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SYNTHESIS OF STABLY DEUTERIATED ABSCISIC ACID, PHASEIC ACID AND RELATED COMPOUNDS

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Key Word Index—Abscisic acid; phaseic acid; deuteriation; internal standards; mass spectrometry; isomerization.

Abstract—Methyl esters of abscisic acid (ABA) analogues and metabolites dissolved in 0.1–1.0 M sodium methoxide in MeO^2H replaced four of their sidechain ^1H atoms with ^2H (C-4 and C-6) over a period of 4 to 8 weeks. The esters were hydrolysed by adding ^2H_2O to provide $^2H_4$ labelled free acids. ABA, 1′,2′-epoxy-β-ionylidene acetic and phaseic acids have been labelled in this way. The method can be used to make ^3H labelled materials.

INTRODUCTION

Selected ion monitoring of phytohormones is one of the most accurate methods for determining their concentrations. The method is well documented [1–4]. The use of methane negative ion chemical ionization mass spectrometry (NICI-MS) coupled with the formation of suitable derivatives enables quantities to be measured down to the attamolar range [5]. If appropriate amounts of the compound, stably labelled with a heavy isotope, are added as an internal standard during the initial extraction, then the procedure gives an estimate of the amount of material present in the tissue that is unaffected by losses during purification.

The *de novo* synthesis of abscisic acid labelled with two or three ^2H or ^{13}C atoms is a complex and costly procedure. The preparation of the labelled metabolites is so much more so that it has not been attempted, other than by feeding suitably labelled ABA to plants and recovering labelled metabolites [6, 7]. The disadvantage of this method is that the labelled samples contain some endogenous, unlabelled material.

The most readily obtainable labelled form of ABA is the hexa-deuterio material produced by exchange in M $[^2H]NaOH$ in $[^2H]H_2O$ [2, 6]. There are dangers inherent in using it because the deuterium is lost by exchange above pH 11, and although this is little appreciated, it is also removed by acidic conditions [8]. Thus great care has to be taken to avoid extremes of pH at all times. [C-6-$^2H_3$]ABA (revised numbering [9]) is more difficult to synthesize [3] but the presence of the three extra mass units places its parent ion well away from that of the unlabelled molecules and the compound labelled in this way has considerable utility as the deuterium atoms are stable in this position even at high pH. However, as noted earlier [10], the deuterium at C-6 appears to be lost very slowly from ABA Me esters. We have now investigated the process and found that the exchange mechanism can be exploited to produce $^2H_4$ labelled samples of analogues and metabolites of ABA which are difficult to synthesize *de novo*.

RESULTS AND DISCUSSION

Treatment of MeABA with 0.1–1.0 M MeONa completely exchanged the four hydrogen atoms at C-2 and C-6 of the ABA and the Me 2-*trans*-ABA formed from it. The C-2 double bond underwent base catalysed isomerization to give a 7:13 ratio of *cis*:*trans* isomers under these conditions. This isomerization also occurred with the other compounds, resulting in a 13:12 ratio of *cis*:*trans* isomers for phaseic acid (PA) and a 1:1 ratio for the 1′,2′-epoxy-β-ionylidene acetate.

It was noted that there was no observable isomerization in conditions that did not cause exchange, which indicates that isomerization and exchange occur together. A mechanism for the exchange of the sidechain is shown in Fig. 1, it accounts for exchange of the C-2 and C-6 hydrogen atoms as well as the observed isomerisation.

The concentrations of the analogues of ABA used were found to affect the amounts of deuteriated material recovered. All experiments were carried out on a 1–2 ml scale with the exception of that with phaseic acid, which was dissolved in 100 μl. Preliminary experiments with low concentrations of MeABA (0.5 mg or less per ml) resulted in less than 0.1% of deuteriated MeABA recovered after 2 months exchange in both 100 and 300 mM MeONa. When the concentrations were increased the yields increased to the values shown in Table 1. The low yield of MePA may result from losses during purification, however, approximately 15 other products were observed during HPLC purification which indicates that other reactions had occurred.

2-*trans*-ABA has been used as a quasi-internal standard for GC [11] but the potential for highly alkaline surfaces to cause significant isomerization when experiments were carried out at the nanogram level or lower suggests that *trans*-ABA should not be used in this way. We have also found that MeABA stored in EtOH at −20° for up to 6 months can *trans*-esterify resulting in up to 5% Et-ABA. Consequently the addition of Et-ABA to

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solutions containing MeABA, as a quasi-internal standard for GC [12] must also be treated with caution. We have shown, by using [$^2H_4$]MeOH, that the methyl ester group of ABA, under the conditions used for exchange, completely *trans*-esterifies with methanol.

