Study of Podophyllotoxin Biosynthesis in

Podophyllum Species

by

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(ABSTRACT)

Lignans are a structurally diverse group of phenylpropanoid metabolites widely distributed throughout the plant kingdom. Their biogenetic pathway has generally been viewed to occur via coupling of two monomeric moieties, with subsequent modifications as required to afford the various lignan skeleton structures. Podophyllotoxin is a tetrahydronaphthalene lignan found in *Podophyllum* species, and its derivative etoposide is widely used medicinally for skin cancer and venereal warts (*Condyloma acuminatum*).

In this investigation, the biogenetic pathway to podophyllotoxin was investigated using whole plants, as well as callus and rhizome tissue. In contrast to previous claims in the literature, no active metabolism leading to podophyllotoxin formation *in vivo* in callus culture was observed. Similar findings were also noted for rhizome tissue. With whole plants, experiments investigating podophyllotoxin formation have been limited to employing lignan substrates, labeled specifically with carbon-14 and tritium. No stable isotopes, *e.g* carbon-13 or deuterium, have been used because of low incorporations. To overcome this difficulty, plants were grown hydroponically for 6 to 9 weeks in an aseptic medium containing either [U-^{14}C-]L-phenylalanine or [1-^{13}C-]L-phenylalanine as precursors. Thus, following administration of [U-^{14}C-]L-phenylalanine to intact P. peltatum plants grown hydroponically, a high in-

corporation into podophyllotoxin was observed (3.30 to 10.23 % absolute incorporation). Next, [1-^{13}C-]L-phenylalanine was administered to the *Podophyllum* plant for 90 days. Following isolation of podophyllotoxin, conversion to its acetate derivative, and subsequent analysis by ^{13}C NMR, it was established that [1-^{13}C-]L-phenylalanine was intactly incorporated into podophyllotoxin. This finding was based upon the carbon-13 enhancement of two signals at 71.3 ppm (C-9′) and 173.6 ppm (C-9). This is the first report of carbon-13 enhancement in a lignan using a carbon-13 specifically enriched precursor.

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Introduction

Lignans are a naturally occurring class of aromatic compounds widely distributed in the plant kingdom and are products of the shikimate/chorismate pathway. By 1978 over 200 lignans had been identified in a wide variety of species in the plant kingdom;[1] since then, many more have been discovered. In 1936 Hartworth first defined lignans as dimers of phenylpropanoid "C_6C_3" compounds coupled at the β-carbons of their propanoid side chains (see Figure 1).[2] More recently, the definition has been expanded to include other natural dimers formed from differently substituted allylphenols by oxidative coupling.[3,4] Lignans of linkages other than "$\beta\beta$-linked" are called neolignans, e.g. cedrusin 1 from *Cedrus deodara* (see Figure 2).[5] For simplicity this discussion will focus on Hartworth's classical lignan definition of "$\beta\beta$-linked" carbon skeleton substitutions, some of which are classified as: (A) diarylbutanes, e.g. phyllanthin 2, (B) diarylbutyrolactones, e.g. arctiin 3, (C) tetrahydrofurans, e.g. liovil 4, (D) tetrahydrofuranofurans, e.g. pinoresinol 5, and (E) tetrahydronaphthalenes, e.g. podophyllotoxin 6 (see Figure 3).

While lignans are found in the bark, leaves, stems, roots, and rhizomes of many plant species, their actual functions are not fully understood. It is speculated that

plants use lignans as insect, fungal, and bacterial deterrents, since many of these compounds have physiological effects such as antiviral, antimitotic, and cytotoxic activity.[6] Interestingly, folk medicines dating back over 1000 years have used crude lignan drugs from plant extracts. For example, an early English text dating from 900-950 AD, the Leech Book of Bald, mentions the use of root extracts from chervil, (*Anthriscus* species, of the parsley family which is presumably lignan rich) as a salve for cancer.[7] Notably, the closely related species *Anthriscus sylvestris* contains deoxypodophyllotoxin 7;[1] this lignan has antitumor activity.[6] Indians in North America and the Himalayas used root extracts from the perennials *Podophyllum peltatum* and *P. hexandrum*, respectively, which contains the lignan podophyllotoxin 6, as treatment for warts and other ailments.[8,9] Currently podophyllotoxin 6 derivatives are used to combat *Condyloma acuminatum* (venereal warts), a variety of leukemias, and skin cancer. Wilson and Friedkin in 1967 were the first to determine the antimitotic mechanism of podophyllotoxin 6[10] in an experiment using grasshopper embryo tubulin, where they determined that podophyllotoxin 6 is a competitive inhibitor of colchicine 8 binding to tubulin. Like the colchicine-tubulin complex, the podophyllotoxin-tubulin complex inhibits microtubular assembly during mitosis.[11] By itself podophyllotoxin 6 is too toxic for clinical use. However, a synthetic derivative, etoposide 9 (VP-16-213, 4-demethyl epipodophyllotoxin ethylidene-β-D-glucoside) is used as an antitumor agent. Both podophyllotoxin 6 and etoposide 9 inhibit nucleoside transport, even though etoposide 9 does not have the antimitotic properties of podophyllotoxin 6. Etoposide 9 can also induce single stranded breaks in DNA which explains its cytotoxicity[12, 13] (see Figure 4). While a detailed discussion of this topic is beyond the scope of this thesis, the reader is referred to the comprehensive review by MacRae and Towers covering the biological activities of lignans.[6]

Although lignans are an important class of natural products, little is known about their biosynthesis. It is generally thought that lignan formation *in vivo* involves the phenolic oxidative coupling of two "C_6C_3" monomers, *i.e.* via free radical coupling in a manner similar to that envisaged for the initial stages of lignin biosynthesis.[14] By contrast, lignin is a high molecular weight polymer that is an important component of the cell wall of vascular plants. Its several functions include strengthening the cell wall allowing plants to conduct water and to grow into large, upright structures;[15] and also, protecting the plant from biological, chemical, and physical attacks. Lignification is a well documented process and is a major offshoot of the phenylpropanoid pathway.[16]

Shikimic Acid Pathway

In 1885, Eykmann isolated shikimic acid 10 from the Japanese plant shikimi-no-ki (*Illicium religiosum*).[17] In 1955, Bernard Davis reported that shikimic acid 10 was a common precursor of the aromatic metabolites phenylalanine 11, tyrosine 12, tryptophan 13, p-aminobenzoic acid 14, and p-hydroxybenzoic acid 15 in mutants of *Eschericha coli* and *Aerobacter aerogenes* (see Figure 5). These mutants were "aromatic polyauxotrophs" which require traces of p-aminobenzoic acid 14 and p-hydroxybenzoic acid 15, and three amino acids; phenylalanine 11, tyrosine 12, and tryptophan 13 to exist. Davis discovered that the mutant strains could survive on media where shikimic acid 10 was substituted for the amino acids and trace benzenoid compounds. Moreover, shikimic acid 10 accumulated in other bacterial strains when the pathway beyond shikimic acid 10 was blocked.[18]

Shikimic acid 10 biosynthesis involves a series of enzyme catalyzed reactions starting with phosphoenolpyruvate (PEP) 16 and D-erythrose-4-phosphate 17, both of which are derived from D-glucose via glycolysis and the pentose pathway, respectively. These intermediates couple together in a reaction catalyzed by 3-deoxy-D-arabino-heptulosonate-7-phosphate, (DAHP) synthase which results in the formation of 3-desoxy-D-arabinose heptulosonic-7-phosphate (DAHP) 18 and orthophosphate (see Figure 6 and Table 1).[19] *Nicotiana silvestris*[20] and mung bean[21] both contain two distinct DAHP synthase isozymes, DAHP synthase-Mn and DAHP synthase-Co. The isozymes have different properties of regulation and distinct characteristics, such as the ability of DAHP synthase-Co to substitute Mg^{++} for Co^{++} at high Mg^{++} concentrations. DAHP synthase-Co is inhibited by caffeic acid 19. In the cytosol of higher plants it was observed that DAHP synthase-Co is able to substitute D-glyceraldehyde-3-phosphate for D-erythrose-4-phosphate 17 in the condensation with PEP 16 forming 2-keto-3-deoxy-D-threo-hexulosonate-6-phosphate (DTHP). However, the metabolic fate, if any, of this compound is not known. On the other hand, DAHP synthase-Mn does not substitute D-glyceraldehyde-3-phosphate for D-erythrose-4-phosphate 17. This enzyme is only activated by the cation Mn^{++} and is inhibited by arogenic acid 20.[22]

The cyclization of DAHP 18 to 3-dehydroquinate 21 is envisaged to occur through a complex sequence of reactions. It is speculated that 3-dehydroquinate synthase enables this conversion to occur by an oxidation, β-elimination, reduction, and an intramolecular aldol condensation (see Figure 7).[23] In *E. coli*[24] and in etiolated *Phaseolus mungo* seedlings,[25] the enzyme requirements are Co^{++} and NAD^{+}. Following cyclization, 3-dehydroquinate dehydratase eliminates water from 3-dehydroquinate 21 to form 3-dehydroshikimate 22 in a reversible reaction via a syn elimination.[26, 27]

Shikimic acid 10 formation is catalyzed by 3-dehydroshikimate reductase from 3-dehydroshikimate 22 (see Figure 6 and Table 1). Originally this reversible reductase enzyme was thought to exclusively use nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor. However, in 1986 three isozymes of 3-dehydroshikimate reductase were isolated from *Pinus sylvestris* which could use both NADPH and nicotinamide adenine dinucleotide (NADH).[28] Shikimic acid 10 is then phosphorylated to shikimic acid 3-phosphate 23 by adenosine triphosphate (ATP) and shikimate kinase.[29] A second molecule of phosphoenol pyruvate 16 is attached to shikimic acid 3-phosphate 23 resulting in 5-enolpyruvylshikimate-3-phosphate 24 in a condensation reaction catalyzed by 5-enolpyruvylshikimate-3-phosphate synthase. 5-enolpyruvylshikimate-3-phosphate 24 undergoes loss of orthophosphate (H_3PO_4) in a stereochemically controlled reaction yielding chorismic acid 25 and is catalyzed by chorismate synthase. In 1978 Hasen *et al.* discovered chorismate synthase activity in the bacteria *Bacillus subtilis*.[30] Since then, this enzyme has been detected in pea (*Pisum sativum*) tissue extracts and chloroplasts.[31]

Chorismic Acid Pathway

Chorismic acid 25 is an important metabolic branching point leading to several aromatic compounds among which are various vitamins, folic acid, lignins, lignans, tryptophan 13, flavonoids, and coumarins.[16] Chorismic acid 25 derives its name from the Greek word "chorismic" meaning to branch or separate.[32] Gibson and Gibson (1964) were the first to detect and identify chorismic acid 25 using mutant strains of *Aerobacter aerogenes*. These strains were incapable of synthesizing phenylalanine

11 and tyrosine 12, but accumulated an unknown compound, later identified as chorismic acid 25. Upon further investigation, Gibson and Gibson established that chorismic acid 25 was formed from shikimic acid 10, and that it could be enzymatically converted into three major compounds: anthranilic acid, prephenic acid 26, and p-hydroxybenzoic acid 15.[32] Beyond chorismic acid 25 this review will only discuss the branch to phenylalanine 11 and tyrosine 12 from prephenic acid 26.

In a Claisen-like rearrangement, prephenic acid 26 is enzymatically synthesized from chorismic acid 25 by chorismate mutase (see Figure 8 and Table 2). In *Nicotiana silvestris*, two distinct isozymes of chorismate mutase have been found, plastidic CM-1 and cytosolic CM-2.[33, 34] The CM-1 isozyme is activated by tryptophan 13, and inhibited by phenylalanine 11 and tyrosine 12. However, CM-2 is not regulated by aromatic amino acids, but is inhibited by caffeic acid 19. Similar pairs of compartmentalized isozymes have also been found in potato tuber.[35, 36]

Originally, the accepted pathways for phenylalanine 11 and tyrosine 12 biosynthesis consisted of the conversion of prephenic acid 26 into phenylpyruvic acid 27 or 4-hydroxyphenylpyruvic acid 28, by catalysis of prephenate dehydratase and prephenate dehydrogenase, respectively. Following this, these intermediates underwent an enzymatically-controlled transamination to afford phenylalanine 11 and tyrosine 12 (see Figure 9 and Table 2). However, in 1974 a novel enzyme, prephenate aminotransferase, was detected in blue-green bacteria[37]; the blue-green bacteria converted prephenic acid 26 into arogenic acid 20, a then newly discovered intermediate which was subsequently converted to tyrosine 12. Since then, arogenate dehydratase and arogenate dehydrogenase have been found which transform arogenic acid 20 into phenylalanine 11 and into tyrosine 12, respectively (see Figure 9 and Table 2). For instance, *N. silvestris* cell cultures contained arogenate dehydratase activity which converted arogenic acid 20 into phenylalanine 11; how-

ever, prephenate dehydratase activity was not detected.[38] Likewise, arogenate dehydrogenase was detected in *Sorghum bicolor X S. sudanesis*, while prephenate dehydrogenase activity was not detected for the formation of tyrosine 12 from arogenic acid 20.[39]

In higher plants more proof is accumulating which suggests that arogenic acid 20 is the major, if not the sole, intermediate for the biosynthesis of phenylalanine 11 and tyrosine 12 from prephenic acid 26. Indeed, Jensen has claimed that evidence cited in older literature may not be valid concerning the *in vivo* pathways of phenylpyruvic acid 27 and 4-hydroxyphenylpyruvic acid 28 for higher plants.[22] For example, Widholm[40] had previously demonstrated that by adding labeled phenylpyruvic acid 27 and 4-hydroxyphenylpyruvic acid 28 to growth media, they could be converted into phenylalanine 11 and tyrosine 12, respectively. However, Jensen points out that labeled compounds many have been transaminated by non-specific aminotransferases.[22]

Phenylpropanoid Pathway

The phenylpropanoid pathway is the transformation of either phenylalanine 11 or tyrosine 12 to the cinnamic acids: p-coumaric 29, ferulic 30, and sinapic 31 acids.[16] The sequence of reactions is described as follows. Both phenylalanine 11 and tyrosine 12 can undergo stereospecific deamination to afford E-cinnamic acid 32 and E-p-coumaric acid 29, respectively. In the plant kingdom the conversion of tyrosine 12 to p-coumaric acid 29 has only been demonstrated in grasses.[41] Tyrosine ammonia-lyase usually catalyzes the reaction; although, in maize phenylalanine ammonia-lyase has the ability to deaminate both phenylalanine 11 and tyrosine 12.[42]

In plants other than grasses, phenylalanine ammonia-lyase is the only enzyme known. Phenylalanine 11 is converted to E-cinnamic acid 32 by a stereospecific anti-elimination of the pro-3S hydrogen and ammonia.[43, 44, 45] Phenylalanine ammonia-lyase (PAL) activity is highly regulated by the physiological state of the plant, where stimulants such as light, pathogens, and plant growth regulators control PAL expression and activity.[46]

Cinnamic acid 32 is enzymatically converted to its derivatives, p-coumaric 29, ferulic 30, and sinapic 31 acids. The conversions begin with the hydroxylation of cinnamic acid 32 to p-coumaric acid 29 by cinnamate 4-hydroxylase, an enzyme specific for the trans isomer.[47] Caffeic acid 19 is then formed by the hydroxylation of p-coumaric acid 29 with p-coumarate-3-hydroxylase.[48] Subsequently, caffeic acid 19 is methylated via a meta-directing O-methyltransferase and S-adenosyl-L-methionine to form ferulic acid 30.[49]

In principle, sinapic acid 31 could be formed in two possible ways. First, the hydroxylation of caffeic acid 19 affords 3,4,5-trihydroxycinnamic acid 33, which could then be subsequently methylated specifically at the 3- and 5- hydroxy positions. However, 3,4,5,-trihydroxycinnamic acid 33 has never been isolated from plants. Alternatively, ferulic acid 30 could first be hydroxylated at C-5 to give 5-hydroxyferulic acid 34, and subsequently methylated to yield sinapic acid 31 (see Figure 10 and Table 3).[50] In support of this latter pathway, 5-hydroxyferulic acid 34 was found in cell walls of *Zea mays* and *Hordeum vulgare*,[51] and ferulic acid 5-hydroxylase has been isolated from *Populus* X *euramericana*.[52] Since the 5-hydroxyferulic acid 34 pathway has now been demonstrated to occur, similar lines of evidence are required if a pathway involving 3,4,5-trihydroxycinnamic acid 33 is to be considered further.

Lignin Biosynthesis

Prior to lignification, the cinnamic acids 29-31 are first enzymatically converted to the three E-monolignols: p-hydroxycinnamyl 35, coniferyl 36, and sinapyl 37 alcohols (see Figure 11 and Table 3). Beginning with 4-coumarate CoA synthase,[53] the hydroxycinnamic acids 29-31 are activated first as their adenosine monophosphate (AMP) esters, and then as their CoA thioesters. The CoA thioesters are converted to aldehydes with cinnamyl CoA NADPH reductase in the presence of NADPH.[54] Finally, the aldehydes are reduced with NADPH in a reversible reaction to the respective monolignols by cinnamyl alcohol dehydrogenase. It is interesting to note that cinnamyl CoA NADPH oxidoreductase is classified as a "type B" enzyme and cinnamyl alcohol dehydrogenase is classified as a "type A", even though both enzymes utilized NADPH as a reducing co-factor. The distinction results from the manner in which hydrogen is abstracted from the NADPH. The type A abstraction removes the "R" hydrogen, while the type B abstraction removes the "S" hydrogen (see Figure 12).[55]

It has been documented that the monolignols can be converted to cinnamyl alcohol glucosides,[15] and these glucosides may act as precursors to lignin in a variety of species.[56] However, the exact role of the cinnamyl alcohol glucosides in lignification is uncertain. The speculative roles of the glucosides are for either storage (*i.e.* lignin precursor reservoir[54]) or monolignol transport to the site of lignification where the glucose is cleaved by a β-glucosidase.[57] However, in a study with *Picea abies*, the β-glucoside, coniferin 38, only accounted in part for the lignin synthesized perhaps indicating that its main role may be in storage capacity.[58] Somewhat in agreement with this, lignifying tissue possesses all the enzymes needed to synthe-

size monolignols from phenylalanine 11.[55] Hence, all monolignols apparently do not need to be glucosylated prior to lignification.

