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THE CONSTITUENT CUTIN ACIDS OF CRANBURY CUTICLE

RODNEY CROTEAU* and IRVING S FAGERSON

Department of Food Science and Technology,
University of Massachusetts, Amherst, Massachusetts 01002, U S A

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Abstract—Purified cutin from cranberry (*Vaccinium macrocarpon*, var Howes) skin was selectively degraded, and the cutin acids, as methyl esters, separated by TLC into seven classes including monobasic acids, dibasic acids, monohydroxy monobasic acids, monohydroxy epoxymonobasic acids, *vic*-dihydroxy dibasic acids, dihydroxy monobasic acids and trihydroxy monobasic acids Of the 41 components identified in cranberry cutin by GLC and MS analysis, 18-hydroxyoctadec-*cis*-9-enoic acid (9 4%), 18-hydroxy-*cis*-9,10-epoxyoctadecanoic acid (7 5%), 10,16-dihydroxyhexadecanoic acid (16 7%) and *threo*-9,10,18-trihydroxy-octadecanoic acid (43 7%) were shown to be the major constituents

INTRODUCTION

PLANT cuticle consists of two parts, the surface wax and the underlying cutin polymer [1] Cutin is, primarily, a crosslinked polyester of C_{13} to C_{18} hydroxylated fatty acids[2,3] although a small proportion of alkyl peroxide and ether linkages have been noted in the polymer.[4] The role of the cuticle in the protection of the plant has been reviewed by Martin [5]

A number of earlier investigators[3,4,6,7] have studied the cutin acid composition of several plant cuticles with moderate success, but not until the publication of Eglinton and Hunneman[8] was a truly comprehensive analysis of cutin carried out These investigators utilized combined gas chromatography-mass spectrometry of cutin acid methyl ester TMS ethers for the unequivocal identification of these materials The position of double bonds in unsaturated cutin acids, however, cannot be determined directly from their mass spectra [9] These investigators,[8] therefore, employed stereospecific hydroxylation of cutin acid double bonds with osmium tetroxide, followed by silylation of the resulting *vicinal* diol and identification through mass spectrometry to locate the original unsaturation.

The cuticular waxes of cranberry cuticle have been studied previously in this laboratory.[10] This investigation is directed toward determining the cutin composition of this material, using the techniques developed by Eglinton and Hunneman [8]

* Present address Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

[1] J VAN OVERBEEK, *Ann Rev. Plant Physiol* **7**, 355 (1956)
[2] E A BAKER and J T MARTIN, *Nature, Lond* **199**, 1268 (1963)
[3] E A BAKER, R F BATT and J. T MARTIN, *Ann Appl Biol* **53**, 59 (1964)
[4] C E CRISP, *The Biopolymer Cutin*, Ph D Thesis, Univ of California, Davis (1965)
[5] J T MARTIN, *Ann Rev Phytopathology* **2**, 81 (1964)
[6] G D MEAKINS and R SWINDELLS, *J Chem Soc* 1044 (1959)
[7] E A BAKER and J T MARTIN, *Ann Appl Biol* **60**, 313 (1967)
[8] G EGLINTON and D H HUNNEMAN, *Phytochem* **7**, 313 (1968)
[9] R RYHAGE and E STENHAGEN, *Arkiv Kemi* **13**, 523 (1959)
[10] R CROTEAU and I S FAGERSON, *Phytochem* **10**, 3247 (1971)

RESULTS

Cutin membranes were prepared from wax-free cranberry skins by successive treatment with dilute acid, oxalic acid, and $ZnCl_2$–HCl [3,11] The IR spectrum of a purified cutin membrane showed a moderate band at 3450 cm^{-1} (ν (OH), intra H-bonding), a strong band at 1740 cm^{-1} (ν (C=O), ester), a weak band at 1725 cm^{-1} (ν (C=O), acid) and two weak bands at 1100 cm^{-1} and 1020 cm^{-1} (ν (CR_2O) and ν s(CH_2O) respectively) The cutin was hydrolyzed with methanolic KOH and peroxide and ether linkages reduced with NaI and HI respectively [4] The resulting cutin acids were methylated with diazomethane and the methyl esters separated by preparative TLC on silica gel [8] As indicated in Table 1, seven

