Production of Justicidin B, a Cytotoxic Arylnaphthalene Lignan from Genetically Transformed Root Cultures of *Linum leonii*

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Callus and hairy root cultures of *Linum leonii* 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. Calli and hairy roots accumulate the arylnaphthalene lignan justicidin B as a major constituent. Hairy roots produce 5-fold higher yields of justicidin B (10.8 mg g^{-1} DW) compared to calli. Justicidin B shows strong cytotoxicity on the chronic myeloid leukemia LAMA-8 and K-562 cell lines and on the chronic lymphoid leukemia SKW-3 cell line with IC_{50} values of 1.11, 6.08, and 1.62 μM, respectively. Apoptotic properties of justicidin B are reported for the first time.

Justicidin B is an arylnaphthalene lignan that exerts cytotoxic,[1] antiviral,[2,3] fungicidal, antiprotozoal,[4] and antiplatelet properties.[5] The potent bone resorption inhibitor justicidin B was used as a lead compound for the design of new antirheumatic drugs.[6] 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 remodeling, bone destruction, and cancer pain.[7] 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. Justicidin B was first isolated from *Justicia* spp. (Acanthaceae) and *Haplophyllum* spp. (Rutaceae).[8,9] Justicidin B has further been isolated from different *Phyllanthus* species (Euphorbiaceae).[10,11] It was shown that cell cultures of *Linum austriacum* produce justicidin B, which was the first report on the occurrence of arylnaphthalene lignans in a species of the Linaceae.[8] Since there is a growing interest in justicidin B due to its various pharmacological effects, the sustainable biotechnological production of this valuable lignan would be a feasible alternative to the supply from natural sources.

Results and Discussion

Our preliminary experiments exhibited that justicidin B is the main cytotoxic principle in the methanolic extract of calli of *Linum leonii* F.W. Schulz. (Linaceae).[12] Therefore, we decided to establish hairy roots from this species in the hope of producing 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*.

Calli cultures were developed as previously described.[12] The genetically modified cultures demonstrated a typical hairy root phenotype: intensive branching, hormone autotrophy, and lack of geotropism. After 9 days, since no bacterial growth was observed, cefotaxime was not added in the medium and the hairy roots showed vigorous growth.

No report on the induction of hairy roots from *L. leonii* has been published so far. We performed analysis of the transferred DNA (T-DNA) from *Agrobacterium rhizogenes* to *L. leonii* cells in order to confirm the hairy root transformation.[13] The T-DNA has left (T_L) and right (T_R) borders. The T_L region in plasmid T-DNA of agropine-type strain *A. rhizogenes* 15834 contains 18 open reading frames including several loci called *rol* (root loci).[14] 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.[15] PCR analysis showed that hairy roots from *L. leonii* contain *rol* C and *rol* A genes (Figure 1, lanes 3 and 4) corresponding to the positive controls obtained by DNA from *A. rhizogenes* ATCC 15834 (lanes 7 and 6). Untransformed calli served as a negative control (lane 2). The second gene system necessary for T-DNA transfer consists of virulence (*vir*) genes encoded by a region outside the T-DNA. The protein coded by *vir* D_2 provides an endonuclease that initiates the transfer process by nicking T-DNA at a specific site.[13] *vir* D_2 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.

The main component in hairy roots of *L. leonii* was isolated by preparative TLC and consequent recrystallization in cold MeOH. This isolate was analyzed by means of GC-MS and NMR. The EIMS of the isolated compound showed an ion at *m*/*z* 364, which is consistent with the data for an arylnaphthalene lignan.[8] Further NMR experiments were performed in order to distinguish between justicidin B and isojusticidin B (Figure 2), as these two isomers have slightly different MS fragmentation patterns. The ^{1}H NMR spectrum revealed that the proton signals at δ 7.12 and 7.05 appeared as singlets, which is reconcilable with 4,5-dimethoxy substitution only.[8] The resonances at δ 7.12 and 7.05 were assigned to H-6 and H-3, respectively, due to the shielding effect of the piperonyl group from the pendant ring. Thus, the isolated compound was unambiguously identified as justicidin B. The ^{1}H NMR data are in full agreement with literature data.[9]

