Conifer Seed Cone Proanthocyanidin Polymers: Characterization by ^{13}C NMR Spectroscopy and Determination of Antifungal Activities

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Proanthocyanidin polymers (condensed tannins) in extracts from *Picea glauca*, *Pinus banksiana*, *Pinus nigra*, *Pinus ponderosa*, and *Pinus taeda* seed cones were isolated by chromatography over Sephadex LH-20 and characterized by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Ranging in molecular weight (MW_n) from 1520 to 2460, the seed cone proanthocyanidins were comprised primarily of procyanidin monomer units with the 2,3-*cis* stereochemistry predominating in the heterocyclic rings. Incorporated into agar media, a proanthocyanidin polymer preparation inhibited the growth of fungal cultures of *Ceratocystis coerulescens* and *Schizophyllum commune* but not *Trametes versicolor*. However, under conditions more representative of those found in nature, the polymer preparation did impart significant decay resistance to *T. versicolor* in wood test specimens normally susceptible to decay by this fungus. It is apparent from these results that proanthocyanidin polymers contribute to the natural resistance of conifer seed cones to fungal degradation.

Keywords: *Pine cones; condensed tannins; proanthocyanidins; procyanidins; ^{13}C NMR spectroscopy; molecular weight; stereochemistry; fungal decay*

INTRODUCTION

Proanthocyanidins (condensed tannins) are phenolic oligomers and polymers comprised of C-4 to C-8 (or C-6) linked flavan-3-ol units (Czochanska et al., 1979). Found in the wood, bark, leaves, and fruits of many trees, they appear to function as a means of defense against microbiological agents of disease and decay (Scalbert, 1991). For plant-pathogenic fungi, which excrete extracellular enzymes (*e.g.*, cellulases, lignases) to digest plant cell walls, proanthocyanidins may provide fungal resistance by forming complexes with these proteins (Laks et al., 1988; Porter and Hemingway, 1989). Alternatively, they may inhibit cell wall digestion by binding to specific cell wall polymers (*e.g.*, cellulose), thereby reducing the accessibility of the degradative enzymes to their substrates (Zucker, 1983). Proanthocyanidin toxicity may also be related to the disruption of metabolic processes on membranes and the complexation of metal ions (Scalbert, 1991).

Considerable differences in molecular weight, monomer composition, and stereochemical features have been revealed for proanthocyanidin polymers from different plant species, as well as from tissues within the same species (Hemingway, 1989; Porter, 1988, 1989a). However, the relationships between different proanthocyanidin structures and their capacity to function as preservatives in particular plants, or tissues, are poorly understood. Thus far, increasing molecular weight has been shown to correlate with an increasing capacity to bind proteins (Field et al., 1989; Kumar and Horigome, 1986; Porter and Woodruffe, 1984). Although the exact mechanisms are unclear, building unit composition, stereochemical features, and more recently described conformational features undoubtedly influence the biological activities of proanthocyanidins (Asquith and Butler, 1986; Fronczek et al., 1993; Hemingway et al., 1983; Spencer et al., 1988; Steynberg et al., 1992).

In woody tissues, natural durability (decay resistance) is usually attributed to the deposition of fungitoxic extractives. Research has shown that after application of the extractives obtained from a durable wood (*Robinia pseudoacacia*), a normally decay-susceptible sapwood (*Populus grandidentata*) can exhibit decay resistance (Smith et al., 1989a,b). Accordingly, the apparent decay resistance observed for conifer seed cones may also be due to the presence of potentially fungitoxic extractives (*e.g.*, proanthocyanidins). To provide the first insight into the nature of proanthocyanidins in seed cones, these polymers were isolated from crude extracts and characterized by ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Since the vast majority of efforts to identify the biological activities of proanthocyanidins have relied on very crude extracts (Scalbert, 1991), screenings for inhibition of wood decay fungi were then conducted with the isolated polymers.

MATERIALS AND METHODS

Plant Materials. Mature seed cones of white spruce [*Picea glauca* (Moench) Voss], jack pine (*Pinus banksiana* Lamb.), black pine (*Pinus nigra* Arnold), and ponderosa pine (*Pinus ponderosa* Laws.) were obtained from sources located in Wisconsin. Loblolly pine (*Pinus taeda* L.) cones were collected in Mississippi. After a rinse with distilled water, *P. glauca*, *P. nigra*, *P. ponderosa*, and *P. taeda* cones were dried in a warm oven (35 °C). The serotinous cones of *P. banksiana* were soaked in distilled water for 30 min and then warmed in an oven (50 °C) to initiate scale opening and concomitant seed release. After the removal of any attached seeds, the dry cones were ground in a Wiley mill (Model 2, 4-mm mesh).

