JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. 2002; **37**: 1258–1265
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jms.392

JMS

An unusual rearrangement of Zofenopril, a new ACE inhibitor drug: mass spectrometric and conformational studies†

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Received 31 May 2002; Accepted 25 September 2002

Zofenopril (1) is a new ACE inhibitor, used in therapy for hypertension and post-myocardial infarction. The protonated quasi-molecular ion (*m/z* 430) of 1, obtained under positive electrospray ionization conditions, loses a benzoic acid molecule (*m/z* 308), which in turn decomposes via loss of CO (*m/z* 280) when low-energy collisional-induced dissociation (CID) and in-source experiments are performed. This rearrangement is the main fragmentation process and can be observed both in-source and in the product ion tandem mass spectra, using either an ion trap or a triple quadrupole instrument.

Other known diastereoisomers of 1, an impurity with an acetyl in the place of the benzoyl group (2) and an impurity with two propanoyl chains in series (3), give the same rearrangement. On the other hand, the mass spectra of the methyl ester (4) and an impurity with two proline moieties (5) do not show this unusual fragmentation. Time-resolved CID spectra of 1 show that the rearrangement occurs after about 2 ms, a time scale comparable to those of the other non-rearrangement cleavages. These experiments suggest a conformation in the gas phase for 1 in which the benzoyl group is close to the hydroxyl of the carboxylic acid group, from which the rearrangement could readily occur. Since compounds 4 and 5 do not show the same behaviour, the presence of a carboxylic acid in the proline ring seems to play a crucial role in the rearrangement, probably due to an intramolecular hydrogen bond. To confirm this hypothesis, deuterium exchanges in mass spectrometric experiments and a conformational analysis via computational methods were performed.

KEYWORDS: ACE inhibitor; Zofenopril; electrospray ionization mass spectrometry; tandem mass spectrometry; rearrangement

INTRODUCTION

ACE (angiotensin-converting enzyme) inhibitors are drugs with different structures and activities used to treat heart failure and hypertension.[1] Zofenopril (**1**, Fig. 1) is an oral pro-drug, yielding via metabolism a free active sulphydryl compound, Zofenoprilat, which acts as an ACE inhibitor.[2]

An important issue in the analytical development of a drug substance is the structural characterization of drug impurities, degradation products and metabolites. It is well documented that in many cases this task is successfully performed using a combination of different mass spectrometric (MS) techniques, sometimes supported by NMR spectroscopy and computational methods.[3]

From a mass spectrometric point of view, a significant improvement in this field of pharmaceutical research was given by the introduction of the atmospheric pressure ionization (API) techniques, such as electrospray (ESI) and atmospheric pressure chemical ionization (APCI).[4–6] In fact, their main characteristics, i.e. sensitivity, capability of analyzing many classes of organic compounds and easy interfacing with high-performance liquid chromatography (HPLC) and capillary electrophoresis, render them particularly suitable for the detection of pharmaceutical compounds and related unknowns, even in trace amounts and in complex matrices. Since under normal operating conditions the API techniques only give the analytes' protonated (or deprotonated in negative ion mode) quasi-molecular ions, structural information is generally obtained using tandem MS techniques (MS/MS). Typically, fragment ions of the analytes are obtained via collision-induced decomposition (CID) with an inert gas, and analysed in a second stage of mass analysis. It is well known that the fragmentation processes involving simple cleavages are the most useful for structural elucidation purposes; however, rearrangement processes during mass analysis are described, which may be

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†Paper presented at the 20th Informal Meeting on Mass Spectrometry, Fiera di Primiero, Italy, 12–16 May 2002.

