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Glycerol and glyceryl esters of ω-hydroxyacids in cutins

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Abstract

Cutins from the leaves and fruits of seven plant species were depolymerized by $NaOCH_3$-methanolysis. The monomers that were released mostly included C16 and C18 ω-hydroxyacids with mid-chain oxygenated substitutions, namely epoxy and hydroxyl groups. Glycerol was also solubilized as a monomer in quantities that ranged from 1 to 14% of the methanolysates. Partial depolymerization of three cutins by CaO-methanolysis released the same monomers as had been obtained in the previous reaction, as well as small quantities of 1- and 2-monoacylglyceryl esters of ω-hydroxyacids. Molar proportions of glycerol permit the esterification of a significant part of the aliphatic ω-hydroxyacids, thereby possibly playing a major role in the polyester structure of cutin. Glycerol had not previously been known to form part of the cutin polymer.

Keywords: Cutin; Cuticle; Methanolysis; Glycerol; Glyceryl esters of ω-hydroxyacids

1. Introduction

Plants are enveloped by specialized cells that confine and protect internal tissues. Protection is needed to defend the plants from pathogens and to avoid uncontrolled water loss. Primary plant tissues, such as leaves and fruits, are protected by an epidermis, whereas secondary plant tissues, like tree trunks, are protected by the peridermis. The protective properties of these tissues are largely due to specific biopolymers: cutin in the epidermis and suberin in the peridermis. Knowledge of the molecular structure of these biopolymers is essential to an understanding of plants' relationships with their environment. This knowledge is relevant to issues of major economic importance, such as the application of pesticides to crops or the conservation of plant foodstuffs.

Cutin is present in the cuticle—the outer layer of the epidermal cells—where it is partly embedded in a matrix of polysaccharides and is mixed with waxes. On the outside, the cutin-rich cuticle is covered with a thin layer of extractable (''epicuticular'') waxes (Holloway, 1982a). Suberin is part of the cell wall of the phellem cells, which constitute most of the peridermis. In some plant species the phellem cell walls are highly ''suberized''—one example is *Quercus suber* bark, which contains up to 50% suberin (Pereira, 1988). Some cell layers in internal tissues, like the endodermis, also have suberin-rich cell walls (Kolattukudy, 1980; Schreiber et al., 1999).

Cutin is known to be a polyester polymer: it is insoluble, and ester-breaking reactions release a mixture of monomers with hydroxyl and carboxylic functions. In isolated cuticles, obtained by enzymatic treatments that partially remove the polysaccharides and from which the extractives were also removed, solubilized cutin products released after de-esterification can be up to 80% of initial weight (Holloway, 1982b). Cutin depolymerization products are mostly long-chain C16 and C18 ω-hydroxyacids, with hydroxyl or epoxy groups in secondary positions. Their primary functional groups have been found to be mostly ester-linked, but with only partially esterified secondary hydroxyls (Deas and Holloway, 1977; Kolatukudy, 1977). The results of these studies led to tentative models for the cutin polymer, based on the inter-esterification of ω-hydroxyacids, both head-to-tail in a linear form, and cross-linked via the

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secondary hydroxyls (Kolattukudy, 1977; Zlotnik-Mazori and Stark, 1988; Tegelaar, 1990).

Suberin is a similar type of polyester polymer, with ω-hydroxyacids and α,ω-diacids as its long-chain aliphatic monomers. Suberin has recently been shown to include glycerol in sufficient quantities to esterify most of the carboxylic groups of acid monomers (Graça and Pereira, 1997; Moire et al., 1999). In addition, glycerol has been found esterified with aliphatic acids and a poly(acylglycerol) structure has been proposed for the suberin polymer (Graça and Pereira, 1997, 2000c).

In suberins, glycerol has been analyzed along with the long-chain monomers following a $NaOCH_3$-catalyzed methanolysis (Graça and Pereira, 1999, 2000a), and the acylglycerol esters were obtained after a partial depolymerization methanolysis (Graça and Pereira, 1997, 2000b). These same techniques were applied here to isolated cuticles from the epidermis of leaves and fruits of several species. Glycerol and acylglycerol esters were also found, and their significance for cutin polymer constitution and macromolecular structure is discussed.

2. Results and discussion

2.1. Cutin monomer analysis

Cutins from isolated cuticles of the leaves of five different species and from the fruits of two further species were depolymerised by $NaOCH_3$-catalyzed methanolysis. High yields of depolymerised material were obtained, ranging from ca. 53% of the extractive-free initial weight in *Citrus aurantium* up to 86% in *Hedera helix*. The cutin methanolysate mixtures were mostly comprised of long-chain aliphatic ω-hydroxyacids that are known to include the cutin polymer (Table 1). Examples of the three types of cutins that have been defined on the base of the ω-hydroxyacid monomer composition (Holloway, 1982b) were found: "C16 cutins" in *Lycopersicon esculentum* and *Citrus aurantium*, where the 10(8-10),16-dihydroxyhexadecanoic acids are the major monomers; "C18 cutin" in *Hedera helix*, where the 9-epoxy-18-hydroxyoctadecanoic and the 9,10,18-trihydroxyoctadecanoic acids are the major monomers; and "mixed-type C16 and C18 cutins" in the remaining cutins that were analyzed, in which the main C16 and C18 acids are present in significant amounts.

The other long-chain aliphatic monomers found in cutins include saturated ω-hydroxyacids, alkanoic acids and alkan-1-ols. In most cases they account for less than 5% of the monomer mixtures (Table 1). The exception is the *Juglans regia* cutin, in which these saturated chain monomers represent approximately 30%. An aromatic compound—coumaric acid—was present in all the cutins, but in most cases it amounted to less than 1% (Table 1). Some of the cutins that were analyzed have been studied before and the monomer composition values found in this study were close to those reported. This was the case with *Lycopersicon esculentum* (Baker and Holloway, 1970), *Hedera helix, Prunus laurocerasus* (Holloway et al., 1981), and *Capsicum annuum* (Holloway, 1982b).

However, in this study glycerol was found to be an additional relevant monomer of cutin. Glycerol was present in all seven cutin samples analyzed and represented between 1 and nearly 14% of the monomers released from the cutin polymers by methanolysis (Table 1). We are only aware of one reference to glycerol being found in epidermal tissues, after depolymerization by methanolysis and detection by TLC (Carvalho, 1993). As in suberin, to date glycerol has been overlooked as an important cutin monomer—something that is largely due to the methods used to recover such monomers.

After depolymerization, cutin monomers were usually extracted from aqueous solutions using organic solvents, and the aqueous phase containing the glycerol was discarded. The methanolysis reaction used here was carried out with low $NaOCH_3$ concentrations and without aqueous/organic phase partitioning and the GC analysis was carried out using all of the depolymerized material. This technique, which was recently developed in order to analyze suberized materials, allows the simultaneous quantification of glycerol and aliphatic acids in both suberins (Graça and Pereira, 2000a) and cutins, as is shown herein.

