Fragmentation Studies on Tetronasin by Accurate-Mass Electrospray Tandem Mass Spectrometry

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We report here the first full fragmentation study of tetronasin **1**. Fragmentation was carried out by high-resolution ESI-CID-MSⁿ. The formulae of the fragment ions were determined by accurate mass measurements. It is demonstrated that the fragmentation routes observed derive essentially from a first loss of water via two different mechanisms. One minor route consists of a charge remote neutral loss and the second major route occurs via the formation of a carbocation. The fragments obtained from this carbocation were produced by subsequent complex neutral eliminations and the structures were inferred, in some cases, by carbocation stability. (J Am Soc Mass Spectrom 2004, 15, 325–335) © 2004 American Society for Mass Spectrometry

Polyether ionophores are a group of natural products that distinctively possess a carboxylate group along with several tetrahydropyran or tetrahydrofuran rings. The oxygen atoms from these groups serve as ligands for the complexation of inorganic ions [1] forming stable complexes, typically with alkali and alkaline earth metal cations [2]. The metal cations are, to a degree, encapsulated by the coordinating ether and carbonyl oxygen atoms [2]. Polyethers often exhibit biological activity as antibiotics, antiparasitic, and growth promotion agents in ruminants and poultry [3]. The biological activity of these compounds is related to their strong affinity for alkali metals and their ability to solubilize and transport metals across cell membranes as either undissociated acids or neutral complexes [2, 4].

In recent years, the legislative demands for the analysis of veterinary antibiotic residues has increased, leading to the development and publishing of methods for the detection of drug metabolites in food [5]. Important methods for the detection of polyether ionophore residues and metabolites in a range of animal products employ HPLC-MS and LC-MS/MS [5]. This fact has prompted the present systematic investigation of the fragmentation of different polyether ionophores carried out by this group. Studies published so far are for monensin [6], and lasalocid acid [7] along with tetrona-

sin **1** (Figure 1) which is presented in this study. A further study of salinomycin and narasin is also being undertaken [8].

Tetronasin **1** (previously known as M139603 and ICI139603) is a polyether ionophore antibiotic which was first isolated as a white crystalline solid from the aerobic fermentation of cultures of *Streptomyces longisporus* (NCIB11462) [3]. Comparison of tetronasin **1** with the other common polyether ionophores shows that it contains a biosynthetically rare acyl tetronic acid moiety along with a cyclohexyl group [9]. The structure of tetronasin **1** was determined crystallographically from the sodium salt of the 4-bromo-3,5-dinitrobenzoyl derivative **2** (Figure 1) [9]. It was observed that in the crystalline phase the sodium ion was hexa-coordinated through five oxygen atoms from the molecule (two of which come from the tetronic acid moiety) and a water molecule which occupies the sixth position. The sodium ion was observed to be at the center of a very distorted octahedron, with the oxygen from the C28 hydroxyl group not participating in the coordination.

The NMR studies of the conformation of tetronasin **1** in solution showed that the sodium is still at the center of a distorted octahedron of oxygen atoms with the C28 hydroxy group being hydrogen bonded to the C5 oxygen. The water molecule involved in the coordination of tetronasin with sodium was found to be hydrogen bonded at one end to the C28 hydroxy group and at the other to the C27 (methoxy) oxygen [10].

Tetronasin shows very high affinity for sodium cations, which normally limits mass spectrometric studies to these ions. Previous fragmentation studies [11] have shown that complementary structural data can be obtained from the analysis of protonated ions as well as

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Figure 1. The structure of tetronasin and its dinitrobenzoyl derivative.

metallated ions. The addition of ruthenium (III) chloride to ionophore solutions has been demonstrated as a highly efficient method for the generation of protonated ions [12].

In this study the main fragmentation pathways of protonated tetronasin sodium salt $[(M - H + Na) + H]^+$ and protonated tetronasin $[(M + H)]^+$ are investigated using the conditions previously optimized in our laboratory [13]. The fragmentation analyses were performed on an FT-ICR instrument to produce accurate-mass data and to enable the elucidation of fragmentation routes by multistage tandem mass spectrometry (MS^n).

