Podophyllotoxin

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RIJKSUNIVERSITEIT GRONINGEN

Podophyllotoxin

A Study of the Biosynthesis, Evolution, Function and Use of Podophyllotoxin and Related Lignans.

Proefschrift

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Our goal is to discover that we have always been where we ought to be. Unhappily we make the task exceedingly difficult for ourselves.
Aldous Huxley, in: The Doors of Perception

We need a plan.
Col. H. Smith

Voor Jan
Voor Niesko

Contents

I. Introduction

Based on: Albert Koulman, Wim J. Quax and Niesko Pras, Podophyllotoxin and Related Lignans Produced by Plants. Will be published as a chapter in Biotechnology: Medicinal Plants, K. G. Ramawat (ed.).

GENERAL INTRODUCTION

Lignans form a group of important plant metabolites. This group receives much attention in the field of natural product chemistry ever since the discovery of podophyllotoxin. Lignans are defined as ãã-dimers of phenylpropanoid derivatives, and are widely spread throughout the plant kingdom. The present interest for this group of natural products is based on their application in the fields of pharmacy and nutrition.

One group of lignans, those with an enterolactone-like structure, has drawn the attention as food additives. These lignans may have a preventive role in breast cancer, due to their phytoestrogenic activity. Different studies claim this cancer preventive activity based on *in vitro* and animal experiments (Cho *et al.*, 1999) and epidemiological data (Adlercreutz, 1999).

The attention of pharmacists for lignans in general and podophyllotoxin in particular is due to the pronounced cytotoxic activity of a number of these compounds. Several plant species accumulate the 2,7'cyclolignan podophyllotoxin, which is used as the starting compound for the production of three semi synthetic anticancer drugs. Most commonly used due to its antineoplastic properties is etoposide, which was developed in 1966 and received FDA approval in 1983. A phosphate analogue, etopophos, produced by Bristol-Myers Squibb Co. was FDA approved in 1996. The third derivative is teniposide, which is less frequently used for chemotherapy in comparison with etoposide (Hande, 1998). Podophyllotoxin is one of the eye-catching results from medicinal plant research.

However, the isolation of podophyllotoxin from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae) plants is not a very ideal production system. The *P. hexandrum* rhizomes may contain ca. 4% of podophyllotoxin on a dry weight basis. The supply becomes increasingly limited due to both intensive collection and lack of cultivation (Choudhary *et al.*, 1998). *Podophyllum peltatum* contains lower amounts of podophyllotoxin (Rai *et al.*, 2000). The species *Podophyllum hexandrum* is listed in appendix II of CITES (convention for international trade in endangered species). This appendix lists species that are not necessarily now threatened with extinction but that may become so unless trade is closely controlled (World Conservation Monitoring Centre, 2001).

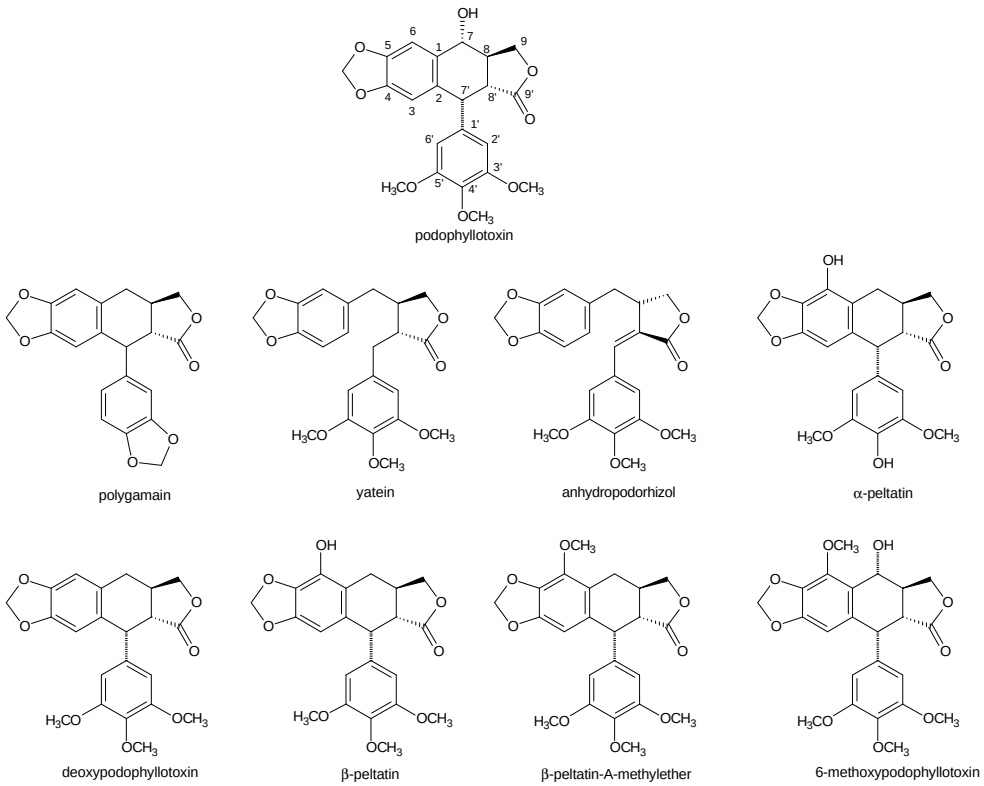
This supply problem forms the drive for a large number of scientists to search for alternative sources of podophyllotoxin. The chemical synthesis of podophyllotoxin is possible (Hadimani *et al.*, 1996; Medarde *et al.*, 1996), but largely hampered by the complicated stereochemical ring closure necessary to attain this compound. Synthetic production therefore only yields restricted quantities at high costs. An ideal resource would be a fast growing, easy to cultivate plant, with a high lignan content, preferably in the aerial parts. A biotechnological approach, aimed at expressing lignan synthetic enzymes in an efficient host cell, might also solve the supply

problem. The third option would be the semi synthetic production of lignans via organic synthesis in combination with plants enzymes that are able to catalyse certain complex reaction steps.

In this chapter we will give an overview of the current status of research dealing with plants producing podophyllotoxin and related lignans. Related lignans can be either lignans with 2,7'-cyclolignano-9,9'-lactone skeleton (following the IUPAC nomenclature by Moss, 2000) without any further oxidation of the 2,7'-ring, or lignans that are considered to be precursors in the podophyllotoxin biosynthesis. The chemical structures of podophyllotoxin and a number of important related lignans are depicted in Fig. 1. There are many more lignans and neolignans but these are not discussed in this thesis.

The subjects discussed range from pharmacological activity, pharmaceutical analysis, taxonomy of lignan-producing plants, and the biosynthesis of lignans and their ecological role to finally the design of biotechnological production systems.

Fig. 1.
Podophyllotoxin and its related lignans



PHARMACOLOGY AND USE OF PODOPHYLLOTOXIN AND DERIVATIVES

Background

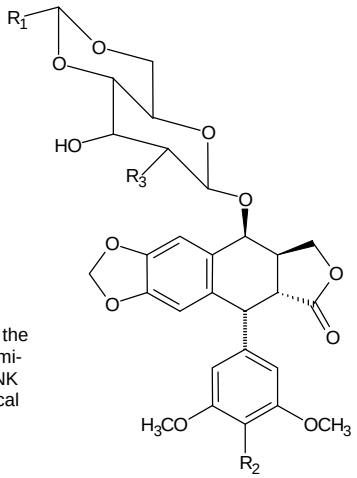
As early as in the first century A.D., Pliny the Elder mentions that the smaller species of *Juniperus* could be used, among other things, to stop tumours or swelling ("tumores" in Latin; König and Hopp, 1993). Dioscorides mentions the use of the oil of *Juniperus* species (*J. sabina*, *J. phoenicea* and *J. communis*) for the treatment of ulcers, carbuncles and leprosy (Gunther, 1959). Generally, dried needles, called savin, or the derived oil was used. In 47 A.D., Scribonius Largus wrote that savin oil was used to soften "hard female genital parts" (Sconocchia, 1983). Later references indicated the use of savin to treat uterine carcinoma, venereal warts and polyps (Hartwell and Schrecker, 1958). At present, we know that the pharmacological activity of *J. sabina* needles is to be ascribed to the lignans deoxypodophyllotoxin and podophyllotoxin (see Fig.1; Hartwell *et al.*, 1953a).

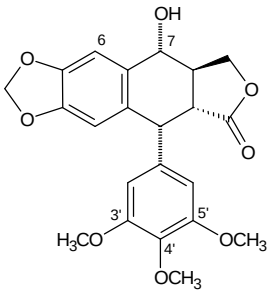
The Leech book of Bald, 900-950 A.D., an early English medicinal book, has reported on the use of root of *Anthriscus sylvestris*. These roots contain podophyllotoxin related lignans such as deoxypodophyllotoxin and were used in ointments prepared from a large number of plants and plant extracts like savine to cure cancer (Cockayne, 1961). In Japan and China the roots of this plant were also used as a kind of crude drug called "Zengo" in Japan (Kozawa *et al.*, 1982) and "qianhu" in China (Kozawa *et al.*, 1978).

Surprisingly a Chinese article states that: "the root is soaked in water, and then crushed and pulverised in a dry atmosphere for use as food. In addition, the young aerial part of this plant is sometimes used for food" (Kozawa *et al.*, 1982). Even though this was still practised in the eighties, the authors do not mention any form of intoxication.

The plant species that are currently used for the extraction of podophyllotoxin, are *Podophyllum peltatum* (May apple or American mandrake) and *Podophyllum hexandrum* (syn. *P. emodi*). *P. peltatum* is indigenous to the eastern part of the United States of America and Canada. *Podophyllum hexandrum* is found in the higher parts of the Himalayan Mountains and is also referred to as the Indian *Podophyllum*. The Natives of the Himalayas as well as the American Indians independently discovered that extracts of the rhizomes possessed a cathartic action. The Indians introduced podophyllin, a resin obtained by ethanolic extraction of the *Podophyllum* roots and rhizomes, to colonists for the use as a catharic, an anthelmentic and misuse as a lethal poison. The main constituents in podophyllin are the lignans podophyllotoxin, 4'-demethylpodophyllotoxin and á- and â-peltatin (see Fig. 1). The colonists also used this resin as an emetic and cholagogue. Podophyllin was included in the first U.S.

Pharmacopoeia, dating from 1820, as a cathartic and cholagogue. Because of its severe toxicity the drug was removed from the 12th edition of this Pharmacopoeia, that appeared in 1942 (Ayres and Loike, 1990; Horwitz and Loike, 1977). In the same year however, it was reported that venereal warts (*Condyloma acuminata*) could be selectively destroyed by the topical application of podophyllin. Nowadays, this resin is still in use to treat this disease (Frega *et al.*, 1997), but also pure podophyllotoxin is applied (Gross, 2001). The use of pure podophyllotoxin, in creams (like Wartec®) and gels (Condylox®), is nowadays recommended instead of podophyllin. The main advantage of pure podophyllotoxin over podophyllin is the higher efficacy and the absence of quercetin and kaempherol that may be responsible for several side-effects (von Krogh *et al.*, 2000; Wiley *et al.*, 2002). In the Netherlands genital warts represent one of the most common sexual transmitted diseases (SOA Stichting, 2003) and without treatment this infection may lead to cervical cancer and other anogenital malignancies (Wiley *et al.*, 2002). Podophyllotoxin is therefore an important weapon in the fight against sexually transmitted diseases.





podophyllotoxin

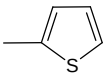
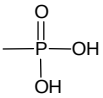
	R_1 =	R_2 =	R_3 =
etoposide (VP 16-213)	—CH_3	—OH	—OH
teniposide (VM 26)		—OH	—OH
etopophos	—CH_3		—OH
NK 611	—CH_3	—OH	—$N(CH_3)_2$

Fig. 2.
The clinically applied cytostatics etoposide, teniposide and etopophos. Podophyllotoxin serves as the starting material for the semi-synthesis of these drugs. NK 611 is currently in the clinical trials used as a new anti-cancer agent

Development of Podophyllotoxin based cytostatics

In 1947 the first reports appeared dealing with the inhibition of growth of cancer in animals by podophyllin (Horwitz and Loike, 1977). Due to their high cytotoxicity the constituents of podophyllin induce severe gastrointestinal side effects, as is also the case with their glycosides, thus limiting their clinical use as a cytostatic (Stähelin and Von Wartburg, 1991). With the aim to find clinically applicable drugs, related to these naturally occurring lignans, several series of glycoside and aglycon derivatives have been prepared by chemical synthesis by Sandoz. This resulted in the development of etoposide in 1966.

This semi-synthetic entered clinical trials in 1971 and it took another 12 years(!) before etoposide became FDA approved. By that time Bristol-Myers Squibb Co. took over license for both etoposide and teniposide (see Fig. 2). In 1996 the phosphate analogue etopophos (see Fig. 2) was also approved. A detailed history of these compounds has been recently described (Imbert, 1998). Etoposide is still used, often in combination with cisplatin and for instance bleomycin, for the treatment of metastatic testicular germ-cell tumours (Flechon *et al.*, 2001). Etoposide alone is also used for the treatment of small cell lung cancer (Mascaux *et al.*, 2000).

The mode of action of Podophyllotoxin

Podophyllotoxin inhibits the formation of the microtubules. *In vitro* it binds to tubulin dimers giving podophyllotoxin-tubulin complexes. This stops the further formation of the microtubules at one end but does not stop the disassembly at the other end leading to the degradation of the microtubules. Cells treated with podophyllotoxin are arrested in the metaphase of the mitosis. Its mode of action is comparable to the alkaloid colchicin and for their mode of action these compounds are called spindle poisons. Other spindle poisons in clinical use are paclitaxel and vincristine-like alkaloids. These cause the cells to enter the mitosis, but the duplicated chromosomes will not be separated. In this way the cells cannot duplicate and growth stops. The specific interaction of these compounds with the microtubuli growth is, however, different as they stop degradation and not assembly (Ayres and Loike, 1990; MacRea and Towers, 1984; Stähelin and Von Wartburg, 1991).

Some structure activity relationship studies have been carried out using several podophyllotoxin analogues, showing that the core structure of deoxypodophyllotoxin is responsible for the cytotoxicity. The extra methoxy group (6-methoxypodophyllotoxin, Fig. 1) on the 6-position does not significantly change in the *in vitro* cytotoxicity compared to podophyllotoxin. Also the methyl group on the 4'-position of the pendent ring is of little effect on the cytotoxicity (Hadimani *et al.*, 1996; Middel *et al.*, 1995).

The mode of action of etoposide

The clinically applied podophyllotoxin-derivatives etoposide, teniposide and etopophos have a completely different mode of action. These compounds are topoisomerase II inhibitors. Topoisomerase II is an enzyme that cleaves double-stranded DNA and seals it again after unwinding. It is crucial in the processes of DNA replication and repair. For its function topoisomerase II binds covalently to the broken DNA. Etoposide and other derivatives stabilise the DNA-topoisomerase II complex in such a way that resealing of the DNA strands becomes impossible. Cells that are duplicating their DNA for the mitosis are very sensitive for this mechanism. The overall effect of these anticancer drugs is the arrest of the cells in late S or early G2 phase of the cell cycle (Hande, 1998; Stähelin, 1973). A major advantage of the newly introduced etopophos (etoposide phosphate) is the improved solubility in water. Etopophos is a pro-drug of etoposide. After administration the phosphate group is hydrolysed in the human body to yield etoposide, which is bioactive. Because of its hydrophilic property etopophos can be administered much easier (Witterland *et al.*, 1996). Several new derivatives of etoposide are currently in the clinical phase of studies. For instance NK 611 (Fig. 2) has an improved water solubility and has also a topoisomerase II blocking activity (Hanauske *et al.*, 1995; Mross *et al.*, 1996).

Recently, a Swedish company (Conpharm) started clinical trials with a new podophyllotoxin derived drug CPH82 (Reumacon®) for the treatment of rheumatoid arthritis. Reumacon® is a mixture of two podophyllotoxin glucosides (podophyllotoxin-4,6-O-benzylidene-â-D-glucopyranoside, AS 3738 and 4'-demethylpodophyllotoxin-4,6-O-benzylidene-â-D-glucopyranoside, AS 3739; Carlstrom *et al.*, 2000).

It can be concluded that nowadays lignans are compounds of interest for the pharmaceutical industry. Podophyllotoxin is an important starting compound to prepare semi-synthetic cytostatics. An investigation of related lignans may lead to new cytostatic compounds, which can be at the basis of new anti-tumour drugs.

More detailed information on the pharmacological activity of lignans and their derivatives can be found in a number of reviews (Bernasconi, 1989; Damayanthi and Lown, 1998; Hande, 1998; Imbert, 1998).

ANALYSIS OF PODOPHYLLOTOXIN AND RELATED LIGNANS

Background

There can be several reasons to develop an analytical procedure for podophyllotoxin and related lignans. First there is a demand for quantitative and qualitative analytical procedures for profiling lignans during production, for instance for testing podophyllotoxin-producing plants. The second main field of podophyllotoxin analysis is concentrated on the clinic, i.e. the body fluids of patients treated with semi-synthetic analogues of podophyllotoxin.

In this part we will mainly focus on the first topic. Different techniques are available; some of which are developed for the quantitative analysis of lignans while others are more suitable for qualitative detection.

Most commonly used are thin layer chromatography (TLC) and high performance liquid chromatography (HPLC), in lesser extend also liquid chromatography coupled to mass spectrometry (LC-MS), gas chromatography (GC) and GC coupled to mass spectrometry (GC-MS). Micellar electrokinetic capillary chromatography (MECC) can also be used for the analysis of lignans but this new method is until now rarely applied.

TLC

Thin layer chromatography (TLC) is a suitable method for the rapid screening of large numbers of samples, for example for monitoring the isolation procedure of a certain compound or for the selection of high producing plants. Generally a polar stationary phase is used such as silica 60. The mobile phase consists of apolar solvent (e.g. $CHCl_3$) mixed with a more polar solvent (MeOH) in ratio of 10 to 1 or 25 to 1 (Broomhead and Dewick, 1990a; Van Uden *et al.*, 1989; Van Uden *et al.*, 1990d). Most lignans can be detected in these systems but the discrimination between yatein and deoxypodophyllotoxin is not possible (see Fig. 1 for the structures). The lignans can be detected using 254 nm UV light in combination with coated plates. It is possible to immerse the plate in a mixture of MeOH and concentrated H_2SO_4 followed by heating at 100°C for 10 minutes. The lignans are then carbonised leaving grey or purple spots (Wichers *et al.*, 1991; Woerdenbag *et al.*, 1990).

For the isolation of lignans preparative thin layer chromatography is still commonly practised (Jiao *et al.*, 1998; Rahman *et al.*, 1990b).

HPLC

High performance liquid chromatography (HPLC) is the most common and widely applied method for the quantitative and qualitative analysis of lignans. Almost all published methods use a reversed phase (RP-18) stationary phase. Some of the older publications mention the use of straight phase columns, which involved silica 60 columns with a eluens consisting of heptane, $CHCl_3$, and MeOH (Van Uden *et al.*, 1989; Wichers *et al.*, 1990). The routinely applied reversed phase columns; e.g. Lichrosorb or Nucleosil are combined with an isocratic mobile phase consisting of water and MeOH or MeCN. In some cases a gradient is used to enable changes in the H_2O / MeOH or H_2O / MeCN ratios.

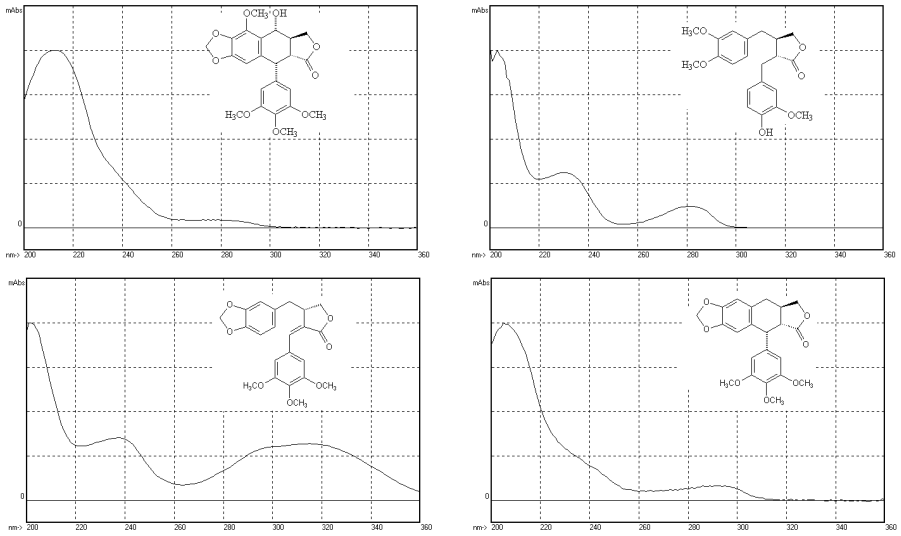


Fig. 3.
The UV spectra of 6-methoxypodophyllotoxin (top left) arcteginin (top right) anhydropodorhizol (bottom left) and deoxypodophyllotoxin (bottom right). These spectra were taken with a Shimadzu diode array detector, with: H_2O:MeCN (60:40) eluens and a RP-18 column.

For the detection UV-spectrophotometry is used. Lignans have a very distinct UV absorption spectrum. The UV spectra of 2,7'-cyclolignans have generally a maximal absorbance around 290 nm. For qualitative analysis a diode array detection (DAD) system is very suitable. The substitutions at the aromatic ring have a clear effect on the UV spectrum and some examples are shown in Fig. 3. Also an extra double bond is easily recognised in the UV-spectra. Podophyllotoxin and most of the related lignans can be identified with the aid of modern software combined with DAD and an UV-spectrum library.

Fewer methods have been published dealing with LC-MS for lignans. LC-MS was used for the first time in clinical chemistry to analyse

etoposide in human serum. This LC system consisted of a standard HPLC system as described above coupled to an atmospheric pressure ionization-electronspray inlet for the mass spectrometer, selectively scanning for ions with a *m/z* of 589 in the positive mode (Chen and Uckun, 2000).

Another method, that was initially developed for the detection of phenolic constituents in *Krameria triandra* roots, should basically be applicable for the detection of podophyllotoxin or related lignans in plant material. This method showed clearly the presence of other phenylpropanoids like flavonoids and neolignans. Here also a standard HPLC-system with a gradient of water/MeOH to MeCN was used. The HPLC was coupled to fast atomic bombardment MS/MS (FAB-MS/MS) using xenon as bombarding gas (Facino *et al.*, 1997).

Recently a study was published in which Wong and co-workers analysed plant material with an atmospheric pressure ionisation tandem mass spectrometer coupled to HPLC (API-LC/MS/MS). The authors analysed different samples of *Podophyllum emodi*. A MeOH water gradient (from 35% MeOH to 65% MeOH over an hour) with a Hypersil BDS column was applied. It was possible to detect podophyllotoxin, its glycoside and several derivatives, e.g. podorhizol â-D-glycoside (Wong *et al.*, 2000). This system is extremely useful for the analysis of plant material, because it yields qualitative and quantitative data. Such systems can play a crucial role in the elucidation of the lignan biosynthesis.

Seidel and co-workers used LC-MS to monitor the metabolic fate of ^{13}C – labelled phenylpropanoids in cell cultures of *Linum album*. For the quantification of incorporation they used an HPLC system connected to a triple-quadrupole tandem mass spectrometer. Samples were analysed by electrospray ionisation (ESI) employing the Micromass Z-Spray source. Elution was carried out on an RP-18 Luna column (50 mm long, 2 mm i.d.; 3 µm particle size; isocratically at a flow rate of 0.2 ml min^{-1} using two solvent mixtures: A (60% water, 40% acetonitrile; v/v) and B (50% water, 50% acetonitrile; v/v). Mass spectra were recorded in the positive and/or negative mode using multiple reaction monitoring (MRM). Ion transitions for MRM (collision energy 10-15 eV) were selected by tandem mass spectrometry (MS/MS) experiments (product ion scans) with reference compounds (Seidel *et al.*, 2002).

The combination of HPLC and ^{1}H-NMR is suitable for the qualitative analysis of plant material. It has been applied on leaves and needles of *Torreya jackii* that produce matairesinol derivatives (Zhao *et al.*, 1999). LC-NMR can yield some additional information about the stereochemistry. For a definite structure elucidation compounds still have to be isolated and subjected to thorough ^{13}C and 2-dimensional NMR techniques.

GC

Gas chromatography is rarely used for the detection of lignans. The main reason is that lignans as such are not really suited for GC-analysis. Lignans like podophyllotoxin are not volatile due to the molecular mass of over 400 and their polarity. However, the advantage of GC analysis is that it can be

combined with electron impact ionisation mass spectrometry. This allows analysing unknown lignans and to detect and interpret structural modifications after bioconversion experiments with enzyme systems or metabolic degradation.

GC-MS is used in the field of nutritional research, e.g. to investigate the metabolic fate of the phytoestrogenic lignans matairesinol and arctigenin. It is possible to analyse these compounds with a GC-MS system after derivatisation with for instance N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). This will shield the free hydroxyl groups of the lignan molecule with a trimethylsilyl moiety, making the lignan apolar enough for GC analysis. For the analysis a silica coated capillary column is used with a high temperature programme (140° to 245°C) coupled to electron ionisation mass spectrometry (EIMS; Morton *et al.*, 1999).

The use of GC-MS for the analysis of lignans is further discussed in chapter III.

Capillary chromatography

There are only two publications on the use of capillary chromatography for analysis of lignans. Micellar electrokinetic capillary chromatography (MECC) was used to separate the lignans present in *Podophyllum* resin (Ganzera *et al.*, 1999). A recent study showed that one of the advantages of MECC could be the separation of diastereomeres of podophyllotoxin. (Liu *et al.*, 2002). Maybe the combination of MECC with MS can be an alternative for LC-MS with respect to the analysis of lignan glycosides in the near future but only under the provision that a higher resolution can be reached in comparison to LC.

PLANTS PRODUCING PODOPHYLLOTOXIN AND RELATED LIGNANS

Occurrence in the Plant Kingdom

Based on the classification of Cronquist (Cronquist, 1988) podophyllotoxin can be found in the order Pinales of the Gymnospermae (Table 1) and in the four orders of the Magnoliopsida (flowering plants: Table 2). In total there are 13 families distributed over the whole plant kingdom producing podophyllotoxin and related lignans. Presently, at least 35 different plant species are cited in the literature to produce podophyllotoxin (see Table 1 and 2). Another 20 different species do not produce podophyllotoxin but only related lignans like certain species of the *Bursera* genus, which do produce deoxypodophyllotoxin and â-peltatin-a-methylether (see Table 2). There is no data of any prokaryotic organism that is able to produce lignans like podophyllotoxin. Also within the plant kingdom the biosynthesis of podophyllotoxins seems restricted to the vascular plants. There are more lignans with the 2,7'-ring closed like the justicidines, originally isolated from *Justicia procumbens* (Fukamiya and Lee, 1986). These compounds type of arylnaphthalene lignans are also isolated from different *Linum* species (Koulman and Konuklugil, 2003; Mohagheghzadeh *et al.*, 2002), *Haplophyllum patavinum* (Innocenti *et al.*, 2002), *Cleistanthus collinus* (Fukamiya and Lee, 1986) and other *Justicia* species (Day *et al.*, 1999; Navarro *et al.*, 2001; Rajasekhar *et al.*, 1998).

The actual number of podophyllotoxin producing plant species might be much higher, since only a small number has been phytochemically analysed. Even during re-examination podophyllotoxin was found in some species that had already been studied on their lignan content (Kozawa *et al.*, 1978; Kuo *et al.*, 1999). A detailed survey of the vascular plants for the presence of podophyllotoxin could significantly extend Tables 1 and 2.

There are, for instance, 230 species in the *Linum* genus (Van Uden *et al.*, 1994) and on the basis of the work of Konuklugil (Konuklugil, 1996) one might assume that a high number of these species produce podophyllotoxin and 6-methoxypodophyllotoxin. These podophyllotoxin producing species are not confined to certain sections of the *Linum* genus. The section *Syllinum* has the most species that produce these lignans. Konuklugil reported also the presence of podophyllotoxins in species from the sections *Linum*, *Dasylinum* and *Linopsis*. Further phytochemical analysis of this genus combined with phylogenetic data would be very interesting. In Table 1 and 2 all published results are summarised.

Table 1. The occurrence of podophyllotoxin and related lignans in the order Pinales of the gymnosperms. See Fig. 1 for the chemical structures.

Plant	Plant part[1]	DOP[2]	PT[2]	PAM[2]	6MPT[2]	Reference:
Fam. Cupressaceae						
Callitris columellaris	L / S	0.062				(Aynehchi, 1971)
Callitris drummondii	N		1.4			(Kier *et al.*, 1963)
Juniperus chinensis	Calli		0.005			(Muranaka *et al.*, 1998)
Juniperus procera	L	X		X		(Muhammad *et al.*, 1995)
Juniperus lucayana	?		0.10			(Hartwell *et al.*, 1953a)
Juniperus phoenicae	L	X		X		(Cairnes *et al.*, 1980)
Juniperus sabina	N	X	0.20	X	X	(Arturo *et al.*, 1990; Hartwell *et al.*, 1953a; San Feliciano *et al.*, 1989)
Juniperus sabina var. *tamariscifolia*	?		0.14			(Hartwell *et al.*, 1953a)
Juniperus scopulorum	?		0.17			(Hartwell *et al.*, 1953a)
Juniperus thurifera	N	0.04		0.004		(San Feliciano *et al.*, 1992)
Juniperus virginiana	?		0.10			(Hartwell *et al.*, 1953a)
Thuja occidentalis	L	0.0014				(Chang *et al.*, 2000)
Fam. Pinacea						
Libocedrus decurrens	L/S ?	0.04				(Kupchan *et al.*, 1967)

[1]: L= leaf, N= needles, S= stem, Sd= seeds, ?= plant part not known.
[2]: The concentration in weight percentage based on dry weight. YAT: yatein; DOP: deoxypodophyllotoxin; PT: podophyllotoxin; 6MPT: 6-methoxypodophyllotoxin. X: detectable amounts.

Table 2. The occurrence of podophyllotoxin and related lignans in flowering plants. Taxonomy based on the classification of Cronquist (Cronquist, 1988), in the four orders of Magnoliidae, Dilleniidae, Rosidae and Asteridae. See Fig. 1 for the chemical structures.

Plant	Plant part[1]	POL[2]	YAT[2]	DOP[2]	PT[2]	â-PEL[2]	PAM[2]	6MPT[2]	Reference:
Ord. **Magnoliidae**									
Laurales									
Fam. Hernandiaceae									
Hernandia nymphaeifolia	B		0.00007 x-OH[3]	0.00008					(Chen *et al.*, 1996) (Chen *et al.*, 1997)
Hernandia ovigera	Sd			X			X		(Yamaguchi *et al.*, 1982)
Hernandia sonora	Sd			X	X	X	X	X	(Udino *et al.*, 1999)
Piperales									
Fam. Piperaceae									
Piper cubeba	F		X						(Badheka *et al.*, 1986)
Ranunculales									
Fam. Berberidaceae									
Diphylleia cymosa	L				0.54				(Broomhead and Dewick, 1990b)
Diphylleia grayi	Rt				1.27	0.126			(Broomhead and Dewick, 1990b; Murakami and Matsushima, 1961)
Podophyllum hexandrum	Rt/Rh			0.017	4.27	0.01			(Broomhead and Dewick, 1990b)
Podophyllum peltatum	Rt/Rh			0.023	0.25	0.33			(Broomhead and Dewick, 1990b)
Podophyllum pleianthum	Rt/Rh			0.01	0.135				(Broomhead and Dewick, 1990b)
Podophyllum / Dysosma versipellis	Rt/Rh			X	0.32	1.35			(Broomhead and Dewick, 1990b; Yu *et*

Table 2. The occurrence of podophyllotoxin and related lignans in flowering plants. Taxonomy based on the classification of Cronquist (Cronquist, 1988), in the four orders of Magnoliidae, Dilleniidae, Rosidae and Asteridae. See Fig. 1 for the chemical structures.

Plant	Plant part[1]	POL[2]	YAT[2]	DOP[2]	PT[2]	â-PEL[2]	PAM[2]	6MPT[2]	Reference:
									al., 1991)
Fam. Rananculaceae									
Pulsatilla chinensis	WP					X			(Mimaki *et al.*, 1999)
Ord. **Rosidae**									
Linales									
Fam. Linaceae									
Sect. *Syllinum*									
Linum album	Rt				0.02	0.04		0.86	(Weiss *et al.*, 1975)
Linum arboreum	Rt				0.02	0.04		0.86	(Konuklugil, 1996)
Linum campanulatum	Rt				0.12	0.16		0.82	(Konuklugil, 1996)
Linum capitatum	Rt				0.02	0.10		0.03	(Broomhead and Dewick, 1990a; Konuklugil, 1996)
Linum cariense	Rt				0.10	0.08		0.12	(Konuklugil, 1996)
Linum elegans	Rt				0.10	0.04		2.30	(Konuklugil, 1996)
Linum flavum	Rt				0.02	0.04		0.65	(Broomhead and Dewick, 1990a; Konuklugil, 1996)
Linum flavum var *compactum*	Rt				0.02	0.04		2.38	(Konuklugil, 1996)
Linum flavum spp *flavum*	Rt				0.06	0.04		0.38	(Konuklugil, 1996)
Linum flavum spp *scabrinerve*	Rt				X	X		0.11	(Konuklugil, 1996)
Linum mucronatum spp *armenum*	Rt				0.02	0.04		0.04	(Konuklugil, 1996)
Linum nodiflorum	L				X	0.01		0.25	(Konuklugil, 1996)
Linum pamphylicum	Rt				0.19	0.02		0.06	(Konuklugil, 1996)
Linum tauricum	Rt				0.06			1.32	(Konuklugil, 1996)
Linum thracicum	Rt				0.06	0.01		0.20	(Konuklugil, 1996)
Sect. *Linum*									
Linum austriacum	Rt				X	0.01		0.05	(Konuklugil, 1996)
Linum lewisii	Rt				X	X		X	(Konuklugil, 1996)
Linum meletonis	Rt	X							(Koulman and Konuklugil, 2003)
Linum monogynum	Rt				X	X		0.01	(Konuklugil, 1996)
Linum sibiricum	Rt				X	X		0.01	(Konuklugil, 1996)
Sect. *Dasylinum*									
Linum hirsutum	Rt				X	X		X	(Konuklugil, 1996)
Linum viscosum	Rt				X	X		X	(Konuklugil, 1996)
Sect. *Linopsis*									
Linum corymbulosum	Rt				X	X		X	(Konuklugil, 1996)
Polygalales									
Fam. Polygalaceae									
Polygala polygama	WP	X		X	0.076				(Hokanson, 1978) (Hokanson, 1979)
Sapindales									
Fam. Burseraceae									
Bursera morelensis	E			0.31					(Jolad *et al.*, 1977)
Bursera fagaroides	WP						X		(Bianchi *et al.*, 1969)
Bursera permollis	S/B			0.004			0.006		(Wickramaratne *et al.*, 1995)
Bursera simaruba	Res	0.05							(Peraza-Sanchez and Pena-Rodriguez, 1992)

Table 2. The occurrence of podophyllotoxin and related lignans in flowering plants. Taxonomy based on the classification of Cronquist (Cronquist, 1988), in the four orders of Magnoliidae, Dilleniidae, Rosidae and Asteridae. See Fig. 1 for the chemical structures.

Plant	Plant part[1]	POL[2]	YAT[2]	DOP[2]	PT[2]	â-PEL[2]	PAM[2]	6MPT[2]	Reference:
Bursera microphylla	S			0.09					(Jolad *et al.*, 1977)
Commiphora incisa	Res	0.5							(Provan and Waterman, 1985)
Fam. Rutaceae									
Haplophyllum cappadocicum	WP	X							(Gözler *et al.*, 1996)
Apiales									
Fam. Apiaceae									
Anthriscus sylvestris	Rh		0.0126	0.007	X				(Kozawa *et al.*, 1978; Ikeda *et al.*, 1998a)
Chaerophyllum aureum	L			X					(Rollinger *et al.*, 2002)
Cicuta maculata	Rh			?[4]					(Hartwell *et al.*, 1953b)
Ord. **Asteridae**									
Lamiales									
Fam. Lamiaceae									
Eriope macrostachya	S.					X			(Raffauf *et al.*, 1987)
Hyptis verticillata	W.P				0.25	X			(Kuhnt *et al.*, 1994)

[1]: B= bark, F= fruit, E= exudate, L= leaf, Rh= rhizome, Rt= root, S= stem, Sd= seeds, Res=resin or exudtae, WP= whole plant.
[2]: The concentration in weight percentage based on dry weight. POL: polygamain; YAT: yatein; DOP: deoxypodophyllotoxin; PT: podophyllotoxin; â-PEL: â-peltatin; PAM: â-peltatin-A-methylether; 6MPT: 6-methoxypodophyllotoxin. X: detectable amounts.
[3]: x-OH= is a hydroxylated derivative of YAT.
[4]: Spectroscopic data hint to the presence of deoxypodophyllotoxin but this is not yet confirmed.

Chemotaxonomy

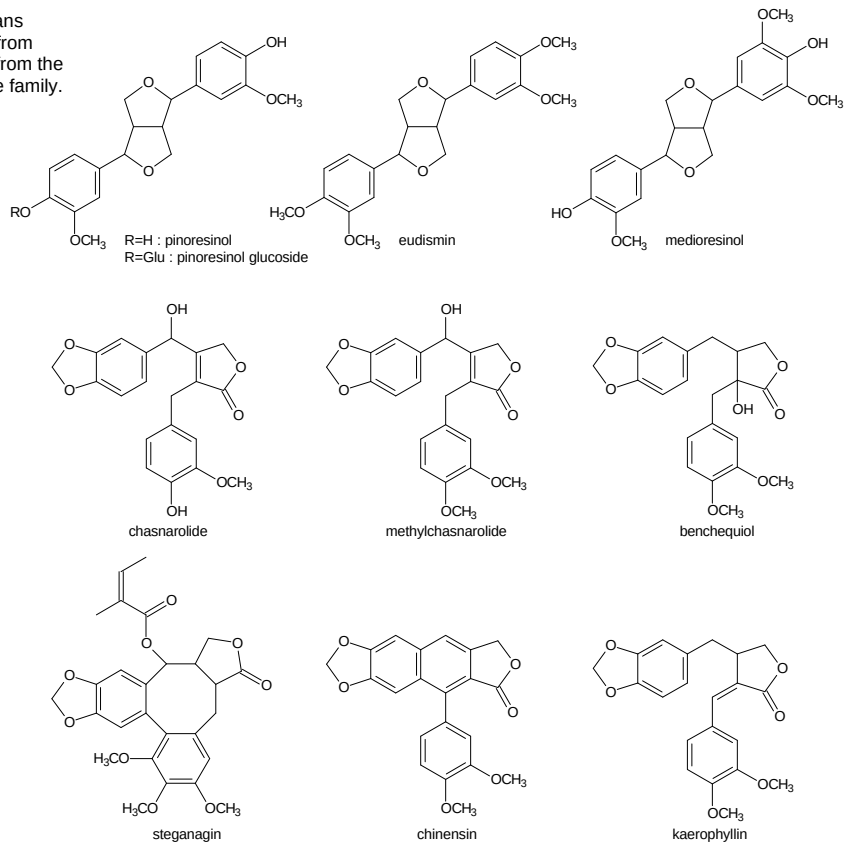
The Tables 1 and 2 show that lignans are widely spread over the plant kingdom. There are some marked qualitative differences between the different plant families with respect to their lignan content. All the studied species in the *Linum* genus that produce podophyllotoxin also produce 6-methoxypodophyllotoxin. In the *Juniperus* genus there is only one species (*J. sabina)* that produces 6-methoxypodophyllotoxin while 8 species produce podophyllotoxin or peltatins. Also the biosynthesis of polygamain seems restricted to the Order of the Rosidae.

The genus *Anthriscus* of the Apiaceae family contains the high lignan producing species *Anthriscus sylvestris* that accumulates mainly deoxypodophyllotoxin but also podophyllotoxin in small amounts. Within the same genus *Anthriscus cerefolium* does not produce any detectable amounts of deoxypodophyllotoxin or podophyllotoxin (see chapter III). There are further only a limited number of species of the Apiaceae family reported to produce lignans (see Fig. 4). One of the oldest articles points to the presence of probably deoxypodophyllotoxin in *Cicuta maculata* (see Fig. 1; Hartwell *et al.*, 1953b). Recently this same lignan was found in *Chaerophyllum aureum*. We could detect the very simple lignan pinoresinol in *Pastinaca sativa* (see chapter III). The *O*-â-D-gyucopyranoside of this pinoresinol (see Fig. 4) is also reported from *Angelica furcijuga* (Matsuda *et al.*, 2000). *Chaerophyllum maculatum* accumulates kaerophyllin (see

Fig. 4; Mikaya *et al.*, 1983). This is also one of the lignans accumulated by *Bupleurum* species (Khetwal *et al.*, 1993), producing derivatives like chasnarolide, methylchasnarolide, chinensin (see Fig. 4; González *et al.*, 1990). *Bupleurum salicifolium* produces also pinoresinol derivatives like eudismin and medioresinol (see Fig. 4; González *et al.*, 1989). The genus *Steganotaenia* produces dibenzocyclo-octadiene lactone lignans like steganagin (see Fig. 4; Wickramaratne *et al.*, 1993). The phytochemistry of the Apiaceae is quite well studied, but mainly for their volatile components and coumarines. It is difficult, if not impossible, to conclude from these studies whether the studied Apiaceae members do not produce any lignans. Therefore it is not possible yet to have a clear picture of the presence of lignans in this family. From the limited data we have, there seems no clear correlation between the phylogeny of the Apiaceae (Downie *et al.*, 2000) and their lignan chemistry.

This limited data on lignan profiles of the species belonging to the Apiaceae family, makes this class of phenylpropanoids not suitable as a chemotaxonomical marker.

Fig. 4. The lignans isolated from species from the Apiaceae family.



BIOSYNTHESIS OF PODOPHYLLOTOXIN AND RELATED LIGNANS

Background

The biosynthetic pathway of podophyllotoxin and related lignans is still a matter of debate. Due to the large number of lignan producing plant species this research field is scattered rather than concentrated on one model species. Different research groups focus on different species, therefore it is not clear to which extend there are similarities in the biosynthesis of podophyllotoxin in the different species. General conclusions can not be drawn as yet and it is only possible to speculate about major parts of the podophyllotoxin biosynthesis.

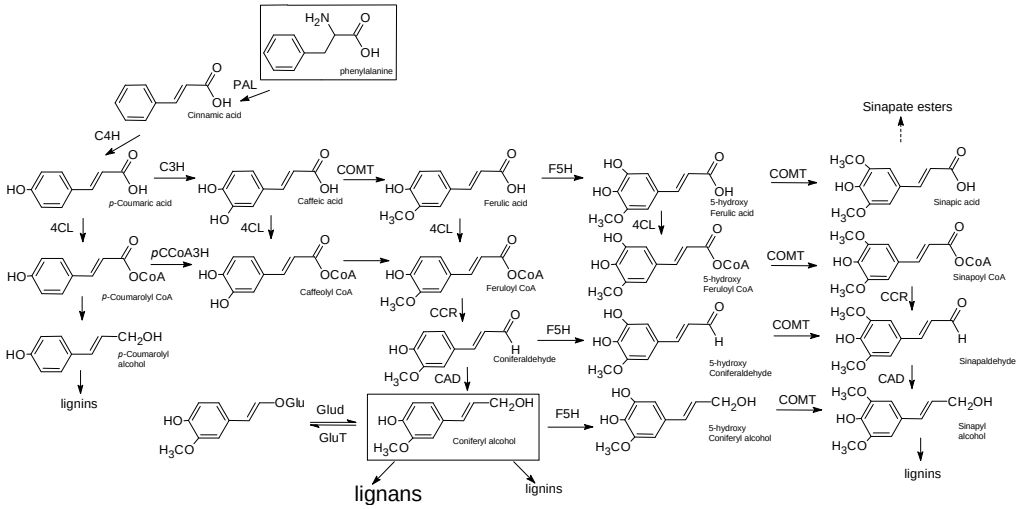


Fig. 5.
The phenylpropanoid biosynthesis in *Pinus* (Li *et al.*, 2000) The enzymes involved are caffeoyl CoA *O*-methyl-transferase (CCoOMT), cinnamate 4-hydroxylase (C4H), cinnamoyl alcohol dehydrogenase (CAD), cinnamoyl CoA reductase (CCR), *p*-coumarate 3-hydroxylase (C3H), 4-(hydroxyl) cinnamoyl CoA ligase (4CL), ferulate 5-hydroxylase (F5H), *p*-coumaroyl CoA 3-hydroxylase (*p*CCoA3H), phenylalanine ammonia lyase (PAL), caffeic acid/5-hydroxylferulic acid/5-hydroxyconiferyl alcohol and aldehyde *O*-methyltransferase (COMT), Glucosyl transferase (Glut), Glucosidase (Glud).

