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THE STEREOCHEMISTRY OF THE HYDROGEN ATOMS AT C-5′ OF ABSCISIC ACID

R. D WILLOWS and B. V MILBORROW

School of Biochemistry, University of New South Wales, P.O. Box 1, Kensington, New South Wales, 2033, Australia

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Abstract—Slices of unripe avocado fruit placed in ^2H_2O (45 atom %) synthesized abscisic acid enriched 18.7% with ^2H at C-5′. 500 MHz ^1H NMR showed that the signal of the axial, 5′-*pro-S* H atom was decreased by 20% The signal of the uncoupled, equatorial H atom was 9 Hz upfield owing to shielding from its *geminal* ^2H partner, the reciprocal shielding was 8.9 Hz. The H^+ added during cyclization, therefore, is at the C-5′-*pro-S* position of ABA. Unlabelled ABA leaked into the medium, the newly synthesized, ^2H labelled ABA was retained in the tissue

INTRODUCTION

Investigations of the biosynthesis of ABA have sought to find a clear, qualitative difference between its stereochemistry and that of carotenoids. The work has established the source of all the carbon and almost all the hydrogen atoms from mevalonate which are incorporated into ABA [4, 5, 6, 8] The stereochemistry of the C-6′ *geminal* Me groups has been deduced by NMR using ^{13}C-ABA synthesized by the fungus *Cercospora rosicola* and from this the cyclization of the precursor of ABA, at present unknown, has been postulated to occur with the attack of a proton on the *re* face of what will become the C-5′ of ABA. The mechanism proposed predicts that the H atom derived from the 4-*pro-R* position of mevalonate will occupy the equatorial 5′-*pro-R* position of ABA and consequently the one derived from the medium will come to occupy the 5′-*pro-S* position [6]. However, the biosynthetic pathway to ABA in the fungus appears to be different from that in higher plants, consequently it is possible that the cyclization mechanism could occur as the mirror image.

The stereochemistry of the hydrogens at C-5′ of ABA, synthesised by higher plants, is now shown to be the same as the proposed stereochemistry for the C-5′ H atoms of ABA synthesized by the fungus. This was confirmed by ^1H NMR and GC/MS analysis of ABA formed by avocado fruit slices immersed in ^2H enriched water

RESULTS

The avocado tissue (100 g) leaked 180 μg of ABA into the 45% ^2H_2O (130 ml) during the 48 hr of the experiment. No ^2H was detected in this sample which must represent a large proportion of the free ABA present initially. The ABA extracted from the mesocarp was shown by GCMS Me NICI to be 14.9% enriched with one, 3.8% with two and 0.44% with three ^2H atoms. It is not yet known if this figure represents isotopic discrimination against ^2H, imperfect equilibration of the ^2H_2O with the water of the tissue or dilution with previously cyclized precursors.

The signal at δ2.276 was identified as that of the uncoupled 5′-*pro-R* H atom superimposed on the upfield peak of the 5′-*pro-S* (Fig 1). This was established by its disappearance, together with a concomitant increase in the coupled signal at δ2.311 and an increase in the signals of the *pro-R* ^1H atom, when the deuterium was removed by exchange.

The biosynthetically deuterated ABA was analysed by He CI GCMS, which gives spectra closely similar to those obtained by EI The base peak, by EI, at 190 *m/z* is formed by the loss of the C-4′ ketone plus C-5′, C-6′ and C-8′ and 9′ [2]. It contained no deuterium as did that at *m/z* 125 (side chain) This is compatible with the ^2H's being at C-5′. The fragment ion at *m/z* 152 in the Me NICIMS is derived from the ring and tertiary hydroxyl group [7]. The deuterated sample showed a peak at 153 *m/z* enriched with 14.9% ^2H, as expected.

The position of the uncoupled signal (δ2.276) of the equatorial 5′-^1H atom in [5′-$^2H_1$] Me ABA was 9.0 Hz upfield of the position of the decoupled signal (δ2.294). The upfield shift is attributed to shielding from the *geminal* ^2H atom and, although somewhat high, is of the order seen in other compounds [3]. The reciprocal experiment showed similar shielding of the axial ^1H by an equatorial ^2H atom (8.9 Hz). This sample was made by completely exchanging both 5′ H atoms in M NaO^2H in ^2H_2O, 3 hr and then exchanging out the ^2H from the 5′-*pro-S* position in ^1H_2O–EtOH (9 1) at pH 11.50, 20 hr. Unlike the replacement of the axial 5′-^1H by ^2H, 20% of which occurred during 3 hr at pH 10.55 (as measured by a conventional pH meter), the deuterium was hardly affected over 3 days at pH 11.55. This strong bonding may account for the order of loss of the ^1H atoms of ABA in strongly basic ^2H_2O The 5′ atoms exchange first, followed by the three ^1H atoms of the 7′ Me group Then the C-3′ H exchanges. Perhaps the resistance to exchange of the ^2H methyl prevents the shift of the 2′-3′ double bond to 2′-7′ and causes the loss of its ^1H atom.

