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Phenolic Extractives from the Bark of *Pinus sylvestris* L. and Their Effects on Inflammatory Mediators Nitric Oxide and Prostaglandin E_2

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The anti-inflammatory properties of phenolic pine (*Pinus sylvestris* L.) bark extract were studied. The pine bark extract was fractionated by liquid−liquid extractions and semipreparative high-performance liquid chromatography to reveal the most potent constituents. The phenolic compositions of three pine bark samples obtained, a crude extract, a chloroform fraction, and a semipreparative fraction, were analyzed using high-performance liquid chromatography with UV diode array detection and/or electrospray ionization mass spectrometry. In addition, eight compounds were isolated and identified by NMR and MS techniques. In total 28 phenolic compounds were identified. The effects of the three pine bark samples on the synthesis of two proinflammatory mediators, nitric oxide and prostaglandin E_2, were measured. It was shown that pine bark contains compounds that inhibit the production of these proinflammatory mediators.

KEYWORDS: Bark; HPLC−DAD; HPLC−ESI-MS; inflammation; macrophages; nitric oxide; nitric oxide synthase; phenolic compounds; *Pinus sylvestris* L.

INTRODUCTION

Plant phenolic compounds have been of growing interest on account of their suggested advantageous health effects and the possibility to use them as food ingredients. During the past decade an increasing interest has been placed on evaluating the functional properties of plant extracts, in particular with regard to their phenolic compositions. Pine bark extract has been traditionally used as a folk medicine (*1*), and it is still used therapeutically as a dietary supplement in Europe. In a study where the antioxidant activities of phenolic extracts from edible and nonedible Finnish plant materials were examined, *Pinus sylvestris* bark was ranked among the most potent plant sources for natural phenolic antioxidants (*2*). In another study where the antimicrobial activities of extracts prepared from Finnish plant materials against selected microbes were examined, pine phloem extract was again found to be one of the most active extracts (*3*). Previously, several low molecular weight phenolics, e.g., monoarylic phenols, stilbenes, lignans, and flavonoids, have been identified from inner pine bark (*4*). Pine bark has also been found to be rich in condensed tannins (*5*, *6*).

The current research was undertaken as part of an extensive cooperative project evaluating the functional properties of extracts from different natural materials. *P. sylvestris* bark was selected as one of the materials to be subjected to extensive studies. In this study, pine bark extract was fractionated to reveal the most potent constituents. The characters of three obtained pine bark samples, a crude extract, a chloroform fraction, and a semipreparative fraction, were tested, and their effects were measured on the synthesis of two proinflammatory mediators, i.e., nitric oxide (NO) and prostaglandin E_2 (PGE_2). NO is a gaseous signaling molecule with a short half-life. It is produced by three different NO synthase (NOS) enzymes: inducible NOS (iNOS), endothelial NOS (eNOS), and neuronal NOS (nNOS). eNOS and nNOS are calcium-dependent enzymes and are responsible for the low physiological production of NO. iNOS is the inducible calcium-independent form of NOS enzyme. It is produced at high amounts in inflammatory cells in response to cytokines and/or bacterial products (e.g., lipopolysaccharide, LPS). In various inflammatory diseases, NO is produced in increased amounts and it is known to act as a proinflammatory and cytotoxic mediator (*7*−*9*). Cyclooxygenase (COX) enzyme catalyzes the synthesis of prostaglandins from arachidonic acid. Two isoforms of COX have been characterized, i.e., constitutively expressed COX-1 and inducible COX-2. COX-2 is responsible for the elevated production of prostaglandins during

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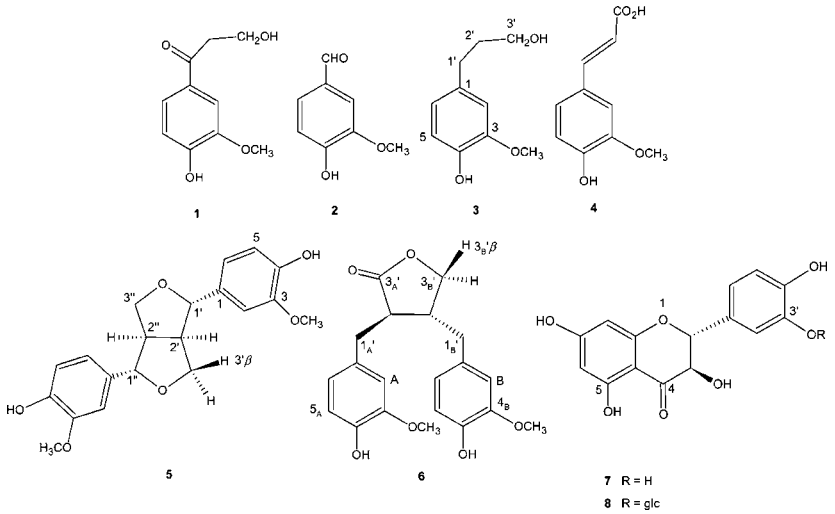

Figure 1. Structures of the compounds β-hydroxypropiovanillone (**1**), vanillin (**2**), dihydroconiferyl alcohol (**3**), ferulic acid (**4**), pinoresinol (**5**), matairesinol (**6**), taxifolin (**7**) and taxifolin 3′-*O*-β-D-glucoside (**8**) isolated from *P. sylvestris* together with the atom numbering and ring designation. The relative configuration of the stereocenters for **5**–**8** is also indicated; however, the absolute stereochemistries are not inferred.

inflammation (*10*−*12*). In the present study, we measured the effects of the pine bark crude extract, chloroform fraction, and semipreparative fraction on NO production through the iNOS pathway and PGE_2 production through the COX-2 pathway in LPS-treated macrophages. The aim of this study was to clarify further the interesting anti-inflammatory properties of pine bark extract and identify the phenolic compounds responsible for these effects.

MATERIALS AND METHODS

Plant Material. *P. sylvestris* L. (Scots pine) bark and phloem were collected in January 2001 in Honkajoki, Siikainen, Finland, air-dried, and ground by Ravintorengas Oy.

Extraction, Fractionation, and Isolation Procedure. Pine bark powder (150 g) was extracted with 70% aqueous acetone (4 × 1500 mL), and the combined extracts were taken to dryness. The solid residue was dissolved in water, and the insoluble material was discarded. Six batches of bark powder were extracted in the same manner and the aqueous solutions lyophilized. The lyophilized residues were combined, weighed (208.7 g, 23.2% of the bark dry weight), and then redissolved in water to a concentration of 0.15 g/mL. The water-soluble crude extract was then first washed with *n*-hexane (3 × equal volume) and then extracted with chloroform (3 × equal volume), yielding a yellow fraction. The chloroform fraction was evaporated to dryness, and the residue (2.071 g, 0.23% of the bark dry weight) was dissolved in 50% aqueous ethanol to a concentration of 40 mg/mL. A part of the chloroform fraction (0.8 g) was fractionated by semipreparative HPLC on the grounds of retention time: semipreparative fraction $R_t = 41-76$ min. The obtained fraction was evaporated to dryness, and the residue (0.472 g, 0.14% of the bark dry weight) was dissolved in 50% aqueous ethanol to a concentration of 20 mg/mL. The crude extract and obtained fractions (chloroform fraction and semipreparative fraction) were analyzed by HPLC−DAD and HPLC−ESI-MS. A further six compounds were isolated by semipreparative HPLC from the chloroform fraction: **1** (3.3 mg), **2** (1.3 mg), **3** (5.5 mg), **4** (2.6 mg), **5** (2.6 mg), and **6** (1.8 mg). In addition, **7** (11.5 mg) and **8** (24.2 mg) were isolated from the crude extract (**Figure 1**).

