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Biomimetic oxidation studies of monensin A catalyzed by metalloporphyrins: identification of hydroxyl derivative product by electrospray tandem mass spectrometry

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Abstract: Monensin A is an important commercially available natural product isolated from *Streptomyces cinnamonensins* that shows antibiotic and anti-parasitic activities. This molecule has a significant influence in the antibiotic market, but until now there are no studies on putative metabolite formations. Bioorganic catalysts applying metalloporphyrins and mono-oxygen donors are able to mimic the cytochrome P450 reactions. This model has been employed for natural product metabolism studies affording several new putative metabolites and *in vivo* experiments confirming the relevance of this procedure. In this work we evaluated the potential of 10,15,20-tetrakis (pentafluorophenyl) porphyrin metal(III) chloride [Fe(TFPP)Cl] catalyst models to afford a putative monensin A metabolite. Oxidation agents such as *meta*-chloroperoxy benzoic acid, iodosylbenzene, hydrogen peroxide 30 wt.% and *tert*-butyl hydroperoxide 70 wt.%, were used to investigate different reaction conditions, in addition to the analysis of the influence of the solvent. The quantification of total monensin A conversion and the structure of the new hydroxylated putative metabolite were proposed based on electrospray ionization tandem mass spectrometry analysis. The porphyrin tested, afforded moderate conversions of monensin A in all reaction conditions and the selectivity was found to be dependent on the oxidation/medium employed.

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Introduction

Monensin A (MonA) (Figure 1) is an important, commercially available polyether ionophore drug (isolated from *Streptomyces cinnamonensins*) that shows antibiotic and anti-parasitic activity. It is widely used as a coccidiostat in avian species and as a growth promoter in cattle (Agtarap et al., 1967; Huczyński, 2012). Ionophores show the ability to form stable, electrically neutral complexes with alkali metal cations and their antibiotic activity is related to their ability to transfer ions across the cell membrane barrier (Pressman et al., 1967; Lowici & Huczyński 2013; Huczyński, 2012). MonA is characterized by a narrow safety margin and several accidental poisonings have been described in animals (Nebbia et al., 1999). Nebbia and co-workers (1999) conducted a study with liver microssomes to characterize which P450-isoenzyme is involved in the biotransformation of the ionophore and also investigated how this process may be affected by tiamulin and other chemotherapeutic agents know to produce toxic interactions with MonA. In this study it was defined that P450 3A is important for the oxidative metabolism of MonA and helps to define toxic interactions. The P450 3A subfamily is the major P450 enzyme present in the human liver and is involved in the metabolism of approximately half of the drugs commercially available. In this respect the possibility that humans could ingest residues of MonA from foodstuffs of animal origin stimulated the investigation of the phase 1 metabolization (Nebbia et al., 1999). Recent reports of significant levels of polyether antibiotic residues in chicken meat and eggs have lead to concerns about antibiotic resistance and other possible health problems (Harris et al., 1998; Rosen, 2001). These two separate issues have encouraged the development of studies to

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better understand the mechanism of metabolization. The commercial importance of the polyether as a feed additive in poultry farming has also driven the search for new derivatives (Elliot et al., 1998).

The cytochromes P450 (CYP450) enzymes are well known for their roles in the oxidative metabolism in living organisms and for their involvement in the formation of a wide variety of xenobiotic metabolites (Mansuy, 2007; Montellano, 2004). Several *in vitro* procedures can be use to predict the phase 1 reactions carried out by P450 and synthetic metalloporphyrins, in the presence of oxygen donors, are able to mimic oxidation and oxygenation transformations. Initially, these procedures were successfully applied for drug development (Macleod et al., 2007; 2008) and recently started to be use as a model to understand natural products phase 1 metabolization.

