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Identification of a new isomer from a reversible isomerization of ceftriaxone in aqueous solution

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ABSTRACT

A reversible isomerization of ceftriaxone in aqueous solution was observed, and the structure of the isomer was determined by mass spectrometry and various 1D and 2D NMR techniques. The mechanism of isomerization was also discussed. Finally, molecular docking simulations were performed and the antimicrobial activities of the isomers were measured. This showed that the biological activity of ceftriaxone was stronger than that of its isomer. The results reported in this article may be important to quality control requirements and to the stability of ceftriaxone products.

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1. Introduction

Ceftriaxone is a third-generation semisynthetic cephalosporin antibiotic with potent antimicrobial activity against the majority of Gram-negative and Gram-positive bacteria. The most remarkable features of ceftriaxone are its excellent stability against beta-lactamase activity and very long elimination half-life [1]. Extensive steric and constitutional isomerization occurs for many cephalosporins, which can lead to different biological properties [2]. The stereoisomers of cephalosporins mainly include 7-epimers [3,4], epimers with an amino acid at the C7 position [5], *syn*/*anti* isomers of the *N*-oxime group [2,3], and epimers involving the amide group at the C7 position [6]. Furthermore, constitutional isomerization of Δ^3 – cephalosporins to the Δ^2 – isomer can occur, which leads to a great loss of antibacterial activity [2]. *Syn*/*anti* isomers of the *N*-oxime group in ceftriaxone have also been observed [2,7]. In this study, a reversible isomerization of ceftriaxone was observed. The structure of the isomer and the mechanism of the reversible isomerization and the antimicrobial activity of the isomers were studied.

2. Materials and methods

2.1. Chemicals and reagents

The following reagents were used in our study: ceftriaxone sodium RS from the National Institutes for Food and Drug Control (Beijing, China); HPLC-grade acetonitrile and methanol from Fisher Scientific (Fairlawn, NJ, U.S.); and Nutrient Agar medium from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All analytical-grade reagents (HCl, CH_3COOH, HCOOH, $HCOONH_4$, CH_3COONH_4, $CH_3(CH_2)_7NH_2$, and H_3PO_4) were obtained from Beijing Chemical Works (Beijing, China). All solutions were prepared in doubly distilled water. Experimental strains for determination of the minimum inhibitory concentration (MIC) value were provided by the National Center for Surveillance of Antimicrobial Resistance (Beijing, China).

2.2. Instruments

The following instruments were used in our study: a P680 HPLC pump, ASI-100 autosampler, PDA-100 diode-array detector (Dionex, Sunnyvale, CA, U.S.), Q-Trap 3200 mass spectrometer (Applied Biosystems, Foster City, CA, U.S.), Bruker Inova-600 NMR spectrometer (Bruker, Zurich, Switzerland), Discovery Studio 2.2.5 software (Accelrys Software Inc., San Diego, CA, U.S.), Mettler Toledo electronic balance (Mettler Toledo, Greifensee, Switzerland), WGP-600 electro-heating standing-temperature cultivator, MLS-3750 autoclave (Sanyo, Moriguchi, Osaka, Japan), and

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Table 1
Gradient program for LC method (analytical).

Time (min)	Mobile phase A (%, v/v)	Mobile phase B (%, v/v)	Mobile phase C (%, v/v)
0	100%	0%	0%
5	100%	0%	0%
15	0%	100%	0%
30	0%	100%	0%
35	0%	85%	15%
45	0%	85%	15%
50	100%	0%	0%
70	100%	0%	0%

GRP-9270 Water-jacket thermostatic incubator (Shanghai Samsung Laboratory Instrument Co., Ltd., Shanghai, China).

2.3. Mass spectrometry conditions

Mass measurements were made on a Q-Trap 3200 mass spectrometer (Applied Biosystems, Foster City, CA, U.S.). The mass spectra of ceftriaxone were obtained using the following optimized MS conditions. Electrospray ionization (ESI) was performed in positive ionization mode, the declustering potential (DP) was 50 V, the entrance potential (EP) was 10 V, the collision energy (CE) was 40 V, curtain gas was flowed at 20.0 L h^{-1}, ion source gas 1 was flowed at 65.0 L h^{-1}, ion source gas 2 was flowed at 60.0 L h^{-1}, the ion spray voltage (IS) was 5500 V, and the temperature (TEM) was 500.0 °C, with the interface heater on. Enhanced MS (EMS) and enhanced product ion (EPI) spectra were acquired from m/z 50 to m/z 1200 in 0.1 amu steps with a 2.0 s dwell time. The Analyst software (version 1.5.1) was used for data acquisition and processing.

