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

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A new arylnaphthalene 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 alkaloids from shoot cultures.[3]

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 ($C_{36}H_{40}O_{19}$) was determined by ^{1}H- and ^{13}C-NMR analyses, and (high resolution) electrospray ionization mass spectroscopy ((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 ^{1}H- and ^{13}C-NMR spectra, typical signals due to the arylnaphthalene lignan, diphyllin (**3**), were observed[9] 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 ^{1}H-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 ^{13}C glycosidation shift for the β-D-apiofuranosyl residue with the anomeric proton at

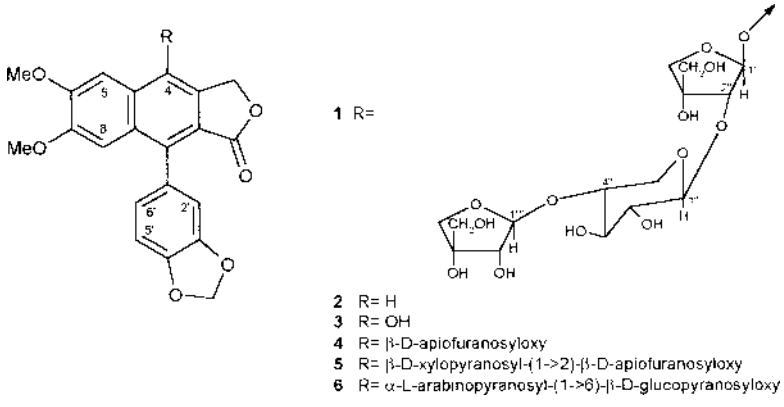

Fig. 1. Chemical Structures of Lignans **1**—**6**

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Table 1. ^{1}H- and ^{13}C-NMR Data (δ Values) of Lignans **1**, **3**—**5** in CD_3OD

Position	**1**		**3**		**4**		**5**	
	δ_C mult.	δ_H mult. (J, Hz)	δ_C mult.	δ_H mult. (J, Hz)	δ_C mult.	δ_H mult. (J, Hz)	δ_C mult.	δ_H mult. (J, Hz)
1	137.0	—	132.6	—	137.1	—	136.9	—
2	120.2	—	123.4	—	126.5	—	126.4	—
3	130.4	—	122.1	—	130.1	—	130.2	—
4	146.1	—	143.4	—	146.4	—	146.1	—
5	101.9	7.67 s	101.2	7.47 s	101.7	7.75 s	102.2	7.65 s
6	153.2	—	151.3	—	153.8	—	153.4	—
7	151.8	—	150.2	—	152.1	—	151.7	—
8	107.7	7.07 s	106.3	7.06 s	107.0	7.11 s	107.5	7.06 s
9	131.8	—	130.5	—	132.0	—	131.8	—
10	128.5	—	123.8	—	128.6	—	128.3	—
1′	129.9	—	128.5	—	130.1	—	129.9	—
2′	108.9	6.97 d (1.7)	110.9	6.83 d (1.5)	111.7	6.85 d (2.0)	109.0	6.96 d (1.5)
3′	149.1	—	147.5	—	149.2	—	149.2	—
4′	149.1	—	147.4	—	149.2	—	149.2	—
5′	111.6	6.81 d (8.0)	108.1	6.97 d (8.0)	108.7	6.99 d (8.0)	112.1	6.78 d (8.0)
6′	124.7	6.78 dd (1.5, 8.0)	123.8	6.79 dd (1.5, 8.00)	124.7	6.81 dd (2.0, 8.0)	124.8	6.76 dd (1.5, 8.0)
CH_2O	68.6	5.51 d (15.5)	66.2	5.24 s	68.7	5.54 d (13.0)	68.3	5.50 d (15.4)
		5.53 d (15.5)				5.60 d (13.0)		5.52 d (15.4)
6-OMe	56.4	4.05 s	56.1	3.99 s	56.2	4.06 s	56.5	4.03 s
7-OMe	55.7	3.74 s	55.8	3.72 s	55.7	3.77 s	55.2	3.73 s
OCH_2O	102.7	6.07 s	99.5	5.92 s	102.4	6.07 s	102.8	6.07 s
		6.09 s		5.98 s		6.09 s		6.09 s
C=O	172.3		170.2		171.8		172.3	
1″	111.2	5.67 d (1.8)			112.9	5.57 d (1.5)	111.7	6.65 d (1.8)
2″	85.9	4.74 d (1.8)			78.5	4.56 d (1.5)	85.8	4.75 d (1.8)
3″	80.7	—			79.5	—	81.0	—
4″	75.6	3.93 d (10.0)			75.7	3.97 d (10.0)	75.6	3.93 d (10.0)
		4.34 d (10.0)				4.38 d (10.0)		4.35 d (10.0)
5″	63.7	3.75 d (11.0)			64.2	3.71 br s	63.7	3.75 d (11.0)
		3.79 d (11.0)						3.79 d (11.0)
1‴	105.9	4.57 d (7.5)					106.3	4.56 d (7.5)
2‴	75.1	3.37 dd (7.5, 9.0)					75.3	3.33 dd (7.5, 9.0)
3‴	76.0	3.50 dd (9.0, 9.0)					77.4	3.40 dd (9.0, 9.0)
4‴	77.0	3.59 ddd					71.1	3.50 ddd
		(4.5, 9.0, 11.0)						(4.5, 9.0, 11.0)
5‴	64.7	3.32 dd (11.0, 11.0)					67.2	3.27 dd (11.0, 11.0)
		4.02 dd (4.5, 11.0)						3.84 dd (4.5, 11.0)
1⁗	109.2	5.06 d (2.0)						
2⁗	78.0	3.93 d (2.0)						
3⁗	80.3	—						
4⁗	75.0	3.81 d (10.0)						
		4.13 d (10.0)						
5⁗	64.7	3.60 br s						