TABLE 1 R_f VALUES FOR CRANBERRY CUTIN ACID METHYL ESTERS

	R_f*
Monobasic acids	0 95
Dibasic acids	0 85
Monohydroxy monobasic acids	0 60
Monohydroxy epoxymonobasic acids	0 52
vic-Dihydroxy dibasic acids	0 35
Dihydroxy monobasic acids	0 30
Trihydroxy monobasic acids	0 15

(Silica gel G, diethyl ether hexane methanol, 40 10 1)

fractions were obtained and characterized as methyl esters of (a) monobasic acids, (b) dibasic acids, (c) monohydroxy monobasic acids, (d) monohydroxy epoxymonobasic acids, (e) *vic*-dihydroxy dibasic acids, (f) dihydroxy monobasic acids, and (g) trihydroxy monobasic acids

Monobasic Acids

This fraction consisted of the methyl esters indicated in Table 2. Components were identified by comparison of GLC retentions and mass spectra to reference standards. The unsaturated esters, methyl oleate and methyl linoleate were not well resolved on SE-30, but did show GLC retention coincidence with authentic standards The peak containing the unsaturated C_{18} acids was scanned (GLC–MS) on the upside and downside of the GLC peak and the presence of both methyl oleate and linoleate confirmed Similar analysis confirmed the identity of methyl palmitate and methyl palmitoleate The unidentified component in this fraction had an apparent molecular weight of 280 and was not a fatty acid methyl ester

Dibasic Acids

GLC showed this fraction to contain six components (Table 2). Dimethyl hexadecane-1,16-dioate and dimethyl octadecane-1,18-dioate were identified by comparison of GLC retentions and mass spectra with authentic standards. Dimethyl hexadec-9-ene-1,16-dioate was identified on the following bases. (a) all the components in this fraction on TLC have the same R_f value as long chain diesters, (b) for analogous compounds on SE-30 the order of elution is: di-unsaturated, mono-unsaturated, saturated; (c) the mass spectrum of this

[11] P J HOLLOWAY and E A BAKER, *Plant Physiol* **43**, 1878 (1968)

TABLE 2 CONSTITUENT CUTIN ACIDS OF CRANBERRY CUTIN

Compound*		% of total cutin acids†	% of the fraction
Monobasic acids			
Tetradecanoic		0 11	2 0
Pentadecanoic		0 12	2 3
Unidentified		0 18	3 4
Hexadec-*cis*-9-enoic		0 38	7 2
Hexadecanoic		0 23	4 5
Heptadecanoic		0 07	1 4
Octadec-*cis*-9-enoic / Octadeca-*cis*-9,12-dienoic		0 19	3 6
Octadecanoic		0 15	2 8
Nonadecanoic		0 07	1 3
Eicosanoic		0 25	4 8
Heneicosanoic		0 08	1 6
Docosanoic		0 19	3 6
Tricosanoic		0 17	3 2
Tetracosanoic		0 46	8 8
Pentacosanoic		0 29	5 5
Hexacosanoic		0 52	9 9
Heptacosanoic		0 12	2 3
Octacosanoic		0 60	11 4
Nonacosanoic		0 14	2 6
Triacontanoic		0 78	14 8
	Total	5 25	97 0
Dibasic acids			
Hexadec-9-ene-1,16-dioic		0 05	6 4
Hexadecane-1,16-dioic		0 24	29 4
Octadeca-9,12-diene-1,18-dioic		0 02	2 9
Octadec-cis-9-ene-1,18-dioic		0 34	42 3
Octadecane-1,18-dioic		0 10	12 1
Eicosane-1,20-dioic		0 06	7 5
	Total	0 80	100 6
Monohydroxy monobasic acids			
16-Hydroxyhexadec-*cis*-9-enoic		0 31	2 0
16-Hydroxyhexadecanoic		1 95	11 8
18-Hydroxyoctadeca-9,12-dienoic		0 81	4 9
18-Hydroxyoctadec-*cis*-9-enoic		9 36	56 7
18-Hydroxyoctadecanoic		1 93	12 3
20-Hydroxyeicosanoic		0 25	1 5
Unidentified		1 89	11 6
	Total	16 75	100 8
Monohydroxy epoxymonobasic acids			
Unidentified		1 03	9 0
18-Hydroxy-*cis*-9,10-epoxyoctadec-12-enoic		2 82	24 8
18-Hydroxy-*cis*-9,10-epoxyoctadecanoic		7 47	65 8
	Total	11 32	99 6

