SYNTHESIS OF LABELLED PRECURSORS OF PODOPHYLLOTOXIN

by

Anie Jose Pullockaran

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APPROVED:

D.G.I. Kingston, Chairman

J.F. Wolfe

N.G. Lewis

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I. INTRODUCTION

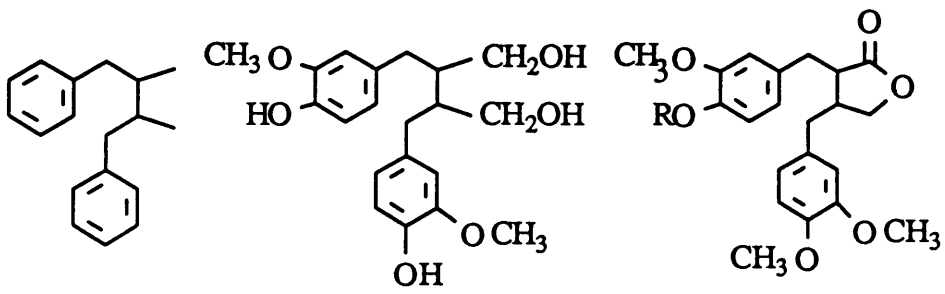
I.1 Lignans

Lignans are natural products widely distributed in the plant kingdom. More than 200 of these natural products have been isolated, their structures elucidated and synthesis developed.[1] Lignans are formed by dimerization of two phenylpropanoid units linked by the ß-carbon of their side chains.[2,3] Naturally occurring dimers that exhibit linkages other than this β-β type linkages are known as neolignans.

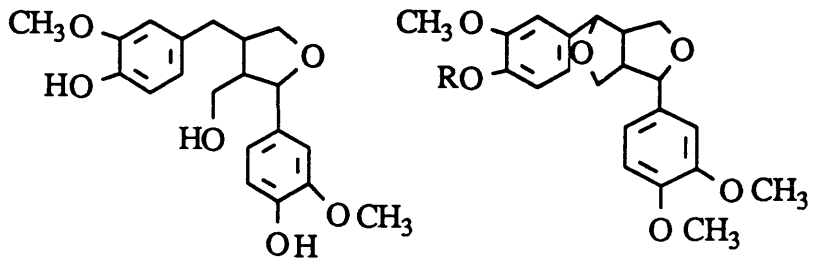
Lignans have been isolated from all parts of the plant material - roots, leaves, bark-wood and resinous exudates. It has been observed that they play a role by accumulating in the zone separating sound from infected wood in combatting or containing bacterial infection, fungal attack, wounding and insect predation.

A great diversity in the chemical assembly of the two characteristic phenylpropanoid units, as well as the degree of oxidation and types of substituents is apparent. Lignans can be classified on the basis of the carbon skeleton **1** into simple diols e.g. secoisolariciresinol **2**, lactones e.g. arctiin **3**, tetrahydrofuran derivatives, e.g. lariciresinol **4**, bistetrahydrofurans e.g. phillyrin **5**, or tetrahydronaphthalene compounds, e.g. podophyllotoxin **6** (**Fig. 1**).

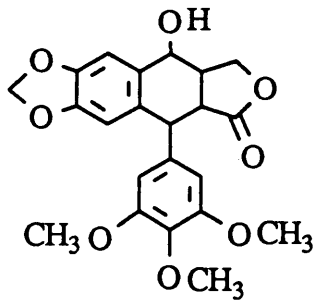
The breadth of the biological activities of these compounds is impressive and has come to be appreciated relatively recently. Lignans possess a variety of pharmacological actions in man, though the most interesting of these are subtle and not easily studied. At the molecular level some, like podophyllotoxin (**6**) are known to bind to the tubulin of microtubules, to interrupt nucleotide transport and DNA synthesis and to be specific inhibitors of certain enzymes.[4,5]



1 2 Seco-isolariciresinol 3 R=glucosyl Arctin



4 Lariciresinol 5 Phillyrin



6 Podophyllotoxin

Fig. 1 Main types of lignans

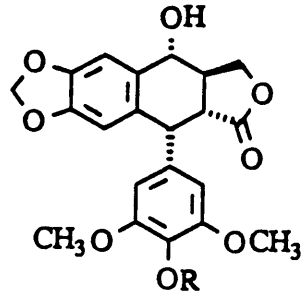
Lignans have aroused considerable interest because some of them eg. the *Podophyllum* lignans display antitumor activities. More systematic investigations of their biological activities and their stereochemical specifications should further our understanding and uncover new uses for lignans.

I. 2 Podophyllotoxins

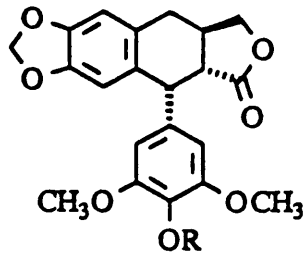
A wide range of aryltetralin lignans and glycosides having cytotoxic activity has been isolated from the *Podophyllum* species.[6] *Podophyllum* lignans are unique in their antimitotic and tumour damaging activity, which is closely associated with their unique configuration at C-2, C-3 and C-4 as in podophyllotoxin with its highly strained, trans-fused -lactone system. **Figure 2** lists the structures of naturally occurring podophyllotoxins which are constituents of podophyllin. The chemistry and anti-neoplastic activity of many of these were actively investigated and discussed by Hartwell.[6]

Podophyllotoxins are commercially isolated from two species of plants namely *Podophyllum peltatum* Linnaeus, the North American plant commonly known as the American mandrake or May apple and the related Indian species *Podophyllum emodi* Wallich or *P. hexandrum* Royle. The dried roots and rhizomes of *P. peltatum* and *P. hexandrum* is known as podophyllum and when the podophyllum is extracted with alcohol, the resin produced is called podophyllin.

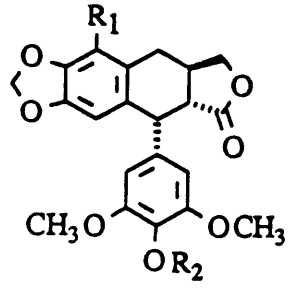
The aryltetralin lignans of the *Podophyllum* may be divided into two biogenetically distinct groups according to the substitution pattern in the pendant aryl ring, which may carry 3,4,5-trimethoxy or 4-hydroxy-3,5-dimethoxy substituents.[7] The other aromatic ring in general contains only 3,4-methylenedioxy substitution (disregarding the C-1-C and C-6-C linkage), although an extra



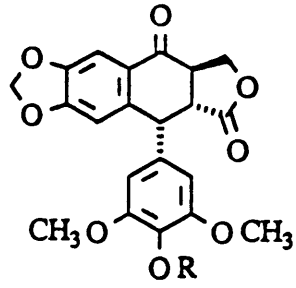
6 R=CH_3 Podophyllotoxin
7 R=H 4'-Demethylpodophyllotoxin



8 R=CH_3 Desoxypodophyllotoxin
3 R=H 4'-Demethyldesoxy podophyllotoxin



9 R_1=OH, R_2=CH_3 β-Peltatin
10 R_1=OH, R_2=H α-Peltatin

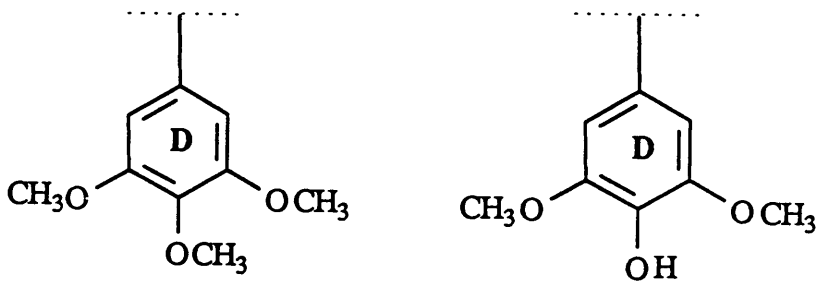


11 R=CH_3 Podophyllotoxone
12 R=H Demethylpodophyllotoxone

Fig. 2 Structures of naturally occuring podophyllotoxins in podophyllin

5-hydroxy is observed in the peltatins.

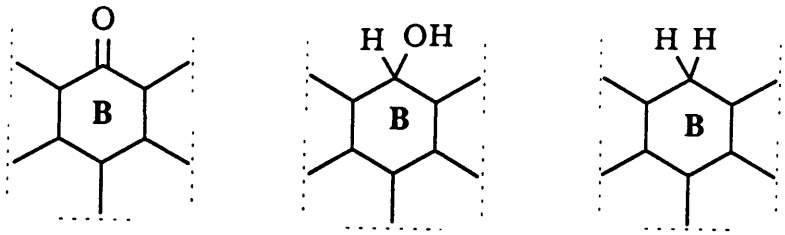
The trimethoxy substituted pendant ring containing lignans are derived from the oxidation of desoxypodophyllotoxin (8) to podophyllotoxin (6) and podophyllotoxone (11). Similarly, 4'-demethylpodophyllotoxin (7) and 4'-demethylpodophyllotoxone (12) are derived via 4'-demethyldesoxypodophyllotoxin (13) and are representatives of the group of lignans containing a 4-hydroxy-3,5-dimethoxy substituted pendant ring. Quantitative estimation shows that in general the trimethoxy series of lignans is present in greater amounts than the corresponding 4'-demethyl series.



Substitution pattern of pendent aryl ring

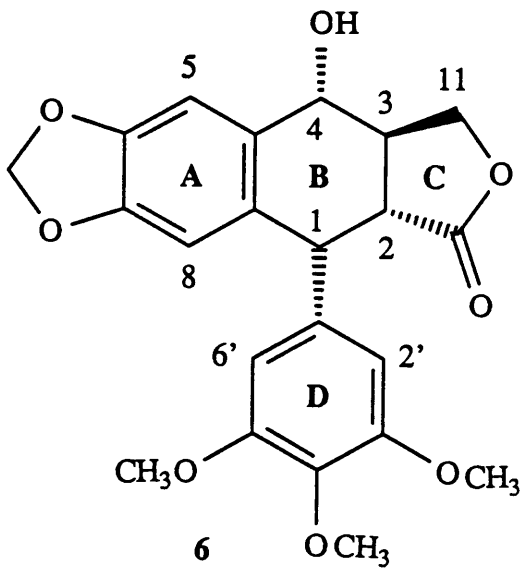
Podophyllum lignans may again be subdivided into three main categories depending on the oxidation state of carbon-4. These are the 4-desoxy series, e.g. desoxypodophyllotoxin (8) and the peltatins (9 & 10), in which there is a methylene group at carbon-4; the 4-hydroxy series, e.g. podophyllotoxin (6), where carbon-4 is partially oxidized to a secondary alcohol; and the 4-keto series, e.g. podophyllotoxone (11), with a carbonyl group at the 4-position. All these compounds have the same stereochemistry at C-1, C-2 and C-3 namely 1α,2α,3ß.

Podophyllotoxin (6) is a major lignan of the *Podophyllum* species and its chemical structure is as shown in the figure. It has a trimethoxy substituted pendant aromatic ring and a secondary



Oxidation states of ring B

alcohol at C-4. This colorless crystalline substance was first isolated from the podophyllin of *P. peltatum* and was named by Podwyssotzki.[8] Its structure was proposed independently by Borsche and Nieman[9] and by Spath *et al* and was revised by Hartwell and Schrecker,[10] who proposed



the formula **6** which has a cis 1:2, trans 2:3 and trans 3:4 stereochemistry. A subsequent analysis of the crystal structure of 2'-bromopodophyllotoxin by Petcher *et al* confirmed the original assignment of absolute configuration.[11]

Table 1 gives the lignan content of *P. hexandrum* and *P. peltatum* root/rhizomes.[14] Of particular significance is the observation that the relative proportions of lignans present are markedly different in both species. Although *P. hexandrum* and *P. peltatum* contain the same range of

Table 1

Lignan Content of *Podophyllum* Species

Lignan	*P.hexandrum*	*P.peltatum*
Podophyllotoxin (6)	128.0	7.6
4'-Demethylpodophyllotoxin (7)	13.6	0.2
Desoxypodophyllotoxin (8)	0.6	0.7
4'-Demethyldesoxypodophyllotoxin (13)	0.3	0.2
β-Peltatin (9)	0.3	10.0
α-Peltatin (10)	0.2	7.6
Podophyllotoxone (11)	1.7	0.6
4'-Demethylpodophyllotone (12)	0.4	0.2
Isopicropodophyllotone (14)	1.0	0.2
4'-Demethylisopicropodophyllone (16)	0.2	0.1

expressed as mg per 10g dried root rhizome

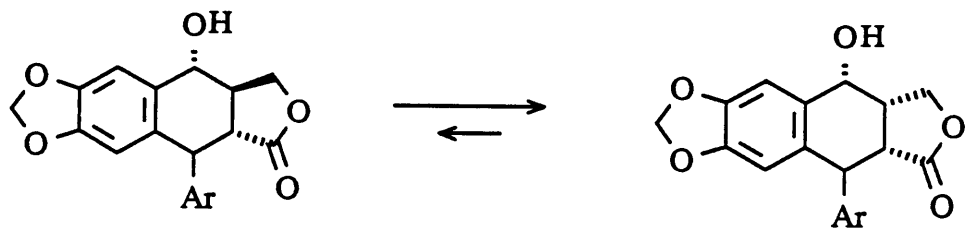
lignans, *P. peltatum* produces podophyllotoxin (**6**), α-peltatin (**10**) and ß-peltatin (**9**) in roughly equal amounts while in *P.hexandrum* the amount of α- and ß-peltatins are quite small, principally podophyllotoxin (**6**) and 4'-demethylpodophyllotoxin (**7**).

4'-Demethylpodophyllotoxin (**7**) is (with podophyllotoxin) one of the main compounds found in Indian podophyllin that produced damage in animal tumors.[12,6] It occurs to the extent of 1.7% and has not been isolated from any other variety of podophyllin. α-Peltatin (**10**) is one of the tumor-necrotizing components of American podophyllum in which it occurs to the extent of 5.5%. ß-Peltatin (**9**) is the 4'-monomethyl ether of α-peltatin (**10**) and occurs to the extent of 5.7% in American podophyllum. It is also biologically active against tumors.[15]

Variation in lignan pattern between individual plants is noted, but in general, dormant plants contain podophyllotoxin (**6**), and α- and ß-peltatin, with ß-peltatin (**10**) usually predominating. Material in the dormant plant appears to be metabolized during growth, then resynthesized during senescence.[14] Podophyllotoxin (**6**) levels are not affected in the same way. It is perhaps possible that α- and ß-peltatins may be utilized for lignin biosynthesis, the phenolic grouping allowing further oxidative coupling processes which are denied to podophyllotoxins.

Picropodophyllotoxin (**17**) is obtained by the mild base-catalyzed conversion of trans-fused lactone to the more stable cis-fused form at carbon-2 of podophyllotoxin.[15] This equilibrium is observed in the presence of mild base catalysis, when podophyllotoxin epimerizes smoothly to picropodophyllotoxin (**17**). Irreversible formation of the enolate of protected **17** followed by removal of the protecting group with dilute acid was used for the first total synthesis of podophyllotoxin by Gensler and Gatonis.[13] The picropodophyllotoxin derivatives display a specific antimitotic effect but are less effective than the corresponding trans compounds.

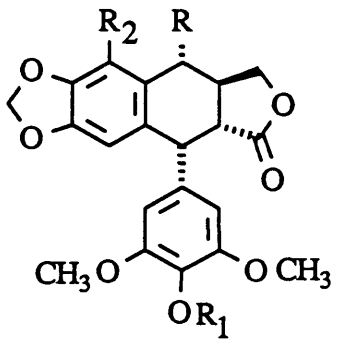
Picropodophyllotoxin glucoside (22) is one of the first lignan glycosides found in a plant of the *Podophyllum* genus,[12,15] and occurs in Indian *Podophyllum* to the extend of 1.8%. It also occurs in American podophyllin. It is inactive against Sarcoma 37 in mice. Its structure is represented by the systematic name 1-O-(ß-D-glucopyranosyl)-picropodophyllin; it is possibly formed by the epimerization of podophyllotoxin glucoside.



6 17

A systematic search for biologically active constituents of water-soluble fractions from *Podophyllum* rhizomes led to the isolation and identification of four new lignan glycosides.[11,17] **Figure 3** shows the well characterized lignan glycosides isolated from *Podophyllum* species. Podophyllotoxin glucoside (18), ß-peltatin glucoside (20), 4'-demethylpodophyllotoxin glucoside (21) and the α -peltatin glucoside (19) were separated from *P. peltatum* in yields ranging from 0.5-1%. The rhizomes of *P. hexandrum* contained podophyllotoxin glucoside (18) and 4'-demethylpodophyllotoxin glucoside (21), but none of the peltatin glucosides. The occurrence of the glycosides in the two species thus parallels that of the respective aglycones.

Podophyllin from any source is a complex mixture chemically which may on further study yield further substances of whose existence we are not yet aware. The implications of these facts for biological and clinical research is that in working with this material due account should be taken of its non-homogeneity and non-uniformity of composition.

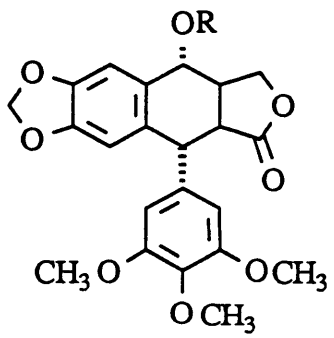


18 R=-O-β-D-glucosyl, R_1=CH_3, R_2=H. Podophyllotoxin glucoside

19 R=R_1=H, R_2=-O-β-D-glucosyl. α-Peltatin glucoside

20 R=H, R_1=CH_3, R_2=-O-β-D-glucosyl. β-Peltatin glucoside

21 R=-O-β-D-glucosyl, R_1=R_2=H. Demethylpodophyllotoxin glucoside

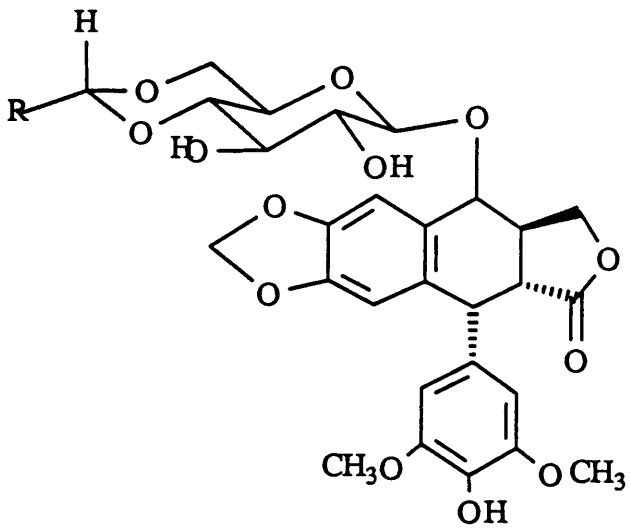


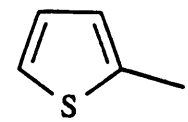
22 R=-O-β-D-glucosyl. Picropodophyllotoxin glucoside

Fig. 3 Lignan glycosides isolated from ***Podophyllum*** species

II. MEDICINAL IMPORTANCE

Crude natural products have been used in the treatment of cancer since ancient times, but it is only in the last 40 years that serious scientific study of naturally occurring anticancer agents has been carried out. Large numbers of anticancer agents of plant origin have been discovered which have had promising activities in animal trials, but only a few of these compounds have shown clinical activity. The most useful compounds in this class are the podophyllotoxin derivatives VP16-213 (etoposide **24**) and VM 26 (teniposide **23**), which have been introduced into medical practice.[4,18] Both are effective in the treatment of a variety of leukemias and solid tumors.





23 R=

24 R=CH_3

II.A. History

The North American plant *P.peltatum* and the related Indian species *P.hexandrum* have long been known to possess medicinal properties. Records of its use as a purgative and emetic extend back to several hundred years. Catesby in his "Natural History of the Carolinas" published in 1731 noted that the root is "said to be an excellent emetic and is used as such in the Carolinas".[19] Jacques Cartier, however, knew of the root both as a mortal poison and as an antidote for snake venom when applied topically to the bite.[20] North American Indians considered the roots as both a medicine and a poison.

Podophyllum was included as a cathartic and cholagogue (an agent that promotes the flow of bile into the intestine especially as a result of contraction of the gall bladder) in the first United States pharmacopeia published in 1820. Although podophyllum and podophyllin were popular in the 19th century as medicinals, they have been replaced in modern medicine by less drastic purgatives and more effective cholagogues. Kaplan's paper in 1942 reporting the cure of condyloma acuminatum by topical application of podophyllin revived the interest in the chemistry and pharmacology of podophyllin and its constituents.[21] Today, the cytotoxic action of podophyllin forms the basis of scientific interest in the drug and of its therapeutic use.

II. 2. Development into clinical drugs

Podophyllotoxins are a particularly instructive class of natural products for consideration in the design and synthesis of potential anticancer agents based upon natural product prototypes. *Podophyllum* lignans are unique in their activity with their configuration as in podophyllotoxin, with its highly strained and trans-fused γ-lactone system.

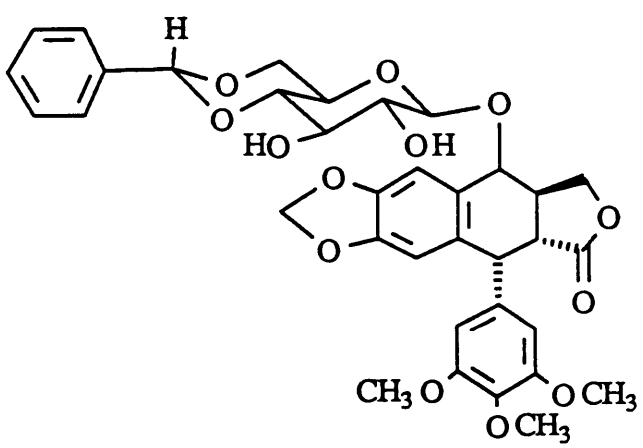
Podophyllotoxin (**6**) itself is a potent antimitotic agent, but it proved to be too toxic to be useful in the treatment of human neoplasms.[15] Its clinical application as a chemotherapeutic agent has been limited by severe toxic side effects during systemic administeration of the drug. In an attempt to discover less toxic analogs, a variety of podophyllotoxin derivatives has been isolated from natural sources or prepared by partial synthesis. The antimitotic activities of many of these analogues have been determined by measurement of their cytotoxicity in different cell lines, their association constants for binding to tubulin, and their relative abilities to inhibit *in vitro* microtubule assembly.

Leiter reported that podophyllotoxin (**6**), 4'-demethylpodophyllotoxin (**7**) and α- (**10**) and ß-peltatins (**9**) all produced damage to Sarcoma 37 in mice.[5] A number of materials structurally related to podophyllotoxin were tested by Leiter and Hartwell for their action on Sarcoma 37 implants. The acetyl derivative of podophyllotoxin and the acetates and methyl and ethyl ethers of the peltatins damaged the tumor. The acetyl derivative of picropodophyllin, like the parent compound, had no effect. Several benzoic acid derivatives, which were structurally similar products formed by oxidative degradation of podophyllotoxin or the peltatins were inactive.

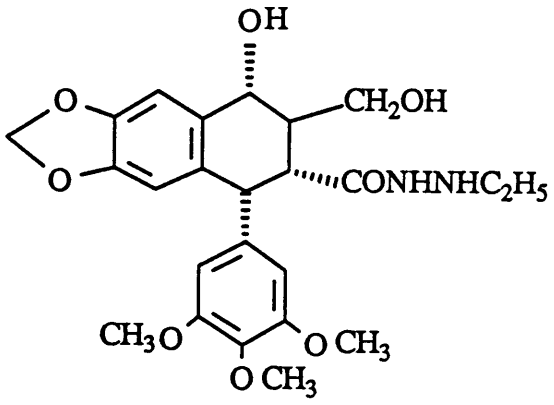
The solubility of podophyllotoxin in water is only 0.01%. Water-soluble derivatives with ionic groups have been synthesized from podophyllotoxin and the α- and ß-peltatins. However, all

these substances are inferior to podophyllotoxin in a chemotherapeutic respect. The glycosides of podophyllotoxin (**18**), α- (**19**) and ß-peltatins (**20**) and 4'-demethylpodophyllotoxin (**21**) have been isolated. These glycosides were found to be 10^2-10^4 times less active in the inhibition of mitosis than their aglycones, although they did not produce the noxious side effects of the corresponding aglycones. However, these glycosides did not act satisfactorily in clinical trials because of non-specific side effects.

Complete esterification of the glucose residue produced derivatives which were, in contrast to the glycosides, only slightly soluble in water and were resistant to enzymatic hydrolysis. The antimitotic activity of these derivatives was considerably lower than that of the non-esterified glycosides. The products of condensation of podophyllotoxin glucosides with benzaldehyde, however, were found to have similar specific antimitotic activities to those of the glucosides, but with remarkably low side effects. The clinically tested drug SP-G (**26**) contains the cyclic acetal podophyllotoxin benzylidene-ß-D-glucoside as the main component and another drug, podophyllic acid ethylhydrazide, designated as SP-I (**27**), has been studied in detail *in vitro* and *in vivo*.[23] Both



26 SP-G

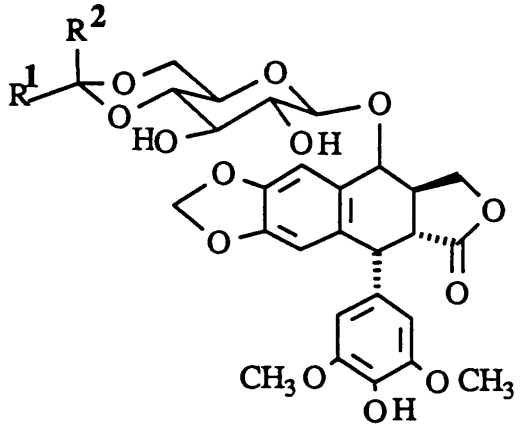


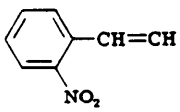
27 SP-I

drugs underwent clinical trials but were found to have minimal antitumor activity and were too toxic for general clinical use.

