Differentiation of Cefaclor and its delta-3 isomer by electrospray mass spectrometry, infrared multiple photon dissociation spectroscopy and theoretical calculations

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Dear Sir,

Cephalosporins are an important class of β-lactam antibiotics and exhibit broad-spectrum activity against Gram-negative and Gram-positive bacteria with low occurrence of adverse effect; they are one of the most prescribed antibiotics in the clinic. New generations of cephalosporins continually hit the market since the emergence of the multi-drug-resistance (MDR) problem.[1,2] The basic structure of cephalosporins consists of a dihydrothiazine ring condensed on a four-membered β-lactam ring (Table 1), which is essential for antibacterial activity. Due to their highly strained basic structure, cephalosporins are very reactive species, and isomerism may occur under certain circumstances.[3]

The C2—C3 double bond migration isomers of Cephaslosporins, also known as the Δ-3 isomers (Table 1), were found to have less or no microbiological activity.[4] Cefaclor, a second generation cephalosporin, bears a D-α-phenylglycine at the C-7 side chain (Table 1).[1] Although Δ-3 Cefaclor is not very toxic, as indicated by Zebrafish embryo toxicity testing,[5] it is considered as a meaningful impurity indicator in drug quality control. For this reason, some pharmacopoeias[6,7] have limited the amount of this impurity in active pharmaceutical ingredients (API) and dosage forms.

For differentiating chemical isomers, in particular, mass spectrometry could be helpful if specific product ions can be successfully assigned.[8] The fragmentation behavior of cephalosporins in both positive and negative ion mode has been studied before,[9–11] and the cleavage of the β-lactam ring was considered to be universal. However, the fragmentation behavior of Δ-3 isomers is rarely seen, and the comparative study on the differentiation of these positional isomers by mass spectrometry has not yet been reported.

In this study, we investigate the fragmentation patterns of Cefaclor, its Δ-3 isomers and other cephalosporin analogues (Table 1) by tandem mass spectrometry in the positive mode. We found that Cefaclor exhibits a diagnostic ion, which could enable the distinction from Δ-3 Cefaclor. The chemical structure of the diagnostic ion was probed by gas-phase IR spectroscopy integrated to tandem mass spectrometry which has recently emerged as a powerful technique for characterizing the structure of mass-selected ions,[12] in particular when isobaric species and/or isomers may coexist.[13] Quantum chemical calculations were performed for deriving the IR absorption spectra of possible isomers, and for characterizing the possible fragmentation pathway for the better understanding of the unusual mechanism.

All the chemical reference standards were obtained from the National Institutes for Food and Drug Control (NIFDC). The structures of these compounds were characterized by ^{1}H-NMR; the configuration of the chiral center at the C2 position of Δ-3 Cefaclor was further characterized by 1D-NOE, which is in good agreement with data previously reported in the literature[3] (Fig. S1 in the Supporting Information).

All the compounds were prepared at the concentration of 10 μg/ml. The collision-activated dissociation (CAD) experiments were performed on an Applied Biosystem Sciex 3200 QTrap triple-quadrupole linear ion trap mass spectrometer (Toronto, Canada) equipped with a Turbo V ESI source. Sample solutions were introduced into the mass spectrometer at a flow rate of 10 μl/min. High-resolution CAD mass spectra of the product ions were measured on an Applied Biosystem Sciex Triple TOF 5600 system with an ESI source. Calibrant Delivery System (CDS) was used for introducing calibration solution to the mass spectrometer for external calibration. Nitrogen gas was used as both the nebulizing and collision gas. IRMPD spectra in the mid-IR (900–1900 cm^{-1}) region were recorded using the Free-Electron Laser (FEL) coupled with a modified commercial hybrid FT-ICR (7T Bruker APEX IV) located at Centre Laser Infrarouge d'Orsay (CLIO) in France. Details on the IRMPD instrumental setup can be found elsewhere.[14,15] The molecular simulation method using CHARMm force field implemented in Discovery Studio 3.1.0 (Accerlys Inc, San Diego, USA) followed by the density functional theory (DFT) method was applied to explore the chemical structures, vibrational frequencies and thermal energies in the gas phase. Geometries and theoretical harmonic frequencies of the diagnostic ion were obtained at the B3LYP/6-311G(d,p) level of theory. The calculated frequencies were scaled by a factor of 0.98[12,16] for comparison to the experimental IR spectra. To evaluate the possible fragmentation pathways, the optimized geometries and thermochemistry data were obtained using long-range corrected hybrid density functional ωB97X[17] with def-TZVP basis set. All the DFT calculations were carried out with the GAMESS software.[18] The detailed sample preparation methods, the IRMPD experimental procedure and the theoretical calculation methods can be found in the Supporting Information.