These biomimetic procedures showed good transformation yields of natural products such as quinones (Pires et al., 2011; Niehues et al., 2012), alkaloids (Schaab et al., 2010), lignans (De Santis Ferreira et al., 2012) and chrologenic acid derivatives (Santos et al., 2005; 2008), but revealed no effect on flavonoid oxidation or oxygenation. Sequential *in vitro* reactions with CYP450 enzymes and *in vivo* analysis confirm the presence of the obtained putative metabolites and validate the procedure. Taking together these results shows the great versatility of metalloporphyrins as biomimetic models to understand phase 1 metabolism of natural products. In addition, metalloporphyrin catalysts exhibit a few advantages over standard *in vitro* methods (Bernadou & Meunier, 2004; Lohmann & Karst, 2008), like the production of sufficient amounts of the metabolites with good enough purity to allow for their structural characterization, as well as pharmacological and toxicological testing. This drastically reduces the number of live animals needed for metabolization investigations (Bernadou & Meunier, 2004). Taken together, the above facts have stimulated this current investigation of the *in vitro* oxidation of MonA in homogeneous medium by various oxidants, catalyzed by [Fe(TFPP)Cl] and to compare the products obtained furhter in *in vivo* analysis. The most important assays for determination of monensin A which have been published use high-performance liquid chromatography. Since the ionophores do not possess any significant UV absorbance, it is preferable to use techniques which have the highest possible specificity, and this generally means some type of mass spectrometry (Blanchflower & Kennedy, 1996). Applications of tandem mass spectrometry coupled with HPLC in the identification and determination of drug metabolites are the most promising technique for a sensitive detection, positive identification and quantitation of metabolites *in vitro* metabolism studies (Holčapek et al., 2008).

Materials and Methods

Material

MonA was purchased from Sigma-Aldrich. The porphyrin 5,10,15,20-tetrakis(pentafluorophenyl) porphyrin, H2TFPP, was acquired from Mid-Century. Metal insertion into this free base-porphyrin was performed out using the method of Adler et al. (1970). Hydrogen peroxide (30% solution in water) was stored at 5 °C and titrated periodically for purity assessment. *Tert*-butyl hydroperoxide (*t*-BuOOH), 70% solution in water and meta-chloroperoxy benzoic acid (*m*-CPBA) was acquired from Acros Organics. Iodosylbenzene (PhIO) was synthesized by hydrolysis of iodosylbenzenediacetate using a procedure adapted from the literature (Saltzman et al., 1973). The solid was carefully dried under reduced pressure and kept at 5 °C; its purity was periodically controlled by iodometric titration (Lucas et al., 1955). Solvents such as dichloromethane (CH_2Cl_2), dichloroethane ($C_2H_4Cl_2$), acetonitrile (MeCN) and methanol (MeOH) were HPLC grade. All compounds used in this study were commercially available from Aldrich or Sigma and were of analytical grade purity unless otherwise stated.

General oxidation procedure

Initially the reaction conditions were optimized by varying the concentration of catalysts and oxidizers. All reactions were performed in an Eppendorf flask (2 mL) under mechanical stirring (Vibrax VXR agitator) at 25 ˚C. The reactions were performed in an atmosphere of air for 24 h. After this time the products were analyzed by silica gel analytical TLC using a mixture of $CHCl_3$:MeOH (93:7) as eluent. After elution, the plates were revealed with solution of vanillin-sulfuric acid (1% vanillin and 1% H_2SO_4 in ethanol) for visualization of the products. In a typical experiment of our group (Macleod et al., 2008; Dos Santos et al., 2011) the ideal proportions obtained catalyst/oxidant/substrate molar ratio was 1:20:20 achieved by adding 0.3 mM:6 mM:6 mM in the CH_2Cl_2 reaction medium (no product formation in the blank sample). After determination of the conditions of the reaction, and its proportions to a study conducted by varying the types of reaction mediuns (CH_2Cl_2, $C_2H_4Cl_2$, MeCN and MeOH), the oxidant used (*m*-CPBA, PhIO, *t*-BuOOH and H_2O_2) total of sixteen reactions were analyzed by HPLC-ESI-MS. Control reactions were carried out in the absence of catalyst under the same conditions as the catalytic runs and no products were detected.

HPLC-ESI-MS analysis

The analysis of products formed in oxidation reactions was developed by liquid chromatography

coupled to mass spectrometry with ESI ionization using Varian chromatograph LC-MS 1200L triple quadrupole, in the positive ion mode with a capillary voltage of 3.2 kV, a cone voltage of 40 V, source temperature 40 ˚C and N2 dessolvation temperature of 250 ˚C, monitored from *m/z* 610 to 800. Table 1 shows the conditions employed in the chromatographic analyses. The conversion of MonA was quantified by comparison of the retention time and unit mass, based on a calibration curve from 10 to 100 µg/mL.

