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Short communication

# Characterization of a novel impurity in bulk drug eprosartan by ESI/MS$^n$ and NMR

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ABSTRACT

A simple and sensitive liquid chromatography tandem multi-stage mass spectrometry (HPLC/MS$^n$) method suitable for eprosartan analysis was developed, by which an unknown impurity in bulk drug eprosartan was detected. The fragmentation behavior of eprosartan and the impurity in negative mode was investigated. Two molecules of $CO_2$ lost from eprosartan precursor ion were observed, while four molecules of $CO_2$ were extruded from the deprotonated molecular ion to the MS$^3$ product ions of the impurity. Furthermore, a characteristic fragmentation ion at $m/z$ 335 was observed in both eprosartan and the impurity indicated that the impurity might have two eprosartan units. The unknown impurity was initially proposed to be eprosartan dimer connected via methylene unit at the thiophene moiety on the basis of the multi-stage mass spectrometric and exact mass evidences, and it was finally elucidated as 4,4′-(5,5′-(1E,1′E)-3,3′-(4,4′-methylenebis(thiophene-4,2-diyl))bis(2-carboxyprop-1-ene-3,1-diyl)bis(2-butyl-1H-imidazole-5,1-diyl))bis(methylene) dibenzoic acid by NMR experiments including 1D ($^1$H NMR, $^{13}$C NMR, DEPT135$^0$) and 2D ($^1$H-$^1$HCOSY, HMQC and HMBC) data.



## 1. Introduction

Eprosartan (4-[2-butyl-5-(2-carboxy-3-thiophen-2-yl-propenyl)-imidazol-1-ylmethyl]-benzoic acid) is a new antihypertensive agent as an angiotensin II receptor antagonist that is highly selective for the typeI (AT1) receptor and that is able to elicit a higher reduction in systolic blood pressure (SBP) than other antihypertensive drugs [1–3]. Eprosartan has been authorized to be used in treatment of hyperpiesia, congestive heart failure and renal failure [4], and can be used to treat hypertension disease effectively with good tolerance due to small hazard of serious ill reactions and serious pharmic reactions, and it also has curative effect on secondary preventing cardiovascular and cerebrovascular diseases [5].

A series of separation and detection techniques have been applied to the analysis of eprosartan, such as high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) [6] or mass spectrometric method [7], and solid phase extraction-high-performance liquid chromatographic method [8,9]. Most of these methods were used to monitor eprosartan concentration in biological fluids. Only a few methods have been described for impurity testing of eprosartan [10], while no suitable method for the separation of eprosartan and related substances, compatible with online HPLC/MS detection, has been presented.

The impurity profile of a drug substance is critical to its safety assessment and its manufacturing process [11]. As the International Conference on Harmonization (ICH) guidelines referred: it is mandatory to know the structural details of impurities that exceed 0.1% in the bulk drugs [12]. Since the impurities are usually process-related compounds, they are most probably structurally similar to the synthesized target drugs. Liquid chromatography in combination with multi-stage mass spectrometry (HPLC/MS$^n$) is useful for characterizing impurity structure due to its capability to afford both of molecular mass and structural information [13,14]. In this paper, we develop an MS-compatible HPLC method suitable for the separation of eprosartan and its related substances, and elucidate trace-level impurity on the basis of multi-stage mass spectrometry and Fourier transform ion cyclotron resonance ESI mass spectrometry (FTICR/ESI/MS). To ascertain the structure of the unknown impurity clearly, 1D and 2D NMR techniques were carried out after preparative isolation.

## 2. Experimental

### 2.1. Materials

The sample of eprosartan was obtained from JianYuan Inc. (Hangzhou, China). Analytical grade triethylamine, phosphoric acid, and formic acid were purchased from Beijing Xudong Chemical Co. (Beijing, China). HPLC-grade acetonitrile was obtained from Merck Co. (Darmstadt, Germany) and water was purified by a Milli-Q purification system (Millipore, Bedford, MA, USA).

