)	3.66 (m)	3.64 (m)
23		1.71 (m)	1.82 (m)	1.75 (m)	1.70 (m)
24		1.68 (m)	1.63 (m)	1.66 (m)	1.65 (m)
25		3.24 (m)	3.07 (m)	3.23 (m)	3.24 (m)
27		2.70 (dd, $J=5, 17$)		2.72 (dd, $J=5, 17$)	2.72 (dd, $J=5, 17$)
27		2.60 (dd, $J=8, 17$)		2.62 (dd, $J=8, 17$)	2.62 (dd, $J=8, 17$)
28		3.67 (m)		3.69 (m)	3.67 (m)
29		1.78 (m)		1.81 (m)	1.82 (m)
30		1.64 (m)		1.62 (m)	1.82 (m)
31		3.26 (m)		3.24 (m)	3.07 (m)
N-acetyl					1.99 (s)

Structure elucidation

Two known compounds (**1**, **2**) and three novel compounds (**3**–**5**) were obtained from the fermentation broth of *S. qinlingensis* finally. Compounds **1** and **2** were identified as ST-F acid and ST-D acid on the basis of the M.P., IR, MS and NMR data.^{7,8}

Compound **3** was obtained as a colorless amorphous powder. The molecular formula $\text{C}_{25}\text{H}_{48}\text{N}_{10}\text{O}_{10}$ was determined by HR-ESI-MS at m/z 649.3630 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{25}\text{H}_{49}\text{N}_{10}\text{O}_{10}$, 649.3628). The IR spectra showed the characteristic absorption bands at 1654 and 1556 cm^{-1} , which revealed the existence of a peptide bond. The NMR spectral data (Tables 1 and 2) suggested the presence of two amido groups, one carboxylic acid group, one carbamoyl group and one guanidino group. The spectral characteristics were very similar to those of STs. By comparing the ^1H and ^{13}C NMR data of **3** with those of ST-D acid (**2**), we found that the chemical shift of C-4, C-5 in the ^{13}C NMR spectra and the chemical shift of H-4 and H-5 in the ^1H NMR spectra were not different. Compound **3** was assumed to be the ST acid type compound. The hypothesis is also proved by the results of ESI-MS/MS; m/z 171, which was assigned to streptolidine, was a characteristic ion peak of STs, whereas the corresponding ion was observed at m/z 189 in the spectrum of **3**. This indicated that streptolidine lactam was converted into acid for compound **3**. The differences between the ^1H and ^{13}C NMR data of **3** and **2** were also observed; we found a chemical shift of C-9 downfield 2.6 p.p.m., C-10 upfield 2.2 p.p.m., C-11 upfield 1.1 p.p.m. and C-12 downfield

3.6 p.p.m. in the ^{13}C NMR spectrum, as well as a chemical shift of H-10 upfield 0.9 p.p.m. and H-12 downfield 0.5 p.p.m. in the ^1H NMR spectrum. These differences may be attributed to the variety of the substitution position of the carbamoyl group attached to D-gulosamine in which the substitution position of carbamoyl was transferred from C-10 to C-12. This presumption was confirmed by the heteronuclear multiple-bond correlation (HMBC) experiment. In the HMBC spectra of **2**, the protons of H-9, H-10 and H-11 were correlated with the carbonyl of the carbamoyl group. However, the carbonyl of the carbamoyl group was correlated with the protons of H-10, H-11 and H-12 in the HMBC spectra of **3**. Thus, the carbamoyl group of **3** was substituted at C-12, and the compound was identified as 12-carbamoylstreptothricin E acid. To our best knowledge, only four 12-carbamoylstreptothricins, 12-carbamoylstreptothricin F, D, C and B, have been reported; so compound **3** is a novel 12-carbamoylstreptothricin antibiotic (Figure 1).^{9,11}

Compound **4** was obtained as a colorless amorphous powder. The molecular formula $\text{C}_{31}\text{H}_{60}\text{N}_{12}\text{O}_{11}$ was determined by HR-ESI-MS at m/z 777.4576 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{31}\text{H}_{61}\text{N}_{12}\text{O}_{11}$, 777.4577). The IR spectra showed the characteristic absorption bands at 1654 and 1556 cm^{-1} , which revealed the existence of a peptide bond. The NMR spectral data (Tables 1 and 2) suggested the presence of three amido groups, one carboxylic acid group, one carbamoyl group and one guanidino group. By comparing the ^1H and ^{13}C NMR data of **4** with those of **2** and **3**, we found that the chemical shift of C-4, C-5,