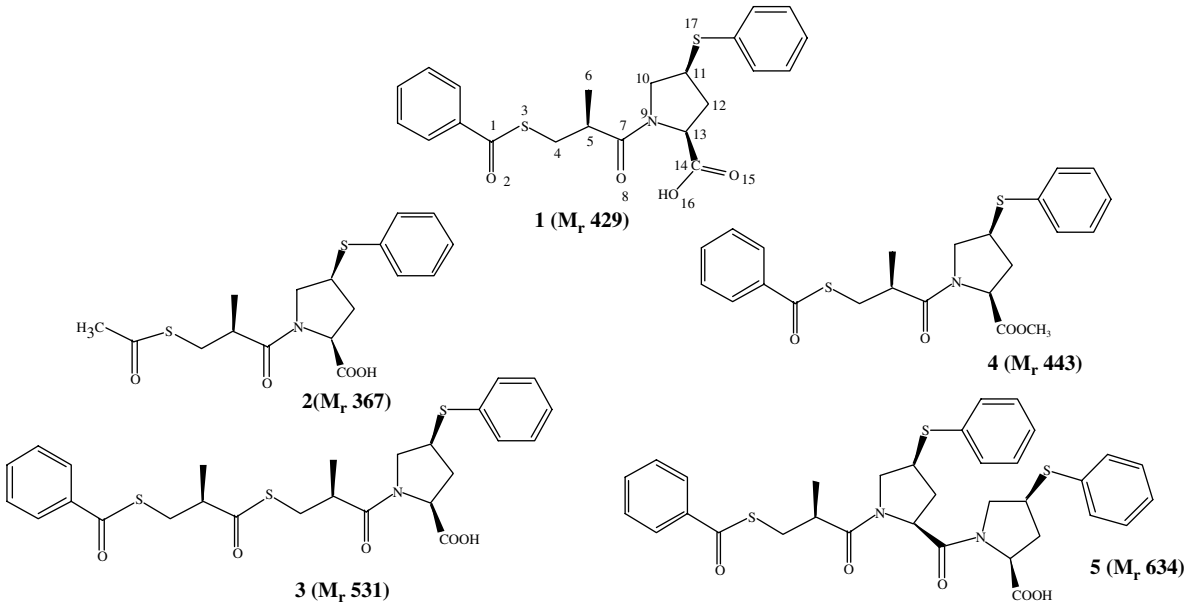


Figure 1. Structural formulae of compounds **1–5**.

misleading in determining the structure of an unknown, if not thoroughly understood. Some recent examples describe rearrangements involving P—N to P—O bond migration in *N*-diisopropyloxyphosphoryl amino acids,[7] internal imidazole elimination in a farnesyl transferase inhibitor,[8] formamide extrusion in aminoacylbenzylamines,[9] C-terminal to N-terminal migration in a modified octapeptide[10] and N to O acyl shifts in modified cyclosporins.[11]

In this paper, we report a detailed mass spectrometric analysis of protonated **1**, its diastereoisomers and related compounds (Fig. 1 and Table 1). Low-energy CID and in-source CID were carried out with ion trap and triple and single quadrupole mass spectrometers in the positive ion ESI mode. The study shows that **1** and all the molecules with a carboxylic acid at position 13 (Fig. 1) undergo an unusual gas-phase rearrangement in which the loss of benzoic acid (**1** and **3**) or acetic acid (**2**) is observed.

EXPERIMENTAL

Materials

Compound **1** was synthesized at Guidotti (Pisa, Italy). Compounds **2**, **3** and **5**, SQ 31904, SQ 32018, SQ 32156, SQ 31905, and SQ 32373 were kindly provided by Dr J. Bertini (Analytical Research Department of Menarini Manufacturing, Logistics and Services, Florence, Italy).

Compound **4** was prepared as follows: to a solution of the calcium salt of **1** (40 mg, 85 μmol) (Guidotti) in anhydrous CH_2Cl_2 (90 mL) (Aldrich) were added 10 mL of anhydrous CH_3OH (Fluka), 4-(dimethylamino)pyridine (3 mg, 8.2 μmol) and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (18 mg, 94 μmol) (Aldrich). After stirring for 24 h at room temperature, the reaction mixture was washed with water and the organic phase was dried on anhydrous Na_2SO_4. The crude material was purified by flash chromatography (IST bulk/Isolute sorbent, mean particle size 40–70 μm, average pore size 60 μm) using hexane–ethyl acetate (1 : 1) as the eluent to give an oil (purity >98%, HPLC, 254 nm). ESI-MS: $[\mathbf{4} + H]^+ = m/z$ 444. ^{1}H NMR spectrum at 500 MHz, in $CDCl_3$ (300 K). Chemical shifts (δ, ppm): 7.93, 2H, *ortho* S-Ph; 7.57, 1H, *para* S-Ph; 7.44, 2H, *meta* S-Ph; 7.26–7.37, 5H, benzoyl; 4.52, 1H, proline α; 4.08, 1H, proline δ; 3.73, 3H, OMe; 3.70, 1H, proline γ; 3.48, 1H, proline δ; 3.25–3.14, 2H, S-CH_2; 2.86, 1H, C*H*Me; 2.63, 1H, proline β; 1.99, 1H, proline β; 1.29, 3H, CH*Me*.

Mass spectrometry

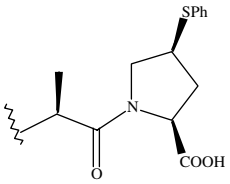
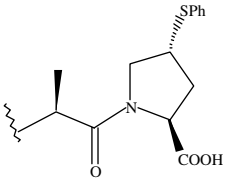
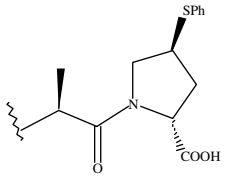
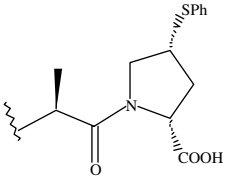
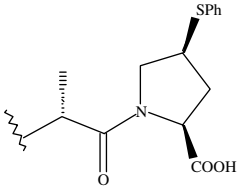
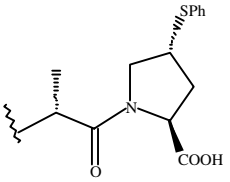
The samples, dissolved in acetonitrile–10 mM ammonium acetate in water (1 : 1), about 10 μg ml^{-1}, were introduced into the source in the infusion mode at a flow-rate of 7 μl min^{-1}. In the deuterium exchange experiments, the sample was dissolved in CD_3OD containing 10 mM deuterated ammonium acetate (CD_3COOND_4).

The experiments described were performed using an ion trap, a triple quadrupole and a single quadrupole mass spectrometer.