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### 2.2. High-performance liquid chromatography (HPLC)

The analyses were performed on an Agilent 1100 series LC system with UV detector (Agilent Technologies, Palo Alto, CA, USA). Hypersil ODS2 column (4.6 mm × 250 mm, 5 μm) was used for the separation. The column temperature was maintained at 30 °C with the detection wavelength of 234 nm. Mobile phases consisted of a mixture of triethylamine buffer adjusted to pH 3.0 with phosphoric acid (A) and acetonitrile (B). The gradient with a flow rate of 0.8 mL $min^{-1}$ started at 30% B for 4 min, followed by a linear increase from 30% to 50% B in 13 min, then maintained 50% of B for 10 min. The bulk drug sample was prepared in acetonitrile and buffer solution (50:50) at 0.5 mg $mL^{-1}$, and 20 μL of sample was injected for analysis.

### 2.3. HPLC/MS and FTICR/ESI/MS analysis

An Agilent 1100 series HPLC system was coupled to a Bruker Esquire 3000$^{plus}$ ion trap mass spectrometer (Bruker-Franzen Analytik GmbH, Bremen, Germany) with an ESI source. A Hypersil ODS2 column was used as above. The mobile phase consisted of a mixture of 30 mM triethylamine buffer adjusted to pH 3.0 with formic acid (A) and acetonitrile (B) (55:45, v/v) at a flow rate of 0.8 mL $min^{-1}$.

The samples were infused into the mass spectrometer from the HPLC system though a T-junction with a splitting ratio of 2:1. The ion source temperature was set at 250 °C and the ESI needle voltage was set at 4.0 kV. Nitrogen was used as the drying gas at a flow rate of 8 L $min^{-1}$ and as the nebulizer gas at a pressure of 25 psi. Helium was introduced into the ion trap with an estimated pressure of $6 \times 10^{-6}$ mbar to improve trapping efficiency, and acted as the collision gas for $MS^n$ experiments. To maximize the ion current, the mass spectrometer was optimized within the collision energy range of 0.5–1.05 V. The isolation width of precursor ions was 2.0 mass units.

The accurate mass spectrometric experiments were performed on an Apex III (7.0 Tesla) Fourier transformation ion cyclotron resonance (FTICRMS) mass spectrometer equipped with electrospray ionization source (ESI) (Bruker, Billerica, MA, USA). XMASS software version 6.1.1 was used for instrument control, data acquisition and processing. The spray voltage was 4.5 kV. The temperature of the capillary was 250 °C. Nebulizing gas and drying gas ($N_2$) were set 35 psi and 30 units, respectively. Product ions were generated in the collision cell and argon was used as the collision gas. Loop injections were made into a mobile phase stream consisting of $H_2O/CH_3CN$ (50:50, v/v) at a flow rate of 0.18 mL $h^{-1}$.

### 2.4. Semi-preparative HPLC

The impurity was prepared on a Varian Prostar semi-preparative high-performance liquid chromatography (Varian Co., USA). A reversed-phase Shim-pack PRC-ODS column (20 mm × 250 mm, 10 μm) was used for the preparation, and a mixture of 30 mM triethylamine buffer adjusted to pH 3.0 with formic acid and acetonitrile (62:38, v/v) as eluate. The collected fractions were evaporated to dryness for further offline MS and NMR experiments.

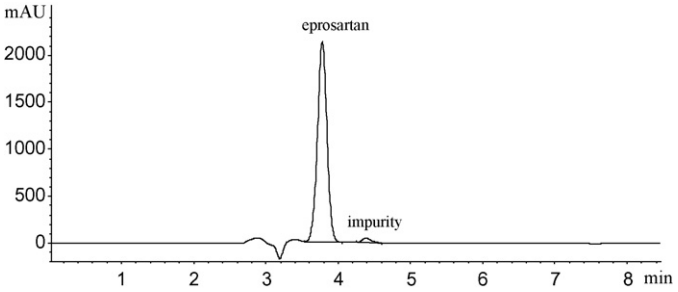



**Fig. 1.** HPLC chromatogram of the bulk eprosartan.

### 2.5. Nuclear magnetic resonance (NMR) spectrometer

NMR spectra including 1D ($^1$H NMR, $^{13}$C NMR, DEPT135$^0$), 2D ($^1$H-$^1$HCOSY, HMQC and HMBC) were recorded with a Bruker Advance DMX 500 instrument with a QNP probe head at ambient temperature, using DMSO as solvent. The data were acquired on Silicon Graphics O2 workstations using XWINNMR version 2.1 (Bruker Analytik, GmbH, Germany).

