Structure Elucidation

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A Sesquineolignan with a Spirodienone Structure from *Pinus sylvestris* L.**

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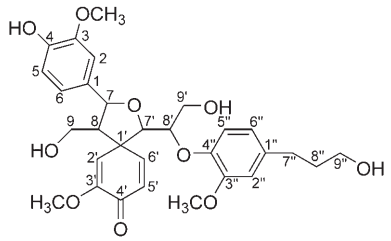
Pine (*Pinus sylvestris* L.) bark extract has been traditionally used as a folk medicine and it is still used therapeutically as a dietary supplement in Europe. Pine bark extract has shown strong antioxidant[1] and antimicrobial[2] activities, and a bioactive fraction has been isolated from pine bark extract.[3] This phenolic fraction was shown to have anti-inflammatory activity, that is, it inhibited the production of two pro-inflammatory mediators, nitric oxide and prostaglandin E_2,[3] as well as good antioxidant activity against the oxidation of liposomes and low-density lipoprotein (LDL) particles.[4] This fraction had minor effects on the permeability of metoprolol in Caco-2 experiments, but not on the other model drugs tested, and it was not mutagenic or toxic to the Caco-2 cells or macrophages.[4] Therefore, it can be regarded as a bioactive and safe material for possible applications in functional foods.

Earlier studies of this fraction resulted in the isolation of ferulic acid, a novel dihydroflavonol, and lignans.[4–6] Continuing our work on this active fraction, we report herein the structural elucidation of the novel sesquineolignan 4,9,9′,9″-tetrahydroxy-3,3′,3″-trimethoxy-7,7′-epoxy-8′,4″-oxy-8,1′-sesquineolignan-4′-one (**1**) with a spirodienone structure (Scheme 1) for which we propose the name pinobatol. Spiro-lignan structures are extremely rare; to the best of our knowledge only one sesquineolignan with a spiro skeleton has been reported thus far. This compound, named woorenol, was extracted from the rhizomes of *Coptis japonica* var. *dissecta*.[7]

Interestingly, the spirodienone structure has been proposed as a logical intermediate formed through a β-1 cross-coupling mechanism during lignin (bio)synthesis.[8,9] Further evidence of a spirodienone structure as a precursor of lignin was observed recently by NMR spectroscopic analysis of an acetylated spruce milled wood lignin.[10,11] However, the actual monomeric structure has not been reported as yet. Therefore, the complete assignment of this spirodienone sesquineolignan nicely accompanies the proposition of the role of spirodienone structures in the polymeric lignin structure and will serve as valuable data for future research not only on lignan but also on lignin.



Scheme 1. The structure and numbering of pinobatol (**1**).

The structure of **1** was identified by MS and NMR experiments. The assignment of all the ^{1}H and ^{13}C NMR signals was achieved by the combination of DQF-COSY, CH_2-edited HSQC, HMBC, NOESY, and selective 1D-TOCSY techniques. The molecular formula of **1** was determined to be $C_{30}H_{36}O_{10}$ by high-resolution electron ionization (EI) mass spectrometry. The accurate mass of the $[M]^+$ ion of **1** at m/z 556 was determined using PFK as a reference compound (found: 556.2321; calcd for $C_{30}H_{36}O_{10}$: 556.2308, deviation: 2.3 ppm).

Initial analysis of the ^{1}H NMR spectrum of **1** indicates signals from three 3,4-substituted aryl rings (one doublet with a small coupling constant of 2.0–2.5 Hz, one with a large coupling constant of 8.0–9.8 Hz, and one doublet of doublets coupled with the first signals). However, one of them has exceptional chemical shifts: $\delta = 6.09$, 6.28, and 7.04 ppm. The corresponding ^{13}C NMR shifts (assigned by HSQC) are also very different from those of a typical lignan aryl-substitution pattern (3-OCH_3, 4-OH). Furthermore, the ^{13}C NMR spectrum shows a signal at $\delta = 181.3$ ppm (C-4′) which has an HMBC correlation with protons that resonate at $\delta = 6.28$ (H-2′) and 7.04 ppm (H-6′). Thus, one of the three proposed 3,4-substituted aryl groups is not actually aryl but is a cyclohexadienone group. The HMBC spectrum also shows a correlation between the signals at $\delta = 6.09$ ppm (H-5′) and 55.9 ppm (C-1′), which is a quaternary carbon atom (from HSQC). This result confirms the presence of a spiro group.

In addition to aromatic signals, the ^{1}H NMR spectrum shows three methoxy singlets, a typical pattern for a 3-hydroxypropyl group at $\delta = 1.77$ (H-8″), 2.59 (H-7″), and 3.55 ppm (H-9″), which usually exists in lignans, and six other signals at $\delta = 2.8$–5.2 ppm. The ^{13}C NMR spectrum reveals, in

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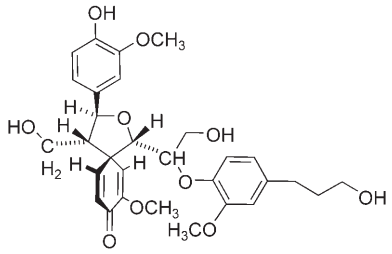
addition to aromatic signals, three signals at $\delta = 82.2$–84.5 ppm, four signals at $\delta = 59.9$–61.7 ppm, three methoxy signals at $\delta = 55.0$–56.3 ppm, and the signals at $\delta = 32.5$ and 35.6 ppm corresponding to C-7″ and C-8″, respectively, of the hydroxypropyl group. It is easy to see from the HSQC spectrum which protons and carbon atoms are directly bound, and by using edited HSQC techniques it can be seen which correlations involve CH_2 groups. There are three CH_2 correlations around $\delta = 60$ ppm, which show the presence of three CH_2OH moieties.

