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MAJOR RESIN ACIDS OF *PINUS NIGRA* NEEDLES

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Key Word Index—*Pinus nigra*; Pinaceae; needles; labdane diterpenes; resin acids; 10-nonacosanol; isoabienol.

Abstract—Labdane diterpene acids were found to be the major resin acid components in *Pinus nigra* needles of various seed sources. The major constituents have been identified as 4-epiimbricataloic acid, manoyl oxide 19-oic acid, 4-epicommunic acid, and 15-monomethyl pinifolate. A GC method was developed to analytically differentiate pinifolic acid from its monomethyl ester in an admixture of both compounds. A minor resin acid was identified as 18-acetoxy-8(17)-labden-15-oic acid. 10-Nonacosanol and isoabienol were identified as major constituents of the needle and cortex extractives, respectively.

INTRODUCTION

The xylem oleoresin of *P. nigra* consists primarily of the common tricyclic abietane, pimarane, and isopimarane resin acids [1–4]. Cortical oleoresin is similar to the xylem, except that the tricyclic neoabietic acid predominates; small amounts of labdanes are also present [5]. Only the tricyclic resin acids have been reported to be present in *P. nigra* foliage [6]. However, in that study of foliage (branches), the xylem along with the smaller cortex contribution obviously overshadowed that of the needles. Preliminary data of our investigation indicated large differences between the xylem and the needles, and between the needles of different sources [7].

In a previous paper [8], the needle resin acid composition of *Pinus resinosa* and *Pinus nigra* putative crosses were reported to differ significantly in labdane composition from that of the parents. *Pinus nigra* needles from a variety of sources—i.e. different geographic origin of seeds—were further investigated for resin acid composition. Four labdane resin acids were found to predominate. Distinctive differences in the composition of these resin acids were observed among the sources.

RESULTS AND DISCUSSION

Identification of resin acids

Needles from various *P. nigra* Arnold sources were extracted and the resin acids were analyzed as the methyl esters by GC (Tables 1 and 2). Because of the differences in resin acid composition among the sources, several were then selected for isolation and identification of the major components.

Table 1. Retention characteristics of *P. nigra* needle resin acid methyl and ethyl esters on a methyl silicone capillary column*

Resin acid	Methyl†	Ethyl‡
Pimaric	1.00	1.00
Manoyl oxide acid	0.96	0.98
Sandaracopimaric	1.06	1.06
Isopimaric	1.20	1.16
Levopimaric	1.25	1.23
Palustric	1.25	1.23
Dehydroabietic	1.35	1.32
Abietic	1.60	1.53
Neoabietic	1.89	1.90
4-Epiimbricataloic	1.72	1.69
Methyl ketone	2.20 (**3c**)	—
Ethyl ketone	—	2.91§
Pinifolic	2.35	3.04
Monomethyl pinifolate	2.35	2.40
Compound **5**	2.82	—

*Methyl silicone = DB-1, a bonded phase (J. & W. Scientific, Inc., Rancho Cordova, CA, U.S.A.) on a fused silica column; length = 15 m, i.d. = 0.25 mm, and film thickness = 0.1 μm; oven temperature = 190°; He u = 40 cm/sec.

†$r_{\text{Me pimarate}}$; $t'_{\text{Me pimarate}}$ = 3.56 min (cf. [30] for SE-30).

‡$r_{\text{Et pimarate}}$; $t'_{\text{Et pimarate}}$ = 4.33 min.

§**3c** where R,R′ = Et, $[M]^+$ 362. Forms slowly on diazoethylation of **3a** in Et_2O–EtOH).

One source contained manoyl oxide acid (**1a**) in significant amounts. The extract from this source was separated by DEAE-Sephadex and the resulting acids fraction recrystallized to yield **1a**. The spectral, physical, and GC characteristics of the methyl ester (**1b**) agreed with those reported previously [8].

