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OCCURRENCE OF POSITIONAL ISOMERS OF DIHYDROXYHEXADECANOIC ACID IN PLANT CUTINS AND SUBERINS

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Abstract—Positional isomers of dihydroxyhexadecanoic acid have been found in cutin and suberin hydrolysates and identified using combined gas chromatography–mass spectrometry of their TMS derivatives. The acid is usually a mixture of the 10,16-, 9,16-, 8,16- and 7,16-isomers which are eluted as a single peak using packed GLC columns. The major isomers are commonly 10,16- and 9,16- with the other isomers occurring in smaller quantities. The implications of the results in the biosynthesis of cutin and suberin are discussed.

INTRODUCTION

10,16-DIHYDROXYHEXADECANOIC acid was first isolated by Matic[1] in 1956 from the mixture of aliphatic acids obtained by alkaline hydrolysis of *Agave americana* cutin. Other workers have subsequently shown that the acid is the most common constituent of Angiosperm cutins and may comprise up to 70 per cent of the total cutin acids of some leaves and fruits.[2,3]

The compound has been identified mainly by chromatographic methods which include reverse-phase paper chromatography,[1,4,5] TLC on silica gel[2,3,6] and more recently GLC using the diacetoxy methyl ester,[7] *bis*-trimethylsilyl (TMS) ether methyl ester[8,11] or *bis*-trifluoroacetyl (TFA) methyl ester[3] derivatives. However, the location of the secondary hydroxyl group at the 10- position has only been confirmed in the acid obtained from *Agave*,[1,7,10] olive[12] and *Vicia faba*[13] leaf and Cox apple fruit[9] cutins. The hydroxyl position is determined from the cleavage products obtained by oxidation[1,7,10] or more conveniently by using combined gas chromatography–mass spectrometry (GLC–MS) of either the TMS ether methyl ester derivative[9,10] or the alkanetriol produced by hydrogenolysis of cutin with lithium aluminium hydride.[13]

This paper describes an examination by GLC–MS of a number of compounds obtained from cutin and suberin hydrolysates which were identified as 10,16-dihydroxyhexadecanoic acid using conventional chromatographic techniques. The mass spectral fragmentations of the TMS ether methyl ester and TMS ether TMS ester derivatives were studied.

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RESULTS

Total cutin and suberin acid mixtures and dihydroxyhexadecanoic acid fractions obtained from them by preparative TLC were studied and characterized, before GLC–MS analysis, using TLC and analytical GLC. A GLC peak which co-eluted with a reference sample of 10,16-dihydroxyhexadecanoic acid was present in all samples when examined as either the TFA methyl ester (on E301 and OV210), the TMS ether methyl ester (on OV1, OV225 and OV210) or the TMS ether TMS ester (on OV1 and OV225) derivatives. Mass spectral scans were carried out on the apices of the GLC peaks which corresponded with methyl 10,16-bis-TMS hexadecanoate and 10,16-bis-TMS hexadecanoic acid TMS ester. However, all the mass spectra obtained, including that from the reference compound, showed that the 10,16-isomer was associated with varying amounts of positional isomers having the secondary hydroxyl group in the 7–9 positions. Although these isomers have not been previously reported by workers using mass spectrometry they are unlikely to be artefacts produced in the mass spectrometer or concentration effects resulting from scanning poorly resolved GLC peaks. Using identical techniques and the same derivatives we have found similar isomerism in some, but not all, classes of hydroxy acid found in cutins and suberins. These results will be published later. Two positional isomers of dihydroxyoctadecanoic acid have been reported in *Agave* cutin[7] using oxidative degradation.

