DOI:10.1021/jf1015297

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Linear and Branched Poly(ω-hydroxyacid) Esters in Plant Cutins

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Poly(ω-hydroxyacid) ester oligomers were obtained from the cutin of four plant cuticles by partial depolymerization methanolysis. The structure of these oligomers was determined by electrospray ionization coupled to tandem mass spectrometry (ESI-MS/MS) analysis of their lithium-adducts, showing which monomers were present and their relative position. Dimers up to heptamers were identified, in a total yield of oligomers of up to 50% of the total cutin content. Two main structural types of poly(ω-hydroxyacid) ester oligomers were found. Poly(10,16-dihydroxyhexadecanoic acid) esters were the main oligomers in the cutin of *Lycopersicum esculentum* fruit, with a predominantly branched structure. The 1D and 2D NMR analysis of these oligomers showed that inter-monomer linkages were mostly in secondary midchain hydroxyls, in a ratio of 4.5 to 1, compared with esterification in the primary ω-hydroxyls. In *Hedera helix* leaves cutin, poly(9-epoxy-18-hydroxyoctadecanoic acid) esters were the dominant oligomers, composed of monomers linearly linked head-to-tail. It is proposed that the cutin polyesters have different poly(ω-hydroxyacid)ester domains, either built of linear chains, where the C_{18}-epoxy ω-hydroxyacids are dominant, or forming a highly branched network, where the C_{16}-dihydroxy ω-hydroxyacids predominate. It is hypothesized that the C_{18} linear polyester can account for the ordered lamellae seen at ultrastructural level in *H. helix* cuticle, and that the C_{16} mostly branched polyester will be the basis of the reticulate structure seen in *L. esculentum* cuticle.

KEYWORDS: Cutin; poly(ω-hydroxyacid) esters; cutin oligomers; cuticle; *Lycopersicum esculentum*; *Citrus aurantium*; *Prunus laurocerasus*; *Hedera helix*

INTRODUCTION

The cuticle is a thin membrane that covers the aerial organs of plants of primary origin, namely, leaves and fruits. The cuticle acts as a skin, protecting against biological attack and weather variability, and allowing controlled exchanges, namely of water vapor, with the environment (*1*, *2*). Besides its vital role in plants, the cuticle is also of economic importance. In agricultural crops, the cuticle avoids premature desiccation and rotting and is the frontier biocide or surface treatments must deal with. Cuticles have a thickness of up to a few micrometers and are attached to the underlying epidermis cells by a network of polysaccharide fibrils (*3*), from which they can be separated by enzymatic hydrolysis of the latter. The main structural component of cuticles is cutin, a polymeric matrix which is embedded and covered with lipophilic waxes. Studies on the structure and composition of cutin have been carried out in isolated cuticles, after separation from the underlying epidermis, and removal of the cuticular waxes with organic solvents.

Cutin is a polyester biopolymer. When any ester-breaking reaction is applied to the extracted cuticles, up to 80% of the material depolymerizes as long-chain aliphatic ω-hydroxyacids. Co-depolymerized are much smaller quantities of alkanoic acids, glycerol, and coumaric acid (*4*). The residue that remains after cutin depolymerization includes polysaccharides, polyaromatics, and sometimes a nonesterified aliphatic fraction known as cutan (*5*). How the long-chain ω-hydroxyacids and other monomers are assembled to build up the cutin polyester is mostly unknown. The structure of the polyaromatics and cutan associated with cutin in cuticles are also very poorly understood.

Cutin composition is variable depending on plant organ and botanical species (*6*, *7*). Cutin ω-hydroxyacids have C_{16} and C_{18} chain lengths, some are saturated, but more frequently they are oxygenated at, or close to, midchain. The C_{16} ω-hydroxyacids predominantly have a secondary hydroxyl group substituted in varying positions, C-7 to C-10, while C_{18} ω-hydroxyacids have either an epoxy or vicinal diol group at midchain. There are cutins mostly composed of C_{16} ω-hydroxyacids, while others are dominantly build up by C_{18} ω-hydroxyacids, with many combinations in between being found (*6*). In this way, "C_{16}", "C_{18}", and mixed type "C_{16}/C_{18}" family types of cutins were defined based on their monomer composition (*6*).

A key point for the discussion of the macromolecular structure of cutin is whether ω-hydroxyacids are interesterified only in their primary hydroxyls, giving rise to linearly developed chains, or also in their secondary hydroxyls, forming branched structures. Studies done on cuticles on the solid state, by ^{13}C NMR and FTIR, and characterization of oligomers obtained after enzymatic and chemical depolymerization, by mass spectrometry and NMR, have

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 Published on Web 08/05/2010

shown that both primary and secondary esters were present in cutins (*8*−*10*). Working models for cutin structure were drawn showing ω-hydroxyacids interesterified both ways (*9*, *11*, *12*). However, which types of linkages are dominant, how they relate with the different monomer compositions, and how the polyester grows three-dimensionally remain unsolved questions.

In order to elucidate how ω-hydroxyacids are interlinked in cutins, in this work we have depolymerized partially the cutin from different plant sources, looking for oligomeric fragments still carrying their original ester linkages. Four cutins with contrasting monomer compositions were studied: C_{16} cutins, from tomato (*Lycopersicumm esculentum*) fruit and *Citrus aurantium* leaves; a mostly C_{18} cutin from the ivy (*Hedera helix*) leaves; and a cutin with mixed C_{16}/C_{18} composition from *Prunus laurocerasus* leaves. The structure of the cutin oligomers was analyzed by electrospray ionization coupled to tandem mass spectrometry (ESI-MS/MS). To see if interesterification of ω-hydroxyacids was in primary or secondary positions, fractions enriched in cutin oligomers were isolated and analyzed by 1D and 2D homo- and heteronuclear high-resolution solution NMR. The implications for the cutin macromolecular structure of the identified ω-hydroxyacid oligomers are discussed, as well as their eventual relations with the supramolecular arrangements seen at the ultra-microscopic level in cuticles.

MATERIALS AND METHODS

Plant Cuticles. Cuticles from the leaves of common ivy (*Hedera helix*), bitter orange (*Citrus aurantium*), and cherry laurel (*Prunus laurocerasus*) and from the fruit of tomato (*Lycopersicum esculentum* [also known as *Solanum lycopersicum*]) were collected and isolated as described (*4*). The isolated cuticles were air-dried and further dried in a vacuum oven at 40 °C before extraction. The dried cuticles were extracted successively in dichloromethane, ethanol, and water, in Soxhlet apparatus (18 h each solvent), to remove waxes and other small-molecule soluble extractives, affording cutin-enriched residues. The extracted cuticles were dried as above, before depolymerization reactions.