Towards Lignans

All lignans are ãã-dimers of phenylpropanoid derivatives. In the shikimic acid route the aromatic amino acid phenylalanine is produced. The biosynthesis from phenylalanine to coniferyl alcohol is not a straight route but a complex metabolic grid, which is depicted in Fig. 5 (Humphreys *et al.*, 1999; Whetten *et al.*, 1998). Coniferyl alcohol is stored in the cell as

coniferin (its â-D-glycoside). *Linum flavum* cell suspension cultures are able to accumulate over 12% of coniferin on dry weight basis, which shows that the coniferin pool can be an important factor in the lignan biosynthesis (Van Uden *et al.*, 1990a). Coniferyl alcohol can also be converted into 5-hydroxy coniferyl alcohol by ferulate 5-hydroxylase (Humphreys *et al.*, 1999). This compound can be further converted into sinapyl alcohol by the action of an O-methyl transferase (Li *et al.*, 2000). Like coniferyl alcohol and *p*-coumaryl alcohol, sinapyl alcohol is used as a building block for the lignin biosynthesis. Different peroxidases generate radicals from these building blocks allowing them to polymerise. To what extend this polymerisation is a controlled process is still a matter of discussion (Lewis, 1999).

The dirigent protein

For the formation of lignans plant peroxidases catalyse the conversion of coniferyl alcohol into its free radical form. *In vitro* the free radicals will dimerise to yield a racemic mixture. However, in the plant the dimerisation is controlled and the formation of one stereoisomer is favoured. This observation led to the discovery of the dirigent protein (Davin *et al.*, 1997). The dirigent protein does not catalyse the reaction as a standard enzyme. There is no active site in the protein and it is not clear how the protein binds (covalently or non-covalently) to the substrates or how it directs the coupling of two monomer radicals in a stereospecific way. Davin and Lewis claim that homologues genes are present in all the plant species they examined, a.o.: *Forsythia* species, *Thuja plicata*, *Populus tremuloides*, *Oxyza sativa*, *Linum usitatissimum*, and *Nicotiana tabacum* (Davin and Lewis, 2000).

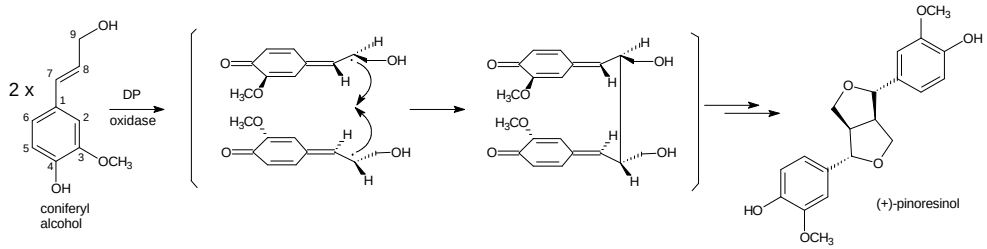


Fig. 6. Dirigent-mediated formation of (+) pinoresinol. Dirigent protein (DP) in *Forsythia intermedia* according to Davin and Lewis (Davin *et al.*, 1997).

The function of each of these dirigent proteins might differ between species. The coniferyl alcohol monomers can couple not only through an 8-8' linkage (see Fig. 6) but also through an 8-5' or 8-4' linkage, which leads to the biosynthesis of neolignans as present in *Piper aequale* or *Persea obovatifolia* (Maxwell *et al.*, 1999; Tsai *et al.*, 1998). This biosynthesis also proceeds in a stereo-controlled way, which suggests the involvement of a dirigent protein. *Eucommis ulmoides* produces a derivative of pinoresinol, which is probably not a dimer formed from two coniferyl alcohols but from two sinapyl alcohol monomers. Again this occurs in a stereospecific way and from this plant a gene encoding a homologue of the dirigent protein has been cloned (Davin and Lewis, 2000).

A recent study with ^{13}C labelled phenylpropanoids fed to cell suspension cultures of *Linum album* showed that ferulic acid was converted into podophyllotoxin (Seidel *et al.*, 2002). The presence of a ^{13}C atom in ferulic acid yielded podophyllotoxin with a mass of M+2. Doubly labelled ferulic acid yielded M+4. Additional experiments with labelled dimethoxycinnamic acid and sinapic acid also yielded podophyllotoxin with an M+1 and M+2. The feeding experiments with labelled methylenedioxycinnamic acid yielded only podophyllotoxin M+1 (see fig. 7; Seidel *et al.*, 2002). It is not clear, whether, the fed substrates are converted back to ferulic acid before they enter the podophyllotoxin biosynthetic pathway.

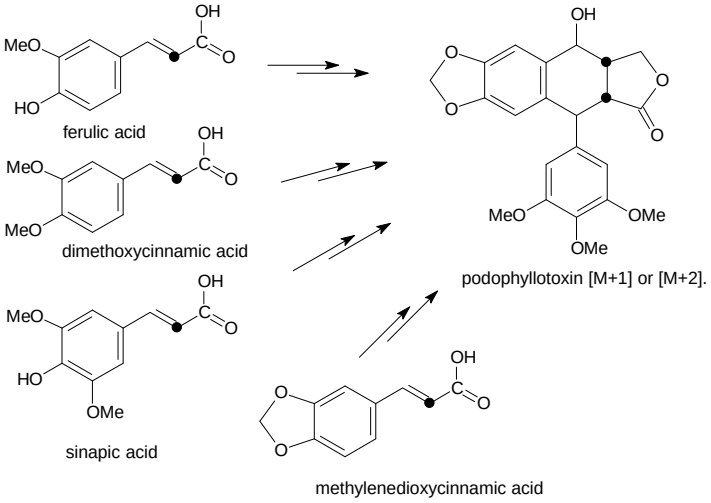


Fig. 7.
Feeding of ^{13}C labeled phenyl-propanoids to cell suspension cultures of *Linum album*. The black dot indicates the presence of the ^{13}C atom. The label can end up.on the indicated positions in the product depending on the substrate (Seidel ***et al.***, 2002).

From Pinoresinol to Matairesinol

From pinoresinol the lignan biosynthesis will split up into different directions leading to a great diversity of lignans. For the biosynthesis leading towards podophyllotoxin the first number of steps have been proven to result in matairesinol (see Fig. 8). These steps were first elucidated in *Forsythia intermedia* (Katayama *et al.*, 1993; Rahman *et al.*,

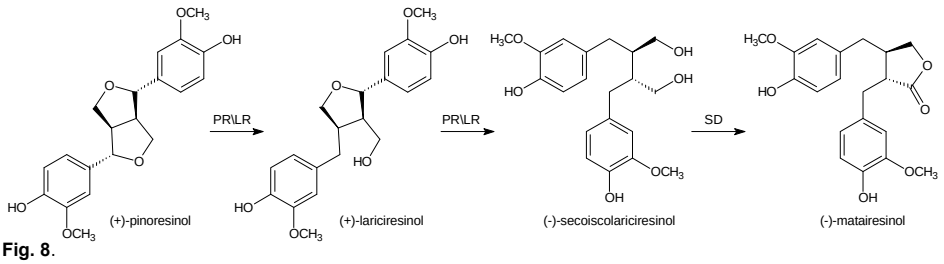


Fig. 8.
The biosynthesis of matairesinol starting with pinoresinol. The enzymes involved are pinoresinol - lariciresinol reductase (PR\LR) and secoisolariciresinol dehydrogenase (SD) (Rahman ***et al.***,1990b).

1990a; Umezawa *et al.*, 1991). Pinoresinol is enantiospecifically reduced in two steps leading to secoisolariciresinol. The same steps have been confirmed to occur in *Linum flavum* (Xia *et al.*, 2000).

From Matairesinol to Podophyllotoxin

Two theories have been published about the biosynthesis of podophyllotoxin starting from matairesinol. The first theory dates back to half way the 80's and was published by Dewick and co-workers. It was proven for *Podophyllum* that matairesinol, yatein, and deoxypodophyllotoxin can be converted into podophyllotoxin. Labelled possible precursors of podophyllotoxin were fed to roots of *Podophyllum hexandrum* and the formation of metabolites was monitored. The results show that *Podophyllum* could convert matairesinol, yatein and deoxypodophyllotoxin into podophyllotoxin. The oxidised analogue of yatein, anhydropodorhizol was not converted into podophyllotoxin. Figure 9 summarises the precursor feeding experiments (Jackson and Dewick, 1984b; Kamil and Dewick, 1986a). From these results it can be concluded that at least 6 steps are necessary to convert matairesinol into podophyllotoxin:

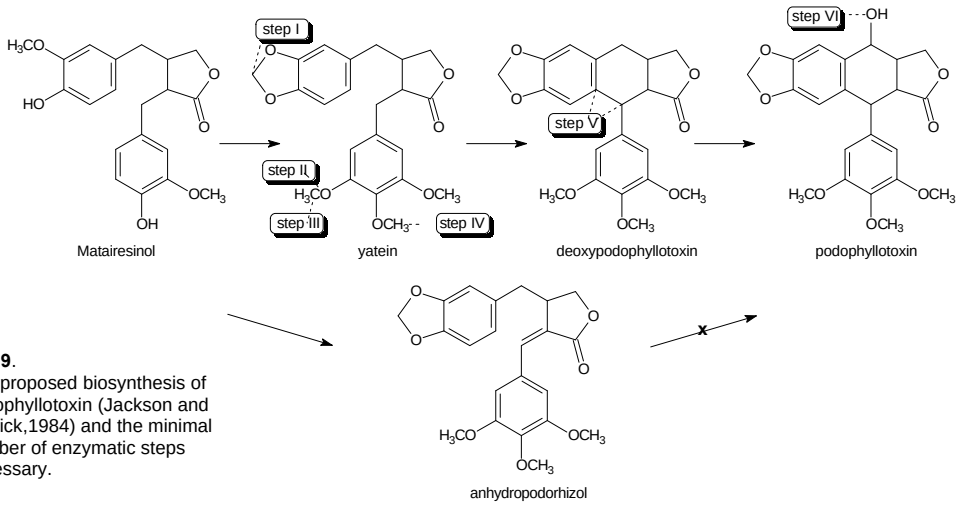


Fig. 9.
The proposed biosynthesis of podophyllotoxin (Jackson and Dewick,1984) and the minimal number of enzymatic steps necessary.

Four steps for the formation of yatein from matairesinol, while one step is needed for the conversion of yatein to deoxypodophyllotoxin and one for the formation of deoxypodophyllotoxin into podophyllotoxin.

Van Uden and co-workers delivered further evidence for this theory by feeding deoxypodophyllotoxin to *Linum* cells, which resulted in the formation of 6-methoxypodophyllotoxin (Van Uden *et al.*, 1995) and much smaller amounts of podophyllotoxin (unpublished results). Cell suspension cultures of *Linum flavum* are able to convert large amounts of deoxypodophyllotoxin into the glycoside of 6-methoxypodophyllotoxin (Van Uden *et al.*, 1997). Recent studies showed that the first step from deoxypodophyllotoxin towards 6-methoxypodophyllotoxin is carried out

by deoxypodophyllotoxin 6-hydroxylase, a cytochrome P450 mono-oxygenase, in *Linum flavum* (Molog *et al.*, 2001). This enzyme converts deoxypodophyllotoxin into â-peltatin.

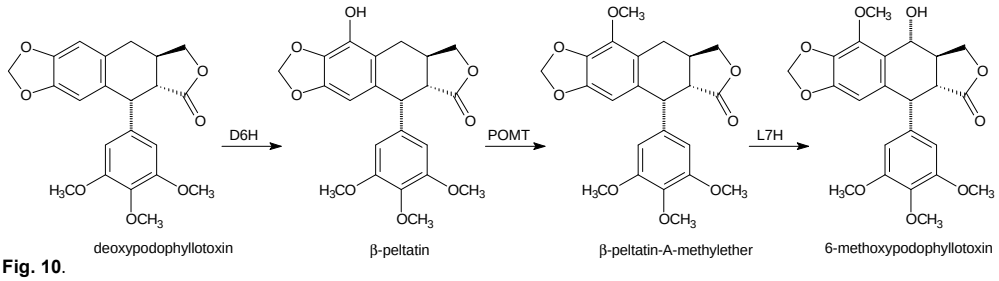


Fig. 10.
The biosynthesis of 6-methoxypodophyllotoxin according to van Uden and Petersen (Molog *et al.*, 2001; Van Uden *et al.*, 1997). The enzyme abbreviations are deoxy-podophylotoxin 6-hydroxylase (D6H), peltatin SAM-dependent *O*-methyl-transferase (POMT), putative lignan 7-hydroxylase (L7H).

On the other hand deoxypodophyllotoxin is hydroxylated at the 7 position yielding podophyllotoxin and its glycoside. Enzyme assays showed that there is an S-adenosyl-L-methionine (SAM)-dependent *O*-methyltransferase that converts â-peltatin into â-peltatin-A-methylether. Cell cultures are able to convert this intermediate rapidly into 6-methoxypodophyllotoxin (see chapter VIII). The biosynthesis of 6-methoxypodophyllotoxin from deoxypodophyllotoxin is shown in Fig. 10.

The second theory is based on the observation that the feeding of matairesinol to roots of *L. flavum* by Xia and co-workers resulted in formation of 7-hydroxymatairesinol. This compound was not yet known from *Linum* species. The feeding of synthetic labelled (^{13}C) 7-hydroxymatairesinol to *Linum flavum* roots yielded labelled 6-methoxypodophyllotoxin as is shown in Fig. 11 (Xia *et al.*, 2000). This is in contradiction with the results previously discussed and it seems very unlikely that this is the normal biosynthetic pathway in *Linum flavum*.

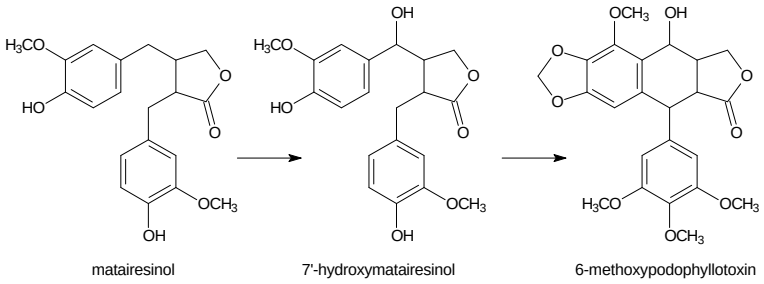


Fig. 11.
The proposed biosynthesis of 6-methoxypodophyllotoxin according to Xia et al, 2000.

Under standard conditions 7-hydroxymatairesinol is not found in *Linum flavum*, only after the feeding of an “overdose” matairesinol to the roots 7-

hydroxymatairesinol is accumulated. One could argue that if 7-hydroxy-matairesinol were really an intermediate, it would be very difficult to detect, since it is then rapidly converted in the biosynthesis of 6-methoxy-podophyllotoxin. This in analogy with the rapid conversion of deoxypodophyllotoxin to 6-methoxypodophyllotoxin by *L. flavum* cells where deoxypodophyllotoxin cannot be measured under standard conditions.

ECOLOGICAL FUNCTION OF PODOPHYLLOTOXIN AND RELATED LIGNANS IN PLANTS

Background

The ecological or biological function of podophyllotoxin and related lignans is scarcely investigated. The high cytotoxicity of podophyllotoxin and related lignans has lead to the generally well-accepted theory that these compounds protect the plant against herbivores.

Insecticidal and other activities

Japanese research dating from the early eighties shows clearly that lignans present in roots of *Anthriscus sylvestris* have insecticidal activity. Deoxypodophyllotoxin is the main responsible compound for this lethal activity on a number of different insect larvae e.g. *Culex pipiens*, *Epilachna spara* and adult insects such as *Blatella germanica* (Kozawa *et al.*, 1982). A further study showed that deoxypodophyllotoxin has an effect on larvae of the Silkworm *Bombys mori* (Inamori *et al.*, 1983).

During a bioguided isolation based on the insecticidal activity against *Drosophila melanogaster*, it was shown that podophyllotoxin was the main constituent of *Podophyllum hexandrum* that was responsible for its insecticidal activity (Miyazawa *et al.*, 1999).

Further *in vitro* and *in vivo* studies showed that podophyllotoxin and related lignans are active against viruses (Charlton, 1998) and different kinds of tumour cells (Damayanthi and Lown, 1998). For other lignans germination inhibitory, antimicrobial and antifungal activity has been reported, but there is no evidence for podophyllotoxin or related lignans concerning these activities (MacRea and Towers, 1984). From the results reported so far, it is not possible to define the exact ecological role of these compounds. The insects used in the above mentioned studies do probably not interact with the plants *in situ* and there are no field studies available involving podophyllotoxin or deoxypodophyllotoxin producing plants.

Induction and elicitation

Plants like *Anthriscus sylvestris* and *Podophyllum hexandrum* do not produce just one lignan but usually accumulate a range of different lignans. For instance *A. sylvestris* accumulates next to deoxypodophyllotoxin also significant amounts of yatein and anhydropodorhizol, as well as smaller amounts of other lignans (see chapter VI). There is also a large variation in the concentration of these compounds within this plant species (see chapter III). There are different theories to explaining why plants produce such a diverse spectrum of compounds in stead of just one main compound. On

the one hand a combination of compounds could have a synergistic effect, on the other hand a large variation in the profile of these metabolites will probably make it more difficult for predators to adapt to the bioactivity of these compounds (Shelton, 2000).

The ecological role of natural products is often characterised by the induction of the biosynthesis of these compounds in the plant after contact with natural enemies like micro-organisms, predators or stress conditions (heat, dryness, as discussed by Abraham *et al.*, 1999). Thus far there are no studies showing that the lignan biosynthesis is induced or changed by stress or predation.

The induction by ecological factors can be compared with elicitation in plant cell cultures. Elicitation is defined as an increase of the anabolism of bioactive compounds caused by stress factors like exposure to micro-organisms. Cell cultures derived from podophyllotoxin producing plants did not increase the lignan production after elicitation. Many different experimental set-ups have been followed in order to try to elicit the lignan biosynthesis in different cell suspension cultures of *Podophyllum hexandrum*, *Linum flavum* and *Anthriscus sylvestris*, but without any success. There are very little data in the literature on the elicitation of the lignan biosynthesis. Only *Juniperus chinensis* callus cultures had an elevated podophyllotoxin production after the addition of chito-oligosaccharides which mimic fungal cell wall constituents (Muranaka *et al.*, 1998). Because this is the only publication on the elicitation of the lignan biosynthesis, it might be assumed that all other experiments in this field failed. Therefore, in most plants the lignan concentration does not seem to be effected by eliciting factors. Still there are examples of metabolites that are not induced but still prevent predation, like the furanocoumarins in *Pastinaca sativa* (Zangerl and Berenbaum, 1997).

Chemical-ecological research has become increasingly important for plant sciences. For molecular biological research it is of great help when a so-called on-off situation is found. By comparison of a production situation with a non-production situation of a plant species that accumulates interesting secondary metabolites it is easier to find the genes that play a role in the biosynthesis. Such genes can be detected and identified with DNA-chips or by subtractive analysis. The availability of these genes is of utmost importance for further study of the regulation of biosynthetic pathways or the biotechnological production of secondary metabolites using recombinant DNA technology.

BIOTECHNOLOGICAL PRODUCTION OF PODOPHYLLOTOXIN AND RELATED LIGNANS

Background

Different research groups investigate a plant-biotechnological approach for solving the supply problem of podophyllotoxin for the production of etoposide and related drugs. Experiments with *Podophyllum* plants in India did not result in a cultivation system with viable agricultural prospects (Choudhary *et al.*, 1998). An approach of selective plant breeding is therefore not a successful option.

A production system, based on plant cell cultures might therefore be the useful alternative. The most commonly followed approach is the use of plant cell cultures. As an alternative microbial systems are studied such as certain strains of fungi. Presently, no prokaryotic cells are able to produce lignans. The different production systems that are available are discussed below.

Plant Cells and organ cultures

The production of fine chemicals or drugs with *in vitro* cultured plant cell systems has been the subject of study for many years. Given the totipotency of the plant cell, implying that all the genetic information present in the plant is essentially available, it seems feasible to produce most, if not all, secondary metabolites using *in vitro*-grown cultures. The successful production of antibiotics by fungi and bacteria was a stimulating example for researchers in the early days of plant cell cultures. Since then, it has become clear that plant cell cultures do not always accumulate (either qualitatively or quantitatively) the same compounds found in the parent plant from which they were established.

However different products can be produced with plant cells, like paclitaxel, L-DOPA (Pras *et al.*, 1993), rosmarinic acid (De-Eknamul and Ellis, 1985) or coniferin (Van Uden *et al.*, 1990a). Rarely these systems are applied for the industrial production of these compounds. At the moment there are no *in vitro* production systems for podophyllotoxin that are of commercial interest.

The most direct approach for the biotechnological production of podophyllotoxin is the use of a cell suspension culture in a large-scale fermentor. This system would produce podophyllotoxin directly (See Table 3). A suitable candidate for fermentation could be a *Linum album* suspension culture. Experiments are underway using large-scale fermentors (200 l) with these cells, but it is questionable whether an economically feasible system can be developed, due to the low production and the

instability of these cells (< 0.5% podophyllotoxin based on dry weight, pers. com. with Prof. Alfermann).

Table 3. Cell cultures producing podophyllotoxin or 6-methoxypodophyllotoxin.

Species	Culture	Medium[1]	Lignans[2]	Reference:.
Callitris drumondi	Suspension	MS + 4 mg l^{-1} NAA	0.1% PT	(Van Uden *et al.*, 1990b)
Juniperus chinensis	Callus	SH 3 mg l^{-1} NAA + Kinetin 0.2 mg l^{-1}	0.005% PT	(Muranaka *et al.*, 1998)
Podophyllum hexandrum	Callus	B5 + 4 mg l^{-1} NAA	0.3% PT	(Van Uden *et al.*, 1989)
	Suspension	B5 + 4 mg l^{-1} NAA	0.1% PT	(Woerdenbag *et al.*, 1990)
Podophyllum peltatum	Callus	MS + 1 ppm 2,4D + 0.2 ppm kinetin	0.71% PT	(Kadkade, 1982)
	Suspension	½ MS + hormones	0.32-0.36% PT	(Kutney *et al.*, 1991)
Linum flavum	Roots	MS + 2 µM 2,4-D	0.7-1.3% 6MPT	(Berlin *et al.*, 1988)
	Suspension	MS + 3 mg l^{-1} NAA	0.004% 6MPT	(Van Uden *et al.*, 1990d)
	Suspension with DOP	MS + 4 mg l^{-1} NAA + 4 mg l^{-1} BAP + 0.8 g l^{-1} DOP	4.41% 6MPT-glu	(Van Uden *et al.*, 1997)
Linum nodiflorum	Suspension	MS + 0.4 mg l^{-1} NAA	0,6% 6MPT + 0.2%PT	(Konuklugil *et al.*, 1999)
Linum album	Suspension	MS + 0.4 mg l^{-1} NAA	0.2-0.5% PT and 6MPT	(Smollny *et al.*, 1998)

[1]: Medium used for culturing. The kind of culture media (MS: Murashige and Skoog (Murashige and Skoog, 1962), SH: Schenk and Hildebrand (Schenk and Hildebrandt, 1972), B5: Gamborg's B5 (Gamborg *et al.*, 1968)), and used phytohormones (NAA: naphtalene acetic acid, 2,4D: 2,4-dichlorophenoxy acetic acid, BAP: 6-benzylaminopurine).
[2]: The concentration in weight percentage based on dry weight; PT: podophyllotoxin; 6MPT: 6-methoxypodophyllotoxin.

Next to the use of undifferentiated cell cultures it is also possible to use organ cultures. Phytohormones are able to initiate differentiation into organs like roots and shoots. Organ cultures are sometimes able to accumulate higher amounts of lignans; for instance the root cultures of *L. flavum* produce higher amounts of 6-methoxypodophyllotoxin than undifferentiated cell cultures (see Table 3). Root cultures, or the usually faster growing hairy root cultures obtained through transformation with *Agrobacterium rhizogenes*, may provide us in the future with an alternative biotechnological production system for podophyllotoxins (Arroo *et al.*, 2002).

Bioconversion systems

Plant cells may form an attractive biocatalytic system, since precursors can be supplied directly to the culture. Several reports dealing with the production of valuable compounds by adding precursors to various culture species have been published. Often the precursor undergoes more than one bioconversion, resulting in complex mixtures of (unknown) products, or the substrate is metabolised via unknown mechanisms (Kawaguchi *et al.*, 1988). Nevertheless, a number of one-step bioconversions by freely suspended cells has been described, for instance the production of codeine with a *Papaver somniferum* culture fed with codeinone (Furuya *et al.*, 1984).

The same approach is possible for the production of 6-methoxy-podophyllotoxin, the 6-methoxy derivative of podophyllotoxin. Cell suspension cultures of *Linum flavum* are able to convert deoxypodophyllotoxin (see Table 3) into the glycoside of 6-methoxy-

podophyllotoxin (Van Uden *et al.*, 1997). For several reasons, this system has not yet reached the stage of commercial application. Firstly, etoposide and other analogues cannot be synthesised from 6-methoxy-podophyllotoxin. Secondly, the isolation of deoxypodophyllotoxin is a rather expensive and time-consuming process. Deoxypodophyllotoxin can be found in the roots of *Anthriscus sylvestris*. This plant is a common weed in Northwest Europe and its roots contain up to 2.5% deoxypodophyllotoxin. The selection of a high deoxypodophyllotoxin producing strain could facilitate a cheaper isolation procedure. This would make *A. sylvestris* a potential crop and interesting starting point for conversion system.

Fungi can also be used for the bioconversion of deoxypodophyllotoxin in to podophyllotoxin-analogues; these experiments with *Aspergillus niger* cultures yielded a racemic mixture of podophyllotoxin (Kondo *et al.*, 1989; Kondo *et al.*, 1990). The use of fungal systems for the production of plant secondary metabolites is poorly developed, but might become of interest in the future.

Recently, Pras and Woerdenbag reviewed the production of secondary metabolites by bioconversion with plants and plant cells (Pras and Woerdenbag, 1999).

THE SCOPE OF THIS THESIS

Podophyllotoxin is an important plant compound that has found its way into different therapies. Up to the present the only source for podophyllotoxin is the *Podophyllum* plant, which is mainly collected from the wild. If we want to save this species from extinction we have to develop a new and sustainable way of producing podophyllotoxin. A better understanding of its biosynthesis is crucial in this development. The aims of this thesis are to improve our knowledge of the biosynthesis of podophyllotoxin and related lignans and develop new strategies for their production.

II. De inleiding van de Nederlandse samenvatting

INLEIDING

Vier jaar spitten

Dit proefschrift geeft slechts een eenzijdig beeld van wat ik de afgelopen 4 jaar aan onderzoek heb uitgevoerd. Het is op de eerste plaats geschreven om aan een wetenschappelijk publiek mijn ideeën uit te leggen en de daaruit voortvloeiende onderzoekingen en onderzoekresultaten te bespreken. Die ideeën heb ik onder meer gekregen door, in het laboratorium, met planten in de weer te zijn. Gelukkig heb ik niet alleen op het lab gewerkt, maar kon ik af en toe ook naar buiten om fluitenkruid uit te spitten en te kijken hoe die plant groeit en bloeit in het open veld. Tijdens mijn promotie onderzoek heb ik veel met fluitenkruid gewerkt. Fluitenkruid bevat verschillende stoffen die nodig waren voor mijn onderzoek. Deze stoffen zijn nergens te koop, maar wel uit het plantenmateriaal te isoleren.

Bij sommige van mijn onderzoekingen had ik wel een kilo gedroogde, vermalen, fluitenkruidwortel nodig voor een paar milligram zuivere eind-product.

De meeste ideeën heb ik opgedaan door een andere manier van spitten, namelijk in de wetenschappelijk literatuur. Wereldwijd is al veel onderzoek gedaan op mijn onderzoeksterrein. Door deze resultaten te bestuderen kon ik aanvullende experimenten bedenken en dus ook aanvullende waar-nemingen doen. Alle literatuur die ik heb gebruikt staat achter in dit proefschrift in de literatuurlijst.

Nederlandse inleiding

Voor leken zal de inhoud van dit proefschrift niet altijd even duidelijk zijn. Voor hen en voor degenen die bij het lezen van de eerste regel van het hoofdstuk I denken: “Waar gaat dit over?”, heb ik een samenvatting gemaakt. Hierin staat een inleiding tot het onderzoek en vervolgens wordt bij elk volgend hoofdstuk een korte samenvatting gegeven.

De plant als geneesmiddelbron

Planten maken verschillende stoffen (vaak ook verbinding genoemd) voor vele doeleinden. Ze maken stoffen die worden gebruikt om insecten te lokken en stoffen om zich te beschermen tegen belagers en/of buren. Soms kunnen deze, meestal giftige, verbindingen in de juiste concentratie toegediend, voor de mens als geneesmiddel dienen.

Algemeen wordt aangenomen dat sinds het begin van de mensheid planten en plantenextracten gebruikt werden om ziektes te bestrijden. Pas

in de laatste honderd jaar is de mens in staat gebleken zelf geneesmiddelen te ontwikkelen buiten de plant om. Maar nog steeds spelen stoffen uit planten verkregen een essentiële rol in de farmacie.

Voor een aantal geneesmiddelen wordt een plantenstof chemisch nagemaakt, zodat men niet meer afhankelijk is van de plant. Zo maakt bijvoorbeeld Bayer aspirines.

Andere plantenstoffen hebben model gestaan voor geneesmiddelen, bijvoorbeeld het bloedverdunnende (beter gezegd: de bloedstolling remmend) middel acenocoumarol (sintrom). Het was bekend dat koeien dood konden gaan als het gevolg van het eten van rottend honingklaver. Deze koeien stierven door inwendige bloedingen. De verbinding verantwoordelijk voor die bloedingen heeft model gestaan bij de ontwikkeling van acenocoumarol.

Bij sommige geneesmiddelen worden de benodigde verbindingen direct uit de plant gehaald, een voorbeeld is morfine uit de papaver. In dit geval is de chemische structuur van de werkzame stof zo complex, dat namaken ervan op industrieel niveau onmogelijk is gebleken. Farmaceutische biologie is de naam die gegeven wordt aan het onderzoek naar het gebruik van planten en natuurstoffen in de geneeskunde.

Biosynthese

Planten maken suikers uit zonlicht, water en koolzuur (CO_2 = koolstofdioxide). Deze suikers worden door planten gebruikt als energiebron en om andere stoffen mee te maken, bijvoorbeeld aminozuren. Door aminozuren en suikers te combineren en te veranderen maken planten weer andere verbindingen. Dit proces vindt plaats in de plant met behulp van enzymen. Ook deze enzymen zijn weer opgebouwd uit aminozuren.

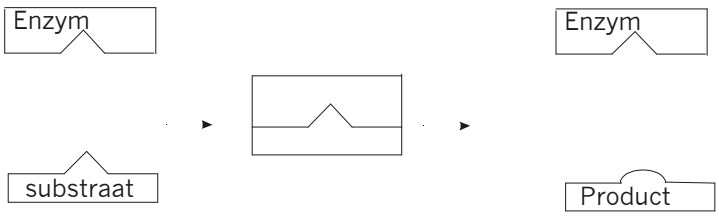


Fig. 1.
Enzymen zijn het chemisch gereedschap van planten. Elke soort enzym is meestal in staat om één soort reactie uit te voeren met één bepaald substraat, waardoor een product wordt gevormd. Dit product kan weer het substraat zijn voor een ander enzym (zo ontstaat een biosynthese). Een enzym kan dezelfde reactie vele malen opnieuw uitvoeren.

Elke plantensoort heeft eigen enzymen (dit ligt vast in de genen van de plant) en maakt eigen combinaties van en veranderingen aan verbindingen. Het grote aantal plantensoorten dat er bestaat zorgt voor een enorme variatie in chemische verbindingen binnen het plantenrijk.

Een mooi voorbeeld is het aminozuur fenylalanine. Dit komt in alle planten voor (zie fig. 2). De kruidnagelboom maakt van fenylalanine de geurstof voor kruidnagel, terwijl de muskaatboom van fenylalanine een de

stof maakt die de geur geeft van noodmuskaat. Ook de geur van anijs en kervel wordt gemaakt van fenylalanine.

Veel andere geurstoffen zijn ook dit aminozuur gemaakt (kaneel, venkel, koffie ect.). Maar het gaat nog verder dan geurstoffen: rodekool dankt aan fenylalanine de paarse kleur en sinaasappelen de oranje kleur. De typische geur van gemaaid gras komt ook door een afgeleide van hetzelfde aminozuur.

Het proces waarbij planten bouwstenen omzetten in andere stoffen noemt men biosynthese. Biosynthese wordt, zoals hierboven uiteengezet, mogelijk gemaakt door enzymen. Enzymen zijn het chemisch gereedschap van planten (en alle andere levende wezens).

Enzymen die biosynthese mogelijk maken zijn meestal gespecialiseerd en slechts in staat om in dit chemische proces een specifieke stap uit te voeren met een specifieke stof.

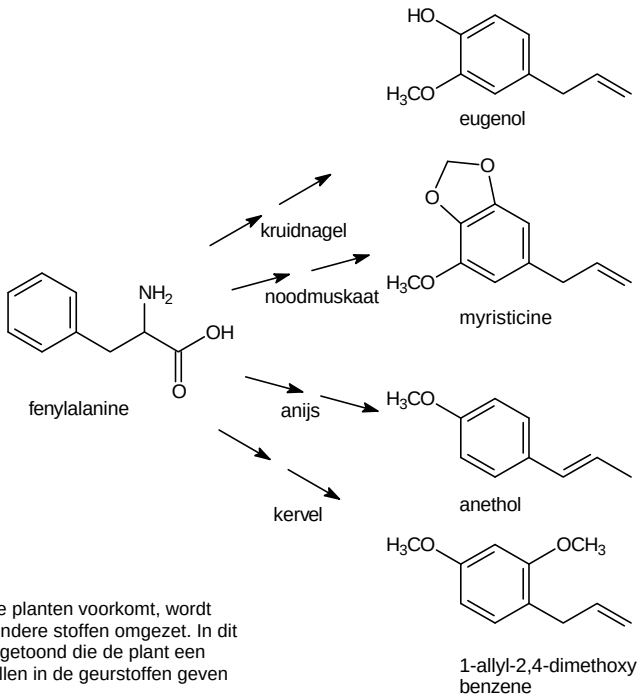


Fig. 2.
Het aminozuur fenylalanine, dat in alle planten voorkomt, wordt door sommige plantensoorten in bijzondere stoffen omgezet. In dit figuur worden een aantal geurstoffen getoond die de plant een specifieke geur geven. Kleine verschillen in de geurstoffen geven grote verschillen in geur.

De biosynthese van fenylalanine naar de geurstof van kruidnagel (eugenol; zie fig. 2) bestaat uit 6 stappen, met andere woorden: de kruidnagelboom heeft voor dit omzettingsproces 6 verschillende enzymen nodig.

Onderzoek aan de biosynthese van plantenstoffen levert informatie over de variatie aan chemische verbindingen en stoffen in het plantenrijk en is

noodzakelijk indien we iets willen veranderen aan de structuur van een stof of een plantensoort meer van deze stof willen laten produceren.

Podofyllotoxine

Er zijn op dit moment drie geneesmiddelen afgeleid van de plantenstof podofyllotoxine.

Deze geneesmiddelen zijn: etoposide, teniposide en etopophos (zie fig. 2 hoofdstuk I). Deze middelen worden gebruikt bij de behandeling van een verschillende vormen van kanker zoals testis carcinoom en kleincellig longkanker. Het zijn giftige middelen met nare bijwerkingen, maar zonder deze middelen zouden die ziektes vrijwel niet te behandelen zijn.

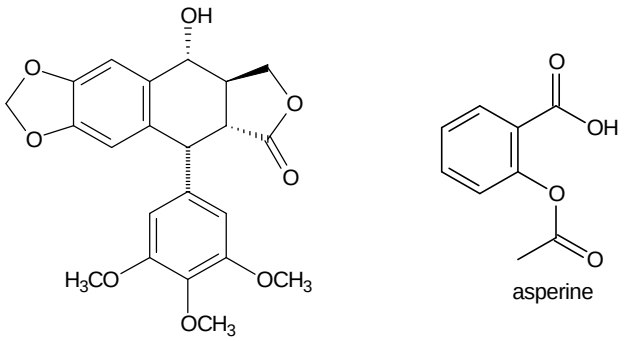


Fig. 3.
De chemische structuur van podofyllotoxine en aspirine.

Om de genoemde stoffen te kunnen maken is de industrie volledig afhankelijk van de plant die podofyllotoxine maakt.

De belangrijkste plantensoort waarin podofyllotoxine wordt gevonden is het groot voetblad, *Podophyllum hexandrum*. Deze plant groeit in het voorgebergte van de Himalaya en is tot nu toe vrijwel niet in cultivatie gebracht. Dat houdt in dat vrijwel alle te gebruiken podofyllotoxine afkomstig is van in het wild verzamelde planten. Er is grote behoefte aan de stof waardoor het niet verwonderlijk is dat deze plantensoort bijna met uitsterven bedreigd wordt. Dit probleem heeft er toe geleid dat men naar andere mogelijkheden is gaan zoeken om aan podofyllotoxine te komen. De meest voor de hand liggende methode is om podofyllotoxine synthetisch na te maken, zoals men dat indertijd voor aspirine heeft gedaan. Maar de chemische structuur van podofyllotoxine is complexer dan die van aspirine. Namaken lukt tegenwoordig wel, maar is erg duur. Een ander benadering is daarom vereist.

Podofyllotoxine wordt ingedeeld bij de lignanen. Lignanen vormen een belangrijke groep van plantenstoffen. Deze stoffen kan men bij veel plantensoorten tegenkomen. Tot de lignanen horen alle stoffen die ontstaan door het samenvoegen van twee fenylalanine-derivaten.

Hoeveel verschillende lignanen er zijn is niet bekend, maar waarschijnlijk meer dan duizend. De kleinste verandering in de structuur kan bepalend zijn voor de werking van de stof. In figuur 1 van hoofdstuk 1 staan de

structuren van een aantal lignanen weergegeven welke erg lijken op de structuur van podofyllotoxine. Al deze lignanen zijn giftig maar hebben een verschillende sterkte van werking. Op het ogenblik wordt alleen podofyllotoxine gebruikt in de farmacie.

Plantencelcultures en biotechnologie

Het is al langer bekend dat men elke willekeurige plantencel kan laten uitgroeien tot een volledige plant. Sinds de jaren zestig is bekend dat onder laboratorium condities het mogelijk is om plantencellen buiten de plant te groeien.

Plantenhormonen kunnen, onder bepaalde condities, cellen laten uitgroeien tot, bijvoorbeeld wortels of bloemen, of een compleet plantje. Het grote voordeel van deze laboratoriummethodes is dat men planten geheel gecontroleerd kan kweken en dat men niet afhankelijk is van seizoenen of andere milieufactoren. Bij een constante temperatuur worden in glazen kolven de plantencellen geschud. Deze cellen zitten in water met daarin opgelost, suiker (als energiebron), zouten, nutriënten en planten hormonen.

Plantencelcultures van het groot voetblad (*Podophyllum*) zouden in staat moeten zijn om podofyllotoxine te produceren. Alhoewel dit theoretisch tot de mogelijkheden behoort blijkt het in de praktijk niet mee te vallen om podofyllotoxine producerende cultures te realiseren.

Om van de biotechnologische benadering gebruik te kunnen maken moet men eerst een goed beeld hebben van de wijze waarop de plant de gewenste stof maakt. Dit proces, de biosynthese, is een van de onderwerpen die ik heb onderzocht voor dit proefschrift.

Vanaf het begin van de jaren tachtig is men al op zoek naar een systeem gebaseerd op plantencelcultures om podofyllotoxine te produceren. Helaas lukt het nog niet om deze methode op industriële schaal toe te passen. Er zijn systemen gevonden die een snelle groei van Podophyllum cellen mogelijk maakten maar weinig productie opleverden en systemen met hoge productie die echter slecht groeien. Dit gebrek aan goede resultaten heeft er toe geleid dat onderzoekers zijn gaan nadenken over andere strategieën. Zou het, bijvoorbeeld, mogelijk zijn om de celcultures genetisch te veranderen zodat er meer geproduceerd wordt? Zou men bepaalde grondstoffen aan de productie kunnen toevoegen welke omgezet worden in podofyllotoxine?

Deze vragen kunnen alleen beantwoord worden als men reeds een goed beeld heeft van de biosynthese van podofyllotoxine. Wat is bekend over podofyllotoxine en andere lignanen?

Lignanen zijn altijd opgebouwd uit twee eenheden, die beide afgeleid zijn van fenylalanine. Om de koppeling van deze twee eenheden mogelijk te maken moet fenylalanine eerst worden veranderd. Daarvoor wordt fenylalanine in de plant via 7 stappen omgezet tot coniferylalcohol. Er zijn 7 verschillende enzymen nodig voor de uitvoering van dit proces. Het product van de koppeling van twee coniferylalcoholeenheden noemen we een lignaan.

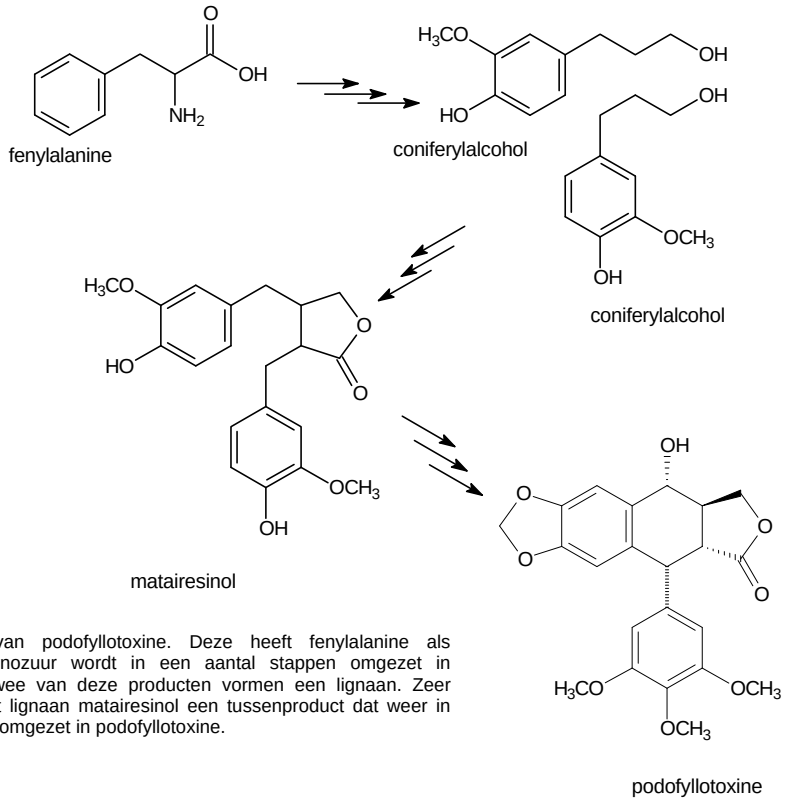


Fig. 4.
De biosynthese van podofyllotoxine. Deze heeft fenylalanine als grondstof. Dit aminozuur wordt in een aantal stappen omgezet in coniferylalcohol. Twee van deze producten vormen een lignaan. Zeer waarschijnlijk is het lignaan matairesinol een tussenproduct dat weer in vele stappen wordt omgezet in podofyllotoxine.