The cyclic acetals of epipodophyllotoxin-ß-D-glucopyranoside revealed no dramatic increase in biological activity. However, some cyclic acetals and ketals of 4'-demethylepipodophyllotoxin-ß-D-glucopyranoside not only exhibited high activity in *in vitro* tests (P-815 mastocytoma cells of mouse in culture) but also gave a significant survival time increase in the mouse lymphocytic leukemia L-1210 test. Two of the most outstanding of these derivatives were VM 26 (teniposide, **23**) and VP16-213 (etoposide, **24**). **Table 2** gives the antimitotic and antitumor activities of acetals of the glucosides.[22]

Antimitotic and antitumor activities of acetals of the glucosides.[22]

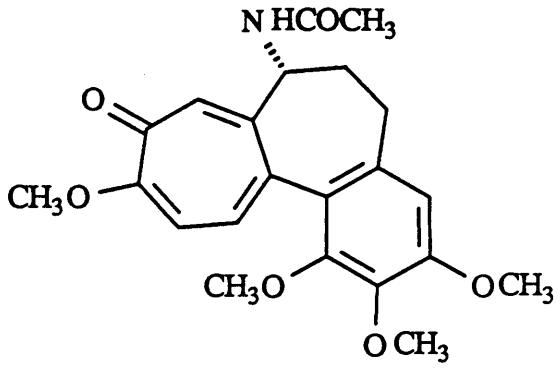


R^1	R^2	P-815 Mastocytoma cells of the mouse, *in vitro* ED_{50}, mg/liter	Mouse leukemia L-1210, survival time increase (%)
CH_3	H	0.031	167
C_2H_5	H	0.0085	97
$CH_3CH = CH$	H	0.016	121
$(CH_3)_2CH$	H	0.0055	121
$C_2H_5CHCH_3$	H	0.0055	84
$(CH_3)_2CHCH_2$	H	0.0048	36
n-C_4H_9	H	0.0062	85
C_5H_9	H	0.0047	39
	H	0.018	136
	H	0.0048	121
C_6H_5	H	0.0068	97
p-$H_3CC_6H_4$	H	0.0086	64
	H	0.0093	29
1-Naphthyl	H	0.013	95
CH_3	CH_3	0.015	106
C_2H_5	CH_3	0.0060	69

II.3. Mode of Action

II.3.1. Podophyllotoxin

Desoxypodophyllotoxin (8) blocks cell division by the inhibition of microtubule assembly in the mitotic apparatus. The mechanism of action of podophyllotoxin is very similar to that of colchicine (28).[15] It disrupts the assembly and function of microtubules and produces metaphase arrest in dividing cells. Depending on the antimitotic activity of podophyllotoxin derivatives, they inhibit microtubule assembly *in vitro* and competitively inhibit colchicine to a greater or smaller degree.



28 Colchicine

Mouse brain tubulin was chosen because podophyllotoxins had been extensively analysed in mouse tumors *in vitro* and *in vivo*. The high activities of podophyllotoxin (6), desoxypodophyllotoxins (8), 4'-demethylpodophyllotoxin (13) and ß- peltatin (9) were demonstrated in mouse bearing Sarcoma 37 (**Table 3**). The relative inactivities of many other analogues such as epipodophyllotoxin (16) and picropodophyllin (17) were also demonstrated in this system.

The dissociation constants for the binding of podophyllotoxin to purified microtubule protein from mouse brain have been measured. These data suggest that tubulin-binding drugs disrupt mitosis by preferential binding to only a small fraction of tubulin molecules which are critical for polymerization.

Table 3

Inhibition of *in Vitro* Microtubule Assembly by Podophyllotoxin Derivatives[25]

	Inhibition Constant K_i
Podophyllotoxin	0.51
Epipodophyllotoxin	1.20
Desoxypodophyllotoxin	0.54
β-Peltatin	0.12
β-Peltatin-A-methyl ether	0.57
4'-Demethylpodophyllotoxin	0.65

K_i determined for the inhibition of [^{3}H]-colchicine binding to mouse brain tubulin

II. 3.2. Teniposide and Etoposide

A feature of etoposide (**24**) and teniposide (**23**) is the unique mechanism of their action. While possessing the capacity for inhibiting cell growth, neither of the drugs delays mitosis. Both drugs act on the cell cycle at stages preceding mitosis and they arrest cells in the late S or G_2 phase of the cell cycle and have no effect on tubulin assembly.

They differ from podophyllotoxin and other mitotic poisons by their capacity for cleaving DNA within cells. Furthermore, etoposide inhibits the transport of nucleotides through the cell membrane somewhat more intensively than podophyllotoxin. The difference in the mechanism of action of podophyllotoxin and the majority of its derivatives on the one hand, and etoposide and teniposide on the other hand, are obvious.

The chemical structures of etoposide (**24**) and teniposide (**23**) are as shown and they differ from each other only in the nature of the substituent on the glucose ring. Etoposide is the cyclic acetal prepared from 4'-demethylepipodophyllotoxin-ß-D-glucopyranoside and acetaldehyde (as its dimethyl acetal) while teniposide is the cyclic acetal prepared when 2-thiophene carboxaldehyde replaces acetaldehyde.

In vivo experiments on leukemia L-1210 indicate a considerable superiority of the acetal derivatives of the glucosides over podophyllotoxin. Although etoposide and teniposide have similar chemical structures and the same type of mechanism of action, their action spectra *in vivo* are different. Etoposide is generally less active *in vitro* and *in vivo* than is teniposide, but it does show a more pronounced therapeutic effect in leukemic mice.

Teniposide is more active on plasmocytoma and on Walker's carcinoma. On the other hand, etoposide is more active on sarcomas 37 and 180 and also on leukemia L-1210. In the case of the latter, in contrast to teniposide, a stable increase in the survival time was observed on oral administration as well. The method of administering these drugs also has a substantial influence on their *in vivo* efficacy.

For a better understanding of the course and results of the clinical testing of teniposide and etoposide one must know that the modern chemotherapy of malignant neoplasms, as a rule, uses several antitumor drugs for each case. Monotherapy - with a single drug - is used mainly in the clinical trials of new medicinal substances. In the majority of cases, a new drug is given to patients whose cancers have proved refractory to previous treatment regimens.

Both compounds are sparingly soluble in water, but medicinal forms are obtained with the aid of solubilizing agents. This extremely instructive fact must be borne in mind before renouncing the experimental study of a new preparation because of its poor water solubility. The modified podophyllotoxin derivatives etoposide and teniposide have demonstrated significant clinical activity in the treatment of several tumors. Teniposide and possibly etoposide is one of the most active single agents in small cell lung cancer with a composite single agent response rate of 40% in 262 patients and a 6% complete response rate.[24]

Etoposide also shows pronounced activity in the treatment of testicular cancer, monocytic or myelomonocytic leukemia, non-Hodgkin's lymphomas and hepatocellular carcinoma. Teniposide has a role in the treatment of Hodgkin's disease, non-Hodgkin's lymphomas and neuroblastoma. Both drugs have meaningful activity in pediatric refractory neuroblastoma with up to 50% efficacy. Also these compounds are not very myelosuppressive.

It has been reported that no clear difference is shown between them clinically and it is also assumed to be desirable to seek for new derivatives of podophyllotoxin. The precise mechanism of action of etoposide and teniposide when known, should make it possible to design and synthesize compounds which are more effective agents. With further modification, the latter may give derivatives possessing new and unique properties.

III. STRUCTURE-ACTIVITY RELATIONSHIP

Several podophyllotoxin derivatives are known to possess antimitotic activity. This is illustrated by their ability to arrest cultured cells at metaphase and to bind to purified tubulin preparations.[6] Several structure-activity studies have been carried out on the antimitotic activity of podophyllotoxin type lignans and a number of conclusions can be drawn.

There is apparently no difference in the tubulin-binding activity of podophyllotoxin and desoxypodophyllotoxin, which suggests that the C-4 hydroxyl group is of little consequence,(**Table 3**). Epipodophyllotoxin (**16**), the C-4 stereoisomer of podophyllotoxin, and 4'-demethylepipodophyllotoxin (**29**) are less efficient at binding tubulin than podophyllotoxin. This illustrates the influence of the stereochemical requirement of the C-4 proton at the receptor site for interaction with tubulin.

The above observations taken together suggest that the increased tubulin-binding activity of the potent antitumor agent ß-peltatin is related to the presence of a hydroxyl group on ring A than to the lack of one on ring B. This is further supported by the decreased activity to approximately that of podophyllotoxin, of ß-peltatin-A-methyl ether relative to ß-peltatin. **Table 3** lists the tubulin binding affinities for podophyllotoxin analogues with substitutions in ring A, B and C.[26,27]

Glycosylation of the 4-hydroxyl group markedly reduces cytotoxic activity. Reducing the polarity of the C-4 glucose moiety results in marked increase in cytotoxic potency. It is especially interesting that the synthetic epipodophyllotoxin derivatives etoposide and teniposide used in cancer chemotherapy have no detectable effect on microtubule assembly.[27] This is in contrast to their antitumor activity which is quite appreciable. This indicates that it is the polarity of the C-4 substituent rather than its size that is the more important factor in relation to antitumor activity,

although the nature of the sugar moiety also affects biological activity.

The stereochemistry about C-2 / C-3 appears to be of importance. Picropodophyllin (**17**) displays a much reduced ability to bind tubulin and picrophyllic acid (**18**) has no detectable activity. The 3, 4, 5-trimethoxy group does not appear to be a required feature determining antitumor activity. 4'-Demethyl compounds are found to be more active than trimethoxy compounds. 4'-Demethyldesoxypodophyllotoxin is, in fact, the most active compound among the podophyllotoxins.

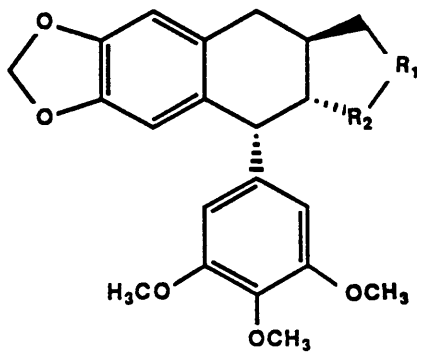
Whereas substitutions in ring A of desoxypodophyllotoxin to give the peltatins do not alter the activity to a great extent, substitutions in ring C of podophyllotoxin decreases the activity[24] (**Table 4**). However, the ring C cyclic ether derivatives of both of these compounds still retain considerable activity. Although the lactone group of ring C is not required for activity, substitutions at position 12 in ring C of compounds with a methylene group at position 13 show considerable losses of activity, which become more severe as the substituents become more bulky. This suggests strict steric requirements for the interaction of position 12 with tubulin.

Although correlations exist between the antitumor activity and the antimitotic activity of podophyllotoxin derivatives, suggesting a common mode of action, this is true for only a limited number of compounds. Noteworthy exceptions are etoposide and teniposide. They have little effect upon tubulin polymerization but do have a demonstrable effect on some types of tumor cells.

Thus it is possible to conclude that the B and C rings are involved in their interaction with tubulin. Specifically, the activity of these compounds is sensitive to the configuration, size and /or hydrophilic character of substituents at the C-4 position in the B ring and to the steric features of the substituents at the 12 position of the C ring.

Table 4

Tubulin-Binding Affinities and Inhibition of *in Vitro* Microtubule Assembly by Podophyllotoxin Derivatives Modified in Ring C[25]



	R_1	R_2	Ki*	ID_{50}**
Podophyllotoxin cyclic ether	O	H_2	5.2	1.0
Desoxypodophyllotoxin cyclic ether	O	H_2	-	0.8
Desoxypodophyllotoxin-cyclopentane	H_2	H_2	-	5.0
Desoxypodophyllotoxin-cyclopentanone	C=O	H_2	-	5.0
Podophyllotoxin cyclic sulfide	S	H_2	-	10
Desoxypodophyllotoxin cyclic sulfide	S	H_2	-	10

* K_i determined for the inhibition of [^{3}H]-colchicine binding to mouse brain tubulin

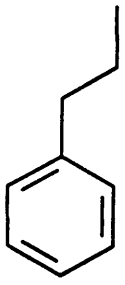
** Chiken brain tubulin was incubated with various concentrations of drug to determine the ID_{50}

IV. BIOSYNTHESIS

Despite the widespread occurrence of lignans in nature and long-established hypotheses concerning their origins, the biosynthetic pathways to these compounds are poorly investigated and little definitive information is available. Their biosynthesis is poorly understood even for medically important compounds such as demethylpodophyllotoxin, used widely in cancer chemotherapy.

Although fairly common as heartwood constituents, lignans have hardly been studied from the biosynthetic viewpoint, largely because of the practical difficulty of finding an active synthesizing system. Also, their formation by oxidative coupling between two substituted cinnamyl alcohols is very obvious from the structural variation encountered in this series.

Most of the pathways proposed involve phenolic oxidative coupling of suitable phenylpropanoid units (**30**) via a free-radical or equivalent two electron process in a manner analogous to those predicted in lignin biosynthesis. The detailed mechanism of the coupling and the separation of this process from the more random polymerization of the same monolignol units to yield lignin are not as yet clear.



30

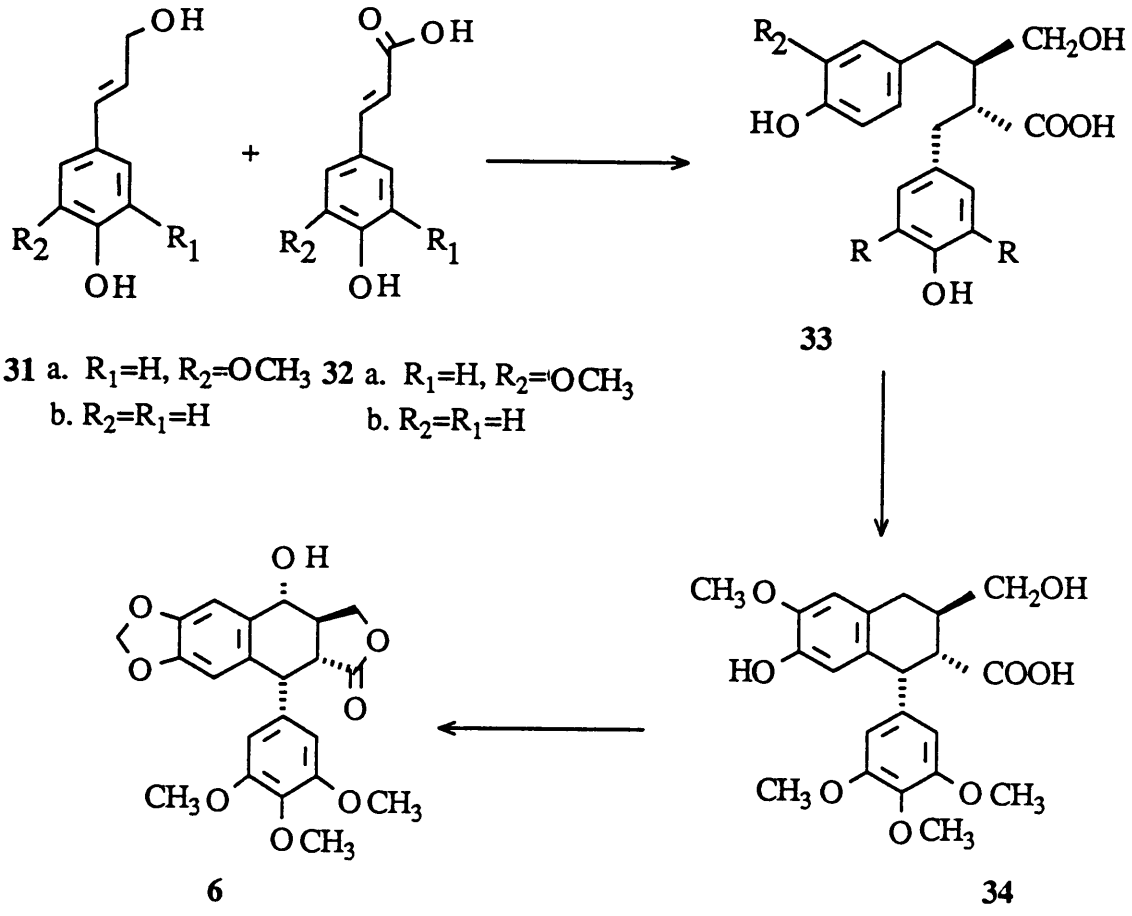
Both lignin and lignans are closely related abundant secondary metabolites produced from the shikimate/chorismate pathway. Several sub-structures of isolated lignin samples are made up of

lignan molecules e.g. isolariciresinol. The point of separation between lignin and lignan biosynthesis is not known. However, it is assumed that different biochemical mechanisms are in operation. This is because lignans are most often obtained as pure enantiomers rather than as a racemic mixture, suggesting that phenylpropanoid coupling reactions giving optically active lignans are enzymatically controlled.

At present, a number of theories exist on the biosynthesis of these phenylpropanoids including the proposed dimerization of two C_6-C_3 units, stereospecifically coupled at the ß-carbon atoms of their side chains. For example, a free-radical coupling reaction (or an equivalent two-electron process) between a hydroxycinnamyl alcohol and a substituted hydroxycinnamic acid, followed by oxidative modification to podophyllotoxin could occur as shown in **Scheme 1**. However, direct experimental evidence for this hypothesis is lacking.

Such a scheme was in fact explored recently in attempts to understand the biosynthesis of podophyllotoxin. Feeding experiments with *P.hexandrum* have demonstrated the incorporation of radioactivity from phenylalanine [^{14}C], cinnamic acid [3-^{14}C] and ferulic acid [2-^{14}C] into podophyllotoxin in confirmation with the proposed pathway.[28,29] An experiment with [Me-^{14}C] ferulic acid yielded podophyllotoxin in which both halves were equally labelled, implying that coupling involves two phenylpropane units of the same substitution pattern, possibly the ferulic acid (**32b**) pattern. Since plant lignans are optically active, these dimerization reactions should proceed by an enzyme catalyzed stereospecific coupling of *p*-hydroxy C_6-C_3 units having a double bond in the side chain, to give an optically active dimer which could give rise to the known lignans by further oxidation (and dehydration) steps.

Structural analysis of the range of *Podophyllum* lignans encountered in *P.hexandrum* and *P.peltatum* has indicated that two groups of lignans are present, those containing 3,4,5-trimethoxy



31 a. R_1=H, R_2=OCH_3 **32** a. R_1=H, R_2=OCH_3
b. R_2=R_1=H b. R_2=R_1=H

Scheme 1 Postulated biosynthetic pathway to podophyllotoxin

substituted pendant ring and those with 4-hydroxy-3,5-dimethoxy substitution. Relationships among these aryltetralin lignans were in fact explored.

Administering desoxypodophyllotoxin (**8**) into *P. hexandrum* has indicated that podophyllotoxin is derived by the hydroxylation of desoxypodophyllotoxin (**Scheme 2**).[29] However, it should be noted that it was not incorporated into 4'-demethylpodophyllotoxin (**7**) or α-peltatin (**10**). Thus *Podophyllum* lignans may be subdivided biogenetically into two groups by their substitution pattern in the pendant ring, which presumably arise from an as yet unidentified common precursor.

Feeding experiments showed very good incorporation of demethyldesoxypodophyllotoxin (**13**) into 4'-demethylpodophyllotoxin (**7**).[29] Thus, methylation of a 4'-demethyl compound to give the corresponding compound in the trimethoxy series does not occur. However, 4-hydroxylation of demethyldesoxypodophyllotoxin (**13**) occurs, analogously to the observed conversion of desoxypodophyllotoxin to podophyllotoxin (**6**).[29]

The stereospecific benzylic hydroxylation of desoxypodophyllotoxin (**8**) is mechanistically acceptable and the sequence proposed is supported by the observed distribution of lignans in nature.[30] Thus desoxypodophyllotoxin has been isolated from a whole range of higher plants whereas podophyllotoxin has a much more restricted distribution and podophyllotoxone (**11**) has only been found in *Podophyllum* species.

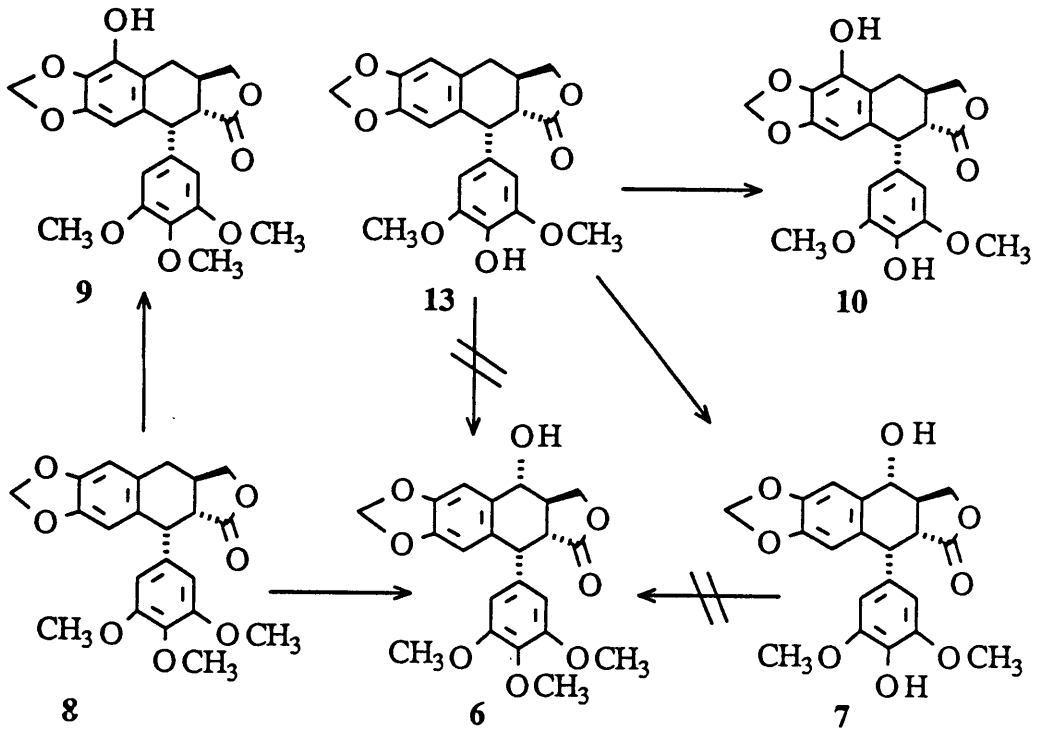
The hydroxylation of desoxypodophyllotoxin does, however, invalidate a number of hypotheses for the biosynthesis of podophyllotoxin and related compounds, in which the 4-hydroxyl is introduced soon after oxidative coupling of the phenylpropane units by addition to a quinone methide intermediate e.g. **Scheme 3**. Such schemes do not reflect the close biosynthetic relationship observed among the three major lignans of *P.hexandrum* namely, podophyllotoxin (**6**), desoxypodophyllotoxin (**8**) and podophyllotoxone (**11**). These compounds are chemically related

therefore by the oxidation level at carbon-4 and may be expected to be related biosynthetically by simple oxidation/reduction process. This would suggest that the production of 4-desoxy and 4-oxy aryltetralin lignans diverges at an early stage.

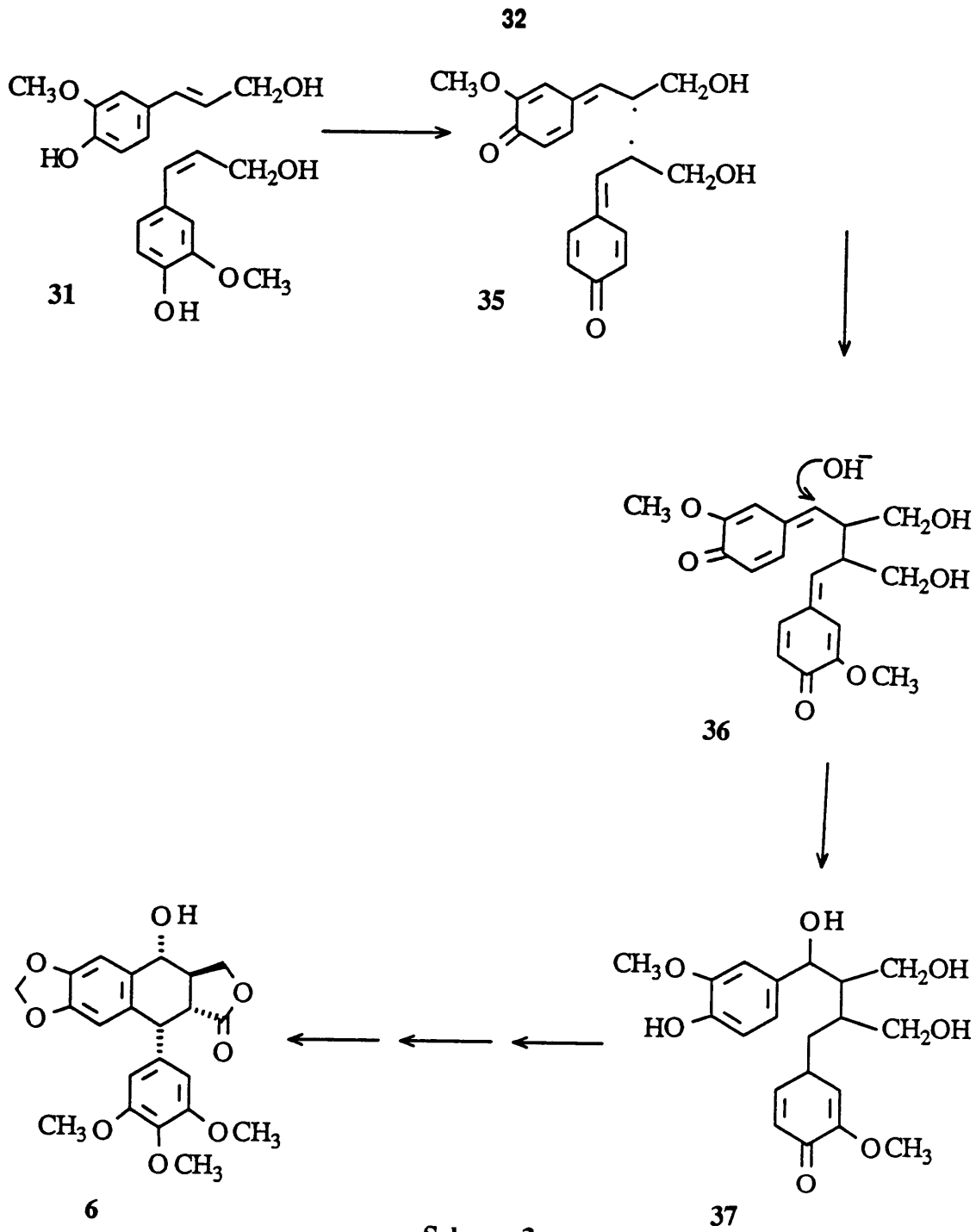
The position of the peltatins has also to be established. In **Scheme 2**, a possible relationship has been indicated, by 5-hydroxylation as opposed to 4-hydroxylation giving podophyllotoxin derivatives. The significant difference in proportions of constituents of *P.hexandrum* (principally podophyllotoxin and 4'-demethylpodophyllotoxin) and *P.peltatum* (principally α- and ß-peltatins) could then be accounted for by preferential hydroxylation at C-4 or C-5 respectively. Hydroxylation could of course occur earlier, at the same time as other substitution patterns are established in the two aromatic rings being built up from the initial ferulic (4-hydroxy-3-methoxy) pattern.

It should be noted however that the incorporations observed in the work cited were very low and the results should thus be regarded with some reservation in the absence of confirmation data. Administering specifically labeled precursors with stable isotopes to both intact plants and callus cultures in larger doses and for extended periods will enable one to monitor the exact biochemical transformations of these precursors.

