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Influence of substituent groups at the 3-position on the mass spectral fragmentation pathways of cephalosporins

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The structural fragment ions of nine cephalosporins were studied by electrospray ionization quadrapole trap mass spectrometry (Q-Trap MS^n) in positive mode. The influence of substituent groups in the 3-position on fragmentation pathway B, an α-cleavage between the C7−C8 single bond, coupled with a [2,4]-trans-Diels-Alder cleavage simultaneously within the six-membered heterocyclic ring, was also investigated. It was found that when the substituent groups were methyl, chloride, vinyl, or propenyl, fragmentations belonging to pathway B were detected; however, when the substituents were heteroatoms such as O, N, or S, pathway B fragmentation was not detected. This suggested that the $[M–R_3]^+$ ion, which was produced by the bond cleavage within the substituent group at the 3-position, had a key influence on fragmentation pathway B. This could be attributed to the strong electronegativity of the heteroatoms (O, N, S) that favors the production of the $[M–R_3]^+$ ion. Moreover, having the positive charge of the $[M–R_3]^+$ ion localized on the nitrogen atom in the 1-position changed the electron density distribution of the heterocyclic structure, which prohibits a [2,4]-reverse-Diels-Alder fragmentation and as a result fragmentation pathway B could not occur. The influence of the substituent group in the 3-position was determined by the intensity ratio (e/d) of ions produced by fragmentation pathway A, a [2,2]-trans-Diels-Alder cleavage within the quaternary lactam ring, including the breaking of the amide bond and the C6−C7 single bond (ion d), and fragmentation pathway B (ion e). The results indicate that the electronegativity of the substituent group was a key influencing factor of pathway B fragmentation intensity, because the intensity ratio (e/d) is higher for a chlorine atom, a vinyl, or a propenyl group than that of a methyl group. This study provided some theoretical basis for the identification of cephalosporin antibiotics and structural analysis of related substances in drugs.

Cephalosporin antibiotics are a series of compounds with characteristic structures. Their matrix structure is composed of a β-lactam ring and a six-membered heterocyclic ring containing nitrogen and sulfur atoms, while the substituent groups are mainly located in the 2-, 3- and 7-positions.

The MS fragmentation regularities of these types of compounds have been reported,[1–6] and the general fragmentation pathways of cephalosporins in positive mode mainly proceed via pathways A and B, which were absent in negative mode,[6] see Fig. 1.[3]

Pathway A involves breaking of the amide bond and the C6−C7 single bond in the quaternary lactam ring, and a [2,2]-trans-Diels-Alder cleavage occurs within the quaternary lactam ring. As a result, fragment A1 containing group R_1 and fragment A2 that represents the six-membered heterocyclic structure are produced.

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Pathway B involves breaking of the C7−C8 single bond within the quaternary lactam ring along with the N1−C6 and C5−S6 single bonds within the six-membered heterocyclic ring. This proceeds by an α-cleavage between

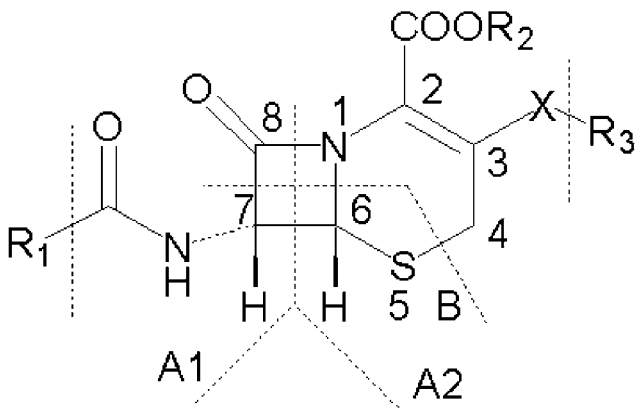
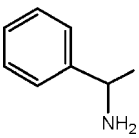
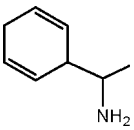
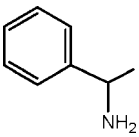
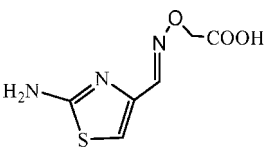
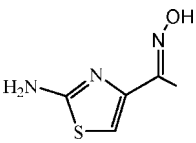
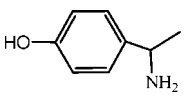
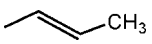
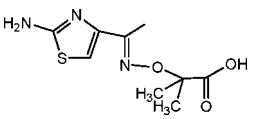
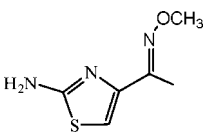
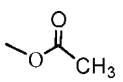
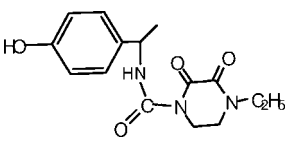
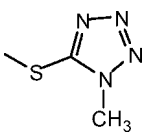


