ARTICLE

OBC
www.rsc.org/obc

Evidence for gas-phase redox chemistry inducing novel fragmentation in a complex natural product

Norberto P. Lopes,[a,b] Christian B. W. Stark,[a,c] James Staunton[a] and Paul J. Gates*†[a]

[a] *University of Cambridge, Department of Chemistry, Lensfield Road, Cambridge, UK CB2 1EW. E-mail: Paul.Gates@bristol.ac.uk.; Fax: +44 (0)117 9251295; Tel: +44 (0)117 9546967*

[b] *Universidade de São Paulo, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Via do Café S/N, CEP 14.040-903, Ribeirão Preto-SP, Brazil*

[c] *Institut Für Chemie – Organische Chemie, Freie Universität Berlin, Berlin, Germany*

Received 28th September 2003, Accepted 19th November 2003
First published as an Advance Article on the web 24th December 2003

The fragmentation of monensin A, in the presence of calcium, barium, silver and copper salts was studied by electrospray ionisation tandem accurate-mass mass spectrometry. The results showed that the calcium, barium and silver complexes of monensin A showed no significant alteration in their fragmentation to that previously observed for the sodium salts. However, the fragmentation of the copper(II) salt resulted in new fragmentation routes. We propose that the copper might be initiating a novel gas-phase redox reaction resulting in a series of highly diagnostic ions. This methodology is demonstrated by locating the change in structure between the naturally occurring analogues monensin A and B.

Introduction

Extensive investigations have been performed on various solution-phase factors, such as solvent composition, solution pH and metal competition in electrospray ionisation (ESI) mass spectrometry (MS).[1–3] Previous studies of Cu^{II}, Fe^{III} and Ag^{I} complexes in ESI, revealed that it is possible to change the oxidation state of the metal cation in the gas-phase.[4] The energetics involved to reduce Cu^{II} to Cu^{I} in negative ESI were low, and in positive ESI oxidation has also been observed.[4] The same reduction process for Ag^{I} to Ag^{0} can occur but was found to require higher energy.[4] Investigations of gas-phase interactions show that copper can form diligand complexes with CO, C_2H_2 and C_2H_4 near to room temperature.[5] In addition, Cu^{I} gas-phase reactions with small acyclic ketones showed that Cu^{I} prefers to retain its oxidation state when σ bonded to carbon. This was demonstrated by its ability to abstract CH_3^- from ketones.[6] Gas-phase reactions of Cu^{I} with diethyl ether and tetrahydrofuran produced copper hydride ($Cu^{I}H$), and the corresponding ether carbonium ion *via* a soft-acid and soft-base reaction.[6] The fragmentation of small peptides and sugars in the presence of the transition metal ions Cu, Co and Mn has shown different types of fragmentation to that observed normally[7,8] and the production of radical species has also been proposed.[8]

Recently, it was demonstrated that the antibiotic polyether ionophores can form complexes with transition metals in the gas-phase.[9] Polyether ionophores characteristically contain a carboxylate group and 2 to 5 ether oxygen atoms which act as ligands for the complexation of cationic species.[10] Since their discovery, polyether antibiotics have found widespread use as feed additives in poultry farming.[10] The economic importance of these compounds has resulted in a high number of studies in different areas of research.[9–15] Monensin A (see Fig. 1) is the most investigated polyether by mass spectrometry.[9–15] The fragmentation of monensin generally occurs at both termini of the molecule and additionally involves the opening of the spiro ketal moiety.[12,13] Previous studies defined fragments as type A (containing the metal cation and the carboxyl terminus) and as Type F (containing the metal cation and the hydroxyl terminus).[14,15] The established fragmentation patterns and the ability of monensin A to form complexes with transition metals suggests that it would be a good test compound to examine its Lewis acid properties and the possibility of redox reactions to generate novel gas-phase products.

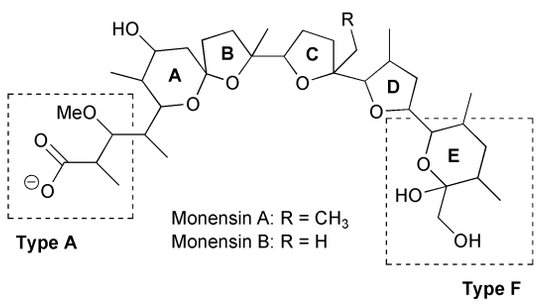


Fig. 1 The structure of the monensin anion.

