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THE BIOSYNTHESIS OF ABSCISIC ACID IN *CERCOSPORA ROSICOLA*

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Key Word Index—*Cercospora rosicola*; abscisic acid; 1′-deoxyabscisic acid; biosynthesis of abscisic acid; mevalonic acid.

Abstract—1′-Deoxyabscisic acid (1′-deoxy-ABA) has been isolated from cultures of *Cercospora rosicola* which are actively synthesizing abscisic acid (ABA). Mevalonic acid is incorporated into ABA and 1′-deoxy-ABA by this fungus. The identity of this compound has been confirmed by synthesis. ^{3}H-labelled 1′-deoxy-ABA is efficiently converted into ABA by *C. rosicola*. Thus it is proposed that 1′-deoxy-ABA is the immediate biosynthetic precursor of ABA.

INTRODUCTION

The plant pathogenic fungus *Cercospora rosicola* has been shown to produce relatively large amounts of the plant growth inhibitor abscisic acid (ABA) (**9**) [1]. This organism may prove very valuable for studies into the pathway of ABA biosynthesis. Studies with plants have so far revealed no details of the intermediates involved in ABA biosynthesis. The levels of ABA found in plants are too low to permit the labelling and isolation of possible intermediates to be successful without some prior knowledge of the likely nature of these compounds. We have initiated research into the pathway of ABA biosynthesis in *C. rosicola* with a view to applying information gained with this organism to the problem of ABA biosynthesis in plants. This paper describes the isolation and identification of *cis,trans*-3-methyl-5-(4′-oxo-2′,6′,6′-trimethylcyclohex-2′-en-1′-yl)-2,4-pentadienoic acid (1′-deoxy-ABA) (**7**) from *C. rosicola* and the chemical synthesis of this compound. Evidence is presented that 1′-deoxy-ABA is the immediate precursor of ABA in the fungus.

RESULTS AND DISCUSSION

ABA was identified as a natural metabolite of *C. rosicola* by GC/MS of the methyl ester after purification on systems A and C. The time-course of ABA production is shown in Fig. 1. Mycelial growth was extensive until *ca* day 7, by which time ABA production had started.

The distribution of radioactivity from [2-^{3}H]-mevalonolactone in the acid–ether fraction after HPLC is shown in Fig. 2. The peak of radioactivity at R_t 16 min co-chromatographed with ABA in systems A and B and with methyl-ABA in systems C and E and on GLC with no change in specific activity. The peak of radioactivity at R_t 21.5 min was collected, methylated and examined by GC/MS. Two components were observed. The minor of these was not radioactive and was identified as methyl palmitate by its MS and R_t. The second component, eluting just after methyl palmitate, contained all the radioactivity and exhibited a base peak at *m/z* 125 with strong ions at *m/z* 174 and 146. The specific activity of the material in this peak was estimated to be 0.1 mCi/mmol (based on the same ε_{max} as ABA) while that of the ABA was estimated at 0.06 mCi/mmol.

In a large-scale extraction *ca* 40 μg of this compound was isolated and purified by HPLC in systems A and B. After methylation it was finally purified by HPLC on system D. This endogenous UV-absorbing material co-chromatographed with the radioactive component of the [2-^{3}H]mevalonolactone feeding experiment on systems A and B and, after methylation, on systems C and D and on GLC with no change in specific activity. The UV spectrum of the methylated compound showed strong absorbance at 267 nm (λ_{max}) with a shoulder at 240 nm. About 15 μg of the methyl ester was analysed by GC/MS. Repetitive scanning of this material indicated the presence of only one component. The MS exhibited peaks at *m/z* (rel. int.): 262 (5.3), 206 (1.0), 189 (3.8), 188 (2.2), 187 (3.3), 174 (22.1), 147 (20.9), 146 (44.8), 145 (13.6), 125 (100), 119 (14.7), 112 (9.2), 105 (6.8), 91 (9.5). The presence of a base peak at *m/z* 125 immediately suggested a compound with an ABA-like side-chain. A MW of 262 suggested that the compound contained one oxygen atom less than ABA. The absence of a M-18 ion suggested that the oxygen from the 1′ position of ABA had been lost. This was supported by the presence of ions at *m/z* 206, 174, 119, 105 and 91 which were presumed to have been formed via a fragmentation pathway analogous to that for methyl-ABA [2], and by the UV spectrum. Thus it was concluded that the compound was methyl-1′-deoxy-ABA (**3**). The 360 MHz ^{1}H NMR spectrum of the purified compound is shown in Table 1. The NMR spectrum was further analysed by decoupling and confirmed the structure proposed from the MS. The presence of the relatively low field doublet at δ 7.72 (J = 14 Hz) indicated that the 2,3 double bond in the side-chain was in the *cis* configuration [3]. The NMR spectrum showed evidence of a minor component which was probably an isomer (not the *trans, trans*) of methyl-1′-deoxy-ABA. The possible origin of this impurity will be discussed later. The ORD of the isolated methyl-1′-deoxy-

