Hsu, M. C., and Woody, R. W. (1971a), *J. Amer. Chem. Soc. 93*, 3515.
Hsu, M. C., and Woody, R. W. (1971b), *Biopolymers 10*, 1421.
Kasha, M. (1963), *Radiat. Res. 20*, 55.
Kasha, M., Rawls, H. R., and El-Bayoumi, M. A. (1965), *Pure Appl. Chem. 11*, 371.
Kim, Y. D., and Ke, B. (1970), *Arch. Biochem. Biophys. 140*, 341.
Kreutz, W. (1970), *Advan. Bot. Res. 3*, 53.
Labaw, L. W., and Olson, R. A. (1970), *J. Ultrastructure Res. 31*, 456.
Olson, J. M. (1966), *in* The Chlorophylls, Vernon, L. P., and Seely, G. R., Ed., New York, N. Y., Academic Press, p 413.
Olson, J. M. (1971), *Methods Enzymol. 23*, 636.
Olson, J. M., and Thornber, J. P. (1971), *Photochem. Photobiol. 14*, 329.
Philipson, K. D., Tsai, S. C., and Sauer, K. (1971), *J. Phys. Chem. 75*, 1440.
Rhodes, W. (1961), *J. Amer. Chem. Soc. 83*, 3609.
Robinson, G. W. (1966), *Brookhaven Symp. Biol. 19*, 16.
Sauer, K., Dratz, E. A., and Coyne, L. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 17.
Sauer, K., Lindsay Smith, J. R., and Schultz, A. J. (1966), *J. Amer. Chem. Soc. 68*, 2681.
Schultz, A. J., and Sauer, K. (1972), *Biochim. Biophys. Acta* (in press).
Seely, G. R., and Jensen, R. G. (1965), *Spectrochim. Acta 21*, 1835.
Simpson, W. T., and Peterson, D. L. (1957), *J. Chem. Phys. 26*, 588.
Thornber, J. P., and Olson, J. M. (1968), *Biochemistry 7*, 2242.
Tinoco, Jr., I. (1962), *Advan. Chem. Phys. 4*, 113.
Tinoco, Jr., I. (1963), *Radiat. Res. 20*, 133.
Vredenberg, W. J. (1965), Ph.D. Thesis, University of Leiden, Leiden, The Netherlands.
Vredenberg, W. J., and Amesz, J. (1966), *Brookhaven Symp. Biol. 19*, 62.
Weier, T. E., Stocking, C. R., and Shumway, L. K. (1966), *Brookhaven Symp. Biol. 19*, 353.

Determination of the Structures of Cutin Monomers by a Novel Depolymerization Procedure and Combined Gas Chromatography and Mass Spectrometry†

T. J. Walton and P. E. Kolattukudy*

ABSTRACT: A convenient novel technique for the structural analysis of the hydroxy fatty acid polymer cutin, the major component of plant cuticle, is described. Treatment of cutin powder with $LiAlH_4$ in tetrahydrofuran gave high yields (80–95%) of ether-soluble reduced monomers which were quite stable and suitable for direct analysis by a combination of gas–liquid chromatography and mass spectrometry (glc-ms). For structural studies this mixture was analyzed by silica gel G and argentation thin-layer chromatography in conjunction with glc-ms analysis. The $LiAlH_4$-susceptible functions of cutin were identified by a similar analysis of the $LiAlD_4$-cleavage products of cutin. The diagnostic fragmentations exhibited by the trimethylsilyl ethers of the individual components together with their deuterium labeling pattern enabled rapid identification of the cutin monomers. This method was applied to cutin isolated from the fruits of peach (*Amygdalus persica*), pear (*Pyrus communis*), papaya (*Malabar papaiarnarum*), apple (*Malus pumila*), and grape (*Vitis vinifera*) and the leaves of *Senecio odoris* and apple. All the cutins examined contained a similar complement of minor components (usually <2%) consisting of fatty acids, alcohols, and α,ω-diols, as well as monohydroxy fatty acids which typically constituted 8–17% of the cutin. However, there were striking differences in the structure of the more polar cutin acids, and on this basis two types of cutin were recognized. One type, typified by papaya, contained dihydroxypalmitic acid as the major component (>74%) and the other contained various proportions of 9,10-epoxy-18-hydroxystearic acid (6.5–32%), 9,10,18-trihydroxystearic acid (5–25%), and their Δ^{12}-monounsaturated counterparts (14–30 and 2–6%, respectively) in addition to dihydroxypalmitic acid (13–45%). In the dihydroxypalmitic acid fraction both 9,16 and 10,16 isomers were present; in papaya the former isomer predominated and in the other cutins examined the latter isomer was dominant. The presence of positional isomers in the dihydroxy-C_{16} acid fraction and the absence of unsaturated C_{16} acids from cutin are consistent with a direct hydroxylation mechanism for the introduction of the in-chain hydroxyl group. On the other hand, the occurrence of unsaturated ω-hydroxy-C_{18} acid, 9,10-epoxy-18-hydroxy-C_{18} acid together with 9,10,18-trihydroxy-C_{18} acid suggests that oleic acid and linoleic acid undergo hydroxylation at C-18, epoxidation at Δ^9, followed by hydration of the epoxide to yield these cutin components.

The plant cuticle is comprised of cutin, a lipid polymer, which is a relatively rigid meshwork of interesterified hydroxy fatty acids, embedded in a layer of waxy material. The structures of the cutin and the waxes largely determine the physical and chemical properties of the plant cuticle, which plays an important role in the interaction between the plant and its en-

† From the Department of Agricultural Chemistry, Washington State University, Pullman, Washington. *Received November 29, 1971.* Scientific paper 3787, project 3001, College of Agriculture, Washington State University. This work was supported in part by a grant from National Science Foundation GB 23081.

* Author to whom enquiries should be made.

vironment. In recent years the chemistry and biosynthesis of many wax components have been studied quite extensively (Kolattukudy, 1970a; Eglinton and Hamilton, 1967; Mazliak, 1968). However, the polymer cutin had been largely ignored until quite recently. Early workers analyzed alkaline hydrolysis products of the cuticle and characterized it as containing hydroxy fatty acids (*e.g.*, Fremy, 1882, Lee, 1925, and Legg and Wheeler, 1929). In a more recent study, five hydroxy acids were identified as the major components of *Agave americana* cutin (Matic, 1956). Since then several studies on cutin composition have been reported and many of them identified components by gas–liquid chromatography (glc) (Crisp, 1965; Baker and Holloway, 1970; de Vries, 1969, 1970a). The application of a combination of glc and mass spectrometry (ms) is the most suitable technique for the location of the hydroxyl groups in the fatty chains. With such a technique Eglinton and Hunneman (1968) determined the structures of many components including minor ones of apple cutin hydrolysate.

Selective degradation techniques showed that ether and peroxide linkages are present in cutin (Crisp, 1965). Due to the presence of these alkali-stable bonds a substantial portion (10–40%) of the cutin is left behind after alkaline hydrolysis (Crisp, 1965; Matic, 1956). For biosynthetic studies a convenient method for a more complete cleavage of the polymer is needed, and such a method has been developed (Kolattukudy, 1970b). This novel procedure involves exhaustive hydrogenolysis of powdered cutin with $LiAlH_4$ in tetrahydrofuran. Since ester bonds and peroxide bridges are reductively cleaved in the same reaction mixture this method gave high yields of reduced monomers which can be conveniently analyzed by thin-layer chromatography (tlc) and glc-ms techniques. However, functional groups susceptible to $LiAlH_4$ such as epoxide and carbonyl, in addition to carboxyl present in the cutin would be converted into hydroxyl groups which cannot be distinguished from the naturally occurring hydroxyl groups. We have resolved this difficulty with the use of $LiAlD_4$ followed by glc-ms. In this paper we describe the structural analysis of cutin of several plant species with the new hydrogenolysis–glc-ms technique. Determination of the structures of the cutin monomers described in this communication lays the foundation for biosynthetic studies which are in progress in this laboratory.

Experimental Section

Isolation of Cutin. Peel from fresh fruits of apple, pear, papaya, and grape were collected. Peach fruits were boiled in water before peeling. These peels and leaves of *Senecio odoris* and apple were boiled with an aqueous solution of oxalic acid (4 g/l.) and ammonium oxalate (16 g/l.). The cuticle was physically separated from the mixture by passing through style A nylon cloth (W. G. Runkles Machinery Co., Trenton, N. J.), and extracted with $CHCl_3$–CH_3OH (2:1, v/v) overnight. The sheets of cuticle were then Soxlet extracted with $CHCl_3$ for 24 hr, dried, and treated with a solution of *Aspergillus niger* cellulase (5 g/l.) and fungal pectinase (Sigma Chemical Co.) (1 g/l.) in pH 4.0 acetate buffer (0.05 M) at 30° for 14 hr. The crude cutin sheets were recovered by filtration and were thoroughly washed with water. Solvent extraction followed by enzyme digestion was repeated twice.

The cutin sheets obtained by the above treatment were dried and ground in a Wiley mill, and the powder which passed through a 40-gauge sieve was collected.

Hydrogenolysis of Cutin. Cutin powder (500 mg) was suspended in dry, redistilled tetrahydrofuran (30 ml), an excess (2.5 times by weight) of $LiAlH_4$ (Pierce Chemical Co.) or $LiAlD_4$ (Merck, Sharp and Dohme, 99% min atom %D) was added, and the mixture was refluxed for 24–72 hr.

At the conclusion of hydrogenolysis, excess $LiAlH_4$ was decomposed by the cautious addition of the reaction mixture to water (100 ml) which was then acidified with concentrated hydrochloric acid (4 ml) and extracted exhaustively with diethyl ether (five 150-ml portions). The ethereal solution was dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure.

Alkaline Hydrolysis of Cutin. Cutin powder was suspended in 95% ethanol (30 ml) and potassium hydroxide (3 g) added. The suspension was refluxed (16–24 hr) under a nitrogen atmosphere. The resulting solution was acidified (concentrated HCl) and the hydroxy fatty acids isolated as described above.

Thin-Layer Chromatography. The hydrogenolysis products were chromatographed on silica gel G with diethyl ether–hexane–methanol (system A, 8:2:1, v/v) as developing solvent. Saturated and unsaturated components were separated by chromatography on silver nitrate impregnated silica gel G plates (4.0 g of $AgNO_3$/30 g of the gel) with appropriate solvent mixtures (monools: Et_2O–C_6H_{14}, 4:1, v/v; diols: system A, 20:5:1, v/v; triols: system A, 8:2:1, v/v; tetraols, system A, 4:1:1, v/v). The alcohols were visualized either by H_2SO_4 charring, or when the products were to be recovered, by spraying with 0.1% solution of dichlorofluorescein in ethanol.

Gas–Liquid Chromatography and Mass Spectrometry. Trimethylsilyl (Me_3Si) ethers of either unresolved alcohol mixtures obtained by hydrogenolysis, or alcohol fractions purified by tlc, were prepared by reaction with *N,O*-bis(trimethylsilyl)-acetamide (0.5 ml; Pierce Chemicals) at 90° for 20 min. Excess reagent was evaporated under a stream of nitrogen, and the sample was dissolved in chloroform and injected into a gas chromatograph. A coiled glass column (147.0 × 0.31 cm o.d.) packed with 3% OV-1 on Gas Chrom-Q, at 235° with a flow rate of about 60 ml/min was used for analysis of the Me_3Si ether mixtures. In the case of fractions separated by tlc, the temperature was varied to give optimum resolution and convenient retention times.