Table 2 ^{13}C NMR data of compounds 1–5 (125 MHz, in D_2O)

Position	1	2	3	4	5
1	173.53	173.45	172.05	174.26	173.10
2	56.74	56.73	57.05	57.18	56.83
3	61.33	61.31	61.54	61.55	61.27
4	67.97	67.96	67.96	67.98	67.96
5	40.59	40.61	40.61	40.61	41.32
6	158.46	157.84	158.26	158.35	158.46
7	79.04	79.09	79.14	79.14	79.13
8	48.95	48.92	48.68	48.92	48.93
9	66.42	66.46	69.03	67.98	66.74
10	69.98	69.99	67.76	67.76	69.97
11	73.37	73.38	72.27	72.28	73.32
12	60.19	60.21	63.79	63.79	60.26
13	157.85	158.45	158.99	158.99	157.82
14	171.97	172.11	171.82	172.08	173.82
15	36.26	36.80	36.61	36.58	41.32
16	48.25	48.67	48.46	48.43	47.17
17	28.97	29.46	29.41	29.37	31.37
18	22.85	24.28	24.27	24.25	24.85
19	38.89	38.76	38.77	38.79	38.96
20		171.82	171.62	171.62	171.62
21		36.59	36.82	36.58	36.58
22		48.66	48.49	48.43	48.46
23		29.38	29.07	29.06	29.42
24		22.88	22.89	24.25	24.30
25		38.76	38.91	38.79	36.77
26		171.61		171.62	171.62
27		36.56		36.58	36.85
28		48.45		48.43	48.70
29		29.06		29.06	29.07
30		22.89		22.90	22.90
31		38.89		38.88	39.00
N-acetyl					173.10, 22.10

C-10, C-12 in the ^{13}C NMR spectra, and the chemical shift of H-4, H-5, H-10, H-12 in the ^1H NMR spectra were similar; so **4** also belongs to 12-carbamoylstreptothricin acid. The molecular weight of **4** is 128 Da higher than that of **3**, which corresponds to a molecule of β -lysine. On the basis of the ^1H and ^{13}C NMR data and HMBC spectra, **4** was identified as 12-carbamoylstreptothricin D acid.

Compound **5** was obtained as a colorless amorphous powder. The molecular formula $\text{C}_{33}\text{H}_{62}\text{N}_{12}\text{O}_{12}$ was determined by HR-ESI-MS at m/z 819.4683 $[\text{M}+\text{H}]^+$ (calcd for $\text{C}_{33}\text{H}_{63}\text{N}_{12}\text{O}_{12}$, 819.4683). The IR spectra showed the characteristic absorption bands at 1651 and 1554 cm^{-1} , which revealed the existence of a peptide bond. The NMR spectral data (Tables 1 and 2) suggested the presence of four amido groups, one carboxylic acid group, one carbamoyl group and one guanidino group. By comparing the ^1H and ^{13}C NMR data of **5** with those of **2–4**, we found that the chemical shift of C-4, C-5 in the ^{13}C NMR spectrum, and H-4, H-5 in the ^1H NMR spectrum were similar to **3**; the chemical shift of C-10, C-12 in the ^{13}C NMR spectrum, and H-10, H-12 in the ^1H NMR spectrum were similar to **2**; so **5** belongs to ST acid. The ^1H NMR spectrum of **5** showed a singlet (3H) at δ 1.99 in addition to all the signals observed in that of **2**, and the signal of the methyl group was observed at δ 22.10 in the ^{13}C NMR spectrum of **5**. In the HMBC spectrum of **5**, we could find that the methyl group is attached to a carbon (δ 173.10) of carbonyl group, which was absent in the ^{13}C NMR spectrum of **2**. The protons

of H-15 and H-16 were correlated with the carbonyl group at δ 173.10, indicating that the acetyl group was attached to the amino group of C-16. Thus, **5** was identified as N-acetylstreptothricin D acid.

