Arylnaphthalene Lignans from in Vitro Cultures of *Linum austriacum*†

Abdolali Mohagheghzadeh,‡,§ Thomas J. Schmidt,⊥ and A. Wilhelm Alfermann*,‡

Heinrich-Heine-Universität Düsseldorf, Institut für Entwicklungs- und Molekularbiologie der Pflanzen, and Institut für Pharmazeutische Biologie, Universitätsstrasse 1, D-40225 Düsseldorf, Germany

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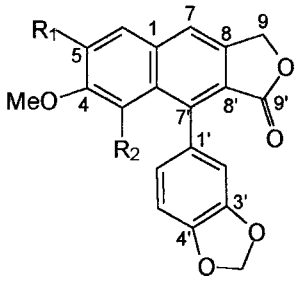
Callus, suspension, and normal and hairy root cultures of *Linum austriacum* produced a new arylnaphthalene lignan, 3,4-dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-dieno-9,9′-lactone (**1**, 0.03−0.73% dry wt), together with justicidin B (**2**, 0.18−1.69% dry wt). The structure of **1** was established using spectroscopic methods.

There is a growing interest in lignans and their synthetic derivatives due to application in cancer chemotherapy and various other pharmacological effects. These include anti-HIV, immunosuppressive, hypolipidemic, antifungal, and antiasthmatic activities and antagonism of platelet-activating factor.[1,2] Previous work has shown that in vitro cultures of *Linum* species (Linaceae) are useful for the production and accumulation of the pharmaceutically important lignans podophyllotoxin and 6-methoxypodophyllotoxin.[3] In this paper we describe the establishment of in vitro cultures of *L. austriacum* L. (callus, cell suspension, root, and hairy root cultures) as well as the isolation, structure elucidation, and quantification of two arylnaphthalene lignans, **1** and **2**, hitherto unknown to occur in the genus *Linum* and the Linaceae.



1 $R_1 = H$ $R_2 = OCH_3$

2 $R_1 = OCH_3$ $R_2 = H$

In a primary screening procedure for the distribution of lignans in *Linum* species, two "lignan-type" components were detected in callus extracts of *L. austriacum* by HPLC due to their UV spectra. Similar HPLC profiles were found in extracts of cell suspension cultures and untransformed and hairy root cultures. Chromatographic separation by preparative HPLC from extracts of the untransformed roots resulted in the isolation of compounds **1** and **2**.

Compound **1** showed a UV spectrum very similar to **2** but appeared in the HPLC chromatogram at a somewhat higher retention time. After isolation by preparative HPLC, GC-EIMS analysis revealed its nature as an isomer of **2** ([M]$^+$ at *m/z* 364). It showed a slightly different fragmentation pattern (see Experimental Section), indicating a difference in the substitution pattern of the aromatic systems.

As a major difference from **2**, the ^{1}H NMR spectrum of **1** lacked the singlet signals for H-3 and H-6 and showed two *ortho*-coupled proton resonances (δ 7.72 and 7.50 ppm, J = 9 Hz) instead, indicating either a 3,4- or a 5,6-dimethoxy substitution. Indicative of 3,4-substitution, the resonance of one of the two methoxy groups was shifted upfield by 0.74 ppm, from δ 4.07 to 3.33, which can be explained by shielding due to the ring current of the piperonyl group. Comparison of the data with those of the corresponding *retro*-lactone derivative (known from *Justicia hyssopifolia*[4]), where the lactone carbonyl is at C-9 instead of C-9', clearly showed that the −OCH_2O− is at 3′,4′ in **1**. As in **1**, the proton signal of C-3-OMe in the *retro*-lactone displayed a considerable upfield shift (δ 3.31 ppm) and H-5 and H-6 resonated as a pair of doublets with J = 9 Hz. At the same time, the most conspicuous difference was the resonance of H-7, which appeared at δ 7.82 in the case of **1**, while it occurred at much lower field (δ 8.4 ppm) in the *retro*-compound,[4] due to anisotropic deshielding by the carbonyl group.

To obtain ^{13}C NMR data for **1** despite the small sample amount available, a HMQC experiment was carried out, allowing ^{13}C NMR shift assignments for all protonated carbons. From an HMBC experiment, some resonances of quaternary carbons could also be assigned. All data taken together proved the structure of **1** as the 5-demethoxy-3-methoxy isomer of justicidin B ("isojusticidin B"), i.e., 3,4-dimethoxy-3',4'-methylenedioxy-2,7'-cycloligna-7,7'-dieno-9,9'-lactone.[5] This compound, to the best of our knowledge, has not been described before.

Compound **2** was identified by mass-spectrometric (GC-EIMS) and NMR spectroscopic analyses (^{1}H, ^{13}C, COSY, HMQC, and HMBC) as the arylnaphthalene lignanolide justicidin B, previously known from *Justicia* (Acanthaceae)[6,7] and *Haplophyllum* (Rutaceae)[8] species.