Mass spectra were recorded (Perkin-Elmer Hitachi RMU 6D, 70 eV ionizing voltage) directly on the glc effluent (Bieman separator interphase), scans being obtained at the top of each peak on the glc recorder. In order to test whether a glc peak represented incompletely resolved components, spectra were also taken at various intervals during the elution of a single peak.

Preparation of Octadecane-1,12-diol and Octadec-9-cis-ene-1,12-diol. 12-Hydroxystearic acid (50 mg) and 12-hydroxy-9-*cis*-octadecenoic acid (ricinoleic acid, 50 mg) were each dissolved in 20 ml of dry redistilled tetrahydrofuran. $LiAlH_4$ (120 mg) was added to each solution and the mixture refluxed (14 hr). The excess $LiAlH_4$ was decomposed by cautious addition of the reaction mixture to water and the acidified aqueous solution was extracted with ether as described above. The diols were purified by repeated silica gel G tlc with system A (20:5:1, v/v) as the developing solvent.

Location of the Double Bond in the Octadecenetriol Fraction. The C_{18}-triol fraction isolated by silica gel G chromatography (10 mg) was dissolved in dichloromethane (3 ml) and *m*-chloroperbenzoic acid (40 mg) was added. The solution was stirred rapidly at 20° for 30 min. Excess sodium sulfite was added, and the mixture was transferred to ether. The ethereal solution was washed with saturated sodium bicarbonate solution and water, dried over anhydrous Na_2SO_4, and evaporated to dryness. The product was dissolved in dry tetrahydrofuran

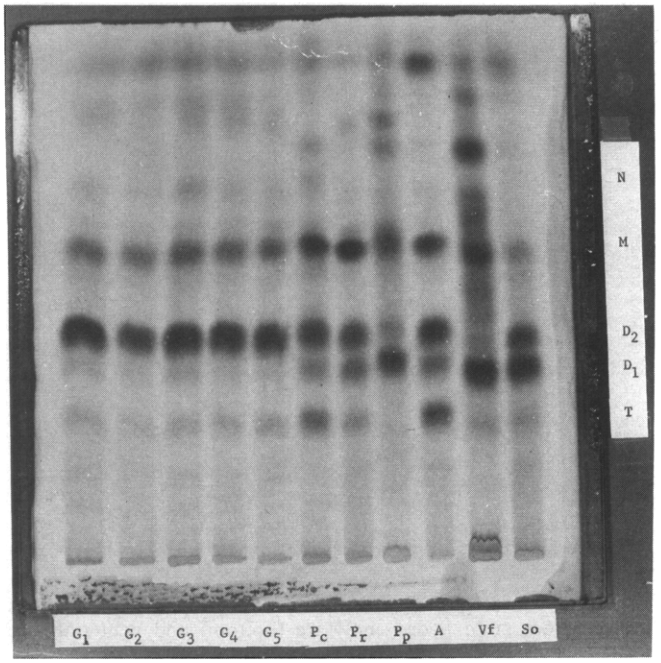


FIGURE 1: Thin-layer chromatography of hydrogenolysis products of several plant cutins. G_1-G_5, berries of White Rose, Thompson, Kendaya, Gamay, and Muskat grape, respectively; Pc, peach fruit; Pr, pear fruit; Pp, papaya fruit; A, apple fruit, Vf, *Vicia faba* leaf; So, *Senecio odoris* leaf. Chromatogram was developed in diethyl ether–hexane–methanol (8:2:1, v/v) and visualized by H_2SO_4 charring. N, monools; M, diols; D_1, hexadecanetriols; D_2, C_{18}-triols; T, tetraols.

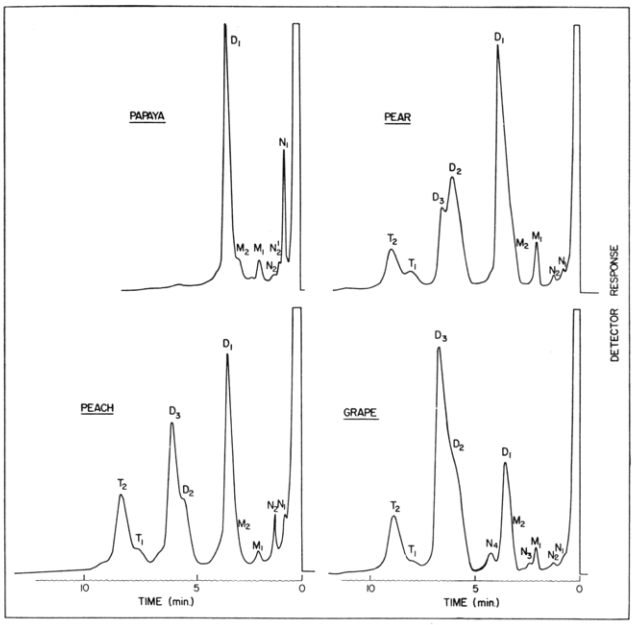


FIGURE 2: Gas–liquid chromatography of the trimethylsilyl (Me_3Si) ethers of several cutin hydrogenolysis products. Peaks were assigned on the basis of mass spectral data to the Me_3Si ethers of: N_1, hexadecanol; $N_{2'}$, octadecenol; N_2, octadecanol; M_1, hexadecanediol; N_3, eicosanol; M_2, C_{18}-diol; D_1, hexadecanetriol; N_4, docosanol; D_2, octadecenetriol; D_3, octadecanetriol; T_1, octadecenetetraol; T_2, octadecanetetraol. Gas chromatography with coiled glass column (147 × 0.31 cm o.d.) packed with 3% OV-1 on 80–100 mesh Gas Chrom Q at 235° and 60 cm³/min of carrier gas (He).

(15 ml), $LiAlH_4$ (50 mg) was added, and the sample was hydrogenolyzed as described above. The alcohol mixture was isolated as described previously, and the Me_3Si ethers were prepared and analyzed by glc-ms.

Results

Recovery of Cutin Hydrogenolysis Products. The yield of ether-soluble hydrogenolysis products from fruit cutins was typically at least 80%, the rest being recovered as a solid. However, in the case of peach, the yield of the hydrogenolysis products was much lower being consistently around 25%. Apple fruit cutin gave 87–94% of the polymer as ether-soluble hydrogenolysis products, whereas alkaline hydrolysis of the same batch of apple fruit cutin indicated a recovery of 74%. In the case of leaf cutins, however, contamination of the cutin preparation by lignified vascular tissue occurred at times because of overdigestion with oxalate–oxalic acid, and in such cases the yield of ether-soluble products was diminished.

Thin-Layer Chromatography. The thin-layer chromatographic separation of the alcohols obtained by hydrogenolysis of several plant cutins is shown in Figure 1. A point of particular interest is the separation of hexadecane-1,7,16-triol from the mixture of saturated and unsaturated C_{18}-1,9,18-triol homologs. This preliminary analysis showed contrasting patterns of alcohol products from the cutins examined. For example, all five varieties of grape analyzed were qualitatively very similar, and octadecanetriol was the dominant component. The peach and apple cutins yielded significantly more tetraols than the other cutins examined. Hydrogenolysis products of apple leaf cutin gave a tlc pattern identical with that of apple fruit cutin. In the case of *V. faba* and papaya the C_{16}-triol was the major component with significant amounts of monools, the C_{18}-triol and -tetraols being virtually absent from these hydrogenolysates. Pear fruit and *S. odoris* leaf cutin yielded comparable amounts of C_{16}- and C_{18}-triols and smaller amounts of tetraols. Thus this preliminary tlc fractionation revealed major differences between cutin composition in the various plant tissues examined. These differences lay primarily in the triol and tetraol regions.

Gas–Liquid Chromatographic Analysis. The glc separation of the Me_3Si ethers of the alcohol mixture derived from four cutins, each of significantly different types as revealed by tlc, is shown in Figure 2. Identification of each component was based upon its mass spectral fragmentation pattern, and the composition of the cutin hydrogenolysis products calculated as described later. Glc of the Me_3Si ethers of the alcohols derived by hydrogenolysis of these cutins revealed similar compounds in the fatty alcohol, and α,ω-diol fractions, while in the more polar derivatives two striking patterns of monomer distribution were evident. While peach, pear and grape fruit cutin hydrogenolysis products each contained varying amounts of C_{18}-triols and C_{18}-tetraols in addition to C_{16}-triols, C_{18} polyhydroxy alcohols were absent from papaya cutin hydrogenolysate.

Mass Spectrometric Identification of Reduced Monomers of Cutin. ALKANOLS AND ALKENOLS. The spectra of the Me_3Si ethers of the octadecanol derived from cutin by hydrogenolysis and deuterolysis were typical of these derivatives of the alkanol fraction (Figure 3). A weak parent ion (M^+, m/e 342) and an intense fragment ion at m/e 327 ($M^+ - CH_3$) were the only significant ions in the high-mass region of the spectrum, as previously observed (Sharkey *et al.*, 1957). These spectra contained the typical rearrangement ions of Me_3Si ethers which include m/e 73 (i), m/e 75 (ii), and m/e 89 (iii), and the diagnostic α-cleavage ion at m/e 103 (iv) characteristic of the Me_3Si ethers of primary alcohols (see Scheme I).

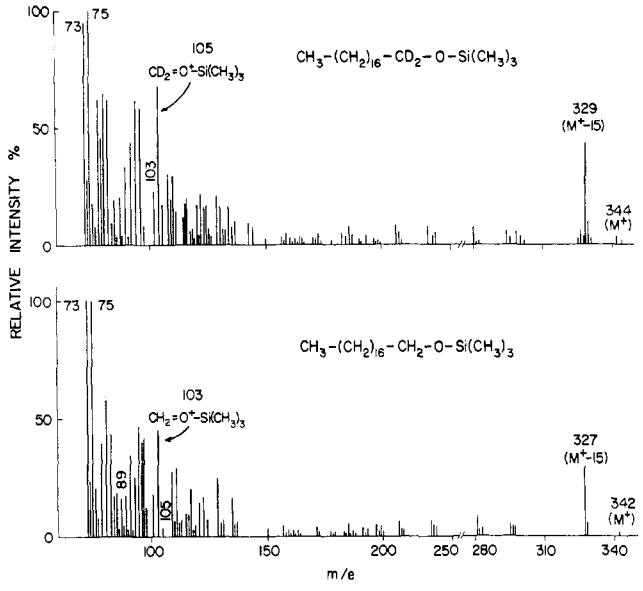


FIGURE 3: Mass spectra of Me_3Si ethers of octadecanols derived from cutin hydrogenolysate (bottom) and deuteriolysate (top).