Another needle source had a major component that appeared to be 4-epicommunic acid (**2a**) based on GC data. The main component in the acid fraction was purified by recrystallization and confirmed as **2a** by comparison of the NMR for the methyl ester **2b** with that reported [9, 10]. The acid **2a** has been isolated from *P. densiflora* [11].

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Table 2. Composition of resin acids in the needles of *Pinus nigra*

Source		Percent of total resin acids*								
Tree designation†	Seed origin	Sand (1.05)	MOA (0.97)	Ecom (1.18)	L/P/I (1.21)	DeAb (1.36)	Ab (1.58)	Eimb (1.73)	Neo (1.89)	Pin (2.28)
IFG										
Ni-45	Unknown	1.1	62.0	—	4.0	4.6	2.4	9.7	9.2	1.0
Ni-52	Unknown	3.3	47.3	—	11.1	4.3	2.5	12.2	13.1	1.2
Ni-58	Turkey	3.7	22.7	—	14.0	1.1	0.9	12.2	11.5	28.5
Ni-70	Croatia	1.1	3.1	—	23.1	3.2	1.1	23.5	26.4	11.8
NiCa-82	Corsica	11.5	—	76.0	tr	tr	tr	7.5	tr	2.5
NiCar-12	Cyprus	2.5	—	—	14.3	10.1	17.5	17.5	29.6	4.2
NiCe-V6	Cevennes Mts.	9.3	—	89.0	tr	—	—	—	—	—
NiM-N3	Algeria	1.1	—	—	4.8	0.9	1.2	45.9	27.2	7.2
Ni-N7	Unknown	tr	60.8	—	4.8	3.8	3.0	7.5	10.0	3.8
NiPa-17	Yalta	1.0	1.7	—	11.5	3.8	2.5	45.9	27.2	7.2
Ni-W11	Unknown	1.2	—	—	17.1	7.5	4.3	11.2	15.8	34.0
UW										
var. *caramanica*	Crimea	3.2	21.9	—	8.3	1.1	1.1	15.4	6.2	31.0
var. *caramanica*	Crimea	3.9	25.4	—	4.1	2.2	0.6	16.5	4.8	37.5
cv. '*Hornibrookana*'	Unknown	1.0	tr	—	24.0	2.6	1.8	29.8	12.0	17.2
cv. '*Pendula*'	Unknown	5.9	—	51.0	40.0	tr	tr	tr	tr	tr
arboretum specimen	Unknown	6.5	39.6	—	14.1	7.8	3.4	13.3	8.3	6.0
KF‡										
402	Spain	7.4	—	85.7	1.2	0.4	0.5	—	0.1	2.4
415	Yugoslavia	1.4	—	—	3.6	4.8	2.6	46.5	10.1	20.3

*By GC of methyl esters on an SE-30/EGiP packed column [18], oven temp. 200°; supplemental data obtained with a DEGS packed column. Sand = sandaracopimaric, MOA = manoyl oxide acid, Ecom = epicommunic, L/P/I = levopimaric/palustric/isopimaric (unresolved), DeAb = dehydroabietic, Ab = abietic, Eimb = epiimbricataloic, Neo = neoabietic, and Pin = pinifolic. Values for Eimb are the sum of the epiimbricalaloate and artifact methyl ketone **3c**. Values for Pin represent the sum of pinifolic acid and monomethyl pinifolate; reanalysis of the methylated needle resin acids from several KF 415 trees on a methyl silicone glass capillary column (conditions per Table 1) showed that monomethyl pinifolate was 70–80% of total pinifolate. Minor components of the order of 5–10% total were also present. GC retention ratios relative to methyl pimarate are given in ( ) for SE-30/EGiP.

†IFG = Institute of Forest Genetics, Placerville, CA, U.S.A.; UW = University of Wisconsin Arboretum, Madison, WI, U.S.A.; KF = Michigan State University Kellogg Forest near Augusta, MI, U.S.A.

‡Average of three trees from 402 seed source, and of two non-MOA trees from 415 seed source [5].

