JOURNAL OF MASS SPECTROMETRY
J. Mass Spectrom. **34**, 611–621 (1999)

Fragmentation of Methyl Abscisate and Pentafluorobenzyl Abscisate in Methane Electron Capture Negative Ionization Tandem Mass Spectrometry

A. G. Netting* and R. O. Lidgard
Facility for Advanced Quantitative Environmental Assessment, School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney, New South Wales 2052, Australia

The methyl and pentafluorobenzyl esters of the plant hormone abscisic acid were subjected to methane chemical ionization tandem mass spectrometry (MS/MS). The spectra obtained allow the unambiguous identification of abscisic acid in a few milligrams of plant material. In full-scan mode pentafluorobenzyl (PFB) abscisate (ABA) gave three significant ions at *m/z* 263 ($[M - PFB]^-$), *m/z* 219 ($[M - PFB - CO_2]^-$) and *m/z* 153 ([M − PFB − side chain]$^-$). Each of these was subjected to MS/MS and structures were assigned to the product ions using the labelled analogues, PFB[1′-^{18}O]ABA, PFB[4′-^{18}O]ABA, PFB[side-chain-$^2H_4$]ABA and PFB[ring-$^2H_6$]ABA. Similarly, in full-scan mode, methyl abscisate gave three significant ions at *m/z* 278 (M^-), *m/z* 260 ($[M - H_2O]^-$) and *m/z* 245 ($[M - H - CH_3OH]^-$) and in MS/MS the use of the methyl esters of the above labelled analogues, and [^{2}H]methyl abscisate, allowed structures to be assigned to the product ions. Using these results it will be possible to dissect abscisic acid so that most labelled atoms from ^{13}C-labelled substrates will be able to be uniquely identified from a few nanograms of ^{13}C-labelled abscisic acid. If sufficient incorporation of ^{13}C-labelled substrates can be obtained it should be possible to investigate the pathway(s) of abscisic acid biosynthesis using less than 1 g of plant material.

KEYWORDS: tandem mass spectrometry; electron capture negative ionization; abscisic acid, pentafluorobenzyl abscisate; methyl abscisate

INTRODUCTION

Our continuing work on the biosynthesis and physiology[1–3] of the plant stress hormone abscisic acid (ABA) now requires both the quantification and the unambiguous identification of the hormone and its precursors in very small amounts of plant material. Mass spectrometry is the method of choice for quantitation since deuteriated ABA is available as an internal standard[4] and we can obtain high sensitivity in electron capture negative ionization (ECNI) by converting ABA into its pentafluorobenzyl (PFB) ester.[5] If tandem mass spectrometry (MS/MS) is available, the required unambiguous identification can be obtained from the combined data of precursor and product ions. This technique is also useful for elucidating biosynthetic pathways by monitoring the incorporation of precursors labelled with stable isotopes. This paper extends our earlier observations of the ECNI and positive chemical ionization mass spectrometry of methyl abscisate (MeABA) and Me-(2*E*)-ABA[6] to the fragmentation of both MeABA and PFBABA in ECNI MS/MS using an instrument containing an ion trap with an external ion source.

* *Correspondence to*: A. G. Netting, Facility for Advanced Quantitative Environmental Assessment, School of Biochemistry and Molecular Genetics, University of New South Wales, Sydney, New South Wales 2052, Australia.
E-mail: a.netting@unsw.edu.au

EXPERIMENTAL

Chemicals

Unless indicated otherwise, chemicals were obtained from Fluka (Buchs, Switzerland). Solvents for use in the Prep-Station and in gas chromatography/mass spectrometry (GC/MS) were of Omnisolv grade from EM Science (Gibbstown, NJ, USA).

Labelled compounds

The expanded numbering system for ABA[7] is shown in Fig. 1 and the methods for the synthesis of the labelled compounds follow.

[1′-^{18}O]ABA. This sample was originally prepared by Gray *et al.*[8] but because it has been in storage for over 20 years a considerable proportion of it has isomerized to (2*E*)-ABA. Consequently, the remaining material was dissolved in acetone and isomerized in the light to give an approximately equimolar mixture of [1′-^{18}O]ABA and its 2*E* isomer. The two isomers were

CCC 1076–5174/99/060611–11 $17.50

Received 27 April 1998
Accepted 8 December 1998

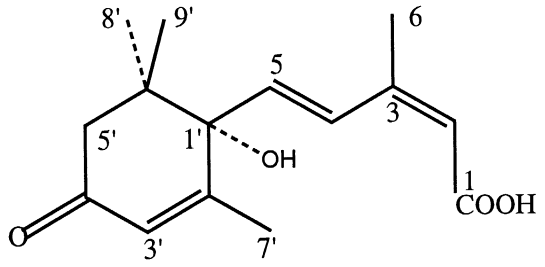


Figure 1. Structure and numbering system for abscisic acid (ABA).

separated by multiple development on a silica gel 60 TLC plate (Merck, Kilsyth, Victoria, Australia) with toluene–ethyl acetate–acetic acid (60 : 30 : 4) containing butylated hydroxytoluene. The band containing [1′-^{18}O]ABA was scraped off and extracted with ethanol, the latter being evaporated. The desired [1′-^{18}O]ABA was purified and quantified by high-performance liquid chromatography (HPLC) (HP 1090, Hewlett-Packard, Waldbronn, Germany) on a Zorbax SB-C18 column (9.4 × 250 mm, 5 μm; Activon Scientific, Thornleigh, NSW, Australia) using MeOH–0.2% aqueous acetic acid (7 : 3) at 2 ml min^{-1}. Diode-array detection was used with the pilot wavelength at 254 ± 2 nm. An aliquot was methylated with CH_2N_2 to give Me[1′-^{18}O]ABA and the PFB ester prepared as described below.

[4′-^{18}O]ABA. ABA (0.5 mg) was suspended in 50 μl of $H_2{}^{18}O$ (97% ^{18}O; Novachem, South Yarra, Australia) with 1 μl of acetic acid to give a final pH of 3.5. The mixture was incubated in the dark at room temperature for 72 h. Evaporation under N_2 gave [4′-^{18}O]ABA and methylation with diazomethane resulted in Me[4′-^{18}O]ABA. Incubation of 0.5 mg of MeABA with the same mixture of $H_2{}^{18}O$ and acetic acid gave Me[4′-^{18}O]ABA.

[^{2}H]MeABA. ABA (100 μg) was dissolved in C^2H_3OH (0.2 ml) and diazomethane (5 ml) that had been distilled from CH_3O^2H (1 ml) was added. After standing for 20 min the diazomethane was evaporated to give the desired [$^2H_1$]MeABA. Any Me[5′-^{2}H]ABA produced as a by-product of this procedure was back-exchanged by incubation for 30 min at room temperature in MeOH–H_2O (pH 9.0) (1 : 1).

[Side-chain-^{2}H]ABA. [Side-chain-^{2}H]ABA ([sc-$^2H_4$]ABA) was prepared by an adaption of the method described previously.[4] Approximately 10 mg of ABA (Sigma-Aldrich, Castle Hill, NSW, Australia) were methylated with CH_2N_2 and, after evaporation and drying under vacuum, dissolved in dry (molecular sieve) CH_3O^2H (2.5 ml). Then 0.6 M $NaOCH_3$ in CH_3O^2H was prepared from Na pieces and dry CH_3O^2H and added to the MeABA to give a 0.3 M solution of $NaOCH_3$. After flushing with N_2 this was stored in the dark for ~2 months. After an equal volume of ^2H_2O had been added the MeABA was extracted immediately three times with hexane. If H_2O had been used there was a possibility that deuterium in the Me[sc-$^2H_4$]ABA could have exchanged out owing to the presence of MeOH from the MeO^{2}H–H_2O equilibrium. Since the ^{2}H tends to exchange out of MeABA on standing, the hexane solution was dried over Na_2SO_4, evaporated and chromatographed[9] immediately on a Chiracel OD column (4.6 × 250 mm) from Daicel Chemical Industries (Tokyo, Japan) using propanzol–hexane (1 : 9) at 0.8 ml min^{-1}. The fractions containing (+)-, (2*E*) and (−)-MeABA were collected separately and immediately saponified with 60% KOH–EtOH (1 : 2) at 40 °C for 30 min. After acidification and dilution with water, the (+)-, (2*E*)- or (−)-ABA was extracted three times with diethylether, the latter being dried over Na_2SO_4. The ABA enantiomers/isomers were chromatographed using four developments on thin layers using the conditions mentioned above and the appropriate bands removed. After extraction from the silica the ABA enantiomers/isomers were finally purified and quantified by HPLC as described above. Further [sc-$^2H_4$]ABA could be obtained by isomerizing the (2*E*)-[sc-$^2H_4$]ABA in sunlight and separating the two isomers by TLC. Aliquots of the resulting [sc-$^2H_4$]ABA were converted to PFB[sc-$^2H_4$]ABA (see below) or to Me[sc-$^2H_4$]ABA with CH_2N_2 for GC/MS.

