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ISOLATION AND MOLECULAR STRUCTURE OF AN OLIGOMER PRODUCED ENZYMATICALLY FROM THE CUTICLE OF LIME FRUIT

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Key Word Index—*Citrus aurantifolia*; Citrus; lime; fruit; cuticle; cutin; molecular structure; mass spectrometry; NMR.

Abstract—The selective enzymatic cleavage of primary ester linkages with porcine pancreatic lipase has been used to investigate the structural framework of *Citrus aurantifolia* (lime) cutin, a cross-linked protective biopolymer obtained from the outer fruit skins. Among the soluble oligomeric products isolated from this reaction is a novel 1307-amu pentamer based on C_{16} fatty acids and hydroxyfatty acids and assembled solely with linkages between the esters of secondary alcohols. This compound was isolated by high-performance preparative thin-layer chromatography, and a structure was proposed using evidence from electron-impact mass spectrometry supported by solution-state NMR spectroscopy. The building blocks of the pentamer include 10,16-dihydroxyhexadecanoic acid, hexadecanoic acid, 10-hydroxyhexadecanoic acid, and 1,7,16-trihydroxyhexadecanol, which can participate in polymer chain elongation as well as the formation of cross-link structures. Three of the four building blocks of the pentamer are among the major monomeric constituents of citrus cutins, but ω-hydroxyoxo acid monomers are absent from the soluble oligomers produced by enzymatic reaction. The resistance of fruit cutins to these breakdown procedures suggests that despite a predominance of primary ester linkages, a support network of secondary ester cross-links may limit the accessibility of interior ester bonds to the lipase enzyme. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Cutin, the support polymer of leaf and fruit cuticle in higher plants, is an insoluble biopolyester composed of epoxy- and hydroxyfatty acids [1]. The integrity and resiliency of this membrane are essential to the role of plant cuticle in waterproofing and for protection against fungal attack. During the last two decades, numerous investigations of cutin's molecular structure have focused upon the monomeric constituents derived from chemical depolymerization procedures [2, 3]. Although these studies provide a firm foundation for understanding cutin's polymeric structure and physiological function, they give no direct information on cross-links or other essential covalent connectivities. Even the compositional information may be incomplete, since a substantial unreacted residue often remains after the degradative treatments.

Alternatively, it has been possible to circumvent the intractability of intact cuticular polymers by using newly accessible investigative tools such as high-resolution solid-state nuclear magnetic resonance (NMR) and Fourier transform infrared spectroscopy (FT-IR) [4–7]. Such direct approaches have yielded both confirmatory and new information regarding the esters and aromatic groups present in plant cuticular materials; NMR relaxation measurements have provided a unique probe of dynamic properties related to cuticular resiliency [8]. Nevertheless, the spectral overlap often exhibited by biopolymers may obscure subtle structural features, and neither NMR nor FT-IR spectroscopy methods can readily verify the presence of covalent linkages in solid compounds that lack isotopic labels.

The present work takes a middle course in the investigation of plant cuticular structure at the molecular level. These studies draw on early suggestions that enzymatic hydrolysis might prove useful for structure elucidation [9] and prior reports that oligomers are formed when fruit cutin is treated with *Fusarium* cutinase or porcine pancreatic lipase [10, 11]. Using the latter enzyme, which is specific for esters of primary alcohols and has a requirement for substrate aggregates [12], a mixture of monomeric and oligomeric materials has been obtained from the fruit of limes. A novel pentamer that retains cutin cross-links has been

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identified using mass spectrometry (MS) and solution-state NMR spectroscopy, and the molecular structure of this compound has been compared with the reported monomeric constituents of fruit cutins [2, 3, 13].

RESULTS

Isolation and preliminary structural analysis

Up to 11% by weight of the lime cutin was hydrolyzed by porcine pancreatic lipase, and the typical yield of isolated soluble products was 2%. This degree of breakdown was smaller than that observed with industrial lipase treatments of apple cutin, for which up to 16% hydrolysis has been achieved using *Pseudomonas fluorescens* lipase and an activating detergent [14]. Since the specific activities of our lipases met or exceeded those enzymes for which some cuticular breakdown has been demonstrated previously [14], this result was unanticipated. The lower yield obtained upon enzymatic treatment of our material was nevertheless in line with the known resistance of lime cutin to alkaline hydrolysis, especially when compared with other fruit cutins [13].