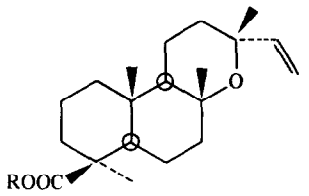
A third source had two major components, one unidentified and the other apparently pinifolic acid (**4a**); two minor components were also evident. Separation of the two major acids by silica gel chromatography was unexpectedly difficult and only partially successful. Isolation of the major unknown acid (in its methyl ester form) from an enriched fraction was achieved by a sequence involving DEAE-Sephadex, methylation with diazomethane, and chromatography on neutral alumina. Spectral data for the unknown isolate showed it to be methyl 4-epiimbricataloate (methyl 15-oxo-8(17)-labden-18-oate, **3b**). Thus, the naturally occurring acid **3a** is the C-4 epimer of imbricataloic acid found in *Pinus elliottii* [12]. Dimethyl pinifolate (**4c**) was also isolated in high purity from the alumina column.

Pinifolic acid has been isolated from *P. sylvestris* needles by Enzell and Theander [13] as the dicarboxylic acid, and by Bardyshev and Degtyarenko [14] as a monomethyl ester. Because diazomethane methylation forms the same dimethyl pinifolate derivative from both pinifolic acid and monomethylpinifolate, the identity of the compound(s) as present in the needles needed further definition. On chromatography of the *P. nigra* acids on silica gel, two elution bands for "pinifolate" were observed by GC monitoring of the column effluent. The material in the first of these elution bands was further purified on silica gel and was shown to be a monomethyl ester by ^{1}H NMR, but the location of the ester methyl could not be established. ^{13}C NMR of the monomethyl ester in CD_3OD showed it to be identical with the monomethyl ester isolated by Bardyshev and Degtyarenko as the 18-oate (18-methyl hydrogen 8(17)-labden-15,18-dioate, **4b**) [15]. The C-15 and C-18 carboxyl groups, however, could not be distinguished by ^{13}C NMR in $CDCl_3$ or CCl_4. Pinifolic acid was tentatively identified in the second elution band based on elution sequence. This identification was verified by comparative GC of methyl and ethyl esters of the *P. nigra* needle resin acids (*vide infra*).

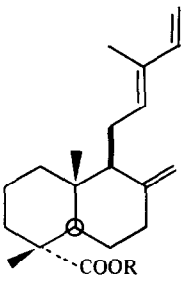
One of the minor acid components seen in the GC analysis of a third source was enriched along with **3a** and **4b** on silica gel. A selected fraction containing the acid was further purified on DEAE-Sephadex and, after diazomethane methylation, by silica gel chromatography. The methylated acid was identified as methyl 18-acetoxy-8(17)-labden-15-oate (**5**) (M_r 378 by MS) by ^{1}H NMR comparison with data reported for its enantiomer [16].

Quantitative determination of resin acid composition

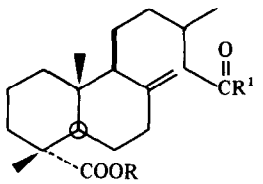
In a limited survey of *P. nigra* needles, a number of



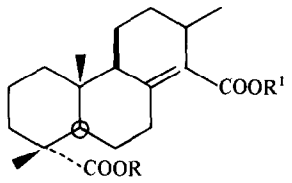
1a R = H
1b R = Me



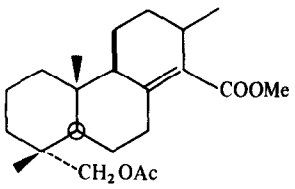
2a R = H
2b R = Me



3a R, R¹ = H
3b R = Me, R¹ = H
3c R, R¹ = Me



4a R, R¹ = H
4b R = Me, R¹ = H
4c R, R¹ = Me



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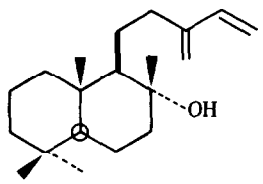

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samples were obtained from various sources to provide representation of the range of the species [17]. The GC analyses of the resin acid methyl esters, obtained by packed columns, are shown in Table 2. Because analyses were done on the methyl esters, the dimethyl pinifolate consists of the contributions of both pinifolic acid and monomethyl pinifolate in the original acid mixtures.