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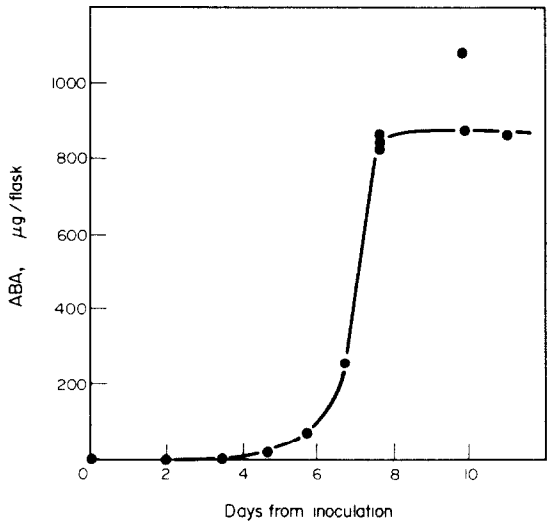


Fig. 1. ABA production by *C. rosicola* grown on solid Miller's medium.

ABA (**3**) showed a negative Cotton effect and was qualitatively identical to that of methyl-(*S*)-ABA. Too little material was available for an accurate determination of the specific rotation to be made. However, it was concluded that the 1′-deoxy-ABA (**7**) had the same absolute configuration at (*S*)-ABA. Thus, if this compound is an intermediate in ABA biosynthesis, the final hydroxylation step must take place with retention of configuration.

Methyl-1′-deoxy-ABA (**3**) and its *trans,trans* isomer (**4**) were prepared by the allylic oxidation of *cis,trans* (**1**) and *trans,trans* (**2**) methyl-α-ionylidenacetates. This route was chosen because it was found that 1′-deoxy-ABA (**7**) and its *trans,trans* isomer (**8**) could not be separated on any HPLC or TLC system tried, although their methyl esters could be separated on an analytical scale by GLC, or by HPLC on system D. Thus for preparative purposes it was necessary to separate isomers (**1**) and (**2**) prior to the oxidation. This was easily achieved by CC on Si gel. Base hydrolysis of methyl-1′-deoxy-ABA (**3**) did not yield 1′-deoxy-ABA (**7**). Methylation and GC/MS analysis of the hydrolysis products indicated a mixture of products, a minor component of which was probably methyl-1′-deoxy-ABA (**3**). The MS of the two major components of the mixture were essentially identical and were qualitatively the same as that of methyl-1′-deoxy-ABA (**3**). However, both of these components exhibited a molecular ion at *m/z* 262 as the base peak. ^{1}H NMR analysis of the hydrolysis mixture after methylation showed some of the features noted in the spectrum of the minor component of the isolated material. Although the identities of the methylated rearrangement products have not been fully established yet, they are clearly isomers of methyl-1′-deoxy-ABA (**3**) [not the *trans,trans* isomer (**4**)] and presumably arise via a rearrangement involving the doubly allylic 1′-proton. We have concluded that the minor component in the isolated material arises as a result of exposure of the extracted material to high pH (>10) during the extraction process. The presence of this component also explains why the molecular ion in the MS of the naturally occurring material is of significantly higher intensity than in the synthetic compound.

