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EPICUTICULAR WAX OF *PINUS RADIATA* NEEDLES

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Key Word Index—*Pinus radiata*; Pinaceae; Monterey pine; epicuticular waxes; cotyledon, primary and secondary needles; composition; alkyl esters; estolides; secondary alcohols; secondary diols; diterpene acids; MS.

Abstract—Epicuticular wax isolated from the cotyledons and primary needles of 10-week-old *Pinus radiata* seedlings is similar in composition and contains 86% neutral compounds, viz. alkyl esters (25%, C_{24}–C_{64}), nonacosan-10-ol (52%), heptacosane-5,10-diol (2%), nonacosane-4,10-diol, nonacosane-5,10-diol, and nonacosane-10,13-diol (total 12%) and estolides, MW *ca* 800 (2%), MW *ca* 1100 (6%), and MW *ca* 1500 (1%). The acidic fraction (14%) contains *n*-acids (78%, C_{12}–C_{32}) and diterpene acids (22%, mainly abieta-8,11,13-trien-18-oic, with lesser amounts of pimara-8(14),15-dien-18-oic, isopimara-7,15-dien-18-oic and hydroxylated aromatic, diene and mono-ene acids). Wax isolated from primary needles of 1-yr-old seedlings had a similar neutral fraction composition, but the acidic fraction contained predominantly the diterpene acid mixture, with only trace amounts of *n*-acids. The wax from 1-yr-old secondary needles from *P. radiata* forest trees aged 5 yr and 40 yr contained an acid fraction (12% 5 yr, 17% 40 yr trees) comprising the diterpene acid mixture, with trace amounts of *n*-acids together with ω-hydroxy acids (C_{12}, C_{14} and C_{16}). The neutral fraction from both young and old trees had a similar composition containing alkyl esters (7%, C_{24}–C_{66}), estolides (90%, MW 566–*ca* 1500), nonacosan-10-ol (2%) and the heptacosane and nonacosane diols (1%). During growth and maturation of *P. radiata*, the nonacosan-10-ol content of the needle wax decreases while the proportion of estolides and diterpene acids increases, the latter probably being located around the stomatal pore.

INTRODUCTION

Needle epicuticular waxes from several pine species have been investigated. Bougault and Bourdier [1] isolated sabinic (12-hydroxydodecanoic) and juniperic (16-hydroxyhexadecanoic) acids from hydrolysates of *Pinus sylvestris* wax and they first used the term estolide to describe a linear polyester formed from the interesterification of these ω-hydroxy acids. More recently, needle wax of *P. sylvestris* has been been re-examined and alkanes (C_{21}–C_{33}, predominantly C_{27} and C_{29}), alcohols (C_{22}–C_{33}, predominantly C_{28} and C_{30}) and esters were identified [2]. The wax of *P. thunbergii* needles was shown to consist of estolides composed of fatty acids (C_{12}, C_{16}, C_{18}), ω-hydroxy acids (C_{16}), and alcohols (C_{16}, C_{18}) interesterified with short chains [3]. Minor components were identified as hexadecane-1,16-diol, dodecane-1,12-diol, triacontan-1-ol and a C_{31} secondary ketonic alcohol [4]. From needle wax of *P. koraiensis*, besides estolides and fatty acids, nonacosane, nonacosan-10-ol, and nonacosan-10-one were identified [5].

ω-Hydroxy acids from the hydrolysed estolide fraction of *P. radiata* epicuticular wax have been examined by Herbin and Sharma [6]. They used old, fading needles, extracted the crude wax by brief immersion of the needles in chloroform and isolated the estolide fraction by its insolubility in petrol. The estolide was hydrolysed with alcoholic alkali, unidentified non-saponifiable material removed and the hydroxy acids were isolated, methylated and analysed by GLC to give 20% C_{12}, 37% C_{14} and 43% C_{16} ω-hydroxy acids [6].

Recent investigation of the surfaces of *Pinus radiata* secondary needles by scanning electron microscopy has shown differences in epicuticular wax morphology, especially in the vicinity of the stomata, with tree age [7]. The wax layer over the cuticle was smooth, and appeared thicker in the vicinity of the stomata, especially on needles from older trees. Wax having a micro-tubular form covered the walls of the guard cells of young-tree needles and, at the rim of the pore, the wax had a granular appearance. The stomatal pore of mature-tree needles was shown to be occluded with wax and it was suggested that this occlusion could be partly responsible for the resistance of mature trees to the needle pathogen *Dothistroma pini*, by preventing growth of the fungal hyphae into the stomata [7]. In contrast to the smooth wax layer of secondary needles, the cotyledons and primary needles of 10-week-old *P. radiata* seedlings are covered with profuse tufts of microtubular and rod-like wax, which appeared more dense in the vicinity of the stomata. As the seedlings matured (*ca* 1 yr, with secondary needles emerging from the axillary buds), the wax in the vicinity of the stomata became amorphous [8]. These scanning electron microscope observations suggested that the needle epicuticular wax underwent significant changes in composition as the tree grew.

This paper describes results of a re-investigation of the composition of *P. radiata* needle epicuticular wax using 1-yr-old green, healthy needles sampled from 5- and 40-yr-old trees, and an investigation of the constituents of cotyledon and primary needle wax of seedlings. The possibility of epicuticular wax composition being an index of tree maturity which could be used for selection and identification of precociously mature trees for breeding for disease resistance [9] added impetus to this study.