Five bands corresponding to nonpolar materials (and a portion remaining at the origin) were eluted with high-performance thin-layer chromatography using methylene chloride/*n*-hexane as a developing solvent. Examination of these materials by LC-MS yielded molecular ions in the range of 1200–1500 amu. For the two fastest migrating bands, the presence of ester carbonyl groups was suggested by FT-IR bands at 1725 cm^{-1} as well as by ^{1}H NMR resonances at 2.05 ppm ($CH_2C{=}O$) and 4.3 ppm ($CHOC{=}O$) [15]. Together with prior compositional analyses of cutin monomers [2, 3], these results supported tentative identification of the hydrolysis products as ester oligomers. As in more exhaustive chemical treatments of the lime fruit cutin, no phenolics were evident among the soluble products of lipase hydrolysis [1, 2, 13]. The band that eluted with an R_f value of 0.78, corresponding to the second least polar component, was selected for further study, since it was relatively abundant and migrated as a single spot using several developing solvents.

High-mass region of the mass spectrum

The electron-impact mass spectrum of the oligomer (Fig. 1) showed a cluster of peaks at *m/z* 1308, 1309, 1310, and 1311, expanded in Fig. 2, top. The abundant peak at *m/z* 1308 was assigned as the protonated molecular ion $(M+H)^{+\bullet}$, corresponding to the empirical formula $C_{80}H_{154}O_{12}$. This result may be attributed to facile protonation of any of several exposed ester carbonyl functions (*vide infra*). Isotopic abundance peaks corresponding to this latter ion were expected and found at *m/z* 1309 $(MH+1)^{+\bullet}$ and 1310 $(MH+2)^{+\bullet}$, respectively. The theoretical intensities of ions for the molecular formula $C_{80}H_{154}O_{12}$ are 100% (1307), 92% (1308), 44% (1309), and 15% (1310), in reasonable agreement with what was observed experimentally for the protonated molecule.

The proposed molecular structure of the oligomer was deduced from the mass spectra in Figs 1 and 2 by reference to Scheme 1. As will be demonstrated below, its pentameric zig-zag ester structure is composed of C_{16} fatty-acid monomers [1, 2] with masses 273 (A), 270 (B and D), 254 (C), and 239 (E) amu, respectively. (A pentamer containing two C_{18} monomers may also be present at a level of about 4%, corresponding to a molecular ion at *m/z* 1335 observed in the mass spectrum.)

For short-chain esters, $R^{+\bullet}$ ions are typically prominent in the mass spectrum; for the long-chain analogs, a more common diagnostic peak is $R{-}C{\equiv}O^{+\bullet}$ [15]. Thus the high-mass spectral patterns shown in Fig. 2 (bottom) were attributed to the formation of such $R{-}C{\equiv}O^{+\bullet}$ ions by the branched cutin oligomer. Following Scheme 1, for instance, cleavage [k] generates the moiety $(B+C+D+E)^{+\bullet}$ which, upon loss of two molecules of water from primary alcoholic groups [15], accounts for the major peak at *m/z* 996 $(B+C+D+E{-}2H_2O{-}H)^{+\bullet}$. (A second oligomer, which eluted with an R_f value of 0.85 and would correspond to (B+C+D+E), also displayed *m/z* 996 $(B+C+D+E{-}2H_2O{-}H)^{+\bullet}$ as the most prominent peak in its mass spectrum.)

The $C{\equiv}O$ functionality of the fragment $(B+C+D+E{-}2H_2O{-}H)^{+\bullet}$ is then lost to produce an ion at *m/z* 968. The observed fragmentation pattern was thus consistent with the presence of two primary alcoholic groups within the tetramer fragment $(B+C+D+E)^{+\bullet}$. The remaining fragment (A) has a mass of 273; it loses three protons readily from its alcohol groups and appeared in the mass spectrum at *m/z* 269. A plausible C_{16}-based structure for A contains two primary alcohol groups, as shown. Precedent for this structure comes from lower plants, in which a positional isomer of monomer A has been reported [16]. Our proposed placement of the mid-chain hydroxyl group is discussed below.

