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Utilisation of electrospray time-of-flight mass spectrometry for solving complex fragmentation patterns: application to benzoxazinone derivatives

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In this paper we describe the application of electrospray time-of-flight mass spectrometry (ESI-TOFMS) to structural elucidation of the fragment ions formed from a range of natural and synthetic allelochemical derivatives. The extensive mass spectrometric characterisation of ten non-glucosylated benzoxazinone derivatives using this method is described here for the first time. The analytes include six naturally occurring 1,4-benzoxazin-3(4*H*)-one derivatives, including the hydroxamic acids DIMBOA [2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and DIBOA [2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one], lactams HBOA [2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one] and HMBOA [2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one], benzoxazolinones BOA [benzoxazolin-2(3*H*)-one] and MBOA [6-methoxy-benzoxazolin-2(3*H*)-one] and four synthetic variations, 2′H-DIBOA [4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one], 2′OMe-DIBOA [2-methoxy-4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one], 2′H-HBOA [2*H*-1,4-benzoxazin-3(4*H*)-one] and 2′OMe-HBOA [2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one]. Assignments of the mass spectral fragments were aided by elemental composition calculation results, comparison of structural analogues and background literature, and acquired knowledge regarding feasible structures for the compounds. The influence of substituents on the chemical reactivity of the compounds with respect to the observed MS behaviour over varying nozzle potentials is addressed and, through comparison of the structural analogues, generic fragmentation patterns have also been identified.

KEYWORDS: electrospray time-of-flight-mass spectrometry (ESI-TOFMS); structural elucidation; allelochemicals; benzoxazinones; benzoxazolinones; fragmentation patterns

INTRODUCTION

The benzoxazinones are a family of compounds produced by a broad range of plants, including commercially important cereal crops such as maize, rye and wheat. These compounds have been implicated in the bioactive and allelopathic properties (natural defence mechanisms) of these plants and as such are of significant scientific interest.[1,2] The benzoxazinones are present in the plants as complex mixtures of the relatively non-toxic glucoside derivatives (Fig. 1, R1 = Glc).[3,4] Upon injury of the plant, enzymatic deglucolysation occurs to release biologically active aglucones (Fig. 1, R1 = OH).[5] Rapid conversion of these compounds occurs to give the benzoxazolinones (Fig. 1),[6] and further metabolic conversion of these compounds in soil has also been described.[7–9]

The biological activity is associated in particular with the DIMBOA (2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) and DIBOA (2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one) derivatives (R1, R2 = OH).* These compounds possess the combination of both the cyclic hemiacetyl and the hydroxamic acid moieties, the instability of which leads to their high reactivity. However, varying degrees of biological activity are known for a broad range of non-glucosylated derivatives,[1,7,8,10,11] demonstrating the highly advanced nature of the mechanism by which plants utilise the benzoxazinones and their subsequent derivatives to perform their allelopathic function.

The relatively high enzymatic, hydrolytic and oxidative instability of the benzoxazinones provides a mechanism for

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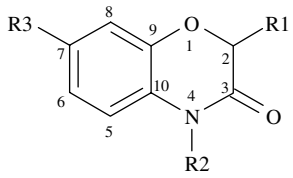
*Acronyms derived from the IUPAC chemical nomenclature are commonly adopted to describe the benzoxazinone derivatives for simplicity, and these will also be used here, i.e.
DIBOA = 2,4-**di**hydroxy-2*H*-1,4-**b**enz**o**x**a**zin-3(4*H*)-one;
DIMBOA = 2,4-**di**hydroxy-7-**m**ethoxy-2*H*-1,4-**b**enz**o**x**a**zin-3(4*H*)-one.



Naturally occurring Derivatives:

Hydroxamic acids (R2 = OH)		
R1 = OH	R3 = H	DIBOA
R1 = OH	R3 = OCH_3	DIMBOA
Lactams (R2 = H)		
R1 = OH	R3 = H	HBOA
R1 = OH	R3 = OCH_3	HMBOA

Benzoxazolinones	
R = H	BOA
R = OCH_3	MBOA

Synthetic Derivatives:

Hydroxamic acids (R2 = OH)		
R1 = H	R3 = H	2′H-DIBOA
R1 = OCH_3	R3 = H	2′OMe-DIBOA
Lactams (R2 = H)		
R1 = H	R3 = H	2′H-HBOA
R1 = OCH_3	R3 = H	2′OMe-HBOA

Figure 1. Structures of benzoxazinones investigated.

their biological activity and this high chemical instability also translates to their behaviour under mass spectrometric (MS) conditions, with a high level of fragmentation observed. However, characterisation of the fragments of the biologically active non-glucosylated benzoxazinone and benzoxazolinone derivatives has not yet been described, and was the primary objective of this investigation. Application of atmospheric pressure chemical ionisation tandem mass spectrometry (APCI-MS/MS) to a range of glucosylated derivatives has been reported,[12,13] with this pioneering work demonstrating the applicability of atmospheric pressure ionisation (API-) MS methods to the structural elucidation of the benzoxazinone class.

Electrospray time-of-flight mass spectrometry (ESI-TOFMS) offers added advantages over this technique as information about molecular formulae can also be obtained through the accurate mass data and also allows the identification of unknown compounds with a sufficient high resolution enabling unequivocal assignment in many cases.[14–17] Therefore, in this study, the mass spectrometric behaviour of a range of benzoxazinones was investigated for the first time using ESI-TOFMS, whereby providing further information regarding the MS behaviour of these compounds, with the aim of further understanding degradation processes and to provide an advanced strategy for screening for as-yet undiscovered analogues and metabolites.

EXPERIMENTAL

Materials

Synthesised DIMBOA, DIBOA, HBOA [2-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one), HMBOA [2-hydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) and the non-naturally occurring derivatives 4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (2′H-DIBOA), 2-methoxy-4-hydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (2′MeO-DIBOA), 2*H*-1,4-benzoxazin-3(4*H*)-one (2′H-HBOA) and 2-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (2′ MeO-HBOA) were purchased from Prof. D. Sicker (University of Leipzig, Germany). DIMBOA (maize extract) was received from Prof. D. Sicker, Dr. S. Chilton (University of North Carolina, USA) and Dr. F. Macias (University of Cadiz, Spain), and BOA [benzoxazolin-2(3*H*)-one] and MBOA [6-methoxybenzoxazolin-2(3*H*)-one] were purchased from Sigma-Aldrich and Fluka, respectively. The high purity of all compounds was predetermined by a range of analytical methods including HPLC-UV, HPLC/MS,[18] NMR and TLC. Pure standards of extracts were obtained by solvent extraction, chromatography and recrystallisation, such that the purity of all compounds was >96%. The synthetic compounds were used for the diagnostic investigations. These were purified by recrystallisation and their purity checked by TLC, ^{1}H and ^{13}C NMR, where at least 99% purity was indicated for all compounds. Spectral quality of the FIA-ESI-TOFMS spectra obtained here was ensured by comparison with ESI-MS spectra obtained following online HPLC chromatography in an independent study.[18] No discrepancies were found between the spectra, either for the synthetic versus extracted standards, or for the FIA versus online HPLC measurements.

