Identification of Ceftiofur Oxidation Products by High-Performance Liquid Chromatography/Electrospray Ionization/Tandem Mass Spectrometry

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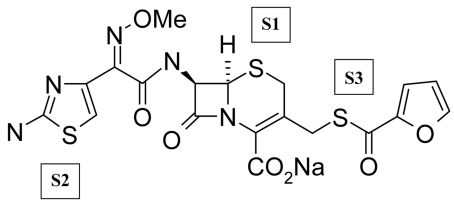
Abstract: Oxidation products of ceftiofur were formed in hydrogen peroxide solution. The structures of the ceftiofur oxidation products were characterized by high-performance liquid chromatography/electrospray ionization/tandem mass spectrometry (HPLC/ESI/MS/MS). The products were identified as compounds oxidized at the sulfur of a cephem ring. For further analysis, experiments were performed using O^{18}-labeled hydrogen peroxide. In addition, density-functional calculations were carried out for six possible oxidation products to support the experimental results.

Key words: Ceftiofur, Oxidation, ESI, Density-functional Calculation, Hydrogen Bonding

Introduction

Ceftiofur sodium is the salt of (6R,7R)-7{[(2-amino-4-thiazolyl)-Z-(methoxyimino) acetyl]-amino}-3-{[(2-furanylcarbonyl)thio]methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0] oct-2-ene-2-carboxylic acid (Scheme 1).

Ceftiofur, a third-generation cephalosporin antibiotic used widely in the treatment of bacterial infections, is currently administered by intramuscular injection for the treatment of certain respiratory diseases in cattle or swine. Ceftiofur is active against both Gram-positive and Gram-negative bacteria, including betalactamase-producing bacterial strains.[1] Its broad spectrum of activity is attributable in part to its resistance to inactivation by bacterial β-lactamase due to the presence of a bulky imino methoxy side chain.[2] Cephalosporin studies, including hydrolysis,[3] quantitative analysis,[4–9] and determination of aqueous stability,[10] have been performed, and the oxidation of the sulfides of penicillin and cephalosporin into sulfoxides by several oxidation reagents, including hydrogen peroxide, have been studied.[11,12] According to previous reports,[11,12] the oxidation of the sulfide moiety into sulfoxide led to R and S configurations of penicillin and cephalosporin, and the oxidation products were isolated and identified by nuclear magnetic resonance (NMR).



Scheme 1. Structure of ceftiofur sodium.

The present study elucidated the structures of ceftiofur oxidation products in hydrogen peroxide solution by high-performance liquid chromatography/mass spectrometry (HPLC-MS) and high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). Electrospray tandem mass spectrometry was used for structural identification. The optimized geometries and stabilities of the theoretically possible oxidation products were investigated using density-functional calculations.

Experimental

Materials

Ceftiofur was commercialized by LG Life Sciences, Ltd. (commercial name: LG Accent; Seoul, Korea). Trifluoroacetic acid (TFA), Hydrogen peroxide solution (30–35.5% in H_2O), hydrogen peroxide-$^{18}O_2$ solution (0.2% in H_2O, Icon Isotopes Co., Summit, NJ, USA), and deionized water were commercially available.

Chromatographic separation

Chromatography was carried out using a Hewlett-Packard HPLC system (HP1100; Palo Alto, CA, USA). The HPLC column was a Capcellpak (4.6 mm ID × 250 mm L, 5 μm, hexadecyl-bonded silica; Shiseido, Tokyo, Japan).

Mobile-phase component I was 0.1% aqueous TFA, and mobile-phase component II was acetonitrile. The column was maintained at 94% of component I for 5 min, after which the composition was linearly increased to 20% of component II for 18 min. The mobile-phase composition was then increased to 80% of component II over 60 min. The flow rate was 1.0 mL/min, and the ultraviolet (UV) detector was set at a wavelength of 254 nm.

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Mass spectrometry

Mass-spectrometric analyses were performed on a Q-Tof2 (Micromass, Manchester, UK) and an LCQ ion-trap mass spectrometer (Finnigan, Fremont, CA, USA) equipped with an electrospray interface. Data were processed and analyzed using MassLynx (Rev. 3.5, Micromass) and Xcaliber software (Rev. 1.2; Finnigan).

Mass spectrometric parameters for Q-Tof2 were set at a capillary voltage of 2.7 kV, source block temperature of 100 °C, desolvation temperature of 200 °C, nebulizer gas flow rate of 10 L/h, scans of *m/z* 50–650 Da, and collision energy of 20 eV. For the LCQ ion-trap mass spectrometer, capillary voltage was 4 kV, capillary temperature was 200 °C, sheath gas flow rate was 60 mL/min, scans were in the range of *m/z* 100–1500 Da, and collision energy was 30%. The mass analyzer was used in positive-ion mode.