To synthesize 1′-deoxy-ABA it was necessary to avoid the base hydrolysis step. Using *t*-butyl esters in place of the methyl esters it was possible to effect the removal of the *t*-butyl group by acid-catalysed elimination. The use of this route resulted in the production of 1′-deoxy-ABA (**7**) and its *trans,trans* isomer (**8**) pure as judged by the mass and ^{1}H NMR spectra of their methyl esters.

The synthesis of radioactively labelled 1′-deoxy-ABA (**7**) and its *trans,trans* isomer (**8**) was easily achieved by introducing tritium atoms into the side-chain methyl group of α-ionone via keto/enol exchange. After the Wittig reaction (which resulted in some loss of label) these tritium atoms are no longer exchangeable [4].

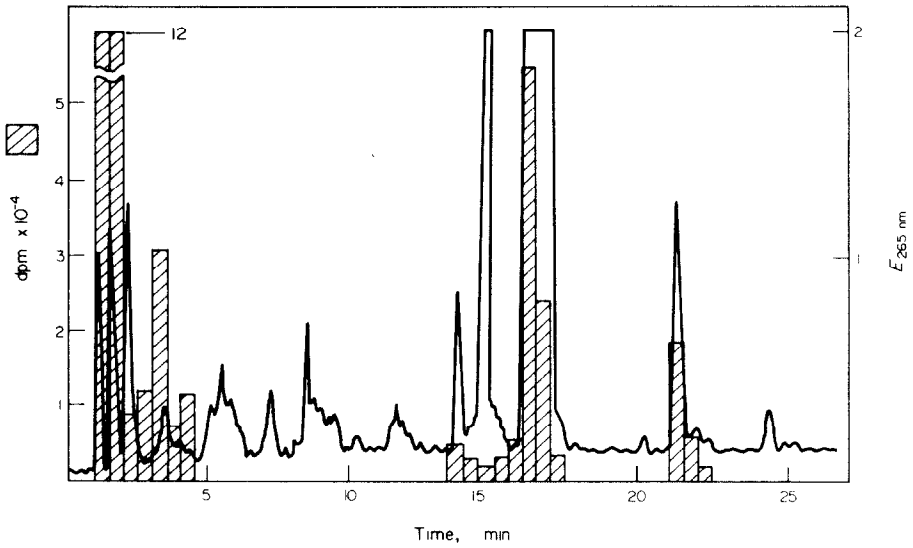
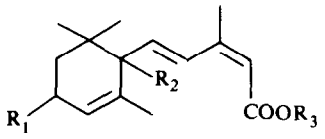


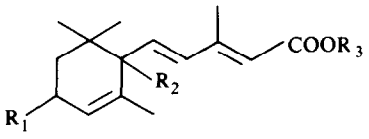
Fig. 2. HPLC of acid ether fraction from [2-^{3}H]mevalonolactone feeding experiment using system A. ☐, dpm/fraction (2.5 ml); ·, E_{265nm}.

Table 1. ^{1}H NMR spectrum of methylated 1′-deoxy-ABA (**3**) isolated from *C. rosicola*

Protons	Resonance	Integral (H)
Me-6′	0.97 (*s*)	3
Me-6′	1.07 (*s*)	3
Me-2′	1.93 (*s*)	3
Me-3	2.0 (*s*)	3
Me-5′	2.14 (*d*, J = 16 Hz)	1
H-5′	2.38 (*d*, J = 16 Hz)	1
H-1′	2.72 (*d*, J = 9 Hz)	1
CO$_2$Me	3.71 (*s*)	3
H-3′	5.7 (*s*)	1
H-2 and H-5	5.93 (*m*)	2
H-4	7.72 (*d*, J = 14 Hz)	1