It has recently been reported that callus cultures of the organism *P.peltatum* produce podophyllotoxin and related compounds; the yields of these substances were about one percent on a dry weight basis.[31] This finding indicates that the enzymes involved in the biosynthesis of these lignans are present in the callus cultures as well as in the intact plant. When culture conditions are adequately developed, significant incorporation levels of the precursors into the lignan podophyllotoxin might be achieved. This is because tissue cultures do not normally have as severe translocation difficulties of the precursors to the site of biosynthesis as do intact plants.



Scheme 2 Lignan interconversions demonstrated in *P. hexandrum*

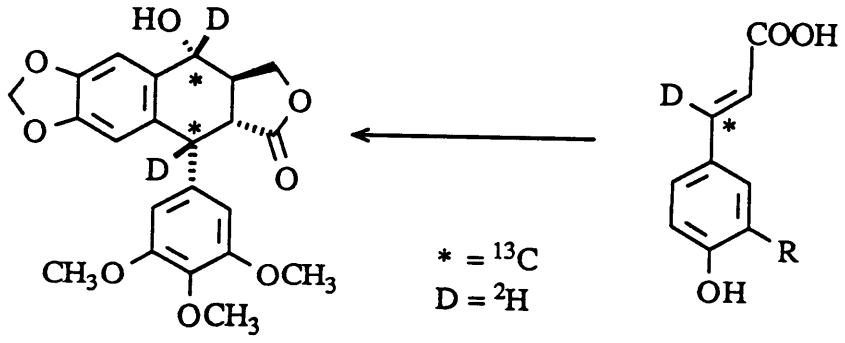

32
CH3O
CH2OH
HO
CH2OH
OCH3
OH
31
35
OH
CH3O
CH2OH
CH2OH
OCH3
36
37
6

Scheme 3

Specifically labelled phenylpropanoid precursors of podophyllotoxin can show the identity and the oxidation state, if any, of these precursors. Feeding desoxypodophyllotoxins diastereomerically labelled at C-4 will prove beyond doubt if the oxidative hydroxylation to podophyllotoxin is stereospecific. One of the diastereomers is expected to be preferentially incorporated with retention of the label, while the doubly labelled desoxypodophyllotoxin will show the extent of incorporation possible in the experiment.

The use of plant tissue cultures in principle makes possible the isolation of sufficient metabolites to carry out analysis by stable isotope measurements by nmr techniques and mass spectral analysis. The use of precursors labelled with stable isotopes(^{13}C, ^{2}H) and dual labelled ($^{13}C/^{2}H$, $^{2}H/^{2}H$) samples makes possible a much more detailed analysis of the biosynthetic pathway than has ever been possible.[32,33,34]

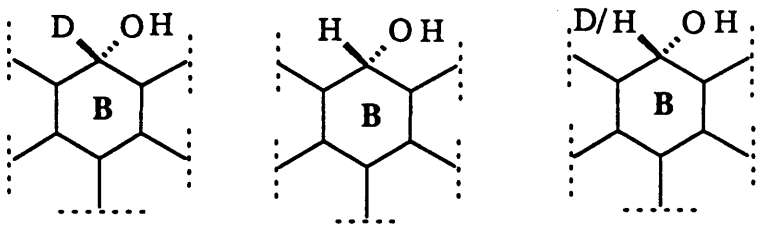
We can follow not only the fate of precursors labelled specifically with ^{13}C, but also the deuterium atoms at the same enriched carbon atom, since this double isotopic technique is a powerful aid to understanding the details of the biosynthetic reactions. Feeding the labeled p-coumaric acid precursor indicated should result in the labelling pattern as shown.



Incorporation of labelled precursors

Labelling of carbon-1 with both deuterium and carbon-13 will unequivocally demonstrate whether or not the methine carbon is derived from p-coumaric acid and if the precursors are incorporated intact with retention of deuterium. This labelling pattern can be proven using nuclear magnetic resonance spectroscopic techniques.

A similar type of labelling pattern should also be evident at C-4. In the case of podophyllotoxin, the stereospecific introduction of the hydroxy group could occur in several ways. The labelling pattern observed should distinguish between possibilities I-III.



I II III

Labelling pattern in ring B

I. In this case deuterium is retained because addition of the oxygen to the double bond (p-coumaric acid) is completely stereospecific.

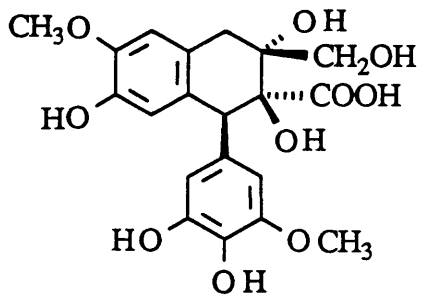
II. The oxygen atom is added to form a carbonyl functionality which on stereospecific reduction offers the corresponding alcohol. In this case there would be no retention of deuterium.

III. In this case the intermediate prior to podophyllotoxin has a methylenic functionality (desoxypodophyllotoxin) at C-4. The stereochemical consequences of this could range from total to partial loss of deuterium.

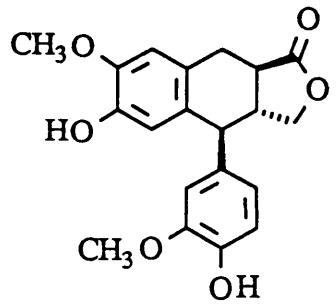
The biologically active desoxypodophyllotoxin is found to be an immediate intermediate of the biosynthetic pathway of podophyllotoxin and its hydroxylation should involve enzymes. Most *in*

vivo hydroxylations, for example, hydroxylation of Brefeldin C to Brefeldin A proceed stereospecifically.[36] A study of this enzymatic hydroxylation is of importance in defining the mechanism of enzymatic hydroxylation and if it proceeds with retention or inversion of configuration. This will also give insight into the type of enzyme needed for the operation and the stereochemical requirement for the presence of three chiral centers located on ring B at C-2, C-3 and C-4 with regard to its effect upon biological activity.

In assessing possible biosynthetic pathways for lignans, it is noteworthy that the anticancer agent podophyllotoxin **6**, α-conidendrin **38** and plicatic acid **39** are structurally similar and their postulated biosynthetic origins are closely parallel. An investigation of aryltetrahydronaphthalene lignan biosynthesis should also provide fundamental information concerning the biogenesis of other types of lignans e.g. those containing diarylbutane, diarylbutyrolactone and substituted furan skeletons.



39 Plicatic acid



38 α- Conidendrin

The information available on the biosynthesis of lignans is incomplete and fragmentary. Recent advances in both plant tissue culture and nmr techniques coupled with the use of stable isotopes present an unusual opportunity to make substantive progress in this important field of biosynthesis

V. RESULTS AND DISCUSSION

V.1 Rationale

Podophyllotoxin is a tetrahydronaphthalene lignan and anticancer agent produced by *Podophyllum* and some other species. Lignans are widespread in plants and are a structurally diverse class of compounds. They have been found in 46 families, 87 genera and 146 species. The biosynthetic pathways to these lignans are poorly investigated and little definitive information is available. Studies that have been reported to date have concentrated on the demonstration of the incorporation of C_6-C_3 units into lignans and on the nature of the preferred units. Little is known about the nature of transformations or the immediate products of coupling.

Desoxypodophyllotoxin is the penultimate intermediate of the podophyllotoxin pathway and its hydroxylation at C-4 should involve enzymes since it is optically active. These observations suggest that the conversion of desoxypodophyllotoxin to podophyllotoxin is enzymatically controlled. If true, then stereospecific removal of one of the diastereotopic C-4 hydrogens from desoxypodophyllotoxin to podophyllotoxin should occur during the hydroxylation *in vivo*. This assumption can be confirmed by isotopic labelling experiments.

Stereospecific hydroxylation of the diastereotopic C-4 position with retention of configuration is consistent with many other examples showing that biological hydroxylations mediated by enzymes involve the stereospecific replacement of a C-H bond by a C-OH bond. The observation of that mechanism would reveal the nature of the enzyme involved in the conversion of desoxypodophyllotoxin to podophyllotoxin. An example of studies involving ^2H labelling is found in some studies of antibiotic biosynthesis.[36]

The work contributed here has been to the synthesis of two desoxypodophyllotoxins diastereotopically labelled with ^{2}H at C-4. These can be used for the C-4 hydroxylation studies, to determine if in fact the conversion occurs with retention of configuration or not. We anticipate that one of these compounds would lose most of its ^{2}H label during hydroxylation. Doubly ^{2}H labelled desoxypodophyllotoxin at C-4 was also prepared which can be used for a control experiment to show the extent of incorporation of label to be expected (**Fig. 4**). Thus incorporation into podophyllotoxin would have to be verified by mass spectral and nmr analysis or both.

A second part of the work has been the synthesis of p-coumaric acid, a known lignan precursor, doubly labeled with ^{2}H or ^{2}H and ^{13}C at the C-3' position. This labeling was selected because monomeric coupling and oxidative modifications of lignan precursors occur at this position, and the availability of a C-3' labeled monomeric precursor would thus make possible the isolation and study of dimeric compounds labeled at the key positions (C-4 and C-1).

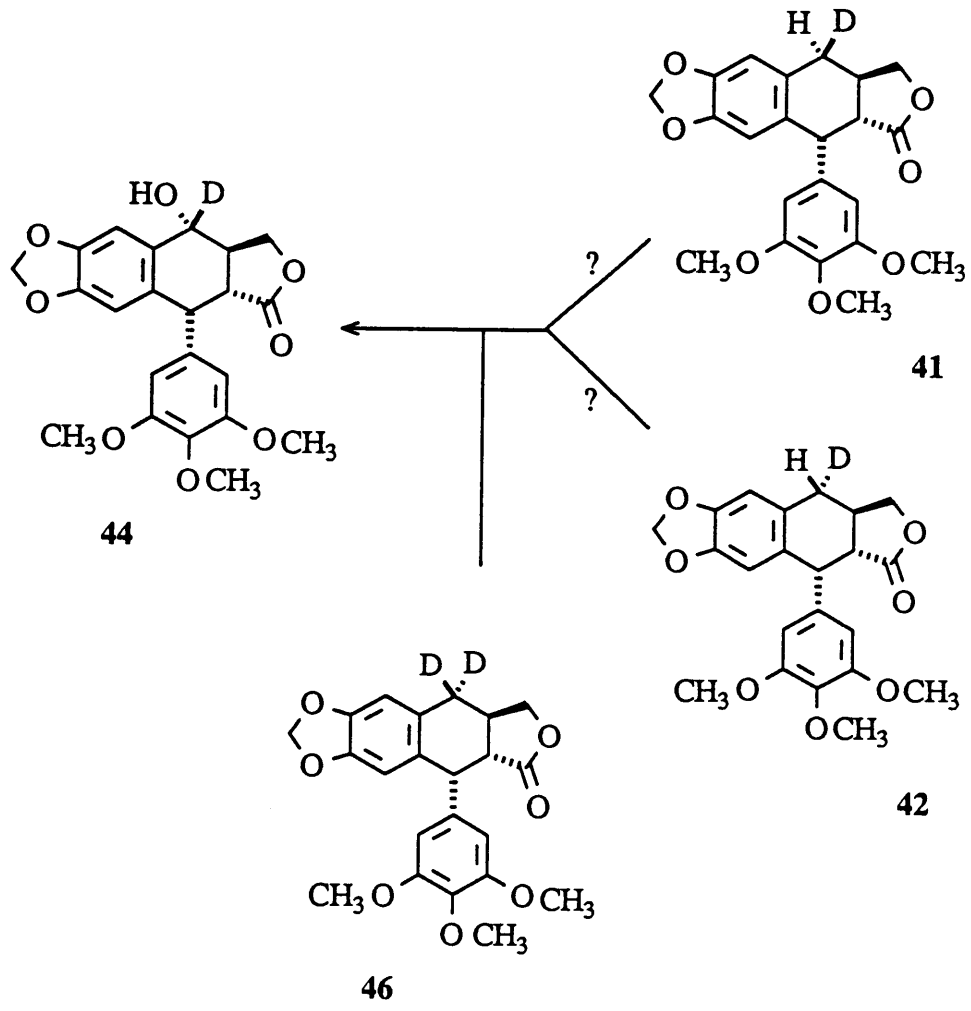
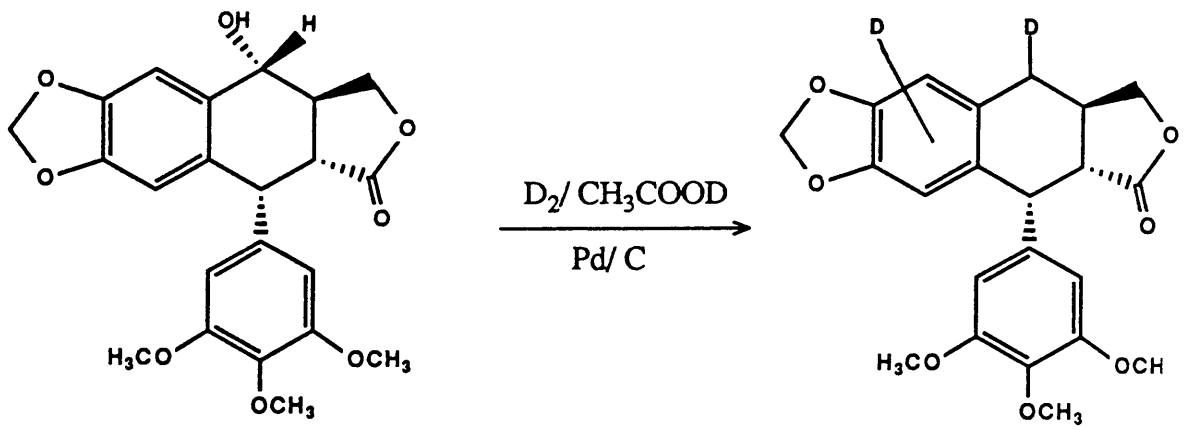


Fig. 4 Incorporation of labelled desoxypodophyllotoxins

V. 2. Synthesis of Desoxypodophyllotoxins

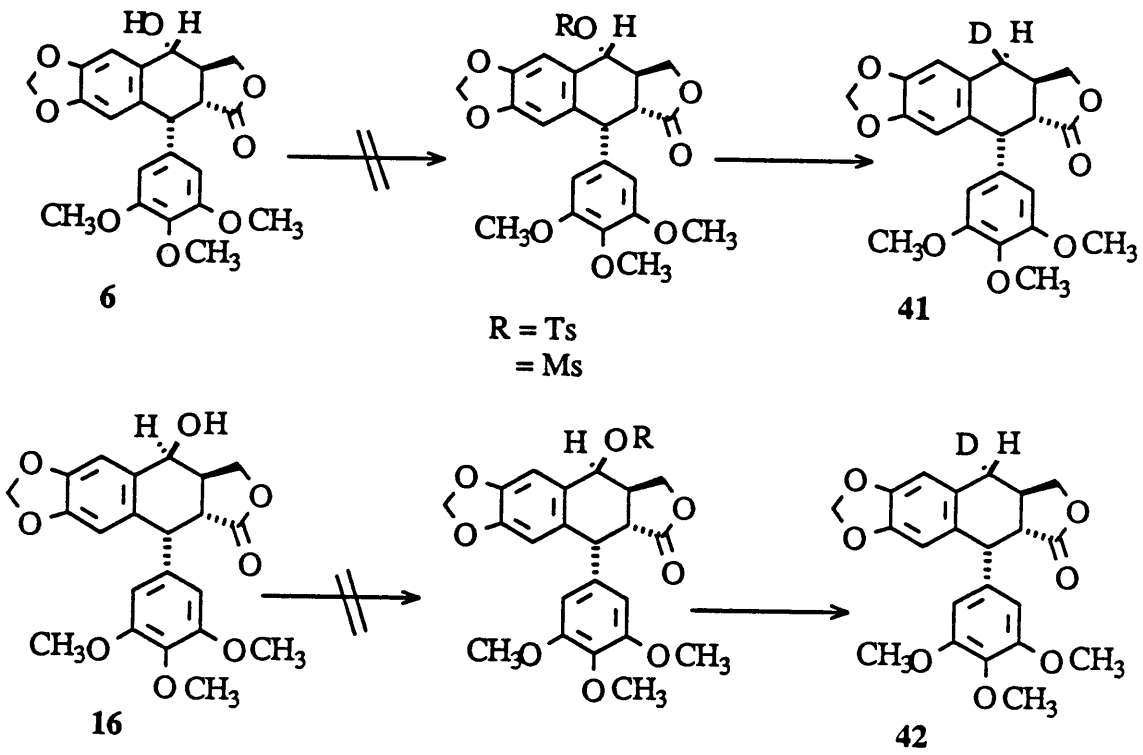
The goal of this work is to make stereospecifically [4-^{2}H]-labeled desoxypodophyllotoxin. Our initial approach was by catalytic deuteration of podophyllotoxin. Catalytic hydrogenation of podophyllotoxin to desoxypodophyllotoxin is a known reaction, and catalytic deuteration could thus potentially provide a simple route to the preparation of labeled desoxypodophyllotoxin.[29] However, it was found to be inappropriate because ring deuteration occurred and the reaction proved not to be stereospecific.



Catalytic hydrogenolysis of podophyllotoxin

A two-step process of converting podophyllotoxin to a 4-derivative which could be reduced stereospecifically to a [4-^{2}H$_1$]-desoxypodophyllotoxin was then attempted. Our initial venture was at making a 4-tosylate (or mesylate) since both podophyllotoxin and epipodophyllotoxin are available and thus both stereoisomers could be made in a stereochemically unambiguous manner. However,

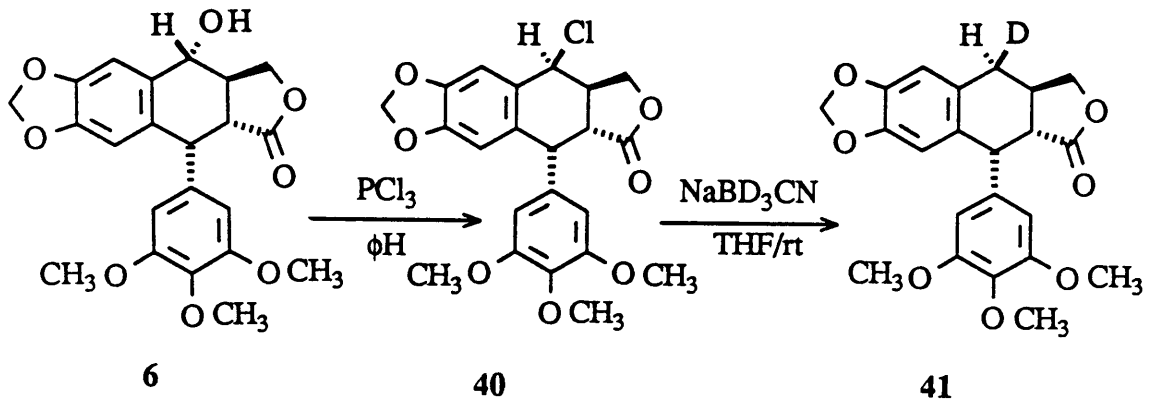
the 4-sulfonates proved to be too unstable and could not be prepared and characterized (**Scheme 4**). We then turned to the 4-chloro derivatives, which have been prepared previously. The literature suggests that both podophyllotoxin and epipodophyllotoxin yield the same chloride.[10] We were able to verify this finding and use it to develop a synthesis of both diastereomers of [4-$^{2}H_{1}$]-desoxypodophyllotoxin.



Scheme 4

V. 2. 1 Synthesis of [4β-$^2H_1$]-Desoxypodophyllotoxin (41).

Scheme 5 shows the synthetic scheme for the preparation of the target compound. Podophyllotoxin (6) was isolated from the commercially available extract podophyllin, derived from *P. peltatum*. This is converted into the chloride on treatment with phosphorus trichloride using the procedure reported by Hartwell and Schrecker.[10] Reaction of podophyllotoxin with either thionyl chloride, phosphorus trichloride or acetyl chloride gives the same podophyllotoxin chloride, but phosphorus trichloride was found to give the purest material and was therefore the halogenating agent of choice for preparative purposes.



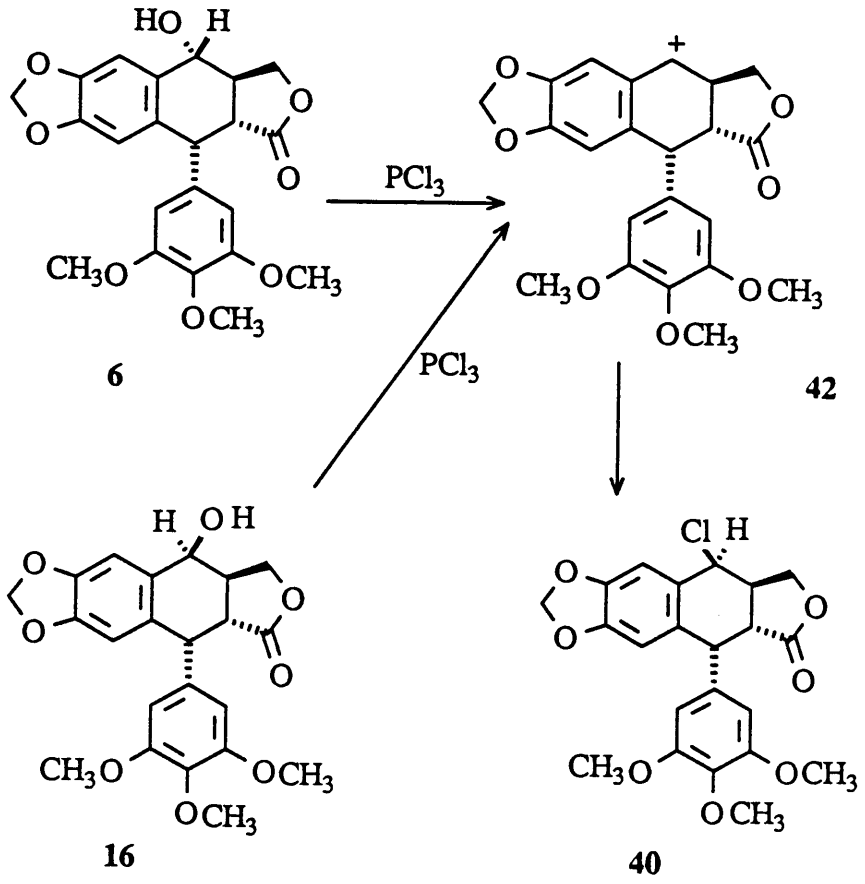
Scheme 5 Synthesis of [4β-$^2H_1$]-desoxypodophyllotoxin

Anhydrous podophyllotoxin was refluxed with PCl_3 in benzene and the solution was decanted off of the $P(OH)Cl_2$ and evaporated *in vacuo* to yield white amorphous podophyllotoxin chloride (**40**). The reaction of podophyllotoxin under these conditions has very high stereospecificity and only one product was obtained. The chloride was soluble in benzene and acetone, sparingly

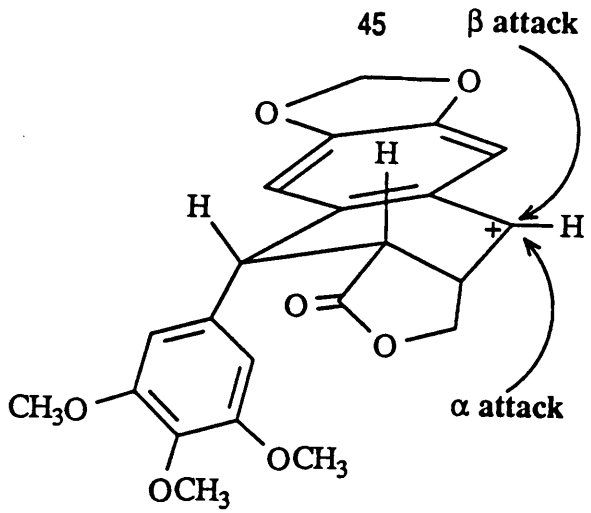
soluble in ether, and insoluble in hexane. In the presence of water or alcohol, it decomposed readily yielding the C-4 epimeric alcohol (**16**) or the epi-ether, presumably by an ionic mechanism. Similarly, ionic character in the displacement at C-4 can be induced by the interaction or complexation of the hydroxyl-group with phosphorus trichloride.

Investigation of the C-4 hydroxy-group displacement reaction established a tendency for net inversion in the reactions of podophyllotoxin and for retention of configuration in those of its C-4 epimer (epipodophyllotoxin) with reagents of a similar type.[10,37] This was tested by treating epipodophyllotoxin (**16**) with phosphorus trichloride under similar conditions. The same podophyllotoxin chloride (**40**) was the resulting product in both cases (**Scheme 6**). This is presumed to arise through a mechanism involving a common C-4 carbocation (**42**), stabilized by mesomeric interaction with the methylene-dioxy group on aromatic ring A and subsequent attack by the nucleophile from the less hindered ß-face (as that remote from the pendant aryl substituent. Carbocation (**42**) would be expected to be very stable, since the aryl ring carries two alkoxy substituents.

Sodium cyanoborodeuteride in THF provides a convenient and selective system for the reductive displacement of the chloride (**40**).[38] Reagents that are capable of reducing a given functional group in the presence of various other sensitive functional groups have been prepared by modifying the reducing power of complex metal hydrides. For example, substituted borohydrides are a particularly successful modification. The steric and electronic effects of substituents greatly influence the reactivity of the borohydride ion. Thus, sodium cyanoborohydride, with its strongly electron withdrawing cyano group, is a milder and more selective reducing agent than sodium borohydride.



Scheme 6 Mechanism of chlorination



α and β faces of carbocation **42**

Sodium cyanoborohydride is an extremely useful reagent for the selective reduction of organic functional groups. The superior selectivity possible for the reductive displacement of halides is demonstrated by its inertness toward almost all other functional groups and even such sensitive groups as lactones and epoxides. Finally, the solubility in polar aprotic solvents has further enhanced its utility as a reducing agent. **Table 5** shows the solubility of sodium cyanoborohydride in various solvents.

Commercially available sodium cyanoborodeuteride (98 atom % D) was dried *in vacuo* and dissolved in freshly dried THF. The podophyllotoxin chloride (**40**) was dissolved in benzene and stirred with excess cyanoborodeuteride in THF at room temperature. The reductive displacement was relatively slow and the residual cyanoborodeuteride was decomposed using ethyl acetate, followed by water and methanol. The deuterated desoxypodophyllotoxin was then extracted with chloroform and purified by column chromatography on a silica gel column. The incorporation of deuterium in the product was found to be variable (30-95 atom % D) and was generally lower than the starting sodium cyanoborodeuteride (98 atom % D).