Table 1. Chromatographic conditions used to quantify the yield of conversion of monensin in the biomimetic reactions.

Chromatographic conditions			
Analytical Column	Xterra MS-C18, 150 mm x 2.1mm, 5µm		
Solvent A	Water (0.1% formic acid)		
Solvent B	MeOH (0.1% formic acid)		
Flow rate	0.3 mL/min		
Mobile phase gradient	Time	%A	%B
	0:00	30	70
	20:00	2	98
	21:00	30	70
	30:00	30	70
Injection volume	10 µL		
Sample concentration	50 µg/mL		

Product elucidation by tandem mass spectrometry

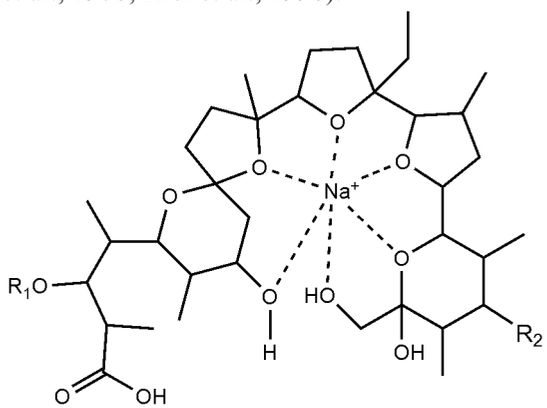
The fragmentation studies were made in accordance with previous analyses by this group (Lopes et al. 2001; 2002a, b). The low resolution MS/MS spectrum of the metabolite product (*m/z* 709) was obtained using a Waters Quattro-LC triple quadrupole mass spectrometer, with Z-spray ESI source operating in the positive ion mode. The samples were directly introduced into the spectrometer through an infusion pump (Harvard Apparatus Holliston MA 01746) at a flow rate of 10 µL/min. The capillary temperature and voltage were maintained at 250 ˚C and 3 kV, respectively and the cone energie was 25kV. Nitrogen was used for spraying and drying, and argon was used as the collision gas. MS/MS analysis of the precursor ion was performed with collision energies in 20-70 eV range.

Accurate-mass analyses were performed on an Apex IV (7 Tesla) Fourier-transform ion cyclotron resonance instrument (Bruker Daltonics, Coventry UK). Solutions were infused from a Cole-Parmer syringe pump into the Apollo ESI source at 120 µL/h. The source temperature was 200 ˚C and the source potential was 4.6 kV. Fragmentation was performed on the isolated precursor ions by sustained off-resonance irradiation (SORI) CID using CO_2 collision gas. The SORI-CID parameters were tuned to fragment all of the precursor ions to maximize product ion generation. Accurate-masses were obtained by a post-acquisition application of a calibration based on the MS/MS analysis of Erythromyicn A obtained with the same cell pressure and SORI-CID conditions.

Results and Discussion

Oxidation reactions

Metabolism studies of MonA have been performed using several species (steers, rats, calves and chickens). The results show that this drug is biotransformed extensively to a large number of metabolites that are qualitatively similar (Donoho et al., 1978; Kiel et al., 1998). Some metabolites have been tentatively identified previously (for example from steers, rats, calves and chickens) which appear to be the result of an *O*-demethylation and/or hydroxylation at various positions along the carbon backbone of the ionophore molecule (Figure 1) (Davison 1984; Donoho et al., 1978; Kiel et al., 1998).



Mon. A	(*m/z* 693)	R1=CH_3, R2=H
M1	(*m/z* 679)	R1=H, R2=H
M2	(*m/z* 709)	R1=CH_3, R2=OH
M3	(*m/z* 695)	R1=H, R2=OH

Figure 1. Monensin A and the main metabolites found in *in vivo* systems.

