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ORIGINAL ARTICLE

Isolation, identification and characterization of related substances in furbenicillin

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Furbenicillin is a broad-spectrum semisynthetic penicillin with strong antibacterial activity against Gram-negative bacteria. In this study, three impurities in furbenicillin, including an unknown epimer, were determined. On the basis of a complete analysis of the spectrum (MS, ^{1}H,^{13}C, 2D NMR and CD) and the results of chemical methods, the unknown epimer impurity was identified as 10-epi-furbenicillin (impurity 1). Isolation and structure elucidation of impurity 1 was also reported here for the first time.
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INTRODUCTION

The central molecular feature of penicillins consists of two fused rings (a four-membered β-lactamic ring and a five-membered thiazolidinic ring) with three chiral centers (2*S*, 5*R*, 6*R*). The addition of a 6-acylamino side chain may result in the introduction of an additional chiral center, such as in amoxicillin, mezlocillin or furbenicillin.

Remarkably, the chiral center at C-6 or C-10 in the 6-side chain can isomerize under specific manufacturing or storing conditions.[1–4] The crucial point is that alteration of the configuration of any of the chiral centers in 6-aminopenicillanic acid or in the 6-side chain can result in partial or total loss of activity. In the case of ampicillin, the C-10 epimers differ in aqueous solubility and activity; furthermore, the activity ratio (*R*/*S*) ranges between two- and fivefold depending on the microorganism used for the test.[5,6] Similarly, the antibiotic activity of the *R*-epimer of sulbenicillin is ~40 times more potent than that of the *S*-epimer.[7] In contrast, in the case of carbenicillin, it was reported that the individual epimers display only minor differences in activity, and that they undergo rapid epimerization in solution.[5,6]

Furbenicillin, initially synthesized by the American company Bristol-Myers in 1969, is a broad-spectrum semisynthetic penicillin with strong antibacterial activity against Gram-negative bacteria, for example, *Hemophilus influenza*, *Pseudomonas aeruginosa* and *Escherichia coli*, and apparently in current clinical use in China for the treatment of the respiratory tract, urinary tract and intestinal tract infections caused by these Gram-negative bacteria.[8–10] It was also shown that furbenicillin is more stable than carbenicillin, mezlocillin and azlocillin against the action of specific enzymes in *P. aeruginosa*.[11] However, a nontrivial synthesis and the poor quality of the final product have strongly limited the clinical use of furbenicillin. Previous studies showed that furbenicillin for injection contained a relatively large amount of an unknown isomeric impurity.[12,13] In this work, in order to provide the knowledge to improve the quality of the final product and the process of manufacture, three impurities, including an unknown isomer of furbenicillin in drug substance, were isolated, identified and characterized by liquid chromatography-MS, CD, NMR and chemical methods.

RESULTS AND DISCUSSION

Structure of the impurities

Our liquid chromatography measurements showed that there were three impurities with a relatively high content (Figures 1 and 2). The area percents of the impurity **1**, **2** and **3** were 19.5%, 9.3% and 0.9%, respectively. Reverse > d-phase C18 HPLC on the 350 mg of furbenicillin afforded 40.0 mg of impurity **1** and 13.5 mg of impurity **2**.

Impurity **1** showed ESI-MS peaks at m/z 487 $[M+H]^+$ and 509 $[M+Na]^+$. The data obtained with LC-MS/MS were virtually the same

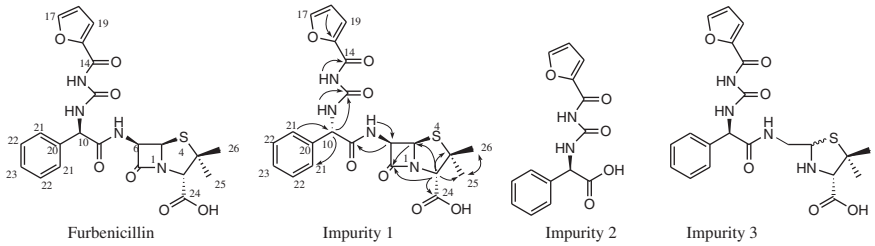

Figure 1 Structure of furbenicillin and impurities **1**, **2** and **3** (arrows on impurity **1** show HMBC correlations).