In similar fashion, the trimer $(C+D+E)^{+\bullet}$ results from cleavage [1] of the oligomer; loss of water from a primary alcohol is then consistent with the appearance of prominent peaks in the mass spectrum at *m/z* 744 $(C+D+E{-}H_2O{-}H)^{+\bullet}$ and 743 $(C+D+E{-}H_2O{-}2H)^{+\bullet}$. Coupled with our observation of the species $(B+C+D+E{-}2H_2O)^{+\bullet}$, it was concluded that monomer B contained one primary alcohol group.

Cleavage [n] would generate a tetramer $(A+B+C+D)^{+\bullet}$, but the mass spectrum suggested that cleavages [k] and [n] occurred together to form a more stable trimer. This latter fragment can eliminate two molecules of water, consistent with the observation of a strong peak at *m/z* 761 $(B+C+D{-}2H_2O+2H)^{+\bullet}$. As described above, this fragment loses $C{\equiv}O$ to produce a peak at *m/z* 733.

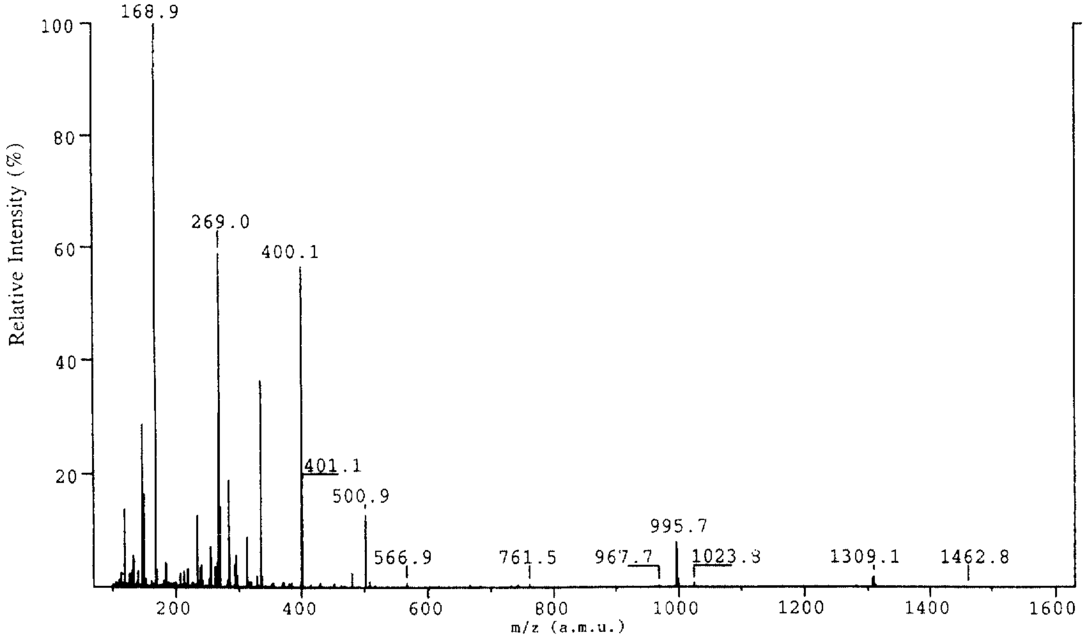


Fig. 1. Electron-impact mass spectrum (EI-MS) of a lime cutin oligomer, obtained with an accelerating potential of 5 keV to span the range *m/z* 100–1500 atomic mass units (amu). The peak intensities are normalized with respect to the base peak (100%) at *m/z* 169.

Since B has one primary alcohol group, the other such moiety must reside on either C or D. As discussed earlier, the fragment $(A-3H)^{+\cdot}$ resulting from cleavage [k] appeared at *m/z* 269. Fragment E from cleavage [n] cannot lose or gain protons easily; hence it appeared in the mass spectrum at *m/z* 239. The hexadecanoic acid monomer (E) has been isolated previously from several fruit cutins [13, 17].

