Phytochemistry, 1975, Vol. 14, pp. 123–128. Pergamon Press. Printed in England.

THE STEREOCHEMISTRY OF CYCLIZATION IN ABSCISIC ACID

B. V. MILBORROW

Shell Research Limited, Milstead Laboratory of Chemical Enzymology, Sittingbourne Research Centre, Sittingbourne, Kent ME9 8AG, U.K.

(*Received* 23 *May* 1974)

Key Word Index—*Persea gratissima*, avocado, Lycopersicon esculentum; solanaceae tomato; abscisic acid; cyclisation; phaseic acid; mevalonate; biosynthesis; Kuhn–Roth oxidation.

Abstract—The hydroxylation of the *pro*-6′-(*R*)-methyl of (+)-abscisic acid, which then cyclises to phaseic acid, was used to define the origin in mevalonate of the 6′-methyl groups. Abscisic acid (ABA), biosynthesised from [2-^{14}C, 2-$^{3}H_2$]-mevalonate, was metabolized to phaseic acid by tomato shoots. The slight loss of [^{3}H] from the phaseate, and to a lesser extent from the ABA, suggested that the unlabelled 6′-methyl was hydroxylated. This was confirmed by Kuhn–Roth oxidation of methyl phaseate to give [^{14}C, ^{3}H]-acetate. The data also suggest that ABA is converted to dihydrophaseate via free phaseate, the conjugates being formed from each free acid.

INTRODUCTION

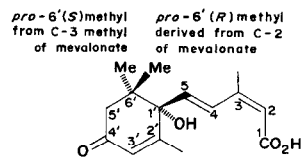
The labelling pattern of abscisic acid (ABA) derived from mevalonic acid stereospecifically tritiated at the 2, 4 and 5 positions [1, 2] is consistent with its formation by the well-known reactions of terpenoid biosynthesis [3]. Furthermore, the [^{14}C]:[^{3}H] ratios in ABA biosynthesized from doubly labelled mevalonate indicate that the three isoprene residues of which the carbon skeleton of abscisic acid is composed are derived equally from the same pool of mevalonate. The stereochemistry in mevalonate of all the [^{3}H] atoms of that molecule which are retained in ABA has now been determined but two stereochemical features of abscisic acid have not been investigated. These are: the stereochemical position at C-5′ of ABA occupied by the pro-4-*R* hydrogen of mevalonate and the direction of cyclisation and hence the stereochemistry at C-6′ in ABA of the methyl groups derived from C-2 and C-3′ of mevalonate. This latter feature has now been investigated and the results are reported below.

RESULTS AND DISCUSSION

Determination of the direction of cyclisation from ^{14}C:^{3}H *ratios*

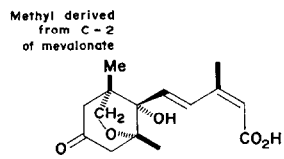
The first experiments to identify which of the two 6′-methyl groups of ABA is derived from C-2 of mevalonate attempted to make use of the loss of tritium that would be expected to occur on hydroxylation of a tritiated methyl group. (+)-Abscisic acid (**1**), but not the (−) enantiomer [4, 5], is hydroxylated on one of the C-6′ methyls and this reaction is stereospecific because the rearranged product of the reaction, phaseic acid (**2**), occurs as one isomer only [6].

Abscisic acid is biosynthesized from [2-^{14}C] and [2-^{3}H] labelled mevalonate by avocado fruit and



(**1**) (+)-(*S*)-abscisic acid

The absolute configuration of natural (+)-(*S*)-ABA has recently been revised [7–11] and is as shown.



(**2**) Phaseic acid

The methylene group of phaseic acid is derived from the *pro*-6′-(*S*)-methyl of abscisic acid which originated from the C-3′-methyl of mevalonate. The absolute configuration of phaseic acid has been deduced from NMR analysis of the epimeric dihydrophaseic acids [12].

