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DOUGLAS-FIR POLYPHENOLS AND HEARTWOOD FORMATION

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Key Word Index—*Pseudotsuga menziesii*; Pinaceae; Douglas-fir; sapwood; heartwood; duraminisation; phenolic compounds; dihydroquercetin.

Abstract—Phenolic extractives have been isolated from Douglas-fir sapwood and heartwood and identified by NMR and mass spectroscopy. Pinocembrin, [5,5′]-bisdihydroquercetin and two lignans, pinoresinol and 2,3-dihydro-2-(4′-*O*-*β*-glucopyranosyl-3′-methoxyphenyl)-3-hydroxymethyl-5-(3-hydroxypropyl)-7-methoxybenzofuran are reported for the first time in these tissues. The chemical structures of these phenolic extractives show that heartwood formation is characterized by both primary (biosynthetic) and secondary reactions. Secondary reactions include hydrolysis of phenol glucosides and oxidative polymerization. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Since the end of the nineteenth century, many attempts have been made to introduce foreign tree species into European plantations. Douglas-fir is among the species of North American origin, one of the few to have gained real economic importance in Europe. In France, it occupies an area of 220 000 ha which might increase to 500 000 ha in the years 2010–2020 [1]. Larger Douglas-fir resources are thus being made available to the European wood industry.

However, some properties of Douglas-fir wood limit its utilization. One is its red-orange colour which makes the derived mechanical pulp hard to bleach. The colour of wood often relies on the presence/absence of some phenolic extractives [2, 3]. This led us to re-examine the polyphenol extractives present in Douglas-fir wood. Their purification and characterization in both sapwood and heartwood is reported, and their occurrence is discussed in relation to the formation of heartwood.

RESULTS

Phenolic extractives were determined in various wood samples of increasing age along a transversal section of a Douglas-fir trunk. Total phenols represent about 0.7% of the sapwood dry weight (Fig. 1). Their content increases up to 2.4% at the sapwood/heartwood transition. Proanthocyanidins were detected in sapwood by the BuOH–HCl assay and gave upon thiolysis [4] 3,4-*trans*-benzylthioepicatechin as main product showing that they are procyanidins largely made of (−)-epicatechin units **1** structurally related to those characterized in inner bark [5]. Their content, estimated as 0.1 and 0.03% by the BuOH–HCl assay and thiolysis, respectively (Fig. 1), is in good agreement with data previously reported by Barton and Gardner [6]. No proanthocyanidins could be detected in heartwood by

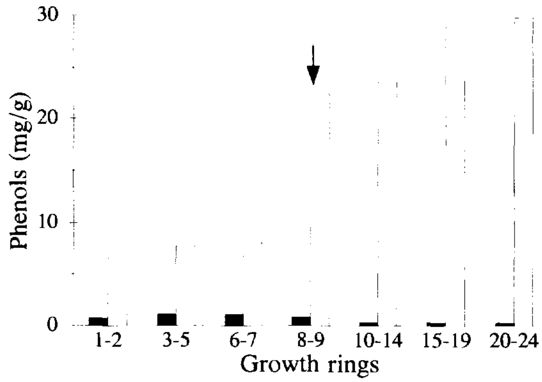

Fig. 1. Contents of total phenols and proanthocyanidins in sapwood and heartwood along a transversal section of a Douglas-fir trunk. Empty bars: total phenols; black bars: proanthocyanidins. The arrow indicates the sapwood/heartwood transition.

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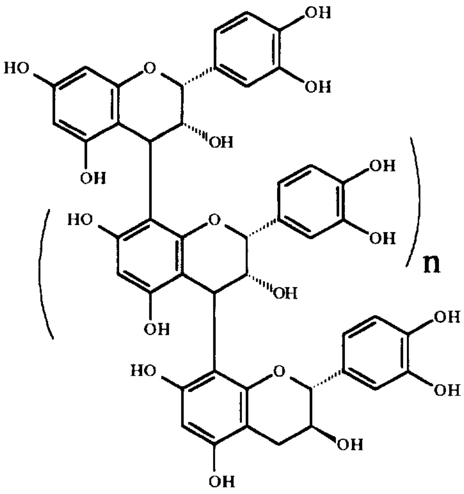

