The Large-Scale Isolation of Deoxypodophyllotoxin from Rhizomes of *Anthriscus sylvestris* Followed by Its Bioconversion into 5-Methoxypodophyllotoxin β-D-Glucoside by Cell Cultures of *Linum flavum*

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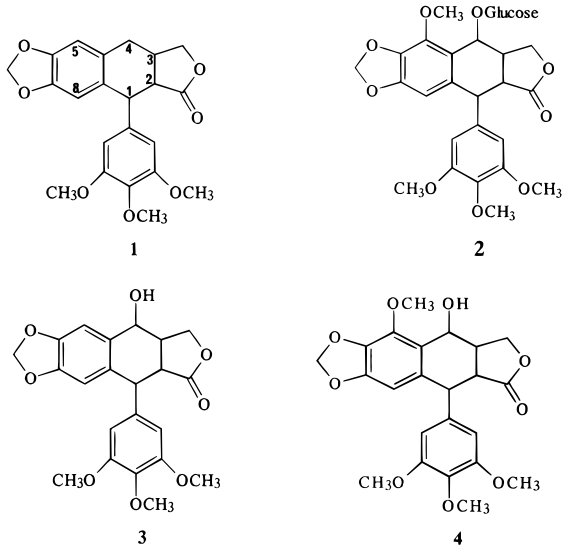
Dried rhizomes of *Anthriscus sylvestris* contained 0.39% deoxypodophyllotoxin (**1**). An isolation procedure with an extraction efficiency of 51.2% was developed for this commercially unavailable lignan. The isolated **1** was subsequently complexed with dimethyl-β-cyclodextrin and fed to cell suspension cultures of *Linum flavum* by which it was bioconverted into 5-methoxypodophyllotoxin-β-D-glucoside (**2**). After 7 days the cells contained 4.41% of this product on a dry-weight basis. An isolation procedure for compound **2** was developed, with an extraction efficiency of 83.4%.

Podophyllotoxin (**3**) is a naturally occurring lignan, that is extracted from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae). It serves as a starting compound for the preparation of the semisynthetic cytostatics etoposide (VP-16-213) and teniposide (VM-26).[1] The supply of *P. hexandrum* rhizomes, which contain ca. 4% of compound **3** on a dry-weight basis, has become limited due to both intensive collection and lack of cultivation.[2] Therefore, the production of **3** and related lignans by means of biotechnological procedures would be an interesting alternative. In the biosynthetic pathway for aryltetralin lignans as proposed by Broomhead *et al.*,[3] the direct precursor of **3** is deoxypodophyllotoxin (**1**).

Recently, **1** was chosen as a substrate for bioconversion experiments with cell suspensions of *P. hexandrum*, which normally accumulate lignan **3**. Because **1** is poorly water soluble, it was added to the cell cultures as a dimethyl-β-cyclodextrin complex. In addition, similar experiments were performed with cell suspensions of *Linum flavum* (Linaceae; yellow flax), which normally accumulate 5-methoxypodophyllotoxin (**4**).[4] Compound **4** has a cytotoxicity that is comparable to that of **3**, so it may also be of interest as a starting compound for new or improved cytostatics.[5] The *P. hexandrum* cell suspensions converted **1** into **3**, while in the *L. flavum* cultures 5-methoxypodophyllotoxin β-D-glucoside (**2**) was formed. The contents as measured after bioconversion were high, respectively: 2.9% and 2.4% on a dry-weight basis.

Because lignan **1** is commercially unavailable, it must be isolated from a suitable natural source for use as a substrate in bioconversion studies. Compound **1** occurs in the rhizomes of *Anthriscus sylvestris* (Apiaceae; wild chervil) a common perennial indigenous to The Netherlands.[6,7]

In this paper, a large-scale isolation procedure of compound **1** from the rhizomes of *A. sylvestris* is presented. In addition, its bioconversion into the glucoside **2** by cell cultures of *L. flavum* and the subsequent isolation of this product is described.



1 **2** **3** **4**

The *A. sylvestris* rhizomes lost 71% H_2O upon drying and contained 0.39 ± 0.02% of **1** on a dry-weight basis ($n = 10$), corresponding to 6.83 g in the total amount of 1.75 kg of plant material. After Soxhlet extraction, 86.1% of **1** originally present in the plant material was extracted. In the extracted rhizomes **1** could not be detected anymore, thus 13.9% of **1** was lost during the extraction procedure, probably due to decomposition.