Figure 1. Partial fragmentation pathways of cephalosporins.[3]

Table 1. Lots, contents and chemical structures of nine cephalosporins

No.	Name (MW)	Lot.	Content	Substitutent groups			
				R_1	R_2	X	R_3
I	Cefalexin[#] (347)	130408 -200710	94.4%		-H	$-CH_3$	—
II	Cefradine[#] (349)	130427 -200306	91.8%		-H	$-CH_3$	—
III	Cefaclor[#] (367)	130481 -200503	93.2%		-H	-Cl	—
IV	Cefixime[#] (453)	130503 -200803	85.2%		-H		—
V	Cefdinir[#] (395)	130502 -200302	90.4%		-H		—
VI	Cefprozil[*] (389)	GOD341	87.2%		-H		—
VII	Ceftazidime[#] (546)	1304823 -200303	89.3%		-H	$-CH_2-$	
VIII	Cefotaxime[#] (455)	130420 -200304	96.5%		-H	$-CH_2-$	
IX	Cefoperazone[#] (645)	130484 -200502	84.2%		-H	$-CH_2-$	

[#] These standard references were obtained from NICPBP in China, and the contents were calculated by the 'as is' method.
[*] This standard reference was purchased from USP, and the contents were calculated by the 'as is' method.

the C7–C8 single bond, which is induced by the carbonyl group in the 8-position, while a [2,4]-trans-Diels-Alder cleavage occurs simultaneously within the six-membered heterocyclic ring. As a characteristic fragmentation pathway, the produced fragment ions provide the structural information of substituent R_1 and the matrix.

For this study, the structural fragmentations of the nine cephalosporins were investigated in positive mode. Based on the elucidation of those ions, the influence of the substituent in the 3-position on fragmentation pathway B was evaluated. This study provided some theoretical basis for the identification of cephalosporins and for the structural analysis of related substances in drugs.

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EXPERIMENTAL

Instrument and reagents

The Q Trap 3200 MS/MS spectrometer was from Applied Biosystems (Framingham, MA, USA). Methanol of HPLC grade was provided by Thermo Fisher Co. (Waltham, MA, USA). Deionized water was provided by WaHaHa Co. (China).

Materials and sample preparation

The lots, contents, chemical structures and source information of nine different cephalosporin antibiotics used in this study are summarized in Table 1. The cephalosporin standards were each accurately weighed (10 mg) into a 25 mL volumetric flask and dissolved in 25 mL of methanol/water (1:1) solution. Test solutions were prepared by mixing 100 μL of the standard solution in 900 μL of methanol/water (1:1) solution. The MS^n analysis was carried out using direct injection mode.

Mass conditions

Flow rate: 10 $\mu L \cdot min^{-1}$; scan range 50–700; ion type: positive; curtain gas: 10 $L \cdot min^{-1}$; IS: +4500 V; TEM: 0.00°C; GS1: 10 $L \cdot min^{-1}$; GS2: 0$L \cdot min^{-1}$; CAD: medium; declustering potential (DP): +50.00 V.

RESULTS AND DISCUSSION

As shown in Table 2, the characteristic precursor ions and fragment ions that were produced by the nine cephalosporin antibiotics were assigned according to the cleavage pathways depicted in Fig. 1. The mass spectrometry results suggest that fragmentation of compounds I–VI proceeds via pathway B; however, these fragmentations via pathway B were not detected for compounds VII to IX suggesting an alternative pathway.

After a careful comparison of the chemical structures, fragmentation pathway B was found to be influenced by the substituent in the 3-position, where the X substituent group in the 3-position of compounds I–VI was either a methyl, chloride or double-bond group and without R_3 groups; while the X group of compounds VII–IX was a methylene connected to R_3 groups that contain various N, O and S heteroatoms.

The structural fragment ions of the nine cephalosporins were analyzed and identified by electrospray ionization quadrapole trap mass spectrometry (ESI Q-Trap MS^n) in positive mode. The individual fragmentation pathways were assigned according to the general fragmentation pathway illustrated in Fig. 1. For example, the mass spectra and fragmentation pathways for compounds I, IV and VII are shown in Figs. 2(1), 2(2), 3(1), 3(2), 4(1) and 4(2), respectively; and the spectra of compounds VIII and IX are shown in Figs. 5 and 6, respectively. It was found that a $[M–R_3]^+$ ion (fragment ion f in Figs. 4(1), 5, 6; corresponding *m/z* ratios are shown in Table 2) was produced initially during the mass analysis of compounds VII–IX, but this ion was not detected in compounds I–VI. Based on the electronegativity equalization theory,[7] when a C−Y σ bond (C=carbon; Y=O, S, N, etc.) is formed, the electron cloud of the σ bond flows from the carbon atom which has a high chemical potential (weak electro-negativity) to the heteroatom which has a low chemical potential (strong electronegativity), and ultimately, the chemical potentials of the bonding atoms become equal. The movements of the electron cloud decrease the bond energy, which makes the C−Y bond easier to cleave than the C−H bond. Therefore, the existence of R_3 heteroatom groups in compounds VII–IX may have contributed to the formation of the observed $[M–R_3]^+$ ion, whereas no $[M–R_3]^+$ ions were detected in compounds I–VI which only have either a methyl, chloride or vinyl substituent in the 3-position.

