Phytochemical and Biosynthetic Studies of Lignans, with a Focus on Indonesian Medicinal Plants
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Chapter 8

Production of a cytotoxic arylnaphthalene lignan using genetically transformed root cultures of *Linum leonii*

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Abstract

Callus and hairy roots cultures of *Linum leonii* F.W. Schulz. were established. The genetic transformation in hairy roots was proven by PCR analysis, which showed integration of *rol A* and *rol C* genes into the plant genome. Callus and hairy roots accumulated the arylnaphthalene lignan justicidin B as a major constituent. Hairy roots produced 5-fold higher yields of justicidin B (10.8 mg g\(^{-1}\) DW) compared to callus. Justicidin B demonstrated strong cytotoxicity to the chronic leukemic cell lines LAMA-8, K-562 and SKW-3 with IC\(_{50}\) values of 1.1, 6.1 and 1.6 μM, respectively. Apoptotic properties of justicidin B were reported for the first time.
Introduction

Justicidin B is an arylphenanthrene lignan which exerts cytotoxic (Joseph et al., 1988, MacRae et al., 1989), antiviral (Gertsch et al., 2003), fungicidal, antiprotozoal (Chen et al. 1996) and antiplatelet properties (Baba et al., 1996). Several tumor types including sarcomas and breast, prostate, and lung carcinomas grow in or preferentially metastasize the skeleton where they proliferate, and induce significant bone remodelling, bone destruction, and cancer pain (Mohagheghzadeh et al., 2002). Thus, justicidin B may have significant clinical utility as a lead compound in the management of bone cancer and osteoclastogenesis, due to its cytotoxic and bone resorption inhibitory properties. The potent bone resorption inhibitor justicidin B was used as a lead compound for design of new antirheumatic drugs (Sabino et al., 2002).

Justicidin B was first isolated from Justicia spp. (Acanthaceae) and Haplophyllum spp. (Rutaceae) (Okigawa et al., 1970, Pettit and Schaufelberger, 1988). Justicidin B has further been isolated from different Phyllanthus species (Euphorbiaceae) (Bachmann et al., 1993). It was shown that cell cultures of Linum austriacum produce justicidin B, which is the first report on the existence of arylphenanthrene lignans in a species of the Linaceae (Mohagheghzadeh et al., 2002). Since there is a growing interest in justicidin B due to its various pharmacological effects, the sustainable biotechnological supply of this valuable lignan would be a feasible alternative.

Our preliminary experiments exhibited that justicidin B is the main cytotoxic principle in the methanolic extract of callus of Linum leonii F.W. Schulz. (Linaceae) (Vasilev and Ionkova, 2005). Therefore, we decided to establish hairy roots from this species in hope of produce justicidin B in high yields. This paper describes the isolation, structure elucidation and cytotoxic evaluation of the major lignan produced by conventional and genetically transformed cultures of L. leonii.

![Fig. 1. Chemical structure of justicidin B.](image)

Material and methods

General experimental procedures

NMR spectra measurements were carried out on a Bruker WM 400 (400 MHz) and a Bruker DRX 500 (500 MHz) spectrometer in CDCl$_3$. TMS was used as an internal standard. GC analysis was performed on a Hewlett Packard 5890 series II gas chromatograph equipped with a 7673 autosampler, and a Hewlett Packard 3365 Chemstation software A10.02 under the following conditions: column: WCOT fused silica CPsil 5 CB lowbleed/MS, # CP7810; 15 m x 0.25 mm ID; film thickness 0.10 μm; (Varian Middelburg, The Netherlands); temperature program: 150°C – 320°C at 15°C min$^{-1}$ injector temperature 250°C; detector (FID) temperature 300°C; carrier gas: helium; inlet pressure 125 kPa; linear gas velocity 40 cm s$^{-1}$; split ratio 100:1; injected volume 1 μL. GC-MS analysis was performed on a
Shimadzu QP5000 GC-MS system equipped with a 17A GC, an AOC-20i autoinjector, and the GC-MS solution software 1.10. GC conditions: WCOT fused silica CPsil 5 CB lowbleed/MS, # CP7810, film thickness 0.10 μm; 15 m x 0.25 mm ID (Varian Middelburg, The Netherlands). Temperature program: 150°C – 320°C at 15 °C min⁻¹. Injector temp.: 275°C; inlet pressure: 75 kPa; column flow: 2.1 mL min⁻¹; linear velocity: 75.5 cm sec⁻¹; split ratio: 20:1; total flow: 46.2 mL min⁻¹; carrier flow (He): 46.2 mL min⁻¹; injection volume: 2 μL. Temperature program 35 min at 90°C, 90-170 °C at 4°C min⁻¹. MS conditions: ionization energy, 70 eV; ion source temperature, 250°C; interface temperature, 250°C; scan speed, 2 scans s⁻¹; mass range, 34-600 u