Experimental

Materials

Methanol (HPLC grade) and ruthenium (III) chloride were obtained from Aldrich (Gillingham, UK). Deuterated water and methanol- d_4 were obtained from Fluorochem (Derbyshire, UK). Labeled water ($H_2^{18}O$ 99at-om%) was obtained from Amersham International (Amersham, UK). Deionized water was used throughout the study. Tetronasin sodium salt was isolated from the growth of *Streptomyces longisporus*, through an ini-

Table 1. The masses and identities of the ions observed in the ESI mass spectrum of tetronasin

Formula	Identity	Observed Mass
$C_{35}H_{51}O_7Na_2^+$	$[(M - H + Na) + Na]^+$	647
$C_{35}H_{52}O_7K^+$	$[(M - H + K) + H]^+$	641
$C_{35}H_{50}O_6Na_2^+$	$647^+ - H_2O$	629
$C_{35}H_{54}O_8Na^+$	$[(M - H + Na) + H]^+$	625
$C_{35}H_{52}O_7Na^+$	$625^+ - H_2O$	607

Figure 2. (a) ESI/MS spectrum of tetronasin; (b) MS/MS spectrum of m/z 625; (c) MS/MS spectrum of m/z 603.

tial methanol mycelial extraction followed by flash chromatography of the crude product using diethyl ether/light petroleum ether (1:1) as eluent to separate some of the non-polar co-metabolites until tetronasin began to be collected. The column was eluted with ethyl acetate plus 1% acetic acid to remove the remaining tetronasin [14]. For the mass spectrometry studies a stock solution of tetronasin (10 mg mL^{-1}) was prepared in methanol and stored at 4°C . Dilute standards were prepared prior to each analysis in methanol/water (1:1) with 0.25 mg mL^{-1} as the final concentration. To generate the protonated ion of tetronasin ($m/z = 603$) 1

Table 2. The formula, identity, calculated mass, observed mass, mass error, and number of incorporated ^{18}O atoms for the fragment ions observed in the MS³ spectrum of m/z 607 (Figure 4a).

Formula	Identity	Calculated mass	Observed mass	Error (ppm)	No. of ^{18}O atoms
$\text{C}_{35}\text{H}_{52}\text{O}_7\text{Na}^+$	Parent Ion	607.3605	607.3644	+6.42	
$\text{C}_{35}\text{H}_{50}\text{O}_6\text{Na}^+$	$607^+ - \text{H}_2\text{O}$	589.3500	589.3476	-4.07	1,2
$\text{C}_{35}\text{H}_{48}\text{O}_5\text{Na}^+$	$589^+ - \text{H}_2\text{O}$	571.3394	571.3406	-2.10	1,2
$\text{C}_{31}\text{H}_{48}\text{O}_4\text{Na}^+$	E3	507.3445	507.3449	+0.79	1
$\text{C}_{30}\text{H}_{48}\text{O}_4\text{Na}^+$	D2	495.3445	495.3450	+1.01	1
$\text{C}_{31}\text{H}_{46}\text{O}_3\text{Na}^+$	K2	489.3339	489.3347	+1.63	1
$\text{C}_{30}\text{H}_{46}\text{O}_3\text{Na}^+$	D3	477.3339	477.3346	+1.47	0,1,2
$\text{C}_{27}\text{H}_{36}\text{O}_4\text{Na}^+$	K1	447.2506	447.2507	+0.22	2
$\text{C}_{21}\text{H}_{28}\text{O}_5\text{Na}^+$	H1	383.1829	383.1826	-0.78	2
$\text{C}_{21}\text{H}_{26}\text{O}_4\text{Na}^+$	G3 & H2	365.1723	365.1711	-3.29	2
$\text{C}_{18}\text{H}_{30}\text{O}_3\text{Na}^+$	E2	317.2087	317.2081	-1.89	0
$\text{C}_{17}\text{H}_{22}\text{O}_4\text{Na}^+$	E1	313.1410	313.1407	-0.96	2
$\text{C}_{14}\text{H}_{24}\text{O}_2\text{Na}^+$	G4	247.1669	247.1670	+0.40	0
Average error (r.m.s.)				1.93	
Mean residual error				+0.23