TABLE 2 —*Continued*

Compound*	% of total cutin acids†	% of the fraction
vic-Dihydroxy dibasic acids		
Unidentified	0 04	12 1
8,9-Dihydroxyheptadecane-1,17-dioic	0 19	50 1
Threo-9,10-dihydroxyoctadecane-1,18-dioic	0 14	37 8
Total	0 37	100 0
Dihydroxy monobasic acids		
10,16-Dihydroxyhexadecanoic	16 71	78 1
10,18-Dihydroxyoctadec-12-enoic	1 11	5 2
10,18-Dihydroxyoctadecanoic	1 41	6 6
Unidentified	0 17	0 8
Unidentified	2 02	9 4
Total	21 42	100 1
Trihydroxy monobasic acids		
9,10,18-Trihydroxyoctadecanoic	0 58	1 3
Threo-9,10,18-trihydroxyoctadecanoic	43 67	98 7
Total	44 25	100 0

* Compounds in each group are given in order of GLC elution as methyl esters and TMS ethers

† Based on total ether soluble material

compound (Fig 1) is similar to, but not identical with, dimethylhexadecane-1,16-dioate; and (d) micro-osmylation of this compound[8] followed by silylation gave MS with very large fragments at *m/e* 259 ($CH_3O_2C(CH_2)_7CHOTMS$) and *m/e* 231 ($CH_3O_2C(CH_2)_5CH$-OTMS), indicating that the original double bond was at the C-9 position

Dimethyl octadec-9-ene-1,18-dioate was identified on the basis of similar considerations The MS of this component was identical to a published spectra of the authentic compound [8] This component was treated with OsO_4 and silylated On MS analysis, this compound gave a spectrum identical to that of dimethyl *threo*-9,10-dihydroxyoctadecane-1,18-dioate, bisTMS ether[12] and confirmed the position of the original double bond at the C-9 position. The MS of the osmylated compound was identical with the MS of the *threo*-isomer, but the GLC retention index (2732) was less [12] As the *erythro*- precedes the *threo*-isomer under

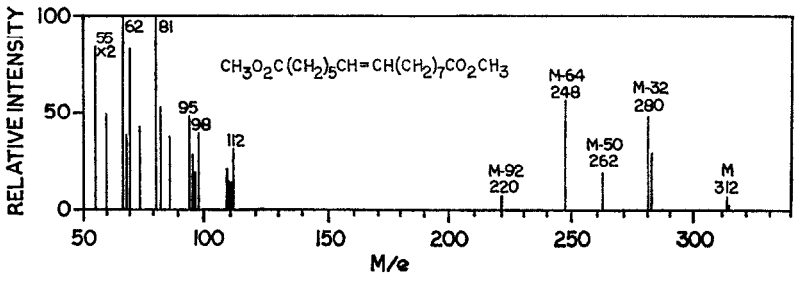

FIG. 1.

[12] G EGLINTON, D H HUNNEMAN and A MCCORMICK, *Org Mass Spectr* **1**, 593 (1968)

these conditions, the *erythro*-assignment was made, indicating that the original double bond at the C-9 position was *cis*.

Dimethyl octadeca-9,12-diene-1,18-dioate was identified on the basis of its chromatographic behavior and by comparison of its MS to that of the authentic published standard.[8] Too little material was available for osmylation, but the available evidence seemed to justify this identification.

Dimethyl eicosane-1,20-dioate was identified on the basis of chromatographic behavior, and by comparison of the MS to that of a published standard.[13]

Monohydroxy Monobasic Acids

GLC analysis (as the TMS derivatives) showed this fraction to contain seven components, six of which were identified (Table 2). Methyl 16-hydroxy-hexadecanoate, TMS ether and methyl 18-hydroxyoctadecanoate, TMS ether were identified by comparison of their GLC retentions and mass spectra to authentic standards and to published data.[8,12] Methyl 20-hydroxyeicosanoate, TMS ether was identified on the basis of chromatographic behavior and by its MS which showed fragmentation similar to the comparable C_{16} and C_{18} compounds but with ions corresponding to the C_{20} homologue

Methyl 18-hydroxyoctadec-9-enoate, TMS ether and methyl 18-hydroxyoctadeca-9,12-dienoate, TMS ether showed GLC behavior which indicated that they were the mono- and di-unsaturated analogues of the saturated C_{18} compound. The MS of these two compounds were identical to published standards.[12] The di-unsaturated compound occurred at a level too low to permit osmylation studies The monounsaturated compound was osmylated and silylated and gave a MS identical to methyl *threo*-9,10,18-trihydroxyoctadecanoate, trisTMS ether,[12] further confirming the placement of the double bond and the identification. The retention index of the osmylated-silylated compound was less than that of the authentic *threo*-isomer (2754 vs 2794) and so was assigned the *erythro* configuration, indicating that the original double bond was *cis*.