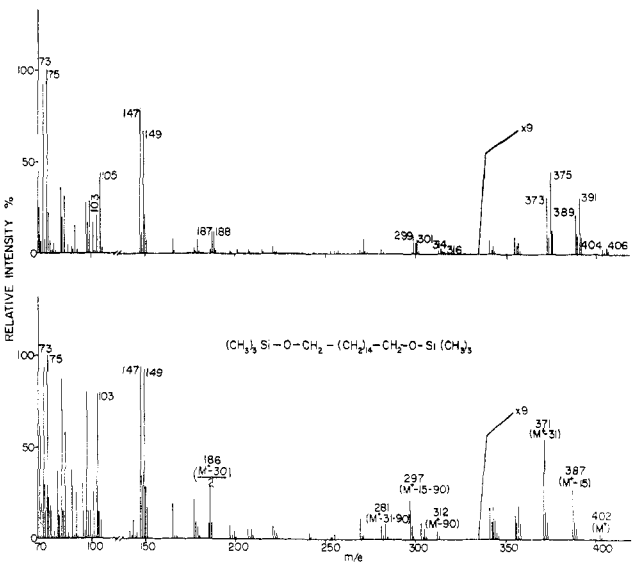


FIGURE 4: Mass spectra of the Me_3Si ethers of hexadecanediols derived from cutin hydrogenolysate (bottom) and deuteriolysate (top).

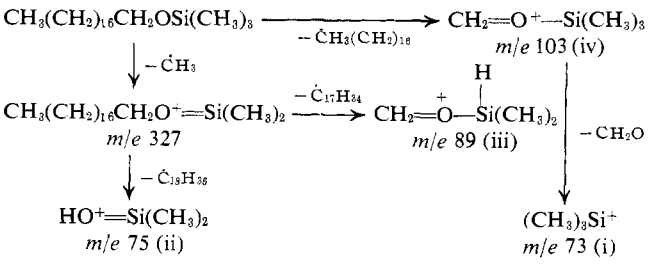
In the case of the deuterolysis derivative, both the parent ion (*m/e* 344) and the principal fragment ion due to loss of methyl (*m/e* 329) occurred 2 amu higher than in the hydrogenolysis derivative, indicating the incorporation of two deuterium atoms into the major component. These ions, together with the shift of the intense α-cleavage ion from *m/e* 103 (iv) in the spectrum of the hydrogenolysis product by 2 amu to *m/e* 105 in the case of the deuterolysis product confirmed the structure of the labeled alkanol as octadecan-1-ol-1-d_2 which is obviously derived from stearic acid in cutin.

In the case of the octadecenol derivative the parent ion (M^+, *m/e* 340) was somewhat more prominent than in the saturated analog, while the fragment ion (*m/e* 325; $M^+ - CH_3$) was still the most prominent feature of the spectrum. The deuterium-labeled derivative had the parent ion at *m/e* 342 and fragment ions at *m/e* 327 ($M^+ - CH_3$) and *m/e* 105, indicating the structure of the alcohol as octadecen-1-ol-1-d_2, confirming its origin in cutin as an octadecenoic acid, most probably oleic acid.

Similar observations confirmed the presence of palmitic, stearic, oleic, eicosanoic, and docosanoic acids in peach, pear, and grape cutins, while papaya contained palmitic, stearic, oleic, and eicosanoic acids.

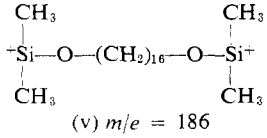
The presence of a significant ion at *m/e* 327 in the spectrum of the octadecanol derived from deuteriolysis of cutin indicated the presence of naturally occurring octadecanol in cutin. Similar indications were obtained for the presence of trace amounts of hexadecanol, octadecenol, eicosanol, and docosanol in the cutin preparations we have examined. Small amounts of very long-chain alkan-1-ols (C_{28}–C_{36}), typical of cuticular wax, were also found in coffee leaf cutin preparations (Holloway *et al.*, 1972). Presumably these alcohols were esterified to carboxyl groups of cutin, suggesting the possibility of covalent linkages between wax components and the polymer cutin. Considering the exhaustive solvent extractions used in the preparation of cutin, it is unlikely that these alcohols represent incomplete removal of wax.

SCHEME I



ALKANEDIOLS AND ALKENEDIOLS. Glc of this fraction revealed two saturated components and an unsaturated component in the case of peach, pear, grape, and apple fruit cutin hydrogenolysates, while that of papaya contained a single saturated diol.

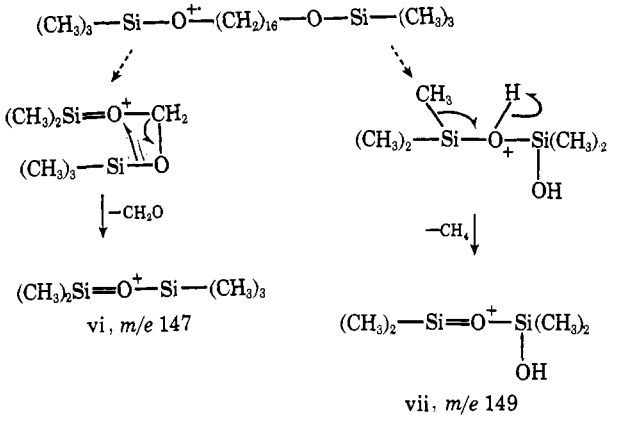
The mass spectrum of the Me_3Si ether of the saturated diol (Figure 4) which was present in all cutin hydrogenolysates examined, had a barely discernible parent ion, but the presence of significant fragment ions at *m/e* 387 ($M^+ - CH_3$), *m/e* 371 ($M^+ - CH_3 - CH_4$), *m/e* 312 ($M^+ - HOSi(CH_3)_3$, *m/e* 297 ($M^+ - CH_3 - HOSi(CH_3)_3$), and *m/e* 281 ($M^+ - CH_4 - CH_3 - HOSi(CH_3)_3$) confirmed the molecular weight as 402, corresponding to the Me_3Si ether of a hexadecanediol. The significant ion at *m/e* 186 ($M^+ - 30/2$), which was accompanied by a first isotope peak at *m/e* 186.5, was assigned to the doubly charged species (v), of the type previously described (McCloskey *et al.*, 1968), which further confirmed the molecular weight of the derivative and identified the diol as α,ω-hexadecanediol. In the case of the deuterated derivative



(v) *m/e* = 186

(Figure 4), the characteristic fragment ions occurred in pairs at *m/e* values 2 and 4 amu higher than the hydrogenolyzed analog (*m/e* 391, 389; 375, 373; 316, 314; 301, 299; and 285, 283, respectively). Further, the presence of two doubly charged ions at *m/e* 187 and 188 of approximately equal intensity and accompanied by half-mass first isotope peaks at

SCHEME II



m/e 187.5 and 188.5, respectively, indicated that two species of deuterated hexadecanediol were obtained by deuteriolysis of cutin, one containing two deuterium atoms (mol wt 404) and the other four deuterium atoms (mol wt 406). The absence of intense ions expected from cleavage of bonds α to an in-chain ether function, the ratio of *m/e* 103:105 in the $LiAlH_4$ and $LiAlD_4$ derivatives, and the characteristic doubly charged ions indicated the structures of these species as hexadecane-1,16-diol-1-d_2 and hexadecane-1,16-diol-1-d_2-16-d_2. These structures are consistent with the presence of both 16-hydroxypalmitic acid and hexadecanedioic acid in the cutin preparation.

The intense ions at *m/e* 147 (vi) and *m/e* 149 (vii) (see Scheme II) present in these spectra were found in all the spectra subsequently discussed and are characteristic of $(Me_3Si)_n$ ethers and have been formulated as arising by reactions which bring the silicon containing functions to within close proximity of each other where they may undergo relatively facile rearrangement, involving expulsion of the alkyl chain (Diekman *et al.*, 1968).

The presence of both 18-hydroxystearic acid and octadecanedioic acid was indicated by the mass spectra of octadecanediols formed by $LiAlH_4$ and $LiAlD_4$ treatment of peach, pear, grape, and apple fruit cutins. This component was absent from the papaya alkanediol fraction.

The spectra of the Me_3Si ether of the unsaturated diol (Figure 5), obtained by hydrogenolysis of all the cutins examined except that of papaya, had an intense parent ion (M^+ *m/e* 428) and exhibited a fragmentation pattern similar to that of the saturated counterpart. The doubly charged ion at *m/e* 199, accompanied by an ion at *m/e* 199.5 confirmed the molecular weight of the principal component as 428. In the case of the deuterium-containing derivative, an intense ion at *m/e* 430 was present, and the fragmentation pattern was characteristic of α,ω-$(Me_3Si)_2$ ether. A major doubly charged ion occurred at *m/e* 200, accompanied by a half-mass ion at *m/e* 200.5, indicating the molecular weight of the principal component as 430, corresponding to the Me_3Si ether of octadecene-1,18-diol-1-d_2, derived from an ω-hydroxyoctadecenoic acid, probably ω-hydroxyoleic, in cutin. In this spectrum there was also a significant ion at *m/e* 201 with an accompanying ion at *m/e* 201.5, indicating the presence of a deuterated diol species containing four deuterium atoms. This component was probably derived from an octadecenedioic acid in cutin.

In the spectrum of the Me_3Si ether of each diol obtained by deuteriolysis, the presence of the corresponding nonlabeled α,ω-diol species was indicated by a weak doubly charged ion and its half-mass first isotope peak of appropriate *m/e* ratio.

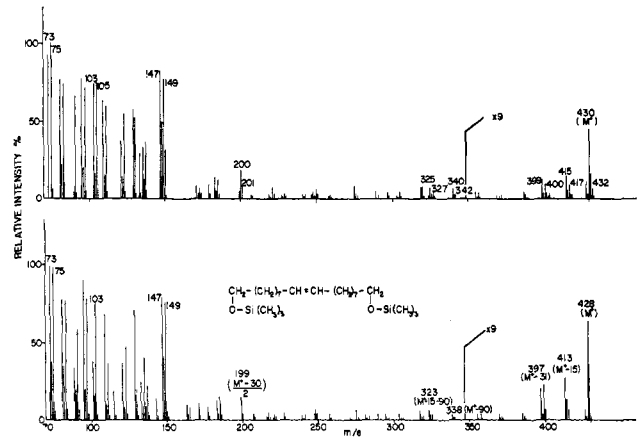


FIGURE 5: Mass spectra of the Me_3Si ethers of the octadecenediols derived from cutin hydrogenolysate (bottom) and deuteriolysate (top).

These observations suggest that traces of naturally occurring α,ω-hexadecanediol, α,ω-octadecenediol, and α,ω-octadecanediol were present in the cutin preparations analyzed. Since α,ω-diol is a component of some cuticular lipids, (Mazliak, 1968), the possibility exists that the diol in the cutin may arise by esterification of free carboxyl groups of cutin and diols of wax.