For ion trap mass measurements, we used a ThermoFinnigan (San Jose, CA, USA) LCQ instrument, equipped with standard ESI ion source, operated in the positive ion mode, with a nebulizer voltage 4.5 kV and a capillary temperature of 200 °C. Nitrogen was used as the nebulizing gas, with a sheath gas flow-rate of 70 (arbitrary units). MS/MS and MS^n product ion scans of the protonated molecular ions were performed at a collision energy of 1.5 V peak-to-peak of resonance excitation r.f. voltage, isolation width = 2–4 u, $Q_z = 0.20$, activation time 30 ms.

Triple quadrupole mass spectra were measured using a VG Quattro instrument (Micromass, Altrincham, UK) with a standard ESI ion source, operated in the positive ion mode, with a nebulizer voltage of 3.8 kV and a cone voltage of 20 V. The source temperature was 85 °C. Nitrogen was used as the nebulizer gas at a flow-rate of 10 l h^{-1} and as the desolvation gas at a flow-rate of 150 l h^{-1}. Tandem mass spectra were acquired at a rate of 400 u s^{-1} using argon as the collision gas, at a collision energy of 10–40 V, in the multi-channel mode.

Table 1. Partial structures, relative abundances and ratios of *m/z* 308 to 280 of **1** and the diastereoisomers studied

Compound	Partial formula	Relative abundance (%) *m/z* 308	*m/z* 280	*m/z* 308/280 ratio
1		36	100	0.36
SQ 31904		100	48	2.01
SQ 32018		100	50	2.00
SQ 32156		33	100	0.33
SQ 31905		33	100	0.33
SQ 32373		100	46	2.17

The single quadrupole mass spectrometer was a ZMD instrument (Micromass) with a standard ESI ion source, operated in either the positive or negative ion mode with a nebulizer voltage of 3.67 kV and a cone voltage of 10 V. The source temperature was 100 °C. Nitrogen was used as the nebulizer gas at a flow-rate of 98 l h^{-1} and as the desolvation gas at a flow-rate of 488 l h^{-1}. Spectra were acquired at a rate of 400 u s^{-1} in the multi-channel acquisition mode.

Computational methods

Computational studies were performed using molecular mechanics and *ab initio* methods as implemented in Sybyl 6.8 (Tripos St. Louis, MO, USA) and PC Spartan Pro 1.0.5 (Wavefunction, Irvine, CA, USA) respectively. A systematic conformational search for the exocyclic single bonds of **1** was carried out. The conformers were optimized by employing the MMFF94[12] force field with a dielectric constant of 1.0 and without non-bonded cutoff. The 45 structures obtained in a range of 4 kcal mol^{-1} (1 kcal = 4.184 kJ) above the minimum were optimized using HF/STO-3G and then HF/6–31G* methods and four of them were used to study the proton–molecule complex of Zofenopril. Nine different potential protonation sites were evaluated for each conformer in the regions of electrostatic potential minima. These were identified using the Surface module of PC Spartan Pro. The geometries of the complexes were then optimized with the HF/STO-3G method.

RESULTS AND DISCUSSION

CID mass spectrum of 1

Low-energy CID and in-source CID of $[\mathbf{1}+\mathrm{H}]^+$ (*m/z* 430) experiments were performed with ion trap and triple and single quadrupole spectrometers in the positive ESI mode. In all the spectra acquired, $[\mathbf{1}+\mathrm{H}]^+$ loses a benzoic acid molecule (*m/z* 308), which in turn decomposes via loss of CO (*m/z* 280). Figure 2(A) shows the product ion tandem mass spectrum of $[\mathbf{1}+\mathrm{H}]^+$ as obtained with the ion trap instrument.

Other fragmentation processes observed in the mass spectra involve the loss of formic acid (*m/z* 384) and simple cleavages leading to the substituted prolinium ion at *m/z* 224, the acylium ion at *m/z* 207 and the benzoyl ion at *m/z* 105.

Less abundant ions are observed at *m/z* 178 and 161. To clarify the origin of all the ions observed, MS^n experiments were performed. MS^3 of *m/z* 430–308 (Fig. 2(B)) confirms that the rearranged ion decomposes exclusively by loss of CO. MS^3 of *m/z* 430–280 (Fig. 2(C)) indicates that *m/z* 178 originates from *m/z* 280; *m/z* 161 arises from loss of ammonia from a rearranged form of *m/z* 178, as shown by MS^4 of *m/z* 430–280-178 (Fig. 2(D)). The *m/z* 178 ion also loses thiophenol to give protonated pyrrole at *m/z* 68. All these fragmentation processes are described in Fig. 3, in which the formal structures of the ions are indicated.

It is interesting that the loss of ammonia was not observed with the triple quadrupole instrument upon CID of mass-selected *m/z* 178, obtained in-source at a high declustering potential (cone voltage). Since the deposition energies involved in ion formation are of the same order of magnitude for both instruments, the loss of ammonia might be due to the longer activation time scale in an ion trap (10^2–10^{-3} s) compared with a triple quadrupole (10^{-4} s),[13] increasing the possibility of observing fragmentation processes implying extensive rearrangement of the ions,[14] such as loss of ammonia from the originally cyclic *m/z* 178 ion.

