Phytochemistry, Vol. 26, No. 6, pp. 1631–1634, 1987.
Printed in Great Britain.

0031–9422/87 $3.00 + 0.00

2,7-DIMETHYL-OCTA-2,4-DIENEDIOIC ACID, A POSSIBLE BY-PRODUCT OF ABSCISIC ACID BIOSYNTHESIS IN THE TOMATO

ROBERT S. T. LINFORTH, W. RUSSELL BOWMAN,* DAVID A. GRIFFIN,† PETER HEDDEN,‡ BRIAN A. MARPLES* and IAN B. TAYLOR§

Department of Physiology and Environmental Science, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, Leics LE12 5RD, U.K.; *Department of Chemistry, University of Technology, Loughborough, Leics LE11 3TU, U.K.; †ICI, Plant Protection Division, Jealott's Hill Research Station, Bracknell, Berks RG12 6EY, U.K.; ‡Long Ashton Research Station, Long Ashton, Bristol BS18 9AF, U.K.

(*Revised received* 7 *November* 1986)

Key Word Index—*Lycopersicon esculentum*; abscisic acid; biosynthesis; 2,7-dimethyl-octa-2,4-dienedioic acid.

Abstract—The abscisic acid deficient tomato mutant *flacca*, compared to isogenic non-mutant controls, produces higher amounts of a previously unidentified acid. This has been synthesized and shown to be 2,7-dimethyl-octa-2,4-dienedioc acid (ODA).

INTRODUCTION

Recent evidence strongly supports earlier proposals [1] that plants are able to synthesize abscisic acid (ABA) (1) indirectly following degradation of a C_{40} precursor, e.g. violaxanthin (2) (scheme 1) [2]. Full details of the ABA biosynthetic pathway in plants have yet to be elucidated, but the current position has recently been summarized [3].

It has been shown that the ABA deficient tomato mutant, *flacca*, can accumulate abnormally high levels of an unidentified component of the acid–ether fraction (see Experimental) [4]. An EIMS (probe) of a partially purified sample of the unknown was reported [4]. It was suggested that this compound might be a biosynthetic precursor of ABA, accumulating behind the *flacca* genetic lesion [4]. The identification and synthesis of the unknown must be carried out before this proposal can be reasonably evaluated.

RESULTS AND DISCUSSION

A sample of the unknown compound was obtained for GC-MS, following purification of an 80% methanolic extract of 500 g of *flacca* tissue (see Experimental). The methyl ester (*ca* 10 μg) was submitted to GC-MS analysis (see Experimental). One major (*ca* 70%) and three minor components were noted on GC-MS which had mass spectra clearly related to that obtained for the previously isolated unknown acid [4]. The four mass spectra were very similar and the three minor constituents are therefore likely to be isomers. The presence of a putative molecular ion at *m/z* 226 appeared to indicate the formation of the dimethyl ester of the previously isolated dicarboxylic acid (M^+ at *m/z* 198). Further details of the mass spectrum of the dicarboxylic acid and its dimethyl ester are given in the Experimental.

A larger sample of the underivatized unknown was purified from 6-week-old *flacca* mutant tomato plants (see Experimental). Analysis of the partially purified acid fraction by ^{1}H NMR (400 MHz) spectroscopy indicated the presence of four compounds. One impurity was identified from the ^{1}H NMR spectrum as salicylic acid (see Experimental). The ^{1}H NMR spectrum also indicated the presence of a short chain aldehyde (see Experimental). Both impurities were removed by further purification by normal phase HPLC (see Experimental) and the ^{1}H NMR (400 MHz) spectrum of the remaining material was consistent with it being a mixture of 2,7-dimethyl-octa-2,4-dienedioic acid (ODA, 3) and 2,7-dimethyl-oct-4-enedioic acid (OEA, 4) (Fig. 1). The final purified extract contained *ca* 900 μg ODA and 600 μg OEA.