Table 1. Loss of tritium from C-2 of mevalonate during the biosynthesis of abscisic acid

Ratio of $^{14}C:^{3}H$ in mevalonate normalized to		3:6·000
Expected ratio of $^{14}C:^{3}H$ after base-catalysed exchange		3:3·000
Observed $^{14}C:^{3}H$ ratio in ABA after base-catalysed exchange	Experiment 1	3:2·484
	Experiment 2	3:2·174
% of tritium lost by the action of isopentenylpyrophosphate isomerase	Experiment 1	20·5%
	Experiment 2	24·2%

The data are from Expts 1 and 2.

the labelling pattern and $^{14}C:^{3}H$ ratios are consistent with one of the *geminal* 6′-methyl groups carrying, proportionally, two tritium atoms while C-4 and C-3′ carry one [2]. The isopentenyl pyrophosphate isomerase of avocado fruit causes considerable racemization of the hydrogen derived from the *pro*-2-(*R*) and *pro*-2-(*S*) positions of mevalonate but an isotope effect operates and relatively little tritium is lost. Previous experiments [1, 2] have shown that the three isoprene residues which comprise ABA are derived equally from mevalonate added to the avocado fruit, consequently the amount of [^{3}H] initially present at C-6′ methyl, C-3′ and C-4 of a precursor of ABA would be expected to be equal, but half of the tritium from the latter two centres is lost when the double bonds are formed (Table 1).

Although the unsaturated precursor of ABA would be expected to carry slightly more ^{14}C compared with ^{3}H than the added mevalonate, the ratio of the tritium in the C-6′ methyl to that at C-3′ and at C-4 of ABA would be expected to be 2:1:1. The hydrogen at C-3′ of ABA can be removed by base-catalysed exchange [6] and the overall $^{14}C:^{3}H$ ratio normalized to 3:3.

If this abscisic acid were fed to tomato plants and metabolized to phaseic acid by the hydroxylation of the C-6′ methyl carrying tritium then a change in the $^{14}C:^{3}H$ ratio from 3:3 to 3:2·333 would be expected, provided that no isotope effect operated on the hydroxylation mechanism. If the C-6′ methyl derived from C-3′ of mevalonate were hydroxylated then no change in the $^{14}C:^{3}H$ ratio would be expected.

The specific activity of the mevalonate is such that very few methyl groups contain two tritium atoms; consequently, there is a considerable opportunity for an isotope effect to favour the removal of protium and discriminate against the removal of tritium. An unchanged $^{14}C:^{3}H$ ratio is not, therefore, rigorous proof of hydroxylation of the C-6′ methyl of ABA derived from C-3′ of mevalonate.

The results in Tables 2 and 3 show that there is a small difference in $^{14}C:^{3}H$ ratio between the abscisic acid supplied to the tomato plants and the

Table 2. The $^{14}C:^{3}H$ ratio of abscisic acid and the phaseic acid formed from it (Expt 1)

	[^{14}C] (dpm)	[^{3}H] (dpm)	$^{14}C:^{3}H$ ratio normalized to mevalonolactone
Mevalonolactone supplied to an avocado fruit (0·5% of total)	9632	25548	3:6·000
ABA after removal of the 3′-^{3}H before feeding to tomato plants (10%)	1622·6	1781·6	3:2·484
ABA recovered from tomato plants	868·1	943·4	3:2·458
Phaseic acid isolated from tomato plants	5338	5524	3:2·341

The labelled precursor supplied to an avocado fruit was a mixture of (±)-[2-^{14}C] and (±)-[2-$^{3}H_2$] labelled mevalonolactone ([2-^{14}C] 10·5 μCi/μM, [2-$^{3}H_2$] 90 μCi/μM).

Table 3. The $^{14}C:^{3}H$ ratio of abscisic acid and the phaseic acid formed from it (Expt 2)

	[^{14}C] (dpm)	[^{3}H] (dpm)	$^{14}C:^{3}H$ ratio normalized to mevalonate
Mevalonolactone (0·1% of total) supplied to an avocado fruit	2292·8	17817	3:6·000
ABA fed to tomato shoots after exchange in base (10% of sample)	1206·9	3398·4	3:2·174
ABA recovered from tomato (total)	242·7	648·3	3:2·063
ABA released from tomato extract by hydrolysis at pH 13 (total)	(209)	(60)	—
Phaseic acid (total sample)	1258·3	3013·8	3:1·850
Phaseic acid released by hydrolysis (total sample)	120·1	273·7	3:1·760
Dihydrophaseic acid from tomato (50% of sample)	(141·6)	(48·3)	—
Dihydrophaseic acid released by hydrolysis	0	0	—