Sample preparation

Approximately 100 mg of ceftiofur was weighed and dissolved in 20 mL of deionized water. A tenfold excess of hydrogen peroxide was added, and the solution was stored in an oven at 50 °C for 4 h. The solution was diluted five times with 4 : 1 (v/v) water:acetonitrile.

Computational details

Density-functional calculations were performed to optimize the geometry of possible oxidation products. There are three oxidizable sulfur atoms in ceftiofur, S*n* (*n* = 1–3), as shown in Scheme 1. A total of six diastereomers, *n*R and *n*S (*n* = 1–3), could be formed as oxidation products. Calculations were carried out using the generalized gradient approximation (GGA) based on density-functional theory (DFT), using the DMol^3 package.[13] In GGA, the Becke exchange[14] and Perdew correlation correction functions[15] were used with the double-numerical-basis set, including polarization functions (DNP).[16] DNP is similar to the 6-31G** basis in Gaussian, but the basis functions are numerical atomic orbitals augmented by polarization functions. Althougu this basis does not include diffuse functions, it exhibits long Slater-type tails.

Results and Discussion

Ceftiofur undergoes oxidation in hydrogen peroxide solution. Figure 1 shows the LC/UV chromatograms of (a) ceftiofur and (b) oxidized ceftiofur. The oxidation products eluted at 23.0 min (oxidant B) and 23.9 min (oxidant A). Oxidant A was observed as a major peak. Oxidants A and B produced molecular ions at *m/z* 540 ($[M+H]^+$) in positive-ion mode. Because the molecular ions of the oxidants differed by 16 Da from the molecular ions of ceftiofur (*m/z* 524), the oxidants were expected to be oxidized compounds of ceftiofur. Oxidants A and B may be isomers of one another. To determine the structure of the oxidants, the fragmentation pathway of ceftiofur was studied. Ceftiofur generated fragment ions in MS/MS mode using the Q-Tof mass spectrometer. The structures of

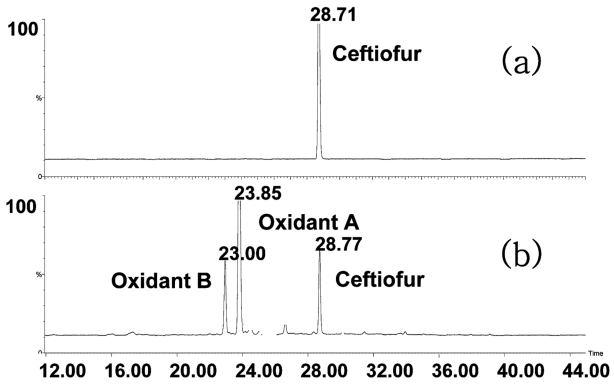


Figure 1. LC/UV chromatograms: (a) ceftiofur and (b) oxidized ceftiofur.

the fragment ions at *m/z* 396, 285, 284, 241, 156, and 126 were identified. Each fragment ion was interpreted based on its structure, as shown in Figure 2. Collision-induced dissociation (CID) spectra of ceftiofur in H_2O and D_2O also supported the fragment ion interpretation. Ceftiofur has four hydrogens that can be readily exchanged with deuterium (hydrogens of an amine, amide, and acid). The ions at *m/z* 400 and 245 of ceftiofur in D_2O are counterparts of the ions at *m/z* 396 and 241 of ceftiofur in H_2O.

The oxidants of ceftiofur also generated fragment ions in MS/MS mode using the Q-Tof mass spectrometer (Figure 3). The ion at *m/z* 522 differed by 18 Da from the molecular ion and can be assigned to H_2O loss. The fragment ion that differed by 18 Da was not observed in the MS/MS spectrum of ceftiofur. The formation of the ion at *m/z* 522 was assumed to be due to H_2O loss, including the oxygen of sulfoxide. The ions at *m/z* 241, 156, and 126 appeared in the MS/MS spectra of ceftiofur and the oxidants. The ion at *m/z* 301 differed by 16 Da from the ceftiofur ion at *m/z* 285, indicating that the fragment ion contained an oxidized sulfur of the cephem ring. Oxidants A and B showed very similar CID and MS/MS results. Thus, the oxidants were likely diastereomers containing the oxidized sulfur of the cephem ring (1R and 1S sulfoxides of ceftiofur). In MS^n

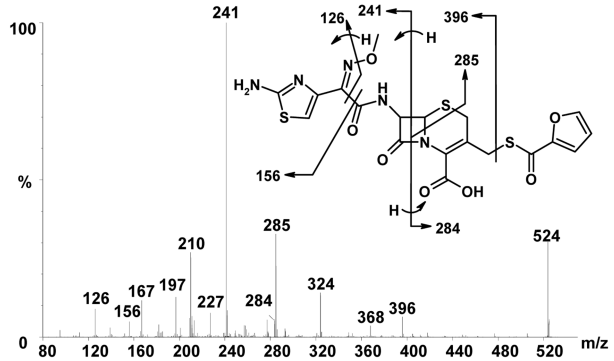


Figure 2. MS/MS spectrum of ceftiofur by Q-Tof: structure and fragmentation.

