Patavine, a New Arylnaphthalene Lignan Glycoside from Shoot Cultures of *Haplophyllum patavinum*

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A new aryl naphthalene lignan glycoside, patavine (1), together with five known lignans, justicidin B (2), diphyllin (3), tuberculatin (4), majidine (5), and arabelline (6) were isolated from shoot cultures of *Haplophyllum patavinum*. The structure of the new compound was elucidated by extensive one-dimensional (1D) and two-dimensional (2D) NMR experiments and mass spectrometry. The cytotoxicity of compounds 1, and 3—6 against LoVo human colon carcinoma cells was investigated.

Key words Haplophyllum; tissue culture; lignan; cytotoxicity

In a number of recent reports,1—3) we described the structures and distributions in vivo and in vitro of different secondary metabolite classes as coumarins, alkaloids, and lignans produced by *Haplophyllum patavinum* (L.) G. Don fil. (Rutaceae), the only species of this genus growing in Italy in a very small disjointed range on the Euganean Hills (Padova, Italy). In the course of the establishment of a protocol for the regeneration of this endangered plant,4) the biosynthetic capabilities of shoot cultures were also evaluated. In a previous report we identified furoquinoline and quinolone alkaloids3) from shoot cultures. We now report the isolation from MeOH extract shoot cultures of a new arylnaphthalene lignan glycoside, patavine (1), together with five known lignans. The known compounds 2—6 were identified as justicidin B (2), diphyllin (3), tuberculatin (4), majidine (5), and arabelline (6) by comparison of their spectral data with those of previously reported data.5—9) These known compounds were also detected in the native plants of this species.2)

Compound 1, obtained as an yellowish amorphous powder, presented a UV maximum absorption spectrum and a bluish fluorescence under UV light similar to lignans 2—6 indicating an arylnaphthalene nucleus.10) Its molecular formula (C36H40O19) was determined by 1H- and 13C-NMR analyses, and (high resolution) electrospray ionization mass spectrometry ((HR)-ESI-MS), which gave a molecular adduct [M + Na]+ at m/z 799.2752 and fragments at m/z 645 and 381 due to the cleavage of one and three pentose units, respectively. In the 1H- and 13C-NMR spectra, typical signals due to the arylnaphthalene lignan, diphyllin (3), were observed9) along with signals due to a sugar portion (Table 1). The structure of the oligosaccharide unit was determined by one-dimensional total correlation spectroscopy (1D-TOCSY) and two-dimensional (2D)-NMR experiments. The 1H-NMR spectrum of 1 showed three anomeric proton signals at δ 5.67 (1H, d, J = 1.8 Hz), 5.06 (1H, d, J = 2.0 Hz), and 4.57 (1H, d, J = 7.5 Hz). The 1D-TOCSY spectrum obtained by irradiating the signal at δ 4.57 clearly showed a spin system typical of a β-D-xylopyranosyl unit, while the 1D-TOCSY spectra obtained by selectively irradiating the signals at δ 5.67 and 5.06 revealed only another signal at δ 4.74 (1H, d, J = 1.8 Hz) and 3.93 (1H, d, J = 2.0 Hz), respectively. The double quantum filtered correlation spectroscopy (DQF-COSY) experiment allowed the complete sequential assignment of all sugar proton resonances that were correlated by the heteronuclear single quantum coherence (HSQC) experiment to the corresponding carbon signals. These data support the presence of one β-D-xylopyranosyl and two β-D-apiofuranosyl units in 1. The absence of any 13C glycosidation shift for the β-D-apiofuranosyl residue with the anomeric proton at...
δ 5.06 suggested that this sugar was terminal; the interglyco-
sidic linkages were established at C-20 of the β-D-apio-
furanose with the anomeric signal at δ 5.67 (δ 85.9) and at C-4 of the β-D-xylopyranose residue (δ 77.0) on the basis of the
downfield shift exhibited by these carbon resonances com-
pared with the corresponding shifts in unglycosylated mod-
els.11) The location of each sugar unit was deduced from the
heteronuclear multiple bond correlation (HMBC) experiment
which showed diagnostic long-range correlations between
the proton signal at δ 5.67 (H-100) and the carbon resonance
at δ 146.1 (C-4), the proton signal at δ 4.57 (H-1-) and the
carbon resonance at δ 85.9 (C-2), and the proton signal at δ
5.06 (H-100) and the carbon resonance at δ 77.0 (C-4). Thus
patavine (I) was characterized as 4-O-β-β-D-apiofuranosyl-
(1→4)-O-β-D-xylopyranosyl-(1→2)-O-β-D-apiofuranosyl-
diphyllin).