The ODA and OEA in the mixture were converted into their trimethylsilyl esters and submitted to GC-MS analyses (see Experimental). Single isomers of OEA and ODA were observed with retention times of 7.32 and 8.76 min, respectively. The mass spectra of the trimethylsilyl esters confirmed that the molecular weights of ODA and OEA were 198 and 200, respectively.

Of the two components identified by H^1 NMR spectroscopy only the ODA would have been detected on HPLC using a UV detector (252 nm). Therefore, the unknown component, characteristically accumulating in the ABA deficient mutant [4], is presumed to be ODA, a previously unknown C_{10} dicarboxylic acid. The metabolic relation-

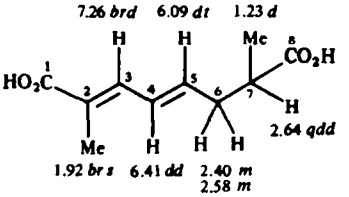
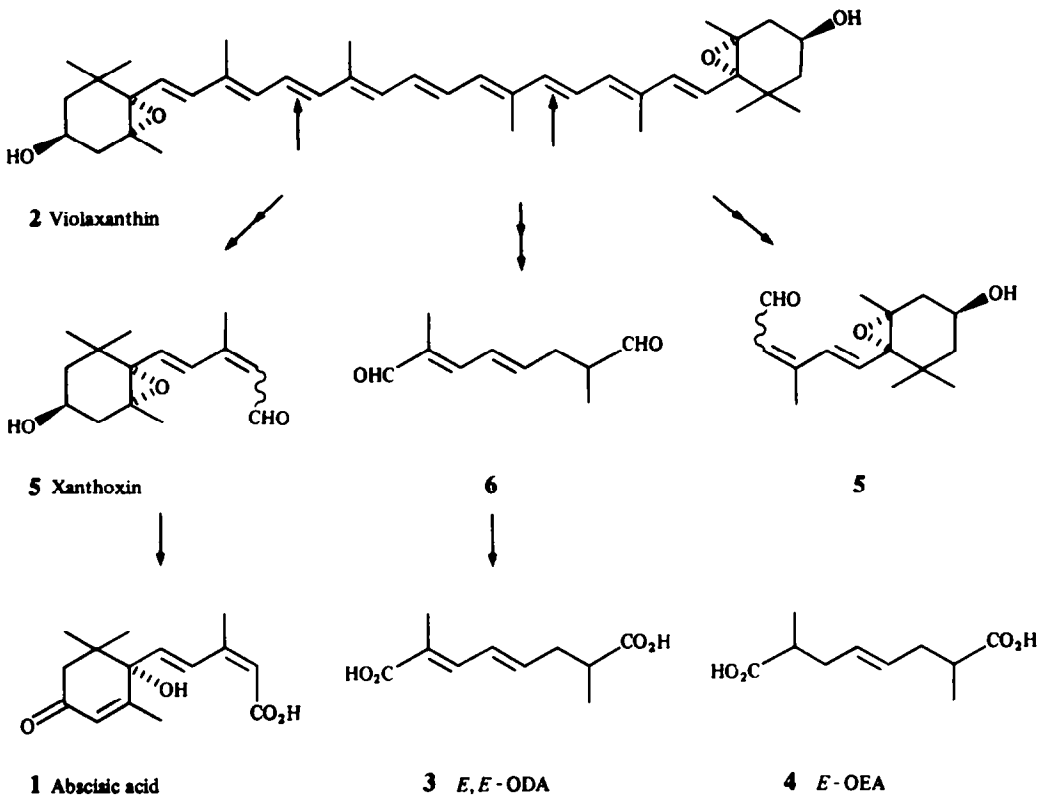


Fig. 1. Interpretation of ^{1}H NMR spectrum of ODA (3).

§To whom correspondence should be sent.