RESULTS

Wax of cotyledons and primary needles of 10-*week-old seedlings*

The crude waxes isolated from cotyledons and from primary needles of 10-week-old *P. radiata* seedlings were each obtained as slightly odoriferous, white solids, each in 0.2 % yield (fr. wt.). The crude wax was separated, after ion-exchange chromatography, into acidic (14 %) and neutral (86 %) compounds. GLC of the Me esters derived from the acidic fraction showed this to be a mixture of 78 % *n*-acids (C_{12}–C_{32}) and 22 % of diterpene acids, mainly abieta-8,11,13-trien-18-oic (dehydroabietic) acid. Lesser amounts of pimara-8(14),15-dien-18-oic (pimaric), isopimara-7,15-dien-18-oic (isopimaric) acid were present, together with a complex mixture of hydroxylated aromatic, diene, and mono-ene diterpene acids, as shown by GC–MS. The composition of the acidic fraction is shown in Table 1.

The neutral fraction was analysed by TLC (petrol–$CHCl_3$, 1:3) and gave 3 major spots (R_f 0.95, 0.4, 0.25), and 3 minor, diffuse ones (R_f 0.85, 0.6, 0.1). The major components were purified by column chromatography and PLC. The least polar (R_f 0.95) was a mixture of alkyl esters (25 % of neutral fraction), showing IR carbonyl absorption at 1735 cm^{-1} and, after hydrolysis and GLC, giving a mixture of *n*-acids (C_{12}–C_{32}) and *n*-alkanols (C_{12}–C_{32}) (Table 2). The main component of the neutral fraction (R_f 0.4, 52 %) was identified as nonacosan-10-ol. The IR spectrum had OH absorption at 3600, 3400 cm^{-1} and the PMR spectrum showed only CH_2 signals (δ 1.35) and a one-proton multiplet at δ 3.85 ($>$C$\underline{\mathrm{H}}$OH). GC–MS of the TMSi ether gave the expected fragmentation ions, viz. *m/e* 369 [M$^+$-127] (100 %) and 229 [M$^+$-240] (67 %) [10]. Other ions recorded in the MS of the fraction, 453 [M$^+$-43] (8 %) and 439 [M$^+$-57] (2 %), suggested that trace amounts of nonacosan-4-ol and nonacosan-5-ol may also be present. The intense polar spot (R_f 0.25) on TLC of the neutral fraction (14 %) showed OH absorption by IR and a CH_2 envelope (δ 1.35) and a methine proton multiplet at δ 3.9 ($>$C$\underline{\mathrm{H}}$OH) in the PMR spectrum. GC–MS showed this fraction to comprise a mixture of nonacosane-4,10-diol, nonacosane-5,10-diol, nonacosane-10,13-diol and heptacosane-5,10-diol. GLC showed that the C_{27} diol comprised 14 %, the C_{29} 4,10 and 5,10 diols *ca* 45 and 30 % respectively and the C_{29} 10,13 diol 11 % of the total diol mixture. The primary MS fragment ions of these diols (as TMSi ethers) are shown in Table 3, and represent cleavages α to the —OTMSi groups. Only nonacosane-4,10-diol *bis*-TMSi ether showed a M$^+$ at *m/e* 584

Table 1. Composition (%) of acidic fractions from ion-exchange chromatography of crude waxes of *P. radiata* cotyledons and primary needles (10-week-old seedlings), primary needles (1-yr-old seedlings) and secondary needles (5- and 40-yr-old trees)

Carbon No.	Cotyledon and primary needle (10-week-old seedling)	Primary needle (1-yr-old seedling)	Secondary needle
n-ACIDS			
12	1.0	tr*	0.8
14	2.8	tr	tr
16	9.8	0.3	—
18	5.7	tr	—
20	1.0	—	—
22	3.3	—	—
24	17.3	—	—
26	15.1	—	—
28	11.1	0.7	1.4
30	10.9	0.4	1.9
32	tr	tr	tr
ω-HYDROXY ACIDS			
12	—	—	0.9
14	—	—	0.9
16	—	—	3.1
DITERPENE ACIDS			
Pimaric	tr	1.1	4.0
Isopimaric	1.0	14.9	12.6
Dehydroabietic	14.1	39.7	57.1
Hydroxy diterpene acids	6.9	42.9	17.3

* tr < 0.1 %.

Table 2. Composition (%) of alkyl esters from *P. radiata* cotyledons and primary needles (10-week and 1-yr-old seedlings) and secondary needles

Carbon No.	Cotyledons and primary needles		Secondary needles*	
	n-acid	*n*-alkanol	*n*-acid	*n*-alkanol
12	1.2	2.4	20.0	2.5
14	2.0	2.3	9.3	3.5
16	2.8	4.6	13.4	—
18	1.3	3.9	14.1	3.2
20	3.9	12.7	7.4	—
22	0.8	2.3	1.9	1.3
24	3.2	4.7	2.2	7.2
26	7.2	15.2	3.1	7.4
28	5.1	10.8	4.4	14.1
30	30.2	24.3	8.8	18.5
32	42.6	16.9	11.3	21.9
34	—	—	4.0	—

* Also contained C_{29}-10-ol 11.5 % and C_{29} diol 3.3 %.

