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Identification of Streptothricin Class Antibiotics in the Early-stage of Antibiotics Screening by Electrospray Ionization Mass Spectrometry

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Abstract Several streptothricin antibiotics have been studied by tandem mass spectrometry. The dominant product ions were derived from the C_7-N bond cleavage which lead to lose streptolidine from the $[M+H]^+$. The fragmentation pathways of key ions were described and certained by parent scan. According to the generalized principles, streptothricin isomers could be distinguished easily by the difference of CID spectra. A facile method based on ion-pair RP-HPLC coupled with electrospray ionization tandem mass spectrometry has been established for the analysis of streptothricins in the fermentation broth of *Streptomyces qinlingensis*. A total of 19 streptothricin-like compounds were identified or tentatively characterized based on their mass spectral data, and in which 11 were the first reported compounds. This could be used as an important de-replication method in the programs of screening novel antibiotics.

Keywords streptothricins, electrospray ionization, tandem mass spectometry, structural characterization

Introduction

Streptothricins (STs) are the earliest antibiotics with broad antimicrobial spectrum found in actinomycetes and had been studied extensively [1, 2]. In addition to a potent inhibitory activity for prokaryotic protein synthesis, streptothricins have cytotoxicity which prevents their clinical or veterinary use [3]. However, this may be useful in the situations in which toxicity may not be a problem, such as in agriculture or prevention of microbial contamination. For example, streptothricins have been registered as an agrochemical fungicide in China in 1990s [4].

With more and more bioactive compounds were isolated from microorganism metabolite, it has become an apparent obstacle that some known antibiotics were rediscovered frequently. Streptothricins are the most abundant antibiotics, and found in about 10% of randomly collected soil actinomycetes [5]. To improve the efficiency of discovering new bioactive lead compounds, quick distinguishing known antibiotics from microorganism metabolites in the early stage of screening is a useful strategy.

Terekhova LP *et al.* reported a methodology to detect streptothricins at the early stages of screening new antibiotics [6]. These approaches were mainly based on chromatographic and electrophoretic mobility of streptothricins and the products of their hydrolysis in the extracts from agar cultures of actinomycetes. The microbe cross-resistance to specific groups of antibiotics was also used to identify these compounds [7]. Mehta RJ *et al.* developed a protocol for early identification of streptothricins, in which *E. coli* K802N/*E. coli* K802N *strn*^{R} pair and TLC system were incorporated as a routine

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method for presumptive detection of streptothricins [8].

Liquid chromatography coupled with mass spectrometry is a powerful technique that is amenable to dereplication of natural products. Liquid chromatography is efficient in separating chemical compounds in a mixture, and MS provides abundant information for structural elucidation of the compounds when tandem mass spectrometry (MS^n) is applied. Therefore, the combination of HPLC and MS facilitates rapid and validate identification of known antibiotics in microorganism metabolites. Electrospray ionization (ESI) is a preferred source due to its high ionization efficiency for water soluable and moderately polar compounds.

The structures of streptothricins consist of a streptolidine, a carbamoyl-D-gulosamine and a β-lysine chain which varies from one to six units in streptothricins F to A, respectively, and streptothricin X includes seven β-lysines [9, 10]. More recently, other members of the family, which are chemically closely related to streptothricin, have been described in references [11, 12] and our laboratory [13]. Ando reported the structure elucidation of *N*-acetyl-streptothricins by application of NMR and fast atom bombardment mass spectrometry (FAB-MS), in which the fragmentation ions were used to determine the position of the acetyl group [14]. The application of mass spectrometry in the screening of streptothricin class antibiotics has not been investigated thoroughly.

Here we report the collision induced dissociation (CID) characters and the fragmentation patterns of the $[M+H]^+$ of several stretptothricins produced by elecetrospray ionization (ESI), utilize them in the differentiation of streptothricins class antibiotics isomers. The analysis of streptothricins in the fermentation broth of *Streptomyces qinlingensis* using ion-pair RP-HPLC-MS/MS is also presented.

Experimental

Chemicals

Pure streptothricins (STs) antibiotics, isolated from the fermentation broth of *Streptomyces qinlingensis* sp. nov., were provided by the Institute of Pesticide Science, Northwest A&F University, China. All above compounds were identified by IR, NMR and HR-MS, and their purities were checked by HPLC. AR grade Acetonitrile (CH_3CN) and trifluoacetic acid (TFA) were purchased from Tedia corporation, USA.