According to the mass fragmentation regularities,[8–10] the cleavage of compounds mainly consists of a charge/free radical site, and induced cleavage. The prerequisite of mass fragmentation analysis is determining the charge and free radical sites. Based on the ionization potential theory, the charge in the molecule is most likely to localize in the atom whose electron ionization energy is the lowest. Furthermore, the electron ionization energy of molecular RX (R = substituent group; X = atoms with low electron ionization energy, e.g. N, S, O) depends on the induction effects of the R group. The ionization of the electron in the X atom is facile when the induction effect of R is large, because induction effects can make the RX^+ ion stable as it is produced by RX.[11]

The $[M–R_3]^+$ ion was formed by cleavage of the R_3 side chain from the quasi-molecular ion $[M+H]^+$ of compounds

Table 2. Main fragment ions from $[M+H]^+$ of nine cephalosporins in positive mode

Chemical No.	M+H (a)	Fragment ions (No.)					
		R_1 (b)	A_1+H (c)	A_2+H (d)	B (e)	[M-R_3] (f)	Others
I	348	106	191	158	192[b]	-	286,242,207,186, 146,140,114
II	350	108	193	158	194[b]	-	333,305,187,176, 140,136,114
III	368	106	191	178	192[b]	-	340,260,243,215, 160,118,114
IV	454	200	285	170	301	-	335,329,257,241, 210,182,126
V	396	-	227	170	243	-	271,209,183, 152,126
VI	390	122	207	184	208[b]	-	373,345,239,190,166,114
VII	547	228	313	-	-	468[a]	440,424,396,365, 277, 167,139,126
VIII	456	156	241	-	-	396	428,412,368,324, 277,230,211,167
IX	646	290	375	-	-	530	502,388,360, 318,186,143

[a] This ion was produced by $[M+H–R_3]$; [b] These ions were produced by $[B–NH_2]$.
— not detected.

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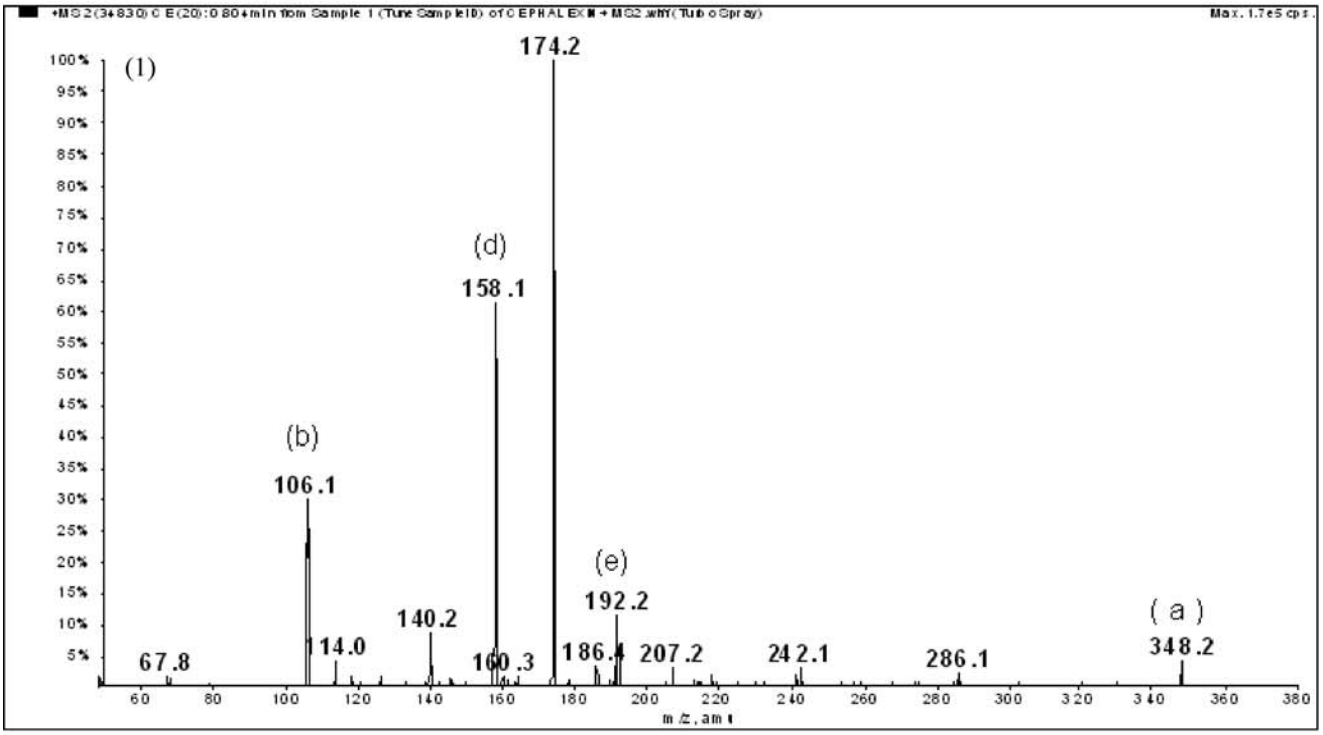