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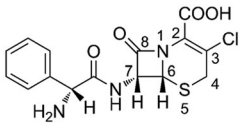
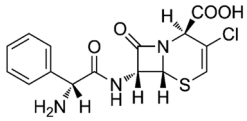
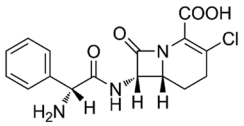
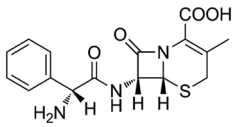
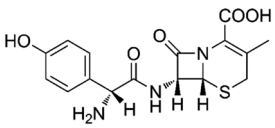
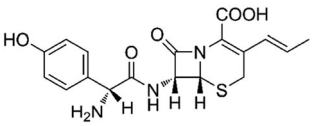
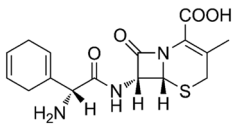
Table 1. Chemical structures of cephalosporins and Δ-3 isomers

Compound	Structure
Cefaclor	
Δ-3 Cefaclor	
Loracarbef	
Cefalexin	
Cefadroxil	
Cefprozil	
Cefradine	

As shown in Fig. 1, Cefaclor, Δ-3 Cefaclor and the analogue Loracarbef have the same kind of product ions related to the cleavage of the amide bond of the β-lactam ring, and these characteristic ions were denominated as A_1^+ and A_2^+ ions,[10] respectively. The ion at *m/z* 192, however, which was assigned as $[B+H-NH_2]^+$ ion,[10] was observed exclusively for Cefaclor. This ion can thus be considered as diagnostic ion for the differentiation of Cefaclor and Δ-3 Cefaclor. The elemental compositions of these product ions were further supported by high-resolution MS/MS experiment (Table 2). The MS/MS experiment and precursor ion scan clearly showed that the ion at *m/z* 192 originated from the ion at *m/z* 351, namely, the deamination ion $[M-NH_3]^+$. Based on this knowledge, we proposed that the $[B+H-NH_2]^+$ ion was generated by the cleavage of both the β-lactam ring and the dihydrothiazine ring followed by a proton transfer reaction (Fig. 1). The same kind of $[B+H-NH_2]^+$ ion was also found in other cephalosporin analogues, such as Cefalexin, Cefadroxil, Cefprozil and Cefradine (Table 1, Fig. S2 and Table S1 in the Supporting Information). It should be stressed that the fragmentation spectrum of protonated Loracarbef, which bears the methylene group instead of the sulfur atom in the six-membered ring as for the cephalosporin analogues, lacks the $[B+H-NH_2]^+$ ion. These results suggest that the sulfur atom, as well as the C=C double bond position, plays a crucial role in the fragmentation process leading to the formation of the $[B+H-NH_2]^+$ ion.