## 3. Results and discussion

During the routine impurity profiling of the bulk eprosartan through HPLC/UV analysis, an unknown impurity with a level of about 1.5% was detected. Since the mobile phase employed consisted of nonvolatile phosphoric acid, and was not able to be injected into LC/MS directly. A suitable method for LC/MS analysis was investigated. The LC/$MS^n$ analysis using the modified method described above was performed and the unknown impurity was detected with molecular mass of 860 both by positive and negative ion mass spectrum (Fig. 1).

### 3.1. Study of the fragmentation behavior of eprosartan

Since the impurity probably shared some common structural moiety with eprosartan. A profound study of the fragmentation pattern of the parent drug was important for elucidating impurity structure by comparison of their fragmentation pathways and neutral loss.

The product ion spectra of deprotonated eprosartan (Fig. 2) indicated that the precursor ion lost two molecules of $CO_2$ continuously, and yielded product ions at $m/z$ 379 and at $m/z$ 335, respectively. The ion at $m/z$ 281 resulted from the loss of 2-methylthiophene and the ions at $m/z$ 237 corresponded to the subsequent loss of $CO_2$ from the product ion of $m/z$ 379. The proposed fragmentation mechanism for eprosartan was described in Scheme 1, which was supported by the accurate mass obtained on FTICR/MS/MS (Table 1).

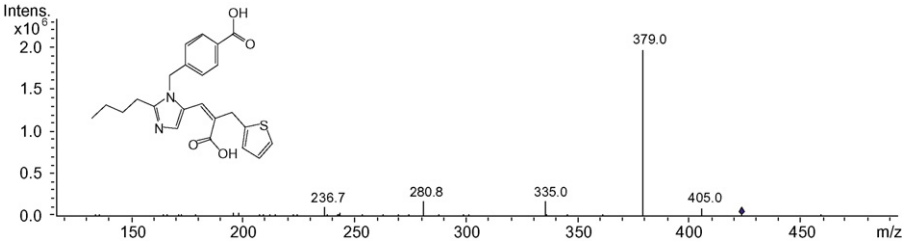
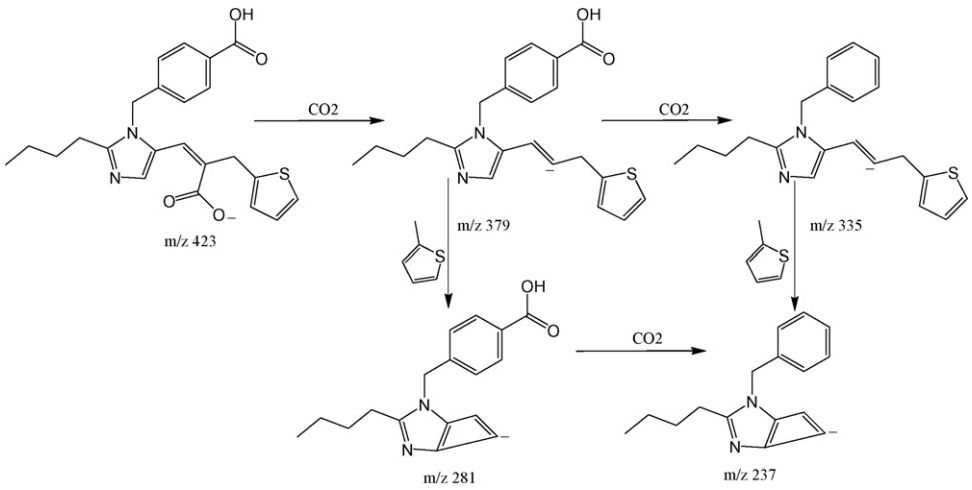



**Fig. 2.** MS/MS spectrum of deprotonated eprosartan.




**Scheme 1.** Proposed fragmentation pathway of eprosartan in negative mode.

**Table 1**
Summary of accurate mass measurements for the precursor and product ions of eprosartan (*m*/*z* 423) and impurity (*m*/*z* 859).