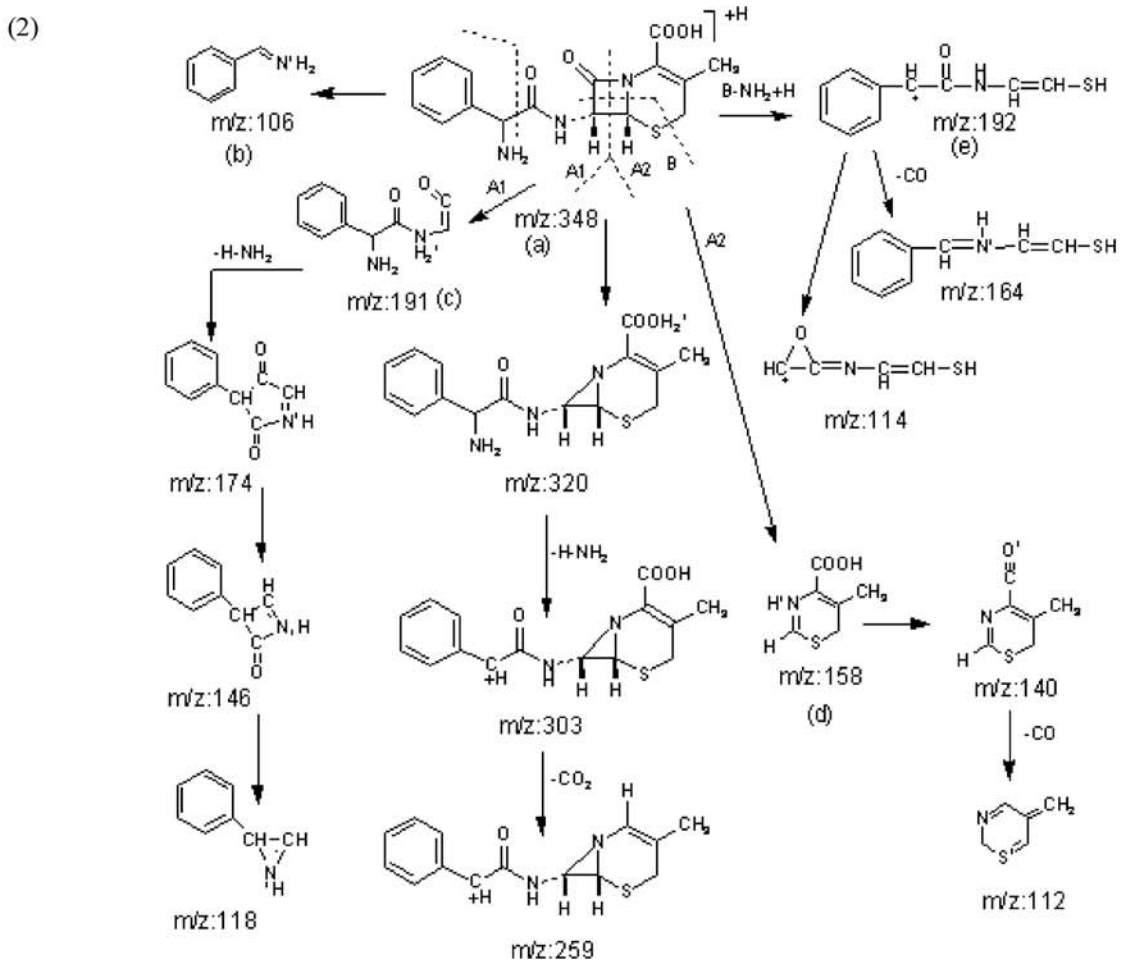

Figure 2. (1) The product ion (MS/MS) spectrum of the $[M+H]^+$ ion of cefalexin (I). (2) The main fragmentation pathways of cefalexin (I).