**Plant material**

The seeds of *Linum leonii* F.W. Schulz. (Linaceae) were a kind gift (No 1636; No com. 253/1999) from the botanical garden Nancy (France). Callus cultures were initiated and grown as described previously (Vasilev and Ionkova, 2005). Hairy roots were induced by direct incubation of segments from sterile grown plants with *Agrobacterium rhizogenes* strain ATCC 15834 cultured in YMB medium in the presence of 20 μM acetosyringone for 2 days in the dark, which increased susceptibility toward infection. The fast growing hairy roots were further maintained under permanent dark on a rotary shaker (80 rpm) and refreshed by a new medium every two weeks. Hairy root cultures were maintained at 25 ± 1 °C as described in Mohagheghzadeh et al. (2002).

**DNA analysis**

DNA isolation was conducted from the dry plant material of the intake plant, calli and hairy roots according to a protocol for rapid isolation from dry and fresh samples (Khanuja et al., 1999). The isolation of DNA of *A. rhizogenes* ATCC 15834 was performed following the instructions of Qiaprep spin miniprep kit from Qiagen (Westburg, BV, Leusden, The Netherlands). The integration of *rol A* and *rol C* genes from *A. rhizogenes* into the plant genome, which is the genetic evidence for hairy roots transformation, was proven by PCR reaction. Therefore the following specific primers were designed (Nader et al., 2004): for *rol A* gene, nucleotide positions 21-42 (5’-CGTTGTCGGAAT-GGCCAGACC-3’) and 268-246 (5’-CGTAGGTCTGAATAT-TCCGGTCC-3’), totally 248 bp; for *rol C* gene, positions 51-70 (5’-TGTGCAAGCAGCGATGAGC-3’) and 550-531 (5’-GATTGCAAAACTTGCAGACTCG-3’), a fragment of 490 bp totally. *Vir D2* gene is not involved in the plant genome during the transformation. The specific primers for the detection of *vir D2* are: primer A (5’-ATGCCCGATCGAGCTCAAGT-3’) and primer E (5’-CCTGACCCCAACATCTC-GTGGCCA-3’), ending in a fragment of 338 bp (Hass et al., 1995). All PCR reactions were performed in a Mastercycler® gradient thermocycler (Eppendorf) with recombinant taq DNA polymerase (Fermentas GMBH, St. Leon-Rot, Germany). The PCR program was 5 min at 95°C, 35 cycles of 30 s at 95°C, 40 s at 50°C, 2 min at 72°C and a final step of 5 min at 72°C.

**Extraction, isolation and quantification**

Air-dried plant material from hairy roots (10 g) was extracted with 80% MeOH (200 mL for 1 h sonification at 25°C). The extract was separated with 3 x 200 mL dichloromethane. Dichloromethane layers were filtered (Na₂SO₄ was used as a drying agent), combined, concentrated under reduced pressure at 50°C, dried and kept at -20°C. The initial amount of hairy roots yielded 780 mg dry dichloromethane extract. The dichloromethane extract was subjected to preparative TLC separation using silica gel 60 F254 (Merck): 10 x 20 cm, 2 mm, toluene: acetone 10:1, development length: 9 cm and λ = 254 nm. The most abundant fraction (Rₜ = 0.45) was pooled and evaporated to dryness consequently. The residue was further purified by recrystallisation in cold MeOH to yield 3.1 mg justicidin B. Quantitative determination of justicidin B in callus and hairy roots was performed by GC
analysis as described by Koulman et al. (2001). Cinchonidine was used as an internal standard. The response factor (RF) was calculated using 3 concentration ratios (3:1, 1:1, 1:3) between justicidin B and cinchonidine; RF=1.80 (CV=1.11%, n=5). The limit of detection (LOD) was established as the amount of analyte that provided a signal-to-noise ratio of 3. LOD was 0.1 µg mL$^{-1}$. The limit of quantification (LLOQ) was defined as the lowest calibration standard that could be quantified with an accuracy of 90-110% and a precision of 15%. LLOQ of justicidin B was 1 µg mL$^{-1}$. Intraday (n=6) and interday (n=5) coefficients of variations (CV) were determined. Intra-day CV from callus and hairy roots determinations are 7.4 and 4.9% respectively, and inter-day variations for callus and hairy roots analyses are 1.8 and 3.4% respectively.

**Leukemic cell lines and culture conditions**

The three leukemic cell lines LAMA-84, K-562 and SKW-3 were supplied from the German Collection of Microorganisms and Cell Cultures (DSMZ). The culture conditions are as previously described (Vasilev et al., 2005).