Cutin Partial Depolymerization. The dried and extracted cutin-enriched cuticles were partially depolymerized by methanolysis reactions, either catalyzed by CaO or $Ca(OH)_2$. The extracted cuticles were cut into a few millimeter-sized pieces with a scissor, and mixed with CaO or $Ca(OH)_2$ in proportions that ranged from 1:1 to 1:2 (w/w); methanol was added in a proportion of ∼1 mL for 2.5 mg of cuticle material. These suspensions in methanol, typically with 100−500 mg of extracted cuticle, were refluxed by heating in an oil bath at 75 °C. The CaO-catalyzed methanolysis were run for 6 h, and the ones catalized by $Ca(OH)_2$ for 1 h. In *L. esculentum* and *H. helix* cuticles, three successive partial methanolysis were carried out, to detect eventual differences in the solubilized monomers and oligomers. After methanolysis reactions, the mixtures were filtered in 0.2 μm pore PTFE filters, and aliquots of the filtrates were taken for gas chromatography-mass spectrometry (GC-MS) and ESI-MS analysis. The remaining of the methanolysates was solvent-evaporated and the residue was dried for the gravimetric determination of the solubilized material by the partial methanolysis depolymerization reactions.

Cutin Complete Depolymerization. For monomer analysis and total cutin content determination, the complete depolymerization of cutin was carried out. The dried extracted-cuticles (100−200 mg) were refluxed in 0.1 M $NaOCH_3$ in methanol (10−25 mL) for 3.5 h. The methanolysis mixtures were filtered in 1 μm pore PTFE filters, and the filtrates were acidified to pH 6 with 0.2 M H_2SO_4. The solvent of the acidified solutions was removed under reduced pressure and the residue was partitioned in CH_2Cl_2:H_2O (25 mL each). The water phase was washed twice with CH_2Cl_2, and the accumulated organic phases were further washed twice with H_2O. Aliquots of the organic phases were taken for GC-MS analysis to access the monomer composition of cutins. The non-depolymerized residues of the methanolysis reactions were dried and weighed for the determination, by mass-loss, of the total cutin content of the cuticles.

ESI-MS/MS Analysis. The analysis of the cutin oligomers obtained by the partial depolymerization of cutins was made by ESI-MS/MS. Electrospray solutions were prepared by dissolving aliquots of the partial depolymerisates in methanol/chloroform 4:1 (v/v) in a concentration of 50 μg of dry matter per mL. To promote ESI ionization, these solutions were prepared with concentrations of 2 mM CH_3CO_2Li (to obtain lithium adducts) and 0.5 M NH_4OH (for Na^+ and NH_4^+-adducts and protonated molecules in positive mode, and deprotonated molecules in negative mode). These electrospray solutions were injected with an infusion pump at rates of 5−10 μL/min in a MicroTof-Q (Bruker, Bremen, Germany), with MS/MS QqTOF analyzer geometry, for high mass accuracy and resolution, and in a Quattro LC (Micromass, Manchester, UK) with QqQ geometry, with unit mass resolution. Ionization source capillary voltages of 4.5 kV were used in the Bruker equipment, and cone voltages of 20−80 V in the Micromass equipment. For the latter case, typically, Na^+-adducts, NH_4^+-adducts, and protonated ions optimized their intensities at cone voltages in the 20−30 V range, and the Li^+ adducts at 50−80 V. MS/MS analyses were carried out in parent-to-daughter mode, by collision-induced dissociation with argon as the collision gas (CID-MS/MS). Argon pressure (0.4−0.5 bar) and voltages in the collision cells were optimized to get the more informative MS/MS fragmentation patterns, with voltages of 60−70 V for the Li^+-adduct precursor ions, 20−40 V for the NH_4^+-adducts and protonated molecules, and ∼40 V for the deprotonated molecules in negative mode.

GC-MS Analysis. Cutin monomers in the complete and partial depolymerization methanolysates were analyzed by gas-chromatography coupled to electron-impact mass spectrometry (GC-MS). Aliquots of the complete and partial methanolysates were dried under a flow of N_2, further in a vacuum oven, and derivatized for trimethylsilyl derivatives with BSTFA (*N*,*O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane) and pyridine in a ratio of 1:1 (v/v). The derivatized solutions were analyzed in a GC-MS (Agilent 5973 MSD) with a DB5-MS column (60 m, 0.25 mm i.d., 0.25 μm film thickness), with the GC conditions: injector 320 °C; oven temperature program, 100 °C (5 min), rate of 8 °C/min up to 250 °C, rate of 2.5 °C/min up to 320 °C (20 min). The MS source temperature was 220 °C, quadrupole 150 °C, and the energy of the electron-impact 70 eV. Monomer identification was based on their mass spectra as described (*13*). Monomer quantification and cutin composition were calculated from the integrated areas of the corresponding peaks in the CG-MS ion chromatograms.

NMR Spectroscopy Analysis. Fractions enriched with dimeric and trimeric esters of the 10,16-dihydroxyhexadecanoic acid, isolated by TLC as described below, were analyzed by 1D (^1H, ^{13}C), and 2D homo- (COSY) and heteronuclear (HSQC, HMBC) solution NMR techniques. All spectra were acquired on a AVANCE III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a four channel 5 mm inverse detection probe head with pulse-field gradients along the Z axis. Spectra were run at 25 °C using standard Bruker pulse programs. ^1H and ^{13}C chemical shifts are referenced to tetramethylsylane. ^{13}C spectra were recorded at 201.24 MHz using the APT (attached proton test) sequence. The modulation of peak sign, to distinguish methyl and methyne from methylene signals, was achieved using a delay of 6.89 ms for the evolution of $^1J_{CH}$. Proton decoupling was applied during the acquisition stage using the WALTZ-16 sequence (*14*).

Standard Bruker pulse programs were used to acquire the homonuclear $^1H-^1H$ (COSY) and heteronuclear $^1H-^{13}C$ two-dimensional correlation spectra. In the $^1H-^{13}C$ heteronuclear single quantum coherence (HSQC) spectrum, a delay of 3.45 ms was used for evolution of $^1J_{CH}$, while in the heteronuclear multiple bond connectivity (HMBC) spectrum a delay of 73.5 ms was used for evolution of long-range couplings. In the HSQC, proton decoupling was achieved using the GARP4 sequence (*15*).

NMR chemical shifts predictions were made with ChemBio Draw Ultra 11.0 (CambridgeSoft, USA) and the spectra were processed and analyzed with MestreNova 6.0 (MestreLab Research, Santiago de Compostela, Spain).

