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Isolation and structure characterization of related impurities in etimicin sulfate by LC/ESI-MS^n and NMR

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ABSTRACT

Etimicin sulfate is a semi-synthetic aminoglycoside which is modified from gentamicin C1a, used as an antibiotic effective both to Gram-positive and Gram-negative bacteria infections. Ten impurities were detected in the bulk drug by LC-ELSD and LC–ESI-MS^n. Weakly acidic cation exchange resin, CM-sephadex and silica gel column chromatography were used for the isolation of six impurities. Based on LC/ESI-MS^n and NMR analysis, related impurities were characterized as gentamicin C1a (**1**), 1-N-ethyl garamine (**2**), 2″-N-ethyl gentamicin C1a, or 6″-N-ethyl gentamicin C1a (**3**), 1-N-ethyl-3′-N-demethyl gentamicin C2b (**4**), 3-N-ethyl gentamicin C1a (**5**), 6″-deamino-6″-hydroxyl etimicin (**6**), 1,3-N, N-diethyl garamine (**7**), 1-N-ethyl gentamicin C2b (**8**), 2″ or 6″-N-ethyl etimicin (**9**), 1,3-N, N-diethyl gentamicin C1a (**10**). **10** is a new aminoglycoside, the ^{1}H and ^{13}C NMR data of compounds **5–7**, **10** are first reported in the paper. The possible mechanism for the formation of impurities is also discussed.

1. Introduction

Etimicin, which is usually used as sulfate salt, is a new aminoglycoside antibiotic modified from gentamicin C1a by ethyl group [1]. It is used in the treatment of infections caused by both Gram-positive and Gram-negative bacteria. Etimicin was effective for the treatment of respiratory tract infection, urinary tract infection, skin and tissue infections similar to netilmicin [2–4]. The oto- and nephro-toxicity are the main side effect of aminoglycoside. Compared to gentamicin C1a, the toxicity of etimicin is lower and is considered to be safe [5,6].

Many methods were developed to analyse etimicin sulfate and its impurities. Due to the lack of conjugated moiety, etimicin and its related substances cannot be detected by UV detector. Reversed-phase liquid chromatography with pre-column derivatization with o-phthalaldehyde (OPA) and with 1-fluoro-2,4-dinitrobenzene was used to the assay of etimicin [7,8]. However, the reactions between aminoglycoside and derivatization reagents would yield new degradations. Evaporative light-scattering detector (ELSD) is used as an universal detector to analyse nonvolatile samples and was widely used to determine the content and possible impurities of aminoglycoside [9–11]. Reversed-phase ion-pair LC method with pulsed amperometric detector (PAD) on a gold electrode was also used, but the low stability and reproducibility limited the wide use of the detector [12,13]. LC–MS^n has been used for many years to identify the structures of minor compounds in aminoglycosides [14,15]. However, in some complicated situations, LC/MS technique by itself cannot furnish final structures; more evidences should be provided by nuclear magnetic resonance (NMR). Due to the high polarity and strong basicity of aminoglycosides, the separation of related substances from aminoglycoside bulk drugs is a challenge. Column chromatography with weakly acidic cation exchange resin, carboxymethyl (CM) sephadex and silica gel were main tools to isolate minor aminoglycosides [16–18].

In the analysis of etimicin sulfate bulk drug, ten related impurities were observed. In view of the fact that the impurity levels were above the acceptance limits of 0.1%, it is necessary to study the structures of related impurities. In this paper, the related impu-

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rities in etimicin sulfate bulk drug were isolated and their structures elucidated by LC/ESI-MS^n and NMR.

2. Experimental

2.1. Materials and reagents

The investigated etimicin sulfate bulk sample was kindly provided by Wuxi Shanhe Pharmaceuticals Group. All related substances (purity >95%) were prepared by column chromatography in our laboratory from etimicin sulfate bulk drug. HPLC grade methanol was purchased from Jiangsu Hanbang Chemical Co. (Nanjing, China); other reagents were analytical reagent grade and purchased from Nanjing Chemical Co. (Nanjing, China). Water was obtained using a Millipore Milli Q-Plus system (Millipore Corp., MA, USA).