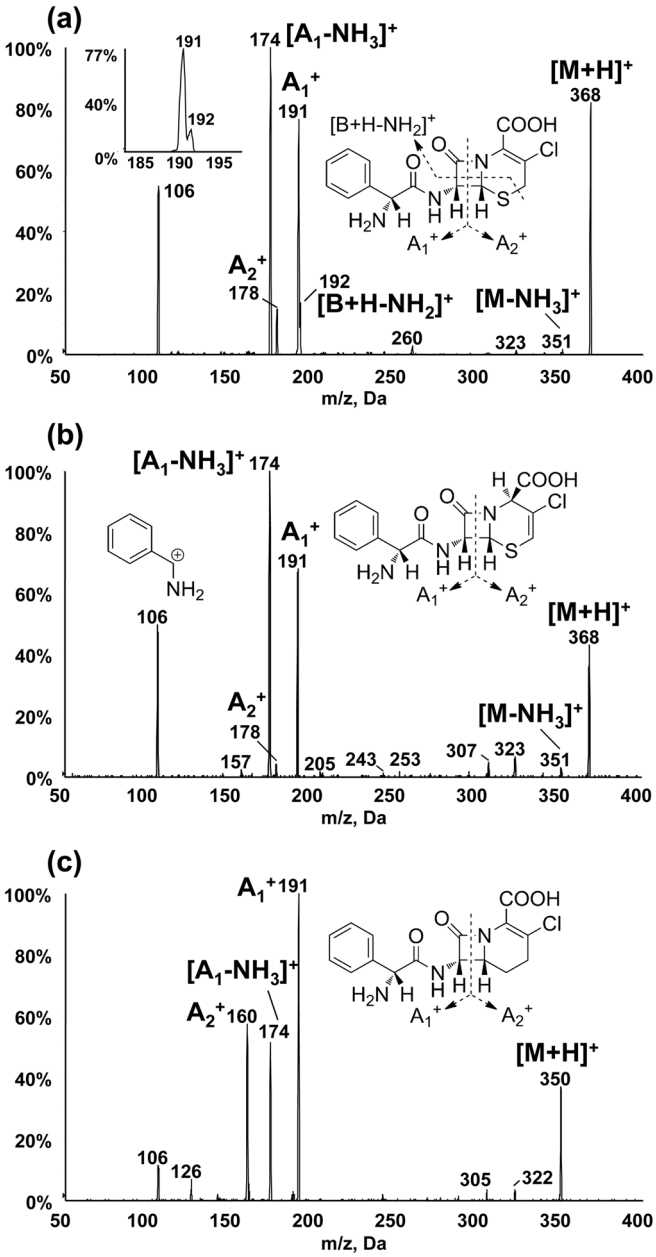


Figure 1. Positive ion ESI-MS/MS spectra of (a) Cefaclor, (b) Δ-3 Cefaclor and (c) Loracarbef.

In order to provide more insight into the structure of the ion at *m/z* 192 of Cefaclor, its IRMPD spectrum was recorded and compared with the theoretical IR spectra (Fig. 2). In the infrared 'fingerprint' region, a good agreement was found between the IRMPD spectra and the theoretical IR spectra of the global minimum conformer **192-a**. The conformer **192-a** has a unique six-membered ring protonated at the carbonyl oxygen atom. Six strong bands are predicted in this mid-IR region for this isomer, these bands stem from the O—H (1239 cm^{-1}) and vinyl C—H (1332 cm^{-1}) bending, carbonyl C^+—OH stretch (1368 cm^{-1}), carbonyl C—C(α) stretch (1488 cm^{-1}), vinyl C—C stretch (1591 cm^{-1}) and amide C—N stretch (1658 cm^{-1}), according to the vibrational modes determined at the B3LYP level. As can be seen in Fig. 2, each strong peak obtained from calculations coincides with the band observed in the IRMPD spectra one to one, the only exception is the two predicted IR absorption peaks at 1332 and 1368 cm^{-1} correspond to the

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Table 2. The accurate masses of product ions of Cefaclor, Δ-3 Cefaclor and Loracarbef