Hexadecanetriol. Silver nitrate chromatography of the ubiquitously distributed hexadecanetriol fraction failed to detect any unsaturated component, and a single peak was observed when this material was analyzed by glc. The mass spectrum of the Me_3Si ether of the hexadecanetriol fraction obtained by hydrogenolysis of peach cutin (Figure 6) showed a weak parent ion (M^+ *m/e* 490) and characteristic fragment ions at *m/e* 475 ($M^+ - CH_3$), *m/e* 400 ($M^+ - HOSi(CH_3)_3$), and *m/e* 385 ($M^+ - CH_3 - HOSi(CH_3)_3$) in addition to the normal low-mass ions. This fraction, from all the cutins examined, except papaya, exhibited strong ions of equal intensity at *m/e* 275 and 317 arising from cleavage of the C-6,7 and

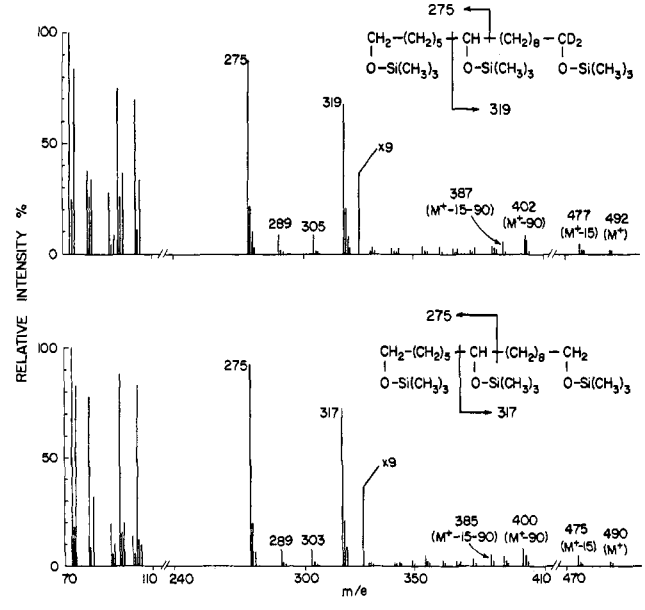


FIGURE 6: Mass spectra of the Me_3Si ethers of the hexadecanetriols derived from peach cutin hydrogenolysate (bottom) and deuteriolysate (top).

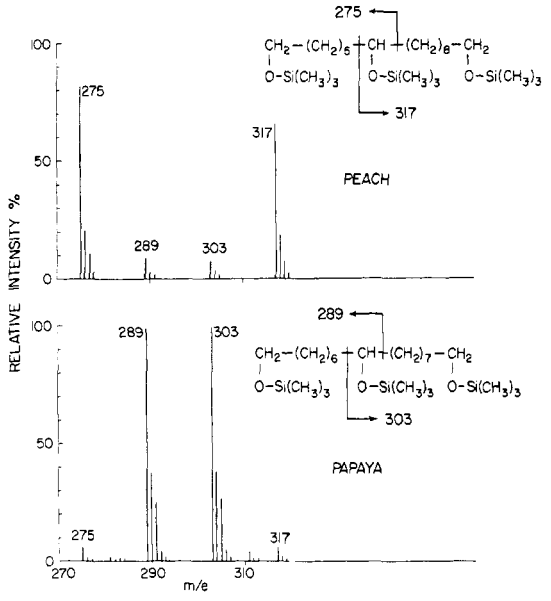


FIGURE 7: Partial mass spectra showing the α-cleavage ions of the Me_3Si ethers of the hexadecanetriols derived from the hydrogenolysates of peach and papaya cutin.

C-7,8 bonds α to the in-chain ether function, while much weaker ions at *m/e* 289 and 303 indicated that a small amount of a positional isomer were also present. The parent ion (M^+ *m/e* 492) and the fragment ions at *m/e* 477, 402, and 387 of the corresponding deuteriolysis product (Figure 6) all showed an increase of 2 amu in their mass, indicating the incorporation of two deuterium atoms. In this case the intense α-cleavage ions occurred at *m/e* 275 and 319, while the weaker α-cleavage ions were at *m/e* 289 and 305. The upward shift of the two α-cleavage ions by 2 amu is consistent with the location of both deuterium atoms at C-1 of a mixture composed principally of the Me_3Si ethers of hexadecane-1,7,16-triol-16-d_2 and a small amount of hexadecane-1,8,16-triol-16-d_2. These structures obviously arise from 10,16-dihydroxypalmitic acid and its positional isomer, 9,16-dihydroxypalmitic acid in peach cutin. Similar mass spectra were obtained for the hexadecanetriol fraction obtained from all cutins except papaya. In this case the relative intensity of the α-cleavage ions was reversed (Figure 7), *m/e* 289 and 303 being the major ions in the hydrogenolysis products, indicating that the major dihydroxymonobasic acid of the cutin of this fruit is 9,16-dihydroxypalmitic acid, whereas in all the other cutins analyzed, 10,16-dihydroxypalmitic acid was the major dihydroxyhexadecanoate. While the glc conditions used failed to resolve the Me_3Si ethers of the isomeric triols, the ratio of the α-cleavage ions in mass spectra determined at several times during elution of this fraction from the column indicated that the early eluent may have been slightly enriched in the hexadecane-1,8,-16-triol isomer.

This is the first time 9,16-dihydroxypalmitic acid was found as a major component of cutin. While this paper was in preparation, we obtained information that 9,16-, 8,16-, and 7,16-dihydroxypalmitic acids were found in cutins and suberins of several species of plants (Holloway and Deas, 1972). Recent glc-ms analysis of the hydrogenolysate of sewage sludge, which contains many different plant residues, showed nearly equal amounts of 1,7,16- and 1,8,16-hexadecanetriol, suggesting the widespread occurrence of positional isomers in the dihydroxy-C_{16} acid fraction of plant cutins (P. E. Kolattukudy and R. E. Purdy, unpublished results). The possible biosynthetic implications of these results are discussed later.

Octadecanetriol. The C_{18}-triol fraction could be separated into two components by argentation tlc. The mass spectrum of the Me_3Si ethers of the less polar component (Figure 8) had a weak parent ion (M^+ *m/e* 518) and fragment ions at *m/e* 503 ($M^+ - CH_3$), *m/e* 428 ($M^+ - HOSi(CH_3)_3$) and 413 ($M^+ - CH_3 - HOSi(CH_3)_3$). The most intense ions in the high-mass region were observed at *m/e* 303 and 317 and assigned to α-cleavage on either side of an in-chain ether substituent at C-9. In the case of the deuteriolysis derivatives, the parent ion occurred at *m/e* 521, 3 amu higher than the parent ion of its hydrogenolysis analog. The fragment ions at *m/e* 506, 431, and 416 also showed upward shifts of 3 amu when compared to their hydrogenolysis counterparts. The introduction of three deuterium atoms into the molecule suggested the presence of a further $LiAlH_4$ sensitive functionality, such as an oxirane, ketone, or aldehyde function in the cutin component which gave rise to this fraction.

In the spectrum of the deuterated product, (Figure 8) four intense ions of approximately equal intensity at *m/e* 303, 305, 318, and 320 were present. These α-cleavage fragment ions indicated that the third deuterium atom in the molecule was not located on an ether substituted carbon atom.

If the third deuterium was introduced by $LiAlD_4$ reduction of a keto group this deuterium would be located on a carbon atom carrying the in-chain ether substituent. If reduction of an aldehyde function gave rise to the third deuterium it would be impossible for any α-cleavage ion of the Me_3Si ether to contain all three deuterium atoms. On the other hand, deuteriolysis of 18-hydroxy-9,10-epoxystearic acid in the cutin would introduce three deuterium atoms, two on the carboxyl carbon and a third on either C-9 or C-10 of the acid (Figure 8), giving rise to octadecane-1,9,18-triol molecules with two different isotope distributions. Since chemical reduction of the oxirane ring involves equal probability of cleavage of the two C–O bonds, the two octadecane-d_3-triols should be formed in equal quantities. The α-cleavage of the Me_3Si ethers of these two octadecane-d_3-triols would give ions of approximately equal intensity at *m/e* 303, 305, 318, and 320. These intense ions together with fragment ions observed in the spectrum, indicated that 9,10-epoxy-18-hydroxystearic acid was the major source of the octadecanetriol in all the cutins examined which yielded this product. However, the presence of small amounts of naturally occurring 9,18-dihydroxystearic acid and 10,18-dihydroxystearic acid (predicted M^+ at *m/e* 520, expected α-cleavage ions in deuterated derivative at *m/e* 305 and 317 and *m/e* 303 and 319, respectively) could not be excluded by these results.

Octadecenetriol. The mass spectrum of the Me_3Si ether of the more polar component obtained from the C_{18}-triol fraction by argentation tlc suggested that this component contained one double bond (Figure 9). In order to determine the position of the double bond, the C_{18}-triol fraction obtained by hydrogenolysis of apple cutin was rigorously purified by repetitive tlc, the double bond epoxidized, and the products reduced with $LiAlH_4$. The glc-ms analysis of the Me_3Si ethers of the reduction products showed a tetraol fraction (M^+, *m/e* 606, fragment ions at *m/e* 591 ($M^+ - CH_3$), *m/e* 516 ($M^+ - HOSi(CH_3)_3$), *m/e* 501 ($M^+ - CH_3 - HOSi(CH_3)_3$) derived from the unsaturated C_{18}-triol, in addition to the saturated C_{18}-triol in the starting material. The complex fragmentation pattern observed in the spectrum of the Me_3Si ether of this tetraol fraction (Figure 10) contained ions which are consistent with α-cleavage fragmentation of a mixture of

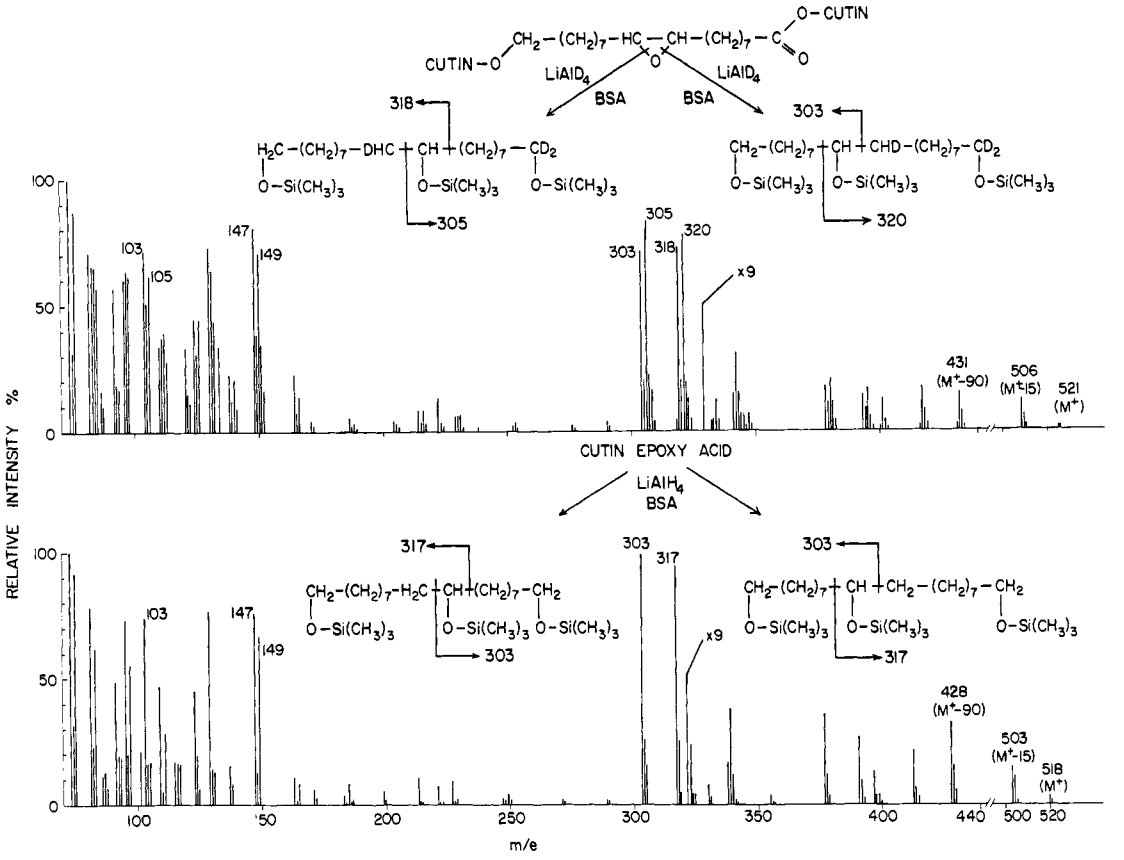


FIGURE 8: Mass spectra of the Me_3Si ethers of the octadecanetriols derived from cutin hydrogenolysates (bottom) and deuteriolysates (top).