Manoyl oxide ester was found in characteristic amounts in the GC of seven sources, while methyl 4-epicommunate highly predominated in three (excluding the *Pendula* cultivar). Mixtures of methyl 4-epiimbricataloate and total pinifolates were characteristic in most all of the sources analysed. The relative amounts of pinifolic acid and monomethyl pinifolate in needle resin acids can be determined by forming esters other than methyl for GC. This was accomplished by preparation of ethyl esters using diazoethane. Thus, diethyl pinifolate is formed from pinifolic acid and ethyl methyl pinifolate from monomethyl pinifolate. As an application of the diazoethane esterification method, needle resin acids from several trees were reanalyzed on a methyl silicone capillary column. Monomethyl pinifolate was found to comprise 70–80% of total pinifolates.

The use of diazoethane has the additional advantage of avoiding alkyl ketone artifacts from aldehydes such as occur in the diazomethane methylation of imbricataloic [12] and 4-epiimbricataloic acids. Whereas resin acid esterification with excess diazoethane is quantitative in ethyl ether within 5 min, equivalent esterification with diazomethane requires the addition of methanol to the solvent [18]. However, methanol enhances formation of the methyl ketone; complete conservation of 4-epiimbricataloate to methyl ketone **3c** can be effected within 15 min in 1:1 methanol–ethyl ether containing excess diazomethane.

Although the number of trees examined was limited, certain patterns of chemical groupings of *P. nigra* were evident based upon major needle resin acid trends (Table 2): a manoyl oxide acid (**1a**) group; a 4-epicommunic acid (**2a**) group; and a 4-epiimbricataloic acid (**3a**)/monomethyl pinifolate (**4a**) group. The biological oxidation of a C-4 methyl to carboxylic acid for the labdane resin acids in a pine tissue generally is restricted to producing either C-18 or C-19 acids [7]. For the closely related *P. nigra* and *P. sylvestris* [5], however, C-18 and C-19 labdane acids co-occur in the needles. Nevertheless, only one of the C-4 epimers of a structural type is present—e.g. communic and 4-epicommunic acids do not co-occur. Although much is yet to be done, the use of needle resin acids as chemotaxonomic and genetic indicators offers an excellent potential in *P. nigra* and other pines.

Some neutrals in needles and cortex

DEAE-Sephadex separation of *P. nigra* needle extracts gave a neutrals fraction from which a major component readily crystallized on solvent removal. The compound was identified as 10-nonacosanol, an alcohol previously found in a number of conifers including pines [19–22].

In our previous publication [5], we reported that a neutral component interferes in the GC analysis of the resin acid esters in methylated cortical oleoresin. On isolation and purification, the interfering compound was identified as isoabienol [13(16),14-labdadien-8-ol, **6**], an alcohol reported to occur in *P. sylvestris* needles [23, 24] but not in the cortex [25]. In our work with *P. nigra* needles, isoabienol did not pose a problem because neutrals were removed by DEAE-Sephadex before the resin acids were analyzed.

EXPERIMENTAL

Needle and branch (cortex) samples were obtained from the Institute of Forest Genetics, Placerville, Calif. (IFG), the University of Wisconsin Arboretum, Madison, Wis. (UW), and Michigan State University's W. K. Kellogg Forest near Augusta, Mich. (KF).

GC was done with a Hewlett–Packard model 5750 or 5840 gas chromatograph equipped with FID. ^{1}H NMR and ^{13}C NMR were recorded at 250 and 62.89 MHz, respectively ($CDCl_3$ soln, unless noted otherwise, and TMS as int. standard). EIMS (CIMS supplementary) were run on Finnegan model 4510 at 70 and 40 eV and reported as *m/z* (rel. int. %).