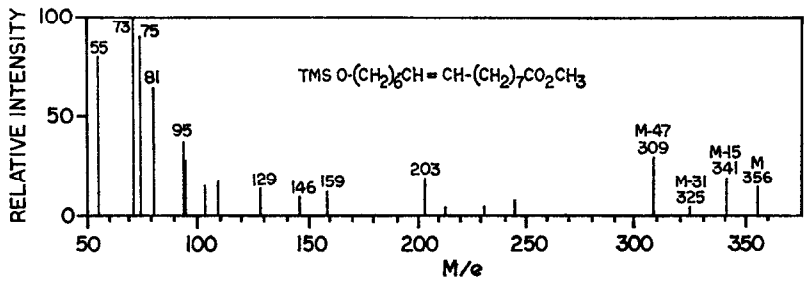

FIG 2

Methyl 16-hydroxyhexadec-*cis*-9-enoate, TMS ether was identified on the basis of its mass spectrum (Fig 2) which was similar to that of the comparable monounsaturated C_{18} compound.[12] This component was osmylated and silylated. The mass spectra and GLC retention of this derivative were identical with those of an authentic sample of methyl-*erythro*-9,10,16-trihydroxyhexadecanoate, TrisTMS ether, thus confirming the identity of the original compound

[13] G EGLINTON, D H HUNNEMAN and K DOURAGHI-ZADEH, *Tetrahedron* **24**, 5929 (1968)

Monohydroxy Epoxy Monobasic Acids

TLC behavior of this fraction indicated components with polarity intermediate between monohydroxy monobasic acid methyl esters and *vic*-dihydroxy dibasic acid dimethyl esters. IR analysis of this fraction showed (in addition to the anticipated ester, hydrocarbon and hydroxyl associated bands) a very weak band at 3000 cm^{-1}, an additional weak band at about 1255 cm^{-1}, weak bands at 920 and 760 cm^{-1} and a medium intensity band at 842 cm^{-1} All of these bands can be attributed to epoxy compounds [14] The 3000 cm^{-1} band is due to C—H stretch of a methine group in a strained ring The 1250 cm^{-1} band is attributed to symmetrical epoxy ring breathing (the so-called 8 μ band) A C—H bending vibration is responsible for the weak band at 760 cm^{-1} (the 12 μ band) The band at 842 cm^{-1} is characteristic of the asymmetric ring bend of a *cis*-epoxide within a long chain solid (the 11 μ band)

GLC of this fraction as the silyl derivatives showed three components of which two were identified (Table 2) Methyl 18-hydroxy-*cis*-9,10-epoxyoctadecanoate, TMS either was identified by comparison of its mass spectrum with that of a published standard [12]

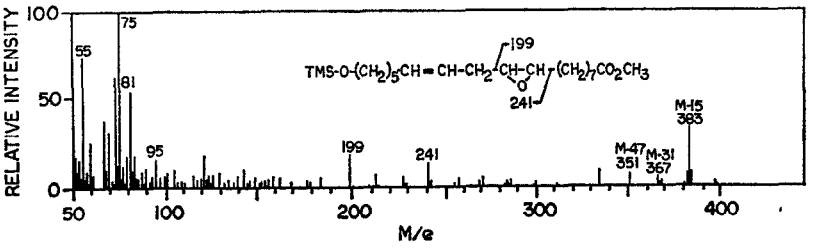

FIG 3

Methyl 18-hydroxy-*cis*-9,10-epoxyoctadec-12-enoate, TMS ether was identified on the following bases· (a) all the components in this fraction on TLC have the same R_f value of an epoxy compound of established identity, (b) the presence of *cis*-epoxide was demonstrated by IR analysis, (c) monounsaturated, compounds precede their saturated analogues on SE-30; (d) the mass spectrum of this compound (Fig 3) was similar to that of the saturated compound, with fragments indicating a component with molecular weight of two less mass units, (e) the mass spectrum showed fragmentations that could be rationalized on the basis of known mechanisms of epoxy long-chain compounds[15] and further indicated, by a large ion at *m/e* 241 (TMSO-C_8H_{14}-CH—CH) that the unsaturation was between the epoxide

and terminal end of the chain, and (f) on osmylation, silylation and mass spectral analysis, this compound showed large ions at *m/e* 261 (TMSO-$(CH_2)_5$CH-OTMS) and *m/e* 315 (TMSO-CH-CH_2-CH—CH-$(CH_2)_7$-CO_2CH_3), consistent with the proposed structure and

confirming the location of the double bond in the C-12 position.

