Phytochemistry, 1963, Vol. 2, pp. 289 to 296. Pergamon Press Ltd. Printed in England

ACETYLENIC FATTY ACIDS IN SEEDS AND SEEDLINGS OF SWEET QUANDONG

J. D. BU'LOCK and G. N. SMITH*
Department of Chemistry, The University of Manchester, Manchester 13, England

(*Received* 14 *February* 1963)

Abstract—The ready estimation of polyacetylenes by spectroscopic means is convenient for the study of fatty acid composition in *S. acuminatum*. A marked difference between seed (reserve) and seedling (somatic) lipids has been observed when the seeds germinate, and the somatic lipids in different parts vary as the seedlings develop. New polyacetylenic acids are observed, including (V) and probably (VI). Independently of photosynthesis, different seedling parts and also the discarded endosperm can incorporate acetate-1-^{14}C uniformly into fatty acids including polyacetylenes; non-acetylenic acids may be the primary synthesis products.

INTRODUCTION

BY STUDYING the germination of seeds with fatty reserves there is an opportunity to investigate fat metabolism in a developing system. During the time that the reserve fats of the seed are mobilized, the less well-known somatic lipids of the young plant make their first appearance. These somatic lipids may be quite different from those in the seeds, and may also show considerable variation in different parts of the same plant, although there are relatively few data on these matters. Rather laborious experimental techniques are general required for an adequate study of such problems, so that the exceptional work of Crombie on the oil-palm[1] and the water-melon[2,3] is all the more remarkable. However, in certain plants, differences in fatty acid composition arise less from differing proportions of common fatty acids than from the presence, in varying amounts, of more distinctive acids. Here experimental work may be considerably facilitated, and species with polyacetylenic fatty acids[4] offer particular advantages, since in these the conjugated unsaturation gives rise to characteristic and intense ultra-violet absorption spectra which can be measured directly on the crude extract.

In the order Santalales, families Olacaceae and Santalaceae, many species contain ximenynic acid (I) as a major component of the seed glycerides. Recently Hatt, Triffett, and Wailes[5,6] examined somatic fats from other parts of such plants and found that ximenynic acid was largely or wholly replaced by more unsaturated acids, namely the dienyne (II), the enediyne (III), and the dienediyne (IV). All these acids contain the "azelaic" end-group $\cdot(CH_2)_7\cdot CO_2H$ but are readily distinguished by their absorption spectra. With the assistance of Dr. J. R. Price of the C.S.I.R.O. we secured some seeds of Sweet Quandong, *Santalum acuminatum* D.C. (syn. *Eucarya acuminata*), one of the Australian species studied by Hatt *et al.*,[6] and examined their germination. The fatty acids from different parts and stages were examined and the incorporation of acetate-1-^{14}C investigated. The results throw

* Present address: Department of Chemistry, The University, Glasgow, Scotland.

[1] S. G. BOATMAN and W. M. CROMBIE, *J. Exp. Botany* **9**, 52 (1958).
[2] W. M. CROMBIE and R. COMBER, *J. Exp. Botany* **7**, 166 (1956).
[3] W. M. CROMBIE and E. E. HARDMAN, *J. Exp. Botany* **9**, 239 (1958).
[4] N. A. SORENSEN, *Proc. Chem. Soc.* 98 (1961).
[5] H. H. HATT, A. C. K. TRIFFETT, and P. C. WAILES, *Australian J. Chem.* **12**, 190 (1959).
[6] H. H. HATT, A. C. K. TRIFFETT, and P. C. WAILES, *Australian J. Chem.* **13**, 488 (1960).

some light on the differentiation of somatic lipids and also on the biosynthesis of acetylenic fatty acids.

