Pergamon

PII: S0031-9422(96)00665-6

Phytochemistry, Vol. 45, No. 2, pp. 257–260, 1997

0031-9422/97 $17.00+0.00

THE CONVERSION OF 2-*CIS*-[^{14}C]XANTHOXIC ACID INTO [^{14}C]ABA

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(*Received* 2 *July* 1996)

Key Word Index—*Persea americana*; abscisic acid; 2-*cis*-xanthoxic acid; avocado; biosynthesis; 1′,4′-diol, xanthoxal.

Abstract—2-*cis*-[2-^{14}C]Xanthoxic acid prepared from natural violaxanthin was supplied to a sliced ripening avocado (*Persea americana* cv. Hass) fruit together with "cold traps" of the 1′,4′-*cis*- and 1′,4′-*trans*-diols of ABA. 16% of the labelled xanthoxic acid was recovered as ABA and its diols (which, it was found later can be formed *in vivo* by reduction of added [^{14}C]ABA). The recent finding that xanthoxal is the substrate of the molybdenum containing aldehyde oxidase in avocado fruit makes 2-*cis*-xanthoxic acid one of the last precursors on the biosynthetic pathway to ABA. The results reported here establish that 2-*cis*-xanthoxic acid is readily converted into ABA in avocado fruit. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The experiments reported here were carried out in 1972 using [^{14}C]xanthoxic acid (**1**) in an attempt to establish the absolute configuration of ABA(**2**) by finding if a cold trap of the 1′,4′-*cis*- or 1′,4′-*trans*-diol(**3**) of ABA added to an avocado fruit became labelled. The results were capable of another explanation, namely that the labelled ABA was formed from xanthoxic acid by another route and then reduced preferentially to the 1′,4′-*trans*-diol.

(±)-[^{14}C]ABA added to the fruit with cold traps of the diols also labelled the 1′,4′-*trans*-diol preferentially, so the results were ambiguous (see Table 3). The recent interest in xanthoxal and the demonstration [H. S. Lee, and B. V. Milborrow, unpublished results] that the molybdenum-containing aldehyde oxidase involved in the biosynthesis of ABA oxidises xanthoxal to xanthoxic acid renewed interest in the results which establish that xanthoxic acid is readily converted into ABA. Rock and Zeevaart [1] have suggested that in higher plants the 1′,4′-*trans*-diol is not a normal metabolite and Vaughan and Milborrow's [2] finding that considerably more (−)-[^{14}C]ABA than (+)-[^{14}C]ABA was reduced to the *trans*-diol gave further support to the suggestion that the diols are not formed by a normal reaction.

RESULTS AND DISCUSSION

Although the [^{14}C]ABA and diols formed from [^{14}C]xanthoxic acid would be entirely (+), the renewed interest in 2-*cis*-xanthoxic acid as a normal intermediate of the pathway of biosynthesis of ABA provided the stimulus to publish these data showing that 2-*cis*-xanthoxic acid is converted into ABA in avocado fruit slices.

The data in Table 1 show that the avocado fruit readily converts xanthoxic acid into ABA. Furthermore Milborrow and Noddle [3] found that (±)-[^{14}C]epoxyionylidene acetic acid [3] was oxidized to ABA, but a proportion of the labelled material was recovered as 1,2′-*epi*-xanthoxic acid (**4**). Milborrow and Garmston [4] postulated that the original racemic material was hydroxylated at C-4′ by the plant to give two epimers of xanthoxic acid–the natural at 1′,2′(N) and the unnatural at 1′,2′(U): this latter was not converted into ABA. Thus the secondary alcohol dehydrogenase that was presumed to oxidize the 4′-OH to a ketone was stereoselective for the 1′,2′-epoxy group in the (N) configuration, and so the 4′-hydroxy molecule could then be converted *in vivo* by the same enzymic reactions into ABA as those by which Burden and Taylor [5, 6] converted xanthoxal into ABA. Namely, oxidation of the C-4′ hydroxyl group to a ketone caused the molecule to rearrange to abscisic

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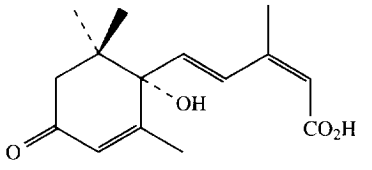

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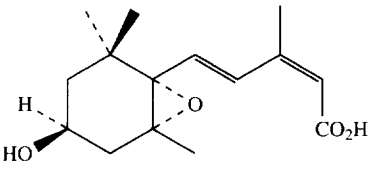