The smallest component of this fraction, eluting first on SE-30, was not identified This compound had an apparent molecular weight of 392 as it showed the appropriate M-15,

[14] O D SHREVE, M R HEETHER, H B KNOGHT and D SWERN, *Analyt Chem* **23**, 277 (1951)
[15] R RYHAGE and E STENHAGEN, *Arkiv Kemi* **15**, 545 (1960)

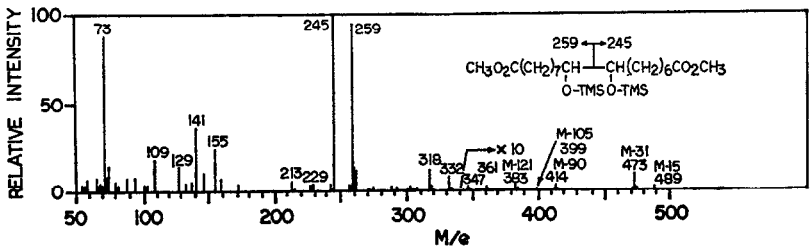

FIG 4

M-32 and M-47 ions (in a ratio associated with TMS ether-methyl esters containing added functionality) [12] The spectrum did not show ions that were readily associated with internal epoxides One could speculate that this component was the TMS ether-methylester of a hydroxy-epoxy tetraunsaturated monobasic C_{18} acid. The apparent molecular weight and GLC behavior are consistent with this identification as is the GLC disappearance of this peak on osmylation and silylation (a nona-TMS ether derivative would not elute under the conditions of the analysis)

vic-Dihydroxy Dibasic Acids

This fraction was silylated and on GLC analysis revealed three components, two of which were identified (Table 2). Dimethyl-*threo*-9,10-dihydroxyoctadecane-1,18-dioate, bisTMS ether was identified by comparison of retention index (2762) and mass spectra with data from the literature [12] Dimethyl 8,9-dihydroxyheptadecane-1,17-dioate, bisTMS ether was identified on the basis of its chromatographic behavior and by examination of its mass spectrum (Fig. 4), which was similar to that of the C_{18} compound[12] and which showed two large fragments at *m/e* 245 and *m/e* 259 due to cleavage between the two carbon atoms (C-8 and C-9) bearing TMS groups.

One component of this fraction was not identified and mass spectral analysis gave indication that this component was not a TMS ether-methyl ester.[12]

Dihydroxy Monobasic Acids

GLC of this fraction as the silyl derivatives revealed five components (Table 2) The major component, methyl 10,16-dihydroxyhexadecanoate, bisTMS ether, was identified by comparison of GLC retention and mass spectrum with those of the authentic compound and with the literature [12]

Methyl 10,18-dihydroxyoctadecanoate, bisTMS ether was identified on the basis of

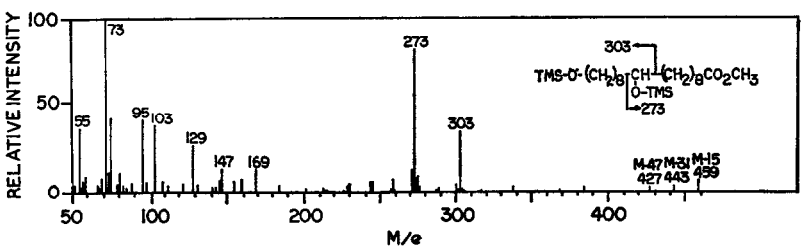

FIG. 5.

chromatographic behavior and its mass spectrum (Fig. 5) which showed a fragmentation mode comparable to the C_{16} compound.[12]