Stock solutions (1 mg mL^{-1}) of individual standards were prepared by dissolving accurate amounts of pure standards in methanol (MeOH). Working standard solutions (1 ng μL^{-1}) were obtained by serial dilution of the stock solutions with 60 : 40 : 1 MeOH/H_2O/HOAc. HPLC-grade solvents [water (H_2O), MeOH and acetonitrile (ACN)] and 98% pure acetic acid (HOAc) purchased from Merck (Darmstadt, Germany) were used for all sample preparations and analyses.

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Analysis

Measurements were performed on a Mariner ESI-*orthogonal*-TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) under the following operating parameters: (A) Source: spray tip potential, 3.5 kV; SCIEX heater, 280 °C; (B) ESI interface: skimmer 1 potential, 10 V; quadrupole DC potential, 7 V; deflection voltage 0.24 V; single lens potential, −25; quadrupole RF voltage, 600; quadrupole temperature, 140 °C; (C) Analyzer: accelerating potential, 4 kV; reflector potential, 1.55 kV; detector voltage, 2.2 kV; path length, 1 m; push pulse potential, 470 V; pull pulse potential, 200 V; pull bias potential, 14 V. An elution solvent of 50 : 50 H_2O/ACN at a flow rate of 200 μL min^{-1} was used. The mass axis was externally calibrated using a mixture of peptides over the range m/z 300–1000. Pure solutions (1 μg mL^{-1}) of the benzoxazinones were analysed by flow injection analysis (FIA) in negative ionisation mode. The instrument was run in continuum mode with samples (20 μL) injected multiple times under varying orifice voltages (−20 to −220 V). Spectra were obtained as background-subtracted averages taken over the entire sample injection. Spectra were acquired over the range m/z 80–300 at a scan rate of 1 s per spectrum. Elemental composition calculations were performed offline using the Applied Biosystems Data Explorer software, version 4.0.0.1. Potential assignments were calculated using the monoisotopic masses with specifications of a tolerance of 20 mDa or 100 ppm deviation and both odd- and even-electron states possible.

RESULTS AND DISCUSSION

A broad variation in stability under API-MS conditions was observed for the range of benzoxazinones investigated. Certain derivatives, namely the hydroxamic acids such as DIBOA and DIMBOA, demonstrated extreme instability in the spectrometer, parallel with the known increased chemical instabilities and biological efficacies of these derivatives.[1,2,10] Consequently, the $[M-H]^-$ ions for these analytes gave only negligible response, and a large number of fragments were observed. In contrast, the benzoxazolinone derivative BOA was highly stable and showed extremely limited fragmentation under the very strongest of fragmentor voltage conditions.

The ESI-TOFMS spectra obtained for the benzoxazinone DIMBOA, over a range of different fragmentor voltages, are shown in Fig. 2. The mass spectrometric (MS) behaviour of this derivative is synonymous with its known reactivity, as is demonstrated by the high level of fragmentation observed and low contribution of the $[M-H]^-$ ion (m/z 210) even at extremely low nozzle potential (−20 V, Fig. 2(a)). Two main fragment ions, at m/z 164 and 149, can be observed, the former being dominant at intermediate fragmentor voltages,

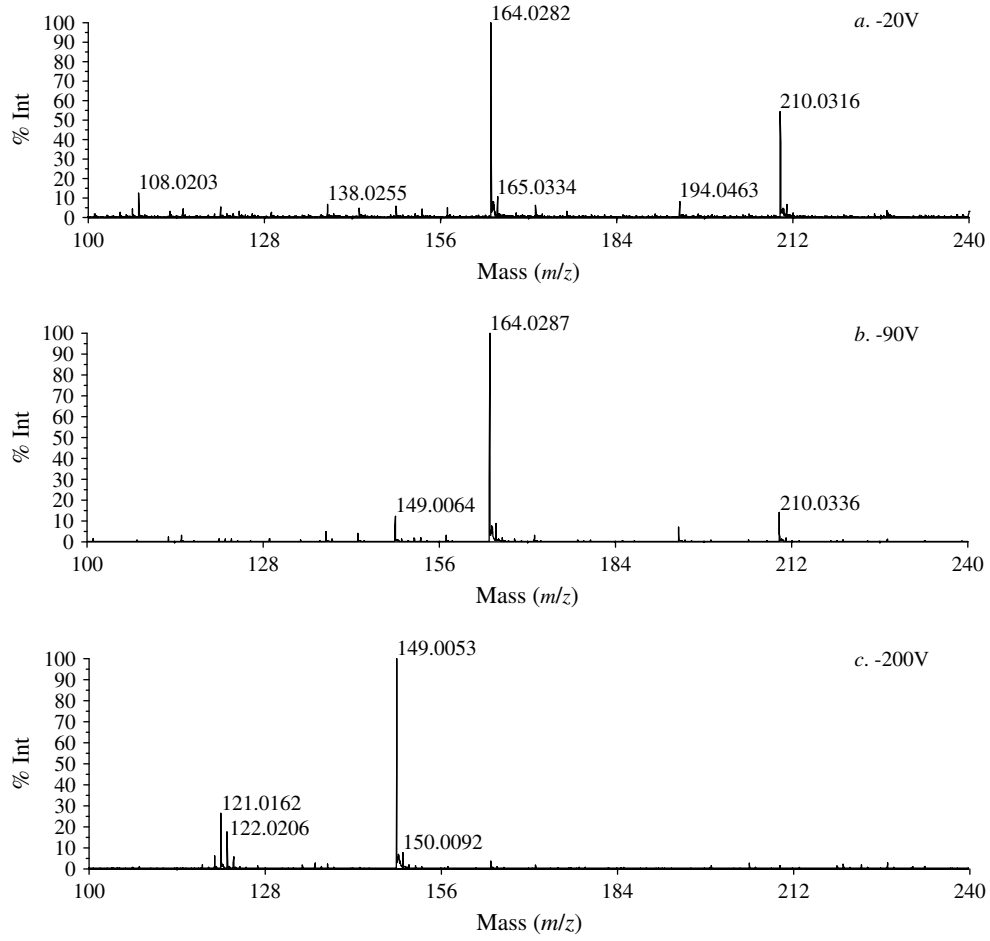


Figure 2. ESI-TOFMS(-) spectra of DIMBOA (snapshots) over the range m/z 100–240 at varying nozzle potentials: (a) −20; (b) −90; and (c) −200 V.

and the latter at higher values. The ion at m/z 164 can be assigned to the fragment arising from loss of the C^2R1H and R2 moieties (refer to compound numbering depicted in Fig. 1). Analogous fragmentation for the non-methoxylated DIBOA derivative to yield an ion at m/z 134 is observed (data not shown) supporting the assignment. The DIMBOA fragment ion at m/z 149 corresponds to subsequent loss of 15 from the m/z 164 ion, assignable to cleavage of a methyl radical. Confirmation of this assignment is further provided by absence of an analogous fragment in the spectra of the non-methoxylated derivatives (data not shown). The dominant ions observed for the hydroxamic acid derivatives, DIMBOA (m/z 164 and 149) and DIBOA (m/z 134), were equivalent to those observed for their respective natural metabolites, MBOA and BOA, supporting the assignment of these fragment ions to benzoxazolinone structures. However, despite the hydroxamic acids showing the same major ions as the corresponding benzoxazolinones, the former also show additional ions not observed for the more stable benzoxazolinones; namely, the ions at m/z 122 and 121 observed for DIMBOA and the ions at m/z 108 and 107 from DIBOA, indicating that the more reactive hydroxamic acid structure leads to higher fragmentation, and that these ions are formed at higher voltages from the deprotonated parent molecule rather than the deprotonated benzoxazolinone intermediate.