The proportion of the monomer acid groups that can potentially be linked by glycerol hydroxyls is determined by their respective molar quantities. The ratio of the molar quantities of hydroxyl groups derived from glycerol to the number of moles of carboxylic acid groups from acid monomers was approximately 13% in *Lycopersicon esculentum*, 25% in *Hedera helix* and *Juglans regia*, 40% in *Prunus laurocerasus*, 55% in *Capsicum annuum* and *Stephanotis floribunda* and above 100% in *Citrus aurantium*. These results show that significant proportions of the acid monomers can be ester-linked to glycerol. However, one has to be careful when comparing the proportions of the different functional groups, because the aliphatic acids are probably underestimated.

The internal standard used for the quantification of aliphatic acids—12-hydroxyoctadecanoic acid methyl ester—was calibrated against the 16-hydroxyhexadecanoic acid methyl ester, and its response factor was used for all ω-hydroxyacids. Evidence exists that the response factors for the secondary oxygenated acids are much higher than those for their saturated chain counterparts (Graça and Pereira, 2000a). Cutin monomers obtained by $NaOCH_3$-methanolysis and quantified using the internal standards were rarely more than 50% of the total loss of mass determined by weighing. This

Table 1
Cutin monomers released from isolated cuticles of seven plants species after $NaOCH_3$-catalyzed methanolysis (acids analyzed as methyl esters, hydroxyl groups as trimethylsilyl ethers)

	Stephanotis floribunda[a]		*Prunus laurocerasus*[a]		*Hedera helix*[a]		*Juglans regia*[a]		*Citrus aurantium*[a]		*Capsicum annuum*[b]		*Lycopersicon esculentum*[b]	
	% w/w	$\mu g.cm^{-2}$	% w/w	$\mu g.cm^{-2}$	% w/w	$\mu g.cm^{-2}$	% w/w	$\mu g.cm^{-2}$	% w/w	$\mu g.cm^{-2}$	% w/w	$\mu g.cm^{-2}$	% w/w	$\mu g.cm^{-2}$
Glycerol	4.9	7.8	3.8	9.1	2.0	5.4	1.7	0.8	13.8	9.4	4.7	71.1	1.1	7.9
Coumaric acid	0.4	0.6	0.3	0.7	0.4	1.1	0.1	0.1	0.1	0.1	1.6	24.2	0.2	1.4
Alkan-1-ols	(0.7)	(1.1)	(0.1)	(0.2)	(2.4)	(6.6)	(4.4)	(2.2)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)	(0.0)
Hexadecanol						0.1	0.3							
Octadecanol						0.1	0.3							
Eicosanol						0.8	2.2							
Docosanol						0.7	1.9	2.4	1.2					
Hexacosanol	0.7	1.1	0.1	0.2	0.4	1.1	1.4	0.7						
Octacosanol						0.2	0.5	0.6	0.3					
Triacontanol						0.1	0.3							
Alkan-1-oic acids	(0.2)	(0.3)	(0.7)	(1.7)	(0.9)	(2.4)	(19.4)	(9.5)	(6.4)	(4.4)	(1.5)	(22.7)	(0.0)	(0.0)
Hexadecanoic acid	0.2	0.3	0.4	1.0	0.2	0.5	17.8	8.7	6.4	4.4	1.2	18.2		
Octadecanoic acid			0.1	0.2			1.1	0.5			0.3	4.5		
Docosanoic acid			0.2	0.5	0.7	1.9	0.5	0.3						
ω-Hydroxyacids, saturated chain	(3.1)	(4.9)	(3.1)	(7.4)	(0.7)	(1.9)	(6.7)	(3.3)	(2.6)	(1.8)	(3.7)	(56.0)	(4.7)	(33.7)
16-hydroxyhexadecanoic acid	1.6	2.5	2.0	4.8	0.7	1.9	6.7	3.3	2.6	1.8	3.5	53.0	4.2	30.1
18-Hydroxyoctadecadecanoic acid	1.5	2.4	1.1	2.6							0.2	3.0	0.5	3.6
ω-Hydroxyacids, C16 "mid-chain" oxygenated	(23.3)	(36.8)	(31.1)	(74.8)	(8.8)	(23.9)	(34.2)	(16.8)	(72.4)	(49.2)	(65.4)	(989.5)	(84.2)	(603.7)
9(10),16-Dihydroxyhexadecanoic acid[d]	23.3	36.8	30.7	73.8	8.8	23.9	34.2	16.8	69.9	47.5	64.4	974.4	81.5	584.4
16-Hydroxy-9(10)-oxohexadecanoic acid			0.4	1.0					1.4	1.0	1.0	15.1	2.0	14.3
9(8),10(9),16-Trihydroxyhexadecanoic acid									1.1	0.7			0.7	5.0
ω-Hydroxyacids, C18 "mid-chain" oxygenated	(47.9)	(75.7)	(43.6)	(104.6)	(72.6)	(197.5)	(15.1)	(7.3)	(0.0)	(0.0)	(11.8)	(178.5)	(2.9)	(20.8)
11,18-Dihydroxyoctadec-?-enoic acid[e]	1.4	2.2	2.0	4.8	1.9	5.2	0.6	0.3			0.4	6.1		
9(10),18-Dihydroxyoctadec-?-enoic acid[e]	4.6	7.3	6.3	15.1	2.0	5.4	0.6	0.3			1.1	16.6		
9(10),18-Dihydroxyoctadecanoic acid			0.3	0.7							1.6	24.2	1.0	7.2
9-Epoxy-18-hydroxyoctadecanoic acid	22.4	35.4	10.2	24.5	44.0	119.8	1.9	0.9			1.0	15.1		
9,10,18-Trihydroxyoctadec-?-enoic acid[e]	2.3	3.6			1.8	4.9	0.6	0.3						
9,10,18-Trihydroxyoctadecanoic acid	17.2	27.2	24.5	58.8	22.0	59.8	10.7	5.2			5.6	84.7	1.2	8.6
9,12,18-Trihydroxyoctadec-?-enoic acid[e]			0.3	0.7	0.9	2.4	0.7	0.3			2.1	31.8	0.7	5.0
Unidentified[f]	19.5	30.8	17.3	41.5	12.2	33.2	18.4	9.0	4.7	3.1	11.3	171.0	6.9[c]	49.5
Total	100	158	100	240	100	272	100	49	100	68	100	1513	100	717

[a] Leaf cuticle.
[b] Fruit cuticle.
[c] Includes other identified acids, the 9(10),15-dihydroxypentadecanoic acids (1.0%) and the 7(8)-hydroxyhexadecane-1,16-dioic acids (3.2%).
[d] The 10,16-isomer is in general predominant, with smaller proportions of the 9,16- isomer and also minor quantities of the 8,16- isomer.
[e] Tentative identification, the position of the double bond not confirmed.
[f] Mostly compounds with EIMS compatible with poly-hydroxylated aliphatic acids.

can be attributed to the limitations in the quantification of acid monomers discussed above. Alternatively, some of the compounds released by depolymerization may not be volatile enough to be analyzed under the GC conditions used here.