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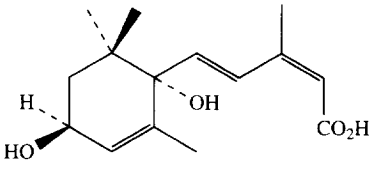

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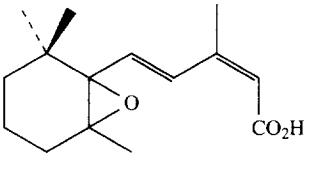

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Table 1. Radioactivity derived from 2-*cis*-[2-^{14}C]xanthoxic acid present in a "cold trap" of the 1′,4′-*cis*- and 1′,4′-*trans*-diols of abscisic acid and in ABA after 20 hrs incubation in avocado slices

Compound	dpm[^{14}C]
(+)-[2-^{14}C]Xanthoxic acid added to avocado mesocarp	3 910 000
Abscisic acid isolated	616 400
1′,4′-*cis*-diol of ABA	373
1′4′-*trans*-diol of ABA	10 171

Half of the final, purified samples were counted at 81% efficiency until the standard deviation fell to 1%. For experimental details, see text

aldehyde. However, as pointed out by Parry *et al.* [7] there is a potential reticulum of reactions between xanthoxal and ABA and it is possible that *in vivo* the 1′,2′-epoxy group could be isomerized enzymically to the C-2′ double bond and the 1′-tertiary hydroxyl group before the 4′-hydroxyl was oxidized to a ketone.

Although the overall yield of [^{14}C]ABA from [^{14}C]xanthoxic acid was 16%, this was measured after a very rigorous isolation procedure which would have lost a considerable proportion of the diols and ABA. The experiment was designed to measure the ^{14}C in the diols unambiguously and the exact percentage conversion was not a concern. Some [^{14}C] for example, was detected in phaseic acid, presumably formed after the xanthoxic acid had been converted into ABA.

The results do show that xanthoxic acid is readily metabolized to ABA in avocado fruit. Sindhu and Walton [8] claimed that an enzyme preparation from bean leaves oxidized xanthoxal to ABA but was unable to oxidize xanthoxic acid to ABA. In spite of this, their data show that while 241 n mol of xanthoxal were converted into ABA, 24 n mol of xanthoxic acid were converted into ABA (10%) by the enzyme preparation.

Xanthoxal now appears to be the first free C_{15} molecule in ABA biosynthesis, its increase (2.6-fold) when the aldehyde oxidase activity is blocked by tungstate suggests that it is the C-1 aldehyde group of xanthoxal that is oxidized as the next step. The rise in [^{14}C]xanthoxal would not be expected to occur if a step further along the pathway were inhibited. This observation makes 2-*cis*-xanthoxic acid the next intermediate and removes AB-aldehyde as a precursor of ABA. Future work will aim at finding if the 1′,2′-epoxy group is isomerized, or even if this occurs enzymically, or if oxidation of the secondary alcohol group at C-4′ so unstabilizes the molecule that it spontaneously isomerizes to ABA.

Recent work of Netting and Milborrow [9, 10] suggests that the latter stages of ABA biosynthesis occur with the future ABA residue bound to a larger jig molecule or template so that the intermediates are not

Table 2. R_fs of compounds used and related products

Compound	Free acid	Methyl ester	Esters after oxidation with MnO_2	O-acetyl methyl ester
Xanthoxic acid	0.6	0.62	(0.62)	0.75
Abscisic acid	0.63	0.60	(0.60)	(0.60)
Phaseic acid	0.57	0.53	(0.53)	(0.53)
1′,4′-*trans*-diol	0.55	0.52	0.60	(0.60)
1′,2′,4′-Trihydroxy xanthoxic acid	0.52	0.42	(0.42)	(0.55)
1′,4′-*cis*-diol	0.40	0.35	0.60	(0.60)

R_fs of probable contaminants and the R_fs of the compounds described in the text, after methylation, oxidation and acetylation, to show how the diols can be converted into products with different chromatographic properties. The R_fs quoted are the positions of the mid parts of the quenched zones when the fluorescent silica gel TLC plates were examined under screened UV illumination (254 nm). The visible spread of the zones was 1–1.5 mm on each side of the quoted R_f after the sequence of multiple developments (ie the zones were 0.014–0.02 R_f units wide). ^{14}C was detected in all these compounds after the first chromatography. Free acids were chromatographed in toluene, ethyl acetate, acetic acid, the Me esters in hexane, ethyl acetate.