The mass spectra of the dihydroxyhexadecanoic acid obtained from *Avena sativa* and *Coffea arabica* cutins are shown in Fig. 1. The mass spectrum of methyl-bis-TMS hexadecanoate (Fig. 1A and B) is typical of aliphatic hydroxy acids[10] showing a base peak at *m/e* 73 and peaks corresponding with M-15, M-31 and M-47. The major fragment ions (Table 1) represents cleavage α to the secondary TMS ether group giving a TMS ether methyl ester fragment (I) and a diTMS ether fragment (II). The TMS ether methyl ester

TABLE 1. PRINCIPLE FRAGMENT IONS OBTAINED FROM TRIMETHYLSILYL DERIVATIVES OF DIHYDROXYHEXADECANOIC ACID

Isomer	Ion I x	Ion I *m/e*	Ion II y	Ion II *m/e*	Ion III z	Ion III *me*
10,16-	8	273	6	275	8	331
9,16-	7	259	7	289	7	317
8,16-	6	245	8	303	6	303
7,16-	5	231	9	317	5	289

I $CH(OSi(CH_3)_3)-(CH_2)_x-CO_2CH_3$ II $CH(OSi(CH_3)_3)-(CH_2)_y-O-Si(CH_3)_3$ III $CH(OSi(CH_3)_3)-(CH_2)_z-CO_2-Si(CH_3)_3$

fragments show the more intense peaks in the mass spectrum and give smaller peaks corresponding with (I)-29 and (I)-104. However, TMS ether methyl ester fragments are not unambiguous as their *m/e* also agrees with methoxyl TMS ether and monounsaturated diTMS ether fragments. They can be confirmed by comparison with the corresponding TMS ether TMS ester derivative. The mass spectrum of bis-TMS hexadecanoic acid TMS ester (Fig. 1C) is similar to the methyl ester but shows strong M-15, M-90-15 and M-90-31 peaks and prominent peaks at 204 and 217. Mass spectral cleavage is also similar but gives a TMS ester TMS ether fragment (III) and the same diTMS ether fragment (II).

The TMS derivatives of the positional isomers of dihydroxyhexadecanoic acid give well defined fragments, e.g. the 10,16-isomer *m/e* 273,275 as the TMS ether methyl ester