Compound	Ions	Measured	Observed	Elemental composition	Error (ppm)
Cefaclor	A_1^+	191.0821	191.0815	$C_{10}H_{11}N_2O_2^+$	3.1
	$[A_1 - NH_3]^+$	174.0555	174.0550	$C_{10}H_8NO_2^+$	2.9
	A_2^+	177.9728	177.9724	$C_5H_5NO_2SCl^+$	2.2
	$[B + H - NH_2]^+$	192.0480	192.0478	$C_{10}H_{10}NOS^+$	1.0
Δ-3 Cefaclor	A_1^+	191.0816	191.0815	$C_{10}H_{11}N_2O_2^+$	0.5
	$[A_1 - NH_3]^+$	174.0550	174.0550	$C_{10}H_8NO_2^+$	0.0
	A_2^+	177.9725	177.9724	$C_5H_5NO_2SCl^+$	0.6
Loracarbef	A_1^+	191.0818	191.0815	$C_{10}H_{11}N_2O_2^+$	1.6
	$[A_1 - NH_3]^+$	174.0551	174.0550	$C_{10}H_8NO_2^+$	0.6
	A_2^+	160.0157	160.0160	$C_6H_7NO_2Cl^+$	−1.9

broad and unresolved band centered at 1347 cm^{-1}. Thus, the IR spectrum in the 900–1900 cm^{-1} is a fingerprint of this special six-membered ring. On the basis of the IR data, low-energy rotamers such as **192-a** and **192-b** can be distinguished. The energy of conformer **192-b** is predicted only 5.3 kcal/mol above conformer **192-a**. However, these rotamers can be distinguished considering, in particular, the position of the O—H bending (1239 and 1143 cm^{-1} for conformers **192-a** and **192-b**, respectively). In addition, it can be noticed that no strong IR active mode is predicted around 1500 cm^{-1} for the **192-b** isomer, while conformer **192-a** is predicted to have a strongly IR active mode at 1488 cm^{-1}, which nicely matches with the observed band near 1489 cm^{-1}. Other conformers are predicted higher in energy and are likely not being formed under our experimental conditions. In addition, their IR absorption spectra are significantly different from the experimental spectra (Fig. 2 and Fig. S3 in the Supporting Information). It can thus be concluded that under our experimental conditions, structure **192-a** is a likely structure for the fragment ion at *m/z* 192.

In light of the structure of **192-a**, a possible fragmentation mechanism (Scheme 1) has been investigated theoretically. DFT calculations indicated that the nitrogen atom of the amino group is the most thermodynamically favored protonation site for Cefaclor in the gas phase, and the lowest corresponding conformer (**Conformer 1**) is the reference energy in Scheme 1. On the basis of our calculation, the rate limiting step for the formation of *m/z* 351 fragment is **TS2** associated with the elimination of NH_3, which is predicted 34.4 kcal/mol higher in energy than **Conformer 1**. Considering that **TS2** looks like an S_N2 TS and that DFT consistently underestimates the corresponding activation energy barrier, one can consider that the calculated barrier is a lower bound value. A three-step reaction mechanism is proposed for this process. Although one of the corresponding transition states could not be located, a relaxed potential energy scan (PES) suggests that the barrier leading to **Conformer 2** (4.6 kcal/mol) is estimated to be 6.2 kcal/mol, which is consistent with earlier reports on similar systems.[19] The energy barrier for the subsequent amide bond *trans–cis* isomerization (**TS1**) is 13.6 kcal/mol which is in agreement with the amide bond isomerism of peptides.[20,21] Transition state **TS2** is associated to the C—NH_3 bond breaking which is assisted by the concerted nucleophilic attack of the sulfur atom leading to the formation of a six-membered ring.