**Cytotoxicity assay**

The MTT-dye reduction assay was carried out as described by Mossmann (1983) with some modifications (Konstantinov, 1999). The clinically applied epipodophyllotoxin derivative etoposide was used as reference cytotoxic drug. Briefly, 100 µL aliquots of cell suspension (1x10$^5$ cells mL$^{-1}$) were seeded in 96-well microplates. Following 24 h incubation at 37°C the cells were exposed to the newly isolated lignan or to etopoide for 72 h. After the incubation period MTT solution (10 mg mL$^{-1}$ in PBS) was added (10 µL/well) and the plates were further incubated for 4 h at 37°C. Thereafter the formazan crystals formed were dissolved through addition of 100 µL/well 5% formic acid in 2-propanol (Merck) and the absorption of the samples was measured with an ELISA reader (Uniscan Titertec) at 580 nm. 100 µL RPMI 1640 medium (Sigma), 10 µL MTT stock and 100 µL 5% formic acid in 2-propanol served as a blank solution. The results were expressed as survival fraction (% of untreated control). All values were expressed as the mean ± SD (n=8). The data processing included the Student’s t-test with P ≤ 0.05 taken as significance level, using Microsoft EXCEL and OriginPlot software for PC.

**Apoptosis assay**

The DNA extraction and horizontal gel electrophoresis procedures were performed as previously described (Vasilev et al., 2005). About 5x10$^6$ SKW-3 cells – treated with justicidin B (at 0.25 or 0.5 µM) and untreated controls, were washed in PBS and spun at 1,800 x g for 5 min. The cell pellets were resuspended in 0.25 mL PBS and lysed through addition of 0.5 mL buffer containing 0.5% Triton X-100, 20 mM Tris-HCl and 1mM EDTA (pH = 7.4). Samples were incubated at 0°C (on ice) for 5 min and thereafter spun at 11,000 x g for 20 min. The supernatants were transferred into 2 mL ‘safe lock’ test tubes and then 0.937 mL 2-propanol as well as 0.187 mL 6 M solution of NaCl was added to each sample. The tubes were gently agitated and incubated at −20°C for 12 h in order to allow precipitation of the hydrophilic DNA. The samples were centrifuged for 20 min at 11,000 g, the supernatants were decanted and DNA was washed in 1 mL ice cold 70% ethanol and then air dried. The isolated DNA was redissolved in 20 µL distilled water and analyzed by gel electrophoresis in 0.8% agarose gel. Finally DNA was stained with ethidium bromide and visualized using an UV transilluminator and photographed with a fixed digital camera (Bio Doc ITTM system).
Results and discussion

Callus cultures were developed as previously described (Vasilev et al., 2005). The genetically modified cultures demonstrated typical hairy roots phenotype: intensive branching, hormone autotrophy and lack of geotropism. The most vigorous growth was observed when the bacterial growth stopped and there was no further necessity of antibiotic.

To our knowledge, no reports on the induction of hairy roots from L. leonii have been published till now. We performed DNA analysis in order to confirm the hairy roots transformation. The TL region in plasmid T-DNA of the agropine-type strain A. rhizogenes 15834 contains 18 open reading frames including several loci called rol (root loci). The products encoded by rol A and rol C genes were found to have a synergistic effect on root induction and induce increased sensitivity to auxin (Slightom et al., 1996, Day et al., 1999). PCR analysis showed that hairy roots from L. leonii contain rol C and rol A genes (Fig. 2, lanes 3 and 4) corresponding to the positive controls obtained by DNA from A. rhizogenes ATCC 15834 (lanes 7 and 6). Untransformed callus served as a negative control (lane 2). Vir D2 was not detected in the hairy roots (lane 5), thus showing that T-DNA is incorporated in the plant genome and it is not a residual bacterial contamination.

![Fig. 2. PCR analysis of L. leonii roots transformed by A. rhizogenes ATCC15834. Lane 1 – DNA marker; lane 2 – untransformed L. leonii plantlets; lanes 3 and 4 – DNA from L. leonii hairy roots in which rol C of 490 bp and rol A of 248 bp integration was positive; lane 5 – DNA from hairy roots not expressing vir D2; lanes 6, 7 and 8 – positives controls of A. rhizogenes DNA showing roal A, rol C, and vir D2 (338 bp) respectively.](image)

We undertook isolation and identification of the main component in hairy roots of L. leonii by preparative TLC and subsequent recrystallisation in cold MeOH. This isolate was analyzed by means of GC-MS and NMR. The EI-MS of the isolated compound showed a m/e value of 364 and mass fragmentation, that is consistent with the data for an arylnaphthalene lignan (Okigawa et al., 1970). Further NMR experiments were performed in order to distinguish between justicidin B and isojusticidin B as these two isomers have no different MS fragmentation pattern. A closer look at the $^1$H NMR spectrum showed that the proton signals at $\delta$ 7.12 ppm and $\delta$ 7.05 ppm appeared as singlets, which is indicative for 4,5-substitution (Okigawa et al., 1970). Therefore the resonance signals at $\delta$ 7.12 ppm and $\delta$ 7.05 ppm were assigned to H-6 and H-3 respectively, due to the shielding effect of the piperonyl group on H-3. Thus the isolated compound was unambiguously identified as justicidin B. The $^1$H NMR data is in full agreement with a previous report of justicidin B (Pettit and Schaufelberger, 1988).