TLC Isolation of Cutin Oligomers. To obtain fractions enriched in oligomeric esters of the 10,16-dihydroxyhexadecanoic acid, a preparative quantity (200 mg) of the partial depolymerisate of *L. esculentum* cutin was obtained applying a methanolysis catalyzed by $Ca(OH)_2$ to ∼500 mg of its extracted fruit cuticle. The recovered partial depolymerisate was applied to silica 0.5 mm thick TLC plates (Riedel-de-Haën, Germany) in a LINOMAT IV apparatus (Camag, Switzerland) and eluted with dichloromethane/ethyl acetate (7:3, v/v). Four bands were recovered, at the baseline (F_0), and at R_f's of 0.1 (F_1), 0.2 (F_2), and 0.4 (F_3). The TLC

bands were scraped and extracted with dichloromethane/ethyl acetate (5:5, v/v). The extracted materials were analyzed by GC-MS and ESI-MS: F_0 (23% of the recovered mass) included mostly monomers as free acids, unidentified compounds and very few oligomers; F_1 (20%) included higher-order oligomers and a small quantity (~15%) of the more polar monomers, namely, the 9,10,18-trihydroxyoctadecanoic acid (as methyl ester); F_2 (22%) was dominantly composed of dimers and trimers of the 10,16-dihydroxyhexadecanoic acid and had practically no monomers; and F_3 (35%) included the main *L. esculentum* cutin monomer, the 10,16-dihydroxyhexadecanoic acid (as methyl ester) (~ 60%) and dimer oligomers of the latter (~40%). Fractions 2 and 3 were analyzed by NMR spectroscopy as described above.

RESULTS AND DISCUSSION

Partial Depolymerization of Cutins. The partial depolymerization of cutins was achieved by methanolysis reactions using CaO or $Ca(OH)_2$ as catalysts, solubilizing between 10 and 60% of the cuticle residues. The cutins with more contrasting monomer composition, the C_{16} *L. esculentum* and the C_{18} *H. helix,* were further analyzed using the $Ca(OH)_2$ methanolysis, which typically gave higher oligomer yields. The $Ca(OH)_2$-catalyzed partial methanolysis applied to the cuticle of *L. esculentum* fruits and *H. helix* leaves solubilized 20.5% and 48.8% of the cuticle residues, respectively. These partial methanolysates represented 25% and 60% of the respective total cutin contents, which were ~78% for *L. esculentum* and ~81% for *H. helix,* as determined after complete depolymerization with $NaOCH_3$-catalyzed methanolysis. When successive partial depolymerization reactions were applied to the residues of the preceding ones, declining yields were obtained. Three consecutive partial methanolysis applied to the *L. esculentum* and *H. helix* cuticles, gave successive yields of 20.5%, 6.4%, 1.9% and 48.8%, 16.6%, and 6.7%, respectively. In the case of *H. helix*, the cumulative value of the three partial methanolysis extracts (72.1%) was 92% of its total cutin content, and in *L. esculentum* the accumulated extracts (28.8%) represented 37% of its cutin content. These differences in the depolymerization behavior, namely, the fact that *H. helix* cutin was "easier" to depolymerise compared to the *L. esculentum* cutin, under the mild methanolysis conditions used, might reflect differences in their polyester structure, as will be discussed later.

The analysis of these partial depolymerisates by GC-MS and ESI-MS showed the presence of cutin monomers and ester oligomers, whose identification is discussed below. An approximate calculation was made of the relative proportion of monomers and oligomers in the partial depolymerisates. On the basis of the TLC preparative results of the *L. esculentum* cutin, oligomers represented between 50 and 65% of the mass of extracted materials by the partial depolymerization reactions. This result, together with the cumulative yields obtained after successive partial methanolysis, showed that the detected oligomers were a significant part of the overall cutin structure.

Cutin Monomer Analysis by GC-MS. The methanolysates from the partial and complete depolymerization of cutins were analyzed by GC-MS to assess their monomer composition. Analysis of the complete depolymerization extracts showed cutin compositions similar to previous results (*4*, *16*, *17*). The analyzed cutins were composed mostly of aliphatic ω-hydroxyacids with C_{16} and C_{18} chain-lengths (more than 90% of all monomers) with small quantities of straight-chain fatty acids (0−7%), fatty alcohols (0−2.5%), glycerol (1−5%), and coumaric acid (less than 0.5%). The structural formula of the main ω-hydroxyacids detected is presented in **Figure 1**.

The expected patterns in monomer composition were recognized in the analyzed cutins. *L. esculentum* and *P. laurocerasus* were confirmed to be "C_{16} cutins", with C_{16} ω-hydroxyacids

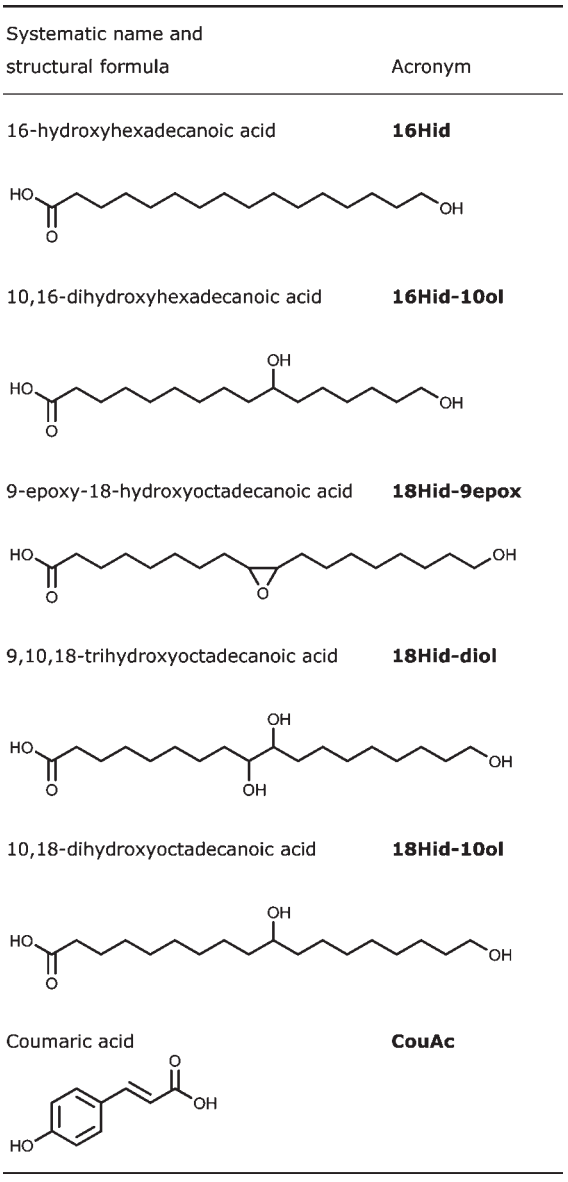


Figure 1. Cutin monomers: building blocks of the ester oligomers found in the partial depolymerisates of cutins.

accounting for 90% and 75%, respectively, of all detected monomers after complete depolymerization. In *L. esculentum*, the *n*,16-dihydroxyhexadecanoic acids represented ca. 93% of all C_{16} ω-hydroxyacids, while the saturated chain counterpart (16-hydroxyhexadecanoic acid) accounted for 5%. The remaining 2% of the C_{16} ω-hydroxyacids in *L. esculentum* cutin was the keto-substituted 16-hydroxy-9(10)-oxohexadecanoic acid.

