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CONSTITUENT ACIDS OF *LIMONIA ACIDISSIMA* LEAF CUTIN

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Key Word Index—*Limonia acidissima*; Rutaceae; leaf cutin; monoacids, diacids; hydroxy acids; aromatic acids; *p*-hydroxy benzaldehyde; capillary GC-MS.

Abstract—The leaf cutin of *Limonia acidissima* was found to comprise *n*-alkanoic (C_{12}), α,ω-alkanedioic (C_3–C_{16}), hydroxyalkanoic (C_3–C_{16}), dihydroxy alkanoic (C_4–C_{20}), hydroxy α,ω-alkanedioic (C_{14}–C_{16}) and aromatic acids, together with *p*-hydroxy benzaldehyde and heptadecane diol. The main constituents were 9, 16- and 10, 16-dihydroxyhexadecanoic acids (*ca* 30%), 10,20-dihydroxyicosanaic acid (*ca* 10%) and 7-hydroxyhexadecane-1,16-dioic acid (*ca* 15%).

INTRODUCTION

The family Rutaceae is very rich in economically important plants [1]. Structural elucidation on secondary metabolites [2] as well as investigation on leaf surface chemistry [3] are well documented. However, the composition of aliphatic monomers in the lipid biopolymer cutin which constitutes the structural component of the cuticle, is not well studied in the leaves of Rutaceae [4–7].

The chemical composition of plant cutins is important because these polyester biopolymers prevent diffusion of moisture and other molecules. Agricultural chemicals interact with the polyester barrier and associated waxes [8–10] and therefore such interaction plays a crucial role in their formulation. It also acts as a major protective barrier towards some pathogenic microorganisms which are unable to synthesize the enzyme cutinase. Moreover the degree of cross linking, the presence of reactive groups, such as epoxides in the biopolymer very much influence the development and structure of the cuticular membrane. Although the composition of the polymer matrix differs between the different plant parts within a species, it has been observed that cutin from both angiosperms and gymnosperms [4, 11] contain C_{16} or C_{18} hydroxyacids as the main components.

The present investigation conducted on the leaf cutin of *Limonia acidissima* describes the identification of its constituent monomers using capillary GC-MS. This programme is a part of the investigation relating to studies on leaf structure properties namely cutin [12] and cuticle [Das and Thakur, unpublished data] of some Indian species.

RESULTS AND DISCUSSION

Identification of the cutin monomers liberated by alkaline hydrolysis has been made through direct comparison of mass spectral data using computerized library searching and by analysing the mass spectral fragmentation patterns. The composition of *L. acidissima* leaf cutin is given in Table 1, whilst the percentages of the monomers and some of their important mass spectral fragmentations are presented in Table 2. The relative amount of each cutin acid as determined by measuring the area under the GC peak of the corresponding TMSi ether TMSi ester.

Table 1. Composition of *L. acidissima* leaf cutin monomer acids and other classes as determined by hydrolysis

Monomers	Relative percentage
Alkanoic acids	0.1
Alkanedioic acids	3.7
Hydroxyalkanoic acids	15.0
Dihydroxyalkanoic acids	40.4
Hydroxyalkandioic acids	20.4
Aromatic acids	4.8
1,17-Heptadecane diol	1.4
4-Hydroxybenzaldehyde	0.2
Unidentified	14.1

The distribution of the components derived from depolymerization are (i) fatty acids having carbon numbers less than 14, 16.9%; (ii) fatty acids having carbon numbers higher than 14, 62.6%; (iii) aromatic acids, 4.8%; (iv) other compounds, 1.6% and (v) unidentified compound, 14.1%. Although in the analysis of cutin acids greater emphasis is usually given to acids having a carbon number greater than 14, the identification of lower acids to an extent of 17% prompted analysis (Table 1) of this fraction also. The presence of alkanoic, alkanedioic, hydroxyalkanoic and dihydroxyalkanoic acids with a slight preponderance of even carbon fatty acids (9.3%) over odd (7.6%) were noted in fraction (i). Although it is generally proposed that branched acids are not present in cutin polymer [11] the presence of 11% branched hydroxyalkanoic acid in this leaf cutin is notable.