In this study, the oxidation of MonA was performed in the presence of the [Fe(TFPP)Cl], which is a commercially available second generation metalloporphyrin well established in the literature as a good catalyst for drug oxidation (Macleod et al., 2007; 2008; Faria et al., 2008; Da Silva et al., 2011). The reactions were carried out in different media as the solubility of the different metabolite products was unknown. The substrate conversion was determined by a calibration curve (Y=0.2176X+4.535, where X= concentration and Y=peak area, r=0.993). Table 2 shows the effect of different solvents and oxidants on MonA oxidation catalyzed by [Fe(TFPP)Cl]. The best conditions for conversion/oxidation of MonA achieved was 63% conversion using the following system PhIO/MeCN.

The drug metabolites were more soluble in either dichloromethane or dichloroethane, and high catalytic activity was obtained to Reaction 10, 6 and 1 as show Table 3. The efficiency of the catalyst in methanol was lower compared to acetonitrile and chlorinated solvents, this fact can be explained by the alcohol acting as a substrate, thus competing with MonA for the catalytic species, leading to the formation on unwanted by-products (Macleod et al., 2008). In other solvents, which do not compete with MonA, the catalyst was able to oxidize the drug with yields as high as 63%. Such high yields are due to the higher ability of MeCN to stabilize the intermediate catalytic species since this solvent has a high donor number (DN=14.1) this system was also the most efficient in several others reports (Bahramian et al., 2006; Macleod et al., 2007; 2008). Another factor that influences the yields of the reactions is the viscosity of the solvent used. The highest yields when comparing $C_2H_4Cl_2$ with CH_2Cl_2 is due to higher viscosities. Viscous solvents favour "in cage" over "out of cage" of MonA with porphyrin (Smith et al., 2006).

PhIO was the first oxidant to be used as an oxygen donor in this study because it is considered a standard and simple oxidant that contains only one oxygen atom and is well-adapted for the selective and clean formation of metal-oxo intermediates (Groves et al., 1997; Mansuy, 2007; Bahramian et al., 2006). The initial step in the mechanism involves the abstraction of a hydrogen atom from the substrate by this intermediate to form a caged substrate radical and FeIV hydroxide complex. This step is followed by recombination of the hydroxy fragment with the substrate radical thus generating a hydroxylated metabolite, corresponding to the oxygen rebound model (Mansuy, 2007; Bahramian et al., 2006). The oxidation of the substrate led to the preferential formation of the hydroxylated product at *m/z* 709. M-CPBA, H_2O_2 and *t*-BOOH were also employed. These oxidants can undergo heterolytic cleavage upon coordination to the metalloporphyrin central metal ion, which gives rise to the active species: oxoferryl porphyrin π-cation radical, $Fe^{IV}(O)P^{\bullet+}$ or $Fe^{V}(O)P$. Homolytic cleavage of the O-O bond may also occur leading to formation of a less reactive intermediate, Me (OH) IVP, as well as the formation of

Table 2. Total conversion of monensin A (%) by different oxidants and in different media catalyzed by Fe(TFPP) analyzed by LC-ESI-MS.

	Reaction	%	Reaction	%	Reaction	%	Reaction	%
m-CPBA	1	57	5	36	9	39	13	23
PhIO	2	49	6	61	10	63	14	15
H_2O_2	3	36	7	18	11	19	15	2
t-BUOOH	4	44	8	44	12	18	16	21
Media	CH_2Cl_2		$C_2H_4Cl_2$		MeCN		MeOH	

*Control reaction in the absence of Fe(TFPP), 0% conversion.

Table 3. The effect of different solvents and oxidants on products obtained in biomimetic reactions of monensin A.

Biomimeticreaction	Reaction medium	Oxidants	*m/z*			
			679	695	709	707
1	CH_2Cl_2	*m*-CPBA	X	-	X	-
2		PhIO	-	-	X	-
3		H_2O_2	-	-	X	X
4		*t*-BUOOH	X	X	-	X
5	$C_2H_4Cl_2$	*m*-CPBA	X	-	X	-
6		PhIO	-	-	X	-
7		H_2O_2	-	-	X	X
8		*t*-BUOOH	X	X	-	X
9	MeCN	*m*-CPBA	-	X	X	X
10		PhIO	-	-	X	-
11		H_2O_2	-	X	-	X
12		*t*-BUOOH	-	X	-	X
13	MeOH	*m*-CPBA	-	X	-	X
14		PhIO	-	-	-	-
15		H_2O_2	-	-	-	-
16		*t*-BUOOH	-	X	-	X