Our conclusion regarding the presence of alcoholic groups on monomers C and D was confirmed by a crosscheck within the mass spectrum. According to Scheme 1, simultaneous cleavages [l] and [n] generate a dimer than can lose water, giving $(C+D+H-H_2O)^{+\cdot}$ at *m/z* 508 as observed. The primary alcohol group that accounts for this fragmentation pattern could thus reside on either C or D.

Information regarding the primary alcohol structures also came from several diagnostic β-cleavages. For instance, cleavage [m] produces a trimer with *m/z* 799 $(A+B+C+H)^{+\cdot}$. Then, a prominent ion at *m/z* 667 is produced by cleavages β to the alcohols, 1,3-hydrogen transfers, and elimination of resonance-stabilized vinylic alcohols. Since the trimer $(A+B+C)^{+\cdot}$ has *m/z* 798, the observed loss of 132 amu confirms the presence of three primary alcohol groups within this fragment. We know independently that one and two of these groups are derived from B and A, respectively (*vide supra*). Thus monomer C has no primary alcohol groups and D must have one. Considered together, these features of the mass spectrum are consistent with the oligomer structure depicted in Scheme 1.

Several fragments of the isolated oligomer from lime cutin may also undergo the McLafferty rearrangement, a diagnostic transformation for carbonyl-containing compounds with γ-hydrogens [15]. Although the oligomer has four secondary ester linkages that could be sites for this process, the only unconstrained moieties within its zig-zag structure are monomers A and E. The palmitoyl group is a particularly favorable candidate for the rearrangement, since it can rapidly form a six-membered transition state with an appropriately located heteroatom and an abstractable hydrogen. As detailed in Scheme 1, McLafferty rearrangements of monomer E, both within the pentamer and in the fragment $(C+D+E)^{+\cdot}$, account for the observed peaks at *m/z* 1023 and 567, respectively.

Low-mass region of the mass spectrum

In addition to indicating the presence of molecular fragments as discussed above, the ions at low mass provided essential confirmatory information regarding the monomeric constituents and cross-link structures of lime cutin. Thus the intense peak at *m/z* 269 was attributed to the species $HOCH_2-(CH_2)_5-C(O)-(CH_2)_8C{\equiv}O^{+\cdot}$, which was derived from fragments A, B, and D. The incorporation of monomers B and D within the lime cutin matrix is expected, since we and others have identified 10,16-dihydroxyhexadecanoic acid as one of its major monomeric constituents [13, 18]. An isomer of mono-