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for impurity **1** and furbenicillin (Figure 3), confirming that impurity **1** is an isomer of furbenicillin. Impurity **1** was isolated according to the method described in section 2.2.2 and characterized using CD, NMR and chemical methods. The ^{1}H-NMR spectrum of impurity **1** (Table 1) displayed resonance for a monosubstituted benzene ring (δ_H 7.45 (2H, d, J=7.2 Hz, H-21), 7.39 (2H, t, J=7.2 Hz, H-22) and 7.32 (1H, t, J=7.2 Hz, H-23)], a monosubstituted furan ring (δ_H 8.00 (1H, d, J=0.6 Hz, H-17), 6.71 (1H, dd, J=0.6, 3.6 Hz, H-18) and 7.65 (1H, d, J=3.6 Hz, H-19)], a penicillin nucleus (δ_H 4.23 (1H, s, H-2), 5.50 (1H, d, J=4.2 Hz, H-5), 5.41 (1H, dd, J=4.2, 7.8 Hz, H-6), 1.48 (3H, s, H-25) and 1.62 (3H, s, H-26)). Besides proton-bearing carbon resonances corresponding to the above units, the ^{13}C NMR and the DEPT spectra showed resonances for three quaternary carbons. The comparison of the NMR data of impurity **1** and furbenicillin (Table 1) revealed that the order of the resonances for H-5 and H-6 in impurity **1** has an inverse pattern ($\delta_{H\text{-}5} > \delta_{H\text{-}6}$), which in turn is different from the one of common penicillin ($\delta_{H\text{-}6} > \delta_{H\text{-}5}$);[8–10] in addition, H-5, H-25, H-26, H-6 and H-10 of impurity **1** were shifted significantly by $\delta_{\Delta H}$ +0.11, +0.08, +0.07, −0.14 and −0.07 p.p.m., respectively, compared with those in furbenicillin. This structure of impurity **1** was further supported by analysis of the 2D NMR data from HSQC, ^{1}H–^{1}H COSY and HMBC experiments. In particular, the correlations between H-2 and C-3, C-5, C-24, C-25, C-26, H-5 and C-6, C-7, H-6 and C-5, H-8 and C-6, and H_3-25 and C-2, C-26 from the HMBC experiments demonstrated the moiety of the 6-aminopenicillanic acid penicillin

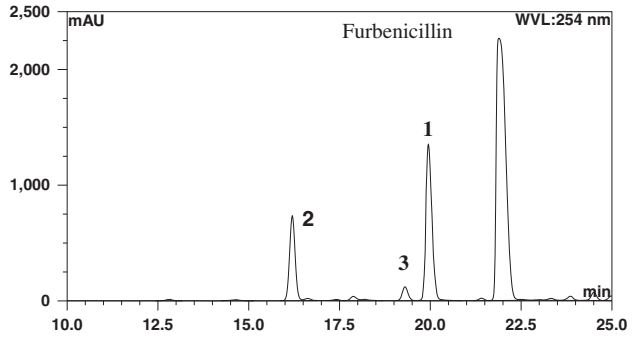

Figure 2 HPLC chromatograms of furbenicillin (**1**: 10-epi-furbenicillin (impurity **1**) 19.5%; **2**: impurity **2** 9.3%; **3**: impurity **3** 0.9%).