The procedure was as for Table 1 except that the specific activities of the mevalonolactones were 5·03 mCi/mM [2-^{14}C]; 500 mCi/mM [2-$^{3}H_2$].

phaseic acid formed from it but the unmetabolized ABA recovered from the tomatoes also shows an alteration in the same direction which indicates that there is a slight leakage of tritium from the molecule (Table 4). Similarly the phaseic acid recovered by saponification of the aqueous residue shows an even greater loss (β-D-glucosyl abscisate has been indentified in this fraction and the phaseic acid also is probably released from a glucose ester).

The results suggest, therefore, that the untritiated 6′-methyl group of ABA, derived from C-3′ of mevalonate, is hydroxylated. However, they do not exclude the possibility that the 6′-methyl group derived from C-2 of mevalonate is hydroxylated but a strong isotope effect favours the retention of tritium.

Determination of the direction of cyclisation by Kuhn–Roth oxidation

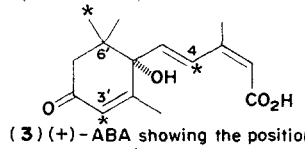
A more conclusive result was obtained by subjecting methyl phaseate to modified Kuhn–Roth oxidation [13]. The methyl phaseate was formed by tomato shoots from ABA biosynthesized from [2-^{14}C]-mevalonate in avocado mesocarp as before and therefore the ABA carried [^{14}C] in one of the two *geminal* 6′-methyl groups. Kuhn–Roth oxidation converts methyl groups, and the carbon atoms to which they are attached, into AcOH but

Table 4. Loss of tritium from ABA fed to, and recovered from, tomato plants and from phaseic acid formed from it

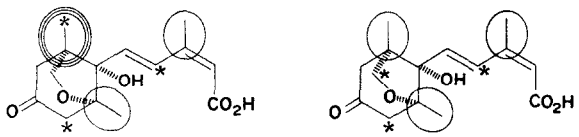
	Expt 1	Expt 2
$^{14}C:^{3}H$ ratio in base-exchanged ABA before feeding to tomato shoots	3:2·484	3:2·174
$^{14}C:^{3}H$ ratio in ABA recovered from tomato shoots	3:2·458	3:2·062
Percentage of ^{3}H lost from ABA	1·1%	5·2%
$^{14}C:^{3}H$ ratio in phaseic acid	3:2·341	3:1·849
Percentage of ^{3}H lost from phaseic acid	5·8%	15·0%
$^{14}C:^{3}H$ ratio in phaseic acid recovered by hydrolysis of tomato aqueous residue	—	3:1·760
% of ^{3}H lost from phaseic acid after saponification	—	19·0%

The data are from Tables 2 and 3 and the $^{14}C:^{3}H$ ratios are normalized to 3:6·000 in mevalonate.

neither of the other two carbon atoms of ABA derived from C-2 of mevalonate can become part of AcOH. Hence the presence or absence of [^{14}C] in the acetate from methyl phaseate shows whether or not the 6′-methyl group of ABA remaining in phaseic acid had been derived from the C-2 of mevalonate (Scheme 1).



(3) (+)-ABA showing the positions (marked *) at which label from [2-^{14}C] mevalonate occurs.



(4) (5)

Scheme 1. Possible labelling patterns of phaseic acid derived from [2-^{14}C]-mevalonate. The carbon atoms ringed are expected to be oxidised to AcOH. Only the acetate derived from C-2 of mevalonate (triply ringed in **4**) can carry [^{14}C] label.

The *p*-bromophenacyl ester of the acetate was made, recrystallized twice and subjected to scintillation counting. It contained [^{14}C] (Table 5). When correction was made for quenching of the scintillator by the *p*-bromophenacyl acetate, and for losses during chromatography and crystallization, approximately 30% of the theoretical yield of [^{14}C] at one position was obtained.