Table 3. Primary fragments in the MS of TMSi ethers of the C_{27} and C_{29} diols from *P. radiata* needle wax

Diol	*m/e*	Ion assignment	Rel. int.
C_{27} 5,10-diol	499	$C_{23}H_{45}(OTMSi)_2$	24
	341	$C_{18}H_{36}OTMSi$	61
	317	$C_{10}H_{19}(OTMSi)_2$	67
	159	$C_5H_{10}OTMSi$	100
C_{29} 4,10-diol	541	$C_{26}H_{51}(OTMSi)_2$	39
	369	$C_{20}H_{40}OTMSi$	100
	317	$C_{10}H_{19}(OTMSi)_2$	92
	145	C_4H_8OTMSi	77
C_{29} 5,10-diol	527	$C_{25}H_{49}(OTMSi)_2$	35
	369	$C_{20}H_{40}OTMSi$	75
	317	$C_{10}H_{19}(OTMSi)_2$	80
	159	$C_5H_{10}OTMSi$	100
C_{29} 10,13-diol	457	$C_{20}H_{39}(OTMSi)_2$	13
	359	$C_{13}H_{25}(OTMSi)_2$	15
	327	$C_{17}H_{34}OTMSi$	70
	229	$C_{10}H_{20}OTMSi$	100

(1 %). Fragmentation schemes (major ions) for nonacosane-5,10-diol and nonacosane-10,13-diol *bis*-TMSi ethers are shown later in Scheme 1. To confirm the structures assigned to the long chain diols, the mixture was oxidised with chromic acid [11] to give diketones (ν_{max} 1715 cm^{-1}). GLC did not separate the diketones as well as the diol TMSi ethers, but probe MS of the diketone mixture demonstrated the presence of nonacosane-4,10 and -5,10 diones. The position of substitution was shown by ions at *m/e* 71 (57 %) for the 4-ketone, *m/e* 85 (82 %) for the 5-ketone and at *m/e* 295 (88 %) for the 10-ketone, formed by cleavage of the 4,5, 5,6 and 9,10 bonds respectively. No ions formed by cleavage of the 3,4 and 4,5 bonds were observed, analogous to the MS fragmentation of C_{31} tri- and tetra-ketones [12]. Cleavage of the 10,11 bond gave an ion *m/e* 169 (17 %). The base peak at *m/e* 184 arises from cleavage of the 11,12 bond, while cleavage of the 2,3 and 3,4 bonds gave ions of *m/e* 86 (20 %) and *m/e* 100 (26 %). A weak M$^+$ was observed at *m/e* 436.

The minor neutral compounds in cotyledon and primary needle epicuticular wax all had IR absorption at 1735 cm^{-1} and yielded *n*-acids, ω-hydroxy acids and α,ω-diols on hydrolysis. They were estolides with MW of *ca* 800 (2 %), 1100 (6 %), and 1500 (1 %) for the TLC spots of R_f of 0.85, 0.6 and 0.1 respectively.

Wax of primary needles of 1-*yr-old seedlings*

The crude wax extracted from primary needles of 1-yr-old *P. radiata* seedlings, obtained as an off-white solid in 0.2 % yield (fr. wt.), was subjected to ion-exchange chromatography as before. The neutral fraction was composed of the same mixture of compounds as that obtained from 10-week-old seedlings, as shown by TLC. GLC and GC–MS of the Me esters of the acidic fraction showed that it comprised mainly diterpene acids, with only trace amounts of *n*-acids (Table 1).

Wax of secondary needles of 5- *and* 40-*yr-old trees*

Secondary needles from 5- and 40-yr-old trees yielded on chloroform extraction, crude wax, off-white to light tan in colour in 0.2–0.3 % yield. Ion-exchange chromatography isolated the acidic fraction, which, after methylation, had a similar composition to that obtained from primary needles of 1-yr-old seedlings, viz. diterpene acids, with trace amounts of *n*-acids (Table 1) but also ω-hydroxy acids, identified by GC–MS of their Me ester TMSi ethers: *m/e* C_{12}, 302 [M$^+$] (1 %), 287 [M$^+$-15] (67 %); C_{14}, 330 [M$^+$] (8 %); C_{16}, 358 [M$^+$] (12 %). The wax extracted from secondary needles of 40-yr-old trees always had a higher acid content (17 %) than that of 5-yr-old trees (12 %) [7].

In an attempt to further resolve the hydroxylated diterpene acid fraction, the Me esters of the secondary needle acid fraction were analysed by PLC and separated into 5 bands. The IR spectra of the mixtures obtained from all bands had IR absorption at 1735 cm^{-1} (ester) and the 3 most polar bands afforded mixtures having IR absorption at 3500 cm^{-1}, indicating the presence of OH groups. The PMR spectra had, in addition to the expected CH_2 and OMe signals, multiplets at δ 7 (aromatic H), δ 5–5.5 (vinyl H), and δ 2.9 (allyl and benzyl H).