Calli 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} dry weight (DW)) than the conventional cultures of calli (2.01 mg g^{-1} DW). This content of justicidin B produced by *L. leonii* hairy roots after a 14-day period is very close to justicidin B levels produced for 30 days in normal root cultures (12.5 mg g^{-1} DW) and transformed roots (16.9 mg g^{-1} DW) of *L. austriacum*.[8] However, hairy roots from *L. leonii* produced a lower amount of justicidin B, compared to the highest yields in the wild plants: 3−4% in *Phyllanthus piscatorum*.[4] Therefore further optimization

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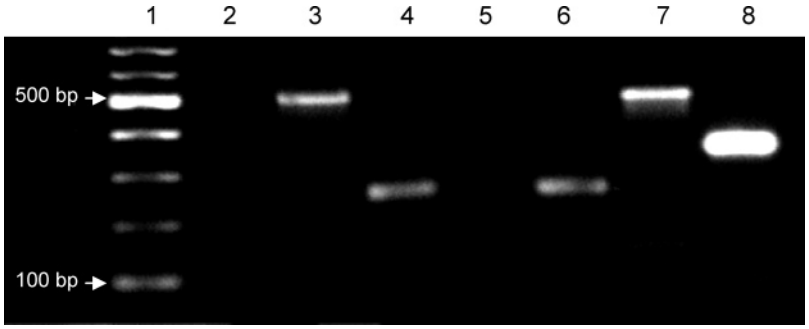

Figure 1. PCR analysis of *L. leonii* roots transformed by *A. rhizogenes* ATCC15834. Lane 1, DNA marker; lane 2, untransformed *L. leonii* plantlets; lanes 3 and 4, DNA from *L. leonii* hairy roots in which *rol* C of 490 bp and *rol* A of 248 bp integration was positive; lane 5, DNA from hairy roots not expressing *vir* D_2; lanes 6, 7, and 8, positive controls of *A. rhizogenes* DNA showing *rol* A, *rol* C, and *vir* D_2 (338 bp), respectively.

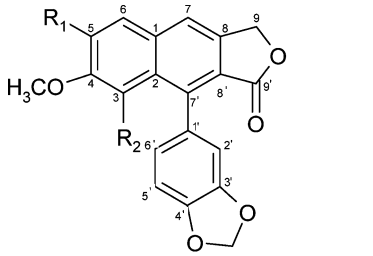

Figure 2. Structures of justicidin B and isojusticidin B.

Table 1. Relative Potency of Justicidin B and Etoposide

		IC_{50} value (μM)	
cell line	cell type	justicidin B	etoposide
LAMA-84	chronic myeloid leukemia	1.11	0.79
K-562	chronic myeloid leukemia	6.08	1.87
SKW-3	chronic lymphoid leukemia	1.62	0.82

of hairy root cultures is needed to reach the levels of justicidin B produced by the wild plants.

In addition to the current data, we investigated further cytotoxicity of justicidin B and etoposide on two chronic myeloid leukemia-derived LAMA-84 and K-562 cell lines, which show a lower response to cytotoxic drugs due to the strong expression of the fusion oncoprotein BCR-ABL (a nonreceptor tyrosine kynase), and on the chronic lymphoid leukemia SKW-3 cell line. The IC_{50} values of screened leukemic cell lines were determined (Table 1). As evident from the results (Figure 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 than etoposide with respect to relative potency. At the higher concentrations, however, it inhibited the proliferation of malignant cells to the same extent as the reference drug etoposide.

The electrophoretic analysis of DNA, isolated from the cytosolic fraction of SKW-3 after 24 h treatment of the cells with 0.5 and 0.25 μM justicidin B, showed oligonucleosomal DNA fragmentation (Figure 4). The observed DNA laddering is a consequence of the action of specific nucleases that 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 high biosynthetic capacity. The major active constituent justicidin B can be easily isolated in reasonable amounts from the genetically transformed root cultures. This is the first report of justicidin B having been 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 may be used as a template for the development of potential new therapeutic agents.