During lignification, the monolignols are converted into their free radical forms by peroxidase in the presence of H_2O_2 to afford five main mesomeric hybrids based on electron spin densities (see Figure 13). By random coupling there could be 25 types of interunit linkages between the monomers; however, not all interunit linkages are found in lignin since some are unstable (e.g. a-a linkage) or are sterically hindered due to low electron-spin density, (e.g. coupling of a and e only when R ≠ H). The phenoxy radicals have the highest electron-spin density at the phenolic oxygen. Since the a-a linkage is unstable, the phenolic oxygen preferentially couples with the second highest electron-spin density site at the β position on the propanoid side chain. Hence, the β-O-4 linkage (see Figure 14) is the most favored linkage in lignin and is believed to encompass approximately 50% of the interunit linkages in lignin.[57] As mentioned previously, the monolignol radicals apparently couple randomly. This appears to be supported by analysis of isolated lignins which are racemic and optically inactive.

In contrast to the optical inactivity of lignins, in several plant species only one of the enantiomeric forms of a lignan is found. For instance, (+)-sesamin is found in the bark of *Zanthoxylum acanthapodium* and and the seeds of *Piper longum*. However, the (-)-enantiomer (-)-sesamin has been isolated from the bark of *Xanthoxylum piperitum* and the leaves of *Ruta montana*.[1] Only a few lignans have been isolated as racemic mixtures (e.g. (±)-syringaresinol has been isolated from *Vinca minor* and *V. major*[1,59]). The optical activity typically observed for these isolated lignans suggests strict enzymatic control of the dimerization coupling for two "C_6C_3" units.

Lignan Biosynthesis Literature Review

In 1969, Ayres published the first experimental evidence that lignans are formed *in vivo* by coupling of two phenylpropanoid monomers.[60] In this work [U-^{14}C]-phenylalanine 11 was wick-fed to a 5-year old *P. hexandrum* plant. A 1.4% incorporation of phenylalanine 11 was reported in the lignan podophyllotoxin 6. In 1981 Ayres *et al.*[61] conducted a similar experiment administering DL-[β-^{14}C]-phenylalanine 11 to *P. hexandrum*. Following isolation and oxidative degradation of podophyllotoxin 6, they determined that two "C_6C_3" monomers were equally incorporated into podophyllotoxin 6 (see Figure 15).

Independently, Stöckigt and Klischies demonstrated in 1977 [63] that both lignan glucosides, arctiin 3 and phillyrin 39, are derived from the coupling of two phenylpropanoid monomers. In their experiments 3,4-[4-O-C^3H_3]-dimethoxycinnamic acid 40 and the doubly labeled precursors, [β-^{14}C,-OC^3H_3]-glucoferulic acid 41, [β-^{14}C,-OC^3H_3]-coniferyl aldehyde 42 and [β-^{14}C,-OC^3H_3]-coniferin 38 were individually administered to shoots of a *Forsythia* species, thought to be *F. suspensa*. 3,4-[4-O-C^3H_3]-dimethoxycinnamic acid 40 was not incorporated into the lignans, suggesting that the 4-hydroxyl group may be necessary for the postulated phenolic oxidative coupling mechanism. On the other hand the doubly labeled arylpropane derivatives 38, 41 and 42 were incorporated into arctiin 3 and phillyrin 39 with no significant change in the $^3H/\ ^{14}C$ ratios (see Figure 16 and Table 4) suggesting that each was incorporated discretely into the lignans of interest. In Stöckigt and Klischies original paper, *F. suspensa* was identified as the species used in their experiments. However, the identification may be in error since arctiin 3 is not present

in this species.[63,64] However, both *F. intermedia* [64] and *F. koreana* [65] contain arctiin 3 and phillyrin 39.

In 1984[66] Jackson and Dewick investigated precursor relationships to podophyllotoxin 6 in *P. hexandrum*. In their experiments, they administered DL-[1-^{14}C-] phenylalanine 11 and the sodium salts of ^{14}C labeled cinnamic acids (32, 30-31, and 43-44, Figure 17) to the roots of 2-3 year old pot grown plants. Phenylalanine 11 and cinnamic acid 32 were found to be reasonably good precursors of podophyllotoxin 6, while ferulic acid 30, sinapic acid 31, 3,4-methylenedioxycinnamic acid 43, and 3,4,5-trimethoxycinnamic acid 44 were poorly incorporated (< 0.1 %) into podophyllotoxin 6 (Table 5). Although [3-O^{14}CH$_3$]-ferulic acid 30 was poorly converted into podophyllotoxin 6, it appears that both halves of podophyllotoxin 6 may have been derived from this precursor. This possibility was investigated in two reactions where the molecule was degraded and the products were analyzed (Figure 18). Podophyllotoxin 6 was first converted to the diphenol 45 using boron trichloride, where it was found that the specific activity was approximately 50 % of the original specific activity indicating that approximately 50 % of the activity was present in the methylenedioxy functionality. In the second reaction, podophyllotoxin 6 was acetylated to afford podophyllotoxin acetate 46 and then treated with alkaline permanganate. The benzoic acid fragment (3,4,5-trimethoxybenzoic acid 47) of podophyllotoxin 6 was shown to possess approximately 50 % of the original carbon-14 radiolabel. This precursor experiment suggests that two phenylpropane compounds containing a pendant ring substitution similar to ferulic acid 30 may be precursors of podophyllotoxin 6 (see Figure 18 and Table 6).

Recently, precursor experiments were conducted by Rahman *et al.*[67] with young shoots of *F. intermedia* to investigate the biosynthetic pathways leading to

epipinoresinol 48, phillygenin 49, and arctigenin 50. [β-^{14}C,γ-^{3}H]-Coniferyl alcohol 36 (^{3}H:^{14}C ratio 8.93) was administered to *F. intermedia* shoots which were then allowed to metabolize for 72 hours. [β-^{14}C,γ-^{3}H-Coniferyl alcohol 36 (see Figure 19) was poorly incorporated into arctigenin 50 (0.12%), and its incorporation into phillygenin 49 and epipinoresinol 48 was almost negligible (< 0.05 %). While a marked increase of the ^{3}H:^{14}C ratio was observed (see Table 7), the ^{3}H:^{14}C ratio should not have changed in phillygenin 49 and epipinoresinol 48 (an expected ^{3}H:^{14}C ratio of 8.93); at least half of the ^{3}H label should have been lost in arctigenin 50 due to the formation of the lactone ring (an expected ^{3}H:^{14}C ratio of 4.43). Rahman *et al.*[67] speculate that the measured increase results from a "primary isotope effect" of coniferyl alcohol 36 when in reversible oxidation-reduction reactions among cinnamic acids and alcohols as depicted in Figure 20. This remains to be proven.

Kamil and Dewick (1986)[68] investigated the biosynthetic relationships between different classes of lignans to determine any possible interrelationship between aryltetralin lactone and dibenzylbutyrolactone lignans. The [4'-methyl-^{14}C]-labeled dibenzylbutyrolactone lignans, yatein 51, podorhizol 52, epipodorhizol 48 and anhydropodorhizol 53, were administered to the roots of *P. hexandrum*. Only a small amount of yatein 51 (0.19 %) was converted into podophyllotoxin 6. Podorhizol 52, epipodorhizol 48, and anhydropodorhizol 53 were not incorporated at all into podophyllotoxin 6 (0.004%, 0.003%, and 0.001%, respectively; negligible amounts). Figure 21 illustrates Kamil and Dewick's[68] proposed biosynthetic scheme. Matairesinol 54 is postulated to be a precursor to yatein 51; yatein 51 is the speculated branch point to the biosynthesis of podophyllotoxin 6, podorhizol 52, and anhydropodorhizol 53. However, no data supports the hypothetical interrelation among yatein 51 to podorhizol 52 and anhydropodorhizol 53, and the interrelation of yatein 51 to matairesinol 54.

Jackson and Dewick demonstrated in 1984[69,70] the interrelationships that exist among the *Podophyllum* lignans. In *P. hexandrum* and *P. peltatum*, there are two groups of aryltetralin lignans distinguished by the substitution pattern of the pendant ring. The two groups are the 4-hydroxy-3,5-dimethoxy substitution series (e.g. 4′-demethylpodophyllotoxin 55), and the 3,4,5-trimethoxy substitution series (e.g. podophyllotoxin 6). The divergence between podophyllotoxin 6 and 4′-demethylpodophyllotoxin 55 is thought to occur early in the biogenesis, and there is no cross over between the two groups (see Figure 22).

Even though much is being discovered about the biosynthetic pathways leading to lignans, data from past studies have been limited by the restricted period in which precursor incorporation data may be collected. Work by Jackson and Dewick [66] demonstrates this limitation in an experiment where radiolabeled L-phenylalanine 11 was administered to pot grown *P. hexandrum* plants at monthly intervals from beginning to the end of *P. hexandrum*'s growth cycle (see Table 8). Their study indicated that a maximum incorporation of 1.19% occurred during midseason, in July during fruit development. Incorporation earlier or later in the season resulted in an amount less than 0.1%, except for June 12 with 0.13% incorporation. This data illustrates that the opportunity to accumulate data for lignan biosynthesis may be limited to plant development and/or to seasonal response.

As an alternative means for studying lignan formation, plant tissue culture could be used. Although there have been few publications on lignan formation in plant tissue culture, Kadkade (1982) described podophyllotoxin 6 production in *P. peltatum* callus. According to his results *P. peltatum* callus biosynthesized podophyllotoxin 6 (0.71 mg/g dry cell weight) under optimum conditions.[71] More recently, van Uden *et al.* observed podophyllotoxin 6 accumulations of as much as 0.3%, based on a dry tissue weight basis with an average of 0.017% in undifferentiated callus- and

suspension-grown cells derived from *P. hexandrum* roots.[72] In addition, root cultures of flax, *Linum flavum*, have produced 5-methoxypodophyllotoxin (~0.7-1.3% on a dry weight basis).[73,74]

Matairesinol 55 and epipinoresinol 48 production have been reported in callus and suspension cultures of *F. intermedia*.[75] The lignan formation in these cultures depends upon the media used. Consequently, two cell lines were developed. One accumulates matairesinol 54 (11.0 mg/g dry cell wt.) as the predominant lignan when grown on one-third strength Murashige and Skoog medium supplemented with casein hydrolysate (500 mg/L). The other cell line produces epipinoresinol 48 (6.2 mg/g dry cell wt.) when grown on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D; 1 mg/L). Noteworthy, the lignan formation of these cell lines differ markedly from the original explant. The leaf explant of *F. intermedia* forms arctigenin 50 as the predominate lignan, followed by epipinoresinol 48 and phillygenin 49; only low levels of matairesinol 54 are formed.

In summary, the study of lignan biosynthesis has emphasized lignan precursor relationships and the interrelationship of some lignans. Data for these studies have come from *in vivo* plant systems; however, these studies have been limited to carbon-14 and tritium radiolabeled experiments because of low precursor incorporations. Some lignan producing plant tissues have been developed in tissue culture systems (callus and cell suspension), but little work has been reported on use of these techniques to study lignan biosynthesis.

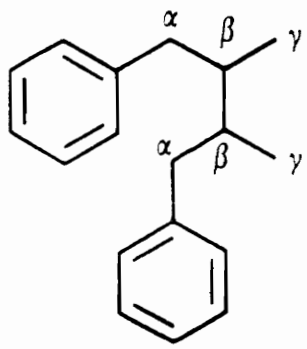


Figure 1. **Basic Lignan Skeleton:** Coupling of Two "Phenylpropane" Units at the β carbons

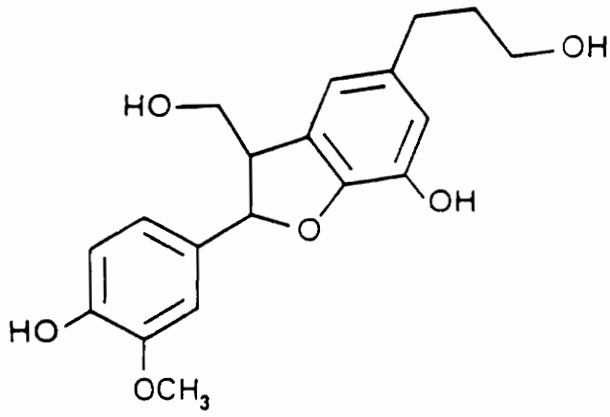


Figure 2. **Cedrusin 1:** A neolignan

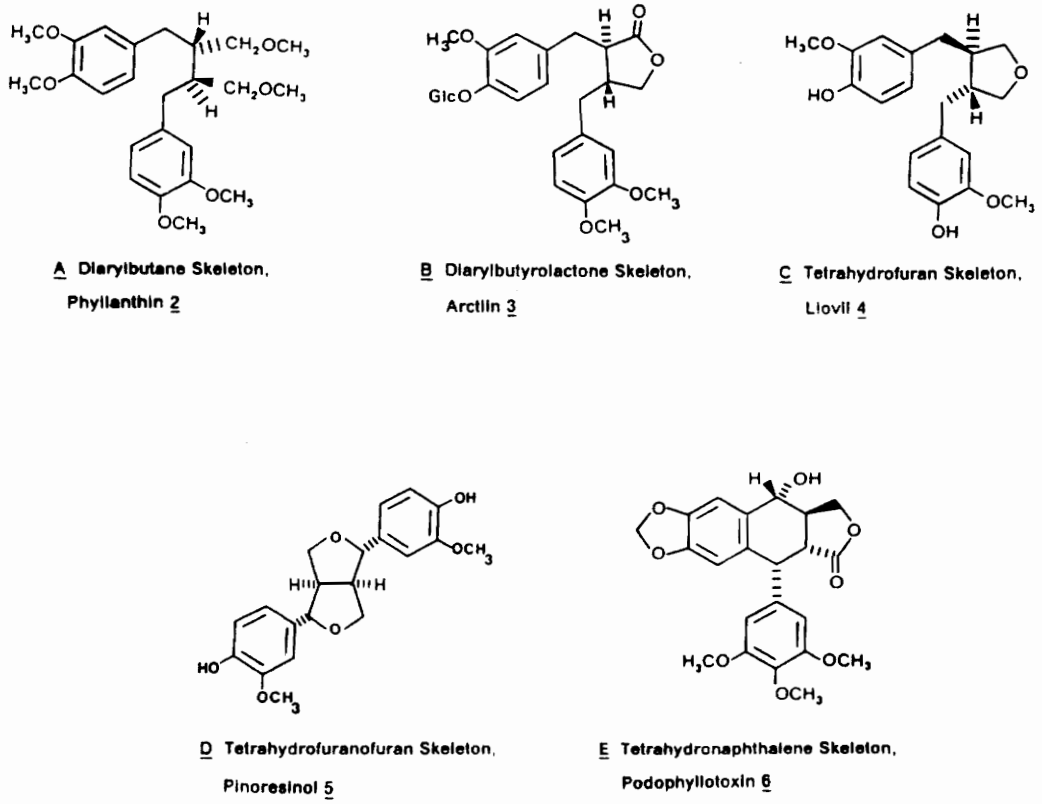
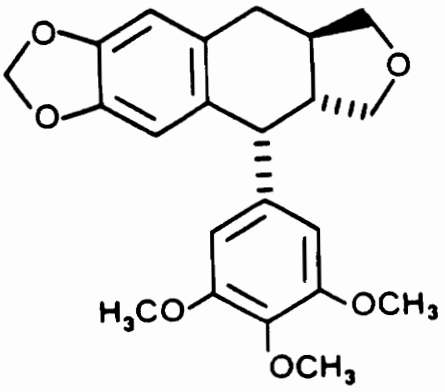


Figure 3. Classification of "β-β" Linked Lignans: Structural Types



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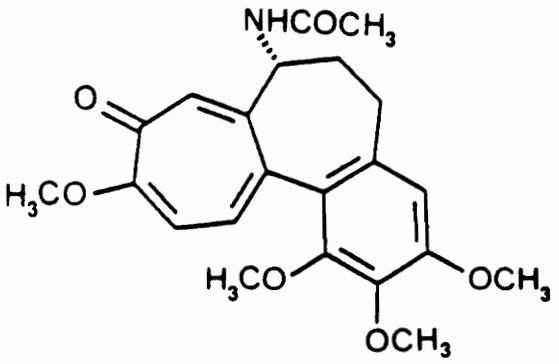

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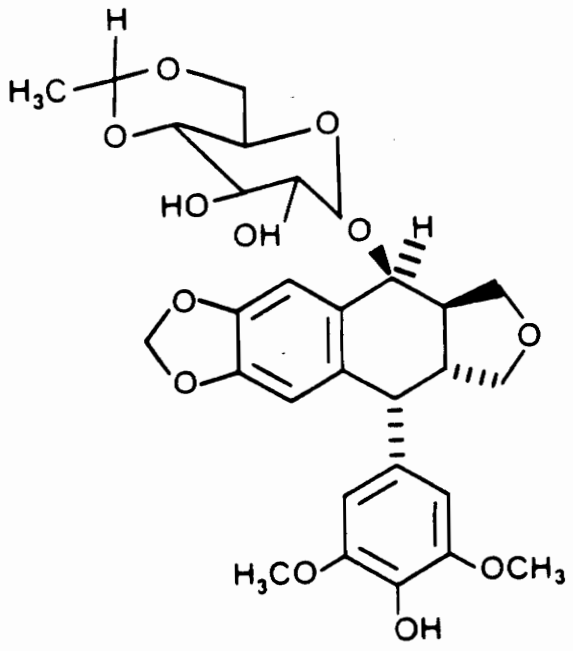

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Figure 4. Deoxypodophyllotoxin 7, Colchicine 8, and Etoposide 9