HPLC Analysis. HPLC separations were conducted on a Merck-Hitachi L-6200A pump connected to a Perkin-Elmer LC-235 UV diode array detector and a Perkin-Elmer GP-100 graphics printer. Analytical HPLC was performed on two systems. The HPLC−DAD system consisted of a Merck-Hitachi L-7100 pump, a Merck-Hitachi L-7250 autosampler, a Merck-Hitachi L-7455 diode array detector, and a Merck-Hitachi D-7000 interface. A PE SCIEX API 365 LC/MS/MS system was connected to a Perkin-Elmer series 200 HPLC system with a UV/vis detector and Analyst Software 1.1 data system. Two eluents, acetonitrile (A) and water/formic acid (99:1, v/v) (B), were used in HPLC. The elution profile was 0−5 min, 100% B (isocratic); 5−60 min, 0−30% A in B (linear gradient); 60−70 min, 30−70% A in B (linear gradient); and 70−80 min, 70% A in B (isocratic). The detection wavelength was 280 nm. Semipreparative HPLC was performed using a 250 × 10 mm i.d., 10 μm, LiChrospher 100 RP-18 column (Merck, Darmstadt, Germany). The constant flow rate was 6.0 mL/min, and the injection volume was 400 μL. Analytical HPLC was performed using a 250 × 4 mm i.d., 5 μm, Spherisorb ODS-2 column (Merck). The flow rate was 1 mL/min, and the injection volume was 20 μL. Phenolic compounds were quantified by comparison with an external standard of ferulic acid. In HPLC−ESI-MS the ionization technique was an ion spray (pneumatically assisted electrospray). The mass spectrometer was operated in both negative and positive modes. Negative mode conditions: spray needle voltage, −4000 V; heated nitrogen gas temperature, 310 °C; orifice plate voltage, −35 V; ring voltage, −220 V; nebulizer gas (purified air) set at 10; curtain gas (N_2) set at 12. Positive mode conditions: spray needle voltage, +5200 V; heated nitrogen gas temperature, 310 °C; orifice plate voltage, +45 V; ring voltage, +220 V; nebulizer gas (purified air) set at 10; curtain gas (N_2) set at 12. Masses were scanned from 60 to 2800 amu in steps of 0.3 amu. The split ratio was 7:3 prior to introduction into the ionization chamber.

NMR Analysis. NMR spectra were recorded on a JEOL Alpha 500 NMR (or JEOL Lambda 400) spectrometer equipped with either a 5 mm normal configuration tunable ($^{13}C\{^1H\}$) probe or a 5 mm inverse *z*-axis field-gradient ($^1H\{X\}$) probe operating at 500.16 (399.78) MHz for ^1H and 125.78 (100.54) MHz for ^{13}C. Spectra were acquired at 25 or 30 °C in CD_3OD or DMSO-d_6, and both ^1H and ^{13}C spectra were referenced internally to TMS (0.00 ppm for both). The spectral widths of the 2-D spectra were appropriately selected from the 1-D spectra and acquired with an adequate level of resolution. All experiments were performed using standard, vendor-supplied pulse sequences.

1-D ^1H spectra were acquired with single-pulse excitation, a 45° flip angle, a pulse recycle time of 9.5 s, and a spectral width of 7 kHz consisting of 64K data points (digital resolution 0.11 Hz/point), zero-filled to 128K prior to Fourier transformation. Spin analysis was performed using PERCH software (*13*) for the extraction of ^1H chemical shifts and J_{HH} coupling constants. NOE difference measurements were acquired using saturation times of 6−8 s at a reduced level of resolution (3.9 Hz/point); 1 Hz of exponential weighting was usually applied prior to Fourier transformation. Both field-gradient double-quantum-filtered COSY and field-gradient NOESY spectra were acquired in phase-sensitive mode and processed with zero-filling (2×, 4×) and exponential weighting (1−3 Hz, 3−5 Hz) applied in both dimensions prior to Fourier transformation. For NOESY spectra, mixing times of 200−800 ms were utilized.

Table 1. Phenolic Compounds in the Pine Bark Crude Extract[a]

compound	$[M - H]^-$ ion *m/z*	fragment ion *m/z*	ref
7, taxifolin 3-*O*-β-D-glucoside	465	303 $[A]^-$, 285 $[A - H_2O]^-$	*4*
8, taxifolin	303	285 $[M - H_2O]^-$	*4*
9, *p*-hydroxybenzoic acid glucoside	299	137 $[A]^-$	*4*
10, procyanidin trimer	865		*5*, *6*, *64*
11, vanillic acid glucoside	229	167 $[A]^-$	*4*
12, catechin glucoside	451	289 $[A]^-$	*4*, *6*
13, procyanidin dimer	577		*5*, *6*, *64*
14, catechin derivative[b]	465		*4*, *46*
15, catechin	289		*4*, *6*
16, procyanidin trimer	865		*5*, *6*, *64*
17, β-hydroxypropiovanillone glucoside	357	177 $[A - H_2O]^-$	compound **1**, *23*
18, ferulic acid glucoside	355	193 $[A]^-$	compound **4**, *4*
19, procyanidin trimer	865		*5*, *6*, *64*
20, epicatechin	289		*6*
21, procyanidin dimer	577		*5*, *6*, *64*
22, lignan glucoside	507	345 $[A]^-$	*4*, *65*
23, procyanidin tetramer	1153		*5*, *6*, *64*
24, procyanidin trimer	865		*5*, *6*, *64*
25, lignan xyloside	495	363 $[A]^-$	compound **3**, *4*, *65*
26, procyanidin trimer	865		*5*, *6*, *64*
27, procyanidin dimer	577		*5*, *6*, *64*
28, lignan rhamnoside	491	345 $[A]^-$	*4*, *65*

[a] The numbers of the compounds refer to the peak numbers in **Figure 2**. A = aglycon. [b] Coeluted together with an unidentified compound.

1-D ^{13}C spectra were acquired with single-pulse excitation, broadband ^1H decoupling, a 45° flip angle, a pulse recycle time of 3.5 s, and a spectral width of 30 kHz consisting of 64K data points (digital resolution 0.46 Hz/point), zero-filled to 128K and with 1 Hz exponential weighting applied prior to Fourier transformation. DEPT 90° and 135° spectra were acquired with spectral windows and processing similar to those utilized for the 1-D carbon spectra but with a postacquisition delay time of 3 s. Field-gradient HMQC, field-gradient HMBC, and HMBC−BIRD experiments were all acquired in magnitude mode and processed with zero-filling (2×, 4×), a $\pi/(3 - 8)$-shifted sine bell function, and exponential weighting (3−5 Hz, 5−25 Hz) applied in both dimensions prior to Fourier transformation. Phase-sensitive HSQC−PMG (*14*) spectra were processed with zero-filling (2×, 4×) and exponential weighting (3−5 Hz, 5−25 Hz) applied in both dimensions prior to Fourier transformation; the lengths of the purge pulse (typically 0.7 ms) and BIRD relaxation delay (typically 400 ms) were optimized on the incoming FID. Phase-sensitive CHSHF spectra with partial homonuclear decoupling in f1 were processed with zero-filling (2×, 4×) and exponential weighting (5 Hz, 5 Hz) applied in both dimensions prior to Fourier transformation. For all $^1H-^{13}C$ correlation experiments, $^1J_{H,C}$ was variously optimized to a value of 145 or 220 Hz or a compromise value of 180 Hz, while the HMBC correlations were optimized for a long-range $^nJ_{H,C}$ coupling of 8 or 5.5 Hz.