[Ring-^{2}H]ABA. ABA (0.1 mg) was dissolved in ~1 M NaO^2H in ^2H_2O (1.0 ml), allowed to stand overnight at room temperature and then acidified with oxalic acid. Dilution with water, extraction with diethylether and evaporation gave the desired [ring-$^2H_6$]ABA (principally [3′-^{2}H, 5′-$^2H_2$, 7′-$^2H_3$]ABA). Again, an aliquot was methylated to give Me[ring-$^2H_6$]ABA.

Automated synthesis of pentafluorobenzyl esters

The following procedure was completed on a PrepStation SPE Module (Hewlett-Packard, Wilmington, DE, USA).

The appropriately labelled ABA was taken up in 10 μl of dimethylacetamide (DMA)–tetramethylammonium hydroxide (TMA-OH) (5 : 1). This was followed by 10 μl of DMA–PFB bromide (10 : 3) whereupon the total was mixed for 1 min. Water–butanol–hexane (2 : 1 : 10) was added, mixed and the hexane layer extracted and evaporated to give PFBABA. A silica column (0.1 g) was washed with hexane and the PFBABA was transferred to it using hexane. The column was then washed with dichloromethane and the PFBABA eluted with 0.15 ml ethyl acetate, which was evaporated. Full details of the methods for extracting ABA and its precursors from plant material, for their derivatizations on the PrepStation and for their quantification by GC/MS will be reported elsewhere.

Gas chromatography/mass spectrometry

A ThermoQuest (Austin, Tx, USA) GCQ gas chromatograph/mass spectrometer operating in the electron capture negative ionization mode was used. The multiplier gain was set at 100 V above the optimum obtained by tuning in the electron impact mode (typically 1300–1400 V). Methane was used as the reagent gas for all the experiments reported here.

A standard split/splitless injector was used in the splitless mode and 1 μl of sample containing ~500 pg of analyte for MeABA or ~100 pg for PFBABA was injected. For MeABA samples, the injector temperature was 200 °C and the column (BPX5, 220 μm × 25 m, from SGE, Ringwood, Victoria, Australia) was held at 80 °C for 1 min

and then programmed at 25 °C min^{-1} to 210 °C, held for 1 min, programmed at 8 °C min^{-1} to 250 °C, held for 2 min, and finally programmed at 25 °C min^{-1} to 300 °C and held for 2 min. The source temperature was 150 °C. Electronic pressure control was used so that a constant linear velocity of helium of 400 mm s^{-1} was obtained. When the instrument was operated in the full-scan mode spectra were acquired from *m/z* 50 to 350. In the MS/MS mode, the ion of interest was held in the trap and fragmentation induced with a collision energy of 0.4–0.85 (arbitrary units) as indicated and then spectra acquired from *m/z* 100 to 300. In general terms, a product ion was considered to be significant if it constituted more than 10% of the base peak in the tandem mass spectrum in a majority of the analogues used.

For PFBABA samples the injector temperature was 250 °C and the same column was held at 180 °C for 3 min and then programmed at 10 °C min^{-1} to 280 °C, held for 2 min and then programmed at 20 °C min^{-1} to 300 °C and held for 2 min. The source temperature was 200 °C. The same conditions of operation with constant linear velocity as used for MeABA were used in the full-scan mode. Similar conditions to those used for MeABA were also used in the MS/MS mode where the collision energy was 0.5–0.7 as indicated. Similar criteria were used to assess the significance of product ions.

RESULTS

The isotopic composition of the labelled analogues of MeABA and of PFBABA used in this study are listed in Table 1. Note that some exchange occurs from PFB[4′-^{18}O]ABA and from PFB[ring-$^{2}H_6$]ABA during the synthesis of the PFB esters.

The full-scan ECNI mass spectrum of PFBABA is given in Fig. 2. It is similar to that presented previously[5] with the base peak at *m/z* 263 ($[M - PFB]^-$). However, in the present instrument fragments at *m/z* 219 and particularly at *m/z* 153 are apparent. The former ion is believed to arise by the loss of the carboxyl group as CO_2 because

Table 1. Isotopic composition (%) of ABA standards (based on % isotopic composition of M^- for MeABA and $[M - PFB]^-$ for PFBABA)

[1′-^{18}O]ABA	Both esters,	[$^{18}O_1$], 35; [$^{18}O_0$], 65
[4′-^{18}O]ABA	Me ester,	[$^{18}O_1$], 48; [$^{18}O_0$], 52
	PFB ester,	[$^{18}O_1$], 13; [$^{18}O_0$], 87[a]
[sc-$^{2}H_4$]ABA	Me ester[b],	[$^{2}H_5$], 4; [$^{2}H_4$], 33; [$^{2}H_3$], 10; [$^{2}H_2$], 10; [$^{2}H_1$], 15; [$^{2}H_0$], 27
	PFB ester[b],	[$^{2}H_6$], 1; [$^{2}H_5$], 8; [$^{2}H_4$], 55; [$^{2}H_3$], 27; [$^{2}H_2$], 6; [$^{2}H_1$], 1; [$^{2}H_0$], 2
[ring-$^{2}H_6$]ABA	Me ester,	[$^{2}H_8$], 1; [$^{2}H_7$], 8; [$^{2}H_6$], 69; [$^{2}H_5$], 17; [$^{2}H_4$], 3; [$^{2}H_3$], 1; [$^{2}H_0$], 1
	PFB ester,	[$^{2}H_8$], 1; [$^{2}H_7$], 8; [$^{2}H_6$], 62; [$^{2}H_5$], 23; [$^{2}H_4$], 4; [$^{2}H_3$], 1[a]
[$^{2}H_1$]MeABA	Me ester,	[$^{2}H_3$], 4; [$^{2}H_2$], 17; [$^{2}H_1$], 69; [$^{2}H_0$], 10

[a] This was the corresponding free acid to that used to synthesize the Me ester. Presumably ^{18}O or ^{2}H exchanged during the synthesis of the PFB ester.
[b] From different samples of [sc-$^{2}H_4$]ABA. The sample of Me[sc-$^{2}H_4$]ABA lost some ^{2}H during storage as the Me ester.

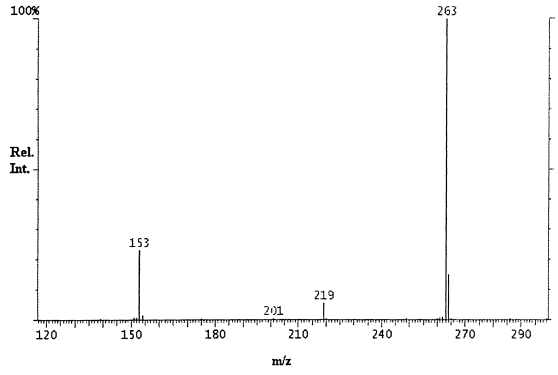


Figure 2. Full-scan methane ECNI mass spectrum of pentafluorobenzyl abscisate (PFBABA). GC and MS conditions are given under Experimental.

neither PFB[1′-^{18}O]ABA nor PFB[4′-^{18}O]ABA shows the loss of ^{18}O atoms in their full-scan spectra. The latter ion arises by the loss of the side-chain because all of the deuteriums are lost in the formation of this ion from PFB[sc-$^{2}H_4$]ABA. The PFB ester of (2*E*)-ABA shows a very much less abundant ion at *m/z* 153 and this, together with differences in retention time, allows an unambiguous distinction between the two isomers in plant extracts.

