Carbon-14 Biolabeling of (+)-Catechin and Proanthocyanidin Oligomers in Willow Tree Cuttings

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Proanthocyanidin polymers, oligomers, and the structurally related monomer (+)-catechin were labeled by incorporation of radioactive precursors in shoots of willow tree (*Salix caprea* L.). [1-^{14}C]-Acetate and [U-^{14}C]-phenylalanine precursors were fed through the cut stems or petioles of leaves. Optimization of several parameters such as the nature and origin of the plant material, leaf maturity, nature, and quantity of radioactive precursor applied and the duration of metabolism led to incorporation yields of 3.2% and to specific activities of 500 μCi/g. Detailed characterization of the products (polymerization degree, procyanidin/prodelphinidin ratio, specific activities) and purification by chromatography are reported. Some sugars bound to radiolabeled proanthocyanidin polymers were removed by enzymic treatment with a mixture of glycosidases. A radioactive purity close to 100% and specific activities suitable for bioavailability studies were obtained.

Keywords: *Proanthocyanidins; Salix caprea; willow tree; biosynthesis; radiolabeling; bioavailability*

INTRODUCTION

Polyphenols are widespread in most fruits and vegetables regularly consumed, either raw or transformed (Swain, 1962; Shahidi and Naczk, 1995). It is generally assumed that we ingest 1 g of polyphenols per day with our diet (Kühnau, 1976). Their antioxidant properties have raised considerable interest over the past years. They may contribute to explain the general protective effects of fruit and vegetable consumption against cancers (Steinmetz and Potter, 1991) and of a moderate consumption of wine against cardiovascular diseases (Hertog, 1997).

The diversity of polyphenol structures is enormous. They are classified into several classes, the most important in our food being phenolic acids and flavonoids (Shahidi and Naczk, 1995). Proanthocyanidins (PAs, *syn*. condensed tannins) differ from other flavonoids by their polymeric nature and are probably the most abundant polyphenols in our diet. They might well represent over 50% of the 1 g of polyphenols consumed every day (Kühnau, 1976). PAs are responsible for the astringency and to some extent the bitterness of many fruits (grape, apple, persimmon, etc.) and derived beverages (wine, cider). For example a glass of red wine (125 mL) contains 30−60 mg of PA dimers and trimers (Ricardo da Silva et al., 1991, 1992) and probably as much or more polymers.

PAs are polymers of flavan-3-ol units linked by a carbon−carbon bond between the benzylic C-4 carbon of the heterocyclic ring and principally the C-8 carbon of the flavanol A ring of another unit but also the C-6 carbon. Their molecular weight most often varies between 600 (dimers) and 3000. They also differ by their hydroxylation pattern and the stereochemistry of C-3. The main PAs found in food are procyanidins, polymers of catechin units (**5**, R = H), and to a lesser extent prodelphinidins, polymers of gallocatechin units (**5**, R = OH) (Shahidi and Naczk, 1995). Two types of units can be distinguished according to their stereochemistry: (gallo)catechin with a 2,3-trans configuration and (gallo)epicatechin with a 2,3-cis configuration.

This high structural complexity makes PAs difficult to analyze and estimate. Furthermore, pure PA molecules needed for biological studies are not commercially available. For these reasons and in contrast to simpler molecules such as quercetin, catechin or genistein, their nutritional effects and their bioavailability have been poorly studied and remain largely unknown.

Isotopic labeling has been an invaluable tool in pharmakinetic studies of drugs or natural products to trace the parent compounds or their metabolites. Labeled polyphenols have been prepared either through synthetic routes (full synthesis or aromatic proton exchange) or by incorporation of a commercial radiolabeled precursor in plant tissues known to accumulate the molecule of interest (Déprez and Scalbert, 1999). Few studies on the bioavailability of PAs have been reported, and still fewer are carried out with labeled substrates. PAs have been radiolabeled by incorporation of $^{14}CO_2$, ^{14}C-acetate, or ^{14}C-phenylalanine in *Vitis vinifera* (Laparra et al., 1977; Harmand and Blanquet, 1978), *Sorghum bicolor* (Reddy and Butler, 1989; Jimenez-Ramsey et al., 1994), and *Lotus pedunculatus* (Terrill et al., 1994). However, only crude mixtures of oligomers or polymers have been prepared, and bioavailability studies did not allow one to determine precisely the effect of the polymerization degree on their absorption (Laparra et al., 1977; Harmand and Blan-

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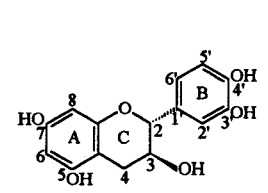
quet, 1978; Jimenez-Ramsey et al., 1994; Terrill et al., 1994).

We report here the preparation of pure radiolabeled PA oligomers and polymers by incorporation of ^{14}C-acetate or ^{14}C-phenylalanine in willow tree cuttings. Male catkins of willow tree were reported to contain high quantities of dimer B3 **2**, trimer C2 **4**, PA polymers **5** and the related (+)-catechin monomer **1** (Thompson et al., 1972). After checking that leaves contained



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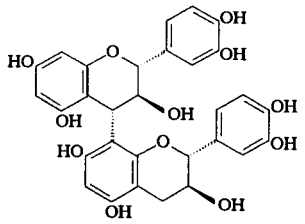

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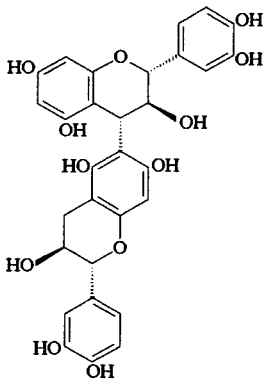

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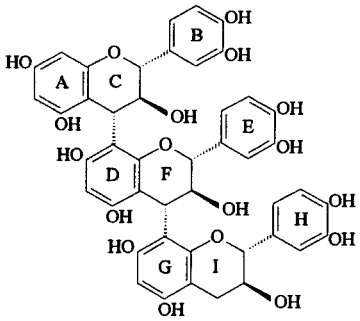

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similar amounts of the same compounds, willow cuttings were selected to prepare the radiolabeled molecules otherwise difficult to obtain by synthetic routes. Various parameters, such as selection of plant material, nature of precursor, and its mode of administration, were optimized and methods of purification of the radiolabeled products developed.

MATERIALS AND METHODS

General. (+)-Catechin was purchased from Fluka, D-glucose, D-arabinose, D-xylose, D-sucrose from Prolabo, D-galactose from Serlabo, D-fucose and chlorogenic acid from Sigma, and D-fructose from Merck. (+)-Gallocatechin was kindly provided by Dr. Alan P. Davies (Unilever, Bedford, U.K.). Radioactive precursors were purchased from Isotopchim for labeling experiments ([1-^{14}C]-acetic acid, sodium salt, 59 mCi/mmol; L-[U-Ring-^{14}C]-phenylalanine, 450 mCi/mmol) and from ICN for toxicity experiments ([1-^{14}C]-acetic acid, sodium salt, 10 mCi/mmol dry). Other radiochemicals were purchased from Sigma ([UL-Ring-^{14}C]-atrazine, 5−25 mCi/mmol dry) and Amersham ([1,4-^{14}C]-putrescine dihydrochloride, 114 mCi/mmol in 2% EtOH). β-Glucosidase from almonds, cellulase Onozuka, and AR2000 pectinase from *Aspergillus niger* were obtained from Fluka, Yakult Pharmaceutical, and Littorale Oenologie (Gist-Brocades), respectively. ^{1}H NMR spectra were recorded by Kate Hervé du Penhoat (CERMAV, Grenoble) at 400 MHz on a Bruker DRX 400 spectrometer at 300 K; chemical shifts (δ) were referenced to TMS. ^{13}C NMR spectra were recorded at 100 MHz on a Bruker AC300 instrument at 303 K; chemical shifts (δ) were referenced to solvent signal.

Analysis of Flavanols and Thiolysis Products of Proanthocyanidins by HPLC. Thiolysis was carried out as described before and used to estimate polymers in leaf extracts (Matthews et al., 1997; Déprez et al., 1999). Analytical RP-HPLC was carried out on a Lichrospher 100 RP-18 column (5 μm, 250 × 4 mm i.d., Merck) with the following elution conditions: solvent A, 0.1% H_3PO_4 in water; solvent B, MeOH; linear gradient, 15−30% B in 30 min for flavanols and 30−90% B in 30 min for thiolysis products; flow rate, 1 mL/min. Detection was carried out at 280 nm or with an ESI-MS Platform LCZ spectrometer (Micromass) operating in negative mode. For ESI-MS detection, 1/5 of the eluent was admitted in the source, masses were scanned from *m*/*z* 150 to 1000, and a cone voltage of −30 V was applied. Retention times of benzylthioethers were 3,4-*cis*-benzylthiocatechin (**6**), 18.7 min; 3,4-*trans*-benzylthiocatechin (**7**), 17.5 min; 3,4-*trans*-benzylthioepicatechin (**8**), 20.8 min; 3,4-*cis*-benzylthiogallocatechin (**9**), 14.6 min; 3,4-*trans*-benzylthiogallocatechin (**10**), 13.4 min; 3,4-*trans*-benzylthioepigallocatechin (**11**), 16.5 min (Déprez et al., 1999).