Streptothricin F acid is the most investigated compound of ST acids, and was first prepared from ST-F by partial hydrolysis, whereas the other ST acids bearing more β -lysine residues were not readily obtained chemically.^{7,8} The results may be attributed to that, although STs possess easily hydrolyzable bonds, such as ester and amido bonds, but the hydrolysis is strongly influenced by the number of β -lysine. In 2006, Hamano *et al.*⁶ isolated a novel ST-resistance gene (sttH) from *S. albulus*, which could catalyze the transformation of ST-D and ST-F from lactam to acid *in vitro*. The results of HPLC-MS/MS analysis also indicated the distribution of ST acids in the metabolites of *S. qinlingensis*. We therefore hypothesized that a similar resistance gene may exist in this actinomycete, the research of which is under way in our laboratory.

Antibacterial assay

Antimicrobial activities of compounds **1–5** against *B. subtilis*, *S. aureus*, *E. coli*, *B. cereus* and *P. aeruginosa* were determined by the micro-broth dilution method, and the results are showed in Table 3. In a comparison of the MICs of STs F and D with those of **1–5**, the antimicrobial activities of **1–5** were decreased significantly, but the extents varied with the structures. The structural activity relationship of STs has been described in previous research, but that of ST acids was not investigated thoroughly.^{12–15} To our best knowledge, only the antibacterial activities of ST acids F and D were reported up to now. ST-D acid exhibited higher activity against *B. subtilis* and *S. aureus* than did ST-F acid, and this indicates that the number of β -lysine plays an important role in the antibacterial activity for this type of ST acid antibiotics. The activity of **4** was slightly lower than that of **2**, and the only difference between them is the substituted position of the carbamoyl group in the D-gulosamine moiety, which implies that their activities were also influenced by the substituted position of the carbamoyl group. It is noticeable that acetylation of β -lysine almost resulted in the loss of activity, and this phenomenon was identical with that of classical ST antibiotics.^{12,13}

METHODS

Microorganism and fermentation

The producing strain *S. qinlingensis* was isolated from a soil sample collected in Qinling Mountain, Shannxi Province, China, and identified by its morphology, physiology, biochemistry and 16S rRNA gene sequence. The voucher specimen of this streptomycete was deposited at the China General Microbiological Culture Collection Center as CGMCC1381, and the 16S rDNA sequence was registered in GenBank with the accession no: AM167521 in the National Center for Biological Information.

The spores of *S. qinlingensis* grown on Gause's No. 1 agar were used to inoculate into a 250 ml flask containing 50 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 r.p.m. for 24 h at 28 °C. Ten milliliters of the seed culture were transferred to 250 ml flasks containing 50 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 r.p.m. for 96 h 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 min. The acidic broth was filtered, adjusted to pH 8.0, and passed through a column of HD-2 (Na^+ , 2 l; Shanghai Huazhen Sci. & Tch. Co., Shanghai, China). Active principle adsorbed on the column was eluted

Figure 1 Structures of compounds 1–5.

Table 3 Antibacterial *in vitro* activities of 1–5 by micro-broth dilution

Compounds	MIC ($\mu\text{g}\cdot\text{ml}^{-1}$)				
	<i>B. subtilis</i> (1.0088)	<i>S. aureus</i> (1.0089)	<i>E. coli</i> (1.1636)	<i>B. cereus</i> (1.1846)	<i>P. aeruginosa</i> (1.2031)
Streptothricin F	6.3	12.5	3.1	25.0	50.0
Streptothricin D	3.1	6.3	3.1	25.0	50.0
1	25.0	50.0	50.0	>100.0	>100.0
2	12.5	25.0	12.5	25.0	50.0
3	25.0	>100.0	25.0	50.0	>100.0
4	25.0	50	12.5	50.0	>100.0
5	50.0	>100.0	50.0	>100.0	>100.0