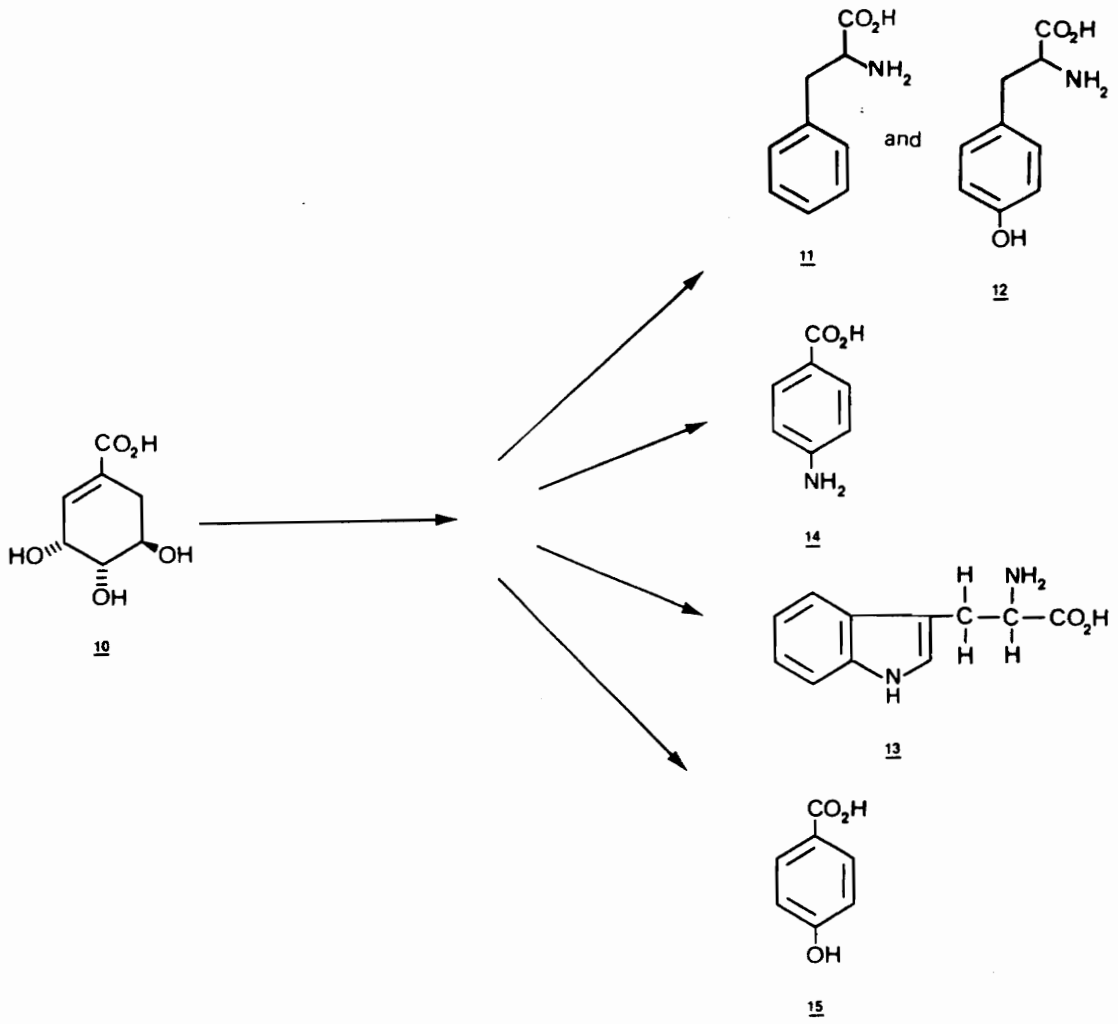


Figure 5. Metabolites Derived from Shikimic Acid 10: Phenylalanine 11, Tyrosine 12, Tryptophan 13, p-Aminobenzoic Acid 14, and p-Hydroxybenzoic Acid 15

Table 1. Enzymes of the Shikimic and Chorismic Acid Pathways

Reaction Step	Enzyme
a	DAHP synthase
b	3-Dehydroquinate synthase
c	3-Dehydroquinate dehydratase
d	3-Dehydroshikimate reductase
e	Shikimate kinase
f	3-Phospho-5-enolpyruvylshikimate synthase

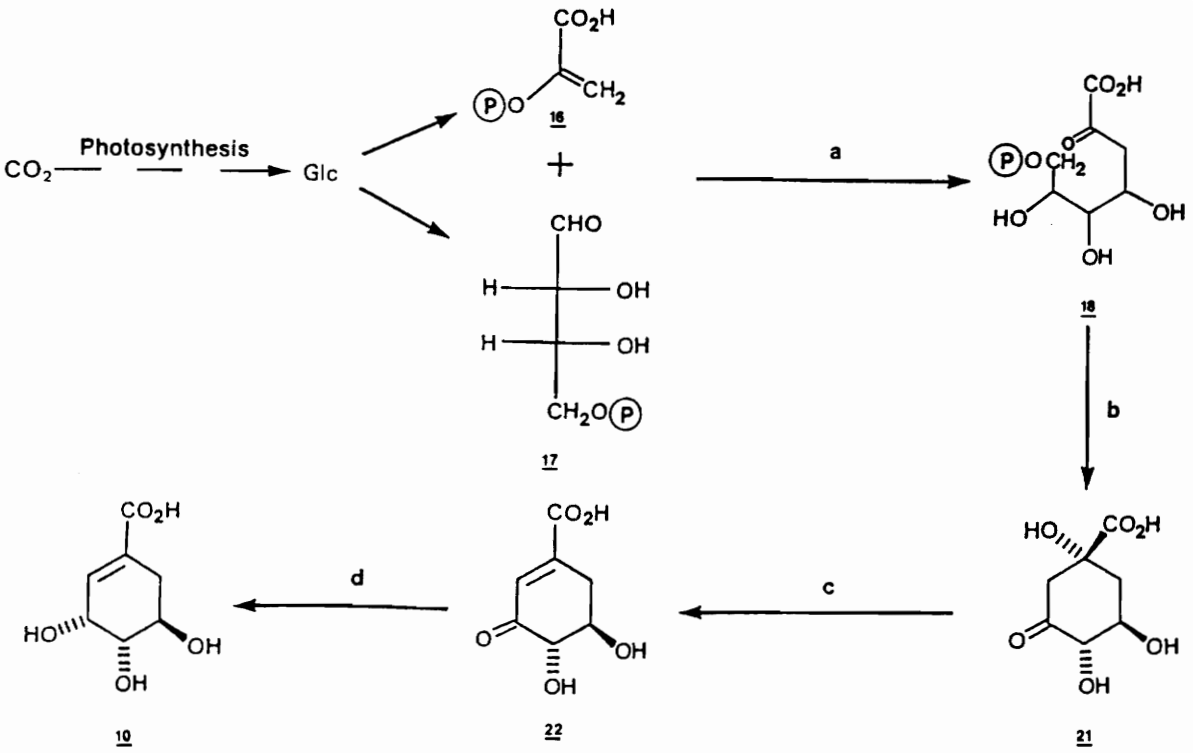


Figure 6. The Shikimic Acid Pathway: CO_2 to Shikimic Acid 10

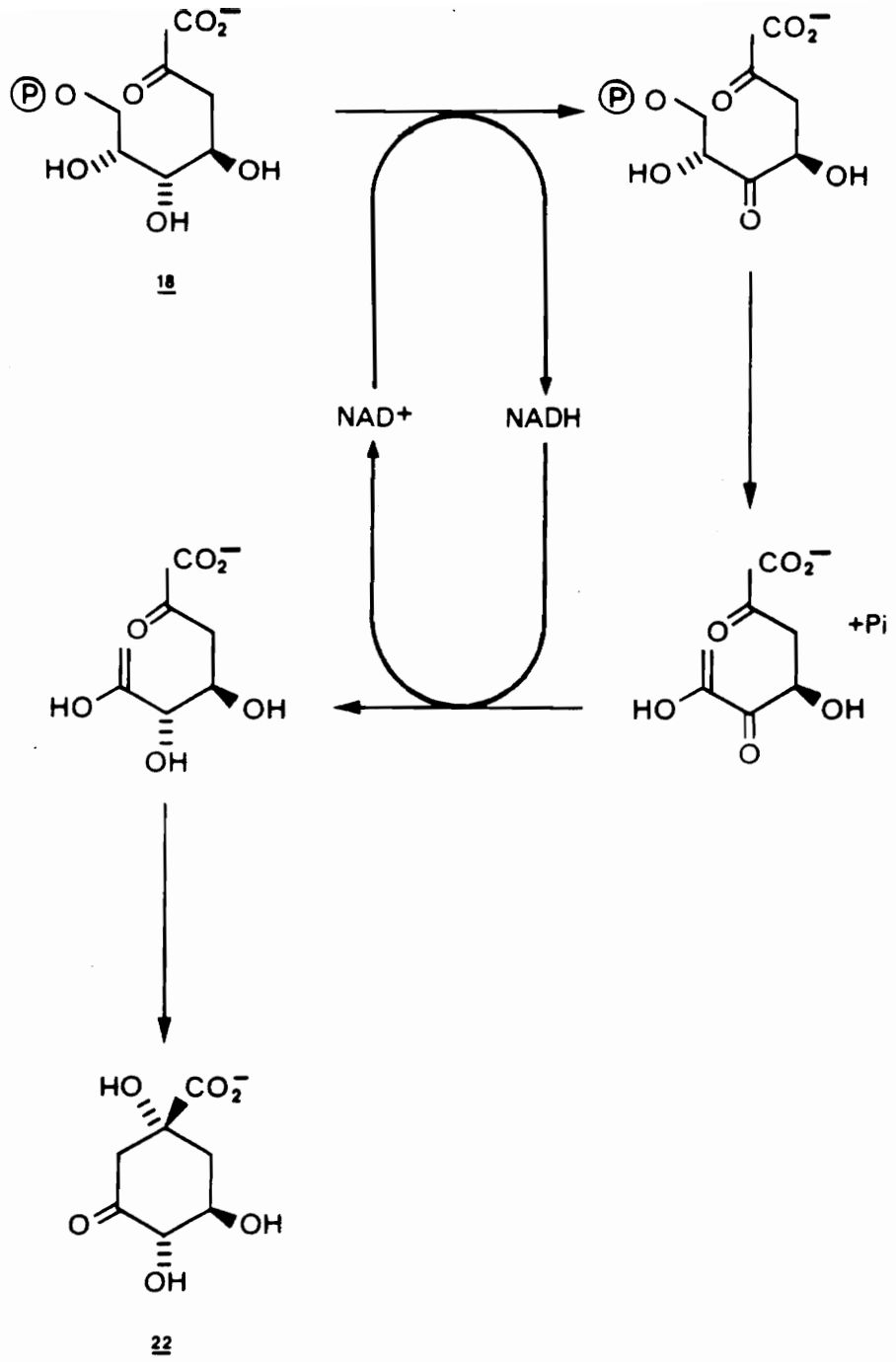


Figure 7. Speculated Cyclization of 3-Dehydroquinate[23]: Transformation of 3-Deoxy-D-Arabino Heptulosonate-7-Phosphate to 3-Dehydroquinate

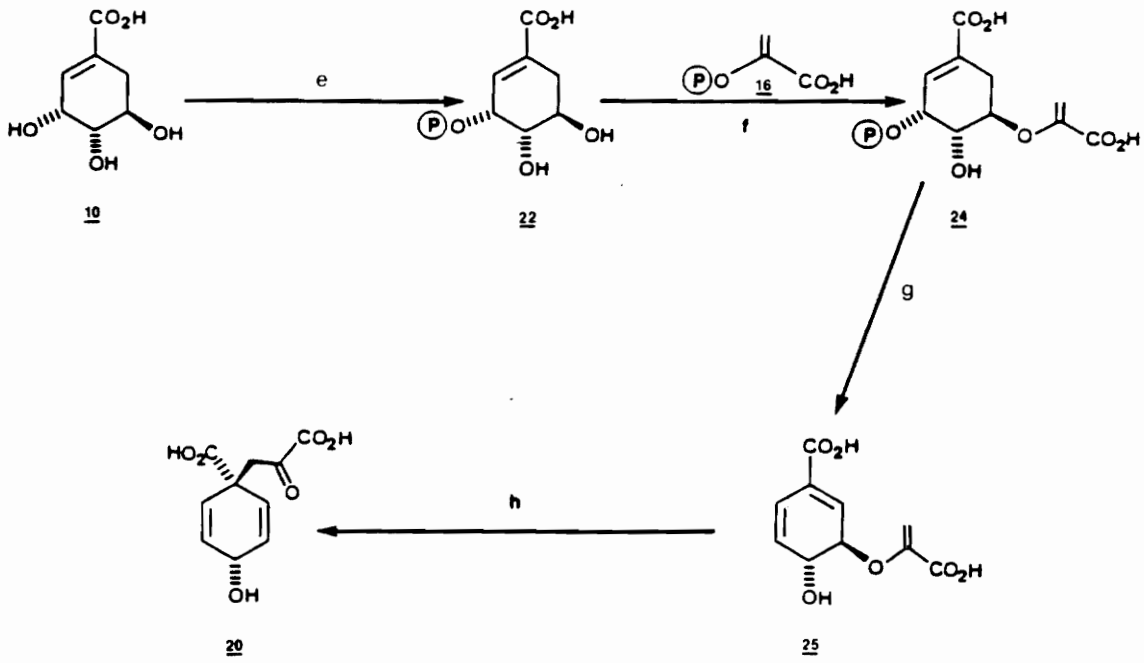


Figure 8. The Chorismic Acid Pathway: From Shikimic Acid 10 to Chorismic Acid 25

Table 2. Enzymes of the Phenylalanine/Tyrosine Pathway from Chorismic Acid

Reaction Step	Enzyme
g	Chorismate synthase
h	Chorimate mutase
i	Prephenate dehydratase
j	Prephenate dehydrogenase
k	Prephenate aminotransferase

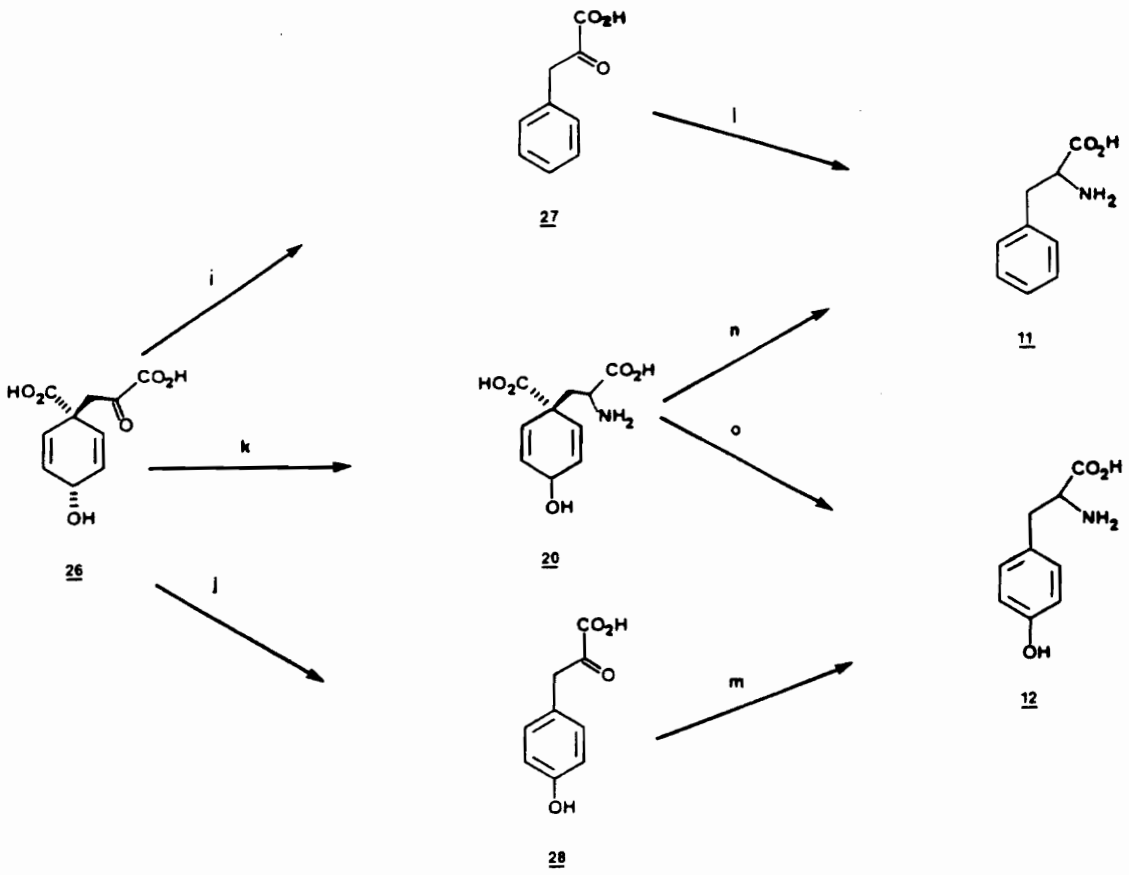


Figure 9. The Phenylalanine/Tyrosine Pathway: From Chorismic Acid 25 to Phenylalanine 11 and Tyrosine 12

Table 3. Enzymes of the Phenylpropanoid Pathway and the Reduction of Activated p-Hydroxycinnamic Acids to Hydroxycinnamyl Alcohols

Reaction Step	Enzyme
l	Phenylalanine aminotransferase
m	Tyrosine aminotransferase
n	Arogenate dehydratase
o	Arogenate dehydrogenase
p	Phenylalanine ammonia-lyase (PAL)
q	Tyrosine ammonia-lyase (TAL)
r	Cinnamate 4-hydroxylase
s	p-Coumarate 3-hydroxylase
t	O-Methyltransferase
u	Ferulate-5-hydroxylase
v	4-Coumarate CoA synthase
w	Cinnamyl-Co NADPH synthase
x	Cinnamyl alcohol dehydrogenase