The Kuhn–Roth oxidation experiment was repeated with doubly-labelled phaseic acid recovered from ^{14}C:^{3}H experiments. The exchanged ABA would be expected to carry two [^{3}H] atoms on C-6′ to one on C-4. However, the ratio of [^{14}C] to [^{3}H] expected in acetate derived by Kuhn–Roth oxidation from the C-6′ methyl remaining in phaseic acid cannot be calculated exactly. This is because a small proportion of the [^{3}H] is lost from the molecule between its being fed to tomato shoots as ABA and its recovery as methyl phaseate (Table 1). The loss of [^{3}H] could occur from either or both of these positions. Contamination of the *p*-bromophenacyl acetate by material containing [^{14}C] only could also account for the apparent decrease in the proportion of [^{3}H] but this is considered unlikely because the *p*-bromophenacyl acetate was recrystallized twice and while chromatography of the mother liquors separated minor impurities none carried label.

The occurrence of a ^{14}C:^{3}H ratio (Table 6) in the *p*-bromophenacyl acetate close to the expected value defines the origin of the labelled acetate as the 6′-methyl of phaseic acid (phaseic acid is numbered here according to the numbering of the atoms in ABA).

In both Kuhn–Roth experiments the recovery of acetate was almost quantitative but the yield of labelled acetate was 30% of the expected amount. The yield of AcOH from methyl groups attached to quaternary carbons is often low.

Table 5. The occurrence of [^{14}C] in acetate derived from the 6′ methyl group of phaseic acid biosynthesized, via abscisic acid, from [2-^{14}C]-mevalonate

	[^{14}C] (dpm)	Weight (mg)
dpm in ABA isolated from avocado fruit supplied with (±)-[2-^{14}C]-mevalonolactone	60000	
dpm in methyl phaseate (3·6 mg) formed from the [^{14}C]-ABA by tomato shoots. This sample was subjected to Kuhn–Roth oxidation	5783	
Expected weight of AcOH produced		62
Titration equivalent of acetate collected by distillation after Kuhn–Roth oxidation (60 mg unlabelled carrier AcOH added before oxidation)		42
Expected wt of *p*-bromophenacyl acetate from 42 mg acetate		183
Wt of *p*-bromophenacyl acetate isolated		148
dpm in twice recrystallized *p*-bromophenacyl acetate (73 mg) corrected for self-quenching and adjusted to 148 mg	579	

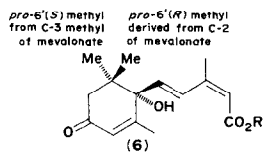
The methyl phaseate was degraded to acetate by Kuhn–Roth oxidation.

Table 6. The occurrence of [^{14}C] and [^{3}H] in acetate derived from the 6′-methyl group of phaseic acid biosynthesized, via abscisic acid, from [2-^{14}C, 2-$^{3}H_2$] mevalonate

	[^{14}C] (dpm)	[^{3}H] (dpm)	Ratio ^{14}C:^{3}H	Ratio normalized to 3:3 in phaseate
Methyl phaseate before oxidation	3786	7401	1:1·955	(3:3)
Expected ratio in *p*-bromophenacyl acetate	—	—	1:3·910	(1:2)
dpm in *p*-bromophenacyl acetate (51·6 mg) after two recrystallizations	294·8	943·2	1:3·200	1:1·637
Total dpm in *p*-bromophenacyl acetate (72·1 mg obtained, 77·1 mg theoretical)	378·9	1212·2	—	—
Expected dpm in *p*-bromophenacyl acetate	1262	4934	1:3·910	(1:2)

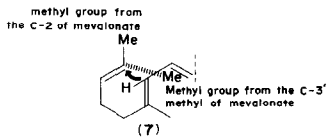
The methyl phaseate was degraded to acetate by Kuhn–Roth oxidation. The ^{14}C:^{3}H shows that although less tritium than expected was present in the acetate the amount retained shows unambiguously that the acetate from the C-6′-methyl group of phaseate is derived from the C-2 of mevalonate. 93·5% of the expected amount of acetate was obtained, 30% of the expected amount of [^{14}C].