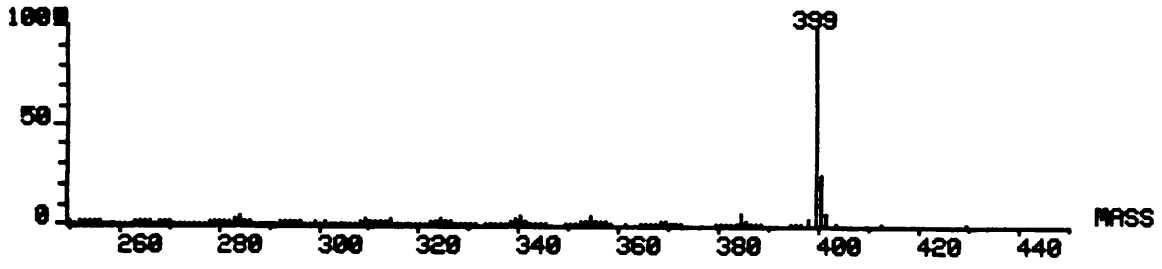
Table 5

Solubility of Sodium Cyanoborohydride in Various Solvents

Solvent	Temperature (°C)	Solubility (g/100g Solvent)
THF	28	37.2
	46	41.0
	62	42.2
Water	29	212.0
	52	181.0
	88	121.0
Methanol	24	very soluble
Ethanol	24	slightly soluble
Diglyme	25	17.6
Isopropylamine	25	slightly soluble

Since the decrease in the ^{2}H incorporation was variable, an effort was made to identify the contributing factors. When hydrochloric acid was used in the decomposition of residual sodium cyanoborodeuteride the lowest ^{2}H incorporation was observed. The substitution of ethyl acetate for hydrochloric acid in the decomposition of the reagent increased the deuterium content of the product tremendously. The other factor contributing to the variable incorporation of deuterium is the purity of THF. The use of unpurified or of aged purified THF resulted in a lower deuterium incorporation. The products using freshly purified THF had the highest deuterium incorporation. In summary, if aprotic ethereal solvents such as THF or diglyme have to be used, freshly purified solvent and a large excess of cyanoborodeuteride have to be employed to avoid isotope exchange and to ensure complete reduction.

The yield of the product was 74%. The isotopic content of the resulting [4β-$^{2}H_{1}$]-desoxypodophyllotoxin (41) was determined with a mass spectrometer in the EI mode to be 95.6% of deuterium incorporation as presented in the mass spectrum of [4β-$^{2}H_{1}$]-desoxypodophyllotoxin . Proton nmr spectroscopy revealed that deuterium is present only at C-4 in the product and that it has a 4-ß or epi configuration (41, **Fig. 5**). This is consistent with metal hydride reduction of the chloride from the less hindered (ß face) of the molecule.



Mass spectrum of [4β-$^{2}H_{1}$]-desoxypodophyllotoxin (41)

Thus the quartet at δ 2.2 due to 4α-H has become a broad doublet and what used to be a quartet at δ 1.88 due to 4ß-H is not seen. The assigned stereochemistry was substantiated by the small coupling constant measured, $J_{3,4\alpha}$ 5.3Hz, consistent with the cis 3-Hα, 4-Hα alignment. Thus the product is [4β-$^{2}H_{1}$]-desoxypodophyllotoxin (**41**) and has the (1β,2β,3α,4α) configuration.

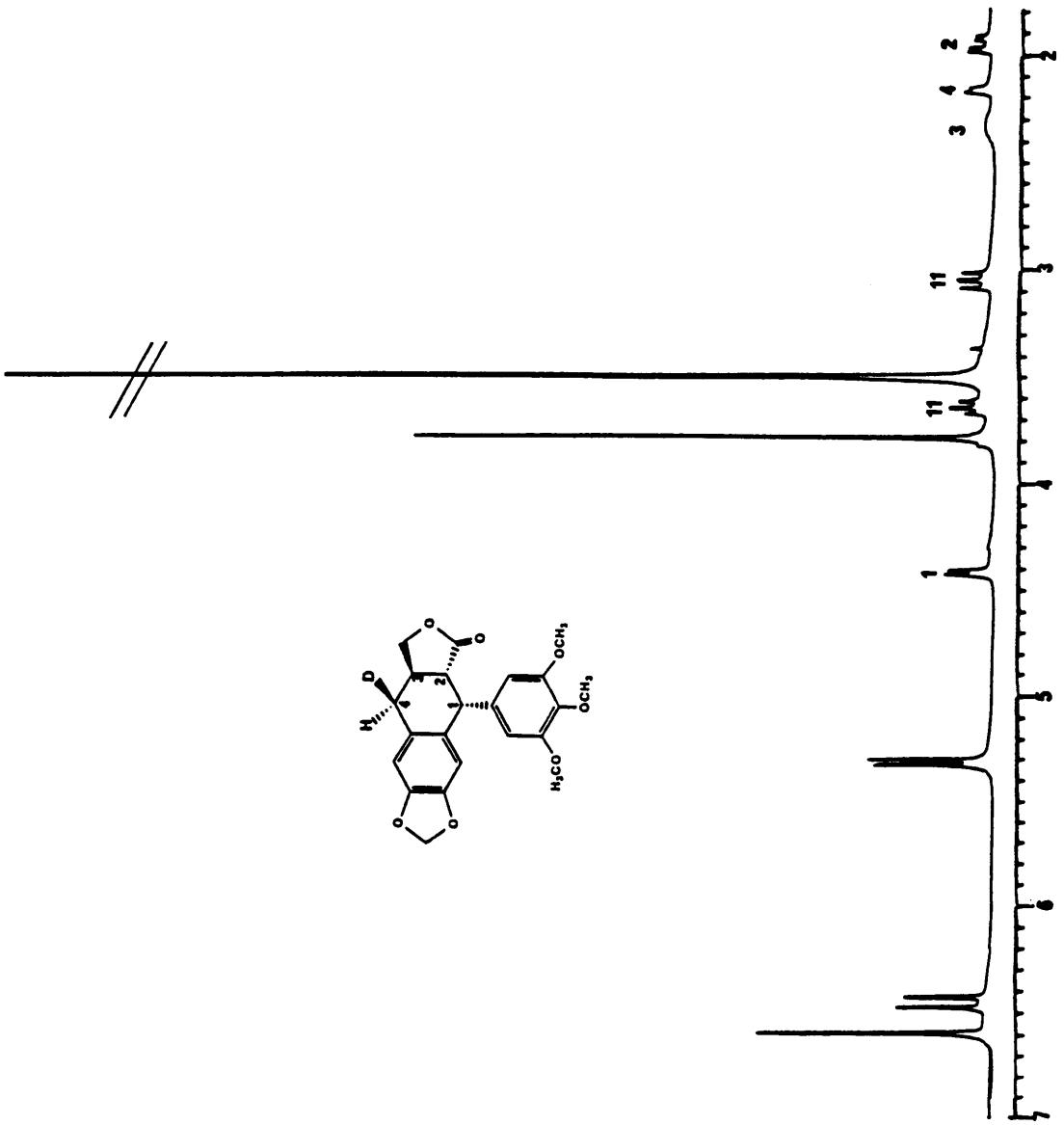


Fig. 5 Proton nmr of [4β-$^2H_1$]-desoxypodophyllotoxin (41)

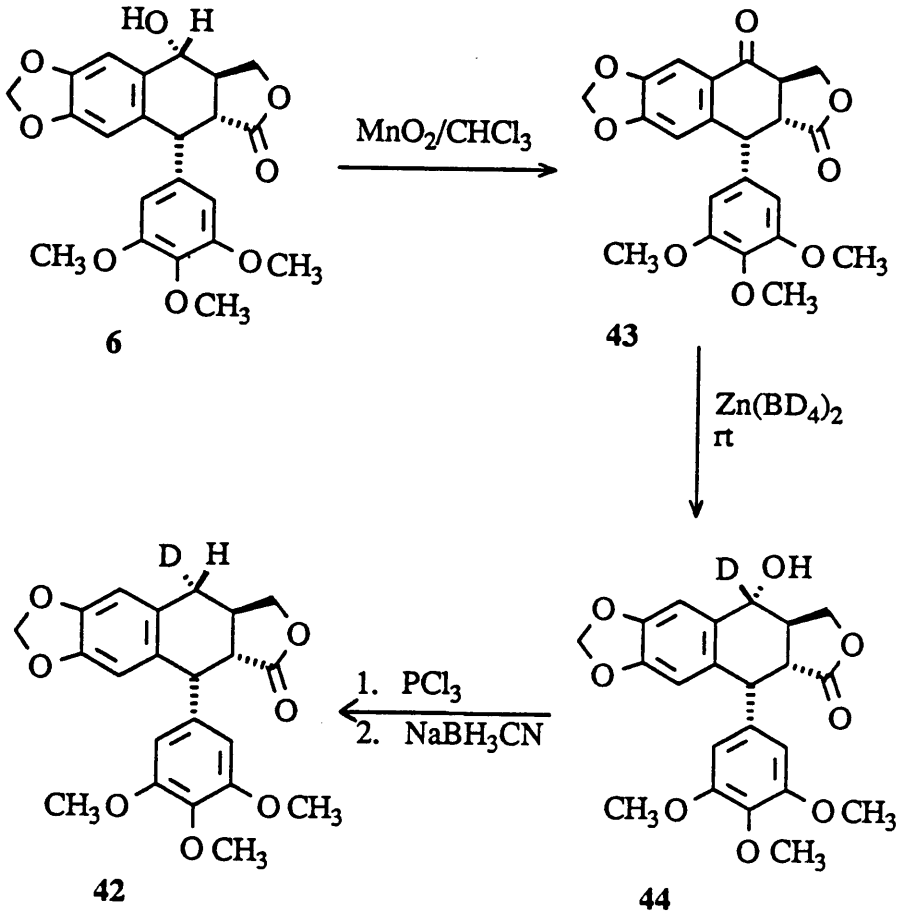
V. 2. 2. Synthesis of [4α-^{2}H1] Desoxypodophyllotoxin (42)

The preparation of [4α-$^2H_1$]-desoxypodophyllotoxin (**42**) was accomplished using the same reaction sequence used for the synthesis of the epimeric compound, replacing the sodium cyanoborodeuteride with sodium cyanoborohydride and starting with deuterated podophyllotoxin (**44**) (**Scheme 7**).

Podophyllotoxin (**6**) can be converted to the deuterated form by oxidation to the corresponding ketone (**43**) and subsequent reduction of the resulting ketone with zinc borodeuteride to afford [4β-$^2H_1$]-podophyllotoxin (**44**).[39] It has previously been reported that podophyllotoxone can be reduced with zinc borohydride to afford podophyllotoxin as the sole alcoholic reduction product. The interconversion between podophyllotoxin and podophyllotoxone is known and has been fully discussed by Gensler *et al.*[39] Thus manganese dioxide is proved to be effective for the oxidation where various other oxidizing reagents like potassium dichromate, chromic anhydride, ethylene with copper chromate catalyst and acetone with aluminum isopropoxide failed.

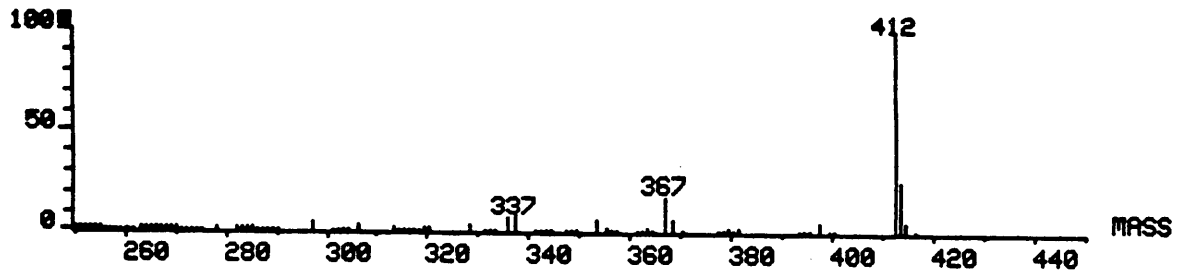
On the basis of the reported preparation, podophyllotoxone (**43**) was obtained by boiling podophyllotoxin with manganese dioxide (commercial grade) in chloroform solution. The resulting ketone was purified using column chromatography on silica gel column to yield 73% of pure compound. The proton nmr spectrum in chloroform solution is identical to the reported spectrum of desoxypodophyllotoxin in the same solvent.[42] The mass spectrum of the compound gave the characteristic mass spectrum of podophyllotoxone.

A satisfactory reagent for the reconversion of the ketone to podophyllotoxin with the preservation of the trans lactone function and avoiding any epimerization at C-2, which is alpha to the lactone carbonyl, was found in ether-soluble zinc borohydride.[39] Zinc borodeuteride is not



Scheme 7 Synthesis of [4β-$^2H_1$]-desoxypodophyllotoxin

commercially available but can easily be prepared from sodium borodeuteride and zinc chloride. Anhydrous zinc chloride (Aldrich) was dissolved in ether and the ethereal solution was added to a suspension of sodium borodeuteride (98 atom %D) in ether. The clear solution of zinc borodeuteride was then stored under inert atmosphere and was found to be very stable.



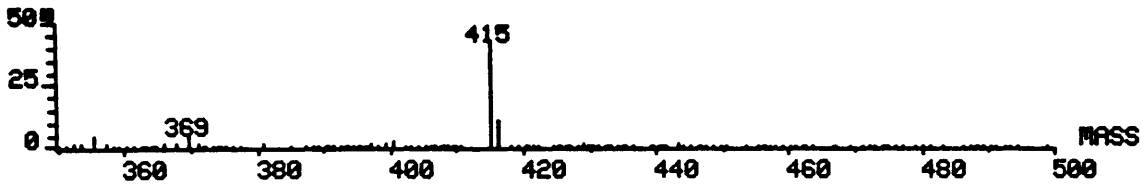
Mass spectrum of podophyllotoxone (43)

Zinc borodeuteride is a very specific reducing agent and milder than sodium borodeuteride. With this reagent the keto lactone podophyllotoxone (43) gave [4β-$^2H_1$]-podophyllotoxin (44) as the sole alcoholic reduction product. The podophyllotoxone was dissolved in benzene and added to excess reagent and stirred at room temperature to yield 65% of the purified product (44). Despite the fact that the reduction of ketone with these reagents in ethereal solvents is relatively slow and incomplete, the use of such solvents in the synthesis of deuterated alcohols is widely practiced for high isotope incorporation and for economic use of the reagent.

The confirmation that podophyllotoxone yields the alcohol as the reduction product provides a consistent stereochemical picture of the metal hydride reduction of the rigid trans lactone, with hydride attacking from the less hindered side, away from the pendant aryl ring of the molecule. The

evidence for the stereochemistry of the alcohol provides further support for this view.

The identity of the reduction product was established by direct spectral comparison with authentic sample. Its ^1H nmr spectrum in chloroform is comparable to the known spectrum except that the 4-H resonance at δ 4.77 is not seen due to deuteration at that site. The isotopic content of the resulting podophyllotoxin was determined by monitoring the ions at *m/z* 414 for unlabelled and *m/z* 415 for monodeuterated material. Thus nmr and mass spectra confirmed the structure of the product as [4β-$^2H_1$]-podophyllotoxin (44) and gave a figure of greater than 99% for the extent of deuteration.



Mass spectrum of [4β-$^2H_1$]-podophyllotoxin (44)

The deuterated podophyllotoxin chloride (45) was prepared by treating deuterated podophyllotoxin (44) with phosphorus trichloride, followed by reductive displacement with sodium cyanoborohydride, in analogy with the reaction of chloride (40) with sodium cyanoborodeuteride. As predicted the product was confirmed to be [4α-$^2H_1$]-desoxypodophyllotoxin (42) by spectral studies and mass spectral analysis. The assignment was substantiated by the distinct pattern observed in the nmr spectrum of the product (42, Fig. 6). The deuterium is present only at C-4 and it has a broad doublet at δ 1.88 due to 4β-H while what used to be a quartet at δ 2.2 due to 4α-H is not seen. The coupling constant of $J_{3,4\beta}$ 11.4Hz, measured further proves the assigned stereochemistry of protons 3 and 4.

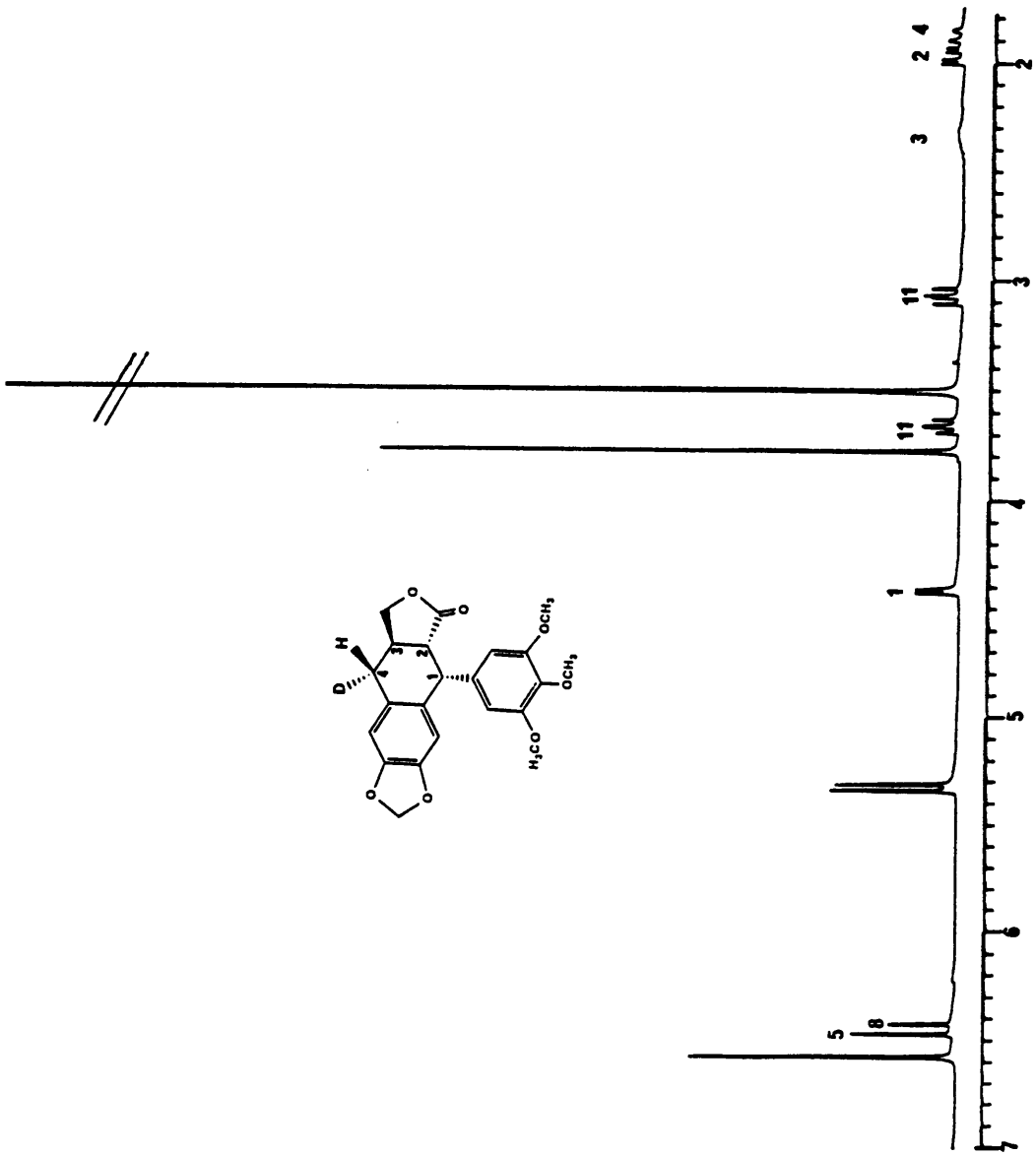
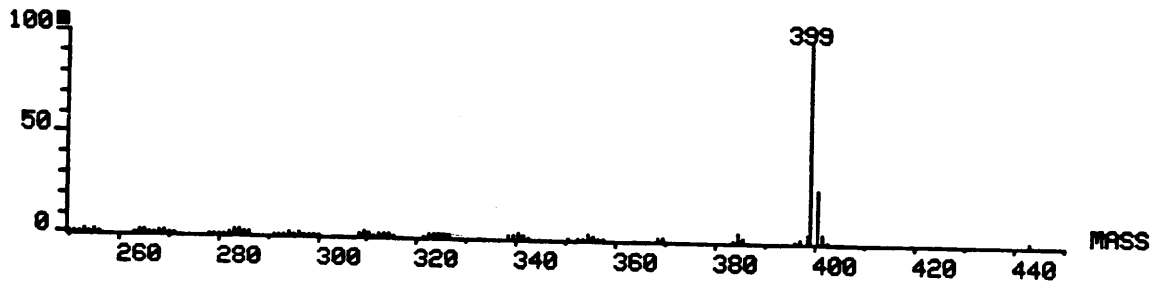


Fig. 6 Proton nmr spectrum of [4α-$^2H_1$]-desoxypodophyllotoxin (42)



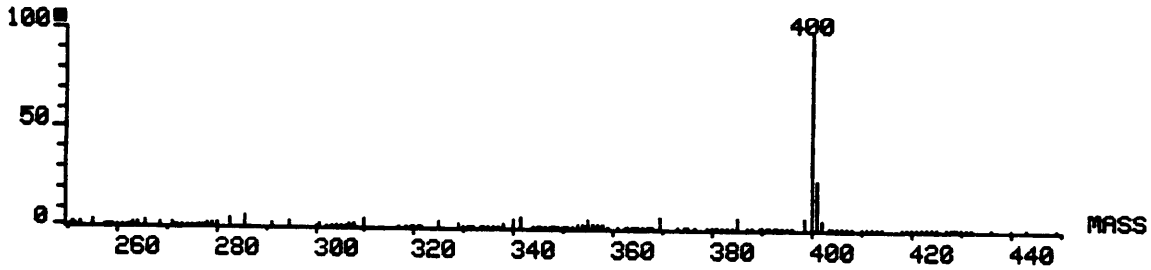
Mass spectrum of [4α-$^2H_1$]-desoxypodophyllotoxin (42)

The mass spectrum of the product showed that the isotopic content of the product was greater than 95% D. An FTIR spectrum of the desoxypodophyllotoxin (42) as a KCl wafer gave the known characteristic spectrum of desoxypodophyllotoxin and showed in addition a deuterium stretching band. Mixed melting points and tlcs proved that the compound is identical with authentic desoxypodophyllotoxin.[53] Thus the evidence is consistent with the product stereochemistry of (1β,2β,3α,4β) for [4α-$^2H_1$]-desoxypodophyllotoxin (42).

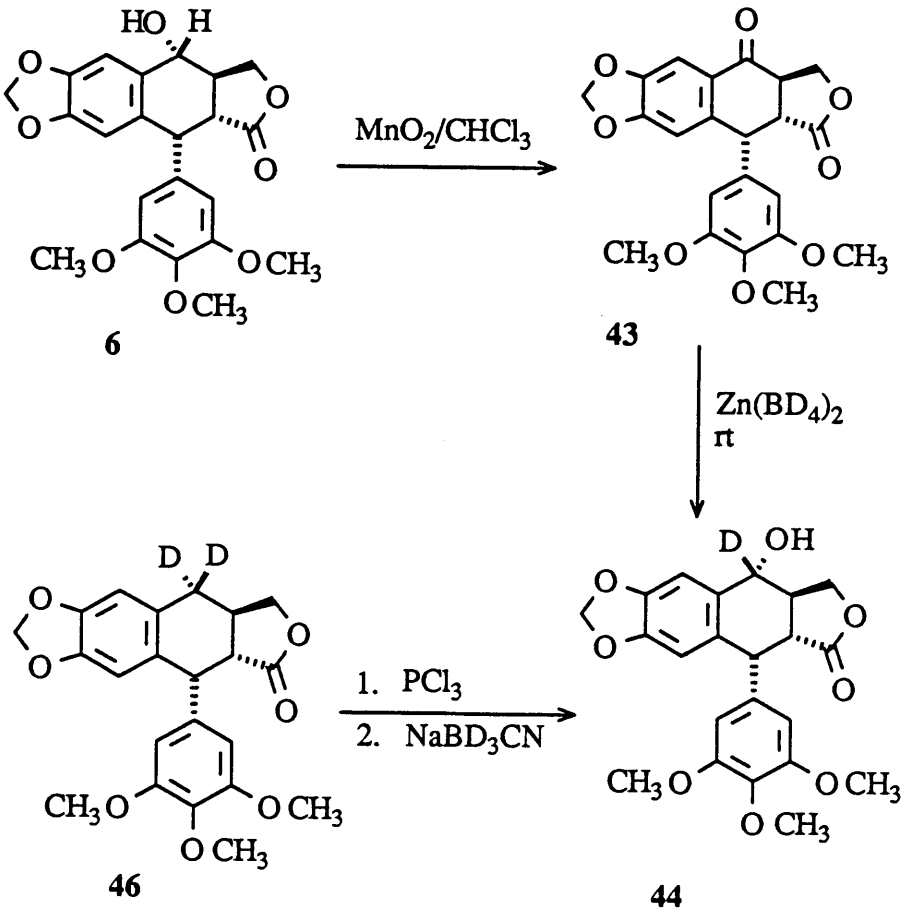
V. 2. 3. Synthesis of [4-$^2H_2$] Desoxypodophyllotoxin (46)

The synthetic **Scheme 8** for [4-$^2H_2$]-desoxypodophyllotoxin (**46**) is a combination of pathways for the preparation of the monodeuterated compounds. The podophyllotoxin (**6**) was oxidized to the keto lactone podophyllotoxone (**43**) using manganese dioxide, and this was converted stereospecifically to [4β-$^2H_1$]-podophyllotoxin (**44**) by zinc borodeuteride reduction as previously described. Treatment of **44** with PCl_3 yielded the corresponding deuterated chloride (**45**) which on subsequent reductive displacement with sodium cyanoborodeuteride yielded the target compound **46**.