MeABA gave a full-scan ECNI mass spectrum (Fig. 3) similar to that recorded previously.[6] The abundances of the ions at *m/z* 245 and 260 are similar and virtually independent of the source temperature. However, the abundance of the *m/z* 278 ion declines with increasing source temperature from about 7 × *m/z* 260 at 150 °C to about equal to *m/z* 260 at 200 °C. Consequently, all mass spectra of MeABA and its analogues reported in this paper were obtained with a source temperature of 150 °C.

MS/MS of the PFB esters of ABA and their heavy isotope analogues

Three significant ions (Fig. 2), namely at *m/z* 263, 219 and 153, are available for MS/MS of PFBABA. In MS/MS the ion at *m/z* 263 (Table 2) gives only two significant product ions at *m/z* 219 and 153 as observed in the full-scan spectrum. It seems clear that the loss of 44 u represents CO_2 from the carboxyl group since ^{18}O atoms are not lost from either PFB[1′-^{18}O]ABA or PFB[4′-^{18}O]ABA. It can also be seen that the product ion at *m/z* 153 is unlabelled

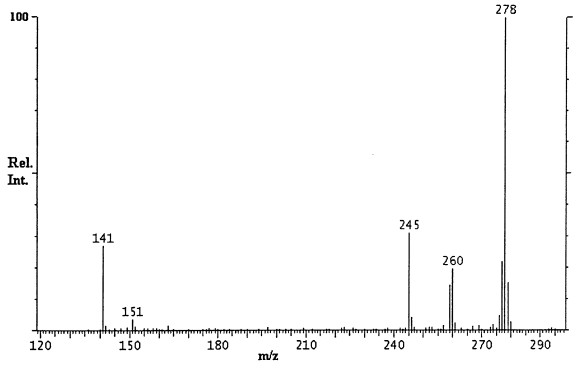
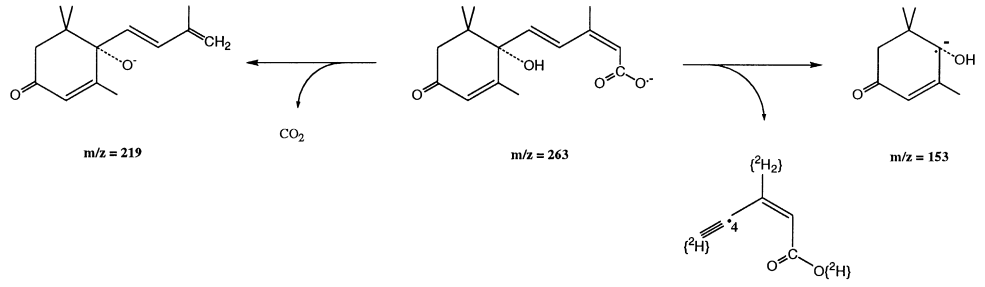


Figure 3. Full-scan methane ECNI mass spectrum of methyl abscisate (MeABA). GC and MS conditions are given under Experimental.

Table 2. Fragmentation of PFBABA ($[M - PFB]^-$) isotopomers in MS/MS (*m/z* with relative abundance (%) in parentheses)[a]

Species	PFBABA (ce = 0.5)	PFB[1′-^{18}O]ABA (ce = 0.7)	PFB[4′-^{10}O]ABA (ce = 0.5)	PFB[sc-$^2H_4$]ABA (ce = 0.7)	PFB[ring-$^2H_6$]ABA (ce = 0.5)
$[M - PFB]^-${1}	263 (6)	265 (7)	265 (8)	267 (trace)	269 (6)
$[\{1\} - CO_2]^-$	219 (32)	221 (32)	221 (36)	223 (39)	225 (33)
$[\{1\} - sc]^-$	153 (100)	155 (100)	155 (100)	153 (100)	159 (100)

[a] ce = Collision energy, arbitrary units.

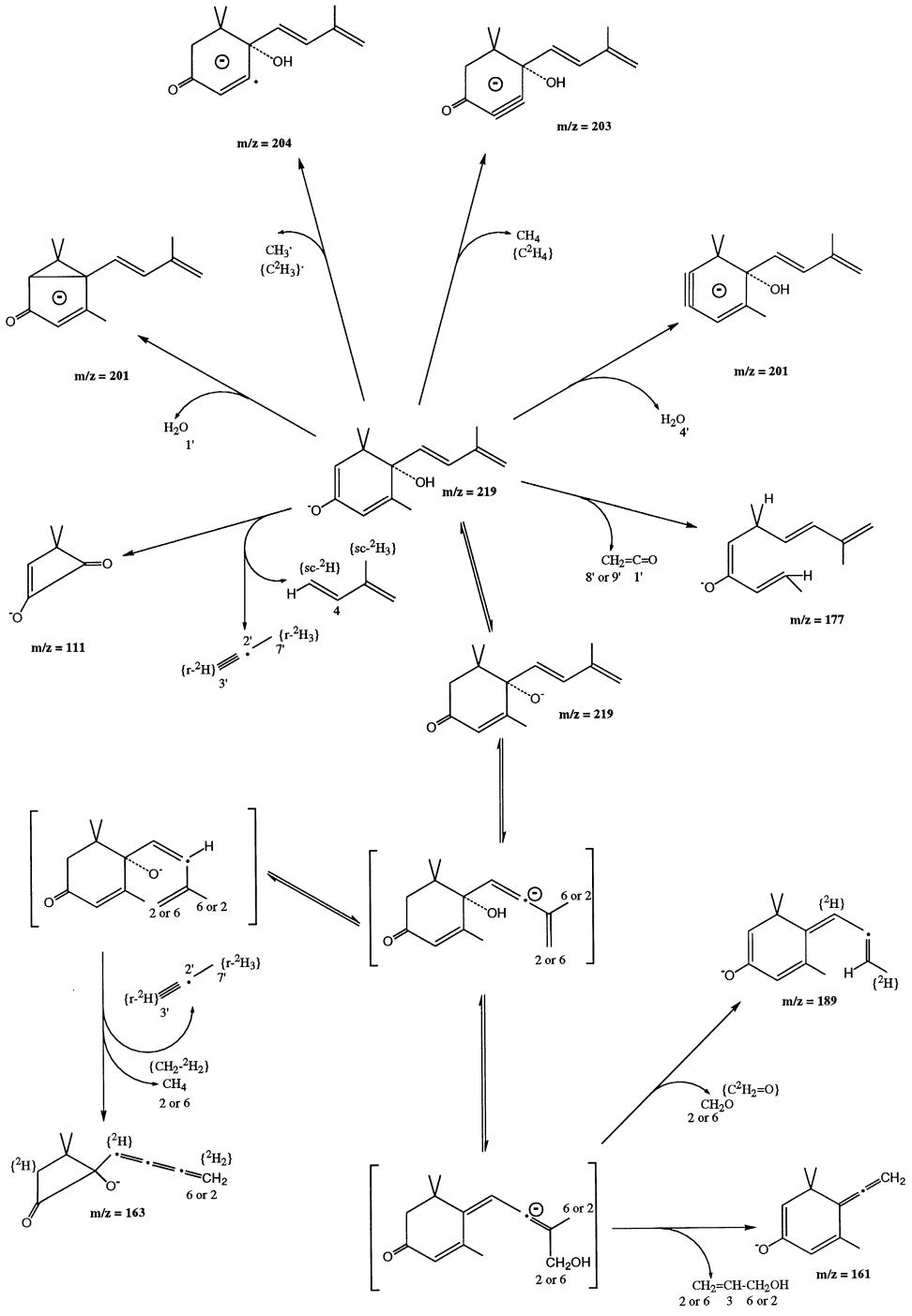


Scheme 1. Rationalization of the product ions obtained in methane ECNI MS/MS of pentafluorobenzyl abscisate (PFBABA) from the precursor ion, *m/z* 263, and its heavy isotope analogue ions. Mass to charge ratios of unlabelled ions are given in all cases. As an aid to interpretation, labelled atoms in some significant ions and neutral fragments have been added in brackets. GC and MS/MS conditions are given under Experimental.

when the analyte is PFB[sc-$^2H_4$]ABA. This indicates that the side-chain is lost in the formation of the *m/z* 153 ion. These fragmentations are rationalized in Scheme 1.

