Lignans of *Linum flavum* var. *compactum*

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**Abstract** A new dibenzylbutyrolactone lignan 7,6'-dihydroxybursehin, together with six known lignans (pinoresinol, lariciresinol, secoisolariciresinol, α-peltatin, β-peltatin, 5-methoxypodophyllotoxin) were isolated from the methanol extracts of *Linum flavum* var. *compactum*. The enantiomeric analysis of pinoresinol and lariciresinol isolated from the species, which are upstream lignans in the lignan biosynthetic pathway, indicated that they are not optically pure, which is in accordance with our recent findings on lignans occurring in other plant species.

**Key words** Lignan · 7,6'-Dihydroxybursehin · *Linum flavum* var. *compactum* · Linaceae · Biosynthesis

**Introduction**

An aryltetralin lignan, podophyllotoxin (Fig. 1), has been isolated from herbaceous perennial *Podophyllum* species and has long been reputed to have antitumor activity. An aryltetralin lignan, podophyllotoxin (Fig. 1), has been isolated from herbaceous *Podophyllum* species and has long been reputed to have antitumor activity. The lignan is exploited commercially as a source of a semisynthetic anticancer drug, etoposide, which is being applied successfully as cancer chemotherapy. Because of the limited supply of *Podophyllum emodi,* much attention has been focused on the availability of the lignan in various plants and its biotechnological production. Several woody plants (e.g., *Juniperus sabina, Thujopsis dolabrata, Callitrus drummondii, Hernandia ovigera*) produce podophyllotoxin, its congeners, or both. Hence, production of the antitumor lignan by woody plants is a challenging subject in the field of wood chemistry and biochemistry. As the first step to accessing biotechnological production it is necessary to understand the biosynthetic mechanisms, but much remains unknown about the biosynthesis of this lignan.

In addition to these woody plants and *Podophyllum* spp., some herbaceous plants such as *Anthriscus sylvestris* and *Linum* spp. (especially those belonging to the section *Syllinum* including *Linum flavum* and *Linum album*) have been known to produce podophyllotoxin and related lignans. In addition, suspension, root, and hairy root cultures of *Linum* species producing significant amounts of 5-methoxypodophyllotoxin (Fig. 1) and related lignans have been established. Thus, *Linum* plants are attractive as plant systems for elucidating the biosynthetic mechanisms of podophyllotoxin congeners, and the detailed knowledge of the mechanisms can be applied to biotechnological production of the lignans in woody plants as well as *Linum* spp. As for *A. sylvestris,* this species exhibits good growth behavior and contains large amounts of deoxypodophyllotoxin (deoxypodophyllotoxin, anthrionic) and yatein (Fig. 1), which were reported to be precursors of podophyllotoxin. Thus, the application of this species to the studies of podophyllotoxin biosynthesis is also of interest; we have reported the lignan analysis in *A. sylvestris* and formation of lignans by an enzyme preparation of the plant.

Recently, enzymatic reactions of upstream steps in the lignan biosynthetic pathway in *L. flavum* have been reported; conversion of pinoresinol to 7'-hydroxymatairesinol (Fig. 1) via lariciresinol, secoisolariciresinol, and matairesinol. However, the enantiomeric compositions of the upstream lignans and other possible precursors of aryltetralin lignans occurring in this *Linum* species remains unknown. Herein we report isolation of a new lignan, 7,6'-dihydroxybursehin, and six known lignans (Fig. 1) from...
Fig. 1. Structures of lignans isolated from *Linum flavum* var. *compactum* (A) and related lignans (B).

*L. flavum* var. *compactum* and the stereochemical characterization of the upstream lignans.

**Experimental**

**Plant material**

Seeds of *Linum flavum* var. *compactum* (Harris Seeds, Rochester, NY, USA) were sowed in a gardening soil and grown in an incubator at 23°C under a day (ca. 20000 lux)/night regime (14/10h) for 3 years.