The product gave only one spot on tlc and co-tlc with desoxypodophyllotoxin. Mass spectra (EI)) gave a molecular ion (M^+, 100%) at 400, two mass units greater than that of desoxypodophyllotoxin (d_0). Its proton nmr spectrum lacked the two quartets due to the two 4-H protons, confirming that the two deuterium atoms are at C-4, (**Fig. 7**). The analytical and spectrometric evidence obtained for the product is compatible with the dideuterated product, [4-



Mass spectrum of [4-$^2H_2$]-desoxypodophyllotoxin (**46**)



Scheme 8 Synthesis of [4-^{2} H_2]-desoxypodophyllotoxin

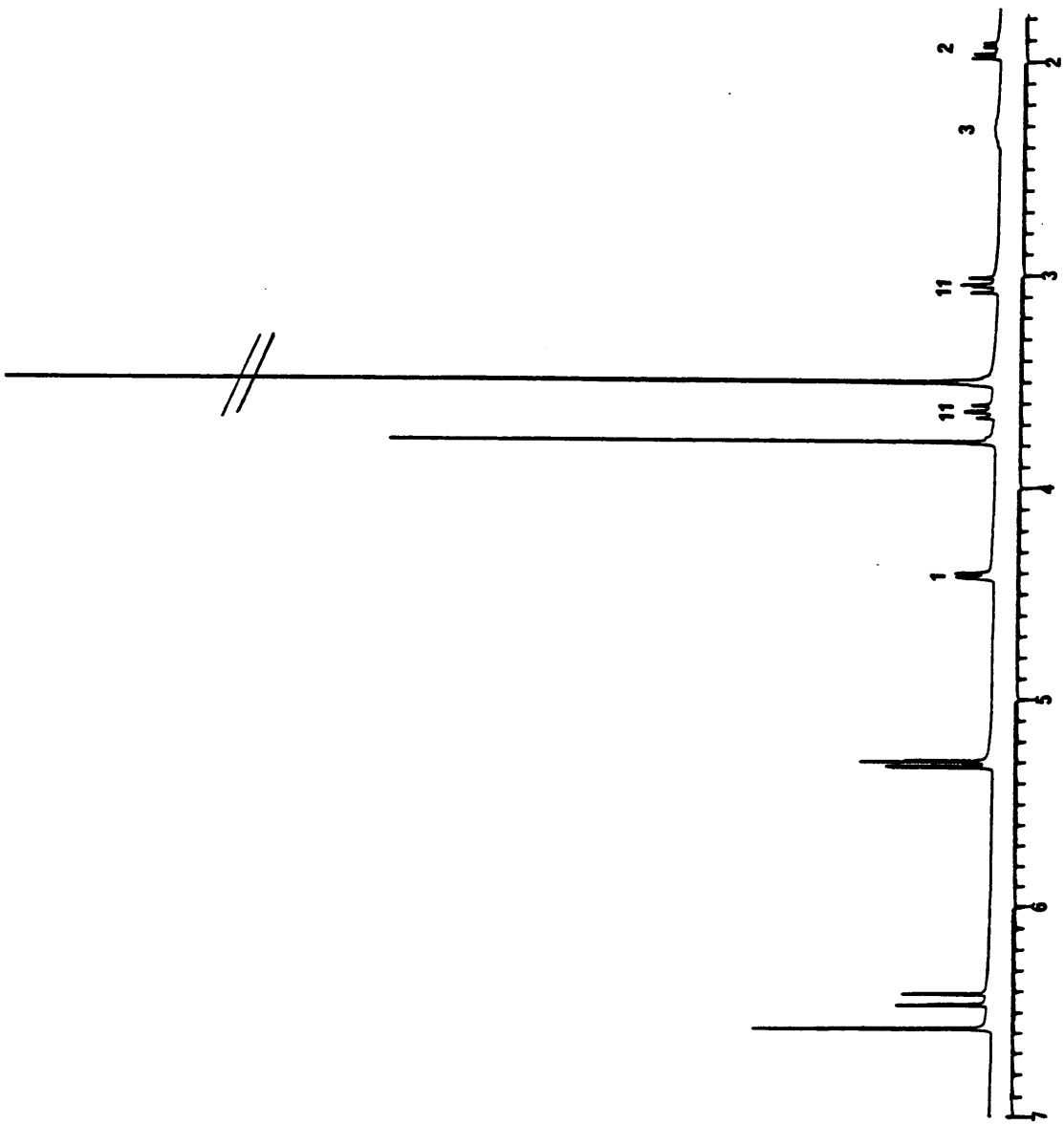


Fig 7 Proton nmr spectrum of [4-$^2H_2$]-desoxypodophyllotoxin (46)

V. 3. Assignment of the proton nmr spectrum of desoxypodophyllotoxin

V. 3.1. Relevance

Proton nuclear magnetic resonance spectrometry is of particular value in the structural elucidation of the aryltetralin lactone lignans of *Podophyllum*. The chemical shifts and coupling constants relating to the alicyclic protons of these compounds are of very characteristic appearance. The spectral pattern is markedly dependent on substitution patterns and stereochemistry, such that easily recognizable sets of signals may be correlated with particular series of compounds.

Changes in stereochemistry, such as epimerization at C-2 to give the picro series, are immediately reflected in the nmr spectrum and further characteristic spectral patterns are produced. Substitution patterns in the aromatic rings may readily be assigned from the chemical shifts of the remaining signals.

Models of podophyllotoxin and its derivatives suggest that their overall structures are similar, with the exception of picropodophyllotoxin. However, the models do not predict the rotational freedom of the D ring with respect to the C ring in the derivatives. They also do not provide unequivocal values of the bond angles in the B and C rings of these compounds. For these reasons, empirical data obtained on the conformational properties of podophyllotoxin and its derivatives can be used to assess the relationship between the structural features of these compounds and their antimitotic activities.

V.3.2. Results and discussion

Biologically active desoxypodophyllotoxin (**8**) and related products have received considerable attention from the spectral and biosynthetic point of view. Complete assignment of the ^{1}H nmr spectrum of desoxypodophyllotoxin is a necessary first step to the characterization of stereospecifically deuterated precursors for biosynthetic studies. Based on the assignments and on comparison with previously reported ^{1}H nmr data, information regarding stereochemistry and conformations of the products under study was obtained.

The first ^{1}H nmr measurements of these lignans were made in 1962.[40] Ayres *et al.* have reported limited chemical shift assignments and coupling constant data for desoxypodophyllotoxin (**8**) along with podophyllotoxin (**6**), epipodophyllotoxin (**16**) and picropodophyllotoxin (**17**) using lower field (100 MHz) measurements and different solvents.[41] Brewer *et al.* have made some chemical shift and coupling constant assignments of podophyllotoxin derivatives.[42] A number of subsequent papers present nmr results, but no complete assignment has ever been made for desoxypodophyllotoxin (**8**).

Our interest has been centered on the geometry of the reduced ring B, as this is linked with the biological activity. The resonances of H-3 and H-2 are close together, despite the effect of the carbonyl group on H-2. The absence of a hydroxyl group at the C-4 position in ring B of desoxypodophyllotoxin produces a substantial chemical shift in the H-4 proton resonance relative to its position in podophyllotoxin.

The ring B protons apart from H-1 are in the complex at δ 2.7-2.8 in deuterochloroform solution (**Fig. 8**). Under these conditions no coupling constants could be measured nor could unequivocal assignments could be made. **Table 6** gives the chemical shift and coupling constant

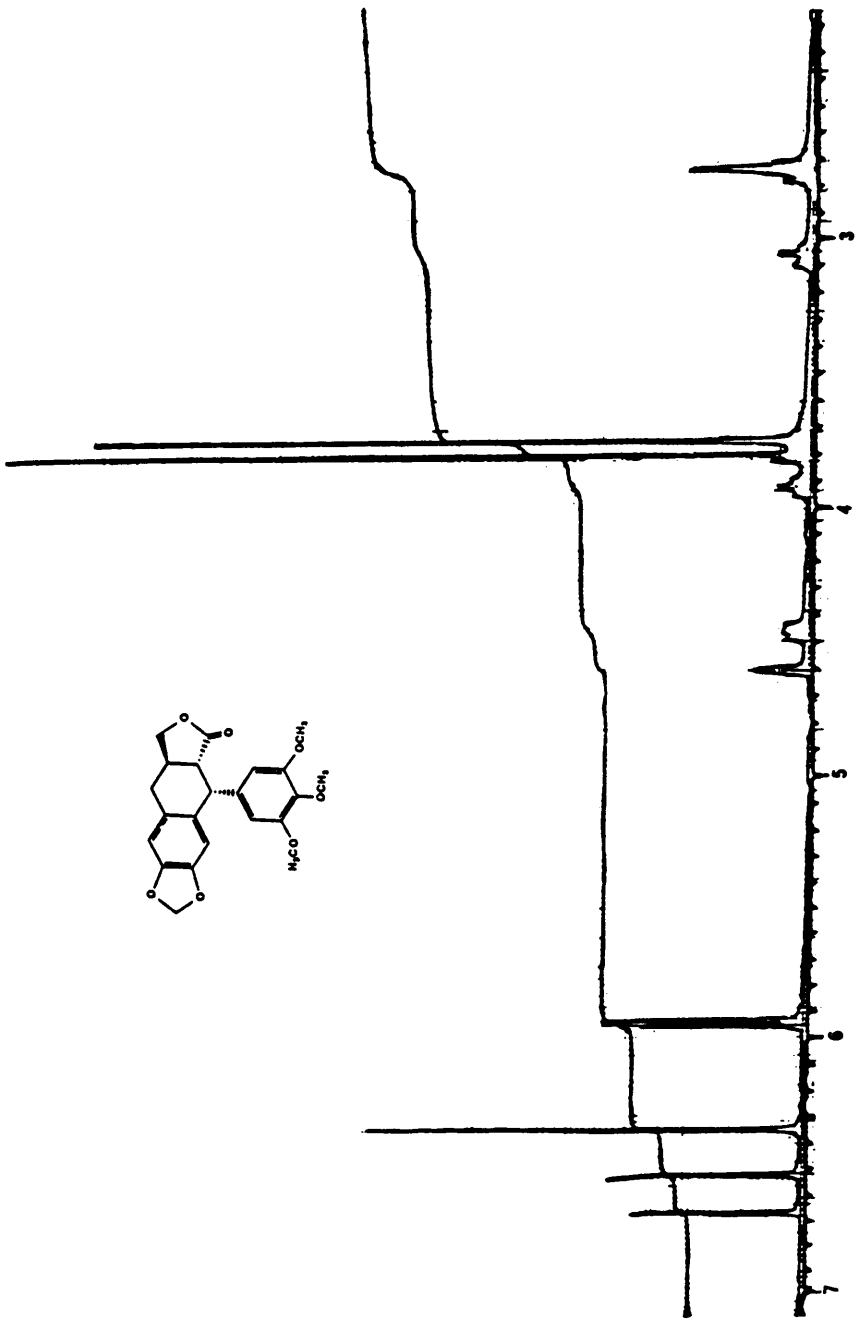


Fig 8 Proton nmr spectrum of desoxypodophyllotoxin in deuterochloroform

Table 6

Proton nmr Data of Desoxypodophyllotoxin in Deuterochloroform: Ring B Protons[42]

Proton	Chemical Shift	Coupling Constant Hz
1	4.60	$J_{1,2}$ = 1.5
2	2.72	$J_{2,1}$ = 1.5
3	2.72	$J_{3,11}$ = 6.1 $J_{3,11}$ = 8.0
4	3.07	–
4	2.78	–

data of ring B protons reported previously for desoxypodophyllotoxin (**8**) in deuterochloroform solution. Aryltetrahydronaphthalene lignans form stable inclusion products with benzene and other solvent molecules.[6] Owing to crowding in the acceptor molecule, the number of possible orientations of the included molecule must be limited. Dissolution in a strongly anisotropic solvent, small enough to be included, should therefore offer the best chance of changing relative chemical shifts. An illustration of this effect and its application to analysis is afforded by a solution of desoxypodophyllotoxin in deuterobenzene rather than in deuterochloroform.

A satisfactory detailed analysis was thus possible on examination of the spectrum of a solution in deuterobenzene. The 270 MHz proton nmr spectrum of desoxypodophyllotoxin is shown in **Fig. 9**. The resonances of the B ring protons and coupling constants are as tabulated in **Table 7**. The proton shifts were assigned by standard chemical shift theory, comparison with previously reported proton nmr spectra, deuterium labelling and specific proton decoupling studies.

The resolution obtained for the protons of ring B from a complex into well defined and well separated peaks in deuterobenzene solution is fascinating. The H-2 quartet was clearly resolved at higher field (δ 1.96) than the H-3 multiplet at a lower field, δ 2.31. Irradiation at δ 1.96 completely decoupled the H-1 doublet at δ 4.4, confirming the assignment of the H-2 protons to the quartet at δ 1.96; $J_{1,2}$ 4.8Hz.

The coupling constant $J_{1,2}$ is most useful for purposes of conformational analysis, whilst the magnitude of $J_{3,4}$ although modified by an electronegative substituent at carbon-4, provides a second point of reference. The broad multiplet for H-3 was not resolvable, since the proton is coupled to five different protons, so the coupling constant $J_{2,3}$ of 13.8Hz was obtained from the H-2 quartet, since $J_{1,2}$ had already been determined.

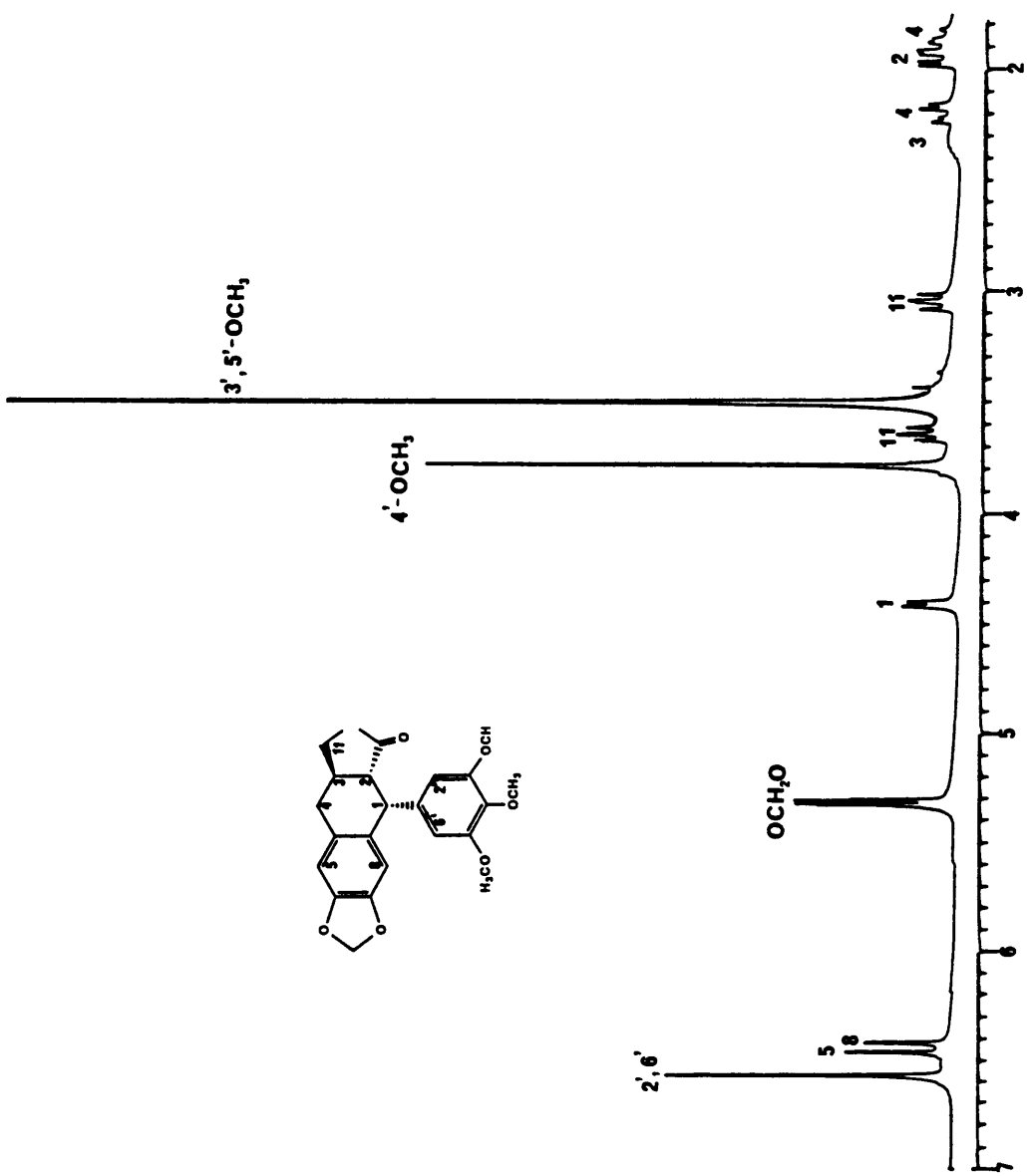


Fig. 9 Proton nmr spectrum of desoxypodophyllotoxin in deuterobenzene

Table 7

Proton nmr Data of Desoxypodophyllotoxin in Deuterobenzene: Ring B Protons

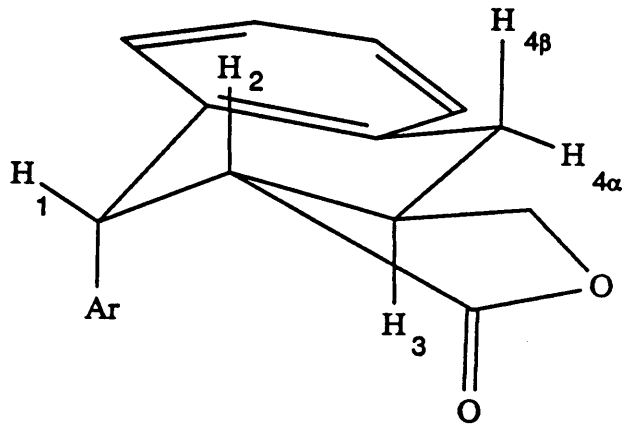
Proton	Chemical Shift	Coupling Constant Hz
1	4.41	$J_{1,2}$ = 4.8
2	1.96	$J_{2,1}$ = 4.8 $J_{2,3}$ = 13.6
3	2.31	$J_{3,2}$ = 13.6 $J_{3,4}$ = 5.3 $J_{3,4}$ = 11.4 $J_{3,11}$ = 7.7 $J_{3,11}$ = 8.5
4	2.2	$J_{4,4}$ = 15.9 $J_{4,3}$ = 5.3
4	1.88	$J_{4,4}$ = 15.9 $J_{4,3}$ = 11.4

Despite this critical resolution the H-4 resonances were too close to the H-3 signal for any ^1H - ^1H decoupling studies. The two H-4 protons have moved upfield with one of them seen at δ 2.2 and the other at δ 1.88, both as double-doublets . In order to obtain information about the stereochemistry of these compounds, their vicinal coupling constants can be converted into corresponding dihedral angles using a modified Karplus equation, which takes into account the effect of electronegativity of substituents on coupling constants.[43] Other effects such as steric factors influence vicinal coupling constants. However, their perturbations are difficult to account for quantitatively and are less important than substituent electronegativity.

Deuterium labelled compounds can be used to simplify the proton nmr spectra. Thus labelled desoxypodophyllotoxin was used to assign the stereochemistry of the C-4 protons. The proton nmr spectrum of the C-4 doubly deuterated [4-$^2H_2$]-desoxypodophyllotoxin (**46**) in benzene solution disclosed the absence of the two quartets at δ 1.88 & 2.2 and confirmed the assignment of these signals to the H-4 protons. The spectrum of the monodeuterated compound [4α-$^2H_1$]-desoxypodophyllotoxin (**42**), (**Fig. 6**) showed that the quartet at δ 1.88 collapsed into a doublet; the quartet at δ 2.2 was absent. The large coupling constant ($J_{3,4\beta}$= 11.4Hz) corresponds to the axial-axial alignment of 3 and 4 protons and proves that the H-4 signal at δ 1.88 corresponds to H-4β. This conclusion is confirmed by a comparison of the spectrum of the compound **42** in deuterochloroform solution to the published spectrum.

The spectrum of [4β-$^2H_1$]-desoxypodophyllotoxin (**41**) also confirmed this assignment, since it showed a doublet for H-4α at δ 2.2 with a coupling constant $J_{3,4\alpha}$ of 5.3Hz, corresponding to an axial-equatorial coupling. The vicinal coupling $J_{4\alpha,4\beta}$ was determined to be 15.9Hz. The high field lactone signal was located at δ 3.06 as a quartet and the low field lactone resonance was seen as a pseudo triplet at δ 3.65. Irradiation at δ 2.31 (3-H) simplified the lactone signals and the

coupling constants were determined as $J_{3,11\alpha}$ 7.7Hz, $J_{3,11\beta}$ 8.5Hz and $J_{11,11}$ 10.5Hz. Assignment of the aromatic proton signals is straightforward. A two-proton singlet at δ 6.57 was assigned to the 2' and 6' protons based on its intensity.



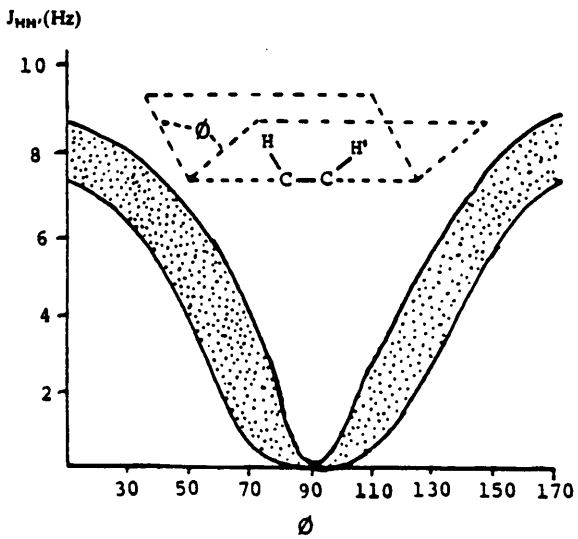
The chemical shift assignments for the methylene-dioxy and methoxy protons of desoxypodophyllotoxin were made on the basis of their chemical shift positions as well as their integrated intensities. The protons of the methylenedioxy group become non-equivalent and were seen as a double doublet at δ 5.33. The less hindered 3',5'-methoxy protons remain equivalent, but are shifted further upfield to δ 3.51 than is the 4'-methoxy group at δ 3.78.

A characteristic feature of desoxypodophyllotoxin and other podophyllotoxin derivatives is that the 2',6'- protons and the 3',5'- methoxy protons give rise to single resonance peaks. An examination of models suggests convincingly that the 2' and 6' protons of the D ring should be fixed at diastereotopic locations. However, at room temperature, the nmr signal for these two hydrogens consists of one sharp two-proton singlet.

The nmr data thus suggests that despite evidence to the contrary from a consideration of models, the endo and exo aromatic protons of the D ring as well as the two methoxy protons are

interchanging positions via rotation about the 1-1' bond between two conformers, at a rate faster than the proton nmr time scale at room temperature. A dynamic nmr study reported corroborates this hypothesis of rapid D-ring rotation at room temperature.[44] The D-ring is essentially perpendicular to the fused A-B-C ring system in the two conformations.

From a knowledge of the relevant coupling constants, the geometry of all the diastereomers can be confirmed by use of the Karplus relationship. Further, an estimate of the conformational equilibrium in flexible models can be obtained. The empirical data on the conformational properties of podophyllotoxins can be used to assess the correlation between the structural features of these compounds and their antimitotic activities.



Karplus relationship

Thus a complete chemical shift and coupling constant assignment of desoxypodophyllotoxin in deuterobenzene solution at room temperature was obtained. The addition of anisotropic solvents during nmr experiments should simplify the intricacy of structure evaluation in other lignans.

V.4. Spectral Data and Characterization

Stable isotopes are essential tools in studies to distinguish between alternative pathways of biosynthesis, in studies of isotope effects and in biological and medical research. The renaissance of interest in stable isotopes of hydrogen, carbon, nitrogen, oxygen and sulphur within the last few years is based upon the development of new instrumentation and on the greater availability of enriched isotopes.

For reasons of cost and ease of synthesis, deuterium has been the most frequently employed isotope, although in principle any other stable isotope can be used. Stable isotope applications still have many limitations of cost, but measurements are simple, inexpensive and without effect on the isotope content. Analytical methods are based on detectable differences among the isotopes.

In considering techniques available for the analysis of stable isotopes, mass spectrometry provides a unique combination of sensitivity with a provision of structural information. It is the most generally applicable and widely used method for isotope analysis and can be applied in different ways. It can very accurately determine the overall ratio of isotopes of an element in a sample following conversion to simple molecules such as CO_2, H_2O or N_2. Alternatively the mass of the molecules themselves and their fragmentation products can be determined - a procedure that often gives structural information on how the isotope is distributed within the molecule, in addition to overall isotopic composition.

Characteristic mass spectra can be obtained from picomole to nanomole quantities of sample depending on the mode of instrument operation. During ionization of the sample, usually by a beam of low energy electrons, molecular ions are produced. This ionized, intact molecule

provides a direct indication of the molecular weight. Thus mass spectrometry is an extremely powerful and sensitive technique both qualitatively and quantitatively.

Nuclear magnetic resonance spectrometry is an indisputably powerful method for determining many structural and conformational parameters. It can also provide simple isotopic concentration values through analysis of peak intensities. The proton magnetic resonance spectrum reflects the environment of protons in a molecule. Deuterium labelling has also been used to simplify the spectra of complicated molecules. Certain classes of compounds also give characteristic spectral patterns and it can also be utilized to reveal stereochemistry of the compound at specific sites. The Karplus relationship has proved to be very useful in determining the correlation between the couplings and the relative alignment.

The presence of characteristic group frequencies in the infra red spectrum and comparison of the spectrum to a known compound is used to obtain structural information. The frequency of absorption depends on the relative masses of the atoms, the force constants and the geometry of the atoms. Hence a shift in C-H can be noticed following deuteration of a compound.

The EI mass spectrum of the three desoxypodophyllotoxins - d_0(a), d_1(b), d_1(c) and d_2(d) are presented in the **Figure 10**. The isotopic incorporation of the these samples are represented in **Table 8**, in terms of their observed intensities and computed intensities. Natural abundance data for C, H and O are taken from Silverstein, Bassler and Morrill. Sample (b) and (c) has the molecular ion peak, of highest intensity at 399 mass units, which corresponds to the molecular formula $C_{22}H_{21}{}^2H_1O_7$, one unit higher than the nondeuterated compound. After correction for the natural abundance, the isotopic content was determined as greater than 95% monodeuterated desoxypodophyllotoxin. The product (d) has the molecular ion peak m/z at 400 corresponding to the elemental composition $C_{22}H_{20}{}^2H_2O_7$ and 94.5% deuterium incorporation.