Manoyl oxide acid (8,13*β*-*epoxy*-14-*labden*-19-*oic acid*, **1a**). Needles (IFG Ni-45 and Ni-52) were cut and extracted with Et_2O. Acids from the DEAE-Sephadex separation [26, 27] were recrystallized from MeOH to yield pure **1a**. Physical and spectral characteristics were consistent with those reported [8].

4-*Epicommunic acid* [8(17), E-12,14-*labdatrien*-18-*oic acid*, **2a**]. Needles (KF #402) were cut, extracted with Et_2O, and the acids separated by base extraction. The acids were recrystallized from MeOH to yield **2a**. Spectral confirmation of the GC identification was made by UV, and comparison NMR; cf. ^{1}H NMR [9] and ^{13}C NMR [10] for methyl *trans*-ozate (*trans*-ozate = 4-epicommunate).

4-*Epiimbricataloic acid* [15-*oxo*-8(17)-*labden*-18-*oic acid*, **3a**]. The Et_2O extract of KF #415 needles was chromatographed on silica gel in a stepwise gradient of Et_2O–petrol (1:9, increasing to 1:4). Fractions enriched in **3a** contained monomethyl pinifolate (**4b**) contaminant, which was removed by chromatography of the methyl esters (CH_2N_2) on neutral alumina III, yielding **3b** as a colourless gum (65 mg, 99% purity), $[\alpha]_D^{20}$ + 14.4° ($CHCl_3$; *c* 1.1). IR ν_{max}^{film} cm^{-1}: 3090, 1645, 890 (exocyclic $=CH_2$), 2885, 2710, 1730, 1385 (CHO), 1730, 1250 (equatorial COOMe) (cf. [28]); MS: 334 $[M]^+$ (5), 301 (2), 274 $[M - HCOOMe]^+$ (8), 257 (5), 180 (11), 161 (12), 121 (100); ^{1}H NMR: *δ*0.70 (Me-20), 0.97 (*d*, *J* = 7 Hz, Me-16), 1.14 (Me-19), 3.66 (OMe), 4.49 and 4.82 (exocyclic $=CH_2$), 9.76 (CHO); ^{13}C NMR: *δ*37.8, 37.0, 38.1, 35.9 (C-1, C-3, C-7, C-12; chemical shift assignments may be interchanged), 18.4, 20.8 (C-2, C-11), 47.7 (C-4), 49.9 (C-5), 26.8 (C-6), 147.0 (C-8), 57.1 (C-9), 39.0 (C-10), 28.8 (C-13), 50.8 (C-14), 202.6 (C-15), 20.1 (C-16), 106.8 (C-17), 179.1 (C-18), 16.5 (C-19), 14.7 (C-20), 51.7 (OMe).

The methyl ketone **3c** [methyl 15-methyl-15-oxo-8(17)-labden-18-oate] was made. The **3b** isolate, in a solution of MeOH–Et_2O (1:1) and excess CH_2N_2, yielded **3c** as the major reaction product within 15 min. MS: 348 $[M]^+$ (1), 288 $[M - HCOOMe]^+$ (9), 175 (5), 161 (12), 121 (100); ^{1}H NMR: *δ*0.70 (Me-20), 0.90 (*d*, *J* = 7 Hz, Me-16), 1.14 (Me-19), 2.12 (COMe), 3.66 (OMe), 4.49 and 4.81 (exocyclic $=CH_2$).

Dimethyl pinifolate (**4c**), formed from **4a** and **4b** on methylation, was isolated from the neutral alumina column (eluting just prior to **3b**) and identified by GC and ^{1}H NMR (cf. ^{1}H NMR of enantiomers in CCl_4 [29] and $CDCl_3$ [16]; note differences in C-17 and C-20 numbering).

Compound **3b** was isolated as a very minor component from the preliminary silica gel chromatography of the *unmethylated* needle extract.