**General experimental procedures**

One-dimensional and two-dimensional nuclear magnetic resonance (NMR) spectra were obtained with a JNM-LA400MK FT-NMR system (JEOL). Chemical shift and coupling constants (*J*) were expressed in δ and Hz, respectively. Low- and high-resolution electron impact-mass spectrometry (EI-MS), gas chromatography-mass spectrometry (GC-MS), and high-performance liquid chromatography (HPLC) were conducted as previously described. Freeze-dried aerial parts (6.3 g) of *L. flavum* var. *compactum* were pulverized and extracted six times with hot MeOH (total 260 ml). To the suspension of the MeOH extracts (1.3 g) in 100 ml of 0.1 M NaOAc buffer (pH 5.0) were added 500 units of β-glucosidase (Sigma G-0395, 5.0 U/mg, from almond). Following incubation at 37°C for 24 h, the reaction mixture was extracted with CH₂Cl₂. An aliquot (381 mg) of the CH₂Cl₂ extracts (454 mg) thus obtained was subjected to reversed-phase HPLC (solvent system A, B, or C) to afford 7,6′-dihydroxylariciresinol (8.0 mg), α-peltatin (13.0 mg), β-peltatin (13.8 mg), pinoresinol (5.1 mg), lariciresinol (2.3 mg), and secoisolariciresinol (2.2 mg).

**Extraction and isolation**

Freeze-dried aerial parts (6.3 g) of *L. flavum* var. *compactum* were pulverized and extracted six times with hot MeOH (total 260 ml). To the suspension of the MeOH extracts (1.3 g) in 100 ml of 0.1 M NaOAc buffer (pH 5.0) were added 500 units of β-glucosidase (Sigma G-0395, 5.0 U/mg, from almond). Following incubation at 37°C for 24 h, the reaction mixture was extracted with CH₂Cl₂. An aliquot (381 mg) of the CH₂Cl₂ extracts (454 mg) thus obtained was subjected to reversed-phase HPLC (solvent system A, B, or C) to afford 7,6′-dihydroxylariciresinol (8.0 mg), α-peltatin (13.0 mg), β-peltatin (13.8 mg), pinoresinol (5.1 mg), lariciresinol (2.3 mg), and secoisolariciresinol (2.2 mg).

**Enantiomeric compositions of lignans**

The enantiomeric composition of secoisolariciresinol isolated from *L. flavum* var. *compactum* was determined by chiral HPLC analysis, as previously described. Freeze-dried roots (7.0 g) of the plant were pulverized and extracted with hot MeOH as above. The MeOH extracts (2.0 g) thus obtained were treated with β-glucosidase and extracted with CH₂Cl₂ as above. An aliquot (ca. 500 mg) of the CH₂Cl₂ extracts (954 mg) was subjected to repeated TLC to afford 5-methoxypodophyllotoxin (15.0 mg).

7,6′-Dihydroxylariciresinol: *H- and 13C-NMR, H-1H correlated spectrometry (COSY), and 1H-detected heteronuclear multiple-bond quantum correlation (HMBC) are summarized in Table 1. MS m/z (%): 402 (2.1), 400 (3.1), 384 (19.6), 233 (15.6), 166 (13.4), 165 (11.0), 151 (100), 139 (10.0). High-resolution MS m/z (M⁺), calculated for C₂₁₇H₂₂O₂: 402.1315, found: 402.1322. Freeze-dried roots (7.0 g) of the plant were pulverized and extracted with hot MeOH as above. The MeOH extracts (2.0 g) thus obtained were treated with β-glucosidase and extracted with CH₂Cl₂ as above. An aliquot (ca. 500 mg) of the CH₂Cl₂ extracts (954 mg) was subjected to reversed-phase HPLC to afford 5-methoxypodophyllotoxin (15.0 mg).

7,6′-Dihydroxylariciresinol, α-peltatin, and β-peltatin: *H- and 13C-NMR, 1H-detected heteronuclear multiple-bond quantum correlation (HMBC) are summarized in Table 1. MS m/z (%): 402 (2.1), 400 (3.1), 384 (19.6), 233 (15.6), 166 (13.4), 165 (11.0), 151 (100), 139 (10.0). High-resolution MS m/z (M⁺), calculated for C₂₁₇H₂₂O₂: 402.1315, found: 402.1287.