the four isomeric octadecanetetraols produced by reduction of a mixture of 1,9,18-trihydroxy-6,7-epoxyoctadecane and 1,10,-18-trihydroxy-6,7-epoxyoctadecane (Figure 11). The predicted α cleavages for such a mixture are at *m/e* 261, 447, 317, 391 (octadecane-1,6,9,18-tetraol), *m/e* 261, 447, 303, 405 (octadecane-1,6,10,18-tetranol), *m/e* 275, 433, 317, 391 (octadecane-1,7,9,18-tetraol), and *m/e* 275, 433, 303, 405 (octadecane-1,7,-10,18-tetraol). Signals for each of these ions, together with fragment ions corresponding to loss of trimethylsilanol (90 amu) from the more highly substituted α-cleavage ions, *e.g.*, *m/e* 315 (*m/e* 405, $(CH_3)_3SiO(CH_2)_5CHOSi(CH_3)_3(CH_2)_3$-$CHO\cdot{}^+Si(CH_3)_3$ — $HOSi(CH_3)_3$), *m/e* 357 (*m/e* 447, $(CH_3)_3$-$SiO(CH_2)_8CHOSi(CH_3)_3(CH_2)_3CHO\cdot{}^+SiCH_3)_3$ — $HOSi$-$(CH_3)_3$) were present in the spectrum. The significant ion at *m/e* 289 may arise by cleavage of the bond α to the C-9 ether group and β to the C-7 ether group in the Me_3Si ether of octadecane-1,7,9,18-tetraol. This fragmentation pattern therefore showed the presence of a Δ^6 double bond in the octadecenetriols isolated from hydrogenolyzed apple cutin.

In the case of the Me_3Si ether of the octadecenetriol the fragmentation pattern (Figure 9) was strikingly different from that of its saturated analog. The hydrogenolysis product had a weak parent ion (M^+) at *m/e* 516 and relatively weak fragment ions at *m/e* 501 ($M^+ - CH_3$), *m/e* 426 ($M^+ - HOSi$-$(CH_3)_3$), and *m/e* 411 ($M^+ - CH_3 - HOSi(CH_3)_3$) were present as expected. As was the case in the saturated counterpart, the spectrum of the unsaturated deuteriolysis indicated incorporation of three deuterium atoms, the parent ion (M^+, *m/e* 519) and the fragment ions at *m/e* 504, 429 and 414 each occurring 3 amu higher than in the hydrogenolysis derivative.

In the spectrum of the hydrogenolysis product, of the four expected α-cleavage ions, the fragment at *m/e* 317 was dominant but the ions at *m/e* 301, 303 and 315 were weak. Similarly, in the spectrum of the deuteriolysis product, the ion at *m/e* 320 was strong but those at 316, 305 and 301 were weak. These fragmentation patterns are in contrast to those observed in the spectrum of the saturated counterparts in which ions produced by α-cleavage on either side of the carbon bearing the in-chain Me_3Si ether were of equal intensity.

Our present interpretation of these results is that in the case of the Me_3Si ether of 1,9,18-trihydroxyoctadec-6-ene, the ease of expulsion of the neutral allylic radical highly favors the cleavage of the carbon–carbon bond α to both the allylic methylene and the Me_3Si ether substituent, giving rise to the very intense ion at *m/e* 317. The other α-cleavage ion at *m/e* 301 is present but it is very weak. Similarly, in the case of the deuterated derivative an intense ion at *m/e* 320 and a weak ion at *m/e* 301 were observed. If the Me_3Si ether of 1,10,18-trihydroxyoctadec-6-ene, the isomeric hydrogenolysis product (Figure 9), underwent α cleavage on either side of the in-chain Me_3Si ether substituent, intense ions would be present at *m/e* 303 and 315. However, these ions were weaker than expected. One possible explanation would be that expulsion of the allylic free-radical favors cleavage of the carbon–carbon bond β to the in-chain ether function, giving rise to an ion at *m/e* 317 in the hydrogenolysis product and at 320 in the deuteriolysis product.

In order to test the validity of the hypothesis that the ease of expulsion of an allylic free-radical significantly influences the α-cleavage pattern of the Me_3Si ethers of polyhydroxyalkenes, the mass spectra of the Me_3Si ether of authentic octadecane-1,12-diol (I) and octadec-9-ene-1,12-diol (II) (Scheme III) were compared.

In the case of the saturated diol (I) α-cleavage on either side

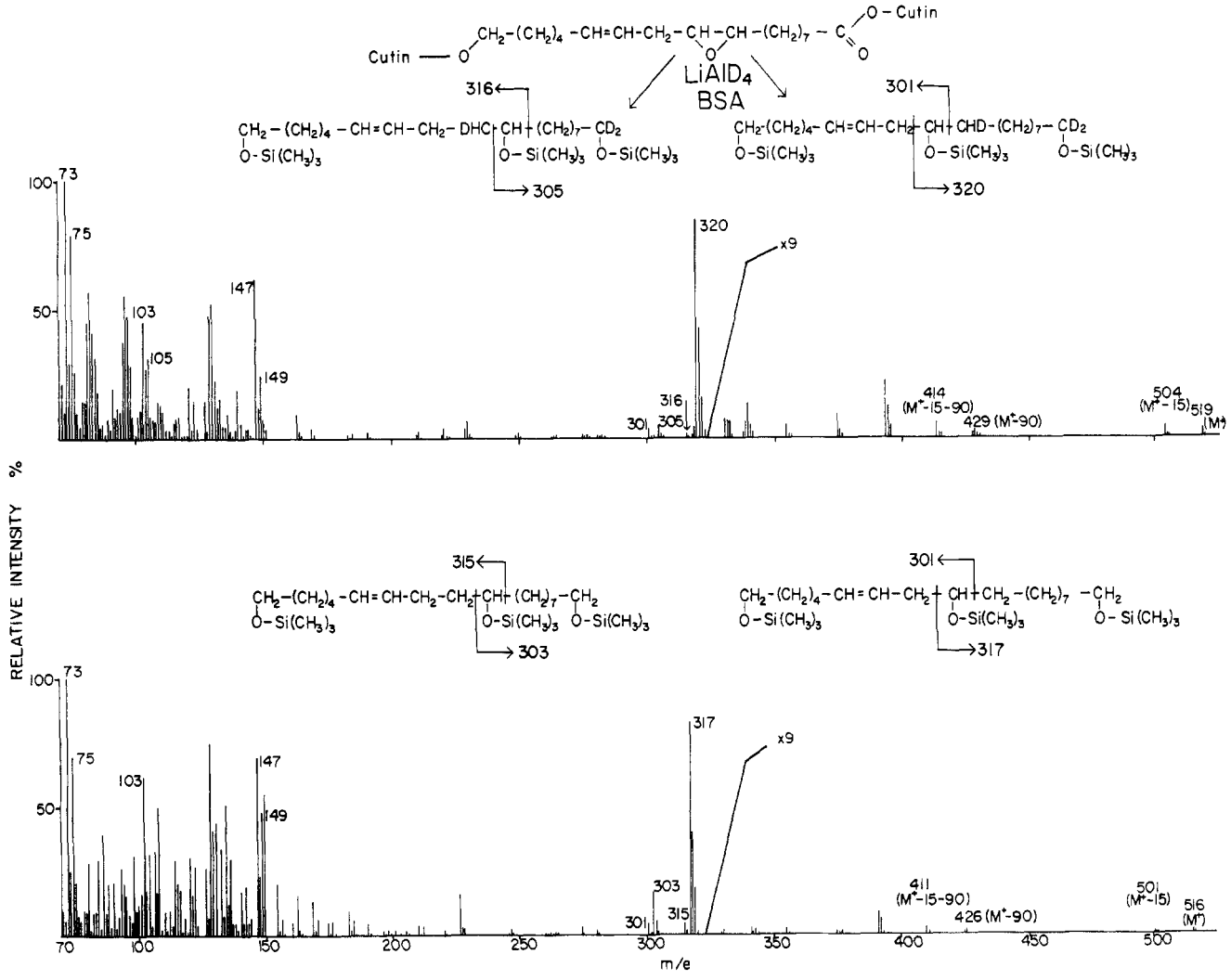


FIGURE 9: Mass spectra of the Me_3Si ethers of the octadecenetriols derived from cutin hydrogenolysates (bottom) and deuteriolysates (top).

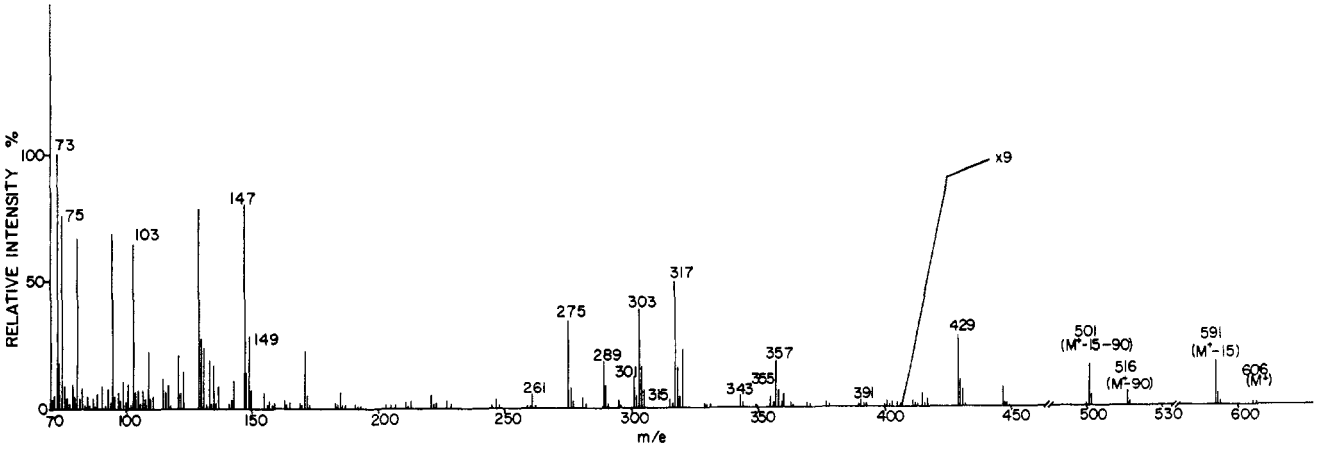


FIGURE 10: Mass spectrum of the Me_3Si ether of the octadecanetetraol fraction derived from the octadecenetriol of apple cutin hydrogenolysate by chemical epoxidation followed by $LiAlH_4$ reduction. The theoretical products and their expected α cleavages are shown in Figure 11.