Scheme 1. Putative biosynthesis of ABA from C_{40} precursors. One possible biosynthetic framework to illustrate the formation of C_{10} by-products as a consequence of ABA synthesis. Precise details of the pathway are not known, although there is good evidence favouring an indirect route.

ship between ODA and OEA remains to be established, along with their functional significance.

Further proof of the structures of ODA and OEA was obtained by synthesis. The spectroscopic data for the synthetic samples closely resembles those of the natural materials (see Experimental). Interestingly, GLC of the synthetic ODA dimethyl ester, prior to separation and purification, showed that it comprised four isomers in much the same proportion as the initially isolated material (see Experimental). The major component of the synthetic and natural ODA gave the same R_t on HPLC (7.75 min, see Experimental).

The major isomer of synthetic ODA was shown by Nuclear Overhauser Enhancement (NOE) to have the *trans*-(*E*)-configuration for the C-2/C-3 olefinic bond. This, and other spectroscopic data suggest that the natural ODA is (*E,E*)-2,7-dimethyl-octa-2,4-dienedioic acid.

The presence of higher levels of ODA in the mutant tomato *flacca*, especially when under water stress, previously suggested some relationship with ABA biosynthesis [4]. It is clearly not an intermediate in the biosynthesis of ABA, but has the same carbon skeleton as the C_{10} middle portion of carotenoids. Therefore it may be a by-product of biosynthesis, if ABA is derived from a C_{40} precursor (Scheme 1). Excessive attempted production of ABA in the mutant may lead to increased cleavage of the C_{40} precursor and results in the accumulation of the excessive quantities of the by-product ODA typically observed [4, 5]. The biosynthesis of ABA from a C_{40} precursor such as violaxanthin (**2**) may produce two molecules of a C_{15} ABA precursor (**5**) and an initial C_{10}-dialdehyde by-product (**6**). This may in turn be oxidized to the C_{10}-dicarboxylic acid (ODA).

It might reasonably be suggested that ODA accumulates in *flacca* plants following the reduction of the related triolefin, OTA, 2,7-dimethyl-octa-2,4,6-trienedioic acid (perhaps a more obvious by-product of ABA biosynthesis). Although OTA has been synthesized (see Experimental) it has not been possible to confirm its presence in plant extracts. The compound should have been readily detectable by GC-MS, particularly as the molecular ion of its dimethyl ester (M^+ at *m/z* 224) is the base peak of the spectrum. Further investigations are necessary to understand the significance of the level of unsaturation in ODA and its relationship (if any) with OEA.

EXPERIMENTAL

Plant material. Tomato (*Lycopersicon esculentum*) plants homozygous for the *flacca* mutation in the cv Ailsa Craig genetic background were grown in the glasshouse for 6 weeks. The plants were then severed from their roots and allowed to wilt for 24 hr before being frozen in liquid N_2 and stored at $-15°$ prior to extraction.

Extraction and purification of a sample of the unknown (ODA) for GC-MS analysis. A bulked sample of 500 g *flacca* tissue (obtained as above) was homogenized and extracted in 500 ml of 80% aq. MeOH containing 2 ml HOAC and 2 mg BHT per l. The extract was filtered and centrifuged (5000 rpm). The supernatant was retained and MeOH removed *in vacuo*. The aq. residue was adjusted to pH 8 and stored at 4° for 4 hr; the resultant ppt was removed by centrifugation (3000 rpm). The supernatant was partitioned 3 × with an equal vol. of Et_2O. The retained aq. phase was adjusted to pH 3 and partitioned 3 × with an equal vol. of Et_2O. The retained organic phase was concd to 25 ml and partitioned 3 × with an equal vol. of 5% $NaHCO_3$. The retained aq. phase was again adjusted to pH 3 and partitioned 3 × against Et_2O, as before. The organic phase was then evapd to dryness and taken up in a final vol. of 1 ml MeOH.