2.2. Equipment and chromatographic conditions

The analysis was performed on an Agilent series 1100 (Agilent Technologies, USA) liquid chromatography system, equipped with a bibasic pump, autosampler and Evaporative Light Scattering Detector (ELSD 2000ES, Alltech, USA). Data were acquired and processed using Agilent Chemstation software. A Gemini NX C_{18} column (150 mm × 4.6 mm ID; 5 μm) size was employed. Phase A consisted of water, ammonia solution (25%, m/m) and acetic acid in the ratio of 96:3.6:0.4 (v/v/v); methanol was chosen as phase B [15]. The flow rate was set at 0.8 ml/min, and injection volume was 10 μl. The following gradient program was used: time (min)/% phase B: 0/25, 5/30, 12/30, 30/60, 35/60, 40/90, 45/90, 50/25 with a post-run time of 10 min. Column oven temperature was 30 °C. The analytes were monitored with ELSD and the impactor position of ELSD was set off. The temperature of drift tube was 105 °C, and the flow rate of carrier gas was 2.6 l/min (from air compressor). The gain control for the ELSD was set at 1.

2.3. Isolation of six impurities by column chromatography

For the isolation, a glass column (7 cm × 60 cm) with weakly acidic cation exchange resin (HD-2) (Shanghai, China), glass column (2 cm × 70 cm) with CM-sephadex gel (GE Healthcare, Sweden) and glass column (2.5 cm × 20 cm) with silica gel (200–300 mesh) (Qingdao, China) were used to prepare impurities. Etimicin sulfate (100 g) was dissolved in water (500 ml) and subjected to weakly acidic cation exchange resin (HD-2) column, then eluted with water (15 l) and diluted ammonia (0.1 M, 0.15 M, 0.2 M, 0.25 M, each 15 l), 14 fractions (A–K) were afforded. Each fraction was detected by thin layer chromatography (TLC) (silica gel plate, Yantai, China) with developing agent consisting of chloroform, methanol and ammonia (25%, m/m) (1/1/1, v/v/v, low layer). The aminoglycosides were visible as brown spots after fumigated in iodine cylinder. Fraction B was further separated with CM-sephadex gel (25 g), eluting with water (600 ml) and diluted ammonia (0.08 M, 1000 ml), then related impurities **7** (37 mg, 1200–1300 ml) and **2** (20 mg, 1400–1500 ml) were separated as pure substance, respectively. Fraction D was further separated with silica gel (30 g), with the lower layer of chloroform, methanol and diluted ammonia (8%, m/m) (2/1/1, v/v/v, 800 ml) as eluent, then **6** (36 mg, 500–700 ml) was obtained. Fraction F containing **10** was chromatographed on CM-sephadex gel (25 g) with diluted ammonia (0.14 M, 3000 ml) as eluent, then the obtained fraction (1600–2000 ml) was subjected to silica gel (30 g) and eluted with the lower phase [$CHCl_3$/CH_3OH/NH_4OH (8%), 2/1/1, 1200 ml]. Finally, **10** (19 mg, 850–900 ml) was afforded through the TLC monitored combination. Fraction G containing **5** was separated with CM-sephadex gel (25 g) using diluted ammonia (0.14 M, 1200 ml) as eluent, then obtained fraction (800–900 ml) was subjected to silica gel (30 g) and eluted with the lower phase [$CHCl_3$/CH_3OH/NH_4OH (10%), 2/1/1, 600 ml], the subsequent fraction (550–600 ml) was further purified with CM-sephadex gel (25 g) eluting with diluted ammonia (0.15 M, 1600 ml) to give **5** (10 mg, 1350–1400 ml). Fraction I was chromatographed on CM-sephadex gel (25 g) with diluted ammonia (0.2 M, 1700 ml) as eluent, the obtained fraction (1000–1200 ml) was subjected to silica gel (20 g) eluting with the lower phase [$CHCl_3$/CH_3OH/NH_4OH (21%), 2/1/1, 700 ml], then impurity **1** (39 mg, 450–550 ml) was finally afforded. These impurities were used as reference substances to identify peaks (Fig. 1) in the liquid chromatography spectra of etimicin sulfate and elucidate the chemical structures by NMR techniques.