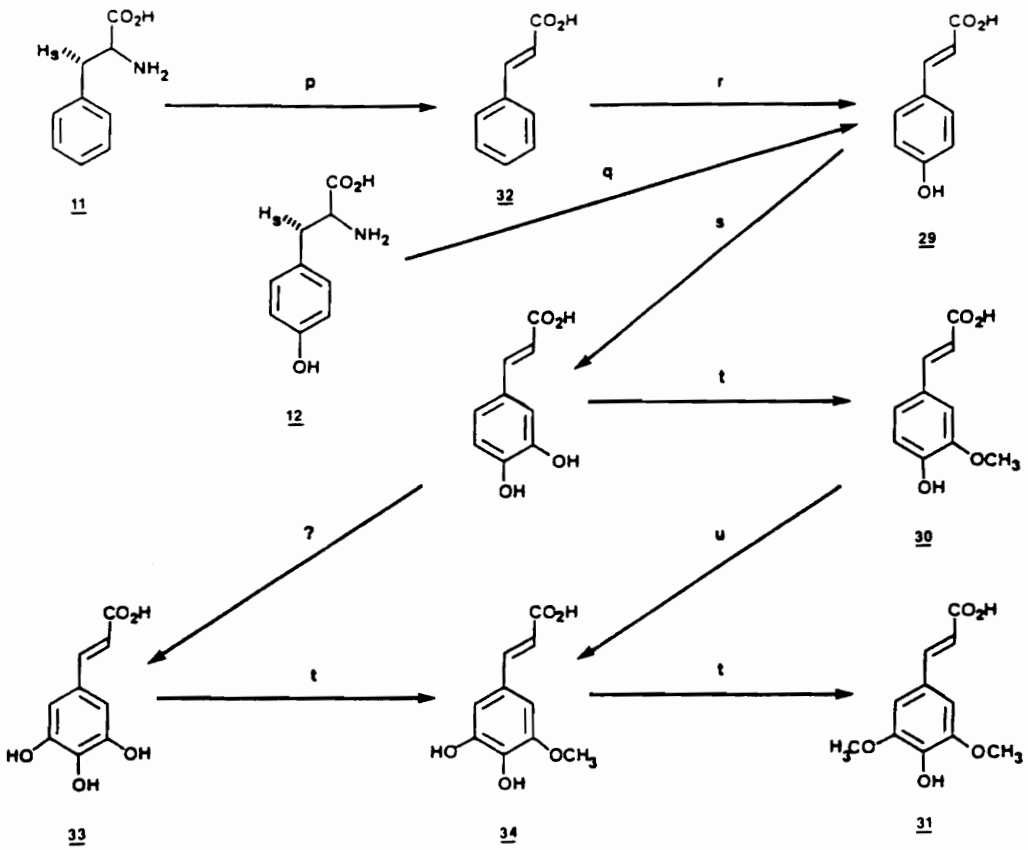


Figure 10. The Phenylpropanoid Metabolic Pathway: Phenylalanine 11 to the Cinnamic Acids 29-31

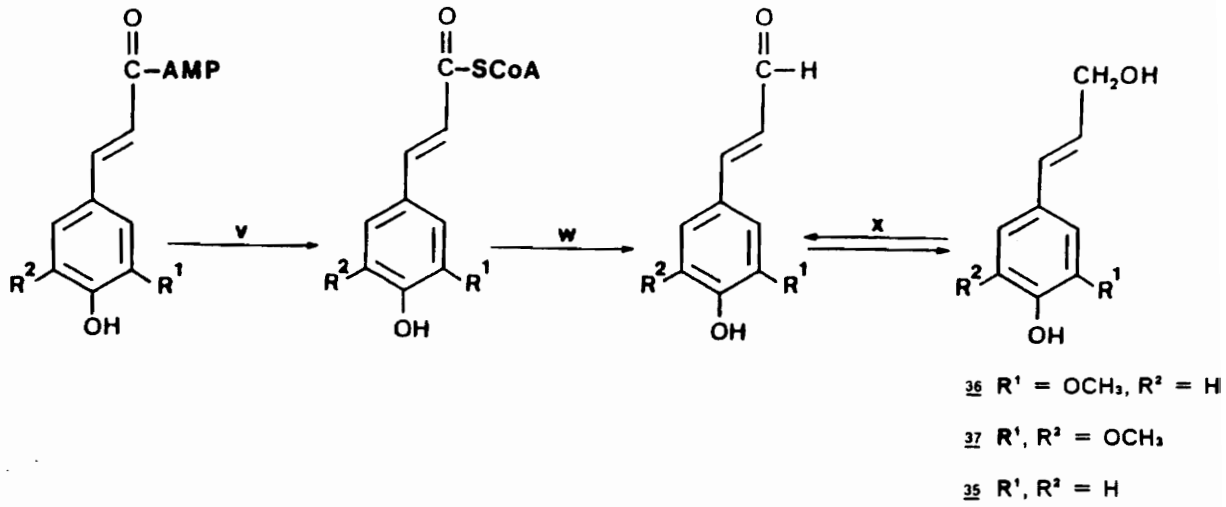


Figure 11. Reduction of Activated Cinnamic Acids: Activated Cinnamic Acids 29-31 to the Corresponding Monolignols 35-37

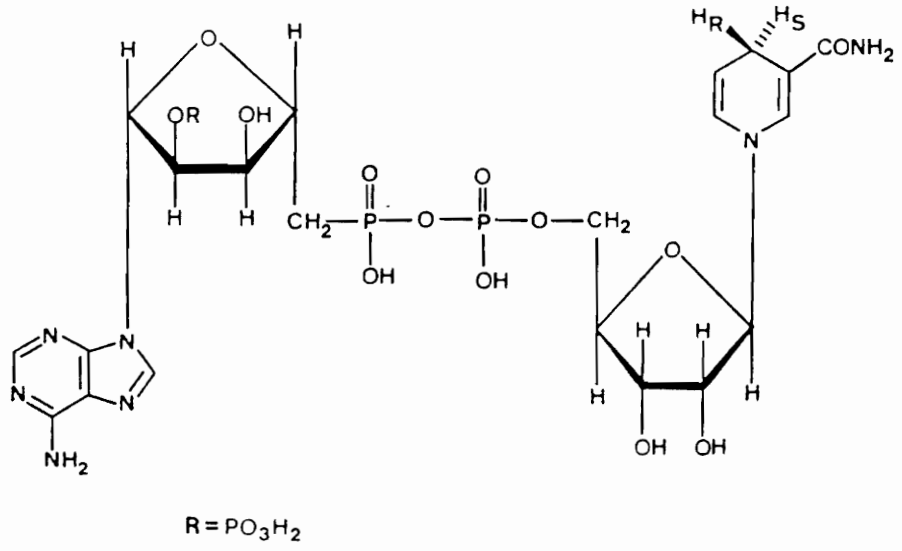
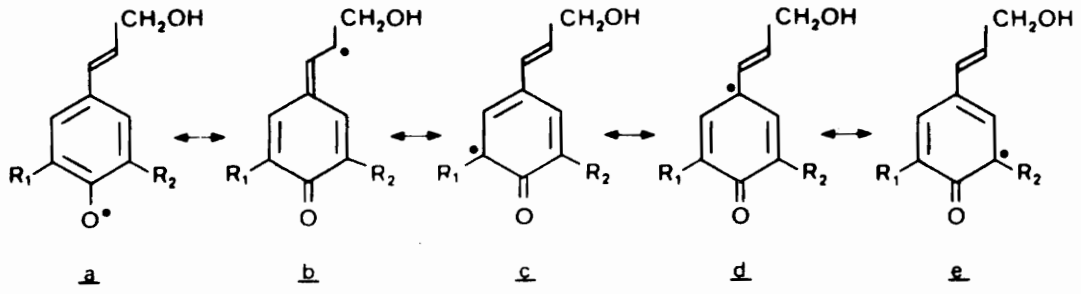


Figure 12. Nicotinamide Adenine Dinucleotide Phosphate: The Reduced Form



Where: • = 1 electron (free radical)
R_1 = H or $-OCH_3$
R_2 = H or $-OCH_3$

Figure 13. Resonance Hybrid Forms of Monolignols

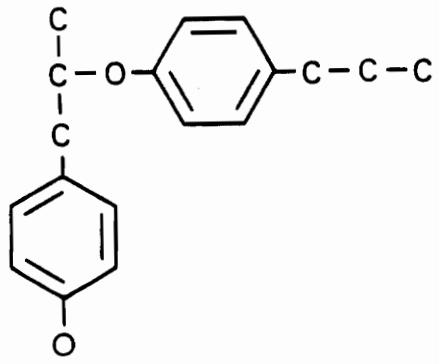


Figure 14. β-O-4 Linkage of Two Phenylpropanoid Units: Presumed the Most Common Phenylpropanoid Linkage Found in Lignin

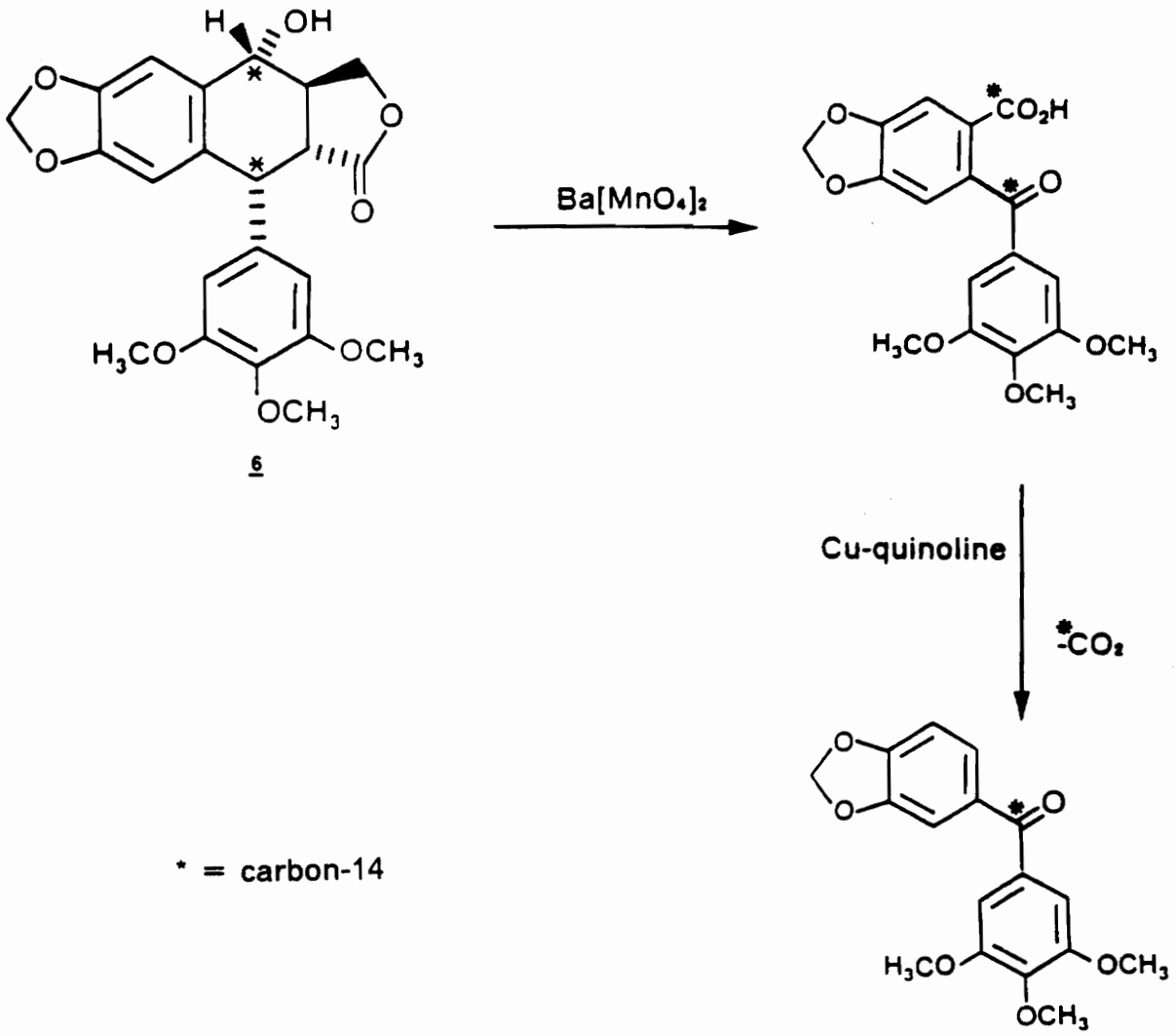


Figure 15. Oxidative Degradation of Podophyllotoxin 6: Ayres, D.C.A., A. Farrow, and B.G. Carpenter, (1981), J. Chem. Soc. Perkin Transaction 1,(7), 2134-2136.

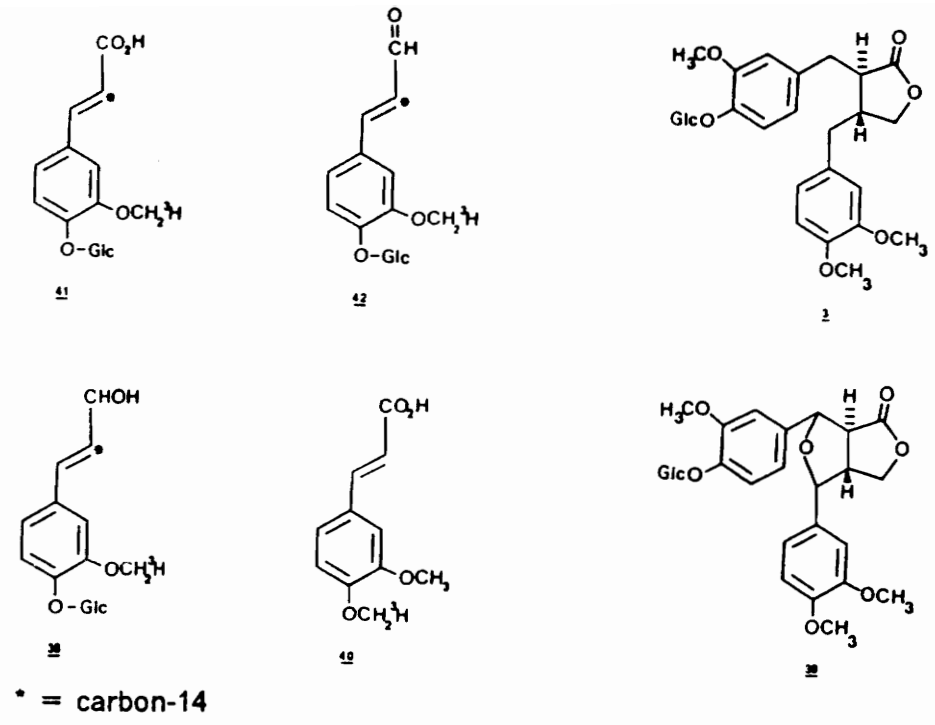


Figure 16. Precursors Administered to Forsythia species, and Arctiin 3 and Phillyrin 39: Stockigt, J., and M. Klischies, (1977), Holzforschung, (31), 41-44.

Table 4. Incorporation of $^{3}H/^{14}C$ Labelled Ferulic Acid Derivatives into Arctiin and Phillyrin in Forsythia species

Precursor	Amount taken up (μmole)	$^{3}H:^{14}C$	Arctiin incorporation absol. (%)	spec. (%)	$^{3}H:^{14}C$	Phillyrin incorporation absol. (%)	spec. (%)	$^{3}H:^{14}C$
Glucoferulic acid 41 (β-^{14}C,-$OCH_2{}^{3}H$)	2.43	10.65:1	3.66	1.9	10.37:1	0.68	1.40	10.11:1
Coniferyl aldehyde 42 (β-^{14}C;-$OCH_2{}^{3}H$)	0.41	12.80:1	1.37	1.2	11.63:1	0.20	0.4	11.27:1
Coniferin 38 (β-^{14}C;-$OCH_2{}^{3}H$)	1.41	12.90:1	0.74	0.7	11.48:1	0.16	0.4	12.25:1

[Stockigt, J., and M. Klischies, (1977), Holzforschung, 30, 41-44.]

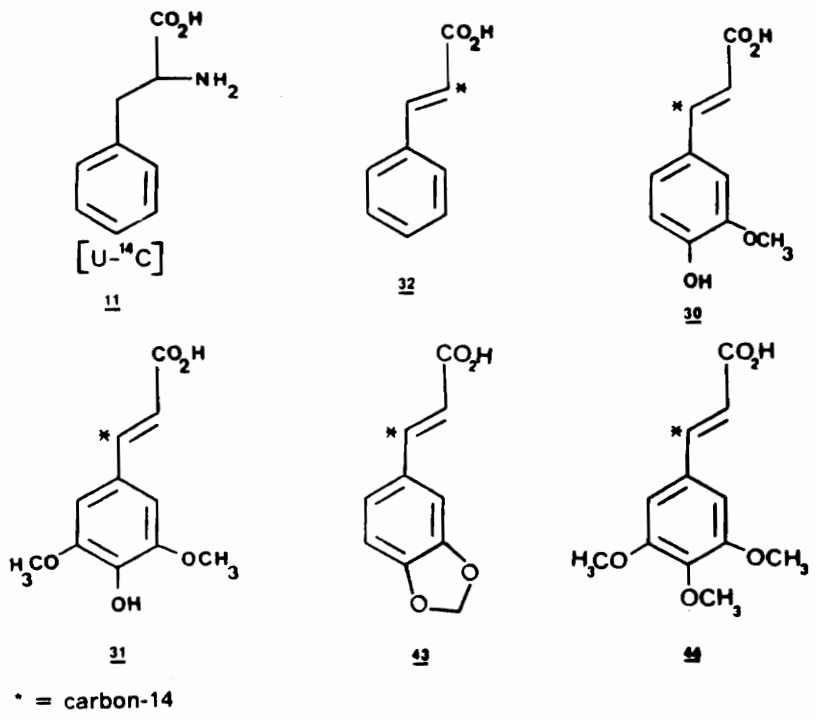


Figure 17. [^{14}C] Labelled Precursors Administered to P. hexandrum: Jackson, D. E., and P. M. Dewick, (1984), Phytochemistry, 23, (5), 1029-1034.