These experiments have shown that the unhydroxylated 6′-methyl group of ABA (which is retained as the 6′-methyl of phaseic acid) is derived from C-2 of mevalonate. The absolute configuration of phaseic acid is now known to be as shown in **2** [12] and consequently the stereochemistry of the 6′-methyl groups of ABA can be deduced to be as in **6.** Hence the direction of cyclisation of the hypothetical uncyclised precursors of ABA is as shown in **7.**



(6)

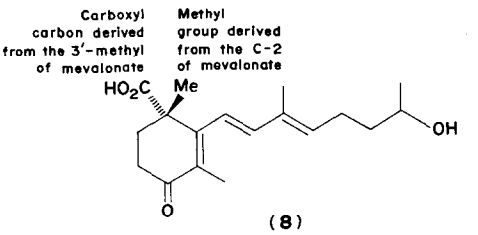
Derivation of the 6′-geminal methyl groups of abscisic acid.



(7)

Arrangement during cyclisation of the 6′-*geminal* methyl groups of abscisic acid showing their derivation from mevalonate.

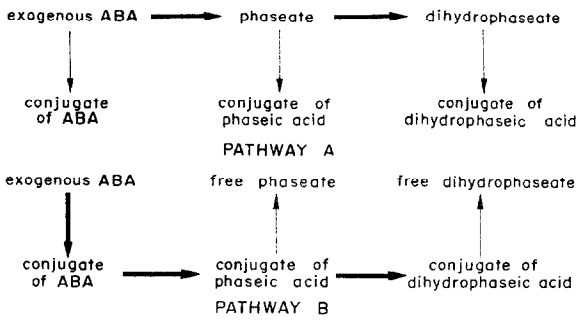
The stereochemistry of the methyl groups of α-carotene is not known at present; whether the 2′-double bond of ABA is first formed in the α or β position is also unknown. However, the investigations by Bu'Lock *et al.* [14] of trisporic acid (**8**) have defined the stereochemistry of the *geminal* methyl groups derived from C-2 and C-3′ of mevalonate and hence their arrangement in β-carotene. It has now been found that those of abscisic acid have the same stereochemistry. This is yet another observation that shows that ABA and carotenoids are synthesized in avocado fruit, if not by a common pathway, then by enzymes which operate with the same stereochemistry. It is possible that the enzymes of ABA biosynthesis have evolved from those of carotenoid biosynthesis.



(8)

Trisporic acid showing the position in mevalonate of the two carbon atoms that are derived from the *geminal* methyl groups of β-carotene.

Finally the data in Table 4 can be taken to indicate that the pathway of metabolism of (+)-ABA added to tomato shoots proceeds to dihydrophaseate via the free acids rather than via their conjugates. The small amounts of radioactivity in ABA and phaseic acids, and its absence from dihydrophaseic acid, when these were released by alkaline hydrolysis, suggest that the conjugated forms of these compounds are formed from the free acids (Scheme 2, pathway A) rather than by metabolism of the terpenoid moieties while conjugated (Scheme 2, pathway B).



Scheme 2.

EXPERIMENTAL

Compounds and solvents and silica gel TLC plates were as described previously [2]. The radioactivity of the [^{3}H] and [^{14}C] labelled materials were measured simultaneously by scintillation counting in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375. The labelled compounds were dissolved in a scintillation solution made up by dissolving 2,5-bis-(5-*t*-butylbenzoxazol-2-yl) thiophen (BBOT) (6 g) and naphthalene (18 g) in a mixture of toluene–methoxyethanol (2:3) to 1 litre. The preparation, when counted in glass vials, gave 15·5% efficiency for [^{14}C] and 18·9% for [^{3}H] in one channel and 49·9% [^{14}C], 0·88% [^{3}H] in the other as determined by counting labelled toluene standards (Packard Instrument Co., Downers Grove, Illinois). The background was 35 cpm in the [^{3}H] and 8 in the [^{14}C] channel. All samples were counted sequentially, with standards, at least three times until the standard deviations of all samples fell to 1% or less.