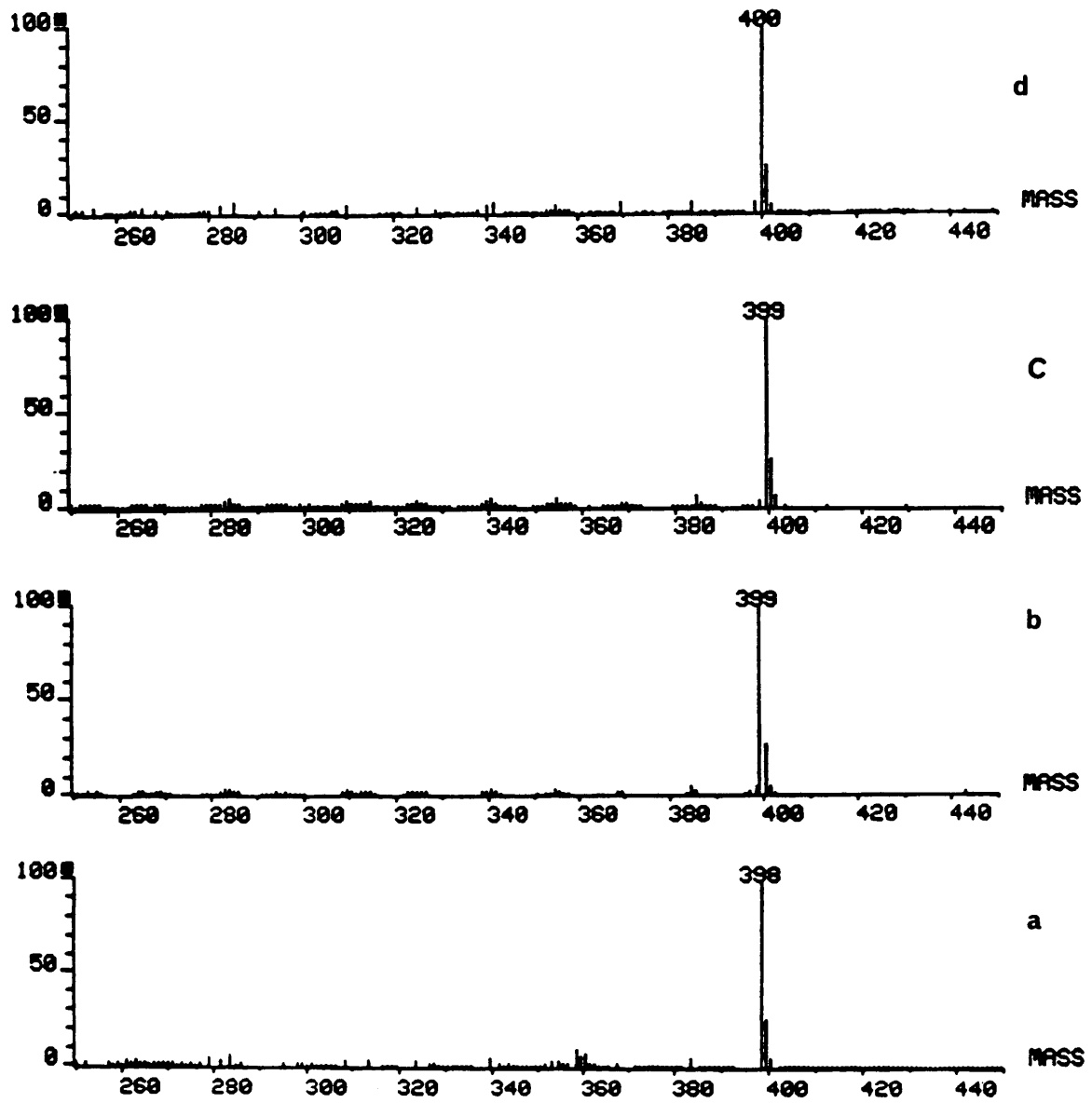
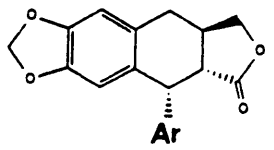
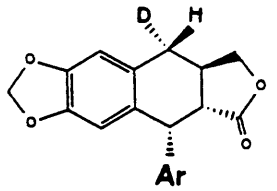
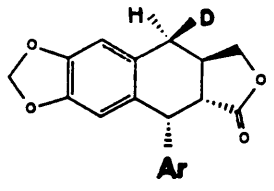
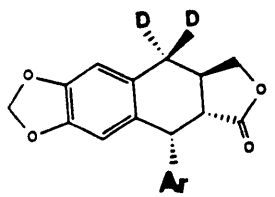


Fig. 10 Mass spectrum of the desoxypodophyllotoxins - d_0(a), d_1(b), d_1(c) and d_2(d)

Table 8

Mass Spectra of Desoxypodophyllotoxins

M/z	Observed Intensity %	Corrected Intensity* %	
398	100.0	100.0	
399	24.8	0.0	
400	4.3	0.0	
398	4.5	4.3	
399	100.0	95.4	
400	25.2	0.0	
401	4.3	0.0	
398	3.0	2.9	
399	100.0	97.1	
400	24.1	0.0	
401	4.6	0.0	
399	5.7	5.5	
400	100.0	94.5	
401	24.5	0.0	
402	4.2	0.0	

* Corrected for the contributions to peak intensities from natural abundance ^{13}C and ^{18}O isotopes.

The sector of the proton nmr of interest for the deuterated as well as the nondeuterated desoxypodophyllotoxins (1-4) in deuterobenzene are displayed in the **Figure 11. Table 9** gives the proton nmr data of these desoxypodophyllotoxins. These spectra of the three specifically labelled products confirm the stereochemical assignments. Even though the proton nmr itself is necessary to characterize these compounds, they have been identified independently by other analytical and spectrometric techniques.

The tlc and co-tlcs of these samples showed that these samples are identical to the authentic sample. Further, FTIR analysis of the samples of nondeuterated and monodeuterated desoxypodophyllotoxins as a KCl wafer exhibit comparable spectra to the known authentic sample, (**Fig. 12-14**). It reveals strong carbonyl absorption of the lactone carbonyl group and the deuterium stretching. Mixed melting point determinations of the products and the authentic desoxypodophyllotoxin showed no depression. Thus the identity and stereochemistry of these samples were determined.

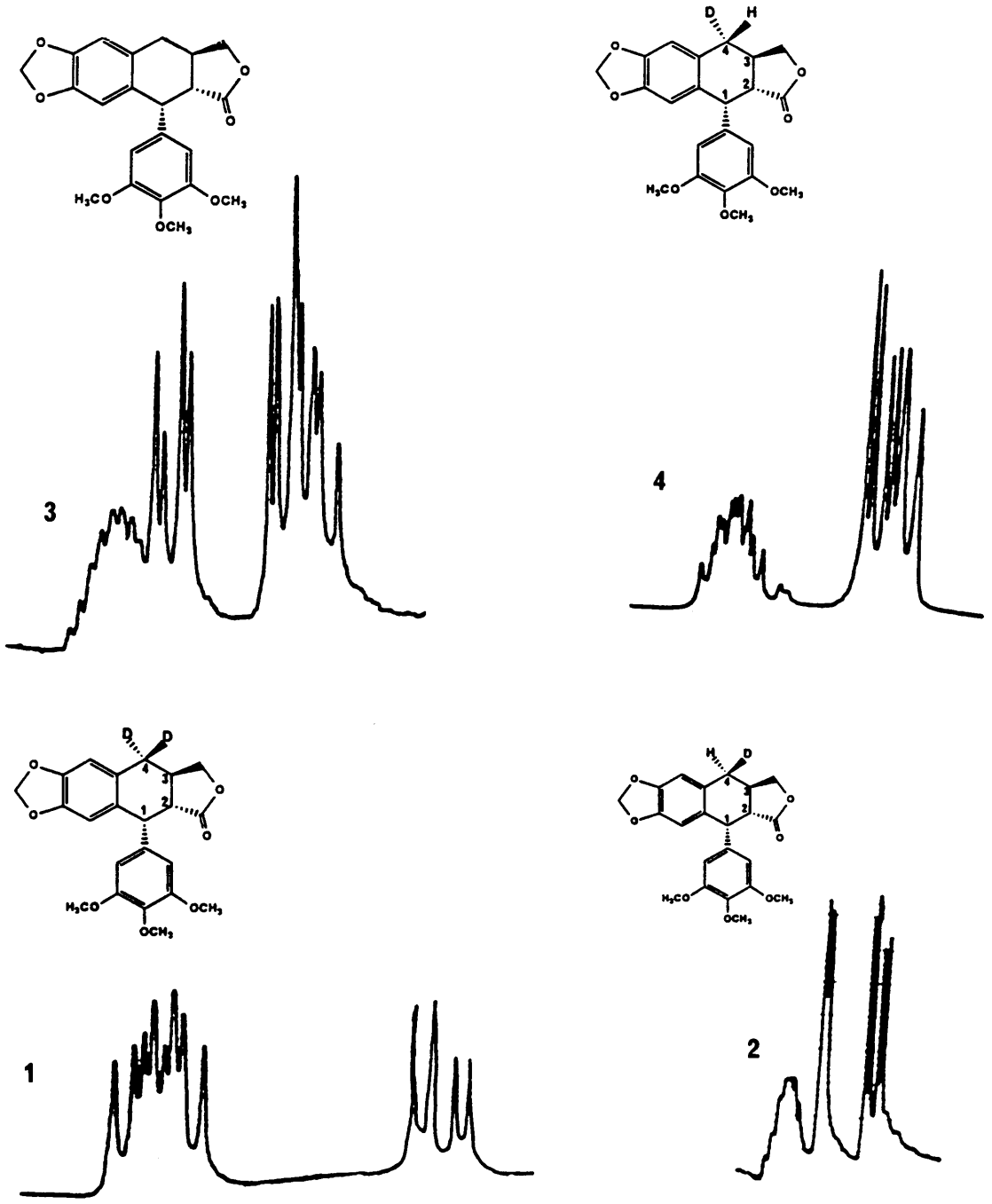


Fig. 11 Protons of the proton nmr spectra of desoxypodophyllotoxins (1-4) in deuterobenzene (δ 2-1.5)

Table 9

Proton nmr data of desoxypodophyllotoxins

Proton	Chemical Shift			
	do	4 α-d_1	4β· d_1	4 - d_2
1	4.41	4.41	4.41	4.41
2	1.96	1.96	1.96	1.96
3	2.31	2.31	2.31	2.31
4	2.20	___	2.18	___
4	1.88	1.89	___	___

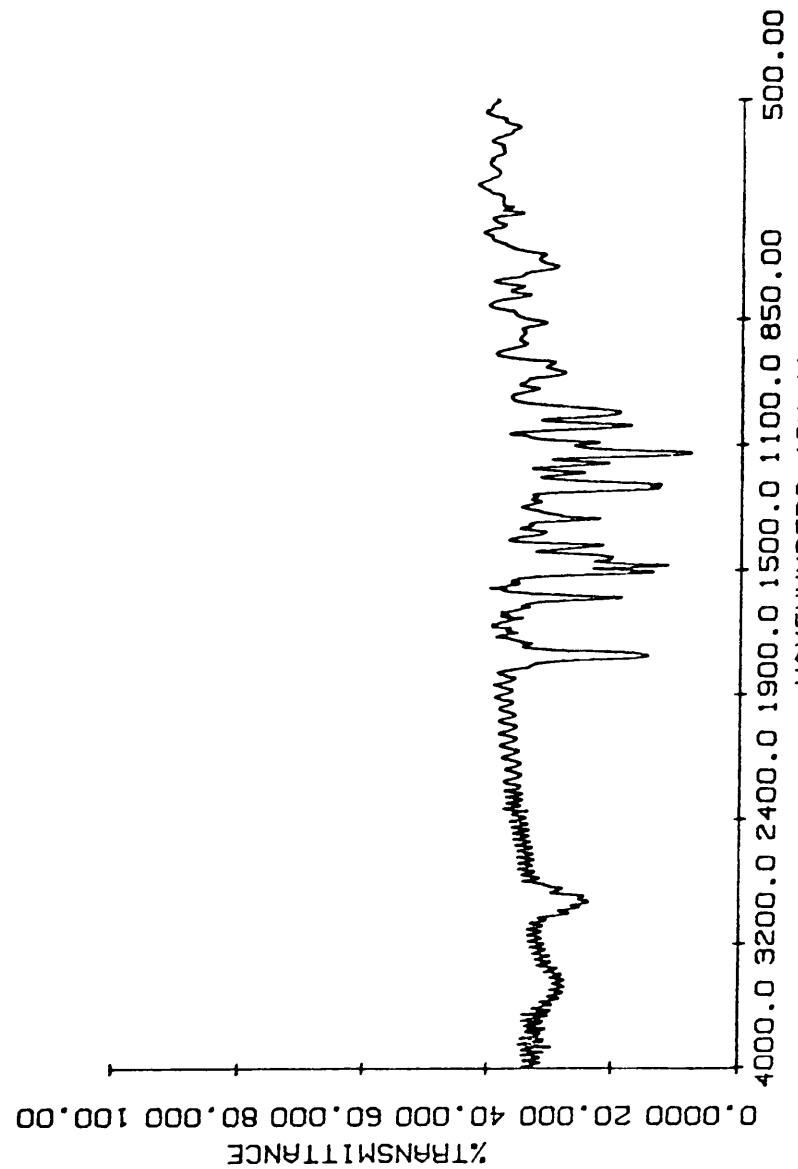


Fig 12 FTIR spectrum of [4β-$^2H_1$]-desoxypodophyllotoxin

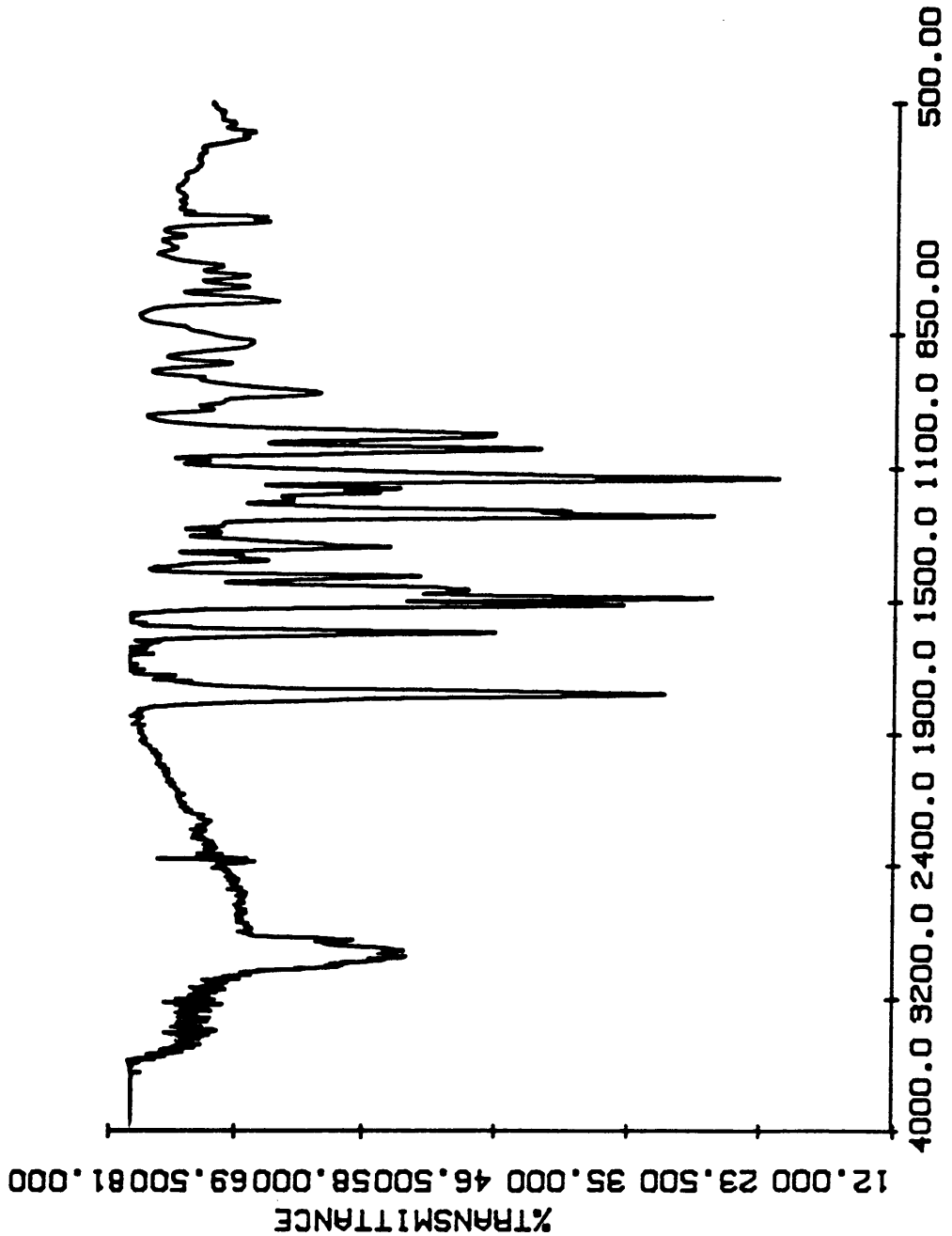


Fig 13 FTIR spectrum of [4α-$^2H_1$]-desoxypodophyllotoxin

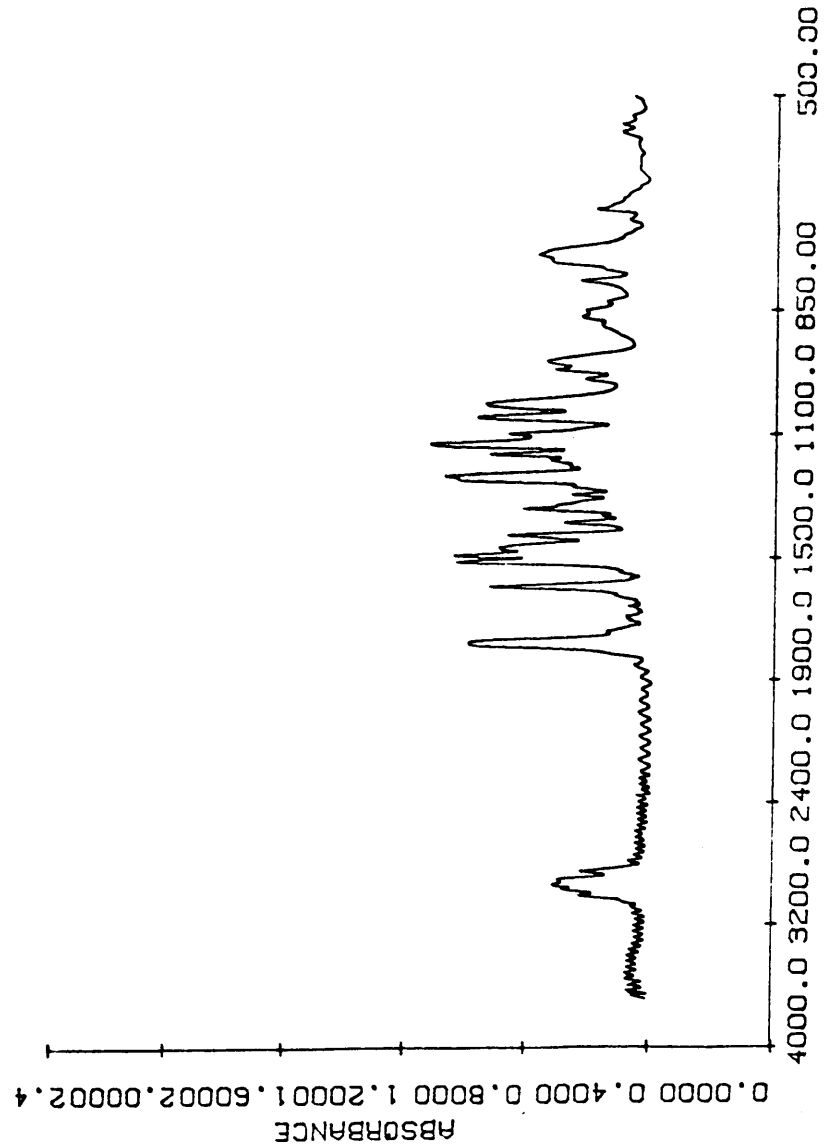


Fig 14 FTIR spectrum of desoxypodophyllotoxin

V. 5. p-Coumaric Acids

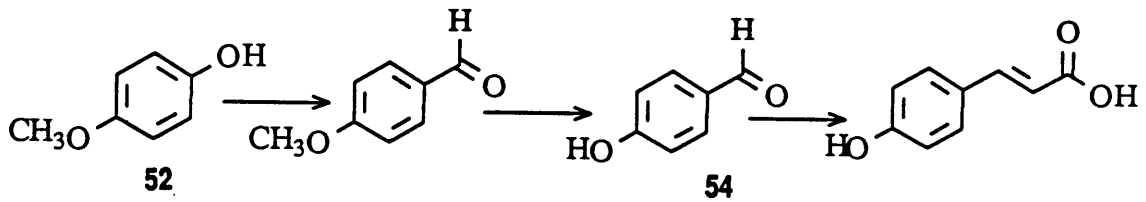
The biosynthesis of podophyllotoxin and other related lignan lactones from *Podophyllum* species have received considerable attention in regards to their value as cancer chemotherapeutic agents. To get an understanding of lignan biogenesis, the proposed approach involves administering various specifically labelled (^{13}C,^{13}C/ ^{2}H,^{2}H) lignan precursors such as p-coumaric acid and ferulic acid into intact plants and tissue cultures. In this way, the exact biochemical transformation of these precursors will be monitored.

Several phenylpropanoid precursors like p-coumaric acid, ferulic acid, cinnamic acid and phenylalanine are found to be incorporated, showing that these monomers are involved in the biogenetic coupling reaction, yet no conclusive evidence is available on the exact nature of the intermediates in the coupling reaction. Feeding experiments with singly and doubly labelled precursors will unambiguously establish as to whether or not p-coumaric acid is incorporated intact with retention of labels and also on the origin of the units and the stereochemistry of the site where coupling occurs.

p-Coumaric acids with specific labels were thus prepared as labelled precursors. The approach for synthesis and labelling of this acid with either ^{2}H or ^{13}C/ ^{2}H labels was from the labelled intermediate 4-hydroxybenzaldehyde. In this synthesis 4-hydroxybenzaldehyde is the key compound for the synthesis of the cinnamyl derivatives, since each is ultimately derived from this intermediate. The Knoevenagel reaction with malonic acid and hydroxybenzaldehyde yielded the corresponding p-coumaric acids.[48]

Our first approach was at making the key intermediate, 4-hydroxybenzaldehyde (**54**) in two steps using Gatterman's aldehyde synthesis.[45,46] Anisole (**52**) was treated with zinc cyanide and

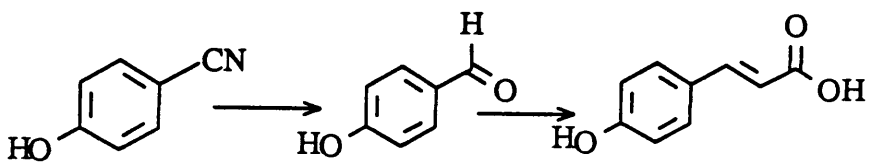
aluminum chloride in the presence of acid to yield 4-methoxybenzaldehyde (**53**) which on demethylation gave **54** (**Scheme 9**). This reaction was laborious since extreme care had to be taken to keep the reaction mixture anhydrous and also to prevent any escape of lethal HCN gas. In addition, the yields were inconsistent and low and we opted for another method to make the hydroxyaldehyde (**54**).



52 **54**

Scheme 9

The photochemical reaction of 4-cyanophenol **48** to **54** in one step was found to be the method of choice since the reaction was simple and gave high yields of the product consistently[47] (**Scheme 10**). The labels could be introduced in high isotopic enrichment and readily available starting



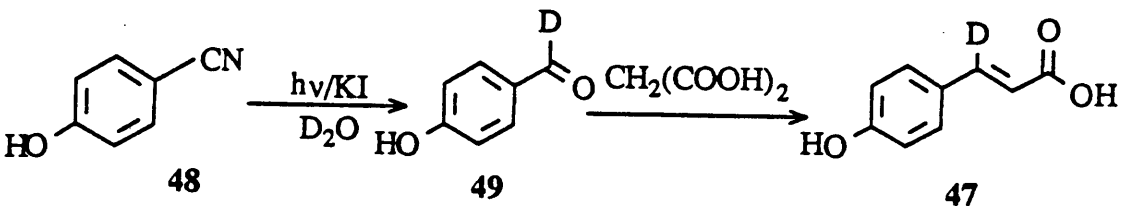
48 **54**

Scheme 10

materials are used. Two specifically labelled coumaric acids, [3'-$^{2}H_{1}$]-4-hydroxycinnamic acid (**47**) and [3'-^{2}H1 / ^{13}C]-4-hydroxycinnamic acid (**48**) are synthesized starting with 4-bromophenol. The 3'-labelling was adopted since that is presumed to be the site of coupling between the two phenylpropanoid units, and is thus of most interest from a biosynthetic perspective.

V. 5. 1. [3'-^{2}H$_1$]-4-Hydroxycinnamic acid (47)

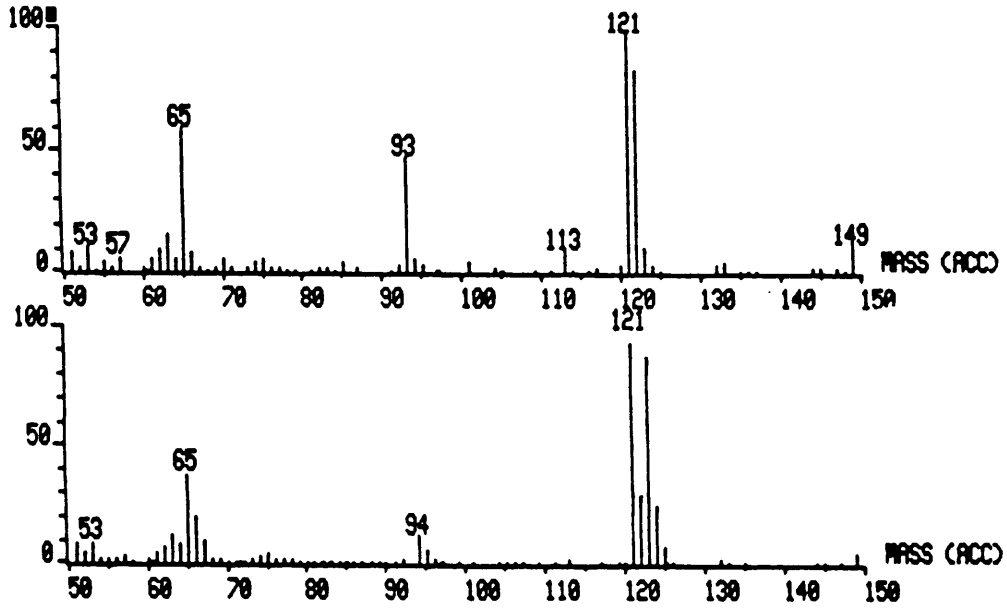
The p-coumaric acid was prepared from commercially available p-cyanophenol by the photochemical reduction of the nitrile group to the deuterated aldehyde.[47] Reduction of 4-cyanophenol to [carbonyl-^{2}H]-4-hydroxybenzaldehyde (49) proceeded photochemically in high yield in deuterium oxide solution when iodide ion was used as the electron donor. An important feature of this conversion is the direct transformation of 4-cyanophenol to the carbonyl labelled aldehyde in one step; this method circumvents the experimental problems associated with the microscale use of the metal hydride reducing agents or active metal catalysts.[49,50]



48 49 47

Scheme 11

The yield was found to be highly dependent on the concentration of 4-cyanophenol. The highest conversion to products was noticed with a dilute solution of the starting material, and the substitution of D_2O and KOD made the reaction time several fold longer than for the reaction in aqueous solution. Use of excess iodide ion made the reaction mixture brown due to the iodine released during the reaction and the yields were low. A mass spectrum of the product revealed the extent of isotopic incorporation, demonstrating that the product is monodeuterated and that the deuterium is incorporated on the carbonyl carbon.



Mass spectrum of 4-hydroxybenzaldehyde (std) and [carbonyl-^{2}H]-4-hydroxybenzaldehyde (49)

Condensation of the deuterated aldehyde with malonic acid by the Knoevenagel synthesis yielded [3'-^{2}H]-4-hydroxycinnamic acid (47) (**Scheme 11**). The aldehyde was condensed with malonic acid in pyridine in the presence of aniline and piperidine. The product was obtained by neutralizing the pyridine and the acid was crystallized from boiling water. The residual iodine which was carried from the former reaction was removed from the product by recrystallization.