Analysis of Free and Bound Sugars. Free sugars in leaves (0.1 g leaf dry wt.) extracted with acetone/water 7:3 (20 mL) were redissolved in water (2 mL). A fucose internal standard was added and the solution (0.1 mL) applied to a Sep-Pak C18 cartridge (Waters) preliminary washed with MeOH and conditioned in water. Bound sugars were hydrolyzed from PA polymers (1 mg) either by acid hydrolysis or enzymatic treatment. Acid hydrolysis was performed by treatment at 100 °C for 3 h in EtOH/2 N aq. HCl 1:1 (0.6 mL). After cooling, acid was neutralized with $NaHCO_3$ and filtered (Harborne, 1965). After evaporation of EtOH, the aqueous phase was applied to a Sep-Pak C18 cartridge as described above. Enzymatic treatment was carried out at 37 °C with β-glucosidase, cellulase Onozuka or AR2000 pectinase in water (1 mL) with stirring and for 18 h (reaction time at which yields reached a plateau). Water was then partially evaporated under reduced pressure, and acetone (0.6 mL) was added to precipitate enzymes. After centrifugation and evaporation of acetone, compounds were redissolved in water and applied to a Sep-Pak C18 cartridge as described above. Sugars were analyzed by HPLC on a DIONEX 4500I series liquid chromatograph equipped with a pulsed amperometric detector (PAD-2). A CarboPac PA1 column (250 × 4 mm i.d.) was used with the following elution conditions: solvent A, water; solvent B, NaOH 50 mM; sequential isocratic elution of 92% A (27 min) and 40% A (8 min); flow rate, 1 mL/min. PAD was employed with a gold electrode and triple-pulse amperometry. Addition of NaOH (300 mM, 1.7 mL/min) to the column effluent increased the PAD sensitivity and minimized baseline drift. Peak identification and quantification were based on retention times, and response factors were determined with reference compounds.

Thin-Layer Chromatography. Bidimensional chromatography was carried out with high-performance cellulose plates on aluminum foils (10 × 10 cm, 0.1 mm, Merck). Eluents for PAs were *t*-BuOH/AcOH/water 3:1:1 (by vol., eluant A), AcOH/water 6:94 (eluant B) and for sugars (Markham, 1982) EtOAc/pyridine/AcOH/water 36:36:7:21 (eluant C) and *n*-BuOH/toluene/pyridine/water 5:1:3:3 (eluant D). Plates were sprayed with a mixture of vanillin (2% m/v) and toluene-*p*-sulfonic acid (1% m/v) reagent in EtOH and heated at 60 °C (PAs) or a mixture of equal volumes of aqueous $K_3Fe(CN)_6$ (2% m/v) and $FeCl_3$ (2% m/v) (all phenolic compounds). Sugars were detected by spraying plates with aniline hydrogen phthalate (aniline, 0.2 mL, phthalic acid, 0.4 g in 25 mL *n*-BuOH/Et_2O/H_2O 24:24:1) and heating at 100 °C (brown spots) (Partridge, 1949). Phenylalanine was revealed by spraying ninhydrine (0.2 g in 100 mL EtOH) and appeared as a purple spot after heating at 100 °C.

Purification of Unlabeled Proanthocyanidin Oligomers and Polymers. Fresh male catkins (230 g fresh wt.) were collected on *Salix caprea* L. trees in February and extracted by acetone/water 7:3 (2 × 100 mL) with a Waring blender. The solution was filtered, and acetone was removed under reduced pressure from the solution. The aqueous phase

was successively extracted with Et_2O (3 × 100 mL) and EtOAc (5 × 40 mL). The EtOAc extract was dried (4 g), redissolved in EtOH/MeOH 2:1 (6 mL), and chromatographed on a Sephadex LH-20 column (55 × 3 cm i.d.; 100 μm; Pharmacia) in absolute EtOH. Four fractions were successively eluted and contained (+)-catechin **1** (470 mL, 444 mg), B3 dimer **2** (540 mL, 250 mg), B6 dimer **3** (670 mL, 55 mg), and C2 trimer **4** (950 mL, 187 mg). The fraction containing C2 trimer was further purified by preparative RP-HPLC (Scalbert et al., 1990) with H_3PO_4/H_2O/MeOH 1:859:140 as eluant. MeOH was removed from the fractions containing C2 trimer which were applied on a Sephadex LH-20 column (140 × 14 mm i.d.) in water to eliminate H_3PO_4. The adsorbed trimer was eluted with MeOH and freeze-dried (42 mg of pure compound).

Procyanidin and mixed procyanidin/prodelphinidin polymers were purified from leaves of adult trees grown outdoors and young trees grown in the greenhouse, respectively. Leaves (1 g dry wt.) were similarly extracted as catkins. PAs in the residual aqueous phase were purified on the same Sephadex LH-20 column as above. The following eluants were successively applied: water (100 mL), MeOH/water 1:1 (50 mL), and acetone/water 7:3 (150 mL). PAs in the acetone/water eluant were dried by removing the solvants under reduced pressure and freeze-drying.

B3 Dimer 2. The purity was assessed by chromatography and NMR spectroscopy. B3 dimer (15 mg) was dissolved in anhydrous pyridine (500 μL) and dry acetic anhydride (500 μL) and the solution stirred at room temperature for 15 h in the dark. Some toluene was added, and the solvents were removed under reduced pressure. Residual pyridine was eliminated by dissolving the acetylated dimer in CH_2Cl_2 (5 mL) and washing with 10% HCl. The organic phase was neutralized by addition of $NaHCO_3$, filtered, and freeze-dried. Acetylated dimer was recovered as a white powder (80% yield on a molar basis). B3 dimer peracetate: ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.18 (d, J = 8.4 Hz, H-5′B or E), 7.17 (d, J = 8.3 Hz, H-5′E or B), 7.06 (d, J = 1.4 Hz, H-2′B), 7.02 (dd, J = 8.4 Hz, J = 1.4 Hz, H-6′B), 6.97 (d, J = 1.4 Hz, H-2′E), 6.76 (dd, J = 8.4 Hz, J = 1.4 Hz, H-6′E), 6.68 (s, H-6D), 6.54 (d, J = 2.1 Hz, H-8A), 6.52 (d, J = 2.1 Hz, H-6A), 5.66 (t, J = 9.7 Hz, H-3C), 5.05 (m, H-3F), 4.99 (d, J = 8.0 Hz, H-2F), 4.80 (d, J = 10.0 Hz, H-2C), 4.52 (d, J = 9.3 Hz, H-4C), 2.97 (dd, J = 5.6 Hz, J = 16.7 Hz, H-4αF), 2.69 (dd, J = 7.8 Hz, J = 16.8 Hz, H-4βF), 1.9−2.4 (m, 30 H, 10CH_3CO).

C2 Trimer 4. It was peracetylated as above. ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.31 (d, J = 8.4 Hz, H-5′), 7.10 (m, H-6′), 7.13 (d, J = 9.1 Hz, H-5′), 6.90 (d, J = 2.0 Hz, H-2′), 6.84 (d, J = 2.0 Hz, H-2′), 6.70 (s, H-6G), 6.66 (s, H-6D), 6.62 (dd, J = 2.1 Hz, J = 8.5 Hz, H-6′), 6.58 (d, J = 2.4 Hz, H-8A), 6.57 (dd, J = 1.9 Hz, J = 8.7 Hz, H-6′), 6.26 (d, J = 2.3 Hz, H-6A), 5.61 (dd, J = 9.0 Hz, J = 10.1 Hz, H-3C), 5.53 (m, H-3F), 5.29 (sl, H-2I), 5.27 (m, H-3I), 4.79 (d, J = 10.2 Hz, H-2F), 4.62 (d, J = 8.3 Hz, H-4F), 4.67 (d, J = 10.1 Hz, H-2C), 4.18 (d, J = 9.0 Hz, H-4C), 2.60 (m, H-4βI), 2.10 (m, H4αI masked by acetate signals), 1.6−2.4 (m, 45 H, 15CH_3CO). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 171.4−167.0 (CO of CH_3CO), 156.3 (C8aA), 154.8 (C8aD), 151(C8aD),0.151.1 (C8aG), 150−147 (C7, C5, A, D and G), 149.2 (C5A), 142.5−141.1 (C4′, C3′, B, E, and H), 136.6 (C1′H), 135.1 (C1′E), 125.6 (C6′B or E), 124.7−122.3 (C2′, C5′, C6′, B, E and H), 119.5 (C2′B, E, and H), 118.8 (C8D), 117.2 (C4aD), 116.7 (C4aA), 116.6 (C8G), 110.4 (C6G), 109.8 (C4aG), 108.4 (C6D), 108.3 (C6A), 79.7 (C2F), 78.6 (C2C), 76.4 (C2I), 72.1 (C3F), 70.9 (C3C), 66.7 (C3I), 36.7 (C4C), 36.7 (C4F), 29.7 (C4I), 21.2−20.2 (CH_3 of CH_3CO).