Assignment

The nature of the TOF detection method provides sufficiently accurate information regarding molecular ion mass that can be utilised to predict molecular formulae and therefore structures of the observed ions. The exact masses of the fragment ions used in the calculations were the background-subtracted average of 60 individual MS scans, with the masses of the most abundant isotopes used. The error range of all assigned signals of zero up to 10 mDa was within the normal expected range under the chosen experimental conditions (J. Krause, Applied Biosystems, Langen, Germany, personal communication, 28/01/02). The higher errors were in general observed for those ions which can be considered as relatively chemically instable, and thus may also be undergoing decomposition during the TOF process, or for those which could not be resolved.

However, even under these circumstances, the ESI-TOFMS results thus obtained enabled the unequivocal identification of all fragments of the selected allelochemicals, as summarised in Table 1. In this table, the observed monoisotopic masses for each fragment ion and the relative intensities of these ions over the range of voltages investigated are presented. The molecular formulae (MF) and calculated mass of the most likely structures are also given, along with the deviation of the observed monoisotopic mass from the calculated value for this formula. Due to space restraints, only the most likely assignments for each fragment are described in Table 1. These were defined by comparison with elemental composition calculation results and analogies within the structural series, and application of knowledge of the compound structures to define feasible structures.

In order to consider all potential assignments, elemental composition calculations were performed on each fragment ion observed with a broad range of tolerance (20 mDa/100 ppm) and allowing for both odd- and even-electron states. The assignments for the major ions observed in the DIMBOA spectra will be discussed in detail here, with structural elucidation of the other derivatives performed in an analogous manner, and omitted here for reasons of brevity.

Only the $C_9H_8NO_5$ MF, corresponding to the $[M-H]^-$ ion, was possible for the m/z 210.0335 ion in the DIMBOA spectra, whilst within the parameters specified, two possibilities were presented for the m/z 194.0409 ion, $C_9H_8NO_4$ and $C_9H_6O_5$. The former corresponded to the $[M-OH]^-$ ion, and was the more likely assignment (Error = −4.9 mDa), with stabilisation of the ion also potentially provided by resonance rearrangement to the enol. The latter MF could be ruled out as a possible assignment as it gave both a higher error (18.9 mDa) and could only be assigned to a non-charged product—a benzodiketo *p*-dioxixin-type structure that would require loss of three protons and the N^4, and oxygen migration.

Three MFs, $C_8H_7NO_3$, $C_8H_5O_4$, and the C^{13} isotope of $C_8H_6NO_3$, were possible for the minor m/z 165.0328 ion. The former corresponds to an odd-electron ion resulting from cleavage of the N^4-substituted OH and the C^2OH moieties with a H migration to the C^3 carbonyl. This is in higher agreement (Error = −10.2 mDa) than the latter (Error = 13.6 mDa), for which only the unlikely even-electron structure resulting from loss of the C^7-substituted methyl and the N^4OH moieties could be contrived. This is considered unlikely due to the higher mass error, the unprecedented nature of this structure for all other analogues investigated and indications that the N atom is retained according to subsequently observed fragments. A resonance structure via a keto-enol-type rearrangement yielding the negative charge on the O can also be envisaged for the former, more likely, structure. The possibility that this ion is merely the ^{13}C isotope of the major ion observed at m/z 164 can be ruled out on the basis of isotopic calculations, as demonstrated visually in Fig. 3. The intensity of the $C_8H_6NO_3$ (m/z 164) ^{13}C isotope is calculated at 9.4%. In the spectra obtained at −20 V (Fig. 3(a)), −90 V (Fig. 3(b)) and −120 V (not shown), the intensity of the m/z 165 ion was 9.9–10.6% that of the m/z 164 ion, and is thus interpreted as the ^{13}C isotopic contribution. However, at higher nozzle potentials (−200 V, −220 V), the relative intensity of the m/z 165 ion reaches as high as 23.9% (Fig. 3(c)), demonstrating the existence of an additional structure responsible for the ion at this m/z ratio. The overlap of two ions, which were not resolvable, at this m/z ratio is attributed to the relatively high error in the elemental composition calculation ($C_8H_7NO_3$, Error = −10.2 mDa) for the assigned structure.

Three structures were calculated for the m/z 164.0280 ion, $C_8H_6NO_3$, $C_8H_4O_4$ and $C_9H_8O_3$, with errors of −7.2, 16.6 and −19.8 mDa, respectively. The former even-electron species arises from the loss of the N^4-substituted OH and the C^2H(OH) moieties, and is the assigned structure, for which a keto-enol rearrangement can also occur. The second

Table 1. Summary of ESI-TOFMS(-) analysis of benzoxazinones

Compound (MF, M[a])	Observed ions (*m/z*)[b]	Voltage (V)	Rel.Int. (%)	Calculated MF	Calculated *m/z*	Error (mDa)	Electron state
BOA	134.0297	80–220	100	$C_7H_4NO_2$	134.0247	5.0	Even
($C_7H_5NO_2$,	78.0363	200[c]	5	C_5H_4N	78.0348	1.5	Even
135.0320)							
DIBOA	180.0334	40	100	$C_8H_6NO_4$	180.0301	3.3	Even
($C_8H_7NO_4$,		60	70				
181.0375)		80	20				
		120	<5				
	134.0298	40	65	$C_7H_4NO_2$	134.0247	5.1	Even
		60–220	100				
	108.0488	80	<5	C_6H_6NO	108.0454	3.4	Even
		120	10				
		220	5				
	107.0425	120	<5	C_6H_5NO	107.0376	4.9	Odd
		220	10				
HBOA	164.0382	60–120	100	$C_8H_6NO_3$	164.0352	3.0	Even
($C_8H_7NO_3$,		130	50				
165.0426)		140	20				
		220	5				
	136.0441	80	<5	$C_7H_6NO_2$	136.0403	3.8	Even
		120	20				
		130	15				
	134.0297	120	<5	$C_7H_4NO_2$	134.0247	5.0	Even
		130	5				
		140	10				
		220	25				
	118.0373	120–220	20	C_7H_4NO	118.0297	7.6	Even
	108.0472	120	70	C_6H_6NO	108.0454	1.8	Even
		130, 140	100				
		220	60				
	107.0416	220	100	C_6H_5NO	107.0376	4.0	Odd
MBOA	164.0394	80	100	$C_8H_6NO_3$	164.0352	4	Even
($C_8H_7NO_3$,		120	70				
165.0426)		140	10				
		160	<5				
	149.0164	80	<5	$C_7H_3NO_3$	149.0117	4.6	Odd
		120–160	100				
	121.0225	120	<5	$C_6H_3NO_2$	121.0168	5.7	Odd
		140	10				
		160	35				
DIMBOA	210.0335	20	50	$C_9H_8NO_5$	210.0407	−7.2	Even
($C_9H_9NO_5$,		90	15				
211.0480)		120	5				
		200, 220	<5				
	194.0409	20, 90	10	$C_9H_8NO_4$	194.0458	−4.9	Even
		120	5				
		200	<5				
	165.0328	90, 120	10	$C_8H_7NO_3$	165.0431	−10.2	Odd
		200	<5				
	164.0280	20–120	100	$C_8H_6NO_3$	164.0352	−7.2	Even
		200, 220	5				
	149.0059	90	15	$C_7H_3NO_3$	149.0117	−5.8	Odd
		120	45				
		200, 220	100				