It is easiest to assign the signals starting from the hydroxypropyl group. The signals for C/H-7″–C/H-9″ can be assigned from the HSQC and DQF-COSY spectra. From the HMBC and NOESY spectra, it is easy to continue to the aryl moiety and the signals corresponding to C/H-1″–C/H-6″ can then be assigned using all of the above 2D NMR methods (see the Supporting Information). NOESY provides a link between the double-primed moiety to the primed moiety, that is, it shows a correlation between protons at positions 5″ and 8′. H-8′ appears as a doublet of triplets in the spectrum, and by DQF-COSY it is shown to be coupled with signals at $\delta = 4.81$ (H-7′) and 3.70 ppm (H-9′), from which the latter is assigned as a CH_2OH moiety on the basis of the edited HSQC spectrum. H-7′ shows HMBC correlations to the dienone ring carbon atoms C-2′ and C-6′. The rest of the dienone moiety atoms are assigned by the combination of DQF-COSY, HSQC, HMBC, and NOESY techniques. The assignment of the unprimed unit can be started from the aryl group, whose signals are easily identified with the aid of the above-mentioned techniques. HMBC shows a link to H-7, and from this H-8 and H-9 are assigned from the DQF-COSY spectrum. The connection between the unprimed and primed moieties is provided by several NOESY correlations.

Selective 1D-TOCSY experiments were used to determine the coupling constants from the overlapping signals. The H-7, H-7′, and H-8″ signals were selected and the spectrum showed the signals for all the aliphatic protons from the unprimed, primed, and double-primed moieties, respectively. With the aid of these and the above-mentioned 2D spectra, all the signals could be assigned unambiguously.

After the assignment, the stereochemistry of **1** was a further problem. The structure includes five stereogenic carbon atoms (C-7, C-8, C-1′, C-7′, and C-8′) and their relative configurations are discussed next. The protons H-8 and H-7′ showed a strong NOE correlation (see the Supporting Information) and are therefore concluded to be on the same side of the tetrahydrofuran ring. Furthermore, H-6′ showed a strong correlation with both H-8 and H-7′, which illustrates that the dienone ring is oriented perpendicularly to the tetrahydrofuran ring and that H-6′ is on the same side of the plane as H-8 and H-7′. In addition, H-2′ shows NOE correlations with H-9 and H-8′, which are all on the opposite side of the tetrahydrofuran ring. Correlations of H-7 with protons H-9 and H-2′ indicate that H-7 is *trans* to H-8. The large coupling constant J(H-7,H-8) = 9.3 Hz is also in agreement with the *trans* orientation. The relative configurations of C-7, C-8, C-1′, and C-7′ can be determined from the above-mentioned NOE results. However, the relative configuration of C-8′ remains undetermined because of the single bond between C-7′ and C-8′, which allows rotation about that bond and therefore inhibits a reliable interpretation of the NOE results. The stereochemical view of **1** is presented in Scheme 2. The relative configuration can thus be deduced to be 7*R**,8*S**,1′*S**,7′*R**.



Scheme 2. A stereochemical structure of pinobatol (**1**).

In conclusion, the novel spirodienone sesquineolignan (7*R**,8*S**,1′*S**,7′*R**)-4,9,9′,9″-tetrahydroxy-3,3′,3″-trimethoxy-7,7′-epoxy-8′,4″-oxy-8,1′-sesquineolignan-4′-one (pinobatol), was unambiguously assigned on the basis of its NMR correlations. The discovery of this compound in the pine bark speaks strongly for its existence as a monomeric unit in the lignin structure.