In addition, partial cutin depolymerization further complicates the quantification of monomers and the calculation of the molar proportions of their functional groups. FTIR-spectra of the residues left after the $NaOCH_3$-methanolysis showed that the depolymerization of the ester aliphatic structure was incomplete in some cuticles. Analysis of the methanolysis residues of *Citrus aurantium* and *Prunus laurocerasus* revealed significant absorption at 1731–1733 cm^{-1}, as a result of the presence of the ester C=O, and at 2856 and 2930 cm^{-1}, due to the $(CH_2)_n$ chains. It is interesting to observe that in cases in which the ester depolymerization was apparently incomplete, these cutins were exactly those in which the molar proportions of glycerol hydroxyls to carboxylic acids was exceedingly high. This was particularly true in the case of *Citrus aurantium*. It may be that glycerol is easier to remove from the ester structure than some of the long-chain acids, which remained partially non-depolymerized. It is possible to speculate that the non-degradable fraction is largely composed of a cutin characterized by non-ester-bonds, as has been suggested for the cutin in *Clivia miniata* (Riederer and Schönherr, 1988).

2.2. Partial cutin depolymerization

The partial depolymerization of cutins was carried out using cuticles from three species, with a methanolysis reaction catalyzed by CaO, using procedures that had previously been developed for suberized materials (Graça and Pereira, 1997, 1999). The yields obtained were much lower than those achieved via $NaOCH_3$-methanolysis. In *Citrus aurantium*, the CaO-methanolysis products represented 25% of the $NaOCH_3$-methanolysis products, those in *Prunus laurocerasus* 10% and those in *Stephanotis floribunda* only 9%. A GC–MS analysis of the depolymerised materials following CaO-methanolysis basically displayed the same monomers solubilized by the $NaOCH_3$-methanolysis. Glycerol was the major monomer, with ca. 30% of the integrated area in the GC–MS runs, while most of the other components identified were the aliphatic acids that were also solubilized after $NaOCH_3$-methanolysis. Small quantities of "dimeric" compounds, ranging between 1.5 and 3.7% of the chromatogram integrated areas (Table 2) were also identified—namely glycerol esterified to ω-hydroxyacids acids, in the form of 1- and 2-monoacylglyceryl esters. The identification of these compounds via their EIMS spectra is discussed below.

The ω-hydroxyacids that were esterified to glycerol were among the main ones found as monomers. In *Stephanotis floribunda*, the acids found as glyceryl esters were the C18 mid-chain hydroxylated ω-hydroxyacids, namely the 9,10,18-trihydroxyoctadecanoic acid. C16 ω-hydroxyacids linked to glycerol were not obtained from this cutin, although they represent approximately 25% of all monomers. In *Prunus laurocerasus*, the main acylglycerols were the 1- and 2-monoacylglyceryl esters of the 9,10,18-trihydroxyoctadecanoic acid, with smaller quantities of glycerol linked to C16 acids. In *Citrus aurantium*, the main acylglycerol detected was the monoacylglyceryl ester of the 10(9),16-dihydroxyhexadecanoic acid, which is also the acid that dominates the monomer composition. In this cutin the 1-monoacylglyceryl ester of the 9,10,18-trihydroxyoctadecanoic acid was also found, occurring in a proportion that was relatively much higher than that found in the monomer form. In *Stephanotis floribunda* and *Prunus laurocerasus* the absence of the glyceryl ester of the 9-epoxy-18-hydroxyoctadecanoic acid is worth noting, given that it is a major monomer in both cutins. The same was observed in *Quercus suber* cork suberin, in which epoxyacids are the main acid monomers, but were not found as glyceryl esters following the same partial depolymerization procedure that was used here for cutins (Graça and Pereira, 1997).

2.3. Identification of the cutin acid monoacylglyceryl esters

The cutin acid monoacylglyceryl esters were identified via the EIMS spectra of their TMS derivatives. The EIMS spectra of the TMS derivatives of 1- and 2-monoacylglycerols have been studied (Curstedt, 1974; Myher et al., 1974) and the results are used in the discussion below. The 1- and 2-monoacylglyceryl esters of the 16-hydroxyhexadecanoic were synthesized and the mass spectrum of the 1-isomer was shown to be identical to that obtained from cutin. The synthesized 2-isomer displayed the differences reported in the mass spectra of the 1- and 2-isomers and helped to confirm the identification of the 2-isomers found in cutin. Fig. 1 presents the EIMS spectra, together with the fragmentation patterns of the TMS derivatives of three of the main glyceryl esters found in cutins—the 1-monoacylglyceryl ester of the 16-hydroxyhexadecanoic acid, the 1-monoacylglyceryl ester of the 9(10),16-dihydroxyhexadecanoic acids, and the 1-monoacylglyceryl ester of the 9,10,18-trihydroxyoctadecanoic acid.

The molecular ion was absent from all the spectra, but the ion at M-15 permitted molecular mass assignment. The cleavage between the C-2 and C-3 carbons in the glyceryl moiety gives rise to the m/z 103 and M-103 ions, the latter being characteristic of the spectra of the TMS derivatives of 1-monoacylglycerols (Myher et al., 1974). Other diagnostic ions are derived from the glyceryl moiety—i.e. at m/z 205 as a result of the cleavage between the C-2 and C-1 (the esterified carbon), and at

Table 2
Monoacylglycerols[a] of cutin ω-hydroxyacids identified in the products of the partial depolymerization of isolated cuticles by CaO-catalyzed methanolysis (analyzed as trimethylsilyl ethers)

Parts per thousand of the total integrated GC–MS peak areas	*Stephanotis floribunda*	*Prunus laurocerasus*	*Citrus aurantium*
1-Mono(16-hydroxyhexadecanoyl)glycerol		0.6	6.0
2-Mono{10(9),16-dihydroxyhexadecanoyl}glycerol			5.3
1-Mono{10(9),16-dihydroxyhexadecanoyl}glycerol		1.9	13.3
1-Mono(18-hydroxyoctadecanoyl)glycerol	4.4		
1-Mono{9(8–11),18-dihydroxyoctadec-?-enoyl}glycerol[b]	11.8		
2-Mono(9,10,18-trihydroxyoctadecanoyl)glycerol	5.9	2.4	
1-Mono(9,10,18-trihydroxyoctadecanoyl)glycerol	11.9	11.7	
1-Mono(22-hydroxydocosanoyl)glycerol	3.1		

[a] Nomenclature based in the recommended rules for acylglycerols (IUPAC-IUB, 1977).
[b] Tentative identification, the position of the double bond not confirmed.

m/*z* 219 due to the loss of the acyloxy moiety. The same loss of the acyloxy group from $M^{+\cdot}$ and $M\text{-}15^{+}$, but with the H rearrangement, gives rise to the ions at *m*/*z* 218 and 203, respectively (Curstedt, 1974). Other significant ions in the low-mass region occur at *m*/*z* 73 (the TMS group), *m*/*z* 129 (the glycerol carbon backbone with a TMS group [$H_2C{=}CH{-}CH{=}O^{+}{-}Si(CH_3)_3$]) and *m*/*z* 147 (produced by the rearrangement of two TMS groups) (Curstedt, 1974).