(*continued overleaf*)

Table 1. (*Continued*)

Compound (MF, M[a])	Observed ions (*m/z*)[b]	Voltage (V)	Rel.Int. (%)	Calculated MF	Calculated *m/z*	Error (mDa)	Electron state
	138.0239	90	5	$C_6H_4NO_3$	138.0196	4.3	Even
		120	5				
		200	<5				
	123.0230	120	<5	$C_6H_5NO_2$	123.0325	−9.5	Odd
		200	5				
		220	10				
	122.0206	200	20	$C_6H_4NO_2$	122.0247	−4.0	Even
		220	40				
	121.0162	200	25	$C_6H_3NO_2$	121.0168	−0.6	Odd
		220	45				
	93.0279	200	5	C_5H_3NO	93.0220	−6.0	Odd
		220	10				
HMBOA	194.0459	80–140	100	$C_9H_8NO_4$	194.0458	0.1	Even
($C_9H_9NO_4$,		160	15				
195.0532)	179.0208	120	5	$C_8H_5NO_4$	179.0223	−1.5	Odd
		140	20				
		160	5				
	166.0509	120	10	$C_8H_8NO_3$	166.0509	0.0	Even
		140	20				
		160	<5				
	150.0172	140	10	$C_7H_4NO_3$	150.0196	−2.4	Even
		160	5				
	148.0376	120	5	$C_8H_6NO_2$	148.0403	−2.7	Even
		140	15				
		160	5				
	138.0558	120	15	$C_7H_8NO_2$	138.0560	−0.2	Even
		140	60				
		160	15				
	133.0132	140	10	$C_7H_3NO_2$	133.0168	−3.6	Odd
		160	<5				
	123.0272	120	5	$C_6H_5NO_2$	123.0325	−5.2	Odd
		140	90				
		160	60				
	122.0205	140	55	$C_6H_4NO_2$	122.0247	−4.2	Even
		160	100				
2′H-DIBOA	164.0396	80	90	$C_8H_6NO_3$	164.0352	5.8	Even
($C_8H_7NO_3$,		120	60				
165.0426)	134.0305	80	100	$C_7H_4NO_2$	134.0247	5.8	Even
		120	90				
		140	100				
		160	80				
		200	10				
	122.0284	120	30	$C_6H_4NO_2$	122.0247	3.7	Even
		140	20				
	120.0471	80	<5	C_7H_6NO	120.0454	1.7	Even
		120	100				
		140	85				
		160	35				
		200	5				
	118.0380	120	40	C_7H_4NO	118.0297	8.3	Even
		140, 160	100				
		200	45				
	108.0395	140	10	C_6H_6NO	108.0454	−5.9	Even
		160	<5				
		200	5				

Table 1. (*Continued*)

Compound (MF, M[a])	Observed ions (*m/z*)[b]	Voltage (V)	Rel.Int. (%)	Calculated MF	Calculated *m/z*	Error (mDa)	Electron state
	107.0386	120	20	C_6H_5NO	107.0376	1.0	Odd
		140	15				
		160	10				
	106.0344	120	5	C_6H_4NO	106.0297	4.6	Even
		140	5				
	93.0362	120	10	C_6H_5O	93.0345	1.7	Even
		140	45				
		160	65				
		200	100				
	90.0362	200	10	C_6H_4N	90.0348	1.4	Even
2′OMe-DIBOA ($C_9H_9NO_4$, 195.0532)	194.0371	90, 110	100	$C_9H_8NO_4$	194.0458	−8.7	Even
		120	90				
		130	5				
		190	<5				
	178.0403	90	10	$C_9H_8NO_3$	178.0508	−10.5	Even
		110	15				
		120	30				
		130	25				
		190	5				
	134.0196	90	<5	$C_7H_4NO_2$	134.0247	−4.0	Even
		110	25				
		120	60				
		130	55				
		190	20				
2′OMe-DIBOA (contd)	118.0250	90	5	C_7H_4NO	118.0297	−4.7	Even
		110	35				
		120–190	100				
	107.0323	90	<5	C_6H_5NO	107.0376	−5.3	Odd
		110	5				
		120	20				
		130	15				
		190	<5				
	106.0224	90	≪5	C_6H_4NO	106.0297	−7.3	Even
		110	<5				
		120, 130	5				
	90.0295	120	≪5	C_6H_4N	90.0348	−5.3	Even
		130	<5				
		190	25				
2′H-HBOA ($C_8H_7NO_2$, 149.0477)	148.0476	120, 140	100	$C_8H_6NO_2$	148.0403	7.3	Even
		160	85				
		180	15				
	134.0333	120	25	$C_7H_4NO_2$	134.0247	8.6	Even
		140	35				
		160	55				
		180	15				
	120.0524	140	50	C_7H_6NO	120.0454	7.0	Even
		160	100				
		180	40				
	118.0394	120	5	C_7H_4NO	118.0297	9.6	Even
		140	25				
		160	85				
		180	100				
	108.0551	160	15	C_6H_6NO	108.0454	9.7	Even
		180	10				

(*continued overleaf*)

Table 1. (*Continued*)

Compound (MF, M[a])	Observed ions (*m/z*)[b]	Voltage (V)	Rel.Int. (%)	Calculated MF	Calculated *m/z*	Error (mDa)	Electron state
	93.0359	160	30	C_6H_5O	93.0345	1.4	Even
		180	30				
2′OMe-HBOA	178.0588	80–140	100	$C_9H_8NO_3$	178.0510	7.9	Even
($C_9H_9NO_3$,		160	30				
179.0582)		180	10				
	134.0310	120	5	$C_7H_4NO_2$	134.0247	7.3	Even
		140	20				
		160	15				
		180	30				
	121.0301	160	10	$C_7H_5O_2$	121.0294	0.7	Even
	90.0390	180	5	C_6H_4N	90.0348	4.2	Even

[a] Monoisotopic value; [b] Background-subtracted average values; [c] Quad RF voltage 400 V.