2.4. Liquid chromatography conditions

A Capcell Pak C18 MGIII (Shiseido, Tokyo, Japan) with dimensions of 250 mm × 4.6 mm i.d. was used for the separation. The column eluent was monitored at a wavelength of 270 nm. Different mixtures of 0.02 mol/L octylamine solution (pH 6.5, adjusted by phosphate, mobile phase A), 0.02 mol/L octylamine solution: acetonitrile:methanol (75:15:10, pH = 6.5, adjusted by phosphate, mobile phase B), and acetonitrile (mobile phase C) were used as the mobile phase at a flow rate of 1.0 ml/min. The gradient program is given in Table 1.

2.5. NMR measurements

NMR measurements were made on a Bruker-Avance 600 MHz instrument (both for ^{1}H and ^{13}C) at 25 °C in DMSO-d_6. The ^{1}H and ^{13}C chemical shift values are reported on the δ scale in ppm relative to TMS (δ = 0.00) and DMSO-d_6 (δ = 39.5 ppm) as the internal standards, respectively.

2.6. Activity determination

2.6.1. Molecular docking

Docking was performed with Discovery Studio 2.2.5 (Accelrys Software Inc.). The protein complexes were selected from the protein databank (PDB, www.rcsb.org). The 3BEC (PDB ID) structure of a penicillin binding protein (PBP) was selected, which is known to include the site at which cephalosporin binds to kill *Escherichia coli*. For the docking study, the protein was initially prepared by adding hydrogen atoms, and the CHARMm force field was applied. The binding site was defined using the known ligand configuration in the active site. The structures of the cis and trans isomers were built and their geometries were optimized within Discovery Studio, and then they were docked into the protein model with LigandFit.

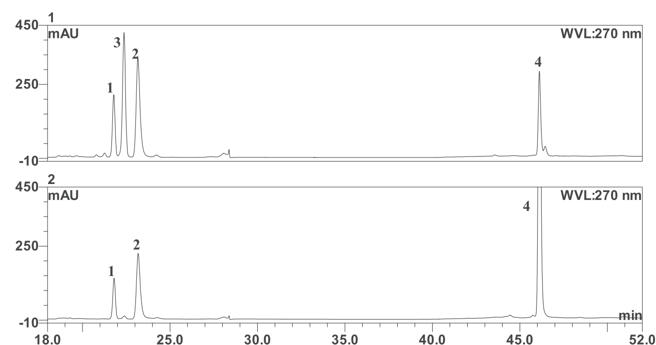

Fig. 1. HPLC chromatograms of ceftriaxone in different solvents: (1) acetic acid-ammonium acetate adjusted with $NH_3{\cdot}H_2O$ to pH 8.0. and (2) acetic acid-ammonium acetate at pH 4.0.

Scoring functions were used to assess which of the two conformations were the best complements to the protein binding site. Each docked compound was assigned a score using the potential of mean force (PMF).

2.6.2. MIC determination

An McF (McFarland) bacterial suspension was prepared with sterile normal saline using the Clinical and Laboratory Standards Institute (CLSI) agar dilution method [8]. 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 (MH) agar plate containing sites with different ceftriaxone concentrations using multi-point inoculators. After culturing of these samples at 35 ± 1 °C for 22–24 h, the MIC values were recorded.

3. Results and discussion

3.1. Ceftriaxone isomerization

Ceftriaxone RS (3.0 mg/mL) in 30 mM ammonium acetate buffer solution adjusted with acetic acid to pH 4.0 was stored for 13 h at room temperature. Four peaks were detected in the chromatogram (Fig. 1(1)). However, as the solution was adjusted with $NH_3{\cdot}H_2O$ to pH 8.0, compound **3** (peak 3) transformed to ceftriaxone again (Fig. 1(2)). The LC–MS results showed that compound 1 (peak 1) with m/z 413.4 was identical to $C_{14}H_{15}N_5O_6S_2$ (Fig. 2 and Figs. S1 and S2 in the electronic supplementary material). Compound 2 (peak 2) with m/z 158.0 was assigned as impurity C of ceftriaxone in Ph. Eur.8.0 (Fig. 2 and Fig. S2 in the electronic supplementary material) [7,9]. Comparison of the mass-to-charge ratios of compound **3** (peak 3) and ceftriaxone (peak 4) indicated that compound **3** was the isomer of ceftriaxone. Thus, these results indicate that ceftriaxone undergoes reversible isomerization in the above solvent system.

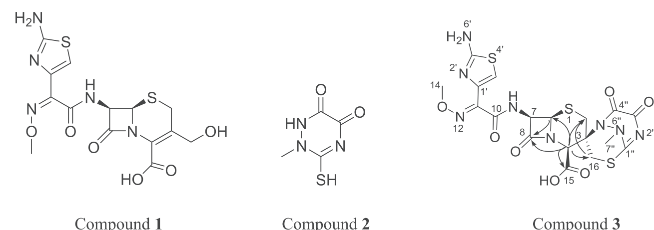

Fig. 2. Structures of Compounds **1**–**3**.

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