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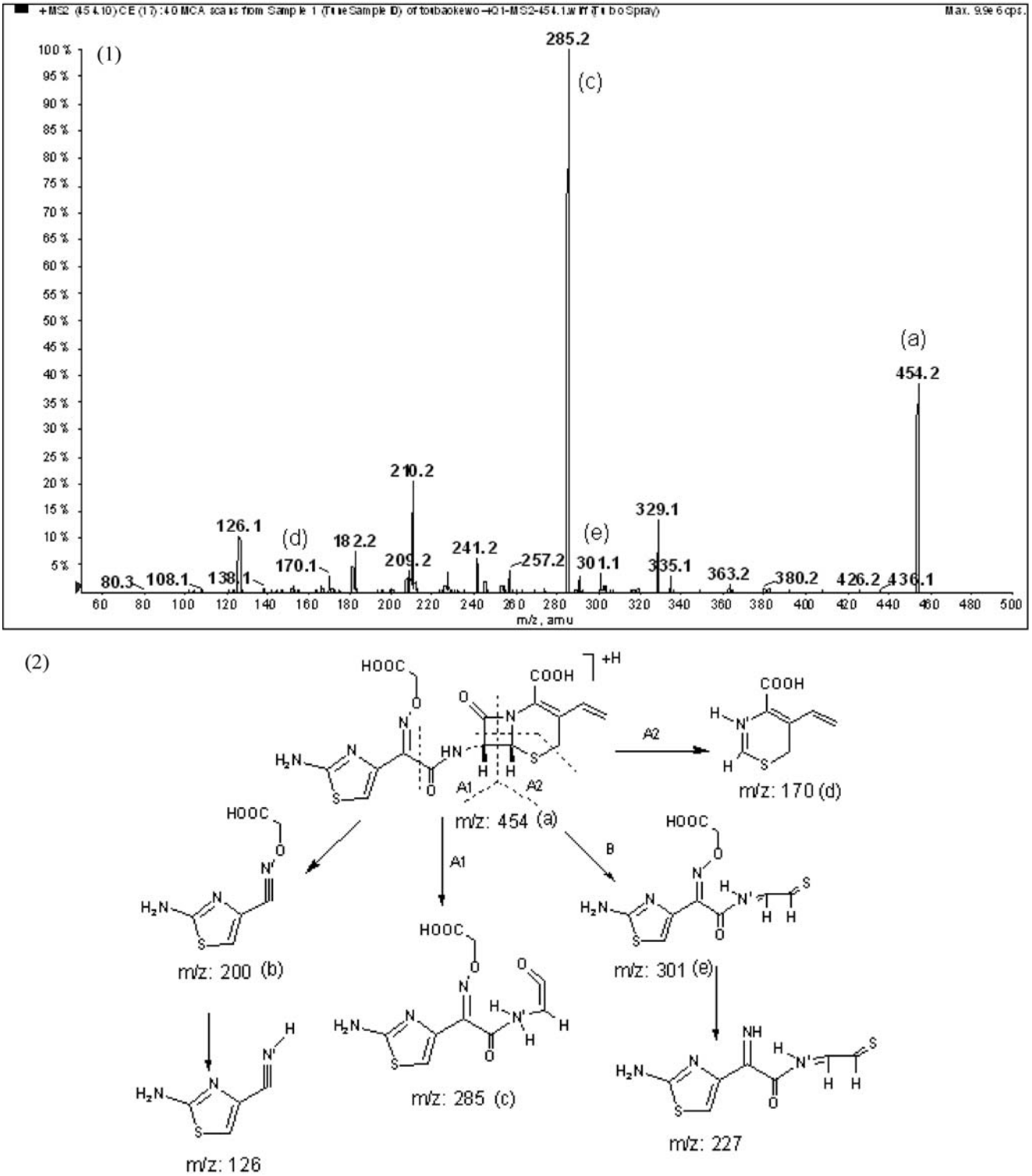

Figure 3. (1) The product ion (MS/MS) spectrum of the $[M+H]^+$ ion of cefixime (IV). (2) The main fragmentation pathways of cefixime (IV).

VII–IX; then due to the electron-withdrawing induction effects from the carbonyl in the 8-position, the nitrogen atom with a lower ionization energy in the 1-position is more liable to be the charge site than the methylene group in the 3-position. Therefore, a new double bond outside the ring is formed and the double bond between C2−C3 is shifted to N1−C2, forming a long-chain conjugated structure that includes the above two double bonds and the carbonyl in the 8-position. As a result, the distribution of electron density in the substrate is changed and the chemical environments for [2,4]-trans-Diels-Alder cleavage are undermined, which prohibits the occurrence of pathway B. The proposed distribution of local electron density of the $[M\text{-}R_3]^+$ ion is illustrated in Fig. 7(a). For compounds I–VI, the distribution of local electron density in the substrate is unchanged since there is no cleavage in the 3-position; hence, fragmentation