The fragmentation of $[M - PFB - CO_2]^-$ (*m/z* 219) is more complex, nine significant product ions being obtained (Scheme 2, Table 3). Two of these appear to involve the loss of a methyl radical and methane from the 7′-position since the PFB[ring-$^2H_6$]ABA loses two or three deuteriums. This and the other fragmentations discussed here are rationalized in Scheme 2. It may be that with PFB[ring-$^2H_6$]ABA some deuterium is lost from the 7′-methyl in the source so that either two or three deuteriums would be lost in the methyl radical from the 7′-position. Unfortunately, the presence of *m/z* 201 as product ions from both PFB[1′-^{18}O]ABA and PFB[4′-^{18}O]ABA indicates that when water is lost it can contain either the 1′- or the 4′-oxygen atom. Either of these losses correspond to *m/z* 207 for PFB[ring-$^2H_6$]ABA. Hence it is not clear precisely which fragmentations result in ions within the range *m/z* 208–204 in PFB[ring-$^2H_6$]ABA. The lack of *m/z* 204, implying the loss of HOD, from *m/z* 223 in PFB[sc-$^2H_4$]ABA suggests that deuterium from the 5-position is not lost as HOD from this latter analyte. However, this may be due to the transfer of deuterium being hampered by the primary isotope effect so that the more labile hydrogens, such as those at the 5′-position, are involved in water loss.

The fragmentation producing the *m/z* 189 ion seems more clear cut but more complex. The presence of this product ion in the spectrum from PFB[1′-^{18}O]ABA indicates that the tertiary hydroxyl is lost, yet the spectrum from PFB[sc-$^2H_4$]ABA suggests that either one or three deuteriums from the side-chain can also be lost. Scheme 2 offers a rationalization of this unexpected loss of formaldehyde. Note that this suggestion implies the isomerization of the side-chain at the 4-position and the transfer of the tertiary hydroxyl to the side-chain before this loss can occur. The ion at *m/z* 177 seems to involve the loss of ketene, the oxygen being derived from the 1′-tertiary hydroxyl since no *m/z* 179 ion is observed in the fragmentation of PFB[1′-^{18}O]ABA. The side-chain is retained since the corresponding fragment from PFB[sc-$^2H_4$]ABA is still fully deuteriated and yet all but one of the ring deuteriums from PFB[ring-$^2H_6$]ABA are also retained. A rationalization of this unexpected fragmentation in which ketene appears to be excised from the centre of the $[M - PFB - CO_2]^-$ ion is given in Scheme 2. Interpretation of the losses to give the product ions at *m/z* 164 plus 163 and 161 is problematic because it is not entirely clear whether ions from the deuterated analogues are related to the former pair or the latter single ion. Nevertheless, it is likely that the ion at *m/z* 163 and *m/z* 164 (*m/z* 163 plus a hydrogen atom) has lost part of the side-chain, as the remaining ion from PFB[sc-$^2H_4$]ABA probably contains either one or three deuteriums, and the 3′,2′,7′-portion of the ring as this fragment from PFB[ring-$^2H_6$]ABA contains only one deuterium. A similar ring fragment to that found with *m/z* 111 (see below) plus a cumulene remnant of the side-chain therefore seems to be a possibility for *m/z* 163 (and *m/z* 164). The ion at *m/z* 161 seems to be derived from the same intermediate as the ion at *m/z* 189: it has lost the tertiary hydroxyl and the side-chain as ions from PFB[1′-^{18}O]ABA and PFB[sc-$^2H_4$]ABA are unlabelled but the ring is intact as the ions from PFB[4′-^{18}O]ABA and PFB[ring-$^2H_6$]ABA are both fully labelled. Thus, the ring conjugated to an allene remnant from the side-chain seems a possibility, as suggested in Scheme 2. Finally, the product ion at *m/z* 111 seems to involve the loss of two fragments, namely a portion of the ring containing the 2′-, 3′-and 7′-carbon atoms and also the side-chain. Certainly, the presence of *m/z* 113 from both of the ^{18}O-labelled analogues suggests that both ring oxygens are retained in this product ion. However, all of the deuteriums are lost from PFB[sc-$^2H_4$]ABA and all but one of the deuteriums from PFB[ring-$^2H_6$]ABA are also lost.



Scheme 2. Rationalization of the product ions obtained in methane ECNI MS/MS of pentafluorobenzyl abscisate (PFBABA) from the precursor ion, $m/z = 219$, and its heavy isotope analogue ions. Mass to charge ratios of unlabelled ions are given in all cases. As an aid to interpretation, labelled atoms in some significant ions and neutral fragments have been added in brackets. The double-headed arrows indicate various formulations for *m/z* 219. GC and MS/MS conditions are given under Experimental.

The fragmentation of the ion at $m/z = 153$ ($[M - PFB - sc]^-$) is also complex and the interpretation of the results from MS/MS of PFB[ring-$^2H_6$]ABA is complicated by the possibility of exchange in the source. Scheme 3, in rationalizing the observed fragments, attempts to account for these difficulties. Thus, the effect of the exchange of three deuteriums from the *m/z* 159 ion of PFB[ring-$^2H_6$]ABA is illustrated. Other than the loss of H_2 to *m/z* 151, the fragmentations of *m/z* 153 seem to fall into two groups: first, those that lose CH_3 or H before and, to some extent, after exchange of deuteriums from PFB[ring-$^2H_6$]ABA and, second, those that lose carbon atoms from the ring itself. The latter fragmentations occur only after exchange of deuteriums from PFB[ring-$^2H_6$]ABA. Presumably these differences reflect the speed at which these two types of fragmentation occur. The rationalization in Scheme 3 suggests that one or both of the 8′,9′-*gem*-methyls are lost as radicals, there being no evidence from PFB[ring-$^2H_6$]ABA for the loss of the 7′-methyl as a radical. The fragment at *m/z* 97 in PFBABA contains both ring oxygens because both PFB[1′-^{18}O]ABA and PFB[4′-^{18}O]ABA

Table 3. Fragmentation of PFBABA ($[M - PFB - CO_2]^-$) isotopomers in MS/MS (*m/z* with relative abundance (%) in parentheses)[a]

Species	PFBABA (ce = 0.7)	[PFB[1′-^{18}O]ABA (ce = 0.7)	PFB[4′-^{18}O]ABA (ce = 0.7)	PFB[sc-$^2H_4$]ABA (ce = 0.7)	PFB[ring-$^2H_6$]ABA (ce = 0.7)
$[M - PFB - CO_2]^-\{2\}$	219 (40)	221 (27)	221 (29)	223 (12)	225 (60)
$[\{2\} - CH_3^\bullet]^-$	204 (94)	206 (100)	206 (100)	208 (90)	210 (5)
$[\{2\} - CH_4]^-$	203 (18)	205 (27)	205 (23)	207 (31)	209 (5)
					208 (29)
$[\{2\} - H_2O]^-$	201 (100)	203 (62)	203 (70)	206 (11)	207 (100)
		201 (30)	201 (74)	205 (100)	206 (62)
				204 (35)	205 (75)
$[\{2\} - H_2C{=}O]^-$	189 (35)	191 (8)	191 (42)	192 (25)	195 (24)
		189 (18)		190 (16)	
				189 (6)	
$[\{2\} - CH_2{=}C{=}O]^-$	177 (12)	177 (9)	179 (18)	181 (10)	182 (8)
					181 (8)
$[\{2\} - CH_2{=}C{=}CH_2 - CH_4]^-$	164 (5)	166 (8)	166 (13)	167 (7)	165 (4)
	163 (2)	165 (3)	165 (5)	166 (1)	164 (8)
				165 (7)	
				164 (9)	
$[\{2\} - CH_2{=}CH{-}CH_2OH]^-$	161 (14)	161 (4)	163 (8)	161 (5)	167 (15)
$[\{2\} - CH_2{=}C{=}CH_2$	111 (8)	113 (5)	113 (15)	111 (4)	112 (19)
$- CH_2{=}CH{-}C(CH_3){=}CH_2]^-$					

[a] ce = Collision energy, arbitrary units.