$CH_3.(CH_2)_5.CH{=}CH.C{\equiv}C.R$ (I) ximenynic acid
$CH_3.(CH_2)_3.(CH{=}CH)_2.C{\equiv}C.R$ (II)
$CH_3.(CH_2)_3.CH{=}CH.(C{\equiv}C)_2.R$ (III)
$CH_3CH_2.(CH{=}CH)_2.(C{\equiv}C)_2.R$ (IV)
$CH_3CH_2.CH{=}CH.(C{\equiv}C)_3.R$ (V)
$CH_2{=}CH.CH{=}CH.(C{\equiv}C)_3.R$ (VI)

Fig. 1. Acetylenic acids from *S. acuminatum* ($R = \cdot(CH_2)_7.CO_2H$)

RESULTS

Germination and growth

Like other Santalales, *S. acuminatum* is semi-parasitic and only germinates with difficulty; in our hands, less than 20 per cent of the seeds developed. The stages observed are similar to those described for *S. album*:[7] the outer shell of the nut splits open and the embryo emerges as a radicle. This lengthens, forming first the root with rootlets and next the hypocotyl; next the cotyledons begin to emerge from the shell and at 12–15 weeks the seedling becomes independent, the cotyledons open or detach, the depleted seed falls away, and the first green parts (plumule) are visible. Until this stage there is no photosynthesis.

Fatty acid composition

Absorption maxima for individual acids are given in Table 1 and were used with the known extinction coefficients for these chromophores[8] for estimation. Overall data for acids in the seeds, root system, and rest of plant, obtained by chromatographic analysis, are given in Table 2, and more approximate data showing the differentiation of the acetylenic acids in different seedling parts, estimated by direct spectrophotometry of lipid extracts, are given in Table 3. Before germination the enyne (I) (ximenynic acid) constitutes about one-half of the total fatty acids in the endosperm, with oleic as the other main component and traces of the dienyne (II). In the depleted seed at 12 weeks the proportion of both acetylenic acids is somewhat greater. At no stage were acids of higher unsaturation than (I) and (II) detected in the endosperm.

Table 1. Absorption maxima of polyacetylenic acids

Compound	Chromophore	Absorption max (mμ)
I	enyne	228
II	dienyne	266, 267
III	enediyne	214, 229, 240, 252, 266, 282
IV	dienediyne	225, 236, 293, 310
V	enetriyne	212, 223, 232, 244, 258, 274, 291, 310, 332
VI	dienetriyne	255, 267, 345*

* Other maxima obscured by those of V also present in samples of this dienetriyne.

The fatty acids from the seedlings are in sharp contrast (Tables 2, 3). At no stage is ximenynic acid detectable outside the seeds. In the radicle at 7–10 days the enetriyne (V) predominates, with the enediyne (III) also present. Later, at 12–15 weeks, there is a systematic

[7] J. Lubbock, *Seedlings* (Kegan Paul, London, 1892) Vol. II, p. 467.
[8] F. Bohlmann, *Angew. Chem.* 67, 389 (1955).

TABLE 2. ANALYSIS OF *S. acuminatum* SEEDS AND 12-WEEK SEEDLING

Plant part	Seed before germination	Discarded seed (12 wk)	Root system (12 wk)	Shoot system (12 wk)
Length (cm)	(diameter ca. 1 cm)		20	10
Dry wt (mg)	380	115	70	350
Total fatty acids (mg)	300	40	1	1
% total as (I)	45	65	absent	absent
„ „ „ (II)	0·3	0·5	1	10
„ „ „ (III)	absent	absent	5	40
„ „ „ (V)	absent	absent	45	10
„ „ „ oleic*	48	35	50	40

Data for seeds refer to the endosperm ("kernel") only; the 12-week seedling was divided into "root system" (roots+rootlets) and "shoot system" (rest of plant) and the recently-discarded seed was also examined. Fatty acids were separated by reversed-phase chromatography and are given as percentages of the total after saponification.

* Including palmitic, etc.

variation in the fatty acids from different parts, revealed directly by spectroscopic examination of crude lipid extracts and summarized by the approximate data of Table 3. It is apparent that the enetriyne (V) makes up nearly all the acetylenic acid content of the rootlets and predominates in the main root, but is a minor component in the hypocotyl and is virtually absent from cotyledons and shoot. Conversely the dienyne (II) is first detectable in the main root but only becomes important in the upper shoot, and predominates in the cotyledons. The acid of intermediate unsaturation, enediyne (III), predominates in the hypocotyl and lower shoot. In the green plumule no acetylenic acids are detectable.