Furthermore, using quantitative HPLC the lignan content of suspension cultures at day 12 and of callus, root, and hairy root cultures at day 30 were determined (Table 1). These findings indicate the presence of **1** and **2** in all cultures. Therefore, we believe that *L. austriacum* cultures, especially the roots, are a potential system for studying the biosynthesis of this type as well as of lignans in general. As stated above, justicidin B (**2**) and similar arylnaphathalenes are known from several different plant families.[9] No such lignan, however, has been isolated from a member of the Linaceae before. Up to present, lignans of the aryltetralin type have been reported in the section *Syllinum*,[3,10]

† In honor of the 64th birthday of Prof. Dr. S. Hadi Samsam-shariat, formerly Professor of Pharmacognosy, Isfahan University of Medical Sciences and Health Services, Isfahan, I. R. Iran.

* To whom correspondence should be addressed: Tel: +49 211 811 4603. Fax: +49 211 811 1466. E-mail: alferman@rz.uni-duesseldorf.de.

‡ Institut für Entwicklungs- und Molekularbiologie der Pflanzen.

⊥ Institut für Pharmazeutische Biologie.

§ Permanent address: Department of Pharmacognosy, Faculty of Pharmacy, Shiraz University of Medical Sciences and Health Services, Shiraz, I. R. Iran.

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Table 1. Lignan Content (mg/g dry wt) in Cultures of *Linum austriacum*

compound	callus 30 days[a] medium I[b]	cell culture 12 days[a] medium II[b]	cell culture 12 days[a] medium III[b]	root 30 days[a] medium IV[b]	hairy root 30 days[a] medium VI[b]
1[c]	0.4	0.4	1.3	7.4	2.5
2	2.9	1.8	6.7	12.5	16.9

[a] Time of subcultivation. [b] Cultivation medium. [c] Compound **2** was used for quantification.

and simple acyclic, furanofuran- and dibenzylbutyrolactone-type lignans are known to be present in *L. usitatissimum* L.[10] (section *Dasylinum*,[11] section *Linum* according to refs 12, 13). Simultanously with the work described here, however, we isolated two compounds from cell cultures of *Linum altaicum* which were identified subsequently as the lignans **1** and **2**.[14] The present work shows for the first time the existence of arylnaphthalene lignans in a species of the Linaceae.

Experimental Section

General Experimental Procedures. UV spectra were measured on-line using a Thermo Quest (Egelsbach, Germany) HPLC system equipped with a Spectra System KO 6000 LP photodiode array detector. The column and the solvent system were as described below. NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on a Bruker DRX500 spectrometer at room temperature in $CDCl_3$. The data are referenced to the solvent signals (7.270 ppm C*H*Cl_3, 77.20 ppm *C*DCl_3). Mass spectra were obtained by GC-EIMS on a Hewlett-Packard MSD 5790 (EI at 70 eV) coupled to an HP 5890A GC. A HREIMS spectrum of compound **1** was obtained with a MAT 8200 spectrometer.

Plant Material. Seeds of *Linum austriacum* L. (Linaceae) were collected at Darestan Forest, Rhoudbar, Guilan Province, Iran, at an altitude of 900 m in June 1999. The plant material was collected and identified by Iraj Mehregan, Department of Pharmacognosy, Faculty of Pharmacy, Shiraz University of Medical Sciences and Health Services. A voucher specimen was deposited at the Institut für Entwicklungs- und Molekularbiologie der Pflanzen, Heinrich-Heine-Universität, Düsseldorf.

Cultures. Callus cultures were initiated from seedlings on the basal medium (MS) according to Murashige and Skoog[15] supplemented with sucrose (3%), naphthalene acetic acid (NAA, 1 mg/L), kinetin (0.5 mg/L), 2,4-dichlorophenoxyacetic acid (0.5 mg/L), and coconut milk (15%), solidified with agar (0.9%), at pH 5.6 before autoclaving. The medium was sterilized in an autoclave at 121 °C for 20 min (medium I). Callus cultures were subcultivated every 4 weeks. Suspension cultures were initiated by transfer of callus tissue into liquid medium (50 mL in 300 mL Erlenmeyer flasks) on a rotary shaker (120 rpm) and subcultivated every 2 weeks. As media were used medium II (= medium I, but without agar) or MS medium including NAA (0.4 mg/L) as the only plant hormone and without coconut water and agar (medium III). Callus and suspension cultures were grown under permanent light (150 μE m^{-2} s^{-1}). Root cultures were initiated from excised roots of seedlings in 1/2 B_5 liquid medium of Gamborg et al. (1962, medium IV).[16] Hairy roots were induced by direct incubation of segments from sterile grown plants with *Agrobacterium rhizogenes* strain LBA 9402 cultured in YMB medium[17] in the presence of 20 μM acetosyringone for 2 days in the dark. After 2 days, the explants were transferred to 1/2 B_5 solid medium (medium V) supplemented with 500 mg/L Claforan (Hoechst Marion Roussel, Frankfurt, Germany). When the "hairy roots" had been induced successfully (2−3 weeks later), they were excised and cultured in the (same, without agar) liquid medium (medium VI) containing the antibiotic, and the cultures were established by several subcultivations. Normal and hairy root cultures were grown under permanent dark on a rotary shaker (80 rpm) and refreshed by the new medium every 2 weeks. All mentioned cultures were maintained at 25 ± 1 °C.