The first column chromatographic purification of the extract caused no loss of **1**, but (colored) impurities were removed. During the second column chromatographic purification 4.8% of **1** was lost. The HPLC chromatogram of the collected fractions now showed two peaks one of compound **1** and one of an unknown compound. The latter was removed on an Amberlite XAD-2 column. The peak ratio of compound **1**:unknown compound increased from 1.28 to 1.75, while the loss of **1** was only 1.5%. Finally, crystallization yielded 3.5 g of **1**, corresponding to an extraction efficiency of 51.2%.

On the basis of HPLC analysis, the isolated **1** had a purity of ca. 98%. TLC showed a spot with the same color and with the same R_f value (0.71) as the reference compound **1** (prepared by chemical reduction of **3**). The melting point of **1** is in agreement with earlier reported values of 167.4−168.3 °C.[11] The IR spectrum as well

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as the ^{13}C-NMR spectrum corresponded with those of the reference **1**.

The presence of cyclodextrin complexed **1** had no effect on the growth of the *L. flavum* cell cultures in terms of packed cell volume, dry weight, fresh weight, and conductance. During the past 5 years, the use of cyclodextrins in order to solubilize apolar substrates in aqueous environments has proven to create smooth conditions for various bioconversions carried out in our laboratory.[11]

Recently, compound **4** and its β-D-glucoside (**2**) were produced in cell cultures of *L. flavum* by bioconversion of cyclodextrin-complexed **1** (1.4 mM).[4] After 7 days 2.38% of combined **2** and **4** on a dry-weight basis was found, corresponding with a production rate of 35.6 mg L^{-1} day^{-1}. On the basis of these results, we harvested the plant cells in the present study after 7 days of incubation with the substrate. The cells contained 4.41% **2** on a dry-weight basis, corresponding with a production rate of 71.2 mg L^{-1} day^{-1} and a bioconversion percentage of 75.3%. This is a very high production rate, taking the structural complexity of the lignan into account.[13] The bioconversion of compound **1** into the glucoside **2** comprises at least four steps: two hydroxylations, a methylation, and a glucosylation.

The percentage of H_2O in the extraction fluid has been described to determine whether **2** is extracted as the glucoside itself or as the corresponding aglucone and can be explained by the presence of a β-glucosidase in the cells.[14] In the present study cells were extracted with different mixtures of MeOH−H_2O. Compound **2** was completely extracted as its glucoside with a MeOH percentage of 70% or higher. Lower concentrations of MeOH increased the amount of aglucone. The total amount of extracted **2** either as aglucone or as glucoside was maximal between 20% and 80% MeOH. On the basis of these results a mixture of 80% MeOH and 20% H_2O was used in order to isolate the lignan **4** completely in its glucosidic form. It was proven that in *L. flavum* cell cultures **1** is bioconverted into **2** and not into its corresponding aglucone. After purification of the extract by column chromatography on Si gel (no loss), the combined fractions containing the glucoside were extracted with CH_2Cl_2. Repeating the extraction 12 times caused a loss of only 1.5%. HPLC analysis showed that the combined fractions did not contain only pure glucoside **2** but that also an unknown impurity was present. Final purification was achieved by column chromatography over Si gel with PhMe−Et_2O−MeOH as the eluent. This step resulted in 15.3% loss of **2**. The yield of **2** with a purity of 99% based on HPLC analysis was 2.0 g, equivalent to an extraction efficiency of 83.4%. The identity of the glucoside was confirmed by adding β-glucosidase to a solution of **2**, which resulted in the formation of **4**. Finally, ^{13}C-NMR data of **2** were in agreement with those published previously for compound **4**,[8,15] and six additional peaks corresponding with the carbon atoms of the attached glucose moiety were present.

In conclusion, a procedure was developed to obtain large quantities of compound **1** from *Anthriscus sylvestris*. This lignan was shown to be a suitable substrate for bioconversion into **2** by cell cultures of *L. flavum* at a high production rate. The bioconversion product was successfully isolated using the procedure described. The unique combination of an unlimitedly available substrate and cell cultures with a high bioconversion capacity offer possibilities for the biotechnological production of the cytotoxic lignans **3** and **4**.