A mechanism for the fragmentation of *m/z* 351 into *m/z* 192 (**192-a**) is also proposed in scheme 1. Critical transition states (**TS3**, **TS4**, **TS5** and **TS6**) for this multiple steps mechanism have been characterized. It is proposed that the C4—S5 bond cleavage occurs through a concerted mechanism (**TS3**). As indicated by the formal positive charge on N1 atom in the resulting **IM3**, a facile

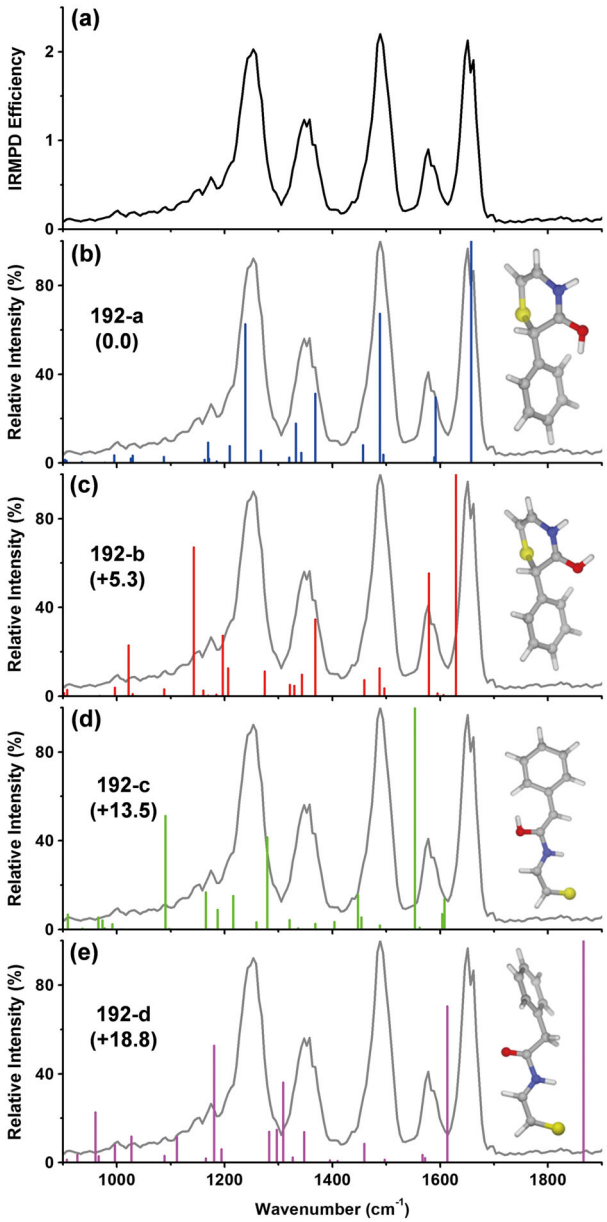
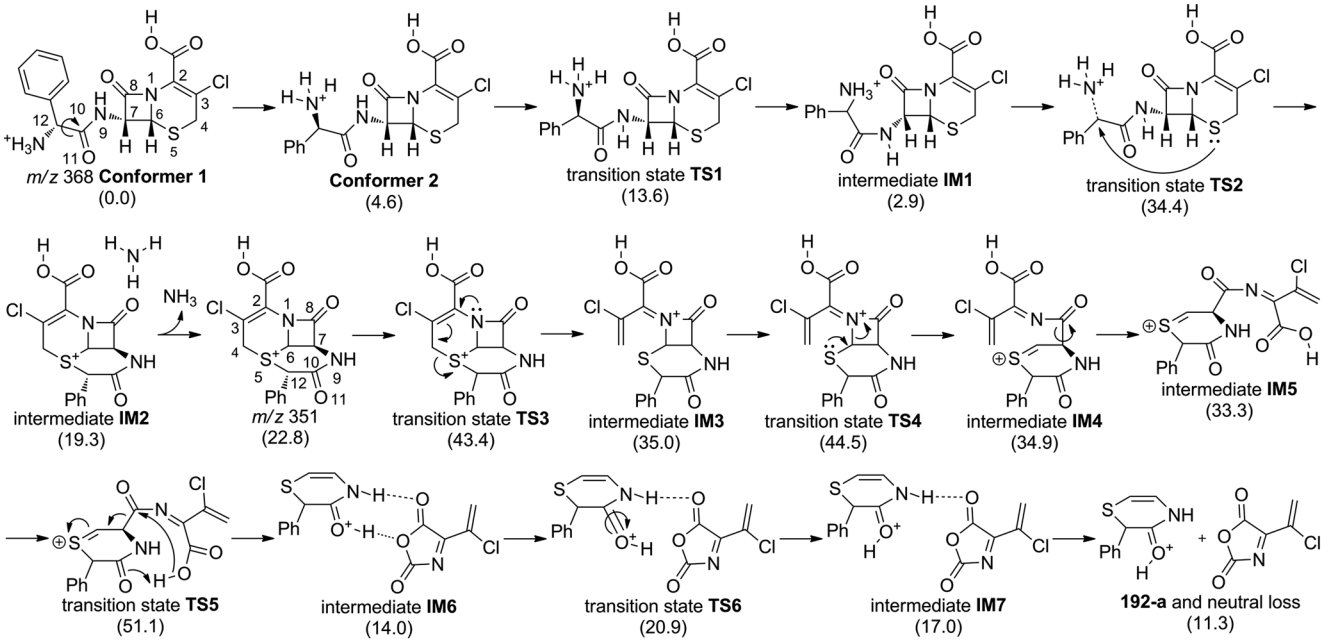