Table 5. Incorporation of [^{14}C] Labelled Precursors into Podophyllotoxin in P. hexandrum

Precursor	mg Isolated	% Incorporation
Phenylalanine 11	22.6	1.19
Cinnamic acid 32	32.2	0.17
Ferulic acid 30	34.0	0.053
Sinapic acid 31	25.3	0.00064
3,4-methylenedioxycinnamic acid 43	5.5	0.016
3,4,5-trimethoxycinnamic acid 44	7.4	0.00039

[Jackson, D. E., and P. M. Dewick, (1984), Phytochemistry, 23, (5), 1029-1035]

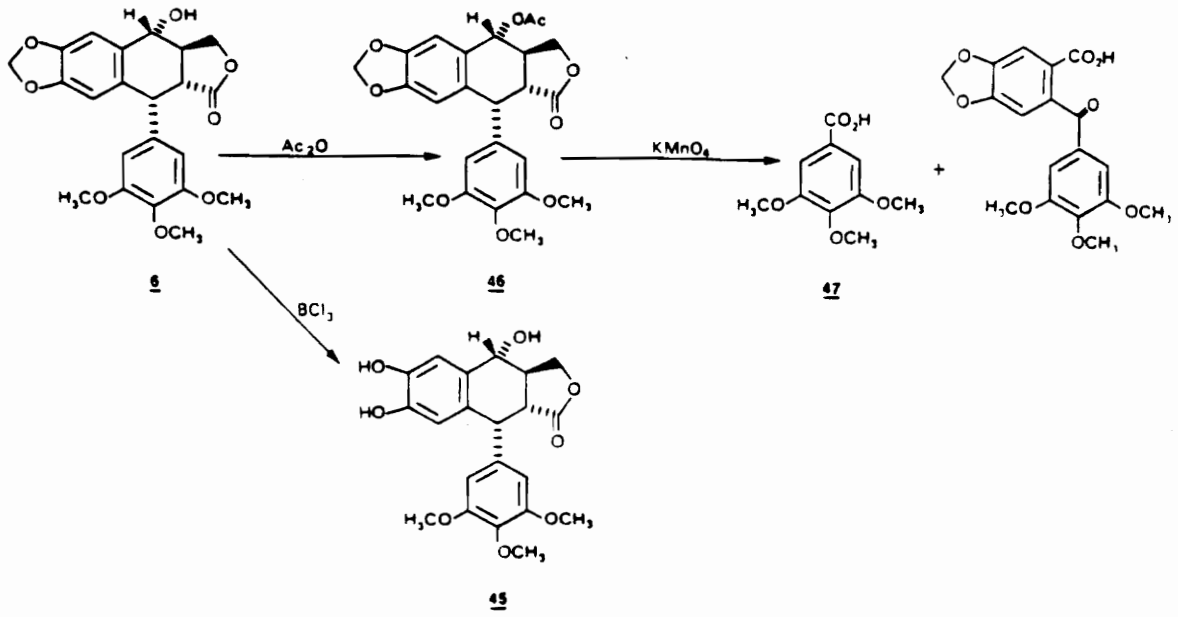


Figure 18. Degradation of Podophyllotoxin 6: Methodologies used by Jackson and Dewick[88] for locating ^{14}C labels in Podophyllotoxin 6

Table 6. Degradation of Podophyllotoxin 6 from [3-O$^{14}CH_3$]-Ferulic Acid 30 Feedings to P. hexandrum

Compound	Specific activity (dpm/mM)	Relative specific activity
Podophyllotoxin acetate 46	$3.25X10^4$	1.00
Podophyllotoxin 6	$3.19X10^4$	0.98
6,7-Demethylenepodophyllotoxin 45	$1.67X10^4$	0.51
Methylenedioxy functionality		0.49
3,4,5,-Trimethoxybenzoic acid 47	$1.69X10^4$	0.52

[Jackson, D., and P. M. Dewick, (1984), Phytochemistry, 23, (5), 1029-1035.]

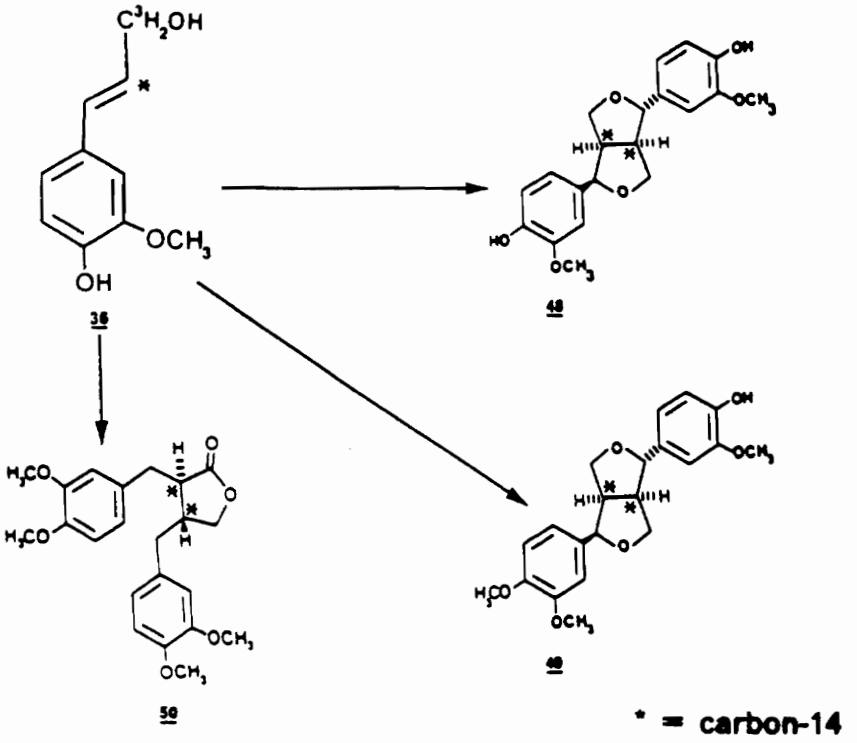


Figure 19. [β-^{14}C, γ-^{3}H] Coniferyl Alcohol 36 Administered to F. intermedia and P. hexandrum

Table 7. Incorporation of [β-^{14}C,γ-^{3}H]-Coniferyl Alcohol into Forsythia intermedia

Plant	^{3}H:^{14}C Ratio of coniferyl alcohol	Lignan Isolated	% Incorp. (^{14}C) Ratio	Measured ^{3}H:^{14}C
Forsythia intermedia	8.93	arctigenin 50 phillygenin 49 epipinoresinol 48	0.12 0.0048 0.029	12.4 27.3 36.4
Forsythia intermedia†	8.83	arctigenin 50 phillygenin 49 epipinoresinol 48	0.074 0.0011 0.050	16.9 15.3 27.0

[Rahman, M. M. A., P. M. Dewick, D. E. Jackson, and J. A. Lucus, "Biosynthesis for Lignans in Forsythia intermedia", Phytochemistry, submitted for publication.]
†Duplicate Experiment

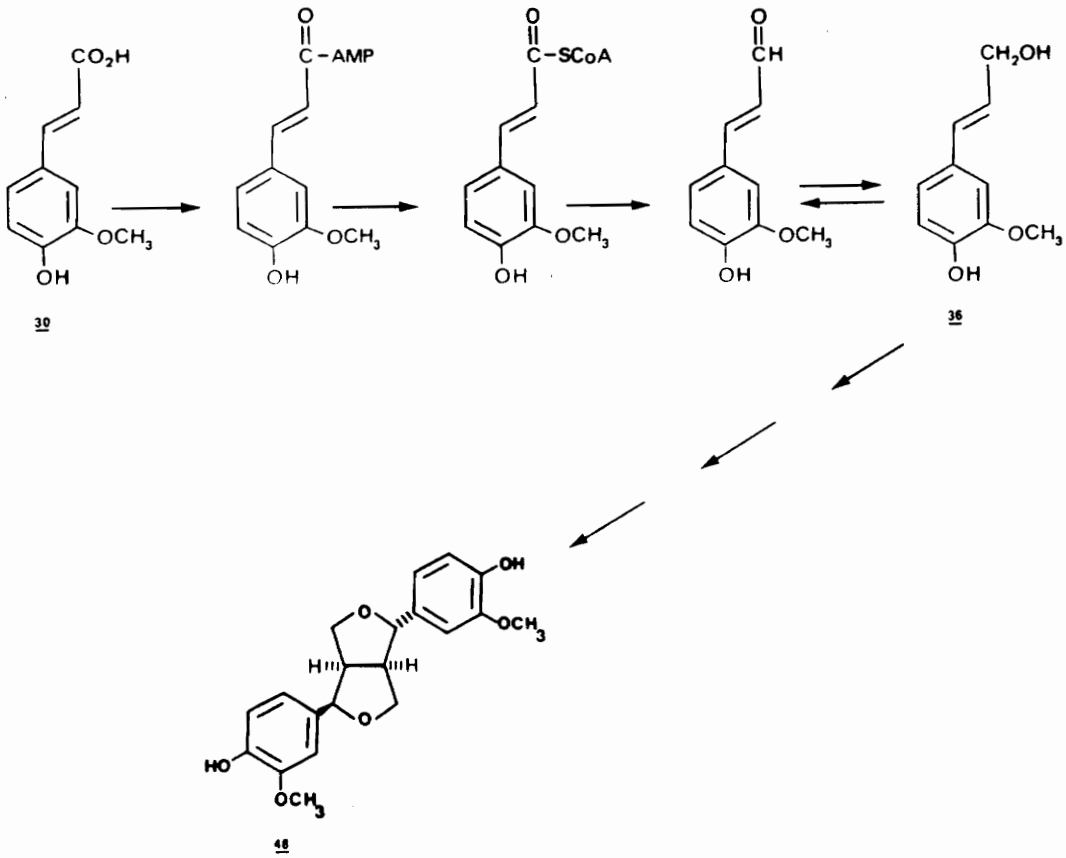


Figure 20. Proposed Pathway for the Incorporation of Ferulic Acid 30 into Epipinoresinol 48

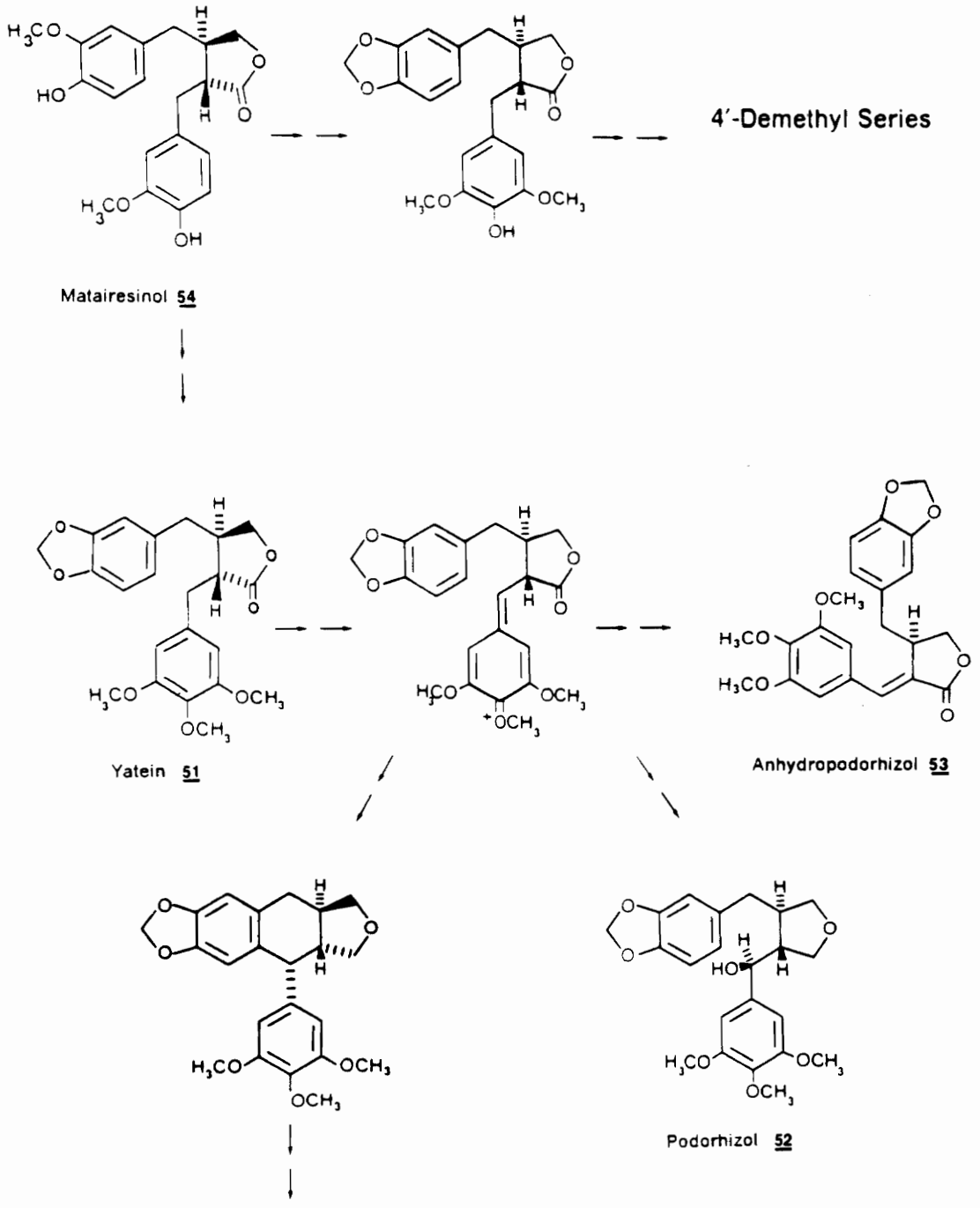


Figure 21. Proposed Biosynthetic Pathway for Podophyllotoxin 6: [Kamil and Dewick[68].]

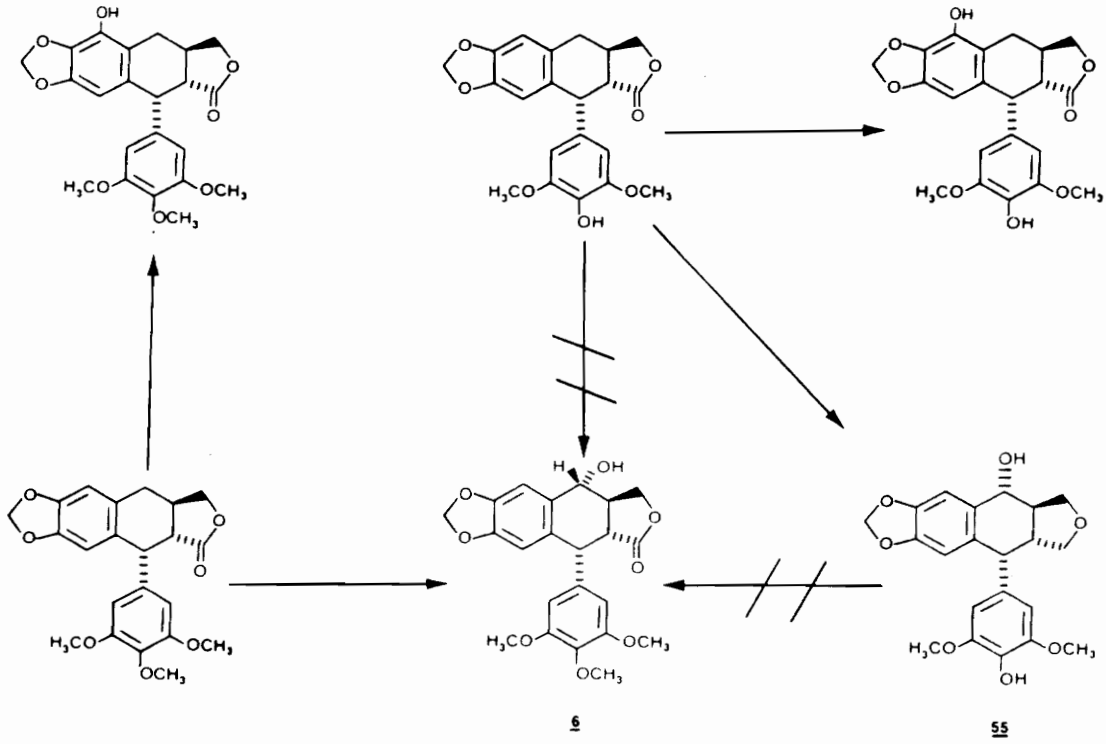


Figure 22. Subdivision of Podophyllum Lignans: Based on Pendant Arylring Substitution; 4'-Demethyl Lignans and 3',4',5'-Trimethoxy Lignans[69,70]

Table 8. Incorporation of DL-[1-^{14}C]-Phenylalanine into Podophyllotoxin 6 in P. hexandrum Plants

Date Fed	mg Isolated	% Incorporation
12 May	12.0	0.04
12 June	12.9	0.13
14 July	22.6	1.19
12 August	2.8	0.08
15 September	57.3	0.13

[Jackson, D. E., and P. M. Dewick, (1984), Phytochemistry, 23, (5), 1029-1035.]

Objectives

The precursor relationship of L-phenylalanine 11 to podophyllotoxin 6 has been well established in *P. hexandrum*; two L-phenylalanine 11 moieties are intactly converted into podophyllotoxin 6. The maximum level of incorporation reported for this precursor is 1.4% of [U-^{14}C]-phenylalanine 11. However, this level of conversion limits precursor experiments to only precursors labeled with the radioisotopes carbon-14 and tritium, since the incorporation levels are insufficient to observe conversion of precursors specifically enriched with carbon-13 and deuterium. If we can optimize uptake of L-phenylalanine 11, then more information can be obtained concerning lignan biosynthesis. By using precursors enriched with carbon-13, deuterium, or dual labeled with carbon-13 and deuterium, we can analyze the precursor conversion into the lignan of interest by carbon-13 nuclear magnetic resonance (NMR) spectroscopy. These types of experiments will give details of the biosynthetic reactions during the biogenesis of lignans such as podophyllotoxin 6.

Current methods used for administering labeled precursors to lignan producing plants such as *P. hexandrum* have not been able to achieve sufficient precursor in-

corporation for carbon-13 and deuterium experiments. The objectives of this thesis are thus two-fold.