| Precursor ions | Product ions | Accurate mass | Exact mass | Formula | Error (ppm) |
|---|---|---|---|---|---|
| 423 | – | 423.1383 | 423.1384 | $C_{23}H_{23}N_2O_4S^-$ | −0.24 |
| | 379 | 379.1493 | 379.1486 | $C_{22}H_{23}N_2O_2S^-$ | 1.84 |
| | 335 | 335.1588 | 335.1587 | $C_{21}H_{23}N_2S^-$ | 0.29 |
| | 281 | 281.1296 | 281.1296 | $C_{17}H_{17}N_2O_2^-$ | 0 |
| | 237 | 237.1394 | 237.1397 | $C_{16}H_{17}N_2^-$ | −1.26 |
| 859 | – | 859.2481 | 859.2481 | $C_{47}H_{47}N_4O_8S_2^-$ | 0 |
| | 841 | 841.2742 | 841.2735 | $C_{47}H_{45}N_4O_7S_2^-$ | 0.83 |
| | 815 | 815.2943 | 815.2943 | $C_{46}H_{47}N_4O_6S_2^-$ | 0 |
| | 797 | 797.2827 | 797.2837 | $C_{46}H_{45}N_4O_5S_2^-$ | −1.25 |
| | 771 | 771.3025 | 771.3044 | $C_{45}H_{47}N_4O_4S_2^-$ | −2.46 |
| | 727 | 727.3146 | 727.3146 | $C_{44}H_{47}N_4O_2S_2^-$ | 0 |
| | 335 | 335.1577 | 335.1587 | $C_{21}H_{23}N_2S^-$ | −2.98 |

### 3.2. Study of the unknown impurity by HPLC/$MS^n$ and FTICR/MS/MS

Analysis of the product ion spectra of deprotonated impurity (Fig. 3a) clearly indicated that the precursor ion proceeded through two fragmentation routes. One route involved loss of $CO_2$ and yielded the product ion at *m*/*z* 815, then lost another molecule of $CO_2$ to yield the product ion at *m*/*z* 771. The $MS^3$ experiment of the ion at *m*/*z* 771 derived from the precursor ion was carried out. The dissociation spectra (Fig. 3b) indicated that the ion at *m*/*z* 771 gave rise to major product ions at *m*/*z* 727 and *m*/*z* 683, corresponding to two neutral losses of $CO_2$. A characteristic ion at *m*/*z* 335 was also observed, which was proposed to be the same as that of eprosartan. While the second route involved loss of a molecule of $H_2O$ first to produce the ion at *m*/*z* 841 and then lost a molecule of $CO_2$ to yield the product ion at *m*/*z* 797. The ions at *m*/*z* 753 and 709 suggested the consequent neutral losses of $CO_2$ in the $MS^3$ spectra of ion *m*/*z* 797 (Fig. 3c). Therefore, four molecules of $CO_2$ extruded from the precursor ion could be observed.

Because the impurity was generated as a byproduct when the bulk compound was synthesized, the impurity might share some common structural moieties with the synthesized target drugs [15]. Upon collision activation of the molecular ion, neutral loss of $CO_2$ is often observed in drugs with carbonyl acid unit [16]. The above analysis suggested that the structure of the impurity probably contained four carboxylic acid units. Therefore, we proposed that the impurity might possess two eprosartan-like structures. To further confirm the impurity structure, we studied the accurate mass by FTICR/ESI/MS (Table 1). The molecular formula $C_{47}H_{48}N_4O_8S_2$ for the impurity was confirmed based on the accurate mass of the precursor ion both in positive and negative modes. To confirm the fragment routes above, we further studied the accurate mass of these fragment ions using FTICR/MS/MS (Table 1), and all the mass values of fragment ions were less than ±3 ppm from the exact mass. The ion at *m*/*z* 335.1577 corresponding to the same ion in Scheme 1 also gave evidence here of the presence of eprosartan unit. Therefore, the structure of the impurity was proposed to be eprosartan dimer connected via methylene unit.

Several kinds of connection modes were proposed, as shown in Fig. 4. It was noted that 2-methylthiophene was easily lost from the dissociation of eprosartan precursor ion. However, the same fragment route was not observed at that of the impurity, which suggested the methylene might connect with thiophene. Furthermore, the fragments ion at *m*/*z* 547 was probably formed due to the loss of 1H-cyclopropabenzene from $MS^3$ experiment of ion *m*/*z* 771, while structure B could be excluded. For structure C, the space hindrance would be much bigger than the other structures. Therefore, structure A was proposed as the rational structure of the impurity, and the proposed fragmentation mechanism was described in Scheme 2. Several kinds of connection mode also presented in the structure A. The correct connection site needs to be determined by NMR technique.