TABLE 3. DIFFERENTIATION OF ACETYLENIC ACIDS IN DEVELOPING *S. acuminatum*

Plant part	Acid			
	I	II	III	V
Seeds	100	+	—	—
10-day radicle	—	+	25	70
12-week seedling rootlets	—	—	+	100
„ „ root	—	+	10	90
„ „ hypocotyl	—	+	70	25
„ „ shoot	—	40	50	+
„ „ cotyledons	—	90	5	+
„ „ plumule	—	—	—	—

Individual acids, estimated from the spectra of crude lipid extracts from small samples of plant tissues, given as approximate percentages (±10) of the total acetylenic acids present (+, trace; —, absent).

Characterization of acetylenic acids

Direct chemical characterization of the somatic acids was not possible because of the minute amounts available. The spectra (Table 1) and elution behaviour (Table 4) leave no doubt that the dienyne and enediyne acids are the acids (II) and (III) of Hatt and co-workers[5,6] and this was confirmed by oxidation of ^{14}C-labelled samples of these acids to give valeric and azelaic acids as expected (Table 5). The main enetriyne acid, for which structure (V)

Table 4. Elution of *S. acuminatum* acids from reversed-phase column

Fractions	MeOH (%)	Chromophore	Comment
5–14	35–40	?	Single λ_{max}, 319 mμ, trace only
15–25	40–45	enetriyne	Trace only
30–40	50	enetriyne	Trace only
35–50	50–53	dienetriyne	Acid VI?: trace only
40–95	53–56–59	enetriyne	Acid V; fractions 50–70 and 71–90, oxidized separately, gave similar products (Table V)
90–110	59	enediyne	Acid III
105–120	59–62	dienyne	Acid II, trace only
120–140	65–68	none	Acid I (ximenynic) runs here if present
135–190	68–80	—	Palmitoleic, palmitic, oleic, stearic (mainly oleic)

Acids (about 1 mg) from saponification of *S. acuminatum* root lipids* separated on paraffin–silicone–keiselguhr column (40×1·1 cm) at 22°, eluting with aqueous methanol at 35 ml/hr and collecting 3 ml samples.

* Chromatography of acids from shoot and cotyledons is similar, the first compound eluted being V (trace only), then III (main component), then II, with I again absent.

is proposed (see below) similarly gave propionic and azelaic acids as expected, but also some acetic acid. Two fractions of the enetriyne (Table 4) were therefore oxidized separately in case the acids were hetergeneous, but both still gave the same oxidation products; this apparent anomaly is discussed below. Also noted in Table 4 are three very minor components of the root system (less than 5 μg altogether); the dienediyne acid (IV) of Hatt *et al.*[6] was not found.

Table 5. Oxidation of individual acids from *S. acuminatum*

Original acid	Wt used (μg)	Activity of Na Salts (counts/sec/mg) C_2	C_3	C_4	C_5	C_6	C_7	C_8	C_9	C_{10}
Dienyne (II)	100	—	—	—	20	1	5	1	2	—
Enediyne (III)	140	—	3	1	25	1	2	1	1	—
Enetriyne (V) (two fractions)	200	12	30	1	2	1	—	—	—	—
	250	15	36	1	3	1	—	—	—	—

Acetylenic acids, from incubation of root slices with acetate-1-^{14}C, oxidized separately with permanganate-periodate reagent.[7] To the oxidation products a mixture of ~ 5 mg of each of the n-alkyl carboxylic acids (to C_{10}) was added and the steam-volatile mixture separated on a column of Amberlite CG50.[8] Azelaic acid was the only labelled dicarboxylic acid produced. The sodium salts of the acids were counted as films of comparable self-absorption.

Incorporation of acetate-1-^{14}C

Overall data for the incubation of 12-week seedling parts with acetate-1-^{14}C (Table 6) show that respiratory breakdown was highest in the shoot (which also released most endogenous, non-labelled CO_2) whilst incorporation into lipids was highest in the depleted seed slices. In all cases the incorporation into acetylenic acids was disproportionately low when compared with the proportion of these acids actually present. The relative incorporation into acetylenic acids was highest in the seed slices, where ximenynic acid predominates; in the more unsaturated shoot acids the relative incorporation into acetylenic acids was noticeably increased in a more prolonged incubation.