The proposed fragmentation in Fig. 3 is supported by deuterium-exchange experiments on **1** (data not shown). As expected from the occurrence of one exchangeable hydrogen in the structure of **1**, the mass of deuterated $[\mathbf{1}+\mathrm{D}]^+$ is shifted to *m/z* 432. CID of this ion gives fragments at *m/z* 384, 309 (+1), 281 (+1), 226 (+2), 207, 179 (+1), 178, 162 (+1), 161, 105 and 69 (+1), where the numbers in parentheses are the mass shifts compared with non-deuterated **1**. The

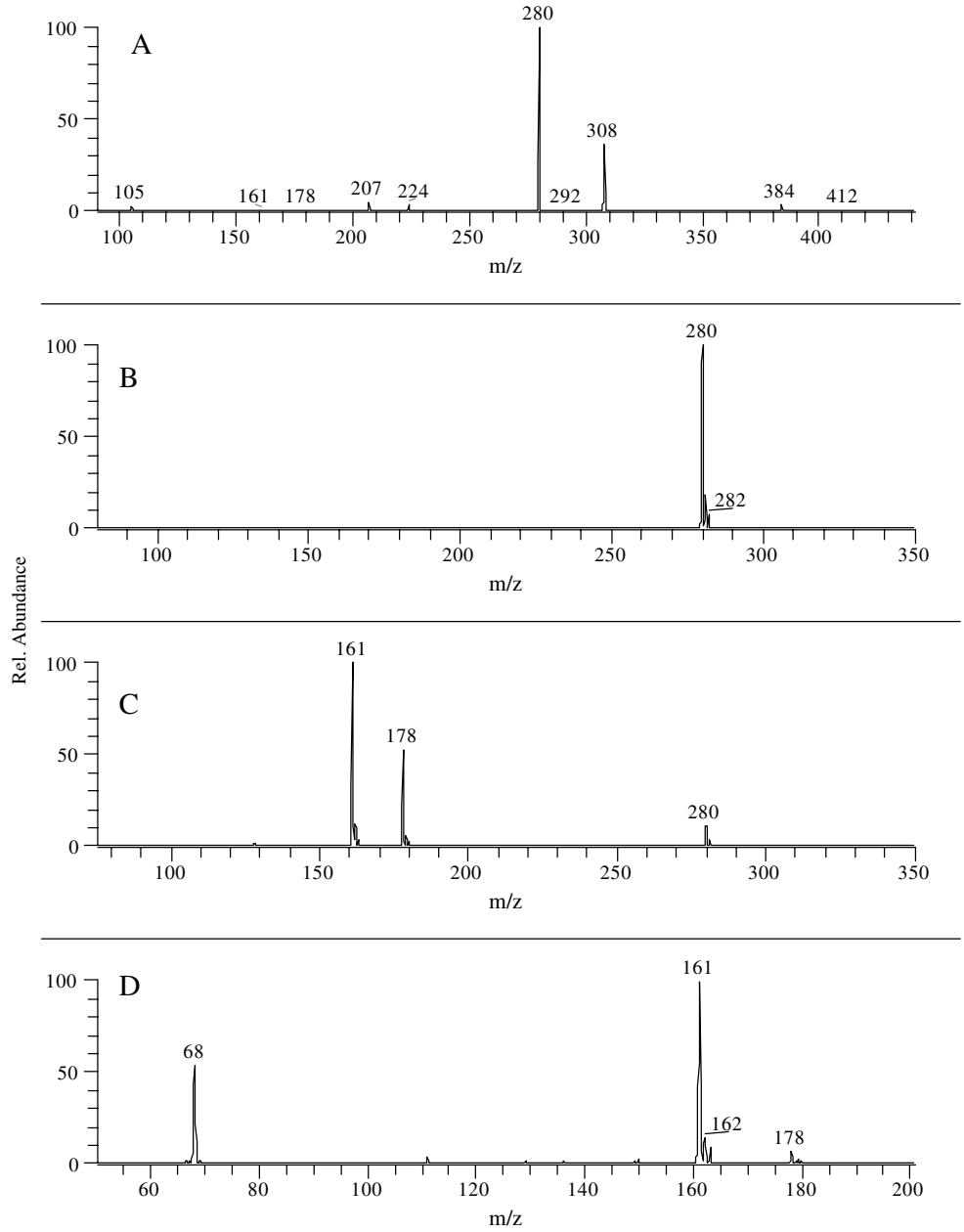


Figure 2. (A) Product ion MS/MS of $[\mathbf{1} + H]^+$ (*m/z* 430); (B) MS^3 of *m/z* 430 − 308; (C) MS^3 of *m/z* 430 − 280; (D) MS^4 of *m/z* 430 − 280 − 178.

occurrence of both deuterated and non-deuterated m/z 178 and 161 ions is noteworthy, suggesting multiple mechanisms for these fragmentation processes, or multiple sites for proton exchange.

Other experiments were carried out using the ion trap instrument: $[\mathbf{1} + H]^+$ was fragmented at increasing collision energies, keeping the excitation time at 30 ms. The relative abundances of the product ions vs the relative collision energies are plotted in Fig. 4. The ions deriving from benzoic acid loss (m/z 308 and 280) are the most abundant at all the energies investigated. In addition, all the relevant fragmentation processes appear at about the same energy (about 20% relative collision energy).