### 3.3. NMR analysis

Two R units were confirmed based on the double proton signals in the $^1H$ NMR (500 MHz, DMSO, $\delta_D$ 2.50) spectrum: two methyl groups $\delta_H$ 0.80 (t, 6H), ten methylene protons [$\delta_H$ 1.28 (m, 4H), $\delta_H$ 1.54 (m, 4H), $\delta_H$ 2.62 (t, 4H), $\delta_H$ 3.90 (s, 4H), $\delta_H$ 5.35 (s, 4H)], and sixteen aromatic protons [$\delta_H$ 6.58 (s, 2H), $\delta_H$ 6.67 (s, 2H), $\delta_H$

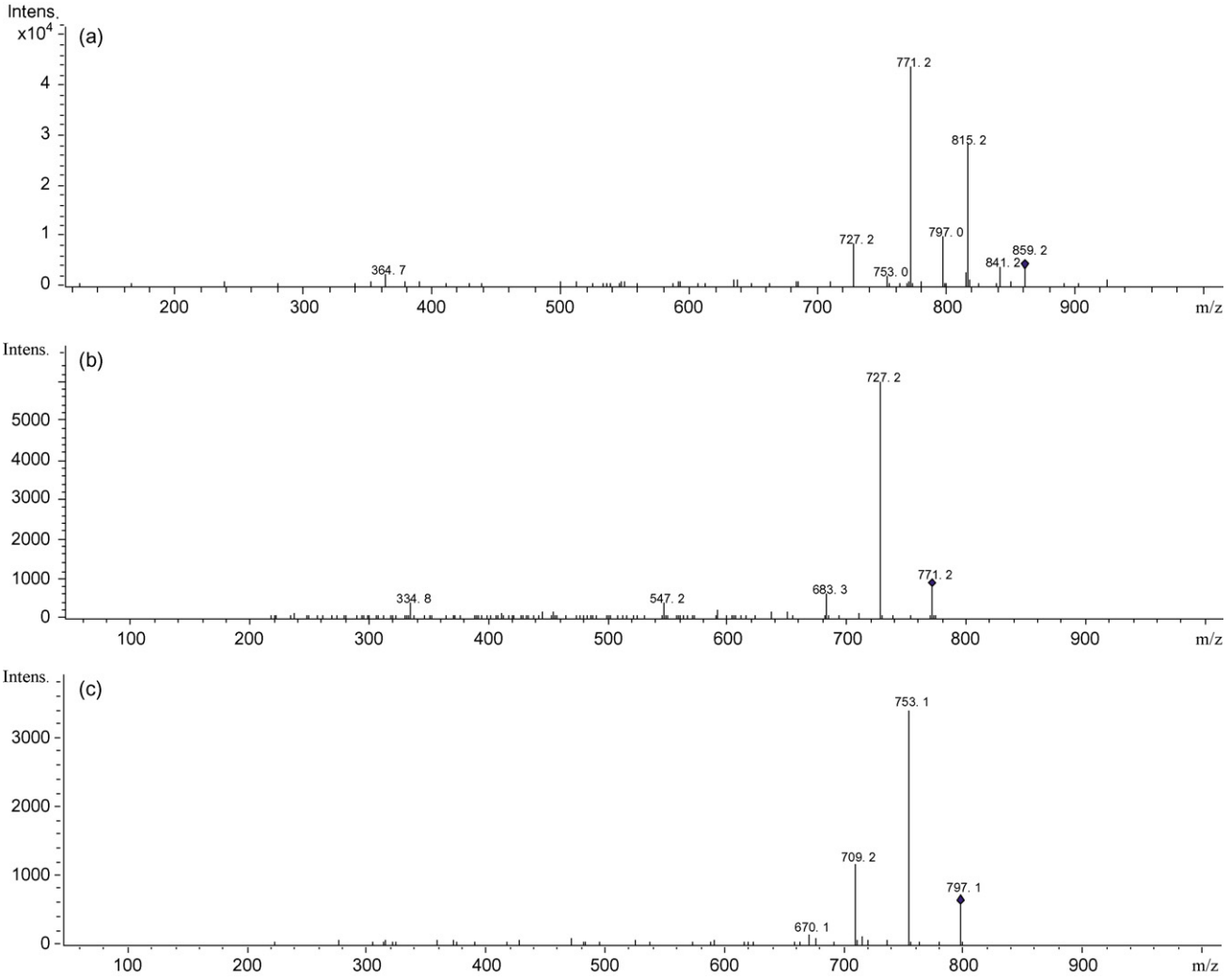



**Fig. 3.** ESI/MS$^n$ spectrum of impurity in negative mode: (a) MS$^2$ of *m*/*z* 859, (b) MS$^3$ of *m*/*z* 771, and (c) MS$^3$ of *m*/*z* 797.