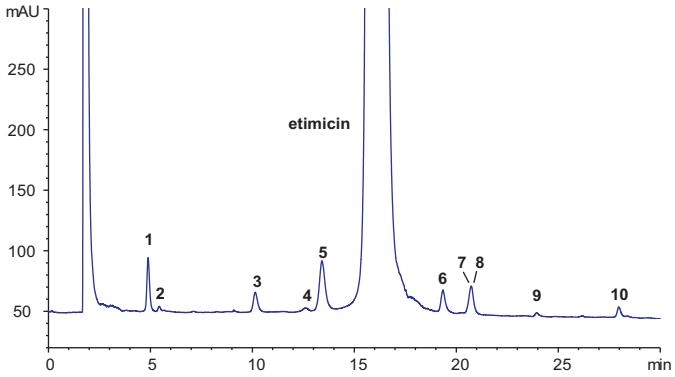

Fig. 1. HPLC-ELSD chromatogram of etimicin sulfate bulk drug (**1** is the starting material, impurities **3**–**6**, **8**, **9** and **10** are produced by synthesis and derived either from gentamicin C1a or its impurities, impurities **2** and **7** are produced by hydrolysis).

2.4. Characterization of impurities isolated from etimicin sulfate

The LC/ESI-MS^n analysis was carried out on an Agilent 1100 series LC-MSD Trap SL mass spectra with an electrospray interface (ESI) (CA, USA). The mass spectra were recorded in positive mode. Ultrahigh pure helium (He) was used as collision gas and high pure nitrogen (N_2) as nebulizing gas. The parameters were as follows: drying gas flow rate, 9 l/min; drying gas temperature, 350 °C; nebulizer, 40 psi; HV capillary voltage, 3300 V. For full scan MS analysis, the spectra were recorded in the range of m/z 100–1500. The isolation width of precursor ions was 4.0 m/z. MS^n data were acquired in the automatic data-dependant mode.

The ^{1}H, ^{13}C NMR spectra of the impurities were recorded on Bruker 300, and 500 MHz spectrometer using D_2O as solvent and tetramethylsilane (TMS) as internal standard. The 2D-NMR experiments (^{1}H–^{1}H COSY, HMQC, HMBC, etc.) were performed using standard Bruker pulse sequences.

3. Results and discussion

3.1. Detection of impurities by HPLC-ELSD and LC/ESI-MS^n

Etimicin sulfate samples were analysed by HPLC-ELSD method described in Section 2.3. Related impurities resulting from chemical synthesis and degradation were detected (Fig. 1). The samples were subjected to LC–ESI-MS^n analysis using the method described in Section 2.4. The chemical structures of the impurities are shown in Fig. 2.

3.2. Structure elucidation of related impurities by LC/ESI-MS^n

For convenience of discussion in the paper, the three rings of the structures were labeled as A, B and C (Fig. 2). Etimicin and

Table 1
The LC/ESI-MS^n data and proposed chemical structures of impurities in etimicin bulk drug (the characterization of impurities **1–3**, **5–7** were already described in [15]).

Substance	t_R (min)	$[M+H]^+$ (m/z)	Fragment ions (m/z)	Proposed chemical structure
1	4.9	450	322, 205, 163, 160	Gentamicin C1a
2	5.4	350	233, 215, 191, 160	1-N-ethyl garamine
3	10.1	478	322, 319, 163, 160	2″-N-ethyl gentamicin C1a/6″-N-ethyl gentamicin C1a
4	12.6	478	336, 333, 191, 146	1-N-ethyl-3′-N-demethyl gentamicin C2b
5	13.4	478	350, 319, 233, 215, 191, 160	3-N-ethyl gentamicin C1a
Etimicin	16.4	478	350, 319, 233, 191, 160	1-N-ethyl gentamicin C1a
6	19.4	479	350, 320, 191, 160	6″-Deamino-6″-hydroxyl etimicin
7	20.7	378	261, 243, 219, 160	1,3-N, N-diethyl garamine
8	20.7	492	350, 333, 233, 191, 160	1-N-ethyl gentamicin C2b
9	23.9	506	350, 233, 215, 191,	2″-N-ethyl etimicin/6″-N-ethyl etimicin
10	28.0	506	378, 219	1,3-N, N-diethyl gentamicin C1a

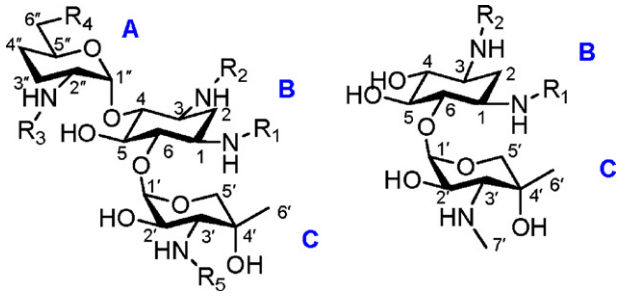

Fig. 2. Chemical structures of etimicin and impurities.

its related impurities followed the similar fragmentation pattern, so it is important to understand the formation of fragment ions of etimicin. In the MS^n spectrum of etimicin, with protonated molecule at m/z 478, the probable fragmetation pathway is shown in Fig. 3.