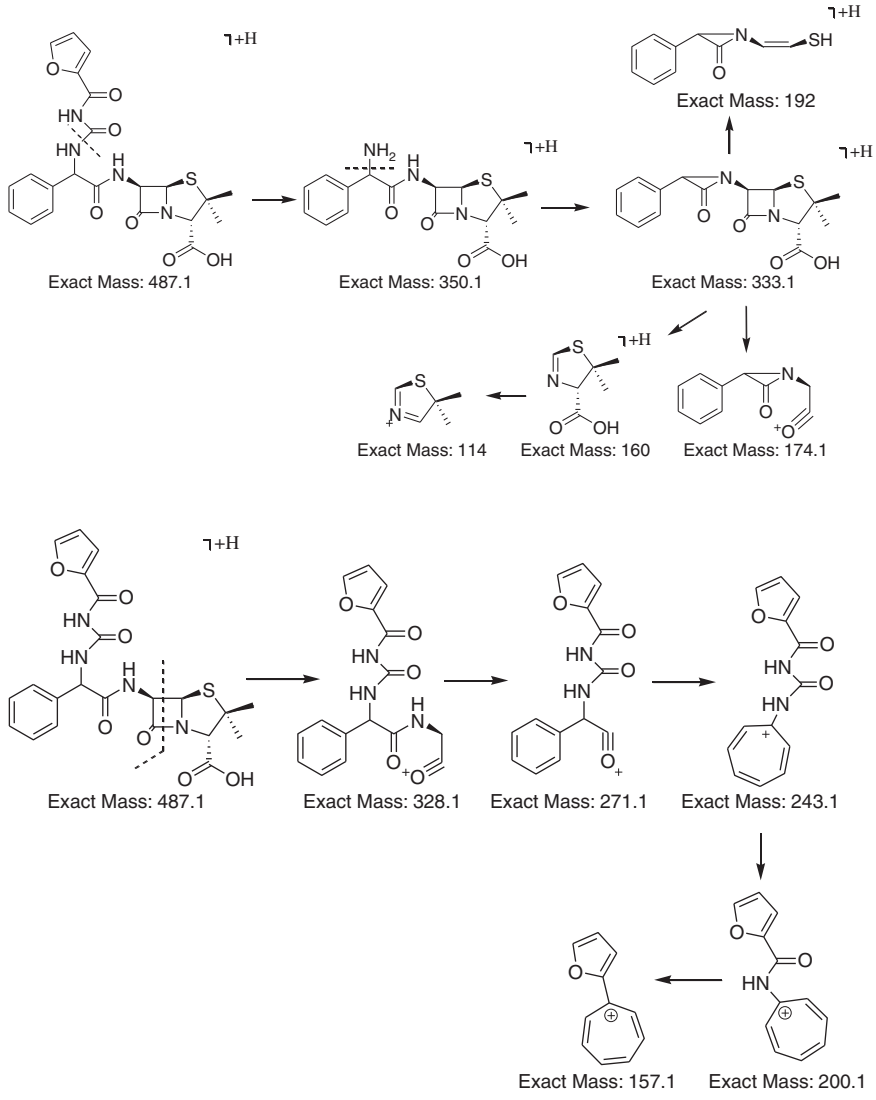

Figure 3 Cleavage pathway of furbenicillin and impurity **1**.

Table 1 NMR Data (δ) of furbenicillin and impurity 1 in DMSO-d_6[a]

	Furbenicillin		*Impurity 1*	
No.	δ_H	δ_C	δ_H	δ_C
2	4.20, 1H, s	70.4	4.23, 1H, s	70.4
3	—	63.7	—	63.9
5	5.39, 1H, d, J=4.2	67.0	5.50, 1H, d, J=4.2	67.1
6	5.55, 1H, dd, J=4.2; 7.8	58.1	5.41, 1H, dd, J=4.2; 7.8	58.7
7	—	173.2	—	172.7
8	9.31, 1H, d, J=7.8	—	9.35, 1H, d, J=7.8	—
9	—	169.6	—	169.7
10	5.75, 1H, d, J=7.8	55.7	5.68, 1H, d, J=7.8	55.8
11	9.39, 1H, d, J=7.8	—	9.35, 1H, d, J=7.8	—
12	—	152.3	—	152.2
13	10.74, 1H, s	—	10.73, 1H, s	—
14	—	158.4	—	158.4
15	—	145.3	—	145.3
17	8.00, 1H, d, J=1.2	147.8	8.00, 1H, d, J=0.6	147.8
18	6.71, 1H, dd, J=1.2; 3.6	112.4	6.71, 1H, dd, J=0.6; 3.6	112.4
19	7.66, 1H, d, J=3.6	117.4	7.65, 1H, d, J=3.6	117.4
20	—	138.2	—	138.4
21	7.43, 2H, d, J=7.8	126.5	7.45, 2H, d, J=7.2	126.7
22	7.36, 2H, t, J=7.8	128.4	7.39, 2H, t, J=7.2	128.5
23	7.31, 1H, d, J=7.8	127.8	7.32, 1H, d, J=7.2	128.0
24	—	168.9	—	168.9
25	1.40, 3H, s	26.6	1.48, 3H, s	26.7
26	1.55, 3H, s	30.3	1.62, 3H, s	30.3

[a]Data (δ) were measured at 600 MHz. Coupling constants (J) are given in Hz. The assignments were based on DEPT, ^{1}H–^{1}H COSY, HSQC and HMBC experiments.