Figure 3. (a) MS/MS spectrum of m/z 627; (b) Two possible mechanisms for the first loss of water from the deuterated sodium salt of tetronasin (m/z 627).

equivalent of ruthenium (III) chloride was added to a solution of tetronasin and the suspension obtained was thoroughly mixed and centrifuged to separate the solid from the liquid. The supernatant was used for mass spectrometric analysis. For more details, see reference [12]. Deuteration was performed by dilution of the stock solution in CD_3OD and D_2O (1:1). The solution

Figure 4. (a) MS³ spectrum for the protonated sodium salt of tetronasin ($625^+ \rightarrow 607^+ \rightarrow$ fragments); (b) MS³ spectrum for protonated tetronasin ($603^+ \rightarrow 585^+ \rightarrow$ fragments).

Scheme 1. (a) An overview of the MS³ fragmentation of 607; (b) an overview of the MS³ fragmentation of 585.

was thoroughly mixed and all exchangeable protons were deuterated. For the ¹⁸O experiments, a solution of tetronasin (1 mg mL⁻¹) was prepared in H₂¹⁸O, in the presence of 1% formic acid. This solution was stored refrigerated for several months to allow the carbonyl oxygen exchange to occur.

Instrumentation

Accurate-mass MSⁿ analyses were performed on a BioApex II (4.7 tesla) Fourier-transform ion cyclotron resonance instrument (Bruker Daltonics, Billerica, MA). Solutions were infused by syringe pump through the Analytica ESI source at 100 μL h⁻¹. Fragmentation analyses were performed on the isolated parent ions by sustained off-resonance irradiation collision induced

dissociation (SORI-CID) using CO₂ collision gas. Precursor ions were selectively isolated through correlated sweep isolation. The cell conditions were tuned to fragment enough of the precursor ion to observe a spectrum of good intensity across the required mass range. Sequential fragmentation steps were usually performed on optimized precursor ions from the previous step. The ESI spectra and isolation experiments were performed at a resolution of approximately 20,000. The MSⁿ spectra had a resolution of approximately 30,000. The mass range for the ESI experiments was from *m/z* 100 to 4000 and for the MSⁿ experiments the mass range was from *m/z* 80 to 1000. Usually eight individual scans were combined to produce a spectrum, but in the cases were weaker signals were observed

Table 3. The formula, identity, calculated mass, observed mass, mass error, and number of incorporated ¹⁸O atoms for the fragment ions observed in the MS³ spectrum of *m/z* 585 (Figure 4b).

Formula	Identity	Calculated mass	Observed mass	Error (ppm)	No. of ¹⁸ O atoms
C ₃₅ H ₅₃ O ₇ ⁺	Parent Ion	585.3786	not observed		
C ₃₅ H ₅₁ O ₆ ⁺	K''	567.3680	567.3657	-4.05	2
C ₃₄ H ₄₉ O ₆ ⁺	M	553.3524	553.3513	-1.99	2
C ₃₅ H ₄₉ O ₅ ⁺	567 ⁺ - H ₂ O	549.3575	549.3589	+2.55	1
C ₃₄ H ₅₃ O ₅ ⁺	N	541.3888	541.3879	-1.66	—
C ₃₄ H ₄₇ O ₅ ⁺	R	535.3418	535.3407	-2.05	2
C ₃₄ H ₄₅ O ₄ ⁺	S	517.3312	517.3339	+5.22	1,2
C ₃₂ H ₅₁ O ₄ ⁺	O	499.3782	499.3801	+3.80	1
C ₃₄ H ₄₃ O ₃ ⁺	517 ⁺ - H ₂ O	499.3207	499.3202	-1.00	1
C ₂₇ H ₃₉ O ₅ ⁺	K1''	443.2792	443.2772	-4.51	2
C ₂₇ H ₃₇ O ₄ ⁺	T	425.2686	425.2663	-5.41	2
C ₂₇ H ₃₅ O ₃ ⁺	V	407.2581	407.2593	+2.95	1
C ₂₆ H ₃₇ O ₂ ⁺	U	381.2788	381.2799	+2.89	0,1
C ₁₇ H ₂₃ O ₄ ⁺	E1''	291.1591	291.1601	+3.43	—
C ₁₆ H ₂₃ O ₂ ⁺	P	247.1693	247.1678	-6.07	1
C ₁₅ H ₂₃ O ⁺	Q	219.1743	219.1728	-6.84	1
C ₈ H ₁₅ O ₂ ⁺	L	143.1067	143.1067	0.00	0
Average error (r.m.s.)				3.40	
Mean residual error				-0.80