1. To develop an aseptic system for administering L-phenylalanine 11 to plant systems that actively produce podophyllotoxin 6, and observe significant incorporation levels of this precursor into the podophyllotoxin 6.

2. To observe the intact conversion of carbon-13 specifically enriched L-phenylalanine 11 into the lignan podophyllotoxin 6 by carbon-13 NMR spectroscopy.

Materials and Methods

General Methods

Proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were recorded on a Bruker WP 270 SY Spectrometer. Samples were dissolved in deuterated chloroform ($CDCl_3$); chemical shifts were reported in parts per million (ppm) downfield from internal standard tetramethylsilane (TMS). Splitting patterns are designated as s (singlet), d (doublet), dd (double doublet), t (triplet), and m (multiplet). Coupling constants are given in Hz. Ultraviolet spectra of samples in ethanol were recorded on a Varian Cary 219 spectrophotometer. Low resolution mass spectra (ms) were determined with a VG 7070EHF mass spectrometer at 70ev. Atomic mass units (amu) with relative abundances, are reported in parenthesis. Infrared spectra were recorded on a Nicolet 5SXCFT-1R spectrophotometer. Melting points were determined in open capillaries with Mel-Temp (50/60 cycles, 110-120 volts, Lab Devices, Cambridge, Mass.) and are not corrected. Thin layer chromatography was carried out on Merck, Kieselgel 60

F_{254}. Flash chromatography was carried out on 60-Kieselgel, 230-400 mesh. Solvents were distilled prior to use.

Liquid scintillation counting employed a Rackbeta 1217 Liquid Scintillation counter using Ecoscint, Aquasol, or Ecolume scintillation cocktails as required. Quenching curves for each scintillation cocktail were developed using ^{14}C-toluene or ^{14}C-hexadecane as standards, with carbon tetrachloride as a chemical quencher.

The high performance liquid chromatography (HPLC) systems had the following configurations: Waters (Milford, MA) trimodule HPLC system comprised of a model 730 data module, a model 721 programmable system controller, 2 model 510 pumps, a model 710 B WISP injector with a model 481 variable wavelength detector, or the Waters system as previously described linked to a Waters 990 photodiode array detector. HPLC grade solvents were filtered with Millipore Durapore® (Millipore Products, MA) 0.45 μm filters. HPLC columns used were either μBondapak C18 (PN 27324) or Novapak C18 (PN 086344). Analysis of eluant was carried out at 290 nm.

P. peltatum rhizomes and seeds were collected from the wild on the campus of VPI & SU, and stored at 4°C until required. *P. hexandrum* seeds were obtained from Thompson and Morgan Seed Company, Jackson, New Jersey. [U-^{14}C-]L-Phenylalanine 11 was obtained from ICN Radiochemicals, Irvine CA. Filter sterilization of incubation media employed Millex-GV, 0.22 μm filter units attached to 5-10 mL disposable, sterile syringes. All aseptic manipulations were conducted under a laminar flow cabinet. Media and water were autoclaved for 25 min. at 120°C (15-20 psi). Plant tissues were lyophilized on a Virtis Model no. 10-010 automatic freeze-dryer.

Isolation and Purification of Podophyllotoxin

Podophyllum hexandrum resin was obtained from William Ranson and Son, Hitchin, Hertfordshire, England. Podophyllotoxin 6 was isolated from 125 g resin, which was suspended in methylene chloride (100 mL). The resulting suspension was applied to silica gel, 60-Kieselgel, 230-400 mesh (700g), 7X60 cm column previously equilibrated in methylene chloride. Subsequent flash chromatography under N_2 consisted of methylene chloride (1 L), methylene chloride-methanol (25:1, 3 L), and methylene chloride-methanol (25:2, 3 L). Fractions containing podophyllotoxin 6 were combined and stripped of solvent under reduced pressure. The podophyllotoxin 6 (35 g) was crystallized from 95% ethanol: m.p. 184°C [lit. 184-185°C[60]]; $[\alpha]^{20}$ -123.67°; mass spec., m/e, 414 ([M^+] 100%), 396 (23%), 189 (10%), 168 (10%), 153 (20%), 115 (10%); UV, λ max (EtOH) nm 288, log ε 3.595; IR υ max (KBr), cm^{-1}, 3484-3444 (OH), 1772 (C=O), 1588, and 1508; ^{1}H NMR ($CDCl_3$) δ: 7.12 (H-2′, 1H, s), 6.51 (H-5′, 1H, s), 6.37 (H-2 and H-6, 2H, s), 5.99 and 5.97 (-OC$\underline{H}_2$O-, 2H, dd, $J_1 = 1.4$, $J_2 = 1.4$), 4.77 (H-7′, 1H, d, J = 8.68), 4.59 (H-7 and H-9′α, 2H, m), 4.08 (H-9′β, 1H, t), 3.82 (4-OC$\underline{H}_3$, 3H, m), 3.76 (3,5-OC$\underline{H}_3$, 6H, m), and 2.82-2.81 (H-8 and H-8′, 2H, m). ^{13}C NMR ($CDCl_3$) δ: 135.43 (C-1), 108.55 (C-2), 152.63 (C-3), 137.58 (C-4), 152.63 (C-5), 108.55 (C-6), 44.15 (C-7), 45.36 (C-8), 174.61 (C-9), 133.41 (C-1′), 106.20 (C-2′), 147.69 (C-3′, C-4′), 110.05 (C-5′), 131.34 (C-6′), 72.71 (C-7′), 40.80 (C-8′), 71.55 (C-9′), 56.33, 60.71, and 101.53 (-O$\underline{C}H_2$O-, and -O$\underline{C}H_3$).

Acetylation of Podophyllotoxin

Podophyllotoxin 6 (52.6 mg; 0.127 mM) was dissolved in freshly distilled acetic anhydride (1 mL). The resulting solution was heated until reflux began, and this was maintained for 1 h under N_2. The solution was cooled (room temperature), and the solvent removed under reduced pressure. The podophyllotoxin acetate 47 (50.8 mg, 0.114 mmol, 89.8% yield) was directly crystallized from 95% ethanol; m.p. 207°C. [lit. 207-209°C [68]]; mass spec., m/e, 456 ([M^+] 100%), 396 (20%), 229 (12%), and 185 (35%); ^{1}H NMR ($CDCl_3$) δ: 6.77 (H-2′, 1H, s), 6.54 (H-5′, 1H, s), 6.39 (H-2 and H-6, 2H, s), 5.99 (-OCH_2O-, 1H, d, J = 1.18), 5.98 (-OCH_2O-, 1H, d, J = 1.39), 5.88, (H-7′, 1H, d, J = 8.63), 4.60 (H-7, 1H, d, J = 4.05), 4.39 (H-9′α, 1H, dd, J_1 = 9.20 and J_2 = 6.72), 4.20 (H-9′β, 1H, t, J_1 = 9.74, and J_2 = 9.29), 3.81 (4-OCH_3, 3H, s), 3.76 (3,5-OCH_3, 6H, s), 2.93 (H-8, 1H, dd, J_1 = 14.42 and J_2 = 4.17), 2.85 (H-8′, 1H, m), and 2.19 (OAc, 3H, s). ^{13}C NMR ($CDCl_3$) δ: 134.78 (C-1), 108.49 (C-2), 152.73 (C-3), 137.57 (C-4), 152.73 (C-5), 108.49 (C-6), 43.80 (C-7), 45.63 (C-8), 173.50 (C-9), 128.42 (C-1′), 106.99 (C-2′), 147.64 (C-3′), 148.16 (C-4′), 109.70 (C-5′), 132.44 (C-6′), 73.70 (C-7′), 38.76 (C-8′), 71.20 (C-9′), 56.24 and 60.67 (-O<u>C</u>H_3), 101.55 (-O<u>C</u>H_2O-), 171.24 and 20.97 (-O<u>C</u>OCH_3).

Initiation of *Podophyllum peltatum* Callus

P. peltatum rhizomes were washed thoroughly with 2% Sparkleen® detergent and tap water. Subsequent manipulations were performed under a laminar flow cabinet. The rhizomes were cut into 10 cm sections and surface sterilized by immersion for 20 min. in household bleach (Clorox®, 100 mL, 30% v/v) containing Tween 20 (1 drop). The rhizomes were then rinsed in autoclaved distilled water (3X80

mL) and bleached. Exposed tissue was aseptically removed by means of a surgical scalpel. The rhizomes were cut into approximately 5X5X5 mm discs. Each disc was placed on 15X100 mm disposable petri dishes containing Murashige and Skoog medium (25 mL). The medium contained 1 mg/L 2,4-D + 0.2 mg/L kinetin + 0.7% Difco Bacto Agar, pH 5.6, (pH adjusted with 1 N NaOH) and had been autoclaved for 25 min. at 121°C at 15-20 psi.

The cultures were maintained at 24°C with a light cycle of 16 h light and 8 h dark. Four weeks after initiation, the callus had developed from the rhizome cambium region. The callus was then separated from the rhizome tissue. The callus cultures were transferred monthly to fresh medium.

Isolation of Podophyllotoxin from *P. peltatum* Callus

P. peltatum callus was wrapped in aluminum foil, frozen (liquid N_2), and lyophilized (72 h). The resulting dry callus was ground in a mortar (120 mm) to a fine powder (8g) and transferred to a cellulose thimble (Whatman, 25X80 mm) in a micro-Soxhlet; the contents of the Soxhlet were continuously extracted for 12 h with distilled acetone. The acetone solubles were cooled to room temperature and stripped of solvent under reduced pressure. The resulting residue (0.255 g) was applied to 2 X 1000 μm silica gel plates (20X20 cm) and eluted with methylene chloride-methanol (100:5). The zone corresponding to podophyllotoxin 6 was carefully removed by means of a single edged razor blade, and eluted with redistilled acetone (25 mL). Following removal of solvent under reduced pressure, the residue was brought to 10 mL in HPLC grade MeOH, 10μL of which was applied to a Novapak C18 HPLC column and eluted with MeOH-H_2O (53:47) at 0.6 mL/min. The podophyllotoxin 6 content was

determined by comparing the retention volume and peak area to authentic podophyllotoxin 6.

Administration of [U-^{14}C-]L-Phenylalanine to *P. peltatum* Callus

[U-^{14}C-]L-Phenylalanine 11 (13.25 μCi, 405 μCi/μmole) was added to L-phenylalanine 11 (1.2 mL, 20 mM) and adjusted to pH 5.6 with 1 N NaOH. The solution was filter sterilized and aseptically transferred (1mL) to freshly autoclaved modified MS medium (100 mL) cooled to approximately 50°C. The autoclaved medium was cooled prior to addition of L-phenylalanine 11 to prevent its thermal decomposition. The medium was then dispensed equally (4X25 mL) to four 15X100 mm disposable petri dishes. The initial medium was composed of 0.2 mM [U-^{14}C-]L-phenylalanine 11 (7.42X10^5 dpm/mg) + 1 mg/L 2,4-D + 0.2 mg/L kinetin + MS medium + 0.7% Difco Bacto Agar at pH 5.6.

Four pieces of callus (~10X10X10 mm) were transferred to each petri dish. The callus grew on the medium containing [U-^{14}C-]L-phenylalanine 11 for 4 weeks at 24°C with a light cycle of 16 h light and 8 h dark. The callus was removed and washed with distilled water, wrapped in aluminum foil, frozen (liquid N_2), and lyophilized. Eight callus pieces were combined and served as a single sample, (520.72 mg). Podophyllotoxin 6 (10 mg) was added to each sample, the whole sample was ground to a fine powder in a mortar (70 mm) with a pestle, and the powder was then transferred to cellulose thimbles (Whatman, 10X15 mm) in micro-Soxhlets. Following continuous extraction for 12 h with 95% ethanol (20 mL), the extracts were cooled and dried under reduced pressure. The resulting residue was partitioned between chloroform (20 mL) and water (20 mL) in a separatory funnel (60 mL). The water layer

was re-extracted with chloroform (3X20 mL). All chloroform extracts were combined and dried under reduced pressure to give a residue (148.74 mg). This was suspended in a minimum volume of acetone (~750 μL), and applied to two 1000 μm silica gel plates (20X20 cm). The plates were air-dried and subsequently eluted with chloroform-methanol (92:8). The band corresponding to podophyllotoxin 6 was carefully removed by means of a single edged razor blade, and the podophyllotoxin 6 was eluted from the silica with methanol. Removal of the solvent under reduced pressure afforded crude podophyllotoxin 6 (21.10 mg).

The isolated podophyllotoxin 6 band was dissolved in acetonitrile-H_2O (40:60, v/v) to give a 0.16% (w/v) solution. An aliquot (200 μL; 2020 dpm/32 μg) was applied to a μBondapak C18 column, which was eluted with acetonitrile-H_2O (40:60, v/v) at a flow rate = 1 mL $min.^{-1}$ with detection at 290 nm. Eluant fractions (0.5 mL) were collected in 20 mL glass scintillation vials, and the radioactivity of each was determined by liquid scintillation spectrometry.

Administration of [U-^{14}C-]L-Phenylalanine to *P. peltatum* Rhizomes

P. peltatum rhizomes, cut into approximately 5X5X5 mm discs, were surface sterilized as described for callus initiation. [U-^{14}C-]L-phenylalanine 11 [11.82 μCi, 405 μCi/μmole] was added to the L-phenylalanine 11 (11.55 mg) previously dissolved in 50 mM potassium phosphate buffer (3.5 mL), and then this solution was filter sterilized. An aliquot (3 mL) of the [U-^{14}C-]L-phenylalanine 11 solution was added to previously autoclaved potassium phosphate buffer (60 mL, 50mM, pH 6.8). 15 rhizome discs (1 sample) were placed in the [U-^{14}C-]L-phenylalanine 11 solution (0.95 mM, 10.5 mL, $3.75X10^8$ dpm/mmol) in a 125 mL Erlenmeyer flask; flasks were shaken at 100 rpm

on an orbit shaker (25°C, in the dark). Rhizomes were incubated in the [U-^{14}C-]L-phenylalanine 11 solution for 0, 24, and 96 h. Procedures for podophyllotoxin 6 extraction and isolation are described in "Administration of [U-^{14}C-]L-Phenylalanine to *P. peltatum* Callus".

The isolated podophyllotoxin 6 band was dissolved in acetonitrile-H_2O (40:60 v/v) to give a 0.16 % (w/v) concentration of crude podophyllotoxin 6. An aliquot (200 μL/450 dpm) was applied to a μBondapak C18 column, which was eluted with acetonitrile-H_2O (40:60 v/v) at a flow rate = 1 mL min.$^{-1}$ with detection at 290 nm. Eluant fractions (0.5 mL) were collected in 20 mL glass scintillation vials, and the radioactivity of each was determined by liquid scintillation spectrometry.

P. peltatum rhizomes were prepared according to the procedures described in "Initiation of *Podophyllum peltatum* Callus". L-Phenylalanine 11 (16.42 mg, 4.0 mM) was dissolved in acetate buffer (10 mL, 5mM)[77] to which [U-^{14}C-]L-phenylalanine 11 (8.43 μCi, 4.05 μCi/μmole) was next added to afford a solution of [U-^{14}C-]L-phenylalanine 11 (1.88X10^8 dpm/mmol, 1.14 X 10^6 dpm/mg). This solution was filter sterilized. For each sample, 50 rhizome discs were placed in the ^{14}C-phenylalanine 11 solution (1 mL) in a 15X60 mm disposable petri dish. Petri dishes were covered with perforated aluminum foil, and the samples were vacuum infiltrated (3 X 600 mm Hg for 30 s; the vacuum was quickly released each time to maximize uptake by the rhizomes). Petri dishes were sealed with parafilm and placed in the dark for incubation periods of 3, 6, and 12 h.

Rhizomes were rinsed with distilled water (25 mL), blotted until dry with Whatman filter paper (qualitative grade 1), wrapped in aluminum foil, frozen (liq. N_2), and lyophilized. Podophyllotoxin 6 (20 mg) was added to each rhizome sample (2079.0 mg), and the whole sample was ground to a fine powder in a mortar (70 mm) with a pestle. Samples were transferred to cellulose thimbles (Whatman 10X15 mm)

in micro-Soxhlets and then continuously extracted for 12 h with redistilled acetone. Acetone solubles were cooled to room temperature and stripped of solvent under reduced pressure. The resulting crude podophyllotoxin 6 eluants were dissolved in a distilled acetone (~500 μL) and applied to two 1000 μm thick silica gel plates (20X20 cm), with methylene chloride-methanol (100:5) as eluant. The band corresponding to podophyllotoxin 6 was carefully removed from each plate with a single edged razor, and the podophyllotoxin 6 was eluted with acetone (25 mL). Samples were dried under reduced pressure to give podophyllotoxin 6 (22.1 mg), and then acetylated with acetic anhydride (1 mL) as described previously. Following solvent removal, recrystallization from 95% EtOH (200μL) afforded podophyllotoxin acetate 47 (18.9 mg). This was further purified by recrystallization.

Administration of Labeled L-Phenylalanine to *P. peltatum* Seedlings

[U-^{14}C-]L-Phenylalanine Administration

P. peltatum seeds were surface sterilized by immersion for 30 min. in household bleach (Clorox®, 50 % v/v, 100 mL) containing Tween 20 (1 drop), rinsed with autoclaved water (2X80 mL), and imbibed on autoclaved moistened Whatman filter paper (qualitative grade 1) in 15X100 mm petri dishes for 24 h. Each seed was subsequently transferred onto 25 mL prepared Potato Dextrose Agar (PDA) in 15X100 mm petri dishes. PDA was prepared by dissolving PDA (39 g; Difco) in distilled water (1L), and the solution was then autoclaved. Seeds were kept on this medium in the dark for two months at 25°C. Since they did not germinate, the embryos were aseptically excised and placed on Murashige and Skoog medium in 15X100 mm disposable petri

dishes. After two months, each seedling was transferred to liquid medium (110 mL) while in a laminar flow cabinet.