When fed to a culture of *C. rosicola* which was actively synthesizing ABA, 1′-deoxy-[3-Me-^{3}H]ABA (**7**) was converted into ABA (**9**) in 11% yield. Exhaustive chromatographic analysis of the labelled product resulted in no change of specific activity. By contrast, there was no detectable conversion of *trans,trans*-1′-deoxy-ABA (**8**) into either ABA (**9**) or its *trans,trans* isomer (**10**). There was no incorporation of 1′-deoxy-ABA into ABA by boiled fungus. The results of these experiments are presented in Table 2. ORD analysis of the residual 1′-deoxy-ABA (**7**) indicated that the material was racemic. The apparent lack of stereoselectivity in the final hydroxylation is not too surprising in the light of the considerable degree of symmetry exhibited by the molecule. This fact has been used to explain why both (*R*)- and (*S*)-ABA are equally biologically active in most tests, and may result in the hydroxylating enzyme accepting both (*R*)- and (*S*)-ABA as substrates [5].





1 $R_1 = H$ $R_2 = H$ $R_3 = Me$

2 $R_1 = H$ $R_2 = H$ $R_3 = Me$

3 $R_1 = O$ $R_2 = H$ $R_3 = Me$

4 $R_1 = O$ $R_2 = H$ $R_3 = Me$

5 $R_1 = O$ $R_2 = H$ $R_3 = t$Bu

6 $R_1 = O$ $R_2 = H$ $R_3 = t$Bu

7 $R_1 = O$ $R_2 = H$ $R_3 = H$

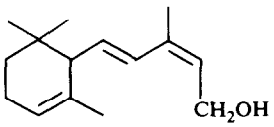
8 $R_1 = O$ $R_2 = H$ $R_3 = H$

9 $R_1 = O$ $R_2 = OH$ $R_3 = H$

10 $R_1 = O$ $R_2 = OH$ $R_3 = H$

11 $R_1 = H$ $R_2 = H$ $R_3 = H$

12 $R_1 = H$ $R_2 = H$ $R_3 = H$



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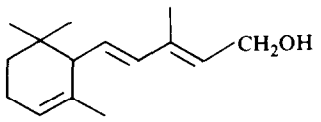

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Table 2. Incorporation of MVA and 1′-deoxy-ABA into ABA by *C. rosicola*

Compound fed			ABA			1′-Deoxy ABA	
				Radioactivity		Radioactivity	
Name	Amount (μg)	Radioactivity (10^{-6} × dpm)	Weight (μg)	(10^{-4} × dpm)	(%) dose	(10^{-4} × dpm)	(%) dose
MVA	6.8	110	830	7.6	0.14	2	0.017
1′-Deoxy-ABA	30	2.5	860	27.5	11	—	—
2-*Trans*-1′-deoxy-ABA	30	2.5	670	0	0	—	—
1′-Deoxy-ABA to boiled fungus	30	2.5	940	0.075	0.03	—	—

It has been suggested previously that 1′-deoxy-ABA (**7**) might be a precursor of ABA (**9**), although the suggestion was dismissed by the apparent observation that 1′-deoxy-ABA (**7**) could be converted non-enzymatically to ABA (**9**) [6]. No details have been published of the methods used to prepare the 1′-deoxy-ABA (**7**) used for these studies. In the light of our findings, if strongly basic conditions were used in the course of the synthesis, the final product may not have been 1′-deoxy-ABA. We have found that 1′-deoxy-ABA (**7**) and its *trans,trans* isomer (**8**) are stable compounds at normal temperatures and physiological pH values. The observation that the *trans,trans* isomer (**8**) is not converted into ABA (**9**) or its *trans,trans* isomer (**10**) and is quantitatively recovered after feeding to the fungus is further evidence against the findings of the previous investigators. Oritani and Yamashita [7] reported that methyl-1′-deoxy-ABA (**3**) was inhibitory to the growth of rice seedlings. It is not clear from their paper that the compound used was pure or why it was not tested as the free acid. It is possible that they observed the same base instability noted here.