MS Analysis. Low-resolution EI^+ and FAB^+ (using PEG or NBA as matrix) spectra were acquired on a VG Analytical ZabSpec instrument using a direct insert probe scanning from 50 to 1500 amu. Accurate mass measurements were performed on this latter instrument using a peak-matching technique with PFK as a reference substance at a resolution of 8000−10000 (at 10% peak height).

Data for β-hydroxypropiovanillone (1): UV λ_{max} (EtOH, nm) 238, 276, 304 (sh); ^1H NMR (CD_3OD, 25 °C) δ H-2 7.44 (d (s1 AB), $J_{H6} = 2.0$ Hz), H-5 6.86 (d, $J_{H6} = 8.2$ Hz), H-6 7.51 (d (s1 AB), $J_{H5} = 8.2$ Hz, $J_{H2} = 2.0$ Hz), H-2′ 3.05 (2H, $J_{H3'} = 6.4$ Hz), H-3′ 3.75 (2H, $J_{H2'} = 6.4$ Hz), OCH_3 3.82 (s); ^{13}C NMR (CD_3OD, 25 °C) δ C-1 128.6, C-2 110.8, C-3 147.2, C-4 151.4, C-5 114.6, C-6 122.8, C-1′ 196.9, C-2′ 40.6, C-3′ 56.9, OCH_3 55.3; $ESI^{+-}MS$ *m/z* 219 $[M + Na]^+$, 197 $[M + H]^+$, 179 $[M - H_2O + H]^+$, 151, 125; $EI^{+-}MS$ (70 eV) *m/z* 196 ($M^{\bullet+}$, 37), 178 ($M - H_2O$, 4), 153 (5), 152 (109), 151 ($M - C_2H_5O$, 100), 123 (12), 108 (4.5); HRMS *m/z* $M^{\bullet+}$ 196.0745 (calcd for $C_{10}H_{12}O_4$ 196.0736), $M - H_2O$ 178.0633 (calcd for $C_{10}H_{10}O_3$ 178.0630), $M - C_2H_5O$ 151.0398 (calcd for $C_8H_7O_3$ 151.0395).

Data for vanillin (2): UV λ_{max} (EtOH, nm) 278, 309.

Data for dihydroconiferyl alcohol (3): UV λ_{max} (EtOH, nm) 235, 278.

Data for ferulic acid (4): UV λ_{max} (EtOH, nm) 242, 293 (sh), 323; HRMS *m/z* $M^{\bullet+}$ 194.0589 (calcd for $C_{10}H_{10}O_4$ 194.0579), $M - CH_3$ 179.0350 (calcd for $C_9H_7O_4$ 179.0344).

Data for pinoresinol (5): UV λ_{max} (EtOH, nm) 236, 278.

Data for taxifolin (7): UV λ_{max} (H_2O, nm) 239, 284; ESI−MS data, see **Table 1**.

Data for taxifolin 3′-*O*-β-D-glucoside (8): UV λ_{max} (H_2O, nm) 236, 285; ESI−MS data, see **Table 1**.

Cell Culture. Murine J774 macrophages were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultured at 37 °C (in 5% carbon dioxide) in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I containing 10% heat-inactivated fetal bovine serum, penicillin (100 units/mL), streptomycin (100 μg/mL), and amphotericin B (250 ng/mL). The cells were harvested with trypsin−EDTA. The cells were seeded in 96-well plates for XTT, in 24-well plates for nitrite measurements, and in 6-well plates for Western blot analysis. Confluent cells were exposed to fresh culture medium containing the compounds of interest.

XTT Test. Cell viability was tested using Cell Proliferation Kit II (Boehringer Mannheim, Indianapolis, IN). The cells were incubated with the tested compounds for 20 h before the addition of sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) (final concentration 0.3 mg/mL) and *N*-methyldibenzopyrazine methyl sulfate (1.25 mM). The cells were then further incubated for 4 h, and the amount of formazan accumulated in the growth medium was assessed spectrophotometrically. Triton-X-treated cells were used as a positive control. The conditions were considered toxic if the cells' ability to metabolize XTT to formazan was lowered by more than 20% in comparison to that of the untreated control.

Nitrite Determinations. Measurement of nitrite accumulation in the culture medium was used to determine NO production. At the indicated times the culture medium was collected, and the nitrite concentration was measured by Griess reaction (*15*).

Prostaglandin E_2 Assays. At the indicated times, the culture medium was collected for prostaglandin E_2 measurement. PGE_2 concentrations were determined by direct radioimmunoassay using reagents from the Institute of Isotopes (Budapest, Hungary).

Western Blot Analysis. After the desired time of incubation the cell pellets were lysed in ice-cold extraction buffer (10 mM Tris base, 5 mM EDTA, 50 mM NaCl, 1% Triton-X, 0.5 mM phenylmethylsul-

fonyl fluoride, 2 mM sodium orthovanadate, 10 μg/mL leupeptin, 25 μg/mL aprotinin, 1.25 mM NaF, 1 mM sodium pyrophosphate, and 10 mM *n*-octyl-β-D-glucopyranoside). After extraction by incubation on ice for 15 min the samples were centrifuged, and the resulting supernatant was diluted 1:4. The supernatant was boiled for 5 min in sample buffer (6.25 mM Tris−HCl, 10% glycerol, 2% SDS, and 0.025% 2-mercaptoethanol) and stored at −20 °C until it was analyzed. The Coomassie Blue method was used to measure the protein content of the samples (*16*). The protein samples (20 μg) were separated by SDS−PAGE on 8% polyacrylamide gel and transferred to a nitrocellulose membrane. iNOS and COX-2 proteins were detected and identified by Western blot analysis using rabbit polyclonal antibody (sc-650 for iNOS and sc-1746 for COX-2) obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Bound antibody was detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Cheshire, U.K.) and the FluorChem 8800 imaging system (Alpha Innotech Corp., San Leandro, CA). The quantitation of the chemiluminescent signal was performed using FluorChem version 3.1 software.

RESULTS AND DISCUSSION

Preliminary fractionation of *P. sylvestris* bark crude extract was performed using liquid−liquid extractions, and further fractionation was carried out using semipreparative HPLC. Samples were analyzed with HPLC−DAD and HPLC−ESI-MS. Since, in some cases, $ESI^{+,-}$ failed to provide a clear indication of $[M + H]^+$ or $[M - H]^-$ ions, the interesting constituents present in these samples were isolated for NMR analysis. A total of eight compounds—β-hydroxypropiovanillone (**1**), vanillin (**2**), dihydroconiferyl alcohol (**3**), ferulic acid (**4**), pinoresinol (**5**), matairesinol (**6**), taxifolin (**7**), and taxifolin 3′-*O*-β-D-glucoside (**8**)—were characterized (**Figure 1**) in this extensive manner. All of them are known, naturally occurring compounds that have been characterized to various degrees previously. However, some of the literature data regarding their characterization is missing or of limited access, and the compilation of the spectral data for some cases here thus represents a step toward addressing this deficiency. Because of the paucity of the literature data, and the varying conditions under which the literature spectra were acquired, full analyses by the application of a set of 2-D NMR experiments was warranted to not only enable structural elucidation, but also effect signal assignment.