Table 4. Fragmentation of PFBABA $\{[M - PFB - sc]^-\}$ isotopomers in MS/MS (*m/z* with relative abundance (%) in parentheses)[a]

Species	PFBABA (ce = 0.5)	PFB[1′-^{18}O]ABA (ce = 0.5)	PFB[4′-^{18}O]ABA (ce = 0.5)	PFB[sc-$^2H_4$]ABA (ce = 0.5)	PFB[ring-$^2H_6$]ABA (ce = 0.5)
$[M - PFB - sc.]^-\{3\}$	153 (99)	155 (100)	155 (100)	153 (56)	159 (100)
$[\{3\} -^n H_2]^-$	151 (17)	153 (13)	153 (18)	151 (17)	156 (33)
$[\{3\} - CH_3^\bullet]^-$	138 (100)	140 (46)	140 (56)	138 (100)	144 (15)
$[\{3\} - CH_3^\bullet - H^\bullet]^-$	137 (55)	139 (22)	139 (26)	137 (44)	143 (30)
$[\{3\} - CH_3^\bullet - H^\bullet - H^\bullet]^-$	136 (20)	138 (9)	138 (13)	136 (22)	
					141 (59)
$[\{3\} - CH_3^\bullet - H^\bullet - CH_3^\bullet]^-$	122 (27)	124 (11)	124 (15)	122 (24)	128 (6)
					127 (6)
					125 (12)
$[\{3\} - CH_2{=}CH{-}OH]^-$	109 (22)	109 (10)	111 (8)	109 (15)	112 (18)
$[\{3\} - (CH_3)_2C{=}CH_2]^-$	97 (30)	99 (15)	99 (16)	97 (31)	100 (7)
					99 (5)

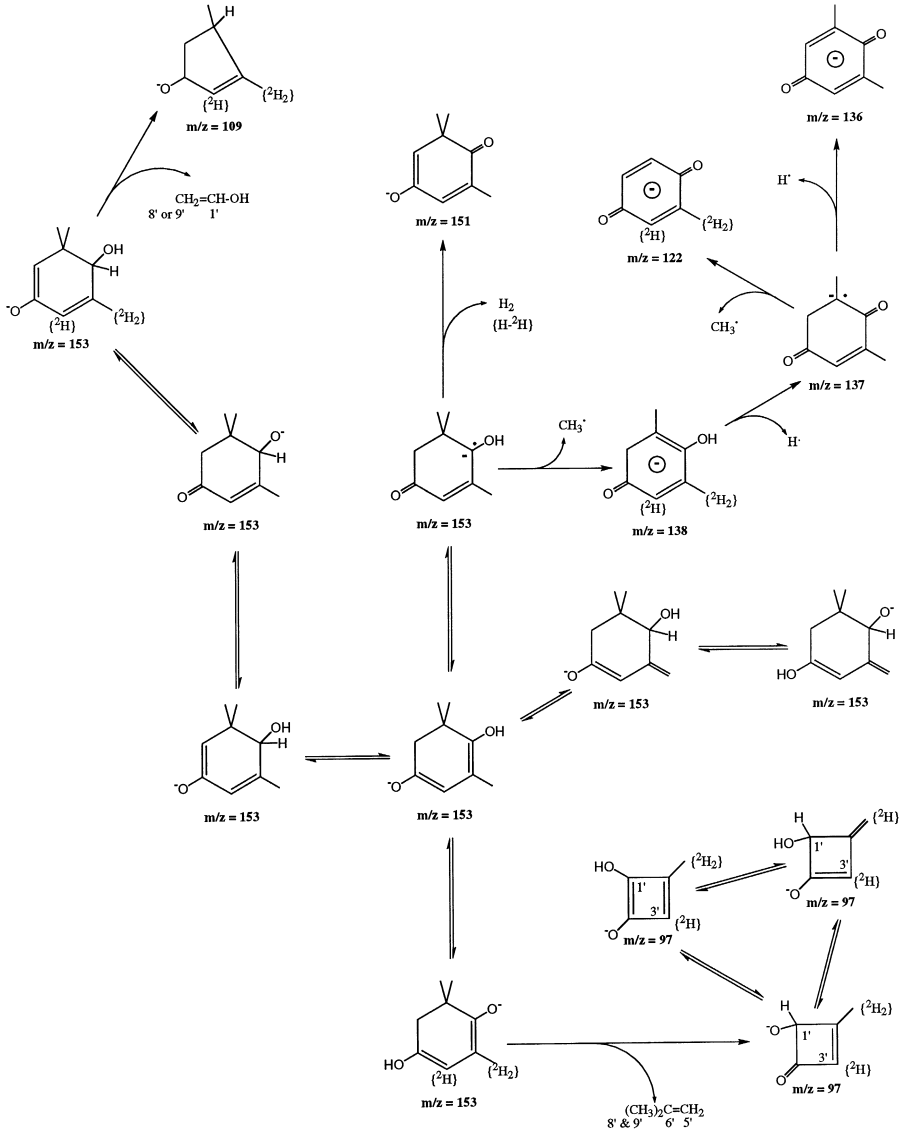
[a] ce = Collision energy, arbitrary units.

give corresponding ions at *m/z* 99. This suggests that 2-methylprop-1-ene containing the 8′-and 9′-methyls is lost. A similar fragmentation has been observed in the electron impact spectrum of MeABA.[8] The other fragmentation listed in Table 4 involves the loss of the 1′-tertiary hydroxyl since the remaining ion from MS/MS of PFB[1′-^{18}O]ABA has the same mass as that obtained from PFBABA. Scheme 3 suggests that either the 8′- or the 9′-methyl is also lost in this fragmentation.

MS/MS of the methyl esters of ABA and their heavy isotope analogues

MeABA (Fig. 3) forms three significant ions for MS/MS, at *m/z* 278 (M^-), *m/z* 260 ($[M - H_2O]^-$) and *m/z* 245 ($[M - H - CH_3OH]^-$), as reported previously.[6] An ion at *m/z* 141, or the corresponding deuterated ion from Me[sc-$^2H_4$]ABA, was observed in the mass spectrum of some MeABA samples. This is due to traces of oxygen in the mass spectrometer adding to the side-chain at the 5-position to give a side-chain fragment.[10] MS/MS was not performed on this ion and it is not discussed further.

The molecular ion of MeABA at *m/z* 278 gave three significant fragments, namely $[M - H]^-$ at *m/z* 277, $[M - H_2O]^-$ at *m/z* 260 and $[M - H - CH_3OH]^-$ at *m/z* 245, although the response was relatively poor and variable in the proportions of product ions produced (Table 5). It is clear from Table 5 that the $[M - H_2O]^-$ ion has lost the tertiary hydroxyl since *m/z* 260 is the base peak with MS/MS of Me[1′-^{18}O]ABA. Although Heath *et al.*[10] have provided evidence that the primary loss of the hydrogen radical is at the 5-position, a rearrangement presumably follows since it appears that the associated hydrogen may come from either the 5- or the 5′-position (Scheme 4). Thus, both Me[sc-$^2H_4$]ABA and Me[ring-$^2H_6$]ABA show the loss of 18 and 19 u. That is, in the case of Me[sc-$^2H_4$]ABA, the loss of HO^{2}H from the tertiary hydroxyl