Deze lignanen kunnen daarna nog op verschillende wijzen worden veranderd en uitgebouwd. Planten kunnen ook meer dan twee coniferylalcoholeenheden aan elkaar koppelen. Dit proces leidt tot de vorming van lignine (hout). Er is een groot aantal stappen nodig om van twee coniferylalcoholeenheden tot podofyllotoxine te komen. Hoeveel stappen nodig zijn is onbekend.

Uit eerder onderzoek is gebleken dat de stof matairesinol, zeer waarschijnlijk, een tussenproduct is. Dat is van belang om te weten want als men matairesinol aan planten of plantencellen toevoegt ontstaat er podofyllotoxine. Recent onderzoek doet vermoeden dat bij celcultures van gele vlas (*Linum flavum*) meerdere routes (= een volgorde van een aantal stappen in de biosynthese) mogelijk zijn, om van matairesinol naar het eindproduct podofyllotoxine en 6-methoxypodofyllotoxine te komen.

Dat een plantensoort dezelfde stof op verschillende wijzen zou kunnen vervaardigen, werd tot voor kort onwaarschijnlijk geacht. Om van matairesinol tot podofyllotoxine te komen zijn er in ieder geval zes stappen nodig (6 enzymen; zie Fig. 9 van hoofdstuk I). Voor elke stap is een enzym nodig dat de stap uitvoert en bij elk enzym hoort een gen. De biosynthese van podofyllotoxine is daarom gebaseerd op meerdere genen. Als er verschillende routes mogelijk zijn, dan zijn daar ook nog meer enzymen

voor nodig en dus even veel genen. Een volgende denkstap is, dat een grotere hoeveelheid genen het resultaat is van een lange evolutie. Tot nu toe dacht men dat de biosynthese van podofyllotoxine zeer recent in de evolutie ontstaan is. Deze conclusie roept de vraag op: “is de biosynthese van podofyllotoxine al heel vroeg in de evolutie ontstaan, of begrijpen wij de biosynthese van deze stof nog niet goed?”

Het antwoord op die vraag zou ons inzicht in de biosynthese van podofyllotoxine bevorderen. Als deze biosynthese al vroeg in de evolutie is ontstaan, dan zou dit betekenen dat de genen voor deze biosynthese in heel veel plantensoorten aanwezig zijn. Dit soort inzichten kan helpen bij het zoeken naar betere productiesystemen.

Fluitenkruid (Anthriscus sylvestris (L.)Hoffm.)

Een van de meest algemene planten in Nederland, die ieder jaar onze bermen en slootkanten voorziet van een witte bloemenzee, is fluitenkruid. Deze plant heeft een wortelstok vergelijkbaar met die van de winterpeen, waar het overigens familie van is. Deze wortels bevatten een redelijk hoge concentratie aan lignanen. De belangrijkste van deze lignanen in fluitenkruid zijn deoxypodofyllotoxine, yateïne en anhydropodorhizol.

Omdat de plant zo makkelijk groeit en zo algemeen is in Nederland vormt het een goede plant voor onderzoek en komt de vraag op of fluitenkruid te gebruiken zou kunnen zijn als grondstof voor de productie van podofyllotoxine.

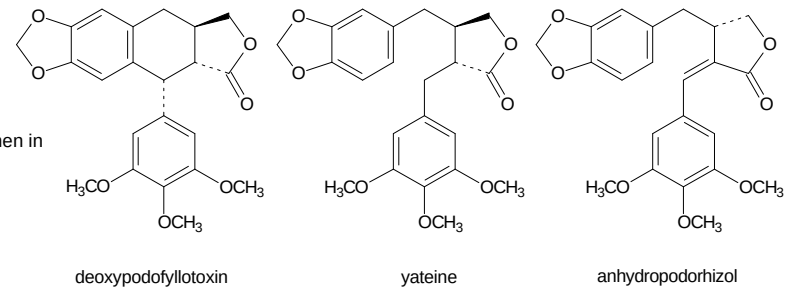


Fig. 5.
De drie belangrijkste lignanen in fluitenkruid.

Het doel van mijn promotie onderzoek

Om podofyllotoxine biotechnologisch te kunnen maken moet eerst de biosynthese van deze stof en van gerelateerde lignanen onderzocht worden en bekend zijn. Mijn onderzoek richt zich dan ook op een beter begrip van deze biosynthese. Daarnaast ben ik ook op zoek gegaan naar andere systemen om podofyllotoxine te kunnen maken. Alhoewel het mijn promotie onderzoek is, heb ik het niet alleen gedaan. Zowel bij het praktische werk als het nadenken en het schrijven van dit proefschrift heb ik hulp gehad van studenten, collega’s en wetenschappers uit verschillende landen.

III. A fast and simple GC-MS method for lignan profiling

Based on: Albert Koulman, Rein Bos, Manuel Medarde, Niesko Pras & Wim. J. Quax (2001) A fast and simple GC-MS method for lignan profiling in *Anthriscus sylvestris* and biosynthetically related plant species. *Planta Med.*, 67: 858-862.

NEDERLANDSE SAMENVATTING

Een GC-MS methode voor de analyse van fluitenkruid en andere planten die lignanen produceren.

Het feit dat fluitenkruid lignanen maakt was bekend door Japans en Koreaans onderzoek. Maar nog onbekend was of fluitenkruid onder alle omstandigheden evenveel en dezelfde lignanen maakt of dat daarin variatie voorkomt. Om de identiteit en de hoeveelheid van lignanen in planten te kunnen bepalen is een betrouwbare analysemethode noodzakelijk.
Verschillende wetenschappers hebben al diverse methodes ontwikkeld, maar deze analyses geven meestal alleen directe informatie omtrent de identiteit of de hoeveelheid van de stof, maar niet beide tegelijk.
Dit is de reden dat we een GC-MS methode hebben ontwikkeld. Deze methode houdt in dat een gas-chromatograaf (GC) wordt gebruikt om een mengsel van stoffen (plantenextract) te scheiden in de verschillende stoffen. Hierbij komen de stoffen een voor een van de GC af (zie figuur 2). Dit scheidingsapparaat is gekoppeld aan een massaspectrometer (MS). Dit apparaat bepaald van elke stof die van de GC komt de molecuulmassa, hetgeen informatie geeft over de identiteit van de stof. Tijdens dit proces breken de moleculen in stukken. Het proces volgt een aantal chemisch-fysische wetten en is specifiek voor elk stof. Door middel van deze bewerking kan men stoffen herkennen en onbekende stoffen identificeren. Vooral lignanen breken op een specifieke manier in stukken waardoor deze makkelijk kunnen worden herkend.

De kwaliteit van de methode hebben we bepaald door te kijken of de methode wel altijd dezelfde gegevens oplevert onder verschillende omstandigheden (zie tabel 2). Onderscheid werd daarbij gemaakt tussen verschillen op één dag (intra day variation) en verschillen tussen verschillende dagen (inter day variation).

De methode hebben we toegepast op fluitenkruid dat op verschillende plekken in Nederland werd verzameld. Dit gebeurde hoofdzakelijk begin april in 1999, op een rit van Zeeland naar Groningen. Bij deze monsters (bestaande uit een aantal planten) werd onderzoek gedaan naar de lignaanprofielen (hoeveelheid en soort). Het bleek dat elke populatie een verschillende hoeveelheid lignanen bevatte.

Planten uit Nieuwegein bleken veel hogere concentraties lignanen te bevatten dan planten uit bijvoorbeeld Diever. Verder bleek dat soortgenoten van fluitenkruid helemaal geen, of heel andere lignanen produceerden. Dit fenomeen wordt verder besproken in volgende hoofdstukken.

ABSTRACT

A new GC-MS method for monitoring lignans was developed to study the variation in plants and elucidate the biosynthetic steps. A simple and fast extraction procedure for lyophilised plant material was developed, giving a lignan rich extract. A GC-MS method was set up using an apolar WCOT fused silica column using a high temperature programme (150°C to 320°C with 15°C min^{-1}). This new GC-MS method gave a clear lignan profile of plant material. It was possible to show the large variation in the concentrations of deoxypodophyllotoxin, yatein and anhydropodorhizol in *Anthriscus sylvestris* (L.) Hoffm. plants growing on different locations using cinchonidin as an internal standard. In contrast with existing GC methods for lignan analysis no derivatisation is needed. It is also possible to use this method for the detection of different classes of lignans in biosynthetically related plant species.

INTRODUCTION

Podophyllotoxin causes the current interest in lignans as a unique starting compound for the production of two widely used anticancer drugs etoposide and teniposide. So far podophyllotoxin has been isolated from plant material because organic synthesis is economically not feasible. The isolation of podophyllotoxin from the rhizomes of *Podophyllum peltatum* L. and *Podophyllum hexandrum* Royle (Berberidaceae) plants is not very ideal for large-scale drug production. The supply of *P. hexandrum* rhizomes, which contain ca. 4% of podophyllotoxin on a dry weight basis, becomes increasingly limited due to both intensive collection and lack of cultivation (Choudhary *et al.*, 1998). Therefore much research effort is devoted to a faster and more economical procedure for the podophyllotoxin production.

Especially the use of biotechnological procedures would be an interesting alternative and *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae; wild chervil) might play an important role in this context. Wild chervil is a common weed in Northwest Europe and its rhizomes contain considerable amounts of the lignans deoxypodophyllotoxin, yatein and anhydropodorhizol (Fig. 1). In the biosynthetic pathway of aryltetralin lignans as proposed by Broomhead and co-workers, the direct precursor of podophyllotoxin is deoxypodophyllotoxin (Broomhead *et al.*, 1991). Cultures of undifferentiated plant cells or fungi can convert deoxypodophyllotoxin into podophyllotoxin (Kondo *et al.*, 1990; Van Uden *et al.*, 1997). The other two compounds could be earlier precursors of podophyllotoxin (Kamil and Dewick, 1986b).

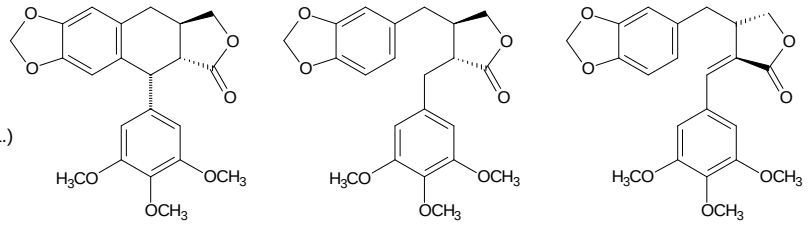


Fig. 1.
The main lignans in *Anthriscus sylvestris* (L.) Hoffm.

Our research is aimed at the possible use of this concept of podophyllotoxin production including *A. sylvestris* as a source of lignan precursors and the identification of enzymes for the bioconversion to podophyllotoxin. An important starting point will be the selection of *A. sylvestris* specimens with a high lignan content for precursor isolation. There are no data available on content and possible variation of lignans in *A. sylvestris*. For these reasons an adequate analytical method is needed which enables a qualitative and quantitative determination of lignans in plant material.

We developed a direct GC-MS analysis including a simple and fast sample preparation that enables the lignan profiling of large numbers of samples.

MATERIALS AND METHODS

Chemicals and reagents

Methanol and dichloromethane (all P.A.) were purchased from Merck (Darmstadt). Cinchonidin was purchased from Fluka (Zwijndrecht, The Netherlands).

Deoxypodophyllotoxin (as published in Van Uden *et al.*, 1997), yatein and anhydropodorhizol were isolated from lyophilised *Anthriscus sylvestris* (L.) Hoffm. rhizomes. Arctigenin $[á]_D$: -22° (MeOH, *c* 5.1) [(Rahman *et al.*, 1990b):-30°] and phylligenin $[á]_D$: +120° (MeOH, *c* 3.2) [(Rahman *et al.*, 1990b):+120°] were isolated from *Forsythia* x *intermedia* Zabel var. Lywood. Structures were confirmed using ^{1}H and ^{13}C NMR. Yatein and anhydropodorhizol were both synthetically prepared as a racemic mixtures (Medarde *et al.*, 1995).

Plant Material

Specimens of *A. sylvestris* (L.) Hoffm. were collected on different locations in the Netherlands and Belgium as summarised in Table 1. Only the rhizomes were collected and thoroughly cleaned from mud, sand, and other roots. Leaves and branches of *Forsythia* x *intermedia* (Zabel) var. Lynwood were collected from a field in Groningen belonging to the University Centre of Pharmacy. Roots of *Anthriscus cerefolium* L. were provided by P.A.V. (Lelystad, The Netherlands). *Pastinaca sativa* L. rhizomes were purchased from the Knollentuin (Dwingeloo, The Netherlands). *Linum flavum* L. was cultured in our laboratory.

Table 1. Collection locations of *Anthriscus sylvestris* (L.) Hoffm. in the Netherlands and Belgium and the calculated total lignan concentration.

Location	Date	Place	Country[a]	North	East	Total Lignan Content (%)[b]
1	17-4-1999	Sth. Bank Pr. Albert Channel	B	50° 48'39"	05°40'16"	0.42
2	17-4-1999	Rochlenge	B	50° 35'15"	05°35'15"	0.52
3	11-4-1999	Zwolle	NL	52° 33'12"	06°10'33"	0.33
4	11-4-1999	Barendrecht	NL	51° 51'45"	05°40'16"	0.62
5	10-4-1999	Koudekerken	NL	51° 41'21"	03°45'52"	1.27
6	11-4-1999	Nieuwegein	NL	52° 00'55"	05°07'16"	2.33
7	10-4-1999	Diever	NL	52° 21'54"	06°18'51"	0.58
8	28-5-1999	Groningen	NL	53°11'32"	06°37'18"	0.48

[a] NL: The Netherlands; B: Belgium.
[b] Defined as the sum of the percentage (w/w) of yatein, anhydropodorhizol and deoxypodophyllotoxin on basis of dry weight.

The plant material was stored at –20°C until lyophylisation. The authors identified all plant material using the Dutch flora (Van der Meijden *et al.*, 1983). Voucher specimens are present at our institute; *A. sylvestris* as Asylv1993 and Asylv1999-1 to 8, *A. cerefolium* as Acere1999, *F.* x *intermedia* Fint1999, *P. sativa* as Psat1999, *L. flavum* as Lflav1989.

Sample preparation

From the 8 locations a number (n = 5 to 7) of at random chosen plants were collected and lyophilised. The dried material was combined, ground, filtered through a 1mm ∅ sieve and homogenised. In case of the more detailed measurements to study the relation between morphology and lignan content or to determine the variation in lignan content within one location, individual lyophilised plants were ground, filtered through a 1mm ∅ sieve and homogenised.

For analysis 100 mg dried and homogenised material was weighed in a Sovirel tube. A 2 ml portion of 80% methanol containing 0.4 g l^{-1} cinchonidin was added and the mixture was sonicated during 1 h. Then 4 ml of dichloromethane and 4 ml of H_2O were added. The tube was closed, vortexed and centrifuged at 1,000 *g* for 6 min. The aqueous layer was discarded and 1.50 ml of the organic layer was transferred into a 1.5 ml micro tube (No./REF 72.690, Sarstedt, Nümbrecht, Germany). The dichloromethane was evaporated using nitrogen and the residue was redissolved in 1.5 ml of methanol. The tubes were centrifuged at 10,000 rpm for 10 min in an Eppendorf centrifuge 5414, and part of the liquid was transferred into a 0.8 ml Crimp Neck Vial (Art. No. 98819, Alltech / Applied Science B.V. Breda, The Netherlands) and closed immediately. The samples were ready for GC-MS analysis.

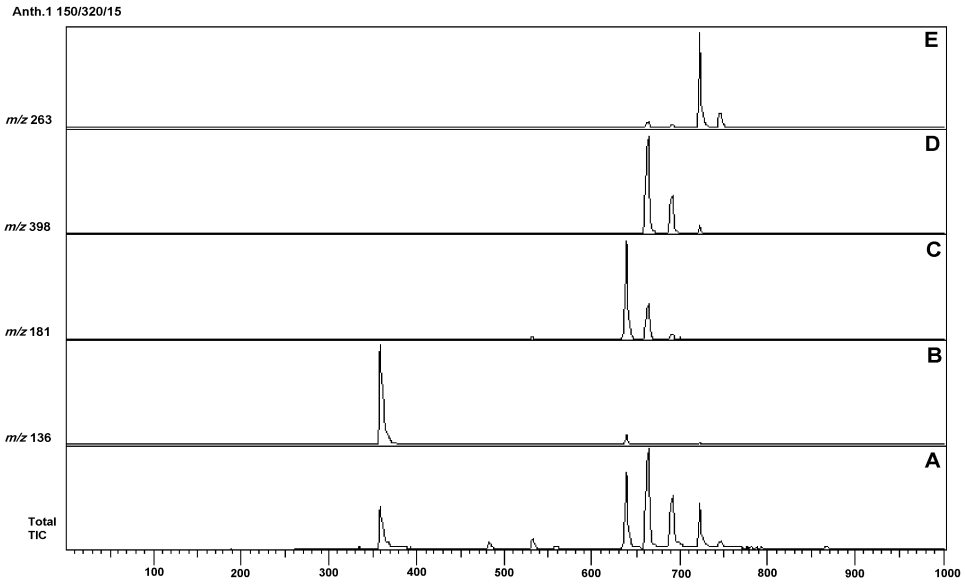


Fig. 2.
Representative total GC-MS chromatogram of an *A. sylvestris* (L.)Hoffm. specimen, collected from location 8 (**A**). The other chromatograms are extracted ion currents; for the I.S. (**B**), yatein (**C**), Deoxy-podophyllotoxin (**D**) and anhydro-podorhizol (**E**). On the x-axes the scan-number and on the y-axes the % intensity of selected ion are given.

GC-MS analysis

GC-MS analysis of the lignans was performed on a Unicam 610 GC-MS. The gas chromatograph was equipped with a WCOT fused-silica CP-Sil 5 CB (15m x 0.31 mm i.d., film thickness, 0.25 μm; Chrompack; Middelburg; The Netherlands). The following oven temperature program was used: 150° to 320°C at 15°C/min and maintained at 320°C for 5 min. The injector temperature was 260°C. The carrier gas used was helium with an inlet pressure of 5 psi, at a linear gas velocity of 32 cm/s and a split ratio of 20:1. The injected volume was 4 μl. To obtain mass spectra electron impact ionisation (70 eV) was used; ion source temperature of 250°C; interface temperature of 280°C; a scan speed of 2 scans/s; mass range 34-600 u. The acquired data were stored and afterwards analysed with Lucy Display V2.70 software. The following characteristic ions were used for single ion monitoring of the compounds: deoxypodophyllotoxin: 398 *m/z*; yatein: 400 *m/z*; anhydropodorhizol: 135 *m/z*. For the internal standard the ion with 136 *m/z* was used for single ion monitoring. The single ion monitoring of the internal standard had no interference of compounds like anhydropodorhizol (*m/z* 135) and Arctigenin (*m/z* 137) due to the large difference in retention time.

Calibration and quantification

For the quantitative determination of deoxypodophyllotoxin, yatein and anhydropodorhizol calibration curves were prepared using methanolic solutions with concentrations ranging from 0.25 μg ml^{-1} to 2.5 μg ml^{-1} of the different lignans. From each solution a 1 ml portion was transferred to a Sovirel tube containing 100 mg of grounded lyophilised *Anthriscus cerefolium* L. (Chervil, Apiaceae) roots. *A. cerefolium* belongs to the same genus as *A. sylvestris* but does not contain detectable amounts of lignans. It was used to check the possible interference of the compounds to be analysed with the biological matrix, i.e. other plant constituents. The mixture was dried using nitrogen and treated further as described in the paragraph Sample Preparation. For each concentration of each of the 3 lignans the calibration was done in triplicate. Linear regression was used for all calibrations. For each compound the limit of detection was determined.

Inter-day and intra-day reproducibility's of the analysis were determined by replicate analysis (n=5) of each lignan at 3 different concentrations in the presence of *A. cerefolium* L. material against a calibration curve. Inter-day and intra-day coefficients of variations (CV) were calculated according to Shah (Shah *et al.*, 1992).

A standard solution of deoxypodophyllotoxin 1 μg ml^{-1} was subjected to the standard sample preparation and was used for control of inter- and intra-day variations and run before and after each series of sample analysis. In case of a high number of samples the standard was also run after each tenth sample.

RESULTS AND DISCUSSION

The presence of a number of lignans in *A. sylvestris* has been proven and confirmed by several authors (Ikeda *et al.*, 1998a). Thus far no analytical procedure has been established to study the lignan composition of *A. sylvestris* rhizomes. Various analytical procedures for 2,7'-cyclolignans and other groups of lignans have been published like HPLC (Lim, 1996) GC (Meagher *et al.*, 1999; Morton *et al.*, 1999), and MECC (Ganzera *et al.*, 1999). These are all quantitative methods, some of which are applied on plant material. In our studies on the biosynthesis of deoxypodophyllotoxin and other lignans in *A. sylvestris* a qualitative as well as quantitative analysis is needed. Therefore we developed a simple and fast sample preparation yielding extracts, which can be directly analysed using GC-MS. We used cinchonidin as an internal standard for the quantification.

The advantage of MS as a detection system lays in the clarity of the mass spectra of lignans. These spectra are easy to interpret and there is extensive literature about the fragmentation (Arimoto *et al.*, 1996). It is also possible to detect other lignans than the previously mentioned. This will give insight in the lignan variation of *A. sylvestris* and the biosynthetic pathway leading to deoxypodophyllotoxin.

The sample preparation as mentioned under the section Materials and Methods turned out to be the way to an optimal extraction of all the examined lignans from the plant material. Different extraction systems were tested; the pH of the methanol and the water phase was varied between 2 and 10. For the sample with the highest lignan content (a specimen of location 6 containing 4.03% on dry weight basis) the water phase was extracted several times with dichloromethane. This organic phase was reduced under vacuum to 1 ml and analysed by GC-MS. Deoxypodophyllotoxin was only present in a very minute amount in the remaining water phase (< 1% of the total amount). The sample preparation can be considered as very fast, because no derivatisation was necessary as in previous described GC-methods for lignans (Meagher *et al.*, 1999; Morton *et al.*, 1999).

The use of cinchonidin as an internal standard resulted in an acceptable inter-day coefficient of variation (generally CV < 5%) for all 3 lignans (see Table 2). In figure 2 an illustrative example of a chromatogram is depicted. The cinchonidin peak has a retention time of 7.6 min and is completely separated from the peaks of the lignans. Yatein has a retention time of 10.1 min., deoxypodophyllotoxin of 10.5 min. and anhydropodorhizol of 10.9 min.

The peak area on basis of the single ion monitoring of each of the compounds in ratio to the internal standard gave a good linearity for all 3 compounds (see Table 2). The ions used for each of the compounds gave the base peaks in the mass spectra. These ions were also present in other mass spectra. For this reason it was necessary to take the retention time into

account for the assignment of the peaks in mixtures. The differences in retention time between the main compounds are sufficient for an unambiguous assignment of the peaks. Only for low concentrations in complex mixtures the interference of other lignans could enhance the limit of detection. In a clear solution of each main lignan the limit of detection was as low as 10 ng ml^{-1}, while in plant samples concentrations below 30 ng ml^{-1} were not detectable due to interference of other compounds.

Table 2. Inter- and intra-day variation, linearity and recovery of the main lignans in a biological matrix consisting of *Anthriscus cerefolium* L. constituents.

Lignans	Variation (CV (%) n = 5)			Linearity (0.025-0.25 µg ml^{-1}) R^2	Recovery (%) Mean ± SD (n = 4)
	Concentration (µg ml^{-1})	Inter-day	Intra-day		
deoxypodophyllotox	0.25	4.8	4.5		
	1.0	3.8	3.1	0.999	105 ± 3
	2.5	3.2	2.2		
yatein	0.25	4.7	4.1		
	1.0	3.3	3.1	0.998	107 ± 2
	2.5	3.1	3.2		
anhydropodorhizol	0.25	5.2	5.1		
	1.0	4.2	3.6	0.996	103 ± 4
	2.5	3.4	3.1		

The inter- and intra-day variances are shown in Table 2. The variation is generally low; yielding CV's of < 5%, meaning an acceptable precision. Near the limit of quantification the CV was somewhat higher than 5% for anhydropodorhizol. From the 3 analysed compounds anhydropodorhizol showed a tendency to decompose in methanolic solution.

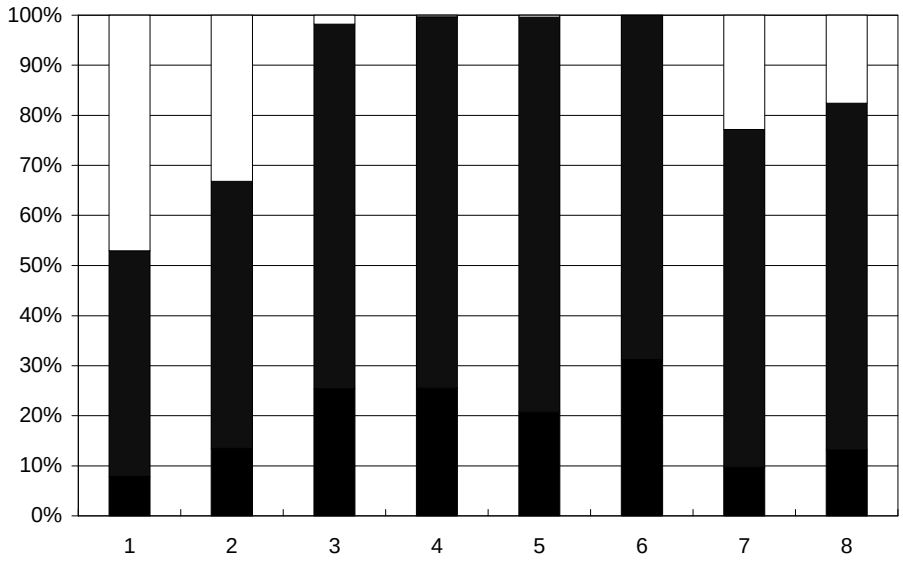


Fig. 3.

The percentage of each of the main lignans yatein ■, deoxypodophyllotoxin □ and anhydropodorhizol □ in *Anthriscus sylvestris* (L.)Hoffm. from the different collection locations as listed in Table 1. Total lignan contents (% DW) are summarised in Table 1.

The recoveries are higher than 100% but do not interfere with the reproducibility. The high recovery is probably caused by working with dichloromethane during sample preparation. The evaporation of this organic phase during pipetting is very rapid and might very well contribute to the high recovery. Whether the instability of anhydropodorhizol influences the recovery is not clear, although the recovery is lower than those of deoxypodophyllotoxin and yatein are, it is still over 100%.

The variation in lignan content between plants of different locations was much larger than anticipated. The total lignan concentration (defined as the sum of the yatein, anhydropodorhizol and, deoxypodophyllotoxin concentrations) differs almost a seven fold between the highest and lowest, resp. no 6 and no 3 in Table 1. There was not only a difference in total lignan concentration, but also in the ratio between different lignans (see Fig. 3). In some specimens (no. 6 Fig. 3) the anhydropodorhizol concentration was not even detectable, while in other specimens the deoxypodophyllotoxin and anhydropodorhizol content was almost equal (no 1 Fig. 3). Statistical analysis showed that in all specimens analysed there is a positive correlation between the yatein and deoxypodophyllotoxin concentration (r^2 = 0.8423; n=39). There is no correlation between the anhydropodorhizol concentration and the concentration of the other two lignans.

The plants on the collection sites no. 8 and 6 (Table 1) were examined in more detail. The mean concentration of deoxypodophyllotoxin differed significantly between these collection sites. The mean deoxypodophyllotoxin content of the analysed plants on location 6 was 1.73% ± 0.72 (on basis of dry weight, *n*=10) while for location 8 it was 0.51% ± 0.35 (on basis of dry weight, *n*=9). The large standard deviation means that within each location there is also a large variation in lignan content. The botanical identity however, was the same for all *A. sylvestris* plants. Differences in root morphology were compared with the lignan content, but no relationship could be found.

Table 3. Mass spectra of lignans found in different plants and the literature wherein these compounds were originally reported.

Compound	Mass spectra m/z (%)	Found in	Reported from[b]
Yatein	77(32) 105(13) 181(100) 400(68)	*A.s.*[a]	*A.s.* (Ikeda *et al.*, 1998a)
Anhydropodorhizol	105(14) 135(74) 176(16) 263(100) 398(23)	*A.s.*	*A.s.* (Ikeda *et al.*, 1998a)
Deoxypodophyllotoxin	173(19) 181(21) 185(16) 398(100)	*A.s*	*A.s.* (Ikeda *et al.*, 1998a)
Arctigenin	137(100) 151(46) 177(7) 372(20)	*A.s., F.i.*	*F.i.* (Rahman *et al.*, 1990b)
Phylligenin	137(22) 151(100) 165(35) 372(89)	*A.s., F.i.*	*F.i.* (Rahman *et al.*, 1990b)
6-Methoxypodophyllotoxin	168(46) 181(18) 426(10) 444(100)	*L.f.*	*L.f.* (Van Uden *et al.*, 1995)
Secoisolariciresinol	135(47) 151(100) 358(30)	*P.s., F.i.*	*F.i.* (Umezawa *et al.*, 1990)
Bursehernin	135(7) 151(100) 208(3) 219(5) 370(42)	*A.s.*	*B.s.*(McDoniel and Cole, 1972)

[a]: *A.s.*: *Anthriscus sylvestris* (L.) Hoffm., *F.i.*: *Forsythia* x *intermedia* Zabel, *L.f.*: *Linum flavum* L., *P.s.*: *Pastinaca sativa* L.. *B.s.*: *Bursera schlechtendalii.*

[b]: Plant and original publication.

The chromatograms of all analysed *A. sylvestris* rhizomes were screened for the presence of any possible intermediates in the biosynthetic pathway leading to deoxypodophyllotoxin. This search resulted in the identification of several compounds that have not yet been described for *A. sylvestris*.

The interpretation of the mass spectra pointed to the lignans arctigenin and phylligenin (see Table 3).

Both compounds were only present in very small quantities and have been previously described for *F.* x *intermedia* Lignan profiling of *F.* x *intermedia* leaves showed clearly high amounts of the same compounds. They were isolated and the MS (see Table 3) and NMR data were consistent with those from arctigenin and phylligenin as published by Rahman (*et al.*, 1990b). The 6th lignan we found in the GC-MS analysis of *A. sylvestris* was bursehernin.

The roots of *Anthriscus cerefolium* L. did not contain any detectable amounts of lignans and were used for the optimisation of the method (see section Materials and Methods). In the roots of *Linum flavum* L. plants it was possible to demonstrate the presence of 6-methoxypodophyllotoxin and β-peltatin and β-peltatin-A-methylether. In the rhizomes of *Pastinaca sativa* L. we were able to detect secoisolariciresinol.

The newly developed GC-MS method makes it possible to profile the lignans in *A. sylvestris*. The developed GC-MS method is applied now to determine the seasonal variation of the lignans in *A. sylvestris* and further studies are focused on the cause of the variation (genetic, abiotic or biotic factors) and finally the selection of a high producing variety. A high lignan producing variety can be used as a source of precursors to be involved in the biotechnological production of podophyllotoxin by plant cell cultures. Bioconversions will be monitored with the developed method.

Furthermore we also could profile the lignan content in other biosynthetically related plant species and screen crops and legumes for lignans with a possible additional nutritional value, like secoisolariciresinol in *P. sativa*. The above mentioned applications demonstrate that the method developed has the potency for a broader application in medicinal plant research.

Acknowledgements

The authors would like to express their gratitude to Ing. Hans van der Mheen for providing the roots of *A. cerefolium* L., to Prof. A.W. Alfermann for providing yatein as reference compound for the guided isolation of larger amounts of this compound. We also like to thank Klaas van Maanen for helping with the collection of *A. sylvestris*.

IV. Volatile components from *Anthriscus sylvestris*.

Based on: Rein Bos, Albert Koulman, Herman J. Woerdenbag, Wim J. Quax & Niesko Pras (2002) Volatile components from *Anthriscus sylvestris* (L.) Hoffm. *J. Chromatogr. A*, 966: 233-238.

NEDERLANDSE SAMENVATTING

De geurstoffen van fluitenkruid

In het onderzoek, dat in dit hoofdstuk staat beschreven, is kervel met fluitenkruid vergeleken. Beide soorten zijn schermbloemige en behoren tot het geslacht *Anthriscus*.

Uit eerder onderzoek is bekend dat kervel (*Anthriscus cerefolium*) veel fenylalanine omzet in methylchavicol and 1-allyl-2, 4-dimethoxybenzene. Deze twee stoffen zorgen voornamelijk voor de karakteristieke smaak en geur van kervel. Kervel maakt echter geen hoge gehaltes aan lignanen. Fluitenkruid (*Anthriscus sylvestris*) gebruikt een belangrijk deel van fenylalanine voor de lignanenproductie. Omdat deze twee soorten zo nauw verwant zijn moeten ze ook voor een groot deel dezelfde genen, enzymen en biosyntheseroutes bevatten. Omdat fluitenkruid al veel fenylalanine omzet in lignanen, wilden wij weten of fluitenkruid fenylalanine ook gebruikt voor de productie van geurstoffen. Daarom hebben we de geurstoffen van fluitenkruid onderzocht.

Op verschillende manieren zijn de geurstoffen van fluitenkruid geïsoleerd en grondig geanalyseerd. De complete lijst van geurstoffen van fluitenkruid staat in tabel 1.

Uit het onderzoek blijkt dat de belangrijkste geurstoffen in fluitenkruid niet van fenylalanine afkomstig zijn. In fluitenkruid is slechts 5 % van de geurstoffen afkomstig van fenylalanine. In kervel is dit wel 90 %.

ABSTRACT

The volatile components of the leaves and roots from *Anthriscus sylvestris* (L.) Hoffm., obtained through hydrodestilation, were analysed by GC and GC-MS. This was compared to dichloromethane extracts of both fresh and dried leaf and root material. The monoterpene fraction (69-70%) dominated, while â-phellandrene (39-45%) was the main component in both the leaf and the root oil. Other components in the leaf oil were â-myrcene (17%), sabinene (6.2%), *Z*-â-ocimene (5.4%) and benzene acetaldehyde (4.1%). In the roots we found *Z*-â-ocymene (16.9%) and á-pinene (4.6%) as other major components. These principle constituents of both essential oils were also present in the dichloromethane extracts of the leaves and the roots, although in much smaller percentages.

INTRODUCTION

The family of the Apiaceae is well known as a source for essential oils and a number of species is especially cultivated for it, like *Pimpinella anisum* and *Anthriscus cerefolium* (Zwaving *et al.*, 1971). The essential oils of the exploited Apiaceae members are often dominated by phenylpropanoid derivatives such as anethol, methylchavicol and 1-allyl-2,4-dimethoxy-benzene.

In our recent phytochemical studies of the Apiaceae member *Anthriscus sylvestris*, we found that this species accumulates high amounts of deoxypodophyllotoxin and related lignans (see chapter III). Lignans also derive from the phenylpropanoid pathway (Lewis and Davin, 1999). A logical question therefore would be if the high lignan content has an influence on the essential oil composition in comparison to that of the essential oils of related species in the Apiaceae family. *Anthriscus sylvestris* accumulates lignans in its roots. These compounds are not found in the leaves. Therefore we studied the essential oil composition of both leaf and root material.

Anthriscus sylvestris (L.) Hoffm., (commonly known as wild chervil or cow parsley) is an abundant weed in Northwest Europe, with a height of 0.15-0.80 m and flowers in Mai-June (Tutin et al., 1968). Due to its high content of lignans, this species might form an interesting alternative for the production of podophyllotoxin (Van Uden *et al.*, 1997).

Next to the composition of the essential oils obtained through hydrodistillation we used other sample preparations and extraction methods to profile the volatile components in *A. sylvestris*. We compared apolar (dichloromethane) extracts of fresh and dried material. We compared drying process consisting out of drying at room temperature or by lyophilisation.

MATERIAL AND METHODS

Plant material

Whole flowering plants were collected near the city of Groningen of June 15, 2001. Voucher specimens have been deposited in our institute (no. Asylv2001). The roots were separated from the aerial parts of the plants, from the aerial parts we only used the leaves. This material was either immediately subjected to hydrodistillation or dichloromethane extraction, or dried at room temperature for 64 h, or lyophilised until dry.

Isolation procedure

Essential oil

The essential oil samples were isolated from fresh material, leaves and roots, by hydrodistillation for 4 h in 500 ml water, according to the determination of the essential oil content in vegetable drugs, using the apparatus described in the *Nederlandse Farmacopee*, 6th edn, 2nd printing (Anonymous, 1966). Xylene (100 µl) was used as the collection liquid, and the oil samples were stored at -20°C until analysed. The oil samples were diluted 50 times with cyclohexane prior to GC analysis; injected volume, 1.0 µl. In addition, for GC-MS analysis, the oil samples were separated into two fractions – with hydrocarbons and oxygen-containing compounds, respectively – by eluting 250 µl of oil on a Bakerbond SPE column, filled with 1 g of silica gel (Mallinckrodt BAKER b.v. Deventer, The Netherlands, # 7086-01), with subsequently 5 ml of *n*-hexane and 5 ml of diethyl ether. After gentle evaporation of the solvent of both fractions, 50 µl of each residue were diluted with 950 µl cyclohexane and then submitted to GC-MS analysis.

Dichloromethane extract

The dichloromethane extracts from fresh material were isolated as follows: 10 g of plant material was extracted three times with 10 ml dichloromethane and dried over Na_2SO_4. The dichloromethane was evaporated carefully and the residue was dissolved in 5 ml of dichloromethane.

The dichloromethane extracts from the dried material were prepared as follows: 2.5 g of dried (at room temperature or lyophilised, respectively) material was extracted three times with 10 ml of dichloromethane. The dichloromethane was evaporated carefully and the residue was dissolved in 10 ml of dichloromethane.

Gas chromatography

GC analysis was performed on a Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 injector and a Hewlett Packard 3365 Series II Chemstation, under the following conditions: column, WCOT fused-silica J&W DB-5 (30 m x 0.249 mm, film thickness 0.25 µm; J&W Scientific, 91 Blue Ravine Road, Folsom, CA 95630, USA); oven temperature program, 60°-300°C at 3°C min^{-1}; injector temperature, 250°C; detector (FID) temperature, 300°C; carrier gas, He; inlet pressure, 17.5 psi; linear gas velocity, 30.8 cm s^{-1}; split ratio, 60:1; injected volume, 1.0 µl for the essential oils and the dichloromethane extracts of the dried material; 4.0 µl for the dichloromethane extract of the fresh material.

Gas chromatography - mass spectrometry

GC-MS (EI) was performed on a Unicam 610/Automass 150 GC-MS system. The GC conditions were: column, WCOT fused-silica CP-Sil 5 CB (25 m x 0.25 mm, film thickness, 0.25 µm; Chrompack, Middelburg); oven temperature program, 50°-290°C at 4°C min^{-1}; injector temperature, 260°C; carrier gas, He; inlet pressure, 5 psi; linear gas velocity, 32 cm s^{-1}; split ratio, 20:1; injected volume, 1.0 µl. MS conditions: ionisation energy, 70 eV; ion source temperature, 250°C; interface temperature, 280°C; scan speed, 2 scans s^{-1}; mass range, 34-500 u.

Identification of the compounds

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{17} *n*-alkanes, and mass spectra with corresponding data from reference compounds and from the literature (Joulain and König, 1998; Adams, 1995). The percentages of the components were calculated from the GC peak areas, using the normalisation method.

RESULTS AND DISCUSSION

Essential oil composition

The yield of the essential oil sample distilled from the fresh leaves was 0.10% (v/w). We could identify 59 components in this oil, amounting to 93% of the total oil (see Table 1). The total percentage of the hydrocarbon fraction in the oil was 79%.

The monoterpene fraction (70%) dominated the essential oil sample. â-Phellandrene (38.8%), â-myrcene (16.7%), sabinene (6.2%), and *Z*-â-ocimene (5.4%) were the main components. The oxygen-containing monoterpene fraction represented 8.8% of the total oil with *trans*-sabinyl acetate as the main component (7.7%). The sesquiterpene was smaller (8.6%) with germacrene-D (4.2%) and *E,E*-á-farnesene (2.5%) as main components (see Table 1.).

The yield of the essential oil sample distilled from the fresh roots was 0.25% (v/w). A total of 50 components were identified, amounting to 80% of the total oil. The total percentage of the hydrocarbon fraction in the oil was 69%.

The essential oil sample was dominated by the monoterpene fraction (69%). â-Phellandrene (45.4%), *Z*-â-ocymene (16.9%), and á-pinene (4.6%) were the main components. The oxygen-containing monoterpene fraction represented 3.4% of the total oil with *trans*-sabinyl acetate as the main component (3.3%). The sesquiterpene fraction was smaller (3.2%) with germacrene-D (4.2%) as a main component.

In the literature no investigations have been found on the essential oils of the roots of *A. sylvestris*, and only three references about the essential oil of the leaves and flowers were found. The oldest study does not give any quantitative data (Kurihara and Kikuchi, 1979), and in the two more recent studies only the emitted volatile compounds were investigated (Borg-Karlson *et al.*, 1993; Valterová *et al.*, 1997). As in our study, in these studies myrcene was identified as the main component as well.

In contrast to the studies of Kurihara and Kukuch (1979) and Valterova and co-workers (1997), we did not find limonene. An other important difference is that in the here presented study â-phellandrene is the most abundant compound while this terpenoid was not found by any of the other studies or only as a minor component (< 2%) (Borg-Karlson *et al.*, 1993). In total we found 47 compounds that have not been previously reported from the essential oils of *A. sylvestris*.

The total percentage of essential in the roots was higher than in the leaves. In general the profile of the root and leaf oils correspond, with only a few marked differences. The most pronounced difference is that in leaves β-myrcene was the second most predominant component, while this is Z-β-ocimene in the roots. Another difference is that the α-pinene concentration

was ten times higher in roots while the sabinene concentration was almost ten times higher in the leaves.

The essential oil of *A. sylvestris* is different from that of the related species *A. cerefolium*. Methylchavicol and 1-allyl-2,4-dimethoxybenzene, which are the most abundant constituents of the essential oil of the *A. cerefolium* leaves, could not be detected in the leaves of *A. sylvestris*. The main monoterpenes in *A. cerefolium* leaves (β-pinene and β-phellandrene) were also present in *A. sylvestris* (Zwaving *et al.*, 1971).

There was no difference in the content of benzene acetaldehyde between the roots and leaves. This is the only major component in the essential oil that is biosynthetically related to lignans. The presence of lignans in the roots has no notable influence on the essential composition in comparison to the leaves, but might explain the absence of phenylpropanoids as main constituents in the essential oil of *A. sylvestris* compared to *A. cerefolium*, which contains only trace amounts of lignans.