The fragmentation of protonated and sodiated monensin produces a substantial number of ions. Typically, ions result from Grob–Wharton type fragmentation or through pericyclic rearrangements as well as simple neutral losses.[12,13] In all cases, though the fragment ions retain the BCD polyether ring section of the molecule intact (Fig. 1). Transition metal ions have more specific binding geometries than alkali metal ions and can involve their d-orbitals in bonding. It was reasoned that exploitation of these differences could lead to changes in the coordination behavior of monensin for the metal ions and that the d-orbital interactions might promote new fragmentation mechanisms.[9] In addition, the low energetics of electron transfer in certain transition metal species (for example Cu^{II}) suggests that gas-phase redox processes might be possible to chemically induce novel fragmentation patterns.

The main aim of this study is to determine the influences of different metal cations (including transition metals) on the fragmentation of monensin A and to compare the fragmentation with that previously reported for monensin A protonated sodium salt $[(M-H+Na)+H]^+$[12] and protonated ions $[M+H]^+$.[13]

† *Current address and address for correspondence:* Dr P. J. Gates, School of Chemistry, University of Bristol, Cantock's Close, Bristol, UK BS8 1TS. E-mail: Paul.Gates@bristol.ac.uk

DOI: 10.1039/b311871a

Results and discussion

To try to generate novel fragmentation mechanisms for monensin A, salts of calcium, barium, silver and copper were added to a solution of the monensin A sodium salt dissolved in methanol : water (7 : 3, 1 mg mL^{-1}).

The MS/MS analyses of the monensin A/Ag^+ complex and the monensin A/Ca^{2+} complex are shown in Fig. 2. In both cases it was observed that the fragmentation pathways were essentially the same as that previously observed for the fragmentation of the sodium salts.[12] Table 1 shows a summary of all the peaks observed in the analyses along with those for a similar analysis of the monensin A/Ba^{2+} complex (spectrum not shown). The ion identities for the equivalent species in the MS/MS analysis of the protonated sodium salt[12] are also shown. The accurate masses (well within 5 ppm average across the whole spectra) provide a very high degree of confidence in the formulae of the ions. This demonstrates that for all three analyses (Ag^+, Ca^{2+} and Ba^{2+}) the fragment ions produced correspond to ions previously observed. The two exceptions were the observation of the **S** + H_2O and **A** + H_2O species. These were not observed in the previous MS/MS analysis of the sodium salts and the presence of these ions in this study is probably evidence that the order of simple neutral losses is altered by changing the cation. They are not structurally significant and can probably be disregarded. The main structural fragments were still produced by a Grob–Wharton type mechanism[14–17] in ring A. The proposed structures of the most important fragment ions are shown in Fig. 3. These results show that the fragmentation mechanisms for the Ag^+, Ca^{2+} and Ba^{2+} complexes are very similar to that previously observed for the protonated sodium salt,[12] and that use of these three metal cations fails to yield the required fragmentation of the core polyether backbone of monensin A (rings BCD) or to produce any new fragmentation routes to those already observed previously.