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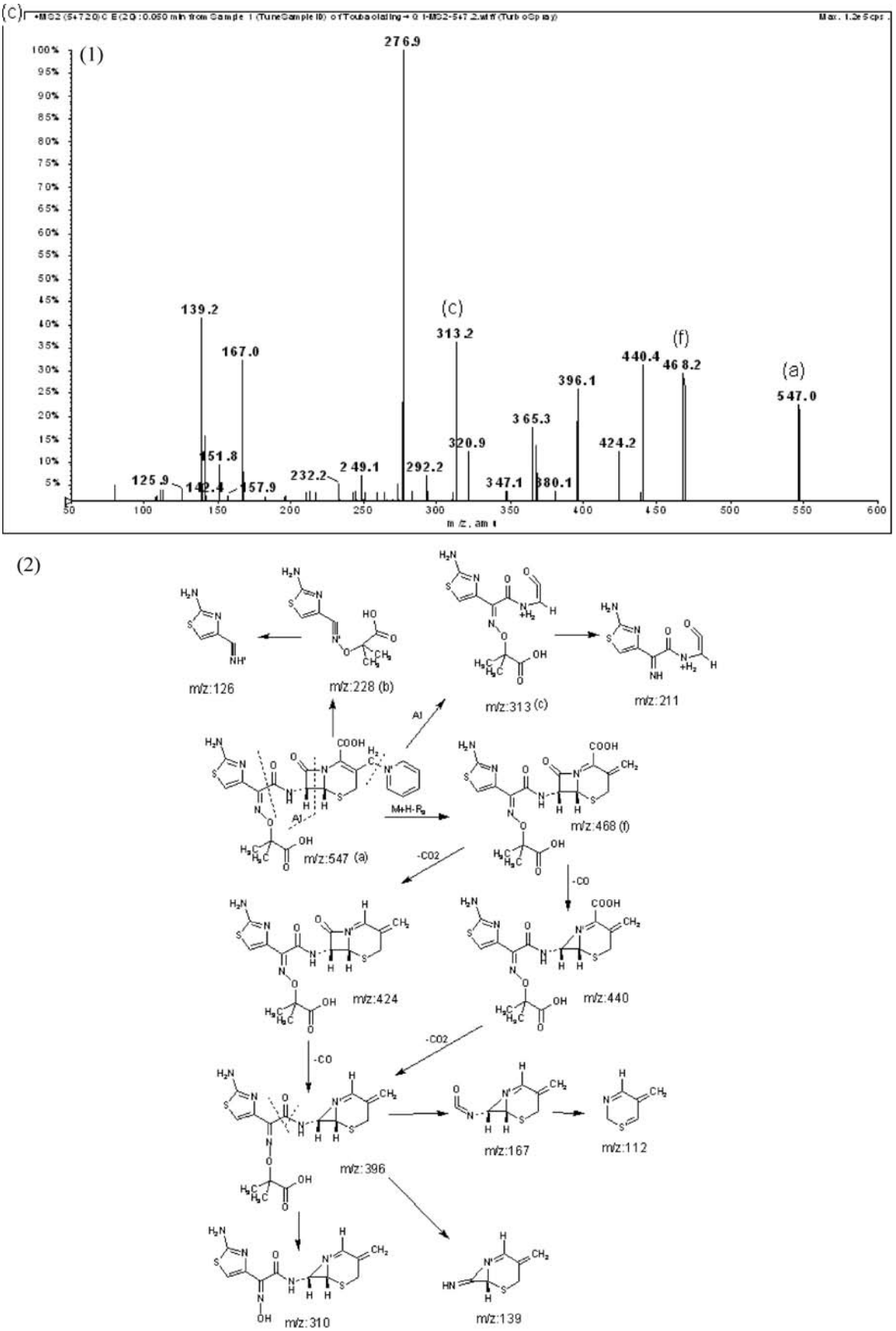

Figure 4. (1) The product ion (MS/MS) spectra of the $[M+H]^+$ ion of ceftazidime (VII). (2) The main fragmentation pathways of ceftazidime (VII).