The feature of the use of [$^2H_4$]MeABA for SIM that is most prone to error is the longer R_t of deuteriated compared with undeuteriated material when long, open bore GLC columns are used to separate the components of an extract. The peak of Me [C-6-$^2H_3$]ABA is delayed some 6 sec in a R_t of *ca* 15 min (0.6%) so all the scans of a light and a labelled peak should be summed before the integrals are calculated and compared.

The exchange of hydrogen atoms at C-2 of carboxylic acid methyl esters in strongly basic methanol has been known for some time but has not been much used for the preparation of deuteriated standards. We have prepared [$^2H_2$]Me palmitate using the same conditions. The advantage of the C-6-Me-2-ene sidechain of ABA and related compounds is that four ^1H atoms are replaced by ^2H so the parent ions of the labelled molecules are separated by 4 mass units and are not superimposed in the mass spectrum on the subsidiary, natural abundance peaks of the unlabelled molecules.

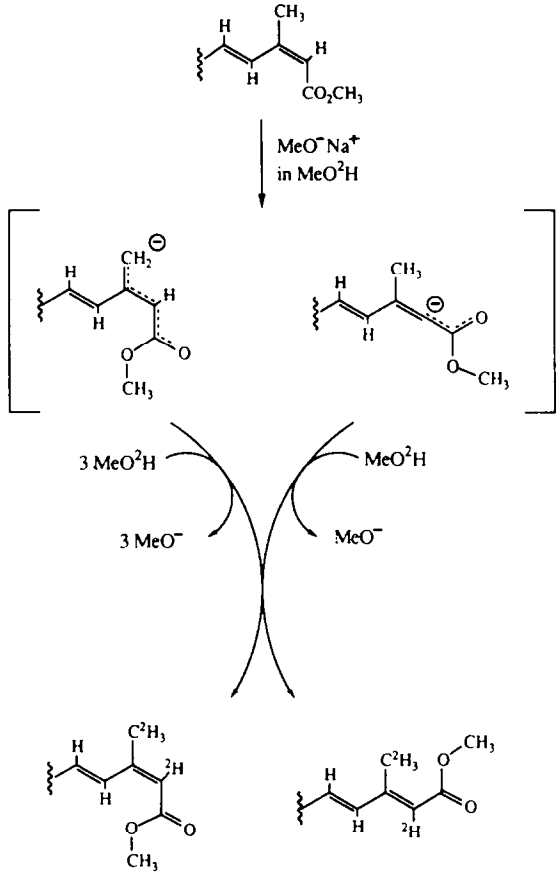

Fig. 1. Proposed mechanism for the exchange of MeABA, MePA and Me 1′,2′-epoxy-β-ionylidene acetate by NaOMe in [^2H]MeOH and the resultant isomerization of the C-2 double bond.

EXPERIMENTAL

General. All solvents were analytical grade or better and, with the exception of the MeO^{2}H (99.8 atom% ^2H), were dried over a 4 Å molecular sieve, Union Carbide. All glasswear was flamed and cooled in a desiccator. ABA, 2-*trans*-ABA and PA were methylated in MeOH with ethereal CH_2N_2, evapd under N_2 and dried overnight at 0.05 mm Hg at 40°. 1′,2′-Epoxy-β-ionylidene acetic acid was also methylated in MeOH with ethereal CH_2N_2 but was dried by azeotroping with dry toluene under N_2. Samples, 2 mg ml^{-1}, were exchanged in MeO^{2}H (99.8 atom% ^2H) under N_2 at various concens of MeONa for 8–10 weeks.

Purification. After exchange in MeO^{2}H the samples were neutralized with a small excess of HOAc, evapd under N_2 dissolved in MeOH, remethylated with CH_2N_2 in Et_2O and evapd under N_2. The samples were dissolved in 5 ml EtOAc (MePA) or 5 ml Et_2O and loaded onto silica Sep-Paks (Waters) and eluted with another 5 ml of solvent, the two frs were combined and evapd under N_2. The MeABA samples were then chromatographed in a Techsil 10 silica gel HPLC column (250 × 8 mm) eluted with *iso*-PrOH–hexane (3:97) at 3 ml min^{-1}. MeABA eluted between 9.5 and 10.5 min and Me 2-*trans*-ABA eluted between 11 and 12 min. The MePA samples were chromatographed in the same system but eluted with *iso*-PrOH–hexane (1:19) at 3 ml min^{-1}. MePA eluted between 13 and 15 min and Me *trans*-PA eluted between 15 and 17 min. Detection and quantification of MeABA and MePA was performed at 263 nm. [$^2H_4$]Me 1′,2′-epoxy-β-ionylidene acetate was not further purified but was analysed for its deuterium content by GC-MS as below. The [$^2H_{10}$]MeABA and [$^2H_{8-10}$]MePA isomers were analysed for their ^2H content by methane positive ion and/or negative ion CIMS [9]. The position of the label in ABA was confirmed by NMR.