5-Methoxypodophyllotoxin, α-peltatin, and β-peltatin: *H- and 13C-NMR (CDCl₃) data coincided with data in the literature. Pinoresinol, lariciresinol, and secoisolariciresinol: *H-NMR (CDCl₃) data were superimposable on those of chemically synthesized authentic samples, respectively.

**Enantiomeric compositions of lignans**

The enantiomeric composition of secoisolariciresinol isolated from *L. flavum* var. *compactum* was determined by chiral HPLC analysis, as previously described. Enantiomeric compositions of pinoresinol and lariciresinol isolated...
Table 1. NMR data for 7,6'-dihydroxybursehernin isolated from *L. flavum* var. *compactum* in CDCl<sub>3</sub>

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<th>H-H COSY&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HMBC&lt;sup&gt;c&lt;/sup&gt;</th>
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NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; HMBC, <sup>1</sup>H-detected heteronuclear multiple-bond quantum correlation

<sup>a</sup> Chemical shifts are δ values; coupling constants (J in parentheses) are given in Hz

<sup>b</sup> Correlation between H-2 and H-6 was not observed clearly

<sup>c</sup> Protons correlated with carbon resonances

<sup>d</sup> May be interchanged

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Fig. 2. Synthetic route for two epimers of (+)-7,6'-dihydroxybursehernins (11a and 11b). Note that only one enantiomer of each compound is shown.

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from the plant were determined by chiral HPLC followed by GC-MS analysis with racemic (+)-[9,9,9',9'-<sup>2</sup>H<sub>4</sub>]pinoresinols and (+)-[9,9,9',9'-<sup>2</sup>H<sub>4</sub>]lariciresinols as internal standards, as previously described. 22-24

Chemical synthesis of authentic lignans

(+)-[9,9,9',9'-<sup>2</sup>H<sub>4</sub>]Pinoresinols 25 (+)-pinoresinols, 15 (+)-[9,9,9',9'-<sup>2</sup>H<sub>4</sub>]lariciresinols, 25 (+)-lariciresinols, 19 and (+)-secoisolariciresinols were prepared previously.

Two diastereomers of (+)-7,6'-dihydroxybursehernins (11a and 11b) were synthesized from piperonal (1) as follows (Fig. 2).

Compound (3). This was synthesized by refluxing a benzene solution of 6-bromopiperonal (2) prepared from 1, 26 ethylene glycol, and p-toluenesulfonic acid with azeotropic removal of water. Yield, 96%. <sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ 4.03 (2H, m), 4.12 (2H, m), 5.96 (2H, s), 5.99 (1H, s), 6.98 (1H, s), 7.06 (1H, s).

6-Hydroxypiperonal (4). Compound (3) in dry Et<sub>2</sub>O was lithiated with n-butyllithium (nBuLi) at −78°C. The resulting phenyllithium was treated with dry O<sub>2</sub> at 0°C to afford crude 2-(6-hydroxypiperonyl)-1,3-dioxolane, which was hydrolyzed with acetone/0.1 N HCl (30:1). The crude product was purified by silica gel column chromatography [eluent, CH<sub>2</sub>Cl<sub>2</sub>–n-hexane (1:2 then 2:1), stepwise elution] to give a mixture of 4 and a by-product 1. The mixture was subjected to the next step without further purification. <sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ 6.00 (2H, s), 6.45 (1H, s), 6.85 (1H, s), 9.61 (1H, s), 11.77 (1H, s).

6-Benzoxypiperonal (5). Compound (4) was benzylated with benzylbromide and K<sub>2</sub>CO<sub>3</sub> in N,N-dimethylformamide at room temperature to afford 5. Yield, 17% from compound (3). <sup>1</sup>H-NMR (CDCl<sub>3</sub>): δ 5.11 (2H, s), 5.97 (2H, s), 6.57 (1H, s), 7.25 (1H, s), 7.36 (5H, m), 10.33 (1H, s).