Sample Preparation

Fermentation broth (0.2 liters) of *S. qinlingensis* filtered at pH 2.0 was adjusted to pH 6.0 and passed through a column of HD-2 (Na^+, 20 ml). Active principle adsorbed on the column was eluted with 0.5 N HCl (100 ml). The eluate was adjusted to pH 6.0 and desalted on a column of active carbon (10 ml). The eluate was filtered through a 0.46 μm membranes before use.

Chromatography

The HPLC instrument used for the analysis was a Finnigan Surveyor equipped with a photodiode array detector. An Atlantis (Registered trademark of Waters, Co.) dC_{18} column (4.6×250 mm, 5.0 μm) and a water/TFA/CH_3CN gradient were used as mobile phase. The gradient started with 5.0% CH_3CN in 0.1% aq TFA for 10 minutes. The percent of CH_3CN was increased to 20% in the next 10 minutes. The detective wavelength was set at 200 nm, and the flow rate was 0.8 ml/minute. The injection volume of the sample solution was 10 μl.

ESI-MS and ESI-MS/MS

The experiments were performed on a Finnigan LCQ Advantage MAX ion-trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with an electrospray interface, and operated in the positive ion mode. The samples were introduced into the electrospray ionization source by a syringe pump at a typical flow rate of 5.0 μl/minute. The mass resolution of ion trap analyzer was typically set to obtain unit resolution. The standard STs solutions were prepared in methanol and analyzed at a concentration of 0.05 μg/μl.

For ESI-MS/MS experiments, the precursor ions produced by the ESI source were selected and fragmented to produce ions of next stage. The collision energy for MS^n was adjusted to 35%, and the isolation width of precursor was 3.0 mass units.

LC-MS and LC-MS/MS

The LC-MS experiments were performed on the LCQ Advantage MAX ion-trap mass spectrometer coupled with a surveyor HPLC. The flow to the ESI source of the mass spectrometer through a fused silica capillary of 50 cm length and 75 μm i.d. was limited to approximately 10% of the LC flow. The MS detector was optimized by injecting STs antibiotics standards (0.05 μg/μl in MeOH) to obtain maximum intensities of $[M+H]^+$. Infusion into the mass spectrometer was performed as follows: the flow of sample solution coming from the built-in syringe pump at a 5.0 μl/minute flow-rate was mixed with mobile phase (100 μl/minute) through a T-piece. A voltage of 4.5 kV applied to the ESI (electrospray ionization) needle resulted in a distinct signal. The temperature of the heated capillary

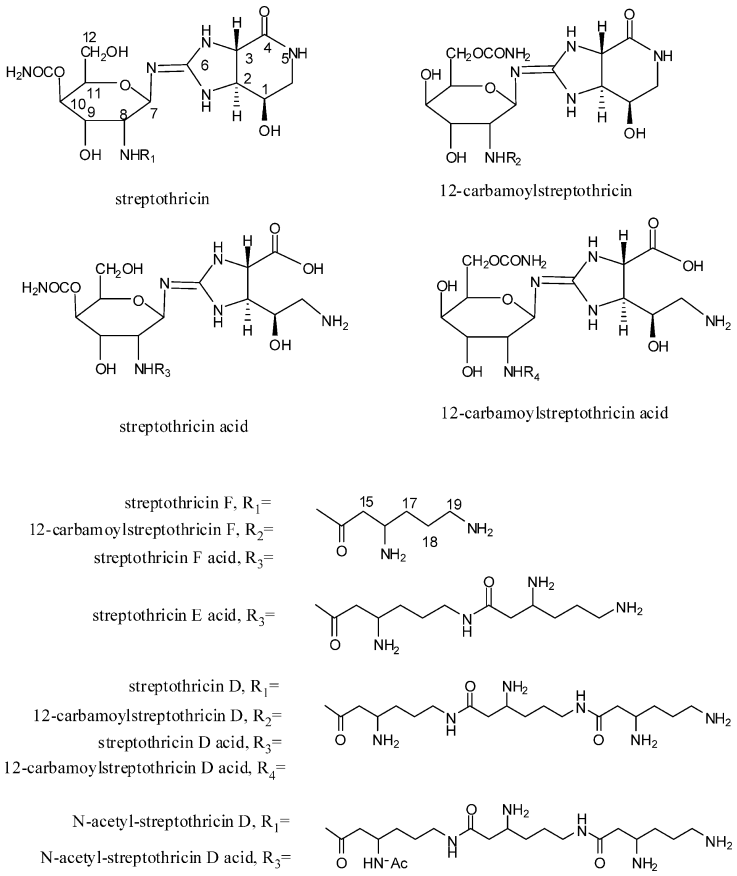


Fig. 1 The structures of the streptothricin-class antibiotics mentioned in this paper.