Aliquots (250 μl) were applied to silica gel GF_{254} TLC plates (1 mm). A crude separation was achieved by developing 3 × in toluene–EtOAc–HOAc (8:1:1). Several broad bands (1 cm) showing strong quenching of fluorescence at 254 nm were eluted with two 15 ml aliquots of MeOH. The presence of the unknown in a band at R_f 0.65 was established by retention time on HPLC following the injection of a 10 μl sample of the MeOH soln. An identical isocratic reverse phase HPLC system was used, to that described previously in the initial discovery of the unknown [4]. This gives an R_t of 7.75 min for the unknown, compared with 7.0 min for ABA. The remaining MeOH soln of the unknown was evapd to dryness and taken up in a series of 250 μl aliquots of MeOH, which were applied to a silica gel GF_{254} TLC plate (0.25 mm). This was developed as before and a strongly quenching band of R_f 0.65 was eluted with two 15 ml aliquots of MeOH. The presence of the unknown was again confirmed by R_t using the isocratic reverse phase HPLC system described previously [4].

The remainder of the sample of the unknown was evaporated to dryness, dissolved in 400 μl MeOH and made up to a 2 ml injection vol. with H_2O prior to purification by HPLC. The entire sample was injected onto an ODS-Partisil 10 μm (250 × 9.4 mm i.d.) column via a 2 ml Rheodyne injection loop. The sample was trapped on column for 10 min by holding the solvent programmer (LC-XP Pye Unicam) at 20% aq. MeOH in 5% HCO_2H. The flow rate was 4 ml/min. The unknown was then eluted with a linear gradient rising from 20 to 100% aq. MeOH in 5% HCO_2H over 25 min. A peak corresponding to the unknown was observed by UV detection (LC-UV Pye Unicam) at 252 nm, eluting 16 min after intiation of the gradient. The eluent containing the compound of interest was collected (25 to 27 min. fraction) and MeOH removed *in vacuo*. The remaining aq. phase was partitioned 3 × with an equal vol. of Et_2O. The retained organic phase was evapd to dryness and the unknown derivatised with excess ethereal CH_2N_2.

The sample was taken up in 50 μl of MeOH and 1 μl was injected via a Grob splitless injection system onto a BP-1 WCOT (25 m × 0.25 mm) capillary column for GC-MS (inlet temp. 250°, source temp. 200°). The carrier gas (He) flow rate was 2 ml/min. The column was heated ballistically from 50° to 200° and the temp. subsequently increased at 5°/min up to 250°.

A major peak (*ca* 70%) was observed at R_t 1.23 min. MS *m/z* (rel. int.): 226 $[M]^+$ (7), 194 $[M-MeOH]^+$ (9), 166 $[M-MeOH-CO]^+$ (9), 162 $[M-2MeOH]^+$ (25), 135 $[M-OMe-MeOH-CO]^+$ (30), 134 $[M-2MeOH-CO]^+$ (81), 125 $[M-101]^+$ (20), 107 $[C_8H_{11}, M-OMe-MeOH-2CO]^+$ (100), 91 $[C_7H_7$, tropylium$]^+$ (46), 79 $[107-CO]^+$ (78), 59 $[CO_2Me]^+$ (59).

Three minor peaks giving the same mass spectrum were also present at R_t 0.71 min (7.5%), 0.83 min (18.5%) and 0.96 min (4%).

Extraction and purification of a sample of the unknown (ODA) for ^1H NMR analysis. A sample of 12 kg *flacca* tissue (obtained as described earlier) was subjected to the extraction and purification methods outlined in the previous section. The procedure was scaled up in proportion to the increase in sample size. Following reverse phase HPLC purification (the sample was not derivatized with diazomethane) the sample containing the unknown (ODA) was taken up in $CDCl_3$ and a 400 MHz ^1H NMR spectrum obtained. Following extensive decoupling work, the extract was shown to consist of a mixture of four compounds.