B6 Dimer 3. It was characterized as a procyanidin dimer by HPLC-ESI-MS (m/z 578) and identified by comparison of its chromatographic characteristics with those described in the literature. Elution order on Sephadex LH-20 column (Thompson et al., 1972), retention time by RP-HPLC (Treutter et al., 1994), and R_f values on bidimensional cellulose HPTLC (Thompson et al., 1972) were all consistent.

Proanthocyanidin Polymers. Procyanidins (**5**, R = H) isolated from leaves of an adult willow tree: ^{13}C NMR (100 MHz, acetone-d_6/D_2O 1:1) δ 157.8−154.4 (C-5, C-7, C-8a), 146.7−144.8 (C-3′, C-4′), 131.3 (C-1′), 120.0 (C-6′), 115.8 (C-2′, C-5′), 106.2 (C-8), 103.9 (C-1″, α or β anomer, sugar), 101.5 (C-4a), 100.3 (C-1″, α or β anomer, sugar), 95.9 (C-6), 82.7 (C-2, 2,3-trans linkage), 76.3−75.6 (C-2, 2,3-cis linkage; C-3″, C-5″, sugar), 73.6−72.8 (C-3; C-2″, sugar), 69.8−69.4 (C-4″, sugar), 65.6 (C-6″, sugar), 37.8 (C-4). Mixed procyanidin/prodelphinidin polymers (**5**, R = H or OH) isolated from leaves of cuttings grown in the greenhouse: ^{13}C NMR (100 MHz, acetone-d_6/D_2O 1:1) δ 156.5−152.3 (C-5, C-7, C-8a), 144.5−143.0 (C-3′, C-4′, C-5′), 129.8 (C-1′), 114.0 (C-2′, C-6′), 105.2, 104.9 (C-8), 103.1 (C-1″, sugar), 99.0 (C-4a), 95.4 (C-6), 81.5 (C-2, 2,3-trans linkage), 76.2−74.0 (C-2, 2,3-cis linkage; C-3″, C-5″, sugar), 72.9−71.0 (C-3; C-2″, sugar), 69.0 (C-4″, sugar), 63.3 (C-6″, sugar), 36.1 (C-4).

Administration of ^{14}C-Labeled Precursors to Willow Shoots. The incorporation chamber (120 (height) × 180 (length) × 40 (width) cm) was equipped with an air extractor. The lighting produced by 18 neon tubes (Mazdafluor, TF 75, 58 W) reached 0.5 mmol photons·m^{-2}·s^{-1} PAR. Light periods lasted 16 h. The air temperature was most often around 25 °C but varied occasionally between 18 and 33 °C. Plates containing 1 M NaOH were placed in the chamber to eventually trap respired $^{14}CO_2$.

Leafy shoots were harvested on an adult willow tree (*S. caprea* L.) at the Arboretum of the Museum National d'Histoire Naturelle of Chèvreloup (Yvelines; shoots harvested from April to September) or on young plants purchased from a nursery and planted in November (the year before the incorporation experiments) in a greenhouse (1 m height, 3 cm stem circumference, shoots harvested from February to October). Four sodium lamps (16 h light period) provided additional illumination. Overall illumination varied between 0.5 and 2 mmol photons·m^{-2}·s^{-1} PAR (400−700 nm).

The stem of the leafy shoot (15−20 cm long; 10−20 leaves) or the leaf petiole was recut under water before administration of the precursor. The cut end was immersed in water containing sodium [1-^{14}C]-acetate (0.4 mCi in 1 mL, pH made to 7 with 1 N HCl). When the solution was almost totally absorbed, more water was added in small aliquots (1 mL) until the labeled precursor was fully absorbed at an average rate of 1 mL/h. The cutting was then fed with larger volumes of water. Shoots were either kept in the growth chamber until full desiccation of leaves (survival before wilting varied between 1 and 6 days) or dried at room temperature for time-course labeling experiments. The same procedure was applied for incorporation of L-[U-Ring]-phenylalanine.

Isolation and Purification of Labeled Flavanols. Dry leaves (1 g, ∼20 leaves) were ground to a fine powder with a porcelain pestle and liquid nitrogen in a porcelain mortar. The powder was extracted with acetone/water 7:3 (3 × 20 mL), and plant debris was filtered off on Whatman No. 4 paper. Extracts were stored at −20 °C until further purification. A similar experiment carried out on similar nonlabeled leaf extracts showed that freezing (1 month) and thawing do not affect PA solubility, as seen by the stable absorbance at 280 nm. Extracts from about 3 g of dry leaves were combined, and water was added to obtain a final 5:95 acetone/water ratio. This solution was applied first to a Sephadex LH-20 column (300 × 20 mm i.d.) to eliminate the remaining [1-^{14}C]-acetate precursor which would otherwise be lost in the rotary evaporator during subsequent sample concentrations. [1-^{14}C]-Acetate was eluted with water (150 mL) together with other polar contaminants (free carbohydrates, phenolic acids). PAs were then eluted with acetone/water 7:3 (150 mL), and acetone was removed from the solution under reduced pressure. This first Sephadex chromatography step was omitted when [U-^{14}C]-phenylalanine was used as a precursor.

The aqueous phase (40 mL) was successively extracted with *n*-hexane (3 × 30 mL) and EtOAc (3 × 30 mL). The EtOAc phase contained (+)-catechin **1**, PA dimers **2**, **3**, and trimer **4**, and the aqueous-phase PA polymers **5**. Several EtOAc extracts corresponding to 11 g of initial dry leaves were pooled, washed with *n*-hexane to remove chlorophylls, and applied to a Sephadex LH-20 column (250 × 20 mm i.d.) in absolute EtOH. Each fraction collected manually (5 mL) was analyzed by

HPTLC using eluant A and sprayed with the vanillin/toluene-*p*-sulfonic acid reagent. Similar fractions were pooled. After evaporation of EtOH and redissolution in water/MeOH 1:1, fractions were analyzed by HPLC and 2D-HPTLC. The still contaminated (+)-catechin and dimer B3 fractions were further purified by cellulose HPTLC (12 × 8 cm, 0.1 mm thickness, Merck); 0.5 μCi in about 40 μL were spotted on a 10 cm width and eluted with eluant B. Labeled flavanols were localized by autoradiography, the cellulose scraped, and the product finally recovered by stirring in acetone/water 7:3. Labeled flavanols were finally dried on phosphoric anhydride under vacuum. This procedure yielded 9.4 mg of (+)-catechin **1** (368 μCi/g), 3.2 mg of B3 dimer **2** (150 μCi/g), and 3.2 mg of C2 trimer **4** (144 μCi/g).

PA polymers **5** in the residual aqueous phase were treated with AR2000, a pectinase preparation used in oenology to free aroma precursors in wine and containing rhamnosidase, arabinosidase, apiosidase, galactosidase, xylosidase, and β-glucosidase activities (Bayonove et al., 1991), to eliminate covalently bound sugars before purification on Sephadex. The enzyme preparation (5 mg) was added to the aqueous phase (30 mL), and the mixture was kept at 37 °C for 18 h under stirring. Water was then evaporated under reduced pressure, and acetone (1 mL) was added to precipitate enzymes. After centrifugation, the acetone-soluble PAs were dried and redissolved in water (5 mL). This solution was centrifuged again to eliminate residual insoluble materials and applied to a Sephadex LH-20 column (250 × 20 mm i.d.) first eluted with water (100 mL) to remove released carbohydrates. Phenolic acids (such as chlorogenic acid) were eluted with MeOH/water 1:1 (50 mL) (Lu and Foo, 1997) and PAs with acetone/H_2O 7:3 (150 mL). The PA solution was concentrated and washed again with *n*-hexane and EtOAc (when residual PA monomers were still detected). Labeled PA polymers (90 mg, 177 μCi/g) were finally dried on phosphoric anhydride under vacuum.