of the in-chain substituent gave ions at *m/e* 187 and 345 in a ratio of 5:1 as expected. The corresponding ions from the unsaturated diol (II) would be at *m/e* 187 and 343. However, the ease of expulsion of the allylic free radical highly favored the cleavage of the carbon–carbon bond α to both the allylic methylene and the in-chain substituted carbon atom, giving ions at *m/e* 187 and 343 in a ratio of 30:1. Thus the presence of a double bond in the proximity of an in-chain Me_3Si ether function can lead to expulsion of an allylic radical and therefore greatly influence the α-cleavage pattern. These results

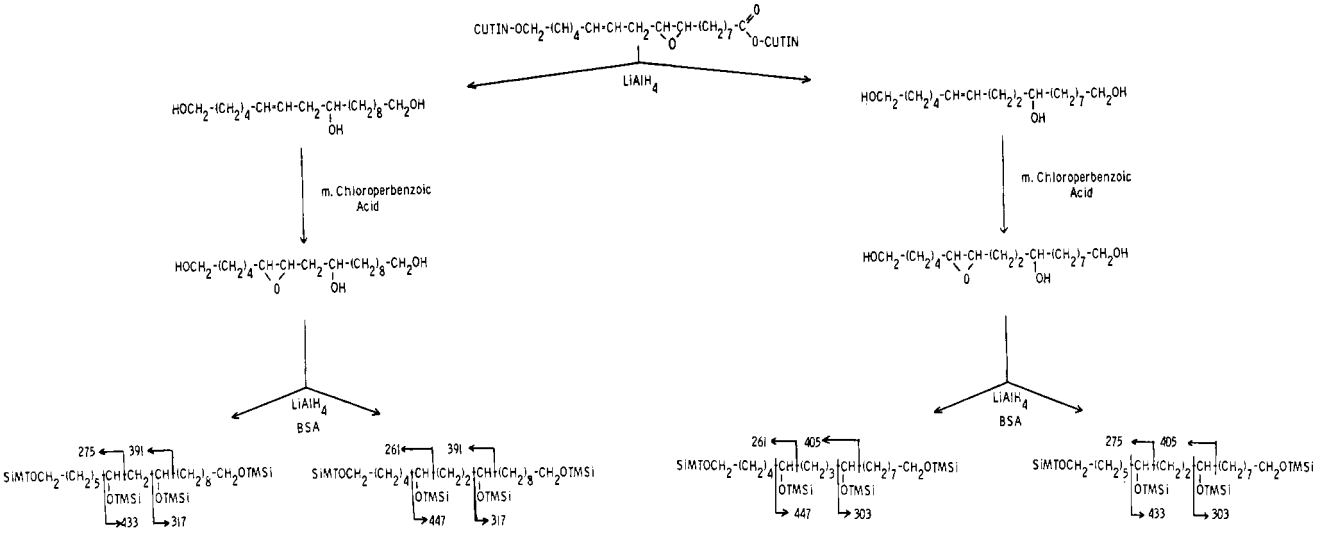
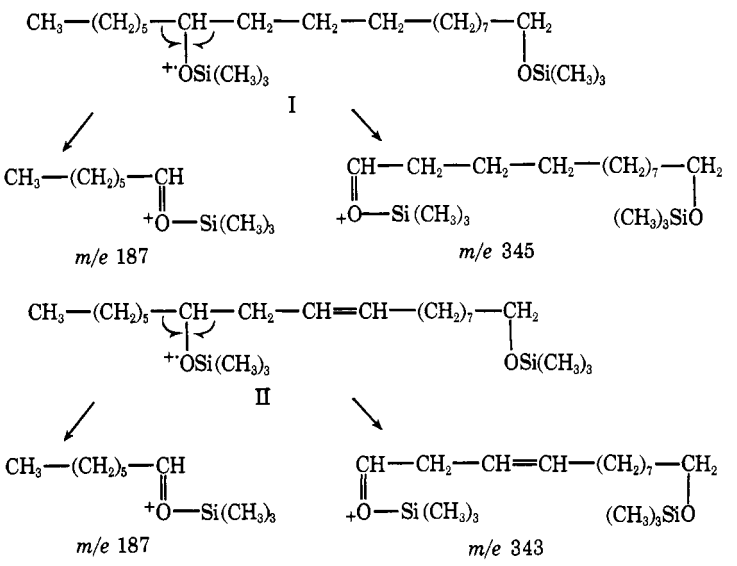


FIGURE 11: The theoretical tetraol products yielded by epoxidation and subsequent $LiAlH_4$ reduction of the octadecenetriol derived by hydrogenolysis of a 9,10-epoxy-18-hydroxyoctadec-12-enoic acid component of apple cutin. The expected α cleavages of the Me_3Si ethers of the components of this tetraol fraction are indicated.

SCHEME III



strongly support our interpretation of the mass spectrum of the octadecenetriol. The mass spectrum of the Me_3Si ether of the alkenetriol and that of the tetraol derived from the chemical epoxidation and reduction of the triol show that 9,10-epoxy-18-hydroxyoctadec-12-enoic acid is a major component of cutin which contains C_{18} polar acids.

Octadecanetetraol. Argentimetric chromatography of the tetrahydroxy alcohol fractions obtained from cutins revealed two components. The mass spectra of the Me_3Si ether of the less polar constituent eluted from argentation chromatography (Figure 12) had a weak parent ion at *m/e* 606 (M^+) and characteristic fragment ions at *m/e* 591 ($M^+ - CH_3$), *m/e* 516 ($M^+ - HOSi(CH_3)_3$), and *m/e* 501 ($M^+ - CH_3 - HOSi(CH_3)_3$). A single intense ion at *m/e* 303 was the base peak in the spectrum and was assigned to cleavage of the carbon–carbon bond between the two in-chain substituted carbon atoms, while a significant weaker ion at *m/e* 405 was assigned to cleavage of the other carbon–carbon bonds α to the substituted in-chain carbon atoms. Fragment ions corresponding to loss of trimethylsilanol from each of the α-cleavage fragment ions at *m/e* 213 (*m/e* 303 − $HOSi(CH_3)_3$) and at *m/e* 315 (*m/e* 405 − $HOSi(CH_3)_3$) were also present in the spectra, indicating the structure of the alcohol derived from cutin as octadecane-1,9,10,18-tetraol. The corresponding tetraol derived by deuteriolysis had the parent ion (M^+) at *m/e* 608, and fragment ions at *m/e* 593 ($M^+ - CH_3$), 518 ($M^+ - HOSi(CH_3)_3$), and *m/e* 503 ($M^+ - CH_3 - HOSi(CH_3)_3$), indicating the presence of two deuterium atoms in the molecule. The intense α-cleavage ion signal was split into two ions of roughly equal intensity at *m/e* 303 and 305, while the less prominent α-cleavage ions were at *m/e* 405 and 407. This fragmentation pattern, together with the increased intensity of the ion at *m/e* 105, indicated the location of both deuterium atoms at C-1 of the molecule. Loss of trimethylsilanol from the α-cleavage ions was evidenced by signals at *m/e* 213 (*m/e* 303 − $HOSi(CH_3)_3$), 215 (*m/e* 305 − $HOSi(CH_3)_3$), *m/e* 315 (*m/e* 405 − $HOSi(CH_3)_3$), and 317 (*m/e* 407 − $HOSi(CH_3)_3$). The structure of the tetraol derived from cutin was therefore confirmed

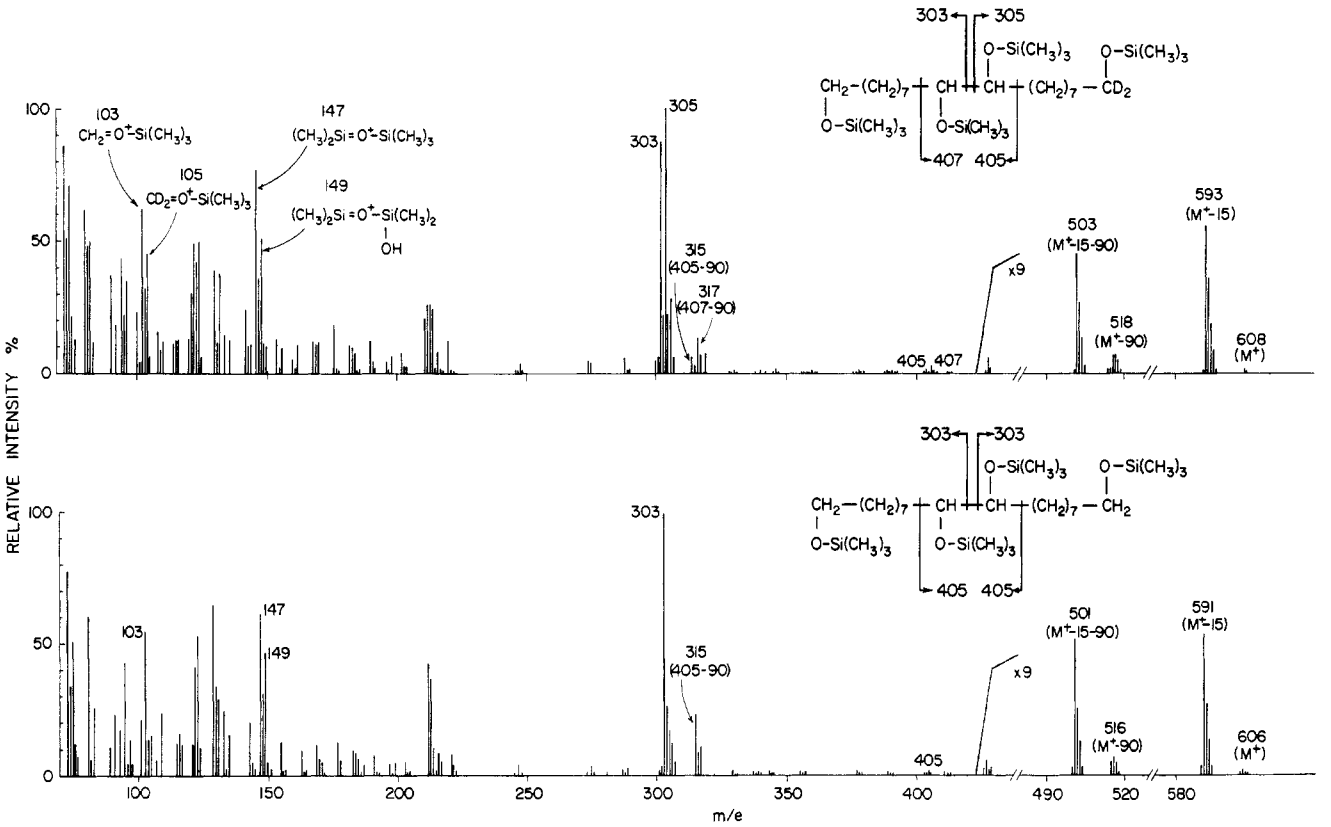


FIGURE 12: Mass spectra of the Me_3Si ethers of the octadecanetetraols derived from cutin hydrogenolysates (bottom) and deuteriolysates (top).