Experimental Section

General Experimental Procedures. Compound **1** (prepared at the Department of Organic and Molecular Inorganic Chemistry, University of Groningen, The Netherlands), compounds **2** (kindly provided by TNO-Zeist, The Netherlands) and **4** (isolated from cell cultures of *L. flavum*[8]) were analyzed by HPLC on a Lichrosorb 7RP-18 column (100 × 3 mm i.d.; Chrompack, Middelburg, The Netherlands) with MeOH−H_2O (45:55; v/v) as the mobile phase at a flow rate of 1.0 mL min^{-1} and detection at 290 nm.[9] For the determination of **1** in the dried rhizomes of *Anthriscus sylvestris*, 50 mg was extracted with 4.0 mL MeOH for 1 h followed by centrifugation (5 min, 1500 *g*). For the determination of **2** in *Linum flavum* cells, 20 mg of dried material was extracted by ultrasonication with 2 mL of various mixtures of MeOH−H_2O for 1 h followed by centrifugation (5 min, 1500 *g*). For the hydrolysis of **2** into its aglucone (**4**), 1.0 mL of the above mentioned extracts of *L. flavum* cells, was evaporated to dryness and redissolved in 0.10 mL MeOH. Then 2 mL of a 2.5% (m/v) solution of β-glucosidase (Sigma, Chemical Co.) in phosphate buffer (0.1 M, pH = 5.0) was added, and the mixture was incubated at 37 °C. Samples of 0.5 mL were taken after 2, 5, 6, and 72 h. After addition of 2.0 mL MeOH they were vortexed and centrifuged (5 min, 1500 *g*). TLC was performed using Si gel F_{254} plates (Merck, cat. no. 5715). The mobile phase was $CHCl_3$−MeOH (100:4; v/v). The spots were visualized after a 10 s bath in H_2SO_4−MeOH (1:20; v/v) and subsequent heating at 110 °C for 10 min. IR spectra (KBr disks) were recorded on a ATI Mattson FTIR infrared spectrophotometer. ^{13}C-NMR spectra (Attached Proton Test) were recorded on a Varian VXR-500 (125 MHz) system with $CDCl_3$ as the solvent. The melting points were determined using an Electrothermal IA 9100 melting point apparatus an are uncorrected.

Extraction and Isolation of Deoxypodophyllotoxin (1). Dried rhizomes of *A. sylvestris* (1.75 kg) were macerated overnight in 18 L of MeOH followed by Soxhlet extraction for 14 h, and the solvent was removed under reduced pressure. The residue was subjected to column chromatography (20 × 11 cm i.d.) over Si gel 60 (Fluka Chemika cat. no. 60741; 1045 g) with *n*-hexane−Me_2CO (2:1; v/v) as the eluent. Fractions containing **1** were pooled and evaporated to dryness using a rotary evaporator. Columm chromatography (120 × 10 cm i.d.; Si gel 60, 3711 *g*) was repeated with the same eluent. Pooled fractions containing **1** were dried under reduced pressure and chromatographed (70 × 5 cm i.d.) over Amberlite XAD-2 (Fluka Chemika cat. no. 06443; 678 g) with MeOH as the eluent. Pooled fractions containing **1** were evaporated to dryness using a rotary evaporator and redissolved in 100 mL MeOH of 60 °C and the volume was slowly reduced to about 50 mL under stirring and a gentle stream of air. After stirring overnight, crystals of compound **1** were harvested by filtration on a Büchner-funnel, washed with a small quantity of ice-cold MeOH and dried at room temperature. The yield was 3.5 g of **1** with a purity of 98% based on the peak heights in the HPLC chromatogram.

Bioconversion of Deoxypodophyllotoxin (1) into 5-Methoxypodophyllotoxin-β-D-glucoside (2). Cell suspension cultures of *L. flavum* were obtained and grown as described previously.[10] For bioconversion, 2.39 g (6 mmol) of the isolated **1** was dissolved in 105 mL MeOH−*n*-BuOH (1/4; v/v). To three sterilized 1-L bottles, 35 mL of this solution was added aseptically through a 0.2-μL filter. The solvent was evaporated in a laminar air-flow cabinet. Culture media were supplemented with 2,6-dimethyl-β-cyclodextrin (Avebe, Veendam, The Netherlands; 4 mM) and autoclaved (121 °C, 20 min). Of this medium 1 L was added to the bottles containing **1**, and they were shaken until **1** had completely dissolved. In 500-mL flasks, 200 mL of medium with **1** as a cyclodextrin complex was inoculated with 125 mL of a 2-week-old cell suspension of *L. flavum*, yielding a final concentration of **1** of 1.23 mM. The cells were harvested after 7 days by filtration and frozen at −20 °C followed by lyophilization.

