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ALKYL ESTERS FROM *PINUS RADIATA* FOLIAGE EPICUTICULAR WAX

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Key Word Index—*Pinus radiata*; Pinaceae; radiata pine; juvenile and mature plant foliage; epicuticular wax; alkyl esters; capillary column GC-MS.

Abstract—Wax esters from the epicuticular wax of juvenile and mature-tree *Pinus radiata* foliage have been shown by capillary column GC-MS to consist mainly of short chain (C_6–C_{12}) alkanols esterified with long chain acids (C_{24}–C_{32}) and long chain alkanols (C_{24}–C_{32}) esterified with short chain acids (C_6–C_{14}) in a non-random manner. Mature-tree foliage wax esters also contained nonacosan-10-ol esterified with dodecanoic and tetradecanoic acids.

INTRODUCTION

An earlier investigation of the alkyl esters obtained from the epicuticular wax of *Pinus radiata* needles proposed a mixture of esters C_{24}–C_{64} for the juvenile, and C_{24}–C_{66} for the mature foliage, the ester composition being based on GC analysis of the derivatized acid and alkanol fractions from the whole ester fraction hydrolysis [1]. The intact wax esters were not analysed directly in that study. The capability of carrying out GC and GC-MS analysis of wax esters [2–5] by employing high temperature stable stationary phases and cool on-column injection of the sample [6] prompted a re-investigation of the wax ester compositions directly, without hydrolysis, in order to preserve structures of individual homologues.

RESULTS AND DISCUSSION

The capillary column GC analysis of the alkyl ester fraction from *P. radiata* juvenile foliage showed only four major peaks, due to C_{36}, C_{38}, C_{40} and C_{42} esters, comprising over 90% of the total esters in the mixture (Table 1). Minor amounts of odd carbon chain esters (C_{35}–C_{47}) were also detected, as well as C_{41} and C_{43} branched esters. The chain-length of the esters ranged C_{30}–C_{50}, not C_{24}–C_{64} as suggested by earlier work [1]. This was confirmed by observing $[M+1]^+$ ions in the probe CI mass spectrum of the wax ester mixture (Table 1). EI-GC-MS data for each wax ester homologue is summarised in Table 1. These show that the esters are composed of a short chain (C_6–C_{10}) alkanols esterified with long chain acids (C_{24}–C_{32}) and long chain (C_{24}–C_{32}) alkanols esterified with short chain acids (C_6–C_{12}). The presence of wax ester acids and alkanols with carbon chain lengths less than C_{12} as determined by GC-MS was not compatible with earlier hydrolysis data [1] necessitating a re-examination of these. When the hydrolysate acid methyl esters and alkanol TMSi ethers were analysed by capillary column GC using a temperature range programmed from 30 to 250°, the C_6–C_{10} alkanol TMSi ethers and C_6–C_{12} acid methyl esters were readily detected (as well as the longer homologues described earlier [1]; Table 2). Decanol was the major alkanol (*ca* 27% of total) in agreement with the ester GC-MS analysis (Table 1). Also present in the alkanol TMSi GC were *anteiso*-C_{11} (*ca* 6% of total) and trace amounts of *anteiso*-C_{25}, C_{27}, C_{29} and C_{31} alkanols. Esters containing these minor alkanols were of too low abundance to be observed in the GC-MS analysis, with the exception of a C_{43} branched ester which consisted of *anteiso*-C_{11} alkanol esterified with C_{32} *n*-acid. Small amounts of C_{27}, C_{29} and C_{31} fatty acids were also detected in the hydrolysis products (Table 2). GC-MS analysis showed the C_{31} acid to be alkylated with hexanol to give the C_{37} ester (Table 1). No sterols or triterpene alcohols were found in the hydrolysis products.

The mixture of alkyl esters from *P. radiata* mature-tree foliage wax was shown by GC and GC-MS to be more complex (Table 3) than that from the juvenile plant leaves. A minor group of three short chain esters C_{24}, C_{26} and C_{28} was identified, composed of dodecanoic and tetradecanoic acid, alkylated randomly with C_{12}, C_{14} and C_{16} alkanols. The larger group were C_{36} to C_{50} even, C_{39}, C_{41}, C_{43} odd, C_{41} and C_{43} branched and C_{41} and C_{43} secondary alcohol esters (Table 3). The range of ester M_rs was confirmed by probe CI mass spectrometry of the wax ester mixture (Table 3). The secondary alcohol esters did not display molecular ions in their EI mass spectra, but their presence was confirmed by independent synthesis and GC-MS analysis. Other, monoterpene alcohol esters were present, but structures could not be proposed from their mass spectra. GC-MS analysis of the neutral fraction of the ester hydrolysate suggested that the monoterpene alcohols from the hydrolysate (Table 2) were either pinenol or unsaturated borneol, *viz.* *m/z* 154 $[M]^+$ (12%), 95 (95%), 91 (100%) [7]. Wax esters from the mature tree pine needles were therefore predominantly composed of C_8–C_{12} alkanols, esterified with long chain acids (C_{28}–C_{34}), together with C_{12} and C_{14} esters of nonacosan-10-ol.