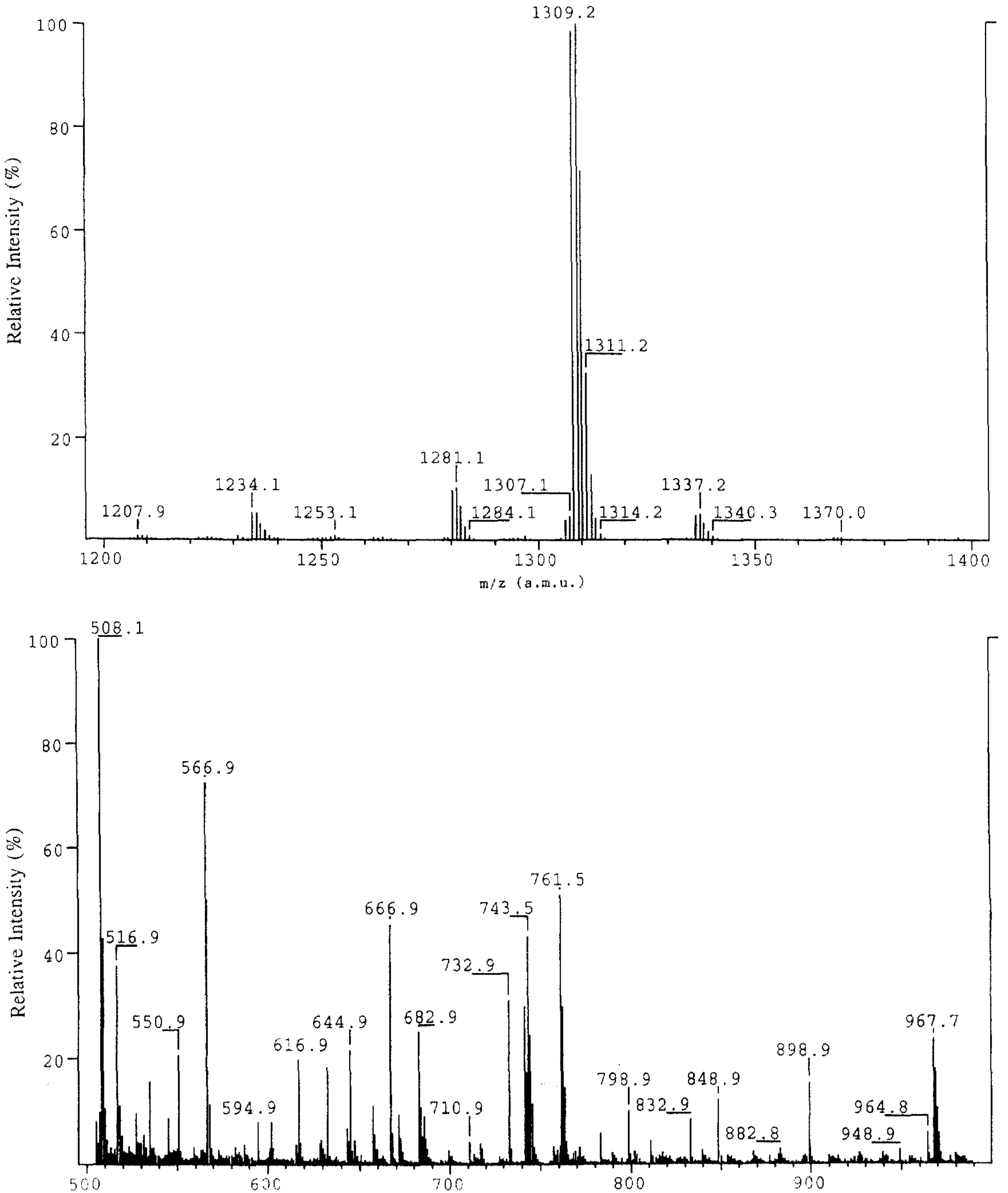
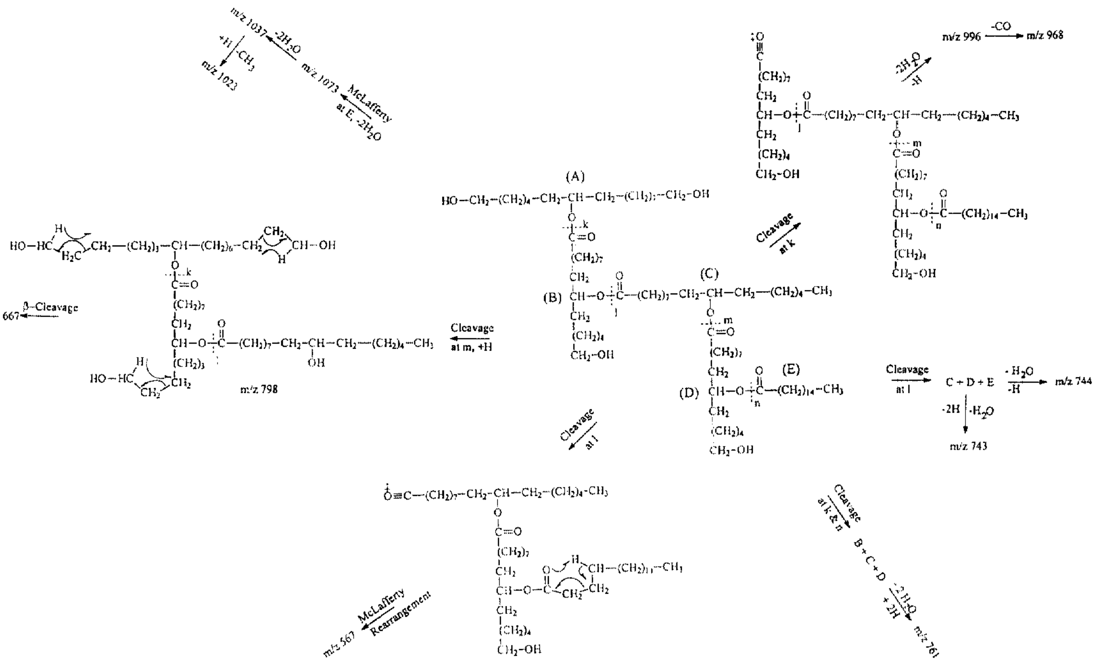