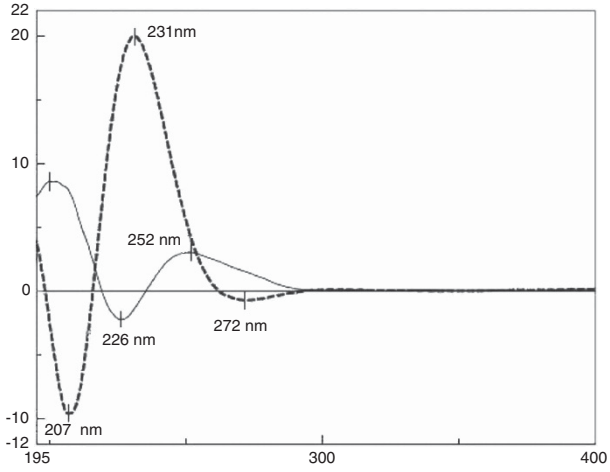

Figure 4 CD spectrum of furbenicillin and impurity **1** [–: furbenicillin; ——: 10-epi-furbenicillin (impurity **1**)].

nucleus. The correlations between H-10 and C-12, C-21, H-11 and C-12, H-18 and C-15, and H-6 and C-9 demonstrated the structure and connection pattern of the side chain, confirming that impurity **1** is characterized by a planar structure identical to that of furbenicillin (Figure 1).

The $J_{H\text{-}5,\,H\text{-}6}$ value in the ^{1}H NMR spectrum of impurity **1** indicated that H-5 and H-6 are in cofacial positions.[8–10] In an NOE experiment of impurity **1**, irradiation of H-2 enhanced the intensity of the H_3-26 signals. Furthermore, the H-2 signals were enhanced upon irradiation of H_3-26, and irradiation of H-5 enhanced the intensities of H-6.

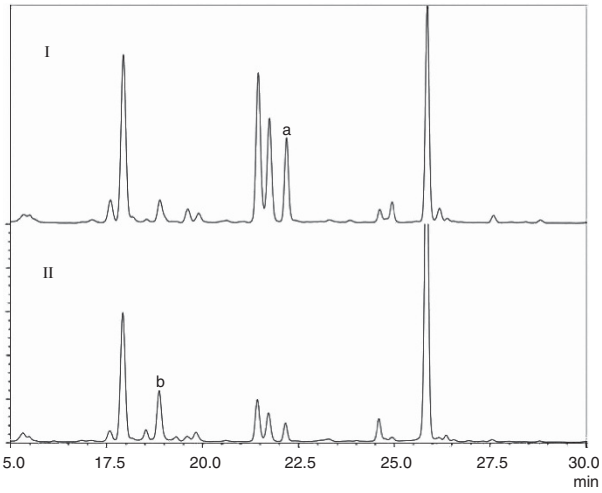

Figure 5 HPLC chromatograms of Marfey's reaction of furbenicillin(I) and impurity 1(II). a: Derivatives of D-amino acid; b: Derivatives of L-amino acid.

Table 2 MIC values of furbenicillin and impurity 1

Sample	*Bacterium*	*MIC50*
Furbenicillin	*Staphylococcus aureus* (ATCC 25923)	4
Impurity **1**	—	>256
Furbenicillin	*Pseudomonas aeruginosa* (ATCC 27853)	4
Impurity **1**	—	>256

These finding showed that the structure of impurity **1**, excluding the 6-side chain, has the same relative configuration as furbenicillin (Supplementary Figures S4 and S11 electronic Supplementary Material).

The CD spectrum of furbenicillin showed a positive Cotton effect at 231 nm and a negative Cotton effect at 207 nm ($n \rightarrow \pi^*$ transition of the β-lactam ring)[14] and a negative Cotton effect at 272 nm ($n \rightarrow \pi^*$ transition of 6-side chian).The predominant difference between the CD data for impurity **1** and furbenicillin (Figure 4) is that for impurity **1**, the Cotton effect due to the 6-aminopenicillanic acid shifted to longer wavelengths by about 20 nm, and the Cotton effect in impurity **1** due to the 6-side chain (~272 nm) was positive in contrast. This suggests that impurity **1** is an epimer of furbenicillin with the opposite configuration at C-10.

The absolute configuration of the phenylglycine residue was further confirmed after hydrolysis of impurity **1** and derivatization HPLC analysis by using Marfey's method.[15,16] The 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide derivative of the hydrolyzate of impurity **1** gave the same HPLC retention time as that of the authentic L-phenylglycine (Figure 5), which showed that the phenylglycine residue in impurity **1** must be assigned the L-configuration. Thus, on the basis of the foregoing evidence, the structure of impurity **1** was established as 10-epi-furbenicillin.

Impurity **2** showed ESI-MS peak at m/z 311 $[M+Na]^+$. By comparation of MS, CD and NMR data with furbenicillin (Supplementary Figures S12–S15 electronic Supplementary Material), impurity **2** was determined to be (*R*)-2-(3-furan-2-carbonylureido)-2-phenylacetic acid.[13] Impurity **3** showed ESI-MS peaks at m/z 461 $[M+H]^+$ and 483 $[M+Na]^+$. By comparation of MS data with furbenicillin (Supplementary Figures S16 and S17 electronic

Supplementary Material), impurity **3** was determined to be 5 (*R*/*S*) furbenicillin penicilloic acid, which represents a common degradation of penicillins.[17,18]

MIC test

The antibacterial activities of furbenicillin and impurity **1** were determined by MIC tests. Impurity **1** was found to be inactive against *Staphylococcus aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) (Table 2).