Evidence that the ^{14}C-labelled fatty acids are uniformly-labelled on the odd-numbered C atoms was obtained by oxidation and decarboxylation of separated acids and is given in Table 7. Using this information, the distribution of radioactivity in a mixture of

TABLE 6. INCUBATION OF *S. acuminatum* PARTS WITH ACETATE-1-^{14}C

	Plant part			
	Seed slices	Root slices	Shoot slices	Intact shoot
Fresh wt (g)	0·3	0·5	1·5	1·4
Incubation time (hr)	6	6	6	40
Per cent ^{14}C used recovered as:				
CO_2	33	50	45	74
tissues after extraction	13	21	17	20
lipids	10	8	4	3
Acetylenic acids as % total fatty acids	66	50	50	50
^{14}C in acetylenic acids as % total lipid ^{14}C	24	2	4	10

Sliced root system, shoot system, and discarded endosperm, and an intact shoot system, all from 12-wk. seedlings, incubated separately with acetate-1-^{14}C (13 μc) at 22° in CO_2-free air, collecting CO_2 as $BaCO_3$ during incubation and subsequently extracting labelled lipids from tissues.

TABLE 7. EVIDENCE OF UNIFORM LABELLING

Original acids	Oxidation products	^{14}C in monobasic acid		^{14}C in CO_2H	
		^{14}C in azelaic acid		^{14}C in whole molecule	
		Found	Theory*	Found	Theory*
Oleic	nonanoic	—	—	0·01	0
	azelaic	—	—	0·19	0·2
C_{16}+C_{18} saturated	—	—	—	0·13	0·125†
Combined acetylenic	azelaic	—	—	0·19	0·2
Enetriyne V	propionic, azelaic	0·17	0·2	—	—
Enediyne III	valeric, azelaic	0·38	0·4	—	—

Labelled fatty acids from incubations of seedling parts with acetate-1-^{14}C separately oxidized with permanganate–periodate reagent, and the oxidation products purified for counting; some oxidation products also subjected to Schmidt degradation.[24]

* For equal labelling of odd-numbered C atoms.

† Calculated for palmitic acid.

TABLE 8. OXIDATION OF MIXED ^{14}C-LABELLED ACIDS

Carrier acid	Total radioactivity (mg carrier × counts/sec)	Original acid (before oxidation)	%^{14}C in original acid*	Approx wt (mg) of original acid
Acetic	4	?		
Propionic	4·5	V	1·7†	0·1
Valeric	20	II, III	2·0	0·5
Heptanoic	105	palmitoleic	7·2	
Nonanoic	1350	oleic	69	0·5
Palmitic	900	palmitic (stearic)	20	
Azelaic	2000	total unsaturated	—	—

Acids (~ 1 mg) from incubation of shoot system with acetate-1-^{14}C oxidized directly with permanganate–periodate and carrier acids (~ 50 mg each) added; steam-volatile acids then separated on Amberlite CG50[9] and palmitic and azelaic acids by recrystallization.

* Assuming uniform labelling of alternate C atoms.

† Assuming CH_3CO_2H also arises from (V); otherwise read 0·9 here.

[24] E. F. PHARES, *Arch. Biochem. Biophys.* 33, 173 (1951).

permanganate–periodate oxidation products, isolated with carriers, can be used to calculate the distribution of radioactivity in the original mixture of fatty acids, as shown for the acids from root slices in Table 8; once again the highest radioactivity was found in the non-acetylenic acids, with 69 per cent in oleic acid.