The product was characterized by spectroscopic methods. Its proton nmr spectrum in a deuterochloroform/ DMSO-d_6 mixture revealed that the 3'-H resonance of the undeuterated compound at δ 7.65 is lacking and that the 2'-H doublet at δ 6.22 had collapsed into a singlet. A mass spectrum in the E.I mode revealed high isotopic incorporation, (**Fig. 15**), with the molecular weight of the compound one mass unit higher than the known p-coumaric acid. Thus the product of condensation is [3'-^{2}H]-4-hydroxycinnamic acid (47).

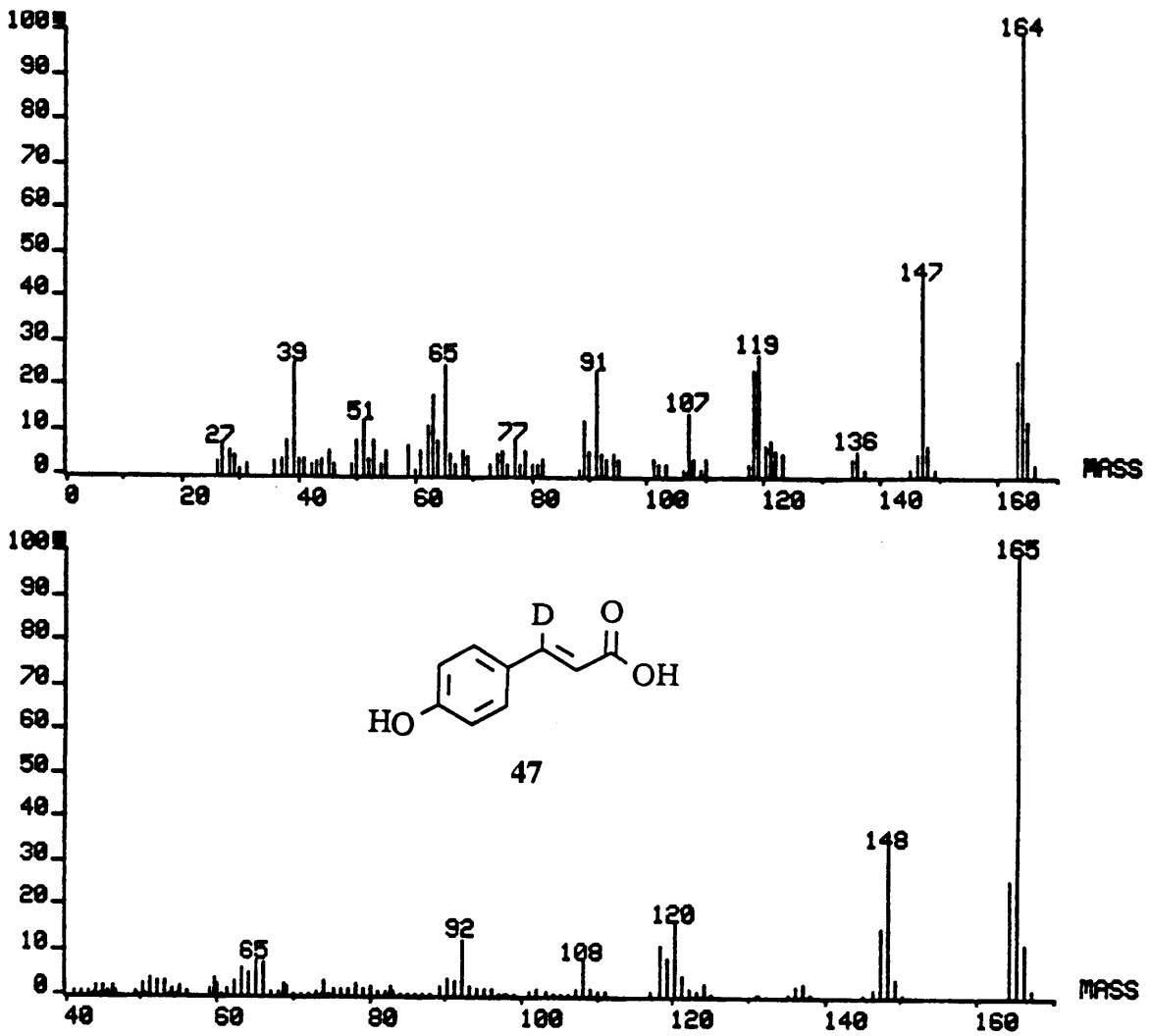
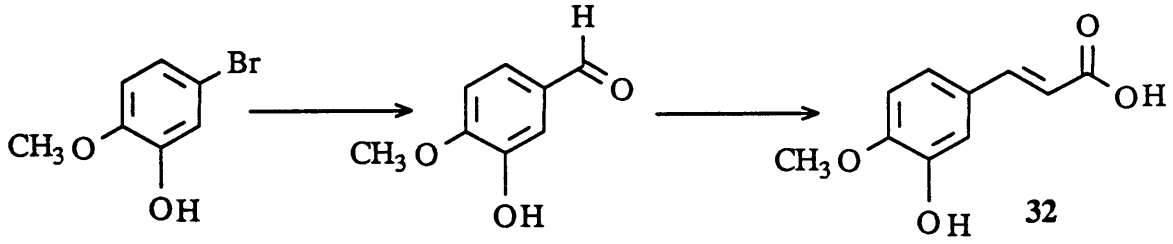


Fig. 15 Mass spectrum of 4-hydroxycinnamic acid (std) and [3'-^{2}H]-4-hydroxycinnamic acid (47)

V. 5. 2. [3'-^{2}H / ^{13}C] Hydroxycinnamic acid (48)

The [3'-^{2}H/ ^{13}C]-4-hydroxy-cinnamic acid (48) was synthesized by an analogous scheme used for p-coumaric acid (47) synthesis. According to **Scheme 12**, [carbonyl-^{13}C / ^{2}H]-4-hydroxybenzaldehyde (51) was condensed with malonic acid and the aldehyde was prepared from 4-bromophenol (49) and Cu^{13}CN by a convenient method which proceeds in only two steps.

The key features of this synthesis are the quantitative conversion of Na^{13}CN to Cu^{13}CN by a standard procedure, the direct conversion of 4-bromophenol to [cyano-^{13}C]-4-cyanophenol (50) without the use of a protecting group and the reduction of the nitrile group to the aldehyde by a photochemical reduction. In experiments with unlabelled material, ferulic acid was also obtained by this route, starting from 3-bromo-5-methoxyphenol.

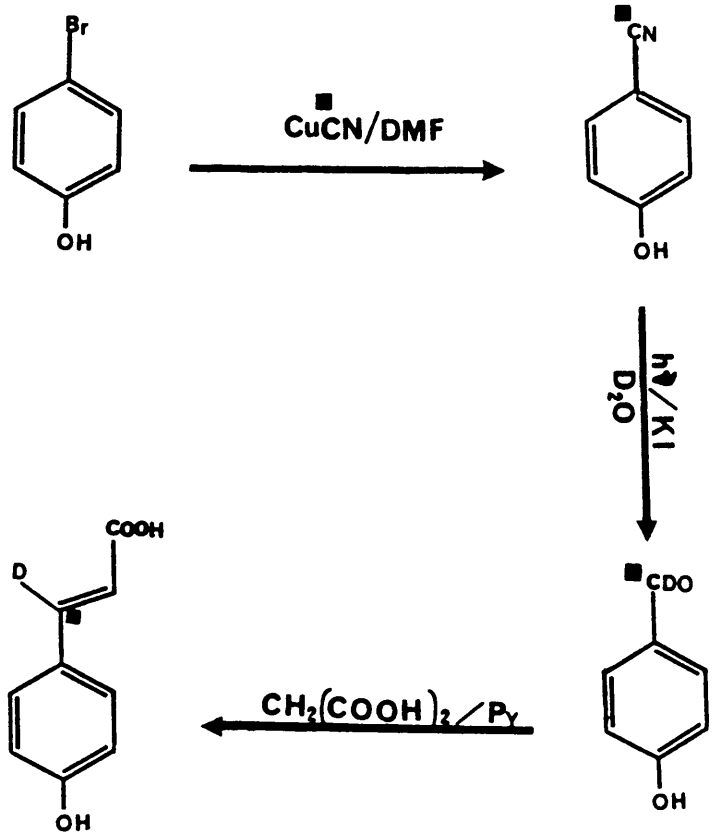


32

Cuprous cyanide was prepared by the excellent and simplified technique by H.J.Barber.[51]

$$2CuSO_4 + 2NaCN + NaHSO_3 + H_2O = 2CuCN + 3NaHSO_4$$

Solid CuCN can be prepared in bulk and kept indefinitely. There is slight frothing and a little SO_2 is evolved, but no appreciable amount of cyanogen or HCN. p-Bromophenol was refluxed with enriched cuprous cyanide in DMF and the cyanophenol was obtained by treating the reaction mixture with acidic ferric chloride solution, followed by extraction with ether. The use of a smaller

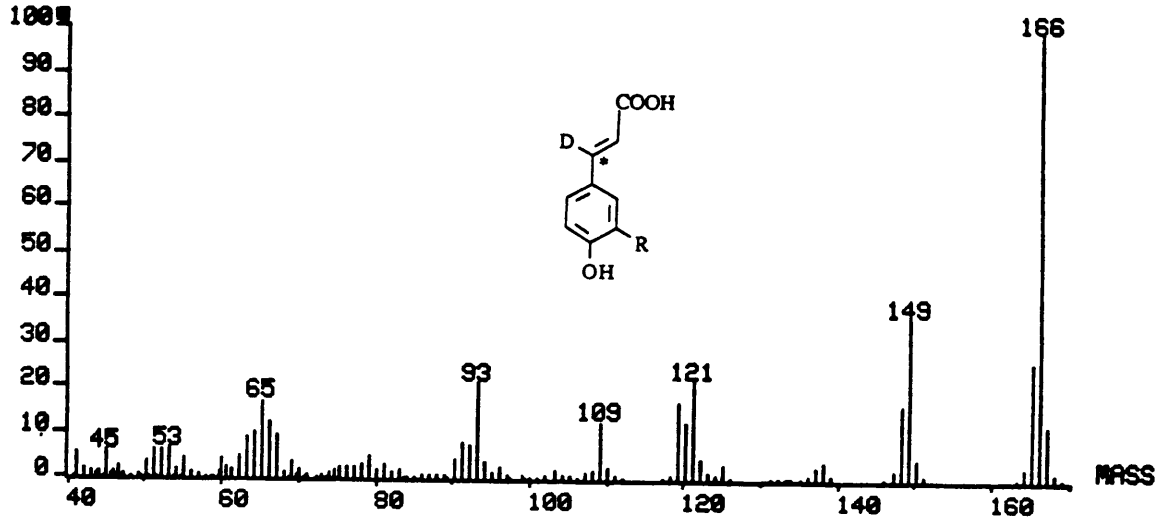


Scheme 12 [3′-$^{2}H_{1}$ / ^{13}C]-4-Hydroxy-cinnamic Acid

amount of solvent has increased the yield from the reported 50% to 89%.[47]

The labelled cyanide was dissolved in alkaline deuterium oxide solution containing KI. U.V. irradiation yielded the doubly labelled aldehyde, [carbonyl-^{2}H/ ^{13}C]-4-hydroxybenzaldehyde (**51**). The final step was the condensation of the carbonyl labelled aldehyde with malonic acid according to the Knovenagel synthesis. Recrystallization of the product yielded [3'-^{2}H / ^{13}C]-4-hydroxy-cinnamic acid (**48**).

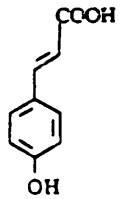
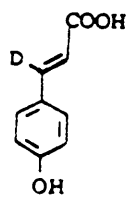
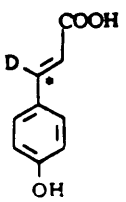
A proton nmr spectrum of the product in $CDCl_3$ / DMSO-d_6 revealed that deuterium is at 3'-C from the singlet observed at δ 6.22 due to 2'-H and 3'-H doublet is not observed. The aromatic protons at C-2 are seen as a quartet due to coupling to the 3'-^{13}C. ^{13}C nmr in the same solvent demonstrate the isotopic enrichment of the 3'-C peak compared to the natural abundance peaks and it is split into a triplet. The mass spectrum of the sample also indicated high isotopic incorporation. The molecule is two mass units higher than the authentic sample of p-coumaric acid



Mass spectrum of [3'-^{2}H/ ^{13}C]-4-hydroxycinnamic acid (**48**)

and the smaller mass units are comparable. Carbon-13 nmr spectroscopy further proves that the product is [3'-^{2}H / ^{13}C]-4-hydroxycinnamic acid (**48**).

Table 10
Mass Spectra of p-Coumaric Acids

	Observed Intensity%	Corrected Intensity%*	
163	25.7	25.7	
164	100.0	97.4	
165	11.3	1.1	
166	2.0	0.9	
163	0.0	0.0	
164	25.7	25.7	
165	100	97.5	
166	11.3	1.1	
167	1.2	0.1	
163	0.0	0.0	
164	2.0	2.0	
165	25.9	25.7	
166	100	97.5	
167	11.3	1.1	
168	1.2	0.1	

* Corrected for the contributions to peak intensities from natural abundance ^{13}C and ^{18}O isotopes.

VI. EXPERIMENTAL

Proton spectra were obtained in the indicated solvent on an IBM WP-270 nuclear magnetic resonance (nmr) spectrometer; chemical shifts are reported in ppm downfield from internal tetramethylsilane. Splitting patterns are designated as s (singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Coupling constants are given in Hertz. Infrared spectra (ir) were recorded on a Perkin-Elmer instrument. Mass spectra were obtained on a VG 7070 instrument operating in the electron ionization (ei) mode. Melting points (uncorrected) were determined in open capillaries. Flash chromatography was carried out on E. Merck silica gel (230-400 mesh). Thin layer chromatography was carried out on E. Merck precoated silica gel plates, 0.2mm. Unless otherwise specified, the solvent for tlc was (CHCl3:i-PrOH,10.7:0.3). Solvents were distilled prior to use.

Podophyllotoxin (6)

5g of Podophyllin (Sigma) was dissolved in hot ethanol for 10 min. and was filtered to remove any residue on the filter paper. Ethanol was then evaporated *in vacuo*. 20ml water was added to the residue dissolved in ethyl acetate and extracted with ethyl acetate (70ml) in a separatory funnel. EtOAc layer was dried with anhy. $MgSO_4$ and evaporated *in vacuo*. Flash column chromatography on silica gel column using ($CHCl_3$:MeOH, 25:1) as eluent yielded (1.56g, 31%) podophyllotoxin after recrystallization from EtOH, m.p. 182°. The sample (tlc and co-tlc) was identical to the authentic sample of podophyllotoxin (Aldrich).

^{1}H nmr ($CDCl_3$) δ 7.12(s,1H,H-5), 6.51(s,1H,H-8), 6.37(s,2H,H-2',6'), 5.99(d,2H,H-OCH_2O,J=1.2Hz), 4.76(d,1H,H-4β,J=9Hz), 4.59(m,H-11α,1), 4.08(t,1H,H-11β), 3.81(s,3H,H-4'-OCH_3), 3.76(s,6H,H-3',5'-OCH_3), 2.9-2.7(m,2H,H-2,3).

Desoxypodophyllotoxin (8)

Podophyllotoxin (12.4mg, 0.0293 mmol) was dissolved in acetic acid (1 ml) and 18mg Pd-C (10%) was added. The reaction flask was then kept under hydrogen in a hydrogenation apparatus at about 95°, with stirring. After 3 hours, the reaction mixture was filtered, the catalyst was washed with 30ml ($EtOH:H_2O$, 1:1) and the solution evaporated to dryness. The crude mixture was then purified by preparative tlc ($CHCl_3$:i-PrOH, 10.7:0.3). The desoxypodophyllotoxin band was eluted to give pure product in 72% yield, m.p. 167.5°. The tlc and co-tlc of the product was identical with an authentic sample of desoxypodophyllotoxin (**8**).

^1H nmr (C_6D_6) δ 6.57(s,2H,H-2',6'), 6.47(s,1H,H-5), 6.42(s,1H,H-8), 5.33(dd,2H,H-OCH_2O), 4.41(d,1H,H-1,*J*=4.8Hz), 3.78(s,3H,H-4'-OCH_3), 3.65($dd_{(ca)}$,1H,H-11α,*J*=7.7 and 10.6Hz), 3.51(s,6H,H-3',5'-OCH_3), 3.06(dd,1H,H-11β,*J*=8.5 and 10.6Hz), 2.31(m,1H,H-3,*J*=13.8,11.4,5.3,8.5 and 7.7Hz), 2.2(dd,1H,H-4α,*J*=15.9 and 5.3Hz), 1.96(d,1H,H-2, *J*=4.8 and 13.8Hz), 1.88(dd,1H,H-4β,*J*=15.9 and 11.4). Eims *m/z* (relative intensity) (**Fig. 10**) 398($M^{+\cdot}$,100), 399(25), 400(4). IR (KBr) (**Fig. 14**) 1776,1484,1238,1126cm^{-1}.

[4β-$^2H_1$] Desoxypodophyllotoxin (41)

A suspension of (250 mg, 0.604 mmol) of podophyllotoxin (dried under vacuum over boiling water for 24 hrs) in dry benzene was refluxed with PCl_3 (0.31 ml of 2M solution in CH_2Cl_2) for about 45 min. An aliquot was taken and checked for the completion of the reaction. The tlc showed a single spot for the chloride and there was no remaining starting material. The clear solution was decanted off from the yellowish residue, which was washed with hot benzene. The combined benzene solutions were then evaporataed and the residue vacuum dried to give white amorphous podophyllotoxin chloride.

The crude podophyllotoxin chloride was dissolved in dry benzene (10 ml) and added dropwise via a cannula to a stirred suspension of $NaBD_3CN$ (excess) in freshly dried THF (15 ml) at 25°C. The reaction mixture was stirred under an N_2 atmosphere for 15 hrs and then EtOAc was added to decompose the unreacted $NaBD_3CN$. When the effervescence had stopped, the organic layer was collected and the aqueous layer was extracted with $CHCl_3$ in a separatory funnel. The combined organic extracts were washed with water and brine and dried over anh. $MgSO_4$. The residue obtained by removing the solvents in *vacuo* was purified by silica gel column chromatography using ($CHCl_3$:i-PrOH,10.7:0.3) as the eluent, to yield 169 mg (70%) of pure, white desoxypodophyllotoxin, which was identical to the authentic sample on tlc and co-tlc.

^1H nmr (C_6D_6) δ 6.57(s,2H,H-2',6'), 6.47(s,1H,H-5), 6.42(s,1H,H-8), 5.33(dd,2H,H-OCH_2O), 4.41(d,1H,H-1,*J*=4.8Hz), 3.78(s,3H,H-4'-OCH_3), 3.65($dd_{(ca)}$,1H,H-11α,*J*=7.7 and 10.6Hz), 3.51(s,6H,H-3',5'-OCH_3), 3.06(dd,1H,H-11β,*J*=8.5 and 10.6Hz), 2.31(m,1H,H-3,*J*=13.8,5.3,8.5 and 7.7Hz), 2.18(d,1H,H-4α,*J*=5.3Hz), 1.96(d,1H,H-2, *J*=4.8 and 13.8Hz). Eims *m/z* (relative intensity) (**Fig. 10**) 398(5), 399(M^+,100), 400(24), 401(4.6). IR (KBr) (**Fig. 12**) 1776,1484,1238,1126cm^{-1}.

Podophyllotoxone (43)

A mixture of (0.5g, 1.207 mmol) of podophyllotoxin, 3.1g MnO_2 and 30 ml $CHCl_3$ was boiled for about 1·5 hrs. The progress of the reaction was checked by tlc. The reaction mixture was filtered to remove the solids and the residue was rinsed with hot $CHCl_3$. The solvent was removed *in vacuo*, and the crude product was purified using column chromatography on silica gel with ($CHCl_3$:i-PrOH, 10.7:0.3) as the eluent. 0.363g (73%) of faintly yellow crystalline podophyllotoxone was obtained, m.p. 180°

^{1}H nmr ($CDCl_3$) δ 7.55(s,1H,H-5), 6.71(s,1H,H-8), 6.39(s,2H,H-2',6'), 6.1(dd,2H,H-OCH_2O, *J*=1.2Hz), 4.85(d,1H,H-1,*J*=1.2Hz), 4.56(dd,1H,H-11α,*J*=9.1 and 10.4Hz), 4.36(dd,1H,H-11β,*J*=9.1 and 7.7Hz), 3.82(s,3H,H-4'-OCH_3), 3.75(s,6H,H-3',5'-OCH_3), 3.53(m,1H,H-3,*J*=10.6,15.5 and 7.7Hz), 3.29(dd,1H,H-2,*J*=15.5 and 4.3Hz). Eims *m/z* (relative intensity) (page # 52) 412(M^+,100), 413(30), 415(5).

[4β-$^2H_1$]-Podophyllotoxin (44)

Podophyllotoxone (0.363g, 0.881 mmol) was dissolved in dry benzene and was added dropwide into a stirred ethereal solution of $Zn(BD_4)_2$ (5 ml) at 25°C. The mixture was stirred for 24 hrs at r.t. in an N_2 atmosphere and then 15ml of water was added to decompose the excess reagent. When effervescence had abated, 5ml of acetic acid in 15ml water was added. The organic layer was collected and the aqueous layer was diluted with water and extracted with $CHCl_3$. The combined ether and $CHCl_3$ solutions were washed with brine, followed by water and dried (anhy. sodium sulfate). Solvents were evaporated *in vacuo*. Purification by flash chromatography on a silica gel column ($CHCl_3$:i-PrOH, 10.7:0.3ml) afforded (0.236g) a 65% yield of [4β-$^2H_1$]-podophyllotoxin (44), m.p. 183°.

^{1}H nmr ($CDCl_3$) δ 7.12(s,1H,H-5), 6.51(s,1H,H-8), 6.37(s,2H,H-2',6'), 5.99(d,2H,H-OCH_2O,*J*=1.2Hz), 4.59(m,H-11α,1), 4.08(t,1H,H-11β), 3.81(s,3H,H-4'-OCH_3), 3.76(s,6H,H-3',5'-OCH_3), 2.9-2.7(m,2H,H-2,3). Eims *m/z* (relative intensity) (page # 54) 415(M^+.,100), 416(25).

Zinc Borodeuteride

A mixture of reagent grade anhy. $ZnCl_2$ (4g, 0.029 mole) with 50ml ether (freshly dried) was boiled under N_2 until most of the solid had dissolved. The mixture was allowed to stand and the supernatent liquid was added dropwise at r.t. to a stirred suspension of 3.068g (0.073 mole) of

$NaBD_4$ in 150ml of ether, using a cannula. The mixture was stirred overnite under N_2. The supernatant ethereal solution of zinc borohydride was transferred into a container and stored at 5^0 under N_2.

[4α-$^2H_1$] Desoxypodophyllotoxin (42)

[4β-$^2H_1$]-Podophyllotoxin (**44**) (250 mg, 0.602 mmol) podophyllotoxin (**44**) was refluxed with phosphorus trichloride (0.05 ml, 1M in CH_2Cl_2) and the resulting chloride was evaporated *in vacuo* and dissolved in dry benzene. The solution was transferred dropwise, via cannula into a stirred suspension of excess $NaCNBH_3$ in freshly dried THF at r.t. The stirring was continued overnight and the excess reagent was removed with ethyl acetate. The mixture was diluted with water and the product extracted with $CHCl_3$ and purified by flash chromatography on a silica gel column ($CHCl_3$:i-PrOH, 10.7:0.3ml) to afford 178 mg (74%) pure [4α-^{2}H1]- desoxypodophyllotoxin (**42**), m.p. 168^0.

^1H nmr (C_6D_6) δ 6.57(s,2H,H-2',6'), 6.47(s,1H,H-5), 6.42(s,1H,H-8), 5.33(dd,2H,H-OCH_2O), 4.41(d,1H,H-1,*J*=4.8Hz), 3.78(s,3H,H-4'-OCH_3), 3.65($dd_{(ca)}$,1H,H-11α,*J*=7.7 and 10.6Hz), 3.51(s,6H,H-3',5'-OCH_3), 3.06(dd,1H,H-11β,*J*=8.5 and 10.6Hz), 2.31(m,1H,H-3,*J*=13.8,11.4,8.5 and 7.7Hz), 1.96(d,1H,H-2, *J*=4.8 and 13.8Hz), 1.89(d,1H,H-4β,*J*=11.4Hz). Eims *m/z* (relative intensity) 398(5), 399($M^{+\cdot}$,100), 400(25), 401(4). IR (KBr) (**Fig. 13**) 1776,1484,1238,1126cm^{-1}.

[4-$^2H_2$]-Desoxypodophyllotoxin (46)

[4-$^2H_1$]-Podophyllotoxin (**44**) (250mg, 0.602 mmol) was treated with 0.05 ml PCl_3 (1M in CH_2Cl_2) as previously described for podophyllotoxin to yield the deuterated chloride. This dried crude product was dissolved in benzene and added dropwise to a suspension of excess $NaCNBD_3$ in freshly dried

THF and stirred overnight at r.t. After decomposing the excess reagent with EtOAc, the product was obtained by extraction with $CHCl_3$ and was purified by flash column chromatography ($CHCl_3$:i-PrOH, 10.7:0.3ml). A total of 169 mg (70%) of pure **46** was obtained, m.p. 168°.

^1H nmr (C_6D_6) δ 6.57(s,2H,H-2',6'), 6.47(s,1H,H-5), 6.42(s,1H,H-8), 5.33(dd,2H,H-OCH_2O), 4.41(d,1H,H-1,*J*=4.8Hz), 3.78(s,3H,H-4'-OCH_3), 3.65($dd_{(ca)}$,1H,H-11α,*J*=7.7 and 10.6Hz), 3.51(s,6H,H-3',5'-OCH_3), 3.06(dd,1H,H-11β,*J*=8.5 and 10.6Hz), 2.31(m,1H,H-3,*J*=13.8 and 11.4Hz), 1.96(d,1H,H-2, *J*=4.8 and 13.8Hz). Eims *m/z* (relative intensity) (**Fig. 10**) 399(6), 400(M^+,100), 401(25), 402(4).

[Carbonyl-^{2}H]-Hydroxybenzaldehyde(49)

A solution of 0.5g p-cyano phenol (0.0042 mol) and 12.6g KI in 200ml of KOD (0.09M) was irradiated for 70hrs. in a quartz tube with a 400W Hg lamp. (Philips, Westinghouse lamps,400 Watt. The glass covering of the lamp was removed so that it would not filter off the u.v. light). A water condenser was attached to the reaction vessel to prevent the loss of material due to heating. The reaction mixture was then acidified with 10% HCl to pH 4.5 and extracted with EtOAc. The extract was washed with aqueous 1M $Na_2S_2O_3$ solution followed by water, and dried with anhy. Na_2SO_4. The solvent was removed *in vacuo*. The crude product was purified using flash column chromatography with 90:10,$CHCl_3$:EtOAc as eluent. Pure product (0.442g, 0.0036 mols) was obtained in 86% yield. The product was identical (tlc and co-tlc) with an authentic sample (Aldrich). Eims *m/z* (relative intensity) (page # 82) 121(100), 122(28), 123(M^+,89), 124(7).