The fragmentation pathway of the protonated ion at m/z 450 (**1**) (Fig. 1) was identical to gentamicin C1a [15]. Since gentamicin C1a is the starting material of the synthesis of etimicin, it is reasonable to propose **1** as gentamicin C1a.

The impurity **2** (Fig. 1) with protonated ion at m/z 350 was characterized as ethyl group substituted (ring B) garamine (**2**) (Fig. 2) according to literature [15]. However, the position of ethyl substitution cannot be confirmed by LC–MS^n only, further analysis by NMR was necessary.

Impurity **3** (Fig. 1), with the protonated ion at m/z 478, is an isomer of etimicin. By referring to the published data [15], the compound was deduced to be ethyl substituted (ring A) gentamicin C1a (**3**) (Fig. 2); the proposed structure could be 2″-N-ethyl gentamicin C1a or 6″-N-ethyl gentamicin C1a.

Compound **4** (Fig. 1) with the $[M+H]^+$ ion at m/z 478, produced the most abundant ion at m/z 336 by loss of ring A (−142 Da). Compared with the loss of 128 Da due to ring A in the fragmentation of etimicin, ring A in this compound was 14 m/z units higher, which is probably due to the presence of an additional methyl group. Further fragmentation of the ion at m/z 336 yielded ions at m/z 191 and 146. This was produced by glycosidical cleavage between ring B and C by loss of 145 Da and 190 Da, respectively. The presence of ion at m/z 333, which is formed by glycosidical cleavage between ring B and C of the ion m/z 478 by loss of 145 Da, indicated that methyl group was absent in ring C compared to etimicin. Thus, the probable structure of 4 is 1-N-ethyl-3′-N-demethyl gentamicin C2b (Fig. 2).

Related impurity **5** (Fig. 1) has the same molecular weight as etimicin with the protonated ion at m/z 478. The fragmentation pattern was identical to that of etimicin (Table 1). It could be concluded that ethyl group is substituted on N-3 position of ring B (**5**) (Fig. 2), compared to etimicin with the ethyl group substituted on N-1 position of ring B.

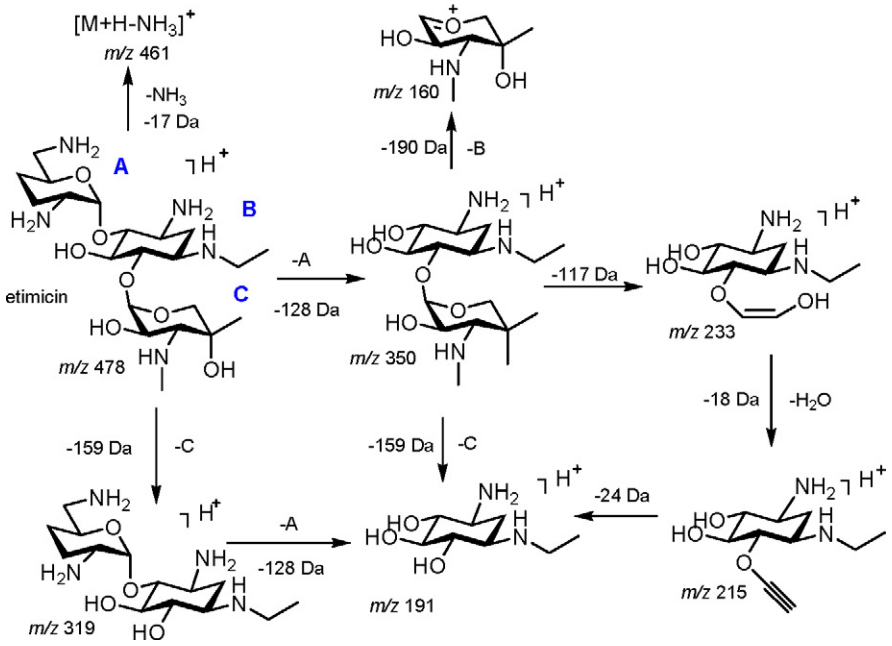

Fig. 3. The probable fragmentation pathway of etimicin.