Measurement of Radioactivity. The radioactivity of PAs and their thiolysis products was determined with a Berthold liquid scintillation spectrometer (LB 506 C) on-line with RP-HPLC. The liquid scintillation cocktail, Optiflow Safe 1 (EG&G Berthold) was delivered at the same flow rate as the HPLC eluent (1 mL/min). The counting efficiency in the Z cell (vol. 2 mL) was on average 50%. Counts were corrected for background (estimated at 20 cpm). The detection threshold was 50 dpm. Specific activites of PAs were determined by reference to the UV absorbance (280 nm) also measured on-line and compared to the molar extinction of purified nonlabeled reference compounds. The specific activity of the labeled precursors was controlled with a Packard Tri-Carb 1500 liquid scintillation analyzer (liquid scintillation cocktail: Ecolite, ICN); the counting efficiency was 95%.

Electronic autoradiographies of thin-layer chromatograms were obtained with an Instant Imager (Packard) and a Storm 860 (Molecular Dynamics) electronic system. Dried cellulose plates were imaged for 3−24 h to obtain a maximal error coefficient of 2%. To test the sensitivity and linearity for carbon-14 of the Instant Imager, 5 μL aliquots of a series of [1,4-^{14}C]-putrescine dilutions were applied to a cellulose TLC plate and imaged for about 2 h. The counting efficiency was 1.2% and detection threshold 0.015 cpm/mm^2. Calibration of the Storm system was systematically performed with [U-^{14}C]-phenylalanine.

The radioactivity in solid tissues was determined by combustion. Dried tissues stored in a desiccator containing silica gel were placed in combusto-cones containing cellulose and combusted for 4 s in an oxidizer (Packard, model 307). The $^{14}CO_2$ produced was trapped in Carbosorb (Packard Instrument Co.), and Permafluor (Packard) was added. The combustion and scintillation counting efficiency was estimated at 85−90% with solutions of ^{14}C-atrazine.

RESULTS

Proanthocyanidins in Willow Tree Leaves. A crude acetone/water extract of leaves harvested on an adult willow tree was analyzed by HPLC-ESI-MS. HPLC traces on selected ions clearly show the presence of (+)-catechin **1** (Figure 1B), two main dimers (Figure 1C), and three trimers (Figure 1D). The main dimer and trimer eluted first on HPLC chromatograms and not separated from each other were purified by chromatography on Sephadex LH 20 and RP-HPLC and identified as dimer B3 **2** and trimer C2 **4** by comparison of the NMR spectra of their acetylated derivatives with those of the literature (Balas and Vercauteren, 1994; Balas et al., 1995). The second dimer was identified as dimer B6 **3**, according to its HPLC retention time and previous identification in the same extract (Thompson et al., 1972).

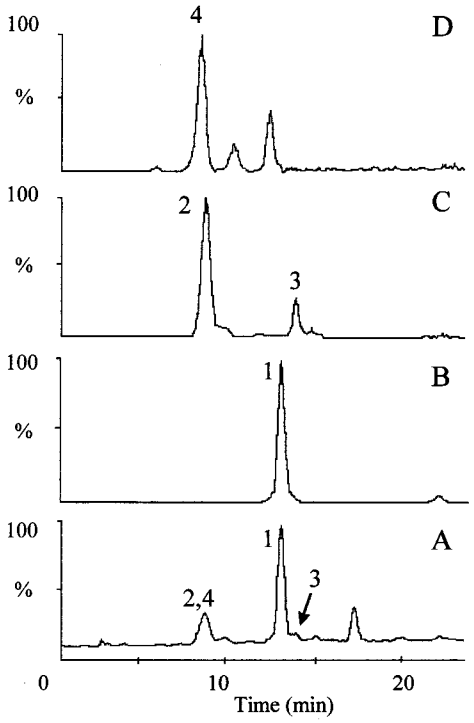

Figure 1. HPLC-ESI-MS chromatograms (negative ionization) of an acetone/water extract of leaves harvested on an adult willow tree: (A) total ion current; (B) flavanol monomers (m/z = 289); (C) flavanol dimers (m/z = 577); (D) flavanol trimers (m/z = 865). (1) (+)-Catechin; (2) dimer B3; (3) dimer B6; (4) trimer C2.

Radiolabeled Flavan-3-ols Produced by Incorporation of ^{14}C-Labeled Precursors in Willow Tree Leaves. ^{14}C-Labeled flavan-3-ols were analyzed in leaf extracts after incorporation of [1-^{14}C]-acetate or [U-^{14}C]-phenylalanine in willow shoots by cellulose bidimensional TLC and RP-HPLC. (+)-Catechin **1**, PA dimers **2** and **3**, trimer **4** (further named "oligomers"), and polymers **5** appearing as a streak from the origin spot are clearly seen on the TLC chromatogram of the crude extract and separated from labeled contaminants such as phenylalanine precursor (^{14}C-acetic acid is too volatile and is removed by chromatography before further analysis, see Experimental Section), sugars, and chlorogenic acid isomers (Figure 2A). Radiolabeled compounds were identified by comparing their retention times and R_f values to those of nonlabeled molecules purified from willow tree catkins. Ethyl acetate/water partition of the crude extract allows one to separate polymers **5** (aqueous phase) from (+)-catechin and PA oligomers analyzed by RP-HPLC (Figure 2B).

To more selectively assess the incorporation of carbon-14 into PA polymers, they were depolymerized by thiolysis (Scheme 1). Radiolabeled benzylthioether prod-

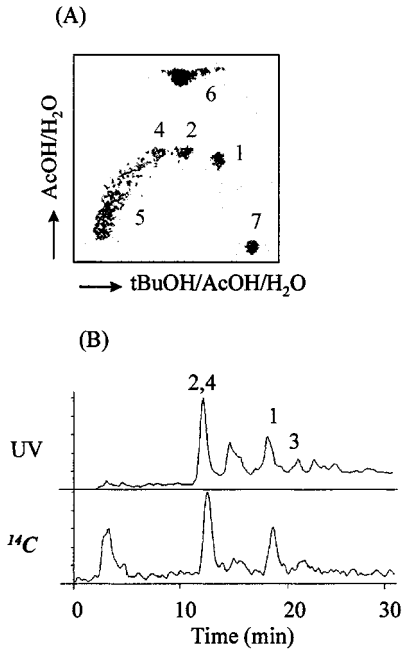

Figure 2. Chromatograms of willow leaf flavan-3-ols radiolabeled with [1-^{14}C]-acetate. (A) Autoradiogram of a bidimensional cellulose thin-layer chromatogram of a crude acetone/water extract. (B) HPLC chromatogram with UV and radioactivity detection of an ethyl acetate extract. (1) (+)-Catechin; (2) dimer B3; (3) dimer B6; (4) trimer C2; 5, proanthocyanidin polymers; (6) sugars; (7) apolar contaminant.

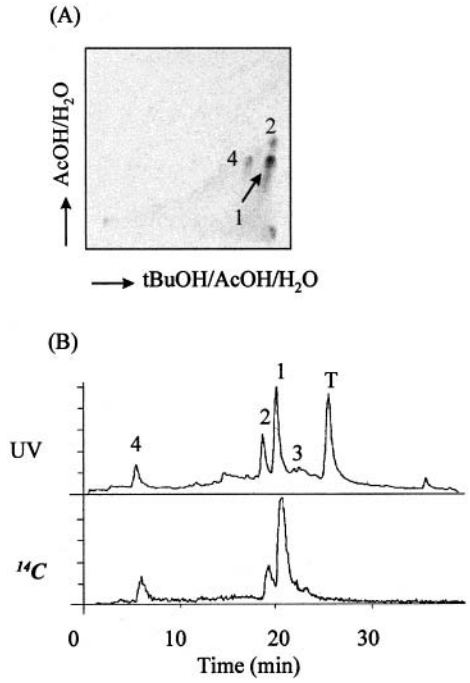

Figure 3. Chromatograms of thiolysis products formed by degradation of willow leaf procyanidins radiolabeled with [1-^{14}C]-acetate. (A) Autoradiogram of a bidimensionnal cellulose thin layer chromatogram; (B) HPLC chromatogram with UV and radioactivity detection. (1, 2) 3,4-*cis* and 3,4-*trans* isomers of benzylthiocatechin; (3) 3,4-*trans*-benzylthioepicatechin; (4) (+)-catechin; (T) benzylmercaptan.

ucts were analyzed by cellulose bidimensional TLC or RP-HPLC (Figure 3). More details on the analysis of prodelphinidin thiolysis products have already been published (Déprez et al., 1999).