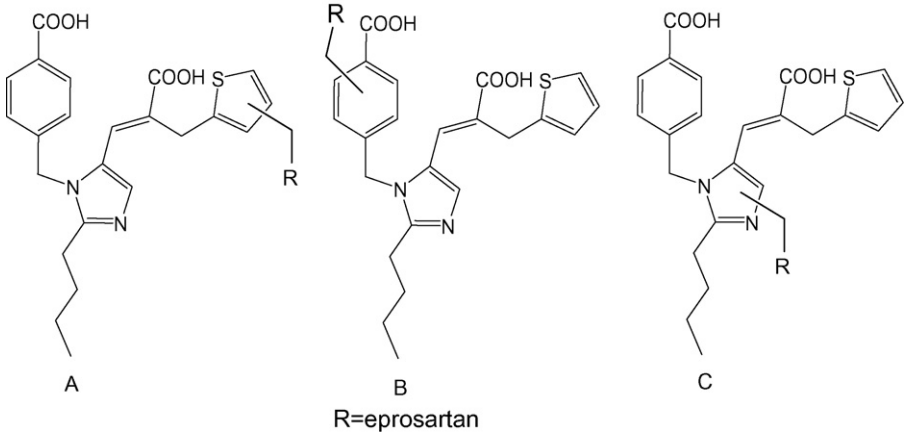
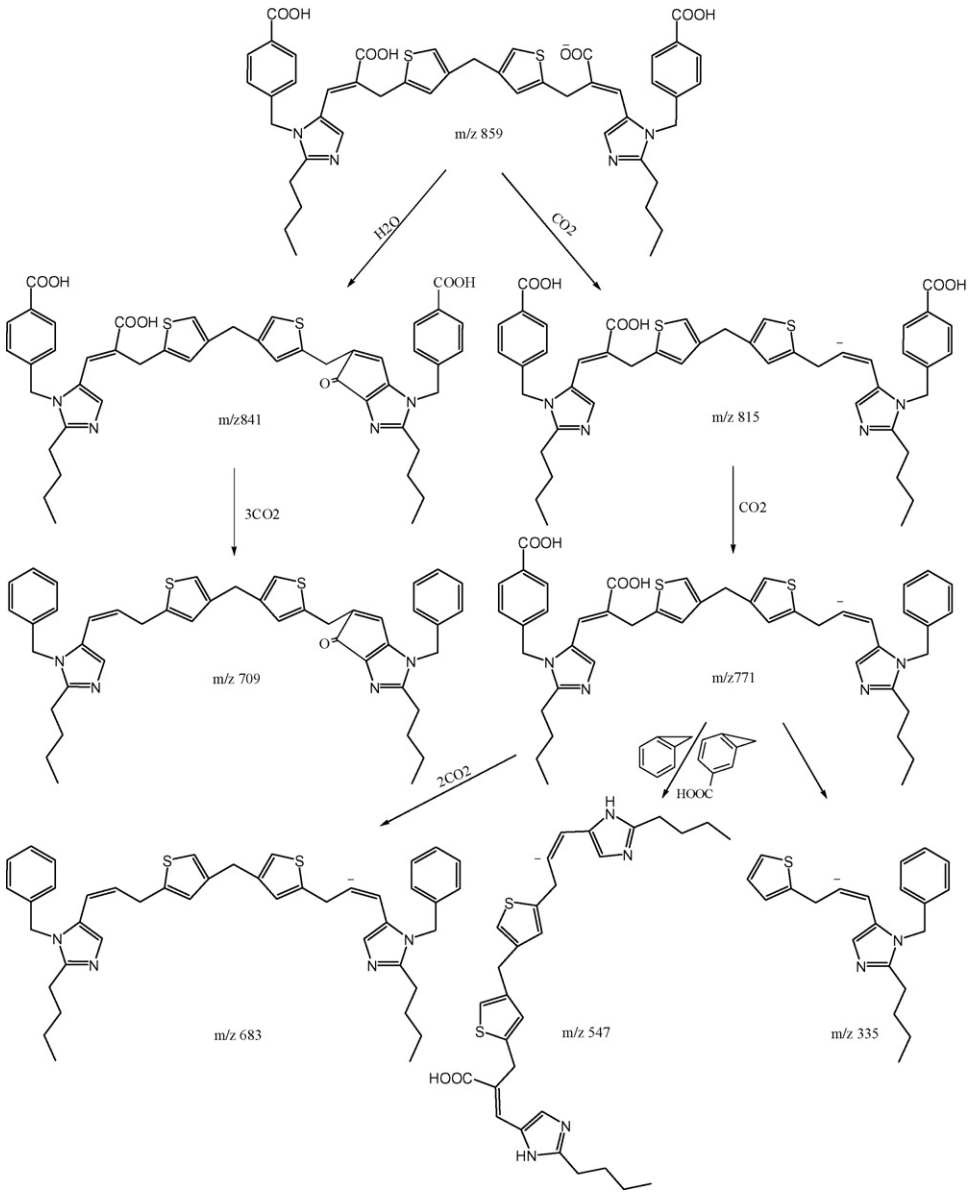