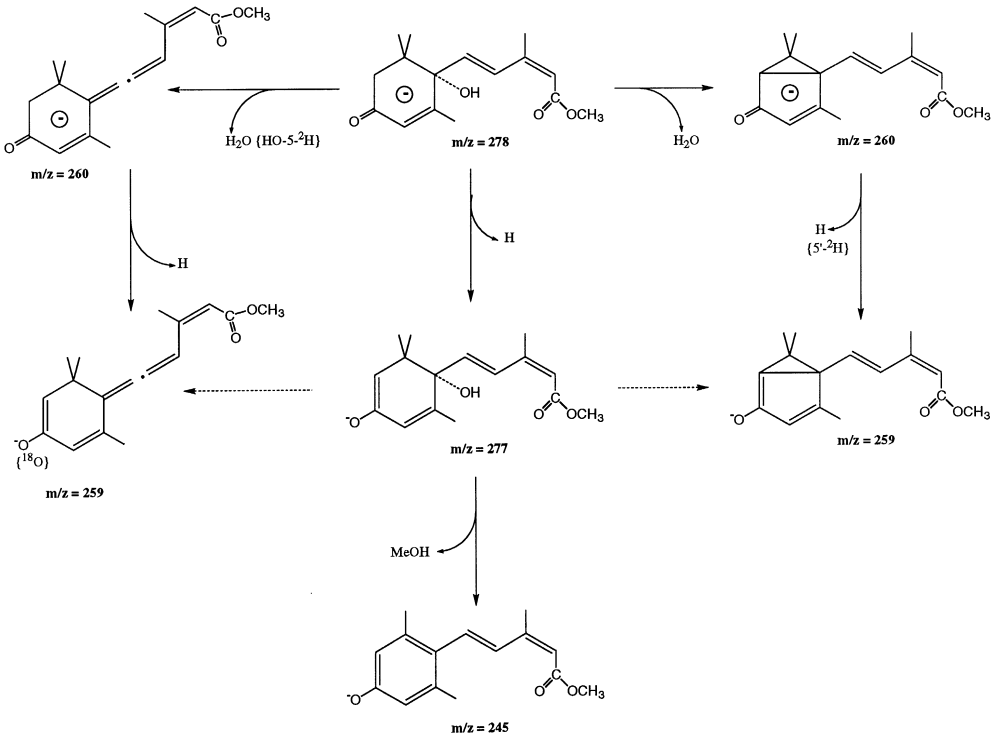
Scheme 3. Rationalization of the product ions obtained in methane ECNI MS/MS of pentafluorobenzyl abscisate (PFBABA) from the precursor ion, *m/z* = 153, and its heavy isotope analogue ions. Mass to charge ratios of unlabelled ions are given in all cases. As an aid to interpretation, labelled atoms in some significant ions and neutral fragments have been added in brackets. The double-headed arrows indicate various formulations for *m/z* 153 and 97. GC and MS/MS conditions are given under Experimental.

Table 5. Fragmentation of MeABA ($[M]^-$) isotopomers in MS/MS (*m/z* with relative abundance (%) in parentheses)[a]

Species	MeABA (ce = 0.5)	Me[1′-^{18}O]ABA (ce = 0.4)	Me[4′-^{18}O]ABA (ce = 0.5)	Me[sc-$^2H_4$]ABA (ce = 0.5)	Me[ring-$^2H_6$]ABA (ce = 0.5)	[^{2}H]MeABA (ce = 0.5)
$[M]^-$	278 (54)	280 (34)	280 (4)	282 (8)	284 (10)	279 (34)
$[M - H^\bullet]^-$	277 (97)	279 (51)	279 (23)	281 (33)	283 (5)	278 (59)
$[M - H_2O]^-$	260 (<1)	262 (5)	262 (100)	264 (70)	266 (100)	260 (<1)
			261 (28)	263 (57)	265 (37)	
		260 (100)		262 (12)	264 (6)	
$[M - H^\bullet - CH_3OH]^-$	245 (100)	247 (8)	247 (78)	249 (100)	251 (5)	246 (34)
		245 (76)		248 (19)	250 (32)	245 (100)

[a] ce = Collision energy, arbitrary units.

plus the protium/deuterium atom from C-5 followed by enolization to accommodate the charge on the 4′-oxygen, and, in the case of Me[ring-$^2H_6$]ABA, the loss of HO^{2}H from the tertiary hydroxyl plus a protium/deuterium atom from the C-5′ again followed by enolization. The loss of the 5′-hydrogen to give the enolate form at *m/z* 277 could also precede the loss of water, as indicated by the dashed arrows in Scheme 4. The loss of the 5′-hydrogen (*m/z* 277), so that the charge is carried on the 4′-oxygen, presumably catalyses the loss of methanol from either the



Scheme 4. Rationalization of the product ions obtained in methane ECNI MS/MS of methyl abscisate (MeABA) from the precursor ion, $m/z = 278$, and its heavy isotope analogue ions. Mass to charge ratios of unlabelled ions are given in all cases. As an aid to interpretation, labelled atoms in some significant ions and neutral fragments have been added in brackets. GC and MS/MS conditions are given under Experimental.

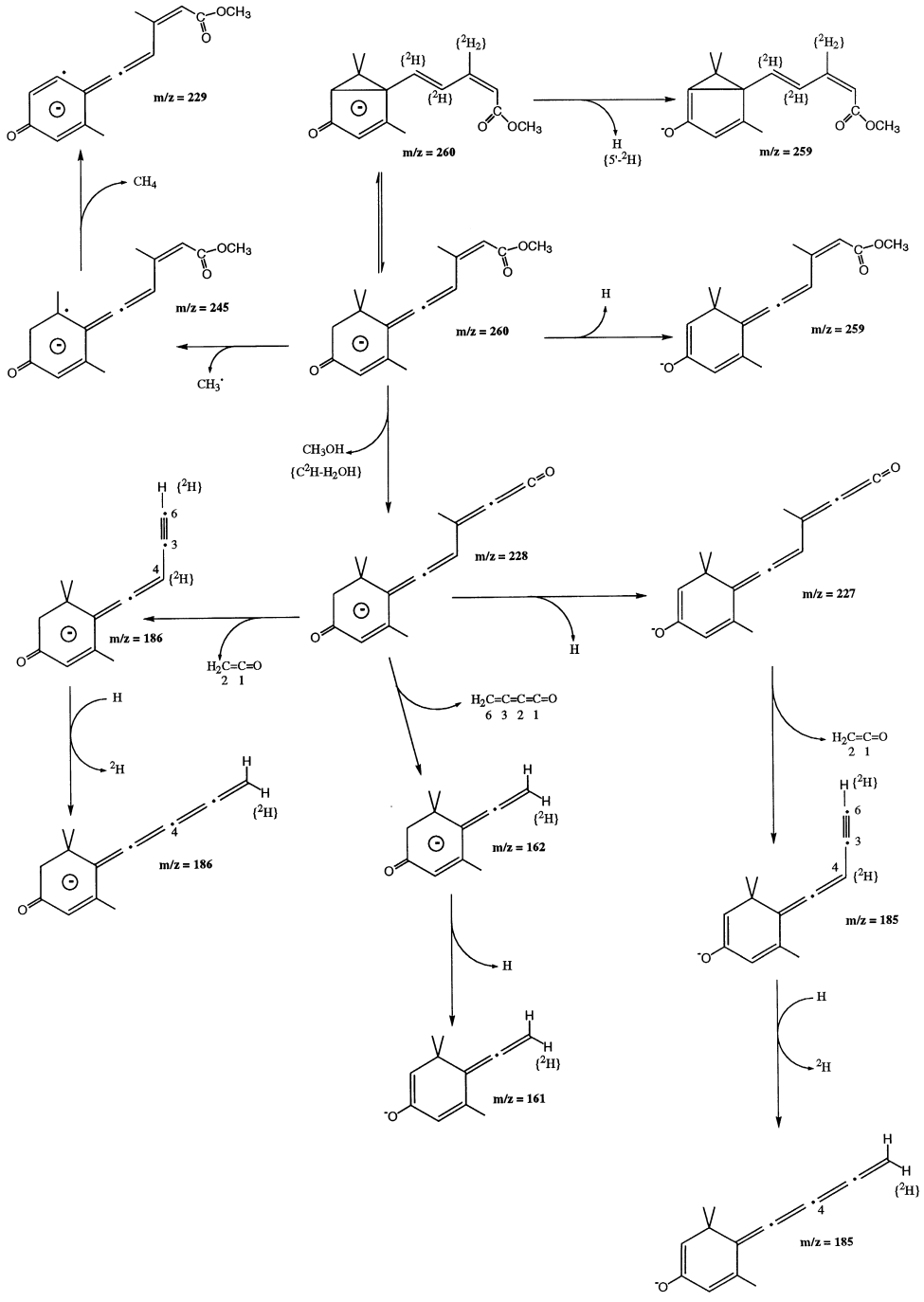
Table 6. Fragmentation of MeABA {$[M - H_2O]^-$} isotopomers in MS/MS (*m/z* with relative abundance (%) in parentheses)[a]