The close structural relationship between 1′-deoxy-ABA (**7**) and ABA (**9**) and the fact that this compound is converted into ABA in good yield by *C. rosicola*, strongly suggests that it is the immediate precursor of ABA in this fungus. These observations have led us to speculate that the pathway of ABA biosynthesis may involve successive oxidations of a 3-methyl-5-(2′,6′,6′-trimethylcyclohex-2′-en-1′-yl)-2,4-pentadienyl intermediate. This intermediate could arise via the cyclization and desaturation of farnesol or a farnesyl derivative. Preliminary studies involving the feeding of **11** and **13** to *C. rosicola* have shown that these compounds are readily converted to 1′-desoxy-ABA and hence to ABA. Their *trans,trans* isomers, **12** and **14**, are readily converted to *trans,trans*-1′-deoxy-ABA (**8**) but not to ABA (**9**) or its *trans,trans* isomer (**10**). These results agree with the findings reported here and suggest that isomerization of the 2,3 double bond precedes, or is concomitant with, the formation of the first desaturated cyclic intermediate.

Milborrow [5] has suggested that dehydrofarnesol may be an intermediate in ABA biosynthesis and that, by analogy with the carotenoids, desaturation precedes cyclization. Unfortunately, although he has stated that dehydrofarnesol is converted into ABA in plant tissues, no experimental evidence has been published.

The biosynthetic route to ABA proposed in this paper is in contrast to a route involving the carotenoid degradation product xanthoxin in which a 1′-en rather than a 2′-en intermediate would most likely be involved. In relation to this it is of interest to note that **11** and **13** have been shown to be active in a rice seedling test, whereas their 1′-en isomers were much less active [8].

EXPERIMENTAL

^{1}H NMR: 360 MHz Bruker.

Materials. *Cercospora rosicola* Passerini (strain No. 138.35 from C.B.S., The Netherlands) was sub-cultured on 50 ml solid modified Miller's medium [9] in 250-ml conical flasks. The medium contained 0.1 concentration minerals and vitamins, 30 g/l sucrose and no hormones. Mycelium was ground with H_2O until a homogeneous suspension was obtained. Aliquots of this soln were then pipetted onto the surface of the agar. The fungus was then maintained at 26 ± 1° under fluorescent lighting (1.5–2 klx).

Counting procedures. Duplicate samples were counted in toluene–Triton-X–PPO (11:0.51:4g) in a liquid scintillation spectrometer.

Chromatography. HPLC was performed using an LDC Model 1601 Gradient Master in conjunction with a Spectrometric III UV-detector and Constametric III pump. HPLC systems, A: ODS-Hypersil (150 × 10 mm), eluted with a linear gradient of 20–100% MeOH in 0.1 HOAc over 40 min, 5 ml/min; B: Hypersil (250 × 10 mm), eluted with $CHCl_3$–HOAc (46:1), 5 ml/min; C: Spherisorb (250 × 10 mm), eluted with hexane–*iso*-PrOH (19:1), 2 ml/min; D: Partisil PAC (250 × 4.5 mm), eluted with hexane–EtOH (100:0.5), 2 ml/min; E: Partisil PAC (250 × 4.5 mm), eluted with hexane–EtOH (20:1), 2 ml/min. GLC: 3% OV-1 columns (1.65 m × 4 mm) at 220° (EC) and 208° (FID); detector temp. 350° (FID) and 300° (EC); N_2 40 ml/min.

Extraction procedures. Agar and mycelium were extracted for 4 hr in 0.2% HOAc in EtOAc (50 ml/flask). The EtOAc was reduced to dryness, dissolved in 2% $NaHCO_3$ and an acid Et_2O fraction prepared. This Et_2O fraction was then analysed by HPLC as described. The time-course of ABA production was followed by extracting duplicate flasks at various times after inoculation. ABA levels were determined in aliquots of the extract, following methylation, by GLC using the EC.