Table 2
^{1}H, ^{13}C NMR data of related impurities (**1, 2** and **5**) of etimicin sulfate.

No.	**1**		**2**		**5**	
	δ_H (J/Hz)	δ_C	δ_H (J/Hz)	δ_C	δ_H (J/Hz)	δ_C
1	2.85–2.94 (1H, m)	52.9	2.50–2.54 (1H, m)	59.8	2.86–3.00 (1H, m)	53.2
2	1.98 (1H, dt, 13.0, 4.1)	37.8	2.16 (1H, dt, 12.6, 3.6)	34.7	2.16 (1H, dt, 13.1, 3.8)	34.8
	1.25 (1H, q, 12.0)		1.02–1.11 (1H, m)		1.09–1.26 (1H, m)	
3	2.85–2.94 (1H, m)	51.7	2.67–2.78 (1H, m)	52.9	2.52–2.68 (1H, m)	57.9
4	3.34 (1H, t, 9.4)	89.1	3.40 (1H, t, 9.2)	80.4	3.46 (1H, t, 9.4)	86.4
5	3.61 (1H, t, 9.4)	76.7	3.31 (1H, t, 9.2)	77.0	3.66 (1H, t, 9.4)	77.2
6	3.27 (1H, t, 9.4)	88.8	3.16 (1H, t, 9.2)	88.2	3.28 (1H, t, 9.4)	89.3
7			1.09 (3H, t, 6.7)	16.2		
8			2.67–2.78 (2H, m)	42.8		
9					1.11 (3H, t, 7.1)	16.6
10					2.72–2.85 (2H, m)	42.6
1′	5.10 (1H, d, 3.9)	102.6	4.97 (1H, d, 3.6)	103.7	5.10 (1H, d, 4.0)	103.0
2′	3.81 (1H, dd, 10.7, 3.9)	71.5	3.83 (1H, dd, 10.6, 3.6)	71.8	3.83 (1H, dd, 10.8, 4.0)	71.7
3′	2.60 (1H, d, 10.7)	65.6	2.61 (1H, d, 10.6)	65.9	2.64 (1H, d, 10.8)	66.0
4′		74.6		74.9		74.8
5′	4.05 (1H, d, 12.4)	69.9	4.04 (1H, d, 12.3)	70.4	4.06 (1H, d, 12.5)	70.3
	3.33 (1H, d, 12.4)		3.35 (1H, d, 12.3)		3.33 (1H, d, 12.5)	
6′	1.22 (3H, s)	23.8	1.23 (3H, s)	24.1	1.23 (3H, s)	24.1
7′	2.53 (3H, s)	39.1	2.54 (3H, s)	39.3	2.64 (3H, s)	39.3
1″	5.21 (1H, d, 3.5)	103.0			5.25 (1H, d, 3.6)	103.0
2″	2.85–2.94 (1H, m)	52.0			2.86–3.00 (1H, m)	52.1
3″	1.67 (1H, dq, 3.7, 13.0)	27.9			1.58–1.87 (2H, m)	28.0
	1.72–1.81 (1H, m)					
4″	1.44 (1H, dq, 3.7, 13.0)	29.6			1.40–1.87 (2H, m)	29.5
	1.72–1.81 (1H, m)					
5″	3.89–3.95 (1H, m)	72.1			3.86–3.94 (1H, m)	72.0
6″	2.77 (1H, dd, 13.4, 4.1)	46.9			2.72–2.85 (2H, m)	46.8
	2.71 (1H, dd, 13.4, 7.3)					

s, singlet; d, doublet; t, triplet; q, quadruplet; m, mutiplet; dd, doublet of doublet; dt, doublet of triplet; dq, doublet of quadruplet; *J*, ^{1}H–^{1}H coupling constants.

As to related substance **6** (Fig. 1) with $[M+H]^+$ ion at *m*/*z* 479, the fragment ions were identical to those of the impurity described in [15] with one amino group in ring A of etimicin replaced by a hydroxyl group. Thus, **6** was identified as in Fig. 2 with the position of hydroxyl group further verified by NMR data.