The mixture of *n*,16-dihydroxyhexadecanoic acids was unresolved in the GC chromatographic conditions used, although they have been separated in normal phase HPLC (*18*). However, the relative abundance of the positional isomers with the secondary hydroxyl substituted in different chain positions could be estimated by the intensities of the corresponding fragment ions in the EIMS mass spectrum of their TMS derivatives, as discussed elsewhere (*19*). In *L. esculentum* the 10-isomer was largely dominant, representing 70−80% of all *n*,16-dihydroxyhexadecanoic acids isomers, which is in accordance with published results (*19*). For this reason, in this work, the 10,16-dihydroxyhexadecanoic acid is represented throughout when the *L. esculentum* monomers and oligomers are discussed, although other isomers, namely, the C-9, could also be present.

In *H. helix* the C_{18} ω-hydroxyacids were the dominant monomers, representing ca. 73% of the cutin composition, meaning that it is a "C_{18} cutin". In this cutin, practically all C_{18} ω-hydroxyacids had oxygen-containing groups at midchain, either an epoxide, a hydroxyl, or two hydroxyls (forming a *vic*-diol group). The 9-epoxy-18-hydroxyoctadecanoic acid was the main monomer, representing 60% of all C_{18} ω-hydroxyacids. However, even in the C_{18} cutins, the *n*,16-dihydroxyhexadecanoic acids are typically present in fair amounts, ca. 9% in *H. helix*, showing that these acids are common to practically all cutins studied so far. Unlike *L. esculentum*, in the *n*,16-dihydroxyhexadecanoic acids of *H. helix,* the position of the secondary hydroxyl was at C-9 and C-10 in similar proportions.

The composition of the *C. aurantium* leaves cutin was dominated by C_{16} ω-hydroxyacids, more than 70% of all monomers, with no C_{18} ω-hydroxyacids detectable. Another relevant feature of the *C. aurantium* cutin is a fairly high proportion of monoacids, namely, hexadecanoic acid, representing more than 6% of its monomer composition. The *P. laurocerasus* leaves cutin showed a mixed composition in terms of C_{16} and C_{18} ω-hydroxyacids, representing 34% and 44%, respectively, of its monomers. The main monomers in this cutin were the *n*,16-dihydroxyhexadecanoic (31% of monomers), the 9,10,18-trihydroxyoctadecanoic acid (25%), and the 9-epoxy-18-hydroxyoctadecanoic acid (10%).

The partial methanolysates of cutins also included monomers in its composition, as shown by GC-MS analysis. The monomer patterns in the four cutins analyzed were similar to the ones obtained after complete depolymerization, with one remarkable exception. The partial depolymerisates showed a higher glycerol content than that measured after the complete depolymerization of cutins. In *L. esculentum* these values were respectively 5% and 1% and in *H. helix*, 4% and 2%. When successive partial methanolysis was applied, very small amounts of glycerol were detected after the first depolymerization reaction. This means that glyceryl ester-linkages in cutins are more prone to be broken either by accessibility or due to less steric constraints within the polyester structure. This preferential solubilization of glycerol in comparison with the long-chain aliphatic acid monomers, under similar partial depolymerization conditions, was also observed in suberins (*20*, *21*).

Cutin Oligomer Analysis by ESI-MS. The oligomers in the partial depolymerisates of cutin were analyzed by ESI-MS. Several ionization-promoting solutions were assayed for the electrospray analysis of cutin oligomers. With 0.1% formic acid, protonated molecules and Na^+ and K^+-adducts were obtained, making the ion chromatograms complex to analyze; also, the Na^+ and K^+-adducts fragmented poorly under CID-MS/MS. Using 0.5 M NH_4OH solutions, besides the $NH4^+$-adducts, the same H^+, Na^+, and K^+ ions were observed. Lithium adducts formed from the 0.2 mM lithium acetate-added solutions, and were shown to be superior both for ESI-MS and MS/MS work. The Li^+-adducts were more intense than all other cations assayed, and although Na^+-adducts were also formed in the lithium acetate solutions, they were somehow suppressed in comparison with the other solutions assayed. Still more important, the Li^+-adducts as precursor ions fragmented very well under CID-MS/MS conditions, producing daughter-ion spectra that were simple to interpret and of high diagnostic content. For these reasons most of the ESI-MS and MS/MS work was done with the Li^+-adducts.

The relative intensity of the Li^+-adduct ions could be seen as indicative of the relative abundance of the ionized oligomers, but care should be taken since different species ionized differently depending on their molecular structure. For instance, the presence of epoxide groups seemed to favor the formation of Li^+-adducts, in comparison with hydroxyl groups. This could be observed in the partial methanolysates where monomers where

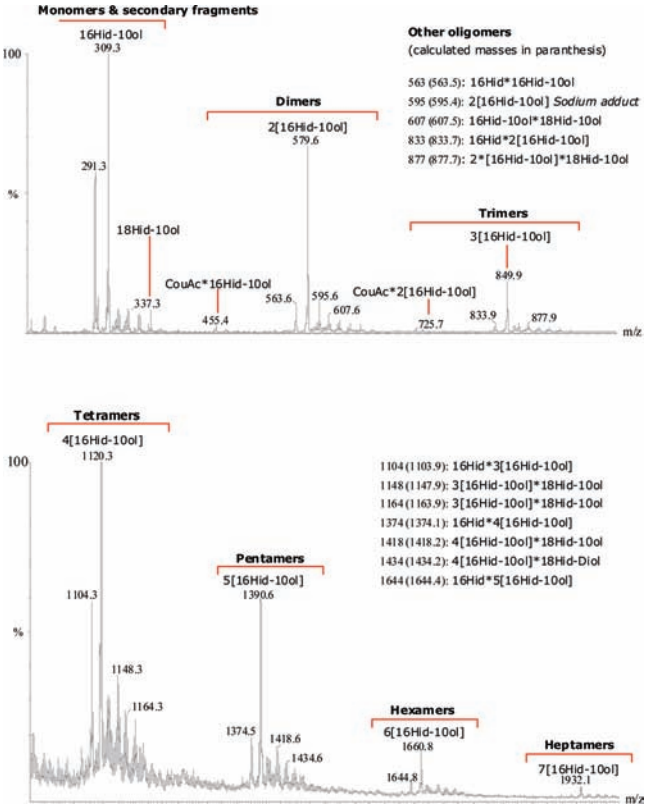


Figure 2. ESI-MS analysis of the partial methanolysis depolymerisate of *Lycopersicum esculentum* fruit cutin. Monomers and oligomers as lithium adducts in positive mode.

detected both by ESI-MS and GC-MS: epoxy monomers showed higher relative peak intensities when analyzed by ESI-MS, in comparison with GC-MS results for the same monomers.

The ESI-MS analysis of the cutin partial methanolysates showed masses corresponding to monomers and ester oligomers (**Figure 2**). The presumed ester oligomers were mostly interesterified cutin ω-hydroxyacids. The monomer composition of the cutin oligomers was identified by the MS/MS analysis, as discussed below. The main monomers found as part of the cutin ester oligomers are structurally represented in **Figure 1**, together with their systematic names and acronyms used here. To represent the cutin oligomers, a notation was used in which the involved monomers are represented by their acronyms, separated by an asterisk (**Figure 2**). The position of the monomers within the ester oligomer structure can be the one represented or eventually another, when different positional isomers are possible, as discussed below.