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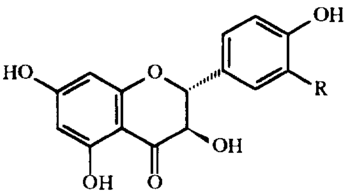
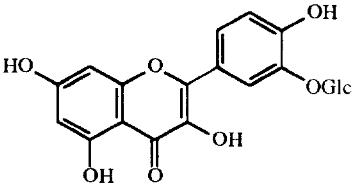


2 R = *O*-*β*-D-glucopyranosyl
3 R = OH
4 R = H



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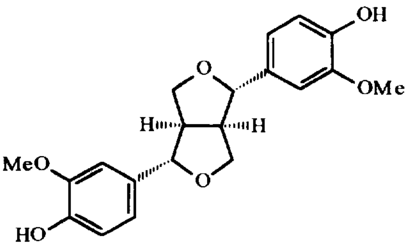

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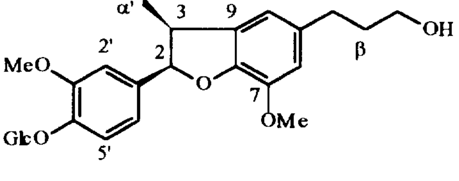

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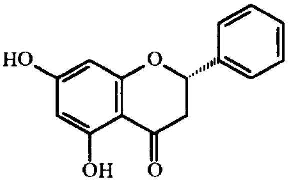

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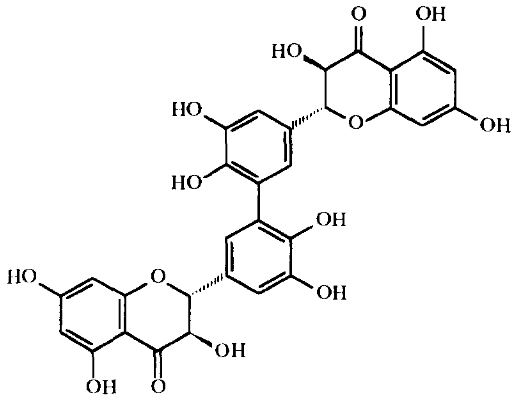
thiolysis. The BuOH–HCl assay applied to the same heartwood extract gave low absorbancies tentatively assigned to proanthocyanidins.

Phenolic compounds were purified by column chromatography and identified by comparison of their NMR and mass spectra with those of the literature. Several of the most abundant compounds have previously been reported in the same tissues: dihydro-



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quercetin 3′-*O*-glucoside **2**, quercetin 3′-*O*-glucoside **5** [7] and dihydroquercetin **3** [8] in sapwood; dihydroquercetin [9] and dihydrokaempferol **4** [10] in heartwood. Their contents are given in Table 1.

Other compounds are reported in Douglas-fir wood for the first time. Two lignans were found in sapwood: pinoresinol **6**, identified by comparison of its ^{1}H NMR [11], ^{13}C NMR [12] and MS [13] spectra with those of the literature, was previously mentioned to occur in the outer bark of the same species [14]. The structure **7** is attributed to the other lignan on the basis of its ^{1}H NMR [15], ^{13}C NMR, short- and long-range homo- and heteronuclear correlation (COSY, COSYLR, HETCOR and INAPT) and NOESY spectra. The exact stereochemistry was established by examination of its CD spectrum. The transformation of the CD spectrum into an ORD spectrum would give two major peaks at 244 and 292 nm, a major trough at 265 nm and zero values at 258 and 286 nm; all values compare well with those of the lignan identified by Lundgren *et al.* [15]. The same (2*R*, 3*R*) stereochemistry as that proposed by Lundgren *et al.* is thus assumed. This lignan glucoside has been previously identified in *Pinus sylvestris* [16, 17], *Larix leptolepis* [18] and *Picea abies* [15]. Two flavonoids were also found in heartwood: pinocembrin **8** identified by its NMR spectra [19, 20] and [5,5′]-bisdihydroquercetin **9**, previously identified in Douglas-fir outer bark [21].