For the peak with retention time 20.7 min (Fig. 1), two compounds were detected in MS spectrum. For the impurity with the protonated ion at *m*/*z* 378 (**7**), which is 28 *m*/*z* units higher than that of 2, indicated that two amino groups were substituted by ethyl groups. Moreover, the fragment ions were the same as those of a known impurity [15]. As a consequence, the probable structure of **7** was elucidated as shown in Fig. 2. Another impurity with the protonated ion at *m*/*z* 492 (**8**) had the most abundant ion at *m*/*z* 350 in MS^2 spectrum. The loss of 142 Da due to ring A is 14 *m*/*z* units higher than that of etimicin (128 Da), and other fragment ions were identical with those of etimicin, indicating an additional methyl group on ring A. In view of the formation of related impurities, the probable structure of **8** was identified as 1-N-ethyl gentamicin C2b (Fig. 2).

Impurity **9** (Fig. 1) had the protonated ion at *m*/*z* 506; the glycosidical cleavage between ring A and B produced the most abundant ion at *m*/*z* 350. The loss of 156 Da due to ring A is 28 *m*/*z* units higher than that of etimicin, indicating an additional ethyl group on ring A. Further fragmentation of *m*/*z* 350 followed same pattern as etimicin. Thus, the probable structure of **9** could be characterized as 2″-N-ethyl etimicin or 6″-N-ethyl etimicin (Fig. 2).

For **10** (Fig. 1) with the protonated ion at *m*/*z* 506, the presence of the most abundant ion at *m*/*z* 378 (−128 Da) which is 28 *m*/*z* units higher than that of etimicin, suggested that N-1 and N-3 positions of ring B were substituted by two ethyl groups. The fragment ion at *m*/*z* 219 (−159 Da) which was formed involving ring B is 28 *m*/*z* units higher than that of etimicin. As a consequence, the proposed structure of **10** was deduced as 1,3-N, N-diethyl gentamicin C1a (Fig. 2).

The LC/ESI-MS^n data and proposed chemical structures of impurities in etimicin bulk drug are shown in Table 1.

3.3. Structure elucidation of related impurities by NMR

The ^{1}H and ^{13}C NMR data (Table 2) of **1** agreed with the literature [17], and the structure was confirmed as gentamicin C1a (Fig. 2). In ^{1}H and ^{13}C NMR data of **2** (Table 2), compared with the data of **1**, the signals of purpurosamine (ring A) could not be observed. Moreover, the chemical shift of C-4 had an upfield shift from 89.1 ppm to 80.4 ppm.

It is reasonable to consider the structure of **2** to consist of 2-deoxystreptamine (ring B) and garosamine (ring C). Two extra signals [δ_C 16.2 (C-7), 42.8 (C-8)] were observed in ^{13}C NMR spectrum, besides, a downfield chemical shift of 7 ppm occurred at C-1, indicating that an ethyl group was substituted on amino group of C-1 (Fig. 2). The assumption was further proved by ^{1}H NMR spectrum. A group of signals due to ethyl moiety [δ_H 1.09 (3H, t, *J* = 6.7 Hz, H-7), 2.67–2.78 (2H, m, H-8)] and an upfield shift of 0.3 ppm at H-1 (ring B) compared to that of **1** were observed.

The ^{13}C NMR spectrum of **5** (Table 2) was similar to that of **1** and had two extra signals [δ_C 16.6 (C-9), 42.6 (C-10)], furthermore, there was a downfield shift of 6 ppm at C-3. In the ^{1}H NMR spectrum, the presence of signals of an ethyl group [δ_H 1.11 (3H, t, *J* = 7.1 Hz, H-9), 2.72–2.85 (2H, m, H-10)], and an upfield shift of 0.3 ppm at H-3 (ring B) compared to that of **1**, indicated that **5** was an isomer of etimicin with the ethyl group substituted on amino group at C-3 (Fig. 2).

The ^{13}C NMR spectrum of **6** (Table 3) was similar to **1** except that the chemical shift of C-6″ was shifted to 66.3 ppm and the presence of an extra ethyl group's signals [δC 15.7 (C-7), 42.5 (C-8)], suggesting that the amino group on C-6″ had been replaced by hydroxyl group. A downfield shift of 6 ppm at C-1 indicated that the ethyl group was substituted on N-1. In ^{1}H NMR spectrum of **6**, the presence of one hydroxylated methylene group's signals [δH

Table 3
^{1}H, ^{13}C NMR data of related impurities (**6**, **7** and **10**) of etimicin sulfate and HMBC data of **6**.