The comparative analysis of lignans produced in vitro and

Table 2. Distribution in Vivo and in Vitro of the Isolated Lignans 1—6

<table>
<thead>
<tr>
<th>Lignan</th>
<th>Native plants</th>
<th>Shoot cultures</th>
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<tbody>
<tr>
<td></td>
<td>Aerial parts</td>
<td>Roots</td>
</tr>
<tr>
<td>Patavine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Justicidin B</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>Diphyllin</td>
<td>3</td>
<td>+</td>
</tr>
<tr>
<td>Tuberculatin</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>Majidine</td>
<td>5</td>
<td>+</td>
</tr>
<tr>
<td>Arabelline</td>
<td>6</td>
<td>+</td>
</tr>
</tbody>
</table>

in vivo by native *H. patavinum* plants from the Euganean Hills sites are reported in Table 2. In previous reports we described the different biosynthetic potentialities between calli and suspension cultures and native plants.1,2,11) In terms of lignan production, only shoot cultures are able to produce
glycosylated derivatives not detected in callus cultures; among these, patavine was not been isolated before in vivo or in vitro. In the spent medium, only glycosylated derivatives are reversed.

The cytotoxicity of compounds 1, and 3–6 against the LoVo cell line (human colon carcinoma) was evaluated. The results are reported in Table 3. All compounds were found to be cytotoxic, but glycosylated compounds were less active than diphyllin (3).

### Table 3. Cytotoxic Activity against LoVo Cell Line in Vitro

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patavine</td>
<td>43.95±4.88</td>
</tr>
<tr>
<td>Diphyllin</td>
<td>7.55±0.75</td>
</tr>
<tr>
<td>Tuberculatin</td>
<td>13.92±1.26</td>
</tr>
<tr>
<td>Majidine</td>
<td>20.22±1.88</td>
</tr>
<tr>
<td>Arabelline</td>
<td>63.21±6.21</td>
</tr>
</tbody>
</table>

Experimental

**General Experimental Procedures** UV spectra in EtOH were recorded on a Perkin-Elmer model Lambda 5 spectrophotometer. IR spectra were recorded on a Perkin-Elmer 1600-FT instrument. Optical rotations were recorded on an Atago Polax-L polarimeter. A Bruker DRX-600 spectrometer operating at 599.19 MHz for 1H and 150.86 MHz for 13C using the XUNMR software package was used for NMR measurements in CD<sub>3</sub>OD solutions. 2D experiments: 1H-1H DQF-COSY, inverse-detected 1H-13C HSQC, and HMBC spectra were obtained by employing the conventional pulse sequences as described previously. The selective excitation spectra in 1D TOCSY were acquired using waveform generator-based GAUSS shaped pulses, with mixing time ranging from 100 to 120 ms and a MLEV-17 spin-lock field of 10 kHz preceded by a 2.5-ms trim pulse. ESI-MS/MS spectra were recorded on an LCQ (Finnigan, San Jose, CA, U.S.A.) ion-trap mass spectrometer equipped with an electrospray ion source and on an ESI-TOF mass spectrometer (Mariner, PE Biosystems). MS spectra were obtained in a positive mode.

**Plant Material** Native plants of *H. patavinum* were collected on the Euganean Hills, Padova (Italy) (a voucher is deposited at the Botanical Garden of the University of Padova, no. PAD 3703).

**Tissue Cultures** Shoot cultures were initiated from callus cultures grown as described previously. Shoots were maintained in hormone-free MS liquid medium and grown under stirring with a 12-h photoperiod (1000 lux) and subcultured on fresh liquid medium every third week.

**Extraction and Isolation** Plant materials (10 g, dry wt.) and fresh shoots (680 g) were exhaustively extracted with MeOH in a Soxhlet apparatus. After solvent evaporation, the MeOH extract was dissolved in H<sub>2</sub>O–MeOH (9 : 1) and successively extracted with CHCl<sub>3</sub> and AcOEt; then the solvent was distilled and the residue dissolved in MeOH was analyzed. The liquid medium of shoot cultures was extracted with EtO<sub>2</sub> (48 h) and then with AcOEt (48 h) in a liquid extraction apparatus; the solvent was distilled, and the residue was dissolved in MeOH and analyzed. The CHCl<sub>3</sub> extract was separated on TLC and yielded justicidin B (2) (5.3 mg), diphyllin (3) (8.6 mg), and tuberculatin (4) (20.2 mg), whereas the AcOEt extract yielded (1) (12.6 mg), majidine (5) (7.0 mg), and arabelline (6) (23.4 mg).

**Cytotoxicity Study** The LoVo cell line (human colon tumor line) was grown as a monolayer in Nutrient Mixture F12 Ham medium (Sigma Chemical Co.) supplemented with 10% fetal bovine serum (FBS; Gibco BRL), 25 mM HEPES buffer, and antibiotic solution (Life Technologies) and incubated at 37 °C in a 5% CO<sub>2</sub> atmosphere. Cytotoxicity of the lignans to LoVo cells was measured by the MTT assay as previously described by Mosmann. The test was performed at least in triplicate. The IC<sub>50</sub> values of compounds 1 and 3–6 are listed in Table 3.

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References