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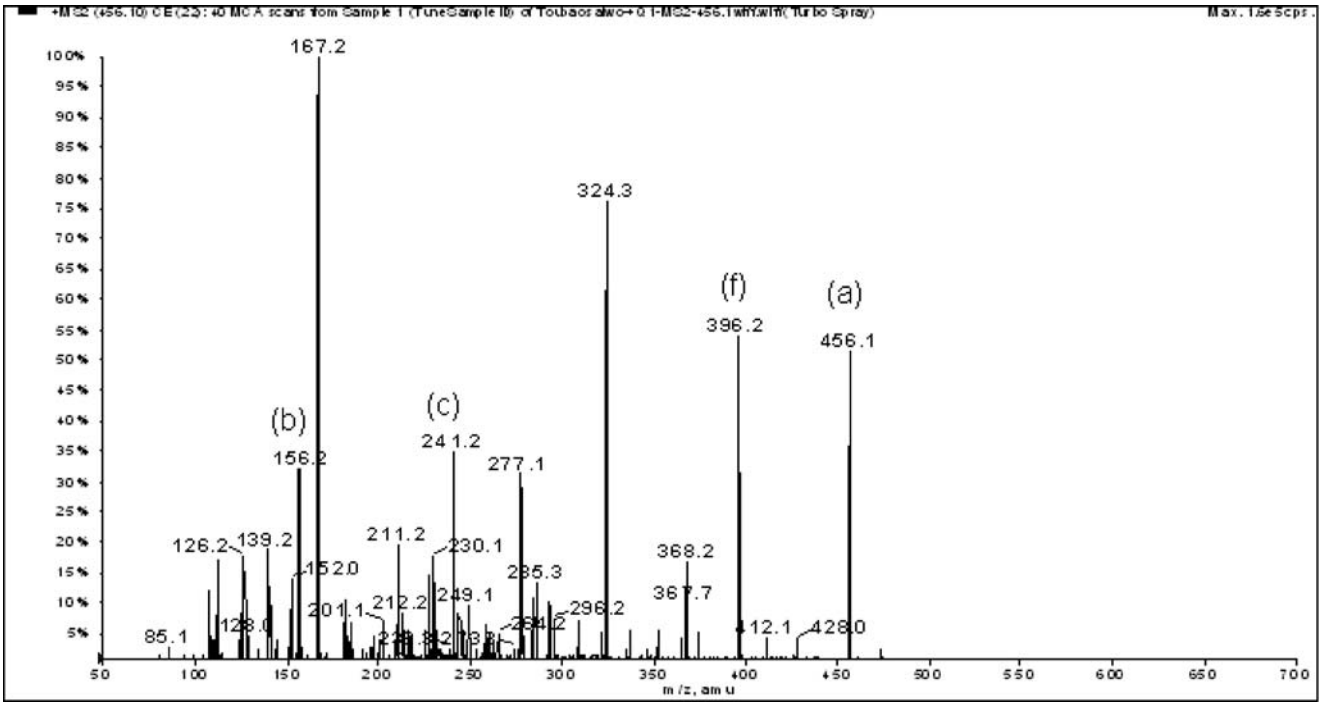

Figure 5. The product ion (MS/MS) spectrum of the $[M+H]^+$ ion of cefotaxime (VIII).

pathway B is able to occur. The proposed distribution of local electron density of the $[M+H]^+$ ion of compound I is illustrated in Fig. 7(b).

Although fragmentation pathway B can occur in compounds I–VI, the intensity of fragment ion e produced by pathway B varies and may also be influenced by the substituent in the 3-position. The effect of the substituent was evaluated by studying the intensity ratio of fragment ions e and d, which are produced by pathways B and A, respectively. This parameter was chosen for the evaluation because pathways A and B are competitive in mass analysis.

As shown in Table 3, the order of intensity ratios of fragment ions e/d from compounds I–VI was VI > III > IV > V > I > II.