Table 1. Composition of the essential oil from *Anthriscus sylvestris*

	Ri[a]	Fresh leaves Ess. Oil (%)	fresh leaves DCM (%)	dry leaves[b] DCM (%)	dry leaves[c] DCM (%)	fresh roots Ess. oil (%)	fresh roots DCM (%)	dry roots[b] DCM (%)	dry roots[c] DCM (%)
Tricyclene	921	t[d]					t	t	
á-Thujene	925	0.3				0.3	0.1		
á -Pinene	932	0.5		0.1		4.6	1.0		
Phenyl acetaldehyde	945	t	0.1			0.1	t	0.7	0.1
Camphene	946	0.1				0.1	t		
Isopropyl benzene	956	0.2				0.1		0.1	
Benzaldehyde	961	t				t			
Sabinene	970	6.2	1.0	0.3		0.8	1.9		
â -Pinene	975	0.4	0.1			0.2	0.2	0.3	0.2
â -Myrcene	990	16.7	3.0	1.8	0.3	0.3	2.5	2.2	0.6
á -Phellandrene	1004	1.3				0.3	0.4	0.3	0.1
Ä[3]-Carene	1010	0.1	0.2	0.1		t	1.5	0.8	0.3
á -Terpinene	1017	0.1				0.1	t		
p-Cymene	1022	t					0.2	0.1	t
â -Phellandrene	1027	38.8	2.3	0.6	0.2	45.4	15.1	10.8	2.6
Z- â -Ocimene	1038	5.4	1.7	0.6	0.1	16.9	4.0	4.3	1.1
Benzene acetaldehyde	1043	4.1	0.9	0.3	0.1	3.8	1.5	1.3	0.4
ã-Terpinene	1055	t				0.1			
cis-Sabinene hydrate	1068	t				t			
Terpinolene	1087	0.1				0.2	0.2	0.1	0.1
trans-Sabinene hydrate	1097	t				t	t		
Undecane	1100	0.1		t		t			
cis-Pinene hydrate	1121	0.1				t			
allo-Ocimene	1130	0.2				t	0.1	0.1	
trans-Pinocarveol	1139	0.6		0.1	0.2	0.1	0.1		
trans-Pinene hydrate	1140	t							
Terpinen-4-ol	1177	0.3				t	t		
á -Terpineol	1187	t					t		
cis-Piperitol	1193	t				t	0.1		
Dodecane	1200	t					t		
trans-Piperitol	1205	t				t	t	0.1	
2-Phenylethyl acetate	1256	t	0.6	0.2	0.1	t	0.2	0.2	
Bornyl acetate	1284	t	0.1				0.1		
trans-Sabinyl acetate	1290	7.7	2.7	1.1	0.4	3.3	2.4	t	
166/82	1296	t	0.1			t	0.2	0.1	
Tridecane	1300						0.1	t	
Isoamylbenzyl ether	1309	t				t			

Table 1. Composition of the essential oil from *Anthriscus sylvestris*

	Ri[a]	Fresh leaves Ess. Oil (%)	fresh leaves DCM (%)	dry leaves[b] DCM (%)	dry leaves[c] DCM (%)	fresh roots Ess. oil (%)	fresh roots DCM (%)	dry roots[b] DCM (%)	dry roots[c] DCM (%)
n-Nonanyl acetate	1311	0.1					t		
E,E-2,4-Decadienal	1314	0.1						t	
cis-Sabinyl acetate	1325	t				t	0.2	0.1	
á -Ylangene	1372	t				t	0.1	t	
â -Bourbonene	1383	t				t	0.1		
â -Cubebene	1390	0.1				t	0.2		
â -Elemene	1391	0.1	0.1			0.1		0.1	
Tetradecane	1400	t				t			
E-Caryophyllene	1418	0.2	0.1	0.1		t	0.1	t	
á -Humulene	1454	t				0.1			
â -Chamigrene	1475	0.1				0.1		0.3	0.1
Germacrene-D	1477	4.2	1.8	1.5	0.8	2.1	0.3	0.2	0.2
ã -Himachalene	1480	t				0.5	0.4	0.2	0.2
A Farnesene	1491	1.2	0.6	0.6	0.3	t	0.2	0.1	0.0
Bicyclogermacrene	1494	t	0.1			0.1	0.1	0.1	0.1
Á -Muurolene	1499	0.1	0.1	0.1		t	0.2		
Pentadecane	1500	t				t			
E,E-á-Farnesene	1505	2.5				0.1	t		
Ä -Cadinene	1521	0.1				0.1	0.1	0.1	0.1
E- ã -Bisabolene	1527	t					0.3		0.2
Cadina-1,4-diene	1527	t				0.2		0.5	
Germacrene D-4-ol	1574	0.2	0.1	0.1		t	0.2	0.2	0.2
epi- á -Cadinol	1643	0.1				t	t		
epi- á -Muurolol	1645	0.2	0.1			t	0.1		
% Identified		92.6				80.1			
Grouped components									
Monoterpene hydrocarbons		70.2				69.3			
Oxygen-containing monoterpenes		8.6				3.4			
Sesquiterpene hydrocarbons		8.6				3.2			
Oxygen-containing sesquiterpenes		0.5				t			
Others									
Oil yield									

[a] Retention index relative to C_9-C_{17} *n*-alkanes on the DB-1 column.
[b] Dried at room temperature.
[c] Freeze-dried.
[d] Trace ((<0.05%).

Dichloromethane extract

The number of identified components in the dichloromethane extracts of leaf material is much smaller compared to the number of compounds in the essential oil obtained through hydrodestilation, 21 versus 59 respectively. The compounds present in the essential oil at low or trace amounts did not appear after the extraction with dichloromethane, with exception for phenyl ethyl acetate. In addition, the number of compounds in the dichloromethane extracts of dried material was much lower. Lyophilisation (9) resulted even in more loss of volatile constituents than drying at room temperature (16). There are also quantitative differences between these extracts. For instance the extract of the freeze-dried material does not show â-phellandrene as the main component.

In the fresh, air dried at room temperature and freeze-dried root material, we could identify respectively 46, 31, and 18 components. In these 3 root extracts, â-phellandrene (15.1%, 10.8%, and 2,6%) is the main component. Other components in the fresh root extract were *Z*- â-ocymene (4.0%), â-myrcene (2.5%), sabinene (1.9%), Ä^3-carene (1.5%), and *trans*-sabinylhydrate (2.4%). The other main components of the both extracts obtained through air drying at room temperature and lyophilisation of root material were â-phellandrene (10.8% and 2.6%), *Z*- â-ocymene with 4.3 and 1.1% together with â-myrcene with 2.2% and 0.6% respectively. In comparison with the root oil the amount of components in the dichloromethane extract from the fresh roots is almost the same, although the same remarks can be made as for the leaves. In general, it can be concluded that from dried plant material only qualitative information about the main volatile components can be obtained.

V. Lignan profiles of indoor-cultivated *Anthriscus sylvestris*.

Albert Koulman, Sieb Batterman, Freeke M. S. van Putten, Rein Bos & Wim J. Quax (2003) Lignan profiles of indoor-cultivated *Anthriscus sylvestris*. *Planta Med.*, accepted.

NEDERLANDSE SAMENVATTING

Lignaangehaltes van binnenshuis gekweekt fluitenkruid

In hoofdstuk III is aangetoond dat fluitenkruidpopulaties van verschillende plekken verschillende lignaangehaltes bevatten. Wanneer fluitenkruid zou kunnen dienen als bron voor lignanen, dan zou het goed zijn om planten te hebben die een hoog gehalte aan lignanen produceren. Het natuurlijk gehalte aan lignanen kan bepaald zijn door genetische, z.g. interne, informatie van de plant, maar het is ook mogelijk dat het gehalte wordt beïnvloed door externe factoren, bijvoorbeeld temperatuur, zonlicht, infecties of de standplaats van de plant (of een combinatie van deze factoren). Wanneer het gehalte aan lignanen bepaald wordt door externe factoren, dan zouden planten, die onder dezelfde omstandigheden worden opgekweekt, hetzelfde gehalte moeten hebben.

Om deze vooronderstelling kunnen toetsen werd zaad van fluitenkruid verzameld van 4 verschillende populaties om daarna onder dezelfde omstandigheden de planten te laten opgroeien.

Fluitenkruidzaad kan niet zomaar worden gezaaid zo is gebleken, dan wil het niet kiemen. Om het zaad te laten kiemen moest het 2 weken bij 4°C op een vochtige ondergrond worden gehouden, met 16 lichturen en 8 uren donker. Na die 2 weken werd de temperatuur verhoogd naar 20°C. Bijna alle zaadje kiemden, na deze behandeling, binnen een week na de temperatuur stijging. De verkregen plantjes hebben we daarna onder exact dezelfde omstandigheden in het laboratorium opgekweekt. Na 5 maanden werden de planten geoogst en het lignaangehalte van alle afzonderlijke planten bepaald. Behalve naar de lignaangehaltes werd ook naar de groeieigenschappen per plant gekeken. Er bleken duidelijke verschillen in groeieigenschappen tussen de planten van de 4 locaties.

De planten, opgekweekt uit Gronings zaad, hadden ongeveer even zware wortels als bladeren, terwijl voor het zaad uit Hoogerheide en Leicester (Engeland) de bladeren veel zwaarder waren dan de wortelen (zie tabel 1). Deze verschillen in groeieigenschappen wijzen erop dat er genetische verschillen moeten zijn tussen de populaties.

De lignaangehaltes daarentegen waren gemiddeld niet verschillend tussen de 4 populaties. Maar er was wel een enorme spreiding in lignaangehaltes tussen de planten onderling van elke populatie. Dit geeft aan dat er binnen de populaties genetische verschillen zijn die leiden tot verschillen in lignaangehaltes.

ABSTRACT

In a previous study we have shown that different populations of *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae) yield significantly different lignan profiles. In this study we collected the seeds of *A. sylvestris* from 4 locations, one in England and three in The Netherlands. The seeds were germinated and grown under identical laboratory conditions. After 5 months the plants were harvested and the lignan profile in the roots and aerial parts was analysed using GC-MS.

The plants from the seeds of the 4 locations showed several significant phenotypic differences in for instance dry-weight and â-sitosterol content. However the 4 groups did not differ significantly in their lignan profile in their roots. The lignans in the roots of *A. sylvestris*, deoxypodophyllotoxin, yatein and anhydropodorhizol reached, on average, equal amounts in all 4 groups. The profiled samples did not contain any other detectable lignans. Within each of the 4 groups, however, a large quantitative variation in lignan profile between the individual plants was observed, pointing to within-population genetic differences between individual plants.

Apparently there is no selection pressure on the lignan concentration in these populations. In the aerial parts of the cultivated plants originating from one location a lignan concentration much higher than in the wild plants and even higher than in the roots was observed.

INTRODUCTION

The use of biotechnological procedures to produce podophyllotoxin would be an interesting alternative and *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae, wild chervil) may play an important role in this context. Wild chervil is a common weed in Northwest Europe and its rhizomes contain considerable amounts of the lignans deoxypodophyllotoxin, yatein and anhydropodorhizol. In the biosynthetic pathway of 2,7'-cyclolignans as proposed by Broomhead and co-workers, the direct precursor of podophyllotoxin is deoxypodophyllotoxin (Broomhead *et al.*, 1991). After feeding, deoxypodophyllotoxin can be converted into podophyllotoxin by cultures of undifferentiated plant cells or fungi (Kondo *et al.*, 1989; Van Uden *et al.*, 1997).

In a previous study we showed that there is a large variation in the lignan profile between different populations of *A. sylvestris*. We compared the concentrations of deoxypodophyllotoxin, yatein and anhydropodorhizol in different populations that were harvested from the wild. There were large quantitative and qualitative differences between the populations. The total lignan concentration differed between 0.33 and 2.33% (w/w) and the percentage of anhydropodorhizol of the total lignans concentration could differ between 45 and 0.5% (see chapter III). We were interested to see if these variations are caused by environmental factors or by genetic differences. Therefore we collected seeds of *A. sylvestris* from 4 different locations to determine the variation in lignan profile after germination and cultivation under identical conditions. This study can help to come to a maximal production of deoxypodophyllotoxin in *A. sylvestris*.

MATERIAL AND METHODS

Plant Material

Ripe seeds of *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae) were collected from plants from 4 locations (see Table 1). Seeds were stored cold (4°C), dry, and dark until the germination procedure started. A voucher specimen is present in our institute coded Asylv1999-1.

Plant cultivation

The seeds germinated after an exposure of two weeks to a day-night regime (16 hours light and 8 hours dark) at 4°C on a humid soil, followed by an increase in temperature to 21°C. Most seeds germinated within one week after the temperature increase. The seedlings were then transferred to potting soil and planted 1 cm deep, with 5 cm distance between the seedlings. The seedlings grew under day-night regime (16 hours light and 8 hours dark) at 23° to 25°C. The plants were watered every other day. The harvesting took place exactly 5 months after the transfer to the potting soil. After harvesting the plants were air-dried, divided in roots and aerial parts, weighted, and ground for analyses.

Analysis

All the plant material was analysed with the previously reported GC and GC-MS methods (see chapter III). With this method â-sitosterol was also analysed (â-sitosterol was purchased from Fluka as reference compound).

Statistics

The Data obtained with the GC-MS analysis was subjected to statistical analysis using SPSS for Windows (version 11.0.1) (SPSS Inc. Illinois, USA).

RESULTS

The seeds of each location were weighed and the weight did not significantly differ between the locations. The average seed weight ranged from 5.2 ± 0.6 mg ($\overline{x}$ ± stdev, n=5, weighed per 10 seeds) for the Hoogerheide location to 5.6 ± 0.2 mg ($\overline{x}$ ± stdev, n=5, weighed per 10 seeds) for the Groningen location.

The method used for germination yielded for all the seeds of the four locations 80 to 90% germination. The final dry weight of the aerial parts of the plants from seeds of the Groningen location (0.46 g ± 0.19; $\overline{x}$ ± stdev, n=6) was significantly lower than the plants from seeds of the Leicester (2.39 g ± 1.34) and Hoogerheide (3.57 g ± 1.43) location. The shoot / root ratios (calculated as dry-weight aerial parts divided by dry-weight roots) differed significantly. In the plants from the seeds of the Groningen location this ratio was low (1.13 ± 0.23) whereas the ratio in the plants from the seeds of the Hoogerheide location was 8.80 ± 3.13. These data show that there were clear phenotypic differences in growth and allocation characteristics between the plants from the seeds originating from the 4 locations.

The GC-MS method used in this study delivered results on three classes of plant metabolites. Except for lignans we were also able to detect plant sterols and prenylated phenylpropanoids like anthriscusin (Ikeda *et al.*, 1998a).

The anthriscusin concentration was significantly higher in the roots than in the aerial parts of the plants originating from the Groningen location. The other locations yielded plants showing the same tendency, but the higher concentrations in the roots were not significantly different. There was no significant difference in total anthriscusin content between the four groups.

One of the main sterols that we identified was â-sitosterol (see Table 1). Significant differences could be observed between the groups in their â-sitosterol content. Plants originating from the Leicester location did not contain any detectable amounts of â-sitosterol. The Groningen and Diever locations yielded plants that contained over 0.03% â-sitosterol in their aerial parts. The plants from the seeds of the Hoogerheide location varied in their â-sitosterol content. Some plants did not contain any detectable amount of â-sitosterol in their roots or aerial parts, while other plants accumulated this steroid in roots or aerial parts or both. These data again show, like for the dry-weight, that there are clear phenotypic differences in â-sitosterol contents between the plants from the seeds of the 4 locations.

The populations originating from the 4 locations did not show any significant difference in lignan content of the roots. The mean concentrations of deoxypodophyllotoxin, yatein or anhydropodorhizol in the roots were similar. Principle component analysis was performed to find correlating factors. There was a correlation between the three lignans. The

mean yatein concentration was 26% ± 17 of the deoxypodophyllotoxin concentration, while the mean anhydropodorhizol concentration was 29% ± 16 of deoxypodophyllotoxin. None of the other peaks in the chromatogram correlated to deoxypodophyllotoxin or one of the other two lignans or any other factor like plant weight or location.

There was a large variation in the total lignan content between the individual plants within each group. The maximum variation within one group was found for the roots of plants from the seeds of the Leicester location. The lowest total lignan content was 0.074% and the highest lignan content was 0.76%. The CV (coefficient of variance) was 108%. The smallest variation in total lignan content was found in the roots of plants from the seeds of the Diever location, with a highest content of 0.32% and a lowest of 0.23% leading to a CV of 18%. The plants of the Groningen population showed intermediate variance (CV = 54% in the roots), in the roots of Hoogerheide population we found a CV of 73%. None of the analysed plants yielded any other detectable lignans.

Table 1. Characteristics of *Anthriscus sylvestris*, cultivated indoor from seeds of 4 locations.

Location [a]	Plant Part [b]		Dry-weight (g)	Anthricusin (%, w/w) [c]	Yatein (%, w/w) [c]	DOP [d] (%, w/w) [c]	AHP [e] (%, w/w) [c]	â-Sitosterol (%, w/w) [c]	Total lignan (%, w/w) [c]
Lei	R	$\overline{X}$	0.45	0.049	0.031	0.232	0.073	0	0.341
		± SD	0.14	0.025	0.052	0.237	0.085		0.370
	A	$\overline{X}$	2.39	0.033	0	0.140	0.018	0	0.158
		± SD	1.34	0.007		0.040	0.017		0.053
Hoo	R	$\overline{X}$	0.47	0.086	0.076	0.247	0.064	0.010	0.387
		± SD	0.28	0.060	0.061	0.166	0.060	0.017	0.282
	A	$\overline{X}$	3.57	0.045	0.041	0.164	0.032	0.017	0.238
		± SD	1.43	0.030	0.047	0.116	0.026	0.018	0.181
Die	R	$\overline{X}$	0.52	0.081	0.025	0.191	0.049	0.006	0.266
		± SD	0.29	0.021	0.008	0.037	0.003	0.011	0.048
	A	$\overline{X}$	1.23	0.059	0.040	0.398	0.071	0.036	0.510
		± SD	0.63	0.028	0.015	0.075	0.019	0.018	0.105
Gro	R	$\overline{X}$	0.43	0.114	0.047	0.186	0.051	0	0.284
		± SD	0.24	0.042	0.015	0.115	0.025		0.153
	A	$\overline{X}$	0.46	0.031	0.030	0.265	0.045	0.033	0.340
		± SD	0.17	0.010	0.039	0.147	0.040	0.017	0.205

[a] Location were seeds were collected; Lei: near Leicester, UK (52°38'54"N, 01°08'19"E), Hoo: near Hoogerheide, NL (51°25'45"N, 04°19'36"E); Die: near Diever (52°21'54"N, 06°18'51"E), NL. Gro: near Groningen, NL (53°11'32"N, 06°37'18"E).
[b] n = 5, R: roots, A: aerial part.
[c] percentage (w/w) based on dry-weight.
[d] DOP: deoxypodophyllotoxin.
[e] AHP: anhydropodorhizol.

DISCUSSION AND CONCLUSION

In contrast to the previous study (see chapter III), the aerial parts of *A. sylvestris* did contain quite high amounts of lignans. Aerial parts of *A. sylvestris* collected from the wild contained only small amounts of deoxypodophyllotoxin, and the total lignan content was significantly lower than in the roots. This was not the case for the in-door grown plants. In the aerial parts of the plants from the seeds of the Diever location the concentration of all the three lignans in aerial parts were significantly higher than the concentrations found in the roots. For the Groningen location this difference was not significant, and for the other two locations the lignan content in the roots was higher. For the aerial parts of the plants we did find a significant difference between two locations for their lignan content. The aerial parts of the plants from the seeds of the Diever location contained significantly more of all three lignans than the aerial parts of the plant originating from the Leicester location. It is not clear why the indoor-cultivated plants accumulate such high concentrations of lignans in their aerial parts, but the presence of lignans in the leaves has been reported before (Kurihara and Kikuchi, 1979). In the previous study on wild populations we only saw a correlation between the deoxypodophyllotoxin and yatein concentration (see chapter III).

The total lignan content of the roots of plants cultivated in-door was comparable to the concentrations found in the wild. In the wild population of *A. sylvestris* of the Groningen location we found a lignan content of 0.51% ± 0.35 (chapter III). This is not significantly different from the content we found in the roots after indoor cultivation (see Table 1). In this previous study of wild populations, we already noted that there was a large variation within each studied location.

In general it can be concluded from these data that there are phenotypic differences between the indoor-cultivated plants from the seeds of four locations as far as growth characteristics and â-sitosterol content are concerned. Because the conditions during the indoor cultivation were equal for all populations it must be assumed that there is a genotypic difference between the seeds from these locations. The genetic difference between the 4 locations does not show up in the lignan content of the roots, due to the large variations in lignan profile within 2 of the 4 groups, which points to within-population genetic variation between each of the individual plants. Apparently there is no selection pressure on the lignan concentration in these populations. The significant differences in lignan content between the populations in the wild point to either ecological factors or differences in development (or both) that influence the lignan profile in the roots. This study does not yet deliver enough data to select and breed for high lignan producing strain of *A. sylvestris*. Further research in this field is necessary.

ACKNOWLEDGEMENT

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VI. A phytochemical study of lignans in whole plants and cell suspension cultures of *Anthriscus sylvestris*

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NEDERLANDSE SAMENVATTING

Onderzoek naar lignanen in planten en celcultures van fluitenkruid

In hoofdstuk III staat beschreven hoe de belangrijkste drie lignanen (deoxypodofyllotoxine, yateïne en anhydropodorhizol) in fluitenkruid kunnen worden geanalyseerd. Bij het ontwikkelen van de analysemethode kwamen wij er achter dat fluitenkruid, naast deze drie ook nog andere lignanen produceert, maar meestal in lagere concentraties.

Hier hebben we op 2 manieren onderzoek naar gedaan. In gedroogde en vermalen wortels van fluitenkruid hebben we gekeken naar welke lignanen er precies in zitten. Daarvoor hebben we een halve kilo gedroogde en vermalen wortels van fluitenkruid gebruikt. Hiervoor heb je ongeveer 3 vuilniszakken gevuld met wortels nodig, wat neerkomt op het omspitten van ruim 20 meter berm. Alle lignanen welke werden aangetroffen, bij de analyses van de extracten, worden vermeld in figuur 2.

Daarnaast zijn er celcultures opgezet van fluitenkruid. Deze zijn gebruikt om de biosynthese van de lignanen te onderzoeken. Dit is de eerste maal dat er van fluitenkruid een celculture is gemaakt. Fluitenkruid tiert welig in Nederland onder bijna alle omstandigheden, maar de door ons opgezette celcultures van deze plant groeiden maar moeizaam en produceerden bijna geen lignanen. Deze cultures hebben we met de drie belangrijkste lignanen gevoed (zie inleiding). Het doel van dit experiment was om te kijken hoe het lignaan deoxypodofyllotoxine wordt aangemaakt in fluitenkruid. In het groot voetblad (*Podophyllum hexandrum*) wordt deoxypodofyllotoxine gevormd vanuit yateïne. Dit omzettingsproces was daarom ook te verwachten bij fluitenkruid. Het bleek echter dat yateïne giftig is voor fluitenkruid cellen. Deze stof werd niet omgezet in deoxypodofyllotoxine. Ook anhydropodorhizol werd niet omgezet maar afgebroken door de celcultures. Heel verrassend was het om te zien dat deoxypodofyllotoxine werd omgezet in podofyllotoxine. Podofyllotoxine zit normaal ook in fluitenkruid maar in extreem lage concentratie terwijl deoxypodofyllotoxine in veel hogere concentraties in de plant voorkomt.

Celcultures zijn niet in staat hoge concentraties deoxypodofyllotoxine op te slaan. Dit komt waarschijnlijk door het feit dat cellen in de celculture anders zijn dan de cellen in de wortels van fluitenkruid. Vermoedelijk missen de cellen in de culture het systeem om deoxypodofyllotoxine op te slaan en wordt het daarom omgezet in podofyllotoxine.

ABSTRACT

In the roots of *Anthriscus sylvestris* 12 different lignans were detected. Arctigenin, dimethyl matairesinol, dimethyl thujaplicatin, podophyllotoxin, 7-hydroxyyatein and 7-hydroxyanhydropodorhizol have not been previously reported to be present in *A. sylvestris*. In the cell suspension culture, which were initiated for this study, trace amounts of deoxypodophyllotoxin could be detected. With these cell suspension cultures we carried out feeding experiments using deoxypodophyllotoxin, yatein and, anhydropodorhizol. Yatein had a toxic effect on the cell cultures and was, like anhydropodorhizol not converted into any detectable product. Deoxypodophyllotoxin, in contrast, was converted into podophyllotoxin, yielding significantly higher concentration than measured in whole plants.

INTRODUCTION

The cytotoxic lignan podophyllotoxin has found its place in pharmacotherapy. It is used topically in the treatment of condyloma (Gross, 2001). Podophyllotoxin also forms a starting compound for the production of three important clinically applied anticancer drugs: etoposide, etopophos and teniposide (Imbert, 1998). Currently the use of podophyllotoxin glycosides against rheumatoid arthritis is under investigation (Carlstrom *et al.*, 2000). These clinical applications demand a reliable and continuous production process of this plant secondary metabolite. However, the main source of podophyllotoxin is the plant *Podophyllum hexandrum* Royle, which is listed in appendix II of CITES (Convention for International Trade in Endangered Species). This appendix lists species that are not necessarily threatened now with extinction but may become so unless trade is closely controlled (World Conservation Monitoring Centre, 2001). This has challenged researchers to consider various other sources for the production of podophyllotoxin. A better understanding of the biosynthesis of podophyllotoxin and of 2,7'-cyclolignans is a prerequisite for this research. Presently, knowledge in this field is still limited and only based on studies with *P. hexandrum* and *Linum flavum* L.. At the moment there is no conclusive data on similarities in the biosynthetic pathway for lignans in these species.

Studies from the early nineties on *P. hexandrum* showed that this species transformed matairesinol into podophyllotoxin through yatein and deoxypodophyllotoxin (see Fig. 1, route a, by Broomhead *et al.*, 1991). Many steps in the biosynthesis of deoxypodophyllotoxin have not yet been elucidated. Recent biosynthetic studies of lignans with a 2,7'-cyclolignan skeleton, which were done with *L. flavum*, may be considered as contradictory. On the basis of feeding experiments with matairesinol to whole plants one group suggested that 7-hydroxymatairesinol is an intermediate (Xia *et al.*, 2000) in the biosynthesis of 6-methoxy-podophyllotoxin (see Fig. 1, route c). In contrast, our group has shown that deoxypodophyllotoxin can be converted into 6-methoxypodophyllotoxin by undifferentiated cell cultures of *L. flavum* (see Fig. 1, route b, by Van Uden *et al.*, 1997). Recently we characterised the enzyme responsible for the 6-hydroxylation of deoxypodophyllotoxin resulting in â-peltatin (see Fig. 1 route b). Due to fact that both these experimental set-ups were carried out with different cell types and used non-physiological amounts of precursor, it is not possible to predict how *L. flavum* produces 6-methoxy-podophyllotoxin under normal conditions. The knowledge we have on the lignan biosynthesis so far is based on studies with various unrelated species. It is not known whether the results obtained in one species can be extrapolated to another species. Therefore, we have decided to study Anthriscus *sylvestris*, the wild chervil.

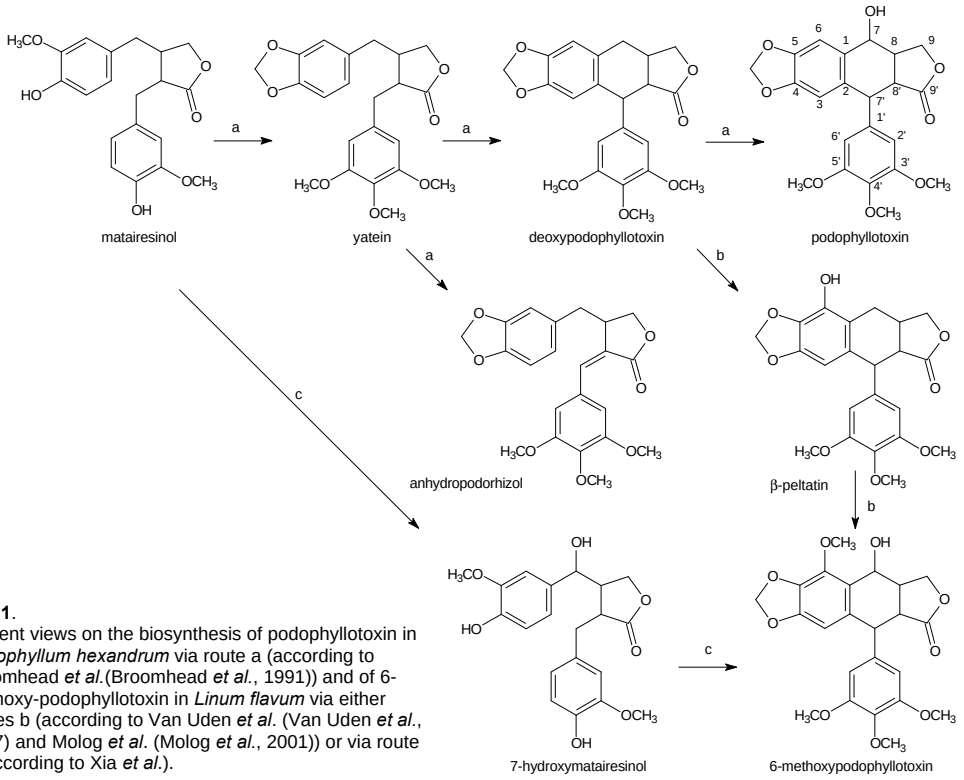

Fig. 1.
Current views on the biosynthesis of podophyllotoxin in *Podophyllum hexandrum* via route a (according to Broomhead *et al.*(Broomhead *et al.*, 1991)) and of 6-methoxy-podophyllotoxin in *Linum flavum* via either routes b (according to Van Uden *et al.* (Van Uden *et al.*, 1997) and Molog *et al.* (Molog *et al.*, 2001)) or via route c (according to Xia *et al.*).

A key step in lignan biosynthesis is the stereospecific ring closure that leads to the formation of 2,7'-cyclolignans. Broomhead and co-workers showed that yatein is a late precursor of deoxypodophyllotoxin in whole plants of *P. hexandrum* (Broomhead *et al.*, 1991). They also showed that anhydropodorhizol is not converted into deoxypodophyllotoxin in this species (see Fig. 1, route a). It is still not known how the crucial ring closure takes place in *L. flavum*, or what kinds of enzymes are involved. Characterisation of such unique enzymes would be a significant step forward in the understanding of lignan biosynthesis.

Anthriscus sylvestris (wild chervil or cow parsley), a member of the Apiaceae family accumulates deoxypodophyllotoxin and at least 7 other lignans in the roots (see Table 1 for references). *Anthriscus sylvestris* is a common plant in Northwest Europe and, hence, an easily accessible source for a further detailed phytochemical study (Van Uden *et al.*, 1997). Due to its high 2,7'-cyclolignan content *A. sylvestris* could become a valuable source for the biotechnological production of podophyllotoxin (Van Uden *et al.*, 1997).

The goal of the present study was to get a more detailed insight into the lignan profile of *A. sylvestris*. There is no data as yet comparing the lignan

biosynthesis in *A. sylvestris* with any of the previous studied species. Therefore we carried out bioconversion experiments using cell suspension cultures of *A. sylvestris*, which were fed with yatein, anhydropodorhizol, and deoxypodophyllotoxin. The combination of these data should help us to further understand the lignan biosynthesis also in relation to other species.

MATERIALS AND METHODS

Chemicals

Podophyllotoxin was obtained from Sigma (St. Louis). Yatein, matairesinol, podorhizol, arctigenin, anhydropodorhizol (See Fig. 3), â-peltatin, and â-peltatin-a-methylether (see Fig. 1) were prepared synthetically (Medarde *et al.*, 1995). Deoxypodophyllotoxin, yatein, and anhydropodorhizol were obtained as optically pure compounds through isolation from *A. sylvestris* roots using the previously described method for deoxypodophyllotoxin (Van Uden *et al.*, 1997).

Plant material

Anthriscus sylvestris (L.) Hoffm. (Apiaceae) roots were collected from the wild near the city of Groningen, the Netherlands on May 21,1999. All plants were flowering or near flowering. After collection the roots were thoroughly cleaned and stored at –20°C prior to freeze-drying. A voucher specimen is present in our institute coded Asylv1999-1.

HPLC-DAD

For routine HPLC analysis of lignans the following system was used: a 4 Lichrosphere 5 RP-18 column (125 x 4 mm i.d.; Chrompack, Middelburg, the Netherlands) with MeCN : H_2O (40:60; v/v) as a mobile phase at a flow of 1.0 ml min^{-1} and diode array detection (200 to 360 nm) with a Shimadzu Photodiode Array UV-VIS SPD-M6A detector (Shimadzu, ‘s Hertogenbosch, The Netherlands).

GC and GC-MS

GC and GC-MS analysis were used for quantitative and qualitative determination of lignans in the plant material. The methods are described in chapter III.

Qualitative analysis of the lignan profile of A. sylvestris roots

Freeze dried roots were ground and 500 g were percolated sequentially with 2 l of dichloromethane and 1 l of methanol. The percolates were combined and the volume was reduced to 100 ml (under reduced pressure). The remaining extract was partitioned with water and the aqueous phase was washed 3 times with 250 ml of dichloromethane. All organic phases were combined and the volume was reduced. The resulting extract was fractionated using column chromatography on a column of 75 x 2.5 cm filled with silica gel 60. Dichloromethane was used as the eluent with a gradually increasing amount of methanol (from 0% to 90%), and eluted at 5 ml min^{-1} with a total volume of 5 litre. Fractions were profiled with the

above-mentioned GC method and those showing similar chromatograms were pooled after which the volume was reduced under vacuum. These concentrated fractions were subjected to GC-MS analysis. The mass spectra of all peaks were examined and were compared with literature data (see Table 1) or reference compounds mentioned in the section C*hemicals*. It should be noted that with EIMS it is not possible to determine the real stereochemistry of lignans, where we are not certain of the stereochemistry compounds.

Initiation of A. sylvestris *callus cultures*

Aerial parts of *A. sylvestris* were collected near the city of Groningen. Leaf material was sterilised by immersion in 70% ethanol for 2 seconds. This was followed by 5 minutes stirring in 5% (w/v) sodium hypochlorite (Boom, Meppel, The Netherlands) solution containing one drop of 1% 7XPF soap (ICN, Ohio, USA). The leaves were washed twice for 10 minutes by stirring in fresh sterile water, followed by a third washing of 20 minutes in fresh sterile water. Subsequently the leaves were cut into squares of 0.5 x 0.5 cm and 5 squares were placed on a petri-dish with solid (0.8% agar, w/v) Linsmaier and Skoog medium (Linsmaier and Skoog, 1965) supplemented with 3% (w/v) sucrose, 0.1 mg l^{-1} indole-3-acetic acid or, 0.1 mg l^{-1} 6-benzylaminopurine. The tissue was cultured at 24°C under a 16 hours light (2000 Lux) and 8 hours dark regime. Every four weeks the callus was subcultured.

Initiation of A. sylvestris *suspension cultures*

Two full-grown callus clumps were placed in 50 ml liquid medium in 100 ml sterile conical flasks. We tested 5 different media. Two were a modification the Murashige and Skoog medium (Murashige and Skoog, 1962). The so-called MS has the addition of 4% (w/v) sucrose, 1.0 mg l^{-1} indole-3-acetic acid and, 1.0 mg l^{-1} 6-benzylaminopurin. The so-called LA has the addition of 4% (w/v) sucrose and 0.4 mg l^{-1} á-naphthalene acetic acid. Two were a modification of Gamborg's B5 (Gamborg *et al.*, 1968). The so-called B5 has the addition of 4% (w/v) sucrose, 2.0 mg l^{-1} 2,4-dichlorophenoxy acetic acid and, 1.0 mg l^{-1} 6-benzylaminopurin. The so-called B5W has the addition of 3% (w/v) sucrose and 4.0 mg l^{-1} á-naphthalene acetic acid. The fifth medium used was MS/B5 with addition of 4% (w/v) sucrose and 3.0 mg l^{-1} á-naphthalene acetic acid. All media were obtained from Duchefa (Haarlem, The Netherlands). The flasks were placed on an IKA KS 501 shaker at a speed of 175 rpm under the same conditions as the callus cultures. After two weeks the cultures were scaled up to a volume of 300 ml in a 500 ml conical flask by the addition of 200 ml fresh medium to 100 ml of a full-grown suspension culture. These were subcultured every 2 weeks, by adding 100 ml of a full-grown culture to 200 ml fresh medium comparable to other systems (Van Uden *et al.*, 1997). All media and supplements used in this study were obtained from Duchefa (Haarlem, The Netherlands).

Bioconversion of lignans

For bioconversion experiments 10 mg of either yatein (Mw 400), anhydropodorhizol (Mw 398), or deoxypodophyllotoxin (Mw 398) were dissolved in a small portion of dichloromethane (± 2.5 ml) and transferred into a 500 ml culture flask. Subsequently three times the amount of 2,6-dimethyl-â-cyclodextrin (Avebe, Veendam, The Netherlands) was added, based on molarity. The 2,6-dimethyl-â-cyclodextrin improves the solubility of the lignans in the medium (Van Uden *et al.*, 1997). Then, 200 ml fresh B5 medium (see Table 2) was added to each flask and then sonicated for 1 h followed by sterilisation (20 min at 120°C). This procedure yielded clear solutions of the compounds to be fed. HPLC and GC analysis showed that no decomposition of the compounds had occurred during this process. To exclude any further chemical degradation or derivation of the lignans during the experiments, we incubated equal amounts of lignans in 300 ml of growth medium for 2 weeks. The analysis of the medium at start of the experiment yielded the same concentration substrate as after 2 weeks. At day zero of the experiment 100 ml of a full-grown (t=14 days) *A. sylvestris* cell suspension was added to these solutions, giving a final volume of 300 ml with a total lignan concentration of 83 µM per litre culture. The cultures were grown for 14 days. During the growth cycle seven 12 ml samples were taken under sterile conditions. The growth parameters (conductivity, pH, fresh weight, and dry weight) were determined as described before (Van Uden *et al.*, 1997). For the determination of the lignans, their precursors and bioconversion products we used the standard sample preparation method (see chapter III). The organic and the aqueous layers were subjected to HPLC analysis to study the metabolic fate of the lignans and their products. We define product here as the sum of lignans formed from the substrate by hydroxylation, methylation, glycosylation, ring closure, or a combination of these reactions as detected by HPLC. Glycosylation was checked through the treatment of the aqueous phase with â-glucosidase as previously described (Van Uden *et al.*, 1997).

RESULTS & DISCUSSION

Phytochemical analyses

Table 1 shows all the lignans we could identify in the roots of *A. sylvestris*. These results are based on GC-MS and HPLC-DAD data compared with reference compounds and with literature data. None of the analytical systems deliver unambiguous information about the stereochemistry of these lignans. It is therefore possible that some compounds we identified are stereoisomers of the originally reported compound.

Table 1. Lignans found in *Anthriscus sylvestris* and their content with the mass spectra and the literature where these compounds have originally been reported (see Fig. 3 for structures).

Compound	Content (%)[a]	Mass spectrum m/z (%)[b]	Reference:
Deoxypodophyllotoxin[c]	0.2 - 1.7	173(19) 181(21) 185(16) 398(100)	(see chapter III)
Yatein[c]	0.1 - 0.6	77(32) 105(13) 135(51) 181(100) 219(6) 264(9) 400(68)	(see chapter III)
Anhydropodorhizol[c]	<0.001 – 0.2	105(14) 135(74) 176(16) 263(100) 398(23)	(see chapter III)
Morelensin[c]	<0.001 – 0.02 [d]	185(12) 253(13) 368(100)	(Jolad *et al.*, 1977)
Hinokinin[c]	<0.001 – 0.04 [d]	135(100) 192(14) 218(8) 219(10) 354(15)	(Lopes *et al.*, 1983)
Bursehernin[c]	<0.001 – 0.01 [d]	135(37) 151(100) 208(3) 219(5) 235(4) 370(100)	(McDoniel and Cole, 1972)
Angeloyl podophyllotoxin[c]	<0.001 – 0.05 [d]	55(19) 83(17) 135(8) 185(23) 229(8) 263(9) 397(15) 496(100)	(Lim *et al.*, 1999)
Arctigenin	< 0.001 [e]	137(100) 151(46) 177(7) 372(89)	(Rahman *et al.*, 1990a)
Dimethylmatairesinol	< 0.001 [e]	151(100) 208(8) 234(5) 235(7) 386(30)	(Lopes *et al.*, 1983)
Podophyllotoxin	< 0.001 [e]	168(6) 181(3) 189(3) 399(3) 414(100)	(Jackson and Dewick, 1984a)
7-Hydroxyanhydropodorhizol	< 0.001 [e]	135(88) 176(17) 207(16) 263(100) 381 (21) 398(51) 414(82)	[f]
7-Hydroxyyatein	< 0.001 [e]	135(88) 151(3) 181(100) 416(19)	(Ziegler and Schwartz, 1978)
Di-methyl thujaplicatin methyl ether	< 0.001 [e]	152(16) 165(3) 167(8) 177(11) 181(100) 219(2) 238(3) 251(5) 264(2) 426(88)	(Badheka *et al.*, 1986)

The GC-MS method does not deliver unambiguous information about the stereochemistry of these lignans. It is therefore possible that some compounds we identified are stereoisomers of the originally reported compound.

[a] The maximum and minimum content found in w/w percentage based on dry weight.

[b] Mass spectra obtained through EI (70 eV) GC-MS, all the main fragments with the relative abundance.

[c] Previously described from *A. sylvestris* (Ikeda *et al.*, 1998a; Ikeda *et al.*, 1998b; Lim *et al.*, 1999).

[d] The maximum and minimum content found in w/w percentage based on dry weight calculated as deoxypodophyllotoxin.

[e] The content was below the limit of quantification but could be detected in concentrated fractions (in w/w percentage based on dry weight calculated as deoxypodophyllotoxin).

[f] See chapter VI.

We could detect deoxypodophyllotoxin, yatein and anhydropodorhizol (see Fig. 2) in all samples of *A. sylvestris* roots. Hinokinin and angeloyl podophyllotoxin (see Fig. 2) were found in variable amounts and not in all samples. Morelensin and bursehernin (see Fig. 2) could only be detected in minute amounts (just above the detection limit) in a few samples. The presence of these lignans in *A. sylvestris* has been reported before (Ikeda *et*

al., 1998b; Lim *et al.*, 1999), but we were also able to detect arctigenin (see Fig. 2) in several samples, a new finding for *A. sylvestris*. Evidence for the presence of other lignans was found, but the concentration of these compounds was too low to be detected using the routine sample preparation (see chapter III).

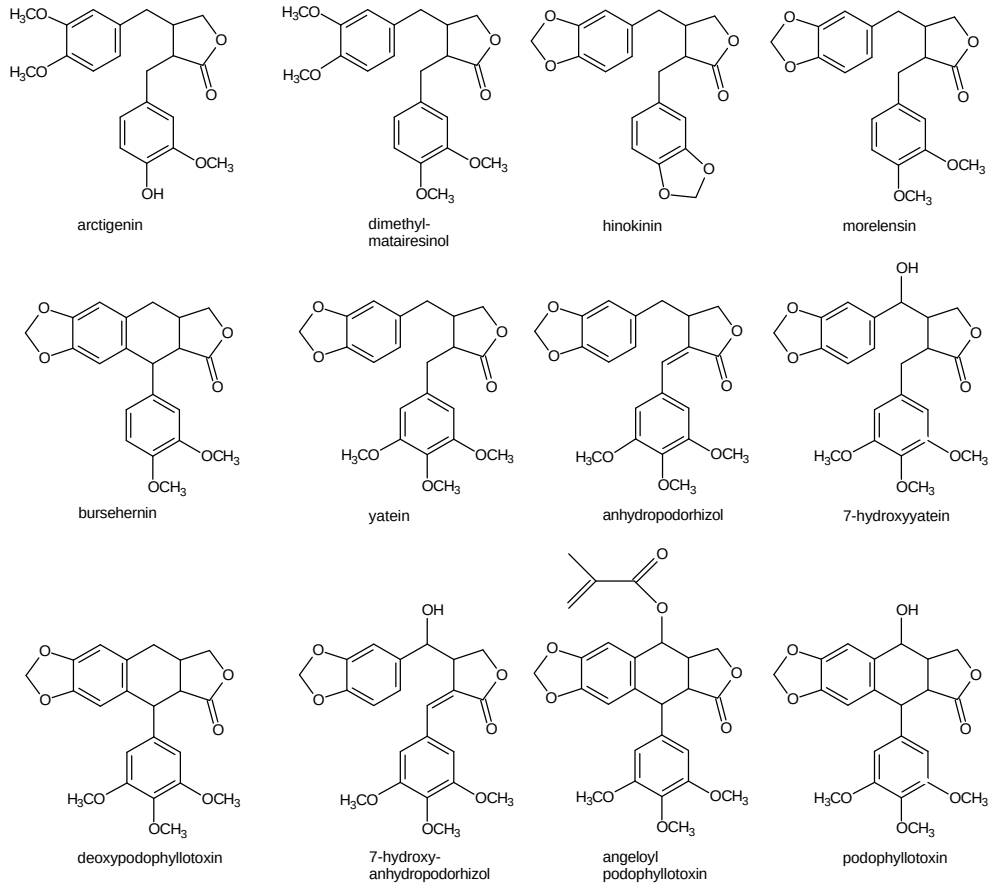

Fig. 2.
The lignans found in the roots *Anthriscus sylvestris*.