Scheme 2. Proposed mechanism for the formation of the putative intermediates (D, E, and F) derived from the carbocation A.

(especially for the protonated ion) 40 or 80 individual scans were combined.

The MS/MS analyses of ^{18}O labeled tetronasin were performed on a quadrupole/time-of-flight (Q-TOF) mass spectrometer (Micromass, Manchester, UK). Solutions were infused by syringe pump into the ESI source at $10\ \mu\text{L}\ \text{min}^{-1}$. Fragmentation was performed using He collision gas at 30–35 normalized energy with a $\pm 1\ m/z$ mass selection window. The acquisition mass range was m/z 100 to 750 with 30 to 50 scans averaged to produce the spectra.

Spectra Calibration and Formula Assignments

Accurate masses for the FTICR experiments were obtained on all fragment ions (to within 5 ppm in most cases) through a post-analysis application of a calibration obtained from the product ion spectrum of eryth-

romycin A acquired under the same instrument conditions. The accuracy of the calibration is shown in the accurate-mass data tables (Tables 2 and 3). In Table 2, the rms of the ppm difference between the observed and calculated masses (mass error) is 1.93 ppm and the mean residual error is +0.23 ppm. In Table 3 the rms error is 3.40 ppm and the mean residual is -0.80 ppm. The rms error is indicative of the peak to peak precision of measurement, whereas the mean residual error gives in indication of any bias or inaccuracy in the overall calibration. The mean residual error should be as close to zero as possible and must be lower than the rms error for the calibration to be considered reliable. With both of these spectra, the mean residual error is an order of magnitude lower than the rms error. The formula assignment of the unknown fragment ions was then achieved by using the formula of the parent ion as the atom constraints for C, H, and O in the elemental

Scheme 3. The fragmentation mechanism starting from intermediate D.

composition calculator. This usually only produced one possible formula match to within 50 ppm i.e., an order of magnitude higher than the rms error of the spectra. In the rare occasion when more than one match was obtained, logical deductions about possible structures from the MS^n data were used to eliminate the extraneous formulae.

Rational and Nomenclature

In producing the schemes, we have attempted to rationalize the fragmentation by use of the simplest explanation consistent with the data obtained. We have tried to avoid invoking high-energy pathways, although some fragments can only be explained by several rearrangements. All fragmentation reactions can be considered as two electron processes with no radical ion involvement. Each fragmentation reaction results in two fragments, one of which retains the charge, the

Scheme 4. The fragmentation mechanisms starting from intermediate E.

other being lost as a neutral. In some cases, the charge can be retained by either fragment producing two sub-fragmentation routes from the same precursor ion. For clarity we have adopted the following fragment ion identification scheme. Structures C, D, E, and F are possible forms of the ion at $m/z = 607$ and are responsible for the majority of the ions observed. For example, the fragment ions E1, E2, and E3 are subfragments of the Isomer E. For the fragmentation of the protonated ion, many of the routes followed were analogous to those observed for the sodium salt, and in these cases the fragments were identified by a double inverted coma after the letter.