Phenolic Composition of the Pine Bark Extract and Fractions. The phenolic profiles of the pine bark crude extract and its two subfractions were investigated using HPLC−DAD and HPLC−ESI-MS (**Figure 2**). Particular phenolic compounds became concentrated in the chloroform and semipreparative fractions. This can clearly be seen by comparing the HPLC chromatograms of the chloroform and semipreparative fractions to that of the pine bark crude extract. A total of 22 individual compounds (**7**−**28**) (**Table 1**) were detected in the crude extract on the basis of their molecular ions and characteristic fragmentation and by comparison of their UV-spectroscopic characteristics and retention times with literature data. The identities of **9** and **11** were further substantiated by comparison of their UV data to those of appropriate aglycon standards. The identities of **17** and **25** were also substantiated by comparison of their UV data to those of compounds **1** and **3**, respectively. Two compounds were isolated by semipreparative HPLC, **7** and **8**, and subjected to NMR analysis. The ^1H NMR data of **7** matched the literature values well (*17*−*19*) as did the ^{13}C NMR data when determined in the same solvent (*18*, *20*, *21*). For **8**, both the ^1H NMR and ^{13}C NMR data matched the reported values extremely well (*19*). Several procyanidin dimers and trimers were detected in the crude extract, and their characterization was consistent with our previous work (*6*). All of the other detected compounds (**7**−**16**, **18**−**28**) have been previously reported to be present in *P. sylvestris* bark, except **17**, which has been reported from *P. sylvestris* needles. In addition to these identified compounds, there were other compounds in the crude extract that were not identified within this study.

The chloroform and semipreparative fractions were found to possess significant biological activity, but the compounds present in these fractions could not be identified on the basis of the HPLC−DAD/ESI-MS data as in all cases analysis by $ESI^{+,-}$ failed to provide a clear indication of $[M + H]^+$ or $[M - H]^-$ ions. HPLC−ESI-MS is widely used for the analysis of plant phenolics, and it generally provides reliable results. At the moment it is not clear why the compounds present in these fractions could not be so analyzed by HPLC−ESI-MS. There can exist various possible reasons for these problems, such as the presence of undetectable compounds interfering with the ionization, causing facile fragmentation, or inhibiting the departure of counterions. Hence, the main compounds of these fractions, **1**−**6**, were isolated and subjected to full spectroscopic and spectrometric analysis. Of these six compounds, four (**1**, **3**, **4**, **6**) have been reported previously from *P. sylvestris* while a further two (**2**, **5**) have been reported to be present in other *Pinus* species. Interestingly, once isolated, recourse to either FAB^+ or EI^+ provided the molecular ions of these six compounds, the latter mode for all compounds **1**−**6**.

The structural determination and assignment of the resonances of compounds **1**−**6** were based mostly on the standard application of COSY, one- and multiple-bond $^1H-^{13}C$ correlation experiments, and NOE difference experiments together with spin analysis using PERCH software (*13*) for the extraction of ^1H chemical shifts and coupling constants. Fragmentation pathways observed in EI^+ were confirmed by daughter or parent ion analysis and/or by accurate mass measurements to help support the candidate structures. Given the fairly modest levels of structural elucidation required in most cases and since they are known compounds, only brief discussions of the structural analyses are presented.

β-Hydroxypropiovanillone (1). 3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)propan-1-one (β-hydroxypropiovanillone, **1**) has been isolated and identified previously once before from *P. sylvestris* bark using thin-layer chromatography and paper chromatography (*22*). In addition, the β-hydroxy glucoside of **1** has been reported from the needles of *P. sylvestris* (*23*). Since it was not possible to locate a paper giving the NMR and MS data, we report them here. The EI mass spectra provided the $M^{\bullet+}$ ion, and consistent with the structure, loss of H_2O and C_2H_5O fragments was observed.

Vanillin (2). 4-Hydroxy-3-methoxybenzaldehyde (vanillin, **2**) has been reported previously from the bark of *Pinus nigra* (*24*). Although the sample was not very pure, the presence of **2** was apparent by the odor of the sample and readily substantiated by NMR. The assigned NMR parameters were found to agree well with the literature values (*25*−*28*) as did the EI mass spectrum (*27*, *28*).

Dihydroconiferyl Alcohol (3). 3-(4-Hydroxy-3-methoxyphenyl)propan-1-ol (dihydroconiferyl alcohol, **3**) has been reported previously from the developing xylem of *Pinus contorta* (*29*), and by paper chromatography from *P. sylvestris* bark (*30*, *31*). The NMR parameters (*32*−*36*) and EI mass spectrum for **3** (*36*−*39*) were consistent with the literature data. MS analysis by either EI^+ or FAB^+ mode provided the $M^{\bullet+}$ ion in the former mode and the $[M + H]^+$ and $[M + Na]^+$ ions in the latter mode; the elemental composition of the $M^{\bullet+}$ ion was substantiated by HRMS.

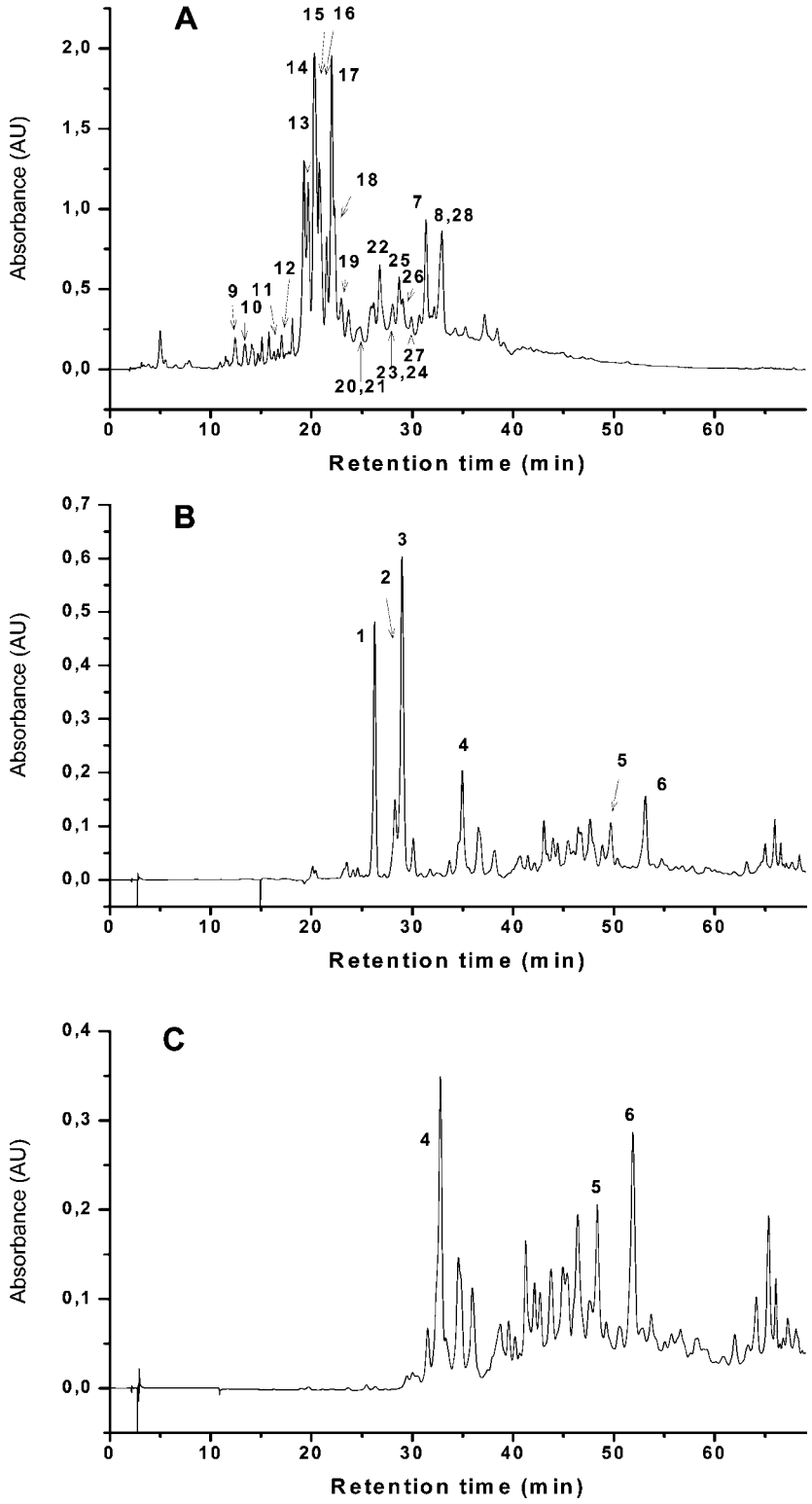

Figure 2. Analytical HPLC chromatograms of pine bark samples: **(A)** crude extract, **(B)** chloroform fraction, **(C)** semipreparative fraction. The peak numbers refer to the numbers of the phenolic compounds in **Figure 1** and **Table 1**.