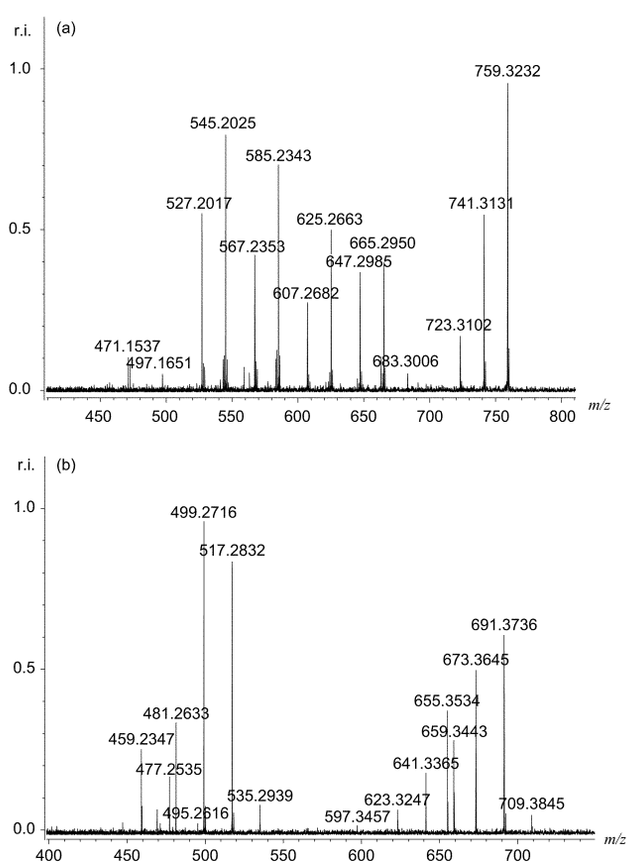


Fig. 2 The MS/MS spectra of monensin A (a) Ag^+ complex (*m/z* 777 precursor ion) and (b) Ca^{2+} complex (*m/z* 709 precursor ion).

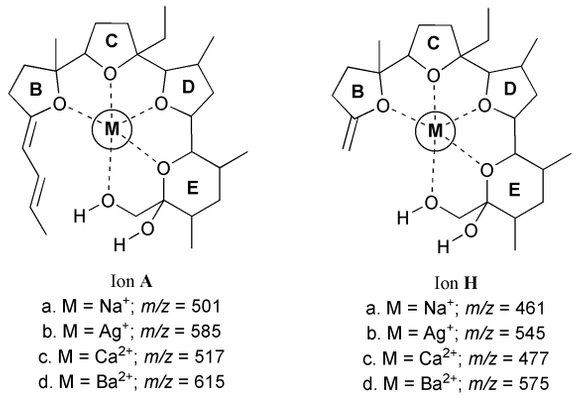


Fig. 3 The two major Grob–Wharton type fragment ions observed in the MS/MS analyses of monensin A complex with Na^+, Ag^+, Ca^{2+} and Ba^{2+}.

The high and low resolution MS/MS analysis of the monensin A/Cu^{II} complex (Fig. 4a) showed a very different set of fragment ions with none of the previously observed Grob–Wharton fragments being observed. A series of ions were produced with even and odd masses (see Table 2). This suggests that two different mechanisms of fragmentation were occurring; one mechanism related to the tendency of Cu^{II} to be reduced to Cu^{I}, with the other mechanism being that of hydride abstraction by the copper cation.[6]

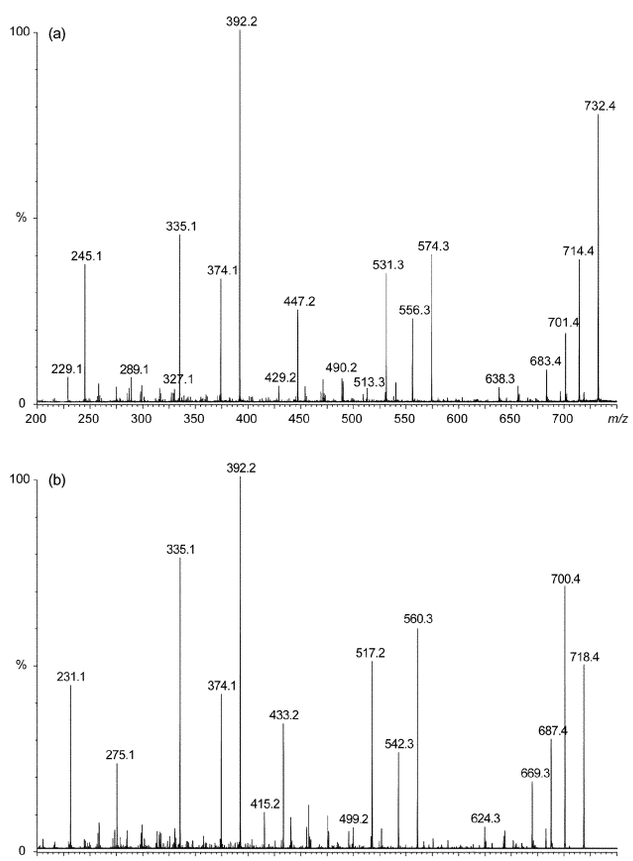


Fig. 4 The MS/MS spectrum of (a) monensin A with one equivalent $CuCl_2$ (*m/z* 732 precursor ion) and (b) monensin B with one equivalent $CuCl_2$ (*m/z* 718 precursor ion).