RO• radicals, thus favoring the occurrence of radical mechanisms (Dos Santos et al., 2011; Meunier & Bernadou 2000; Meunier et al., 2004). The catalytic activity seems to be highly dependent on the nature of the oxidant - PhIO was the most efficient, followed by *m*-CPBA. Reactions using H_2O_2 afforded the lowest yields, which can be explained by the catalyzed dismute process of this oxidant in the presence of iron-porphyrin. Moreover, hydrogen peroxide was chosen because it is considered to be a clean oxidant (water is the only side-product produced) and the high yield was obtained in reaction 3 using CH_2Cl_2 as the solvent.

Table 3 shows the formation of hydroxylated (*m/z* 709), epoxydized (*m/z* 707), *O*-demethylated (*m/z* 679) and *O*-demethylated/hydroxylated compounds (*m/z* 695) as products from the biomimetic oxidation of MonA.

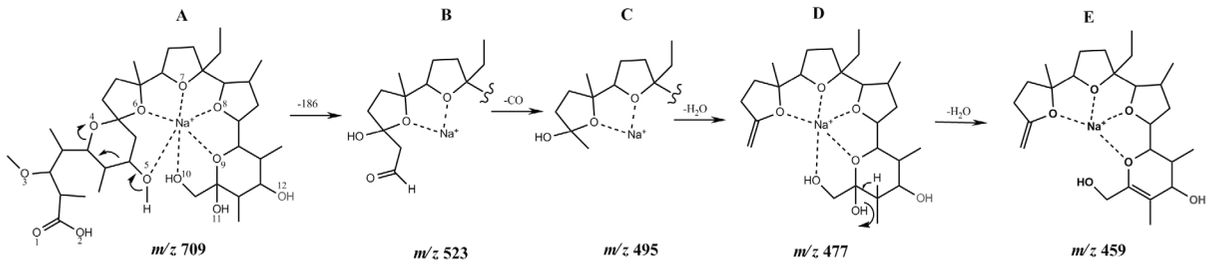
Fragmentation studies of hydroxylated product (m/z *709*)

A previous systematic investigation carried out of MonA by ESI-MS showed that it is possible to detect $[M+H]^+$ only at low pH and high cone voltage (Lopes et al., 2002a,b). The addition of an excess of crown ethers to the solution was shown to increase the relative intensity of this molecular ion through competition of the crown ether for the sodium cation (Lopes et al., 2002a,b). This study revealed some limitations in employing the protonated precursor ion in tandem mass spectrometric studies and revealed the importance understanding the fragmentations pathways of the sodiated sodium salts as observed for others ionophors (Lopes 2002c, Fonseca et al., 2004). Lopes et al., (2002a,b) found that extensive fragmentation of the sodium salts was possible either by increasing the energy of the CID process. The full fragmentation mechanism of MonA sodium salt was proposed and this analysis demonstrated that the common fragmentation ions were produced via a Grob-Wharton type mechanism (Grob & Baumann, 1955; Grob, 1969; Lopes et al., 2002a,b; Wharton & Hiegel 1965). The Grob-Wharton fragmentations occurred at two different sites in the molecule generating two major fragments that produced the remaining ions through either pericyclic rearrangements or by simple neutral losses. The same fragmentation mechanisms occur for the hydroxylated product. In the low resolution ESI-MS/MS spectrum of the hydroxylated product, the structural fragments are possibly resulting in significant fragmentation due to Grob-Wharton type mechanisms and/or elimination of H_2O, as previously proposed by Lopes et al., (2002 a,b). The molecular formula of the hydroxylated ion were obtained by high-resolution accurate-mass MS/MS and the results with errors are shown in Table 4.