*Ferulic Acid (**4**).* 3-(4-Hydroxy-3-methoxyphenyl)-(*E*)-prop-2-enoic acid (ferulic acid, **4**) has been detected previously in *P. sylvestris* bark (*40*). Additionally, the 4-hydroxy glucoside of **4** has also been reported (*4*). The observed ^{1}H and ^{13}C NMR chemical shifts were consistent with the literature values (*41*−*45*) for **4**, and the identification was confirmed by MS analysis (*27*). We found one mistake in the MS assignments of ref *27*, namely, the fragment *m*/*z* 137 is naturally $[M - CH_3]^+$ and not $[M - H - CO]^+$, which in turn is *m*/*z* 123. The elemental composition was substantiated by HRMS. The *trans* nature of the double bond was evident from the observed NOEs from both vinyl protons (H-1′ and H-2′) to the aromatic H-2 and H-6 signals as well as the distinct chemical shifts and vicinal coupling constant (15.9 Hz) of these vinyl protons in comparison to those of analogous *cis* isomers (*19*).

*Pinoresinol (**5**).* 2,6-Di(4-hydroxy-3-methoxyphenyl)-4,8-dihydroxy-3,7-dioxabicyclo[3.3.0]octane (pinoresinol, **5**) has not been identified previously from *P. sylvestris*, but it has been

Table 2. Contents of the Phenolic Compounds as Ferulic Acid Equivalents in the Chloroform and Semipreparative Fractions (Mean ± SEM, μg/mL)

	concn, μg/mL
chloroform fraction	
β-hydroxypropiovanillone	188 ± 15
vanillin	75 ± 6
dihydroconiferyl alcohol	313 ± 21
ferulic acid	118 ± 2
pinoresinol	44 ± 3
matairesinol	81 ± 5
semipreparative fraction	
ferulic acid	203 ± 4
pinoresinol	80 ± 1
matairesinol	147 ± 3

reported from the phenolic extract of the root bark of *Picea abies* and from the leaves of *Pinus armandii* (*46*, *47*). Although a precise spin simulation of the ^{1}H NMR spectrum of **5** for the aliphatic region was not forthcoming, possibly due to the presence of impurities which, although substantial, only slightly overlapped the signals of the main component, we could extract the NMR parameters well enough to find out that they as well as the UV, EI-MS, and HRMS results were consistent with the data reported earlier (*47*, *48*).

*Matairesinol (**6**).* Although (*E*)-3,4-di[(4-hydroxy-3-methoxyphenyl)methyl]dihydro-2(3*H*)-furanone (matairesinol, **6**) has not been reported previously from *P. sylvestris* bark, it was recently found in *P. sylvestris* knot wood (*49*). In addition, it has been reported from the leaves of *P. armandii* (*47*). The observed ^{1}H and ^{13}C NMR chemical shifts and EI-MS and UV data were consistent with the literature values (*50*−*56*), proving also that the ^{1}H chemical shifts were consistent with the *trans* relationship of the two benzyl groups. The assignment of the *pro-R* and *pro-S* protons for atom sites $1_A'$ and $1_B'$ and H-$3_B'\alpha$ and H-$3_B'\beta$ were also based on the assignments of the dimethyl ether (*50*). The identification of the structure was supported by MS analysis, with the sequential fragmentation of the $M^{\bullet+}$ ion starting with loss of one benzyl group substantiated by HRMS.

*Taxifolin (**7**).* The ^{1}H and ^{13}C NMR data for this compound were consistent with those found in the literature (*16*−*18*).

*Taxifolin 3′-O-β-D-Glucoside (**8**).* The ^{1}H and ^{13}C NMR data for this compound were consistent with those found in the literature (*19*).

The contents of phenolic compounds (as ferulic acid equivalents, mean ± SEM, μg/mL) identified in the chloroform and preparative fractions are presented in **Table 2**.

Effects on NO and PGE_2 Production and on iNOS and COX-2 Protein Expression. Flavonoids and other polyphenolic compounds have been shown to possess antioxidant and anti-inflammatory properties (*57*). Some of them also have inhibitory effects on iNOS and COX-2 expression (*58*−*60*). Therefore, we tested the effects of pine bark extracts on NO production and iNOS protein expression as well as on PGE_2 production and COX-2 protein expression in activated macrophages. To rule out possible cytotoxic effects, extracts were first tested using the XTT test to measure cell viability. None of the tested extracts were toxic in the incubation conditions used.

The effects of the pine bark crude extract, chloroform fraction, and semipreparative fraction were tested on NO production. NO production was induced by LPS, and nitrite, a stable end product of NO in culture medium, was measured after 24 h of incubation time. As seen in **Figure 3A**, the chloroform fraction inhibited NO production by 24−32% (at 20 and 50 μg/mL concentrations, respectively) and 50 μg/mL preparative fraction inhibited NO production by 63%. The crude extract did not inhibit NO production at concentrations up to 50 μg/mL (**Figure 3A**).

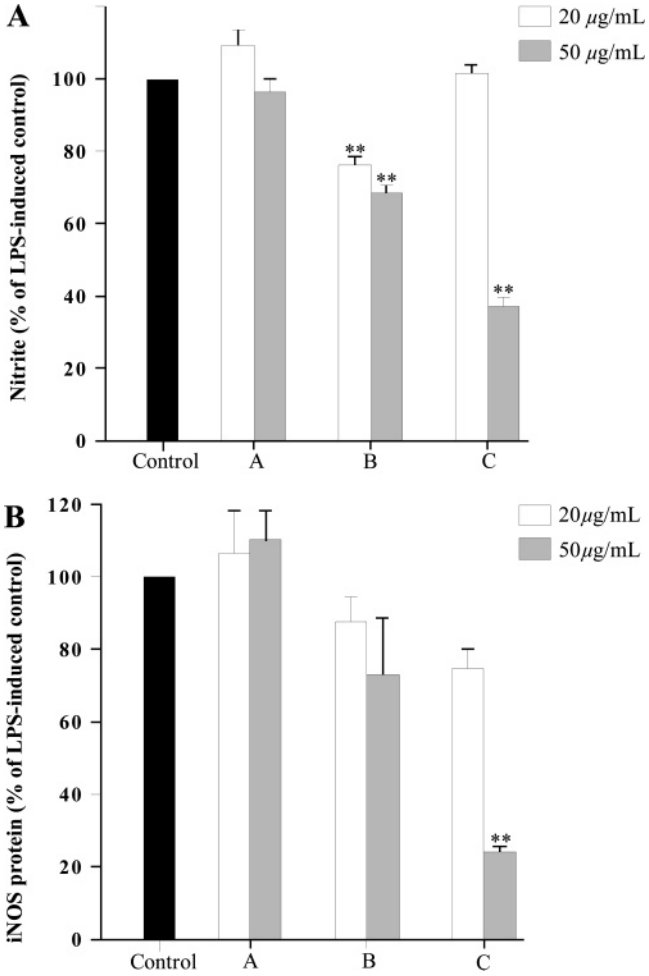

Figure 3. (**A**) Effects of the pine bark crude extract (A), chloroform fraction (B), and semipreparative fraction (C) on LPS-induced NO production. NO production was determined by measuring nitrite accumulation in the culture medium after 24 h of incubation by Griess reaction. The data are given as the mean ± SEM, $n = 6$. Two asterisks indicate $P < 0.01$ in comparison to cells incubated with LPS (control). (**B**) Effects of the pine bark crude extract (A), chloroform fraction (B), and semipreparative fraction (C) on LPS-induced iNOS protein expression. The data are given as the mean ± SEM, $n = 3$. Two asterisks indicate $P < 0.01$ in comparison to cells incubated with LPS (control).