The labelled compounds were then hydrolysed to their free acids in 60% KOH in EtOH for 40 min at 40° which also removed the exchangeable deuteriums at C-3′, C-5′ and C-7′ to give [$^2H_4$]ABA, [$^2H_4$]1′,2′-epoxy-β-ionylidene acetate and [$^2H_4$]PA. The amount of label was determined by remethylating a small amount and obtaining a mass spectrum. ^2H atoms are not lost from C-2 and C-6 of the free acids under these conditions.

Mass spectrometry. Methane positive and/or negative-ion CI spectra: samples were either introduced on the probe which was heated to 300° over 10 sec or were introduced by GC. GC was carried out on a 15 m × 0.32 mm i.d. BP-5 column, film thickness 1 μm, carrier gas was He at 2 ml min^{-1} and the temp. programmed from 150° at 10° min^{-1}.

NICI spectra. [$^2H_{10}$]MeABA; *m/z* (rel. int.): 141 (3.7), 142 (0.4), 143 (16), 144 (87), 145 (100), 146 (4.4), 156 (9.7), 157 (2), 158 (20), 159 (1), 252 (3.3), 253 (0.5), 254 (3.6), 255 (3), 256 (3.5), 267 (5), 268 (12.8), 269 (24), 270 (9), 285 (8.3), 286 (16), 287 (68.3), 288 (92.5), 289 (3.5); [$^2H_4$]MeABA; *m/z* (rel. int.): 141 (2), 143 (11), 144 (48), 145 (100), 146 (6), 152 (13), 248 (3), 249 (7), 250 (2), 262 (6), 263 (12), 264 (5), 279 (2), 280 (6), 281 (12), 282 (9). [$^2H_{10}$]MeABA; *m/z* (rel. int.): 144 (6.5), 145 (100), 146 (10.6), 147 (7.2), 156 (5.5), 157 (5.6), 158 (6), 254 (2), 267 (3), 268 (10), 269 (12), 270 (7), 285 (4), 286 (15), 287 (25), 288 (37), 289 (8.2); [$^2H_4$]Me 2-*trans*-ABA; *m/z* (rel. int.): 143 (6), 144 (27), 145 (100), 146 (8), 152 (15), 248 (3), 249 (10), 250 (2), 262 (9), 263 (18), 264 (6), 280 (4), 281 (18), 282 (11) [$^2H_0$]MePA; *m/z* (rel. int.): 141 (86), 142 (2), 168 (28), 169 (3), 293 (100), 294 (40), 295 (10); [$^2H_8$]MePA; *m/z* (rel. int.): 141 (19), 142 (9), 143 (20), 144 (100), 145 (8), 171 (2), 293 (2), 297 (2), 298 (3), 299 (4); [$^2H_4$]MePA; *m/z* (rel. int.): 141 (24), 142 (5), 143 (16), 144 (100), 145 (24), 168 (23), 293 (32), 294 (12), 295 (10), 296 (11), 297 (11) [$^2H_8$]Me 2-*trans*-PA; *m/z* (rel. int.): 141 (4), 142 (4), 143 (7), 144 (100), 145 (16), 171 (3), 172 (3), 173 (3), 174 (2), 297 (2), 298 (4),

Table 1. Deuteration of the side chain of MeABA, MePA and Me 1′,2′-epoxy-β-ionylidene acetate by various concentrations of NaOMe in [^{2}H]MeOH over an 8–10 weeks period and at a temperature of 20°

	Sodium methoxide (mM)	Time (days)	^2H in side chain (%)*					Recovery (%)†
			$^2H_0$	$^2H_1$	$^2H_2$	$^2H_3$	$^2H_4$	
MeABA	0.1	66	100	0	0	0	0	n.d.
	1	66	100	0	0	0	0	n.d
	10	66	100	0	0	0	0	n.d
	100	54	n.d.	n.d.	n.d.	n.d.	n.d	0–10‡
	100	71	2	0	8	41	47	80–90
	300	71	1	0	7	30	62	50–60
	1000	54	n.d	n.d	n.d	n.d	n.d	0
Me 2-*trans*-ABA	0.1	66	100	0	0	0	0	n.d
	1	66	100	0	0	0	0	n.d
	10	66	100	0	0	0	0	n.d
	100	54	n.d	n.d	n.d	n.d	n.d	0–10‡
	100	71	0	0	0	6	94	80–90
	300	71	0	0	0	3	97	50–60
	1000	54	n.d	n.d	n.d	n.d	n.d	0
MePA	100	71	12	6	13	64	5	10
Me 2-*trans*-PA formed in this exchange			2	2	0	81	15	—
	300	71	n.d	n.d	n.d	n.d	n.d	0
Me 2-*trans*-1′,2′-epoxy-β-ionylidene acetate	100	71	0	0	8	28	64	90–100
	300	71	0	0	0	2	98	90–100

*Deuterium content for starting isomer only is shown.