No well-defined polyphenols soluble in ethyl acetate or water could be recognized in heartwood by either HPLC or TLC. Only orange-brown polymeric materials that do not migrate on cellulose thin layers with either aqueous acetic acid or *s*-butanol–acetic acid–water eluents were found in these fractions. Together with dihydroquercetin, they represent the bulk of polyphenols in this tissue (Table 1). Their NMR spectra were poorly resolved and could not be used for structural analyses. They gave a pink colour with the vanillin–sulphuric acid assay for proanthocyanidins, but no proanthocyanidin-derived ben-

Table 1. Content of the various polyphenols isolated from Douglas-fir sapwood and heartwood (mg/g). Data were obtained by HPLC unless otherwise mentioned

	Sapwood	Heartwood
Flavonoids		
Dihydroquercetin	0.4	15
Dihydrokaempferol	nd	0.3*
Pinocembrin	nd	0.03*
[5′,5′]-Bisdihydroquercetin	nd	0.15
Dihydroquercetin-3′-*O*-glucoside	2.0	nd
Quercetin-3′-*O*-glucoside	0.01*	nd
Procyanidins	1.0†	nd
Lignans		
Pinoresinol	0.2*	nd
Lignan-*O*-glucoside 7	0.02*	nd
Phenolic polymers		5.1*‡

nd: non-detected.

* Yield of purification.

† Determined by the BuOH–HCl assay.

‡ Crude EtOAc (4.2 g) + crude aqueous (3.4 g) extracts.

zylthioethers by thiolysis. They also gave a purple-red colour with zinc–hydrochloric acid [9] suggesting that they contain some bound 3-hydroxyflavanones. The presence of 3-hydroxyflavanones in these polymers was further confirmed by the observation of a peak at 1635 cm^{-1} in the IR spectrum [22]. UV spectra were also closely related to that of dihydroquercetin with a major absorption band at 282 nm (MeOH) that shifted to 325 nm upon addition of sodium hydroxide and 312 nm upon addition of aluminium chloride. The characteristic band I at 375 nm was also clearly observed in the $AlCl_3$ spectrum [23].

DISCUSSION

Various kinds of phenolic extractives, either lignans, stilbenes or flavonoids, are common constituents of woods [24]. Some lignans are reported here for the first time in Douglas-fir wood, but flavonoids (dihydroquercetin and procyanidins) remain the major extractives. Dihydroflavonols (dihydroquercetin and dihydrokaempferol) have been identified in the wood of many gymnosperm species [3]. Their content never exceeds 0.1%, with the exception of *Larix* and *Pseudotsuga* spp. where dihydroquercetin accounts for 0.5–2% of the wood weight.

In Douglas-fir, the transformation of sapwood into heartwood alters the composition of phenolic extractives in three different ways: (i) Some phenols such as dihydroquercetin or dihydrokaempferol, not found or found in much lesser amount in sapwood, are formed at the boundary between sapwood and heartwood. (ii) Phenolic glycosides are no longer observed in heartwood. The same observation has been made with other species such as Atlas cedar, western larch or Sitka spruce [7]. (iii) Some phenols are oxidized to complex polymers.

These polymers are formed by polymerization of dihydroquercetin, procyanidins and probably other phenolic compounds present in sapwood or formed at the sapwood/heartwood boundary. The presence of methoxyl groups in polymeric phlobaphenes isolated from Douglas-fir heartwood [25] is likely explained by the inclusion of lignans in the polymer. Such a polymerization explains the disappearance from heartwood of the lignans and proanthocyanidins identified in sapwood. Similar complex phenolic polymers based on lignans, proanthocyanidins, guaiacylic polymers or stilbenes have been isolated and characterized in heartwood or outer bark of various gymnosperms [26].

The reactions leading to the formation of these polymers are best illustrated by the present isolation of [5,5′]-bisdihydroquercetin **9** formed by oxidative dimerization of dihydroquercetin and the isolation from Douglas-fir outer bark of pseudotsuganol, a biphenyl-linked pinoresinol-dihydroquercetin adduct [27]. The oxidative formation by a polyphenol oxidase of biphenyl or biarylether linkages [28] resistant to acid treatments and involving the catechol B ring of flavonoids, also explains why no benzylthioepicatechin and virtually no cyanidin were formed from these polymers by thiolysis and the butanol–hydrochloric acid reactions, whereas the phloroglucinol A ring of bound procyanidins still reacts with the vanillin–sulphuric acid reagent.