Isolation of Crude Proanthocyanidins. Into an Erlenmeyer flask (500 mL), fitted with a ground glass stopper, were added dry seed cone tissue (20 g) and acetone/water (7:3) to obtain a suspension having a total volume of 500 mL. Purged with N_2, the flask was sealed and kept at room temperature

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in darkness. After 48 h of steeping, the suspension was filtered through a Büchner funnel with a glass fiber filter (Whatman GF/A).

The red-brown filtrate obtained was partially evaporated *in vacuo* (<40 °C) to remove the acetone and gave a milky aqueous suspension which was centrifuged (15 000 *g*, 15 °C, 15 min). The precipitate, suspended in distilled water, was frozen in liquid N_2 and freeze-dried to give a cream-colored powder (precipitate 1, 728 mg). Extraction of the brown supernatant with diethyl ether (3 × 1 vol) gave ether extracts which were combined and evaporated *in vacuo* to afford a yellow oil (174 mg). The aqueous phase, partially evaporated *in vacuo* to remove any residual ether, was centrifuged as before to give a precipitate which was suspended in distilled water and freeze-dried (precipitate 2, 40 mg). The final brown aqueous solution obtained was also freeze-dried to afford a crude proanthocyanidin preparation as a brown fluffy mass (672 mg).

The wet cone tissue from the filtration step above was steeped a second time (48 h) in acetone/water (7:3). Evaporation *in vacuo* of the acetone in the resulting filtrate gave a milky tan suspension which was freeze-dried (148 mg). The extracted cone residue remaining was allowed to stand until the acetone had evaporated and then was oven dried (80 °C) and weighed (18.2 g).

Purification and Analysis of Proanthocyanidin Polymers. Crude proanthocyanidin preparation (672 mg) was dissolved in a minimum amount of methanol/water (1:1) and applied to a Sephadex LH-20 (Pharmacia) column (2.5 cm × 7 cm) equilibrated in the same solvent mixture. While eluting with this solvent mixture (3 L, 1.6 mL/min), the eluates progressively changed in color from brown to yellow until finally they were almost colorless. At this point, the solvent system was changed to acetone/water (7:3) which released the bound proanthocyanidin polymers as a sharp band in a relatively small volume (60 mL) of solvent. After evaporation of the organic solvents from the methanol/water (1:1) and acetone/water (7:3) eluates, the resulting aqueous solutions were freeze-dried to obtain a tan solid (fraction 1, 382 mg) and a brown fluffy mass (fraction 2, proanthocyanidin polymers, 223 mg), respectively.

For the characterization of the proanthocyanidin polymers, samples (80–100 mg) were dissolved in acetone-d_6/D_2O (1:1) and analyzed by ^{13}C NMR spectroscopy using a Bruker AMX-360 instrument equipped with a 5-mm QNP probe and operating at 90.5 MHz. The central solvent signal (29.8 ppm) was used as the internal reference. Spectra were acquired at 300 K over a spectral width of 21 kHz with 70° radio frequency pulses. The data acquisition time was 0.75 s with a total repetition time of 1.25 s. Apodization of the FID by experimental multiplication, with a line broadening of 0.75 Hz, was carried out before Fourier transformation. The proton-decoupled spectra were typically obtained after 4.5×10^4 transients were accumulated.

Screenings of Antifungal Activity. Malt-extract agar medium comprised of 2% malt extract (Difco) and 1.5% agar (Bacto, Difco) was autoclaved and then set aside. Sterile filtered (0.45 μm) stock solutions (80 mg/mL) of fractions 1 and 2 (proanthocyanidin polymers), prepared in ethanol/water (1:1), were added to the still warm medium to obtain the final desired treatment concentrations (50, 100, 200, 1000 mg/L). Precipitate 1 would not completely dissolve in this solvent system, and therefore dimethyl sulfoxide or ethanol/water (3:1) was used as a solvent. After mixing, the media were dispensed into sterile Petri dishes (1.5 cm × 10 cm) and allowed to solidify. Controls were prepared as above using the appropriate amounts of solvents.

Each agar plate was inoculated with a mycelial plug (4-mm diameter) cut from the margins of fungal cultures of *Ceratocystis coerulescens* (Muench) Bakshi (C256), *Schizophyllum commune* Fr. (MAD566S), or *Trametes versicolor* (L.:Fr.) Pilat (MAD697). After days 5, 6, and 7 of incubation at 27 °C, the radial growth of each colony was measured. Inhibition of mycelial growth was determined by comparisons with the controls.