CONCLUSIONS

In this work, a complete spectral analysis (MS, ^{1}H,^{13}C, 2D NMR and CD) and other chemical methods were employed to identify three impurities, including a previously unknown epimer, in the furbenicillin drug substance.

Stereoisomers are typically recognized by the body as distinct chemical entities, and they exhibit different pharmacological activities, toxicities and pharmacokinetics. The epimerization at C10 of furbenicillin can result in the decrease of antibacterial potency.

MATERIALS AND METHODS

Chemicals and reagents

The following reagents were used in our study: furbenicillin sodium substance from Bocom Pharmaceutical Co., Ltd, Taiyuan, China; acetonitrile and methanol from Chromatographic Pure, Fisher (Fairlawn, NJ, USA); 1-fluoro-2-4-dinitrophenyl-5-L-alanine amide and all other reagents (CH_3COCH_3, CH_3COOH, KH_2PO_4, H_3PO_4 and Na_2CO_3) that were commercially obtained were of analytical reagent grade. All solutions were prepared in double-distilled water.

Instruments

The following instruments were used in our study: a Dionex P680 HPLC pump, a Dionex ASI-100 autosampler, a PDA-100 diode-array detector (Dionex, Sunnyvale, CA, USA), Q-Trap 3200 (AB Sciex, Foster City, CA, USA), a Varian INOVA 600 MHz NMR spectrometer (Varian, Palo Alto, CA, USA), and a JASCO-815 CD spectrometer (Jasco, Japan).

LC-MS spectrometry

Chromatographic conditions. A mobile phase containing methanol—10 mmol l^{-1} ammonium formate (pH 3.0, adjusted with CH_3COOH) (55:45, v/v) was used at a flow rate of 1.0 ml min^{-1}. The CAPCELL PAK-C18 column (250 × 4.6 mm i.d., 5.0 μm) was maintained at 25 °C. Samples were prepared in the mobile phase at a concentration of 1.0 mg ml^{-1}. The injection volume was 10 μl. UV detection was performed from 200 to 400 nm (extraction: 254 nm).

MS conditions. The mass spectra of impurities of furbenicillin were obtained using the following optimized MS conditions. ESI was used in positive ionization mode, the declustering potential was 15 V, the entrance potential was 10 V and the collision energy was 15 V. The curtain gas, ion-source gas 1, and ion-source gas 2 flowed at 20.0, 65.0 and 60.0 l h^{-1}, respectively; the ion spray voltage (IS) was 5500 V, and the temperature (TEM) was 500.0 °C, with the interface heater on. Enhanced MS and enhanced product ion spectra were acquired from m/z 50 to m/z 200 in 0.1 a.m.u. steps with a 2.0-s dwell time. The Analyst software (version 1.5.1, AB Sciex) was used for data acquisition and processing.

Preparative liquid chromatography. A Grace Prevail C18, 5 μm, 10 mm × 250 mm i.d. was used for the separation. The column eluent was monitored at a wavelength of 254 nm. Different mixtures of 0.01 mol l^{-1} KH_2PO_4 solution (pH = 3.2), adjusted with phosphoric acid, (mobile phase A) and methanol (mobile phase B) were used as the mobile phases (A:B, 48:52) at a flow rate of 2.0 ml min^{-1}.

NMR measurements. NMR measurements were carried out on a Varian INOVA instrument (for both ^{1}H and ^{13}C) at 25 °C in DMSO-d_6. The ^{1}H and ^{13}C chemical shifts values are reported on the δ scale in p.p.m. relative to TMS ($\delta = 0.00$ p.p.m.) and DMSO-d_6 ($\delta = 39.5$ p.p.m.) as the internal standards, respectively.

MIC determination. The McFarland bacterial suspension was prepared with sterile normal saline using the Clinical and Laboratory Standards Institute agar dilution method.[19] The suspensions were diluted with sterile normal saline in a proportion of 1:100. The final solutions were inoculated onto the surface of a Müller–Hinton agar plate containing sites with different furbenicillin and impurity **1** concentrations using multi-point inoculators. After culturing of these samples at 35 ± 1 °C for 22–24 h, the MIC values were recorded.

ACKNOWLEDGEMENTS

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)