The high-mass region of the spectra shows the expected ions at M-15, M-73, the aforementioned M-103 and M-147 (loss of two TMS groups). The M-103 is very abundant in the case of the saturated chain ω-hydroxyacids, but much less so in the case of the ω-hydroxyacids that carry TMSiO groups in secondary positions. This is due to the competitive fragmentation associated with the latter. The M-103 ion also loses a TMSiOH group to yield the M-103-90 ion. The acyl ion is present, and the rearrangement of the acyl moiety with a TMS group leads to the ion of mass acyl +74 (Curstedt, 1974). Ions of mass acyl-16 and acyl-90 are also significant, the latter being typical of the TMS of monoacylglyceryl esters of ω-hydroxyacids (Graça and Pereira, 1997).

The mass spectra of the monoacylglyceryl esters of ω-hydroxyacids with TMS-derivatized secondary hydroxyls are dominated by the fragment ions that arise from the α-clevages associated with the latter, thus allowing them to be located in the acyl moiety. In the mass spectrum of the TMS derivative of the 1-monoacylglyceryl ester of the 10(9),16-dihydroxyhexadecanoic acids, ions derived from the cleavage of the bonds on both sides of the mid-chain TMSiO-carrying carbon were shown to be present: *m*/*z* 477 and 275 for the 10- isomer and *m*/*z* 463 and 289 for the 9-isomer (Fig. 1b). The latter ions reveal the presence of these two chromatographically unresolved positional isomers, with the TMSiO group located in either the C-9 or the C-10 of the acyl moiety (as observed in the monomers). The fragment ions that include the glyceryl moiety, which are found at *m*/*z* 477 and *m*/*z* 463, also lose a TMSiOH group with a mass of 90, thus producing other significant ions at *m*/*z* 387 for the 10-isomer and at *m*/*z* 373 for the 9-isomer.

The mass spectrum of the TMS derivative of the 1-monoacylglyceryl ester of the 9,10,18-trihydroxyoctadecanoic acid shows the complementary ions, at *m*/*z* 463 and 303, that are due to the cleavage between the mid-chain TMSiO-carrying carbons (Fig. 1c). An abundant ion at *m*/*z* 317 results from the rearrangement of the acyloxy moiety with a TMS group [TMSO-$CO(CH_2)_7CHOTMS$], following the cleavage between the C-9 and C-10 carbons. Most of the other significant ions in this compound's spectrum are assigned as discussed above.

The 2-monoacylglyceryl esters were identified by the similarity between their mass spectra and the 1-isomers, as well as by the conspicuous absence of the M-103 ion (and the consequent M-103–90) (Myher et al., 1974). The 2-isomers display a comparatively smaller ion at *m*/*z* 205, and a *m*/*z* 218 that is more abundant than the *m*/*z* 219. A comparatively abundant ion at *m*/*z* 191, which was attributed to the rearrangement of two TMS groups with one of the "C-1" carbons, is also characteristic of the 2-isomers (Curstedt, 1974).

2.4. Glycerol in the cutin polymer structure

Current models for the structure of the cutin polymer are basically composed of long-chain aliphatic monomers—mostly ω-hydroxyacids. In order to suggest a molecular model for the cutin ester polymer, it is necessary to quantify the relative proportions of carboxylic acid and hydroxyl groups and to know whether they are ester-linked or not. The ω-hydroxyacid monomers possess a carboxylic acid group and a primary hydroxyl group, and most carry one or two additional secondary hydroxyl groups. In the cutins that were studied for this purpose, the carboxylic acids and primary hydroxyl functions were shown to be almost all esterified, but roughly only half of the secondary hydroxyls were found to be ester-linked (Deas and Holloway, 1977;

Kolattukudy, 1977). The inter-esterification of ω-hydroxyacids can occur either through their primary functions, thereby building linear chains, or in the secondary hydroxyls, which results in the cross-linking of the structure. Tentative models for the cutin polymer have been drawn up on the basis of these two types of linking structures (Kollattukudy, 1977; Zlotnik-Mazori and Stark, 1988; Tegelaar, 1990).

More recently, oligomers of the two types of structures have been obtained from cutins and involve the main acids that are found as monomers. Linear dimers were identified in *Lycopersicon* after a partial alkaline hydrolysis (Osman et al., 1995), and ester oligomers with masses compatible with up to eight interconnected ω-hydroxyacids were detected in the same cutin, although it was not determined whether esterification occurred via primary or secondary linkages (Osman et al., 1999). In *Citrus aurantifolia* fruit cutin up to four ω-hydroxyacids ester-linked in linear form were identified after a mild depolymerization with iodotrimethylsilane