The formation of iNOS protein after LPS challenge and the effects of pine bark extracts on it were tested by using Western blot analysis. The crude extract and chloroform fraction had no significant inhibitory action on iNOS protein expression, whereas, in the semipreparative fraction, an inhibitory activity on iNOS protein expression was detected (**Figure 3B**). The preparative fraction at the higher concentration tested (50 μg/mL) inhibited iNOS protein expression by 76% in comparison to the LPS-treated control.

The effects of the pine bark crude extract, chloroform fraction, and preparative fraction on LPS-induced PGE_2 production were tested using radioimmunological assay. After 24 h of incubation the chloroform fraction and semipreparative fraction significantly inhibited PGE_2 production but the crude extract had no effect (**Figure 4A**). The chloroform fraction inhibited PGE_2 production by 71% and 84% (20 and 50 μg/mL, respectively),

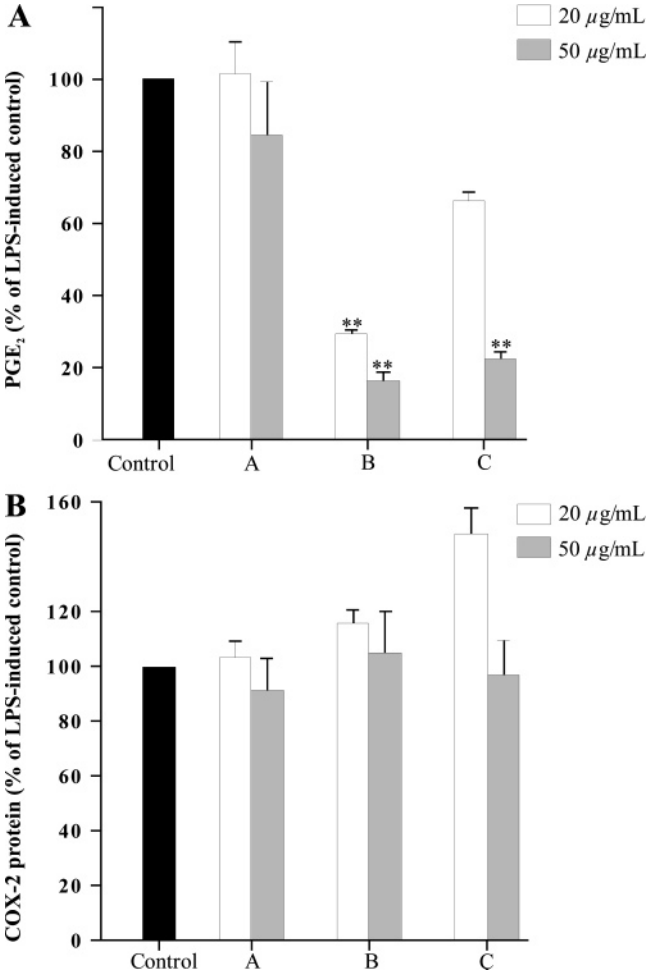

Figure 4. (**A**) Effects of the pine bark crude extract (A), chloroform fraction (B), and semipreparative fraction (C) on LPS-induced PGE_2 production. PGE_2 production was determined by measuring PGE_2 with radioimmunological assay. The data are given as the mean ± SEM, $n = 6$. Two asterisks indicate $P < 0.01$ in comparison to cells incubated with LPS (control). (**B**) Effects of the pine bark crude extract (A), chloroform fraction (B), and semipreparative fraction (C) on LPS-induced COX-2 protein expression. The data are given as the mean ± SEM, $n = 3$.

and the semipreparative fraction inhibited PGE_2 production by 34% and 77% (20 and 50 μg/mL, respectively).

The effects of the pine bark crude extract, chloroform fraction, and semipreparative fraction on COX-2 protein expression was detected by using Western blot analysis. None of the tested extracts had inhibitory action on COX-2 protein expression (**Figure 4B**).

These results show that pine bark contains compounds that inhibit the production of the proinflammatory mediators NO and PGE_2. The active compounds are enriched in the semipreparative fraction. All of the phenolic compounds isolated from the semipreparative fraction are known to have some biological effects, ferulic acid of which is probably the most notable. Ferulic acid has been found to possess antioxidant (*61*), antibacterial, antifungal, antihepatotoxic, antioestrogenic, antitumor, and antimitotic activities (*62*). The antioxidant efficiency of ferulic acid arises both from the methoxy substitution in the *ortho* position to the hydroxyl group and from the presence of the propenoic side chain (*61*). Both matairesinol and pinoresinol have shown inhibitory activity against cyclic adenosine monophosphate phosphodiesterase (*62*). Matairesinol is also known to have a high antioxidative potency similar to that of the well-known antioxidant Trolox (*63*). The effects on NO and PGE_2 production found in the present study may result directly from these isolated compounds, or they could be a consequence of the interactive effects of some or all compounds present in the semipreparative fraction. Earlier, it was implied that the antioxidant activities of knot wood extracts may indicate synergistic effects between the different phenolic compounds present as in many cases the antioxidant potency of the extract was higher than that of the isolated compounds (*63*). It can also be possible that other pine bark compounds present in the extracts but not analyzed within this study are responsible for the detected effects. In the present study, the active compounds in the chloroform and semipreparative fractions inhibit NO production through the iNOS pathway by reducing iNOS expression, whereas, in the case of PGE_2, the active compounds seem to inhibit COX-2 activity and have no effect on COX-2 expression. These results suggest that compounds that have anti-inflammatory effects can be extracted from pine bark.

ABBREVIATIONS USED

CHSHF, CH shift; COSY, correlation spectroscopy; COX, cyclooxygenase; DAD, diode array detection; DEPT, distortionless enhancement by polarization transfer; eNOS, endothelial NOS; EI, electron impact; ESI, electrospray ionization; FAB, fast atom bombardment; FID, free induction decay; HMBC, heteronuclear multiple-bond correlation; HMQC, heteronuclear multiple-quantum correlation; HPLC, high-performance liquid chromatography; HRMS, high-resolution mass spectra; HSQC, heteronuclear single-quantum correlation; iNOS, inducible NOS; LPS, lipopolysaccharide; MS, mass spectrometry; NMR, nuclear magnetic resonance; nNOS, neuronal NOS; NO, nitric oxide; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; PMG, poor man's gradient; UV, ultraviolet.

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LITERATURE CITED

(1) Drehsen, G. From ancient pine bark uses to pycnogenol. In *Antioxidant Food Supplements in Human Health*; Parker, L., Hiramatsu, M., Yoshikawa, T., Eds.; Academic Press: San Diego, 1999; pp 311−322.

(2) Kähkönen, M. P.; Hopia, A. I.; Vuorela, H. J.; Rauha, J.-P.; Pihlaja, K.; Kujala, T. S.; Heinonen, M. Antioxidant activity of plant extracts containing phenolic compounds. *J. Agric. Food Chem.* **1999**, *47*, 3954−3962.