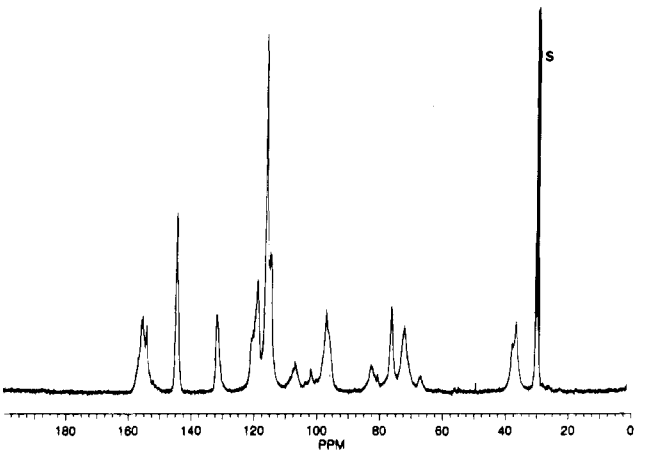


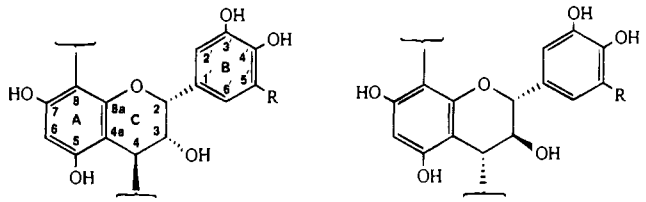
Figure 1. Solution-state ^{13}C NMR spectrum of proanthocyanidin polymer preparation from *P. banksiana* seed cones (S = solvent, acetone-d_6). Chemical shifts and assignments of resonances are given in the text.

Decay resistance imparted by proanthocyanidins was also determined according to a modified version of the ASTM (1992) method used to test the efficacy of wood preservatives. Sapwood (sweetgum, *Liquidambar styraciflua* L.) test blocks (3 mm × 6 mm × 6 mm) were oven dried, weighed, and then treated with a 3% solution (w/v) of either fraction 1 or 2 dissolved in ethanol/water (1:1). A solution of precipitate 1 (3% w/v), used to treat test blocks, was prepared with ethanol/water (3:1). Treated test blocks, along with the controls (solvent-treated only), were air-dried, steam sterilized (30 min), and then transferred to culture bottles containing *T. versicolor* (MAD697). After 5 weeks of incubation (27 °C, 70% relative humidity), adhering mycelium was removed from the test blocks, which were then dried and weighed to determine the extent of decay.

RESULTS AND DISCUSSION

To obtain proanthocyanidin polymers representative of those present in intact plant tissues, extreme care was taken to prevent exposure of the preparations to heat, light, and air. Extractions with acetone/water (7:3), an efficient solvent system for extracting proanthocyanidin polymers (Foo and Porter, 1980), yielded crude extracts which were subsequently fractionated. For the *P. ponderosa* cones, a sample of an initial acetone/water extract was analyzed by ^{13}C NMR spectroscopy (data not shown) and revealed a predominance of methylenic resonances (25–40 ppm) indicative of significant amounts of nonpolar extractives. After the removal of acetone in the extracts *in vacuo*, these nonpolar extractives (*e.g.*, resin acids, fatty acids) were removed as precipitates. Residual amounts of nonpolar extractives remaining in the resulting aqueous solutions were removed by extracting with diethyl ether; for the *P. ponderosa* cones, resin acids present in the diethyl ether fraction were identified by gas–liquid chromatography (data not shown). Once free of organic solvents, each aqueous phase was freeze-dried to give a crude proanthocyanidin preparation from which the proanthocyanidin polymers were isolated by Sephadex LH-20 chromatography according to established methods (Karchesy et al., 1989). The purified proanthocyanidin polymers obtained were then analyzed by ^{13}C NMR spectroscopy.

A representative ^{13}C NMR spectrum from the analyses of the proanthocyanidin polymer preparations is shown in Figure 1. Assignments for the resonances observed were made using those reported in the literature for model compounds and isolated polymer preparations (Czochanska et al., 1980; Karchesy and



2,3-*cis* 2,3-*trans*

Figure 2. Stereochemistries of 4 → 8 linked procyanidin (R = H) and prodelphinidin (R = OH) chain extender units in proanthocyanidins.