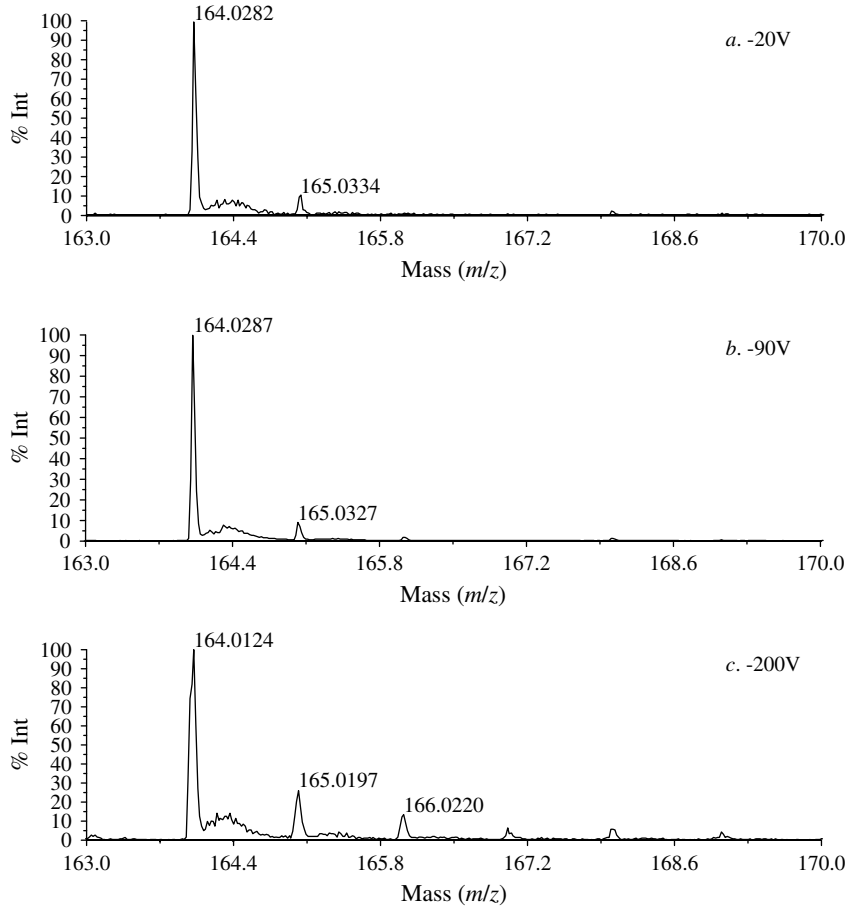


Figure 3. ESI-TOFMS(-) spectra of DIMBOA (snapshots) over the range *m/z* 163–170 at: (a) −20; (b) −90; and (c) −200 V.

MF could be formed by loss of the N^4OH, the C^7 methyl and a H from the C^2HOH, leading to a odd-electron species with O^- and $O^\bullet$ radical moieties; however, this structure is considered unlikely due to the high error and nature of the fragmentations required. The latter of the three possible MFs is also unlikely, where only the uncharged structure resulting from loss of the N^4OH and a C^2-substituted O group with a H migration can be contrived, and the error is very high.

The ion at *m/z* 149.0059 is most likely attributed to $C_7H_3NO_3$ (Error = −5.8 mDa), with the structure corresponding to that resulting from cleavage of a methyl radical from the *m/z* 164 structure given earlier. A keto-enol-type rearrangement is also possible for this structure. An even-electron species $C_8H_5O_3$, corresponding to the structure resulting from cleavage of the N^4OH, the C^2 oxygen and C^7 methyl, also matches the observed *m/z*. However, this structure is ruled out on the grounds of the higher mass inaccuracy (−18.4 mDa) and likelihood of the former assignment according to comparisons between structural analogues, as discussed in the previous section.

JMS

Two MF possibilities are given for the ion at m/z 138.0239, the even-electron species $C_6H_4NO_3$ and the odd-electron species $C_7H_6O_3$, with errors of 4.3 and −8.2 mDa, respectively. The former MF corresponds to a deprotonated nitroso resorcin derivative. A resonance rearrangement product resulting in a structure with the negative charge on the O can also be predicted for this assignment. No likely structures can be envisaged for the latter MF, with possible aldehyde, alcohol and ketone variations all unlikely according to the required rearrangements and the resulting structures and charge distributions.

Only one MF for each of the ions observed at m/z 122.0206 and 121.0162, $C_6H_4NO_2$ and $C_6H_3NO_2$ respectively, was possible, with both assigned to chinonimin structures. The high intensity of the ion at m/z 122 relative to that at m/z 121 can be seen clearly in Fig. 2(c), clearly demonstrating that two structures are responsible for these ions and that this is not a result of ^{13}C isotopic contribution.

Two likely possibilities, C_5H_3NO and C_6H_5O, exist for the ion at m/z 93.0279, with high correlation and relatively little difference in the errors observed (6.0 and −6.6 mDa, respectively). The former assignment, corresponding to the oxycyclopentadiene imine, is considered most likely, and is supported by the observation of an analogous structure for the BOA derivative (m/z 78). The latter, assignable to phenolate, however, was also observed for other analogues, 2′H-DIBOA and 2′H-HBOA, although these derivatives lack the C^7 methoxy substituent. Formation of this ion from DIMBOA would require the addition of two protons to satisfy the formula and is an unlikely fragmentation. Furthermore, so far our own unpublished data from MS/MS investigations performed showed the ion to be formed from the m/z 121 precursor,[18] from which only the C_5H_3NO structure can then be formulated.

From the assignments described here and in Table 1, fragmentation pathways for the derivatives could also be predicted. An example of the fragmentation information obtained is shown schematically in Fig. 4 for DIMBOA. Keto-enol rearrangement products are not shown in the figure for reasons of simplicity; however, they can be formulated for many of the structures presented.

[M − H]⁻ ions

[M − H]⁻ ions were observed for all analytes (Table 1), and these ions are assigned to those generated by loss of the hydrogen substituted at the nitrogen. In previous literature,[13] the structure of the [M − H]⁻ ions was assigned to that arising from loss of H^+ from the hydroxyl group at position C^2 (R1, see numbering in Fig. 1). We propose that this assignment is less likely for three reasons. Firstly, the [M − H]⁻ ions were observed for all analytes investigated here, including those synthetic derivatives with methoxy substituents at position C^2 (R1 = OMe). Secondly, results from the literature cited above describe spectra of C^2-glycosylated hydroxamic and lactam derivatives, all of which show a [M − H]⁻ ion. The ions from these first two examples can only arise from the loss of the nitrogen-substituted hydrogen. Furthermore, in the cited literature, examples of derivatives substituted with methoxy groups at the nitrogen are also given. Consistent with our assignment, no [M − H]⁻ ions are observed for these derivatives; rather, the corresponding $[M - OCH_3]^-$ ions are observed.