(3) Rauha, J.-P.; Remes, S.; Heinonen, M.; Hopia, A.; Kähkönen, M.; Kujala, T.; Pihlaja, K.; Vuorela, H.; Vuorela, P. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* **2000**, *56*, 3−12.

(4) Pan, H.; Lundgren, L. N. Phenolics from inner bark of *Pinus sylvestris*. *Phytochemistry* **1996**, *42*, 1185−1189.

(5) Matthews, S.; Mila, I.; Scalbert, A.; Donnelly, D. M. X. Extractable and non-extractable proanthocyanidins in barks. *Phytochemistry* **1997**, *45*, 405−410.

(6) Karonen, M.; Loponen, J.; Ossipov, V.; Pihlaja, K. Analysis of procyanidins in pine bark with reversed-phase and normal-phase high-performance liquid chromatography-electrospray ionization mass spectrometry. *Anal. Chim. Acta* **2004**, *522*, 105−112.

(7) Alderton, W. K.; Cooper, C. E.; Knowles, R. G. Nitric oxide synthases: structure, function and inhibition. *Biochem. J.* **2001**, *357*, 593−615.
(8) Moilanen, E.; Whittle, B.; Moncada, S. Nitric oxide as a factor in flammation. In *Inflammation: basic principles and clinical correlates*, 3rd ed.; Gallin, J. I., Snyderman, R., Eds.; Lippincott Williams & Wilkins: Philadelphia, 1999; pp 787−800.
(9) Wallance, P.; Leiper, J. Blocking NO synthesis: how, where and why? *Nat. Rev. Drug Discovery* **2002**, *1*, 939−950.
(10) Dubois, R. N.; Abramson, S. B.; Grofford, L.; Gupta, R. A.; Simon, L. S.; Van De Putte, L. B. A.; Lipsky, P. E. Cyclooxygenase in biology and disease. *FASEB J.* **1998**, *12*, 1063−1073.
(11) Vane, J. R.; Bakhle, Y. S.; Botting, R. M. Cyclooxygenases 1 and 2. *Annu. Rev. Pharmacol. Toxicol.* **1998**, *638*, 97−120.
(12) FitzGerald, G. A. COX-2 and beyond: approaches to prostaglandin inhibition in human disease. *Nat. Rev. Drug Discovery* **2003**, *2*, 879−890.
(13) See for example, Laatikainen, R.; Niemitz, M.; Weber, U.; Sundelin, J.; Hassinen, T.; Vepsäläinen, J. General strategies for total-lineshape-type spectral analysis of NMR spectra using integral-transform iterator. *J. Magn. Reson., A* **1996**, *120*, 1−10; see also the program Web site at http://www.perchsolutions.com.
(14) Nuzillard, J.-M.; Gasmi, G.; Bernassau, J.-M. HMQC Experiment with single scan per t_1 value and without field gradient pulses. *J. Magn. Reson., A* **1993**, *104*, 83−87.
(15) Green, L. C.; Wagner, D. A.; Glogowski, J.; Skipper, P. L.; Wishnok, J. S.; Tannenbaum, S. R. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.* **1982**, *126*, 131−138.
(16) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(17) Lundgren, L. N.; Theander, O. The constituents of conifer needles. Part 13. *Cis*- and *trans*-dihydroquercetin glucosides from needles of *Pinus sylvestris*. *Phytochemistry* **1988**, *27*, 829−832.
(18) Shen, C.-C.; Chang, Y.-S.; Ho, L.-K. Nuclear magnetic resonance studies of 5,7-dihydroxyflavonoids. *Phytochemistry* **1993**, *34*, 843−845.
(19) Baderschneider, B.; Winterhalter, P. Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* **2001**, *49*, 2788−2798.
(20) Cotterill, P. J.; Scheinmann, F.; Stenhouse, I. A. Extractives from Guttiferae. Part 34. Kolaflavanone, a new biflavanone from the nuts of *Garcinia kola* Heckel. Applications of carbon-13 nuclear magnetic resonance in elucidation of the structures of flavonoids. *J. Chem. Soc., Perkin Trans. 1* **1978**, 532−539.
(21) Markham, K. R.; Ternai, B. Carbon-13 NMR of flavonoids. II. Flavonoids other than flavone and flavonol aglycons. *Tetrahedron* **1976**, *32*, 2607−2612.
(22) Oksanen, H. Isolation and identification of ω-hydroxypropioguiacone from the bark of pine (*Pinus sylvestris* L.). *Suom. Kemistil.* **1965**, *B38*, 65−67.
(23) Andersson, R.; Lundgren, L. N. Monoaryl and cyclohexenone glycosides from needles of *Pinus sylvestris*. *Phytochemistry* **1988**, *27*, 559−562.
(24) Hafızoğlu, H.; Holmbom, B.; Reunanen, M. Chemical composition of lipophilic and phenolic constituents of barks from *Pinus nigra*, *Abies bornmulleriana*, and *Castanea sativa*. *Holzforschung* **2002**, *56*, 257−260.
(25) Kolehmainen, E.; Laihia, K.; Knuutinen, J.; Hyötyläinen, J. Proton, carbon-13 and oxygen-17 NMR study of chlorovanillins and some related compounds. *J. Magn. Reson. Chem.* **1992**, *30*, 253−258.
(26) Morgan, K. R.; Newman, R. H. Principal values of carbon-13 NMR chemical shift tensors for a collection of substituted benzenes. *J. Am. Chem. Soc.* **1990**, *112*, 4−7.
(27) Tan, J.; Bedranek, P.; Liu, J.; Svatoš, A.; Hahlbrock, K. Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected Arabidopsis thaliana roots and leaves. *Phytochemistry* **2004**, *65*, 691−699.
(28) Graham, B.; Mayol-Bracero, O. L.; Guyon, P.; Roberts, G. O.; Decesari, S.; Facchini, M. C.; Artaxo, P.; Maenhart, W.; Koll, P.; Andreas, M. O. Water-soluble organic compounds in biomass burning aerosols over Amazonia 1. Characterization by NMR and GC-MS. *J. Geochem. Res.* **2002**, *107*, LBA 14/1−14/16.
(29) Savidge, R. A. Dihydroconiferyl alcohol in developing xylem of *Pinus contorta*. *Phytochemistry* **1987**, *26*, 93−94.
(30) Oksanen, H. Paper chromatography of phenolic constituents in the bark of pine (*Pinus sylvestris* L.). *Suom. Kemistil.* **1961**, *B34*, 91−92.
(31) Oksanen, H. Isolation of four phenolic compounds from the bark of pine (*Pinus sylvestris* L.); identification of dihydroconiferyl alcohol, *p*-hydroxybenzoic acid and vanillic acid. *Suom. Kemistil.* **1965**, *B38*, 258−260.
(32) Miyakoshi, T.; Chen, C.-L. Carbon-13 NMR spectroscopic studies of phenylcoumaran and 1,2-diarylpropane type lignin model compounds. Part 2. Substituent effects on carbon-13 chemical shifts of aromatic carbons. *Holzforschung* **1992**, *46*, 39−46.
(33) Pan, H.; Lundgren, L. N. Phenolics from inner bark of *Pinus sylvestris*. *Phytochemistry* **1996**, *42*, 1185−1189.
(34) Manners, G. D.; Penn, D. D.; Jurd, L.; James, L. F. *J. Agric. Food Chem.* **1982**, *30*, 401−404.
(35) Steeves, V.; Förster, H.; Pommer, U.; Savidge, R. Coniferyl alcohol metabolism in conifers—I. Glucosidic turnover of cinnamyl aldehydes by UDPG: coniferyl alcohol glucosyl-transferase from pine cambium. *Phytochemistry* **2001**, *57*, 1085−1093.
(36) Dawidar, A. M.; Ezmiriy, S. T.; Abdel-Mogib, M.; El-Dessouki, Y.; Angawi, R. F. New stilbene carboxylic acid from *Convolvulus hystrix*. *Pharmazie* **2000**, *55*, 848−849.
(37) Ralph, J.; Hatfield, R. D. Pyrolysis-GC-MS characterization of forage materials. *J. Agric. Food Chem.* **1991**, *39*, 1426−1437.
(38) Savidge, R. A.; Förster, H. Coniferyl alcohol metabolism in conifers − II. Coniferyl alcohol and dihydroconiferyl alcohol biosynthesis. *Phytochemistry* **2001**, *57*, 1095−1103.
(39) Kraus, R.; Spiteller, S. Phenolic compounds from roots of *Urtica dioica*. *Phytochemistry* **1990**, *29*, 1653−1659.
(40) Oksanen, H. Paper chromatographic evidence for the presence of ω-hydroxypropioguaiacone and four aromatic acids in the bark of pine (*Pinus sylvestris* L.). *Suom. Kemistil.* **1960**, *B33*, 167−171.
(41) Kelley, C. J.; Harruff, R. C.; Carmack, M. Polyphenolic acids of *Lithospermum ruderale*. II. Carbon-13 nuclear magnetic resonance of lithospermic and rosmarinic acids. *J. Org. Chem.* **1976**, *41*, 449−455.
(42) von Massow, F.; Smith, M. A. R. Indirect carbon-13-hydrogen coupling in asymmetrically trisubstituted benzenes: a carbon-13 nuclear magnetic resonance study. *J. Chem. Soc., Perkin Trans. 2* **1976**, 977−980.
(43) Ternai, B.; Markham, K. R. Carbon-13 NMR studies of flavonoids. I. Flavones and flavonols. *Tetrahedron* **1976**, *32*, 565−569.
(44) Damu, A. G.; Kuo, P.-C.; Shi, L.-C.; Hu, C.-Q.; Wu, T.-S. Chemical constituents of the stem of *Sargentodoxa cuneata*. *Heterocycles* **2003**, *60*, 1645−1652.
(45) Kujala, T.; Loponen, J. M.; Klika, K. D.; Pihlaja, K. Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds. *J. Agric. Food Chem.* **2000**, *48*, 5338−5342.
(46) Pan, H.; Lundgren, L. N. Phenolic extractives from root bark of *Picea abies*. *Phytochemistry* **1995**, *39*, 1423−1428.
(47) Fang, J.-M.; Lang, C.-I.; Chen, W.-L.; Cheng, Y.-S. Diterpenoid acids from the leaves of armand pine. *Phytochemistry* **1991**, *30*, 2793−2795.
(48) Harrison, L. J.; Sia, G.-L.; Sim, K.-Y.; Tan, T.-W.; Connolly, J. D.; Lavaud, C.; Massiot, G. A ferulic acid ester of sucrose and other constituents of *Bhesa paniculata*. *Phytochemistry* **1995**, *31*, 1497−1500.