$[\mathbf{1} + H]^+$ was also fragmented at increasing excitation times with 30% relative collision energy. The corresponding plot is depicted in Fig. 5: the rearrangement is observed starting from 2 ms, a time-scale comparable to those of the other fragmentation processes. This short time-scale suggests that a significant population of the protonated quasi-molecular ions of **1** already exist in the gas phase in a favourable conformation for the rearrangement to occur.

To explain the unusual rearrangement in which the loss of benzoic acid is observed, a conformation in which the carbonyl group linked to sulphur is close to the hydroxyl of the carboxylic acid group can be proposed. It is plausible to think that this conformation is stabilized by an intramolecular hydrogen bond involving the exchangeable proton of the carboxylic group, as supported by the deuterium exchange experiments on **1**, in which the loss of PhCOOD is observed (m/z 309).

The hypothesis of a hydrogen bond can also be supported by the observation that this unusual rearrangement is not

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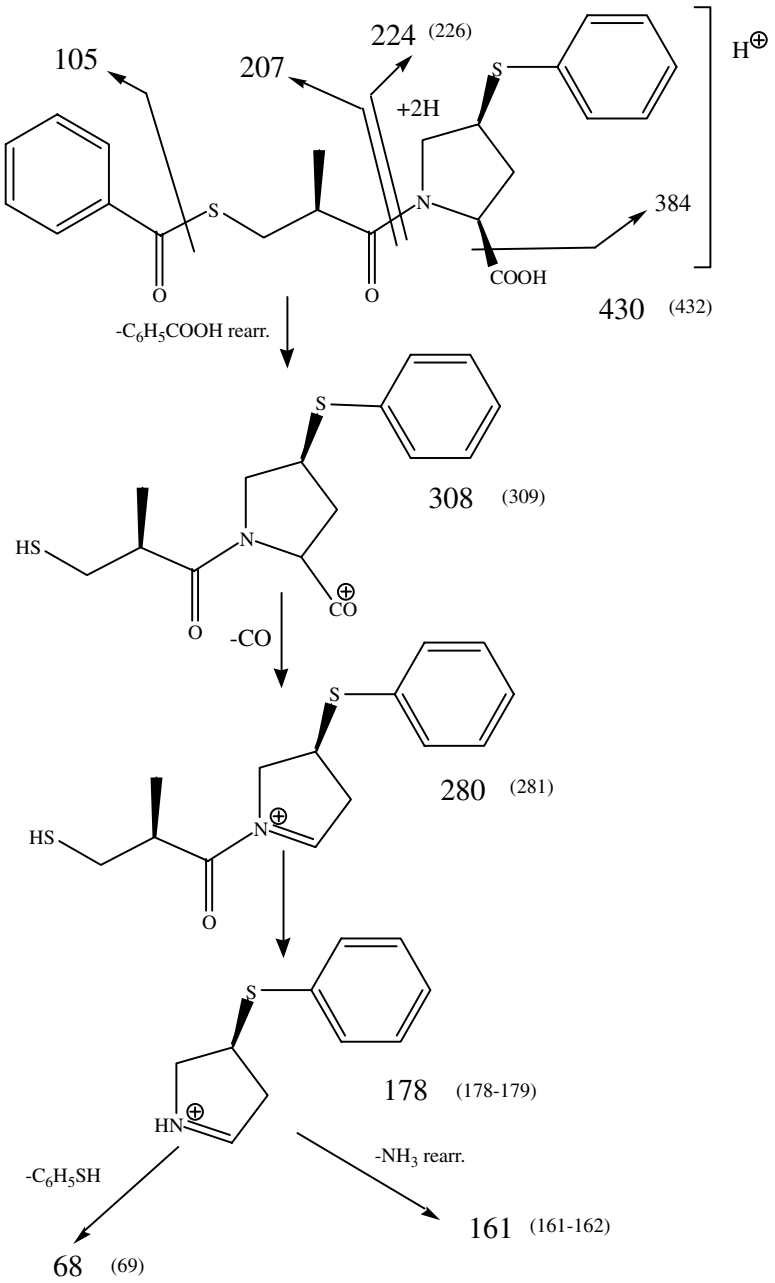

Figure 3. Proposed fragmentation scheme of $[\mathbf{1} + H]^+$. The numbers in parentheses indicate the *m/z* values after deuterium exchange.

observed in negative ESI of **1**, where obviously the carboxylic acid group is deprotonated. The negative ESI spectrum of **1** shows fragments at m/z 290 (loss of thiobenzoic acid), 137 (thiobenzoate anion) and 109 (data not shown).

CID mass spectra of related compounds

The five known diastereoisomers of **1**, namely SQ 31904, SQ 32018, SQ 32156, SQ 31905, and SQ 32373 (Table 1), also show the above-described benzoic acid loss under CID as the main fragmentation process. The main difference lies in the relative abundances of m/z 308 and 280, as shown in Table 1: diastereoisomers with a *cis* configuration on the proline ring have lower m/z 308/280 ratios than *trans* isomers (0.33–0.36 vs 2.00–2.17).