No.	**6**			**7**		**10**	
	δ_H (J/Hz)	δ_C	HMBC(H-C)	δ_H (J/Hz)	δ_C	δ_H (J/Hz)	δ_C
1	2.79 (1H, m)	59.3	C2, C6, C8	2.48–2.68 (1H, m)	59.7	2.50–2.70 (1H, m)	59.5
2	2.18 (1H, dt, 13.0, 3.6)	34.5	C1, C3, C4, C6	2.32 (1H, m)	31.6	2.34–2.38 (1H, m)	31.2
	1.09 (1H, q, 13.0)		C1, C3, C4, C6	0.96–1.20 (1H, m)		0.96–1.19 (1H, m)	
3	2.83 (1H, m)	51.7	C1, C2, C4	2.48–2.68 (1H, m)	59.0	2.50–2.70 (1H, m)	57.8
4	3.33 (1H, t, 9.4)	89.4	C5, C1″	3.47 (1H, t, 9.2)	78.1	3.47 (1H, t, 9.2)	85.8
5	3.63 (1H, t, 9.4)	76.8		3.32 (1H, t, 9.2)	77.3	3.70 (1H, t, 9.2)	77.4
6	3.34 (1H, t, 9.4)	87.8	C5, C1′	3.30 (1H, t, 9.2)	88.0	3.36 (1H, t, 9.2)	88.2
7	1.08 (3H, t, 7.1)	15.7	C8	1.10 (3H, t, 7.2)	16.3	1.10 (3H, t, 7.1)	16.1
8	2.75 (1H, dq, 11.2, 7.2)	42.5	C1, C7	2.75–2.89 (2H, m)	42.9	2.78–2.88 (2H, m)	42.9
	2.49 (1H, dq, 11.2, 7.2)		C1, C7				
9				1.13 (3H, t, 7.2)	16.2	1.11 (3H, t, 7.1)	16.5
10				2.75–2.89 (2H, m)	42.9	2.78–2.88 (2H, m)	42.6
1′	4.98 (1H, d, 4.0)	103.4	C6, C2′, C5′	4.99 (1H, d, 3.8)	103.8	4.99 (1H, d, 3.6)	103.8
2′	3.82 (1H, dd, 10.7, 4.0)	71.6	C3′	3.84 (1H, dd, 10.7, 3.8)	71.9	3.86 (1H, dd, 10.8, 3.6)	71.7
3′	2.59 (1H, d, 10.7)	65.6	C1′, C2′, C6′, C7′	2.62 (1H, d, 10.7)	66.0	2.66 (1H, d, 10.8)	66.0
4′		74.6			74.9		74.7
5′	4.02 (1H, d, 12.7)	70.1	C3′, C4′	4.04 (1H, d, 12.4)	70.4	4.02 (1H, d, 12.6)	70.5
	3.33 (1H, d, 12.7)		C3′, C4′	3.36 (1H, d, 12.4)		3.35 (1H, d, 12.6)	
6′	1.22 (3H, s)	23.8	C3′, C4′, C5′	1.24 (3H, s)	24.2	1.23 (3H, s)	24.1
7′	2.54 (3H, s)	39.0	C3′	2.56 (3H, s)	39.3	2.57 (3H, s)	39.2
1″	5.15 (1H, d, 3.4)	103.2	C4, C2″, C3″, C5″			5.29 (1H, d, 2.8)	102.5
2″	2.90 (1H, dt, 11.7, 4.0)	52.0	C1″, C3″			2.96–3.00 (1H, m)	52.1
3″	1.66 (1H, dq, 3.6, 12.5)	28.0	C1″, C2″, C4″, C5″			1.63–1.82 (2H, m)	27.8
	1.79 (1H, m)		C1″, C2″, C5″				
4″	1.45 (1H, dq, 3.6, 12.1)	28.1	C2″, C3″, C5″, C6″			1.47–1.82 (2H, m)	29.4
	1.70 (1H, m)		C2″, C5″				
5″	3.98 (1H, m)	72.3	C6″			3.84–3.97 (1H, m)	71.6
6″	3.63 (1H, dd, 12.0, 3.8)	66.3				2.78–2.88 (2H, m)	46.7
	3.53 (1H, dd, 12.0, 6.8)		C5″				

s, singlet; d, doublet; t, triplet; q, quadruplet; m, mutiplet; dd, doublet of doublet; dt, doublet of triplet; dq, doublet of quadruplet; *J*, ^{1}H–^{1}H coupling constants.