Selection of Plant Materials for Optimal Proanthocyanidin Radiolabeling. To make plant material available throughout the year for the labeling experiments, young willow plants were grown in a greenhouse. However, flavanols present in leaves were found to differ in several respects from those of leaves harvested on adult trees grown outdoors (Table 1). Adult tree leaves were found to contain high amounts of (+)-catechin, dimer B3, trimer C2, and procyanidin polymers and low amounts of prodelphinidin polymers. In contrast, leaves from plants grown in the greenhouse contained no dimer and trimer but bigger amounts of polymers. Furthermore these polymers differ in their constitutive units and are predominantly prodelphinidins. This observation, first deduced from thiolysis analysis, was con-

Scheme 1. Procyanidin (R = H) and Prodelphinidin (R = OH) Depolymerization by Thiolysis into Stereoisomers of Benzyl Thio(epi)Catechin (R = H) and Benzyl Thio(epi)Gallocatechin (R = OH) and Flavan-3-ols

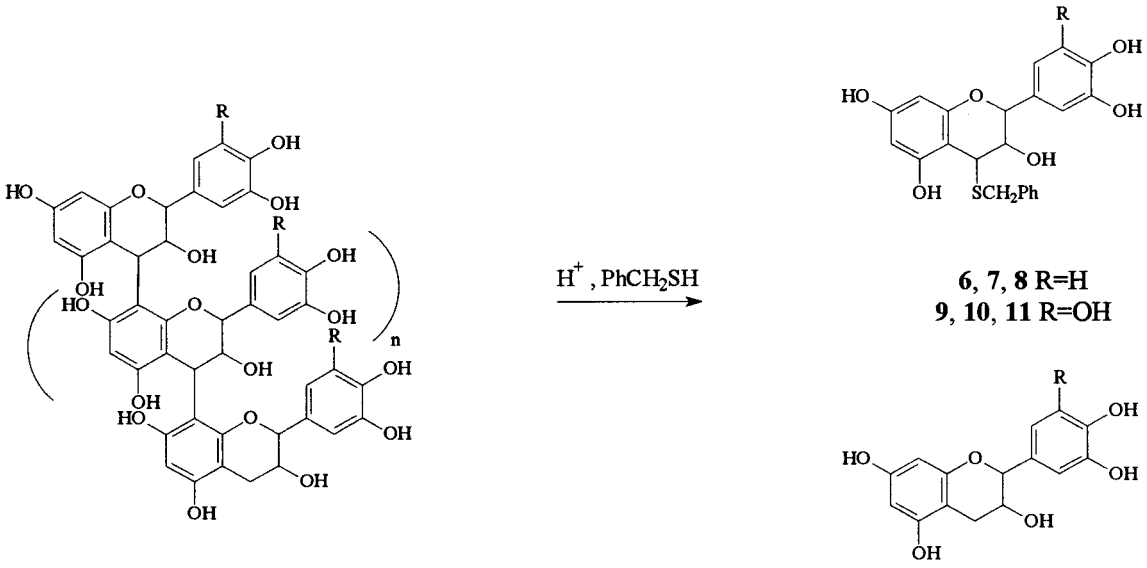

Table 1. Flavan-3-ols in Leaves of Willow Plants Grown Outdoors or in the Greenhouse and Collected in May (% dry wt)

	willows	
	adult tree grown outdoors	young plant grown in the greenhouse
(+)-catechin	0.8	0.1
(+)-gallocatechin	nd[a]	0.1
B3 dimer	0.5	nd[a]
B6 dimer	0.1	nd[a]
C2 trimer	0.2	nd[a]
polymers		
procyanidin units	1.0	0.6
prodelphinidin units	0.1	2.3

[a] nd: not detected.

firmed by ^{13}C NMR spectroscopy (Czochanska et al., 1980): polymers isolated from adult trees showed major signals at 116 (C-2′, C-5′), 120 (C-6′), and 145 ppm (C-3′, C-4′) with a 2:1:2 intensity ratio characteristic of procyanidins, whereas polymers isolated from plants grown in the greenhouse showed signals at 116 (C-2′, C-6′) and 145 ppm (C-3′, C-4′, C-5′) in a 2:3 ratio characteristic of prodelphinidins.

Some variations in the proportion of procyanidins and prodelphinidins were observed according to the sample analyzed: procyanidins represented 70−90% of the total PAs in adult tree leaves and 10−20% in the leaves of young trees grown in the greenhouse. No nonextractible PAs were detected by thiolysis in the residue of extraction recovered after filtration of the crude acetone/water leaf extracts (Matthews et al., 1997).

The content and composition of leaf flavanols also varied with the season of harvest. The PA content in leaves collected on adult trees grown outdoors and estimated by thiolysis coupled with RP-HPLC increased from 0.55% to 1.14% dry wt. from April to July (fully matured leaves). It remained constant between February and August in leaves collected on greenhouse trees.

Shoots harvested from adult trees were thus selected for labeling (+)-catechin **1**, dimer B3 **2**, trimer C2 **4**, and procyanidin polymers **5** (R = H). Young plants grown in the greenhouse were preferred to label PA polymers **5** (largely prodelphinidins) in high yield.

Translocation of Radiolabeled Precursors through the Plant. The radiolabeled precursor solutions were administered by translocation through the cut stem to shoots placed in a culture chamber equipped with neon tubes and an air extractor. Sodium [1-^{14}C]-acetate and [U-^{14}C]-phenylalanine were tested and the amount of radioactivity recovered in the different tissues of the shoots determined (Table 2).

The extent of precursor translocated through the plant varied with the nature of the precursor. With phenylalanine, about 80% of the radioactivity was retained in the stem whereas with acetate the major part of the radioactivity was found in leaves. The amount of precursor reaching the leaves was higher in the lower part of the shoot with both [1-^{14}C]-acetate (Figure 4) and [U-^{14}C]-phenylalanine precursors. In an experiment using the latter precursor, the stem of the shoot and its eight leaves were separated into three segments and analyzed separately (Table 3). The radioactivity measured by combustion of the whole samples was 2−3 times higher in the basal segment (both leaves and stem) as compared to the upper segment. The radioactivity was also higher at the base of the leaves

Table 2. Radioactivity Translocated to Stems and Leaves after Administration of [1-^{14}C]-Acetate or [U-^{14}C]-Phenylalanine to Willow Shoots

	precursor				
	phenylalanine[a]				acetate[b]
labeling duration (h)	48[d]	72[d]	144[d]	72[c]	48[d]
radioactivity (%)[g]					
leaf extract	12	17	16	15	41
*precursor	6.2	0.3	nd[f]	1.7	ND[e]
*flavanol monomers	0.0	0.1	0.2	nd[f]	0.3
*flavanol polymers	0.9	1.4	3.4	0.2	1.1
*sugars	2.8	10.1	8.7	11.8	2.7
*apolar compounds	0.1	0.1	0.2	0.2	0.4
leaf residue	2	5	5	2	21
stem	86	78	79	83	38

[a] 25 μCi. [b] 90 μCi. [c] Shoot collected on an adult tree grown outdoors. [d] Shoot grown in a greenhouse. [e] Not determined due to the volatility of the precursor. [f] Not detected. [g] The total radioactivity recovered was close to that administered to the shoot, and for the sake of comparison, results are expressed as percent of the total radioactivity analyzed.

Figure 4. Autoradiogram of a 20-cm-long shoot collected on a young willow plant fed [1-^{14}C]-acetate (50 μCi) through the cut stem. Full desiccation was reached after 72 h.

Table 3. Radioactivity (μCi/g dry wt. plant material) Found in Different Segments of a Willow Shoot Grown in a Greenhouse, 144 h after Administration of 25 μCi [U-^{14}C]-Phenylalanine. Lower, Intermediate, and Apical Segment Bore Two, Two, and Four Leaves, Respectively

	segment		
	basal	intermediate	apical
stem	75.9	50.5	25.5
leaves	9.4	7.7	4.2

as compared to their apex. When leaves of shoot fed [U-^{14}C]-phenylalanine were cut into three parts—proximal, intermediate, and distal—radioactivity in the proximal part was on average from 1.6 (apical leaves) to 4.1 (basal leaves) times higher as compared to the distal one. Incorporation of [1-^{14}C]-acetate through the cut petiole to excised isolated leaves showed a similar heterogeneity in translocation (Figure 5).

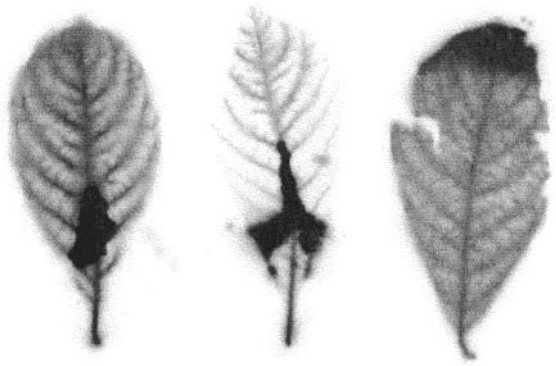

Figure 5. Autoradiogram of excised leaves collected on a young willow plant and fed [1-^{14}C]-acetate (50 μCi) through cut petioles. Full desiccation was reached after 54, 20, and 20 h, respectively, for leaves from the left to the right.