[M − OH]⁻ fragment ions

According to the reasons described above, the [M − OH]⁻ fragment ions observed are assigned to those arising from cleavage of the N^4-substituted hydroxyl group. [M − OH]⁻ fragment ions are observed for all hydroxamic acid derivatives (R1 = OH; R2 = OH), but are not observed for the lactam analogues (R1 = OH; R2 = H). Analysis of the fragment trends for the hydroxamic acid derivatives also indicates that the [M − OH]⁻ fragment ions are not formed by loss of oxygen from the [M − H]⁻ ion but rather directly from the parent molecule. Both ions are simultaneously

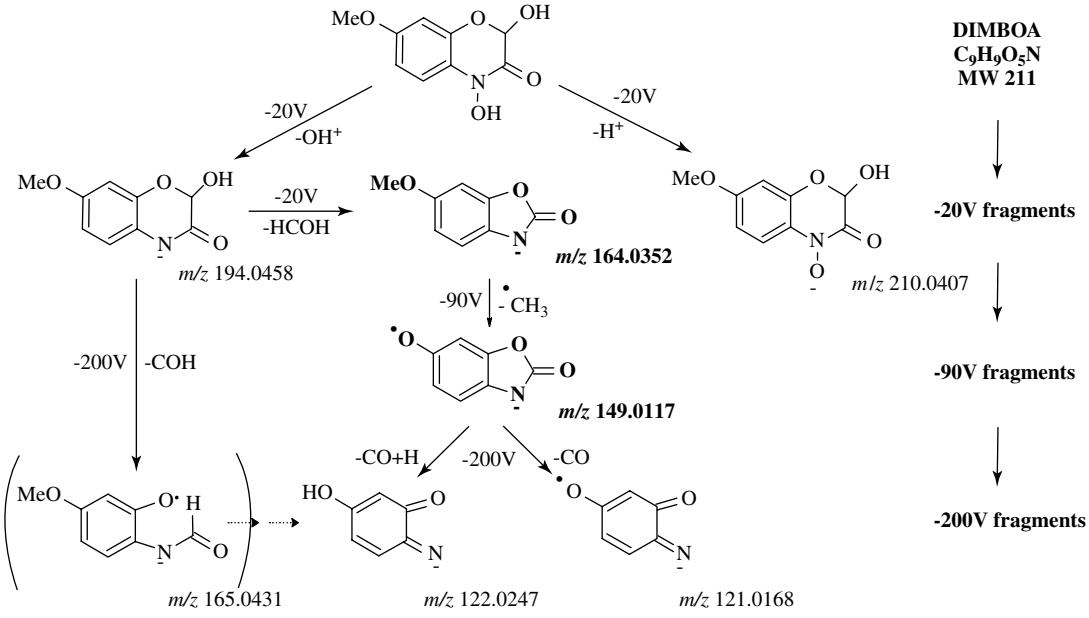


Figure 4. DIMBOA fragments as assigned from ESI-TOFMS(-) spectra at different nozzle potentials.

observed and furthermore the intensity of the $[M - OH]^-$ ion does not increase relative to that of the $[M - H]^-$ ion, as would be expected should the latter be the parent.

Further fragment ions

In Table 2 the structural assignments for all fragment ions observed for the benzoxazinones, according to the calculated MF values given in Table 1, are presented schematically according to common structural type. This shows not only the structural analogues formed from different derivatives, but also demonstrates differences in reactivities for different derivatives according to substitution, in cases where analogous structures are not detected. Resonance structures are also shown where possible (**4**, italicised text), and keto-enol rearrangements (not shown) also exist for all structures **1** and **2** where the negative charge is on the N, i.e. R2 is denoted as -.

Benzoxazolinone-type structures (structures **2**, Table 2) arising from loss of the C^2HR1 and R2 moieties from the parent molecules were observed for all derivatives, including those derivatives with a C^2H_2 moiety (2′H-DIBOA, 2′H-HBOA). Fragment ions resulting from cleavage of the $C^3 = O$ and R2 moieties (structures **3**, Table 2) were observed only for the HBOA and HMBOA lactams and the 2′H-substituted synthetic derivatives under the ESI-TOFMS(-) conditions, showing that such a structure is not likely from derivatives possessing hydroxy (and/or methoxy) substituents at the R1 and R2 positions.

The observation of ions of the same structural type for structural analogues (varying in C^7 methoxy substitution) provides further confirmation of the assignments. These are grouped together for clarity. The m/z 134 fragment (**2**, R2 = −; R3 = H), corresponding to loss of the C^2HR1 moiety, and the N substituent (R2) from the naturally occurring DIBOA and the 2′H-DIBOA and 2′OMe-DIBOA synthetic derivatives were also observed, analogous to the m/z 164 fragment ion of DIMBOA, providing further confirmation of the assignment and fragmentation mechanisms. The weak response of this fragmentation for the lactam derivatives (natural and synthetic derivatives) indicates the importance of the nitrogen-substituted hydroxyl group (R2 = OH) on the reactivity of these compounds.

Comparison of the results obtained for the range of benzoxazinones investigated enabled the elucidation of a generic fragmentation pattern, as presented in Fig. 5. In general, the compounds demonstrated a fragmentation pathway of, firstly, cleavage of the nitrogen substituent (R2), followed by subsequent losses of the C^2HR1 and $C^3 = O$ moieties (as CO groups and otherwise). This scheme is valid for the derivatives investigated, with observed analogies between the structural variants, indicating that the pathway can also be applied to other compounds of the class. As can be seen in Fig. 5, the N^4 substituent (R2), C^2R1H, CO and CH_3 (C^7 methoxylated derivatives) are commonly observed losses for the broad range of derivatives investigated, and parallel fragmentation is thus predicted for other structural analogues.

In general, structures could be assigned for all molecular formulae according to the ESI-TOFMS results obtained and structural information derived from the available literature regarding mass spectrometry of related heterocyclic compounds.[6,12,13,19,20a] However, in the case of the fragment at m/z 90 occurring at higher voltages (−180 to −200 V) for the synthetic derivatives, only the molecular formula $C_6H_4N^-$ was feasible, for which no plausible structure could be assigned with confidence. Evidence indicates, however, that such ion fragments are typical in the MS analysis of benzoxazole derivatives exhibiting structures similar to the benzoxazinones investigated here.[20b] In the EI-MS analysis of BOA, an ion at m/z 91, assigned to the radical cation $C_6H_5N^{+\bullet}$, has been observed, for which no structural assignment could also be given.[21,22] Thus the structure of the m/z 90