Results and Discussion

The ESI/MS spectrum of tetronasin shows the presence of several peaks, corresponding for example, to mono-, disodiated, and potassiated peaks (Figure 2a and Table 1). At low fragmentation energy, the MS/MS spectra of protonated tetronasin sodium salt $[(M - H + \text{Na}) + \text{H}]^+$ ($m/z = 625$) and the protonated tetronasin $[(M + \text{H})]^+$

Scheme 5. First fragmentation mechanism starting from intermediate **F**.

($m/z = 603$) only showed a peak at $m/z = 607$ and $m/z = 585$, respectively (Figures 2b and c). These fragmentations derive from a loss of a water molecule. The results obtained suggest that the first water loss occurs from the hydroxyl group at C28. We propose that this first loss can happen via two different mechanisms. The MS/MS spectrum of the deuterated tetronasin (Figure 3a) shows that one mechanism implicates a charge

remote neutral loss of water by a 1,4-elimination (loss of DOH) as the minor route (Figure 3b) [15]. The second mechanism, the major route, requires that protonation (or deuteration) of the hydroxyl group at C28 occurs leading to the loss of D_2O and formation of an allylic carbocation **A** (Figure 3b), that shows good relative stability through resonance.

The ESI-MS spectrum of the ^{18}O labeled tetronasin showed ions corresponding to the incorporation of ^{18}O ($m/z = 627$ with one ^{18}O and $m/z = 629$ with two ^{18}O). Dissociation of both of these ions showed only a loss of water (H_2O) forming the fragments corresponding to $m/z = 609$ and $m/z = 611$ (Table 2), respectively. This means that the loss of the first water from the hydroxyl at C28 contains no ^{18}O , suggesting that the incorporation of ^{18}O is in the tetronic moiety or carbonyl C5 as expected.

Increasing the collision energies had little effect on the MS/MS spectra of either the protonated sodium salt $[(M - H + Na) + H]^+$ or protonated molecule $[(M + H)]^+$. An additional collision stage (MS^3) was required to produce a full series of fragment ions. This behavior is probably indicative of the stability of the *quasi*-cyclic conformation of the tetronasin. The MS^3 spectra obtained for the protonated sodium salt ($625^+ \rightarrow 607^+ \rightarrow$ fragments) and protonated molecule ($603^+ \rightarrow 585^+ \rightarrow$ fragments), respectively, can be seen in Figure 4. An overview of the different routes of these fragmentation pathways is shown in Scheme 1. The mass accuracy data is contained in Tables 2 and 3.

The fragmentation observed in the MS^3 spectrum of 607 arises from carbocation **A**, as previously explained. We propose that the observed complex neutral eliminations arise from different mechanisms, such as β -eliminations and hydrogen shifts. Some of the fragmentations observed on the MS^3 spectrum of 607 don't occur in the MS^3 spectrum of 585, probably due to the presence of sodium. The ^{18}O labeling experiment, in particular the MS/MS spectrum of the ion at $m/z = 611$ (with two incorporated ^{18}O atoms), supports the proposed structures observed on MS^3 spectrum of 607.

As an initial example, migration of the proton at C12 of the stable conjugated resonance form **A1** (Scheme 2) to the carbonyl at C5, assisted by the lone pair of electrons from oxygen C5, can give the species **C** (Scheme 2) in which the carbonyl at C5 gets protonated. Structure **C** may then be in equilibrium with two different forms, **E** and **F**. The carbonyl at C5 can alternatively attack the form **A1** (Scheme 2) (this process being initiated by one of the lone pair of electrons of the oxygen at C3), as in a Lewis acid-base reaction [16, 17], resulting in the formation of a stable six membered ring in which the charge is now attributable to sodium (see Structure **A2**, Scheme 2). The migration of the proton at C6 to the heterocyclic oxygen followed by opening of the six membered ring at the bond C5-O produces the allene **D**. Structures **D**, **E**, and **F** (Scheme 2) are putative intermediates for the mechanisms discussed in the following section. It is not possible to explain subse-

Scheme 6. Second and third fragmentation mechanisms starting from intermediate F.

quent fragmentation without the existence of these intermediates.