Administration of radioactive compounds. *Ca* 50 μCi [2-^{3}H]mevalonolactone (745 mCi/mmol) or *ca* 1 μCi 1′-deoxy-[3-Me-^{3}H]ABA (8.3 mCi/mmol) was administered to the fungus (1 flask; *ca* 3 g fr. wt fungus) at the onset of the period of rapid ABA production (i.e. after *ca* 6 days). The compounds were dissolved in 2 ml H_2O and pipetted onto the surface of the mycelium. After 24 hr the flasks were extracted and acid–Et_2O fractions prepared as before.

Synthesis of methyl-3-methyl-5(2′,6′,6′-trimethyl-2′-cyclohexen-1′-yl)-2,4-pentadienoates [cis,trans *and* trans,trans *isomers of methyl α-ionylidene-acetate* (**1** *and* **2**)]. A mixture of α-ionone (10 g, 50 mmol) and carbmethoxymethylenetriphenylphosphorane (34 g, 100 mmol) was heated together, with stirring, for 90 min at 180°. After cooling to 100°, the mixture was poured, with stirring, into 300 ml hexane. The mixture was stirred until the triphenylphosphine oxide solidified and allowed to stand at −20° for 12 hr. The triphenylphosphine oxide was removed by filtration and the filtrate concentrated to a viscous oil by rotary evaporation. Distillation at 100–120° (0.5 mm) yielded 8 g of a crude mixture of *cis,trans* (**1**) and *trans,trans* (**2**) methyl α-ionyldenacetates in a 1:2 ratio (by GLC).

Separation of cis,trans (**1**) *and* trans,trans (**2**) *isomers of α-ionylidine acetate.* 1 g of the crude mixture of esters was chromatographed on a 95 × 3 cm column of Si gel eluted with a mixture of hexane–toluene–HOAc (50:50:0.1). 5-ml fractions were collected and analysed by GLC. Pure **1** was obtained in the early fractions, and pure **2** in the later fractions. The relevant fractions were bulked to yield 210 mg of pure **1** and 340 mg of pure **2**. The identities of **1** and **2** were confirmed by their ^{1}H NMR spectra which were identical to those previously reported for these compounds [3].

Cis,trans *methyl-3-methyl-5-(4′-oxo-2′,6′,6′-trimethylcyclohex-2′-en-1′-yl)-2,4 pentadienoate* (*methyl-1′-deoxy-AB4*) (**3**). 100 mg (0.4 mmol) **1** in 20 ml of CH_2Cl_2 was stirred with 2 g of CrO_3(pyridine)$_2$ complex for 10 hr. At the end of this time, another 1 g of the complex was added and the stirring continued for another 10 hr. The mixture was poured into a separating funnel and the reaction flask washed with 3 × 20 ml Et_2O. The combined organic phases were washed successively with satd $NaHCO_3$ (4 × 30 ml), 0.1 M HCl (3 × 30 ml), satd $NaHCO_3$ (1 × 30 ml), and H_2O (1 × 50 ml). The organic layer was dried over dry $MgSO_4$. The material was concentrated by rotary evaporation to a yellow oil and purified by PLC on Si gel developed in CH_2Cl_2–

The major UV-absorbing zone at R_f 0.3 was eluted with Et_2O. Evaporation of the Et_2O gave 58 mg of a yellow syrup. The product showed only a single peak on GLC and on HPLC in system D. MS (GC/MS) 24 eV *m/z* (rel. int.): 262 (1.5), 206 (1.0), 189 (2.6), 187 (3.8), 174 (22.6), 147 (25.8), 146 (56.6), 145 (13.3), 125 (100), 119 (23.2), 112 (15.3), 105 (12.0), 91 (21.2); UV λ_{max}^{MeOH} nm: 267, 249 (s); ^{1}H NMR (360 MHz, $CDCl_3$): δ 0.97 (3 H, *s*), 1.07 (3 H, *s*), 1.93 (3 H, *s*), 2.00 (3 H, *s*), 2.14 (1 H, *d*, J = 16 Hz), 2.38 (1 H, *d*, J = 16 Hz), 2.72 (1 H, *d*, J = 9 Hz), 3.71 (3 H, *s*), 5.70 (1 H, *s*), 5.93 (2 H, *m*), 7.72 (1 H, *d*, J = 14 Hz).