Therefore, starting with 500 g of dried and ground root material of *A. sylvestris*, four more concentrated fractions were prepared with increasing percentages of methanol in dichloromethane. A very concentrated sample of each of the fractions was analysed with GC-MS. We could detect a number of lignans, not yet described for *A. sylvestris* but known from other plants. These include dimethyl matairesinol (see Fig. 2) and dimethyl thujaplicatin methyl ether (see Fig. 2) in the apolar fraction. Both compounds gave specific mass spectra with peaks at *m/z* 151 and 177, the latter being characteristic for the dimethoxyphenyl moiety. Dimethyl

matairesinol has been reported in *Virola sebifera* and it gives M^+ at *m/z* 386 (Lopes *et al.*, 1983). Dimethyl thujaplicatin methyl ether has already been found in *Piper cubeba* where M+ is *m/z* 416 and the base peak at *m/z* 181 corresponds to the trimethoxyphenyl moiety. Fragments at *m/z* 238 and 265 prove that the trimethoxyphenyl moiety is adjacent to the keto function of the lactone ring moiety (Badheka *et al.*, 1986).

The more polar fraction yielded several hydroxylated lignans, of which podophyllotoxin (see Fig. 2) was most abundant. 7-Hydroxyyatein and 7-hydroxyanhydropodorhizol (see Fig. 2) were found in trace amounts. These hydroxylated compounds have all been described in other plant species. Like podophyllotoxin, which is a hydroxylated derivative of deoxypodophyllotoxin, the compounds in this fraction are hydroxylated derivatives of lignans present in much higher amounts in *A. sylvestris*. A hydroxylated yatein has already been described as a by-product in the synthesis of stegane lignans (Ziegler and Schwartz, 1978). We recently found 7-hydroxyanhydropodorhizol after the feeding of anhydropodorhizol to *L. flavum* cell suspension cultures (unpublished results). To the best of our knowledge this is the first time that the latter lignan has been found in plants collected from the wild.

The treatment of an aqueous extract from *A. sylvestris* roots with â-glucosidase, or the use of extraction methods preserving the plants own glycosidases (Canel *et al.*, 2000) did not yield any hydroxylated lignan. Therefore we have no evidence for the presence of lignan glycosides in *A. sylvestris* roots.

With the current knowledge of the possible steps in lignan biosynthesis it must be concluded that not all lignans as detected in *A. sylvestris* roots fit into one single pathway leading to one single end-product. There is no reason to assume that compounds like hinokinin and dimethyl matairesinol are precursors in the route to deoxypodophyllotoxin. Morelensin forms the substrate for the formation of yatein (via a methoxylation) and bursehernin (via a ring closure). Both yatein and bursehernin can form a substrate for the formation of deoxypodophyllotoxin. Via the ring closure of yatein deoxypodophyllotoxin can be formed, while the methoxylation of bursehernin would result in the formation of deoxypodophyllotoxin as well.

Plant cell culture

To study the biosynthesis of lignans in *A. sylvestris* we set up cell suspension cultures from aerial parts of the plant. We generated sterile callus cultures on a solid medium. For the initiation of suspension cultures we used full-grown callus clumps that were inoculated into liquid media of 5 different compositions. MS, LA and MS/B5 media did not result in any growth (as measured by conductivity and fresh weight). The cultures grown on the B5 and the B5W media showed both a clear decrease of conductivity in time (from 3.5 mS to 0.5 mS). The B5 medium yielded the largest increase of fresh weight and this medium was used for further culturing and experiments. This method was also used for the initiation of root cultures, but without success due to the high incidence of infection.

The suspension cultures of *A. sylvestris* had a growth cycle of 14 days. During this time the conductivity decreased from 2.8 ± 0.3 mS to 0.4 ± 0.1 mS, the fresh weight increased from 151 ± 18 g l^{-1} to 238 ± 15 g l^{-1} and the dry weight increased from 7.6 ± 1.7 g l^{-1} to 14.8 ± 0.9 g l^{-1} (mean ± SEM, n=4). The cultures grew in small aggregates, making them very lumpy. This did not seem to effect the growth, but complicated the sampling procedure and the determination of the growth parameters. The growth remained stable for a period of more than one year. The suspension cultures of *A. sylvestris* produced trace amounts of only deoxypodophyllotoxin, which never exceeded the limit of quantification (0.001% w/w, based on dry weight). No other lignans were detected. This established cell suspension culture is the first reported *in vitro* culture of *A. sylvestris*.

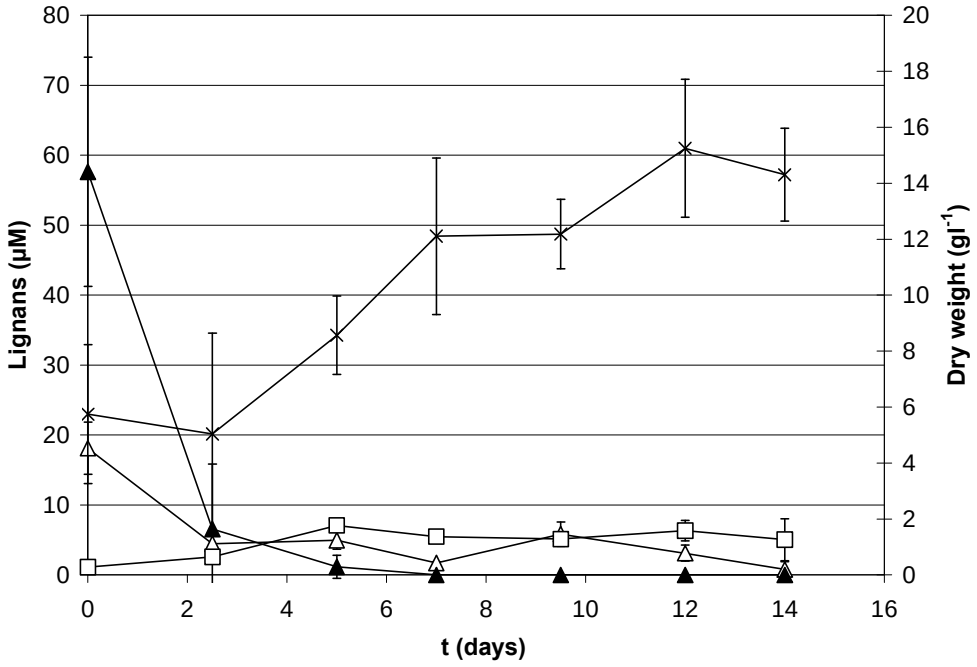

Fig. 3.
The bioconversion experiment of deoxypodophyllotoxin after feeding 10 mg to a 300 ml (84 µmol l^{-1}). The concentration of deoxypodophyllotoxin in the cell (Ä), of deoxypodophyllotoxin in the medium (▲) in the *A. sylvestris* cell culture and the formed product (□) expressed as µM. The growth of cultures is expressed as dry weight (×) (g l^{-1}) (n=3 ± SEM).

Bioconversion experiments

To compare the lignan formation of *A. sylvestris* with that of *L. flavum* and *P. hexandrum in vitro* we fed yatein, anhydropodorhizol and deoxypodophyllotoxin to the newly established cell cultures. The first two

lignans were fed in order to investigate the capacity of *A. sylvestris* cell cultures to perform the ring closure. The third lignan was fed to study the metabolic fate of the possible products formed by the ring closure. The results were compared to control cultures, grown under the same conditions.

Feeding of deoxypodophyllotoxin led, in part, to its conversion into podophyllotoxin (see Fig. 3). The cells took up a larger part of the deoxypodophyllotoxin within one day. Over a period of five days deoxypodophyllotoxin was slowly converted into podophyllotoxin. Additionally, the UV-spectra of some peaks hinted to the presence of traces of other lignan-like products, which could not be further identified. Deoxypodophyllotoxin had, at the used concentration, no significant effect on the growth of the cell cultures.

The feeding of anhydropodorhizol resulted in a rapid uptake by the cells. Within three days the anhydropodorhizol concentration in the media decreased to values below 10 μM. The concentration of anhydropodorhizol in the cells at that point was 25 ± 6 μM. After this point the intra-cellular concentration decreased, but no products could be detected. At the concentration used this lignan had no significant effect on the growth, the dry weight increased in 14 days from 4.7 ± 1.7 g l^{-1} to 16.9 ± 0.7 g l^{-1} (not significantly different to the control).

Feeding of 10 mg yatein to a 300 ml *A. sylvestris* cell cultures had a clear toxic effect. Such an effect was also observed for *L. flavum* cell cultures (data in preparation for publication). Feeding experiments with sub-toxic concentration (8 mg to 300 ml cell culture) did not reveal any detectable bioconversion products.

The feeding of deoxypodophyllotoxin to cell cultures of *A. sylvestris* did not lead to storage of this compound (see Fig. 3). This is in contrast to what is observed *in planta*. It had no toxic effect but was hydroxylated into podophyllotoxin. The podophyllotoxin concentration in the cultures after deoxypodophyllotoxin feeding clearly exceeded normal podophyllotoxin or angeloyl podophyllotoxin concentrations. The second compound was not measured after deoxypodophyllotoxin feeding, while *in planta* the angeloyl podophyllotoxin is present in higher amounts than podophyllotoxin (see Table 1). Physiological and biochemical differences between the root cells *in plant* and the *in vitro* cultured cells used in this study may play an important role in this unexpected bioconversion. This could point to the presence of a specialised system for the storage of deoxypodophyllotoxin and other lignans in the roots of *A. sylvestris*. It can also be the reason for the absence of ring closing activity after feeding of yatein or anhydropodorhizol.

The feeding of anhydropodorhizol and yatein did not yield any hydroxylated products. The high toxicity of yatein to the cells makes it impossible to perform feeding experiments yielding detectable amounts of a product. The feeding of anhydropodorhizol also yielded limited amount of product. It could be explained by the idea that these compounds do not reach the cellular compartment where the hydroxylation takes place. The

disappearance of anhydropodorhizol and yatein from the medium and from the cells is therefore possibly caused by degradation of the lignans to smaller fragments that we cannot detect in the analytical systems used. We did not observe ring closure of fed yatein or anhydropodorhizol. This may attribute to the same fact that we used undifferentiated cells in our experiments.

In general we can conclude that the roots of *A. sylvestris* produce a divers range of lignans. Experiments with cell suspension cultures revealed a hydroxylating activity restricted to deoxypodophyllotoxin. This activity demonstrates that *A. sylvestris* cells cultures are useful bioconversion catalysts for the synthesis of podophyllotoxin from deoxypodophyllotoxin. Induction of this activity *in planta* would result in an interesting new source for podophyllotoxin. The exact biosynthetic pathway *in planta* remains to be elucidated.

ACKNOWLEDGEMENT

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VII. The bioconversion of lignans from the putative 6-methoxy-podophyllotoxin biosynthesis by *Linum flavum* cells

Based on: Albert Koulman, Aäron C. Beekman, Alexandre M. A. Vinhas, Manuel Medarde, Blanca Madrigal, Rein Bos, Niesko Pras, Wim J. Quax The bioconversion of lignans from the putative 6-methoxypodophyllotoxin biosynthesis by *Linum flavum* cells. *J. Nat. Prod.*, submitted.

NEDERLANDSE SAMENVATTING

Het omzetten van lignanen uit fluitenkruid door celcultures van gele vlas

Binnen onze werkgroep is er veel ervaring met celcultures van gele vlas (*Linum flavum*). Deze cultures kunnen het deoxypodofyllotoxine (dat door fluitenkruid wordt gemaakt) snel omzetten in 6-methoxypodofyl-lotoxine. Om het omzettingsproces te versimpelen maakten wij gebruik van een ruw extract van fluitenkruid. Dit ruwe extract werd volgens het principe van koffie zetten gemaakt. Alleen de koffie was gemalen fluitenkruidwortel en in plaats van heet water werd dichloormethaan gebruikt. Dichloor-methaan verdampt heel gemakkelijk. Wat overblijft is geconcentreerd fluitenkruid-extract, met daarin een hoog gehalte deoxypodofyllotoxine en ook allerlei voorlopers uit de lignaanbiosynthese.

Het ruwe extract van fluitenkruid werd aan de celcultures van gele vlas gevoed. Wij verwachtten dat de cultures al het deoxypodofyllotoxine zou omzetten en ook alle voorlopers uit de biosynthese. Als dit proces zou slagen zou er op deze wijze een goedkoop en snel productiesysteem worden verkregen. Het bleek echter dat vrij lage concentraties ruw extract al giftig waren voor de celculture. De cellen gingen binnen enkele dagen dood. Wanneer we concentraties gebruikten, die nog niet giftig waren, bleek dat voornamelijk het deoxypodofyllotoxine werd omgezet en niet het aanwezige yateïne en anhydropodorhizol.

Om dit proces beter te leren begrijpen zijn wij deze drie stoffen steeds apart gaan voeden aan de celcultures van gele vlas. Daarnaast hebben wij ook een aantal andere voorlopers uit de biosyntheseroute gevoed, om zo een beeld te krijgen van de biosynthese. Evenals het ruwe extract van fluitenkruid bleek yateïne giftig te zijn voor de celcultures. Deoxypodofyl-lotoxine werd zonder problemen omgezet in 6-methoxypodofyllotoxine. Het omzettingsproces hebben we verder onderzocht. De resultaten daarvan staan in het volgende hoofdstuk.

Na het voeden van anhydropodorhizol verschenen er bij de analyses van de cellen twee nieuwe pieken die we niet direct konden benoemen. Door verschillende soorten spectroscopie te gebruiken lukte het ons om die pieken identificeren als de gehydroxyleerde vorm van anhydropodorhizol. Gehydroxyleerd betekend dat er een OH groep aan het molecuul is gezet (net als bij deoxypodofyllotoxine naar podofyllotoxine). Heel bijzonder is echter dat de nieuwe stof niet normaal voorkomt in gele vlas. Schijnbaar kan het enzym, dat in natuurlijke omstandigheden de hydroxylering van deoxypodofyllotoxine uitvoert, ook andere stoffen hydroxyleren. Dit is bijzonder omdat men ervan uitgaat dat een enzym maar één reactie kan uitvoeren bij één stof (substraat). Als een enzym meerdere omzettingen kan doen bij meerdere substraten, dan zijn er minder enzymen nodig voor de gehele biosynthese. Als gevolg van deze redenering, zijn er ook minder genen bij betrokken.

ABSTRACT

Undifferentiated cell cultures of *Linum flavum* are a suitable bioconversion system to study the production of 6-methoxypodophyllotoxin and related lignans. A crude extract from the roots of *Anthriscus sylvestris* containing lignan precursors were fed to these cells as well as a range of individual lignan precursors, particularly yatein and closely related compounds. With respect to the production of 6-methoxypodophyllotoxin the feeding of crude extracts had no advantage over the feeding of pure deoxypodophyllotoxin due to the toxicity of yatein. The cells did not convert yatein even at low concentrations, as was also the case for its synthetic analogue naphthayatein. Anhydropodorhizol was converted into 7-hydroxyanhydropodorhizol and its glycoside. Both compounds are not yet described. This 7-hydroxylation seems to be a general reaction in lignan biosynthesis by *L. flavum* cells. Matairesinol, secoisolariciresinol and podorhizol were all glycosylated by the cells. The resulting compounds are not intermediates that fit in the current views on the biosynthetic cascade leading to 6-methoxypodophyllotoxin.

INTRODUCTION

Podophyllotoxin (see Fig. 1) is the starting compound for the production of three important clinically applied anticancer drugs etoposide, etopophos and teniposide (Imbert, 1998). So far podophyllotoxin is isolated from the rhizomes of *Podophyllum peltatum* and *Podophyllum hexandrum* (Berberidaceae) plants because organic synthesis is not economically feasible. The supply of *P. hexandrum* rhizomes however, which contain up to 5% of podophyllotoxin on a dry weight basis, becomes increasingly limited due to both intensive collection and lack of cultivation (Choudhary *et al.*, 1998; Rai *et al.*, 2000). Therefore much research effort is devoted to a faster and more economical procedure for the production of podophyllotoxin. Especially the use of bioconversion technology would be a promising alternative for the production of podophyllotoxin and related lignans. Previous research has shown that undifferentiated cell cultures of *Linum flavum* (Linaceae; yellow flax) form a very suitable bioconversion system (Van Uden *et al.*, 1997). These cells convert deoxypodophyllotoxin (see Fig. 1) into 6-methoxypodophyllotoxin (see Fig. 1), which may be used as a starting compound for the preparation of synthetic 6-methoxy analogues of etoposide (Saito *et al.*, 1988).

Anthriscus sylvestris (Apiaceae; wild chervil) is a common weed in Northwest Europe and its rhizomes contain considerable amounts of the lignans deoxypodophyllotoxin, yatein (see Fig. 1) and, anhydropodorhizol (see Fig. 1). Broomhead and co-workers showed that yatein is precursor of deoxypodophyllotoxin in *Podophyllum hexandrum* (Broomhead *et al.*, 1991). Undifferentiated cultures of plant cells or fungi are also able to convert deoxypodophyllotoxin into podophyllotoxin after feeding (Kondo *et al.*, 1989). There is no data showing that such systems are able to convert yatein into deoxypodophyllotoxin. To obtain deoxypodophyllotoxin precursor yatein should undergo a complex ring closing reaction. This transformation can be mimicked chemically (Ward, 1990) by acidic cyclisation of lignan derivatives with a 7-hydroxygroup (Medarde *et al.*, 1996). It is also possible via an oxidative cyclisation of 4'-phenolic compounds (Medarde *et al.*, 1998), although the stereochemical outcome of the cyclisation is different from the products in the biosynthetic process of plants.

This study aims to identify the *in vitro* modifications of lignans with an open ring structure by *L. flavum* cell. For this study we used the precursor yatein and structurally related compounds like anhydropodorhizol, matairesinol, secoisolariciresinol and, podorhizol (see Fig. 1), which are putative intermediates or products in the biosynthetic pathway leading to deoxypodophyllotoxin, podophyllotoxin and 6-methoxypodophyllotoxin. Furthermore we used a synthetic analogue of yatein, naphthayatein (see Fig. 1) as well. Moreover putative steps of the biosynthesis can be proven by such direct precursor feeding experiments. Therefore this cell culture

system was used to study the biosynthesis of 6-methoxypodophyllotoxin and its biotechnological potential.

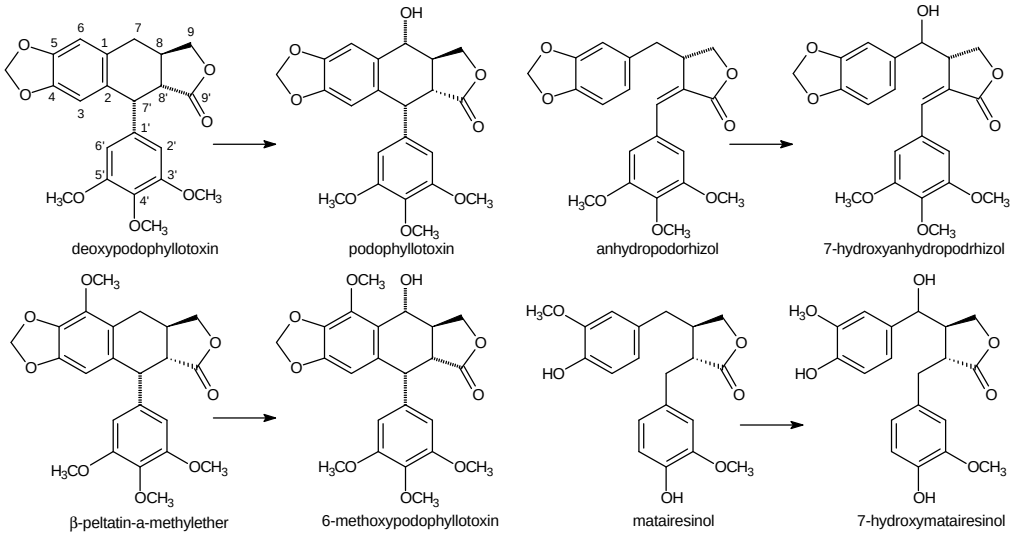

Fig. 1.
Currently known 7-hydroxylation of lignans occurring in *L. flavum* cell cultures, except for the hydroxylation of matairesinol into 7-hydroxymatairesinol that was reported by Xia and co-workers (2000) using whole plants.

MATERIAL AND METHODS

Chemicals

Podophyllotoxin was obtained from Sigma (St. Louis). We obtained yatein, anhydropodorhizol and deoxypodophyllotoxin as optically pure compounds through isolation from *A. sylvestris* roots using a previously described method for deoxypodophyllotoxin (Van Uden *et al.*, 1997). Maceration of 25 g of lyophilised ground *A. sylvestris* roots with 100 ml CH_2Cl_2 was followed by percolation with 300 ml CH_2Cl_2 to make a crude apolar extract.

Sample preparation

For the determination of the lignan precursors and their bioconversion products 100 mg dried and homogenised material was weighed in a Sovirel tube. A 2 ml portion of 80% methanol was added and the mixture was sonicated during 1 h. Then 4 ml of dichloromethane and 4 ml of H_2O were added. The tube was capped, vortexed and centrifuged at 1,000 *g* for 6 min and 2.0 ml of the organic layer was transferred into a 2 ml Eppendorf tube and dried under N_2 and redissolved in 1.0 ml of MeOH. This organic layer and the aqueous layer were subjected to HPLC. Growth parameters of the cell cultures were determined by harvesting 12 ml samples in a calibrated conical tube, followed by centrifugation for 5 min at 1,000 *g*. Conductivity in the resulting supernatant was measured. Cell dry weight was calculated from the weight difference of a glass fibre filter before and after suction filtration of an aliquot cell suspension culture and cell dry weight after subsequent freeze-drying.

Analysis

For routine HPLC analysis the following system was used: a LiChrosphere 100 RP-18 column (5 μm, 125 x 4 mm i.d.; Chrompack, Middelburg, the Netherlands) with MeCN : H_2O (40:60; v/v) as a mobile phase at a flow of 1.0 ml min^{-1} and UV detection (200 to 360 nm) with a Shimadzu Photodiode Array UV-VIS SPD-M6A detector (Shimadzu, 's Hertogenbosch, The Netherlands). For structure conformation a GC-MS method was used (see chapter III).

Bioconversion of lignans

Cell suspension cultures of *L. flavum* were obtained and grown as described previously, under these conditions the cultures produce trace amounts of 6-methoxypodophyllotoxin (<5 μM) (Van Uden *et al.*, 1990a). For bioconversion experiments the different compounds or extracts were dissolved in a small CH_2Cl_2 portion (± 2.5 ml) and transferred to a 500 ml culture flask. To such a flask 2 times the amount of 2,6-dimethyl-â-

cyclodextrin (Avebe, Veendam, The Netherlands) was added, based on molarity. 200 ml of culture medium was added and the flask was sonicated for 1 h followed by sterilisation. This procedure yielded pale brown solutions after the addition of an extract or clear solutions when a pure compound was added. At day zero 100 ml of full grown (t=14 days) *L. flavum* cell suspension was added to the enriched medium and the cultures were grown for 10 days. Each day a 12 ml sample was taken under sterile conditions, the growth parameters were determined and the medium was profiled on the lignan content. For the extraction of lignans the cells in the sample were treated as described above.

Extraction and Isolation of 7-hydroxyanhydropodorhizol

Lyophilised *L. flavum* cells (10.3 g) were incubated with 25 mM potassium phosphate, pH 7.0 as described previously (Canel *et al.*, 2000) and further extracted with 80% MeOH under sonication. The volume of methanolic extract was reduced under vacuum and partitioned between H_2O and CH_2Cl_2. The apolar fraction was subjected to chromatography on a column (25 x 2.5 cm i.d.) with silicagel 60 (63 – 200 mesh, ICN, Zoetermeer, The Netherlands). CH_2Cl_2 was used as the eluent with an increasing amount of MeOH, from 0 to 50%, at a flow rate of 7 ml min^{-1}, with a total volume of 2 L. Fractions containing 7-hydroxyanhydropodorhizol were pooled and the volume was reduced under vacuum. This total fraction also contained a small amount of 6-methoxypodophyllotoxin and was therefore chromtagraphed on a column (100 x 2 cm i.d.) with silicagel 60; hexane : CH_2Cl_2 : MeOH (90:10:1; v/v/v) was used as eluent. This yielded 13 mg 7-hydroxyanhydropodorhizol with a purity of 95% on basis of HPLC/DAD chromatography.

7-hydroxyanhydropodorhizol. Yellowish oil; $[á]^{22}_D$ (*c* –19.5 ,MeOH); ^{1}H NMR ($CDCl_3$, 200 MHz) δ 2.66(1H,d, *J*=4.2 Hz, H-4), 3.57(1H, m, H-3), 3.88 (6H, s, 3',5'OCH_3), 3.90 (3H, s, 4'-OCH_3), 4.26(1H, dd, *J*= 9.0, 2.0 Hz, H-3a), 4.29(1H, dd, *J*= 9.0, 2.0 Hz, H-3a), 7.48 (1H,m,H-1), 5.93,5.94 (each 1H, d *J*= 1,3 Hz, OCH_2O), 6.67 (1H, s, H-8), 6,68(2H, s, H-5, H-9), 6,72 (2H, s, H-2', H-6'); ^{13}C NMR ($CDCl_3$, 200 MHz) δ 51.2 (CH, C8), 2 x 54.6 (CH_3), 59.8 (CH_3), 71.4 (CH_2, C9), 74.6 (CH, C7), 101.9 (CH_2, OCH_2O), 2 x 106.8 (CH, C2',C6') 107.0 (CH, C5), 109.1 (CH, C6), 109.4 (CH, C3), 118.6 (CH, C2), 129.4 (C, C1) 129.7 (C, C8'), 136.5 (C, C1'), 139.0 (CH, C7'), 147.4 (C, C4), 153.1 (C, C4'), 2x 158.1 (C, C3',C5'), 172.8 (C, C9'); EIMS (70 eV) *m/z* [M^+] 414 (82), 398 (51), 381 (21), 263 (100), 207 (16), 176 (17), 135 (88).

Synthesis of lignans

The synthesis of lignans was carried out as racemic mixtures, by the well-known conjugate addition-alkylation process, using benzyl bromides or benzaldehydes as alkylating agents. During this process, phenolic hydroxyl groups were protected as benzyl ethers, with are deprotected in the next step. By Raney Ni desulphurisation (and debenzylation) products type I

and III (Fig. 4) were obtained. By this procedure, known compounds (two steps overall yield): matairesinol (35%), yatein (74%), podorhizol and *epi*-podorhizol (podorhizol and *epi*-podorhizol, 79% combined yield) were isolated after chromatographic purification of the reaction product.

$LiAlH_4$ reduction of matairesinol yielded secoisolariciresinol. Anhydropodorhizol was produced by acetylation of podorhizol followed by elimination with base. All known compounds matairesinol, yatein, anhydropodorhizol, secoisolariciresinol, podorhizol and epi-podorhizol are identical (direct comparison or described spectroscopic data) to those described. The synthetic procedure is detailed below for the new compound naphthayatein.

Synthesis of (±) (3S,4S)-[(2-naphthyl)methyl]-3-[(3',4',5'-trimethoxyphenyl)methyl]tetrahydrofuran-2-one (Naphthayatein.

Conjugate addition-alkylation. To 350 mg (1.4 mmol) of 2-(2-naphthyl)-1,3-dithiane in 14 ml of dry tetrahydrofurane (THF), at –78°C under Argon, 1 ml (1.6 mmol) of *n*BuLi (1.6 M in hexanes) were added. After 45 min 100 µl (1.4 mmol) of 5H-furan-2-one in 2 ml of THF were added dropwise and allowed to react for 3 h. Then, 548 mg (2.1 mmol) of 3,4,5-trimethoxybenzyl bromide in 2 ml of THF, followed by 420 ml (2.8 mmol) of tetramethylethylenediamine (TMEDA), were consecutively added. The mixture was allowed to reach room temperature and quenched by addition of a NH_4Cl saturated solution. By the usual work up 250 mg of dithiane derivative (35% yield) were isolated.

(±) (3S,4R)-4-[2-(2-naphthyl)-1,3-dithian-2-yl]-3-(3,4,5-trimethoxyphenylmethyl)tetrahydrofuran-2-one.

^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.95 (2H, m, S-CH_2-$\underline{CH_2}$-CH_2-S), 2.31-2.75 (2H, m, H-7'), 3.04 (1H, m, H-8), 3.31-3.75 (4H, m, S-$\underline{CH_2}$-CH_2-$\underline{CH_2}$-S), 3.79 (1H, m, H-8'), 3.85 (3H, s, 4'-OCH_3), 3.86 (6H, s, 3',5'-OCH_3) 4.10 (1H, m, H-9a), 4.70 (1H, dd, J=7.0, 4.0 Hz, H-9b), 6.59 (2H, s, H-2', H-6'), 7.53-7.55 (1H, m, naphth.), 7.56 (1H, d, J=8.8 Hz, naphth.), 7.84-7.93 (2H, m, naphth.), 7.94 (1H, d, J=8.8 Hz, naphth.), 7.98 (1H, dt, J=8.8, 1.8 Hz, naphth.), 8.45 (1H, s, naphth.). ^{13}C NMR ($CDCl_3$, 100 MHz) δ 24.4 (CH_2), 2x 27.1 (CH_2), 31.3 (CH_2), 37.1 (CH), 42.7 (CH), 52.0 (C), 56.0 (CH_3), 2x 60.9 (CH_3), 64.5 (CH_2), 2x 104.8 (CH), 125.7 (CH), 125.7 (CH), 127.1 (CH), 127.3 (CH), 127.6 (CH), 128.4 (CH), 129.6 (CH), 132.6 (C), 133.3 (C), 133.9 (C), 135.0 (C), 135.0 (C), 2x 153.4 (C), 178.0 (C).

Reduction with Ni Raney. By addition of an excess of Ni Raney onto 200 mg of the latter product in 60 ml of THF, followed by stirring and reflux for 2 h, after filtration through silicagel, 130 mg (82% yield) of naphthayatein (**7**) were obtained.

(±) (3S,4S)-[(2-naphthyl)methyl]-3-[(3',4',5'trimethoxyphenyl)methyl] tetrahydrofuran-2-one. (7). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 2.62-2.87 (4H, m, H-7,7'), 2.93 (1H, m, H-8), 3.75 (6H, s, 3',5'-OCH_3), 3.82 (3H, s, 4'-OCH_3), 3.87 (1H, m, H-8'), 3.93 (1H, m, H-9a), 4.22 (1H, m, H-9b), 6.31 (2H, s, H-2', H-6'), 7.11 (1H, d, J=8.4 Hz, naphth.), 7.46-7.49 (2H, m, naphth.), 7.47 (1H, s, naphth.), 7.74 (1H, d, J=8.4 Hz, naphth.), 7.73-7.82 (2H, m, naphth.); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 35.2 (CH_2), 38.7 (CH_2),

40.9 (CH), 46.6 (C), 2x 56.0 (CH_3), 60.9 (CH_3), 71.4 (CH2), 2x 106.3 (CH), 125.9 (CH), 126.5 (CH), 126.8 (CH), 127.2 (CH), 127.5 (CH), 127.7 (CH), 128.5 (CH), 132.2 (C), 133.5 (C), 133.5 (C), 135.6 (C), 135.6 (C), 2x 153.3 (C), 178.7 (C); FAB-MS *m/z* [M^+]406(40), 181(100).

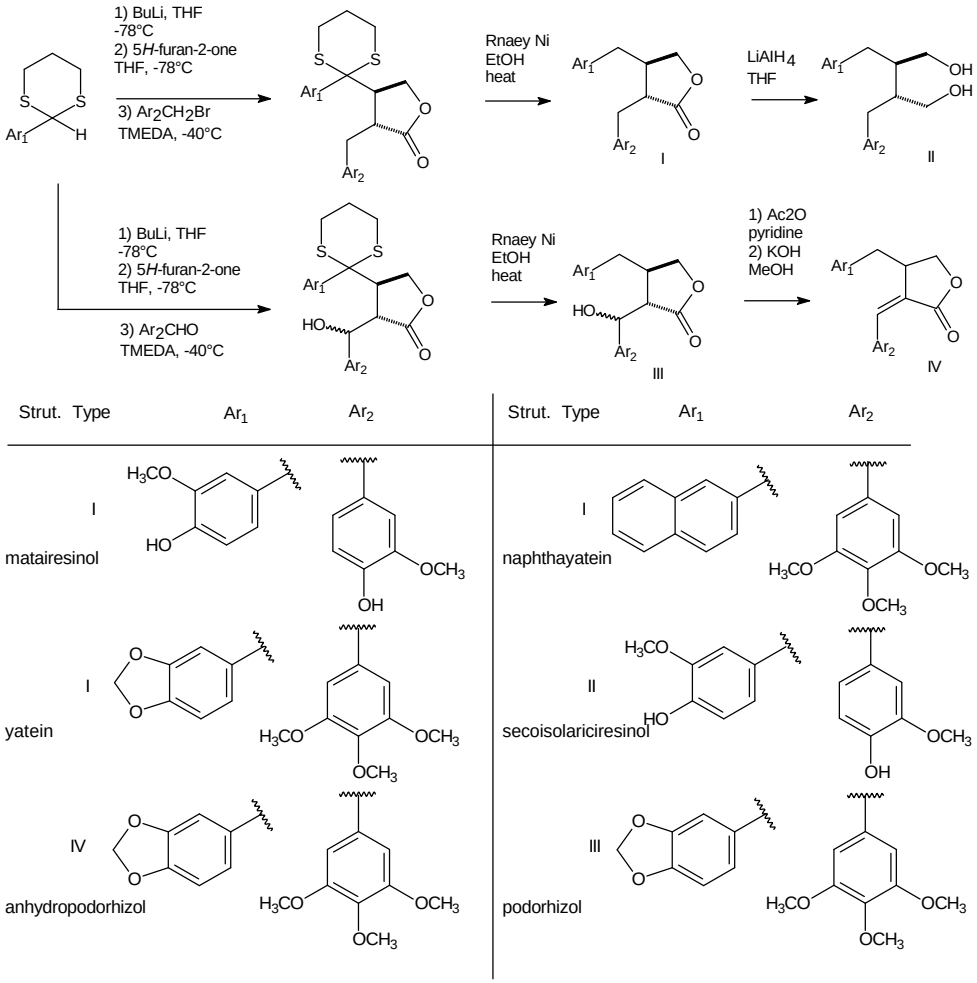

Fig. 4.
Chemicel synthesis of lignans used in this study.

RESULTS AND DISCUSSION

Bioconversion of crude extracts from A. sylvestris

Although 6-methoxypodophyllotoxin can be easily produced through the bioconversion of deoxypodophyllotoxin by *L. flavum* suspension cultures, it demands a complete isolation of deoxypodophyllotoxin (Van Uden *et al.*, 1997). In order to circumvent this time-consuming isolation we tried a more direct approach for the production of 6-methoxypodophyllotoxin by preparing a precursor containing crude extract of *A. sylvestris*. The roots of this plant (25 g) were percolated with dichloromethane. The percolate was reduced under vacuum to finally yield 560 mg crude extract containing 23% (± 0.7) deoxypodophyllotoxin, 7% (± 0.9) yatein, and 8% (± 1.5) anhydropodorhizol and 6% other lignans (like hinokinin, bursehernin, and morelensin; Ikeda *et al.*, 1998b; Ikeda *et al.*, 1998a; Lim *et al.*, 1999). Different amounts of the crude extract were complexed with 2,6-dimethyl-â-cyclodextrin and fed to the *Linum flavum* cell cultures.

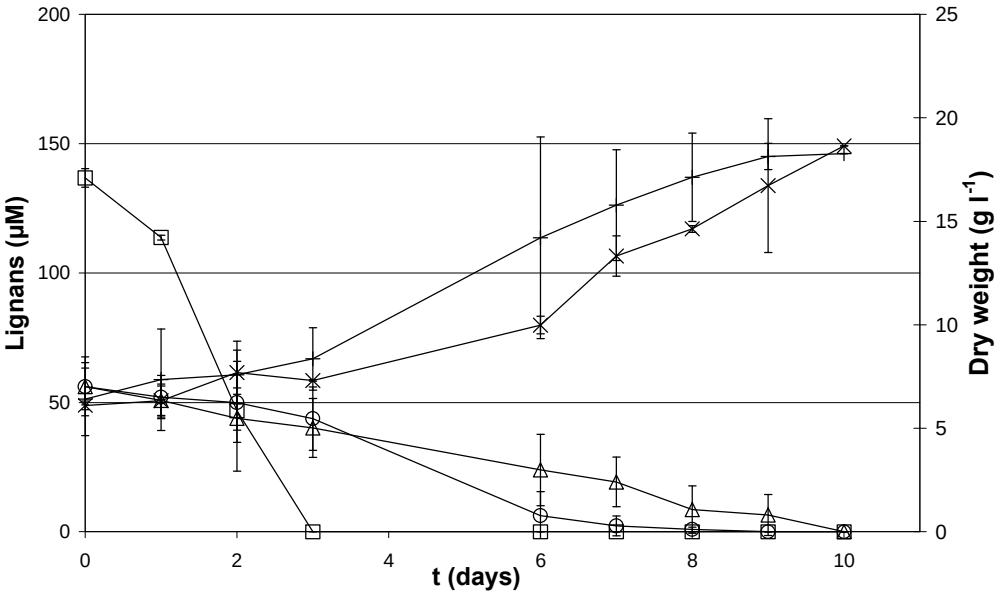

Fig. 2a.
The fate of the main lignans deoxypodophyllotoxin (□), yatein (Ä), anhydropodorhizol (○) of 75 mg crude extract *A. sylvestris* after feeding to a 300 mL *L. flavum* cell culture expressed as µM lignan per liter cell culture.The increase of dry weight of the culture fed with 75 crude extract (×) and the control culture (+) shows the viability of the used cell cultures (g L^{-1}) (n=3).

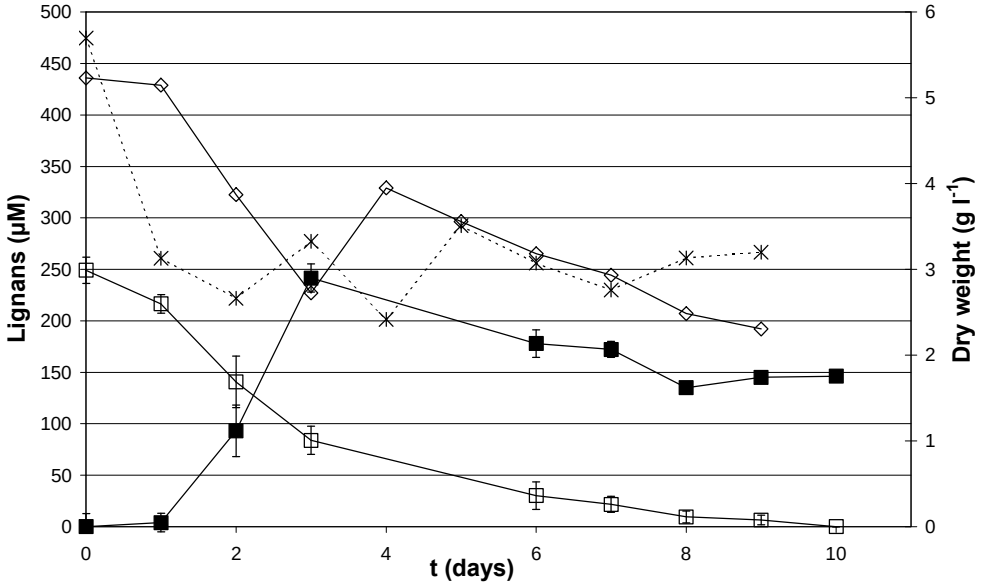

Fig. 2b.
The total lignan concentration of the 75 mg (□) crude extract, the total concentration of products formed (■) and 125 mg crude extract (◇) *A. sylvestris* after feeding to a 300 mL *L. flavum* cell culture; all expressed as µM lignan per liter cell culture. The decrease of dry weight shows the lack of viability of the cell cultures fed with 125 mg crude extract (æ) (g L^{-1}) (75mg, n=3, 125 mg, n=1).

The time course of the concentration of the main lignan precursors after feeding 75 mg crude extract is depicted in Figure 2a. In the same figure the dry weight of the culture is shown as well as that of a control culture. The culture fed with 75 mg crude extract showed growth similar to the control. The conductivity decreased, the fresh weight increased up to 280 g l^{-1} and the dry weight increased up to 18 g l^{-1}. Figure 2b shows clearly the lack of growth for the culture fed with 125 mg of the crude extract. The dry weight of this 125 mg culture decreased from 5.7 g l^{-1} to around 3 g l^{-1}. The crude extract of *A. sylvestris* seemed to contain a component that was toxic to the *L. flavum* cell culture. Also experiments with 150 mg and 250 mg of extract resulted in a total lack of growth. Lignans are very cytotoxic compounds. In cytotoxicity assays, using human tumour cell lines, the bioactivity of deoxypodophyllotoxin, yatein and anhydropodorhizol is comparable (Middel *et al.*, 1995). Based on a 44% lignan content 125 mg of crude extract will result in a total lignan concentration of 399 µM in the cell culture. Cell cultures of *L. flavum* are able to grow in medium with a concentration of deoxypodophyllotoxin of over 1200 µM and convert this lignan further to 6-methoxypodophyllotoxin (Van Uden *et al.*, 1997). Therefore, the direct cytotoxicity of the lignans seems not likely to cause the growth decrease.

The *L. flavum* cell culture takes up deoxypodophyllotoxin very quickly, see Figure 2a. The cells converted it into 6-methoxypodophyllotoxin and

its glycoside; the sum of the concentrations of 6-methoxypodophyllotoxin, its glycoside and podophyllotoxin is depicted as product in Figure 2b. This bioconversion is in agreement with previous results (Van Uden *et al.*, 1997). However, the uptake of yatein and anhydropodorhizol is much slower as can be seen in Figure 2a. Although yatein is the expected direct precursor of deoxypodophyllotoxin (Broomhead *et al.*, 1991), it was taken up even slower than anhydropodorhizol. The concentration of the yatein and anhydropodorhizol decreased to below the detection limit at the final stage (after t = 7 days) of the bioconversion experiments. There was no indication of any other product formation and therefore it seems likely that these compounds are degraded. In summary, the *L. flavum* cells are able to bioconvert added substrates like deoxypodophyllotoxin and also â-peltatin-a-methylether (see Fig. 1) very efficiently (see next chapter) but putative biosynthetic intermediates before deoxypodophyllotoxin are not taken up as efficient by the cells and not converted into detectable products.

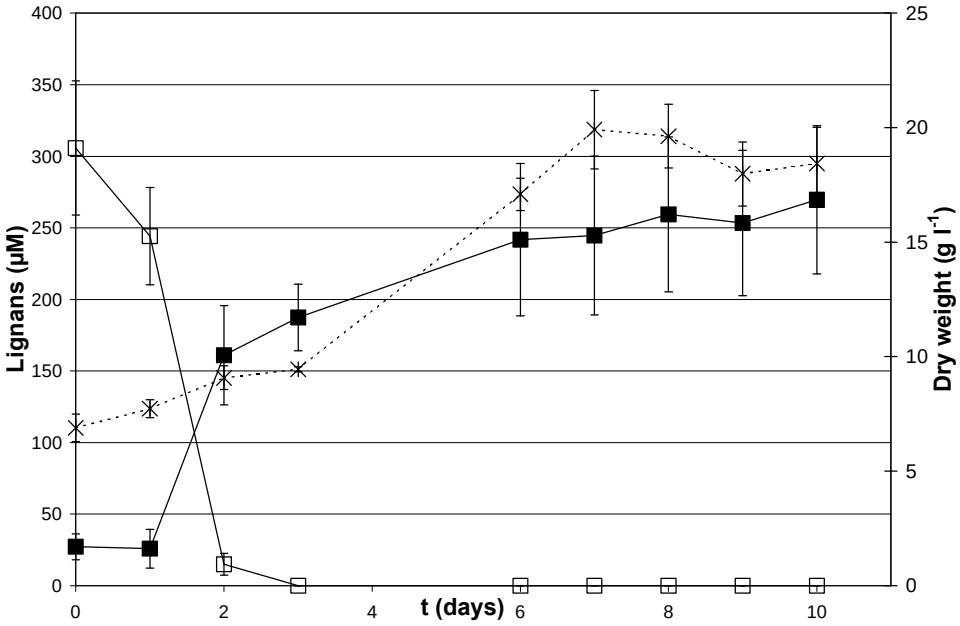

Fig. 3a.
The bioconversion of deoxypodophyllotoxin (□) after feeding to a 300 mL *L. flavum* cell culture and the sum of products formed (■) expressed as µM lignan per liter cell culture. The increase of dry weight (×) shows the viability of the used cell cultures (g L^{-1}) (n=3).