Since $[R'-1]^+$ fragments were not observed for long-

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Table 1. Diagnostic mass spectral ions for acid ($RCO_2H_2^+$) and alkanol ($[R'-1]^+$) fragments, and composition of alkyl esters (RCO_2R') from *Pinus radiata* juvenile foliage

Carbon no.	% of total	EI $[M]^+$ observed	CI $[M+1]^+$ observed*	$RCO_2H_2^+$ fragment	$[R'-1]^+$ fragment	Composition		Approx. % within homologue
						Alkanol	Acid	
30	0.1		453	369		6	24	100
32				145		24	8	tr†
	0.4	480	481	369	112	8	24	12
				397	84	6	26	88
34	1.2	508	509	117		28	6	16
				145		26	8	tr
				173		24	10	tr
				369	140	10	24	5
				397	112	8	26	46
				425	84	6	28	33
35	0.1							
36	23.1	536	537	117		30	6	2
				145		28	8	25
				173		26	10	tr
				397	140	10	26	1
				425	112	8	28	5
				453	84	6	30	67
				481		6	32	tr
37	0.3	550	551	467		6	31	100
38	21.8	564	565	145		30	8	20
				173		28	10	7
				425	140	10	28	9
				453	112	8	30	28
				481	84	6	32	36
39	0.2							
40	25.5	592	593	173		30	10	9
				201		28	12	11
				453	140	10	30	55
				481	112	8	32	25
41 br‡	0.4		607	453	154	11 br	30	100
41	0.4		607	173		31	10	100
42	24.3	620	621	173		32	10	tr
				201		30	12	19
				481	140	10	32	81
43 br	1.6		635	481	154	11 br	32	100
43	0.3		635	201		31	12	100
44	0.2			201		32	12	100
45	0.1							
46	0.1							
47	tr							
48	tr							
50	tr							

* $[M-1]^+$ also observed.

† tr, 0.1.

‡ br, Branched.

chain alkanols in the GC-MS analysis of both juvenile and mature *P. radiata* foliage wax esters, the within-homologue distribution of the wax esters could not be calculated using the formula of Aasen *et al.* [8]. Also, since the intensity of characteristic ions for wax esters is dependent on carbon chain length [8], the within-homologue distribution of individual esters (Tables 1 and 3), calculated from integration of $RCO_2H_2^+$ ion chromatograms, is approximate.

Most plant epicuticular waxes contain saturated long-chain wax esters having carbon numbers from C_{32} to C_{64} [9]. The occurrence in *P. radiata* of C_{30}–C_{50} wax esters containing unusually high amounts of short-chain saturated alkanols, and short-chain fatty acids, is thus quite unusual. Further work is needed to establish whether they are common in the Pinaceae, or in other conifers. The latter possibility is suggested by the identification of C_8 and C_{10} alkanols in wax esters of the conifer *Juniperus scopulorum* [10]. Short chain fatty acids, however, were not detected in that species.

From the compositional data in Tables 1 and 3, it is apparent that the fatty acids and alcohols are combined in

Table 2. Acid and alkanol composition from hydrolysates of wax esters of *Pinus radiata* juvenile and mature-tree foliage

	Juvenile foliage		Mature foliage	
Carbon no.	Alkanol	Acid	Alkanol	Acid
6	2.6	3.2	0.1	tr*
8	3.0	4.8	0.6	0.7
10	26.5	7.1	19.3	3.3
Monoterpene 10			8.5	
a-11	6.5		3.0	
11			0.3	tr
12	2.5	9.2	3.5	23.9
13			0.3	
14	1.7	2.6	3.8	4.9
16	1.3	1.3	0.9	2.5
18	1.3	0.4	0.8	1.2
20	2.1	0.1	0.6	0.5
22	4.7	1.1	1.1	0.3
24	7.1	3.7	6.9	0.2
25	0.4	0.6	tr	
26	8.5	10.7	4.8	8.8
27	0.5	0.5	0.3	
29-10-ol			9.4	
28	11.0	9.8	10.2	10.9
29	1.0	0.5	0.7	
30	11.5	24.7	11.9	14.6
31	1.1	1.9	0.5	
32	6.7	17.8	12.4	19.4
34				3.8

*tr, $< 0.1\%$.

a non-random manner in both the mature and juvenile foliage epicuticular waxes. Random combinations would have produced significant quantities of both shorter chain C_{12}–C_{20} and longer chain C_{52}–C_{64} wax esters, but these were not observed. Few wax ester distributions have been studied in sufficient detail to establish whether the combinations present are random or not, but available data suggest that in many plants [9] and bacteria [11], wax ester synthesis is non-selective. Non-random esterification has been reported for the wax esters of a grass, *Echinochloa crus-galli* [12], which contains smaller amounts of the longer chain esters than would be predicted from a random combination.