In the four cutins analyzed, clusters of ions corresponding to dimers up to heptamers were detected. In **Figure 2**, the ESI-MS chromatogram of the partial depolymerisate of *L. esculentum* cutin is presented, with the identified monomers and oligomers. The monomeric residues found as part of the ester oligomers corresponded to the main monomers obtained after complete methanolysis. The mixtures of oligomers found in the partial depolymerisates of the C_{16} cutins of *L. esculentum* and *C. aurantium* were dominated by the poly(10,16-dihydroxyhexadecanoic acid) esters. In *H. helix*, the more intense ESI-MS peaks corresponded to poly (9-epoxy-18-hydroxyoctadecanoic acid) oligomers. However, oligomers of mixed composition were present in all cutins analyzed, including ω-hydroxyacids of different chain lengths, and with different types of midchain substituents. This was particularly the case in the C_{16}/C_{18} cutin of *P. laurocerasus*, reflecting its more complex monomer composition.

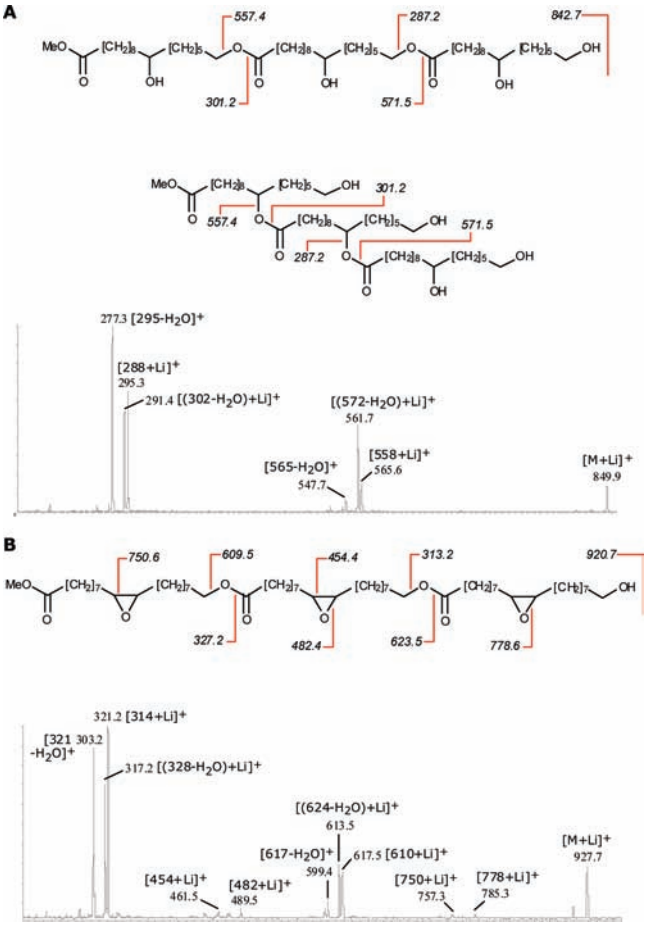


Figure 3. ESI-MS/MS spectrum of the (**A**) tri(10,16-dihydroxyhexadecanoic acid) ester oligomers from *Lycopersicum esculentum* fruit cutin and (**B**) tri(9-epoxy-18-hydroxyoctadecanoic acid) ester oligomer from *Hedera helix* leaves cutin.

Structural Characterization of Cutin Oligomers by ESI-MS/MS. The CID-MS/MS analysis of cutin oligomers, in the form of lithiated adducts, provided the structural information necessary to identify its monomeric composition. There were oligomers composed of only one ω-hydroxyacid, but others included different monomers in its composition. When different monomers were part of the oligomer, several isobaric positional isomers were possible, and the Li^+ MS/MS spectra also permitted to detect which ones were present and in which relative positions. An important characteristic that helped the positional location of monomers within the oligomer structure, was that the carboxylic acid end groups were obtained as methyl esters, due to the methanolysis reactions used, giving rise to fragments of specific mass for those terminal monomers.

The fragmentation pattern of the lithiated adducts of ω-hydroxyacid ester oligomers is exemplified by the MS/MS spectrum of the 10,16-dihydroxyhexadecanoic acid trimer from *L. esculentum* cutin, shown in **Figure 3A**. All intermonomer ester bonds cleaved at the C−O−C linkage, producing two fragment ions with either the hydroxyl or carboxylic acid moieties regenerated. In this way, each monomer and all possible sets of interesterified ω-hydroxyacids within the oligomer gave rise to fragment ions, which appeared as Li^+-adducts in the MS/MS spectra. Referring to the trimer in **Figure 3A**, the moieties where the acidic group is regenerated gave rise to the Li^+-adducts at m/z 565 (right dimeric moiety) and m/z 295 (free acid monomer). The latter daughter ions showed little secondary fragmentation, typically only one neutral loss of 18 Da, presumably as water, producing the ions at m/z 547 and 277, respectively. The moieties where the hydroxyl group were regenerated (which include the methylated carboxylic acid end group) originated very small intensity ions, but after a nominal loss of water (18 Da), gave strong ions at m/z 561 (methylated left dimeric moiety) and 291 (monomer methyl ester). What this MS/MS spectrum did not reveal, however, was the position of interesterification between the 10,16-dihydroxyhexadecanoic acid monomers, which could be in the primary or secondary hydroxyl. Because the MS/MS fragmentation occurred only at the ester bonds, both primary (linear)- and secondary (branched)-linked monomers gave rise to the same daughter ions (**Figure 3A**). This fragmentation pattern of the lithiated ester oligomers is consistent with previous results obtained with dimeric model compounds of ω-hydroxyacids resynthesized from suberin monomers (*21*).

The MS/MS spectrum of the Li^+-adduct of the tri(9-epoxy-18-hydroxyoctadecanoic acid) ester oligomer from *H. helix* cutin is shown in **Figure 3B**. These oligomers built only from the C_{18} 9-epoxy ω-hydroxyacid cannot be branched due to the lack of secondary hydroxyl groups, so they must develop as linear chains. Besides the fragments associated with the breaking of the ester bonds equivalent to those discussed above, other ions were formed from fragmentation in the epoxide groups. Apparently, two bonds of the oxirane ring were broken, and the moiety that kept the oxygen after the rearrangement appeared as a lithiated ion in the MS/MS spectrum (m/z 461, 489, 757, 785). These ions, although of small intensity, were diagnostic of the position of the epoxide groups and so of the monomer residues of which they were part. These diagnostic ions were particularly useful in the analysis of mixed-monomer oligomers. An example is shown for the trimer composed of two monomer units of 9-epoxy-18-hydroxyoctadecanoic acid and one unit of 9,10,18-trihydroxyoctadecanoic acid, found in the *P. laurocerasus* cutin partial depolymerisate. Three different positional isomers were possible (assuming the structure as a linear ester), and the analysis of the mixed MS/MS spectrum showed that all three isomers were present.