was set at 300°C. The number of ions stored in the ion trap was regulated by the automatic gain control. High-purity nitrogen (N_2) was used as sheath and auxiliary gas. The flow rate of the sheath and the auxiliary gas was set at 75 and 10 (arbitrary units), respectively. Ultrahigh-purity helium (He) was used as the damping and collision gas at a pressure of 0.133 Pa. Voltages across the capillary and the octapole lenses were tuned by an automated procedure to maximize the signal for the interesting ions. The standard LCQ software package was used for instrument control, data acquisition and processing.

A data-dependant 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.

Results and Discussion

ESI-MS^n Analysis of Streptothricins

A total of 10 streptothricin antibiotics (Fig. 1) were included in this study. They were divided into four groups: classical streptothricins, 12-carbamoylstreptothricins, streptothricin acids and 12-carbamoylstreptothricin acids. Most of known streptothricin antibiotics belong to classical streptothricins. 12-carbamoylstreptothricins are the isomers of the corresponding streptothricins, and only two compounds were reported before. All 12-carbamoyl-streptothricins involved here are novel compounds. Streptothricin and 12-carbamoylstreptothricin acids are the partial hydrolysis products of its corresponding antibiotics, these compounds were not investigated thoroughly until now. Inamori [15] and Taniyama [16] have independently reported that ST-F-acid was chemically prepared from ST-F, but other streptothricin acids which have two and three β-lysine moieties in the molecules were not obtained successfully. Hamano isolated a novel enzyme which can catalyze the hydrolysis of the amide bond of streptolidine lactam, ST-F-acid and ST-D-acid were prepared from ST-F and ST-D *in vitro*, respectively [17]. Most of streptothricin acids involved here are novel compounds. The collision induced dissociation (CID) spectra of protonated streptothricin F, 12-carbamoylstreptothricin F, streptothricin D, and 12-carbamoylstreptothricin D are shown in Fig. 2 as