with 0.5 M HCl (81). The eluent was adjusted to pH 7.0 with NaHCO_3 and concentrated *in vacuo* to a small volume (about 300 ml). The concentrated solution was diluted with MeOH (3:1) to precipitate sodium chloride. The MeOH was removed on a rotary evaporator, and the aqueous solution was applied on a column of CM-Sephadex C-25 (0.5 l, Na^+). The column was eluted with a linear gradient of water and 1.0 M NaCl solution (1 l each). The eluents were collected in 50 ml fractions and analyzed by HPLC-MS/MS. The analyses were performed on a Finnigan LCQ Advantage MAX LC/MS (Thermo Finnigan, San Jose, CA, USA) equipped with a Waters (Waters, Milford, MA, USA) Atlantis dC18 column (4.6 $\times$ 250 mm, 5 μm), and a water/TFA/acetonitrile gradient was used as mobile phase. The gradient started with 5% acetonitrile in 0.1% aqueous TFA for 10 min. The percent of acetonitrile was

increased to 20% in the next 10 min. The flow rate of the mobile phase was 0.8 ml min^{-1} and the injection volume of sample was 10 μl . The eluate from the HPLC was introduced into a splitter to provide a 0.1 ml min^{-1} flow into the mass spectrometer. An electrospray ionization (ESI) interface with positive-ion mode was used. The ESI conditions were as follows: source voltage 4.5 kV, capillary temperature 300°C , sheath gas flow 75, auxiliary gas flow 10, capillary voltage 10 V. A data-dependent acquisition was used in the LC-MS/MS experiments. The collision energy for MS/MS was adjusted to 35% and the isolation width of precursor ions was 3.0 mass units. The results showed that ST acids were distributed in fractions 25–28 and 72–77, and these fractions were combined with fractions A and B, respectively. The two fractions were further desalted by chromatography on active carbon columns and concentrated under reduced pressure to a small volume (about 10 ml). The concentrated fractions were further separated on a Shimadzu (Tokyo, Japan) 6AD HPLC apparatus equipped with a column of Hypersil ODS-BP (20 $\times$ 250 mm, 10 μm , flow rate 3.5 ml min^{-1}), monitored by a Shodex (Kawasaki, Japan) RI-101 detector. Fraction A was eluted with 6.0% MeOH containing 2.0% TFA; fraction B was eluted with 8.0% MeOH containing 6.0% TFA. Five fractions were collected according to the peaks in the HPLC profiles, and each fraction was lyophilized, redissolved in water and passed through a column packed with 717 anion ion exchange resin (Cl^- , 50 ml; Shanghai Huazhen Sci. & Tch. Co.) to exchange TFA for chloride. The solutions were lyophilized to yield five compounds, with 1 (45 mg), 2 (83 mg) and 3 (51 mg) being from fraction A, and 4 (32 mg) and 5 (36 mg) being from fraction B, respectively.

Structure elucidation

The structures of these compounds were elucidated on the basis of extensive 1D and 2D NMR experiments and high-resolution mass spectrometry, and confirmed by comparison of their ESI-MS/MS data with those of LC-MS/MS. Melting points were measured on an X4 apparatus and uncorrected. IR spectra

were determined on an IR-450 instrument (KBr plate). ^1H , ^{13}C NMR, DEPT, COSY, heteronuclear single quantum coherence and HMBC spectra were taken on a Bruker Avance 500 MHz (500 MHz for ^1H and 125 MHz for ^{13}C , respectively) spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) in D_2O solution with TMS as an internal standard. The ESI-MS/MS and HR-ESI-MS spectra were obtained on a Finnigan LCQ LC-MSⁿ (Thermo Finnigan) and a Bruker APEX II mass spectrometer (Bruker Biospin GmbH) using glycerol as the matrix. Optical rotation was measured in methanol solution on a Perkin-Elmer 341 Polarimeter (Perkin Elmer, Fremont, CA, USA).

Antibacterial assay

Minimum inhibitory concentration of compounds 1–5 against *B. subtilis*, *S. aureus*, *E. coli*, *B. cereus* and *P. 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% sheep blood agar medium in Mueller-Hinton broth, and adjusting to a 0.5 McFarland standard (approximately 1.5×10^8 cells ml^{-1}). 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 a concentration of 1000 $\mu\text{g ml}^{-1}$ and used immediately or stored in working samples at -20°C until used. Doubling 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 bacterium were delivered to wells of a 96-well plate. The final concentration of inoculum in each well was 3.7×10^5 cells ml^{-1} . Minimum inhibitory concentration end points were read after 18 h 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 contained only bacteria. Three replications were carried out for each sample.

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