Hemingway, 1980; Newman et al., 1987; Porter et al., 1982). Accordingly, resonances between 153 and 156 ppm correspond to C-5, C-7, and C-8a of the aromatic A-ring of the flavan-3-ol units (see Figure 2). Unsubstituted carbons in this aromatic ring, C-8 and C-6, are assigned to the signal at 96.7 ppm. The typical interflavanoid linkage of C-4 to C-8 is represented by the resonance at 106.8 ppm assigned to substituted C-8. In each case, the ^{13}C NMR spectra revealed that the proanthocyanidins were predominantly of the procyanidin type; the signal at 144.5 ppm is therefore attributed to C-3′ and C-4′ in the B-ring of the procyanidin flavan-3-ol units. Other aromatic resonances are assigned as follows: 101.8 ppm, C-4a; 114.4−114.8 ppm, C-2′; 116.0 ppm, C-5′; 118.8 ppm, C-6′; 131.8 ppm, C-1′.

Generally, proanthocyanidins possess a 2*R* absolute configuration with exceptions in several families of monocotyledonous plants (Ellis et al., 1983). With respect to the aromatic B-ring attached at C-2, the orientation of the hydroxyl at C-3 can be either *cis* or *trans* as shown in Figure 2. Previous reports of the *cis*:*trans* ratios for proanthocyanidin polymers isolated from bark tissues of various pine and spruce species coincide with the general predominance of the 2,3-*cis* configuration (Porter, 1989a; Samejima and Yoshimoto, 1982). An exception is the procyanidin polymers from *Pinus radiata* middle bark, where the 2,3-*trans* configuration is more abundant (Czochanska et al., 1980). In comparison, the proanthocyanidin polymers of *P. radiata* phloem (Czochanska et al., 1980) and male seed cones (Porter, 1989a,b) are both comprised of similar amounts of procyanidin and prodelphinidin units and have the typically more common 2,3-*cis* configuration. These observations exemplify the structural variability of proanthocyanidins that can occur between tissues within the same species. For this reason, it was extremely important to determine the extent to which the structure of the proanthocyanidins in seed cones differed from those in bark.

The relative amounts of the two stereochemical configurations (2,3-*cis* or *trans*) in seed cone proanthocyanidin polymers were determined from the ^{13}C NMR spectra by integrating the resonances at 82.5 and 76.0 ppm corresponding to C-2 in 2,3-*trans* and 2,3-*cis* chain extender units, respectively. The *cis*:*trans* ratios calculated and shown in Table 1 indicate that for the seed cone proanthocyanidins analyzed, the more common 2,3-*cis* configuration predominates. Consistent with these data, the signal at 36.5 ppm corresponding to C-4 showed increasing amplitude of its downfield shoulder with the increasing proportion of 2,3-*trans* units. For the terminal units, a 2,3-*trans* (catechin) stereochemistry was typically shown by a small resonance at 80.6 ppm corresponding to C-2 and the upfield signal (66.9 ppm) corresponding to C-3. Accordingly, both the chain extender and terminal unit stereochemistries reflect those previously shown in conifer bark tissues.

Table 1. Conifer Seed Cone Proanthocyanidin Polymer Preparation 2,3-*cis*:2,3-*trans* Ratios and Number-Average Molecular Weights (MW_n)

species	2,3-*cis*:2,3-*trans*	MW_n
P. banksiana	65:35	2460
P. nigra	81:19	1520
P. ponderosa	74:26	1560
P. taeda	75:25	1840
P. glauca	66:34	1690

In addition to potential differences in structural units and stereochemical features, proanthocyanidins also show variability in their molecular weight distributions, as demonstrated by gel permeation chromatography of the respective peracetate derivatives (Williams et al., 1983). Measurements of number-average molecular weight (MW_n) of proanthocyanidins from different sources by ^{13}C NMR spectroscopy have also revealed a wide range of values (Foo and Porter, 1980; Porter, 1989a). We determined the MW_n of the seed cone proanthocyanidin polymers by integration of the signals at 71.9 and 66.9 ppm corresponding to C-3 in chain extender and terminal units, respectively. The range of molecular weights obtained (1520−2460) were typical of those obtained previously for pine and spruce bark proanthocyanidins (Czochanska et al., 1980; Porter, 1989a; Samejima and Yoshimoto, 1982). Thus, results obtained from the ^{13}C NMR spectroscopic analyses suggest that the proanthocyanidins in conifer seed cones are similar to those in conifer bark tissues.