Fig. 2. Expansions from Fig. 1: the molecular-ion region of the mass spectrum (top) and the fragment ions with *m/z* 500–1000 (bottom). Each spectrum has peak intensities normalized to the largest peak in the region.

mer A has also been reported as a constituent of some plant cutins [16].

The position of secondary alcohol linkages involving monomers A, B, and D was confirmed by diagnostic α-cleavages to produce the species $^{+}\mathrm{O{\equiv}C{-}(CH_2)_8{-}CHO}$, the base peak in the mass spectrum at *m/z* 169. The unusual monomer C and its linkage to D were evidenced by the strong peak at *m/z* 400, corresponding to the species $[\mathrm{H_3C{-}(CH_2)_6{-}CH_2{-}O{-}C(O){-}(CH_2)_8{-}CH(OH){-}(CH_2)_5{-}CH_2OH}]^{+\bullet}$. In our proposed scheme, this latter ion is produced by simultaneous cleavages [1] and [n] to form (C + D), followed by α-cleavage of the resulting dimer. Alternatively (C+D) may fragment to yield C (*m/z* 255); the location of the secondary alcohol at the 10 position is supported by observation of the species



Scheme 1. EI-MS fragmentation patterns for an oligomer isolated after treatment of lime cutin with lipase enzymes.

$^{+}O\equiv C-(CH_2)_8-CHO$ at *m/z* 169, though a smaller peak observed at *m/z* 154 could correspond to $^{+}O\equiv C-(CH_2)_7-CHO$ and thus be derived from a 9-hydroxyhexadecanoic acid monomer.

Proton NMR spectrum

As noted above, prelimiinary ^1H NMR spectral data for the cutin oligomer were consistent with the presence of ester functional groups. Detailed structural analysis was precluded by the small amount of available oligomer and its likely molecular complexity, but tentative identifications were made for the major functional groups present in the pentamer. The peak assignments followed chemical-shift trends reported for phosphodiesters, triglycerides, and natural waxes [19–21]. ^1H resonances were observed corresponding to methyl groups, bulk methylenes, methylenes situated at α and β positions to esters, methylenes of primary alcohols, and methines bound to oxygen. Neither alkenes nor aromatics were evidenced in the NMR spectrum. Additional magnetic inequivalences were observed for many proton types, as expected for a pentamer with four chiral centers and the possibility of hydrogen-bonding interactions and/or conformationally ordered structures. All of these observations were consistent with the fragments deduced from our EI-MS data and the known monomeric constituents of lime cutin [13, 18].

DISCUSSION

This report represents the first detailed structural characterization of an oligomer from the natural polyester of lime cuticle. Using a strategy first suggested by Purdy and Kolattukudy [9, 10], we have relied on the selectivity of enzymatic hydrolysis to produce soluble cutin fragments that nevertheless retain essential cross linkages of the original structure. In order to determine the molecular structure of one such oligomeric product, both EI-MS and NMR experiments have been carried out.

The high-mass region of the mass spectrum provides the molecular weights of the pentameric products as well as the trimers and tetramers formed by cleavage of its ester bonds. Both the covalent connectivities between dimeric fragments and the positions of mid-chain hydroxylation within the monomers have been deduced from ions in the mass range 500–1000. The presence of primary alcohol groups, which are expected to result from enzymatic hydrolysis of primary esters in the cutin polymer, has been confirmed by loss of water and diagnostic β-cleavages in the MS fragmentation patterns of the oligomer. As demonstrated previously for the high-molecular-weight estolides found in coniferous waxes [22], structural elucidation of these plant materials may be accomplished using MS as the primary diagnostic tool. Taken together, the EI-MS data for lime fruit cutin are accounted for by a pentameric zig-zag structure composed entirely of secondary ester linkages. This finding represents the most direct verification to date of covalent cross-links present in this class of cuticular polymers.