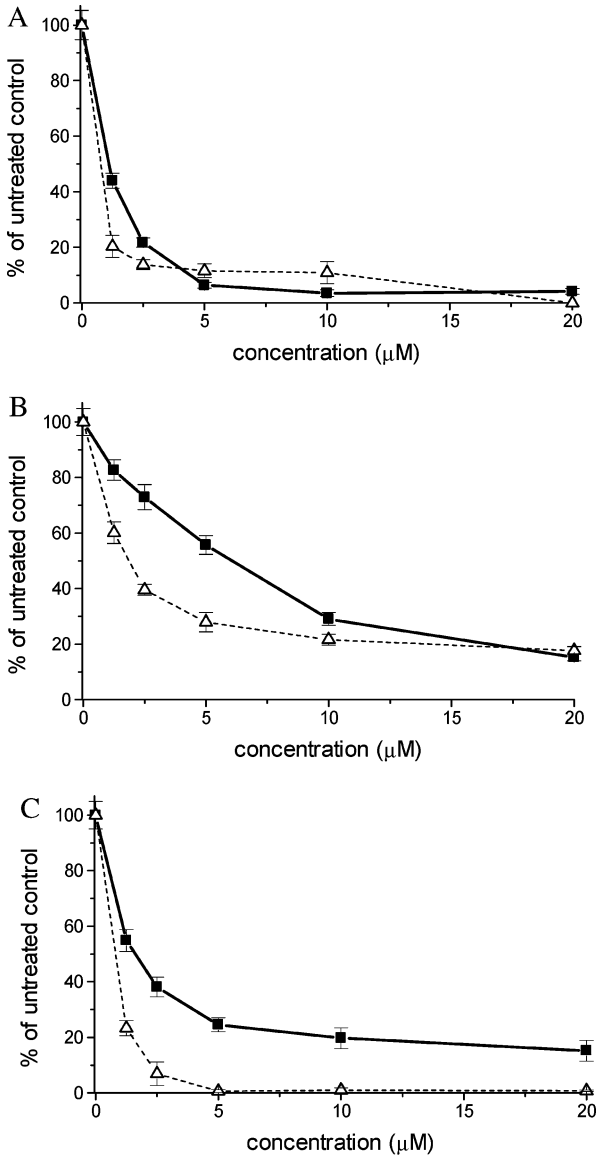

Figure 3. Concentration response curves for justicidin B and etoposide following 72 h treatment of LAMA-84 (A), K-562 (B), and SKW-3 (C), as assessed by the MTT-dye reduction assay. Each data point represents the arithmetic mean of at least 8 independent experiments. The error bars indicate the corresponding standard deviation.

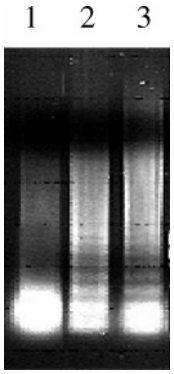

Figure 4. Imaging of DNA-laddering induced by justicidin B treatment. DNA was extracted from the cytosolic fraction of untreated 5 × 106 SKW-3 cells (1) or following 24 h exposure to justicidin B at 0.25 μM (2) or 0.5 μM (3).

Experimental Section

General Experimental Procedures. NMR spectra measurements were carried out on Bruker WM 400 (400 MHz) and Bruker DRX 500 (500 MHz) spectrometers in $CDCl_3$. TMS was used as an internal standard.

GC. GC analysis was performed on a Hewlett-Packard 5890 series II gas chromatograph equipped with a 7673 autosampler and Hewlett-Packard 3365 Chemstation software A10.02 under the following conditions: column, WCOT fused silica CPsil 5 CB lowbleed/MS, 15 m × 0.25 mm i.d.; film thickness, 0.10 μm (Varian Middelburg, The Netherlands); temperature program, 150−320 °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. GC-MS analysis was performed on a Shimadzu QP5000 GC-MS system equipped with a 17A GC, an AOC-20i autoinjector, and the GCMS solution software 1.10. GC conditions: WCOT fused silica CPsil 5 CB lowbleed/MS, film thickness, 0.10 μm; 15 m × 0.25 mm i.d. (Varian Middelburg, The Netherlands); temperature program, 150−320 °C at 15 °C min^{-1}; injector temperature, 275 °C; inlet pressure, 75 kPa; column flow, 2.1 mL min^{-1}; linear velocity, 75.5 cm s^{-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. Cultures. The seeds of *L. leonii* 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.[12] 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 as described earlier.[8]