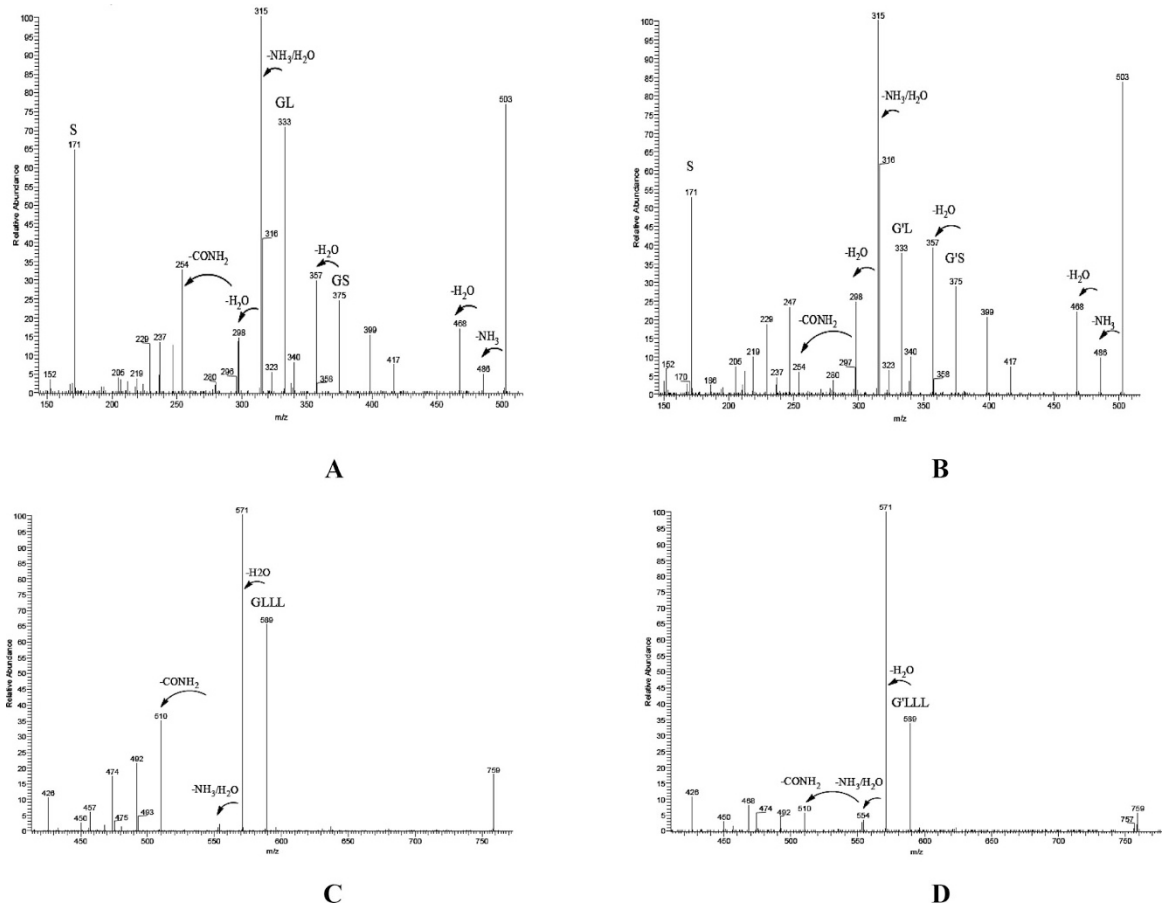


Fig. 2 The CID spectra of the $[M+H]^+$ of streptothricin F (**A**), 12-carbamoyl streptothricin F (**B**), streptothricin D (**C**), and 12-carbamoylstreptothricin D (**D**). The collision energy of the experiments was approximately 40%.

examples. The product ions and their relative abundances of all compounds are tabulated in Table 1. All streptothricins consist of a carbamoylated D-gulosamine to which the β-lysine homopolymer (1 to 7 residues) and the amide form of the unusual amino acid streptolidine (streptolidine lactam) are attached. The dissociations of the antibiotics in the CID experiment occurred dominatly at the the C-N bond between streptolidine lactam and D-gulosamine and amide bond of β-lysine chain. For convenience of discussion, carbamoylated D-gulosamine, β-lysine and streptolidine lactam in the structures are coded as G, L and S, respectively. For streptothricin acid and 12-carbamoylstreptothricin, streptolidine and 12-carbamoylated D-gulosamine are coded as S′ and G′, respectively. For *N*-acetylstreptothricins, the *N*-acetyl-β-lysine is coded as L′. The product ions of the protonated antibiotics are also coded in the aboved pricinple. For instance, the ions losing streptolidine lactam from streptothricins F and D were called GL and GLLL because D-gulosamine is attached by one and three β-lysine, respectively.

The letter coding of the product ions was expanded in Table 1 to cover the formed ions from water or ammonia losses. For example, the (GL-18/17) ion referred to the ion formed from the ion GL through a loss of water or ammonia molecule.

The fragmentation pathways shown in Scheme 1 were the major routes for the formation of the product ions. Some individual ions could also be formed from other intermediates. All pathways in Scheme 1 were confirmed by parent ion scans.

Compared with classical streptothricin antibiotics, the heterocyclic ring in the structure of streptothricin acids was changed from streptolidine lactam to amino acid. The mass shifts reflecting the changes in the product ions containing the modified structural moieties supported the structural assignments of the product ions in Scheme 1. For instance, the S′ ion of streptothricin F acid which contained the heterocyclic ring, was found at *m*/*z* 189 *versus* *m*/*z* 171 for the S ion of streptothricin F. The GL and (GL-H_2O) ions of streptothricin F acid were found to have the same *m*/*z*