The first two PLC bands (R_f 0.85, 0.8) contained mainly the *n*-acids and pimaric, isopimaric and dehydroabietic acids, identified as the Me esters by co-chromatography with standards and comparison of their MS with published spectra [13]. The remaining PLC bands (R_f 0.5, 0.45, 0.4) were mixtures of the same hydroxy diterpene acids when analysed as their Me esters TMSi ethers by GLC. Of the 7 main peaks, satisfactory MS were obtained for 5. The major GLC peak had the following MS: *m/e* 420 [M$^+$] (67 %), 377 [M$^+$-43] (100 %), 330 [M$^+$-90] (48 %), 302 [M$^+$-15-43-60] (36 %), 287 [M$^+$-43-90] (52 %), 259 [M$^+$-43-90-28] (65 %) and 195 [M$^+$-225] (63 %). Since the base peak was M$^+$-43 (loss of isopropyl), it is possible this compound is an abiet-8(14)-ene derivative as this carbon skeleton favours loss of isopropyl by allylic cleavage [13]. The other ions in the MS show loss of 90 (TMSiOH), 60 (Me formate from ring-A C_4 ester), and 28 amu (CO expulsion from ring). An ion of *m/e* 195 is possibly a ring-A fragment formed by fission of the 6,7 and 9,10 bonds [22], but with a carbonyl group also in ring A. This compound would therefore appear to be an abiet-8(14)-en-18-oic acid derivative, with a carbonyl substituent in ring A and an OH substituent in rings B or C.

The second largest GLC peak in this group had *m/e* 402 [M$^+$] (14 %), 387 [M$^+$-15] (100 %), 327 [M$^+$-15-60] (12 %), 312 [M$^+$-90] (25 %), 237 [M$^+$-15-60-90] (31 %), and 131 [M$^+$-271] (10 %). Two lesser peaks had *m/e* 402 [M$^+$] (61 and 100 %), 387 [M$^+$-15] (100 and 42 %), 327 [M$^+$-15-60] (9 and 81 %), 312 [M$^+$-90] (61 and 23 %), and 237 [M$^+$-15-60-90] (67 and 38 %), respectively. The compounds with M$^+$ of *m/e* 402 also showed an ion at *m/e* 121 [$C_9H_{11}{}^+$] (12–30 %), characteristic of diterpene acids [13]. Since the M$^+$ was *m/e* 402, it is suggested that they are all hydroxy-dehydroabietic acid derivatives. One of these 3 showed an ion *m/e* 131, possibly a TMSi isopropyl ether fragment [$Me_2CHOTMSi^+$], suggesting that the original compound was 15-hydroxyabieta-8,11,13-trien-18-oic acid.

The fifth GLC peak had *m/e* 404 [M$^+$] (100 %), 361 [M$^+$-43] (18 %), and 314 [M$^+$-90] (28 %). Since the M$^+$ was also the base peak, it is possible that this compound is an hydroxy abieta-7,13-dien-18-oic (abietic) acid derivative [13]. Unfortunately, sufficient information could not be obtained from GC–MS of these hydroxy diterpene acid Me ester TMSi ethers to enable definite structures to be proposed and further work on these compounds is planned.

When analysed by TLC, the neutral fraction of *P. radiata* secondary needle wax separated into 8 spots (R_f 0.95, 0.85, 0.6, 0.45, 0.4, 0.25, 0.2, 0.15) but with considerable streaking. The neutral fraction was chromatographed on a column of alumina using petrol–$CHCl_3$ mixtures as eluants. Further purification of the collected fractions was achieved by PLC, although most of the fractions still produced diffuse bands. Readily characterised was a mixture of alkyl esters (7 % of neutral fraction), which after hydrolysis, ion-exchange chromatography and GLC consisted of *n*-acids (C_{12}–C_{34}), *n*-alkanols (C_{12}–C_{32}) and also nonacosan-10-ol and nonacosane-5,10-diol (Table 2). Also present were free nonacosan-10-ol (2 %) and a mixture of the heptacosane and nonacosane diols (1 %) previously isolated. The 5 remaining fractions obtained from column chromatography and PLC (R_f 0.85, 0.6, 0.45, 0.2, 0.15) were estolides (90 %), with mps in the range 62–72° [7]. Their IR spectra had absorption at 1735 cm^{-1} (ester) and their PMR spectra, in addition to the CH_2 envelope at δ 1.4, had signals at δ 2.3 (t, J = 7 Hz, —CH_2—CO—), and δ 4.1 (t, J = 7 Hz, —$C\underline{H}_2$—OH). The estolide fractions, numbered 1–5, comprised 16, 19, 25, 21 and 9 % of the total neutral

Table 4. Composition (%) of the hydrolysis products of the five estolide fractions isolated from *P. radiata* secondary needle wax

Carbon No.	Estolide fraction 1	2	3	4	5
n-ACID					
12	37.9	22.5	16.3	6.2	8.4
14	0.6	4.4	tr*	tr	tr
16	0.5	—	—	—	—
18	0.3	—	—	—	—
ω-HYDROXY ACIDS					
12	8.4	23.5	31.3	38.5	45.3
14	11.2	14.5	15.8	19.2	14.4
16	7.6	11.8	12.0	13.1	18.1
n-ALKANOL					
12	0.2	0.1	—	—	—
14	0.4	0.2	—	—	—
18	0.9	0.6	—	—	—
22	0.5	0.1	—	—	—
24	0.8	0.6	tr	—	—
26	1.2	0.6	tr	—	—
28	9.8	1.0	tr	—	—
30	1.9	0.8	tr	—	—
32	2.0	0.3	—	—	—
α,ω-DIOLS					
12	1.0	1.5	3.0	8.0	—
14	12.9	13.9	15.4	12.8	—
16	2.1	2.3	2.3	1.5	—