From the results of the feeding of crude apolar extracts of *A. sylvestris,* two main conclusions can be drawn. First there are toxic components in the crude extract of *A. sylvestris* roots which limit the amount of extract that can be fed to *L. flavum* cell culture. This makes a cheap and efficient production system less easy to achieve. A production system using pure deoxypodophyllotoxin yields much higher amounts of 6-methoxy-

podophyllotoxin. The second conclusion that can be drawn is that after feeding, yatein and anhydropodorhizol are not directly converted into deoxypodophyllotoxin and subsequently 6-methoxypodophyllotoxin.

Bioconversion of individual lignans

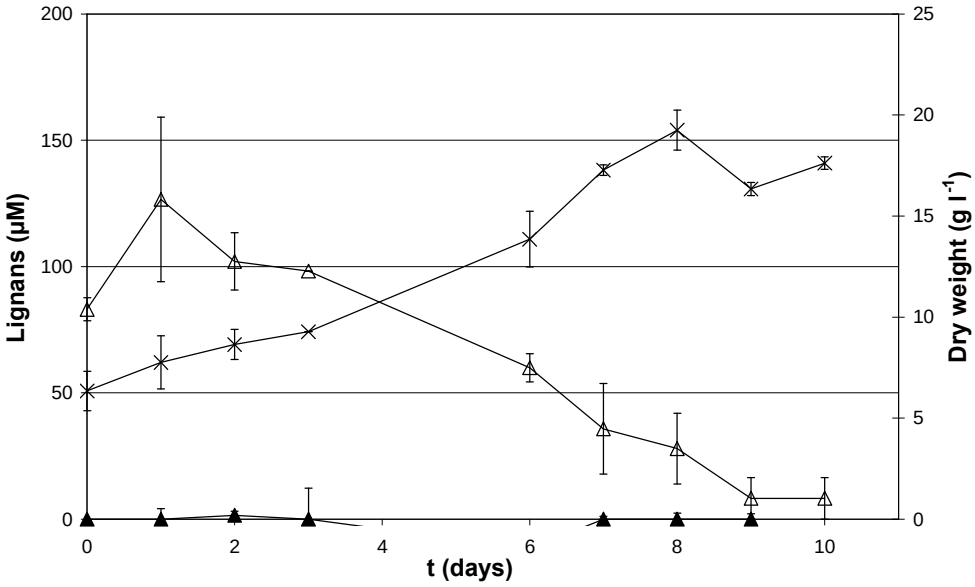

Fig. 3b.
The bioconversion of **3** (Ä) after feeding to a 300 mL *L. flavum* cell culture and the sum of products formed (▲) expressed as µM lignan per liter cell culture. The increase of dry weight (×)shows the viability of the used cell cultures (g L^{-1}) (n=3).

It was especially the last conclusion that raised some questions. To study this phenomenon in more detail we started feeding experiments using the individual main lignan precursors from *A. sylvestris*: yatein, anhydropodorhizol and deoxypodophyllotoxin. Lignan deoxypodophyllotoxin was isolated from *A. sylvestris*. The other two precursors yatein, anhydropodorhizol and compounds matairesinol, naphthayatein, secoisolariciresinol, podorhizol and epi-podorhizol were obtained through organic synthesis (see Fig. 3 and material and methods). We fed these compounds at a low concentration of 120 µM, comparable to twice the concentration of yatein and anhydropodorhizol in the 75 mg crude extract feeding experiment.

To exclude any chemical degradation or derivation of the lignans during the experiments, we treated equal amounts of lignans in the same way and incubated these compounds in 300 ml of growth medium for 2 weeks. The analysis of the medium at start of the experiment yielded the same concentration substrate as after 2 weeks. Lignan deoxypodophyllotoxin was converted into 6-methoxypodophyllotoxin and its glycoside within 3 days (see Fig. 3a). Cell growth was not affected. The detailed

bioconversion kinetics of deoxypodophyllotoxin including intermediates and product monitoring is currently being investigated.

Compound yatein slowly disappeared from the medium when added to the culture (see Fig. 3b). Cell growth was not negatively influenced at this concentration. No significant amount of product could be detected intracellularly or in the medium. In this study we define product as the sum of lignans formed from the substrate by hydroxylation, methylation, glycosylation, ring closing, or a combination of these reactions. The feeding was repeated with increased concentrations of yatein up to 32 mg to one culture, giving a final concentration of over 270 µM. For this experiment we used optically pure yatein, in order to exclude the

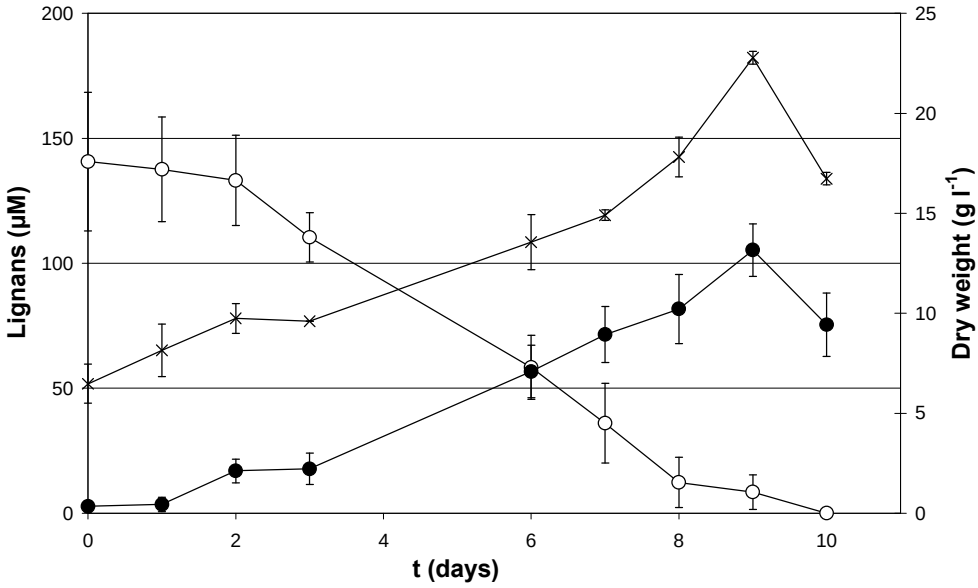

Fig. 3c.
The bioconversion of anhydropodorhizol (○) after feeding to a 300 mL *L. flavum* cell culture and the sum of products formed (●) expressed as µM lignan per liter cell culture. The increase of dry weight (×) shows the viability of the used cell cultures (g L^{-1}) (n=3).

possibility that the non-natural isomer, as present in the synthetically prepared yatein, is responsible for the extreme low bioconversion rate. The optically pure yatein was isolated from the roots of *A. sylvestris*. This experiment yielded no detectable bioconversion. However a clear decrease in growth was observed, similar to the effect of 125 mg of crude extract. Therefore it seems that yatein is a compound that causes severe toxicity and yatein could be one of the toxic components in the crude extract of *A. sylvestris* that inhibits the growth of the culture. There is another observation that can be deduced from these experiments as well; only the last part of the lignan biosynthesis in *L. flavum* starting with deoxypodophyllotoxin apparently is or becomes active in plant cell culture.

The ring closure of yatein leading to deoxypodophyllotoxin does not take place in *L. flavum* cell cultures. It is possible that the expression of the responsible earlier enzyme(s) is not induced after addition of this precursor in the culture medium. Alternatively yatein may not be reaching the enzyme in the cell.

The synthetic yatein-like precursor naphthayatein was fed to *L. flavum* cells at a concentration of 35 mg l^{-1} (86 μM). Compound naphthayatein is structurally very close to yatein, with a naphthalene moiety replacing the 1,3-benzodioxole system, both similar in size, reactivity and intermolecular binding properties. The compound slowly disappeared from the medium and no bioconversion products could be detected. The cell growth was comparable to that of the control cultures during the 14 days experiment, in agreement with its reported lower toxicity (Medarde *et al.*, unpublished results).

The feeding of anhydropodorhizol resulted in the appearance of two new peaks in the HPLC chromatogram, starting from day 3 in the growth cycle. Cell growth seemed to be affected in this experiment, but not significantly. The UV spectrum of these new peaks that occurred at retention times of 7.0 and 1.8 minutes respectively showed a high degree of similarity to that of the substrate anhydropodorhizol. Figure 3c shows the time course of the total product concentration (sum of the peaks at 7.0 min and 1.8 min) that was calculated as μM using a mass of 414 for the peak at 7.0 min and 594 for the 1.8 min. The same figure shows the disappearance of the precursor (anhydropodorhizol) and the dry weight. Using the GC-MS method recently published (see chapter III) we could obtain a mass spectrum of the 7.0 minute peak, which gave a spectrum with its base peak at *m/z* of 263 and a highest mass with a *m/z* of 414. The other major fragment had a *m/z* of 135. Based on the MS and the UV spectrum we presumed that this compound is a hydroxylated derivative of anhydropodorhizol. The hydroxylation has taken place at the 7 position, so the compound is named 7-hydroxyanhydropodorhizol (Fig. 1). We obtained further evidence for the structure by an extra feeding experiment with anhydropodorhizol at a two-fold higher concentration. The cells were harvested at the end of the growth cycle (t = 14) and the hydroxylated derivative 7-hydroxyanhydro-podorhizol was isolated using column chromatography. Its ^{1}H-NMR spectrum, in comparison with that of anhydropodorhizol, clearly showed that one proton at the 7 position has disappeared. The rest of the proton NMR spectrum was consistent with that of anhydropodorhizol, confirming that this compound is really 7-hydroxyanhydropodorhizol.

To check if 7-hydroxyanhydropodorhizol was also present as a glycosidic form the water phase of the cell extract was treated with â-glucosidase as previously described (Van Uden *et al.*, 1989). The HPLC analysis of the water phase did not show any lignan aglycon. The treatment with â-glucosidase yielded 7-hydroxyanhydropodorhizol, showing that this compound is also present as its glycoside. From these experiments it is not clear what kind of sugar moieties are coupled to the lignan. Further research will be necessary to determine if except for glucose also other

sugars are coupled to anhydropodorhizol. Therefore we will use the more general term glycoside.

A second proof for the presence of the glycoside of 7-hydroxyanhydropodorhizol was delivered by the application of an extraction method where glycosidases from plant cells remain active (Canel *et al.*, 2000). The cells were harvested at day 13 of the growth cycle and extracted with 25 mM potassium phosphate, pH 7.0 for 1 hour and then further extracted with MeOH. This method yielded a much higher amount of 7-hydroxyanhydropodorhizol in the organic phase then after the standard extraction method. This again shows that in the cells 7-hydroxyanhydropodorhizol is also present as its glycoside.

We suspected that 7-hydroxyanhydropodorhizol could be a natural lignan. However a literature survey did not reveal 7-hydroxyanhydropodorhizol or its glycoside as naturally occurring lignans. Recently Xia and co-workers published the conversion of matairesinol into 7-hydroxymatairesinol by *Linum flavum* roots (see Fig. 1; Xia *et al.*, 2000). This compound was also not previously reported and only discovered after bioconversion experiments. *L. flavum* cells seem to be able to hydroxylate deoxypodophyllotoxin, â-peltatin-a-methylether, matairesinol and anhydropodorhizol all at the 7-position (see Fig. 1). Of the products only 6-methoxypodophyllotoxin is found in high amounts in *L. flavum* plants and podophyllotoxin is only found in trace amounts in the roots (Broomhead and Dewick, 1990a).

One could argue that there are four very specific 7-hydroxylating enzymes (see Fig. 1) each responsible for the 7-hydroxylation of one of the structurally related lignan precursors. The presence of a hydroxylating enzyme with a broader substrate specificity for this type of precursors seems more plausible to the authors. This enzyme could have a high affinity for â-peltatin-a-methylether and its broader specificity is only observable when artificially high amounts of intermediates are fed. For example Xia co-workers used concentrations of 220 μM of matairesinol while this compound is not detectable under natural conditions in *L. flavum*. The 7-hydroxy products of this enzyme are therefore undetectable under natural conditions in the plant. On the contrary, the feeding of yatein did not result in its 7-hydroxy form. The combination of the toxic properties of yatein to the *L. flavum* cells with the very poor up-take of yatein by the cells probably inhibits this conversion. Based on the reaction type the hydroxylating enzyme is very probably a cytochrome P450 monooxygenase, which is a membrane-bound enzyme. The substrate has to be taken up by the cells and transported across the membrane to enable a bioconversion. It is not yet clear why yatein is differently metabolised.

The feeding of matairesinol (see Fig. 1) to the *L. flavum* cell cultures did not result in the same product (7-hydroxymatairesinol) as reported by Xia and co-workers. Cell growth was not affected and the cells rapidly took up matairesinol but did not hydroxylate it further (see Fig. 1), instead this compound was glycosylated. This was proven by the treatment of the cells after the bioconversion experiment with â-glucosidase as mentioned above.

The aglycon matairesinol was indeed formed. Not only matairesinol was directly glycosylated, podorhizol, and secoisolariciresinol had the same fate.

At present there are two published biosynthetic routes towards 6-methoxypodophyllotoxin in *L. flavum*. A recent study showed that 7-hydroxymatairesinol is an intermediate originating from matairesinol. At the moment there are no intermediates known between 7-hydroxy-matairesinol and 6-methoxypodophyllotoxin. Several other studies proved a route that has deoxypodophyllotoxin as an intermediate. The steps from deoxypodophyllotoxin towards 6-methoxypodophyllotoxin have been mapped out. The enzyme responsible for the hydroxylation of deoxypodophyllotoxin has been recently characterised. It is P450 mono-oxygenase that is able to convert deoxypodophyllotoxin into ß-peltatin (Molog *et al.*, 2001). The enzyme able to convert â-peltatin into â-peltatin-a-methylether, has also been characterised and is an S-adenosyl-L-methionine dependent methyltransferase (Kranz and Petersen, 2001). The last step in the putative biosynthesis of 6-methoxypodophyllotoxin is not enzymatically characterised but the feeding of â-peltatin-a-methylether very rapidly results in 6-methoxypodophyllotoxin (unpublished results).

This study did not result in the identification of the precursors of deoxypodophyllotoxin, and neither did it correspond with the theory that 7-hydroxymatairesinol is an intermediate of the 6-methoxypodophyllotoxin biosynthesis. The results found for *Podophyllum hexandrum,* showing yatein as a direct precursor of deoxypodophyllotoxin, have not yet been confirmed for *L. flavum.* From the combination of our results and those reported in the literature, it seems possible that the biosynthesis of lignans in *L. flavum* is a more flexible system, using several routes (at least 2). The biosynthetic route, or better, cascade towards 6-methoxypodophyllotoxin needs further study.

From these series of bioconversion experiments a number of conclusions can be drawn. The feeding of anhydropodorhizol results in hydroxylation at the 7 position followed by glycosylation. The 7-hydroxylation seems to be a general reaction in lignan biosynthesis in *L. flavum* and could point to a hydroxylating enzyme with broad substrate specificity. This should be definitely proven by testing individual precursors on this putative cytochrome P450 monooxygenase that should be isolated from the cells or preferably from a heterologous expression host. The precursor yatein**,** which is the expected direct precursor of deoxypodophyllotoxin (Broomhead *et al.*, 1991), underwent no ring-closing and was not converted at all as was the case for the synthetic analogue naphthayatein; no ring closing reaction could be detected.

The present study shows that *L. flavum* cells were able to modify a limited number of putative precursors of deoxypodophyllotoxin. Unexpectedly the resulting compounds are not intermediates that fit in the current views on the biosynthetic cascade leading to 6-methoxy-podophyllotoxin.

ACKNOWLEDGMENT

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VIII. The bioconversion process of deoxypodophyllotoxin with *Linum flavum* cell cultures.

Based on: Albert Koulman, Aäron C. Beekman, Niesko Pras & Wim J. Quax, The bioconversion process of deoxypodophyllotoxin with *Linum flavum* cell cultures, *Planta Med*., accepted.

NEDERLANDSE SAMENVATTING

De omzetting van deoxypodofyllotoxine door de celcultures van gele vlas

In het vorige hoofdstuk is beschreven dat na het voeden van deoxypodofyllotoxine aan celcultures van gele vlas 6-methoxypodophyllotoxine ontstaat. Dit gaat niet in één omzetting (stap), maar er zijn meerdere stappen voor nodig. Om het omzettingsproces beter te kunnen begrijpen hebben we een aantal experimenten uitgevoerd waarbij verschillende concentraties deoxypodofyllotoxine aan celcultures van gele vlas werden gegeven. Vervolgens werd heel precies nagegaan wat er met deze stof gebeurde.

Bij een lage concentratie deoxypodofyllotoxine werd de stof snel omgezet in 6-methoxypodophyllotoxine en in de glycoside hiervan. Een glycoside is dezelfde stof met een suikermolecuul eraan gekoppeld. Plantencellen koppelen graag aan giftige stoffen een suikergroep (glycoside). De suikergroep maakt deze beter oplosbaar in water. Dit heeft voor de plantencel het voordeel dat de geglycosileerde stof makkelijker kan worden opgeslagen. Zo kunnen plantencellen giftige stoffen opslaan, zonder er zelf last van te krijgen.

Behalve 6-methoxypodophyllotoxine maakten de cellen ook â-peltatine van het gevoede deoxypodofyllotoxine. Dat werd weer verder omgezet in 6-methoxypodophyllotoxine. Bij hogere concentraties verliep het proces trager. Opmerkelijk was dat, in de dagen na het toevoegen van deoxypodofyllotoxine er een hoeveelheid van de stof verdween.

Beter gezegd, bijna al het deoxypodofyllotoxine werd opgenomen en omgezet door de cellen, maar we konden maar een deel van de producten meten. Na verloop van tijd verdween dit verschil weer. Dit kwam doordat er weer meer 6-methoxypodofyllotoxine onstond.

Door verschillende extractietechnieken toe te passen kwamen wij er achter dat een deel van de deoxypodofyllotoxine werd omgezet in â-peltatine, dat tijdelijk in de vorm van glycoside werd bewaard.

Naast de eerder genoemde producten werd in dit experiment ook steeds een kleine hoeveelheid podofyllotoxine gevonden. Dat ook dit lignaan in deze cultures werd aangemaakt kan worden verklaard uit de aanwezigheid van het enzym met een brede substraatspecificiteit, zoals in het vorige hoofdstuk al is gepostuleerd.

ABSTRACT

The *in vitro* cell suspension culture of *Linum flavum* is able to convert high amounts of the 2,7'-cyclolignan deoxypodophyllotoxin into 6-methoxypodophyllotoxin 7-*O*-glucoside. We studied this conversion in detail by monitoring the intermediates and side-products after feeding different concentrations of deoxypodophyllotoxin. At a low concentration (0.1 mM) deoxypodophyllotoxin is rapidly converted into 6-methoxy-podophyllotoxin 7-*O*-glucoside, 6-methoxypodophyllotoxin and traces of â-peltatin and podophyllotoxin. The feeding of 0.5 and 2.0 mM also shows a rapid conversion into 6-methoxypodophyllotoxin 7-*O*-glucoside, but a delayed formation of 6-methoxypodophyllotoxin and β-peltatin. By using different extraction methods we delivered proof in favour of the hypothesis that a part of the deoxypodophyllotoxin after uptake is temporarily stored as â-peltatin glucoside.

INTRODUCTION

Lignans are plant metabolites originating from the phenylpropanoid pathway. Plants produce these compounds through a stereospecific coupling of two monolignol radicals in the presence of the dirigent protein (Lewis and Davin, 1999). In current pharmacotherapy, only the lignans with a 2,7'-cyclolignan skeleton are applied (i.e. podophyllotoxin, Fig. 1). This lignan is used in the treatment of condyloma (Gross, 2001) and as a starting compound for the production of three important clinically used anticancer drugs: etoposide, etopophos and teniposide (Imbert, 1998). Recently, several clinical studies have been carried out with a semisynthetic podophyllotoxin glycoside against rheumatoid arthritis (Gudmundsdottir and Jonsson, 2000). This medicinal use of lignans demands a reliable and continuous source for production. This has challenged researchers to consider various alternatives for the production of podophyllotoxin. A better understanding of the biosynthesis of podophyllotoxin and of 2,7'-cyclolignans in general, is a prerequisite for this research.

In previous studies we showed that cell suspension cultures of *Linum flavum* are able to convert high amounts of deoxypodophyllotoxin (see Fig 1) into 6-methoxypodophyllotoxin glycoside (see Fig. 1). The lignan deoxypodophyllotoxin can be easily isolated from the roots of *Anthriscus sylvestris*, a common weed in Northwest Europe (Van Uden *et al.*, 1997). This forms a new production system for high amounts of hydroxylated 2,7'-cyclolignans. However, the aglycon of the product (6-methoxy-podophyllotoxin, see Fig. 1) has not yet the same pharmaceutical application as podophyllotoxin (San Feliciano *et al.*, 1993). A detailed survey of this bioconversion system, however, could lead to an improved insight of the metabolic fate of deoxypodophyllotoxin. This knowledge is essential for any experiments aimed at the (genetically) engineering of the biosynthesis that should lead to a higher production of podophyllotoxin. Recently, we have been able to show that the first step in the biosynthesis of 6-methoxypodophyllotoxin glycoside from deoxypodophyllotoxin is the 6-hydroxylation by a cytochrome P450 mono-oxygenase leading to â-peltatin (see Fig. 1; Molog *et al.*, 2001). In this study we will look further into the product formation after feeding deoxypodophyllotoxin to *L. flavum* cell suspension cultures.

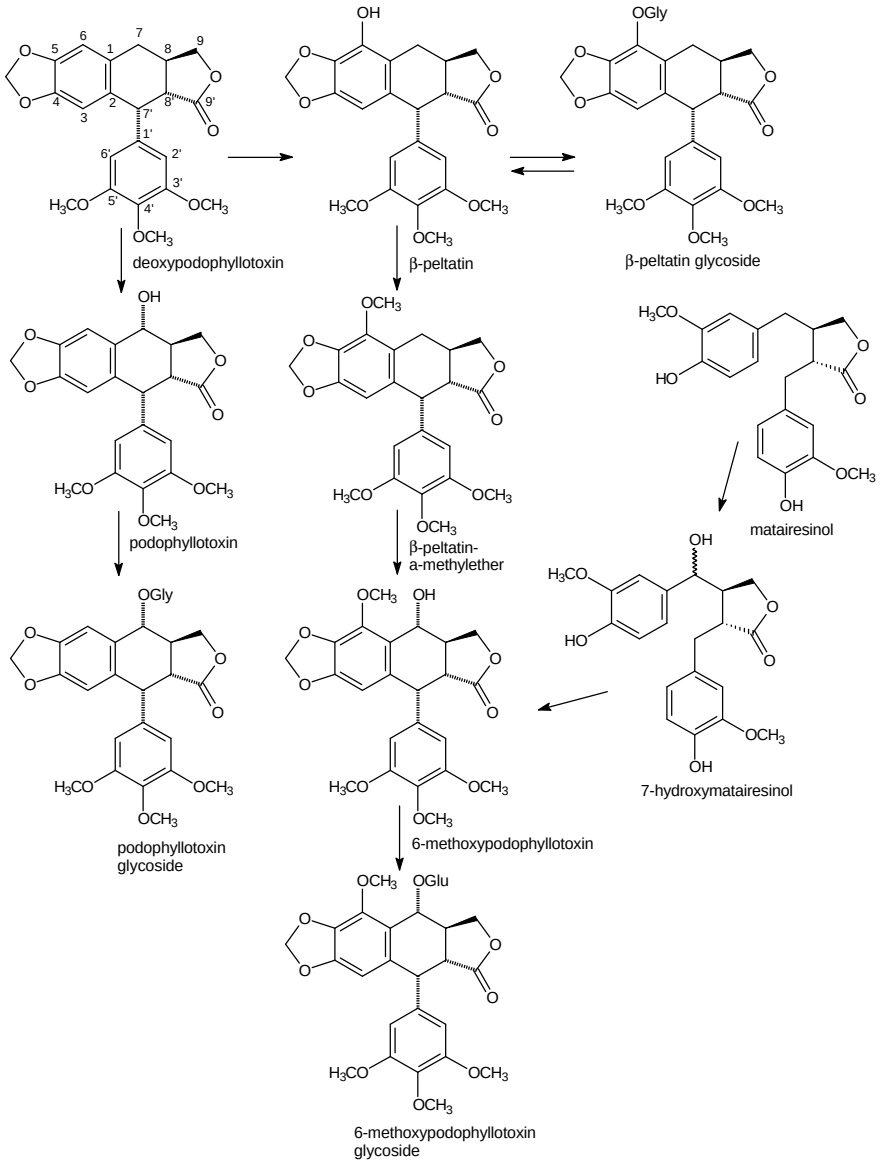

Fig. 1.
The bioconversion of deoxypodophyllotoxin into â-peltatin, podophyllotoxin, â-peltatin-a-methylether, â-peltatin glycoside, podophyllotoxin glycoside, 6-methoxypodophyllotoxin, podophyllotoxin and 6-methox-ypodophyllotoxin 7-*O*-glucoside as found in this study. Additionally, the route proposed by Xia and co-workers (2000) is shown, starting from matairesinol via 7-hydroxy-matairesinol into 6-methoxy-podophyllotoxin.

MATERIALS AND METHODS

Chemicals

Podophyllotoxin was obtained from Sigma (St. Louis), β-peltatin and, β-peltatin-A-methylether were prepared synthetically (Medarde *et al.*, 1995). Deoxypodophyllotoxin was obtained as an optically pure compound through isolation from *A. sylvestris* roots using the previously described method (Van Uden *et al.*, 1997). 6-methoxypodophyllotoxin and 6-methoxypodophyllotoxin glycoside were obtained through isolation from *L. flavum* cultures after bioconversion of deoxypodophyllotoxin as previously described (Van Uden *et al.*, 1997).

Plant cell culturing

Linum flavum plant cell cultures were grown as previously described, using 500 ml culture flasks with a total cell culture volume of 300 ml (Van Uden *et al.*, 1997).

Addition of deoxypodophyllotoxin to cell cultures

Deoxypodophyllotoxin (M_w 398) was added in three different concentrations (0.1, 0.5 and 2 mM) with twice as much dimethyl β-cyclodextrin (M_w 1331) in a molar ratio. Deoxypodophyllotoxin and dimethyl β-cyclodextrin were transferred to a bottle, and 160 ml MS medium was added and sterilised for 25 min. This method did not result in any degradation or in formation of side-products. Without the addition of the cells to the medium, the deoxypodophyllotoxin concentration remained stable for the whole culture period (14 days).

The cooled down medium was transferred to a sterile 500 ml Erlenmeyer flask and inoculated with 80 ml *Linum flavum* suspension culture (day 14 culture). Deoxypodophyllotoxin feeding did not alter the growth characteristics of the cell cultures. The cell material was filtered on a Büchner funnel and lyophilised overnight.

Extraction of lignans from lyophilised cells, the standard method

During the growth cycle eight 12 ml samples were taken under sterile conditions. The growth parameters (conductivity, pH, fresh weight, and dry weight) were determined as described before (Van Uden *et al.*, 1997). For the determination of the lignans, their precursors and bioconversion products we used standard sample preparation methods (see chapter III). These consist of an extraction of 100 mg lyophilised and ground plant material with 2 ml 80% MeOH for 1 hour under sonication. The addition of 4 ml CH_2Cl_2 and 4 ml of H_2O leads to a two-layer system. The organic and the aqueous layers were subjected to HPLC analysis to study the metabolic fate of the lignans and their products.

Extraction of lignans from lyophilised cells, phosphate buffer method

This method is equal to the method above (= standard method), except for the step where 80% methanol was replaced by 25 mM potassium phosphate buffer pH 7. The cells are turned or rocked slowly for 30 min. This method is based on the work of Canel and co-workers (Canel *et al.*, 2000).

Method of analysis

The HPLC system used in this study consisted of an autosampler (Kontron HPLC 360), a pump (Pharmacia LKB 216), a column (LiChroCART 125-4, Lichrospher 100 RP-18; 6 µm) and a detector (Shimadzu Photodiode Array UV-VIS SPD-M6A). The eluens was acetonitrile/water: 40/60 (v/v) + 1 µM phosphoric acid, flow: 1 ml min^{-1} and injection volume: 20 µl. This easy and fast system was used for the quantitative analysis of lignans. The supporting software contained a library of UV spectra of lignans. The characteristic lignan absorption peak is at 290 nm. All compounds could be detected in a single run, 6-methoxy-podophyllotoxin glucoside (4 min), podophyllotoxin (7 min), β-peltatin (8 min), 6-methoxypodophyllotoxin (11 min), deoxypodophyllotoxin (18 min), and β-peltatin A-methylether (26 min). All compounds were present as references and calibration curves were made and run regularly, based on 6 concentration (10, 25, 50, 100, 250 and 500 µM). At the start of every analysis a control sample was used containing all 6 lignans.

To compare the intracellular levels of the compounds with the externally added deoxypodophyllotoxin to *L. flavum* cells, the levels of substrate and product are expressed in µmol l^{-1} of the total cell culture volume. These levels are the sum of the concentration in the medium, the concentration in the organic phase and the concentration in the aqueous phase of cell extract.

Statistics

The student t-test was used to determine significant differences.

RESULTS AND DISCUSSION

The *Linum flavum* cell suspension cultures used in this study only produce a very low amount of 6-methoxypodophyllotoxin (0.01%, based on dry weight). In bioconversion experiments, *L flavum* cell cultures are able to convert the deoxypodophyllotoxin into 6-methoxypodophyllotoxin glucoside, which seems to be the end-product. Most of the intermediates in this process could be detected in the cells of *L. flavum* cells (in the water and organic fraction) and only a very small fraction was found in the medium, probably due to cell lysis. Because *L. flavum* cells were proven to be a very efficient bioconversion system (Van Uden *et al.*, 1997) we studied the fate of the precursor deoxypodophyllotoxin, intermediates and end product in more detail. For these experiments we fed 0.1, 0.5 and 2.0 mM of deoxypodophyllotoxin complexed with dimethyl-â-cyclodextrin.

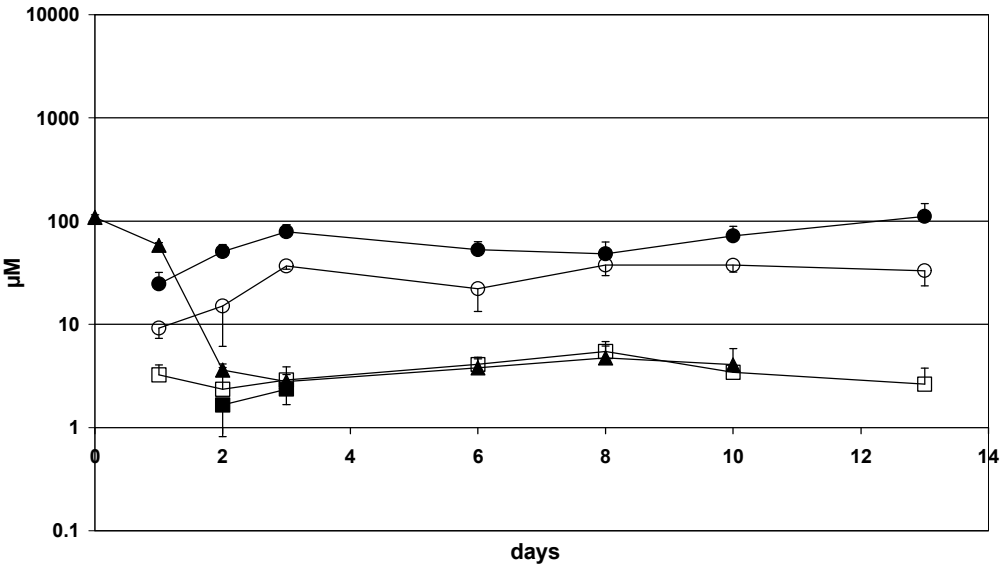

Fig. 2a.
The feeding of 0.1 mM of deoxy-podophyllotoxin. Time course of the deoxy-podophyllotoxin (▲)concentration and the concen-tration of the bioconversion products: â-peltatin (■), podophyllotoxin (□), 6-methoxy-podophyllotoxin (○) and its glucoside (●) after feeding of 0.5 mM deoxy-podophyllotoxin to *Linum flavum* cells. The concentrations are the sum of the concentration in the cell and the concentration in the medium. All concentrations presented in µM ± SEM (n = 4).

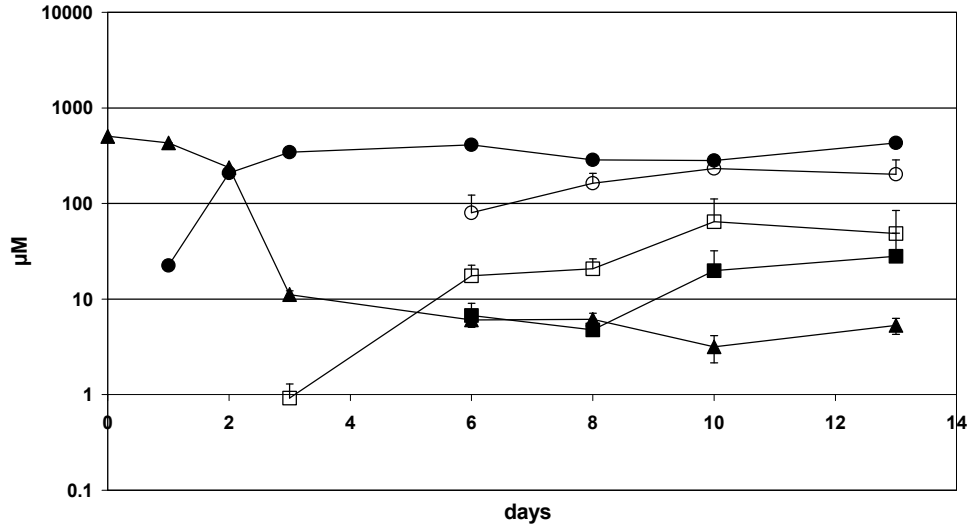

Fig. 2b.
The feeding of 0.5 mM of deoxy-podophyllotoxin. Time course of the deoxy-podophyllotoxin (▲) concentration and the concen-tration of the bioconversion products: â-peltatin (■), podophyllotoxin (□), 6-methoxy-podophyllotoxin (○) and its glucoside (●) after feeding of 0.5 mM deoxy-podophyllotoxin to *Linum flavum* cells. The concentrations are the sum of the concentration in the cell and the concentration in the medium. All concentrations presented in µM ± SEM (n = 4).

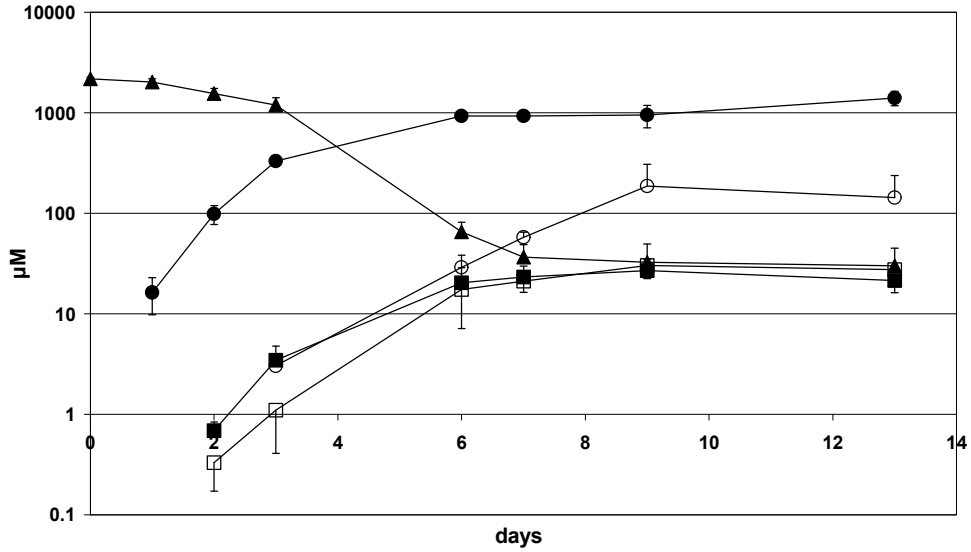

Fig. 2c.
The feeding of 2.0 mM of deoxy-podophyllotoxin. Time course of the deoxy-podophyllotoxin (▲)concentration and the concen-tration of the bioconversion products: â-peltatin (■), podophyllotoxin (□), 6-methoxy-podophyllotoxin (○) and its glucoside (●) after feeding of 0.5 mM deoxy-podophyllotoxin to *Linum flavum* cells. The concentrations are the sum of the concentration in the cell and the concentration in the medium. All concentrations presented in µM ± SEM (n = 4).

At all three concentrations of deoxypodophyllotoxin we could detect 6-methoxypodophyllotoxin glucoside already 24 h after feeding (day 1; see Figs. 2a, 2b and 2c). After an initial increase the 6-methoxypodophyllotoxin glucoside level remains almost constant from day 3 for all three concentrations of deoxypodophyllotoxin. In all experiments there is an increase at day 13, which is however not significant. After the addition of 0.5 and 2 mM deoxypodophyllotoxin, the levels of β-peltatin and 6-methoxypodophyllotoxin rise slower than the level of 6-methoxy-podophyllotoxin glucoside.

Surprisingly, podophyllotoxin is also formed after deoxypodophyllotoxin feeding. β-Peltatin-A-methylether (see Fig. 1) cannot be found in cell material. In comparison with the other concentrations the feeding of 0.1 mM deoxypodophyllotoxin differs in the time point that the products other than 6-methoxypodophyllotoxin glucoside are formed. β-peltatin, podophyllotoxin, and 6-methoxypodophyllotoxin already appear on day 1.

Furthermore, the feeding of 0.1 mM deoxypodophyllotoxin did not lead to a clear increase in the level of podophyllotoxin from day 1 to day 13. For 0.1 and 0.5 mM of deoxypodophyllotoxin there is a tendency for the product levels to exceed the level of added deoxypodophyllotoxin but it should be taken in account that this difference is not significant (see Fig. 3a and 3b).

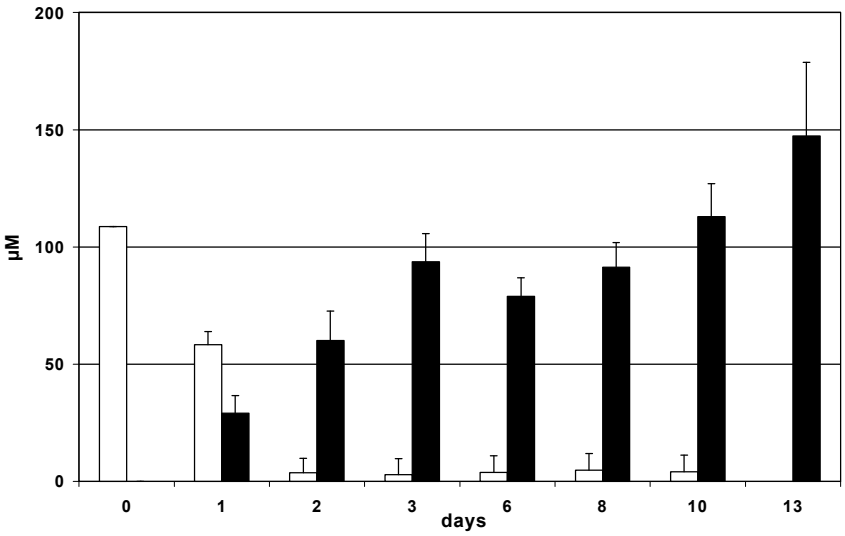

Fig. 3a.
The concentration of the precursor deoxypodophyllotoxin (□) and the total concentration of the biocon-version products â-peltatin, podo-phyllotoxin, 6-methoxypodophyllotoxin and its glucoside (■) during the growth cycle of *Linum flavum* cells after feeding of 0.1 mM deoxypodophyllotoxin. The concentrations are the sum of the concentration in the cell and the concentration in the medium. All concentrations presented in µM ± SEM (n = 4).

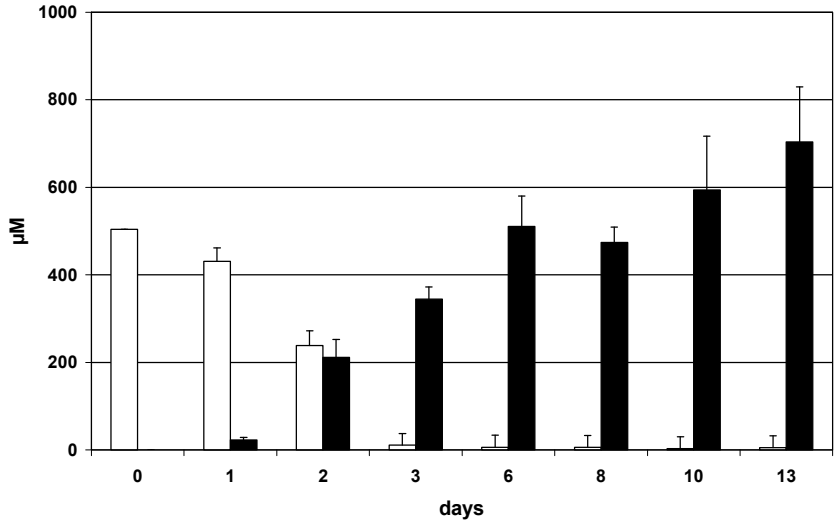

Fig. 3b.
The concentration of the precursor deoxypodophyllotoxin (□) and the total concentration of the bioconversion products â-peltatin, podo-phyllotoxin, 6-methoxypodophyllotoxin and its glucoside (■) during the growth cycle of *Linum flavum* cells after feeding of 0.5 mM deoxypodophyllotoxin. The concentrations are the sum of the concentration in the cell and the concentration in the medium. All concentrations presented in µM ± SEM (n = 4).

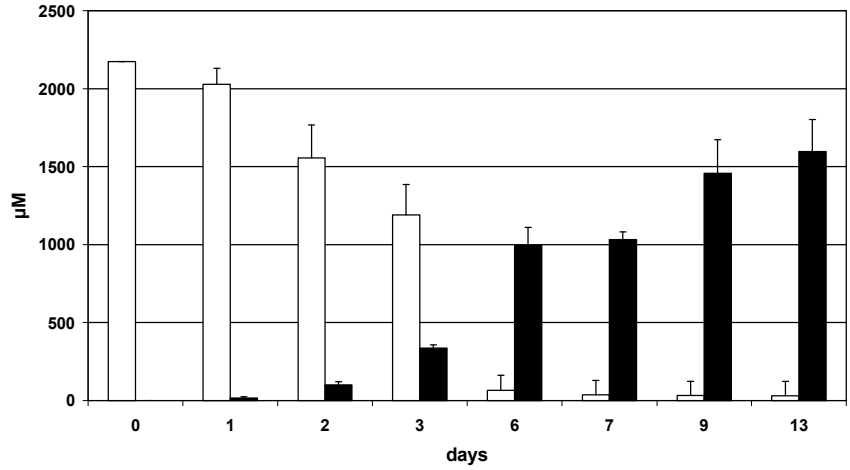

Fig. 3c.
The concentration of the precursor deoxypodophyllotoxin (□) and the total concentration of the bioconversion products â-peltatin, podo-phyllotoxin, 6-methoxypodophyllotoxin and its glucoside (■) during the growth cycle of *Linum flavum* cells after feeding of 2.0 mM deoxypodophyllotoxin. The concentrations are the sum of the concentration in the cell and the concentration in the medium. All concentrations presented in µM ± SEM (n = 4).