6-Benzoxypiperonal (5) was converted to 6 by Stobbe condensation with dimethyl succinate and potassium tert-butoxide in t-butyl alcohol at reflux tempera-
J = 7.6, 9.5), 3.86 (3H, s), 3.87 (3H, s), 3.94 (1H, t, J = 9.0), dd, J = 6.1, 8.8), 4.32 (1H, t, J = 8.4), 5.17 (1H, broad s), 5.40 (1H, dd, J = 6.3, 9.0), 4.32 (1H, t, J = 8.4), 5.17 (1H, broad s), 5.84 (2H, m), 6.20 (1H, s), 6.25 (1H, s), 6.76 (3H, m); 13C-NMR (CDCl3): δ = 33.2, 36.1, 52.4, 55.8, 55.8, 72.5, 72.9, 98.1, 101.1, 108.7, 109.6, 110.9, 115.8, 117.7, 133.5, 141.1, 146.6, 148.3, 148.4, 148.9, 179.1; IR v max (CH2Cl2) cm−1: 1762 (C=O), 3450 (OH), 3600 (OH).

Results and discussion

In the present study, a novel 7-hydroxydibenzylbutyro-lactone lignan, 7,6′-dihydroxybursehernin, along with six known lignans (5-methoxypodophyllotoxin, α-peltatin, β-peltatin, pinoresinol, lariciresinol, secoisolariciresinol) were isolated from Linum flavum var. compactum (Fig. 1). The isolation of the known lignans from this species is in good accordance with previous reports; Linum spp., especially those of section Syllium including Linum flavum and Linum album, produced 5-methoxypodophyllotoxin, podophyllotoxin, α-peltatin, and β-peltatin. Also, production of pinoresinol and lariciresinol in L. album and of pinoresinol in L. flavum were reported.

The novel lignan was identified to be 7,6′-dihydroxybursehernin (Fig. 1) as follows. High-resolution EI-MS exhibited a molecular ion peak at m/z 402.1287, indicating a molecular formula of C21H22O8. Its IR spectrum exhibited strong absorbance at 1750 cm−1, showing the presence of an ester or a lactone ring. The 1H-NMR spectrum (Table 1) gave two triplets, δ 3.95 (1H) and 4.13 (1H), indicating the presence of oxygenated methylene protons. The two dd signals at δ 2.14 and δ 2.37 are assigned to benzylic protons, and the two signals at δ 2.59 (m) and δ 2.69 (dd) are ascribed to two methine protons. The doublet at δ 4.92 is indicative of an oxygenated benzyl group. The singlet at δ 5.86 is indicative of an aromatic methenedioxy group, and two singlets at δ 3.87 and δ 3.88 are assigned to two aromatic methoxyl groups. A group of signals between δ 6.85 and δ 6.97 are assigned to the 1,3,4-trisubstituted aromatic ring, and two singlet signals at δ 6.25 and δ 6.30 are ascribed to the 1,2,4,5-tetrasubstituted aromatic ring. The data together with 13C-NMR (Table 1), H-H COSY (Table 1), C-H COSY (not shown), and HMBC (Table 1) results indicate that the compound is a new 7-hydroxydibenzylbutyro-lactone lignan, 7,6′-dihydroxybursehernin. The structure was further confirmed by total synthesis; and the relative configuration of its C-OH was determined by comparing the synthesized two C-OH epimers (11a and 11b) (Fig. 2).

The spectrometric data of 7,6′-dihydroxybursehernin isolated from L. flavum var. compactum coincided with those of 11a in all respects but not with 11b. A 7-hydroxydibenzylbutyro-lactone, epipodorhizol (Fig. 1), which has intramolecular hydrogen bonding between C=O and lactone C = O and a larger J7,8 value (6.6 Hz), was determined to be 7R,8S isomer; and the other epimer (podorhizol) (Fig. 1), which does not show intramolecular hydrogen bonding and has a smaller J7,8 value (2.2 Hz), was identified to be 7S,8R isomer. The IR spectra of the epimeric alcohols 11a and 11b showed striking differences with respect to the absorption range of OH and the lactone carbonyl groups as in the case of podorhizol and epipodorhizol. Thus, the IR spectrum of 11a in CH2Cl2
exhibited, in addition to a band at 3590 cm\(^{-1}\) (free OH group), a broad and concentration-independent band at 3510 cm\(^{-1}\). In contrast, 11b gave the corresponding absorption bands in 3600 cm\(^{-1}\) and 3450 cm\(^{-1}\); and the latter band disappeared after dilution. The epimer 11b exhibited typical \(\gamma\)-lactone carbonyl absorption at 1762 cm\(^{-1}\), whereas in the case of 11a a distinct shift to a longer wave length (1750 cm\(^{-1}\)) was observed. These data indicate that 11a has hydrogen bonding between C=O and the lactone carbonyl, as in epipodophorizol, shown by Kuhn and von Wartburg.\(^{24}\) In contrast, this is not the case for 11b, like podophorizol.\(^{28}\)