(49) Willför, S.; Hemming, J.; Reunanen, M.; Holmbom, B. Phenolic and lipophilic extractives in scots pine knots and stemwood. *Holzforschung* **2003**, *57*, 359−372.

(50) Chimichi, S.; Cosimelli, B.; Bambagiotti-Alberti, M.; Coran, S. A.; Vincieri, F. F. Complete ^{1}H (600 MHz) and ^{13}C NMR spectral assignments of 3,4-bis[(3,4-dimethoxyphenyl)-methyl]-dihydro-(3*R*-trans)-2(3*H*)-furanone (matairesinol dimethyl ether), a naturally occurring dibenzylbutyrolactone lignan. *Magn. Reson. Chem*. **1993**, *31*, 1044−1047.

(51) Estévez-Braun, A.; Estévez-Reyes, R.; González, A. G. ^{13}C NMR assignments of some dibenzyl-γ-butyrolactone lignans. *Phytochemistry* **1996**, *43*, 885−886.

(52) Chênevert, R.; Rose, Y. S. A chemoenzymatic synthesis of both enantiomers of a cis-lignan lactone. *Tetrahedron: Asymmetry* **1998**, *9*, 2827−2831.

(53) Tiwari, A. K.; Srinivas, S. P.; Kumar, S. P.; Rao, J. M. Free radical scavenging active components from *Cedrus deodara*. *J. Agric. Food Chem.* **2001**, *49*, 1345−1348 and references therein.

(54) Rahman, M. M. A.; Dewick, P. M.; Jackson, D. E.; Lucas, J. A. Lignans of *Forsythia intermedia*. *Phytochemistry* **1990**, *29*, 1971−1980.

(55) Fischer, J.; Reynolds, A. J.; Sharp, L. A.; Sherburn, M. S. Radical carboxyarylation approach to lignans. Total synthesis of (−)-arctigenin, (−)-matairesinol, and related natural products. *Org. Lett.* **2004**, *6*, 1345−1348.

(56) Sicilia, T.; Niemeyer, H. B.; Honig, D. M.; Metzler, M. Identification and stereochemical characterization of lignans in flaxseed and pumpkin seeds. *J. Agric. Food Chem*. **2003**, *51*, 1181−1188.

(57) Middleton, E., Jr.; Kandaswami, C.; Theoharides, T. C. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease and cancer. *Pharmacol. Rev.* **2000**, *52*, 673−751.

(58) Chen, Y.-C.; Yang, L.-L.; Lee, T. J.-F. Oroxylin a inhibition of lipopolysaccharide-induced iNOS and COX-2 gene expression via suppression of nuclear factor-κB activation. *Biochem. Pharmacol.* **2000**, *59*, 1445−1457.

(59) Chen, Y.-C.; Shen, S.-C.; Chen, L.-G.; Lee, T. J.-F.; Yang, L.-L. Wogonin, baicalin, and baicalein inhibition of inducible nitric oxide synthase and cyclooxygenase-2 gene expressions induced by nitric oxide synthase inhibitors and lipopolysaccharide. *Biochem. Pharmacol.* **2001**, *61*, 1417−1427.

(60) Banerjee, T.; Van der Vliet, A.; Ziboh, V. A. Downregulation of COX-2 and iNOS by amentoflavone and quercetin in A549 human lung adenocarcinoma cell line. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2002**, *66*, 485−492.

(61) Natella, F.; Nardini, M.; Di Felice, M.; Scaccini, C. Benzoic and cinnamic acid derivatives as antioxidants: structure−activity relation. *J. Agric. Food Chem.* **1999**, *47*, 1453−1459.

(62) Harborne, J. B., Baxter, H., Moss G. P., Eds. *Phytochemical Dictionary*; Taylor and Francis Ltd.: London, U.K., 1999; pp 478−535.

(63) Willför, S. M.; Ahotupa, M. O.; Hemming, J. E.; Reunanen, M. H. T.; Eklund, P. C.; Sjöholm, R. E.; Eckerman, C. S. E.; Pohjamo, S. P. Holmbom, B. R. Antioxidant activity of knotwood extractives and phenolic compounds of selected tree species. *J. Agric. Food Chem.* **2003**, *51*, 7600−7606.

(64) Friedrich, W.; Eberhardt, A.; Galensa, R. Investigation of proanthocyanidins by HPLC with electrospray ionization mass spectrometry. *Eur. Food Res. Technol.* **2000**, *211*, 56−64.

(65) Popoff, T.; Theander, O. The constituents of conifer needles. VI. Phenolic glycosides from *Pinus sylvestris*. *Acta Chem. Scand., B* **1977**, *31*, 329−337.

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