Capillary GC-MS analysis of the wax esters of *P. radiata* needles highlights the care that must be taken when analysing the acids and alkanols isolated from hydrolysis of the wax esters. Volatile C_6–C_{10} constituents are readily lost, especially after conversion to derivatives such as TMSi-ethers, or methyl esters. Low initial GC temperature settings (e.g. 30°) are also essential to separate these volatile constituents from the solvent peak. Finally, the advantages of capillary columns for these analyses compared with packed columns, particularly for separating odd-chain and branched esters from the usually more abundant even-chain saturated esters, are clearly demonstrated in this study.

EXPERIMENTAL

Wax esters. Alkyl ester fractions from the epicuticular wax of juvenile and mature *P. radiata* foliage were obtained as described previously [1].

Nonacosan-10-ol esters. Nonacosan-10-ol [1] (0.2 mg) was esterified in dry toluene separately with dodecanoyl and tetradecanoyl chloride (1 equiv). The mixtures were heated to *ca* 0° for 15 min, evapd to dryness and taken up in dry CH_2Cl_2 ready for analysis.

Ester hydrolysis and derivatization of acidic and neutral fractions. The wax esters were hydrolysed using MeOH–KOH as before [1], but the fractions from DEAE-Sephadex ion-exchange chromatography of the hydrolysate [1] were not evapd to dryness under red. pres. Concn of fractions was carried out to near-dryness at *ca* 80° at atm. pres. Et_2O solns of the esters of acidic fractions were concd at *ca* 50°, atm. pres. After derivatization of the alkanol fraction (BSTFA, pyridine, 60°, closed tube, 30 min), excess of reagents was not removed; the reaction mixture was diluted with dry CH_2Cl_2 to give *ca* 2 mg/ml of derivative for analysis.

Analysis. Wax esters were dissolved in CH_2Cl_2 for injection into the chromatograph. GC of the wax esters was carried out on a 25 m × 0.3 mm immobilized OV-1 fused silica capillary column programmed from 100 to 300° at 4°/min. The sample was loaded onto the column using an air-cooled on- column syringe injector. Carrier gas was He at $u = 100$ cm/sec. GC of the wax ester hydrolysate acid Me ester and alkanol TMSi ether samples was carried out on 25 m × 0.2 mm OV-1 glass and fused-silica capillary columns maintained isothermally at 30° for 5 min, then temp. prog from 30 to 50° at 10°/min, then 50–250° at 4°/min, or from 100 to 250° at 4°/min. The injector was a purged-spitless type at 280°, FID at 300°. The carrier gas was He at $u = 40$ cm/sec. Peak areas were determined by integration. GC-MS was carried out using quadrupole filter instruments operating at 70 eV, 300 μA or 35 eV, 350 μA electron energy, and an ion source temperature of 250°. Other MS parameters were

Table 3. Diagnostic mass spectral ions for acid ($RCO_2H_2^+$) and alkanol ($[R' - 1]^+$) fragments, and composition of alkyl esters (RCO_2R') from *Pinus radiata* mature tree foliage

Carbon no.	% of total	EI $[M]^+$ observed	CI $[M+1]^+$ observed*	$RCO_2H_2^+$ fragment	$[R'-1]^+$ fragment	Composition Alkanol	Composition Acid	Approx. % within homologue
24	0.5	368		201		12	12	100
26	2.0	396	397	201		14	12	94
				229		12	14	6
28	0.7	424	425	201		16	12	19
				229		14	14	81
36	1.7	536	537	425		8	28	100
38	6.0	564	565	173		28	10	12
				201		26	12	13
				425	140	10	28	31
				453	112	8	30	44
39	0.9		579					
41 s†	14.0		607	201		29 s	12	100
40	17.6	592	593	173		30	10	7
				201		28	12	29
				453	140	10	30	45
				481	112	8	32	19
41 br‡	tr§		607					
41	1.9		607					
43 s	7.2		635	229		29 s	14	100
42	29.3	620	621	173		32	10	2
				201		30	12	29
				229		28	14	1
				453		12	30	2
				481	140	10	32	61
				509	112	8	34	5
43 br	1.7		635	481		11 br	32	100
43	1.4		635	201		31	12	100
44	10.1	648	649	201		32	12	31
				481		12	32	16
				509		10	34	53
45	0.1		663					
46	2.2		677	509		12	34	100
48	0.9							
50	tr							

*$[M - 1]^+$ also observed.

†s, Secondary alcohol (nonacosan-10-ol) and ester.

‡br, Branched (anteiso).

§tr, 0.1%.

chosen to maximize high mass sensitivity. The capillary columns were coupled to the MS ion-source either directly, or through an open-split interface heated maximally at 285°. Spectra were taken at 2.8 sec intervals. CI probe mass spectra were obtained using CH_4 gas at 0.6 torr.

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