Methyl 10,18-dihydroxyoctadec-12-enoate,bisTMS ether showed GLC behavior consistent with its unsaturated nature and produced a mass spectrum similar to that of the saturated C_{18} compound but indicating a molecular weight of two less mass units The ion at *m/e* 303 (TMSO-C_9H_{17}-OTMS) in the spectrum of the saturated derivative was replaced with *m/e* 301 (TMSO-C_9H_{15}-OTMS) in the spectrum of the unsaturated component, indicating that the unsaturation was between the C-10 TMS and the terminal TMS groups Osmylation of this component followed by silylation and mass spectroscopy showed a large ion at *m/e* 261 (TMSO-$(CH_2)_5$-CH-OTMS) indicating that the original double bond was in the C-12 position

Two components in this fraction remained unidentified The largest component of the two, eluting last on SE-30, showed a mass spectrum with very low intensity ions at *m/e* 593 (M-15?) and *m/e* 577 (M-31?) and two larger ions at *m/e* 303 and *m/e* 259. This component could represent a C_{18} methoxy-dihydroxymethyl ester,bisTMS ether (e g methyl 10-methoxy-9,18-dihydroxyoctadecanoate,bisTMS ether) either naturally occurring in cutin or possibly produced during methylation with CH_2N_2

Trihydroxy Monobasic Acids

GLC of this fraction as the TMS derivatives gave results shown in Table 2 Methyl *threo*- 9,10,18-trihydroxyoctadecanoate, TrisTMS ether was identified by comparison of retention index and mass spectrum with those in the literature [12]

The GLC retention of the smaller component indicated that it was probably the unsaturated trihydroxy C_{18} analogue The mass spectrum of this compound was identical to a published spectrum of methyl 9,10,18-trihydroxyoctadecanoate,trisTMS ether.[8] The rearrangement ion at *m/e* 332 confirmed the 9,10-diTMS combination,[12] while the shift from *m/e* 303 in the spectra of the saturated component to *m/e* 301 for the unsaturated component again indicated a double bond between C-10 and the end of the chain As this component occurred in such a small proportion, attempts to locate the double bond by osmylation failed The double bond is probably in the C-12 position as it was in the comparable eighteen carbon epoxy and dihydroxy acids of other fractions

DISCUSSION

Monobasic acids in the range C_{14} to C_{24} have previously been reported in cutin hydrolysates [3,8,16,17] While these acids may be cuticle wax components that have not been fully extracted, there is good evidence that they are actual cutin constituents bound by ester linkages to the hydroxy acid network Electron microscope studies have indicated that some of the cuticular wax components are chemically bound to cutin[18] and the cutin isolation procedure employed would, almost certainly, remove any traces of unbound wax material. The cranberry cuticle wax fatty acids[10] show a much different distribution than the fatty acids derived from cutin. Furthermore, unsaturated acids are found only in the cutin of cranberry, not in the wax

Dibasic acids have been previously reported in the cutin of apple,[8,19] and tomato[17] in

[16] H A M A DE VRIES, *Acta Bot Neerl* **18,** 589 (1969)
[17] J SHISHIYAMA, F ARAKI and S AKAI, *Plant Cell Physiol* **11,** 323 (1970)
[18] P A ROELOFSEN and A L HOUWINK, *Protoplasma* **40,** 1 (1951)
[19] C H BRIESKORN and A J BOSS, *Fette Seifen Anstrichm* **66,** 925 (1964)

which they also constitute a small proportion. The presence of the unsaturated C_{16} compound and saturated C_{20} compound has not been previously reported in cutin, although the C_{20} compound is a common constituent of cork suberin.[20,21]

The ω-hydroxy monobasic acids are common components of plant cutin and chain lengths from C_{10} to C_{18} have been noted.[4,17] 18-Hydroxyoctadecanoic acid has been identified in the cutin of a wide variety of plants[2,7] although the unsaturated C_{18} compounds appear to be less common [8,20] This is the first report of 16-hydroxyhexadec-9-enoic acid. C_{20} hydroxylated acids have been reported in tomato cutin.[17] To our knowledge this is the first report in which 20-hydroxyeicosanoic acid has been specifically identified.

ω-Hydroxy-epoxymonobasic acids have been noted previously Crisp[4] tentatively identified 16-hydroxy-9,10-epoxy hexadecanoic acid in *Agave* cutin An 18-hydroxy-epoxyoctadecanoic acid was reported in apple[19] and tomato[17] cutin Nothing comparable to the unsaturated epoxy acid reported here has been noted previously.