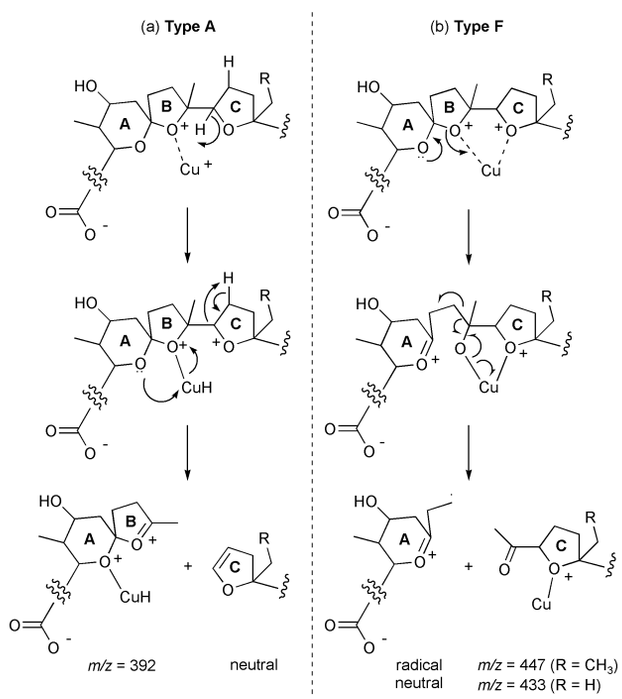
It is proposed that the type A (even-massed) fragment ions are the result of hydride abstraction by Cu^{II} from the α-oxygen position of a neighbouring tetrahydrofuran ring, as shown in Scheme 1(a). In this mechanism, only the fragment ions with the anionic carboxyl group (COO^-) are detected – the Cu^{2+} cation counteracts the negative charge resulting in a singular positively

Table 1 Formulae, calculated mass, observed mass, mass measurement error (ppm) and fragment ion identity (from the MS/MS analysis of the protonated sodium salt [12]) for the ions observed in the MS/MS analysis of monensin A: Ag^+, Ca^{2+} and Ba^{2+} complexes (see Fig. 2)