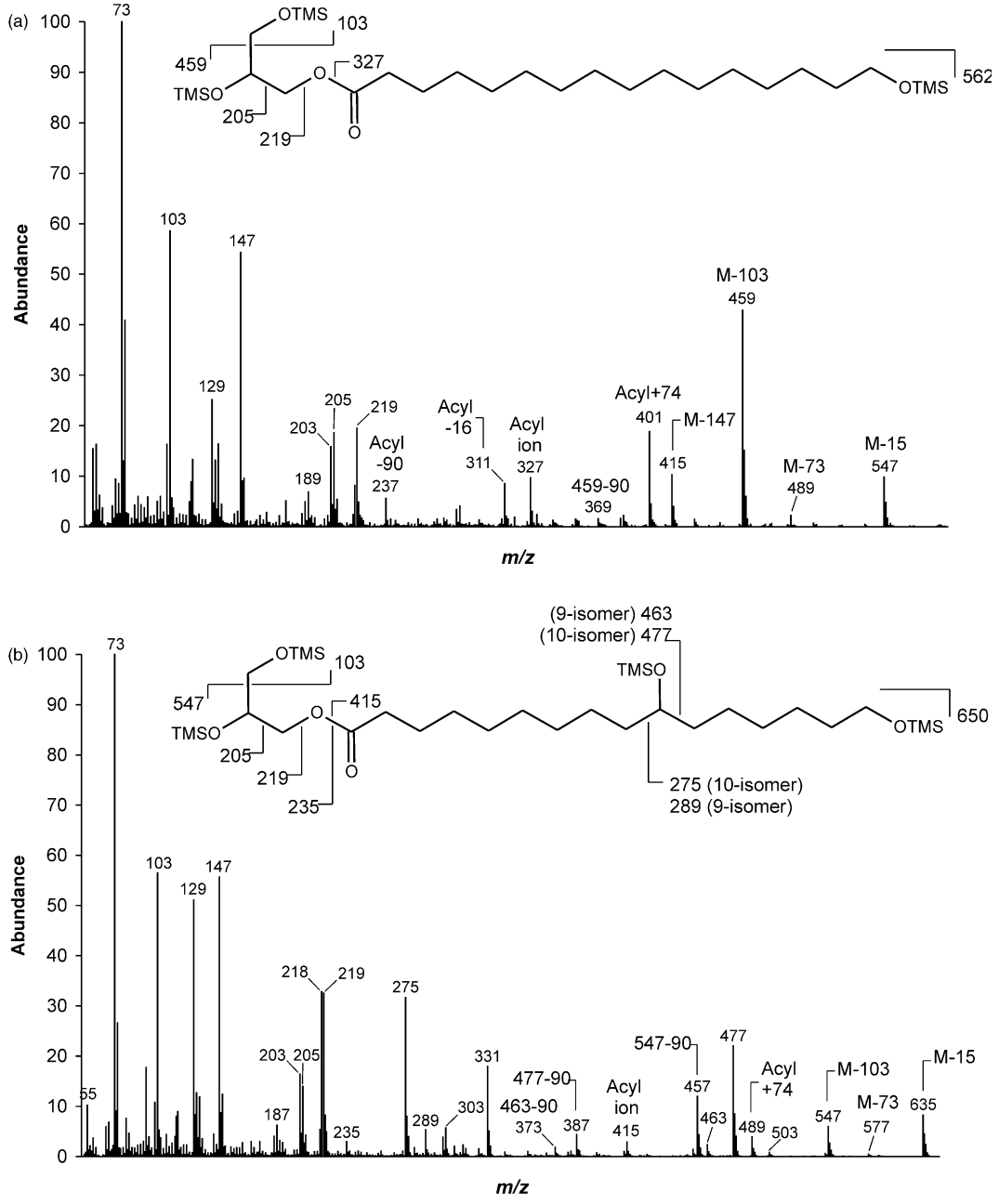

Fig. 1. EIMS spectra and main fragmentation patterns of monoacylglyceryl esters of ω-hydroxyacids obtained from cutins: (a) TMS derivative of the 1-mono(16-hydroxyhexadecanoyl)glycerol [16-trimethylsilanyloxyhexadecanoic acid 2,3-bis(trimethylsilanyloxy)propyl ester]; (b) TMS derivative of the 1-mono{10(9),16-dihydroxyhexadecanoyl}glycerol [10(9),16-bis(trimethylsilanyloxy)hexadecanoic acid 2,3-bis(trimethylsilanyloxy)propyl ester]; (c) TMS derivative of the 1-mono(9,10,18-trihydroxyoctadecanoyl)glycerol [9,10,18-tris(trimethylsilanyloxy)octadecanoic acid 2,3-bis(trimethylsilanyloxy)propyl ester].

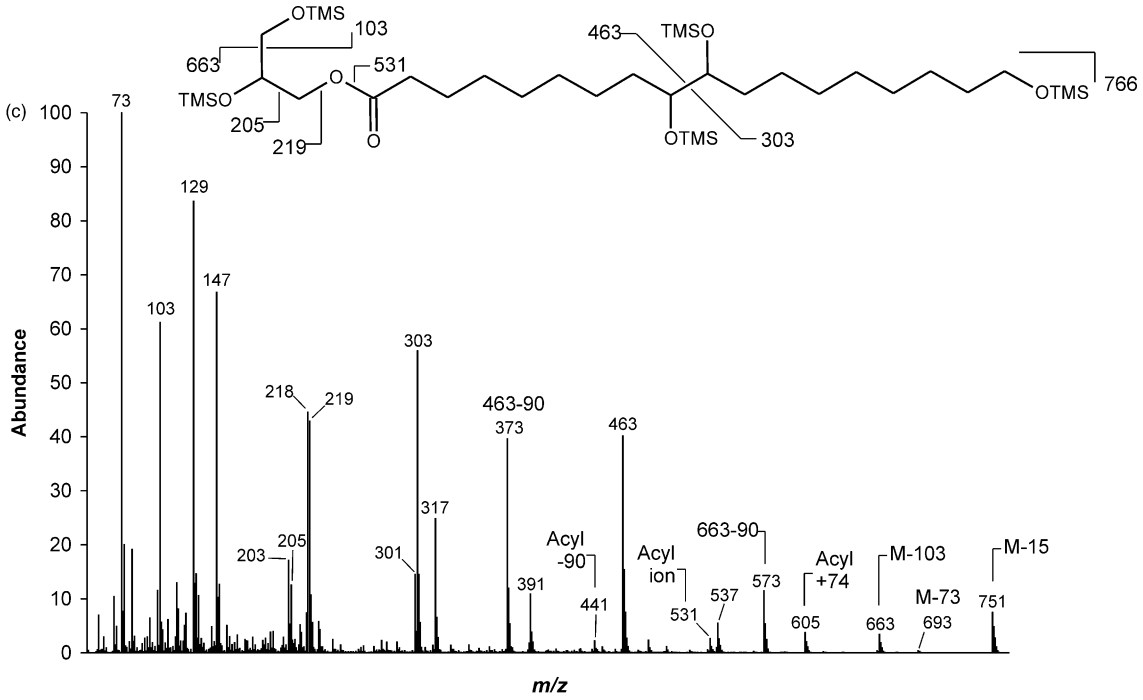

Fig. 1. (*continued*).

(Ray et al., 1998). A five-unit oligomer has also been identified in the products of a lipase enzymatic depolymerization, with ω-hydroxyacids and an aliphatic triol ester-linked through their secondary hydroxyls, thus building a zigzag structure (Ray and Stark, 1998).

In this study, following cutin de-esterification, glycerol was found co-solubilized with the long-chain ω-hydroxyacids. Subsequent to partial methanolysis conditions it was also found ester-linked to the ω-hydroxyacids. Calculation of the molar proportions shows that glycerol can bind a significant number of the carboxylic acid groups present in cutin. The presence of glycerol as an esterifying monomer within the cutin structure is compatible with studies on the intact cutin polymer of the *Citrus aurantifolia* fruit using CP-MAS solid state ^{13}C NMR, which revealed the presence of carbons involved in primary and secondary ester linkages (Zlotnik-Mazori and Stark, 1988; Stark et al., 1988, 1989). Abundant signals were also found for "rigid" carbons involved in secondary ester-linkages. This can be explained by the presence of secondary C-2 esterifications in glycerol, given that it has been postulated that only part of the secondary hydroxyls in ω-hydroxyacids are esterified (Deas and Holloway, 1977; Kolatukudy, 1977).

The evidence presented here for glycerol as an important monomer in cutin and for its linkage to ω-hydroxyacids gives rise to new possibilities for developing models for the molecular structure of the cutin polymer. Glycerol can obviously act as an anchoring point for ω-hydroxyacids, be they linear-linked or cross-linked. Conformation of the glycerol molecule by rotation around single bonds permits macromolecular expansion into several—or even opposite—directions.