Coumaric Acid and Glycerol Ester Oligomers. Besides the poly(ω-hydroxyacid) esters, the ESI-MS analysis of cutin partial depolymerisates showed other types of oligomers, namely, dimeric esters of ω-hydroxyacids both with glycerol and coumaric acid. Glycerol and coumaric acid are known cutin monomers (*4*, *22*), although typically found in small quantities, as the monomer analysis presented above showed. The monoacylglycerol ester of the 10,16-dihydroxyhexadecanoic acid was obtained from the *P. laurocesarus* cutin, also identified by the MS/MS spectrum of its Li^+-adduct (*23*). Glycerol esterified to ω-hydroxyacids was previously found in several cutins, including the ones studied here, but using GC-MS analysis (*4*). Coumaric acid esterified to 10,16-dihydroxyhexadecanoic acid was found in the *L. esculentum* partial depolymerisate (**Figure 2**). The MS/MS spectrum of the corresponding lithiated adduct showed evidence for the coumarate ester structure, but the MS/MS of the same molecular mass in negative mode showed evidence for the coumaryl ester structure, so eventually both were present.

Linear and Branched ω-Hydroxyacid Ester Oligomers: NMR Analysis. To see if ω-hydroxyacids were interesterified in primary or secondary hydroxyl positions, a mixture of oligomers of the 10,16-dihydroxyhexadecanoic acid, from *L. esculentum* cutin, was analyzed by high-resolution NMR spectroscopy. This oligomer fraction, mostly composed by dimers and trimers (as shown by ESI-MS), was isolated by preparative TLC from the partial depolymerisates of the *L. esculentum* fruit cutin. The oligomers were characterized by ^1H and ^{13}C NMR (APT sequence to differentiate methylene and quaternary carbons, from methyne and methyl signals),

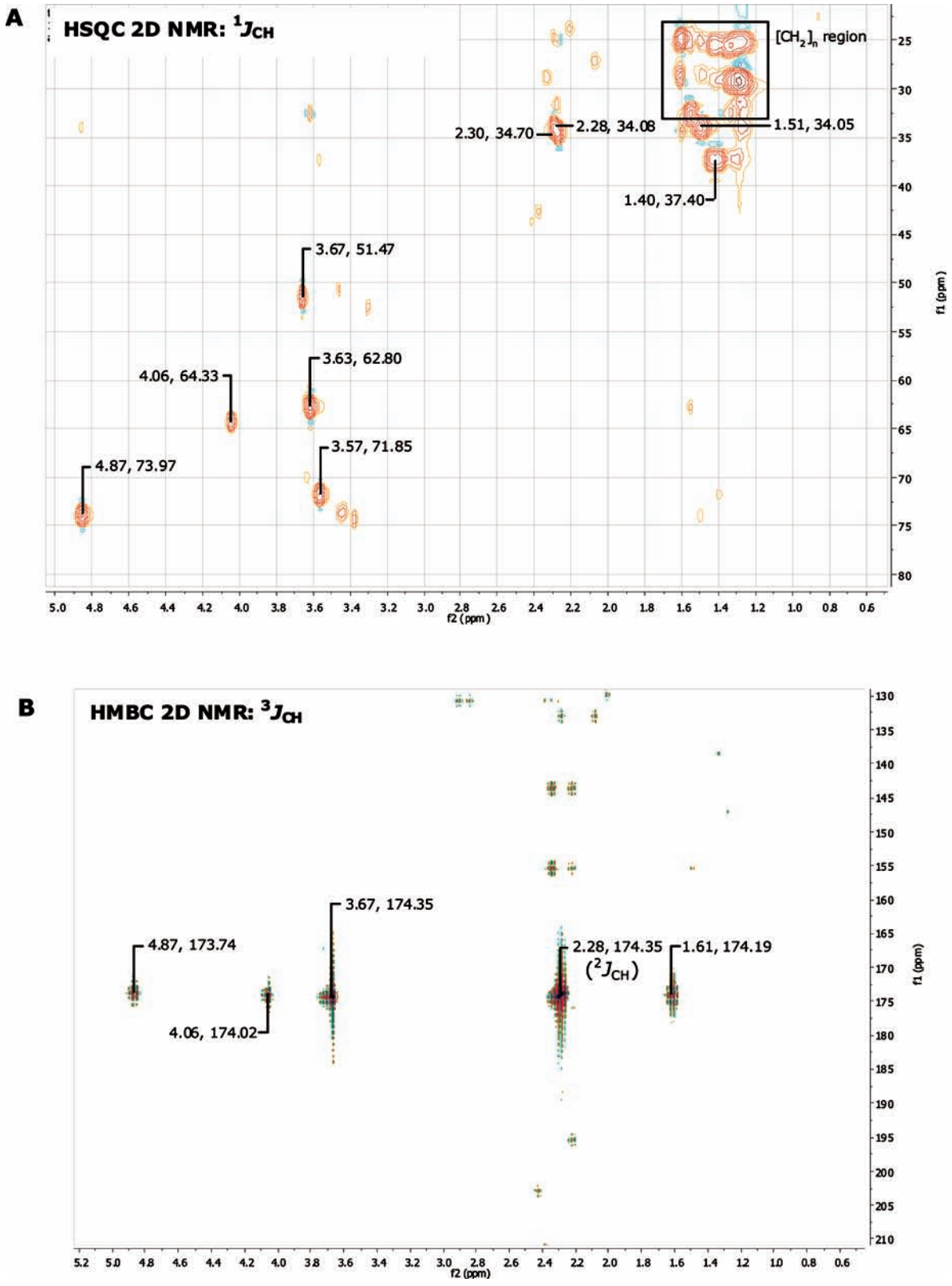


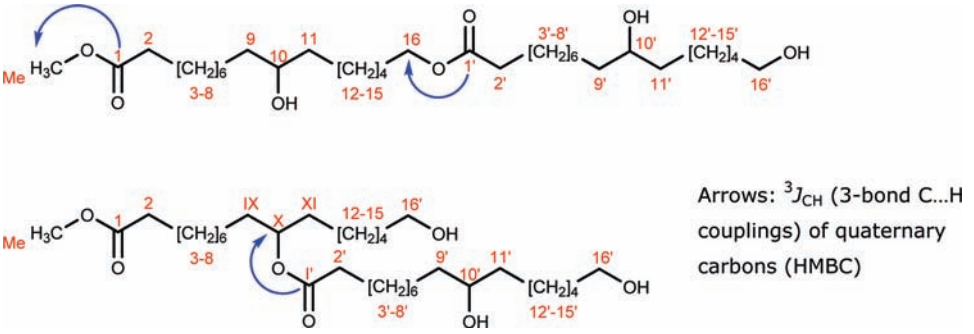
Figure 4. Correlation 2D NMR of the mixture of di- and tri(10,16-dihydroxyhexadecanoic acid) ester oligomers from *Lycopersicum esculentum* fruit cutin: (**A**) HSQC $^{1}H-^{13}C$ direct connectivities and (**B**) HMBC $^{1}H\cdots^{13}C$ three-bond connectivities of quaternary carbonyl carbons.

together with 2D homonuclear COSY (to identify vicinal H atoms), and heteronuclear HSQC (to identify C−H direct bonds, **Figure 4A**) and HMBC (to identify long-range, mainly 3-bond C···H connections, **Figure 4B**). The results presented below showed that both primary and secondary ester linkages were present in the 10,16-dihydroxyhexadecanoic acid oligomers.