Formula	Calculated and observed masses (with ppm error) for the Ag^+ complex	Calculated and observed masses (with ppm error) for the Ca^{2+} complex	Calculated and observed masses (with ppm error) for the Ba^{2+} complex	Ion identity (from Na^+ fragmentation) [12]
$C_{36}H_{62}O_{11}X^+$	Not observed	709.3834 709.3845 (+1.55 ppm)	807.3261 807.3254 (−0.87 ppm)	Precursor Ion (PI)
$C_{36}H_{60}O_{10}X^+$	759.2332 759.3232 (0.00 ppm)	691.3729 691.3736 (+1.01 ppm)	789.3155 789.3161 (+0.76 ppm)	**D**
$C_{36}H_{58}O_9X^+$	741.3126 741.3131 (+0.67 ppm)	673.3623 673.3645 (+3.27 ppm)	771.3050 771.3049 (−0.13 ppm)	**E**
$C_{35}H_{56}O_9X^+$	Not observed	659.3467 659.3443 (−3.64 ppm)	757.2893 757.2859 (−4.49 ppm)	**D** − MeOH
$C_{36}H_{56}O_8X^+$	723.3021 723.3002 (−2.63 ppm)	655.3517 655.3534 (+2.59 ppm)	753.2944 753.3001 (+7.57 ppm)	**E** − H_2O
$C_{35}H_{54}O_8X^+$	Not observed	641.3361 641.3365 (+0.62 ppm)	739.2787 739.2802 (+2.03 ppm)	**D** − MeOH, H_2O
$C_{35}H_{52}O_7X^+$	Not observed	623.3255 623.3247 (−1.28 ppm)	721.2682 721.2699 (+2.36 ppm)	**D** − MeOH, $2H_2O$
$C_{34}H_{56}O_7X^+$	683.3071 683.3066 (−0.73 ppm)	Not observed	Not observed	**D** − MeOH, CO_2
$C_{34}H_{54}O_6X^+$	665.2966 665.2950 (−2.40 ppm)	597.3463 597.3457 (−1.00 ppm)	695.2889 695.2877 (−1.73 ppm)	**D** − MeOH, CO_2, H_2O
$C_{34}H_{52}O_5X^+$	647.2860 647.2885 (+3.86 ppm)	Not observed	Not observed	**D** − MeOH, CO_2, $2H_2O$
$C_{31}H_{50}O_6X^+$	625.2653 625.2663 (+1.60 ppm)	Not observed	Not observed	**S** + H_2O (not observed with Na)
$C_{31}H_{48}O_5X^+$	607.2547 607.2582 (+5.76 ppm)	Not observed	Not observed	**S**
$C_{28}H_{48}O_7X^+$	Not observed	535.2942 535.2939 (−0.56 ppm)	633.2369 633.2377 (+1.26 ppm)	**A** + H_2O (Not observed with Na)
$C_{28}H_{46}O_6X^+$	585.2340 585.2343 (+0.51 ppm)	517.2837 517.2832 (−0.97 ppm)	615.2263 615.2243 (−3.25 ppm)	**A**
$C_{28}H_{44}O_5X^+$	567.2234 567.2253 (+3.35 ppm)	499.2731 499.2716 (−3.00 ppm)	597.2157 597.2178 (+3.52 ppm)	**B**
$C_{25}H_{44}O_7X^+$	563.2132 563.2131 (−0.18 ppm)	495.2630 495.2616 (−2.83 ppm)	593.2056 593.2032 (−4.05 ppm)	**G**
$C_{28}H_{42}O_4X^+$	Not observed	481.2625 481.2633 (+1.66 ppm)	579.2052 579.2030 (−3.80 ppm)	**B** − H_2O
$C_{25}H_{42}O_6X^+$	545.2027 545.2025 (−0.37 ppm)	477.2524 477.2535 (+2.30 ppm)	575.1950 575.1995 (+7.82 ppm)	**H**
$C_{25}H_{40}O_5X^+$	527.1921 527.1917 (−0.76 ppm)	459.2418 459.2437 (+4.14 ppm)	557.1844 557.1856 (−2.15 ppm)	**H** − H_2O
$C_{22}H_{36}O_4X^+$	471.1659 471.1637 (−4.67 ppm)	Not observed	Not observed	**R**
RMS error	1.96 ppm	2.03 ppm	3.05 ppm	
Mean residual	+0.29 ppm	+0.26 ppm	+ 0.32 ppm	

Table 2 Formulae, calculated mass, observed mass, mass measurement error (ppm) and fragment ion identity for the ions observed in the MS/MS analysis of the monensin A/Cu^{II} complex (see Fig. 4a)