Plants used. The avocado (*Persea gratissima* cv. Fuerte) fruits were imported and used when they showed incipient softening. The half fruits were incised almost to the skin in a 3 × 3 mm grid pattern and the solutions were poured into the cavity left by the stone and worked along the cuts. The tomato (*Lycopersicon esculentum* cv. Arastor) seedlings were grown in a glass house (*ca* 20°) and used 4–5 weeks after sowing. The doubly labelled ABA was isolated from avocado mesocarp (253 g, Expt 1; 171 g, Expt 2) using conventional methods [2] 24 hr after a fruit had been supplied with a mixture of (±)-[2-^{14}C]- and (±)-[2-^{3}H$_2$]-mevalonolactones in 0·1 M potassium phosphate buffer, pH 7·2 containing Tween 20 (1%, v/v). The (+)-ABA isolated after two chromatographic separations in toluene–EtOAc–AcOH (25:15:2) was methylated with ethereal CH_2N_2 and chromatographed with multiple development in hexane–EtOAc (2:1). In previous experiments [15] such samples had been shown to contain radiochemically pure ABA. However, in this experiment is was necessary to feed free abscisic acid to the tomato plants and to exchange out the 3′-hydrogen still labelled with tritium from C-2 of mevalonate. These operations were carried out simultaneously by hydrolysing the methyl ester in 2 ml ethanolic KOH (2 vol. EtOH, 1 vol. 10 N KOH in H_2O) at 20° for 1 hr, evaporating in N_2 in 0·2 ml H_2O, then acidifying with 0·5 N H_2SO_4 to pH 3·0 and extracting the exchanged abscisic acid with Et_2O. This ABA (*ca* 0·7 mg) was chromatographed again in toluene–EtOAc–AcOH. 10% of the ABA was kept for counting and the remainder was mixed with 0·5 mg (±)-ABA in acetone (1 ml) and then H_2O was added (50 ml). This solution was taken up by 10 seedling tomato shoots cut at ground level (each 4·2 g fr. wt). The seedlings were supplied with H_2O in dim light for 90 hr and then chopped and extracted with MeOH (2 × 1 litre) plus 2,6-di-*t*-butyl-4-methylphenol (BHT) (2 mg/l.). The acid fraction was isolated by one extraction with Et_2O followed by EtOAc (× 3), it was then treated as before. ABA (R_f), phaseic acid (R_f 0·35) and dihydrophaseic acid (R_f 0·20) were eluted and methylated. The methyl esters were chromatographed separately in hexane–EtOAc (2:1), eluted and counted. After the acid fraction had been removed the aqueous residue was saponified at pH 13 for 30 min at 40°, the pH was readjusted to 3·0 and a hydrolysed acid fraction was extracted with ethyl acetate.

Kuhn–Roth oxidations by a modified procedure. The purified methyl abscisate biosynthesized from (±)-[2-^{14}C]-mevalonate in avocado mesocarp was collected from a number of experiments and hydrolysed (60000 dpm). This material, mixed with 5 mg (±)-ABA, was fed to tomato shoots in 0·005 M potassium phosphate buffer pH 7·4 and the methyl phaseate (2·0 mg) isolated as before. This material was subjected to Kuhn–Roth oxidation with 60 mg cold carrier AcOH added to a solution of CrO_3 (2·5 g) in H_2O (10 ml). After 18 hr under reflux at 110° the AcOH was steam distilled, titrated to pH 7·2 with 0·02 N KOH and evaporated to dryness. The potassium salt was dissolved in dimethylformamide and reacted with a 20% excess of *p*-bromophenacyl bromide at 20°. The *p*-bromophenacyl acetate was chromatographed in hexane–EtOAc (3:1) on silica gel TLC plates and recrystallized twice from hexane. The radioactivity in the final product was measured by liquid scintillation spectrometry and corrected for self-quenching by spiking with a [^{14}C] toluene standard. The remaining parts of the chromatogram were counted but no other radioactivity was present. The experiment was repeated with methyl phaseate (0·5 mg) derived from (±)-[2-^{14}C, 2-^{3}H$_2$]-mevalonolactone but AcOH (10 mg) and BHT (9·3 mg) were added to the flask before oxidation commenced and the recrystallized *p*-bromophenacyl acetate was spiked with a [^{3}H] and then a [^{14}C] standard. Some of the [^{14}C, ^{3}H]-methyl phaseate used was recovered from the experiment recorded in Table 3. The scintillation solution, including the naphthalene, was evaporated under N_2 at 50° after BHT (10 mg) had been added to bind the powdery residue. The methyl phaseate was isolated by chromatography on a silica gel TLC plate in $CHCl_3$–hexane (1:1).

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