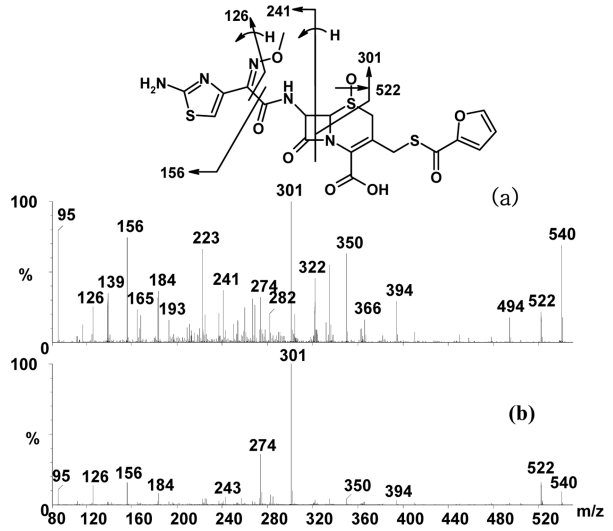
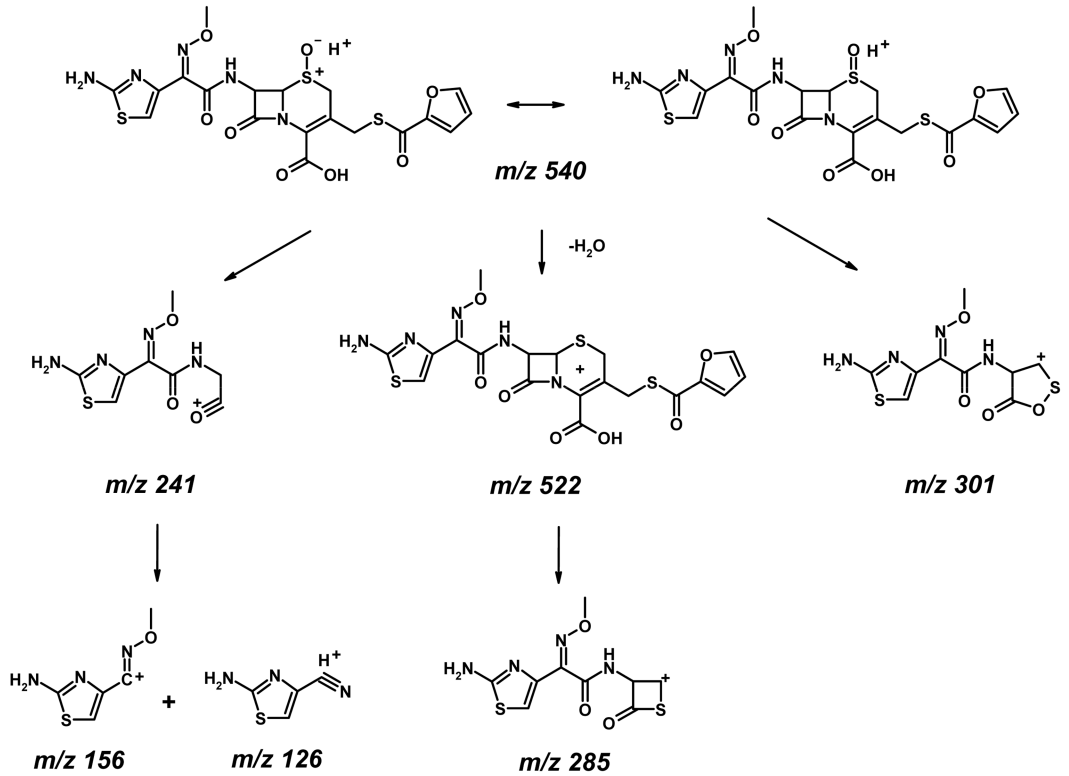


Figure 3. MS/MS spectrum of ceftiofur by Q-Tof: structure and fragmentation of (a) oxidant A and (b) oxidant B.

mode, using the LCQ mass spectrometer, the ion at *m/z* 522 was most abundant in the MS^2 spectrum. The MS^3 spectrum shows that the ion at *m/z* 285 originated from the ion at *m/z* 522. Based on these results, a fragmentation pathway was proposed for the ceftiofur oxidation product (Scheme 2).