Formula	Calculated and observed masses (with ppm error)	Fragment ion identity
$C_{36}H_{61}O_{11}Cu^+$	732.3504 732.3534 (+ 4.10 ppm)	Precursor ion (PI)
$C_{36}H_{59}O_{10}Cu^+$	714.3399 714.3410 (+ 1.54 ppm)	PI − H_2O
$C_{35}H_{58}O_{10}Cu^+$	701.3320 701.3310 (− 1.43 ppm)	PI − MeO
$C_{36}H_{57}O_9Cu^+$	696.3293 696.3274 (− 2.73 ppm)	714 − H_2O
$C_{35}H_{56}O_9Cu^+$	683.3215 683.3217 (+ 0.29 ppm)	701 − H_2O
$C_{34}H_{57}O_8Cu^+$	656.3344 656.3346 (+ 0.30 ppm)	PI − MeOH, CO_2
$C_{34}H_{55}O_7Cu^+$	638.3238 638.3196 (− 6.58 ppm)	PI − MeOH, CO_2, H_2O
$C_{28}H_{47}O_8Cu^+$	574.2561 574.2551 (− 1.74 ppm)	Type A (break D–E)
$C_{28}H_{45}O_7Cu^+$	556.2456 556.2463 (+ 1.26 ppm)	574 − H_2O
$C_{26}H_{44}O_7Cu^+$	531.2378 531.2396 (+ 3.39 ppm)	Type F (break A–B)
$C_{26}H_{42}O_6Cu^+$	513.2272 513.2266 (− 1.17 ppm)	531 − H_2O
$C_{23}H_{39}O_7Cu^+$	490.1986 490.1977 (− 1.84 ppm)	Type A (break C–D)
$C_{23}H_{37}O_6Cu^+$	472.1881 472.1891 (+ 2.12 ppm)	490 − H_2O
$C_{21}H_{36}O_6Cu^+$	447.1802 447.1814 (+ 2.68 ppm)	Type F (break B–C)
$C_{21}H_{34}O_5Cu^+$	429.1697 429.1671 (− 6.06 ppm)	447 − H_2O
$C_{17}H_{29}O_6Cu^+$	392.1255 392.1252 (− 0.77 ppm)	Type A (break B–C)
$C_{17}H_{27}O_5Cu^+$	374.1149 374.1153 (+ 1.07 ppm)	392 − H_2O
$C_{14}H_{24}O_5Cu^+$	335.0914 335.0913 (− 0.30 ppm)	Type A (break A–B)
$C_{16}H_{24}O_3Cu^+$	327.1016 327.1031 (+ 4.59 ppm)	Type F (break C–D)
$C_{10}H_{14}O_2Cu^+$	229.0284 229.0273 (− 4.80 ppm)	Type F (break D–E)
RMS error	2.44 ppm	
Mean residual	−0.30 ppm	

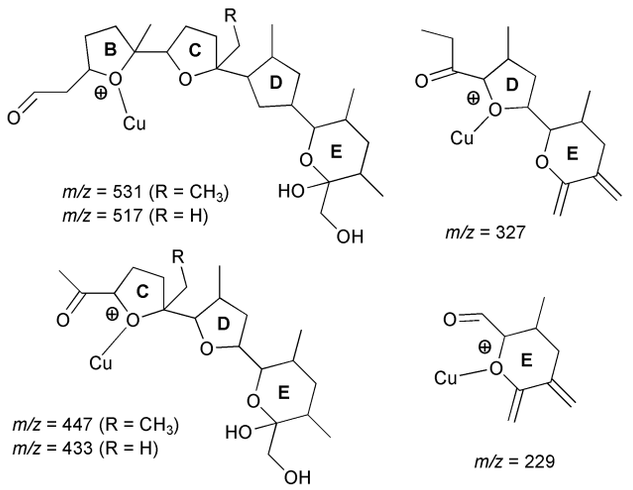


Scheme 1 The proposed mechanisms for the reactions between Cu^{II} and monensin A generating the type A and F fragment ions.

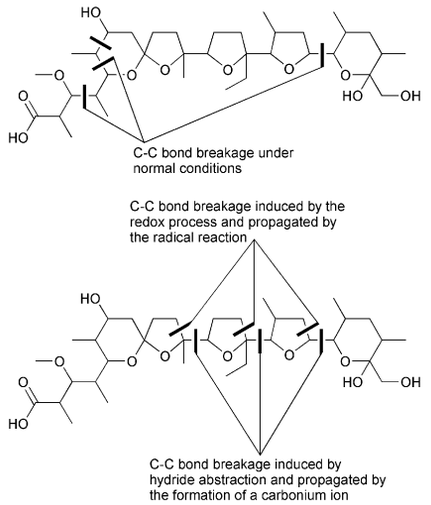
charged ion. The accurate mass-analysis shows that all the even massed fragment ions could be produced by this proposed mechanism resulting in the breakage of the bonds between pyran rings B–C (*m*/*z* 392), C–D (*m*/*z* 490) and D–E (*m*/*z* 574) along with the corresponding neutral losses of water (see Table 2). The remarkable ability of Cu^{II} to abstract an (activated) hydride, *via* a Lewis acid mechanism, thus leads to a novel fragmentation pathway not observed for the other metal cations studied and furnishing a series of ions, each with one less tetrahydrofuran ring.