Isolation and Purification of 5-Methoxypodophyllotoxin β-D-glucoside (2). Dried *L. flavum* cells (55.2 g) were extracted with 2 L of MeOH 80% (v/v) by ultrasonication for 1.5 h. After filtration and squeezing, the cell material was extracted again with 700 mL of 80% (v/v) MeOH. The solvent was removed by rotary evaporation yielding 22 g of raw extract, which was further extracted by ultrasonication with 300 mL H_2O for 40 min. The suspension obtained was chromatographed on a column (20 × 11 cm i.d.), with 850 g Si gel 60; H_2O was used as the eluent at 10 mL/min. Fractions containing **2** were pooled, concentrated to a volume of 600 mL under reduced pressure, and extracted 12 × with 300-mL portions of CH_2Cl_2. After each extraction **2** was determined in the H_2O phase. The CH_2Cl_2 fractions yielded 2.6 g residue that was subjected to column chromatography (97 × 1.8 cm i.d.) over Si gel 60 (95 g) with PhMe−Et_2O−MeOH (10:10:7; v/v/v) as the eluent. Fractions containing **2** were pooled and dried under reduced pressure. The residue was redissolved in 50 mL H_2O, frozen at −20 °C and lyophilized. The yield was 1.0 g of **2** with a purity of 99% on the basis of the peak heights in the HPLC chromatogram.

Deoxypodophyllotoxin (1): white crystals, mp 166.7−168.2 °C (MeOH); IR ν_{max} (KBr) 2937, 2842, 1765 (C=O), 1587, 1504, 1418, 1331, 1225, 1123, 1034, 941, 857, 768 cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 174.8 (C-12), 152.3 (C-3′ + C-5′), 146.9 (C-7), 146.6 (C-5), 136.8 (C-6), 136.1 (C-10), 130.5 (C-1′), 128.1 (C-4′), 110.3 (C-9), 108.3 (C-8), 108.1 (C-2′ + C-6′), 101.0 (C-13), 71.9 (C-11), 60.6 (C-4′ − OCH_3), 56.1 (C-3′ − OCH_3 + C-5′ − OCH_3), 47.3 (C-2), 43.6 (C-1), 33.0 (C-4), 32.6 (C-3).

5-Methoxypodophyllotoxin β-D-glucoside (2): white crystals, mp 155−165 °C (H_2O) with effervescence; IR ν_{max} (KBr) 3425 (OH), 2938, 2902, 2839, 1774 (C=O), 1618, 1589, 1507, 1476, 1420, 1252, 1125, 1071, 1036, 996 cm^{-1}; ^{13}C NMR ($CDCl_3$) δ 174.22 (C-12), 152.36 (C-3′ + C-5′), 149.71 (C-7), 141.89 (C-5), 136.72 (C-6), 134.74 (C-1′), 134.50 (C-4′), 132.64 (C-9), 122. 18 (C-10), 107.98 (C-2′ + C-6′), 104.75 (C-8), 101.66 (C-13), 71.97 (C-11), 69.16 (C-4), 60.60 (C-4′ − OCH_3), 60.01 (C-5 − OCH_3), 56.30 (C-3′ − OCH_3 + C-5′ − OCH_3), 45.79 (C-2), 44.19 (C-1), 38.66 (C-3) glucoside: additional δs at 76.30, 75.65, 74.34, 72.93, 56.15 (CH's), and 61.24 (CH_2).

Plant Material. Plants of *A. sylvestris* Hoffm. (Apiaceae) were harvested on June 2, 1993, from a road side at Lage Meeden, The Netherlands. A voucher specimen is deposited in our institute. The rhizomes were cut from the plants, and the clay was removed by washing with H_2O. The rhizomes were freeze dried, cut into pieces, ground, and sieved (1 mm).

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