As previously indicated, the majority of studies on the toxicity of proanthocyanidins have dealt with very crude extracts. Using the seed cone proanthocyanidin polymers isolated, and characterized, we set out to determine if the detection of antifungal activities for proanthocyanidin polymers would be accentuated upon their purification. Initial toxicity screening experiments were conducted using plates of agar medium containing various concentrations of procyanidin polymers from *P. nigra*. For comparative purposes, some media contained samples of the major crude fractions (precipitate 1 and fraction 1) from which the proanthocyanidin polymers were separated. After inoculation, the radial growth of the fungi on the agar plates was measured and compared with the controls to determine the relative amounts of inhibition. As shown in Table 2, significant inhibition (*ca.* 13%) of the cultures of *C. coerulescens* and *S. commune* by proanthocyanidin polymers occurred at the treatment concentration of 1000 mg/L. When lower levels of proanthocyanidins were used in the medium, lower levels of inhibition were observed for these two fungi. In contrast, *T. versicolor*, a hearty fungus commonly used in the ASTM soil block test method, was not inhibited at any treatment level.

For the fungi *C. coerulescens* and *S. commune*, minimal levels of inhibition were observed from fraction 1, which likely contained low molecular weight proanthocyanidins as well as other water-soluble extractives (*e.g.*, carbohydrates, phenolics). From these data it is apparent that the use of crude extracts could result in an underestimation of the actual antifungal activity of proanthocyanidin polymers due to dilution with relatively nontoxic chemical species.

In addition to the water-soluble extractives, the hydrophobic extractives comprising precipitate 1 were tested for antifungal activity. Incorporation of these materials into the agar medium gave a very finely

Table 2. Radial Growth Inhibition of Cultures of Wood-Damaging Fungi by *P. nigra* Seed Cone Crude Fractions and Proanthocyanidin Polymers

agar treatment	concn (mg/L)	fungal inhibition: *C. coerulescens*, % (SD)	fungal inhibition: *S. commune*, % (SD)	fungal inhibition: *T. versicolor*, % (SD)
precipitate 1	1000	66.7 (2.6)[a]	54.0 (1.6)	68.4 (0.0)
column fraction 1	1000	3.4 (3.9)	1.0 (2.8)	0.0
column fraction 2 (proanthocyanidin polymers)	50	0.0	3.8 (1.8)	0.0
	100	0.0	4.9 (2.8)	0.0
	200	9.0 (2.2)	7.6 (3.3)	0.0
	1000	13.5 (2.0)	12.8 (3.2)	0.0

[a] Each value represents an average of three or four replicates.

divided milky suspension. After inoculation and culturing, it was observed that the growth of the three fungi was less than half that of the controls. Such results suggest that the natural durability of the seed cones is dependent on nonpolar extractives. This has recently been demonstrated in experiments in which specific resin acid types (*e.g.*, abietane) exhibited significant levels of decay inhibition in wood (Eberhardt et al., 1994).

Although screenings on agar media can yield useful information about the potential toxicity of a particular compound, the artificial conditions provided by this method may give misleading results, especially with water-insoluble treatments that can reduce the homogeneity of the media when used at high concentrations. To verify the above experimental results, the seed cone extractives were also screened by the ASTM soil block method. Accordingly, test blocks of sweetgum sapwood were impregnated with solutions (3%, w/v) of either precipitate 1, fraction 1, or fraction 2 (proanthocyanidin polymers). Test blocks were also treated with solvents alone to provide controls. Upon comparison of the weights of the test blocks before and after exposure to cultures of *T. versicolor*, the decay levels were determined as percents of the individual dry weights of the test blocks prior to incubation.

Results from these experiments revealed lower levels of decay for each of the treatments as compared to that for the controls [44.4 ± 3.1% (mean ± SD, $n = 9$)]. Although providing the greatest levels of inhibition during screenings on agar medium, precipitate 1 was the least inhibitive (16.7%) with test block weight losses of 37.0 ± 2.6%. Therefore, the relative contribution of the nonpolar extractives toward inhibiting fungal decay may be significantly lower than that suggested by our initial screenings on agar. Such results exemplify that caution must be exercised when methods used to identify biological activities do not accurately simulate conditions found in nature.

Test specimens treated with fractions 1 and 2 (proanthocyanidin polymers) showed decay levels of 36.6 ± 3.5 and 35.0 ± 3.7%, respectively. The lower yet significant level of inhibition by fraction 1 (17.6%) warrants further investigation to identify the active species. Especially important is the significant level of decay inhibition (21.2%) provided by the proanthocyanidin polymer preparation (fraction 2) in the test block experiments. These results indicate that proanthocyanidin polymers do indeed play a significant role as inhibitors of decay in woody substrates such as conifer seed cones.

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