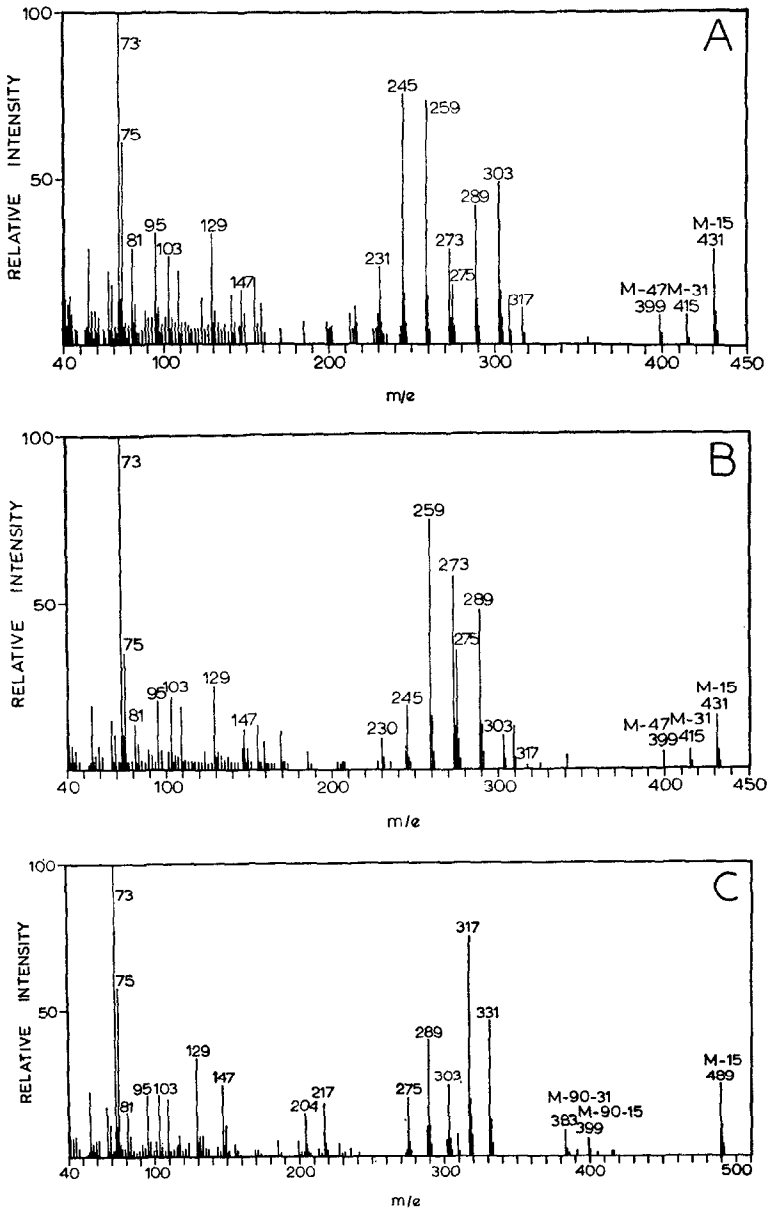

FIG. 1. MASS SPECTRA OF DIHYDROXYHEXADECANOIC ACIDS OBTAINED FROM PLANT CUTINS. A-*Avena sativa* (TMS ETHER METHYL ESTER), B-*Coffea arabica* (TMS ETHER METHYL ESTER), C-*Coffea arabica* (TMS ETHER TMS ESTER).

and 331,275 as the TMS ether TMS ester; the fragments are summarized in Table 1. The isomer contents of the dihydroxyhexadecanoic acids studied were calculated only approximately using the most intense fragment ions of the individual isomers assuming that the sensitivity was the same for each isomer. Good agreement was obtained between measurements of either TMS ether methyl ester, TMS ether TMS ester or diTMS ether fragments.

The results obtained are shown in Table 2 together with the relative peak area of dihydroxyhexadecanoic acid evaluated from the total acid GLC chromatogram.

Positional isomers were present in all the samples of dihydroxyhexadecanoic acid studied in the present investigation. The occurence of the acid in plant suberins and in the cutins of *Betula pendula*, *Coffea arabica*, *Avena sativa* and *Zea mays* has not been previously reported. 10,16-Dihydroxyhexadecanoic acid was the major isomer of several cutins, e.g. *Malus* sp.,

TABLE 2. POSITIONAL ISOMERS OF DIHYDROXYHEXADECANOIC ACID FOUND IN SOME CUTINS AND SUBERINS

Species	Approximate isomer content (%)				Relative peak area (%) for dihydroxyhexadecanoic acid from total acid chromatogram
	7,16	8,16	9,16	10,16	
CUTIN					
Agave americana lutea leaf	3	30	20	47	10·5
**Sansevieria trifasciata* subsp. *laurentii* leaf	4	34	51	11	12·4
Betula pendula leaf	0	5	82	13	20·5
Coffea arabica leaf	3	11	56	30	61·0
**Avena sativa* cv. Blenda leaf	12	38	36	14	12·2
**Zea Mays* leaf	9	13	71	7	17·9
Malus Golden Hornet leaf	2	8	24	66	39·5
Citrus aurantifolia leaf	2	5	30	63	69·3
Euonymus europaeus leaf	4	5	69	22	24·2
Malus Zumi fruit	2	8	21	69	20·5
Rosa canina fruit	1	3	20	76	65·3
Lycopersicon esculentum fruit	2	9	12	77	71·1
SUBERIN					
**Betula pendula* cork	6	14	38	42	3·5
**Ribes Grossularia* cv. Malvern Cross cork	2	7	14	77	3·4

* Fractions from PLC.

Rosa canina, *Lycopersicon esculentum*, and the 9,16-isomer of others, e.g. *B. pendula*, *Z. mays*, *Euonymus europaeus*. In some cutins the predominant isomers were either 10,16- and 9,16-, e.g. *C. arabica* or 9,16- and 8,16-, e.g. *Sansevieria trifasciata*, *A. sativa*. 7,16-Dihydroxyhexadecanoic acid was found in much smaller amounts rarely exceeding 10 per cent of the total isomers.