The presence of intramolecular hydrogen bonding in 11a together with the \(H_2-H_6\) coupling constant \((J_{6\alpha,8\alpha}=7.6)\) of 11a indicates that this compound (and 7,6'-dihydroxyburschernin isolated from \(L.\) flavum var. compactum) has the relative configuration of \(7R^*, 8S^*\).\(^{19,27-29,31}\) indicating that 10a,b and 11a,b are \(H_2-H_6\) trans diastereomers. This was further confirmed by NOE difference measurements; during irradiation of 7,6'-dihydroxyburschernin isolated from \(L.\) flavum var. compactum at \(\delta 3.95\) (\(H_{5\alpha}\)), the signals of \(H_2\) and \(H_5\beta\) but not \(H_6\) were enhanced, whereas \(H_5\alpha\) and \(H_6\beta\) but not \(H_6\) were enhanced when \(\delta 4.13\) (\(H_{9\alpha}\)) was irradiated, confirming the trans configuration of \(H_2\) and \(H_6\) in terms of the \(\gamma\)-butyrolactone ring. Taken together, the relative configuration of 7,6'-dihydroxyburschernin isolated from \(L.\) flavum var. compactum (and 11a) was determined to be \(7R^*, 8S^*, 8'R^*(Fig. 1).\)

Recently, it has been found that there is a great stereochemical diversity in the upstream steps of lignan biosynthesis.\(^{4,20-24,32}\) Therefore, characterization of enantiomeric compositions of the upstream lignans is of importance. Secoisolaricresinol isolated from \(L.\) flavum var. compactum in the present study is an optically pure (\(-\))-enantiomer, whereas pinoresinol and lariciresinol are not, with 65% and 70% enantiomer excess (e.e.) in favor of (+)-enantiomers, whereas pinoresinol and lariciresinol are not, with 65% and 70% enantiomer excess (e.e.) in favor of (+)-enantiomers, respectively (data not shown). The result accords well with the general features of enantiomeric compositions of naturally occurring lignans;\(^{22}\) pinoresinol and lariciresinol are not optically pure. In contrast, Xia et al. reported that optically pure (\(+\))-lariciresinol and optically pure (\(+\))-secoisolaricresinol were formed following incubation of racemic (\(\pm\))-pinoresinols with a crude pinoresinol/ lariciresinol reductase preparation from \(L.\) flavum.\(^{25}\)

However, Xia et al. did not compare the enantiomeric compositions of the enzymatically formed lignans with the corresponding lignans occurring in their \(L.\) linum plant. The fact that lariciresinol isolated from \(L.\) flavum var. compactum in the present study is not optically pure can be accounted for by postulating that this plant has two pinoresinol/ lariciresinol reductase isoforms that reduce the opposite enantiomers of pinoresinol, as in the case of the \(Arctium lappa\) enzymes\(^{20,22}\) and \(Thuja plicata\) recombinant pinoresinol/lariciresinol reductase isozymes\(^{35}\), one has the same stereochemical selectivity in terms of pinoresinol enantiomers as the reductase reported by Xia et al.,\(^{17}\) and the other has the opposite stereochemical selectivity affording (\(+\))-lariciresinol as \(Daphne genkwa\) reductase.\(^{24}\)

In conclusion, we have reported the phytochemical characterization of \(L.\) flavum var. compactum lignans, including isolation of the novel lignan.

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References