In a complementary manner, both the low-mass portion of the mass spectrum and the ^1H NMR data yield structural information regarding the monomeric constituents of the lime cutin oligomer. Both the ^1H

NMR resonances and the prominent MS peak at m/z 269 support the identification of 10,16-dihydroxyhexadecanoic acid (B and D) and 1,7,16-trihydroxyhexadecanol (A). The dihydroxy species has been found previously to be an abundant monomer in fruit and vegetable peels [17, 23], and it has also been identified as one of the major chemical depolymerization products of lime cutin [13]. The triol A could be formed from the dihydroxy acid B following a biosynthetic pathway described for wax esters in leaves of *Brassica oleracea* [3]; one of its positional isomers has been reported as a minor constituent of cutins in lower plants [15]. All functional groups found in monomers A and B are also evident in the solid-state ^{13}C NMR spectra of intact lime cutin [24].

The novel midchain-hydroxylated monomer C, which yields a prominent ion in the mass spectrum only when linked to D and/or E, has been identified as a significant constituent of lime cutin [13]; its C_{15} analog is also present in cutins from conifers and coffee [25, 26]. Finally, monomer E appears in the mass spectrum at m/z 239 and has been identified among the products of several chemical depolymerization procedures [1, 13, 23]. These building blocks of the cutin polymer could be related biosynthetically: hexadecanoic acid (E) could produce 10,16-dihydroxyhexadecanoic acid (B, D) (by ω-hydroxylation and then midchain hydroxylation [27, 28]), and also 10-hydroxyhexadecanoic acid (C) (by midchain hydroxylation). As noted above, both solution-state and solid-state NMR data are consistent with the proposed structures.

Nevertheless, the commonly occurring C_{16} ω-hydroxyoxo acid monomers [13, 18] are notably absent from the structure of our oligomeric fragment. Such constituents can form chain-lengthening primary ester linkages or, if esterified to the secondary alcohol of a different monomer, they might participate in cutin cross-links. In retrospect this negative finding is not surprising: the hydroxyoxo acid monomers may reside within the nine-tenths of the cutin polymer that is inaccessible to porcine pancreatic lipase, or they may be present among our monomeric breakdown products. In independent experiments, we have recovered the major C_{16} ω-hydroxyoxo acid monomer of lime fruit cutin among the soluble dimers produced by treatment of the polyester with iodotrimethylsilane, a mild chemical reagent with a documented preference for cleavage of sterically hindered esters [29–31].

Several conclusions may be drawn from the foregoing enzymatic depolymerization studies of lime fruit cutin. First, the resistance of lime and apple cutins to lipase hydrolysis suggests that despite a preponderance of primary ester linkages [1], covalent cross-links render many potential cleavage sites inaccessible within the cuticular network. The absence of phenolics among the soluble breakdown products indicates their possible involvement in these resistant cross-linkages. The low yield of hydrolysis products could derive from steric hindrance of sites at the polymer interior or from the lack of an aqueous milieu appropriate for vigorous lipase activity. Secondly, despite the modest overall yield of soluble products, the oligomer identified in this work is surprisingly representative of the established lime cutin monomeric constituents. Three of its four building blocks are prominent among the C_{16} hydroxy acids that comprise the biopolyester [13]; conversely, the known monomers appear in the oligomer with one notable exception. The "missing" hydroxyoxo hexadecanoic acids are released only upon more exhaustive chemical treatments with KOH, methanolic BF_3, or iodotrimethylsilane, indicating their possible role in cuticular protective functions. Thirdly, the cross-links that survive lipase hydrolysis are formed invariably between secondary alcohols and primary ester carbonyl groups, underscoring the role of 10,16-dihydroxyhexadecanoic acid as an ideal precursor for the support polyester of leaf and fruit cuticles.