DISCUSSION

The demonstration of positional isomers of dihydroxyhexadecanoic acid has an important bearing on the possible biosynthetic pathways involved in cutin synthesis. Earlier workers have proposed that the various cutin monomers are formed mainly from unsaturated fatty acid precursors via ω-hydroxyalkyl hydroperoxide intermediates.[7,14] Cutin acids such as 18-hydroxyoctadec-9-enoic, octadec-9-ene-1,18-dioic, 9,10,18-trihydroxyoctadecanoic and 9,10-dihydroxyoctadecane-1,18-dioic can clearly be derived from octadec-9-enoic acid by further oxidation or hydroxylation. An analogous series of C_{16} compounds derived from hexadec-9-enoic acid does not occur in cutin although 9,10,16-trihydroxy-

hexadecanoic acid has been reported in minor amounts in tomato[8] and *Agave*[7] cutins. However, direct experimental evidence for the involvement of saturated fatty acids in cutin synthesis has recently been obtained by Kolattukudy.[13] Hexadecanoic acid-1-^{14}C was readily incorporated into the 10,16-dihydroxyhexadecanoic acid component of *Vicia faba* leaf cutin and other evidence suggested the pathway: hexadecanoic → 16-hydroxyhexadecanoic → 10,16-dihydroxyhexadecanoic → cutin. Hexadec-9-enoic acid-10-^{14}C was not incorporated into dihydroxyhexadecanoic acid which suggests a direct secondary hydroxylation system, i.e. substitution of a H atom by an OH group. Both terminal[15] and secondary hydroxylation[16] enzyme systems are known in plants. The pathway is also supported by the very common occurrence in many plant cutins of hexadecanoic and 16-hydroxyhexadecanoic acids and the absence of other C_{16} acids.[3]

The occurrence of positional isomers of dihydroxyhexadecanoic acid in cutin and suberin hydrolysates lends support for a direct hydroxylation synthesis from 16-hydroxyhexadecanoic acid. The hydroxylation system, however, does not appear to be specific, unlike that found in *Ricinus communis* where hydroxylation occurs only at the 12- position of oleic acid.[16] In some of the plants studied hydroxylation occurred predominantly in the 9 or 10 positions and in others either in both 8 and 9, or in 9 and 10 positions. Hydroxylation in the 7 position did not occur to any great extent.

EXPERIMENTAL

Preparation of cutin and suberin acids. Acids were obtained by hydrolysis with 3% KOH[5] in EtOH of isolated cuticular membranes and powdered corks prepared using methods described previously.[3,17]

Isolations of dihydroxyhexadecanoic acid from *Sansevieria trifasciata*, *Avena sative*, *Zea mays* cutins and *Betula pendula*, *Ribes Grossularia* suberins were made using preparative-TLC of the total methyl esters on 0·75 mm layers of Silicagel Woelm TLC using the solvent system $CHCl_3$/EtOAc (6/4). The compound was recovered from the adsorbent by elution by Et_2O.

Methyl esters were prepared using excess CH_2N_2, TFA methyl esters using $(CF_3CO)_2O$[3] and TMS ether methyl esters and TMS ether TMS esters from N,O-*bis*-(trimethylsilyl) acetamide using the method of Eglinton *et al.*[10] modified by the addition of 10% anhydrous pyridine to the reaction mixture.

Chromatographic analysis. Analytical TLC of the total acid mixtures was carried out on layers of silica gel using published methods.[2,3,18] Relative GLC retention data for dihydroxyhexadecanoic acid derivatives was obtained using Hewlett–Packard 5751G and Varian 1840 gas chromatographs fitted with fid. The chromatographic columns were stainless steel 1·5 m × 2 mm, packed with 3% loadings of E301, OV1, OV210 and OV225 on Chromosorb W AW DMCS. Analyses were carried out using temperature programming from 130° to 250° at 6°/min using a flow rate of 30 ml/min N_2. *n*-Tetracosane was used as the internal standard for E301 and OV1 analyses and *n*-octacosane for OV210 and OV225 analyses. Relative peak areas were determined using the triangulation method.

GLS–MS. Mass spectra were obtained on an LKB 9000 combined gas chromatograph–mass spectrometer using the same chromatographic conditions as those used for analytical GLC. The same 3% OV1 and OV225 GLC columns were also used with a flow rate of 30 ml/min of He. The instrument was operated at an ionizing potential of 70 eV with the separator and ion source maintained at 270°. Spectra were recorded in 5 sec (*m/e* 1–600) on the apices of the dihydroxyhexadecanoic acid peaks as determined from the continuous record produced from the total ionization monitor at 20 eV. Peaks in the mass spectra with a relative intensity greater than 1% were recorded on the bar charts.

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