Experimental Section

The bark and phloem from *Pinus sylvestris* L. (Scots pine) were collected, air-dried, and ground by Ravintorengas Oy. The resulting powder (450 g) was then extracted with 70% aqueous acetone. The water-soluble extract was first washed with *n*-hexane (3 × equal volume) and then extracted with chloroform (3 × equal volume) to yield a yellow fraction (1.3 g). The chloroform fraction was partitioned by column chromatography on silica gel. The elution was performed with mixtures of chloroform, methanol, and water in different proportions. Several fractions were obtained. The fraction eluted with chloroform/methanol/water (237:12:1, v/v) was subjected to semipreparative purification on a LiChroCART column (LiChrospher 100 RP-18, 250 × 10 mm I.D., 10 μm, Merck Darmstadt, Germany; linear gradient elution with acetonitrile (solvent A) and water/formic acid (99:1, v/v; solvent B): 0–5 min 100% B followed by 5–60 min 0–30% A in B, and 60–70 min 30–70% A in B with a constant flow rate of 6 mL min^{-1} with detection at 280 nm) to yield **1** (4.3 mg). $[\alpha]_D^{20} = +6.0$ deg cm^3 g^{-1} dm^{-1} ($c = 0.83$ mg cm^{-3} in ethanol); ^{1}H NMR (500 MHz, [D$_6$]acetone, 25 °C, tetramethylsilane (TMS)): $\delta = 1.77$ (m (approx. quintet), 2H; H-8″), 2.59 (br t, ^3J(H-7″,H-8″) = 7.7 Hz, 2H; H-7″), 2.84 (dt, ^3J(H-8,H-9) = 5.7 Hz, ^3J(H-8,H-7) = 9.3 Hz, 1H; H-8), 3.55 (d, ^3J(H-9,H-8) = 5.7 Hz, 2H; H-9), 3.55 (t, ^3J(H-9″,H-8″) = 6.4 Hz, 2H; H-9″), 3.70 (d, ^3J(H-9′,H-8′) = 3.0 Hz, 2H; H-9′), 3.73 (s, 3H; 3′-OCH$_3$), 3.77 (s, 3H; 3″-OCH$_3$), 3.88 (s, 3H; 3-OCH$_3$), 4.19 (dt, ^3J(H-8′,H-9′) = 3.0 Hz, ^3J(H-8′,H-7′) = 8.4 Hz, 1H; H-8′), 4.81 (d, ^3J(H-7′,H-8′) = 8.4 Hz, 1H; H-7′), 5.16 (d, ^3J(H-7,H-8) = 9.3 Hz, 1H; H-7), 6.09 (d, ^3J(H-5′,H-6′) = 9.8 Hz, 1H; H-5′), 6.28 (d, ^4J(H-2′,H-6′) = 2.5 Hz, 1H; H-2′), 6.63 (dd, ^4J(H-6″,H-2″) = 2.0 Hz, ^3J(H-6″,H-5″) = 8.1 Hz, 1H; H-6″), 6.72 (d, ^3J(H-5″,H-6″) = 8.1 Hz, 1H; H-5″), 6.79 (d, ^4J(H-2″,H-6″) = 2.0 Hz, 1H; H-2″), 6.82 (d, ^3J(H-5,H-6) = 8.0 Hz, 1H; H-5), 6.98 (dd, ^4J(H-6,H-2) = 2.0 Hz, ^3J(H-6,H-5) = 8.0 Hz, 1H; H-6), 7.04 (dd, ^4J(H-6′,H-2′) = 2.5 Hz, ^3J(H-6′,H-5′) = 9.8 Hz, 1H; H-6′), 7.21 ppm (d, ^4J(H-2,H-6) = 2.0 Hz, 1H; H-2); ^{13}C NMR (125 MHz, [D$_6$]acetone, 25 °C, TMS): $\delta = 32.5$ (C-7″), 35.6 (C-8″), 55.0 (3′-OCH$_3$), 55.9 (C-1′), 56.0 (3″-OCH$_3$), 56.3 (3-OCH$_3$), 59.9 (C-9), 61.3 (C-9′), 61.5 (C-8), 61.7 (C-9″), 82.2 (C-8′), 82.9 (C-7),

84.5 (C-7′), 110.6 (C-2), 113.2 (C-2′), 113.5 (C-2″), 115.6 (C-5), 119.3 (C-5″), 119.8 (C-6), 121.4 (C-6″), 129.4 (C-5′), 135.3 (C-1), 138.1 (C-1″), 145.3 (C-4″), 146.9 (C-4), 148.4 (C-3), 151.5 (C-6′), 151.9 (C-3″), 153.5 (C-3′), 181.3 ppm (C-4′); UV λ_{max} (EtOH) 238, 259, 278 nm; EI-MS: found: 556.2321; calcd for $C_{30}H_{36}O_{10}$: 556.2308 (deviation: 2.3 ppm).

The HPLC-DAD system consisted of a Merch-Hitachi L-6200A pump connected to a Perkin–Elmer LC-235 UV-diode array detector and a Perkin–Elmer GP-100 graphics printer.

NMR spectra were acquired on a Bruker Avance 500 spectrometer (equipped with a BBO-5 mm-Zgrad probe) operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C spectra. Spectra were recorded at 25 °C in [D_6]acetone with a nonspinning sample in a 5-mm NMR tube. Spectra were processed on a PC with Windows XP operating system and XWin-NMR software. ^{1}H NMR, ^{13}C NMR, DQF-COSY (cosygpmfqf), NOESY (noesygpph), CH_2-edited HSQC (hsqcedetgpsisp.2, with shaped pulses), HMBC (hmbcgplpndqf), and selective 1D-TOCSY (selmlgp.2, spinlock time 200 ms) were all measured by the pulse programs (in parantheses) originally installed by Bruker. More detailed experimental description of the NMR parameters can be found, for example, in reference [6].

EI^+ mass spectra were obtained on a VG ZABSpec instrument (VG Analytical, Manchester, UK) in positive-ion mode using PFK (perfluorokerosine) as a reference compound.

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