Among the fatty acids having chain lengths greater than C_{14},C_{16} fatty acids constitute *ca* 50% of the total cutin monomers whereas C_{18} and C_{20} fatty acids amount

Table 2. Relative percentages and important mass spectral peaks for TMSi ether TMSi ester derivatives of monomeric components present in depolymerized leaf cutin of *L. acidissima*

Components	Relative percentage	Important peaks (*m/z*)
Alkanoic acid:		
Dodecanoic	0.1	272 $[M]^+$, 257 $[M-15]^+$, 117, 75, 73
Alkanedioic acids:		
1,3-propanedioic	0.8	233 $[M-15]^+$, 147(100%), 131, 117, 73
1,5-pentanedioic	0.4	261 $[M-15]^+$, 147, 117, 75, 73
1,6-hexanedioic	0.2	275 $[M-15]^+$, 147, 73
1,9-nonanedioic	0.4	317 $[M-15]^+$, 201, 147, 117, 75, 73
1,10-decanedioic	0.2	331 $[M-15]^+$, 315 $[M-15-16]^+$, 147, 117, 75, 73
1,11-undecanedioic	0.5	345 $[M-15]^+$, 147, 117, 75, 73
*Decenedioic acid	0.2	344 $[M]^+$, 329 $[M-15]^+$, 147, 117, 73(100%)
1,16-hexadecanedioic	1.0	415$[M-15]^+$, 399 $[M-31]^+$, 117, 75, 73
Hydroxyalkanoic acids:		
2-Hydroxypropanoic	1.1	233 $[M-1]^+$, 219 $[M-15]^+$, 147, 117, 73
2-Hydroxy-2-methylpropanoic	7.2	247 $[M-1]^+$, 233 $[M-15]^+$, 147, 131, 117, 73
3-Hydroxy-2-methylbutanoic	3.9	261 $[M-1]^+$, 247 $[M-15]^+$, 147, 117, 73
10-Hydroxydecanoic	1.3	317 $[M-15]^+$ and other characteristic peaks
11-Hydroxyundecanoic	0.5	331 $[M-15]^+$ and other characteristic peaks
10-Hydroxypentadecanoic	0.1	387 $[M-15]^+$, 371 $[M-31]^+$, 331 (α-cleavage), 317 (α-cleavage), 147, 117, 75, 73
16-Hydroxyhexadecanoic	0.9	401 $[M-15]^+$, 385 $[M-31]^+$, 311 $[M-14-90]^+$, 147, 117, 103, 75, 73
Dihydroxyalkanoic acids:		
2,3-Dihydroxybutanoic	0.1	321 $[M-15]^+$, 292 $[M-CO_2]^+$, 220 (McLafferty rearrangement), 200, 147, 117, 73
9,16-Dihydroxyhexadecanoic and 10,16-Dihydroxyhexadecanoic	27.9	489 $(M-15]^+$, 317 (α-cleavage), 289 (α-cleavage), 489 $[M-15]^+$, 331 (α-cleavage), 275 (α-cleavage),
9,18-Dihydroxyoctadecanoic and 10,18-Dihydroxyoctadecanoic	1.5	517 $[M-15]^+$, 331, 317, 303 (all α-cleavages).
10,20-Dihydroxyicosanoic	10.9	331 (100%, α-cleavage).
Hydroxyalkanedioic acids:		
6-Hydroxytetradecane 1,14-dioic and 7-Hydroxytetradecane-1,14-dioic	0.9	475 $[M-15]^+$, 317, 275 (α-cleavages) 475 $[M-15]^+$, 303, 289 (α-cleavages)
8-Hydroxyhexadecane-1,16-dioic and 7-Hydroxyhexadecane-1,16-dioic	4.3	317, 303 (α-cleavages), 147, 117, 73 503 $[M-15]^+$, 331(100%), 289(80%)
7-Hydroxyhexadecane-1,16-dioic	15.2	503 $[M-15]^+$, 331(100%), 289(80%)
Aromatic acids:		
4-Hydroxybenzoic	0.3	282 $[M]^+$, 267 $[M-15]^+$, 223, 193, 91, 73
Phthalic	2.4	310 $[M]^+$, 295 $[M-15]^+$, 149, 73
3-Methoxy-4-hydroxybenzoic	0.1	312 $[M]^+$, 297 $[M-15]^+$, 267, 253, 223, 73
4-Hydroxycinnamic	1.0	308 $[M]^+$, 293 $[M-15]^+$, 267, 249, 233, 219, 73
3-Hydroxycinnamic	1.0	308 $[M]^+$, 293 $[M-15]^+$, 249, 219, 73
Other compounds:		
4-Hydroxybenzaldehyde	0.2	194 $[M]^+$, 179 $[M-15]^+$, 151, 91, 77
1,17-Heptadecanediol	1.4	414 $[M]^+$, 399 $[M-15]^+$, 383 $[M-31]^+$, 301 $[M-15-90]^+$, 192 $[M-30/2]^+$, 149, 147, 117, 75, 73