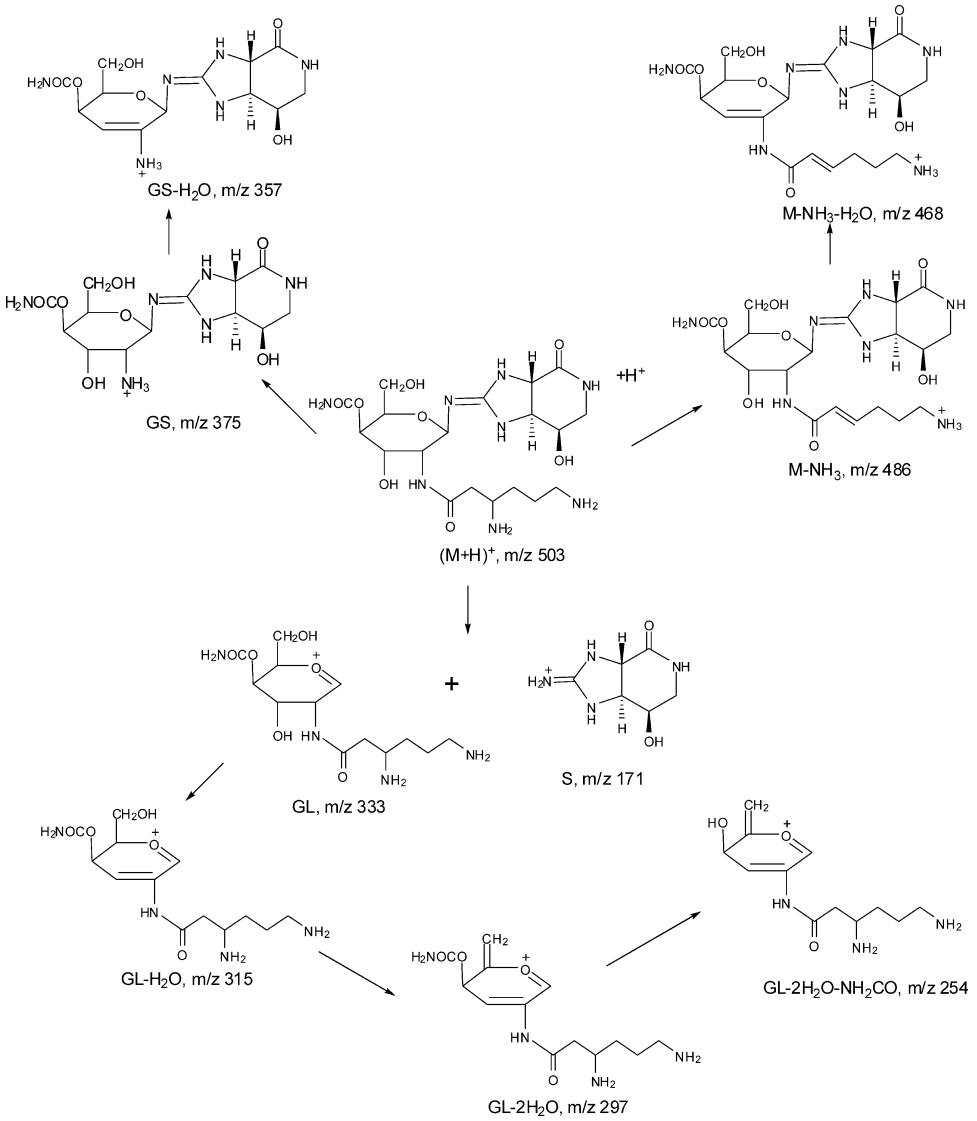
Table 1 The important product ions of streptothricins and their relative abundance

Compound	$[M+H]^+$	GL_n[c]	GL_n-17	GL_n-18	GL_n-18-17	GL_n-18-18	GL_n-18-18-43	S/S′	GS/GS′
ST F[a]	503	333/72	316/47	315/100	298/15	297/13	254/34	171/72	375/57
12′-ST F[b]	503	333/36	316/62	315/100	298/26	297/7	254/7	171/50	375/17
ST F acid	521	333/30	316/17	315/37	298/10	297/20	254/18	189/100	393/6
ST E acid	649	461/85	444/5	443/100	426/3	425/1	382/95	189/2	—
ST D	759	589/66	—	571/100	554/3	553/1	510/36	—	—
12′-ST D	759	589/35	—	571/100	554/3	553/2	510/6	—	—
ST D acid	777	589/70	—	571/100	554/3	553/1	510/56	—	—
12′-ST D acid	777	589/23	—	571/100	554/5	—	510/1	—	—
N-acetyl-ST D	801	631/25	—	613/90	596/2	—	552/100	—	—
N-acetyl-ST D acid	819	631/25	—	613/100	596/5	595/5	552/90	—	—

[a] ST was the abbreviation of streptothricin.

[b] 12′ means 12-carbamoyl.