Plants were maintained in enclosed, aseptic, hydroponic units at 16 h day @24 ± 2°C and 8 h night @20 ± 2°C. Figure 24 illustrates the hydroponic unit which was developed to house the plants in an aseptic environment. Each plant was transferred to a Mason jar (2 quart). Inside the Mason jar, the plant's root was positioned through a teflon contraption which held the plant upright on top of a teflon bottle (125 mL). The roots were placed inside the bottle and immersed in the medium (Hoagland's). The medium was continuously aerated with filter sterilized air, pumped by a Nalgene 8000 aquarium pump through a 0.45 μm air filter connected to teflon tubing. Another 0.45 μm air filter was attached to the top of the Mason jar to prevent build up of air pressure. In addition, a sterile H_2O reservoir was attached to the system to replenish the medium with H_2O which evaporated from the constant aeration.

Hydroponic units were built with all autoclavable parts. A small vial capped with a silicone cork was also attached to the unit for easy sampling of the medium. All components of the hydroponic units were designed so that once the plant was established in the unit, the unit would not have to be opened until termination of the experiment. The hydroponic medium contained [U-^{14}C-]L-phenylalanine 11 and Hoagland's medium, adjusted to pH 5.9 with 1 N NaOH. This medium was prepared by dissolving L-phenylalanine 11 (45.45 mg) and [U-^{14}C-]L-phenylalanine 11 (180 μCi; 405 μCi/μmol) in Hoagland's medium (50 mL), whereupon the solution was filter sterilized. Aliquots (10 mL) of this [U-^{14}C-]L-phenylalanine 11 solution were aseptically transferred to autoclaved Hoagland's medium (4X100 mL portions). The final specific activity of [U-^{14}C-]L-phenylalanine 11 in the Hoagland's medium was 8.8X10^6 dpm/mg Phe (1.45X10^9 dpm/mmol).

During the experiment, aliquots (1.5 mL) of medium were taken from each plant to monitor for microbial contamination, L-phenylalanine 11 concentration, and radioactivity. The L-phenylalanine 11 concentrations were determined by assay with Phenylalanine Ammonia-Lyase (PAL), and the radioactivity was monitored by liquid scintillation counting of aliquots (100 μL). Medium contamination was monitored by transferring 1 mL of the medium on to 25 mL PDA on 15X100 mm petri dishes. If contamination was observed, then the plant was discarded; otherwise, the plants were harvested after 9 weeks.

After 9 weeks, plants developed 2 leaves (spans measuring ~3 cm each), stem (height ~6.5 and ~5.3 cm), and roots (~4, ~4.5, ~7.2, and ~11 cm long). The entire plant was harvested, wrapped in aluminum foil, frozen (liq. N_2), and lyophilized. Podophyllotoxin 6 (10 mg) was added to the dry plant material, and the whole sample was ground in a mortar (70 mm) and transferred to a cellulose thimble (Whatman 25X80 mm) in a micro-Soxhlet; the contents of the Soxhlet were continuously extracted with redistilled acetone (25 mL) for 4 h under N_2. Isolation and acetylation of podophyllotoxin 6 followed the procedures described in "Administration of [U-^{14}C-]L-Phenylalanine 11 to *P. peltatum* Rhizomes".

Assay for Phenylalanine

Phenylalanine 11 ammonia-lyase (PAL) derived from *Rhodotorula glutinis* was obtained from Sigma, (PAL activity 2.6 k units/L). The assay mixture was composed of 2 mL of 0.1 M Trizma base, pH 8.75, 30°C; 0.04 mL PAL; and 0.06 mL L-phenylalanine 11 solution. A standard curve was prepared for each assay with the following L-phenylalanine 11 concentrations: 0.6, 0.48, 0.36, 0.24, 0.12, and 0.06 mM. The assay measured the rate that L-phenylalanine 11 was converted to cinnamic acid,

based on the rate of UV absorbance at 290 nm (AUFS at 0.1, chart display 50 sec/cm or 20 sec/cm). Aliquots (0.06 mL) of hydroponic media (procedure described in "[U-^{14}C-]L-phenylalanine" in "Administration of Labeled L-Phenylalanine to *P. peltatum* seedlings") were assayed for L-phenylalanine 11. Sample concentrations were determined from standard curves based on the initial rate of UV absorbance.

[1-^{13}C]-L-Phenylalanine Administrations

Seed preparation was the same as described previously except that the stratification step was eliminated. The hydroponic unit construction and maintenance were also the same as described previously. The medium was prepared by dissolving [1-^{13}C]-L-phenylalanine 11 (77.97 mg) in Hoagland's medium (35 mL) which was subsequently filter sterilized. Aliquots (5 mL) of [1-^{13}C]-L-phenylalanine 11 solution were transferred to preautoclaved Hoagland's medium (125 mL; to obtain a final 0.5 mM [1-^{13}C]-L-phenylalanine 11 concentration). As previously noted, each plant was aseptically transferred to a hydroponic unit. The medium was exchanged every two weeks, during which time the medium was monitored for contamination. If contamination was detected, the plant was discarded; otherwise, the plants were maintained for 3 months.

At harvest, the plants were frozen in liquid N_2 and lyophilized. Three plants were combined to make a sample (176.8 mg combined dry weight). Podophyllotoxin 6 (5.6 mg) was added, and the whole sample was ground to a fine powder with a mortar (70 mm). The contents were transferred to a cellulose thimble (Whatman, 25X80 mm) in a micro-Soxhlet. The sample was extracted with redistilled acetone (25 mL) under N_2 for 4 h. Acetone solubles (7.65 mg) were cooled to room temperature and stripped of solvent under reduced pressure. The resulting extract was applied to a 1000 μm

thick silica gel plate (20X10 cm) with methylene chloride-methanol (100:5) as eluant. The band corresponding to podophyllotoxin 6 (6.59 mg) was acetylated as described for "Acetylation of Podophyllotoxin" in "General Methods".

The resulting C-13 enriched podophyllotoxin acetate 47 (5.5 mg) was taken up in $CDCl_3$ (250 μL) with TMS added as the standard. This sample was analyzed on a Bruker WP 270 SY Spectrometer and was scanned 21,972 times. Unlabeled podophyllotoxin acetate 47 (5.5 mg) was then scanned 21,737 times under identical conditions for the natural abundance C-13 spectrum. The natural abundance C-13 spectrum was subtracted from the C-13 enriched spectrum.

Results and Discussion

Previous studies have demonstrated that two moieties of L-phenylalanine 11 are intactly converted into the lignan podophyllotoxin 6 in *Podophyllum hexandrum*. Ayres[60] demonstrated this in an experiment in which [U-^{14}C]-phenylalanine 11 was wick-fed to *P. hexandrum* plants; once the precursor was taken up by the plants, the plants were grown for 8 more days. Podophyllotoxin 6 was subsequently isolated from the plants. The incorporation of administered phenylalanine 11 into podophyllotoxin 6 ranged from 0.6% to 1.4%. Ayres verified that the two moieties of phenylalanine 11 were incorporated equally into podophyllotoxin 6. This was demonstrated following oxidative degradation and analysis of the fragments, as depicted in Figure 15.

Jackson and Dewick[66] also investigated the precursor relationships to podophyllotoxin 6 in *P. hexandrum*. They administered radiolabeled phenylpropanoids; phenylalanine 11, cinnamic 32, ferulic 30, sinapic 31, and 3,4,5-trimethoxycinnamic 33 acids in a precursor solution (2 mL in water) were administered individually to the pre-rinsed roots of pot grown *P. hexandrum* plants. Air was blown across the leaves to facilitate the uptake of solution, which took 6 to 7

hours. The roots were then covered with vermiculite, and the plants were placed in a greenhouse for 7 days. After 7 days, the podophyllotoxin 6 was isolated and incorporation of the precursor was determined.

By this method, Jackson and Dewick[66] were able to obtain the incorporation levels as reported in Table 5. Ferulic 30, sinapic 31, and 3,4,5-trimethoxycinnamic acids 33 were poorly incorporated (< 0.1%), while phenylalanine 11 and cinnamic acid 32 had higher incorporations.

In addition, Jackson and Dewick were able to demonstrate that two ferulic acids are converted to podophyllotoxin 6 intact. In this experiment, [3-O^{14}CH$_3$] ferulic acid 30 was administered to the *P. hexandrum* plants. The intact conversion of two units of ferulic acid 30 was verified by the oxidative degradation of podophyllotoxin 6, (see Figure 18 and Table 6). Although ferulic acid 30 was poorly incorporated (0.053%), the data indicate that the 3-methoxyl group of the pendant ring is converted intact to podophyllotoxin, and it is very conceivable that the phenylpropanoid precursors for podophyllotoxin have substitution patterns similar to ferulic acid 30.

The works of Ayres,[60] and Jackson and Dewick,[66] are the only reports known concerning incorporations of phenylalanine 11 and "C_6C_3" precursors into podophyllotoxin in *Podophyllum* species. Phenylalanine 11 has been demonstrated to have a complete conversion into podophyllotoxin 6 with maximum incorporations of 1.19%[66] and 1.4%.[60] For this reason we chose L-phenylalanine 11 for our initial experiments.

Experimental Modification of Isolating and Purifying Podophyllotoxin

In our initial experiments, we employed podophyllotoxin 6 isolation procedures similar to that reported by Kadkade[71] and Hokanson.[76] *P. peltatum* tissue, callus or rhizomes, was dried by lyophilizing; the dried tissue (520 mg) was continuously extracted with 95% ethanol via Soxhlet extraction until no more podophyllotoxin 6 could be extracted. The ethanol solution was subsequently cooled from a boil (86°C) to ambient temperature and was evaporated from the extract containing podophyllotoxin 6 (148.74 mg). This was then taken up in chloroform (20 mL), which was extracted with water (3 X 20 mL). The chloroform solubles, containing podophyllotoxin 6, were dried under reduced pressure. The residue was suspended in a minimum amount of acetone (750 μL) and applied to silica gel plates. The plates were air dried and the sample was eluted in chloroform-methanol (92:8 v/v).[71,76] The band corresponding to podophyllotoxin 6 was eluted from the silica with methanol. The methanol was removed from the sample under reduced pressure. Crystallization of the podophyllotoxin was attempted by taking up the dried sample (21 mg) in 0.5 mL 95% ethanol and slowly cooling the solution (first to 4°, then to -20°C); however, all attempts failed.

We then turned to reversed-phase high performance liquid chromatography to determine the purity of the isolated podophyllotoxin 6. For our μBondapak C-18 column, we used the solvent system acetonitrile-water (40:60 v/v)[77,78] at a flow rate 1 mL min^{-1}, with detection by UV absorbance of the eluant at 290 nm. Podophyllotoxin 6 had a retention volume of 10.3 mL, and the podophyllotoxin content was determined by comparing the retention volume and peak area of known amounts of authentic podophyllotoxin 6.

This procedure was also followed for the experiments in which [U-^{14}C-]L-phenylalanine 11 was administered to *P. peltatum* callus and rhizomes. However, after discovering a report by Buchardt *et al.*,[79] we realized that we were not obtaining a maximum recovery of podophyllotoxin 6 using 95 % ethanol as an extraction solvent. In the procedure just described, podophyllotoxin 6 was extracted from callus and rhizome tissues with 95% ethanol in a Soxhlet extraction apparatus. Buchardt *et al.* reported that podophyllotoxin 6 was converted mostly into picropodophyllotoxin (among other compounds) in a hot ethanol solution. According to their results, 10 to 90 % of podophyllotoxin 6 was lost when heated to 40-80°C in 96 % ethanol (pH 4.5 with lactate buffer). They found that conversion of podophyllotoxin 6 was dependent upon the temperature and duration that the solvent was heated.

Since we were using 95% ethanol at its boiling temperature to extract podophyllotoxin 6, we could be significantly reducing our recovery of podophyllotoxin 6. Therefore, we investigated the extent to which our yield of podophyllotoxin 6 was affected.

Podophyllotoxin 6 (40 mg) was dissolved in 25 mL of 95% ethanol. The temperature was raised until reflux began, and this was maintained for 4 hours. The solution was cooled, dried under reduced pressure, and made up to 50 mL volume in HPLC grade methanol. 10 μL of this solution was applied onto a Novapak C18 column with solvent system MeOH-H_2O (53:47), flow rate 0.6 mL minute^{-1} with detection at 290 nm. The retention volume of podophyllotoxin 6 was 6 mL. The recovery of podophyllotoxin 6 was calculated to be 58.2% and was determined by comparing its retention volume and peak area to authentic podophyllotoxin 6.

For comparison, podophyllotoxin 6 (40 mg) was dissolved in freshly distilled acetone. The acetone solution was treated in the same manner as described above

for the 95% ethanol solution of podophyllotoxin 6. Using acetone, 93.7% of the podophyllotoxin 6 was recovered. Therefore, acetone was used to extract podophyllotoxin from *Podophyllum* tissues in the experiments following the administration of [U-^{14}C-]L-phenylalanine 11 to *P. peltatum* callus and rhizomes. The low yield from the 95% ethanol extraction of podophyllotoxin 6 does not invalidate the data for the callus and rhizome experiments since the extract solution (in 95 % ethanol) was replaced with fresh 95 % ethanol every 4 hours during the 12 hour extraction in these experiments.

We also changed our thin layer chromatography solvent system. Previously, chloroform-methanol (92:8 v/v)[71,76] had been used to isolate podophyllotoxin from the 95% ethanol extract. However, the use of this solvent system resulted in a low recovery of podophyllotoxin 6 (actual amount not determined). When the isolated band containing podophyllotoxin 6 was separated a second time by thin layer chromatography with chloroform-methanol (92:8 v/v), a UV absorbing band traveled with the solvent front (the compound(s) in this band were not identified). Therefore, a new thin layer chromatography solvent system was developed. Different ratios of hexane-acetone, and methylene chloride-methanol were tested; the methylene chloride-methanol (100:5 v/v) resulted in the best resolution of the podophyllotoxin extract (podophyllotoxin Rf = 0.58).

Two types of reverse-phase high performance liquid chromatography columns were used to quantify podophyllotoxin 6 content. The first column used was a μBondapak C-18; the elution system used for this column was acetonitrile-water (40:60 v/v) with a flow rate 1 mL minute^{-1}. This system was used for the [U-^{14}C-]L-phenylalanine 11 administration experiments with *P. peltatum* callus and rhizome. We next employed a Novapak C-18 column, where it was found that the ratios of acetonitrile-water used were inadequate to separate the components iso-

lated from the *P. peltatum* extracts. Methanol-water (53:47 v/v), at a flow rate of 0.6 mL minute^{-1} was found to be efficient for complete separation of the components in the extracts (with a retention volume of 10 mL for podophyllotoxin 6).

Acetylation of Podophyllotoxin

As mentioned previously, crystallization of the isolated podophyllotoxin 6 was difficult using 95% ethanol, so it was converted into the readily crystallizable acetate derivative 46. This was carried out by dissolving podophyllotoxin 6 in freshly distilled acetic anhydride; the resulting solution was refluxed for 1 hour while under a constant flow of nitrogen gas. The acetic anhydride and acetic acid were removed via evaporation leaving podophyllotoxin acetate 46, which was further purified by crystallization (95% ethanol). Because of the high yield of conversion (89.8%), no external catalyst was required. In later experiments, the acetylation of podophyllotoxin 6 became a routine purification procedure.

Initiation of *P. peltatum* Callus

Plant tissue culture is the vegetative maintenance of plants *in vitro*. In culture, plant parts (explants) can be induced to form callus (undifferentiated tissue), single cell suspension cultures, and specific organs. The techniques of plant tissue culture are used for genetic research, to propagate plants, to develop virus-free plant sources, and as a source for specific secondary plant metabolites such as podophyllotoxin 6. Our interest in tissue culture focused on podophyllotoxin 6 biogenesis in *P. peltatum* callus. If the callus actively produces podophyllotoxin 6,

then the metabolic pathway for this compound can be investigated on a year-round basis.

Kadkade[71](1982) described methods for obtaining callus cultures of *P. peltatum* which produce podophyllotoxin 6, where callus growth and podophyllotoxin 6 production had been examined under various culture conditions. These included tissue source, growth regulators, and light.

Kadkade used Murashige and Skoog (MS) media (see Table 9) supplemented with combinations of auxins and cytokinins to induce callus from *P. peltatum* tissue. The auxins studied were 2,4-dichlorophenoxyacetic acid (2,4-D), indoleacetic acid (IAA), and naphthaleneacetic acid (NAA). Each auxin was combined separately with one of the cytokinins. The cytokinins studied were kinetin and N_6-benzyladenine. Both ranged in auxin and cytokinin concentration from 0.1 part per million (ppm) to 5.0 ppm. NAA and/or IAA in the presence of kinetin promoted the most callus growth; however, 2,4-D (1.0 ppm) and kinetin (0.2 ppm) together promoted callus growth and yielded maximum podophyllotoxin 6 formation (0.71 % dry weight callus in 8 week old cultures).

Kadkade[71] observed that podophyllotoxin 6 formation is light dependent. Callus grown in total darkness for 8 weeks produced only 0.24% podophyllotoxin 6, while callus grown with a light cycle of 16 hours light and 8 hours dark formed 0.71 % podophyllotoxin 6. Podophyllotoxin 6 formation was also found to depend on the original tissue from which the callus was developed. Callus developed from rhizomes, roots, leaves, and stems of *P. peltatum* was placed on MS medium which was supplemented with NAA (1.0 ppm) and kinetin (0.2 ppm). After callus development, the callus was transferred to a MS medium containing 2,4-D (1.0 ppm) and kinetin (0.2 ppm), for maximum podophyllotoxin 6 production. After 8 weeks of growth, the podophyllotoxin 6 content was determined. Callus from rhizome and root tissues

contained the highest amounts of podophyllotoxin 6 (0.57 % and 0.54 %). Callus originating from the other tissue sources produced less podophyllotoxin 6: leaf (0.12 %) and stem (0.05 %).

Based on Kadkade's results, similar procedures were adapted for callus growth and podophyllotoxin 6 synthesis in *P. peltatum*. Rhizomes were the explants used for callus source. The medium used for callus initiation was MS medium supplemented with NAA (1.0 ppm) and kinetin (0.2 ppm). Once callus developed, the callus was transferred to MS medium containing 2,4-D (1.0 ppm) and kinetin (0.2 ppm). The callus was grown at 24 °C with a light cycle of 16 hours light and 8 hours dark.