DISCUSSION

The origins from mevalonate onwards of all the H atoms of ABA have now been established with the

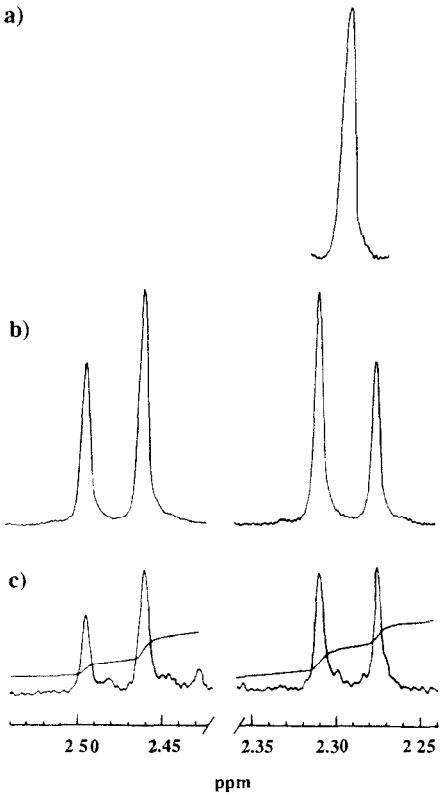

Fig 1 5′-*pro-R* (equatorial, δ2 29) and 5′-*pro-S* (axial δ2 47) regions of the ^{1}H NMR spectrum of MeABA in [^{2}H] $CHCl_3$. (a) Decoupled 5′-*pro-R* H of MeABA by irradiation of the downfield, 5′-*pro-S* H signal, (b) unlabelled MeABA and (c) 20% [5′-*S*-$^2H_1$] MeABA, formed by exchange, with the uncoupled C-5′-*R*-$^1H_1$ signal superimposed on the signal at δ2 276.

demonstration that the H^+, derived from the medium during cyclization, comes to occupy the 5′-*pro-S* position This is as expected from the postulated folding of the precursor, at present unidentified, which gives rise to a pseudo-chair intermediate by the formation of the bond between the future C-6′ and the *si* face of the future C-1′ [6]

EXPERIMENTAL

Plant material Avocado fruits (*Persea gratissima* cv Hass) were obtained from the local market or from New South Wales Department of Agriculture, Tropical Fruit Research Station, Alstonville

Procedure Hard avocadoes were pretreated in air containing *ca* 1% ethylene for 4 hr The mesocarp was cut into slices (10 mm, total 100 g) and immersed in 1 3 vol ^2H_2O (45 atoms %) /wt for 48 hr under laboratory light The excess ^2H_2O was removed by filtration and the ABA extracted separately from both fruit and ^2H_2O

Extraction Aliquots (20 ml) of the ^2H_2O were acidified HOAc (0 2 ml) and chromatographed on three C_{18} Sep Paks (Waters Associates Pty Ltd) in series, eluting first with 0 2% Aq HOAc, then ABA with EtOH–H_2O–HOAc (175 325 1)

The fruit was extracted in Me_2CO–HOAc (99 1, containing 0 01% 2,6-di-*tert* butyl 4-Me phenol), 200 ml + 200 ml, in darkness for 2–7 days The Me_2CO was evapd and the fat removed from the aq residue before evapg to dryness The residue was further purified by TLC (silica gel 60F_{254}, Merck) developed × 4 in toluene–EtAc–HOAc (25 15 2) The ABA was eluted and rechromatographed on a C_{18} Sep Pak as described above

Purification The samples were chromatographed in a Techsil 10 C_{18} HPLC column (250 × 8 mm) eluted with MeOH–H_2O–HOAc (250 250 1) at 3 0 ml/min Detection and quantification of ABA was performed at 263 nm with a Hewlett–Packard diode array detector coupled to a Hewlett–Packard 1B workstation ABA eluted between 6 5 and 7 5 min and was methylated with CH_2N_2 in Et_2O then rechromatographed in the same system MeABA eluted between 12 and 13 min The Me ABA was then analysed for its ^2H content by GC-MS and if it contained more than 10% $^2H_1$ it was further purified for NMR by HPLC in a Techsil 10 silica gel column (250 × 8 mm) eluted with *iso*-PrOH–hexane (3 97) Methyl ABA eluted between 11 and 12 min

Synthesis of deuterated ABA 5′-*S,S*-[5′-$^2H_1$]ABA was synthesized by exchange of 1 mg of ABA in 1 ml of ^2H_2O at pH 10 55, 3 hr, then purified as before (*ca* 20% $^2H_1$ as determined by GC-MS)

[5′-*R*-$^2H_1$] MeABA was synthesized by completely exchanging both 5′H atoms of 1 mg of ABA in 1 ml of M NaO^2H in ^2H_2O, 3 hr, then methylating with CH_2N_2 The deuterated MeABA was then exchanged in ^1H_2O–EtOH (9 1) at pH 11 5, 20 hr The sample was remethylated and purified as before (*ca* 10% of the deuterium in each position was replaced by ^1H)

GC-MS Negative-ion CI spectra of MeABA were obtained using a Finnigan 3200 quadrupole MS, interfaced to an INCOS data system Samples were separated on a 1 5 × 2 mm 3% OV-1 GC column programmed from 150° at 10°/min with CH_4 as the carrier and ionising gas

He was used as the ionizing gas to obtain MS of MeABA equivalent to a conventional EI spectrum Fragment ions above *m/z* 205 were more prominent (*ca* × 5) than in the conventional EI and were increased by 1 mass number The peaks of *m/z* 205 and below in the He spectrum were identical with those obtained by EI MeABA MS He *m/z* (rel int) 279 (23) 261 (100) 247 (18 4) 222 (7 3) 205 (5 5) 191 (2 7) 190 (79 5), 162 (38 7), 147 (6 7), 134 (33 5), 126 (1 4), 125 (26 1) [5′-^{2}H] MeABA (20%) 280 (3 2), 279 (14 1), 263 (6 5), 262 (4 7), 261 (67), 191 (3 4) 190 (100), 126 (0), 125 (30 2)

NMR ^{1}H NMR spectra were recorded at 500 MHz in [^{2}H] $CHCl_3$ in 5 mm tubes using the residual ^{1}H of chloroform as a reference Spectra were recorded with 16 K data points, 5000 Hz spectral width, 90° pulse of typically 6 μsec, and a 3 sec recycle time

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