[c] The ions were formed by losing streptolidine lactam or streptolidine from $[M+H]^+$, the content of "GL_n" varied with different compounds.



Scheme 1 Fragmentation pathway of streptothricin F.

values as the counterpart ions of streptothricin F at 333 and 315, respectively.

N-acetylstreptothricin D acid is a *N*-acetylated derivative of streptothricin D acid at the C_{16}-N of β-lysine. All product ions of this compound formed from losing streptolidin had *m*/*z* values of 42u higher than the corresponding ions produced from streptothricin D acid. For example, the *m*/*z* value of the GL′LL ion was found at 631 corresponding to a shift of 42u from the *m*/*z* value of GLLL ion of streptothricin D acid at 589.

The primary fragmentation was formed from the protonated streptothricin antibiotics by losing streptolidine lactam, which involved the fission of C_7-N bond between gulosamine and streptolidine. Another important fragmentation was produced by the cleavage of amide bond of β-lysine chain, which led to the formation of GS and (GS-H_2O) ions. For all streptothricin antibiotics, the relative abundance of the GL_n ions were more higher than the GS(S′) ions. The relative abundance of the GS(S′) ions was apparently influenced by the number of β-lysine, more β-lysine number would lead to lower abundance. For streptothricin F, the GS ion (*m*/*z* 375) from losing β-lysine was one of the major ions, whereas the corresponding ion of streptothricin D was hardly observed (Table 1).

In the CID spectra of protonated streptothricins, the ions formed from the loss of water or ammonia, and the loss of both water and ammonia were abundant. The ions formed by losing streptolidine lactam were the common fragmentation of all streptothricins, but the tendency of neutral loss of them was varied. For streptothricins F and D, the most abundant ions at *m*/*z* 315 and 571 were produced by losing water from the ions of GL and GLLL, respectively. The difference of them was GL could produce (GL-NH_3) ion at *m*/*z* 316 at the same time, but GLLL almost lost water solely (Table 1). The results indicated that the tendency of neutral loss was strongly influenced by the relative basicity of β-lysine chains, streptothricin D containing three β-lysine molecules making it more basic than streptothricin F. The similar phenomenon was also observed in the MS/MS research of some aminoglycoside antibiotics [18].

The fragmentation pathways were also influenced by the substitution position of the carbamoyl group attached to the D-gulosamine, and this characteristic could be used to identify streptothricins and 12-carbamoylstreptothricins by ESI MS/MS. Because the antimicrobial activities of these isomers were reduced notably compared with that of streptothricins, it would be a useful fast identification method. By comparison of the CID spectra of streptothricin F with that of 12-carbamoylstreptothricin F, the main difference of them was the relative abundance of the ion at *m*/*z* 254, the ion was prominent in streptothricin F with an abundance of 35%, while the abundance of the ion at *m*/*z* 254 was less than 5.0% in 12-carbamoylstreptothricin F. The ion (*m*/*z* 254) in streptothricin F was obtained by elimination of the carbamoyl group substituent at C_{10}-O of D-gulosamine from the (GL-2H_2O) ion (*m*/*z* 297). But in 12-carbamoylstreptothricin F, the (GL-H_2O-NH_3) ion (*m*/*z* 298) was the mainly neutral loss product of GL ion, and the ion (*m*/*z* 254) could not be formed from it. This suggested that carbamoyl group substituted at C_{10}-O made D-gulosamine more basic than substituted at C_{12}-O, and lead to lose two molecules water in streptothricin F, whereas lose one molecule water and one molecule ammonia from D-gulosamine in 12-carbamoylstreptothricin F. The principles summarized here were testified by other MS analysis of streptothricin antibioctics. For example, the (GLLL-2H_2O-$CONH_2$) ion (*m*/*z* 510) was present at an abundance of 35～40% in streptothricin D, whereas its abundance was also less than 5.0% in 12-carbamoylstreptothricin D.

Structure Elucidation of Novel Streptothricins in Microorganism Metabolites

Streptothricins were the most widely distributed antibiotics in soil microorganism, mainly produced by streptomyces. Its isolation was a complex process involved extraction from the fermentation broth using ion exchange resin and further chromatographic purification. In the screening of novel antibiotics, we established an isolation method which was based on ion-pair RP-HPLC and Refractive Index Detector (RID) to purify streptothricins from the fermentation broth of *Streptomyces qinlingensis* sp. nov., then the analysis method based on ion-pair RP-HPLC-MS/MS had also been developed. By this methodology, 19 streptothricin-like compounds were identified in the fermentation broth, in which 11 compounds were reported for the first time (Table 2).