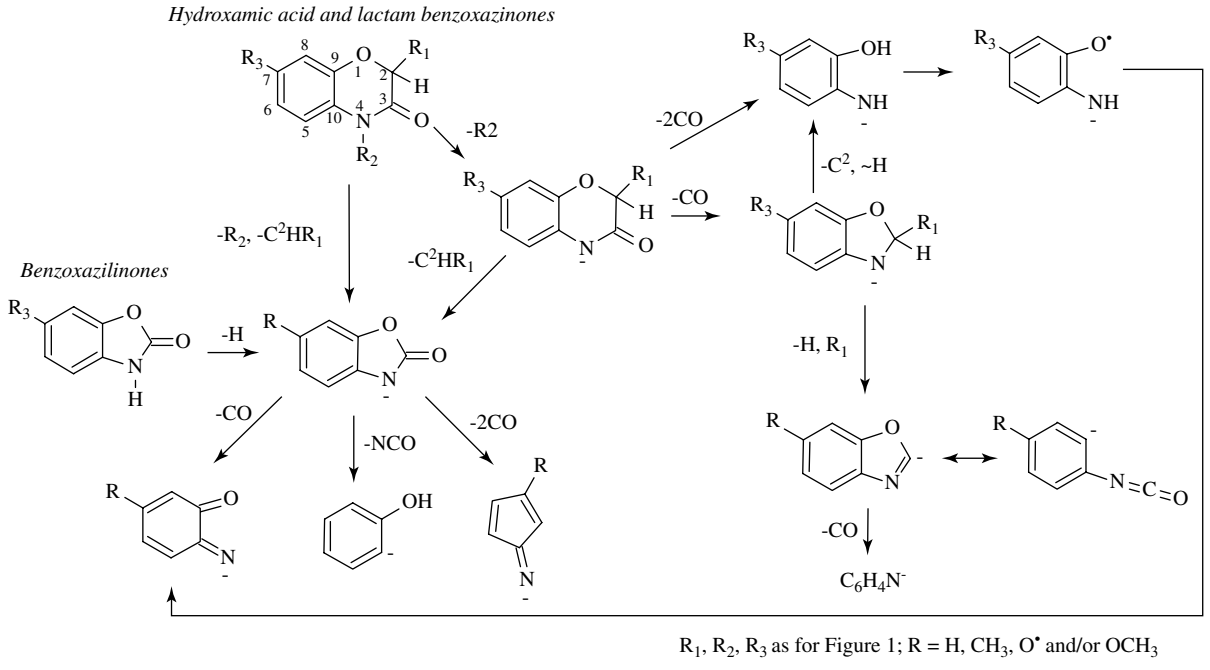
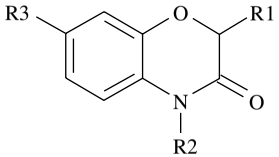
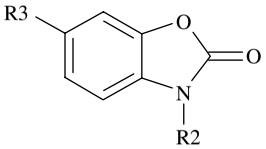
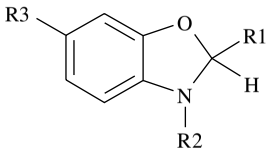
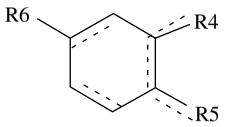


Figure 5. Generic benzoxazinone fragmentation pattern as determined by ESI-TOFMS(-).

Table 2. Structural assignments for the fragment ions observed for the benzoxazinones by ESI-TOFMS(–)[a]

–H/R2 (N substituent)					–C²HR1				–C³ = O					–C²HR1C³ = O/–OC²HR1C³ = O/ –C²HR1C³ = ONR²				
																		
1					**2**				**3**					**4**				
R1	R2	R3	*m/z*	Benz.	R2	R3	*m/z*	Benz.	R1	R2	R3	*m/z*	Benz.	R4	R5	R6	*m/z*	Benz.
OH	O^-	OCH_3	210	DIM	—	OCH_3	164	DIM,M	OH	—	OCH_3	166	HM,DIM[b]	$O^\bullet$	$^-$NCOH	OCH_3	165	DIM
OH	O^-	H	180	DI	—	H	134	DI,B,H,SP1-4	OH	—	H	136	H	*$O^\bullet$*	*$N(CO^-)H$*	*OCH_3*	*165*	[c]
OH	—	OCH_3	194	DIM,HM	—	OH	150	DIM,HM	H	—	H	120	SP1, SP3	—	N=C=O	OCH_3	148	HM
OH	—	H	164	DI[b], H	*H*	*O^-*	*150*	[c]						—	N=C=O	H	118	H,SP1-4
					OH	—	*150*	[c]										
OCH_3	O^-	H	194	SP2	*O^-*	*H*	*150*	[c]						OH	HN^-	OCH_3	138[d]	HM
H	O^-	H	164	SP1										OH	HN^-	H	108	DI,H,SP1,SP3,B[b]
					—	$O^\bullet$	149	DIM,M										
OH	—	$O^\bullet$	179	HM										OH	N=O	O^-	138[d]	DIM[e]
														OH	*$= N - O^-$*	*$= O$*	*138*	[c]
OCH_3	—	H	178	SP2, SP4														
H	—	H	148	SP1[b],SP3										—	N=C=O	$O^\bullet$	133	HM
														OH	HN^-	$O^\bullet$	123	HM,DIM
														O-	H	H	93	SP1,SP3
														OH	—		*93*	[c]
														=O	$=N^-$	OH	122	DIM,HM
														= O	*=NH*	*O^-*	*122*	[c]
														=O	$=N-O^-$	H	122	SP1,SP2[c]
														=O	$=N^-$	$O^\bullet$	121	DIM,M
														$O^\bullet$	HN^-	H	107	DI,H,SP1,SP2,B[b]
														=O	$=N^-$	H	106	SP1,SP2,SP4,B[b]

[a] SP = synthetic products: SP1 = 2′H-DIBOA, SP2 = 2′MeO-DIBOA, SP3 = 2′H-HBOA, SP4 = 2′MeO-HBOA; Additionally observed ions not fitting into these categories: *m/z* 78 (BOA, C_5H_4N), *m/z* 90 (SP1-4, $C_6H_4N^-$), *m/z* 93 (DIMBOA, C_5H_3NO), *m/z* 121 (SP4, $C_7H_5O_2$); [b] APCI-MS(-) mode observed ion; [c] Alternative possible structures; [d] Assignments different (despite structural analogies) due to significantly different observed *m/z* values; [e] This assignment not possible for HMBOA.

fragment ion observed here remains to be determined, elucidation of which requires additional experimental work, e.g. labelling experiments, etc., in the future. Its structure could also not be elucidated applying LC/MS/MS, as described by Bonnington *et al*.[18] However, the generic fragmentation scheme obtained from the interpretation of the ESI-TOFMS data could be confirmed in almost all cases via the observed transitions in LC/MS/MS. In the latter some additional fragments were obtained, e.g. those at m/z 166, 92 and 91, which were not observed in the ESI-TOFMS spectra.

