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Ferulic Acid Esters from Bark of *Pseudotsuga menziesii*[1]

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A mixture of ferulic acid esters was isolated from the *n*-hexane solubles (*n*-hexane wax) of the bark of *Pseudotsuga menziesii* (Mirb.) Franco. Saponification of the isolated esters yielded 1-docosanol, 1-tetracosanol, and ferulic acid. These esters may be related to suberin formation in bark cork cells and in cut potato tubers.

Pseudotsuga menziesii (Mirb.) Franco (Douglas-fir) is the most important commercial timber species on the west coast of North America. How to use the large amount of bark that accumulates after lumber and wood products have been manufactured from *P. menziesii* has always been a concern to the timber industry. For about 40 years there has been commercial interest in the wax from the bark (Hall, 1971). During our studies on the bark of this species, we have investigated *n*-hexane wax (*n*-hexane solubles) and benzene wax (benzene solubles extracted from the *n*-hexane insoluble residue). Previous papers (Laver et al., 1971; Loveland and Laver, 1972a,b; Laver and Fang, 1986) have been concerned with sitosterol, campesterol, fatty acids, wax alcohols, and chemically intact sterol and wax esters. In this paper we report the isolation of chemically intact ferulic acid esters from the *n*-hexane wax and discuss their possible significance in suberin formation in bark cork cells and in cut potato tubers.

EXPERIMENTAL SECTION

Melting points are uncorrected. Thin-layer chromatography (TLC) and preparative TLC (1.0 mm) were performed on silica gel G with the following solvent systems: (A) diethyl ether–*n*-hexane (1:4, v/v); (B) chloroform–carbon tetrachloride (6:1, v/v); (C) diethyl ether–*n*-hexane–methanol (10:40:1, v/v/v). Detection method: ultraviolet light (UV) followed by iodine vapors. Gas–liquid chromatography (GLC) utilized flame ionization detectors and the following column systems: (1) 5% SE-30 on Gas-Chrom Q packed in 1.50 m × 2.159 mm stainless steel, isothermal 250 °C, helium at 30 mL/min, injector heater 245 °C, detector heater 250 °C; (2) 3% OV-17 on Gas-Chrom Q packed in 1.829 m × 2.159 mm, stainless steel, isothermal 210 °C, helium at 30 mL/min, injector heater 240 °C, detector heater 255 °C. Preparative GLC utilized the conditions of column system 2, except the column was 1.829 m × 5.334 mm and the effluent was split 1/6 to detector and 5/6 to collection trap (liquid nitrogen). ^{1}H nuclear magnetic resonance (NMR) spectra were obtained at 100 MHz with tetramethylsilane (TMS) as internal standard. Electron impact mass spectra (MS) (probe) were obtained on a quadrupole instrument at 70 eV. Field desorption mass spectra (FD/MS) were obtained on a magnet instrument equipped with a carbon whisker emitter, +1.8 kV to the field anode (FD emitter) and −6.8 kV to the cathode, emitter heating current 0.0 mA. GLC/MS spectra were obtained on column system 1 and a quadrupole instrument at 70 eV.

Plant Material and Extraction. Bark 3.8–5.1 cm thick was collected from a freshly cut, dominant, 58-year-old Douglas-fir tree in the George T. Gerlinger Experimental Forest near Falls City, OR. The bark (1974.76 g, moisture content 11.2%, ground to pass a screen with holes 1.3 cm square) was Soxhlet extracted with *n*-hexane for 36 h. Solvent evaporation left a light yellow, waxlike solid (100.91 g).

Isolation of Ferulic Acid Esters. An aliquot (17.00 g) of the wax was separated on a silica gel G column with chloroform–*n*-hexane (3:1, v/v). Ten bands were observed under UV light. The second fastest moving band was collected. The solids (4.17 g) were recovered by solvent evaporation and purified by preparative TLC (solvent A, UV detection); mp 67–70 °C. TLC (solvent B) showed a single spot, R_f 0.19. GLC (column systems 1 and 2) showed no peaks either before or after silylation [hexamethyldisilazane and trimethylchlorosilane in pyridine (2:1:10, v/v/v)]. IR (ν_{max}, cm^{-1}; $CHCl_3$): 3500, 1730, 1315, 1269, 1175, 719.

Saponification of Ferulic Acid Esters. Saponification was accomplished by refluxing for 3.75 h in 10% ethanolic potassium hydroxide, extraction with *n*-hexane (unsaponifiables), acidification, and extraction with diethyl ether (saponifiables).

1-Docosanol and 1-Tetracosanol. TLC (solvent C) of the unsaponifiables showed one spot only (R_f 0.70) identical with 1-docosanol and 1-tetracosanol on cochromatography. The unsaponifiables were silylated, and GLC (column systems 1 and 2) showed spectra with two peaks only (retention times 14.0 and 26.5 min, and 6.5 and 12.5 min, respectively), identical with those of silylated authentic 1-docosanol and 1-tetracosanol. GLC/MS of the GLC peak with 14.0-min retention time showed peaks of m/z 383 $[M - CH_3]^+$, 247, 125, 111, 99, 98, 97, 96, 85, 84, 83, 71, 70, 69, 58, 56, 55, 43, 42, 41, and 29. GLC/MS of the GLC peak with 26.5-min retention time showed peaks of m/z 411 $[M - CH_3]^+$ plus the lower mass peaks as for

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the spectrum listed above. The GLC/MS spectra were identical with those from silylated authentic 1-docosanol and 1-tetracosanol.