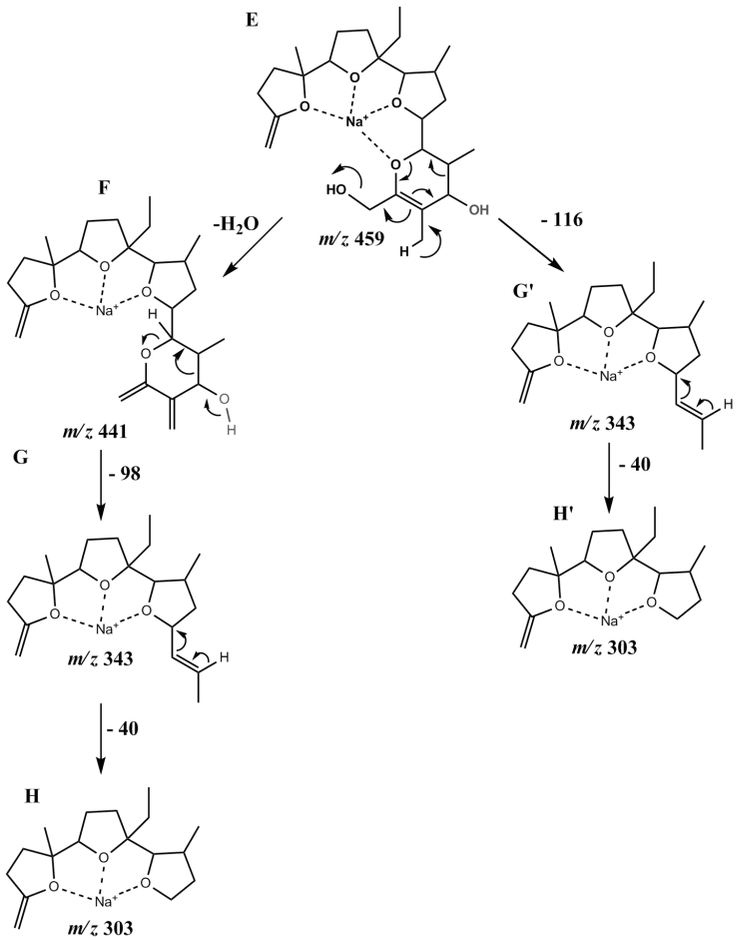
Scheme 1 shows the proposed fragmentation of the hydroxylated product (*m/z* 709; ion A). In this scheme, we have proposal that the site of protonation is the acid function, in accordance with all published data on the three-dimensional structure of MonA in the solid state (Duax et al., 1980; Martinek et al., 2000; Paz et al., 2003). The mechanism is initiated by the neutral elimination of mass 186 via a Grob-Wharton fragmentation involving ring A (O-4) (Grob & Baumann, 1955; Grob, 1969; Lopes et al., 2002a, b; Wharton & Hiegel 1965) resulting in the formation of product ion B (*m/z* 523). Fragment B loses CO and H_2O, resulting in ions C and D (*m/z* 495 and *m/z* 477), which have high intensity in the product ion spectra.

Table 4. Molecular formula, observed and calculated masses, mass error (ppm) and fragment scheme identify for all the fragment ions observed in the accurate-mass ESI-MS/MS spectrum of the metabolite *m/z* 709.