We propose that the fragments **D2** ($m/z = 495$) and **D3** ($m/z = 477$) derive from the same route (Scheme 3) with **D** being the precursor. This route may be explained by an initial 1,3-hydrogen shift of the proton at C7 to C5 with the formation of the diene **D1**, that then gives the allene **D2** ($m/z = 495$) via a simple neutral elimination of the 3-methylene-furan-2,4-dione unit. The sodiated ion **D2** can then undergo a charge-remote neutral loss of one molecule of water leading to **D3** ($m/z = 477$). The MS/MS spectrum of the ion at $m/z = 611$ (with two incorporated ^{18}O atoms) shows the presence of peaks at $m/z = 497$, suggesting that the ^{18}O atoms

where possibly exchanged at the carbonyl at C5 and one of carbonyls of the tetronic moiety. Therefore, $m/z = 497$ corresponds to the ion **D2** with one ^{18}O remaining at C13 after the rearrangement.

Scheme 4 shows the two additional fragmentation routes that may explain the formation of the fragments **E1** ($m/z = 313$), **E2** ($m/z = 317$), and **E3** ($m/z = 507$). Structures **E1** and **E2** may derive from a β -elimination with cleavage at the bond C14–15. The sodium cation can be chelated to both of the resulting neutral fragments leading to the sodiated ions **E1** or **E2**. The ^{18}O labeling experiment shows only a peak at $m/z = 317$ corresponding to the coincidence of **E1** with two exchanged oxygens and **E2** with no exchanged oxygens.

Scheme 7. The fragmentation mechanisms starting from intermediate **K**.

The intermediate **E** can alternatively undergo a simple neutral loss of mass 100 (Scheme 4), corresponding to the tetronic acid unit, to form the ketene **E3**. This loss occurs as mass 102 in the ^{18}O labeling experiments which is in agreement with the proposed mechanisms.

The intermediate **F**, in which the oxygen of the pyran ring is protonated, may dissociate by three different routes (Schemes 5 and 6). The first one implies that the resonance form **G**, in which the ring is opened at the bond C15–O, can undergo a neutral loss of one molecule of water with the formation **G1** (Scheme 5), one of the possible fragments corresponding to $m/z = 589$ ($m/z = 593$ on ^{18}O labeling experiment). An eight membered ring (see **G2**, Scheme 5) can be formed by the attack of a lone pair of electrons of the oxygen of the carbonyl at C5, to the carbocation **G1** as described for **A1** (Scheme 2). A 1,3-hydrogen shift from the proton at C16 to C18 of **G2** followed by a cleavage at the bond C17–18 may produce the ions **G3** ($m/z = 365$) and **G4** ($m/z = 247$). In the ^{18}O labeling experiment, $m/z = 369$ and $m/z = 247$ were observed, corresponding, respectively, to **G3** with

Scheme 8. The fragmentation mechanisms starting from intermediate **C''**.

Scheme 9. The fragmentation mechanisms starting from intermediates I'' and K'' .

two exchanged oxygens and **G4** with no exchanged oxygens.

Another possible resonance form of **F** is Structure **H** (Scheme 6). In this intermediate the ring is opened at the bond C19–O. Fragmentation at the bond C17–18 leads to the carbocation **H1** ($m/z = 383$), that later can lose water producing **H2** ($m/z = 365$). The Structure **H2** shows good relative stability by resonance. In the ^{18}O labeling experiment, the ions corresponding to $m/z = 387$ (**H1** with two exchanged oxygens) and $m/z = 369$ (**H2** with two exchanged oxygens) were observed.

The carbocation **I** (Scheme 6), another resonance form of **F**, may lose a molecule of water with the

formation of **I1** ($m/z = 589$). Migration of the proton at C22 to the carbonyl at C5 produces **J**. The Structure **K** (a resonance form of **J**) can undergo a neutral elimination of the dihydro-furan unit as seen in Scheme 7 giving **K1** ($m/z = 447$). As already observed for the intermediate **E**, **K** (Scheme 4) may also lose the tetronic acid unit, producing the sodiated ion **K2** ($m/z = 489$). In the ^{18}O labeling experiment, the ions corresponding to $m/z = 451$ (**K1** with two exchanged oxygens) and $m/z = 369$ (**K2** with one exchanged oxygen) were observed.