Monomethyl pinifolate [18-*methyl hydrogen* 8(17)-*labden*-15,18-*dioate*, **4b**]. Fractions enriched in **4b** were obtained from the same silica gel column used for preliminary purification of **3a**. These fractions (220 mg, 80% purity), largely contaminated with **3a**, were rechromatographed on silica gel (Et_2O–petrol, 1:4) to yield **4b** as a gum. ^{1}H NMR: *δ*0.70 (Me-20), 0.98 (*d*, *J* = 7 Hz, Me-16), 1.14 (Me-19), 3.66 (OMe), 4.49 and 4.82 (exocyclic $=CH_2$). This data ($CDCl_3$) and that obtained in CCl_4 were similar; previously reported data in CCl_4 is highly suspect, as indicated by the COOMe chemical shift of *δ*3.51 ppm [14; note differences in C-17 and C-20 numbering]. ^{13}C NMR (CD_3OD) was in agreement with the literature, particularly the ^{13}C<u>C</u>OOH of C-15 at *δ*176.4 ppm and the ^{13}C<u>C</u>OOMe of C-18 at *δ*180.2 ppm [15]. In $CDCl_3$ the C-15 and C-18 (carboxylic) carbons had equivalent chemical shifts at *δ*179.3 ppm; in CCl_4 two chemical shifts were seen at *δ*177.9 and 178.6 ppm. For MS, **4b** was methylated (CH_2N_2) to **4c** and the fragmentation pattern found to be consistent with the literature [13; note differences in C-17 and C-20 numbering].

Minor components. The KF #415 needles used for the isolation of **3a** and **4b** also contained two minor components amounting to approximately 5% of the total resin acids. Appropriate fractions from the above preliminary silica gel column, enriched in the minor components but containing **3a** and **4b** as contaminants, were fractionated by DEAE-Sephadex to remove neutrals. Fractions containing the first minor component to elute from a second silica gel column (Et_2O–petrol, 1:4) were methylated (CH_2N_2) and rechromatographed on silica gel (Et_2O–petrol, 1:9) to yield **5** (methyl 18-acetoxy-8(17)-labden-15-oate) as a gum. ^{1}H NMR: *δ*0.70 (Me-20 for equatorial acetoxy substituent at C-18) [16; note differences in C-17 and C-20 numbering], 0.82 (Me-19), 0.93 (*d*, *J* = 7 Hz, Me-16), 2.07 (OAc), 2.35 (2H, *br s*, C<u>H</u>$_2$OAc), 3.66 (OMe), 4.48 and 4.81 (exocyclic $=CH_2$); MS: 378 $[M^+]$ (3), 318 $[M - MeCOOH]^+$ and $[M - HCOOMe]^+$ (70), 305 $[M - CH_2COOMe]^+$ (30), 189 (30), 175 (80), 135 (100). The second minor component was not obtained in sufficient purity for identification.

GC retention data. Pinus nigra resin acids were methylated in Et_2O–MeOH (9:1) with CH_2N_2 [18] or ethylated with diazoethane (CH_3CHN_2). CH_3CHN_2 was prepared in the usual way from *N*-ethyl-*N*-nitrosourea (Aldrich Chemical Co., Milwaukee, WI, U.S.A.). Quantitative ethylation occurred in excess ethereal CH_3CHN_2 within 5 min *without* alcohol present; this was verified by subsequent reaction with CH_2N_2 (Et_2O–MeOH, 9:1) which on GC analysis showed no methyl ester peaks.

Neutrals. Neutrals from the needle extract crystallized upon removal of solvent. The precipitate was washed and recrystallized from MeOH to yield pure 10-nonacosanol, mp 81.5–82.5° (lit. [19] mp 83.5°); MS gave a fragmentation pattern identical to that in the literature [19].

Cortex neutrals were chromatographed on silica gel (Et_2O–petrol, 1:99) to yield an alcohol which recrystallized from pentane, mp 71.5–72° (evap. cap. corr.) (lit. [23] mp 68–68.5°); rotation, UV, and ^{1}H NMR confirmed the identification of this alcohol as isobienol [13(16),14-labdadien-8-ol, **6**] [23].

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