Table 3. Conversion of (±)-[2-^{14}C]ABA into the 1′,4′-*cis*- and 1′,4′-*trans*-diols of ABA in avocado fruit (two experiments)

	Experiment 1	Experiment 2
(±)-ABA added to mesocarp	1 000 000	1 000 000
1′,4′-*cis*-diol isolated	1 369	50 400
1′,4′-*trans*-diol isolated	2 176	171 600

(±)-[2-^{14}C]ABA (1×10^6 dpm 4.9 μCi μmol^{-1}) was supplied to an avocado fruit, together with cold traps of the diols, in the same way as [^{14}C]xanthoxic acid in Table 1 but were chromatographed once as Me esters.

free until the synthesis of the ABA moiety has been completed. The intermediates in the final stages of the pathway, therefore, may not exist as free compounds.

EXPERIMENTAL

An avocado fruit that was just beginning to show signs of incipient softening was divided longitudinally into three sectors and the mesocarp of each was then sliced through to the skin in a criss-cross pattern at 5 mm intervals. 1.30×10^6 dpm [^{14}C]xanthoxic acid (0.38 μCi μmol^{-1}) was added to each avocado third in sodium phosphate buffer (4ml, 0.02 M, pH 6.6 and Triton X-100, 0.5%). A "cold-trap" of a mixture of 1′,4′-*cis*- and 1′,4′-*trans*-diols of (±)-ABA (100 μg of each) in the same buffer solution (2 ml) was added to one avocado third immediately and a similar addition to the other two fruit thirds was made after 4 and 20 hr. The three parts were combined and homogenized in methanol (500 ml containing BHT (2,6-di-*t*-butyl-4-methyl phenol) (20 mg) and saturated sodium bicarbonate solution (20 ml) at 20 hr).

The slurry was centrifuged and the pellet re-extracted with methanol (2×500 ml), which was added and the volume evapd. to 100 ml. The neutral fr. was removed with ether (4×50 ml) extraction and the pH adjusted to 3.5. BHT (2 mg) was added and the acid fr. extracted into ether (4×50 ml), it was applied to the origin of two precoated Merck silica gel GF_{254} $200 \times 200 \times 2$ mm TLC plates and developed in toluene–EtOAc–HoAc (25:25:2) containing BHT (200 mg l^{-1}). The zones opposite markers of ABA and the 1′,4′-*cis*-and *trans*-diols were eluted and each twice rechromatographed in the same way. Before each rechromatography 100 μg of (±)-ABA and 100 μg of the *trans*-diol were added to the plate as a line just below the origin containing *cis*-diol, while (±)-ABA and *cis*-diol (100 μg) were applied just above the line where the *trans*-diol would be placed. This procedure ensured that any contaminating labelled molecules of the alternate diol would be "washed out" as the lower band passed through the upper one as the plate underwent five or more developments (Table 1). After elution from the silica gel the diols and ABA were methylated and rechromatographed twice in hexane–EtOAc (2:1) containing BHT (200 mg l^{-1}). After the silica gel carrying the appropriate diol had been scraped off, segments of the rest of the plate were assayed for radioactivity. Altogether the diols were chromatographed $\times 3$ as free acids, $\times 2$ as methyl esters (after both of these the (±)-ABA and intermediate zones were free from radioactive contaminants). The diol methyl esters were then oxidized to abscisic acid methyl ester by a 10-fold excess of MnO_2 in dry chloroform during 30 min, stirring at 20°. The new ABA methyl ester samples were then rechromatographed, treated with acetic anhydride in pyridine, rechromatographed and the radioactivity in half of the

sample was measured. The remaining halves were reduced with borohydride, as before, to give an approximately equal division of the ^{14}C label between the 1′,4′-diol methyl esters. This confirmed that the label, originally in the diols, had been present in abscisic acid after MnO_2 oxidation. [2-^{14}C]xanthoxic acid was unaffected when subjected to MnO_2 oxidation under identical conditions.

This purification procedure was designed to free the diols from the expected labelled contaminants: unmetabolized xanthoxic acid, abscisic acid, phaseic acid and trihydroxy xanthoxic acid (an acid hydrolysis product) (see Table 2).

Radioactivity. The [^{14}C] content of the samples was measured by scintillation spectrometry in a solution comprising toluene, naphthalene, methoxy-ethanol and 2,5-*bis*-(5-t-butylbenzoxazol-2-yl)thiophen (BBOT) 6g l^{-1} in a Packard "Tricarb" Model 3375 set to give 81% counting efficiency of a standard [^{14}C]toluene source. Other sections of the TLC plates were scraped directly into vials and the scintillation solution (10 ml) added.

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