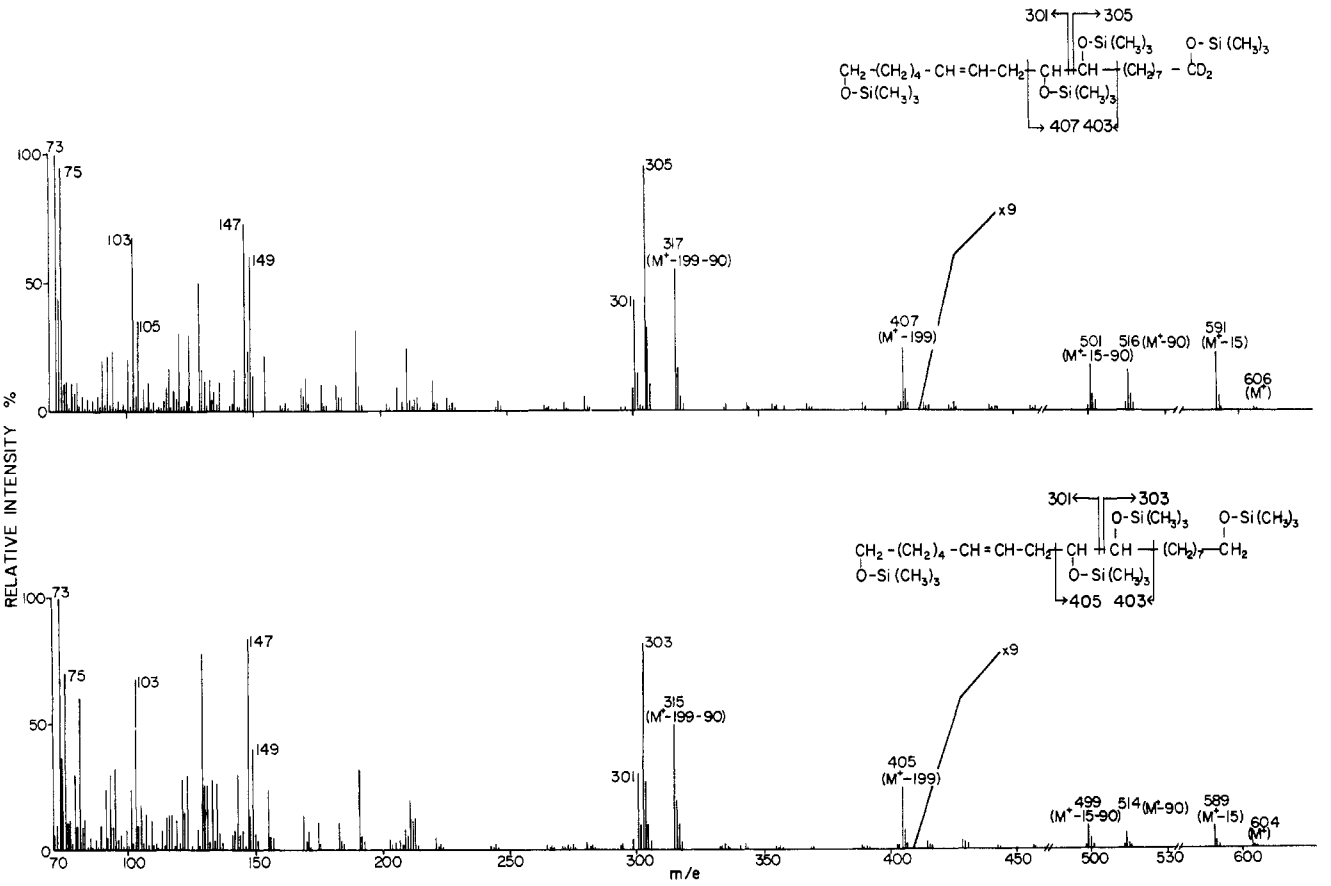


FIGURE 13: Mass spectra of the Me_3Si ethers of the octadecenetraols derived from cutin hydrogenolysates (bottom) and deuteriolysates (top).

as octadecane-1,9,10,18-tetraol-1-d_2, which obviously was formed from 9,10,18-trihydroxystearic acid in the polymer.

Octadecenetetraol. The spectra (Figure 13) of the Me_3Si ether of the unsaturated component separated by $AgNO_3$ chromatography were strikingly different from those of the alkanetetraol derivative. In the case of the hydrogenolysis product, a weak parent ion (M^+, *m/e* 604) and characteristic fragment ions at *m/e* 589 ($M^+ - CH_3$), *m/e* 514 ($M^+ - HOSi(CH_3)_3$), and *m/e* 499 ($M^+ - CH_3 - HOSi(CH_3)_3$ were present. The expected major α-cleavage ions are at *m/e* 303 and 301. However, if the double bond is located at Δ^6 position we would expect the expulsion of an allylic radical to favor the formation of the ion at *m/e* 405 and consequently diminish the intensity of the α-cleavage ion at *m/e* 301. In fact the most intense ion in the α-cleavage region occurred at *m/e* 303 with a weak ion at *m/e* 301, while a relatively intense ion was present at *m/e* 405. The intense fragment ion at *m/e* 315 ($M^+ - 199 - HOSi(CH_3)_3$) represents loss of trimethylsilanol from the fragment at *m/e* 405 (metastable ion at *m/e* 245 ($315^2/405 = 245.00$)). Fragment ions corresponding to loss of trimethylsilanol from the intense α-cleavage ion at *m/e* 303, (*m/e* 213, *m/e* 303 $- HOSi(CH_3)_3$) and the weak α-cleavage ion at *m/e* 301(*m/e* 211, *m/e* 301 $- HOSi(CH_3)_3$) are also present.

In the case of the deuterated analog, the parent ion (M^+, *m/e* 606) and fragment ions at *m/e* 591 ($M^+ - CH_3$) and *m/e* 516 ($M^+ - HOSi(CH_3)_3$) indicate the incorporation of two deuterium atoms. The upward shift by 2 amu of the intense ion *m/e* 305 together with the increase in intensity of *m/e* 105 indicates that both the labeled atoms are located on C-1. Just as in the case of the hydrogenolysis derivative the α-cleavage ion containing the unsaturation, at *m/e* 301, is much weaker, while the ion at *m/e* 407, arising from the expulsion of an allylic radical, was intense. A prominent fragment ion at *m/e* 317 arising from loss of trimethylsilanol from this fragment ($M^+ - 199 - HOSi(CH_3)_3$; metastable at *m/e* 247 ($317^2/407 = 246.902$)) was also present. We therefore conclude that 9,10,18-trihydroxyoctadec-12-ene, previously identified in apple cutin (Eglinton *et al.*, 1968) is a widespread component of cutins containing polar C_{18} acids.

Conflicting reports on the composition of cutin have appeared in the literature (Brieskorn and Reinartz, 1967; Shishiyama *et al.*, 1970), arising from analysis by glc alone. Analysis of the composition of the hydrogenolysis products (Table I) by mass spectrometry in conjunction with glc allowed the simultaneous determination of unequivocal structures for both the major and minor hydroxy acids of cutin. Further, the mass spectrometry data were used to supplement the glc results in instances when the latter alone were insufficient to allow accurate quantitation. Thus the relative amounts of alkanols and alkenols present in the hydrogenolysate were estimated from the characteristic $M^+ - 15$ fragment ion, and the levels of α,ω-octadecanediols and α,ω-octadecenediols based upon the relative intensities of the characteristic doubly charged fragment ion. Also, in this latter case, since the Me_3Si ethers of hexadecanetriol and the octadecanediols had very similar retention volumes under the glc conditions used, the level of the latter fraction was determined by glc analysis of the Me_3Si ethers of the diol fraction separated from the hexadecanetriols by tlc. The results of these determinations are presented in Table I.

TABLE I: Composition of the Reduced Monomers Obtained from Cutin Hydrogenolysis.[a]

	% Composition				
	Peach	Pear	Apple	Papaya	Grape
Hexadecan-1-ol	0.47	0.25	0.29	11.92	T
Octadecan-1-ol	1.42	0.35	1.38	0.45	0.23
Octadecen-1-ol	T	T	T	1.34	T
Eicosan-1-ol	T	T	T	0.45	0.80
Hexadecane-1,16-diol	0.91	2.74	6.62	4.18	1.33
Docosan-1-ol	T	T			T
Unidentified				7.24	
Octadecane-1,18-diol	3.20	3.34	2.51		2.56
Octadecene-1,18-diol	3.69	9.42	7.30		3.81
Hexadecanetriol	32.97	44.76	23.11	74.41	12.65
Octadecane-1,9,18-triol	25.95	6.50	13.11		32.26
Octadecene-1,9,18-triol	13.62	24.94	14.63		29.87
Octadecane-1,9,10,18-tetraol	14.66	4.95	24.88		11.83
Octadecene-1,9,10,18-tetraol	3.10	2.74	6.05		1.99

[a] Compositions are based upon triangulation of the gas–liquid chromatograms obtained when the Me_3Si ethers of the cutin hydrogenolysis products were analyzed on 3% OV-1 on Gas Chrom Q packed in a glass column (147.0 × 0.31 cm O.D.) at 235° with a carrier gas flow rate of 60 cm^3/min. Additional quantitation was based upon the relative intensities of $M^+ - 15$ fragment ions in the mass spectra of the Me_3Si ethers of primary alcohol fractions, and upon the doubly charged ion in the spectra of the α,ω-C_{18}-diol fractions. These data were supplemented by glc analysis of the Me_3Si ethers of the diol fraction separated from the hexadecanetriol by tlc.

Discussion

The novel hydrogenolysis technique, in conjunction with tlc and glc-ms gives a rapid and sensitive method for the determination of the structures of the monomers of cutin. The convenient analytical procedures involved and the intense diagnostic ions encountered in the mass spectra of Me_3Si ethers of polyhydric alcohols derived from the cutin make this a suitable routine procedure. The high yield of reduced monomers obtained from the hydrogenolysis when compared to hydrolysis is very important, especially for biosynthetic studies (Kolattukudy, 1970b; Kolattukudy *et al.*, 1971; Kolattukudy and Walton, 1972).

Me_3Si ethers of methyl esters are commonly used for glc-ms analysis of hydroxy fatty acids in biological samples (Pace-Asciak and Wolfe, 1971; Batt *et al.*, 1971). The method of preparation of these derivatives may give rise to artefact formation (Holloway and Deas, 1971) which can be avoided with the hydrogenolysis method. In addition, the polyhydric alcohol mixtures are quite stable showing no tendency to polymerize unlike the hydroxy acid mixtures obtained by cutin hydrolysis (de Vries, 1970b; Baker and Holloway, 1970). The major disadvantage of this method is the fact that functions such as oxirane and carbonyl that are susceptible to $LiAlH_4$ give rise to hydroxyl groups that can be distinguished from the naturally occurring ones only with the use of $LiAlD_4$.

In the limited number of examples of cutin examined by our procedure, two distinct families of hydroxy fatty acids, one

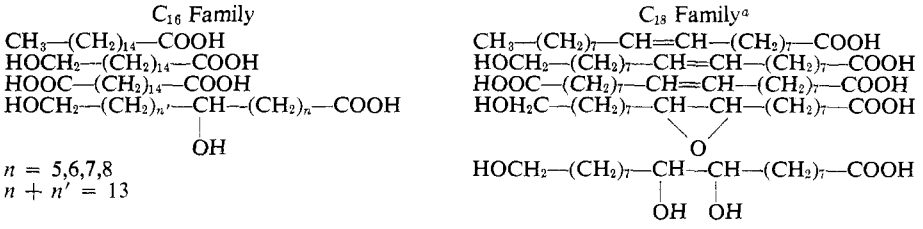


FIGURE 14: The two hydroxy fatty acid families found in cutin. [a] Analogs containing a Δ^{12} unsaturation were also present.

containing C_{16} members and the other C_{18} members were detected (Figure 14).