The silylated compounds with retention times 6.5 and 12.5 min were isolated by preparative GLC. Desilylation with water, extraction with *n*-hexane, and solvent evaporation gave solid samples of 1-docosanol and 1-tetracosanol.

1-Docosanol: mp 71 °C [lit. mp 70.8 °C (Francis et al., 1937)]; IR (ν_{max}, cm^{-1}; KBr) 3340, 2900, 2840, 1450, 1040, 700, identical with spectrum of authentic 1-docosanol. Anal. Calcd for $C_{22}H_{46}O$: C, 80.9; H, 14.2. Found: C, 81.5; H, 14.4.

1-Tetracosanol: mp 75 °C [lit. mp 75.3 °C (Francis et al., 1937)]; IR (ν_{max}, cm^{-1}; KBr) 3340, 2900, 1450, 1260, 1040, 795, 700, identical with spectrum of authentic 1-tetracosanol. Anal. Calcd for $C_{24}H_{50}O$: C, 81.3; H, 14.2. Found: C, 80.4; H, 14.2.

Ferulic acid [3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid]: white solid on diethyl ether evaporation; recrystallization (benzene) yielded ferulic acid; mp 168.5–170 °C [lit. mp 169–170 °C (Kurth, 1950)]; IR(ν_{max}, cm^{-1}; KBr) 3430, 3020, 2900, 1695, 1665, 1620, 1600, 1590, 1510, 1465, 1200, 1174, 1030, 968, 848, 800, identical with the spectrum of authentic ferulic acid; ^1H NMR (acetone-d_6, δ) 3.90 (3 H, s, OCH_3), 6.38 (1 H, d, J = 16 Hz, H-2), 6.86 (1 H, d, J = 8 Hz, aromatic H adjacent to phenolic OH), 7.14 (1 H, d, J = 8 Hz, each doublet further split into two peaks, J = 2 Hz, aromatic H para to OCH_3), 7.32 (1 H, d, J = 2 Hz, aromatic H adjacent to OCH_3), 7.60 (7 H, d, J = 16 Hz, H-3), carboxylic proton off scale, phenolic proton exchanged with deuterium of the solvent; FD/MS, m/z 194 $[M]^+$ no fragment peaks, identical with spectrum of authentic ferulic acid.

RESULTS AND DISCUSSION

The wax yield was 5.75%, similar to the 5.47% reported by Kurth and Kiefer (1950). Manners (1965) reported wax yields of 4.75% for bark from old-growth Douglas-fir and of 4.80% for bark from second-growth Douglas-fir. A yield value near 5% therefore seems consistent.

Silica gel column separation of the wax components resolved 10 distinct bands. The fastest moving band has previously been shown to contain sterol and wax esters (Laver and Fang, 1986). The second fastest moving band yielded the ferulic acid esters described here. It represents approximately 25% of the extract or 1.4% of the bark (dry-weight basis).

TLC and preparative TLC of the collected band yielded one TLC zone. GLC of the material showed no peaks either before or after silylation, indicating no alcohols. IR strongly supported the presence of ester groups. GLC and GLC/MS of the silylated unsaponifiables showed the presence of 1-docosanol and 1-tetracosanol. Silylation, preparative GLC, and desilylation yielded pure, solid 1-docosanol and 1-tetracosanol as shown by melting points, IR, and combustion elemental analyses. The ratio of 1-docosanol to 1-tetracosanol was 1:1.3, as measured by GLC peak areas. The saponifiable portion yielded only ferulic acid as shown by mp, IR, NMR, and FD/MS. Thus, it has been established that the *n*-hexane wax from the bark of *P. menziesii* contained 1-docosanol ferulate and 1-tetracosanol ferulate.

Wax alcohol esters have been reported in tree barks. Kurth (1950) and Hergert and Kurth (1952) reported finding both wax alcohols and ferulic acid after saponification of the hexane extract of Douglas-fir bark. Because ferulic acid is not soluble in hexane, it was expected that it was originally present as wax alcohol esters. However, no specific ester linkages were described. Hergert (1958) showed that ferulic acid was a cell wall constituent of cork and that it must be bound as a polyestolide. Afterward, several articles on these compounds in tree barks were reviewed by Rowe et al. (1969).

Adamovics et al. (1977) reported the isolation of a mixture of ferulic acid esters from a chloroform extract of a cork fraction from the bark of *P. menziesii*. These authors were prompted to study the phenolic compounds of *P. menziesii* bark because of the work by Mizicko et al. (1974), which demonstrated that cork-rich fractions of the bark enhanced the natural healing process of suberization and wound-periderm initiation in cut seed potatoes. Wound-healing of cut seed potatoes is important commercially, and we know of one company (Bohemia Inc., Eugene, OR) that sells ground *P. menziesii* bark to potato growers for the specific purpose of treating freshly cut seed potatoes.