* tr < 0.1%.

fraction, respectively. The estolides were difficult to obtain in a pure form and fractions were obtained from alumina column chromatography and PLC which contained groups of compounds with similar MW. Each estolide fraction was separately hydrolysed, the acids and alcohols quantitatively separated by ion-exchange chromotography and then analysed by GLC and GC–MS. The composition of each estolide fraction is shown in Table 4. These data suggest the estolides were composed of a chain of interesterified ω-hydroxy acids and α,ω-diols, terminated at each end with an *n*-acid. Estolide fractions 1 and 2 also contained *n*-alkanols, mainly C_{28}, C_{30} and C_{32}, which can be linked to ω-hydroxy acids with the chain terminated with an *n*-acid. Osmometric MW determination of the 5 estolide fractions gave for number 1 *ca* 1100, 2 *ca* 1100, 3 *ca* 1200, 4 *ca* 1500, and 5 *ca* 1600. Probe MS investigation of the 5 estolide fractions using evaporation temperatures in the range 250–350° gave satisfactory spectra only for estolide fractions 1 and 2. The MS of estolide fraction 1 (Fig. 1) showed two series of M^+, the major series having *m/e* 876 (2%), 848 (3%), 820 (5%), 792 (10%), and 764 (3%), while the minor series had *m/e* 902 (1%) and weak ions at 874, 846, 818, and 790. The major series showed M^+-200 ions, and a base peak of *m/e* 183. Estolide fraction 2 had M^+ *m/e* 1074 (5%), 1046 (8%), 1018 (12%), 990 (12%), and 962 (6%), M^+-200 ions for each and a base peak of *m/e* 183. The types of fragmentation observed in MS of alkyl esters are: (a) acyl-oxygen fission, (b) alkyl oxygen fission with rearrangement of two H, (c) alkyl-oxygen fission with loss of one H, (d) γ-H transfer, (e) fission of 4,5 bond of alcohol moiety with rearrangement of one H and (f) carbonyl–carbon fission to alkoxy-carbonium ion [14]. Fission of the 3,4 bond of the alcohol moiety, with rearrangement of one H also gives an ion (g) characteristic for wax esters [15]. The estolides obtained from *P. radiata* needle wax undergo similar fragmentation, as shown by the fragmentation of a fraction 1 estolide, M^+ 792, composed of two molecules of dodecanoic acid and one each of 12-hydroxydodecanoic acid and tetradecane-1,14-diol (Fig. 2). The base peak (*m/e* 183) is due to loss of the terminal C_{12} acyl group, which, together with the ion at *m/e* 381, are both (a) type fragmentations. Other prominent ions at *m/e* 201, 394, 399, and 592 are due to (b) and (c) type fragmentations. Another estolide, M^+ 792, composed of two molecules of dodecanoic acid and one each of 14-hydroxytetradecanoic acid and dodecane-1,12-diol gives the same fragment ions from the two terminal ester groups, viz. *m/e* 183, 201, 592, and from the central ester group, ions at *m/e* 409 for (a), 427 for (b), and 366 for (c) type fragmentations. Other estolides, composed of two molecules of dodecanoic acid and