*Position of double bond undetermined.

to 1.5 and 10.7%, respectively. The distribution of such fatty acids, the predominance of dihydroxyhexadecanoic acids (27.9%), low levels of 16-hydroxy hexadecanoic acid (0.9%) and the absence of fatty acids in the range C_{14} to C_{20}, clearly reflect that the cutin in the leaves of *L. acidissima* is well elaborated and developed [13]. The degree of development and polymerization of this cutin is related to the high abundance of dihydroxyalkanoic (40.4%) and hydroxyalkanedioic acids (20.4%). Amongst the dihydroxy alkanoic acids the presence of fatty acids belonging to the members of all the three classes viz. C_{16} (27.9%), C_{18} (1.5%) and C_{20} (10.9%) occur. The presence of alkanedioic acids with chain lengths lower than C_{14} (13.9%) and hydroxyalkanedioic acids amounting to a

total of 20.4% (Tables 1 and 2) is unusual since such high percentage of dioic acids are normally found in suberins [14].

The present investigation revealed the presence of a few less common aromatic acids, viz. 3-hydroxycinnamic, 4-hydroxybenzoic, 3-methoxy-4-hydroxybenzoic and phthalic together with the more common 4-hydroxy cinnamic. The comparative abundance of these less common acids (3.8% of total monomers) is an important feature in the matrix of the cutin biopolymer. Such acids esterified to the polyesters might play a protective role when released by the hydrolytic enzymes excreted by fungi.

The presence of 1, 17-heptadecane diol in *L. acidissima* is probably the first report of its occurrence as a constituent of a cutin. The occurrence of triols covalently attached in the cutin of *Psilotum* [15] has already been reported. The exhaustive extraction of the cutin prior to hydrolysis precludes the possibility that the diol is a component of the cuticular waxes.

The characterization of 4-hydroxy benzaldehyde as a hydrolytic product under basic condition also represents the first report of its occurrence in a cutin. It might have arisen either directly through hydrolysis of the cutin itself or from a *p*-coumaryl residue bonded to the cutin structure. The possible pathway for this formation from the latter source may involve hydration of the double bond, followed by cleavage of the α, β bond with respect to the ester function under the influence of the electron releasing effect of the *p*-hydroxy group.

EXPERIMENTAL

Fresh mature leaves of *L. acidissima* were collected from the University Campus in Sept. Leaf segments (× 250) (10 × 10 mm) were immersed successively in $CHCl_3$ and MeOH (2 hr in each solvent), dried and immersed in 100 ml of $ZnCl_2$–conc HCl (1:2) overnight to separate cuticular membranes. These were washed with H_2O and the $ZnCl_2$–HCl treatment repeated. After thorough washing the cuticles were extd in a Soxhlet with MeOH, dried and boiled with 1.6% ammonium oxalate, 0.4% oxalic acid soln (500 ml) for removal of pectin. The membranes were once again washed, air-dried and extd with MeOH and $CHCl_3$–MeOH (1:1) to remove residual lipids. The cutin obtained was dried *in vacuo* and powdered (presence of ester group indicated from IR spectra).

Depolymerization was achieved by refluxing 50 mg of cutin with 100 ml of 3% KOH in EtOH for 6 hr. After removing the EtOH the resulting soln was acidified (concd HCl) and extracted with Et_2O (5 × 100 ml). The Et_2O soln was evapd to dryness and the solid dried over P_2O_5.

The cutin acids thus isolated were treated with excess *N, O*-bis-trimethylsilyl acetamide and heated at 100° for 30 min in a N_2 atmosphere. Excess reagents were evapd under N_2 and the sample redissolved in $CHCl_3$ and injected directly in to the GC and GC-MS systems. GC was performed on a 30 m DB-5 fused silica capillary column, temp. prog. from 70 to 250° at 10°/min using He as carrier (1 ml/min). GC-MS was performed at 72 eV with a splitless injection system and a computerized library search facility.

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