Stability

The relative stabilities for the $[M-H]^-$ ions were variable for the different structures. At low voltages (−40 to −140 V), the $[M-H]^-$ ions dominated the spectra for all derivatives excluding DIMBOA and 2′H-DIBOA, for which the $[M-H]^-$ ions were not observed as dominant at any voltage (Table 1). The stability of the lactam derivatives over the corresponding hydroxamic acids is clearly evident, both for the natural and synthetic derivatives. The $[M-H]^-$ ion of DIBOA was only observed as dominant at −40 V, as compared with HBOA for which the $[M-H]^-$ ion dominated the spectra up to −120 V. The $[M-H]^-$ ion of 2′H-DIBOA was not dominant at any voltage, but that for the 2′H-HBOA analogue was dominant up to −140 V. The same trend was observed for the analogues methoxylated at the C^2 position, with that of 2′MeO-DIBOA only dominant up to −110 V, but the HBOA analogue dominant to −140 V.

Comparison of the relative intensities of the $[M-H]^-$ ions in the spectra at a medium strength fragmentation voltage of −120 V for all the derivatives (see Table 1) demonstrates the far higher instability of DIBOA and DIMBOA over the other analogues, consistent with reported biological activities. At this fragmentation voltage, the relative intensities of the $[M-H]^-$ ions were very minor at 5% and <5% for DIMBOA and DIBOA, respectively, whilst for the other derivatives they were observed in the range of 60–100%. At −120 V, the $[M-H]^-$ ions dominated the spectra for the BOA, HBOA, HMBOA, 2′H-HBOA and 2′OMe-HBOA derivatives, whilst the relative intensities for MBOA, 2′H-DIBOA and 2′OMe-DIBOA were 70, 60 and 90%, respectively.

These results also demonstrate the enhanced reactivity resulting from methoxy group substitution at C^7 consistent with the relative chemical and biological reactivities of the derivatives.[10] DIMBOA shows higher reactivity over DIBOA, and MBOA over BOA, and this has been ascribed to electronic stabilisation of subsequent intermediates by the methoxy substituent at the C^7 aromatic carbon.[23] This trend is not observed, however, for the HBOA and HMBOA lactam analogues, which are known to exhibit increased stability and thought to undergo a different degradation mechanism.[23]

The $[M-H]^-$ ions of the R1 = H and MeO-substituted DIBOA and HBOA derivatives also showed increased stability over the natural analogues, demonstrating the contribution of the OH group in this position (C^2) to the reactivity of these compounds. Methoxy substitution yielded further improvements to the stability of the $[M-H]^-$ ions over the R1 = H− and OH-DIBOA derivatives; however, once again, the effect of this substitution on the lactam derivatives was not as significant.

The $[M-OH]^-$ ions (hydroxamic acids) were considerably less intense than the $[M-H]^-$ ions (DIMBOA and 2′OMe-DIBOA) and in fact were not observed to any significant level for either DIBOA or 2′H-DIBOA under the described ESI-TOFMS conditions. The ion corresponding to subsequent loss of the C^2HR1 moiety (structure **2**, Table 2), however, was observed at high intensity for all the derivatives, demonstrating the unstable nature of the R1 = OH, R2 = −structure. The possibility of the neutral loss of formic acid from the deprotonated molecule to form these benzoxazolinone-type structures cannot be completely excluded, although the observation of these structures also for the lactam derivatives (R2 = H), for which a formic acid cannot be formed, supports that the fragmentation sequence of R2 followed by C^2HR1 is more likely. Benzoxazolinone-type structures (structures **2**, Table 2) arising from loss of the C^2HR1 and R2 moieties from the parent molecules observed for all derivatives, however, are much more significant ions for the hydroxamic acid analogues. Loss of OH followed by the neutral loss of formaldehyde (C^2HOH) is more likely as the concerted loss of a neutral formic acid would require two simultaneous fragmentation reactions at different sites in the molecule. This was confirmed by independent MS/MS investigations.[18]

Bioactivity and degradation mechanisms

The mechanism for the allelopathic function of the benzoxazinones is, as yet, not clearly defined and is the subject of continued interest and research. The observation of structures possessing the isocyanate moiety, implicated in the photosynthetic inhibiting potential of these compounds,[1] has been described here for the first time (Table 2, **4**; R5 = N═C═O) and could also be proved by applying LC/MS/MS,[18] and thus may provide useful information regarding the allelopathy of these compounds.

Common ions for the hydroxamic acid derivatives and their natural metabolites—the corresponding benzoxazolinone analogues—were observed in the MS results described here, consistent with proposed biodegradation mechanisms,[1] as discussed in previous sections. These ions were of much less significance for the lactam analogues, which followed alternative fragmentation mechanisms, which also provides a potential explanation for the lower biological activity of these derivatives.[10] Fragmentation was most readily observed for derivatives hydroxylated at both the C^2 and N^4 positions (hydroxamic acid derivatives, R1, R2 = OH), consistent with their enhanced bioactivity.[2] Substitution of the hydroxyl group at the C^2 position (R1 = H, OMe) increased stability of the benzoxazinones investigated. This is also consistent with the much lower biological activity known for the naturally occurring C^2-glycosylated benzoxazinones—the form in which these compounds are found in undamaged plants.[13]

The isocyanate and phenol derivatives of structure **4**, shown in Table 2, are of particular interest as these compounds have been implicated in the abiotic and soil degradation of these compounds, respectively, but have as yet not

been isolated. The isocyanate derivatives (**4**; R5 = N═C═O) have been described as intermediates in abiotic degradation studies, with the formation of this compound thought to be the rate-limiting step of the reaction.[1,24] Rapid conversion of the benzoxazolinones in soil has also been described, with the main products thought to be 2,2′-oxo-1,1′-azobenzene (AZOB),[8] corresponding mono- and dimethoxy analogues MAZOB and DIMAZOB,[8] 2-amino-3*H*-phenoxazin-3-one[7] and the trimethoxy analogue 2-amino-4,6,7-trimethoxy-3*H*-phenoxazin-3-one.[7] These derivatives are thought to be formed via the intermediate 2-aminophenol, although no direct experimental evidence has been presented for this to date. A range of structural analogues of 2-aminophenol (**4**; R4 = OH, R5 = NH_2) have been observed as fragments in the MS studies conducted here (**4**; R4 = OH, $O^{\bullet}$, O^-, =O; R5 = NH^-, N = O, $=N^-$, =NH, $=N\text{-}O^-$), offering some support for this intermediate.

CONCLUSIONS

The identification of all fragments of the selected benzoxazinones was achieved applying ESI-TOFMS with use of elemental composition calculation results, where the error for all assigned signals varied in the range 0 to ±10 mDa. Within this range reliable assignment was still possible in conjunction with comparison of data for structural analogues and application of knowledge of feasible structures for the possible assignments. Through the use of the large database of fragmentation results produced it was possible to determine a strategy for structural elucidation. The MS results show the important influence of the C^2-, N^4- and C^7-substituted groups on the stability. Common ions for the derivatives and their natural metabolites as well as parallel trends in stability for the different derivatives were observed.

The similarities and differences in the MS fragmentations described here should thus be useful in future screening for further potential metabolites and analogues. Indeed, further ions were also observed by MS here, which have been implicated in the bioactivity and biodegradation of these compounds, but as yet had not been obtained.

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