The inter-esterification between ω-hydroxyacids may occur mostly in a linear form, at least in parts of the cutin polymer. The linking of these linear chains of ω-hydroxyacids to the same glycerol moiety can position the former in a parallel arrangement, thereby even rendering some kind of ordered structure possible. In this case, the oxygenated groups at the mid-chain of the monomers, such as epoxy, free hydroxyl or oxo, would be close enough to establish hydrogen bonds capable of constituting an important reinforcement in the polymeric structure. Such an arrangement, in which glycerol is linked to linear chains of ω-hydroxyacids in parallel structures, can be prevalent in some parts of the cuticle, thereby accounting, for instance, for the organized lamellae that are observed in the outer part ("cuticle proper") of many cuticular membranes (Holloway, 1982a).

Small quantities of coumaric acid were found solubilized as methyl esters after the $NaOCH_3$-methanolysis in all the cutins that were analyzed. This means that a number of the hydroxyl groups of the ω-hydroxyacids can be esterified to hydroxycinnamic acid aromatic moieties. Small quantities of aromatic monomers—namely coumaric and ferulic acids—have been obtained following cutin depolymerization (Riley and Kollatukudy, 1975; Hunt and Baker, 1980). Solid-state ^{13}C NMR studies on the fruit cutin of *Citrus aurantifolia* have shown significant signals assigned to carbons of esterified hydroxycinnamic acids (Stark et al., 1989). In suberins it

has been suggested that thanks to its esterification to ω-hydroxyacids, ferulic acid may play the role of bridging the aliphatic polyester structure to the abundant polyaromatics in suberized cell walls (Graça and Pereira, 1998, 2000b).

In suberins, glycerol esterified to all types of suberinic acids has been identified after partial methanolysis, and diester oligomers of diglycerol-α,ω-diacid have been obtained from the potato skin suberin. In the light of these results, it has been proposed for suberins that by means of their successive ester-linkage, glycerol and α,ω-diacids make the polyester polymer grow three-dimensionally (Graça and Pereira, 1997, 2000c). The latter structure can at most only constitute a minor part of the cutin structure, since α,ω-diacids are either absent or are only present in small quantities, as in the fruit cuticle of *Lycopersicon esculentum* here analyzed.

Lipids derived from glycerol that is ester-linked to long-chain fatty acids are known to play several essential roles in plants and animals, namely as structural components of cellular biomembranes and as insulating protective coatings (Lehninger, 1975). The suberized and cutinized cells in the outer tissues of plants have similar functions, namely those of protecting internal tissues and regulating mass exchange with the surrounding environment (Schreiber et al., 1996). Cutins and suberins are different from other glycerolipids inasmuch as their monomers are polymerized, thereby forming macromolecules that are largely based on ester linkages. This is possible because these biopolymers are comprised of bifunctional fatty acids, namely ω-hydroxyacids and α,ω-diacids, with at least two ester-linking functional groups in each monomer. Clearly, much more work will be necessary in the future if we are to achieve a complete understanding of the macromolecular structure of these plant biopolyesters.

3. Experimental

3.1. Plant material

Leaves were sampled from plants, some of which were cultivated in greenhouses (*Citrus aurantium* L. and *Stephanotis floribunda* Brongn.) and some in the open (*Hedera helix* L., *Juglans regia* L. and *Prunus laurocerasus* L.) at the Botanical Garden in Würzburg. Two types of fruit (*Capsicum annuum* and *Lycopersicon esculentum*) were bought at the local market in Würzburg. Cuticular membranes of the seven different species were isolated using the method described by Schönherr and Riederer (1986), albeit with small modifications. Disks were punched out from leaves and fruit and were immersed in an enzymatic solution containing 1% (w/w) cellulase (Celluclast, Novo Nordisk, Bagsvaerd, Denmark) and 1% (w/w) pectinase (Trenolin, Erbslöh, Geisenheim, Germany) dissolved in citric buffer (10^{-2} M, pH 3.0). Sodium acid (NaN_3, Sigma Aldrich, Deisenhofen, Germany) that gave a final concentration of 10^{-3} M was added to the enzymatic solution to prevent the growth of microorganisms. After several days fruit cuticles and cuticles from the adaxial leaf sides were collected, thoroughly washed in deionised water and then air-dried under a gentle stream of nitrogen.

Scissors were used to cut isolated cuticles into very small pieces (<1 mm), which were then successively extracted in Soxhlet apparatus with CH_2Cl_2 (6 h), EtOH (12 h), H_2O (18 h) and MeOH (12 h). The amounts extracted by each solvent and the total (in parentheses) are given as% of the dry wt: *Capsicum annuum*, 2.0, 2.7, 8.1, 1.8 (14.6); *Citrus aurantium*, 8.0, 2.5, 11.6, 2.2 (24.3); *Lycopersicon esculentum*, 6.1, 3.4, 11.6, 2.3 (23.4); *Hedera helix*, 16.8, 5.3, 11.7, 1.7, (35.5); *Juglans regia*, 27.1, 5.3, 14.9, 4.0 (51.3); *Prunus laurocerasus*, 22.0, 3.4, 8.9, 2.0 (36.3); *Stephanotis floribunda*, 1.9, 1.8, 7.2, 1.1 (12.0).

3.2. $NaOCH_3$-catalyzed methanolysis

Extracted cuticles (100–200 mg) (dried over P_2O_5 under vacuum at 40 °C) were refluxed for 5 h in 15–20 ml of a 50 mM solution of $NaOCH_3$ in MeOH, which was prepared by dissolving metallic sodium in dry MeOH. The reaction mixtures were filtered in 0.45 μm PTFE filters and aliquots of the methanolysates were taken directly for GC–FID and GC–MS analysis. The residues were dried and weighed to determine the methanolysis yields. Materials extracted by the $NaOCH_3$-methanolysis of two replicates are expressed as % of the extracted cuticles and in μg cm^{-2} (the latter value is based on the average percentage): *Capsicum annuum*, 81.5, 82.7 (1513 μg cm^{-2}); *Citrus aurantium*, 48.8, 52.8 (68 μg cm^{-2}); *Lycopersicon esculentum*, 72.1, 84.7 (717 μg cm^{-2}); *Hedera helix*, 77.0, 85.5 (272 μg cm^{-2}); *Juglans regia*, 58.8, 59.0 (49 μg cm^{-2}); *Prunus laurocerasus*, 56.5, 58.8 (240 μg cm^{-2}); and *Stephanotis floribunda*, 63.7, 64.7 (158 μg cm^{-2}).

3.3. Monomer quantitative analysis

Internal standards were added to the aliquots taken for the GC–FID analysis used to quantify monomers. 1,12-Dodecanediol (0.1–0.15 mg in MeOH soln.) was used as the internal standard for glycerol and 12-hydroxyoctadecanoic acid methyl ester (0.3–0.5 mg in MeOH soln.) for the quantification of the phenolic and long-chain aliphatic monomers. Integrated areas were corrected by response factors of 0.75 for glycerol, 0.9 for coumaric acid methyl ester, 0.76 for alkan-1-ols, 1.14 for alkan-1-oic acid methyl esters and 1.13 for ω-hydroxyacid methyl esters (Graça and Pereira, 2000a).