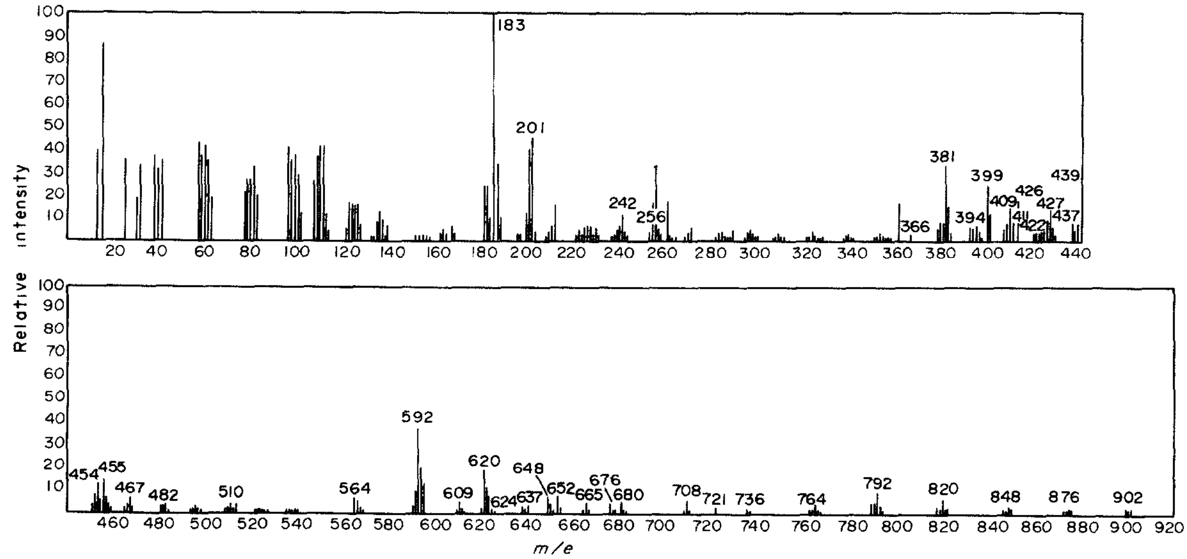
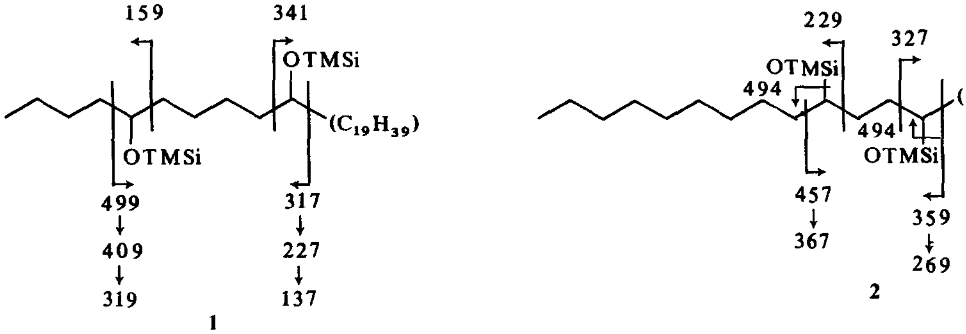


Fig. 1. Mass spectrum of estolide fraction 1. Evap. temp. 350°.



Scheme 1. MS fragmentation of nonacosane-5,10-diol 1 and nonacosane-10,13-diol 2 *bis*-TMSi ethers

C_{12}, C_{14} and C_{16} α,ω-diols and C_{12}, C_{14} and C_{16} ω-hydroxy acids (Table 4) give rise to the homologous series of M^+ starting at *m/e* 764. Further estolides, containing two molecules of tetradecanoic, hexadecanoic acid, etc., or one each of dodecanoic and tetradecanoic acid, etc., combined with the C_{12}, C_{14}, C_{16} α,ω-diols and C_{12}, C_{14}, C_{16} ω-hydroxy acids can be envisaged, but these would be only minor constituents of estolide fraction 1 as shown by hydrolysis (Table 4), and would not be observed in the MS. While the base peak in MS of alkyl esters is due to the $RCO_2H_2^+$ ion, with the acylium ion, RCO^+ *ca* 30–40% rel. int. [14], the reverse is observed in the case of the estolides. Ions of *m/e* 242 and 256 arising from cleavage of the 3,4 [15] and 4,5 [14] bonds of the alcohol moiety also appear more intense than similar ions in the MS of alkyl esters.

The second series of molecular ions starting at *m/e* 790 appear to be from estolides composed of *n*-alkanols (up to C_{32} based on hydrolysis, Table 4) linked to an ω-hydroxy acid with the chain terminating with a C_{12} *n*-acid, e.g. an estolide composed of a C_{12} *n*-acid, C_{12} ω-hydroxy acid and a C_{28} *n*-alkanol (the major *n*-alkanol in fraction 1 estolide, Table 4) gives M^+ of *m/e* 790. Another composed of a C_{12} *n*-acid, C_{32} *n*-alkanol and C_{16} ω-hydroxy acid gives M^+ of *m/e* 902.

Theoretically, the smallest and largest compounds in estolide fraction 1 have MW 566 and 986 respectively, based on hydrolysis (Table 4), but M^+ for these compounds were not observed in the MS. Estolide fraction 1 therefore comprises a mixture of polyesters having two and three ester groups per chain.

Estolide fraction 2, with major compounds having M^+ 962, 990, 1018, 1046 and 1074, is composed of molecules similar to those of estolide fraction 1, except with two ω-hydroxy acid moieties per molecule, and these can be arranged either consecutively or separated by the α,ω-diol. While several structures, both linear [1, 3] and cyclic [16] have been proposed for estolides obtained from various conifers, they have been based on the neutrality or acidity of the compound, hydrolysis and Rast MW determinations. The structures for the constituent molecules of estolide fractions 1 and 2 from *P. radiata* needle wax would appear to be the first definitive ones based on both hydrolysis and MS.

Estolide fractions 3 and 4, containing C_{12} *n*-acid, ω-hydroxy acids and α,ω-diols (Table 4), and with MW *ca* 1200 and 1500 respectively, are probably also composed of molecules similar to those in fractions 1 and 2, but with 3 and 4 ω-hydroxy acids interesterified with one α,ω-diol, with the polyester chain terminated at each end with a C_{12} *n*-acid. The number of possible structures becomes very large as the chain length increases and this will contribute to the considerable streaking observed on TLC of the secondary needle wax.

