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A CUTIN ACID IN *PINUS SYLVESTRIS* MICROSPORES

ANDREW B. CALDICOTT* and GEOFFREY EGLINTON

Organic Geochemistry Unit, School of Chemistry, University of Bristol, Bristol BS8 1TS, England.

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Key Word Index—*Pinus sylvestris*; Pinaceae; pollen; cutin acids; 9,16-dihydroxyhexadecanoic acid; nonacosan-10-ol; chemotaxonomy.

Abstract—Nonacosan-10-ol (0·7%) and the cutin acid, 9,16-dihydroxyhexadecanoic acid (0·3%) are present in *Pinus sylvestris* microspores. The pollen coat hence has some features in common with leaf cuticles.

INTRODUCTION

Dihydroxyhexadecanoic acids are widely distributed in nature, largely as components of plant cutin [1–7]. In these polymerically bound acids a primary hydroxyl function is always present. Biosynthetically derived dihydroxyhexadecanoic acids, other than those found in cutins, possess no primary hydroxyl functions [8].

While cutin is a polymeric component of the outer layer of plant leaves, its presence in microspores has always been held in doubt [9]. A cutin acid is now reported as a bound constituent of *Pinus sylvestris* microspores, a fact which strongly implies that microspores may be cutinized to some extent.

RESULTS

Earlier studies indicate that GC–MS is particularly useful in the analysis of phenolic and cutin acids [1, 4, 10, 11]. In this study, such acids were identified by the GLC retention data and mass spectra of their methyl ester *O*-trimethylsilyl ether derivatives [10–12]. *Pinus sylvestris* microspores were subjected to both ultrasonic and exhaustive Soxhlet extraction with a number of organic solvents. The solvent-stripped spores were saponified with ethanolic KOH. Et_2O-soluble acids which were liberated represent only a minor part of the total spore wt (2·6%, Table 1), the major component being *p*-coumaric acid. Minor amounts of *p*-methoxycinnamic acid probably result as an artefact from reaction of diazomethane with *p*-coumaric acid [3]. Small amounts of isomeric dihydroxyhexadecanoic acids were also present, constituting *ca* 0·3% of the spore by weight.

The mass spectrum of the relevant GC–MS peak (as the methyl ester, di-TMSi ether) is similar to that published [4] for the 9,16-isomer, and is readily rationalized [1]. The molecular ion is absent, though the M-15, M-31 and M-47 series

Table 1. GC–MS analysis of ether soluble acids liberated upon saponification of solvent-stripped *Pinus sylvestris* microspores

Ether-soluble acids*	Retention index SE-33	% Total
p-Methoxycinnamic	1645	9·1
m-Coumaric	1670	1·7
p-Coumaric	1785	69·0
Hexadecanoic	1920	2·6
Octadecadienoic	2080	2·1
Octadecenoic	2090	2·6
9,16-Dihydroxyhexadecanoic†	2480	12·2
10,16-Dihydroxyhexadecanoic†	2480	0·6
8,16-Dihydroxyhexadecanoic†	2480	0·2

* Identified as methyl ester–TMSi ether derivatives (GC–MS).

† Non separable isomers upon GLC. Relative ion intensities have been employed for quantitative estimate (*m/e* 259/289; 273/275; 245/303).

* Present address: The Chemistry Department, The University, Newcastle, England.

Part VI in the series GC-MS studies on Long Chain Hydroxy Acids. For Part V see Caldicott, A. B., Simoneit, B. R. and Eglinton, G., *Phytochemistry*, submitted.

is present, together with a metastable ion for the loss of 32 from the M-15 ion. The ions resulting from cleavage α- to the methine group (carbon 9) bearing the TMSiO-function are found at *m/e* 289 and 259. A further ion resulting from the expulsion of an aldehydic function occurs at *m/e* 230. Loss of a methoxytrimethylsilane moiety from the 259 ion is supported by a metastable ion at 92·8 amu. and a fragment ion at *m/e* 155. Characteristic rearrangement ions are present at *m/e* 159, 147 and 103. The 9,16-dihydroxyhexadecanoic acid was accompanied by traces of the 10,16- and 8,16-isomers, as indicated by very weak α-cleavage ions at *m/e* 273, 275, 245 and 303.

The extractives of the pollen were also saponified, though no 9,16-dihydroxyhexadecanoic acid was identified. However, nonacosan-10-ol is reported for the first time as a pollen constituent. The mass spectra of nonacosan-10-ol and its TMSi ether confirm the absence of positional isomers. In the ether, major ions at *m/e* 229 and 369 result from ready cleavage, α- to the methine function, giving rise to $TMSi\overset{+}{O}{=}CH(CH_2)_8Me$ and $TMSi{-}\overset{+}{O}{=}CH(CH_2)_{18}Me$ respectively. In the alcohol, the M-18 ion results from the loss of water from the molecular ion, while cleavage α- to the methine function gave rise to ions at *m/e* 157 and 229.

DISCUSSION

Kolattukudy and Walton [6] suggest that, in *Vicia faba* leaves, free dihydroxyhexadecanoate is rapidly incorporated into cutin. If such a situation applies to plants generally, it is likely that the bound 9,16-dihydroxyhexadecanoic acid, hitherto unreported [13, 14] in *Pinus sylvestris* microspores (Table 1), is part of a cutinized layer. The absence of such a lipid in the pollen extractives is also consistent with Kolattukudy and Walton's finding for *V. faba* seedlings.