Figure 2. (a) Experimental IRMPD spectrum and ((b), (c), (d) and (e)) scaled theoretical IR spectrum of four conformers (192-a, 192-b, 192-c and 192-d) of the ion at *m/z* 192. Theoretical IR data are shown in vertical lines in (b), (c) (d) and (e), IRMPD spectrum is included for comparison in grey lines. Calculated relative Gibbs free energies (kcal/mol) at the B3LYP level are given in parentheses.



Scheme 1. The proposed fragmentation mechanism of ion at *m/z* 192. The numbers in parenthesis are relative Gibbs free energies (kcal/mol) calculated at the ωB97X level.

β-lactam ring opening through **TS4** may occur leading to **IM4**. Following a low energy conformational change leading to **IM5**, formation of an anhydride (**IM6**) as suggested for protonated peptides[22,23] is proposed through **TS5**. Formation of **192-a** could then occurs following a low energy isomerization within the ion-neutral complex[24] (**TS6** at 20.9 kcal/mol).

It is worth mentioning that this multi-steps fragmentation mechanism is highly dependent on the nature of the Cephalosporin analogs. For Loracarbef, for example, the rate limiting transition state associated with the loss of NH_3 is likely to be higher since no sulfur is available for a concerted nucleophilic attack. In the case of Δ-3 Cefaclor, the route towards the $[B+H-NH_2]^+$ fragment is also impossible because the concerted ring-opening (**TS3**) could not be achieved due to the position of the C=C double bond. The proposed mechanism thus offers a possible interpretation for the selective formation of *m/z* 192 from Cefaclor.

In conclusion, Cefaclor and Δ-3 Cefaclor could be differentiated by positive electrospray tandem mass spectrometry. The key diagnostic fragment ion relevant to both the β-lactam and dihydrothiazine rings breaking was comprehensively analyzed by the IRMPD spectrum and theoretical calculations. Our studies suggest that the diagnostic ion has a special six-membered ring with a protonated carbonyl group. The possible fragmentation pathways are rationalized by the charge-induced dissociation coupled with proton transfer reactions. The approaches and results reported here are useful for elucidating the fragmentation pathways of other kinds of cephalosporins and Δ-3 isomers, which in turn are important for drug screening.

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Yours,

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