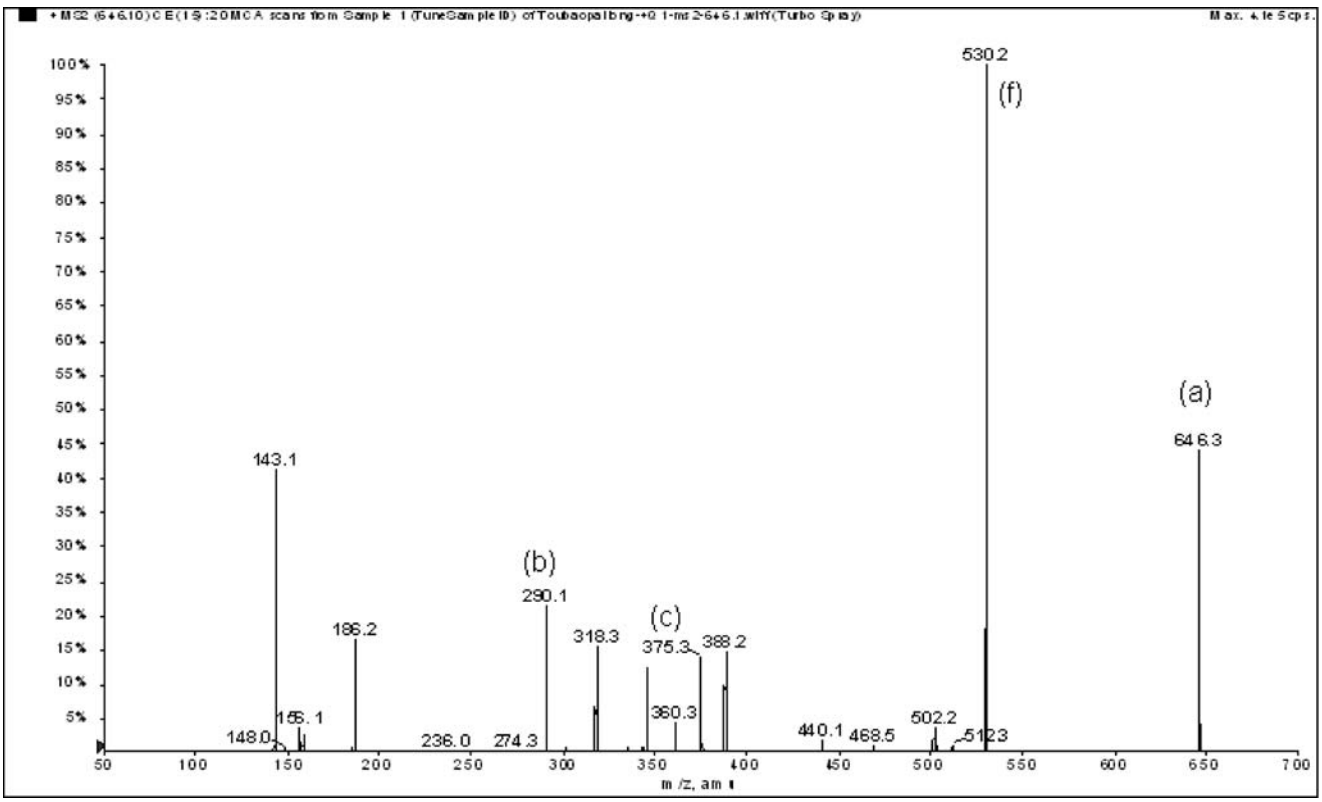

Figure 6. The product ion (MS/MS) spectrum of the $[M+H]^+$ ion of cefoperazone (IX).

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Table 3. The intensity ratio of ion intensity (e/d) of compounds I–VI

	Compound No.					
	I	II	III	IV	V	VI
Group X	methyl	methyl	chloride	vinyl	vinyl	propenyl
Intensity ratio (e/d)	0.20	0.10	2.02	1.06	0.60	3.35

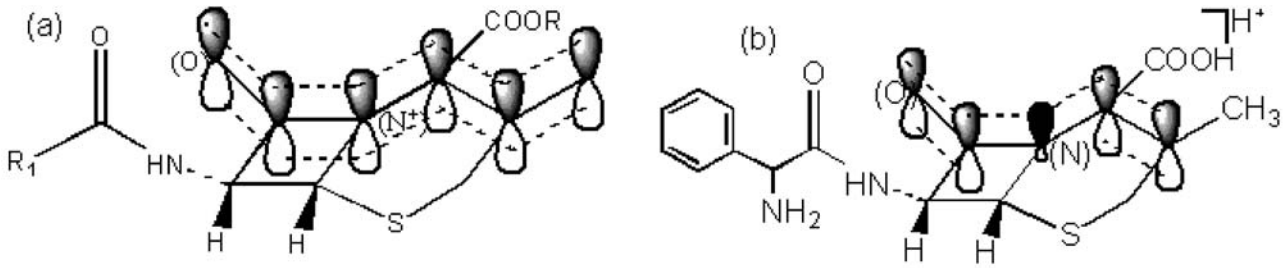

Figure 7. Distribution of local electron density of the $[M–R_3]^+$ ion (a) and the $[M+H]^+$ ion of compound I (b).

The electronegativity of the substituent group X was found to be a key factor influencing the fragmentation of pathway B, because the intensity ratio (e/d) was stronger when X was a chlorine atom, a vinyl group, or a propenyl group as compared to the ratio when X was a methyl group.

CONCLUSIONS

The effect of substituent groups in the 3-position of various cephalosporin analogues on fragmentation pathway B was investigated by Q-Trap MS^n analysis. The results suggest that the $[M–R_3]^+$ ion was a key influence on fragmentation pathway B. As described by the electronegativity equalization theory, the production of the $[M–R_3]^+$ ion was facilitated due to the strong electronegativity of heteroatoms (O, N, S) when compared to other atoms that are less electronegative. According to the ionization potential theory, the positive charge of the $[M–R_3]^+$ ion was located on the nitrogen atom in the 1-position, which changed the distribution of the electron density and prohibited the [2,4]-reverse-Diels-Alder cleavage. As a result, pathway B could not occur. Furthermore, it was found that the fragmentation intensity of pathway B was influenced by the electronegativity of the substituent in the 3-position, which was demonstrated by the stronger intensity ratio of fragments e/d when the substituent was a chlorine atom, a vinyl, or a propenyl group when compared to a methyl substituent. This study provided some theoretical basis for the identification of cephalosporin antibiotics and structural analysis of related substances in drugs.

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