The total product level formed after feeding 2.0 mM deoxypodophyllotoxin was lower than the level of added deoxypodophyllotoxin (see Fig. 3c). These differences in product formation rates in relation to substrate concentration are also reported for other cell cultures. The cell cultures of *Rhodiola sachalinensis* are able to convert up to 3 mmol l^{-1} of tyrosol into salidroside. Higher concentration of tyrosol lead to a decrease of the product formation rate (Xu *et al.*, 1998). The cells are likely to be stressed by the large amount of externally added substrate.

The pattern of the intermediates in these different experiments lead to the question why the intermediates podophyllotoxin, β-peltatin and 6-methoxy-podophyllotoxin are formed *after* the initial increase of 6-methoxy-podophyllotoxin glucoside, and why are these compounds not further converted into 6-methoxypodophyllotoxin glucoside?

To determine whether all of the initially added deoxypodophyllotoxin was transformed, the sum of all product levels was calculated (see Fig. 3a, 3b and 3c). From these graphs it is clear that in the beginning of the growth cycle the levels of products did not correlate with the disappearance of deoxypodophyllotoxin. This phenomenon is dependent on the initial concentration of deoxypodophyllotoxin. After feeding 0.1 mM of deoxypodophyllotoxin, the levels of the substrate and the products are equalised on day 3; for 0.5 mM this is on day 6. For 2 mM the amount of product stays lower than the amount of substrate. Apparently, deoxypodophyllotoxin is stored away somewhere in the cell during the first days of the feeding experiment. After a few days it is re-introduced into the pathway. This re-entering in the pathway appears to happen earlier when the deoxypodophyllotoxin is added in the concentration of 0.1 mM.

These results lead us to the hypothesis that we were missing part of the substrate or product in our analysis. In a series of experiments we compared the standard extraction method with 80% methanol (= method 1), an alternative method using instead 20% methanol (= method 2), the newly reported method with 25 mM potassium phosphate buffer (= method 3). The deoxypodophyllotoxin concentration fed to the cell cultures was 2 mM; all samples were taken on day 6.

Table 1. The extraction yields by using different extraction methods. The results are from day 6 after 2 mM deoxypodophyllotoxin feeding on day 0 (n=3). The mean levels of all compounds are presented as µM ± SEM (standard error of the mean).

Method	DOP[a]	PT[b]	PEL[c]	6MPT[d]	6MPTG[e]	Total product[f]
1[w]	230 ± 96.7	0.49 ± 0.16	5.9 ± 0.44	6.4 ± 1.4	1039 ± 94.0	1049 ± 95.9
2[x]	210 ± 98.2	31.3 ± 7.9	167 ± 39.1	684 ± 95.7	0	883 ± 137
3[y]	201 ± 97.5	46.7 ± 10.2	226 ± 52.6	576 ± 41.0	0	822 ± 94.6
4[z]	201 ± 97.5	46.7 ± 10.2	226 ± 52.6	1104 ± 79.0	0	1377 ± 131

[a] DOP: deoxypodophyllotoxin; [b] PT: podophyllotoxin; [c] PEL: â-peltatin [d] 6MPT: 6-methoxypodophyllotoxin; [e] 6MPTG: 6-methoxypodophyllotoxin glucoside; [f] Total product: sum of products (PT + PEL + MPT + MPTG).
[w] 1: method 1, extraction cell material with 80% MeOH during 1h of sonication.
[x] 2: method 1, extraction of the cell material with 20% MeOH during 1h of sonication.
[y] 3: method 3, extraction of the cell material with a 26 mM potassiumphosphate buffer (pH 7) during 30 min rocking.
[z] 4: method 4; method 3 with the additional extraction of the cell debris with 80% MeOH during 1 h of sonication.

The amount of 6-methoxypodophyllotoxin glucoside isolated with the method 1 (standard method) was comparable with that of Figure 2c. The

alternative methods showed considerably more podophyllotoxin, β-peltatin, 6-methoxypodophyllotoxin (listed in Table 1). This is the result of cellular glucosidases that remain active and yield the lignan aglycones in method 2 and 3 as described by Canel and co-workers (Canel *et al.*, 2000). Still the sum of the total amount of product remained lower with method 2 and 3 than with method 1 (see Table 1). Therefore we combined method 3 and 1. The cell debris that was first extracted with method 3 was extracted again with 80% methanol as in method 1 (this combination = method 4). This additional extraction in method 4 recovered the total expected amount of 6-methoxypodophyllotoxin derived from 6-methoxypodophyllotoxin glucoside (see method 1) and did not yield more of the other lignans. It should be remarked that control *Linum flavum* cells did not show any lignans using the alternative extraction methods.

We suggest that the glycosidic products formed via bioconversion can somehow tightly be bound in the biological matrix and therefore cannot be extracted. The cells seem to store some of the substrate temporarily as non-extractable β-peltatin glycoside (see Fig. 1) and to a lesser extent podophyllotoxin glycoside (see Fig. 1). If β-peltatin and podophyllotoxin glycosides are re-introduced into the pathway the cells may treat these lignan glycosides differently than the externally added deoxypodophyllotoxin. This may explain the finding that β-peltatin and 6-methoxypodophyllotoxin are detectable after the increase of 6-methoxypodophyllotoxin glucoside.

Only when cellular glucosidases are allowed to be active the aglycones can be recovered and analysed. It is obvious that the alternative extraction methods gave a large increase of the yield of these compounds, and consequently a higher bioconversion percentage could be calculated. The bioconversion percentage for 6-methoxypodophyllotoxin lies around 55% ± 4% (SEM, n=5). The sum for all products formed (podophyllotoxin, β-peltatin and 6-methoxypodophyllotoxin) is ca. 69% ± 7 (SEM, n=5). This makes it a very efficient bioconversion system for such high a concentration of substrate in comparison to other undifferentiated cell suspension systems (Pras and Woerdenbag, 1999).

In the light of the recent study of Xia and co-workers (Xia *et al.*, 2000) it becomes clear that there are apparently two different routes leading to 6-methoxypodophyllotoxin. They showed that after feeding of matairesinol (see Fig. 1), this lignan is hydroxylated at the 7 position (see Fig. 1) and then is further converted into 6-methoxypodophyllotoxin. They were not able to show any other intermediate, but it seems very unlikely that deoxypodophyllotoxin or â-peltatin play a part in this route. Therefore it seems most likely that there are at least two routes leading to 6-methoxypodophyllotoxin: one via â-peltatin and the other via matairesinol. It is not clear if these two routes use separate sets of enzymes or whether the same enzymes are involved in both these routes.

An important finding of the feeding of deoxypodophyllotoxin to *Linum flavum* plant cell cultures is the detection of podophyllotoxin next to large amounts of 6-methoxypodophyllotoxin and its glucoside. This very limited formation of podophyllotoxin by *L. flavum* cell culture after the addition of

deoxypodophyllotoxin is difficult to accord with these two pathways. The feeding of matairesinol by Xia and co-workers did not yield any podophyllotoxin. It therefore seems more likely that podophyllotoxin is a kind of by-product in the bioconversion process of deoxypodophyllotoxin (to 6-methoxypodophyllotoxin). This conclusion is of major importance in the development of high production systems for podophyllotoxin with *L. flavum* cells or plants. To reach an increase the 7-hydroxylation of deoxypodophyllotoxin it seems most useful to block the 6-hydroxylation and to prevent the formation of â-peltatin-A-methylether, or to introduce a 7-hydroxylating enzyme from an other plant species with a high substrate specificity for deoxypodophyllotoxin.

ACKNOWLEDGMENT

This project was partly sponsored by the EU; BIO4-CT98-0461 LIGNOCANCER; Plant Cell Factories as a Source of Anti-Cancer Lignans. We would like to thank Prof. Manuel Medarde for providing us with different lignans as reference compounds, Dr. Rein Bos for his advice concerning analytical problems, and Prof. W.A. Alfermann for critically reviewing the manuscript.

IX. Bioconversion of 2,7'-cyclolignans by heterologously expressed human cytochrome P450 3A4.

Based on: Albert Koulman, Arend F. van Peer, Monique Ebbelaar, Rein Bos & Wim J. Quax (2003) Bioconversion of 2,7'-cyclolignans by heterologously expressed human cytochrome P450 3A4, *J. Biotech.*, submitted.

NEDERLANDSE SAMENVATTING

Het maken van podofyllotoxine met menselijke enzymen.

Voor elke omzetting die gebeurt in een plant (maar ook in mens of dier), is een enzym nodig dat deze stap uitvoert (katalyseert). Alles wat leeft heeft enzymen en de genen bepalen hoe deze enzymen er uitzien (zie fig.1 van hoofdstuk II).

Het is mogelijk om een gen uit een plant of een dier in, bijvoorbeeld, een bacterie te zetten. Deze bacterie kan dan het enzym gaan aanmaken dat hoort bij het gen. Zo wordt bijvoorbeeld menselijk insuline gemaakt met behulp van bacteriën.

Voor mijn onderzoek was het gen dat codeert voor het menselijk enzym cytochroom P450 (CYP) 3A4 interessant. Dit enzym komt voornamelijk voor in de lever. De lever is een belangrijk orgaan bij de afbraak van, voor de mens, giftige stoffen (en dus ook van ingenomen geneesmiddelen). In de lever komen meerdere enzymen voor die daarbij een belangrijke rol spelen, zoals enzym CYP 3A4. De lever speelt dus ook een cruciale rol in de afbraak van de antikankermiddelen die zijn afgeleid van podofyllotoxine en hoe dit gaat was al bekend. Wij waren benieuwd of het enzym CYP 3A4 verschillende lignanen op dezelfde manier zou omzetten als die antikanker-middelen.

Het bleek dat het enzym met de lignanen die wij aanboden (zoals podofyllotoxine en â-peltatine) niets deed. Het enzym bleek wel instaat het lignaan deoxypodofyllotoxine te hydroxyleren in podofyllotoxine (zie fig. a).

Het blijkt dus mogelijk om met een menselijk gen een stap uit te voeren van een plantenbiosynthese.

Dit was nog nooit eerder uitgevoerd. De omzetting verliep echter niet snel en had een lage opbrengst. Met de huidige DNA technieken is het mogelijk om enzymen te verbeteren (ook wel evolutie in een reageerbuis). Een verbeterde vorm van dit enzym, CYP 3A4 zou in deze gedachtegang, een goed startpunt kunnen zijn voor de biotechnologische productie van podofyllotoxine met fluitenkruid als grondstof.

Fig. a.
De hydroxylering van deoxypodofyllotoxine door het humane enzym CYP 3A4. Het product van deze reactie is podofyllotoxine.

ABSTRACT

Podophyllotoxin and other 2,7'-cyclolignans are clinically applied plant compounds. In the metabolism of these lignans and related pharmaceuticals, cytochrome P450 (CYP) enzymes play a central role. In plants these enzymes mediate lignan biosynthesis through hydroxylation reactions; in humans they initiate degradation of the podophyllotoxin derived anticancer medicine etoposide and teniposide, by 3-*O*-demethylation. We examined the conversion of 2,7'-cyclolignans by human CYP 3A4, heterologously expressed in *E. coli* DH5á. The enzyme did not perform 3-*O*-demethylation on any of the tested substrates. Instead, CYP 3A4 catalysed the hydroxylation of deoxypodophyllotoxin into podophyllotoxin. The enzyme shows a distinctly different reactivity to the 2,7'-cyclolignan than to their semi synthetic derivatives. Furthermore, the conversion of deoxypodophyllotoxin by this heterologous expression system shows potential for production of specific 2,7'-cyclolignans.

INTRODUCTION

The use of biotechnological procedures would be an interesting option to produce podophyllotoxin and *Anthriscus sylvestris* (L.) Hoffm. (Apiaceae; wild chervil) may play an important role in this context. Wild chervil is a common weed in Northwest Europe and its rhizomes contain considerable amounts of the lignans deoxypodophyllotoxin (see Fig. 1), yatein and anhydropodorhizol. Different publications show that deoxypodophyllotoxin can be converted into podophyllotoxin or 6-methoxypodophyllotoxin by plant cell cultures (Van Uden *et al.*, 1997). However the conversion into podophyllotoxin by cell suspension cultures of *Podophyllum hexandrum* is not very efficient (Van Uden *et al.*, 1995). Until now it is not clear which (kind of) enzyme is responsible for the hydroxylation of deoxypodophyllotoxin yielding podophyllotoxin. It is assumed that this step is carried-out by a cytochrome P450 monooxygenase, but thus far nobody has been able to perform the conversion of deoxypodophyllotoxin into podophyllotoxin without whole cells.

There is a second important relation between cytochrome P450 (CYP) enzymes and lignans. In the liver these enzymes detoxify drugs that are based on lignans. Particularly CYP 3A4 is of interest. This enzyme is responsible for the degradation of approximately 50% of all drugs administered to humans (Guengerich, 1999; Hasler *et al.*, 1999). This includes, the from podophyllotoxin derived medicines, etoposide and teniposide (see Fig. 1; Relling *et al.*, 1992; Relling *et al.*, 1994; Yamasaki *et al.*, 1997; Zhao *et al.*, 1998). Human CYP 3A4 initiates catabolism of etoposide and teniposide via a 3'-*O*-demethylation. Until now, there are no reports on the effect of this enzyme on podophyllotoxin and other plant 2,7'-cyclolignans. Based on the experiments with etoposide and derivatives it can be assumed that 2,7'-cyclolignans containing a 3'-methoxy group might react accordingly. On the other hand there are different reports on the inhibitory effects of other lignans on P450 enzymes. But except for the podophyllotoxin derivative etoposide, that mutually inhibits CYP 3A4 (together with quinine), those lignans are structurally different from the 2,7'-cyclolignans discussed in this report (Lewis and Davin, 1999; Parker *et al.*, 2000; Ueng *et al.*, 2000; Zhao *et al.*, 1998).

The aim of this study is to investigate the relation between the human CYP 3A4 and the 2,7'-cyclolignans.

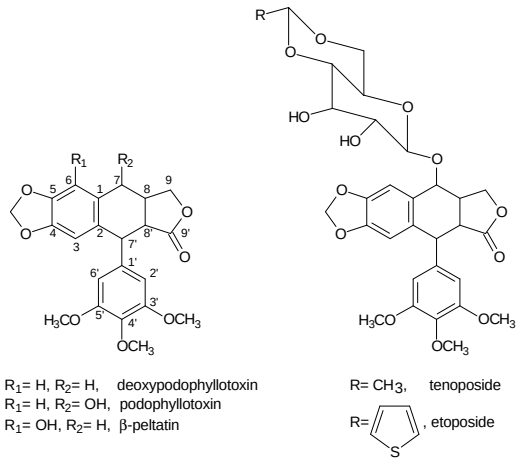

Fig. 1.

MATERIALS AND METHODS:

Chemicals

Methanol, acetonitrile, dichloromethane and glucose were derived from Merck (Darmstadt). Deoxypodophyllotoxin was isolated from *A. sylvestris*, as published in (see chapter III; Van Uden *et al.*, 1997). Other lignans were prepared through chemical synthesis and provided by M. Medarde (University of Salamanca, Spain). Dimethyl-β-cyclodextrin was derived from AveBe (Foxhol, The Netherlands). All other chemicals were purchased from Sigma.

Bacterial strains and plasmids

Expression of CYP 3A4 was performed in *E. coli* DH5á (Gibco BRL, Gaithersburg, MD) with a coexpression plasmid (Parikh *et al.*, 1997) containing the human CYP 3A4 and NADH-P450 reductase gene. Plasmids without the genes were used for control experiments and will be referred to as control plasmid. The group of F.P. Guengerich (Vanderbilt University School of Medicine, Nashville) kindly provided us with these plasmids.

Expression of CYP 3A4

A 10 ml LB DH5á culture (Amp 100 ìg ml^{-1}), containing the expression plasmid, was grown overnight (37º C, 250 rpm) for inoculation (1:100) of 30 ml TB (Amp 100 ìg ml^{-1}) that was additionally grown (37º C, 200 rpm) till an OD_{600} of 0.5. Expression was induced by adding IPTG (1.0 mM), thiamine (1.0 mM) and ä-aminolevulinic acid (0.5 mM) followed by another 24 h of growth (30º C, 200 rpm). Cells were harvested by centrifugation (10 min, 4000 rpm, 4º C) after the determination of the OD_{600}, and washed with potassium phosphate buffer (0.1 M, pH 7.4). Cells were diluted to an OD_{600} of 1.5 in Potassium Phosphate buffer (0.1 M, pH 7.4) containing 20% glycerol and stored in small portions at –70º C, until usage.

Assays

Assays were performed in potassium phosphate buffer (0.1 M, pH 7.4) containing glucose (12.5 mM). In the assays with podophyllotoxin, deoxypodophyllotoxin and â-peltatin (see Fig. 1) we added dimethyl β-cyclodextrin (M_w 1331) in a 1 to 3 molar ratio. Cells with expressed CYP 3A4 were thawed on ice and added to the buffer (200 ìl ml^{-1}) together with the substrate (0.2 mM), NADP (1 mM), glucose-6-phosphate (5 mM) and glucose-6-phosphate dehydrogenase (1 u ml^{-1}). Finally $MgCl_2$ (1 M, 30 ìl ml^{-1}) was added, followed by incubation for 2 h (37 ºC, 250 rpm). All substrates were dissolved in methanol (20 mM stocks). Conversion of the CYP 3A4 substrate testosterone (Parikh *et al.*, 1997; Wang *et al.*, 1997) was performed as a control in all experiments. The conversions with a

volume of 1.0 ml were performed in Pyrex glass tubes, 4 ml and 40 ml conversions were performed in 50 ml and 500 ml glass Erlenmeyer flasks respectively.

Sample preparation

The incubations of the substrate with the enzyme were stopped by addition of acetonitrile (0.5 ml ml^{-1}), vortexing and cooling on ice (10 min). Subsequently, samples were centrifuged (10 min, 4000 rpm, 4º C) and the supernatant was analysed on HPLC.

HPLC

HPLC analysis was performed with a LiChrosphere 100 RP-18 column (5 µm, 125 x 4 mm i.d.; Chrompack, Middelburg, the Netherlands) using MeCN : H_2O (40:60; v/v) as a mobile phase at a flow of 1.0 ml min^{-1} and UV detection (200 to 360 nm) with a Shimadzu Photodiode Array UV-VIS SPD-M6A detector (Shimadzu, 's Hertogenbosch, The Netherlands).

CYP CO-saturation difference analysis

In order to determine the amount of active CYP enzyme in the bacterial cells, potassium phosphate buffer (0.1 M, pH 7.4) was gently mixed with cell culture (1:1) in a 1 ml cuvet. A pinch of sodiumhydrosulfite was added and carefully mixed. After 1 min the baseline absorption (400-500 nm) was recorded. Then the mixture was gassed through with CO for 30 s, with a gas flow of 1-2 bubbles s^{-1}, and again the absorption spectrum of 400-500 nm was recorded.

RESULTS

Expression of CYP 3A4

After expression of CYP 3A4, CO-saturation difference spectra of whole cell cultures were measured to determine CYP 3A4 activity. Expressed but inactive (misfolded) proteins can be distinguished from active enzymes, by their absorption of 420 nm instead of 450 nm.

Cultures containing expressed CYP 3A4 all showed absorbance at 450 nm, whereas no absorption at 450 nm could be detected in cultures containing the control plasmid. However, separately grown expression cultures varied in their expression levels of active CYP.

Conversion experiments with CYP 3A4

Cultures containing the expressed CYP 3A4 / NADH P450-reductase system clearly converted the CYP 3A4 substrate testosterone into 6-â-hydroxytestosterone. The compounds used in the assays (podophyllotoxin, deoxypodophyllotoxin and â-peltatin, see Fig. 1) could all be detected by HPLC-analysis. Cells expressing CYP 3A4 gave only an additional product peak in the HPLC-analysis, when deoxypodophyllotoxin was used as a substrate. However, the conversion rate is lower compared with that of testosterone. Interestingly, CYP 3A4 did not convert podophyllotoxin and â-peltatin, despite the high structural similarity between these compounds and deoxypodophyllotoxin. The product of the deoxypodophyllotoxin conversion was identified as podophyllotoxin, on basis of the retention time and the UV absorbance spectra (200-380 nm), which both matched.

We tried to improve the conversion by varying different parameters. Expression cultures supplied with deoxypodophyllotoxin concentrations of 0.4 mM, 0.2 mM, 0.1 mM and 0.01 mM converted 0.4 mM and 0.2 mM into equal amounts. There was also no difference between the amounts of product formed by conversion of 0.1 mM and 0.01 mM concentrations. However, the experiments with 0.4 mM and 0.2 mM yielded approximately 50% higher amount product than the experiments with 0.1 mM and 0.01 mM deoxypodophyllotoxin.

Longer incubation times of 4 hours and 24 hours had no extra effect on the conversion rates of deoxypodophyllotoxin, compared to a 2 hours incubation time. Additional conversion experiments with varying reaction volumes were also performed. The studied enzyme converted deoxypodophyllotoxin in equal amounts in 1 ml and 4 ml reaction volumes. However, no conversion was obtained when the experiment was performed in a larger volume of 40 ml.

DISCUSSION

The only known interactions between human CYP 3A4 and lignans are inhibitory effects (Lewis and Davin, 1999; Ueng *et al.*, 2000), and 3'-*O*-demethylation of etoposide and teniposide (Relling *et al.*, 1992; Relling *et al.*, 1994). The mechanism of enzyme inhibition is not clear yet and competitive, non-competitive as well as mutual inhibition of CYPs by lignans has been found. *O*-demethylation is a reaction that eliminates a methyl group from the 3'-methoxy group of etoposide and teniposide. This results in a 3'-hydroxy group. Deoxypodophyllotoxin, podophyllotoxin and â-peltatin all three possess methoxy groups at the 3' and 5' position. Therefore we expected a possible *O*-demethylation of these compounds too. The only reaction observed was the hydroxylation of deoxypodophyllotoxin, resulting in podophyllotoxin. We did not observe *O*-demethylation of any of the tested lignans. There could be two reasons for this. Firstly, the tested lignans have a 4'-methoxy group instead of a 4'-hydroxy group, as is present in etoposide and teniposide. This could cause steric or electrochemical intervention of the *O*-demethylation reaction. Secondly, etoposide and teniposide contain a relatively large glycosidic moiety on the 7 position. This moiety effects the chemical property of the compounds to a large extent (for instance: mass, polarity, etc.). The different conversion (hydroxylation instead of *O*-demethylation) is therefore more likely to be caused by alternative binding to the enzyme, such as a different orientation of the substrate in the active site.

Besides, hydroxylation at the 7 position of etoposide and teniposide is difficult since it is occupied by a glycosidic moiety whereas this position is free in deoxypodophyllotoxin.

As discussed above, deoxypodophyllotoxin is converted into podophyllotoxin. The enzyme can not convert the product further, since the enzyme does not affect podophyllotoxin. Furthermore, the experiments suggest an important influence of the presence of hydroxy groups at the 6- and 7-carbon. This could be due to steric or electrochemical intervention of the hydroxy groups with the CYP 3A4-catalysed reaction. In podophyllotoxin, the 7-position is already occupied and in â-peltatin, the hydroxy group is located on the 6-position, very close to the 7-position.

Finally, the conversion of deoxypodophyllotoxin into podophyllotoxin shows that a CYP type of enzyme can perform a hydroxylation at the 7-carbon. This supports the expectation that a plant CYP enzyme is involved with the conversion of deoxypodophyllotoxin into podophyllotoxin during biosynthesis (Henges, 1999).

With the conversion of deoxypodophyllotoxin we demonstrated that such a reaction opens a new alternative for the large-scale production of specific lignans. The study of other production systems has not yet yielded any promising breakthroughs. The chemical synthesis of podophyllotoxin is possible (Hadimani *et al.*, 1996; Medarde *et al.*, 1996), but is hampered by the complicated stereochemical ring closure that has to take place to attain

this compound. Synthetic production therefore only yields restricted quantities at high costs. Bioconversion of lignans by fungi has been accomplished, but either conversion rates were very low, or the process took several weeks (Kamal and Damayanthi, 1997; Kondo *et al.*, 1989; Kondo *et al.*, 1990; Van Uden *et al.*, 1990c) Besides, the mechanisms by which those conversions are performed are poorly understood. Another alternatives for the large-scale production of lignans are plant cell suspension cultures. Several important lignans already have been obtained by this method and some even in relatively high amounts (Berlin *et al.*, 1986; Van Uden *et al.*, 1995; Van Uden *et al.*, 1997). However, considerable improvement of manufacturing both qualitatively and quantitatively, is necessary to achieve commercially favourable production systems.

The usage of a heterologous expression system for specific lignan conformational modifications, as shown in our experiments, can be a strong tool for the manufacturing of lignans. Firstly, it shows that non-plant enzymes can perform specific biosynthetic steps in plant metabolism. This largely increases the arsenal for possible bioconversion tools (enzymes), which can be used for specific conversions. Secondly, since it concerns an inducible system, the disturbing toxicity that several products exhibit towards fungi or bacteria can be by-passed. This enables production of lignans with microorganisms. Application of other inducible expression systems containing CYP 3A4 is possible as well. Of course the conversion of deoxypodophyllotoxin by CYP 3A4 has to be improved for large-scale production of podophyllotoxin. This may be achieved through protein engineering in combination with manipulation of the concerned organism for increased expression of (active) CYP 3A4. Thirdly, deoxypodophyllotoxin is an easy accessible source, due to the fact that it is accumulated in roots of the common weed *A. sylvestris* (Van Uden *et al.*, 1997). Therefore, production of podophyllotoxin from deoxypodophyllotoxin by heterologous expression of (improved) CYP 3A4 might be a good alternative for the near future.

CONCLUSIONS

Deoxypodophyllotoxin is catabolised by a different CYP 3A4 mediated process than etoposide and teniposide. The absence or presence of hydroxy groups at the 6- and 7- carbon atoms of deoxypodophyllotoxin, podophyllotoxin and â-peltatin respectively, influence CYP 3A4 conversion to a great extent.

Heterologous expression of CYP 3A4 in bacteria can be used for alternative production of podophyllotoxin and might be applicable for large-scale production of this compound.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to Prof. F.P. Guengerich for providing us with the plasmids containing the human CYP 3A4 and NADH-P450 reductase gene and Prof. Manuel Medarde for providing us with different lignans as reference compounds. This project is partly sponsored by the EU; BIO4-CT98-0451 LIGNOCANCER; Plant Cell Factories as a Source of Anti-Cancer Lignans.

X. Evolution of lignan biosynthesis in the genus *Bursera*

Based on: Albert Koulman, Wim J. Quax & Judith X. Becerra (2003) Evolution of lignan biosynthesis in the genus *Bursera*. In preparation.

NEDERLANDSE SAMENVATTING

De evolutie van de lignaanbiosynthese in het plantengeslacht *Bursera*

Hoe is de biosynthese van podofyllotoxine ontstaan tijdens de evolutie? Is deze recent ontstaan of al lang geleden. Op deze vragen hebben we een antwoord proberen te vinden. (Met recent bedoelen wij de tijdsperiode dat een soort is ontstaan en met lang geleden de periode dat het hele geslacht of de hele familie is ontstaan.)

Het onderzoek werd mogelijk door samenwerking met een onderzoeksgroep in Arizona (USA) die zich heeft gespecialiseerd in de evolutie van *Bursera* planten. Het geslacht *Bursera* bevat ongeveer 70 soorten. Het zijn struikachtige planten waarvan de meeste soorten in Mexico voorkomen, sommige soorten komen tot in Peru en andere soorten tot in Noord Amerika voor. Met behulp van verschillende DNA technieken is een stamboom gemaakt van dit plantengeslacht, waaruit de mate van verwantschap zou moeten blijken tussen de soorten. Het is inmiddels bekend dat binnen het geslacht *Bursera* twee hoofdsecties zijn te onderscheiden die ieder voor zich weer bestaan uit zo vier subgroepen (zie Fig. 2).

Aan de hand van de lignanen die aanwezig zijn binnen de planten van dit geslacht hebben de planten in groepen verdeeld. Deze groepen hebben we vergeleken met de DNA stamboom.

Van een aantal soorten van dit geslacht is bekend dat deze lignanen produceren. Uit dat onderzoek kwam naar voren dat sommige stappen in de biosynthese in een sectie voorkwamen, en de stamboom volgden (zie Fig 3), terwijl andere stappen in de biosynthese veel minder overeenkomst vertoonden met de stamboom (zie Fig 4).

Deze resultaten maakten het moeilijk om duidelijke conclusies te kunnen trekken uit dit onderzoek. Echter, wat wel naar voren kwam was, dat vroege stappen uit de biosyntheseroutes niet de stamboom volgden, terwijl de stappen die heel specifiek zijn voor de lignaanbiosynthese wel de stamboom volgden. Uit deze gegevens valt te concluderen dat tijdens het ontstaan van de soorten binnen dit geslacht, ook de lignaanbiosynthese is ontstaan. Als deze conclusie geldt voor dit plantengeslacht mag men aannemen dat het ook voor andere plantensoorten opgaat.

ABSTRACT

The occurrence of the 2,7'-cyclolignan biosynthesis in unrelated plant species seems to be a clear example of convergent evolution. Such lignans have also been reported from different species of the genus *Bursera*. Taking advantage of the fact that a molecular phylogeny of the *Bursera* was recently reconstructed, we analysed the lignan profile of thirteen Bursera species belonging to different lineage's within the genus. The comparison of the species' lignan profiles with the species' phylogenetic relationships gave a first insight in the evolution of the 2,7'-cyclolignan biosynthesis. Half of the analysed species do not produce any detectable amount of this type of lignans. This could be explained by the fact that the genes for the lignan biosynthesis are either absent in these species, or present but not all functionally expressed. From the species that produced lignans we were able to conclude that the critical steps in the 2,7'-cyclolignan biosynthesis are confined to one section of the genus. Therefore, it can be assumed that this part of the lignan biosynthesis must have arisen once during the evolution of these species. Other synthetic steps can be explained by the recruitment of enzymes facilitating a convergent and rapid evolution of parts of the lignan biosynthesis.

INTRODUCTION

Plants produce a variety of secondary metabolites. The chemical structure of many of these compounds has been elucidated over the years, but their total number remains unknown. The exact function of these compounds is in most cases poorly understood. The same is true for their evolution and their biosynthesis. It is generally assumed that a certain secondary metabolic pathway arises during the evolution of a plant species through the evolution and recruitment of enzymes. Such systems often make metabolic pathways specific for a certain plant genus or family (Berenbaum, 1995). This has led to the use of chemotaxonomy as an aid in plant classification.

Some biosynthetic routes however do not seem to be restricted to a certain family or genus. These routes occur in different unrelated plant species, like the biosynthesis of the monoterpene carvon, which takes place in the families of the Apiaceae and the Labiaceae (Cseke *et al.*, 1998). This may have occurred through convergent or parallel evolution. The many cases of convergent evolution has been called one of the most remarkable phenomena about the evolution of the secondary metabolism in plants (Pichersky and Gang, 2000).

An outstanding example of convergent evolution is the last part of the biosynthesis of 2,7'-cyclolignan. For instance, the cytotoxic 2,7'-cyclolignan podophyllotoxin (see for structure Fig. 1) can be found in at least 13 different plant families divided over 5 different sub-classes: Pinales (Hartwell *et al.*, 1953a), Magnoliidae (Broomhead *et al.*, 1991), Dilleniidae (Shaari and Waterman, 1994), Rosidae (see chapter III) and Asteridae (Kuhnt *et al.*, 1994). In some of these families there are genera where the majority of the species produce 2,7'-cyclolignans (for instance the *Juniperis*). In other families synthesis seems limited to only one or two species, such as *Anthriscus sylvestris* in the Apiaceae, although it is not clear in which depth all species have been investigated. Therefore, the evolution of the last part of the biosynthesis of 2,7'-cyclolignan is an interesting subject for the study of the convergent evolution of biosynthetic pathways.

The biosynthesis of these lignans is only partially understood, however, synthesis of all lignans starts at the stereo-specific coupling of two monolignol units, usually coniferyl alcohol (Rahman *et al.*, 1990a). A recent study showed that other monolignols could also be coupled and used in the lignan biosynthesis but there is only limited understanding of this process yet (Seidel *et al.*, 2002). For several species such as *Forsythia*, it has been shown that this coupling takes place under direction of the dirigent protein (Davin *et al.*, 1997). Homologous genes of the dirigent protein have been found in many plant species and it is likely to be present in almost all plant species. Although it is not clear whether the function of the dirigent protein is the same in every plant species, its widespread

distribution underscores that the initial steps for lignan formation have spread via divergent evolution.

The product of the coupling of coniferyl alcohol is pinoresinol. This compound is reduced twice leading to secoisolariciresinol. The lactone ring is then closed to give matairesinol, which may be the starting point for all lignans with a lactone ring. This first part of the lignan biosynthesis seems to be present in many plants and from matairesinol many different lignans are formed in many different species, a clear example of divergent evolution (Lewis and Davin, 1999).

Broomhead and co-workers showed that matairesinol is the precursor of podophyllotoxin in *Podophyllum hexandrum* (order of the Magnoliidae; Broomhead *et al.*, 1991). Xia and co-workers showed that for the biosynthesis of 6-methoxypodophyllotoxin in *Linum flavum* (order of the Rosidae) also matairesinol serves as a precursor (Xia *et al.*, 2000).

In this study, we have traced the lignan profile of a number of *Bursera* species onto their phylogeny to get insight into the evolution and the sequence of the biosynthetic steps of the formation of 2,7'-cyclolignans.

MATERIALS AND METHODS

Species involved and sample collection

Bursera comprises about 100 species distributed from the South of the United States to Peru. It reaches its maximum diversity in the tropical dry forests of Mexico, where about 85 species occur (Rzedowski and Kruse, 1979). The genus is relatively well known taxonomically, and it has been divided into two sections that are distinguished, among other traits, by the characteristics of the bark. Section *Bursera* includes species with the peeling and colourful bark, while species in section *Bullockia* have a rough, non-peeling bark (McVaugh and Rzedowski 1965; (Toledo, 1982). A phylogenetic tree of 69 species and 9 outgroups was recently reconstructed using the sequences of the Internal Transcribed Spacer region (ITS), the 5S Non-Transcribed region (5S-NTS), and the External Transcribed Spacer region (ETS) of nuclear ribosomal DNA (Becerra and Venable, 1999); (Becerra, 2003). This phylogenetic analysis confirmed the division of the species into two main clades corresponding to the two sections of the genus. Section *Bursera* was further divided into four main subclades, while section *Bullockia* included two main subclades. Our study includes 13 *Bursera* species that were selected so as to have one or two species belonging to each of the six main subclades found in the molecular phylogeny. Samples of these species were collected from field sites in Mexico and quick dried with silica gel.

Chemicals

Podophyllotoxin was obtained from Sigma (St. Louis). Yatein, matairesinol, podorhizol, arctigenin, anhydropodorhizol, β-peltatin, and β-peltatin-a-methylether were prepared synthetically (Medarde *et al.*, 1995). Deoxypodophyllotoxin, yatein, and anhydropodorhizol were obtained as optically pure compounds through isolation from *A. sylvestris* roots using the previously described method for deoxypodophyllotoxin (Van Uden *et al.*, 1997).

Analysis

The lignan profile of 14 *Bursera* species (Table 1) was determined applying a recently developed GC-MS method (see chapter III) using 100 mg dried leave material. The mass spectra of all peaks were compared with the mass spectra of known lignans. Lignans deliver an unambiguous mass spectrum at 70 eV electric ionisation (Arimoto *et al.*, 1996). The aqueous phase was treated with â-glucosidase to check the presence of any glycosylated lignans, according to the previously reported method (Van Uden *et al.*, 1997).

RESULTS

Of the thirteen species analysed, eight did not contain any detectable amount of lignans (Table 1). All the lignans found in the other species have a dibenzylbutolactone skeleton. In *Bursera fagaroides* var. *elognata* and *Bursera acuminata* we detected 3 different 2,7'-cyclolignans. Morelensin was originally detected in *Bursera morelensis* (Jolad *et al.*, 1977), but we detected this compound also in *Bursera acuminata*. Morelensin was easily identified on basis of its mass spectrum. It has a total mass of 368 and this is also the base peak, smaller fragments have a *m/z* of 185 and 235 (Table 2). In *B. acuminata* and *B. fagaroides* var. *elongata* we found deoxypodophyllotoxin. This lignan has a total mass of 398 and characteristic fragments 173, 181 and 185 *m/z*. In the same species we found also β-peltatin-a-methylether. This compound has a total mass of 428 and its identity as well as that of deoxypodophyllotoxin was confirmed using HPLC-DAD in comparison with reference compounds. Deoxypodophyllotoxin and β-peltatin-a-methylether were originally reported from *Bursera microphylla*.

Table 1. The lignans in *Bursera* species.

Species	BUR[a]	MOR[a]	YAT[a]	DOP[a]	PAM[a]	POLY[a]	SAV[a]	HIN[a]	CUB[a]	IBU[a]	DME[a]	GUA[a]
	Group 1					Group 2	Group 3			Group 4		
B. excelsa										+	+	+
B. submoniliformis							+					
B. cuneata							+	+	+			
B. mcvaughiana												
B. penicillata							+					
B. citronella							+	+				
B. schlechtendalii[b]	+		+									
B. fagaroides var *elongata*	+		+	+	+							
B. chemopodicta												
B. discolor												
B. microphylla[c]				+								
B. suntui												
B. morelensis[d]		+		+								
B. kerberii												
B. lancifolia												
B. acuminata	+	+		+	+							
B. simaruba[e]						+						

[a]: BUR: bursehernin; MOR: morelensin; YAT: yatein; DOP: deoxypodophyllotoxin; PAM: β-peltatin-a-methylether; POLY: polygamain; SAV: savinin; HIN: hinokinin; CUC: cubebinin; IBU: iso-bursehernin; DME: 3,4-dimethoxy-3',4'-methylenedioxylignano-9,9'-epoxylignan-9'-ol ; GUA: Guayadequiol (the structures are depicted in Fig. 1a and 1b).
[b]: literature data (McDoniel and Cole, 1972).
[c]: literature data (Bianchi *et al.*, 1968).
[d]: literature data (Jolad *et al.*, 1977).
[e]: literature data (Peraza-Sanchez and Pena-Rodriguez, 1992).

In other *Bursera* species we found lignans that are new for this genus but are already known from other plant species. Savinin was isolated for the first time from *Juniperus*, but has also been reported from *Zanthoxylum tessmannii* (Rutaceae). Its mass spectrum has a base peak at 135 *m/z* and a total mass of 352 (Table 2). Hinokinin can be found in other plant genera

such as *Piper* (Piperaceae) and *Anthriscus* (Apiaceae) and it has a base peak at 135 *m/z* and a total mass of 354. Cubebinin is a lignan originally found in *Piper cubeba*. It is the reduced form of honikinin, having a free hydroxyl group at the lactone ring, which can be deduced from the 221 *m/z* peak.

Table 2. The mass spectra of lignans found in this study and the genus wherein these compounds were originally reported (some of the names imply a certain stereo-chemistry that cannot be unambiguously determined with the used methods, the compounds found is this study may therefore be stereoisomers of the reported structures).

Compound	Mass spectrum *m/z* (%)	Genus	Reference:
Savinin	77(50) 131(5) 135(100) 159(6) 189(80) 217(160 352(10)	*Zanthoxylum*	(Foyere Ayafor *et al.*, 1984)
Hinokinin	135(100) 192(14) 218(8) 219(10) 354(15)	*Virola*	(Lopes *et al.*, 1983)
Cubebinin	135(100) 161(10) 221(4) 356(12)	*Piper*	(Badheka *et al.*, 1987)
Iso-bursehernin	135(100) 151(75) 192(15) 218(10) 219(3) 370(25)	*Virola*	(Lopes *et al.*, 1983)
DME	135(80) 151(100) 177(24) 203(8) 372(53)	*Aristolochia*	(Rücker and Langmann, 1978)
Guayadequiol	107(26) 135(51) 137(9) 151(100) 386(24)	*Bupleurum*	(González *et al.*, 1990)
Hinokinin	135(100) 192(14) 218(8) 219(10) 354(15)	*Virola*	(Lopes *et al.*, 1983)
Morelensin	185(12) 253(13) 368(100)	*Bursera*	(Jolad *et al.*, 1977)
Bursehernin	135(37) 151(100) 208(3) 219(5) 235(4) 370(100)	*Bursera*	(McDoniel and Cole, 1972)
Yatein	77(32) 105(13) 181(100) 400(68)	*Anthriscus*	(see chapter III)
Deoxypodophyllotoxin	173(19) 181(21) 185(16) 398(100)	*Anthriscus*	(see chapter III)
β-peltatin-a-methylether	168(9) 215(8) 260(13) 369(16) 383(22) 379(38) 428(100)	*Linum*	(Arimoto *et al.*, 1996)

In *Bursera excelsa* we found 3 different lignans that were not known from the *Bursera* genus, 3-(3,4-dimethoxybenzyl)-2-(3,4-methylenedioxy-benzyel)butyrolactone (Lopes *et al.*, 1983) and 3,4-dimethoxy-3,-4desmethylenedioxycubebin (Rücker and Langmann, 1978). According to the new IUPAC rules these compounds should now be named 3,4-dimethoxy-3',4'-methylenedioxylignano-9,9'-lactone and 3,4-dimethoxy-3',4'-methylenedioxylignano-9,9'-epoxylignan-9'-ol. We call the first compound iso-bursehernin, since the only difference between this compound and bursehernin is the placement of the aromatic groups in relation to the lactone ring. This difference can be determined from the mass spectra as discussed in the original article describing this compound (Lopes *et al.*, 1983). We call the second compound DME (3,4-dimethoxy-3',4'-methylenedioxylignano-9,9'-epoxylignan-9'-ol). It resembles cubinin, with one dioxymethelene bridge replaced by two methoxy groups. This compound was originally reported from *Aristolochia triangularis* (Rücker and Langmann, 1978). The third compound is guayadequiol, which was originally reported from *Bupleurum salicifolium* (González *et al.*, 1990).

DISCUSSION

Biosynthesis of lignans in Bursera

The lignans we found in the analysed *Bursera* species can be appended with literature data. There are reports on 5 different lignans from *Bursera* species. These species yielded the same lignans as we found, with two exceptions. Peraza-Sanchez and Pena-Rodriguez isolated polygamain from *Bursera simuruba* (Peraza-Sanchez and Pena-Rodriguez, 1992), which we did not find. There is also a report on the presence of 5'-demethoxy-â-peltatin in *Bursera fagaroides* (Bianchi *et al.*, 1969). However, it is known from the recently established phylogeny that *B. fagaroides* is split into three varieties, which are not all closely related (Becerra and Venable, 1999). The variety used by Bianchi and co-workers has not been determined.

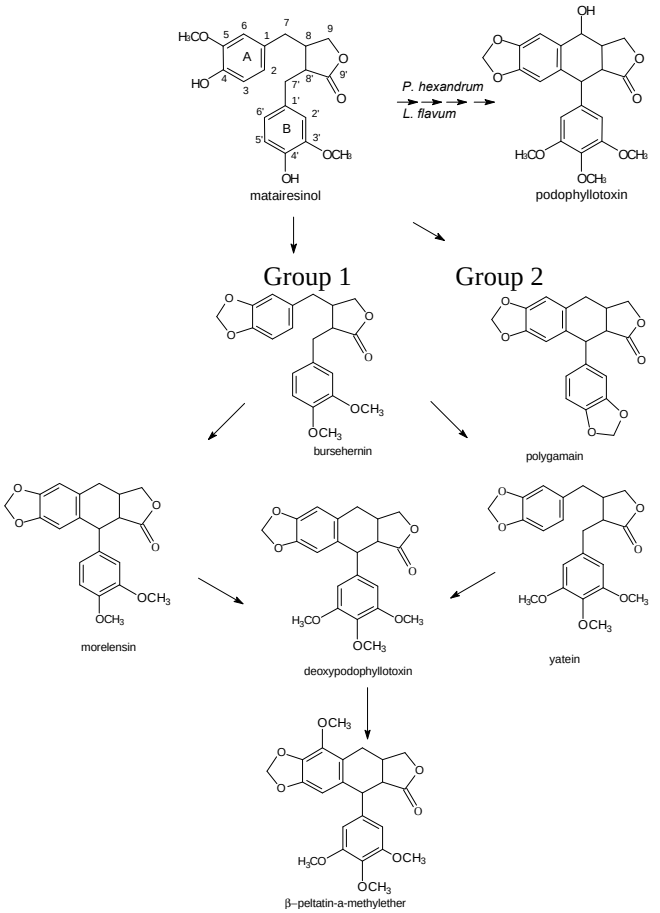

Fig. 1a.
The lignans found in Bursera species. With arrows the possible biosynthetic steps are depicted.