These oxidative reactions may also lead to the partial insolubilization of polyphenol extractives in heartwood [29] or bark [30]. However, in Douglas-fir, thiolysis failed to detect proanthocyanidins bound to the insoluble matrix of sapwood or heartwood, probably because of the formation of acid-resistant linkages as discussed above.

The formation of polyphenols in heartwood is thus the result of both primary (biosynthetic) and secondary reactions. Primary reactions are those corresponding to the *de novo* synthesis of polyphenols [31] such as dihydroquercetin in Douglas-fir. These polyphenols often contribute to increase wood durability [32, 33] and consequently the life span of the tree [34]. Secondary reactions are those resulting from the decompartmentation accompanying the death of parenchymatous cells: polyphenols present in vacuoles and enzymes such as glycosidases and polyphenol oxidases present in other cell compartments would come in contact leading to the formation of aglycones and to the oxidative polymerization of polyphenols. Such reactions may be profitable to the plant by limiting the water solubility and leaching of these protective molecules [30].

EXPERIMENTAL

Polyphenol determination. A transversal section of a 24-year-old Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) was air dried. Seven samples of different ages were roughly ground in a Retsch mill SM1 and then made into thin powders in a Dangoumeau vibratory ball mill for 15 min. Wood powders (200 mg) were extracted by Me_2CO–H_2O 7:3 during 3 hr at room temp. under magnetic stirring. The suspensions were centrifuged and polyphenols analysed in the supernatant: total phenols were estimated by the Folin–Ciocalteu assay and proanthocyanidins by the BuOH–HCl assay with gallic acid and cyanidin, respectively, used as standards [35]. Proanthocyanidins were also analysed by thiolysis: Me_2CO was removed under red. pres. and the aqueous phase extracted with Et_2O and EtOAc. The residual aq. soln was treated in acid in the presence of toluene-α-thiol and the products analysed by HPLC using benzylthioepicatechin as a standard [4]. Other flavonoids were estimated by HPLC on a Lichrospher RP-18 column (5 μm, 250 × 4 mm i.d., Merck) with the following eluents: solvent A, H_2O–H_3PO_4 (990:1), solvent B, MeOH, a linear gradient from 0 to 90% B in 30 min, a speed flow of 1 ml/min and a detection at 280 nm. Retention times of the main flavonoids were 22.6, 22.9, 24.8, 25.8 and 29.8 min for dihydroquercetin-3′-*O*-glucoside **2**, dihydroquercetin **3**, dihydrokaempferol **4**, [5′,5′]-bisdihydroquercetin **9** and pinocembrin **8**, respectively. Appropriate standards were used for their estimation.

Polyphenol extraction. Douglas-fir sapwood and heartwood were ground in a Retsch mill SM1 (particle size less than 60 mesh). Wood powders (1.5 kg) were extracted in Me_2CO–H_2O 7:3 (3 × 4 l). Me_2CO was removed under red. pres. and the aqueous phase successively extracted by Et_2O (3 × 1 l) and EtOAc (3 × 1 l). Aqueous and organic phases were concentrated under red. pres. and freeze-dried. Weights of Et_2O, EtOAc and aqueous extracts were 4.3, 4.1, 15.0 g and 37, 4.2 and 3.4 g for sapwood and heartwood, respectively.

Purification of sapwood polyphenols. The Et_2O extract (4.3 g) was fractionated on a Sephadex LH20 column (70 × 3.5 cm i.d.) with EtOH as eluent to afford two pure products: dihydroquercetin **3** (from 0.7 to 0.9 l; 270 mg) and pinoresinol **6** (from 1.1 to 1.5 l; 540 mg). The EtOAc extract (4.1 g) was fractionated under the same conditions. One of the frs (from 0.7 to 1.0 l; 880 mg) was repurified on a shorter column (30 × 3 cm i.d.) and eluted with EtOH to provide pure dihydroquercetin-3′-*O*-glucoside **2** (440 mg). The aqueous extract (11 g) was fractionated on a Sephadex LH20 column (50 × 5 cm i.d.) and eluted with water containing increasing proportion of Me_2CO (5%, 0.7 l; 10%, 2.4 l; 20%, 1.5 l; 30%, 3.0 l; 40%, 6.3 l). Elution with 10% Me_2CO gave successively the lignan-*O*-glucoside **7** (30 mg) and a yellow oil further purified on a short Sephadex LH20 column (5 × 2.5 cm i.d.) using 15% Me_2CO as eluent to recover dihydroquercetin-3′-*O*-glucoside **2** (840 mg). Elution with 40% Me_2CO gave quercetin-3′-*O*-glucoside **5** (20 mg).