Two types of cutin with different distribution of these hydroxy fatty acid families were encountered. One type, typified by *V. faba* (Kolattukudy and Walton, 1972) and papaya cutin, contained a dihydroxypalmitic acid as the major cutin component with variable amounts of ω-hydroxy and non-hydroxy fatty acids. Apparently rapidly growing plants use the usual end product of fatty acid synthetase, without further elongation or desaturation, for cutin synthesis. The second type of monomer pattern encountered in the cutins of the more slowly growing tissues contained in addition to the C_{16} components, C_{18}-polyhydroxy fatty acids. The results presented above show unambiguously the widespread occurrence of 9, 10-epoxy-18-hydroxystearic acid and its Δ^{12} unsaturated analog in plants. Although the saturated analog had been identified by chemical degradation as a component of apple cutin (Brieskorn and Boss, 1964), in a more recent glc-ms analysis of apple cutin such a component was not detected (Eglinton and Hunneman, 1968). In the latter study the epoxy compounds might have undergone rearrangements during the procedures used and the major unidentified components may represent such rearrangement products.

The structure determinations described in this paper have obvious biogenetic implications. In the C_{16} family of acids neither vicinal diol functions nor double bonds were detected. Therefore the in-chain hydroxyl group may be suspected to originate by a direct hydroxylation mechanism. In fact our biosynthetic studies show that such a mechanism is highly probable (Kolattukudy, 1970c; Kolattukudy and Walton, 1972). The occurrence of a hydroxyl group at C-7, C-8, C-9, or C-10 (Holloway and Deas, 1971) position is also consistent with such a hypothesis.

On the other hand, in the C_{18} family of acids the major components contain a double bond at C-9, an epoxide at the 9,10 position or vicinal diol at the 9,10 position. These results suggest that oleic acid may be the precursor of this family of acids and that epoxidation followed by hydration of the epoxide may be the mechanism of introduction of the in-chain functional groups in these compounds. This hypothesis is strongly supported by our biosynthetic studies (Kolattukudy *et al.*, 1971; T. J. Walton and P. E. Kolattukudy, unpublished results). The results presented in this paper show that the unsaturated epoxy acid and the unsaturated 9,10,18-trihydroxystearic acid contain a double bond at C-12 and therefore these components may originate from linoleic acid by a series of reactions analogous to that suggested for the biosynthesis of the saturated counterpart. Our biosynthetic studies with [1-^{14}C]linoleic acid do indeed support this hypothesis (P. E. Kolattukudy and R. P. S. Kushwaha, unpublished results). Thus the structural studies on cutin monomers reported here give important clues about the mechanisms involved in their biosynthesis.

Acknowledgments

We thank Mrs. Susan Grant, Mrs. Linda Brown, Mr. Lawrence Ashly, and Mr. R. P. S. Kushwaha for technical assistance, Mr. Charles Oldenburg for providing us with the plant materials, and Dr. Milton Zucker for a critical reading of the manuscript.

References

Baker, E. A., and Holloway, P. J. (1970), *Phytochemistry 9*, 1557.

Batt, R. D., Hodges, R., and Robertson, J. G. (1971), *Biochim. Biophys. Acta 239*, 368.

Brieskorn, C. H., and Boss, J. (1964), *Fette, Seifen, Anstrichm. 66*, 925.

Brieskorn, C. H., and Reinartz, H. (1967), *Z. Lebensm.-Unters.-Forsch. 135*, 55.

Crisp, C. E. (1965), Ph.D. Thesis, Univ. of Calif., Davis, Calif.

de Vries, H. A. M. A. (1969), *Acta Bot. Neerl. 18*, 589.

de Vries, H. A. M. A. (1970a), *Acta Bot. Neerl. 19*, 36.

de Vries, H. A. M. A. (1970b), *Acta Bot. Neerl. 19*, 41.

Diekman, J., Thomson, J. B., and Djerasi, C. (1968), *J. Org. Chem. 33*, 2271.

Eglinton, G., and Hamilton, R. J. (1967), *Science 156*, 1322.

Eglinton, G., and Hunneman, D. H. (1968), *Phytochemistry 7*, 313.

Eglinton, G., Hunneman, D. H., and McCormick, A. (1968), *Org. Mass Spectrom. 1*, 593.

Fremy, M. E. (1882), *Ann. Sci. Nature, Ser. 6*, 360.

Holloway, P. J., and Deas, A. H. B. (1971), *Chem. Ind. (London)*, 1140.

Holloway, P. J., and Deas, A. H. B. (1971), *Phytochemistry 10*, 2781.

Holloway, P. J., Deas, A. H. B., and Kabaara, A. M. (1972), *Phytochemistry* (in press).

Kolattukudy, P. E. (1970a), *Annu. Rev. Plant Physiol. 21*, 163.

Kolattukudy, P. E. (1970b), *Plant Physiol. 46*, 759.

Kolattukudy, P. E. (1970c), *Biochem. Biophys. Res. Commun. 41*, 299.

Kolattukudy, P. E., and Walton, T. J. (1972), *Biochemistry 11*, 1897.

Kolattukudy, P. E., Walton, T. J., and Kushwaha, R. P. S. (1971), *Biochem. Biophys. Res. Commun. 42*, 739.

Lee, B. (1925), *Ann. Bot. 39*, 755.

Legg, V. H., and Wheeler, R. V. (1929), *J. Chem. Soc. 31*, 2444.

Matic, M. (1956), *Biochem. J. 63*, 168.

Mazliak, P. (1968), *Progr. Phytochem. 1*, 49.

McCloskey, J. A., Stillwell, R. N., and Lawson, A. M. (1968), *Anal. Chem. 40*, 233.

Pace-Asciak, C., and Wolfe, L. S. (1971), *Biochemistry 10*, 3657.

Sharkey, A. G., Friedel, R. A., and Langer, S. H. (1957), *Anal. Chem. 29*, 770.

Shishiyama, J., Araki, F., and Akai, S. (1970), *Plant Cell Physiol. 11*, 323.

Structure and Biosynthesis of the Hydroxy Fatty Acids of Cutin in *Vicia faba* Leaves†

P. E. Kolattukudy* and T. J. Walton

ABSTRACT: Cutin, the lipid polymer, which is the structural component of cuticle, was isolated from *Vicia faba* leaves by a combination of enzymatic and chemical techniques. Exhaustive hydrogenolysis of powdered cutin followed by thin-layer chromatography and a combination of gas chromatography and mass spectrometry showed that this cutin was composed of 10,16-dihydroxypalmitic acid (77.8%), 9,16-dihydroxypalmitic acid (7.1%), 16-hydroxypalmitic acid (7.1%), palmitic acid (3.6%), stearic acid (2.2%), and oleic acid (0.8%). Disks from young leaves of *V. faba* incorporated sodium [1-^{14}C]acetate (2.4%), [1-^{14}C]palmitic acid (16%), [1-^{14}C]stearic acid (1.5%), and [1-^{14}C]oleic acid (2.7%) into cutin. Only the first two substrates labeled the dihydroxy acids while all four substrates labeled the nonhydroxy and ω-hydroxy acid fractions. The polar acids derived from [1-^{14}C]-palmitic acid were shown to be 16-hydroxypalmitic acid and 10,16-dihydroxypalmitic acid containing a little 9,16-positional isomer by radiochromatographic techniques in conjunction with analysis of acetylated derivatives and chromic acid oxidation products. The time course of incorporation of [1-^{14}C]palmitic acid into cutin acids showed that at all times the dihydroxypalmitic acid contained most of the ^{14}C and that labeled hydroxy acids did not accumulate in the soluble lipids. Biosynthetically labeled 16-hydroxypalmitic acid was incorporated into cutin directly and after conversion into dihydroxypalmitic acid, when the substrate was fed to disks of young *V. faba* leaves in the presence of oxygen. Exogenous labeled dihydroxypalmitic acid was also directly incorporated into cutin under similar conditions. These results suggest that the following reaction sequence is involved in the biosynthesis of cutin in *V. faba* leaves: palmitic acid → 16-hydroxypalmitic acid → 10,16-dihydroxypalmitic acid → cutin. These reactions occurred much less rapidly in old leaves. Neither [10-^{14}C]palmitoleic acid nor [10-^{14}C]palmitelaidic acid was converted into dihydroxypalmitic acid in *V. faba* leaves, showing that a Δ^9 double bond was not involved in the in-chain hydroxylation. A direct hydroxylation was also indicated by the fact that [9,10-^{3}H,1-^{14}C]palmitic acid fed to *V. faba* leaf disks gave 10,16-dihydroxypalmitic acid with a ^{3}H:^{14}C ratio of 78% of that in the substrate. Phenanthroline and bipyridyl inhibited the hydroxylations of palmitic acid and this inhibition could be partially reversed by Fe^{2+}. These results suggest that a mixed-function oxidase-type enzyme catalyzes the direct hydroxylation at C-10 of ω-hydroxypalmitic acid.

Plant cuticle is made of a polymer, cutin, which is embedded in wax. The chemistry and biosynthesis of cuticular wax has been studied quite extensively in recent years (Eglinton and Hamilton, 1967; Kolattukudy, 1970a; Mazliak, 1968). However, cutin has attracted little attention until recently. This polymer on hydrolysis gives a variety of hydroxy-fatty acids (Matic, 1956; Baker and Holloway, 1970; Crisp, 1965; de Vries, 1969). The hydroxy acids of apple fruit cutin have been analyzed by a combination of gas–liquid chromatography and mass spectrometry (Eglinton and Hunneman, 1968). More recently other plant cutins have been subjected to similar analysis (Holloway and Deas, 1971). The major hydroxy acids thus far observed are ω-hydroxypalmitic acid, ω-hydroxyoleic acid, 10,16-dihydroxypalmitic acid, 18-hydroxy-9,10-epoxystearic acid, and 9,10,18-trihydroxystearic acid (Kolattukudy and Walton, 1972).

Alkaline hydrolysis leaves behind a residue which may consist of up to 45% of the cutin (Crisp, 1965). This residue contains ether bonds and peroxide bridges. For biosynthetic studies a more quantitative method of isolating the cutin monomers from a very small quantity of tissue was needed and such a method has been developed (Kolattukudy, 1970b; Walton and Kolattukudy, 1972b; Kolattukudy *et al.*, 1971).

Autooxidative processes involving the leaf lipids were postulated to be the mechanism for cutin biosynthesis (Priestly, 1943; Huelin, 1959). The only experimental evidence concerning cutin biosynthesis consisted of measurements of lipoxidases and other oxidases that were manifested as a result of wounding of leaves (Heinen and Brand, 1963). In this report we describe the results of the first systematic attempts to study the biosynthesis of cutin with the use of specifically labeled substrates, a novel cleavage technique, and a combination of gas chromatography and mass spectrometry.

On the basis of these studies we propose a biosynthetic pathway for cutin biosynthesis in *Vicia faba* leaves.

Experimental Section

Plants. Broad bean (*V. faba*) plants were grown from seed purchased from Burpee Co., California, in a soil–sand–peat-moss (1:1:1) mixture under wide spectrum Growlux (very

† From the Department of Agricultural Chemistry, Washington State University, Pullman, Washington. *Received November 29, 1971.* Scientific paper 3789, project 3001, College of Agriculture, Washington State University. This work was supported in part by a grant from National Science Foundation GB 23081.

* Author to whom inquiries should be made.