The assignments made for ^{1}H and ^{13}C chemical shifts are presented in **Table 1** and associated structures. The NMR spectra showed carbons and protons unambiguously characteristic of the primary esters of the linear structures, and secondary esters of the branched structures. Characteristic of the primary ester was the methylene group (numbered 16) linked to the oxygen atom of

Table 1. ^{1}H and ^{13}C NMR Assignments of the Linear and Branched Ester Oligomers of 10,16-Dihydroxyhexadecanoic Acid (Methyl Esters) from *Lycopersicum esculentum* Fruit Cutin

	structure	^{13}C δ ppm	^{1}H δ ppm
Common			
Me	**CH_3**−O−(C═O)−	51.45	3.67
1	CH_3O−(**C**═O)−CH_2−	174.35	
2	CH_3O−(C═O)−**CH_2**−	34.08	2.28
3−8, 3′-8 & 12−15, 12′-15′	−**CH_2**−	25−32	1.3−1.6
9,11,9′,11′	−**CH_2**−CH(OH)−	37.40	1.40
10, 10′	−**CH**(−OH)−	71.85	3.57
16′	−**CH_2**−OH	62.80	3.63
Linear			
16	−**CH_2**−O−(C═O)	64.33	4.06
1′	−CH_2−O−(**C**═O)	174.02	
2′	−CH_2O(C═O)−**CH_2**−	34.70	2.30
Branched			
IX,XI	−**CH_2**−CH−[−O−(C═O)]−	34.05	1.51
X	−CH_2−**CH**−[−O−(C═O)]−	73.97	4.87
I′	−CH_2−CH−[−O−(**C**═O)]−	173.74	



the ester bond, with chemical shifts of 64.33 and 4.06 ppm, respectively, for the carbon and hydrogen atoms. The corresponding methylene, but connected to the free ω-hydroxyl at end chains (16′), resonates at lower frequencies, 62.80 ppm for the carbon and 3.63 ppm for the protons. Characteristic of the secondary ester structure was the methyne (X) connected to the oxygen of the branched ester bond, with chemical shifts of 73.97 ppm for the carbon and 4.87 ppm for the proton. The corresponding methyne, but linked to the free secondary hydroxyl (10, 10′), had lower frequency chemical shifts of 71.85 ppm for the carbon and 3.57 ppm for the proton. The above assignments made for the linear ester structure are similar to the ones observed in other linearly linked ω-hydroxyacids, also found in cutins (*24*). The assignments for the methyne of the branched ester structure are consistent with the ones made for equivalent structural moieties in synthesized estolides (*25*).

The $^{3}J_{CH}$ (three bond distance C···H) couplings detected in the HMBC spectra, involving the ester carbonyl quaternary carbons, gave further proof for the presence of primary and secondary esters. Three different quaternary carbonyl carbons were present, with small differences in their chemical shifts: the carbon in the end-chain methyl ester (Me), with a chemical shift of 174.35 ppm; the carbon in the linear primary ester (1′), at 174.02 ppm; and the carbon in the secondary branched ester (I′), at 173.34 ppm. These assignments were confirmed by their $^{3}J_{CH}$ connectivities: the methyl ester carbon was coupled with the methyl protons; the primary ester carbon was coupled with the methylene (*16*) protons; and the secondary ester carbon was coupled with methyne (X) proton (**Table 1**, and associated structures). Also proof for the branched ester was given in the COSY spectra, which showed the **H**-C-C-**H** coupling between the methyne proton of the branched structure (X, 4.87 ppm) and their vicinal CH_2 protons (IX and XI, 1.51 ppm).

The relative proportion of linear and branched ester-linkages in the analyzed mixture of cutin oligomers could be estimated by the integrals of the areas of their assigned proton peaks in the ^{1}H spectra: the two methylene protons at 4.06 ppm for the linear primary esters and the methyne proton at 4.87 ppm for the branched secondary esters. In this way, the branched secondary esters were shown to be largely dominant, roughly 4.5 times more than the linear primary esters, in this mixture of 10,16-dihydroxyhexadecanoic acid oligomers from *L. esculentum* cutin.

Cutin Macromolecular Structure. Cutin is a polyester macromolecule, which upon depolymerization by any ester-breaking reaction, releases a mixture mostly composed by aliphatic long-chain ω-hydroxyacids. Early work showed that C_{16} and C_{18} ω-hydroxyacids were the main cutin monomers, and their interesterification was hypothesized as the possible structure of cutin (*1*). Because many of these cutin ω-hydroxyacids had primary and secondary hydroxyl groups, linear and branched interesterification was possible, and working models were drawn including both structures (*9*).

Several studies were carried out to assess how much of the primary and secondary hydroxyls were esterified within the cutin polyester. Starting from the intact cutin, free and esterified hydroxyls were chemically labeled, followed by depolymerization and analysis of the ensuing monomers (*8*). In the fruit cutin of *L. esculentum*, free secondary hydroxyls were oxidized with CrO_3-pyridine, and after a NaOMe-catalyzed methanolysis, the solubilized monomers showed that most primary hydroxyl groups were esterified, but only about 40% of the secondary hydroxyls were ester-linked (*8*). In another approach, the free hydroxyls were mesylated, followed by hydrogenolysis depolymerization with deuterated reagents, thus marking the esterified hydroxyls: about 40% of the secondary hydroxyls were calculated to be free, and practically all primary ones esterified (*9*).

Studies on the intact cutin by ^{13}C solid-state NMR also showed that esterification was possible both at primary and secondary hydroxyl positions. Solid-state NMR dynamics studies in *Citrus aurantifolia* cutin revealed that the primary esters had higher

mobility within the polyester structure, in comparison with the much more rigid secondary ones, as shown by the longitudinal relaxation times of the respective carbons and protons (*11*, *26*). The cuticles from *L. esculentum* fruit (*27*) and *Agave americana* leaves (*28*), after a reaction intended to remove polysaccharides, were analyzed in the DMF-swollen state by high-resolution magic angle spinning (HR-MAS) NMR. Carbons and protons with chemical shifts and couplings assignable to primary and secondary esters were found. Besides, branching in the hydrocarbon chains, located α to the primary ester carbonyls, was shown possible by the NMR data (*27*, *28*).