In this analysis, three 12-carbamoylstreptothricin compounds were detected, and their molecular weights were 520, 690 and 776, respectively. The substitution position of carbamoyl group at the D-gulosamine was certained by the relative abundance of the ion formed from losing streptolidine, two molecules water and one carbamoyl group, the *m*/*z* values of the corresponding ions were at 254, 424 and 510, respectively. The heterocyclic ring structure was confirmed by its fragmentation ion mass, *i.e.*, the mass of streptolidine lactam and amino acid were 170 and 188, respectively. Other streptothricins acids were also distinguished by the rule.

N-acetylstreptothricins were the most familiar derivatives of classical streptothricins. To all of them, the acetylation position was always at the amido of the β-lysine attached to

Table 2. Detected streptothricins in *Streptomyces qinlingensis* metabolites by LC-MS/MS

No.	R.T.	Compound	*m/z*, $[M+H]^+$
1	4.7	Streptothricin F acid	521
2	6.1	Streptothricin E acid	649
3*	6.3	12-carbamoylstreptothricin F acid	521
4*	6.4	*N*-acetyl-10,12-dicarbamoylstreptothricin E acid	734
5	6.9	Streptothricin D acid	777
6	7.6	Streptothricin F	503
7*	9.8	12-carbamoylstreptothricin D acid	777
8*	10.8	12-carbamoylstreptothricin F	503
9	11.7	*N*-acetylstreptothricin F	545
10*	11.7	*N*-acetyl-10,12-dicarbamoylstreptothricin E	716
11*	12.5	*N*-acetylstreptothricin E acid	691
12	15.8	Streptothricin D	759
13*	17.0	Streptothricin C acid	905
14*	19.0	*N*-acetylstreptothricin D acid	819
15	21.5	*N*-acetylstreptothricin D	801
16*	21.5	*N*-methyl-*N*-acetylstreptothricin D acid	833
17*	22.0	*N*-methylstreptothricin D acid	791
18	22.0	*N*-acetylstreptothricin C	929
19*	22.0	*N*-acetylstreptothricin C acid	947

* Compound reported the first time in nature.

the D-gulosamine [19, 20]. For example, the ion (*m/z* 673) observed in the CID spectra of *N*-acetylstreptothricin D was formed from the $[M+H]^+$ losing one molecule β-lysine, which indicated that the distant β-lysine was not be acetylated. This principle was also adapted to certain the acetylation position in the analyses, and the conclusion may be confirmed by the CID spectra in some cases.

Two dicarbamoylstreptothricin compounds, *N*-acetyl-10,12-dicarbamoylstreptothricin E and its acid, were also identified in the fermentation broth. To our best knowledge, this type compound was not reported up to now. The key ion (*m/z* 546) of losing streptothricin from $[M+H]^+$ indicated that it was a streptothricin-like compound. Its molecular weight of 715, was 85u higher than *N*-acetylstreptothricin E, which implicated a carbamoyl and acetyl group. Acetylation usually occurred in the first β-lysine and carbamoylation occurred at the hydroxyl attached to C_{10} and C_{12} of D-gulosamine, so we presumed that the compound was *N*-acetyl-10,12-dicarbamoyl streptothricin E. Another compound was identified as *N*-acetyl-10,12-dicarbamoylstreptothricin E acid in the same way.

Conclusion

The low energy CID of protonated streptothricins produced a simple set of product ions. The dominant product ions were derived from the C_7-N bond cleavage which lead to lose streptolidine from the $[M+H]^+$. The loss of water, ammonia and carbamoyl from the ions above mentioned was another important characteristic of streptothricins. The fragmentation pathway was influenced by the substitution position of the carbamoyl group attached to the D-gulosamine, and streptothricin isomers could be distinguished easily by the differences of CID spectra. The form of neutral loss ion was also influenced by the length of β-lysine chain. By this LC-MS/MS methodology, the 19 streptothricin class compounds were detected from the fermentation broth of *Streptomyces qinlingensis* sp. nov., among of them, 11 are novel compounds found in nature. These results showed that LC-MS/MS have been proved to be an extremely useful technique in the fast screen the streptothricin class antibiotics.

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