Callus and hairy roots of L. leonii retained the capability to produce justicidin B. This finding supports the hypothesis that arylnaphthalene lignans are characteristic of the section Linum. Hairy roots accumulated 5 times higher amounts of justicidin B (10.8 mg g$^{-1}$ DW) than the conventional cultures of callus (Table 1). The content of justicidin B in L. leonii hairy roots after 14 days period is very close to
justicidin B levels produced for 30 days in normal root cultures and transformed roots of *L. austriacum*: 12.5 and 16.9 mg g\(^{-1}\) DW, respectively (Mohagheghzadeh et al., 2002). However, hairy roots from *L. leonii* produced lower amount of justicidin B, compared to the highest yields in intact plants: 3-4 % in *P. piscatorum* (Gertsch et al., 2003). Therefore further optimization of hairy roots cultures is needed to compete with the levels of justicidin B produced by intact plant.

<table>
<thead>
<tr>
<th>Culture type</th>
<th>Justicidin B (mg g(^{-1}) DW)</th>
<th>Variation (CV %)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intraday (n=6)</td>
<td>Interday (n=5)</td>
</tr>
<tr>
<td>Callus</td>
<td>2.01</td>
<td>7.4</td>
</tr>
<tr>
<td>Hairy roots</td>
<td>10.8</td>
<td>4.9</td>
</tr>
</tbody>
</table>

Table 1. Content of justicidin B in cell cultures.

In addition to the current data, we underwent further cytotoxicity examination of justicidin B on three chronic myeloid leukemia-derived cell lines, LAMA-84, K-562 and SKW-3, that show a lower responsiveness to cytotoxic drugs due to the strong expression of the fusion oncoprotein BCR-ABL (a non-receptor tyrosine kinase). The IC\(_{50}\) values of screened leukemic cell lines were determined (Table 2). As evident from the presented results (Fig. 3) both compounds caused concentration-dependent cytotoxic effects in the panel of human tumor cell lines under investigation. Justicidin B proved to be slightly less active in respect to relative potency. At the higher concentrations (10 µM), however, it inhibited the proliferation of malignant cells at the same extend as the referent drug etoposide.

The electrophoretic analysis of DNA, isolated from the cytosolic fraction of SKW-3 after 24 h treatment cells with 0.5 and 0.25 M justicidin B evoked oligonucleosomal DNA fragmentation (Fig. 4). The observed DNA laddering is a consequence of the action of specific nucleases which degrade the higher order chromatin structure during the apoptotic process. Therefore it is firmly established that the primary cytotoxic effect of justicidin B is mediated by activation of the programmed cell death pathways.

Hairy roots of *L. leonii* demonstrated a high biosynthetic capacity. The major active constituent justicidin B can be easily isolated in reasonable amounts from the genetically transformed root cultures. To our knowledge this is the first report of justicidin B isolated from the hairy roots of *L. leonii* as well as the first report on the apoptotic properties of this valuable arylnaphthalene lignan. Therefore optimization of *L. leonii* hairy roots is worthy of further consideration as an alternative production system of justicidin B, which is used as a template for the development of potential new therapeutic agents.

Table 2. Relative potency of justicidin B and etoposide.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>IC(_{50}) value (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Justicidin B</td>
<td>Etoposide</td>
</tr>
<tr>
<td>LAMA-84</td>
<td>chronic myeoid leukemia</td>
<td>1.1</td>
</tr>
<tr>
<td>K-562</td>
<td>pre-B-cell lymphoma</td>
<td>6.1</td>
</tr>
<tr>
<td>SKW-3</td>
<td>chronic lymphoid leukemia</td>
<td>1.6</td>
</tr>
</tbody>
</table>
Fig. 3. Concentration response curves for justicidin B (■) and etoposide (Δ) following 72 h treatment of LAMA-84 (A), K-562 (B) and SKW-3 (C), as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments. The error bars indicate the corresponding standard deviation.
Fig. 4. Imaging of DNA laddering induced by justicidin B treatment. DNA was extracted from the cytosolic fraction of untreated $5 \times 10^6$ SKW-3 cells (1) or following 24 h exposure to justicidin B at 0.25 µM (2) or 0.5 µM (3).

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