**Fig. 4.** The proposed structures of the impurity.

7.01 (d, 4H), $\delta_H$ 7.23 (s, 2H), $\delta_H$ 7.35 (s, 2H), $\delta_H$ 7.88 (d, 4H)]; as well as the double carbon signals of $^{13}$C NMR (125 MHz, DMSO, $\delta_C$ 40.5) and DEPT135° spectra: two primary carbons [$\delta_C$ 14.55], ten secondary carbons [$\delta_C$ 22.65 (2C), $\delta_C$ 27.03 (2C), $\delta_C$ 29.86 (2C), $\delta_C$ 30.16 (2C), $\delta_C$ 46.80 (2C)], sixteen tertiary carbons [$\delta_C$ 124.70 (2C), $\delta_C$ 124.93 (2C), $\delta_C$ 125.61 (2C), $\delta_C$ 126.58 (4C), $\delta_C$ 130.71 (4C), $\delta_C$ 131.45 (2C)], and eighteen quaternary carbons including four carbonyl carbon signals. One methylene proton signal at $\delta_H$ 4.16 (s, 2H) in combination with the carbon signal at $\delta_C$ 30.80 (1C) confirmed the presence of methylene unit. The single peak of aromatic protons at $\delta_H$ 6.58 and 6.67, which was assigned to the thiophene protons, proved the symmetric connection of 4-4′-2-methyl thiophene with methylene group. In addition, Heteronuclear multiple bond correlation (HMBC) for the methy-




**Scheme 2.** Proposed fragmentation mechanism of the impurity in negative mode.

lene proton signals at $\delta_H$ 4.16 with aromatic carbons at $\delta$ 125.61 and $\delta$ 142.04 which were assigned to thiophene carbons, was in agreement with the proposed structure. The 2D NMR spectra, including the analysis of $^1$H-$^1$HCOSY, as well as the heteronuclear multiple quantum correlation (HMQC) and HMBC spectra, allowed the assignment of all proton and carbon signals. Therefore, the impurity was finally elucidated as 4,4′-(5,5′-(1E,1′E)-3,3′-(4,4′-methylenebis(thiophene-4,2-diyl))bis(2-carboxyprop-1-ene-3,1-diyl)bis(2-butyl-1H-imidazole-5,1-diyl))bis(methylene) dibenzoic acid. To the best of our knowledge, this is a novel impurity.

## 4. Conclusion

A rapid and sensitive HPLC/MS$^n$ method was developed for the identification of an unknown impurity in the bulk drug eprosartan. In combination with high resolution mass spectrometric and the NMR evidences, the structure of the unknown impurity was unambiguously confirmed to be 4,4′-(5,5′-(1E,1′E)-3,3′-(4,4′-methylenebis(thiophene-4,2-diyl))bis(2-carboxyprop-1-ene-3,1-diyl)bis(2-butyl-1H-imidazole-5,1-diyl))bis(methylene)dibenzoic acid.

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