Table 4. Incorporation of [1-^{14}C]-Acetate (445 μCi) for 48 h into Leaf Flavanols of a Shoot Harvested on an Adult Willow Tree

	concentration (% dry leaves)	specific activity (μCi/g)	incorporation yield (% radioactivity fed)
(+)-catechin	0.53	79.6	0.11
B3 dimer	0.31	65.9	0.05
C2 trimer	0.19	58.9	0.03
procyanidin polymers	0.55	23.0	0.03

The major part of the activity in leaves (60−83%) was extracted by aqueous acetone, and most of the radioactivity in the extract was shared between some unmetabolized precursor, flavanols, sugars, and some apolar compounds (Table 2). Radioactivity in the precursor was virtually nil 3 days after administration of [U-^{14}C]-phenylalanine, whereas the radioactivity in flavanols, sugars, and apolar compounds increased in the meantime. The total radioactivity recovered in these experiments shows the absence of significative loss through exhaust of carbon dioxide or volatile organic compounds.

Incorporation of Radiolabeled Precursors into Flavan-3-ols. The yield of incorporation of the precursor differed according to the flavanol considered. With shoots collected on adult trees, the precursor [1-^{14}C]-acetate was preferentially incorporated into (+)-catechin, followed by dimer, trimer, and polymers (Table 4). The same results were obtained in all leaves of a same shoot whatever their age (Figure 6). Specific activities of (+)-catechin were also higher than those of dimer B3 and trimer C2. Specific activities of the flavanols were also affected by the position of the leaves on the shoot. With the exception of the younger apical leaves, these activities were higher in the basal leaves than in the upper ones (Figure 6).

Incorporation of the precursors into PA polymers was further examined by thiolysis degradation and the specific activity of the polymer units determined (Table 5). Procyanidins and prodelphinidins were both labeled, and specific activities varied by a factor of 2−3 according to the polymer unit considered.

The season also had a major impact on flavanol labeling. Shoots collected on the adult tree showed maximal yield of incorporation into flavanols (3.2%) in early July. This yield dropped to 0.05% in August. Incorporation yields in shoots grown in the greenhouse were maximal in May (3.4%) and decreased to 2.7% in June as the temperature in the greenhouse increased. The same shoots were then placed outdoors, and incorporation yields measured again in July reached 3.2% and dropped to 0.05% in August as the metabolic activity decreased. When the shoots were brought back to the greenhouse in early September under sodium lamp illumination, new leaves were formed 2 weeks later and the incorporation yield at that time reached 1.0%. However, in the middle of October, yields dropped again to values as low as 0.01%.

The length of labeling largely affected the incorporation yield which regularly increased in the 6 days following administration of [U-^{14}C]-phenylalanine (Table 2). In further labeling experiments, the leafy stems were kept in the incorporation chamber until the leaves fully wilt. However, large variations in the resistance of the shoot to desiccation were observed. In practice, the incorporation length varied from 2 to 8 days.

A final factor affecting the yield of incorporation is the nature of the plant sample, either shoots or excised leaves. The incorporation of [1-^{14}C]-acetate into PAs was 1.5-fold higher when the precursor was fed to excised leaves rather than to leafy shoots, despite the reduction by one-half of the labeling length explained by a more rapid wilting (Table 6).

Scaling-Up of the Incorporation Procedure. Once the incorporation yields have been optimized, an easy way to increase the specific activity of labeled flavanols is to increase the amount of labeled precursor applied (Table 7). No toxicity was observed when up to 200 μCi (0.4 μmol) [U-^{14}C]-phenylalanine was administered. However, some toxic effects on shoots (blackening of the leaf periphery, premature wilting) were observed when too high amounts of sodium [1-^{14}C]-acetate precursor were applied. Administration of 1 mCi acetate (100 μmol in 1 mL) reduced the incorporation yield into PAs and sugars to 0.05% and 0.8%, respectively, both values lower than those reached when only 400 μCi of the same precursor were fed (0.4% and 4.8% respectively). No apparent damage to leaves was observed at this last concentration.

To further examine the toxicity of acetate and its effect on its incorporation into PAs, a low amount of [1-^{14}C]-acetate (5 μmol) was fed to a shoot in the presence or absence of larger amounts of nonlabeled acetate (500 μmol). Addition of this amount of nonlabeled acetate did not induce any leaf blackening but reduced the incorporation of ^{14}C-acetate into PAs from (1.62 ± 0.34)% to (0.91 ± 0.46)%. Neutralization with 10% HCl of the precursor solution before administration only partially restored its incorporation to a (1.13 ± 0.17)% value. The absorption rate of the precursor solution was also reduced from 200 to 75 μL/min and rose to 125 μL/min when acetate was neutralized.

Some typical incorporation results are presented in Table 8. The yields of incorporation into (+)-catechin and PAs reached 3% of the radioactivity fed, and the specific activities varied from 200 to 500 μCi/g depending on the flavanol considered, the radioactivity administered, and the length of labeling.

Contaminants and Purification of Radiolabeled Flavanols. The main radiolabeled contaminants are sugars (free sugars account for 7% of the leaf dry weight; the main sugars were sucrose (3.9%), glucose (3.1%), fructose (0.3%), and galactose (traces)), chlorogenic acid, and nonidentified apolar compounds. Large variations in incorporation yields into these contaminants were

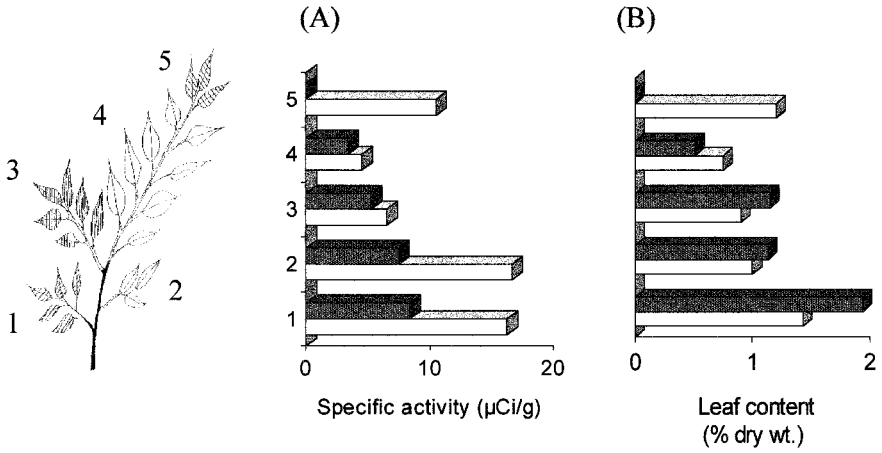

Figure 6. Specific activities (A) and contents (B) of (+)-catechin, B3 dimer and C2 trimer in leaves of a willow shoot harvested on an adult tree and fed [1-^{14}C]-acetate (45 μCi) for 48 h: (white bars) (+)-catechin; (gray bars) B3 dimer and C2 trimer (not separated in the HPLC system used).

Table 5. Incorporation of [1-^{14}C]-Acetate (90 μCi) for 48 h into Leaf Proanthocyanidins of a Shoot Grown in the Greenhouse. Proanthocyanidins Were Submitted to Thiolysis and Radioactivity Determined in Each Product by RP-HPLC

thiolysis products	concentration (% dry leaves)	specific activity (μCi/g)	incorporation yield (% radioactivity fed)
	Prodelphinidin Units		
3,4-*cis*-benzylthiogallocatechin **9**	0.69	41.3	0.21
3,4-*trans*-benzylthiogallocatechin **10**	0.21	23.1	0.03
3,4-*trans*-benzylthioepigallocatechin **11**	0.59	18.8	0.08
	Procyanidin Units		
3,4-*cis*-benzylthiocatechin **6**	0.17	29.6	0.04
3,4-*trans*-benzylthiocatechin **7**	nd[a]	nd[a]	nd[a]
3,4-*trans*-benzylthioepicatechin **8**	0.23	23.8	0.04
	Terminal Units		
(+)-catechin **1**	0.09	13.9	0.01

[a] nd = not detected.