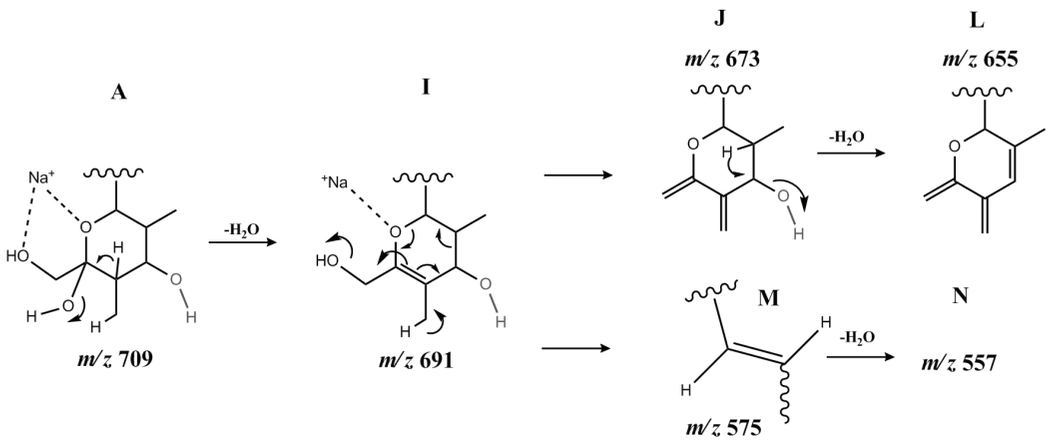
Formula	Observed mass	Calculated mass	Error	Identify
$C_{36}H_{62}O_{12}Na^+$	Not observed	709.4139	-	*m/z* 709
$C_{36}H_{60}O_{11}Na^+$	691.4022	691.4033	-1.59	I
$C_{36}H_{58}O_{10}Na^+$	673.3927	673.3928	-1.48	J
$C_{36}H_{56}O_9Na^+$	655.3829	655.3822	+1.06	L
$C_{31}H_{52}O_8Na^+$	Not observed	575.3560	-	M
$C_{31}H_{50}O_7Na^+$	557.3439	557.3454	-2.69	N
$C_{26}H_{44}O_9Na^+$	Not observed	523.2883	-	B
$C_{28}H_{46}O_7Na^+$	517.3142	517.3141	+1.93	P
$C_{28}H_{40}O_6Na^+$	499.3026	499.3036	-2.00	Q
$C_{25}H_{42}O_7Na^+$	459.2717	459.2723	-1.30	E
$C_{26}H_{42}O_4Na^+$	441.2727	441.2716	+2.49	R
$C_{20}H_{32}O_3Na^+$	343.2268	343.2249	+1.90	G
$C_{17}H_{28}O_3Na^+$	303.1938	303.1936	+6.59	H



Scheme 1. The proposed fragmentation route showing the formation of product ion *m/z* 459 in the positive ion ESI-MS/MS of the hydroxylated metabolite of monensin A.

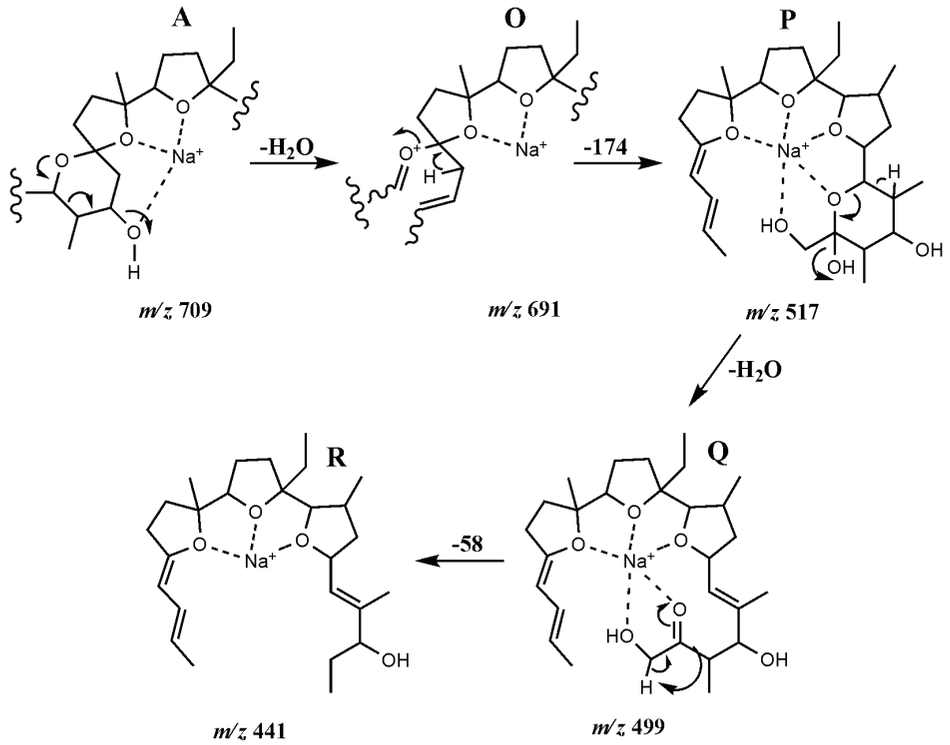


Scheme 2. The proposed fragmentation route showing the formation of product ion *m/z* 303 in the positive ion ESI-MS/MS of the hydroxylated metabolite of monensin A - a crucial ion for the location of the site of hydroxylation.