Species	MeABA (ce = 0.7)[b]	Me[1′-^{18}O]ABA (ce = 0.7)[b]	Me[4′-^{18}O]ABA (ce = 0.7)	Me[sc-$^2H_4$]ABA (ce = 0.7)	Me[ring-$^2H_6$]ABA (ce = 0.5)	[^{2}H]MeABA (ce = 0.7)
$[M - H_2O]^-${4}	260 (11)	260 (11)	262 (13)	264 (11)	266 (58)	261 (16)
	259 (37)	259 (37)	261 (33)	263 (42)	265 (62)	260 (41)
$[\{4\} - CH_3^\bullet]^-$	245 (2)	245 (2)	247 (3)	249 (2)	251 (<1)	246 (2)
	244 (2)	244 (2)	246 (5)	248 (1)	250 (nd)	245 (7)
$[\{4\} - CH_3OH]^-$	229 (35)	229 (35)	231 (21)	232 (5)	235 (6)	229 (32)
	228 (75)	228 (75)	230 (100)	231 (100)	234 (100)	228 (87)
$[\{4\} - CH_3OH - H_2C{=}C{=}O]^-$	186 (27)	186 (27)	188 (19)	187 (16)	192 (nd)	186 (41)
	185 (100)	185 (100)	187 (89)	186 (41)	191 (9)	185 (100)
$[\{4\} - CH_3OH - H_2C{=}C{=}C{=}O]^-$	162 (35)	162 (35)	164 (7)	163 (26)	167 (4)	162 (31)
	161 (9)	161 (9)	163 (36)	162 (15)	166 (nd)	161 (18)

[a] ce = Collision energy, arbitrary units.
[b] Data not available for Me[1′-^{18}O]ABA. Since 1′-^{18}O is lost in forming $[M - H_2O]$, data for MeABA were used.

8′- or the 9′-methyl together with the tertiary hydroxyl to give an aromatic ring in conjugation with the side-chain. Heath *et al.*,[10] however, proposed the loss of a methyl radical from the 8′- or 9′-methyl after the loss of the tertiary hydroxyl plus the 5-hydrogen as an alternative. Certainly the 1′-hydroxyl is lost in this fragmentation because *m/z* 245 is a much more significant ion than *m/z* 247 in the MS/MS of Me[1′-^{18}O]ABA.

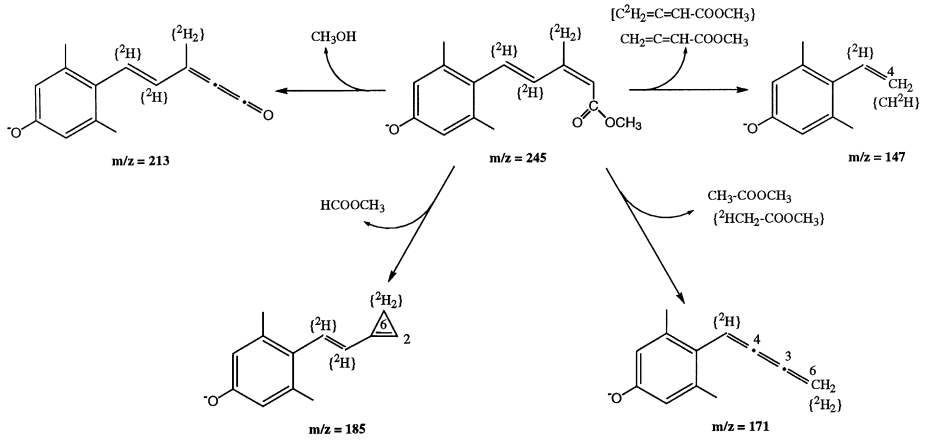
Scheme 5 rationalizes the fragmentation of the ion at *m/z* 260 in MS/MS. The ion at *m/z* 260 is shown as having lost the 5-hydrogen with the tertiary hydroxyl in forming this ion, although it is also possible that the 5′-hydrogen could be lost. This latter loss probably predominates with Me[sc-$^2H_4$]ABA because this analogue contains one ^{2}H at the 5-position. This is illustrated in Scheme 5 to account for the four ^{2}H observed with Me[sc-$^2H_4$]ABA (Table 6). The ion at *m/z* 245 appears to be formed by the loss of a methyl radical from either the 8′-or the 9′-*gem*-methyl, in agreement with Heath *et al.*[10] Certainly the methyl ester methyl, the 6-methyl or the 7′-methyl are not lost in this fragmentation because [^{2}H]MeABA, Me[sc-$^2H_4$]ABA and Me[ring-$^2H_6$]ABA all show a loss of 15 u. The *m/z* 229 ion appears to be formed from the *m/z* 245 ion by the loss of methane to give a quinone-type structure in conjugation with the side-chain. However, some of *m/z* 229 would be from *m/z* 228 containing ^{13}C. This latter ion is formed from *m/z* 260, in which the 5-hydrogen atom has been lost, by the loss of MeOH from the methyl ester since [^{2}H]MeABA contains *m/z* 228 as the base peak, as does MeABA. The *m/z* 228 ion can either lose



Scheme 5. Rationalization of the product ions obtained in methane ECNI MS/MS of methyl abscisate (MeABA) from the precursor ion, $m/z = 260$, and its heavy isotope analogue ions. Mass to charge ratios of unlabelled ions are given in all cases. As an aid to interpretation, labelled atoms in some significant ions and neutral fragments have been added in brackets. The double-headed arrows indicate two alternative formulations for *m/z* 260. GC and MS/MS conditions are given under Experimental.

a hydrogen atom to give *m/z* 227, which further loses ketene from the end of the side-chain to give *m/z* 185, or can lose ketene directly to give *m/z* 186. Note that this product ion from Me[sc-$^2H_4$]ABA only contains one ^2H atom whereas the structures shown for *m/z* 186 and 185 would be expected to contain two ^2H atoms. This can be rationalized as shown in Scheme 5 by assuming exchange during isomerization to the cumulene structure. The ion at *m/z* 162 has also lost part of the side-chain as this ion only contains one ^2H atom from Me[sc-$^2H_4$]ABA and none from [^2H]MeABA. Scheme 5 suggests that it is the 3–4 bond that is ruptured during this fragmentation.

In comparison with the *m/z* 260 ion, the fragmentation of the *m/z* 245 ion is relatively simple, principally because the ring apparently stabilizes as a phenolic anion even if the initial loss of a hydrogen is from the 5-position.[10] In fact, the fragments that are observed are the result of neutral losses of various sizes from the side-chain. Thus the *m/z* 213 ion results from the loss of methanol, *m/z* 185 from the loss of methyl formate, *m/z* 171 from the loss of methyl acetate and *m/z* 147 from the loss of methyl buta-2,3-dienoate, all from the carboxyl end of the side-chain. On the assumption that protium, rather than deuterium, is preferentially transferred in these fragmentations, these



Scheme 6. Rationalization of the product ions obtained in methane ECNI MS/MS of methyl abscisate (MeABA) from the precursor ion, $m/z = 245$, and its heavy isotope analogue ions. Mass to charge ratios of unlabelled ions are given in all cases. As an aid to interpretation, labelled atoms in some significant ions and neutral fragments have been added in brackets. GC and MS/MS conditions are given under Experimental.