DNA Analysis. DNA isolation was conducted from the dry plant material of intake plant, calli, and hairy roots according to a protocol for rapid isolation from dry and fresh samples.[16] The isolation of DNA of *A. rhizogenes* ATCC 15834 was performed following the instructions of Qiaprep spin miniprep kit from Qiagen (Westburg, b.v., 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 root transformation, was proven by PCR reaction. Therefore the following specific primers were designed: [17] for the *rol* A gene, nucleotide positions 21−42 (5′-CGTTGTCG-GAATGGCCCAGACC-3′) and 268−246 (5′-CGTAGGTCT-GAATAT-TCCGGTCC-3′), totally 248 bp; for *rol* C gene, positions 51−70 (5′-TGTGACAAGCAGCGATGAGC-3′) and 550−531 (5′-GATTGCAAACTTGCACTCGC-3′), a fragment of 490 bp totally. The *vir* D_2 gene is not involved in the plant genome during the transformation. The specific primers for the detection of *vir* D_2 are as follows: primer A (5′-ATGCCCGATCGAGCTCAAGT-3′) and primer E (5′-CCTGACCCAAACATCTCG-GCTGCCCA-3′), ending in a fragment of 338 bp.[18] 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 × 200 mL of CH_2Cl_2. CH_2Cl_2 layers were filtered, combined, dried (Na_2SO_4), concentrated under reduced pressure at 50 °C, dried, and kept at −20 °C. The initial amount of hairy roots yielded 780 mg of dry CH_2Cl_2 extract. The CH_2Cl_2 extract was subjected to preparative TLC separation using silica gel 60 F_{254} (Merck): 10 × 20 cm, 2 mm, toluene/acetone, 10:1, development length 9 cm, and λ = 254 nm. The most abundant fraction (R_f = 0.45) was pooled and evaporated to dryness. The residue was further purified by recrystallization in cold MeOH to yield 3.1 mg of justicidin B. Quantitative determination of justicidin B in calli and hairy roots was performed by GC analysis as described.[19] The response factor (RF) was calculated as described earlier,[20] using cinchonidin as an internal standard; 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. LODs were 0.1 μg mL^{-1}. The limit of quantification (LOQ) was defined as the lowest calibration standard that could be quantified with an accuracy of 90−110% and a precision of 15%. LOQ of justicidin B was 1 μg mL^{-1}. Intra-day (n = 6) and inter-day (n = 5) coefficients of variations (CV) were determined. Intra-day CV from callus and hairy root determinations are 7.4 and 4.9%, respectively, and inter-day variations for callus and hairy root 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 obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The culture conditions are as previously described.[21]

Cytotoxicity Assay. The MTT-dye reduction assay was carried out as described by Mossmann[22] with some modifications.[23] The clinically applied epipodophyllotoxin derivative etoposide was used as reference cytotoxic drug. Briefly, 100 μL aliquots of cell suspension (1 × 10^5 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 etoposide for 72 h. After the incubation period MTT solution (10 mg mL^{-1} in PBS) was added (10 μL per well) and the plates were further incubated for 4 h at 37 °C. Thereafter the formazan crystals were dissolved through addition of 100 μL per well of 5% HCO_2H in 2-propanol (Merck), and the absorption of the samples was measured with an ELISA reader (Uniscan Titertec) at 580 nm; 100 μL of RPMI 1640 medium, 10 μL of MTT stock, and 100 μL of 5% HCO_2H 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 $p \leq 0.05$ taken as significance level.

Apoptosis Assay. The DNA extraction and horizontal gel electrophoresis procedures were performed as previously described.[21] About 5 × 106 SKW-3 cells, treated with justicidin B (at 0.25 or 0.5 μM) and untreated controls, were washed in PBS and spun at 2000 rpm for 5 min. The cell pellets were resuspended in 0.25 mL of PBS and lysed through addition of 0.5 mL of buffer

containing 0.5% Triton X-100, 20 mM Tris-HCL, and 1 mM EDTA (pH = 7.4). Samples were incubated at 0 °C (on ice) for 5 min and thereafter spun at 13 000 rpm for 20 min. The supernatants were transferred into 2 mL Safe-Lock test tubes, and then 0.937 mL of 2-propanol and 0.187 mL of a 6 M solution of NaCl were 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 13 000 rpm, the supernatants were decanted, and DNA was washed in 1 mL of ice-cold 70% EtOH and then air-dried. The isolated DNA was redissolved in 20 μL of distilled H_2O 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).

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