The different m/z 308/280 ratios obtained for *cis*- and *trans*-substituted proline could be explained in terms of different activation energies for the elimination of CO from the m/z 308 ions. The product ions (m/z 280) have a quasi-planar, non-hindered structure in both isomers. On the other hand, the starting *cis* m/z 308 ions are expected to have higher internal energy than the *trans*-substituted ions, owing to the steric hindrance of the two substituents on the proline ring. This stereochemistry could favour CO elimination in *cis* isomers.

The unusual rearrangement is also given by **2** and **3** (Fig. 1). Compound **2** carries an acetyl in the place of the benzoyl, and loses acetic acid, as shown in Fig. 6(A). Compound 3, carrying two mercaptomethylpropanoic chains in series, gives two such rearrangements, losing benzoylthiomethylpropanoic acid (m/z 308) and, to a lesser extent, benzoic

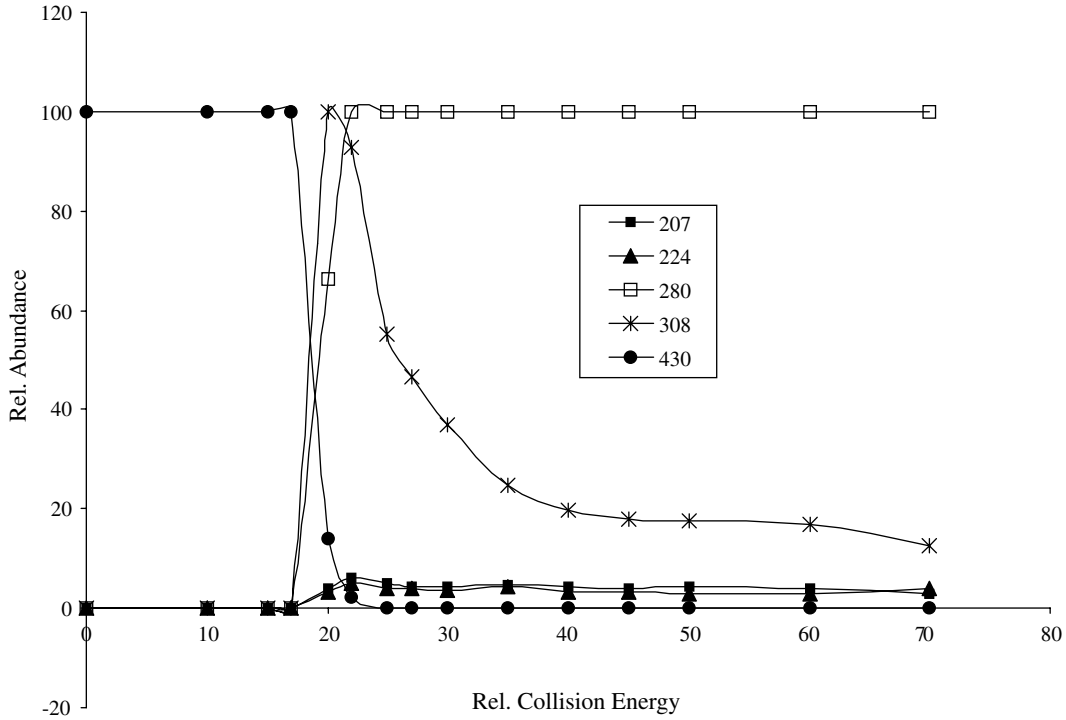

Figure 4. Relative abundances of the product ions of $[\mathbf{1} + H]^+$ vs the relative collision energies.

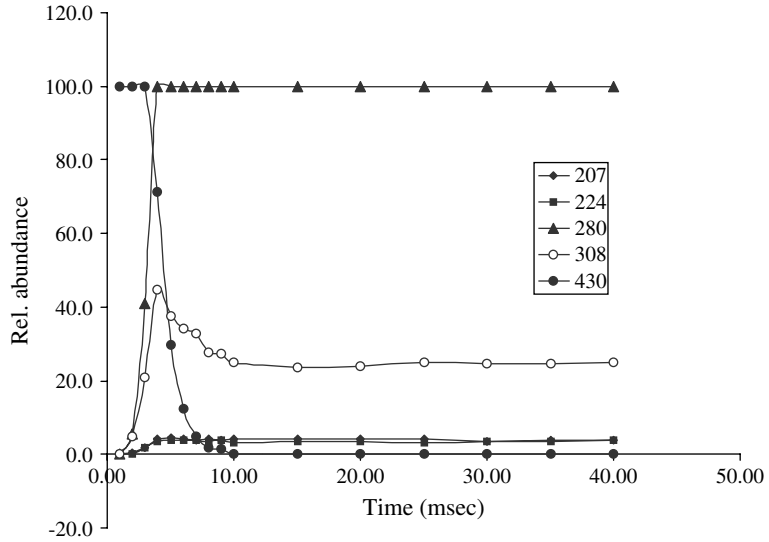

Figure 5. Relative abundances of the product ions of $[\mathbf{1} + H]^+$ vs the activation times.

acid (m/z 410). The product ion spectrum and the proposed fragmentation scheme are shown in Fig. 6(B).