† Approximate total yield for both *cis* and *trans* isomers formed during exchange.

‡ Exchanged in [$^2H_4$]MeOH. MeABA (0.5 mg ml^{-1}). NMR indicates complete exchange and complete *trans* esterification of methyl ester.

n.d. = not determined.

299 (11), 300 (23), 301 (24), 302 (18), 303 (7); [$^2H_3$]Me 2-*trans*-PA; *m/z* (rel. int.): 141 (3), 142 (2), 144 (100), 145 (19), 168 (5), 169 (4), 170 (4), 296 (5), 297 (5).

PICI spectra. [$^2H_0$]Me 1′,2′-epoxy-β-ionylidene acetate; *m/z* (rel. int.): 109 (22), 111 (20), 123 (45), 125 (5), 133 (9), 137 (9), 139 (5), 151 (5), 153 (23), 154 (2), 155 (2), 173 (5), 175 (15), 179 (2), 187 (9), 189 (22), 191 (18), 205 (25), 207 (10), 215 (18), 221 (5), 233 (100), 234 (16), 247 (18), 248 (2), 264 (10), 265 (95), 266 (16); [$^2H_4$]Me 1′,2′-epoxy-β-ionylidene acetate; *m/z* (rel. int.): 109 (5), 111 (6), 123 (7), 125 (5), 126 (9), 137 (4.5), 139 (4.2), 151 (5), 153 (1.5), 154 (1.5), 155 (1.5), 156 (1.6), 157 (7.5), 173 (11), 178 (3.3), 179 (9), 191 (6.7), 192 (7.5), 193 (11), 194 (6), 195 (6), 208 (6), 209 (16), 218 (6), 219 (15.4), 225 (2.3), 235 (13.5), 236 (44.5), 237 (100), 238 (16.2), 250 (6.2), 251 (16.5), 252 (3.4), 267 (9.7), 268 (27.4), 269 (93), 270 (22). [$^2H_0$]MePA; *m/z* (rel. int.): 125 (4), 139 (2), 149 (3), 165 (3), 219 (2), 235 (2.5), 245 (9), 247 (5), 263 (22), 264 (5), 277 (100), 278 (25), 279 (5), 295 (6); [$^2H_8$]MePA; *m/z* (rel. int.): 125 (6), 129 (24), 155 (5), 169 (2), 251 (4), 252 (20), 253 (6), 254 (12), 255 (6), 266 (10), 267 (12), 268 (15), 269 (26), 270 (12), 271 (16), 272 (10), 280 (44), 281 (58), 283 (100), 284 (98), 285 (78), 286 (40), 287 (6).

NMR. ^{1}H NMR spectra 300 MHz in [^{2}H]$CHCl_3$ in 5 mm tubes using the residual ^{1}H of $CHCl_3$ as a reference. Spectra were recorded with 8K data points, 3000 Hz spectral width, 90° pulse of typically 6 μsec, and a 30 sec recycle time. MeABA: **5.753** (*s*, H-2), **2.009** (*s*, Me-3), 7.871 (*dd*, H-4), 6.152 (*dd*, H-5), **1.923** (*d*, Me-2′), **5.942** (*s*, H-3′), **2.288** (*dd*, H_{eq}-5′), **2.478** (*dd*, H_{ax}-5′), 1.014 (*s*, Me_{eq}-6′), 1.110 (*s*, Me_{ax}-6′), 3.706 (*s*, Me ester); Me-*trans*-ABA: **5.854** (*s*, H-2), **2.290** (*s*, Me-3), 6.416 (*dd*, H-4), 6.152 (*dd*, H-5) **1.902** (*d*, Me-2′), **5.948** (*s*, H-3′), **2.302** (*dd*, H_{eq}-5′), **2.467** (*dd*, H_{ax}-5′), 1.022 (*s*, Me_{eq}-6′), 1.112 (*s*, Me_{ax}-6′), 3.731 (*s*, Me ester) The signals in bold represent the hydrogen atoms that were completely exchanged in [$^2H_{10}$]MeABA and [$^2H_{10}$]Me 2-*trans*-ABA.

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