The tendency of Cu^{II} to reduce its oxidation state to Cu^{I} could be applied to explain the type F fragments (odd massed). The oxidation state change could then be initiated by the bonding between Cu^{II} and the ether oxygen atom, as shown in Scheme 1(b). The breakage of the bond between the oxygen (ring B) and the carbon then follows, possibly assisted by another suitable sited oxygen (ring A). Further homolytic breakage of the bond between the copper and the oxygen then results in the reduction to Cu^{I} followed by a radical elimination similar to that observed in electron impact MS of simple ethers. The previously proposed radical species observed in the CID MS/MS in the presence of Cu^{II} strengthens the proposed mechanism.[8]

Alternative reactions at the other possible sites of redox-induced cleavage produce the rest of the type F fragment ions by breakage of the bonds between the rings E–D (*m*/*z* 229), D–C (*m*/*z* 327) and C–B (*m*/*z* 447) (see Scheme 2 and Table 2)



Scheme 2 The structures of the type F ions produced.



Scheme 3 A schematic of the C–C bonds in monensin that are broken under normal conditions and under the influence of Cu^{II}.

with their corresponding losses of water. The accurate masses (2.5 ppm average across the whole spectrum) are in full agreement with the proposed structures and are consistent with the presented mechanism.

Further studies were performed on monensin B (see Fig. 4b) which has a methyl group in place of the ethyl (Fig. 1) to confirm the proposed gas-phase redox process and to demonstrate the application of this methodology to identify the structural differences between monensin A and B. Monensin B showed the same series of even and odd massed fragment ions. The proposed Lewis acid induced bond breakage (rings B–C) produced the same ion at *m*/*z* 392 as observed for monensin A *via* hydride abstraction. Breakage between C–D and D–E produced the expected ions at 14 mass units less than observed for monensin A. When the redox-induced mechanism occurs, the ions produced by breakage between rings E–D and D–C have the same mass as those observed in the monensin A spectrum. Conversely, the ion produced by breakage between rings C–B is at 14 mass units less than the corresponding ion derived from monensin A. These results help confirm the proposed fragmentation pathway and successfully demonstrate a simple application of this methodology.

The formation of the two series of fragment ions, A and F, are the result of two different redox mechanisms of Cu^{II} in the gas-phase. In the first, the oxidation state change, Cu^{II} to Cu^{I}, occurs by abstraction of a hydride and further formation of the even-massed carbocations. In the former, the process involves homolytic breakage of a C–C bond after the radical elimination resulting in the formation of the odd-massed ions.

Conclusions

The results show that we have been able to chemically induce novel fragmentation pathways exploiting the unique Lewis acid and redox properties (propagated by a radical reaction) of Cu^{II} complexes of a natural product in the gas-phase. In the first mechanism (Scheme 1a), the heterolytic bond cleavage yields a carbonium ion and in the second mechanism (Scheme 1b), the homolytic bound cleavage affords an alkyl radical. These two separate features resulted in the formation of the two sets of fragment ions, summarised in Scheme 3, thus enabling the generation of novel gas-phase products, that have not been observed previously (*i.e.* with protonated or sodiated ions).[12,13]

This study presents strong evidence for the occurrence of a gas-phase redox process. The other possible factors that might induce alternative fragmentation (such as cation charge or size) have effectively been ruled out by the observation that the analysis of the Ag^{+}, Ca^{2+} and Ba^{2+} monensin complexes fragment in a very similar way to the Na^{+} complex. This work has significant implications in the study of ionophore metabolites as well as derivatives synthesised from genetically engineered systems.[18,19]

Experimental

Sample preparation

Samples of monensin A were obtained as sodium salts from either Aldrich (Gillingham, Dorset, UK) or from Sigma (Poole, Dorset, UK). LC-MS analysis clearly showed the presence of monensin A and B in the purchased samples. Pure samples of both natural products were obtained by LC-MS fraction collection. To obtain the calcium, barium, silver and copper adducts, one molar equivalent of the appropriate metal chloride was added to a solution of monensin in methanol : water (7 : 3, 1mg mL^{-1}). This resulted in the exchange of the sodium for the appropriate metal ion by at least 50% (as determined by ESI-MS). The resulting solutions were analysed by mass spectrometry without separation or recrystalisation.