In addition to this work, previous research had indicated that ferulates are associated with suberin formation (Kolattukudy and Dean, 1974; Riley and Kolattukudy, 1975). Riley and Kolattukudy (1975) showed that ferulic acid is tightly (possibly covalently) bound to the suberins of vegetable root crops, including potato tubers. The isolation of ferulate esters reported here indicates that these esters may also be associated with suberin formation in tree barks. Litvay and Krahmer (1976, 1977) showed that samples of outer bark of *P. menziesii* contained as much as 35% cork. Cell walls in the cork had large amounts of suberin and a wax-extractive zone. The suberin zone contained layers of wax. There is a close association between the waxes and the suberins of cork cells in *P. menziesii* bark.

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A Comparison of Methods for the Intrinsic Labeling of Wheat Protein with ^{35}S

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A hard red winter wheat variety (Brule), grown to maturity in a greenhouse, was intrinsically labeled with ^{35}S by either stem injection or addition of the isotope to the medium in which detached wheat heads were grown. Two levels of isotope were applied by each method at 5, 10, 15, or 20 days postanthesis. Significantly higher ($P < 0.05$) yield (weight/head) of grain was observed with injection compared to the incubation method. Of the isotope introduced by injection, 77% translocated into the wheat kernel, and of this amount at least 77% of the isotope in the kernel was associated with kernel protein and free sulfur amino acids. Data suggest that injection of wheat stems 15 days after anthesis, with doses up to 10 μCi of ^{35}S, would be a suitable method of obtaining intrinsically labeled wheat protein for bioavailability studies.

The use of intrinsically labeled nutrients offers investigators an effective means of conducting bioavailability studies. Nutrients intrinsically labeled with radioisotopes may be incorporated into diets fed to small animals and monitored throughout the organism.

Selected nutrients in wheat have been intrinsically labeled with radioisotopes with varying efficiency, depending in part on the maturity of the plant when the isotope is administered (McConnell and Ramachandran, 1956; Bilinski and McConnell, 1958; Lee and Wan, 1963; Lee and Reynolds, 1963). Effect of element concentration on uptake may also be a consideration. Starks and Johnson (1985) reported that the total zinc content of whole wheat flour was higher when stems were injected with $^{65}Zn + ZnSO_4$ than with ^{65}Zn alone, showing greater zinc uptake with increased injection load.

Mode of application may also influence results (Weaver, 1985). Two of the most commonly used procedures are injection of isotope solution into the hollow stem of the plant or incubation of detached wheat heads in liquid medium containing added isotope. Using the stem injection method, Lee and Reynolds (1963) obtained a maximum of 39% incorporation of ^{35}S in whole wheat flour.

Studies in which the isotope has been administered by incubation of detached heads have generally utilized short incubation periods, e.g., 12 h (Donovan et al., 1977) or 14 h (Graham and Morton, 1963). These studies were useful in defining the development of the plant at different stages of growth. However, for use in animal studies, a sufficient quantity of fully mature labeled grain similar in composition to grain used for human consumption is necessary. Donovan and Lee (1977) incubated wheat heads (detached at 8 or 20 days after flowering) in nutrient medium for 12 days, resulting in grain development similar to that of field-grown material.

The objective of this study was to optimize incorporation of ^{35}S into wheat kernel protein. Variables examined were concentration of dose, time after anthesis, method of isotope introduction (incubation vs injection), and extension of incubation period until wheat heads were mature.

MATERIALS AND METHODS

Hard red winter wheat (Brule) was grown in the greenhouse after it had been vernalized at 4 °C for 8 weeks. Potting mix contained 75% peat moss, 20% perlite, and 5% vermiculite (v/v/v). Greenhouse conditions: temperature, 16–27 °C; relative humidity, 50–65%; natural daylight conditions, August to January, 155 days. The first wheat head emerged after 48 days in the greenhouse, and anthesis was noted after an additional 19 days.

At 5, 10, 15, or 20 days after anthesis, carrier-free $^{35}SO_4$ ($Na_2{}^{35}SO_4$; New England Nuclear, Boston, MA) was introduced into the wheat heads by injection or incubation with 50 μL of one of two solutions (0.1 or 0.2 μCi/mL in sterilized incubation medium described below).

Stem Injection. An area 5 cm above the last node was cleaned with ethanol. A hole was pierced with an injection needle, and 50 μL isotope solution was slowly introduced with a Hamilton syringe. The hole was then covered with nitrocellulose glue to seal and to prevent infection.

Incubation. Wheat heads were cut above the last node. A slanted cut was then made 11–12 cm from the base of the head while the stem was kept in a 0.5% solution of sodium hypochlorite to prevent infection. Each excised wheat head was inserted into a 50-mL glass vial (25 × 100 mm) through a hole drilled in the cap lined with a 0.2-μm

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