Trans,trans-*methyl*-3-*methyl*-5-(4′-*oxo*-2′,6′,6′-*trimethylcyclohex*-2′-*en*-1′-*yl*)-2,4 *pentadienoate.* 100 mg **2** was oxidized and purified as above to yield 49 mg **4** as a yellow oil. MS (GC/MS) 24 eV *m/z* (rel. int.): 262 (5.0), 206 (13.0), 189 (6.3), 188 (2.4), 187 (7.5), 175 (24.0), 174 (77.9), 147 (37.0), 146 (91.0), 145 (24.5), 125 (100), 119 (23.1), 112 (18.0), 105 (9.6), 91 (21.2); UV λ_{max}^{MeOH} nm: 267, 240 (s); ^{1}H NMR (330 MHz, $CDCl_3$): δ 0.97 (3 H, *s*), 1.07 (3 H, *s*), 1.88 (3 H, *s*), 2.12 (1 H, *d*, J = 16 Hz), 2.27 (3 H, *s*), 2.35 (1 H, *d*, J = 16 Hz), 2.65 (1 H, *d*, J = 9 Hz), 3.71 (3 H, *s*), 5.77 (1 H, *s*), 5.95 (2 H, *m*), 6.20 (1 H, *d*, J = 14 Hz).

Cis,trans-3-*methyl*-5-(4′-*oxo*-2′,6′,6′-*trimethylcyclohex*-2′-*en*-1′-*yl*)-2,4-*pentadienoic acid* [1′-*deoxy*-*ABA* (**7**)]. A mixture of α-ionone (10 g, 50 mmol) and carb-*t*-butoxymethylene-triphenylphosphorane (34 g, 100 mmol) was reacted as described previously to yield, after distillation, 6.2 g of a crude ester mixture. The *cis,trans* and *trans,trans* isomers were separated as described above for the Me esters and the pure isomers oxidized with CrO_3 (pyridine)$_2$ to yield 32 mg **5** and 45 mg **6** as yellow syrups. 10 mg **5** and 10 mg of *p*-toluenesulphonic acid in 5 ml C_6H_6 was refluxed for 60 min. The C_6H_6 was removed by rotary evaporation, the residue dissolved in a small volume of EtOAc and subjected to PLC using toluene–EtOAc–HOAc (50:30:3) as the developing solvent. The major UV absorbing zone at R_f 0.4 was eluted with EtOAc. Evaporation of the EtOAc gave 5 mg colourless glass. Trituration with hexane gave 4.2 mg **7** as a white solid. The identity of the product was confirmed by the fact that the UV, MS and NMR of its methyl ester, prepared with CH_2N_2, were identical to those of **3**.

The *trans,trans* isomer **8** was prepared in an analogous manner and its structure confirmed as above.

^{3}H-*labelled* α-*ionone.* α-Ionone (1 g, 5 mmol) and ^3H_2O (0.2 ml, 5Ci/ml) were dissolved in 10 ml dry dioxane and *ca* 1 mg of metallic Na was added. The mixture was heated in a sealed tube at 80° for 30 min and allowed to stand at room temp. for 24 hr. The mixture was poured into 100 ml Et_2O and dried over Drierite for 3 days. After removal of the solvent the residue was distilled at 1 mm pressure to yield 700 mg ^{3}H-labelled α-ionone (15.4 mCi/mmol). Prior experiments with D_2O under the same conditions had shown that the label was confined to the side-chain methyl group.

^{3}H-*labelled*-3-*methyl*-5-(4′-*oxo*-2′,6′,6′-*trimethylcyclohex*-2′-*en*-1′-*yl*)-2,4 *pentadienoic acids* (^{3}H-7 *and* ^{3}H-8). ^{3}H-labelled **7** and **8** were prepared from ^{3}H-labelled α-ionone by identical methods to those described above except that the initial separation of ^{3}H-labelled **5** and **6** was performed by PLC using the same solvent system as used for the CC. The final products had a sp. act. of 8.6 mCi/mmol.

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