3.4. CaO-catalyzed methanolysis

Extracted and dried cuticles of *Prunus*, *Stephanotis* and *Citrus* (100–200 mg) were mixed with an equal amount of CaO (fine powder, pre-activated at 800 °C), and refluxed in dry methanol (20 ml), with stirring, for 1 h. The reaction mixtures were filtered in 0.2 μm PTFE filters. After solvent removal the methanolysate extracts were dried for derivatization for GC–MS analysis, and for quantitative determination. The materials extracted by CaO-methanolysis (two replicates), expressed in % of extractive-free cuticles, were: *Citrus*, 13.5, 12.7; *Prunus*, 5.3, 5.3; *Stephanotis*, 6.5, 7.1.

3.5. GC–FID and GC–MS analysis

Aliquots taken from the $NaOCH_3$-methanolysate solutions (1–2.5 ml) were solvent evaporated under N_2 and the residue trimethylsilylated with pyridine-BSTFA (1% TMCS) (1:1) at 60 °C for 15 min. Monomers were quantified by GC–FID in a HP 5890 under the following GC conditions: J&W DB5-MS column (60 m×0.25 mm×0.25 μm); split injection, injector, 300 °C, detector, 300 °C; initial temperature, 100 °C (5 min), 10 °C min^{-1} up to 240 °C, 2 °C min^{-1} up to 300 °C (15 min); carrier gas He, at 30 psig of column head pressure. The same GC conditions were used for GC–MS analysis. The dried extracted materials from the CaO-methanolysis were TMS derivatized as above and analyzed by GC–MS. GC conditions: SGE HT-5 column (50m×0.33 mm×0.1 μm): splitless injection, injector, 325 °C; initial temperature, 50 °C (5 min), 10 °C min^{-1} up to 250 °C, 3 °C min^{-1} up to 325 °C (15 min); carrier gas He, at 1 ml min^{-1}.

GC–MS of the $NaOCH_3$-methanolysates and of the CaO-methanolysates were conducted under the GC conditions described above in an Agilent 6890 MSD 5973, and the EIMS spectra were obtained at 70 eV of ionisation energy, source at 220 °C and quadrupole at 150 °C.

3.6. Identification of compounds

Cutin compounds were identified from the EIMS spectra of their TMS derivatives. Long-chain and phenolic monomers were identified on the basis of spectra that have been published and discussed (Eglinton and Hunneman, 1968; Holloway, 1982b). Glycerol was identified by comparing the mass spectrum and GC retention time with an authentic standard (Graça and Pereira, 2000c). Monoacylglyceryl esters of ω-hydroxy-acids (as TMS derivatives) were identified on the basis of previous studies of the mass spectra of this type of compound (Myher et al, 1974; Curstedt, 1974; Graça and Pereira, 1997) and by synthesising the 1- and 2-mono (16-hydroxyhexadecanoyl)glycerol model compounds.

Main fragment ions and relative abundance of the TMS derivatives of monoacylglycerols:

Synthesized 1-mono(16-hydroxyhexadecanoyl)glycerol, trisTMS: molecular mass 562; M-15 (*m*/*z* 547), 26; M-73 (*m*/*z* 489), 2; M-103 (*m*/*z* 459), 100; M-147 (*m*/*z* 415), 7; Acyl + 74 (*m*/*z* 401), 33; *m*/*z* 385 (not assigned), 4; M-103-90 (*m*/*z* 369), 3; Acyl ion (*m*/*z* 327), 14; Acyl-16 (*m*/*z* 311), 11; Acyl-90 (*m*/*z* 237), 9; *m*/*z* 219, 36; *m*/*z* 218, 18; *m*/*z* 205, 19; *m*/*z* 203, 25; *m*/*z* 147, 44; *m*/*z* 129, 34; *m*/*z* 103, 72; *m*/*z* 73, 61.

Synthesized 2-mono(16-hydroxyhexadecanoyl)glycerol, trisTMS: molecular mass 562; M-15 (*m*/*z* 547), 7; M-73 (*m*/*z* 489), 1; M-147 (*m*/*z* 415), 1; Acyl + 74 (*m*/*z* 401), 29; *m*/*z* 385 (not assigned), 11; Acyl ion (*m*/*z* 327), 13; Acyl-16 (*m*/*z* 311), 23; Acyl-90 (*m*/*z* 237), 1; *m*/*z* 219, 29; *m*/*z* 218, 70; *m*/*z* 203, 23; *m*/*z* 191, 20; *m*/*z* 147, 67; *m*/*z* 129, 78; *m*/*z* 103, 93; *m*/*z* 73, 100.

Monoacylglycerols from cutins:

1-Mono(16-hydroxyhexadecanoyl)glycerol, trisTMS: retention time 29.2 min, molecular mass 562; M-15 (*m*/*z* 547), 10; M-73 (*m*/*z* 489), 2; M-103 (*m*/*z* 459), 43; M-147 (*m*/*z* 415), 10; Acyl + 74 (*m*/*z* 401), 19; *m*/*z* 385 (not assigned), 2; M-103-90 (*m*/*z* 369), 2; Acyl ion (*m*/*z* 327), 10; Acyl-16 (*m*/*z* 311), 9; Acyl-90 (*m*/*z* 237), 6; *m*/*z* 219, 19; *m*/*z* 218, 8; *m*/*z* 205, 19; *m*/*z* 203, 16; *m*/*z* 147, 55; *m*/*z* 129, 25; *m*/*z* 103, 59; *m*/*z* 73, 100.

1-Mono(18-hydroxyoctadecanoyl)glycerol, trisTMS: retention time 32.2 min, molecular mass 590; M-15 (*m*/*z* 575), 9; M-73 (*m*/*z* 517), 1; M-103 (*m*/*z* 487), 31; M-147 (*m*/*z* 443), 2; Acyl + 74 (*m*/*z* 429), 14; M-103-90 (*m*/*z* 397), 3; Acyl ion (*m*/*z* 355), 6; Acyl-16 (*m*/*z* 339), 6; Acyl-90 (*m*/*z* 265), 5; *m*/*z* 219, 14; *m*/*z* 218, 8; *m*/*z* 205, 15; *m*/*z* 203, 16; *m*/*z* 147, 44; *m*/*z* 129, 25; *m*/*z* 103, 63; *m*/*z* 73, 100.

1-Mono(22-hydroxydocosanoyl)glycerol, trisTMS: retention time 38.9 min, molecular mass 646; M-15 (*m*/*z* 631), 7; M-73 (*m*/*z* 573), <1; M-103 (*m*/*z* 543), 21; M-147 (*m*/*z* 499), 2; Acyl + 74 (*m*/*z* 485), 9; M-103-90 (*m*/*z* 453), 1; Acyl ion (*m*/*z* 411), 4; Acyl-16 (*m*/*z* 395), 4; Acyl-90 (*m*/*z* 321), 3; *m*/*z* 219, 13; *m*/*z* 218, 12; *m*/*z* 205, 11; *m*/*z* 203, 9; *m*/*z* 147, 24; *m*/*z* 129, 22; *m*/*z* 103, 50; *m*/*z* 73, 100.