Assignments confirmed by HSQC and HMBC experiments.

δ 5.06 suggested that this sugar was terminal; the interglycosidic linkages were established at C-2″ of the β-D-apiofuranose 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 compared with the corresponding shifts in unglycosylated models.[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-1″) 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-1⁗) and the carbon resonance at δ 77.0 (C-4‴). Thus patavine (**1**) was characterized as 4-*O*-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 *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.[1,2,12] In terms of lignan production, only shoot cultures are able to produce

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

		Native plants		Shoot cultures	
		Aerial parts	Roots	Shoots	Medium
Patavine	**1**	−	−	+	+
Justicidin B	**2**	+	+	+	−
Diphyllin	**3**	+	+	+	−
Tuberculatin	**4**	+	+	+	+
Majidine	**5**	+	−	+	+
Arabelline	**6**	+	−	+	+

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

Compound	IC_{50} (μl/ml)
Patavine	43.95±4.88
Diphyllin	7.55±0.75
Tuberculatin	13.92±1.26
Majidine	20.22±1.88
Arabelline	63.21±6.21

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**).

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 ^{1}H and 150.86 MHz for ^{13}C using the UXNMR software package was used for NMR measurements in CD_3OD solutions. 2D experiments: ^{1}H–^{1}H DQF-COSY, inverse-detected ^{1}H–^{13}C HSQC, and HMBC spectra were obtained by employing the conventional pulse sequences as described previously.[11] 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.[13] 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.[12] 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.[3]

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_2O–MeOH (9 : 1) and successively extracted with $CHCl_3$ and AcOEt; then the solvent was distilled and the residue dissolved in MeOH was analyzed. The liquid medium of shoot cultures was extracted with Et_2O (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 $CHCl_3$ 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).

Patavine (**1**): Amorphous powder; $[\alpha]_D^{24}$ −18° (c=1.1, MeOH); UV (EtOH) λ_{max} nm: 205, 224, 262, 290, 313, 350. HR-ESI-MS *m*/*z*: 799.2752 $[M+Na]^+$, 645 $[M+H-api]^+$, 381 $[M+H-2api-xyl]^+$; ^{1}H- and ^{13}C-NMR (CD_3OD): see Table 1.

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_2 atmosphere. Cytotoxicity of the lignans to LoVo cells was measured by the MTT assay as previously described by Mosmann.[14] The test was performed at least in triplicate. The IC_{50} values of compounds **1** and **3**—**6** are listed in Table 3.

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