Table 7. Fragmentation of MeABA {$[M - H - CH_3OH]^-$} isotopomers in MS/MS (m/z with relative abundance (%) in parenthese)[a]

Species	MeABA (ce = 0.7)	Me[1′-^{18}O]ABA (ce = 0.7)	Me[4′-^{18}O]ABA (ce = 0.7)	Me[sc-$^2H_4$]ABA (ce = 0.85)	Me[ring-$^2H_6$]ABA (ce = 0.7)	[^{2}H]MeABA (ce = 0.8)
$[M - H - CH_3OH]^-${5}	245 (14)	245 (11)	247 (16)	249 (1)	250 (27)	246 (tr)
$[\{5\} - CH_3OH]^-$	213 (51)	213 (53)	215 (56)	217 (55)	218 (100)	213 (57)
				216 (55)	217 (18)	
$[\{5\} - H(C{=}O)OCH_3]^-$	185 (26)	185 (24)	187 (28)	189 (42)	190 (35)	185 (21)
				188 (12)	189 (6)	
$[\{5\} - CH_3(C{=}O)OCH_3]^-$	171 (100)	171 (100)	173 (100)	175 (45)	176 (73)	171 (100)
				174 (100)	175 (51)	
				173 (47)	174 (36)	
$[\{5\} - CH_2{=}C{=}CH-(C{=}O)OCH_3]^-$	147 (26)	147 (25)	149 (29)	150 (15)	152 (41)	147 (16)
				149 (26)	151 (14)	
				148 (16)		

[a] ce = Collision energy, arbitrary units.

results are rationalized in Scheme 6. If it is assumed that the presence of deuterium does not affect the position of a fragmentation and that deuterium is only transferred during the loss of neutrals if protium is absent from the given position, an approximate isotopomer composition for Me[sc-$^2H_4$]ABA can be calculated. Thus, for example, the presence of equal abundances of m/z 217 and m/z 216 (Table 7) in the MS/MS of Me[sc-$^2H_4$]ABA suggests that the 2-position is 50% labelled with ^{2}H. Continuing this line of reasoning from the data listed in Table 7 with the fragmentations outlined in Scheme 6 suggests that Me[4-^{2}H, 5-^{2}H, 6-$^2H_2$]ABA and Me[2-^{2}H, 4-^{2}H, 5-^{2}H, 6-$^2H_1$]ABA are the most abundant isotopomers at about 39% and 24%, respectively.

DISCUSSION

In an earlier publication[4] we observed, in ^{1}H NMR, complete exchange of the 2-and 6-positions. Assuming that a maximum of 10% ^{1}H at these positions was at the limits of detection, this implies that at least 80% of the sample was Me[2-^{2}H, 6-$^2H_3$]ABA. This contrasts with the present sample where the calculation described above suggests that Me[2-^{2}H, 6-$^2H_3$]ABA forms about 11% of the total. In the present study we attempted to obtain protium-free [sc-$^2H_4$]ABA for use as an internal standard so we saponified the methyl esters obtained from the reaction mix immediately after isolating (+)-, (2*E*)-and (−)-MeABA from the chiral column. We then chromatographed the free acids on silica thin-layer plates, a procedure that we now suspect leads to the loss of ^{2}H since only [$^2H_4$]ABA is obtainable after this step while more enriched [^{2}H]ABA ($^2H_0$, 3%; $^2H_1$, 5%; $^2H_2$; 11%; $^2H_3$, 28%; $^2H_4$, 18%; $^2H_5$, 27%; $^2H_6$, 7%; corrected for ^{13}C) can be isolated if reversed-phase HPLC of the free acids is substituted for the thin-layer step. Willows *et al.*[4] purified the methyl esters from the reaction mix by silica solid-phase extraction and by normal-phase HPLC so that considerable opportunity was available for the loss of deuterium. It seems likely, then, that the different labelling patterns are due to loss of deuterium from different parts of the ABA side-chain in the purification procedures; perhaps silica TLC of the free acid favours loss from the 2-and 6-positions while normal-phase HPLC favours loss from the 4-and 5-positions.

The results presented in this paper allow us to dissect the ABA molecule to determine where heavy isotopes

from appropriately labelled substrates have been incorporated. For example, if a ^{13}C-labelled precursor were to be incorporated we could determine if C-1 was labelled from the MS/MS of PFBABA: the precursor ion, *m*/*z* 263 would be labelled but the product ion, *m*/*z* 219, would be unlabelled. A similar result would also be obtained if the amount of label in *m*/*z* 213 was compared with that in *m*/*z* 185, both product ions from MS/MS of *m*/*z* 245 from MeABA. Using the data in the schemes and tables we could, in a similar manner, determine if C-2 (*m*/*z* 213 and 171 from *m*/*z* 245 from MeABA), C-6 (*m*/*z* 189 from *m*/*z* 219 from PFBABA with *m*/*z* 185 from *m*/*z* 245 from MeABA) and C-3 (*m*/*z* 147 from *m*/*z* 245 from MeABA with the data for C-6 just mentioned). The C-4 and C-5 atoms remain as a pair in all the fragmentations described here so that any labelling of one or other of these two atoms cannot be distinguished. By similar comparisons it should be possible to determine if C-1′, C-2′ plus C-3′ plus C-7′, possibly C-7′ and therefore C-2′ plus C-3′, C-4′, C-5′ plus C-6′ and C-8′ plus C-9′ are labelled using MS/MS on the two product ions, at *m*/*z* 219 and 153, from PFBABA. Also, the incorporation of ^{18}O into the carboxyl oxygens, the 1′-oxygen and the 4′-oxygen can be distinguished from tandem mass spectra of both MeABA and PFBABA.

Recent evidence[11] suggests that chloroplastic carotenoids are synthesized via the glyceraldehyde phosphate/pyruvate pathway and that ABA is formed from the oxidative cleavage of 9-*cis*-violaxanthin and/or 9-*cis*-neoxanthin[12] via xanthoxal.[13] In fact, Lee and Milborrow have shown[14] that labelled pyruvate is incorporated into carotenoids and ABA in isolated intact chloroplasts. Hence there is little doubt that ABA is synthesized from carotenoids in plastids but there are several observations that suggest that the situation is more complex in stressed plants. The most important of these observations is the extensive incorporation of deuterium from ^2H_2O into ABA in the absence of similar incorporation into carotenoids in stressed tomato seedlings.[15] If sufficient incorporation of an appropriately labelled substrate can be obtained it should be possible to distinguish this deuterium-incorporating pathway from the chloroplastic glyceraldehyde phosphate/pyruvate pathway using the techniques described in this paper.

Acknowledgements

The mass spectrometer used here was provided by the Australian Research Council under the RIEF grants scheme. We are grateful to F. Warganegara, P. Duffield and B. V. Milborrow for the provision of labelled ABA samples and to D. Bourne for his extensive unpublished work on the fragmentation of MeABA and for critically assessing the manuscript.

REFERENCES

1. R. D. Willows, A. G. Netting and B. V. Milborrow, *Aust. J. Plant Physiol.* **21**, 327 (1994).
2. A. G. Netting and B. V. Milborrow, *Aust. J. Plant Physiol.* **21**, 345 (1994).
3. A. G. Netting, M. L. Windsor and B. V. Milborrow, *Aust. J. Plant Physiol.* **24**, 175 (1997).
4. R. D. Willows, A. G. Netting and B. V. Milborrow, *Phytochemistry* **30**, 1483 (1991).
5. A. G. Netting and B. V. Milborrow, *Biomed. Environ. Mass Spectrom.* **17**, 281 (1988).
6. A. G. Netting, B. V. Milborrow, G. T. Vaughan and R. O. Lidgard, *Biomed. Environ. Mass Spectrom.* **15**, 375 (1988).
7. G. L. Boyer, B. V. Milborrow, P. F. Wareing and J. A. D. Zeevaart, in *Plant Growth Substances 1985*, edited by M. Bop p. 99. Springer, Berlin (1986).
8. R. T. Gray, R. Mallaby, G. Ryback and V. P. Williams, *J. Chem. Soc., Perkin Trans. 2* 919 (1974).
9. I. D. Railton, *J. Chromatogr.* **402**, 371 (1987).
10. T. G. Heath, D. A. Gage, J. A. D. Zeevaart and J. T. Wilson, *Org. Mass Spectrom.* **25**, 655 (1990).
11. H. K. Lichtenthaler, J. Schwender, A. Disch and M. Rohmer, *FEBS Lett.* **400**, 271 (1997).
12. S. H. Schwartz, B. C. Tan, D. A. Gage, J. A. D. Zeevaart and D. R. McCarty, *Science* **276**, 1872 (1997).
13. H. S. Lee and B. V. Milborrow, *Aust. J. Plant Physiol.* **24**, 727 (1997).
14. H. S. Lee and B. V. Milborrow, *Aust. J. Plant Physiol.* **25**, 507 (1998).
15. H. M. Nonhebel and B. V. Milborrow, *J. Exp. Bot.* **38**, 980 (1987).