Mass spectrometry

Low-resolution mass spectrometric analyses were performed on either a Quattro-LC or a Q-tof instrument (Micromass, Manchester, UK). Solutions were infused into the Z-spray ESI source at 5 μL min^{-1}. The accurate-mass MS/MS analyses were performed on a BioApex II (4.7 Tesla) Fourier-transform ion cyclotron resonance (FTICR) instrument (Bruker Daltonics, Billerica, USA) and solutions were infused from the ESI source at 100 μL h^{-1}. The MS/MS experiments were also performed on the Q-tof (Micromass, Manchester, UK). The precursor ions of the calcium, barium, silver and copper complexes of monensin were isolated prior to performing collision-induced dissociation (CID).

Acknowledgements

The authors would like to kindly thank Sarah Maslen, of the University of Cambridge, Chemistry Department Mass Spectrometry Service, for performing the Q-tof analyses. This work was supported by grants from the "Fundação de Amparo a Pesquisa do Estado de São Paulo" (Brazil), F. Hoffmann-La Roche Ltd. (Basel, Switzerland), the European Union, the "Biotechnology and Biological Sciences Research Council" (London, UK). Additional financial support was also provided by the "Unilever Safety and Environmental Assurance Centre" (Bedfordshire, UK).

References

1 C. G. Enke, T. L. Constantopoulos and G. S. Jackson, *J. Am. Soc. Mass Spectrom.*, 1999, **10**, 625.
2 M. H. Amad, N. D. Cech, G. S. Jackson and C. G. Enke, *J. Mass Spectrom.*, 2000, **35**, 784.
3 N. P. Lopes, C. B. W. Stark, H. Hong, P. J. Gates and J. Staunton, *Analyst*, 2001, **126**, 1630.
4 C. J. V. Berkel, *J. Mass Spectrom.*, 2000, **35**, 773.
5 M. A. Blitz, S. A. Mitchell and P. A. Hackett, *J. Phys. Chem.*, 1991, **95**, 8726.
6 R. C. Burnier, G. D. Byrd and B. S. Freiser, *J. Am. Chem. Soc.*, 1981, **103**, 4360.
7 D. J. Harvey, *J. Am. Soc. Mass. Spectrom.*, 2001, **12**, 926.
8 I. K. Chu, C. F. Rodrigues, A. C. Hopkinson and K. W. M. Siu, *J. Am. Soc. Mass. Spectrom.*, 2001, **12**, 1114.
9 J. Schen and J. S. Brodbelt, *Analyst*, 2000, **125**, 641.
10 C. T. Elliott, D. G. Kennedy and W. J. McCaughey, *Analyst*, 1998, **123**, 45.
11 D. E. Kiehl, R. K. Julian and A. S. Kennington, *Rapid Commun. Mass Spectrom.*, 1998, **12**, 903.
12 N. P. Lopes, C. B. W. Stark, H. Hong, P. J. Gates and J. Staunton, *Rapid Commun. Mass Spectrom.*, 2002, **16**, 414.
13 N. P. Lopes, C. B. W. Stark, P. J. Gates and J. Staunton, *Analyst*, 2002, **127**, 503.
14 Y. H. Kim, J. S. Yoo, C. H. Lee, Y. M. Goo and M. S. Kim, *J. Mass Spectrom.*, 1996, **31**, 855.
15 D. A. Volmer and C. M. Lock, *Rapid Commun. Mass Spectrom.*, 1998, **12**, 157.
16 C. A. Grob, *Angew. Chem., Int. Ed. Engl.*, 1969, **8**, 535.
17 P. S. Wharton and G. A. Hiegel, *J. Org. Chem.*, 1965, **30**, 3254.
18 P. F. Leadlay, J. Staunton, M. Oliynyk, C. Bisang, J. Cortés, E. Frost, Z. A. Hughes-Thomas, M. A. Jones, S. G. Kendrew, J. B. Lester, P. F. Long, H. A. I. McArthur, E. L. McCormick, Z. Oliynyk, C. B. W. Stark and C. J. Wilkinson, *J. Ind. Microbiol. Biotechnol.*, 2001, **27**, 360.
19 M. Oliynyk, C. B. W. Stark, A. Bhatt, M. A. Jones, Z. A. Hughes-Thomas, C. Wilkinson, Z. Oliynyk, Y. Demydchuk, J. Staunton and P. F. Leadlay, *Mol. Microbiol.*, 2003, **49**, 1179.