3.63 (1H, dd, J = 12.0, 3.8 Hz, H-6″), 3.53 (1H, dd, J = 12.0, 6.8 Hz, H-6″)] and an ethyl group's signals [δH 1.08 (3H, t, J = 7.1 Hz, H-7), 2.75 (1H, dq, J = 11.2, 7.2 Hz, H-8), 2.49 (1H, dq, J = 11.2, 7.2 Hz, H-8)], supported the structure deduced from ^{13}C NMR spectrum. The HMBC spectrum showed significant correlations between H-6″ (δH 3.53) and C-5″ (δC 72.3), and between H-1 (δH 2.79) and C-8 (δC 42.5), indicating that the hydroxyl group was located at C-6″ (ring A) and the ethyl group should be substituted on N-1. Hence, the chemical structure of 6 was verified as shown in Fig. 2.

The ^{13}C NMR spectrum of **7** (Table 3) was similar to **2**. It had extra ethyl group signals [δ_C 16.2 (C-9), 42.9 (C-10)], downfield of 6 ppm at the position of C-3 compared to that of **2**, suggesting that an ethyl group was substituted on N-3 (Fig. 2). The presence of extra ethyl group signals [δ_H 1.13 (3H, t, *J* = 7.2 Hz, H-9), 2.75–2.89 (2H, m, H-10)], coupled with an upfield shift of 0.3 ppm at H-3 (ring B) in ^{1}H NMR spectrum, supported the deduction on the basis of the ^{13}C NMR spectrum. Accordingly, the chemical structure of 7 was elucidated as shown in Fig. 2.

As to **10** (Table 3), two extra signals of ethyl groups [δ_C 16.1 (C-7), 42.9 (C-8), 16.5 (C-9), 42.6 (C-10)] compared to 1 were observed; downfield of 6 ppm both at positions of C-1 and C-3 suggested two ethyl groups substituted on N-1 and N-3, respectively, (Fig. 2). Two pairs of signals of ethyl groups [δ_H 1.10 (3H, t, *J* = 7.1 Hz, H-7), 2.78–2.88 (2H, m, H-8), 1.11 (3H, t, *J* = 7.1 Hz, H-9), 2.78–2.88 (2H, m, H-10)] were observed; both of H-1 and H-3 (ring B) had an upfield shift of 0.3 ppm. Thus, impurity **10** was characterized as shown in Fig. 2.

All ^{1}H and ^{13}C NMR signals of **1**, **2**, **5** were assigned as shown in Table 2, and **6**, **7**, **10** in Table 3, respectively. All of the structure deductions were further confirmed by LC/ESI-MS^n.

3.4. Formation of related substances

Gentamicin C1a (**1**) as the starting material for etimicin synthesis, was detected by LC/ESI-MS^n and confirmed by NMR. Due to the multiple amino groups in gentamicin C1a, impurities such as **3**, **5**, **9** and **10** were produced as by-products in the reaction. Gentamicin C1a was isolated from bacterial fermentation liquor and impurities with similar structure participated in the reaction; as a result, **4**, **6** and **8** were formed. Glycosidic hydrolysis between purpurosamine (ring A) and 2-deoxystreptamine (ring B) of etimicin and **10** yielded **2** and **7**, respectively.

4. Conclusion

Ten impurities of etimicin sulfate bulk drug were detected by LC-ELSD and LC/ESI-MS^n. The structures of impurities were proposed on the basis of LC/ESI-MS^n, fragmentation mechanism and synthetic procedure. Six of impurities (**1**, **2**, **5–7**, **10**) were isolated from etimicin sulfate bulk drug by column chromatography and these structures were confirmed by NMR spectrum. Starting material along with impurities, synthetic byproducts and degradation were the main sources for the formation of these impurities.

However, not all impurities of etimicin sulfate could be identified by LC/ESI-MS^n, or isolated as pure substance and characterized by NMR. The application of LC/NMR would be the development direction.

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