1-Mono{10(9),16-dihydroxyhexadecanoyl}glycerol, tetrakisTMS: retention time 30.6 min, molecular mass 650; M-15 (*m*/*z* 631), 8; M-73 (*m*/*z* 577), <1; M-103 (*m*/*z* 547), 6; M-147 (*m*/*z* 503), <1; Acyl + 74 (*m*/*z* 489), 4; M-103-90 (*m*/*z* 457), 11; Acyl ion (*m*/*z* 415), 3; Acyl-16 (*m*/*z* 399), <1; Acyl-90 (*m*/*z* 325), 1; *m*/*z* 219, 31; *m*/*z* 218, 31; *m*/*z* 205, 14; *m*/*z* 203, 16; *m*/*z* 147, 55; *m*/*z* 129, 50; *m*/*z* 103, 55; *m*/*z* 73, 100; [α-cleavages to the carbon with the secondary TMSiO group, see Fig. 1(b)] *m*/*z* 477, 20; [477–90], *m*/*z* 387, 4; *m*/*z* 463, 3; [463–90], *m*/*z* 373, 2; *m*/*z* 289, 6; *m*/*z* 275, 30.

2-Mono{10(9),16-dihydroxyhexadecanoyl}glycerol, tetrakisTMS: retention time 30.2 min, molecular mass 650; M-15 (*m*/*z* 631), 3; M-147 (*m*/*z* 503), <1; Acyl +

74 (*m*/*z* 489), 5; Acyl ion (*m*/*z* 415), 4; Acyl-16 (*m*/*z* 399), 1; Acyl-90 (*m*/*z* 325), <1; *m*/*z* 219, 20; *m*/*z* 218, 39; *m*/*z* 205, 3; *m*/*z* 203, 13; *m*/*z* 191, 11; *m*/*z* 147, 54; *m*/*z* 129, 38; *m*/*z* 103, 49; *m*/*z* 73, 100; [α-cleavages to the carbon with the secondary TMSiO group] *m*/*z* 477, 6; [477-90], *m*/*z* 387, 6; *m*/*z* 463, 3; [463-90], *m*/*z* 373, 7; *m*/*z* 289,11; *m*/*z* 275, 22.

1-Mono{9(8–11),18-dihydroxyoctadec-?-enoyl}glycerol, tetrakisTMS: retention time 33.6 min, molecular mass 676; M-15 (*m*/*z* 661), 3; M-103 (*m*/*z* 573), 1; M-147 (*m*/*z* 529), <1; Acyl + 74 (*m*/*z* 515), 2; M-103–90 (*m*/*z* 483), 8; Acyl ion (*m*/*z* 441), 3; Acyl-16 (*m*/*z* 425), <1; Acyl-90 (*m*/*z* 351), 1; *m*/*z* 219, 20; *m*/*z* 218, 12; *m*/*z* 205, 9; *m*/*z* 203, 10; *m*/*z* 147, 34; *m*/*z* 129, 38; *m*/*z* 103, 49; *m*/*z* 73, 100; [α-cleavages to the carbon with the secondary TMSiO group] *m*/*z* 489, 1; *m*/*z* 475, 15; [475–90], *m*/*z* 385, 1; *m*/*z* 463, 3; [463–90], *m*/*z* 329, 13; *m*/*z* 315, 38.

1-Mono(9,10,18-trihydroxyoctadecanoyl)glycerol, pentakisTMS: retention time 35.5 min, molecular mass 766; M-15 (*m*/*z* 751), 7; M-73 (*m*/*z* 693), <1; M-103 (*m*/*z* 663), 3; Acyl + 74 (*m*/*z* 605), 4; M-103–90 (*m*/*z* 573), 11; Acyl ion (*m*/*z* 531), 3; Acyl-16 (*m*/*z* 515), <1; Acyl-90 (*m*/*z* 441), 2; *m*/*z* 219, 43; *m*/*z* 218, 44; *m*/*z* 205, 12; *m*/*z* 203, 17; *m*/*z* 147, 67; *m*/*z* 129, 84; *m*/*z* 103, 61; *m*/*z* 73, 100; [α-cleavages to the carbon with the secondary TMSiO group, see Fig. 1(c)] *m*/*z* 463, 40; [463–90], *m*/*z* 373, 40; *m*/*z* 303, 38; *m*/*z* 317, 25.

2-Mono(9,10,18-trihydroxyoctadecanoyl)glycerol, pentakisTMS: retention time 34.6 min, molecular mass 766; M-15 (*m*/*z* 751), 3; Acyl + 74 (*m*/*z* 605), 3; Acyl ion (*m*/*z* 531), 4; Acyl-16 (*m*/*z* 515), 1; Acyl-90 (*m*/*z* 441), 3; *m*/*z* 219, 33; *m*/*z* 218, 60; *m*/*z* 205, 3; *m*/*z* 203, 14; *m*/*z* 191, 10; *m*/*z* 147, 68; *m*/*z* 129, 83; *m*/*z* 103, 72; *m*/*z* 73, 100; [α-cleavages to the carbon with the secondary TMSiO group] *m*/*z* 463, 16; [463–90], *m*/*z* 373, 54; *m*/*z* 303, 47; *m*/*z* 317, 26.

3.7. Synthesis of 1- and 2-mono(16-hydroxy hexadecanoyl)glycerol

The synthesis reaction was performed according to Neises and Steglich (1978). 17.5 mg (65 μmol) of 16-hydroxyhexadecanoic acid (Tokyo Kasei) were dissolved in 2.5 ml of DMF and 5 μmol of 4-(dimethylamino)-pyridine (DMAP) dissolved in 1 ml of CH_2Cl_2, whereupon 18.3 mg (200 μmol) of glycerol (Merck) were added. The solution was stirred at 0 °C and 100 μmol of *N,N′*-dicyclohexylcarbodiimide (DCC) dissolved in 2 ml of CH_2Cl_2 were added. The reaction solution was allowed to reach ambient temperature and react for 24 h. The reaction mixture was concentrated under reduced pressure, dissolved in 2.5 ml of $CHCl_3$, and washed twice with 2 ml of H_2O. Aliquots from the $CHCl_3$ solution were dried and TMS derivatives prepared as described above and analyzed by GC–EIMS.

The yield of monoacylglycerols, which was calculated on the basis of the ion chromatogram integrated areas, was ca. 31.5% for the 1-mono(16-hydroxyhexadecanoyl)-glycerol and 0.5% for the 2-mono(16-hydroxyhexadecanoyl)glycerol.

3.8. FTIR analysis

Dried extracted cuticles and the corresponding residues following $NaOCH_3$-methanolysis were milled in a vibratory ball mill, and 1.5–2 mg of the milled materials were mixed with 200 mg KBr in a pellet device. Absorbance FTIR spectra were obtained using a BioRad FTS 165, as described by Rodrigues et al. (1998).

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