The spectrum of one of the compounds clearly matched that of salicylic acid. A pure sample of salicylic acid was found to have an R_f of 0.7 on the TLC system used in the purification procedure, compared with R_f 0.65 for the unknown (ODA). On reverse phase HPLC (as described previously) salicyclic acid eluted *ca* 20 sec earlier than the unknown. Its presence as a contaminant was therefore not surprising. The extract was further purified by a normal phase HPLC system which gave an R_t of 32.2 min for salicylic acid and an R_t of only 9.0 min for the compound of interest. The extract was evapd to dryness and taken up in a series of 20 μl aliquots of EtOH. These were injected onto a 250 × 4.6 mm i.d. column packed with 10 μm NH_2 Techsil and eluted isocratically using a $CHCl_3$–MeCN–HOAc mixture (110:90:5) at a flow rate of 1.6 ml/min. A 1 min fraction containing the compound of interest (detected at 252 nm, R_t 9.0 min was collected and 15 ml of H_2O added. The organic solvents were removed *in vacuo* and the remaining acidic aq. phase was partitioned 3 × against equal vols. of Et_2O. The organic phase was evapd to dryness and the sample taken up in $CDCl_3$ to obtain a further 400 MHz ^1H NMR spectrum. It was found that as well as removing salicylic acid, the normal phase HPLC purification had also removed a second contaminant which previously gave the following ^1H NMR spectrum: δ1.61 (>6 H, *br d*), 2.36 (2H, *t*, J = 7.5 Hz), 2.43 (2H, *dt*, J = 1.7, 7.5 Hz), 9.77 (1H, *t*, J = 1.7 Hz). This compound was not thought to be of any interest and its structure was not further investigated.

One of the two remaining compounds, later proved by synthesis to be 2,7-dimethyl-octa-4-enedioic acid (OEA, **4**), gave the following 400 MHz ^1H NMR spectrum: δ1.16 (6H, *d*, J = 7 Hz), 2.18 (2H, *ddt*, J = 12.5, 7, 3.8 Hz), 2.33 (2H, *m*), 2.53 (2H, *m*, J = 7 Hz), 5.47 (2H, *tt*, J = 3.8, 1.7 Hz). The ^1H NMR spectrum of the other compound, 2,7-dimethyl-octa-2,4-dienedioic acid (ODA, **3**) is displayed in Fig. 1. Molecular weights of both compounds were confirmed by GC-MS of their TMSi esters (splitless injection 2 μl; He flow rate 2 ml/min; GP-Sil 5 capillary column 12 m × 0.22 mm; column oven temp. 100°, raised to 280° at 15° min).

Synthesis of 2,7-dimethylocta-2,4-dienedioic acid. Dimethyl 2,7-dimethyl-octa-enedioate was prepared by the published procedure [6] and brominated using *N*-bromosuccinimide in refluxing CCl_4 (containing $CaCO_3$ to neutralize liberated hydrogen bromide) and a catalytic amount of dibenzoyl peroxide (radical initiator) and a gave a crude mixture of the 3-bromo- and 3,6-dibromo-octa-4-enedioate. This crude mixture was dehydrobrominated [7] without purification, using lithium bromide and lithium carbonate in hot DMF (120–125°, 2 hr, under N_2) to yield a mixture of dimethyl 2,7-dimethyl-octa-2,4-dienedioate and dimethyl 2,7-dimethyl-octa-2,4,6-trienedioate. The triene was crystalline and most was removed by filtration. The residual oil was further purified by prep. TLC [silica gel, petrol (60–80°)]. GLC (capillary W-COT fused silica column packed with OV-1) indicated that all four geometrical isomers of the diolefin were present with the *trans,trans*-isomer predominant (*ca* 70%).

A sample of the oil, containing di- and tri-olefin esters was hydrolysed with KOH in refluxing aq. dioxan to yield a mixture of the corresponding di-carboxylic acids. The remaining tri-olefin diacid was separated by crystallization from petrol (60–80°)–Et_2O.