Estolide fraction 5 gave no alcohol fraction after hydrolysis, and appears to be an acidic estolide [1] composed of a chain of 4-5 interesterified ω-hydroxy acids terminated at the OH end with a C_{12} *n*-acid,

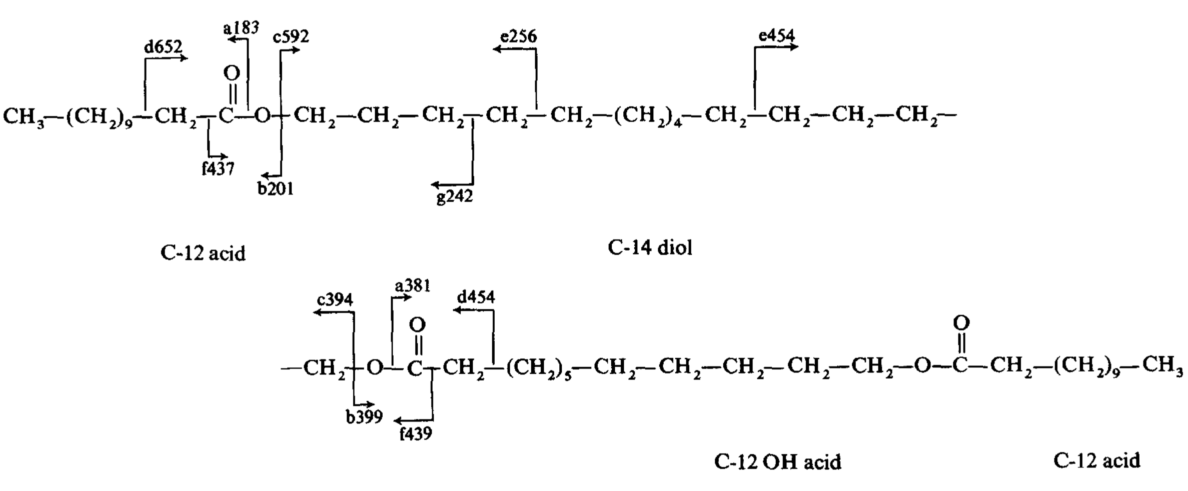


Fig. 2. MS fragmentation of estolide, MW 792, composed of two molecules of dodecanoic acid, one of 12-hydroxydodecanoic acid, and one of tetradecane-1,14-diol. (a, b, c, d, e, f, g, refer to fragmentation described in text).

leaving a free carboxylic acid group at the other. This estolide fraction should have appeared in the acid fraction during the ion-exchange chromatography of the crude wax. Its appearance in the neutral fraction may be due to the long polyester chain not permeating the macroreticular structure of the ion-exchange beads.

DISCUSSION

The epicuticular wax of cotyledons and primary needles of 10-week-old *P. radiata* seedlings, which appears in the form of numerous tufts of minute rods and tubes [8], has now been shown to contain nonacosan-10-ol as the major constituent. There is increasing evidence from chemical analysis of waxes and scanning electron microscopy of plant surfaces for correlating nonacosan-10-ol with the presence of wax tubes on the leaf surface [10, 17]. The present study provides another example of this association. The microtubular wax observed on the walls of the guard cells of 1-yr-old secondary needles of 5-yr-old trees [7] is probably also mainly nonacosan-10-ol. Other long chain compounds found in needle wax are the previously unknown heptacosane-5,10-diol, nonacosane-4,10-, 5,10-, and 10,13-diols. Only *n*-alkanols and α,ω-diols, combined as esters and estolides, and nonacosan-10-ol have previously been recognised as constituents of conifer needle waxes [3-5, 18]. Long chain secondary diols are less well known as constituents of plant waxes [19].

The alkyl ester mixture obtained from primary needles is composed of C_{12}–C_{32} *n*-acids and C_{12}–C_{32} *n*-alkanols, predominantly C_{28}–C_{32}, and theoretically contains 121 different esters. The corresponding mixture derived from secondary needles has, in addition to the above acids and alcohols, C_{34} *n*-acid, and nonacosan-10-ol and nonacosane-5,10-diol in the hydrolysate. The *n*-acid predominating in the secondary needle mixture is C_{12}. Hydrolysis also showed that the secondary alcohols in *P. radiata* secondary needle wax exist in the esterified, as well as the free form. Esterified secondary alcohols have also recently been demonstrated in the wax of *Rosa virgo* [11].

The free acid fraction obtained after ion-exchange chromatography of wax of primary needles from 10-week-old seedlings is composed mainly of C_{12}–C_{32} *n*-acids, together with minor amounts of pimaric, isopimaric, dehydroabietic, and hydroxylated diterpene acids. When seedlings are *ca* 1-yr-old, secondary needles emerge from the axillary buds and the primary needles begin to senesce. At this growth stage, the wax in the vicinity of the primary needle stomatal pores becomes amorphous, as well as partially occluding the pore, while the wax on the remainder of the primary needle surface retains the microtubular appearance [8]. TLC showed the neutral fraction of these primary needles to have the same composition as that obtained from 10-week-old seedlings, viz. alkyl esters, nonacosan-10-ol and the C_{27} and C_{29} diols with minor amounts of estolides. The acidic fraction, however, was shown by GLC of the Me esters to be predominantly diterpene acids, with only trace amounts of *n*-acids. If the appearance of diterpene acids as the major constituent in the acid fraction of these primary needles can be correlated with the change in wax structure in the vicinity of the stomatal pores, then it appears that the diterpene acids are synthesised by the subsidiary cells which form the stomatal pore, while the needle epidermal cells synthesise the neutral compounds, viz. the alkyl esters, estolides, nonacosan-10-ol and the C_{27} and C_{29} diols.