The two vicinal dihydroxydibasic acids identified here are also present in apple cutin.[8]

The saturated dihydroxymonobasic acids are very common cutin constituents and are often found in relatively high concentration.[2,4,7] 10,18-Dihydroxyoctadec-12-enoic acid has not been previously reported.

The trihydroxy monobasic acid 9,10,18-trihydroxyoctadecanoic is of widespread occurrence, having been found in sizable proportion in many cutins examined thus far. The *threo* form is found in *Agave*[20] and cranberry while apple contains both *threo* and *erythro* forms.[8] The unsaturated C_{18} trihydroxy compound has also been reported in apple cutin [8] In neither study was the double bond located, although in cranberry the double bond in related acids was shown to be in the C-12 position. Mikolajczak and Smith[22] have identified *threo*-9,10,18-trihydroxyoctadec-cis-12-enoic acid in *Chamaepeuce* seed oil.

No indication was seen of participation of cuticular wax components, other than the fatty acids, in the cutin complex.

Previous IR studies of intact cutin membranes[8,20,23] have indicated the presence of free hydroxyl groups. The IR spectra of cranberry cutin showed free hydroxyl and possibly free acid groups. On a functional group basis there are 2·3 hydroxyls for each carboxyl group present in cranberry cutin. Thus, even if all carboxyls are bound in ester linkages, the majority of hydroxyls are unbonded (a small proportion of hydroxyls are bound in peroxide and ether linkages)

It appears that the common C_{16} and C_{18} plant fatty acids are the precursors of the cutin acids. The placement of hydroxyls and double bonds in the cutin acids favors this explanation, as does the presence of requisite unsaturated acids in cutin but not in cuticle wax.[10] ω-Hydroxylation or carboxylation of precursors seems to occur at an early stage in cutin synthesis. ω-Hydroxy and ω-carboxy acids are sometimes found in both cuticle wax[24,25] and cutin, but ω-hydroxy and carboxy acids with secondary hydroxyl groups are, apparently, found only in the cutin This would seem to indicate that hydroxylation within fatty acid chains is a mechanism specific to cutin synthesis.

The mechanisms of internal hydroxylation and polymerization are unknown, although

[20] M Matic, *Biochem J* **63,** 168 (1956)
[21] A Gonzalez-Gonzalez, *Acta Cient Compost* **5,** 57 (1968)
[22] K. L Mikolajczak and C R Smith, *Lipids* **2,** 261 (1967)
[23] F E Huelin, *Austral J Biol Sci* **12,** 175 (1969)
[24] P Mazliak, *Phytochem* **2,** 253 (1963)
[25] J A Lamberton, *Austral J Chem* **14,** 323 (1961)

Heinen and Brand[26] and Crisp[4] have postulated that autoxidation or lipoxidation are involved While it is possible, hypothetically, to construct pathways by which these processes might generate a number of individual cutin acids, it is difficult to conceive how these mechanisms can account for the very specific positional hydroxylations generally observed

It seems almost certain that the final conversion of cutin acid units to the polymerized layer of cutin occurs extracellularly in the cuticle itself In view of the great proportion of ester linkages in cutin, it would appear that the final linkage process is largely esterification, an event not likely to be mediated by oxidative processes The indication that small quantities of protein may exist within the cutin[23] suggests the presence of an extracellular enzyme that could be responsible for final cutin esterification *

EXPERIMENTAL

Preparation of cutin [3,11] The skins from 1 kg (representing 6040 cm^2) of commercially ripe cranberries as previously described[10] were extracted with hot $CHCl_3$ to remove cuticle wax and digested with 1% H_2SO_4 to remove flesh, then washed with water After refluxing with 0 4% oxalic acid–1 6% ammonium oxalate to remove pectin, the crude cutin membranes were dried and refluxed 3 hr with excess 50% ZnCl in concentrated HCl to remove cellulose This last procedure was then repeated with fresh reagent The membranes were then washed, dried and exhaustively extracted with MeOH to yield cutin (4 6 g, 765 g/cm^2)

Solvolysis of cutin 300 mg of cutin were saponified by refluxing with 70 ml of 3% methanolic KOH in MeOH for 12 hr The residue (68 5 mg) was washed with MeOH and saponified again for 10 hr with fresh reagent The washed residue (43 5 mg) was then refluxed for 5 min with 50 ml of 4% NaI in 4% v/v HOAc–*iso*PrOH to reduce peroxide linkages[4] (12 μeg/g) This residue (20 7 mg) was saponified a third time for 6 hr as previously described and the residue (13 5 mg) refluxed for 10 min with 1% HI in 4% v/v HOAc–iso-PrOH to reduce ether linkages[4] (final residue, 8 4 mg)