Further crystallization yielded pure 2,7-dimethyl-octa-2,4-dienedioic acid which was recrystallized 3 × from hexane–Et_2O mp 124–126°; (found: C, 60.3; H, 7.1; calc. for $C_{10}H_{14}O_4$: C, 60.6; H, 7.1%); λ_{max}^{EtOH}: 263 nm (ε = 21 620); EIMS (probe) 70 eV, *m/z* (rel. int.): 198(2), 180(4), 162(3), 152(14), 135(13), 134(43), 111(35), 107(66), 91(32), 79(72), 45(80); ^{13}C NMR (20.1 MHz, $CDCl_3$): δ 12.19 (*q*, C-7 Me), 16.51 (*q*, C-2 Me), 36.85 (*t*, C-6), 39.25 (*d*, C-7), 125.42 (*s*, C-2), 128.31 (*d*, C-4), 140.07 and 140.51 (*s* and *s*, C-3, C-5), 174.46 (*s*, C-8), 182.55 (*s*, C-1); ^{1}H NMR (400 MHz, $CDCl_3$): 1.23 (3 H, *d*, J = 7 Hz, 7-Me), 1.92 (3 H, *br s*, 2-CH_3), 2.39 (1 H, *m*, 6-H′), 2.55–2.68 (2 H, *m*, 6-H″, 7-H), 6.08 (1 H, *dt*, J = 15 Hz, J = 7 Hz, 5-H), 6.42 (1 H, *dd*, J = 15 Hz, J = 11 Hz, 4-H), 7.27 (1 H, *dt*, J = 11 Hz, J = *ca* 1 Hz, 3-H). The yields for the synthetic procedure were not optimized.

Dimethyl 2,7-dimethyl-octa-2,4-dienedioate. 2,7-Dimethyl-octa-2,4-dienedioic acid was methylated with CH_2N_2 in dry Et_2O to give a quantitative yield of the pure dimethyl ester, bp 180–190° (0.1 mm Hg); EIMS (probe) 70 eV, *m/z* (rel. int.): 226(3), 195(2), 194(3), 166(5), 163(5), 162(10), 135(17), 134(36), 125(13), 107(55), 91(50), 79(71), 59(95); ^{13}C NMR (20.1 MHz, $CDCl_3$): δ 12.63 (*q*, C-7 Me), 16.78 (*q*, C-2 Me), 37.18 (*t*, C-6), 39.31 (*d*, C-7), 51.66 and 51.77 (*q* and *q*, 2 OMe), 126.02 (*s*, C-2), 129.15 (*d*, C-4), 138.32 and 139.07 (*s*, and *s*, C-3, C-5), 169.10 (*s*, C-8), 176.26 (*s*, C-1); ^{1}H NMR (90 MHz, $CDCl_3$): δ 1.19 (3 H, *d*, J = 7 Hz, C_7-Me), 1.96 (3 H, *br s*, C_2–Me), 2.3–2.7 (3 H, *m*, 6-H_2, 7-H), 3.70 (3 H, *s*, C_3–OMe), 3.78 (3 H, *s*, C_1–OMe), 6.01 (1 H, *dt*, J = 15 Hz, J = 7 Hz, 5-H), 6.43 (1 H, *dd*, J = 15 Hz, J = 11 Hz, 4-H), 7.18 (1 H, *dq*, J = 11 Hz, J = 1 Hz, 3-H).

Acknowledgements—We wish to thank Mr. David Bowen for carrying out the synthesis of ODA, Mr. Mike Harris for ^{13}C NMR and ^{1}H (90 Mz) NMR spectroscopy, Mr. John Greenfield for mass spectroscopy, and Mr. M.A. Kipps and ICI for ^{1}H (400 MHz) NMR spectroscopy. We would also like to acknowledge financial support from the AFRC.

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