On the other hand, the rearrangement is not observed in the CID spectra of the methyl ester of **1** (**4**, Fig. 1), or in **5** (Fig. 1), carrying two prolines, as shown in Fig. 6(C) and (D), respectively. This evidence again supports the occurrence of a hydrogen bond stabilizing a favourable conformation, since **4** does not have a carboxylic group on the proline ring, whereas **5** does, but on the proline residue more distant from the benzoyl group.

Proposed rearrangement mechanism

On the basis of the putative hydrogen bond between the benzoyl oxygen and the carboxyl hydrogen, the mechanism in Fig. 7 can be proposed. It considers an oxazolone structure for m/z 308, according to that reported for peptide b-type ions to which it can be considered structurally similar.[15,16] The formation of this relatively stable cyclic ion and a stable benzoic acid molecule may be the driving force for the rearrangement process. The mechanism implies two acylic nucleophilic substitutions, the first involving nucleophlic attack of the amide oxygen on the carbonyl of the carboxy group, according to the formation mechanism of b-ions.[16] The leaving group, i.e. the hydroxyl, acts as the nucleophile in the second substitution, which occurs on the thioester carbonyl; the hydrogen bond drives the oxygen to the reaction centre, probably through a concerted mechanism. Final proton transfer to the 3-mercapto-2-methylpropanoyl

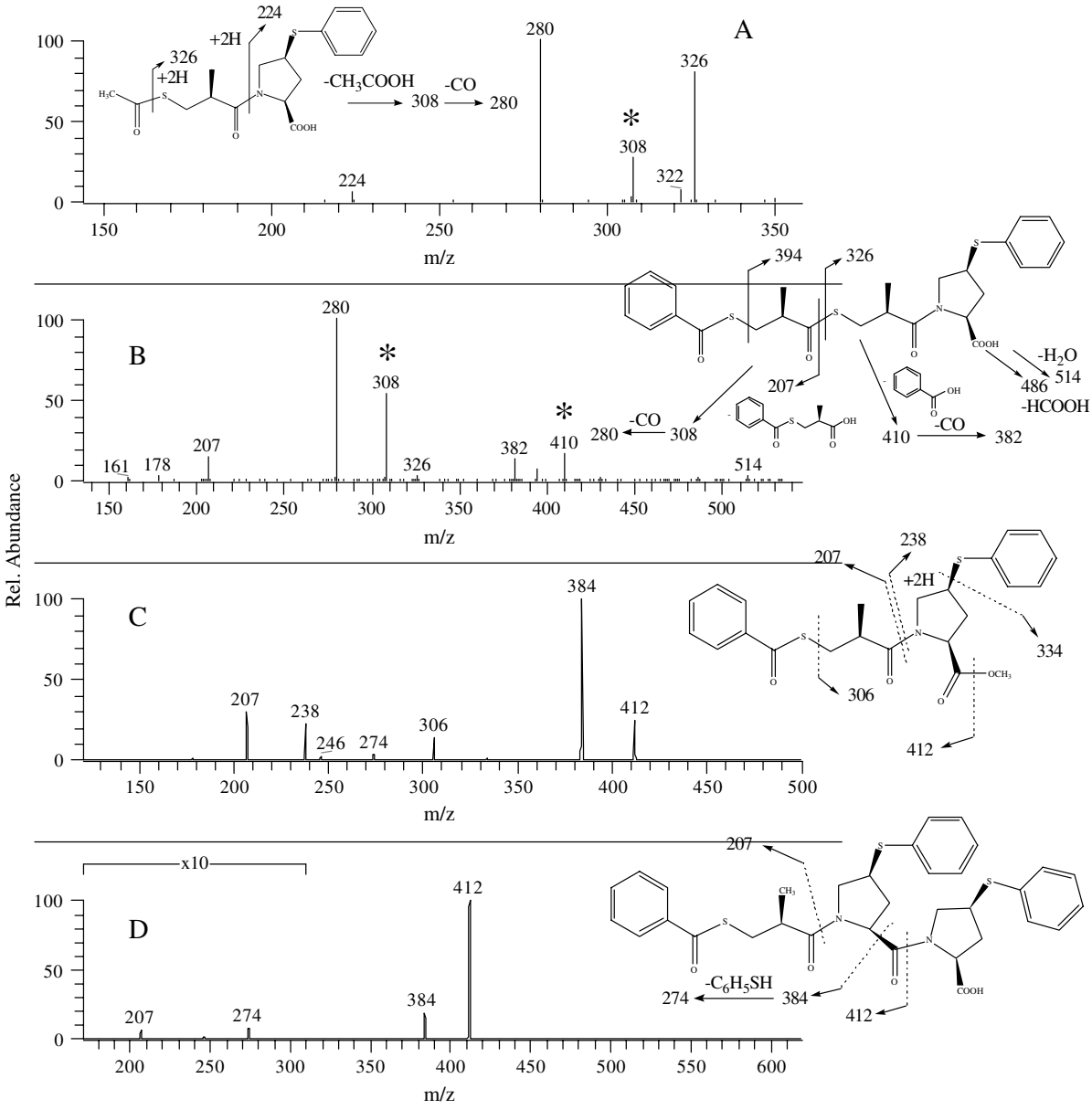

Figure 6. Product ion MS/MS and fragmentation schemes of related compounds: (A) $[\mathbf{2} + H]^+$, *m/z* 368; (B) $[\mathbf{3} + H]$, *m/z* 532; (C) $[\mathbf{4} + H]^+$, *m/z* 444; (D) $[\mathbf{5} + H]^+$, *m/z* 635. The peaks marked with asterisks correspond to the described rearrangement.