To confirm the proposed fragmentation pathway, ceftiofur was oxidized using hydrogen peroxide-$^{18}O_2$ solution. The molecular ion of the oxidant, $[M+H]^+$, was detected at m/z 542. The ion from the sample oxidized using unlabeled hydrogen peroxide was compared with the ion from the sample oxidized using hydrogen peroxide-$^{18}O_2$. In MS/MS mode, the same characteristic ions at *m/z* 241, 156, and 126 were present, as observed in the spectra of ceftiofur and of the oxidants produced using unlabeled hydrogen peroxide solution. The ion at m/z 522 originated from the molecular ion (*m/z* 542) and was assigned to the loss of $H_2{}^{18}O$. The characteristic ion at *m/z* 303, as well as the fragment ion at *m/z* 301, indicated oxidation at the sulfur of the cephem ring. These results were consistent with the proposed fragmentation pathway.

As mentioned earlier, the two peaks in the LC chromatogram of the oxidized ceftiofur sample produced the same molecular ions at *m/z* 540. Based on previous work,[9,10] the oxidants were assigned as 1R and 1S sulfoxides of ceftiofur. The optimized geometries of six diastereomeric oxidants were calculated. The calculated reaction energies, DE = [E(oxidants)+E(H_2O)]–[E(ceftiofur)+E(H_2O_2)], and the relative energies are shown in Table 1. The oxidation reactions of the sulfur of the cephem ring were more thermodynamically favorable than other oxidation reactions by 10-20 kcal/mol, and the 1S sulfoxide was more stable than the 1R sulfoxide by 4.1 kcal/mol. This stability may have been due to the presence of hydrogen bonding between the oxygen and hydrogen of the neighboring amide. Figure 4 clearly shows that the newly formed hydrogen



Scheme 2. Proposed fragmentation pathway of ceftiofur oxidation products.

Table 1. Reactions and relative energies of six possible oxidation products.

Oxidation product	Reaction energy[a] (kcal/mol)	Relative energy (kcal/mol)
1S	−45.32	0.00
1R	−41.32	4.10
2S	−25.05	20.27
2R	−25.75	19.57
3S	−33.17	12.14
3R	−35.89	9.43

[a]Reaction energy, DE = [E(oxidant)+E(H_2O)]–[E(ceftiofur)+E(H_2O_2)]

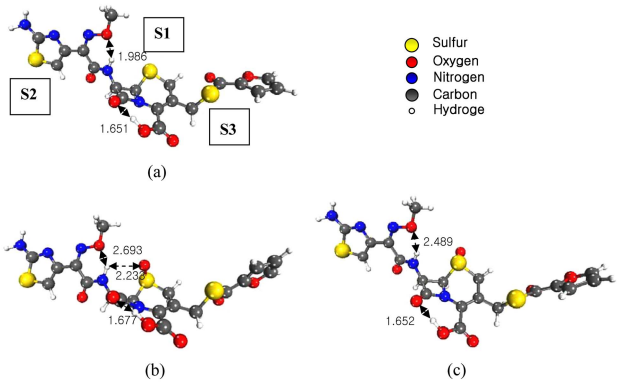


Figure 4. Optimized structure: (a) ceftiofur; (b) 1S oxidant, and (c) 1R oxidant. Hydrogen bonds are shown as dashed lines, and the bond lengths are in Å.

bonding stabilized the 1S sulfoxide.

Conclusions

When ceftiofur was treated in aqueous hydrogen peroxide solution, two primary peaks appeared near the peak surrounding ceftiofur in HPLC. For structural elucidation of ceftiofur oxidation products, LC/MS^n analysis was performed. The products were oxidized forms of ceftiofur assumed to be the R and S forms of sulfoxide (1R and 1S sulfoxides of ceftiofur). A fragmentation pathway was proposed. Fragmentation data obtained using hydrogen peroxide-$^{18}O_2$ solution supported the proposed pathway. Interpretation of the fragment ions at *m/z* 285 (detected ceftiofur and oxidation product) and *m/z* 301 (detected oxidation product) was important for identifying the oxidation site. Based on interpretation of the mass data, the structures of oxidation products were proposed without isolation. The proposed structures were confirmed by geometry optimization and energy evaluation using density-functional theory. Thus, MS/MS spectra and fragmentation of ceftiofur oxidation products can be used to determine and confirm the structures of the related compounds.

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