The extracts and washings were combined, the volume reduced under vacuum, the pH adjusted to 9, and nonsaponifiables extracted with light petroleum No nonsaponifiable material could be detected The extract was then acidified and extracted with Et_2O to give Et_2O-soluble cutin acids (270 mg) Of the original 300 mg of cutin approximately 26 mg was H_2O soluble after solvolysis

Preparation of derivatives The cutin acids were taken up in Et_2C -MeOH (10 1) and methylated with excess CH_2N_2 [27] Hydroxylation of double bonds[8] was carried out by treating approximately 100 μg of cutin acid methyl ester with 60 μl of pyridine–Et_2O (1 8) and 1 mg OsO_4 in 30 μl of Et_2O The solution was allowed to stand for 1 5 hr and then 0 6 ml of a Na_2SO_3 suspension added (1 5 ml 16% aq Na_2SO_3 plus 5 ml MeOH) The mixture was allowed to stand for 1 hr After centrifugation the supernatant was evaporated to dryness and silylated

Silylation of samples was carried out with bis-(trimethylsilyl) trifluoroacetamide plus 1% trimethylchlorosilane using 100 μl reagent per 500 μg of sample The solutions were heated initially to 80° then allowed to stand for several hours before analysis

Preparative TLC of cutin acid methyl esters The cutin acid methyl esters were chromatographed on silica gel G wedge plates with a solvent system containing Et_2O–hexane–MeOH (40 10 1) [8] Seven bands were visualized on spraying with 0 05% aq rhodamine 6G Each band was scraped from the plate and eluted with dry Et_2O The intermediate areas were also eluted and checked via GLC Intermediate zones were devoid of material, however, a small amount of dihydroxy ester was found in the trihydroxy ester fraction and was corrected for during quantitative analysis

IR Spectra were taken with a Perkin–Elmer Model 337 spectrophotometer To obtain spectra of intact cutin, a membrane was pressed between two 1 5 cm AgCl disks Isolated cutin acid methyl ester fractions were analyzed as thin films on the surface of an AgCl disk

GLC The separation, quantitative analysis and semi-preparative trapping of cutin acid fractions was carried out using a Perkin–Elmer 900 gas chromatograph The column employed throughout was a 1 5 m × 3 mm ss column coated with 5% SE-30 on 60/80 mesh acid washed and silanized Chromasorb W, programmed from 180° to 320° at 10°/min A flow-rate of 35 ml/min was employed, and a 15 1 exit port split ratio utilized when trapping subfractions The following internal standards were employed, for Fractions 1 and 2, methyl heptadecanoate, for Fractions 3 and 4, methyl 9-hydroxyoctadecanoate, for Fractions 5 and 6, methyl 8,9-dihydroxyoctadecanoate, and for Fraction 7, methyl 9,10,16-trihydroxyhexadecanoate

* P E Kolattukudy [*Biochem Biophys Res Commun* **41,** 299 (1970)] has now demonstrated that palmitic acid–1–^{14}C is readily incorporated into 10,16-dihydroxy palmitic acid of *Vicia faba* cutin and has suggested the reaction sequence palmitic acid → 16-hydroxy palmitic acid → 10,16-dihydroxy palmitic acid → cutin

[26] W HEINEN and I V D BRAND, *Z Naturforsch* **18,** 67 (1963)
[27] H SCHLENK and J L GELLERMAN, *Analyt Chem* **32,** 1412 (1960)

Peak areas were determined by triangulation, and linear detector response by weight assumed Retention indices were determined by co-injection with *n*-alkanes while programming from 150° to 300° on SE-30

MS Mass spectra were taken with a Hitachi Perkin–Elmer RMU-6A mass spectrometer operated at 70 eV When the GC-MS mode was employed a Varian Model 1200 gas chromatograph was employed as the inlet system The background was subtracted from each spectrum and peaks with a relative intensity of more than 2% tabulated However, for clarity of presentation the spectra in Figs 1–5 show only major peaks or others of particular importance

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Key Word Index—Vaccinium macrocarpon, Ericaceae, cutin acids, hydroxy C-16 and C-18 acids