Purification of heartwood polyphenols. The Et_2O extract (20 g) was fractionated on a Sephadex LH20 column (50 × 5 cm i.d.) with EtOH as eluent. One fraction (from 0.7 to 0.9 l) contained two main products repurified on a smaller column (35 × 1.5 cm i.d.) with the same eluent to give successively dihydrokaempferol **4** (50 mg) and pinocembrin **8** (390 mg). A second fraction (from 1.1 to 1.6 l) gave dihydroquercetin **3** (7.6 g). A third fraction (from 5.4 to 9.2 l) contained one main product further purified on Sephadex LH20 (50 × 1.5 cm i.d.) with Me_2CO–H_2O 1:1 as eluent to afford [5,5′]-bisdihydroquercetin **9** (230 mg).

2,3-*Dihydro-2-(4′-O-β-glucopyranosyl-3′-methoxyphenyl)-3-hydroxymethyl-5-(3-hydroxypropyl)-7-methoxybenzofuran* **7**. ^1H NMR (400 MHz, MeOH-d_4 used as reference signal, $\delta = 3.50$ ppm): δ 7.32 (*d*, $J_{5',6'} = 8.5$ Hz, H-5′), 7.22 (*d*, $J_{2',6'} = 2.0$ Hz, H-2′), 7.12 (*dd*, H-6′), 6.92 (*br s*, $J_{4,6} \approx 1$ Hz, H-6), 6.90 (*d*, H-4), 5.74 (*d*, $J_{2,3} = 6.0$ Hz, H-2), 5.08 (Glc-1), 4.05 (Glc-6a), 4.04 (3H, *s*, OMe_3-7), 4.03 (H-α′a), 4.01 (3H, *s*, OMe_3-3′), 3.93 (*dd*, $J_{\alpha' a,\alpha' b} = 11$ Hz, $J_{\alpha' b,H3} = 7.5$ Hz, H-α′b), 3.87 (≈ *br d*, $J_{Glc6a,Glc6b} \approx 12$ Hz, Glc-6b), 3.75 (2H, *t*, $J_{\beta,\gamma} = 6.5$ Hz, H-γ), 3.68 (Glc-2), 3.66 (H-3), 3.62 (Glc-3), 3.58 (Glc-4), 3.53 (Glc-5), 2.81 (*dd* ≈ *t*, $J_{\alpha,\beta} \approx 7$ Hz and 8.4 Hz, H-α), 2.0 (2H, *m*, H-β). ^{13}C NMR (100 MHz, MeOH-d_4): δ 151.2 (C-3′), 147.9 (C-4′), 147.7 (C-8), 145.5 (C-7), 138.6 (C-1′), 137.4 (C-5), 129.9 (C-9), 119.7 (C-6′), 118.3 (C-5′), 118.2 (C-4), 114.4 (C-6), 111.4 (C-2′), 103.1 (Glc-1), 88.7 (C-2), 78.5 (Glc-5), 78.1 (Glc-3), 75.2 (Glc-2), 71.6 (Glc-4), 65.3 (C-α′), 62.8 (Glc-6), 62.5 (C-γ), 57.0 (OMe-7), 56.97 (OMe-3′), 55.9 (C-3), 36.0 (C-β), 33.2 (C-α). CI-MS (NH_3) *m/z*: 540 $[M+18]^+$. CD: $\Delta\varepsilon_{234}$ −4.1, $\Delta\varepsilon_{275}$ −1.4, $\Delta\varepsilon_{286}$ −1.2 (MeOH: *c* 1.6×10^{-4} g/ml).

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