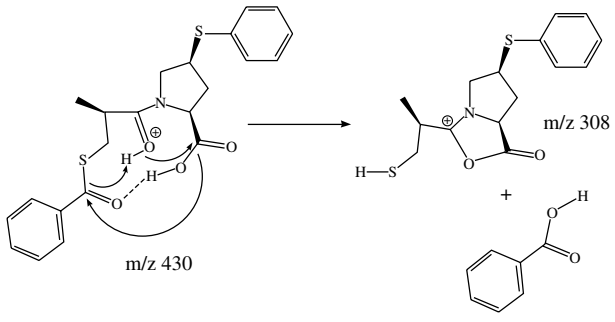

Figure 7. Proposed mechanism of the benzoic acid loss from $[\mathbf{1} + H]^+$.

chain is supported by the above-mentioned deuterium-exchange experiments (see also the scheme in Fig. 3). The protonation at the amide oxygen is given on the basis of *ab initio* calculations (see below), in agreement with literature data which show the CO of the amide group as the thermodynamically favoured protonation site in peptide bonds.[17] However, it is well accepted that intramolecular proton exchange occurs during CID, leading to rapidly interconverting protonated quasi-molecular ions having different protonation sites.[18]

Conformational results

Minima of **1**, obtained at the HF/6–31G* level, were classified into four groups: three of them have different intramolecular hydrogen bonds, involving the carboxylic hydrogen as donor atom. For these groups, hydrogen bond acceptor atoms are as follows (for atom numbering see Fig. 1): O-8 (HBO8 group), N-9 (HBN9 group) and O-2 (HBO2 group). The fourth HBO group contains conformers without intramolecular hydrogen bond. Most stable structures belong to the HBO8 and

HBO2 groups; their relative energies lie in the range 0–1.5 kcal mol^{-1}.

To study proton–molecule complexes, two conformers were selected for each of two energetically favoured groups, HBO8 and HBO2. All of them showed regions of electrostatic potential minima around carboxyl, amide and thioester groups. These regions were analysed as potential protonation sites as described in the Experimental section. For all of the conformers analysed the preferred protonation sites were found near O-8 and O-15 since the other complexes have higher energy (6–40 kcal mol^{-1} above). In Fig. 8 is shown the *ab initio* optimized geometry of a complex obtained from a conformer belonging to the HBO2 group and where proton is in the amide oxygen (O-8) region. Here the relative dispositions of the proton, benzoyl group and carboxyl group are in agreement with the proposed rearrangement mechanism (Fig. 7).

A preliminary NMR conformational study (A. Ettorre, unpublished results) indicates that the structure of **1** in the complex shown in Fig. 8 could also exist in solution. NMR data also suggest an overall conformational flexibility of this molecule, in agreement with x-ray diffraction results[19] that show two very different structures in the unit cell.

CONCLUSIONS

An unusual rearrangement leading to elimination of benzoic acid was observed in the positive ion ESI CID mass spectra of Zofenopril (**1**), a novel ACE inhibitor drug. The same type of rearrangement was also observed in the known stereoisomers of **1**, and also in related compounds bearing a carboxylic group on the proline ring. The rearrangement did not occur, however, in related compounds without a Carboxylic acid group in the above-specified position. To explain these results, the existence in the gas phase of a hydrogen bond involving the hydrogen of the carboxylic group and the benzoyl oxygen was postulated for protonated **1**. A benzoic acid elimination mechanism involving two acylic nucleophilic substitutions was proposed. The existence of stable conformations with the described hydrogen bond was also supported by *ab initio* calculations.

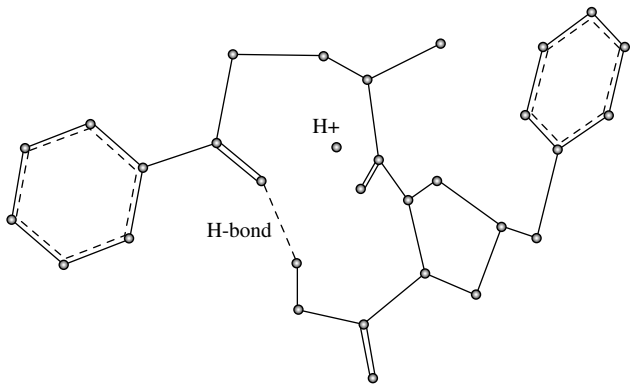

Figure 8. HF/STO-3G structure of **1**. Only carboxylic hydrogen (H-16) is shown. Dashed line indicates H-bond between H-16 and O-2.

Acknowledgements

The authors thank Mr Piero Bacci for helpful discussions, Mr Daniele Bertocci for technical assistance and Dr Piero D'Andrea for the purification of **4**.

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