Direct evidence of the original intermonomer ester linkages had been provided by the structural characterization of oligomers obtained from cutins, although typically obtained in small yields. A partial KOMe-catalyzed hydrolysis applied to *L. esculentum* fruit cutin released oligomers that after ESI-MS analysis showed molecular weights compatible with up to five interesterified *n*,16-dihydroxyhexadecanoic acid monomers (*29*). The linear ester-linked form was attributed to dimers isolated from this oligomer mixture, since only one signal was found for a methyne carbon by ^{13}C APT (at 63.4 ppm) NMR, which was assigned to the methyne linked to the free secondary hydroxyl (*30*). Other ester oligomers were extracted by enzymatic and chemical partial depolymerization methods applied to the cutin of *Citrus aurantifolia*. A tetramer was freed by enzymatic hydrolysis, built from three units of 10,16-dihydroxyhexadecanoic acid and a less common substituted fatty alcohol, to which an all-branched ester structure was attributed, based on its EI-MS fragmentation pattern (*31*). Dimers, trimers, and tetramers of the 10,16-dihydroxyhexadecanoic acid and the structurally related ketone, 10-oxo,16-hydroxyhexadecanoic acid, were isolated after iodo trimethylsilane (*32*), fluoridric acid and enzymatic (*24*) partial depolymerization of the *C. aurantifolia* cutin. On the basis of the ESI-MS and 1D and 2D homo- and hetero correlation NMR results, linear ester linkages were assigned to all these oligomers (*24*, *32*).

In the present work, we have provided direct evidence that the interesterification of ω-hydroxyacids is a representative feature of cutin structure. Poly(ω-hydroxyacid) ester oligomers from dimers up to heptamers were released from four different cuticles, after mild partial methanolysis reactions. Yields were significant, and after three successive partial depolymerization reactions, represented 40−90% of the respective total cutin content. Within these partial depolymerisates, oligomers represented 50−65% of solubilized materials, as discussed above. The ESI-MS/MS analysis of these cutin oligomers showed that in all cases they were composed by the more significant ω-hydroxyacids known as monomers after the complete depolymerization of the respective cutin. Thus, we concluded that the detected oligomers were representative of the polyester structure of the analyzed cutins.

Two main types of poly(ω-hydroxyacid) ester oligomers were found in the analyzed cutins. One based on the 10,16-dihydroxyhexadecanoic acid, found in the C_{16} cutins of *L. esculentum* and *C. aurantium*, and the other based on the 9-epoxy-18-hdroxyoctadecanoic acid, was dominant in the C_{18} *H. helix* leaves cutin. Direct NMR evidence showed that the poly(10,16-dihydroxyhexadecanoic acid) ester oligomers from the *L. esculentum* cutin were mostly, 4.5 to 1, esterified in their C-10 secondary hydroxyl compared to esterification on the primary C-16 ω-hydroxyl. We cannot exclude that ester bond breaking in the partial depolymerization was prevalent in the primary ester position, due to, for instance, easier steric accessibility. However, the significant proportion of secondary esters found in these oligomers showed that, at least in this cutin, the polyester structure was significantly branched. On the contrary, the poly(9-epoxy-18-hydroxyoctadecanoic acid) ester oligomers, dominant in *H. helix* cutin, must form linear structures with monomers linked head-to-tail, considering that the latter only have a primary hydroxyl group. The *H. helix* cutin gave higher yields of solubilized oligomers after the partial methanolysis reactions, compared to the case of *L. esculentum* cutin. Eventually, the midchain ester-branching observed in the latter makes depolymerization more difficult compared with the linear ester-chains dominant in the former.

The two cutin macromolecular arrangements, the highly branched C_{16}-dihydroxy type and the linear C_{18}-epoxy type, can eventually account for the differences that are seen at ultrastructural level in plant cuticles. Polymers made of linear chains can organize themselves in ordered or even crystalline secondary structures, while polymers with extensive chain-branching will typically organize as nonordered reticulate networks. Plant cuticles show, as observed by transmission electron microscopy, different ultrastructural arrangements, namely, in the outer and inner regions of the cuticular membrane (*33*). These regions can be neatly poly lamellate, form a reticulate network of fibrils or be visually amorphous. On the basis of which cases were found in a particular cuticle, different structural types were defined (*34*). The *H. helix* leaves cuticle was classified as a Type 1 cuticle, characterized by a well-delineated poly lamellate outer region and a reticulate inner region (*33*). Eventually, the linearly linked C_{18}-epoxy ω-hydroxyacids shown here to be structurally relevant in this cuticle, organize themselves to build the orderly parallel lamellae. The *L. esculentum* fruit cuticle was defined as a Type 4 cuticle, meaning that all its regions are reticulate (*33*). The highly branched C_{16} polyester shown here to be a relevant structural component of this cutin can be responsible for this nonordered reticulation. In *P. laurocerasus*, which did not show a clear C_{16} or C_{18} dominant monomer and oligomer pattern (discussed above), was also classified as a mostly reticulate type 4 cuticle (*33*).

The macromolecular structure of cutins probably include other features besides the poly(ω-hydroxyacid) ester backbones. Glycerol, although in relative minor proportions, was found to be part of cutins and to be ester-linked to ω-hydroxyacids (*4*). Some cutins also include long-chain α,ω-diacids in its composition, and therefore can be more closely related with suberins, where the latter, together with glycerol and ω-hydroxyacids, are found in significant proportions. Also, dimeric ester oligomers of coumaric acid were detected in our analysis of the partial depolymerisates of *L. esculentum* cutin, eventually both in the coumaryl or coumarate forms. Like in suberins, the esterification of ω-hydroxyacids to aromatic hydroxycinnamic acids can bridge the aliphatic polyester to the other cell-wall polymers, either polyaromatics or polysaccharides. Surely, a lot of extra research work is needed to come to a definite picture of the macromolecular structure of the cutin polyester.

Supporting Information Available: Page 1−2: ESI-MS analysis of *Hedera helix* cutin partial depolymerisate. Page 3−4: ESI-MS analysis of *Citrus aurantium* cutin partial depolymerisate. Page 5−6: ESI-MS analysis of *Prunus laurocerasus* partial depolymerisate. Page 7: ESI-MS/MS spectrum of the lithiated adduct of an oligomer of mixed monomer composition, the trimeric esters 2[18Hid-9epox]*18Hid-diol from *Punus laurocerasus* leaves cutin. Page 8: ESI-MS/MS of dimeric esters of coumaric acid and 10,16-dihydroxyhexadecanoic acid (CouAc*16Hid-10ol) from *Lycopersicum esculentum* fruit cutin. This material is available free of charge via the Internet at http://pubs.acs.org.

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Received for review April 23, 2010. Revised manuscript received July 20, 2010. Accepted July 21, 2010. Work is supported by the Programa Operacional Ciência e Inovação (POCTI) and Fundação para a Ciência e a Tecnologia (FCT) projects POCTI/QUI/70589/2006, REDE/1517/RMN/2005 (NMR spectrometers) and REEQ/204/AGR/2005 (MicroTof-Q mass spectrometer).