The fragmentations observed in the MS^3 spectrum of $m/z = 585$ (Figure 4b) follow a similar mechanism to that which was observed in the MS^3 spectrum of $m/z = 607$, however, due to extra proton, losses of CO_2 , CH_3OH , H_2O and other small neutrals are additionally observed. As described for the sodiated tetronasin spectrum, an ^{18}O experiment was used to support the proposed mechanisms. In this part of the discussion the ^{18}O labeling experiment corresponds to MS/MS of the ion at $m/z = 589$. All the corresponding ^{18}O labeled fragments are listed in Table 3.

To explain some of the fragment ions in the MS^3 spectrum of $m/z = 585$, we propose that the ion assumes the putative Structure **C''** (Scheme 8). This molecule can then be the origin of several different fragment ions by four simple mechanisms. Assuming that the **C''** form is protonated at the oxygen of the pyran ring **F''**, it can later undergo a concerted elimination that is initiated by one of the lone pairs of the oxygen from the furan ring, leading to the formation of the cation **L** ($m/z = 143$). The Structure **C''** may also be in equilibrium with a form in which the protonation occurs in the methoxy group inducing an easy loss of methanol with the production of the carbocation **M** ($m/z = 553$). If **C''** is in equilibrium with a form in which the tetronic moiety is protonated, a loss of carbon dioxide may occur giving **N** ($m/z = 541$), that is observed at very low intensity. The later fragment may then lose COCH_2 by a pericyclic elimination mechanism leading to the fragment **O** ($m/z = 499$). For the fourth mechanism we propose that the protonated species **C''** undergoes a β -elimination at C14–15 with the formation of a fragment **E1''** ($m/z = 291$), that by loss of CO_2 gives the fragment **P** ($m/z = 247$). The diketide resonance form of **P** may then lose CO with the formation of the fragment **Q** ($m/z = 219$).

Two additional mechanisms that may explain the rest of the fragments derived from $m/z = 585$ are described on Scheme 9. One involves a charge-remote neutral elimination of methanol from **K''** ($m/z = 567$) giving fragment **R** ($m/z = 535$). If the oxygen from the furan ring of **R** is protonated, this may then open with the formation of the carbocation **R1** followed by loss of water giving fragment **S** ($m/z = 517$) that is stabilized by resonance. Structure **S** may also correspond to a loss of water from the tetronic moiety of **R**, which explains the peaks observed in the ^{18}O experiment ($m/z = 519$ with one exchanged oxygen and $m/z = 521$ with two exchanged oxygens).

The second mechanism leads to the formation of **K1''**

($m/z = 443$), in a similar manner to what was observed in the MS^3 spectrum of the sodiated tetronasin, for the intermediate I. Fragment T ($m/z = 425$) results from a loss of water and can subsequently eliminate both CO_2 and water to produce the fragments U ($m/z = 381$) and V ($m/z = 407$), respectively.

Conclusions

Complete fragmentation pathways are proposed for protonated tetronasin and the protonated tetronasin sodium salt. The formulae of the fragment ions are confirmed by accurate-mass analysis (see Tables 1, 2, and 3) to within 5 ppm in most cases (with a 1.93 ppm r.m.s. error for MS^3 of 607 and a 3.4 ppm r.m.s. error in the MS^3 of 585). The larger average error in the analysis of the protonated ion is probably the result of fewer ions being measured, resulting in the lower signal-to-noise ratio observed in the spectrum. The analysis performed in deuterated media clearly shows the two possible routes for the first water loss. The ^{18}O labeling experiments also provide useful data to support some of the proposed mechanisms.

The results presented will be of significant use for future identification of tetronasin metabolites and biosynthetically generated derivatives.

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