EXPERIMENTAL

Isolation of lime cutin

The biopolyester was isolated from the skin of limes (*Citrus aurantifolia*) by published procedures [7]. In brief, the procedure involved three steps: (1) peeling and separation of the cuticle by treatment with *Aspergillus niger* pectinase; (2) enzymatic degradation of polysaccharides with cellulase, pectinase, and hemicellulase; (3) exhaustive dewaxing by Soxhlet extraction with methanol, methylene chloride, and tetrahydrofuran. All chemicals were obtained from Sigma or Aldrich chemical companies. Typically, 250 limes yielded 4.5 g of dry cutin.

Enzymatic depolymerization

A Wig-L-Bug amalgamator (Spex Industries, Edison, NJ) was used to produce small cutin flakes. A 500 mg sample of cutin was dispersed in 14.28 ml of 60 mM phosphate buffer (pH = 7.6) and shaken at 150 rpm and 30° for about 2 h. After addition of purified porcine pancreatic lipase (125 units/mg protein, a gift of Dr W. D. Behnke) or *Rhizopus arrhizus* lipase (13,000 units/mg, Sigma Chemical Co.) to obtain a final concentration of 2.5 μg/ml, the reaction mixture was shaken for 20 h at 30° [11] in a thermostatically controlled incubator. Specific activities for the enzymes are quoted as μmol fatty acid produced per min by hydrolysis of tributyrin and olive oil, respectively. Similar degrees of cutin hydrolysis were obtained with both enzyme preparations.

Isolation of soluble products

Upon filtering, the solid polymeric residue was washed with 5% methanol–water. After addition of sodium chloride to the aqueous portion, extraction was carried out with distilled methylene chloride

(3 × 20 ml). The extract was washed with distilled water (2 × 10 ml), kept over anhydrous magnesium sulfate for 1 h, and evaporated to dryness under reduced pressure. A typical procedure afforded 9.1 mg of viscous liquid. A second extraction of the aqueous wash with 2:1 methylene chloride–methanol yielded 2.5 mg of additional material.

High-performance preparative thin-layer chromatography (HPTLC) was conducted using silica gel 60 plates (10 × 20 cm, 0.5 mm thickness) from EM Science, 3:2 methylene chloride–*n*-hexane as the customary developing solvent, and iodine for visualization. Five bands were visible; the band at $R_f = 0.78$ was recovered and eluted with 9:1 methylene chloride–methanol to yield 0.9 mg of final product.

Spectroscopy

Electron-impact mass spectrometry (EI-MS) was carried out at 70 eV using a Finnigan MAT-90 double focusing mass spectrometer. An accelerating potential of 5 keV allowed observation of ions with m/z in the range 40–1500 amu. The probe and sample temperatures were 270° and 250°, respectively. The low-resolution mass spectral data were acquired at a mass resolving power of 2000.

For LC-MS, spectra were obtained with a Finnigan MAT TSQ 70 mass spectrometer interfaced to a Waters 600-MS HPLC system. The studies were carried out with a Waters reverse phase C18 column (25 cm × 2.0 mm i.d., packed with 5-μ particles), a flow rate of 1 ml/min, and a methanol–water (30:70) mixture containing 0.05% trifluoroacetic acid for elution.

^{1}H nuclear magnetic resonance (NMR) spectra were acquired at 400 MHz on a JEOL GX-400 spectrometer. Samples were dissolved in $CDCl_3$ containing 1% tetramethylsilane (Wilmad Glass Company, Buena, NJ) to provide both a field-frequency lock and an internal chemical shift standard. Typical spectra were acquired with 2500 transients, using 16K time-domain points and a spectral width of 5500 Hz. ^{1}H NMR of cutin pentamer: δ 0.9 (CH_3, *t*, 6H); 1.3–1.6 ($(CH_2)_n$, two *br s*, ~ 110H); 2.05 ($C\underline{H}_2COOR$, *m*, 8H); 3.49 ($C\underline{H}_2OH$, *m*, 2H); 3.62 ($C\underline{H}_2OH$, *t*, 2H); 3.75 (H-bonded $C\underline{H}_2OH$, *m*, 4H), 4.05, (CHOCOR, *t*, 1H). 4.3 (CHOCOR, *t*, 2H) and 4.4 (CHOCOR, *m*, 1H) ppm.

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