DISCUSSION

Germination and seedling development

In mature plants of *S. acuminatum* and other Santalales, the fatty acid compositions of seed and somatic lipids are very different;[5,6] this not unusual situation here involves mainly differences in the type of acetylenic acids present. It is now shown that such differences are established in the earliest stage of germination, the principal seed-fat acid being entirely absent in the seedlings and vice versa. The differences in acetylenic acid composition in different parts of the later seedlings is equally striking (Table 3). At first sight the steady decrease in unsaturation from rootlets to plumule might be explained by progressive desaturation of fatty acids during a downward translocation into the root system, but this is not plausible on general grounds. It is well-established[1-3] that in germination seed lipids are broken down to small molecules before translocation and that these small molecules serve in various sites as substrates for respiration and synthesis. At the same time there is little acceptable evidence for the translocation of intact fatty acids. In our experiments (Table 6), the different plant parts are fully capable of independent fatty acid synthesis. We must conclude that the different fatty acid compositions arise *in situ* from detailed differences in enzyme systems and are simply one biochemical aspect of tissue differentiation. Thus our observation that at 7–10 days the emergent embryo contains lipids of the highly-unsaturated kind later confined to the root system, accords with its description as a radicle which is largely presumptive root. The same feature was observed in the oil-palm where early seedling development is rather similar.[1] The regular gradation of unsaturation in the seedling lipids remains unexplained; few comparable data are available but a somewhat similar effect has been noted in the polyacetylenic oils of certain Compositae.[9]

The marked depletion of endosperm lipids as the seedlings develop (Table 2) is of course typical of seeds with fatty reserves; more remarkable is the observation that 12–15 weeks after germination the endosperm, largely exhausted and now detached from the seedling, still incorporates 10 per cent of added acetate-1-^{14}C into fats within 6 hr. This confirms the work of Newcomb and Stumpf on germinating peanuts,[10] in which the rapid turnover of reserve fats after germination was first demonstrated.

Acetylenic fatty acids

The dienyne and enediyne acids are identifiable, by comparison with published data and by oxidation, with the previously-described acids (II) and (III).[5,6] The principal root acid, for which the new structure (V) is proposed, is probably the same as an acid with λ_{max} at 243 and 328 mμ detected by Hatt *et al.*[6] As separated by us, the root acid has the enetriyne chromophore, behaves on the column as expected for a C_{18} enetriyne (Table 4), and gives azelaic acid on oxidation; structure (V) is preferred over the alternative (with chromophore reversed) by analogy with congeners (I)–(III). As well as the expected propionic acid, (V) also gave some acetic acid on oxidation (Table 5), and since heterogeneity of the material in this respect seems to be ruled out (Table 4) we conclude that partial

[9] F. Bohlmann, W. Sucrow, H. Jastrow, and H. Koch, *Ber.* **94**, 3179 (1961).
[10] E. H. Newcomb and P. K. Stumpf, *J. Biol. Chem.* **200**, 233 (1953).

isomerization occurs in the oxidation of (V). The ready prototropic rearrangement of polyacetylenes to more fully hyperconjugated products is a well-known reaction.[11]

Of the three minor polyacetylenes detected in the root acids (Table 4), two are enetriynes more polar than (V), and could be oxygenated derivatives analogous to the 8-hydroxyximenynic acid of other Santalales;[12] the third has the spectrum of a dienetriyne (Table 1) and from its elution behaviour the structure (VI) seems at least probable. The series (I)–(VI) conforms to the known preference for conjugated unsaturation in polyacetylenes.

In fungi, polyacetylenes are formed from acetate[13] with a malonic acid derivative as the homologation reagent,[14] and the mechanisms of synthesis that have been suggested[14,15] are of a kind which is closely related to the biosynthesis of fatty acids by acyl(+malonyl) condensations. The occurrence of polyacetylenic derivatives of stearic acid in plants has always exemplified the closeness of this connection, for which our observations now provide experimental support. The series of acids (I)–(VI) could arise either (a) by a common homologation mechanism adding a saturated chain to six different (C_{12}?) acids, or alternatively (b) by successive dehydrogenation reactions confined to the C_9–C_{18} portion of a C_{18} chain. The first type of pathway, (a), resembles the homologation routes demonstrated for the formation of linoleic acid in the hen oviduct[16] and of oleic acid in *Clostrydium butyricum*[17]; the alternative pathway, (b), could be related to such processes as the dehydrogenation of fatty acids (stearic⟶oleic⟶linoleic) observed by Bloch in yeasts[18,19] or the apparent conversion of oleic into linoleic in soyabean plants.[20] Our data for the acetylenic fatty acids are insufficient to distinguish decisively between homologation and dehydrogenation pathways, but the data of Table 6 do suggest that acetate-1-^{14}C is first incorporated into the non-acetylenic acids and that the ^{14}C reaches the acetylenic acids more slowly. Such a picture would be more simply explained by the dehydrogenation hypothesis than by the homologation mechanism.