The composition of the acidic fraction of the wax from secondary needles is similar to that of primary needles of 1-yr-old seedlings. Dehydroabietic acid comprises over 50% of the mixture. If the subsidiary cells of secondary needles behave in the same way as those of primary needles, the former probably also synthesise the diterpene acid mixture and it may be a surfeit of diterpenoid production by the subsidiary cells as the tree ages which eventually leads to occlusion of the stomatal pores of needles of old trees and renders them resistant to entry by fungal hyphae.

That dehydroabietic acid is the major diterpenoid found is unexpected, since the major diterpene acids found in *P. radiata* wood are pimaric, isopimaric, sandaracopimaric, levopimaric, palustric, abietic and neoabietic [20], with only trace amounts of dehydroabietic [21]. Whether dehydroabietic acid is synthesised by the subsidiary cells *per se*, or whether it arises by extracellular disproportionation, or by allylic hydroxylation–dehydration–aromatisation sequences from diene diterpene acids cannot be answered from the present chemical information. Certainly, the diterpenoids on the needle surface are exposed to air, water and sunlight which could promote the above reactions.

The neutral fraction of secondary needle wax, besides containing alkyl esters, secondary alcohols and diols, was composed largely of estolides. Estolides are also minor constituents of primary needle wax. Probe MS of fraction 1 and 2 estolides from secondary needle wax show these are composed largely of molecules formed from 1 α,ω-diol esterified with 1 and 2 ω-hydroxy acids respectively, with the chain terminated at each end with a C_{12} *n*-acid. The MS of fraction 1 estolide shows that all possible combinations of α,ω-diol and ω-hydroxy acid exist. Minor constituents in these fractions are molecules containing ω-hydroxy acids esterified with C_{12} *n*-acid and *n*-alkanols, mainly C_{26} to C_{32}. Fraction 3 and 4 estolides are probably similar in composition to those of fraction 1 and 2, but in the absence of MS data for these higher estolides, structures can be assigned only on the basis of hydrolysis products and MW. Fraction 5 estolide appears to contain acidic estolides [1] composed of C_{12} *n*-acid and ω-hydroxy acids, although it is possible that this fraction could also contain molecules with cyclic structures consisting of 6 or 7 interesterified ω-hydroxy acids, *cf.* [16].

Pinus radiata needle epicuticular wax therefore undergoes significant changes in composition as the tree grows and matures, with the free fatty acids, nonacosan-10-ol, and the heptacosane and nonacosane diols of the cotyledons and primary needles of seedlings giving way to the diterpene acids and estolides of secondary needles of fully-grown and mature trees. Further work on the needle epicuticular waxes is in progress in order to clarify the site of synthesis of the diterpene acids and neutral compounds.

EXPERIMENTAL

Epicuticular waxes were extracted from 1-yr-old secondary needles of forest trees and from primary needles and cotyledons of seedlings by the method previously described [7]. IR spectra were recorded in $CHCl_3$. PMR (60 MHz) spectra were recorded in $CDCl_3$ solns with TMS as internal standard. Ion-exchange chromatography was carried out on columns of DEAE-Sephadex A-25 as described previously [7]. Analytical TLC was carried out on $CuSO_4,5H_2O$–Si gel (3:97) [23] and PLC on Si gel layers (0.25 mm), *ca* 10 mg of material applied to 520 × 20-cm plates, bands visualised with I_2 and then extracted with $CHCl_3$. Solvent systems used were petrol–$CHCl_3$ (1:1) for diterpene acid Me esters, and petrol–$CHCl_3$ (1:3) for neutral wax constituents. Column chromatography was carried out on Al_2O_3 (Brockmann activity II), using solvent systems: petrol, petrol–$CHCl_3$ 9:1, 4:1, 3:2, 1:1, 2:3, and $CHCl_3$. A Pye LCM2 moving wire LC detector was used to facilitate location and collection of fractions. GLC was carried out on 1.2 m × 2 mm glass columns packed with 3% OV-17, temp. programmed 150–280° at 4°/min. Carrier gas N_2 at 30 ml/min. Injector temp. 180°, FID detector temp. 280°. Carboxylic acids were chromatographed as their Me esters (CH_2N_2) and hydroxy acids as their Me ester TMSi ethers (BSA–C_5H_5N) and alcohols as their TMSi ethers. Compounds were quantified by peak area; detector response for each was not determined. MS (probe) were obtained on a magnetic sector instrument at 300 μA, 70 eV electron energy and ion-source temp. of 230°. GC–MS was carried out using similar chromatographic conditions to above (He carrier gas). The interface was a single-stage glass jet separator maintained at 250°. Spectra were taken at 100 μA, 24 eV electron energy and ion-source temp. of 230°. Hydrolyses were carried out using MeOH–KOH [6]. Work-up involved removal of MeOH under red. pres., addition of H_2O, adjustment of pH to 3–4 by dropwise addition of conc HCl and extraction of organic ppt into Et_2O. The Et_2O layer was H_2O washed, dried and concentrated.

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