4-Hydroxy-[3'-^{2}H]-Cinnamic acid (47)

[Carbonyl-^{2}H]-Hydroxybenzaldehyde (**49**) (0.4420g, 0.0036 mols), malonic acid (0.4264g, 0.0041 moles), 1ml dry pyridine and 1 drop each of piperidine and aniline was stirred under an N_2 atmosphere in an oil bath at about 50°. The yellowish gummy mass was treated with 1N H_2SO_4 to remove pyridine. It was then warmed with 1ml cold water. The solution was cooled and filtered to get yellowish solid. The crude sample was then dissolved in boiling water, and neutral Norit A was added to decolorize it. The charcoal was filtered off while hot and the filtrate was cooled to get white needles of pure **47** (0.3151g) in 53% yield after recrystallization. The product was found to be identical with an authentic sample of p-coumaric acid by tlc and co-tlc, mp 216°.

^{1}H nmr (DMSO-d_6:$CDCl_3$) 7.42(d,2H,H-3,*J*=8.6Hz), 6.80(d,2H,H-2,*J*=8.6Hz), 6.21(1H,s,H-2'). Eims *m/z* (relative intensity) (**Fig. 15**) 164(26), 165($M^{+\cdot}$,100), 166(11).

<u>Cu^{13}CN:</u>

4.4783g $CuSO_4$ was dissolved in 16ml water (40- 50°), 1.4g $NaHSO_3$ was dissolved in 4ml water (50-60°) and 1.0g (0.0 mols) of Na^{13}CN was dissolved in 4ml water (50-80°). The solutions were kept at 60°. The bisulphite solution was run slowly with stirring into the acidified (congo red) copper solution, followed immediately by the cyanide solution. After 10 minutes, the hot solution was filtered, the product washed thoroughly with boiling water and then with alcohol, and dried overnight at 100° to a fine soft powder, 84% (1.5291g) yield.

<u>[Cyano-^{13}C]-4-Cyanophenol (50)</u>

Cu^{13}CN (1.5291g, 0.0169 mol) prepared from Na^{13}CN, (3.2132g, 0.0186 mol) p-bromo phenol and 16.5ml dry DMF (dried by distillation over P_2O_5) were refluxed under N_2 in a 50ml flask for 4hrs. The reaction mixture was then cooled and diluted with 30ml of ferric chloride solution (11.25g

$FeCl_3.6H_2O$ in 22.5ml H_2O + 7.5ml conc.HCl). The mixture was warmed at 50-60° until no oily layer was visible, and extracted with ether while warm. The ether layer was washed with water, dried with anhy.Na_2SO_4 and evaporated *in vacuo*. The crude product was purified using column chromatography with (hexane:ether, 70:30) as eluent. The unreacted starting material came off first and then p-cyano phenol. The solvent was removed using a rotary vaccuum pump leaving the pure p-cyano phenol, white flakes in 89% (1.8098g) yield.

IR spectrum (KBr): 2200 cm-1. Identical on proton nmr, tlc and co-tlc to an authentic sample of 4-cyanophenol (Aldrich).

[3'-^{13}C / ^{2}H]4-Hydroxycinnamic acid (48)

[Cyano-^{13}C]-4-Cyanophenol (**50**) (0.0759g, 0.625 mmol) was reduced photolytically by the same procedure described for 4-hydroxy-[carbonyl-^{2}H]-benzaldehyde to give the title compound **51**. The crude product was purified using flash column chromatography, with $CHCl_3$:EtOAc, 90:10 as eluent. Pure product **51** (0.0667g, 0.5375 mmols), was obtained in 86% yield. It was found to be identical with the tlc and co-tlc of the authentic sample. **51** (0.0533g, 0.4302 mmols) was condensed with malonic acid (0.0537g, 0.5162 mmols) by the procedure described earlier to give the title compound, p-coumaric acid (**48**) (0.0393g),in 55% yield after recrystallization, m.p. 216.5°.

^{1}H nmr (DMSO-d_6:$CDCl_3$) 7.42(q,2H,H-3), 6.8(d,2H,H-2,*J*=8.6Hz), 6.21(s,1H,H-2',*J*=8.6 and 4.4Hz). ^{13}C nmr (DMSO-d_6:$CDCl_3$) 113.7, 114.2, 120.5, 128.1, 142.5, 158.1, 166.5. Eims *m/z* (relative intensity) (page # 87) 165(26), 166(M^+,100), 167(11).

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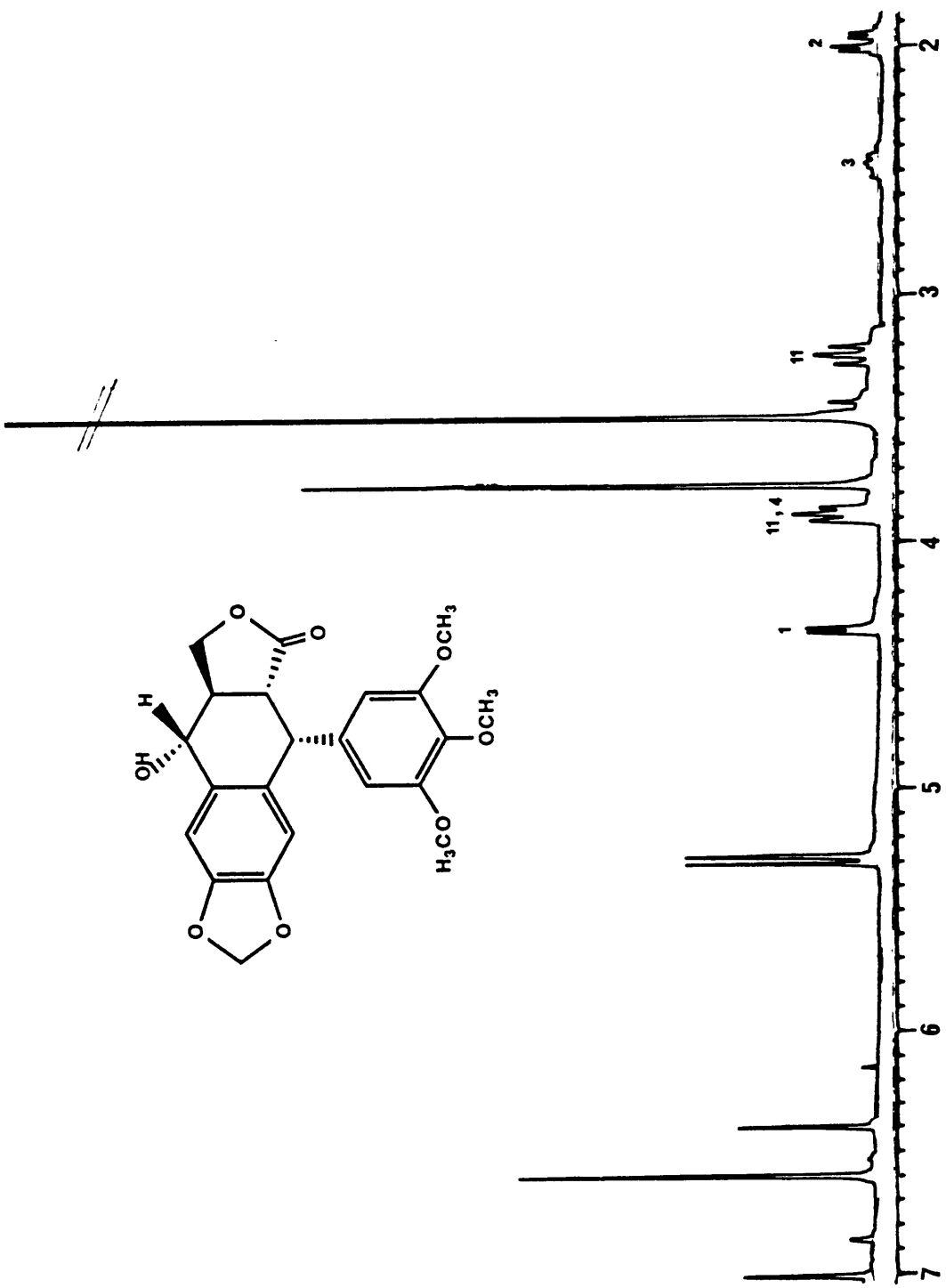
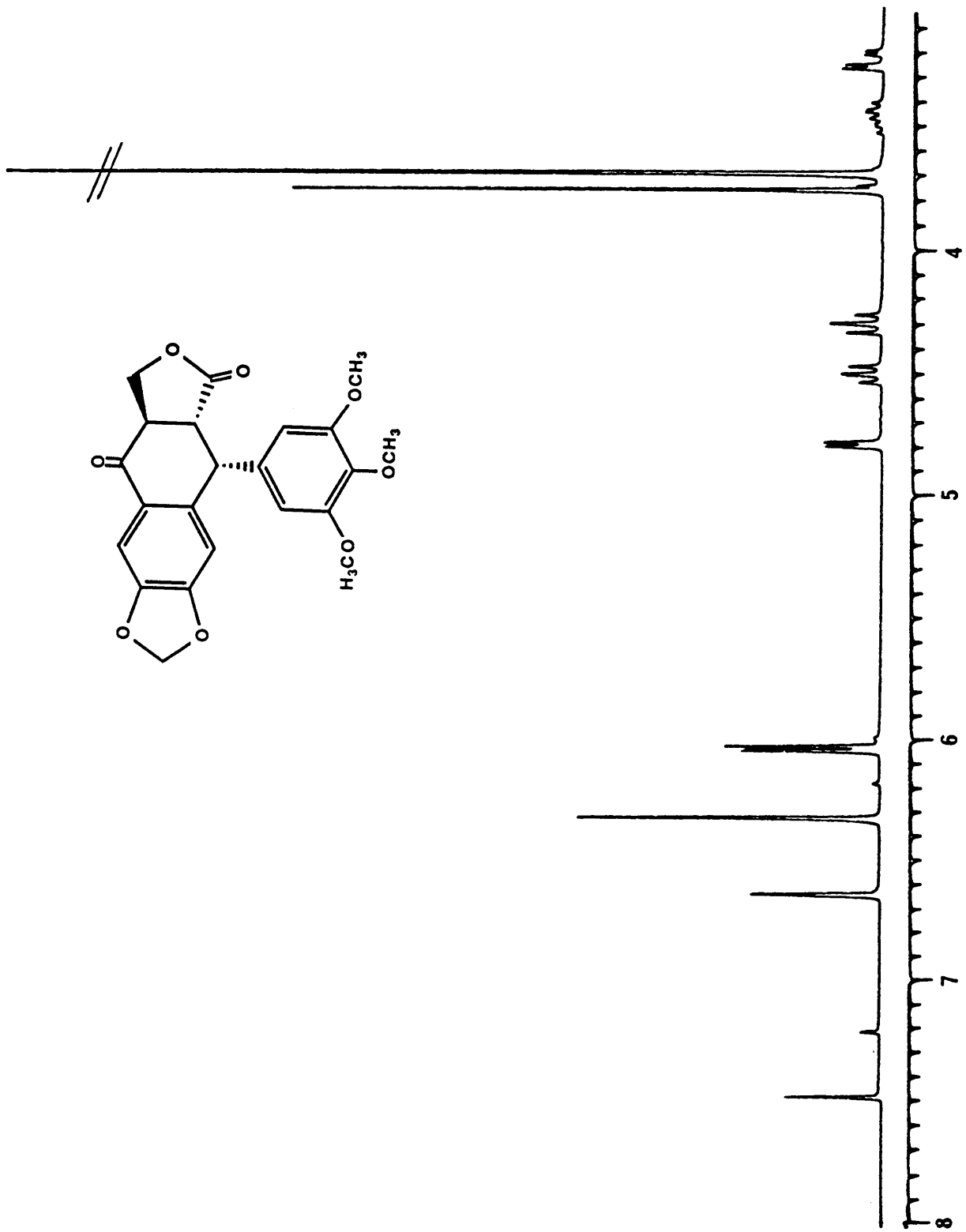
VIII. APPENDIX

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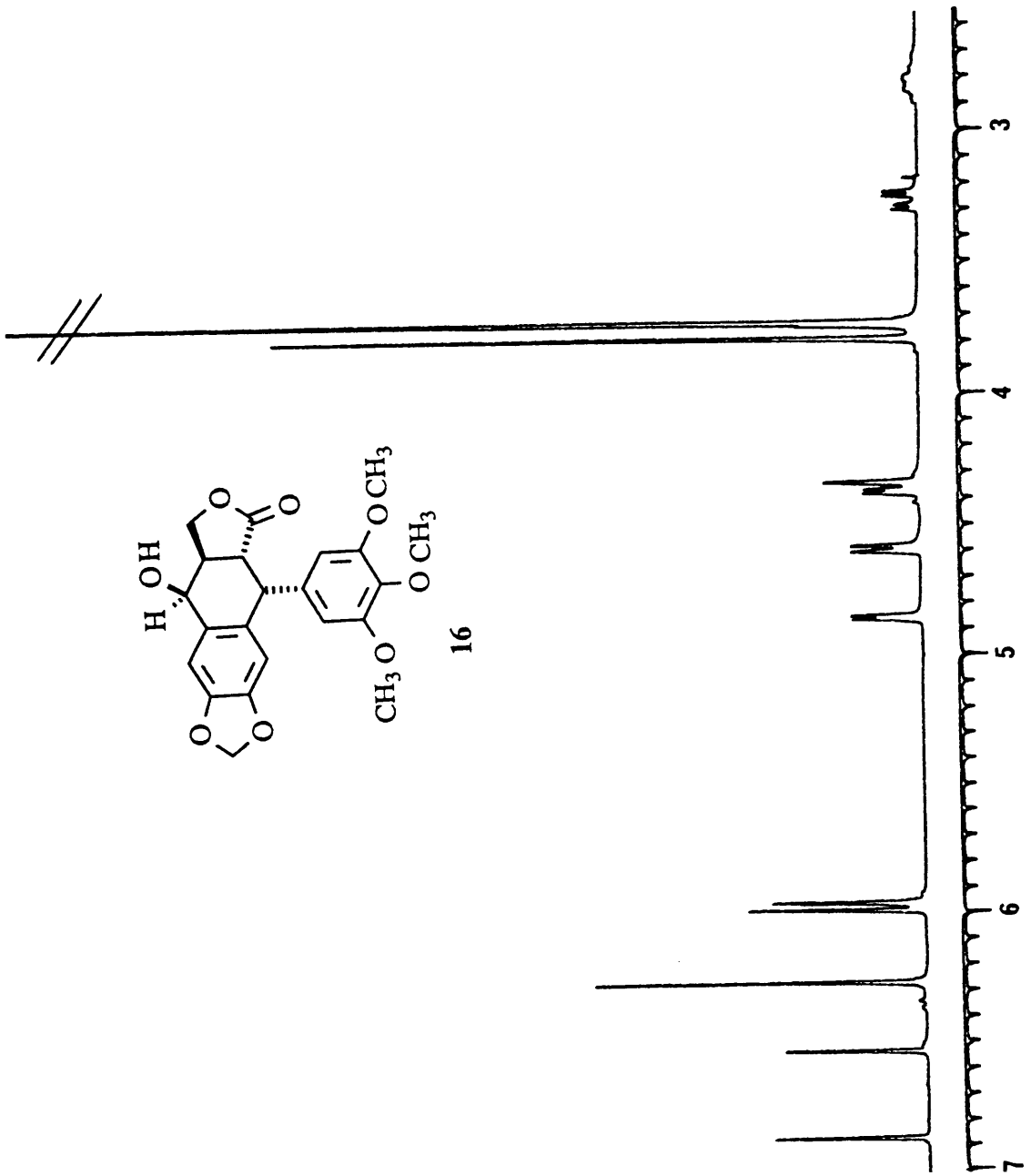

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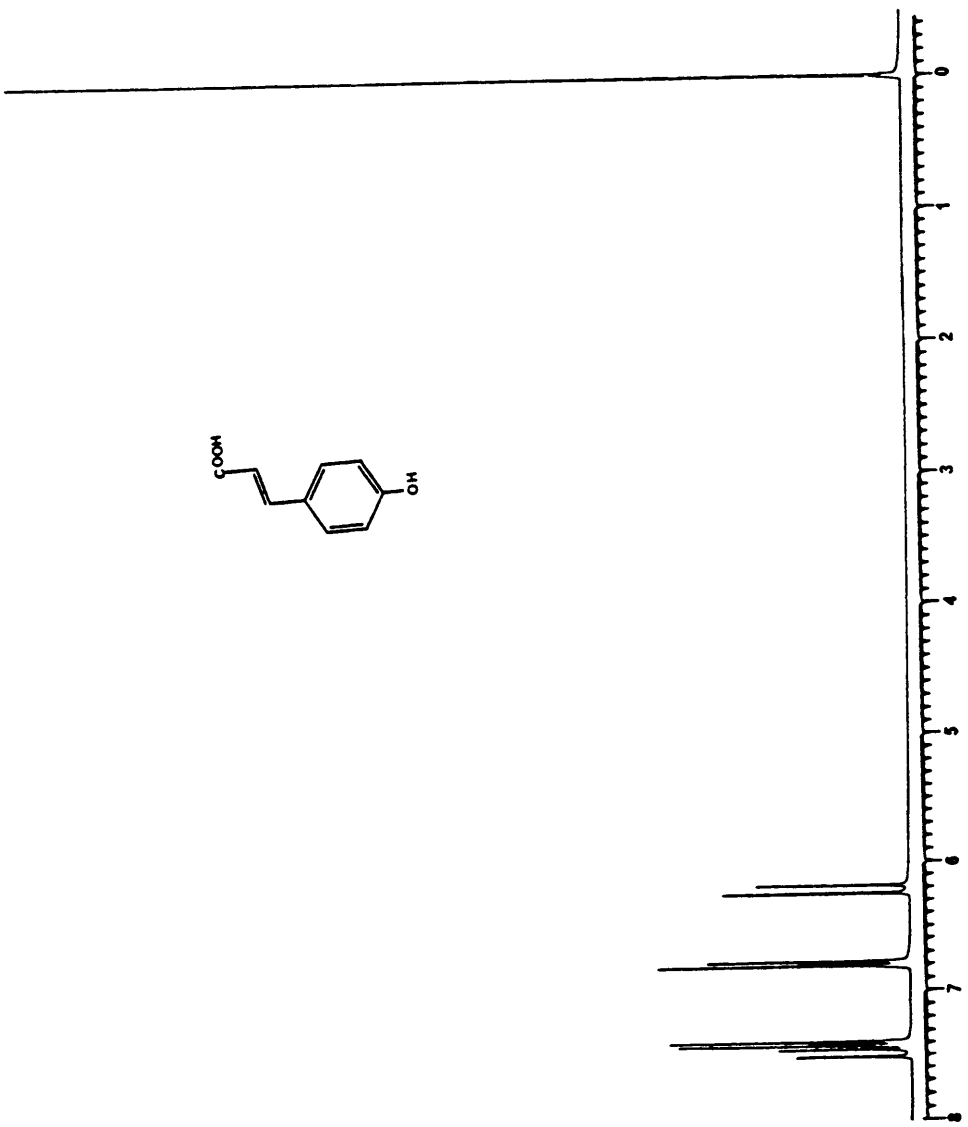
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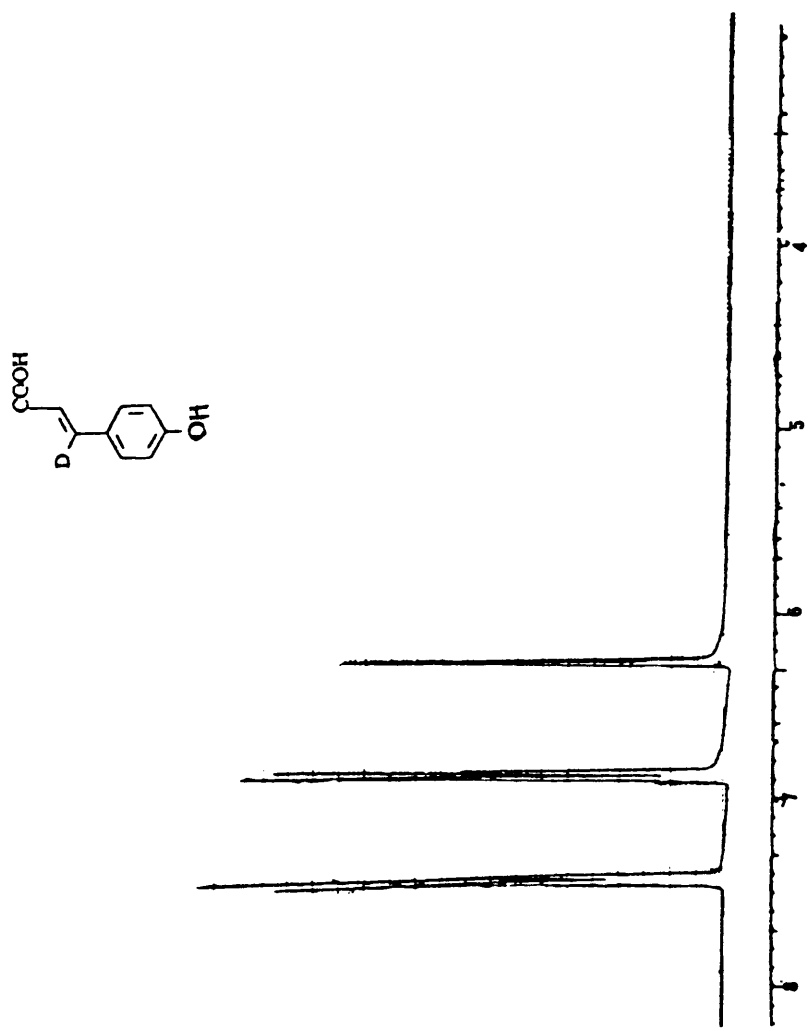
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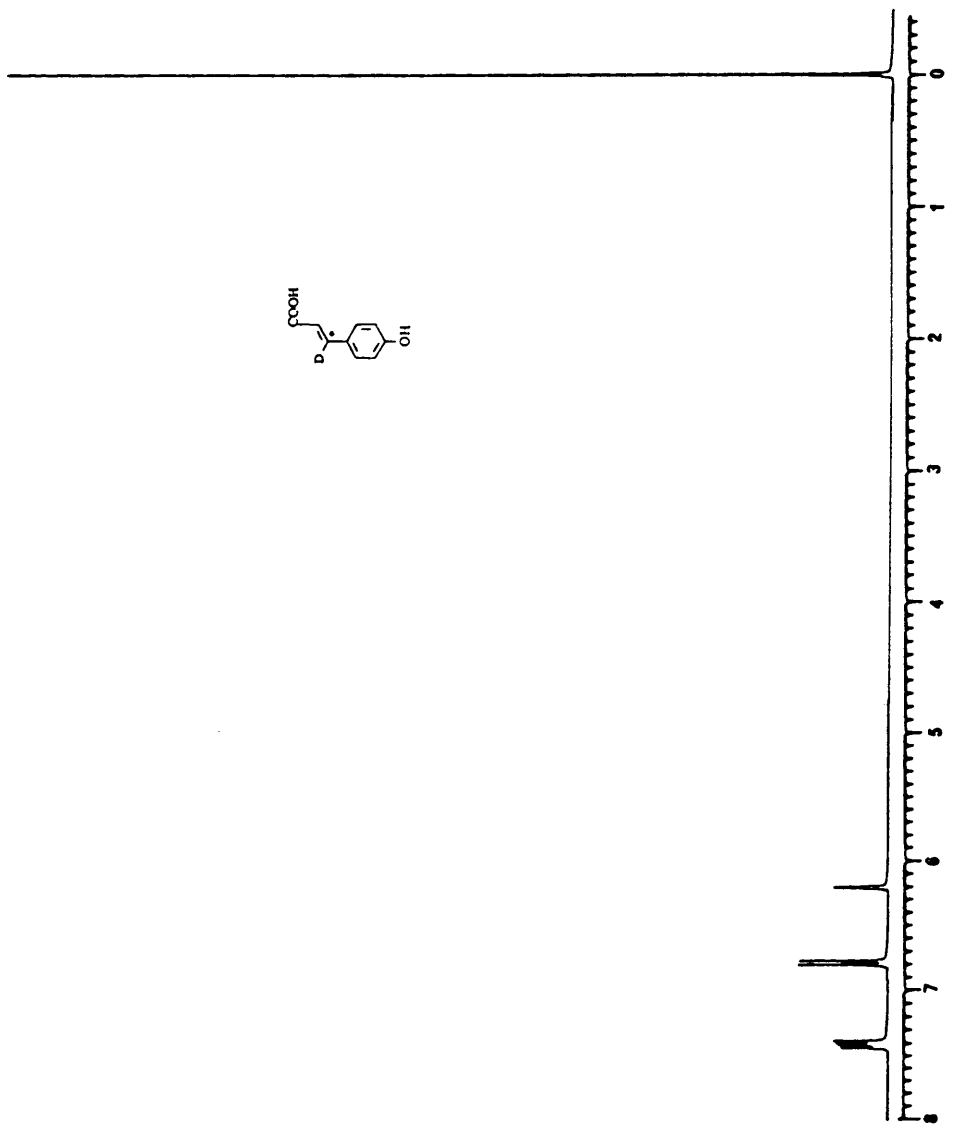
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SYNTHESIS OF LABELLED PRECURSORS OF PODOPHYLLOTOXIN

by

Anie Jose Pullockaran

Committee Chairman: David G. I. Kingston

Chemistry

(Abstract)

Podophyllotoxin is an aryltetralin lignan and anticancer agent produced by the *Podophyllum* and some other species and is extracted from Podophyllin in up to 30 percent yield. Little definitive information is available on the biosynthetic pathways to podophyllotoxin. Administering various specifically labelled precursors into intact plants and tissue cultures can give a much improved insight into the lignan biogenesis. The first part of the work has been to the synthesis of two diastereotopically labelled with ^{2}H at C-4. Desoxypodophyllotoxin is the penultimate intermediate of the podophyllotoxin pathway and specifically labelled compounds can be used for the C-4 hydroxylation studies. Doubly ^{2}H labelled desoxypodophyllotoxin at C-4 was also prepared which can be used for a control experiment. This work also led to the unambiguous assignment of the proton nmr of desoxypodophyllotoxin. A second part of the work has been the synthesis of p-coumaric acid, a known lignan precursor, doubly labeled with ^{2}H or ^{2}H and ^{13}C at the C-3' position. This labeling was selected because the availability of a C-3' labeled monomeric precursor would thus make possible the isolation and study of dimeric compounds labeled at this key positions. These compounds were synthesized, and their identity and stereochemistry were determined by spectroscopic and analytical techniques. The isotopic incorporation of these compounds was 95% d_1 or d_2 (or greater), as determined by mass spectral analysis.