Scheme 3. The proposed fragmentation route showing the formation of product ion *m/z* 655.

The formation of product ions *m/z* 343 and *m/z* 303 (Scheme 2) from product ion E can occur in two different mechanistic pathways. Step one, involves the loss of H_2O of the tetrahydrofuran ring, forming the product ion M (*m/z* 441) which can give rise to the product ion H (*m/z* 303) by a Grob-Wharton type fragmentation, followed by a neutral loss of mass 40 (assumed to be the elimination of 1,4 propyne in a charge remote mechanism). In the second step, the product ion *m/z* 303 is formed by loss of mass 156 from E via a pericyclic rearrangement analogous to the elimination of propyne from dihydropyran affording ion G' which lose the side chain given *m/z* 303. The analysis in this study, combined with our previous fragmentation studies of monensin A, led us to propose the structure of



Scheme 4. Proposed mechanism of fragmentation involving the dihydropyran system and the formation of product ion *m/z* 441 - crucial for locating the site of hydroxylation in the MonA metabolite.

the hydroxylated metabolite shown in schemes 1 and 2.

The second proposed fragmentation pathway of *m/z* 709 (Scheme 3) is initiated by the loss of water involving oxygen O-11 to produce product ion I (*m/z* 691). Ion I can then undergo a pericyclic rearrangement analogous to that in the dihydropyran fragment, to produce product ion M *m/z* 575 or the neutral loss of water involving oxygens O-10 and O-11 to form product ion L *m/z* 655. The route shown is believed to be the most likely due to the formation of the conjugated π-system and because the pericyclic rearrangement leading to a loss of mass 116 is not observed as shown in Scheme 3.

The relationship of the hydroxy group (O-5) and the ether oxygen (O-4) in ion A, induces a loss of water, to produce intermediate ion O (*m/z* 691) resulting in a third possible route for the fragmentation of *m/z* 709 - shown in scheme 4. Ion O can fragment to give product ion P *m/z* 517 via the loss of mass 174.

The fragmentation mechanism proposed in scheme 4 suggests the possibility of a water elimination from the dihydropyran system of product ion P (*m/z* 517) resulting in product ion Q which inturn can go on to fragment by a proton migration in the position α to the carbonyl, to form product ion R (*m/z* 441) by loss of mass 58. The existence of product ion R (*m/z* 441) may prove to be highly significant as it is the product ion that is crucial for determining the position of hydroxylation in the precursor ion – the MonA metabolite.

In conclusion, these results have demonstrated the ability of the [Fe(TFPP)Cl] iron porphyrins to mimic the action of CYP P450 in monensin A oxidation, with the formation of a main products. The formation of these products is highly dependent on the oxidant and reaction medium. Our studies have also demonstrated the potential application of these biomimetic methods as an alternative model in the synthesis/search of new drug derivatives of antibiotics. This should provide samples sufficient for biological tests, as well as aid studies that pursue the elucidation of *in vivo* drug metabolism. In the study, a hydroxylated metabolite of MonA was elucidated by high-resolution accurate-mass tandem mass spectrometry. We also have presented a complete fragmentation pathway for the hydroxylated derivative of monensin and the formulae of all fragments have been proved by accurate-mass analysis. The fragmentation studies presented could be of significant use for future identification of others monensin metabolites and showed that LC-ESI-MS/MS is a powerful tool to study the *in vitro* metabolism of drugs, allowing the identification and structural elucidation of novel metabolites.

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Authors' contributions

JNSJ contributed in metalloporphyrins reactions and structure elucidation. BAR to metalloporphyrins reactions, low resolution LC-MS analysis and manuscript writing. MDA and YI contributed to metalloporphyrins reactions. APFP and LABM contributed to low resolution LC-MS analysis. ARMO contributed in LC-MS analysis, metalloporphyrins reactions and critical reading of the manuscript. NPL contributed to high resolution mass acquisition, data analysis and strutuce elucidation. PJG contributed to high resolution mass acquisition and critical reading of the manuscript. PJG and All authors have read the final manuscript and approved the submission.

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