Extraction, Isolation, and Determination of Lignans. A fine powder (0.5 g) of the lyophilized in vitro cultured normal roots was extracted with ethanol (5 mL) in an ultrasonic bath (two times for 30 s with cooling on ice for 90 s between). Distilled water (15 mL) was added, and the pH was adjusted to 5.0 by *o*-phosphoric acid. After adding β-glucosidase (2.5 mg), the sample was incubated at 35 °C for 1 h. Ethanol (30 mL) was added and the mixture incubated for another 10 min at 70 °C in an ultrasonic bath. After centrifugation, the supernatant was used for HPLC analysis using Thermo Quest (formerly LDC Milton Roy, Egelsbach, Germany) and Waters (Eschborn, Germany) instruments equipped with a Spectro Monitor 3200 UV-detector or a Waters 996 photodiode array detector, respectively.[18] Separation was performed using a Nucleosil 100-C_{18} column (8.0 mm i.d., 40 + 250 mm length, particle size 5 μm) and a gradient system with water (A) and acetonitrile (B) as eluents as follows: 0 to 17 min from 45 to 67% B, from 17 to 18 min to 50% B, and until 25 min back to 45% B. The flow rate was 3.0 mL/min between 0 and 11.0 min, 0.8 mL/min between 11 and 18.0 min, and again 3.0 mL/min between 18 and 25.0 min. Compounds **2** and **1** were collected at t_R 23.08 and 25.35 min, respectively. For quantification of the lignans, samples of 0.2 g of dried callus, cells, or roots were used and HPLC analysis was performed using a similar C_{18} column (4.6 mm i.d.) and gradient system (flow 0.8 mL/min). The detector wavelength was 290 nm. Isolated justicidin B (**2**) was used for quantification.

3,4-Dimethoxy-3′,4′-methylenedioxy-2,7′-cycloligna-7,7′-dieno-9,9′-lactone (isojusticidin B) (**1**): (<1 mg); UV (PDA detector of HPLC) λ_{max} 252, 290 nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 7.82 (1H, s, H-7), 7.72 (1H, d, J = 9.1 Hz, H-2), 7.50 (1H, d, J = 9.1 Hz, H-3), 6.89 (1H, d, J = 7.9 Hz, H-5′), 6.82 (1H, dd, J = 1.6, 7.9 Hz, H-6′), 6.04 (1H, d, J = 1.3 Hz, OCH_2O (a)), 6.03 (1H, d, J = 1.6 Hz, OCH_2O (b)), 5.37 (2H, 2s, $\Delta\nu$ = 1.3 Hz, CH_2-9), 3.97 (3H, s, OCH_3 at C-4), 3.33 (3H, s, OCH_3 at C-5); ^{13}C NMR ($CDCl_3$, 125 MHz) δ 171.5 (s, C-9'), 152.5 (s, C-4), 148.2 (s, C-4'), 147.8 (s, C-5), 139.5 (s, C-8), 126.4 (d, C-2), 123.3 (d, C-6'), 122.4 (d, C-7), 119.7 (d, C-3), 111.4 (d, C-2'), 108.6 (d, C-5′), 101.4 (t, O-*C*H_2-O), 69.2 (t, C-9), 62.5 (q, O*C*H_3 at C-4), 58.8 (q, O*C*H_3 at C-5). GC-EIMS *m*/*z* 364 $[M]^+$ (100), 333 (9), 319 (16), 303 (82), 291 (29), 276 (22),167 (29), 163 (31); HREIMS *m*/*z* 364.09436 (calcd for $C_{21}H_{16}O_6$, 364.09470).

4,5-Dimethoxy-3,4′-methylenedioxy-2,7′-cycloligna-7,7′-dieno-9,9′-lactone (justicidin B) (**2**): UV (PDA detector of HPLC) λ_{max} at 258, 296, 310 nm; all NMR spectroscopic data were in full agreement with reports.[6,7,19] GC EIMS *m*/*z* 364 $[M]^+$ (100), 335 (4), 319 (6), 305 (11), 291 (8), 277 (19), 167 (18), 163 (17).

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