EXPERIMENTAL PROCEDURE

Germination and Growth

The seeds used had been stored for upwards of 12 months. Since various special treatments failed to stimulate germination, the final procedure was simply to place the seeds, with shells intact, in moist earth at 27° in the dark; a proportion then germinated in 30–40 days and grew for up to 15 weeks thereafter. Because germination was sporadic, experiments were made with single seedlings and material from ^{14}C-incorporation studies was used for analytical examination.

Incorporation of ^{14}C

Sliced fresh tissue (0·3 to 1·4 g) or intact seedling parts were incubated with sodium acetate-1-^{14}C (Radiochemical Centre, Amersham) (13 μc, 0·2 mg) in a flask connected to a CO_2 trap containing $Ba(OH)_2$, and a slow stream of CO_2-free air drawn through. After

[11] E. R. H. Jones, G. H. Whitham, and M. C. Whiting, *J. Chem. Soc.* 3201 (1954).
[12] S. P. Ligthelm, *Chem. & Ind., (London)* 249 (1954).
[13] J. D. Bu'Lock and H. Gregory, *Biochem. J.* **72**, 322 (1959).
[14] J. D. Bu'Lock and H. M. Smalley, *J. Chem. Soc.* 4662 (1962).
[15] I. Fleming and J. Harley-mason, *Proc. Chem. Soc.* 245 (1961).
[16] R. Reiser and N. L. Murty, *Biochem. Biophys. Research Commun.* **5**, 265 (1961).
[17] G. Scheuerbrandt, H. Goldfine, P. E. Baronowsky, and K. Bloch, *J. Biol. Chem.* **236**, PC70 (1961).
[18] D. K. Bloomfield and K. Bloch, *J. Biol. Chem.* **235**, 337 (1961).
[19] C. Yuan and K. Bloch, *J. Biol. Chem.* **236**, 1277 (1961).
[20] R. O. Simmons and F. W. Quackenbush, *J. Am. Oil Chemists' Soc.* **31**, 441 (1954).

6 hr (40 hr in one case) at 22° the plant material was washed and the lipids extracted. Respiratory CO_2 trapped as $BaCO_3$ was removed and counted at intervals throughout the incubation.

Radioactivity measurements were made with a thin-window counter of about 5 per cent efficiency, using infinitely-thick films or, where this was impracticable, samples of comparable self-absorption.

Examination of lipids

Chopped plant tissue was extracted with cold ethanol and then with several lots of hexane, the combined extracts then evaporated and the lipids taken up in hexane. After saponification with a small excess of cold N KOH in methanol, total fatty acids were isolated and estimated by titration. In one experiment with ^{14}C incorporation the mixture of fatty acids (about 1 mg) was oxidized directly with permanganate–periodate reagent.[21] In other experiments the mixture was resolved by partition chromatography on a column of silicone-treated keiselguhr supporting liquid paraffin (B.P. grade), eluting with aqueous methanol of graded concentrations (Table 4);[22] elution was followed by titration and/or spectroscopically. Recovery of polyacetylenic acids was over 90 per cent compared with the spectroscopically-determined concentration before saponification.

To the products from permanganate–periodate oxidations of labelled acids, standard mixtures of carrier acids were added (Tables 5, 8), and the steam-volatile and non-volatile fractions separated. The mixture of steam-volatile acid was resolved on an ion-exchange column by the method of Seki,[23] which proved very satisfactory. From the non-volatile fraction azelaic and palmitic acids were separated by crystallization.

For the Schmidt degradation of acids from oxidations, a procedure based on that of Phares[10] was employed.

[21] E. Rudloff, *Can. J. Chem.* **34**, 1413 (1956).
[22] G. A. Howard and J. P. Martin, *Biochem. J.* **46**, 532 (1950).
[23] T. Seki, *J. Biochem.*, (*Tokyo*) **45**, 856 (1958).