Although the biosynthesis of lignans is still not fully understood, the sequence of steps can partly be deduced from the products we found. Lignans found in these *Bursera* species can be divided in four groups. Group 1 (Fig. 1a), the most complex, embraces the 2,7'-cyclolignans with the dioxymethylene bridge on the A-ring, and the di- or trimethoxyphenyl functionality on the B-ring, like deoxypodophyllotoxin. The closely related group 2 (see Fig. 1a) is only formed by polygaimin, also a 2,7'-cyclolignan but with two methylenedioxy bridges. Group 3 (Fig. 1b) is related to polygaimin having two methylenedioxy bridges, but without the 2,7'-bridge. A member of this group is hinokinin. Group 4 (see Fig. 1b) comprises the dimethoxy and methylenedioxy lignans without the 2,7'-bridge such as guayadequiol. These have in contrast to group 1 the methylenedioxy bridge on the B-ring.

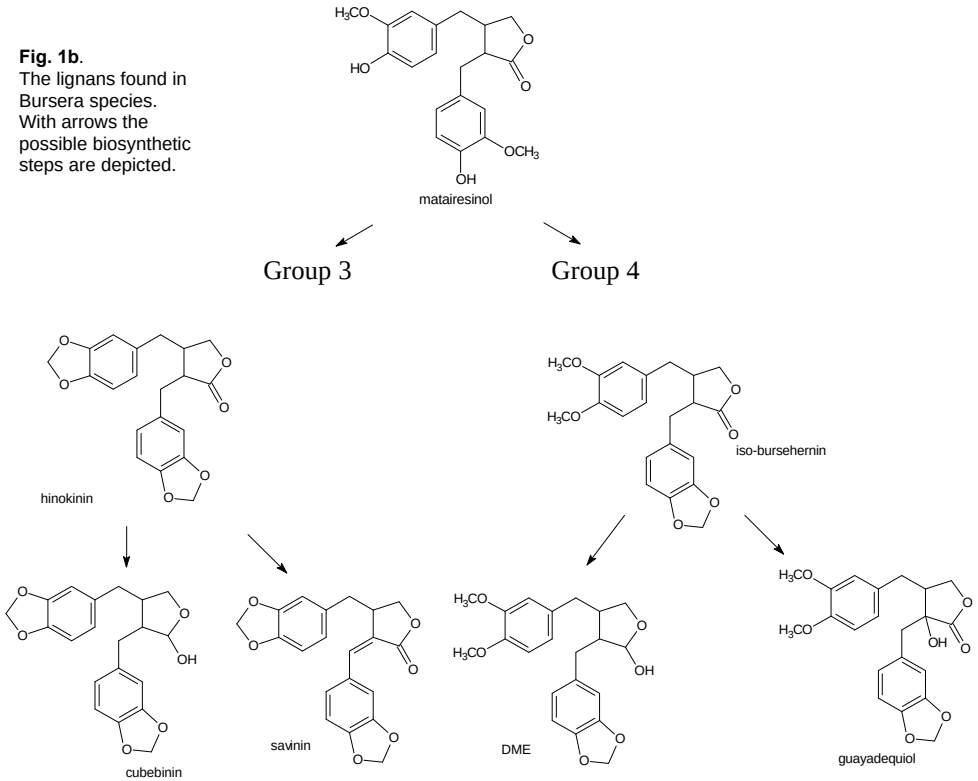

Fig. 1b. The lignans found in Bursera species. With arrows the possible biosynthetic steps are depicted.

Based on the current knowledge about lignan biosynthesis these compounds can all be derived from matairesinol (Fig. 1a and 1b). The initial steps in the biosynthesis after matairesinol are either the formation of methylenedioxy bridge or the methylation of the *para*-hydroxy group or both. With those two steps, all the starting compounds necessary to get the four lignan-groups can be found. For example, bursehernin in group 1, hinokinin in group 2, and 3 and iso-bursehernin in group 4. The following steps differ much more.

In group 1 and 2 the next step can be a ring closure at the 2,7' position, which will yield polygamain in group 2 and morelensin in group 1. In this group it is also possible that yatein is formed via hydroxylation at the 5'-position followed by methylation. These two products can undergo these reactions leading now deoxypodophyllotoxin. The hydroxylated compound, now at the 6-position, and followed by methylation will lead to β-peltatin-a-methylether.

In group 3 and 4 a reduction can take place at the lactone group giving a free hydroxyl. This reaction will produce cubebinin in group 3 and its analogue in group 4. In group 3 an oxidative step will lead to savinin. An extra hydroxylation at the 8'-position will yield guayadequiol in group 4.

Phylogeny and Biosynthesis

The different groups of lignans can be related to the phylogeny of *Bursera*. Groups 1 and 2 are found in *Bursera* section *Bursera* while groups 3 and 4 are found in the *Bullockia* lineage. Group 2 and 4 are specific for one species while group 1 and 3 are found in more than one species. When the results of this study, combined with literature, are parsimoniously traced onto *Bursera*'s phylogeny, it is possible to make some inferences concerning the evolution of lignan biosynthesis in the genus.

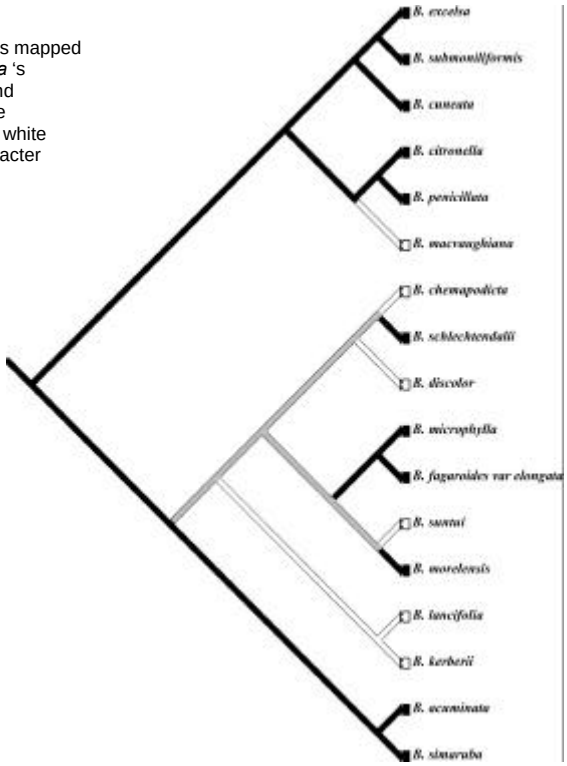

Fig. 2.
The presence of the lignan biosynthesis was mapped onto *Bursera*'s phylogeny. *B. macvaughiana* 's location in the phylogeny follows Cuevas and Rzedowski (1999). Black lines represent the presence of either one or more lignans and white lines indicate their absence. Equivocal character reconstruction is indicated in gray.

Although lignans are found throughout the *Bursera* genus some species do not seem to accumulate any 8,8'-lactonring lignans. The absence of these compounds can be explained by different hypothesis. A first hypothesis would be a total absence of genes and enzymes responsible for the lignan biosynthesis. A second hypothesis would be that the genes for the lignan biosynthesis are present but not expressed by these species under the tested conditions. The third hypothesis is that some genes for the lignan biosynthesis are mutated and therefore dysfunctional leading to a blockage of the lignan biosynthesis. The first hypothesis is unlikely. This would imply that the whole lignan biosynthesis should has evolved independently many times in the *Bursera* genus, otherwise it would be difficult to explain the presence of lignans in *B. schlechtendalii* and not in *Bursera chemapodicta* and *Bursera discolor* (see Fig. 2). The second and third hypothesis, or a combination of both seems most likely. It is also possible that these species only produce lignans under certain condition or in specific organs.

The biosynthesis of these lignans requires a number of different reactions that are based on very different chemical reaction mechanisms. A number of these reactions change the oxidative state of the molecule, such as the reduction of the lactone ring. This reduction only occurs in two closely related species, but with different lignans as a substrate (in *B. excelsa* iso-burseherinin is reduced into DME and in *B. cuneata* hinokinin into cubebinin). It is also possible that an oxidation reaction occurs. Kamil and Dewick proposed a lignan specific oxidation reaction, which can lead to three different products (Kamil and Dewick, 1986b). In their study on lignan biosynthesis in *Podophyllum hexandrum* they proposed that an oxidation reaction of yatein could lead to a quinone like intermediate. This intermediate would than either cyclise (leading to 2,7'-ring closure), with a loss of a proton (leading to a double bond) or an addition of water (leading to an extra OH). Although the presence of such a quinone like intermediate has never been proven it could explain the different lignans in this genus. In the section *Bursera* this reaction could be responsible for the ring closure leading from burseherin to morelensin (*B. morelensis* and *B. acuminata*) or from yatein to deoxypodophyllotoxin (*B. fagaroides* see Fig. 3).

In the *Bullockia* lineage this mechanism would explain the formation of savinin out of hinokinin (in *B. submoniliformis*, *B. cuneata*, *B. penicillata*, and *B. citronella*) and explain the hydroxylation of iso-bursehernin into guayadequiol. However if this is the same mechanism underlying the reactions that takes place in *P. hexandrum* there is a distinct difference. In *P. hexandrum* three different products were formed, while in the species in this study the reaction yields only one product in certain species. It may therefore be possible that in each species a different homologue of the enzyme has evolved that is responsible for these reactions.

Either cytochrome P450-dependent mono-oxygenases or *O*-methyltrans-ferases probably carry out the other reactions that are necessary for the lignan biosynthesis in this genus. The first type would be responsible for

the hydroxylation of the aromatic rings and the formation of methylenedioxy bridge. The second class of enzymes is responsible for the methylation of the *para* hydroxy group of the starting compound matairesinol and the methylation of the free hydroxy group that originates from the just mentioned hydroxylation.

The formation of the methylenedioxy bridge is the only reaction that takes place in all of the studied species that produce lignans. Except for *B. excelsa* all the species are able to form this bridge on the A ring. All of the species of the *Bullockia* group and *B. simuraba* of the *Burser*a subgenus are able to form this bridge on the B ring. There is circumstantial evidence from studies with seeds of *Sesamum indicum* that for the biosynthesis of sasaminin (a different lignan with two methylenedioxy bridges) two different enzymes form the two bridges. Whether this is also true for the *Bursera* genus is not known.

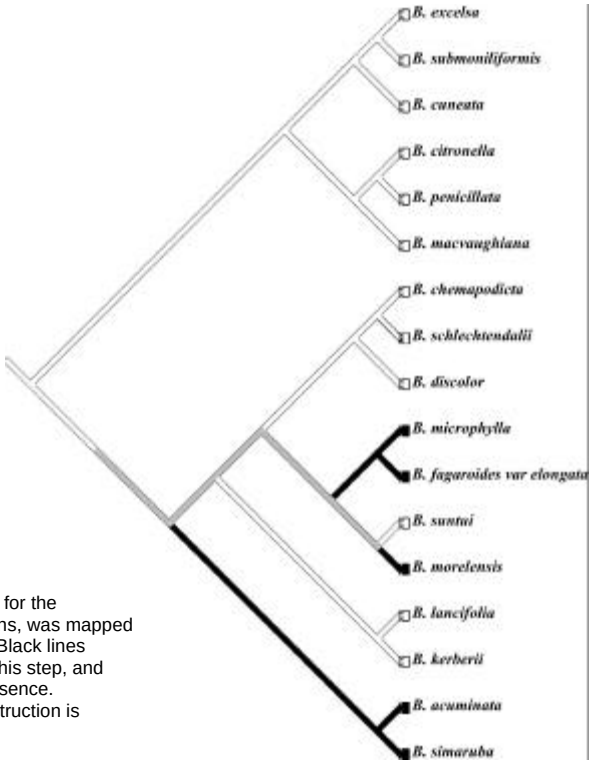

Fig. 3.
The ring closure necessary for the formation of 2,7'-cyclolignans, was mapped onto *Bursera*'s phylogeny. Black lines represent the presence of this step, and white lines indicate their absence. Equivocal character reconstruction is indicated in gray.

Also the *para*-methylation is not restricted to one section of the genus. Though, the position of the reaction differs between the two sections. It seems apparent that this reaction is carried out by an *O*-methyltransferase, probably SAM (S-adenosyl-L-methionine) dependent, but there is limited data on this. A study of a comparable SAM dependent *O*-methyltransferase, methylating anol at the same position into anathol, does not seem to have a very high substrate specificity (Kemmerer and

Reichling, 1996). Also a study with strawberries showed that a comparable methylation, is performed by an enzyme that accepts different substrates (Abbott *et al.*, 2002). On the other hand it has been shown that a single amino acid mutation in an *O*-methyltransferase can change the substrate specificity (Gang *et al.*, 2002).

The other methylation and hydroxylation reactions occur only in the *Bursera* section. The sequence of the reactions taking place seems not to be the same in all species as discussed above. The hydroxylation and subsequent methylation at the 5' position or 6 position are performed on different substrates and in different unrelated species (see Fig. 4).

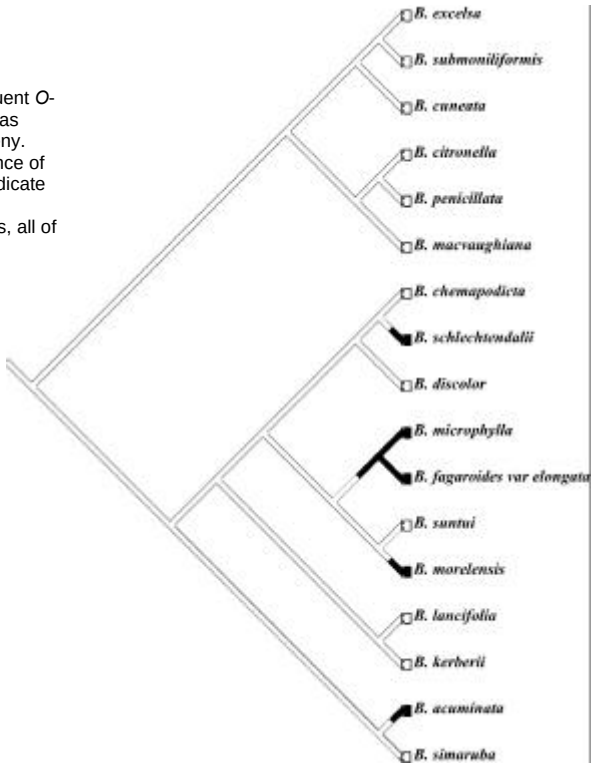

Fig. 4.
The hydroxy-lation and subsequent *O*-methylation at the 5'-position, was mapped onto *Bursera*'s phylogeny. Black lines represent the presence of the two steps and white lines indicate their absence. It has arisen independently at least four times, all of them among species of section *Bursera*.

Analogues of these reactions are present in different biosynthetic pathways. This hydroxylation followed by a methylation at the 5' position shows a high degree of similarity to the monolignol biosynthesis. In the monolignol biosynthesis the hydroxylation of coniferyl aldehyde is carried out by monooxygenase that is also able to hydroxylate other substrates that differ in the aliphatic side chain. In this so-called metabolic grid the following step is an O-methylation. This methylation, with for instance caffeate as substrate, is carried out by caffeate *O*-methyltransferase (COMT). There are different reports showing that COMT has a broad substrate specificity (Li *et al.*, 1997). COMT is also able to accept 5-

hydroxyferulate as a substrate. Studies with other plant OMTs showed that also in other biosynthetic pathways one enzyme is able to carry out two steps in the biosynthesis. This was shown for an OMT from *Catharanthus roseus* that carries out two steps in the flavonoid biosynthesis (Cacace *et al.*, 2003).

From the phylogenetic information and the lignan profiles of the *Bursera* species, it is possible to suggest two scenarios for the evolution of the lignan biosynthesis. For the first scenario one can argue that the lignan biosynthesis up to and including the 2,7'-cyclolignans is conserved and present in all species of the *Bursera* genus (and maybe in all plant species). In some species the genes are not expressed and no lignans can be detected, as discussed before. In the second scenario, the lignan biosynthesis from matairesinol to the different lignans like deoxypodophyllotoxin arises convergently during the evolution of the different species. This explains that the formation of 2,7'-cyclolignans is restricted to only the *Bursera* section, and the biosynthesis of savinin in the *Bullockia* section. Through the participation of enzymes from other routes, different steps can be catalysed facilitating a convergent and rapid evolution of last parts of the 2,7'-cyclolignan biosynthesis. Because the lignan specific reactions, such as 2,7'-ring closure, is specific for one section of the genus, this second scenario explains much better our findings. Still, one can not exclude that a more complicated combination of both scenarios is true.

ACKNOWLEDGMENT

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XI. Abstract, Discussion and Conclusions

ABSTRACT

This thesis covers a broad study on the production of Podophyllotoxins. In this final chapter the synergy of the previous chapters is discussed. Therefore, this discussion will start out with the conclusions of each of the chapters. Finally, an outlook for future research and opportunities in lignan research will be given.

Chapter III: ***GC-MS method***

This study on the production of podophyllotoxin and related 2,7'-cyclolignans started with the development of a new GC-MS method. With this analytical technique it became possible to profile the lignans in *A. sylvestris*. This member of the Apiaceae family accumulates mainly deoxypodophyllotoxin, yatein and anhydropodorhizol. The GC-MS method developed was applied to determine the variation of lignan profiles in different populations of *A. sylvestris*. From these data we could show that in some populations the lignan content was significantly higher than in others. This opens the possibility to screen for a high producing strain of *A. sylvestris* that can be applied as a starting point for a biotechnological production of lignans

Furthermore we also could profile the lignan content in other biosynthetically related plant species. We showed that *Anthriscus cerefolium*, a species closely related to *A. sylvestris*, does not produce any detectable quantity of lignans.

Chapter IV: ***The volatile components of A. sylvestris***

Although *A. sylvestris* and *A. cerefolium* are closely related species, they are clearly different in their lignan content. This observation lead to further study of the metabolic fate of phenylpropanoids in these two species. We could show that the essential oil of *A. sylvestris* was also different. Methylchavicol and 1-allyl-2,4-dimethoxybenzene (originating from the phenylpropanoid pathway), which are the most abundant constituents of the essential oil of the *A. cerefolium* leaves, could not be detected in the leaves of *A. sylvestris*. The main monoterpenes in *A. cerefolium* leaves (β-pinene and β-phellandrene) were also present in *A. sylvestris* (Zwaving *et al.*, 1971). There was no difference between the roots and leaves in the content of benzene acetaldehyde. This is the only major component in the essential oil that is biosynthetically related to lignans. The presence of lignans in the roots has no notable influence on the essential oil composition in comparison to the leaves, but might explain the absence of phenylpropanoids as main constituents in the essential oil of *A. sylvestris* compared to *A. cerefolium*. From this study it can be concluded that *A. sylvestris* does not use its phenylpropanoids for the production of high amounts of essential oils, like *A. cerefolium*.

Chapter V: ***Lignan profiles of indoor-cultivated A. sylvestris.***

When *A. sylvestris* plants from the seeds of four different locations grew under identical indoor condition, they showed phenotypic differences. Therefore, it must be assumed that there is a genotypic difference between the seeds from these locations. The genetic difference between the 4 locations does not show up in the lignan content of the roots. The large variations in lignan profile within 2 of the 4 groups points to genetic variation between each of the individual plants. Apparently there is no selection pressure on the lignan concentration in these populations. From these data it is not yet clear if it is possible to select and breed for high lignan producing strains of *A. sylvestris*. Further research in this field is necessary.

Chapter VI: ***Lignans in A. sylvestris.***

The roots of *A. sylvestris* produce a divers range of lignans, next to the three main lignans deoxypodophyllotoxin, yatein and anhydropodorhizol. These lignans do not fit in a clear biosynthetic route leading to one end-product. Experiments with cell suspension cultures revealed a hydroxylating activity restricted to deoxypodophyllotoxin leading to podophyllotoxin. This activity observed *in vitro* is in contrast to accumulation *in planta* of deoxypodophyllotoxin (see chapter III). The hydroxylating activity demonstrates that *A. sylvestris* cell cultures could be useful biocatalysts for the synthesis of podophyllotoxin from deoxypodophyllotoxin. Induction of this activity *in planta* would result in an interesting new source for podophyllotoxin. The exact biosynthetic pathway *in planta* remains to be elucidated.

Chapter VII: ***Lignans in Linum flavum cell cultures***

L. flavum cell suspension cultures produce only trace amounts of lignans during their normal growth cycle. Previous studies showed that lignan production can be increased to as much as 4.4% after feeding of the precursor deoxypodophyllotoxin. In this study we looked at the possibility of feeding putative precursors of deoxypodophyllotoxin.

From this series of bioconversion experiments a number of conclusions can be drawn. The feeding of anhydropodorhizol results in hydroxylation at the 7 position followed by glycosylation. Precursor yatein**,** which is the accepted direct precursor of deoxypodophyllotoxin (Broomhead *et al.*, 1991), underwent no ring closure and was not converted at all as was the case for the synthetic analogue naphthayatein; no ring closure reaction could be detected.

The present study shows that *L. flavum* cells were able to modify a limited number of putative precursors of deoxypodophyllotoxin. Unexpectedly the resulting compounds are not intermediates that fit into the current views on the biosynthetic cascade leading to 6-methoxypodophyllotoxin.

Chapter VIII: ***Bioconversion of deoxypodophyllotoxin***

An important finding of the feeding of deoxypodophyllotoxin to *Linum flavum* plant cell cultures is the detection of podophyllotoxin next to large amounts of 6-methoxypodophyllotoxin and its glucoside. This study showed that *L. flavum* cells are able to hydroxylate deoxypodophyllotoxin at the C-7 or at the C-6 position. The hydroxylation at the 7 positions yielding podophyllotoxin is much less favoured than the 6-hydroxylation leading to â-peltatin. The 7-hydroxylation performed by the *L. flavum* cells does not only take deoxypodophyllotoxin as a substrate, also â-peltatin-A-methylether and other lignans like matairesinol (Xia *et al.*, 2000), anhydropodorhizol (see chapter VII) are hydroxylated at this position. However there seem to be differences in the efficiency of the reactions. The 7-hydroxylations of deoxypodophyllotoxin and anhydropodorhizol proceeds much slower in cell suspension cultures of *L. flavum* than the 7-hydroxylation of â-peltatin-A-methylether.

It seems that podophyllotoxin is a kind of by-product in the bioconversion process of deoxypodophyllotoxin (to 6-methoxy-podophyllotoxin). This conclusion is of major importance in the development of high production systems for podophyllotoxin with *L. flavum* cells or plants. To increase the 7-hydroxylation of deoxypodophyllotoxin it seems most useful to block the 6-hydroxylation and to prevent the formation â-peltatin-A-methylether, or to introduce a 7-hydroxylating enzyme from an other plant species with a high substrate specificity for deoxypodophyllotoxin.

Chapter IX: ***Bioconversion by Human CYP 3A4***

Until now it is not clear which (kind of) enzyme is responsible for the hydroxylation of deoxypodophyllotoxin yielding podophyllotoxin. It is assumed that this step is carried out by a cytochrome P450 mono-oxygenase (CYP), but thus far nobody has been able to convert deoxypodophyllotoxin into podophyllotoxin without whole cells. Human CYP 3A4 initiates, via a 3'-*O*-demethylation, the catabolism of etoposide and teniposide, semi-synthetic derivatives of podophyllotoxin. Heterologously expressed CYP 3A4 converts deoxypodophyllotoxin differently. This lignan is hydroxylated at the 7 position, yielding podophyllotoxin. The absence or presence of hydroxy groups at the 6- and 7-carbon atoms of deoxypodophyllotoxin, podophyllotoxin and â-peltatin respectively, influence CYP 3A4 conversion to a great extent.

Heterologous expression of CYP 3A4 in bacteria can be used as an alternative for the production of podophyllotoxin and might be applicable for large-scale production of this compound.

Chapter X: ***Evolution of lignan biosynthesis***

Our understanding of the biosynthesis of 2,7'-cyclolignans is still limited. It is striking that many different unrelated plant species are able to produce these lignans. An improved understanding of the evolutionary process

behind biosynthesis will help to increase our knowledge of lignan biosynthesis in general.

From the data that have been obtained on the phylogeny and the lignan profiles of the *Bursera* species it is possible to suggest two scenarios for the evolution of the lignan biosynthesis. For the first scenario one can argue that the lignan biosynthesis up to and including the 2,7'-cyclolignans is conserved and present in all species of the *Bursera* genus (and maybe in all plant species). In some species the genes are not expressed and no lignans can be detected, as discussed before. In other species mutations in the genes encoding enzymes participating in lignan biosynthesis cause differences in the catalysed reactions and therefore different products or only intermediates are formed like the production of yatein by *B. schlechtendalii*. In the second scenario, lignan biosynthesis from matairesinol to the different products, like deoxypodophyllotoxin, arises during the evolution of the different species. This may explain that the formation of 2,7'-cyclolignans is restricted to only the Bursera section. The participation of both mono-oxygenases and methyltransferases from the monolignol biosynthesis in the lignan biosynthesis of *Bursera* could catalyse different steps. For instance the conversions that lead from bursehernin to yatein, from morelensin to deoxypodophyllotoxin, or maybe even from deoxypodophyllotoxin to â-peltatin-A-methylether (see Fig. 3 for the reaction and Fig. 4 for their place in the phylogeny). Thus, the recruitment of these enzymes can facilitate a convergent and rapid evolution of final parts of the 2,7'-cyclolignan biosynthesis. It is this second scenario that better explains our findings. Especially the absence of any 2,7'-cyclolignans in the Bullockia section speaks against the first scenario. Still, one can not exclude that a more complicated combination of both scenarios is used. If the second scenario is true it also means that presence of podophyllotoxin in so many different unrelated plant species is really an example of convergent or parallel evolution.

THE SYNERGY

As stated in the scope of the thesis, the aims of this thesis are to improve our knowledge of the biosynthesis of podophyllotoxin and related lignans and develop new strategies for their production. Although the information has been obtained from many different plant species, the assembled data can be used to extrapolate results from one species to an other.

A large part of the thesis focuses on *Anthriscus sylvestris*. This Apiaceae member accumulates quite high amounts of deoxypodophyllotoxin, yatein and anhydropodorhizol. These are not the only lignans produced by this plant. Chapter VI shows all the lignans we could find in this species. Matairesinol and pluviatolide have been found by Suzuki and co workers in Japanese *A. sylvestris* (Suzuki *et al.*, 2002). Based on the knowledge we have from other species and other biosynthetic routes one can make a hypothesis about the biosynthetic relation between these compounds (see Fig 1).

We found that *A. sylvestris* cell suspension cultures are able to hydroxylate deoxypodophyllotoxin. But this yielded podophyllotoxin concentrations that were much higher than found *in Planta*. What becomes clear from this scheme is that there is not something as one biosynthetic pathway leading to one end product. Another important conclusion coming from this hypothetical scheme is that there are many more enzymatic steps needed to yield all these lignans than the, in the introduction mentioned, minimal of 6. Such a high number of different enzymes makes it difficult to understand how the lignan biosynthesis arose during the evolution. Is the biosynthesis of podophyllotoxin and related lignans made possible by a set of genes that is conserved in all plant species and only expressed in a small number? Or, can this be a matter of repeated or parallel evolution?

These questions are also relevant when we look at the family of the Apiaceae, which does not seem to contain any closely related species producing 2,7'-cyclolignans. Next to parallel and repeated evolution it could also be possible that some genes that take part in this biosynthesis have undergone horizontal gene transfer. This phenomenon is widespread in mico-organisms (Ragan, 2001). Gene transfer is also possible between bacteria and plants and this finding has been the basis of the *Agrobacterium tumefaciens* transformation technique that is used to alter plants genetically. Whether bacteria-mediated horizontal gene transfer is responsible for the spread of lignan biosynthesis is doubtful, because lignans are only found in plants and not in bacteria.

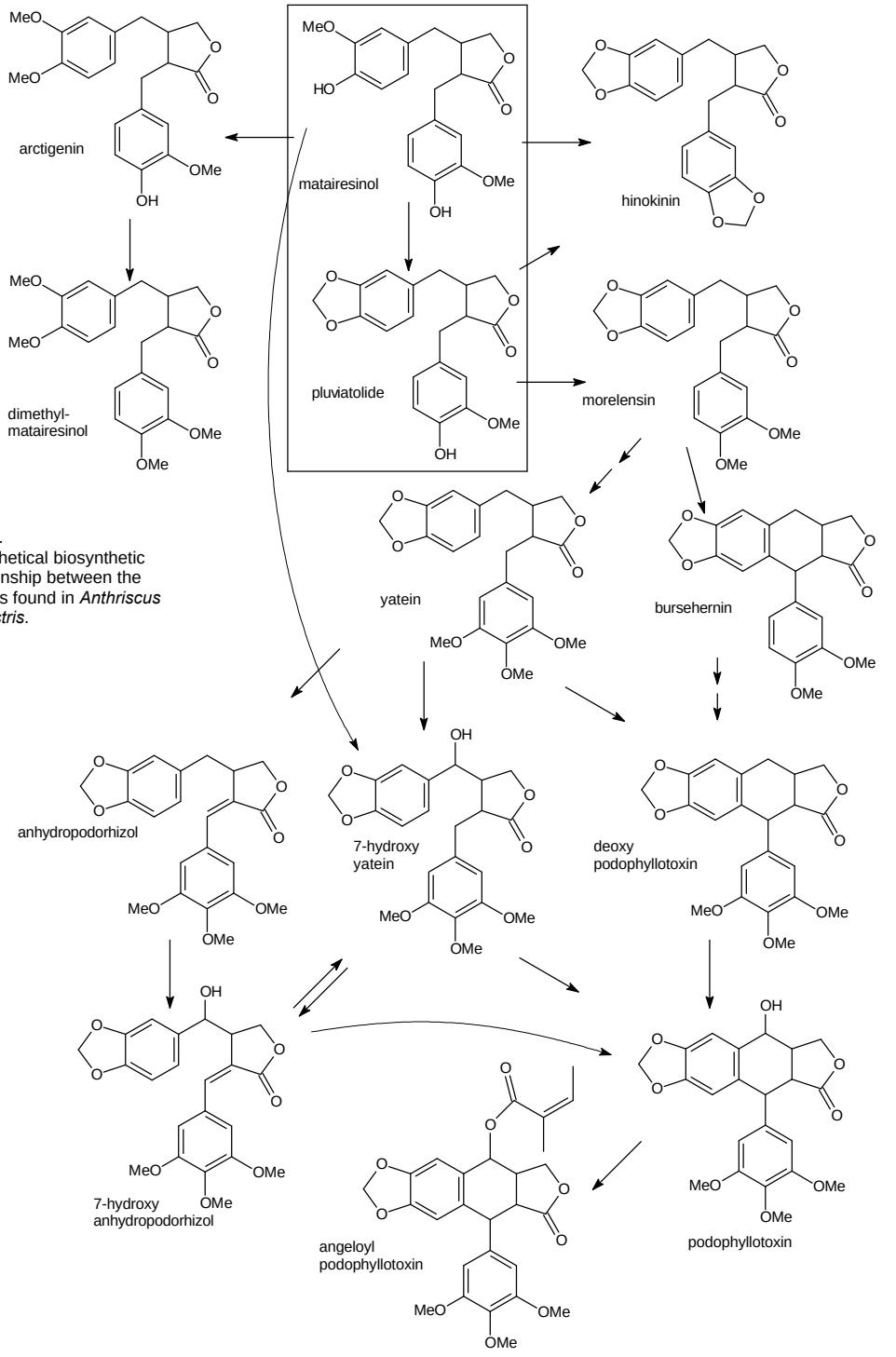


Fig. 1.
Hypothetical biosynthetic relationship between the lignans found in *Anthriscus sylvestris*.

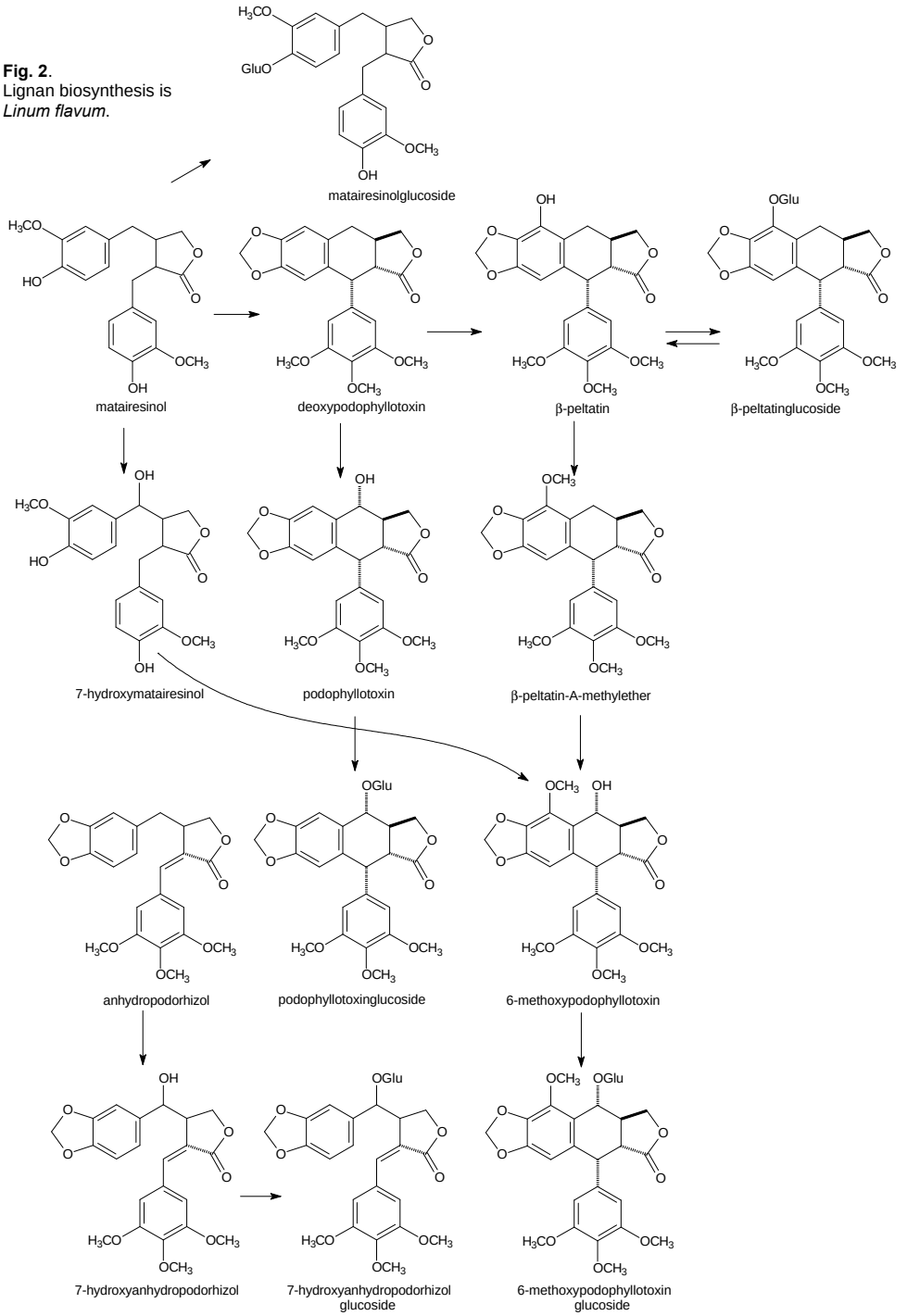
The experiments with indoor-cultivated plants contribute some extra information on this point. The plants that yielded the seeds for these experiments seem to have experienced only little selection pressure on the lignan profiles. If this is true, it becomes difficult to understand that biosynthesis of podophyllotoxin has (recently) evolved. However these large variations in lignan content might be a very successful strategy to prevent any predation. Large variations in these toxic lignans make it very difficult for herbivores to adapt. The genotypical differences between the individual plants yield for the whole population a better chance of survival. This would mean that the lignan biosynthesis in *A. sylvestris* is not only recently evolved but is still evolving. When the biosynthesis of deoxypodophyllotoxin evolved recently in this species this would imply that the biosynthesis also evolved recently in other species like *Linum* and *Podophyllum*.

To tackle this question on the lignan biosynthesis evolution, we studied the *Bursera* genus, for which the phylogeny is well mapped out. It could be possible that the biosynthesis of deoxypodophyllotoxin raised early in the evolution of plants and that this biosynthesis is conserved, but not expressed in all plants. This would explain the presence of this compound in *Anthriscus* and in *Bursera*. However the biosynthesis of deoxypodophyllotoxin follows the phylogeny in *Bursera*, indicating that during the evolution of this genus also the deoxypodophyllotoxin biosynthesis arose.

If the biosynthesis of deoxypodophyllotoxin has arisen during the evolution of the *Bursera* genus it seems likely that the same will be true for the *Anthriscus*. This leaves us with the question how such a high number of different enzymatic steps arose independently during the evolution of the lignan biosynthesis.

This can be explained assuming those one-enzyme catalyses more steps in this biosynthetic scheme. We do not have any data as of yet about the enzymes taking part in this biosynthesis in *A. sylvestris*. But from the results that we have from *L. flavum* (see chapter VII and VIII.) it seems more likely that enzymes with broader substrate specificities (able to catalyse different steps) could take part in the biosynthesis of lignans. In our experiments with *Linum flavum* cells we could show that after feeding of anhydropodorhizol also hydroxylation at the 7-position occurred (see chapter VII). Apparently the 7-hydroxylation of intermediates is quite a common feature in the lignan pathway. An enzyme, which has the capacity to hydroxylate fed anhydropodorhizol, deoxypodophyllotoxin and matairesinol, is probably a less specifically acting cytochrome P450 monooxygenase. This yields compounds which are not all normally accumulated in the plant or present as biosynthetic intermediates. The idea that lignans are formed via different routes is also something we see in *Linum*. *Linum flavum* converts matairesinol into 7-hydroxymatairesinol, which is than further converted in 6-methoxy-podophyllotoxin. It is also able to convert deoxypodophyllotoxin into 6-methoxypodophyllotoxin.

Fig. 2.
Lignan biosynthesis is *Linum flavum*.



A hypothetical biosynthetic

scheme as is given for *Anthriscus* can also be used to come to a better understanding of what happens in *Linum* (see Fig. 2). First of all there is not one straight biosynthetic pathway leading to one end product. The network shows that there are different pathways that can lead to the same product. Such a product might be accumulated, but also converted further into other products. Several enzymatic steps involving the same type of chemical reactions like *O*-methylations may in fact be catalysed by the same enzyme.

As with the biosynthesis of coniferyl alcohol and its analogues, it might be very well possible that there is a limited number of enzyme classes that can produce these ranges of compounds. The minimum number of enzyme classes required are a hydroxylase (cytochrome P-450), an *O*-methyl-transferase, an oxidising enzyme to form the double bond formation, a dioxymethylene bridge-forming enzyme (probably also a cytochrome P-450), and a ring closing enzyme. Probably there are two hydroxylase active, one for the aromatic hydroxylations and one for the aliphatic hydroxylations.

This would give the need for a total number of 6 enzymes with broad substrate specificity to enable the lignan biosynthesis. Differences in affinity for the different substrates could explain the variations in product concentration. The levels of gene-expression, regulation and uptake in cell compartments will also play a role in the accumulation of these compounds. Our hypothetical network may be of support for further studies on the biosynthesis of lignans but also to develop new strategies to genetically modify and improve the lignan production of plants.

From these studies it seems attractive to look for a biotechnological production system outside the plant. Genes encoding plant enzymes might be expressed in fast growing micro-organisms. Possibly also non-plant genes and enzymes, can be used to construct a successful production system. A first step in this field is shown in chapter IX, where a bacterium expressing a human gene is used to convert deoxypodophylloxin into podophyllotoxin.

XII. Nederlandse samenvatting: Conclusie

De biosynthese

Aan de hand van de onderzoekresultaten die in hoofdstuk 9 zijn opgenomen kan worden aangenomen dat de biosynthese van podofyllotoxine in het geslacht *Bursera* recent is ontstaan. Dit betekent, dat die biosynthese meerdere malen onafhankelijk van elkaar ontstaan moet zijn in verschillende plantengroepen. Een recente evolutie van een dergelijke complexe route is beter te begrijpen als men aanneemt dat enzymen uit andere biosyntheseroutes een rol kunnen spelen. Dit kan alleen gebeuren als deze enzymen niet kieskeurig zijn met betrekking tot de stoffen die worden omgezet. Aanwijzingen dat zulke enzymen een rol spelen in de lignaan- biosynthese werd gevonden in het geslacht gele vlas. Daarnaast is het gevonden grote aantal verschillende lignanen in fluitenkruid ook een aanwijzing hiervoor. Vele van deze lignanen zijn aanwezig in zeer lage concentratie en zijn vermoedelijk een bijproduct van de biosynthese van deoxypodofyllotoxine. Een stof die aanwezig bleek te zijn in de hoge concentratie. Ook verklaart de theorie de mogelijkheid van verschillende routes binnen een plantensoort naar hetzelfde eindproduct toe (zie de inleiding).

De productie

Fluitenkruid bevat redelijk hoge concentraties lignanen. Elke fluitenkruidplant die wij hebben onderzocht bevatte een andere hoeveelheid lignanen. Ook planten uit één populatie, van dezelfde locatie, lijken in lignaangehalte niet op elkaar.

Het ziet er echter wel naar uit dat het mogelijk is om een hoog producerende lijn te verkrijgen, maar dat vraagt een goed opgezet kruis- en selectieprogramma. Daarnaast lijkt het interessant om te onderzoeken of gedurende het groeiseizoen de gehaltes veranderen en of er externe factoren zijn vast te stellen die de gehaltes zouden kunnen beïnvloeden.

Een andere interessante strategie is om bij het kruisen niet te selecteren op het gehalte deoxypodofyllotoxine, maar direct op het gehalte podofyllotoxine.

Om tot podofyllotoxine te komen zou het interessant zijn om, uitgaande van het gen voor het menselijk enzym CYP 3A4, een beter producerend enzym te maken (via genetische technieken), dat het deoxypodofyllotoxine uit de fluitenkruidwortels kan gebruiken als substraat.

Fluitenkruid of gele vlas genetisch veranderen heeft echter maar een kleine kans op succes. Deze planten kunnen al podofyllotoxine maken maar zijn niet aangepast aan de opslag van deze stoffen. Zou men een extra hydroxylerend gen in fluitenkruid zetten dan zal dit waarschijnlijk leiden

tot zeer ongezonde planten of planten waarin de biosynthese wordt omgeleid.

Verder onderzoek op dit gebied is hard nodig. Het hier gepresenteerde onderzoek heeft namelijk aangetoond, dat de weg naar de biotechnologische productie van podofyllotoxine zeer vruchtbare resultaten kan opleveren bij voortgaande analyses.

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Koulman A., Batterman S., Van Putten F.M.S., Bos R., Quax W.J. Lignan profiles of indoor-cultivated *Anthriscus sylvestris*. *Planta Medica*. accepted.

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Koulman A., Beekman A.C., Vinhas A.M.A., Medarde M., Madrigal B., Bos R., Pras N., Quax W.J. The bioconversion of lignans from the putative 6-methoxypodophyllotoxin biosynthesis by *Linum flavum* cells. *Journal of Natural Products*. submitted.

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Fu M., Koulman A., Van Rijsel M., Lützen A., De Boer M.K., Tyl M.R., Liebezeit G. Chemical Characterisation of three Haemolytic Compounds from *Fibrocapsa japonica* (Raphidophyceae). *Toxicon*. submitted.

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