Primary long chain alcohols have been identified in gymnosperm microspores [15, 16]. The minor amounts of nonacosan-10-ol, a typical cuticular wax constituent [6], herein reported among *Pinus sylvestris* microspore extractives, further indicate that the exterior of the pollen grains may possess some of the characteristics associated with the surfaces of plant leaves. Examination of pollen exine fine-structure by various specific chemical reagents may yield further evidence of cutinization. Swift and Remsen [17] have partly characterized the cell wall structure of algal spores by chemical degradation in conjunction with electron microscopy. Such methods have also been applied to pollen membranes by Rowley [18], who finds that the pollen endexine layer in both *Chamaenerion* and *Zauschneria* species is readily degraded by acetolysis, saponification and chlorination, whereas the ectexine is resistant. His findings are consistent with the pollen endexine layer being composed partially of cutin, as well as of the widely accepted carbohydrate structure. Dungworth *et al* [19] have subjected the exines of *Pinus pinaster* and *Lycopodium clavatum* pollens to vigorous hydrogenolysis and obtained small amounts of hydrocarbons, predominantly the C_{16} and C_{18} *n*-alkanes: their results are also in accord with the occurrence of cutin [20] in the microspores.

The occurrence of cutin acids in a pollen further implies that such lipids in fossil sediments, including a 5000-yr-old mud from Lake Esthwaite [21], Green River Shale [22] and Brown Coal [4], may not merely derive from fruit, leaf and stem fragments [5], but from pollens and microspores as well. The analysis of microspore-rich sediments, e.g. fiminites and gyttya with the $LiAlD_4$ degradation method [6] may thus prove rewarding.

EXPERIMENTAL

Bound, ether-soluble acids. Pollen grains of *Pinus sylvestris* L. purchased from Cernelle, were stored at $-10°$ until required for use. The pollen (1·727 g) was suspended in 50 ml MeOH–$CHCl_3$ (1:1) and immersed in a Dawe ultrasonic bath for 3 hr. Following ultrasonic treatment, Soxhlet-extraction with MeOH–$CHCl_3$ (1:2), MeOH and H_2O was carried out consecutively for a total period of 72 hr. After air-drying, the bulk (827 mg) of the buff coloured residue (854 mg) was saponified in 6% ethanolic KOH (15 ml). After centrifugation (2000 *g* for 3 hr) the deep-amber supernatant was combined with the ethanol washings (20 ml) and taken to dryness (*vacuo*). The residue was dissolved in water (8 ml), acidified with dil. H_2SO_4 to pH 2 and extracted with Et_2O (4 × 1·5 ml). Combined Et_2O extracts were washed with H_2O and the acids separated from non-saponifiable lipids by extraction into 6% aq. $NaHCO_3$ (3 × 1 ml). The organic layer, free of acids, yielded 1 mg of solids when taken to dryness and was not further examined.

The $NaHCO_3$ extracts were acidified to pH 2 (dil. H_2SO_4) and extracted with Et_2O (4 × 1·5 ml). The combined Et_2O

washings were washed with H_2O (5 × 4·0 ml) and evaporated. Residue (21·4 mg) was methylated with CH_2N_2 in MeOH and an aliquot silylated with N,O-*bis*(trimethylsilyl)-acetamide prior to GC–MS analysis following a previously reported method [5]. Mass spectra were identified by comparison with published data [4, 10].

Solvent-extractable lipids. A further aliquot of *P. sylvestris* microspores (5·509 g) was Soxhlet extracted with toluene–MeOH (1:1) for 24 hr. Solvent removal yielded a brown gum (1·185 g) which was subsequently heated under reflux with 6% ethanolic KOH (25 ml). EtOH was removed (*vacuo*), the residue taken up in water (20 ml) and acidified to pH 2. The solution was extracted with 30% hexane-Et_2O (5 × 16 ml). The organic layers were combined, concentrated to 8 ml and washed free of acids with 6% $NaHCO_3$ soln (3 × 2 ml). Evaporation of the organic layer (*vacuo*) yielded an amber gum (343 mg). An aliquot (28 mg) was subjected to preparative TLC, employing Si gel H (Merck) (0·3 mm) and Et_2O–C_6H_{12}–MeOH (40:10:1). The secondary alcohol fraction (R_f 0·70) was collected and recrystallized from EtOAc. Colourless crystals (*ca* 3 mg) had *mp* 80–81° (Lit [23], 80·2–81°C) for nonacosan-10-ol, representing 0·67% of the spores, M^+, *m/e* 424 (0·8); M-18, 406 (8), 297 (25), 157 (35), 55 (57), 57 (98); 69 (63); 71 (42); 83 (100); 85 (30); 97 (78); 99 (12); 111 (33); 125 (21); 139 (15). The corresponding TMSi ether gave a GLC retention index of 3078 upon OV-1 phase: its mass spectrum displayed the following significant ions: M^+, *m/e* 496 (0·5); M-15 (3·5); M-29 (1·5); M-43 (0·6); *m/e* 369 (58); 229 (100); 129 (14); 103 (20); 97 (28); 73 (60); 75 (56).

GC–MS was carried out upon a Varian MAT CH-7 instrument, as were probe spectra on nonacosanol and its TMSi ether. GLC separations were carried out upon stainless steel 2·5 m × 1·6 mm external dia tubing packed with 1% OV-17 or OV-1 upon a Gaschrom Q support (100–120 mesh), N_2 at 15 ml/min. For GC–MS, a silanised 1·6 mm o.d. glass column was employed packed with 2% SE33 coated upon Gaschrom Q.

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