Table 6. Parameters of the Biolabeling in Excised Leaves and Leafy Shoots Collected on Young Willow Plants and Administered [1-^{14}C]-Acetate (50 μCi), Respectively, through Cut Petioles and Cut Stems. Results Are the Mean of Triplicates

	excised leaf	leafy shoot
labeling duration (h)	31 ± 20	65 ± 35
average absorption rate of the precursor (μL/h)	50	200
Incorporation Yields (% radioactivity fed)		
catechin	0.17 ± 0.12	0.11 ± 0.02
PA oligomers and polymers	2.31 ± 1.39	1.62 ± 0.34
non-PA contaminants	7.75 ± 4.07	7.19 ± 3.25
Specific Activities (μCi/g)		
catechin	498 ± 391	63 ± 30
PA oligomers and polymers	841 ± 566	108 ± 41

observed (Table 2). With [U-^{14}C]-phenylalanine, the radioactivity incorporated into sugars varied from 3.8% to 74.1% of the total activity in the leaf extract; incorporation into chlorogenic acid and other nonidentified compounds reached up to 9.0% and 36.7%, respectively. With [1-^{14}C]-acetate, the major labeled contaminants were sugars (from 20.6% to 79.2% of the total activity in the leaf extract); no labeled chlorogenic acid could be detected.

Labeled dimers and trimer were separated from the polymers by ethyl acetate/water partitioning. Flavan-3-ols in both EtOAc and aqueous extracts were easily separated from labeled sugars by column chromatography on Sephadex LH-20. Some oligomer fractions still contained impurities, which were finally eliminated by cellulose thin-layer chromatography.

Table 7. Effect of the Amount of Radiolabeled Precursor Administered to a Willow Shoot on the Specific Activity of Proanthocyanidin Polymers (labeling duration 48−96 h)

precursor	radioactivity administered (μCi)	proanthocyanidin specific activity (μCi/g)
[U-^{14}C]-phenylalanine	25	30
	200	301
[1-^{14}C]-acetate	90	48
	300	221
	400	261

Purification of polymers required an additional step. Analysis of nonlabeled PA polymers purified by the same procedure and analyzed by ^{13}C NMR spectroscopy showed prominent signals in the 60−105 ppm region attributed to sugars. These sugars, likely covalently linked to PAs (Achmadi et al., 1994), represent about 3−4% of the PA fraction as seen by hydrolysis with HCl (Table 9). The main sugars are glucose, xylose, galactose, and arabinose. To remove these bound sugars from the labeled PAs, the PA fraction was treated with enzymes. The highest yields of hydrolysis were obtained with the AR2000 pectinase preparation. They were close to those obtained by hydrolysis with HCl.

Purification yields are shown in Table 10. Each Sephadex chromatography resulted in a loss of 20−40% of flavanols. The overall yield of purification varied between 30% and 60%.

Table 8. Recovery of ^{14}C-labeled (+)-catechin and Proanthocyanidin Dimer, Trimer and Polymer from Leaves of Willow Cuttings after Three Independent Incorporations of Labelled Phenylalanine or Acetate. Length of Labelling Varied between 72 and 80 h

^{14}C−precursor (amount administered, μCi)	^{14}C−Product[a]	amount in leaf extract (mg)	total activity (μCi)	specific activity (μCi/g)	incorporation yield (% radio fed)
[U-^{14}C]-phenylalanine (200)	polymers[b] (2/8)	18	5.3	301	2.7
[1-^{14}C]-acetate (400)	(+)-catechin	9	4.8	543	1.2
	B3 dimer	11	2.5	234	0.6
	C2 trimer	11	2.2	198	0.5
	polymers (9/1)	70	12.7	180	3.2
[1-^{14}C]-acetate (350)	polymers[b] (2/8)	19	9.5	506	2.7

[a] Values in parentheses represent the ratio of procyanidin to prodelphinidin units in proanthocyanidin polymers. Experiments showing a high procyanidin/prodelphinidin ratio were carried out on shoots collected on an adult tree; others with a low ratio were carried out on shoots grown in the greenhouse. [b] Free of monomer flavanol contaminant but may contain some dimers and trimers.

Table 9. Sugars Removed by Enzymatic and Acid Hydrolyses from the Proanthocyanidins Isolated from Willow Leaves (mg/g proanthocyanidin)

sugar	β-glucosidase	cellulase	AR2000	HCl
glucose	5.4	12.3	14.3	21.9
xylose	0.0	2.8	17.6	7.7
galactose	0.8	1.0	2.1	1.3
arabinose	0.0	0.0	3.0	4.8
total	6.2	16.1	37.0	35.7

DISCUSSION

Various parameters, such as the nature of the plant material, the nature and quantity of the precursor, and the method of incorporation, affect the incorporation yield of the labeled precursor into both polyphenols and nonphenolic compounds. It is essential to optimize these parameters in order to obtain high specific activities and high purity of the labeled polyphenols.

Selection of Plant Material for Proanthocyanidin Radiolabeling. Haslam et al. found high amounts of (+)-catechin **1**, dimer B3 **2**, and trimer C2 **4** in willow tree catkins (Thompson et al., 1972). However, catkins are available for only a short period of the year and could not be used for labeling experiments. Leaves on adult trees showed similar contents of the same molecules, but the season for labeling was still limited to about 4 months (April−July). To further extend this period, young willow trees were grown in a greenhouse, and labeling could thus be carried out from February to October, but the composition of the leaves differed from that of leaves harvested on adult trees by the absence of monomer, dimer, and trimer and the presence of large amounts of PA polymers, largely prodelphinidins. Leaves of these young willow shoots were thus used to label PA polymers **5** and adult tree to label (+)-catechin and PA dimer and trimer.

This difference in flavanol composition was not explained by the change in the environmental conditions. Indeed, the PA pattern in leaves was not changed when cuttings from the adult tree were grown in the greenhouse or when the plants from the nursery initially grown in the greenhouse were moved outdoors. The difference in PA composition is thus explained by genetic polymorphism (Harborne and Turner, 1984). Similarly, two chemomorphs with a prodelphinidin content of 55% and 90% of total PAs in needles have been decribed in Scotch pines (Lebreton et al., 1990).

Translocation of Radiolabeled Precursors through the Plant. Once the plant material has been selected, the incorporation of the precursor into the compounds of interest will depend on its translocation to the metabolically active tissues. Translocation depends on the nature of the precursor. Solutes such as ^{13}C-phenylalanine were easily translocated through the roots of *Fagus grandifoliia*, whereas ^{13}C-ferulic acid fed in similar conditions did not reach the aerial parts of the plant (Lewis et al., 1990). Administration of ^{14}C-phenylalanine to a sorghum panicle showed that 50% of the radioactivity remained in the stem and did not reach the seeds (Reddy and Butler, 1989). Translocation of [U-^{14}C]-phenylalanine and [3-^{14}C]-cinnamic acid fed to the detached leaves of *Polygonum* or wheat was largely limited to their basal part (Barnes and Friend, 1975). In the present study, 78−86% of the radioactivity applied as ^{14}C-phenylalanine fed to willow shoots was found in the stem and did not reach the leaves (Table 2), possibly because of some incorporation into insoluble polymeric lignins. Better translocation was observed with [1-^{14}C]-acetate, but no increase of incorporation in both flavanols and nonphenolic compounds could be observed (compare incorporations at 48 h for both precursors, Table 2). This tends to indicate that a major part of acetate has not been metabolized. There is thus no clear evidence for preferring one or the other precursor on the basis of their translocation.

Incorporation of Radiolabeled Precursors into Flavan-3-ols. Incorporation yields into flavanols depend on the nature of the precursor, on the more or less active biosynthesis of PAs in the plant tissues, and on the competing incorporation of the precursor into nonphenolic products. Specific radioactivities of PAs will depend on the yield of incorporation and on the dilution with preexisting nonlabeled molecules. To increase both the incorporation yield and specific activities, it is important to extend as much as possible the duration of metabolism and to feed high amounts of radiolabeled precursor.

Different studies have shown large variations in incorporation yields according to precursors. $^{14}CO_2$ was 3−5 times less efficient than ^{14}C-acetate to label (+)-catechin in *Uncaria gambir* (Das and Griffiths, 1967). $^{14}CO_2$ was also less effective than ^{14}C-phenylalanine when fed to sorghum to label condensed tannins (Jimenez-Ramsey et al., 1994). On the other hand, shikimic acid was 20 times more effective than L-phenylalanine in the labeling of gallic acid (Zaprometov and Bukhlaeva, 1968). In case of willow shoots, we could not observe any significant difference between phenylalanine and acetate. Since [U-^{14}C]-phenylalanine was about 10 times more expensive than [1-^{14}C]-acetate, the latter was preferred.

Large variations of PA specific activities were observed according to the position of the leaves on the shoot. They were 2−3 times higher in the lower leaves as compared to the upper leaves (Figure 6A). This may be explained by the limited translocation of the precur-

Table 10. Purification Yields of Radiolabeled Flavan-3-ol Monomer and Polymers. Flavanol Monomers Were Isolated from Leaves of an Adult Tree and Polymers from Those of Young Willow Trees Grown in a Greenhouse

flavanol precursor	(+)-catechin [1-^{14}C]-acetate ($n = 2$)[a]	PA polymers [1-^{14}C]-acetate ($n = 6$)[a]	PA polymers [U-^{14}C]-phenylalanine ($n = 2$)[a]
Purification step (% of total labeled flavanol)			
sephadex LH-20[b] + solvent fractionation	59	71	
solvent fractionation			81
enzyme hydrolysis[c] + Sephadex LH-20		76	76
sephadex LH-20	60		
cellulose TLC	83		
All steps (% of total labeled flavanol)	29	45	62
Yield (% leaf dry wt.)	0.1	3.2	2.6

[a] n: Number of repetitions. [b] Residual acetate precursor is removed by a first Sephadex LH-20 chromatography. [c] Polymers are treated by enzymes to remove bound sugars.

sor to the upper leaves (Figure 4, Table 3), the loss of some precursor in the stem (Table 2), or a higher biosynthetic activity in the lower parts of the shoot. The higher concentration of flavanols in the lower leaves (Figure 6B) shows that PAs continue to accumulate once the leaves are fully developed. An attempt to use these lower leaves to directly feed the precursor through the petiole was carried out. The total incorporation yield into flavanols (2.5%, Table 6) was of the same order than the best ones obtained with the experiments on shoots (Table 8). Specific activities were also significantly increased in detached leaves despite the larger dilution by the large content of nonlabeled PAs (Figure 6B).

Yields of incorporation also varied according to the state of maturity of the shoot at the time of harvest. Variations in the nature and content of polyphenols in leaves according to their state of maturity have commonly been reported (Feeny and Bostock, 1968; Tissut, 1968; Scalbert and Haslam, 1987; Koupai-Abyazani et al., 1993; Bohm et al., 1994; Lister et al., 1994). Variations of the biosynthetic activity of the flavanols with time was thus expected. The PA content in leaves of the willow tree grown outdoors increased from April to July (fully matured leaves). The incorporation of [1-^{14}C]-acetate into PAs was highest (3%) in early July.

In general, total incorporation yields into PAs commonly reached 3−5% and specific activities 200−500 μCi/g (Table 8). These values are consistent with the best results reported in the literature. Specific activities of 0.8 (Jimenez-Ramsey et al., 1994) and 290 mCi/mmol (Harmand and Blanquet, 1978) (calculated on a catechin unit basis) were reported for condensed tannins. Our own values of 147 μCi/mmol for PA polymers (calculated on a polymer unit basis) compare well with those values. Previous works on PA labeling show that less than 0.1% of [1-^{14}C]-acetate was converted into procyanidins of the A type (Eastmond and Gardner, 1974) and 0.5% of [U-^{14}C]-phenylalanine into condensed tannins (Reddy and Butler, 1989; see Déprez and Scalbert (1999) for a review).

Heterogeneous Incorporation of Precursors into Proanthocyanidins. Incorporation yields and specific activities vary with the flavanol considered or the unit in the polymer. Specific activities decreased from monomer to polymers (Table 4). In polymers, degradation by thiolysis showed that less radioactivity was incorporated into PA terminal units than into internal units (Table 5). Similar observations were previously made on Douglas fir PAs and led Stafford to propose a biosynthetic scheme for PAs (Stafford and Lester, 1982). This scheme implies the existence of a mode of polymerization consisting of the incorporation of labeled catechin molecules into preexisting PA polymers or oligomers that were unlabeled and would then add progressively more radiolabeled units.

Specific activities in procyanidin or prodelphinidin units of PA polymers were not significantly different, but they did vary with the unit stereochemistry. Thiolysis of the radiolabeled PAs showed that benzylthioethers with a 2,3-trans stereochemistry (catechin **6, 7** and gallocatechin **9**, **10**) were more highly labeled than those with a 2,3-cis stereochemistry (epicatechin **8** and epigallocatechin **11**). These differences reflect a late accumulation of 2,3-trans units as compared to 2,3-cis ones. Similar conclusions were reached by comparing the composition of PAs in leaves of *Onobrychis* at different stages of maturity (Koupai-Abyazani et al., 1993). These differences of specific activities are then the result of the PA biosynthetic pathway and of its varying expression over time which leads to the accumulation of PAs with definite structures.

Contaminants and Purification of Radiolabeled Flavanols. When using radiolabeled molecules as tracers, it is essential to have molecules of high purity, otherwise one might trace the impurities. The use of plants for labeling polyphenols usually results in the formation of large amounts of nonphenolic labeled contaminants which need to be carefully eliminated. No cheap precursor allows, so far, one to channel the radioactivity specifically to PAs or other polyphenols of interest.

The nature of plant materials used for labeling polyphenols affects the relative importance of incorporation of the precursor into polyphenols or contaminants. Incorporation of labeled phenylalanine into sugar contaminants was much lower when the precursor was fed to whole leaves rather than to leaf disks (Hillis and Isoi, 1965). In the present willow tree materials, labeled sugars were the main impurities. They accounted for 4−80% of the radioactivity in the leaf extract depending on the choice of the precursor and the labeling duration and correspond to 2−7 times more radioactivity than labeled flavanols (Table 2). Not only acetate but also phenylalanine was incorporated into sugars. A similar conversion of phenylalanine to sugar was shown in *Eucalyptus* species (Hillis and Isoi, 1965). A more specific labeling of polyphenols with phenylalanine could possibly be acheived with shorter incorporations, but their specific activities would be reduced consequently.

Few authors have paid attention to the purification of radiolabeled PA polymers. In the present study, purification by chromatography on a Sephadex LH-20 column and cellulose TLC led to the isolation of (+)-catechin, PA dimer B3, and trimer C2 with a radio purity close to 100%, as shown by HPLC and bidimensional TLC checking.

Purification of radiolabeled PA polymers required an additional treatment with enzymes to remove some bound sugars which, in contrast to free sugars, could not be removed by simple chromatography on Sephadex LH-20. Glycosylated PAs have been described on several occasions (Porter et al., 1985; Gujer et al., 1986; Zhang et al., 1988; Achmadi et al., 1994). Bound sugars must be removed in order to study the intestinal absorption of these radiolabeled PA polymers which would otherwise be removed by glycosidases in the gut (Griffiths and Smith, 1972; Day et al., 1998) and their absorption confused with that of PAs.

Various commercial glycosidase preparations were tested, and AR2000 was found to release the highest amount of sugars (Table 9). The total amount (3.7%) and the proportion of the four sugars are close to those observed by hydrochloric acid hydrolysis. The higher amount of xylose, liberated by AR2000 enzymes as compared to the hydrochloric acid treatment could be explained by the relative instability of xylose in the conditions of acid hydrolysis (Barton et al., 1982).

Yields of purified PA polymers recovered at the end of the purification process (3.2% and 2.6% leaf dry wt., depending on the precursor, Table 10) can be compared to the initial content of PA polymers as determined by thiolysis (Table 1). These purification yields and the yield of purification at each step of the protocole (Table 10) allow one to calculate the actual content of PA polymers in the leaves, i.e., 7.1% and 4.2% depending on the precursor. These PA contents are higher than those directly determined by thiolysis on leaves (2.9% leaf dry wt., Table 1). This apparent discrepancy is explained by an underestimation of the PA content of leaves when assayed by thiolysis. Yields of depolymerization of PAs by thiolysis varied between 30% and 70% depending on the PA samples due to the presence of thiolysis-resistant interunit bonds (Matthews et al., 1997). It can be concluded that the actual PA content of leaves is closer to 4−7%.

CONCLUSION

Administration of radiolabeled precursors, either acetate or phenylalanine, to willow tree shoots led to the isolation of PA dimer, trimer, and polymer of high radiopurity. Such a method provided labeled molecules which cannot yet be easily obtained through synthetic routes due to their high structural complexity.

Biolabeling methods with excised tissues have, however, their limits, the major one being the limited incorporation of the precursor into the molecule of interest when plant survival does not exceed a few days. An alternative would be to grow different tissues with the appropriate labeled precursor, either plant cells with the same precursors or entire plants grown in pots under a $^{14}CO_2$ atmosphere. These methods require a much higher investment both in time and money to establish and grow the cell cultures or to feed the radiolabeled gas in a closed growth chamber. The method developed here allowed us to obtain in a relatively short delay and at a relatively low cost several pure labeled PAs suitable for bioavailability studies. Current work on their bioavailability in rodents and on their metabolism and absorption by human liver and intestinal cell lines is now being carried out.

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