In our experiments, callus induction was difficult. Bacterial contamination inherent in the rhizome was very difficult to eliminate, and callus development and growth was slow. Kadkade[71] provided no discussion for disinfecting the explant tissue prior to callus induction. We developed a disinfectant procedure by immersing the rhizomes for 20 minutes in household bleach (100 mL, 30 % v/v) containing Tween 20 (1 drop) as a surfactant. Other bleach concentrations and duration of tissue immersion were tested (from 100 % bleach for 5 minutes to 25 % bleach for 20 minutes), but those procedures resulted in either killing the rhizome tissue or bacterial contamination of explant greater than 75 %. The contamination appeared as a cream colored halo surrounding the explant and was evident within the first two weeks after introducing the rhizome to the callus initiation medium. The contaminated cultures were discarded. Approximately 40 callus cultures were retained for continued growth.

The uncontaminated rhizomes began to show signs of callus formation 2 to 4 weeks after initiation. The callus developed from the cambial region of the rhizome. The callus was colored pale brown-green and was hard. Four weeks after introducing the rhizomes to the initiation medium, the callus was separated from the rhizome,

and the rhizome tissue was discarded. The callus was placed on MS medium supplemented with 2,4-D (1.0 ppm) and kinetin (0.2 ppm), and transferred to fresh medium monthly.

The callus doubled its mass in 6 weeks. The callus doubling time was determined over a 3 month period by first measuring the fresh weight of the callus before transferring to fresh medium, and then recording the final fresh weight after 4 weeks of growth. The growth rate of our callus was much slower than that reported by Kadkade. His callus, grown under similar conditions, reportedly doubled its mass within 2 weeks.

Isolation of Podophyllotoxin from *P. peltatum* Callus

The callus was harvested to determine its podophyllotoxin 6 content (25 cultures; final dry weight 8 g). The callus was frozen in liquid nitrogen, lyophilized, and extracted with acetone. The acetone extract containing podophyllotoxin 6 was separated by thin layer chromatography using the solvent system methylene chloride-methanol (100:5 v/v). The band corresponding to podophyllotoxin 6 was carefully removed with a razor blade, and podophyllotoxin was eluted from the silica gel with acetone. Acetone was removed from the podophyllotoxin 6 sample, and the sample was taken up in methanol to analyze podophyllotoxin 6 content by high performance liquid chromatography at 290 nm. The podophyllotoxin 6 in the sample was also compared to authentic podophyllotoxin 6 with respect to retention volume and peak area in determining concentration. Podophyllotoxin 6 content in our callus ranged from 0.0082 % to 0.0032 %. This is markedly different from the amount of podophyllotoxin 6 that Kadkade[71] had reported in his callus (maximum of 0.57 %).

Administration of [U-^{14}C-]L-Phenylalanine to *P. peltatum* Callus

We next turned our attention to inducing podophyllotoxin 6 synthesis in the callus. Kadkade[71] reported that casamino acids (from yeast extracts) stimulate podophyllotoxin 6 production in callus. He found that MS medium supplemented with 2,4-D (1.0 ppm), kinetin (0.2 ppm), and casamino acids (0.05 % w/v) induced the callus to produce 0.71 % podophyllotoxin 6. Callus grown with the same medium, with the exception of casamino acids, yielded only 0.57% podophyllotoxin 6. Kadkade suggested that casamino acids induce podophyllotoxin 6 biosynthesis since casamino acids contain L-phenylalanine 11 which is a precursor for the biosynthesis of podophyllotoxin 6 in intact *P. peltatum* plants.[60,61] Therefore, we investigated the use of phenylalanine 11 to induce podophyllotoxin 6 biosynthesis in our callus cultures.

The callus was placed on medium (Murashige and Skoog) containing [U-^{14}C]L-phenylalanine 11 (0.2 mM, 7.42X10^5 dpm/mg) for the purpose of tracing the L-phenylalanine 11 uptake into the callus and into podophyllotoxin 6. The callus was grown on this medium for 4 weeks at 24 °C with a light cycle of 16 hours light and 8 hours dark.

After 4 weeks, the callus was removed from the medium, frozen in liquid N_2, and lyophilized. Authentic podophyllotoxin 6 (10 mg) was added to the dry callus as a carrier to increase the podophyllotoxin 6 sample size for easier sample handling during the podophyllotoxin 6 extraction and isolation procedures. The callus (plus podophyllotoxin 6 carrier) was ground to a fine powder. Podophyllotoxin 6 was removed from the powdered callus by Soxhlet extraction with 95 % ethanol. The ethanol was removed from the podophyllotoxin 6 extract by evaporation, and the crude podophyllotoxin 6 extract was taken up in chloroform. The chloroform solution

was washed with water to remove compounds more polar than podophyllotoxin 6 from the extract; podophyllotoxin 6 remained in the chloroform solution, while the more polar compounds went into the water. The chloroform was removed from the podophyllotoxin 6 extract, and the extract was further purified by silica gel thin layer chromatography using the solvent system chloroform-methanol (92:8). The band containing podophyllotoxin 6 was carefully removed with a razor blade. Podophyllotoxin 6 was eluted from the silica gel with methanol. Methanol was removed from the sample by evaporation, and the sample (18.6 mg) was taken up in acetonitrile-water (29.8 mL, 40:60 v/v). A portion of the sample (200 μl, 2020 dpm/32 μg) was applied to a reverse phase high performance liquid chromatography column eluted with acetonitrile-water (40:60 v/v).

During the high performance liquid chromatography, the eluants (0.5 mL) were collected from the column in scintillation vials, and the radioactivity of each was determined by liquid scintillation spectrometry. Figure 23 shows the radiochromatogram for the sample. As the data indicate, no radioactivity was associated with the podophyllotoxin peak. If [U-^{14}C-]L-phenylalanine 11 was incorporated into podophyllotoxin 6, then the fractions collected from 10.0 to 11.5 mL and corresponding to podophyllotoxin 6 should have been radioactive. However, the radioactivity of these eluants did not exceed the background; the majority of the radioactivity eluted with the solvent front during the high performance liquid chromatography.

No detectable radioactivity resulting from incorporation of [U-^{14}C]L-phenylalanine 11 into podophyllotoxin 6 was thus observed, although podophyllotoxin 6 was the major component in the sample applied to the column (27 μg podophyllotoxin 6 of the 32 μg sample). The podophyllotoxin 6 content was determined by comparing the retention volume and peak area of this chromatogram to retention volume and peak

area of known concentrations (0.001% to 0.1%) of authentic podophyllotoxin 6. In this experiment, we were therefore unable to detect podophyllotoxin 6 biosynthesis in *P. peltatum* callus tissue [0.00% incorporation of [U-^{14}C-]L-phenylalanine 11 (see Figure 23 and Table 10)]. L-Phenylalanine did not induce podophyllotoxin 6 formation in callus of *P. peltatum*.

Administration of [U-^{14}C-]L-Phenylalanine to *P. peltatum* Rhizomes

We next investigated if podophyllotoxin 6 formation could be detected in a *P. peltatum* rhizomes. Since rhizomes of *P. peltatum* contain as much as 1% podophyllotoxin 6 based on dry weight,[80] we speculated that podophyllotoxin 6 biosynthesis would be detectable in this tissue. Similar to the callus study, carbon-14 labeled L-phenylalanine 11 was used as the precursor to probe podophyllotoxin 6 formation.

During mid-summer (July), the rhizomes were collected from the wild. They were cleaned in a dilute detergent solution (2% Sparkleen) and rinsed thoroughly to remove soil and detergent. The rhizomes were then cut into approximately 2 cm sections and disinfected (see Materials and Methods). While using aseptic conditions, the rhizomes were rinsed twice, and the bleach damaged tissue was removed. The rhizome tissue was then cut into approximately 5X5X5 mm disks under aseptic conditions. This procedure was necessary to minimize sources of microbial contamination, to ensure that the carbon-14 label taken up by the rhizomes arises directly from the [U-^{14}C-]L-phenylalanine 11 administered.

The rhizome disks (15 disks per sample) were incubated in a [U-^{14}C-]L-phenylalanine 11 solution (10.5 mL, 0.95 mM, 2.26X10^{6} dpm/mg, in a 50 mM

potassium phosphate buffer). The disks were continuously shaken in this solution for the incubation period of 0, 24, and 96 hours.

Upon completion of the incubation period(s), the disks were rinsed, blotted to remove excess solution, frozen in liquid nitrogen, and lyophilized. After lyophilization, 10 mg of authentic podophyllotoxin 6 was added to each sample as a carrier to increase the sample size of podophyllotoxin 6 to be isolated and purified from the rhizome tissue. The rhizomes plus added carrier were ground to a fine powder, and extracted with 95 % ethanol. Podophyllotoxin 6 was then isolated (see Materials and Methods).

The isolated podophyllotoxin 6 was purified by high performance liquid chromatography (see Materials and Methods). The eluants from the chromatography column were collected in scintillation vials, and the radioactivity associated with the eluants was determined by liquid scintillation spectroscopy. Podophyllotoxin 6 was the major compound in the sample (28 μg podophyllotoxin 6 of 32 μg total weight applied to column). However, no detectable radioactivity was associated with podophyllotoxin 6 (see Table 11).

It was speculated that we did not detect podophyllotoxin 6 formation in this experiment since the level of carbon-14 administered was too low to observe radioactivity incorporation into podophyllotoxin 6 (the average radioactivity level taken up by the rhizome samples during the 96 hour incubation was $2.25X10^6$ disintegrations per minute for an average rhizome sample dry weight of 372.58 mg). Therefore, we focused our attention on a new experimental design to increase the uptake of the precursor solution.

Liquid absorption was next investigated for the rhizome disks. Rhizome disks were incubated in an aqueous solution consisting of 3 drops of blue food coloring in 10 mL of water (food coloring contents are water, propylene glycol, and 1.5% blue

coloring, propylparaben and sodium metabisulfite). The food coloring was distributed by the Kroger Co., Cincinnati, Ohio. After incubating 24 hours, no dye was detected inside the tissue. To force liquid (diluted blue food coloring) to infiltrate the disks, the disks were placed in a dish containing 1 mL of the blue liquid. The dish was placed in a desiccator which was brought to a vacuum (600 mm Hg). The vacuum was maintained for 30 seconds, then quickly released. This procedure was conducted three times to maximize liquid infiltration. By this method, the disks absorbed dye evenly throughout their tissue.

We also attempted to improve our previous rhizome experiment by following a similar procedure developed by Kojima and Uritani (1971).[81] In their procedures, slices of sweet potato (*Ipomoea batatas* Lam. cv. Norin 1) roots were incubated with 12.5 μmoles of 2-^{14}C trans-cinnamic acid or 2-^{14}C-quinic acid in a 5 mL acetate buffer. Their experiments resulted in incorporation of both cinnamic acid and quinic acid into chlorogenic acid. With the expectation that L-phenylalanine 11 would induce podophyllotoxin 6 formation in the rhizome tissue, we too administered a highly concentrated precursor (4.0 mM [U-^{14}C-]L-phenylalanine 6).

[U-^{14}C]L-Phenylalanine 11 was dissolved in a 5 mM acetate buffer (final solution: 4.0 mM L-phenylalanine 11, 1.14X10^{6} dpm/mg). The rhizomes were prepared as described for the previous rhizome experiment except that the sample size was increased; each rhizome sample consisted of 50 pre-disinfected rhizome disks. Each sample of rhizome disks was placed in a petri dish, and 1 mL of [U-^{14}C-]L-phenylalanine 11 was added to each sample. Immediately after adding the solution, the petri dish was covered with perforated aluminum foil. The sample was infiltrated as previously described for increasing liquid absorption in rhizome disks.

The samples were incubated for 3, 12 and 24 hours. After incubation, the samples were rinsed, blotted to remove excess liquid, frozen in liquid nitrogen, and

lyophilized. Authentic podophyllotoxin 6 (20 mg) was added to each sample for easy isolation and purification of podophyllotoxin 6 from each sample. The samples were ground to a fine powder and extracted with acetone. Podophyllotoxin 6 in each sample was isolated, acetylated, and purified by recrystallization (see Materials and Methods). Podophyllotoxin acetate 46 was recrystallized for purification three times, or until the radioactivity of the samples was not measurable above background (see Table 12, data after 24 hours incubation). In summary, no detectable amount of podophyllotoxin 6 was biosynthesized with the L-phenylalanine 11 administered during the incubation periods tested. In order to achieve incorporation of carbon-14 radiolabeled L-phenylalanine 11 into podophyllotoxin 6, we had to turn our attention to administering the precursor to actively growing *P. peltatum* plants.

Administration of Labeled Phenylalanine to *P. peltatum* Seedlings

[U-^{14}C-]L-Phenylalanine Administration

Since we were unable to observe L-phenylalanine 11 incorporation into podophyllotoxin 6 in either callus or rhizome experiments of *P. peltatum*, an aseptic system for administering labeled precursors to intact plants of *P. peltatum* and *P. hexandrum* was developed. Previous studies using intact plants of *P. hexandrum* resulted with only low incorporations of L-phenylalanine 11 (ranging from 0.04 to 1.19%).[66] These low incorporations were primarily due to the length of time during which the precursor was administered. In our experiment we developed a system which extends the precursor exposure time to the intact plants to afford maximum precursor uptake and intact incorporation into podophyllotoxin 6. The system con-

sisted of *P. peltatum* plants grown in individual aseptic hydroponic units with hydroponic medium containing the L-phenylalanine 11 precursor.

A similar hydroponic system had been developed to study lignin formation in *Leucaena leucocephala* by Lewis *et. al.*.[82] It consists of an aseptic hydroponic system designed to house an intact *L. leucocephala* seedling with a sterile air exchange system and a sterile medium containing isotopic precursors. In that study, carbon-14 precursors were administered to *L. leucocephala* to assure that incorporation into lignin actually took place. After development of the system, 2-^{13}C-ferulic acid was administered to intact plants over a 28 day period. Sufficient incorporation of the ^{13}C precursor was observed for analysis of the tissue (and lignin) by solid state ^{13}C nuclear magnetic resonance. For the first time, precise information about lignin bonding patterns *in situ* were observed. It can thus be envisaged that with a similar experimental design specifically labeled ^{13}C, ^{2}H, and ^{13}C/^{2}H labeled precursors could be administered to *P. peltatum* and *P. hexandrum* so that precise biosynthetic pathway of lignans can be probed.

The aseptic hydroponic system has several outstanding features. Precursors can be administered discretely to intact plants for long time periods, more than 28 days,[82] since the administration of precursors is under aseptic conditions. The elimination of bacterial and fungal contamination prevents the possibility of administering labeled compounds other than those being administered. The system also furnished the plant light for photosynthesis, aeration of medium to supply roots with oxygen, and water.

Figure 24 illustrates the hydroponic unit design. A Mason jar (2 quart) housed each plant. The clear, colorless glass allowed for light infiltration. Inside the Mason jar, the plant was supported atop a teflon bottle (125 mL). The roots were placed inside the bottle and immersed in a medium (Hoagland's, see Table 13) containing the

precursor L-phenylalanine 11. This medium was continuously aerated with filter sterilized air pumped by an aquarium pump, and an air filter was fixed to the top of the Mason jar to prevent build up of air pressure.

The hydroponic units were constructed with materials which could be autoclaved prior to use. The units were constructed under a laminar flow cabinet under aseptic conditions, and were designed so that once the seedling was placed in the unit, it would not need to be opened until termination of the experiment.

Seedlings were obtained from *P. peltatum* and *P. hexandrum* seeds. The seeds were disinfected by immersing the seeds in household bleach (50% v/v, 100 mL) for 30 minutes. The seeds were placed directly on to autoclaved moistened filter paper for 24 hours to imbibe the seeds. The imbibed seeds were then placed on Potato Dextrose Agar to stratify the seeds, forcing germination. After two months the seeds had not germinated; therefore, the embryos were aseptically excised from the seeds. While in a laminar flow cabinet, the embryo of each seed was removed by carefully cutting the seed coat with a scalpel. Each tiny embryo (approximately 1X1X2 mm) was then placed on MS medium that did not contain any growth regulators and placed in a growth chamber maintained at 24°C with 24 hours light. Two months later the embryos had developed into seedlings; each consisted of 2 leaflets (~2 cm span), and a radicle (~2 cm). At this stage, each seedling was transferred to a hydroponic unit within the confines of a laminar flow cabinet.

The seedlings contained in the hydroponic units were maintained under controlled conditions (16 hours illumination (24°C) and 8 hours dark (20°C)). Hoagland's medium (110 mL) with dissolved precursor [U-^{14}C-]L-phenylalanine 11 was supplied for each seedling (0.5 mM L-phenylalanine 11; 1.45X10^{9} dpm/mM). Air was continuously pumped through the medium by a Nalgene aquarium pump through 0.45 micron filters. During the aeration of the medium, water evaporation occurred. Water

was replenished to the medium daily from the sterile water reservoir. This was necessary to maintain the constant specific activity of [U-^{14}C-]L-phenylalanine 11 being administered to the plant.

The medium was tested periodically to assure that the [U-^{14}C-]L-phenylalanine 11 specific activity remained constant during the administration. To test the medium, aliquots of medium were aseptically taken from each unit by pumping a portion of medium into a sampling vial sealed with a silicone cork. Under aseptic conditions, a 1.5 mL aliquot of medium was removed from the sample vial and analyzed for phenylalanine 11 concentration, ^{14}C activity, and microbial contamination. The remaining aliquot was pumped back into the medium.

L-Phenylalanine 11 concentration was determined by assay with phenylalanine 11 ammonia-lyase (PAL) (see Materials and Methods). The carbon-14 present in the medium was measured by scintillation spectroscopy; 100 μL aliquots of the medium were measured in duplicate. To determine the specific activity of the medium, the carbon-14 was divided by the concentration of L-phenylalanine 11. During the experiment, the medium was exchanged two times at 14 and 38 days. For each medium exchange the specific activity of the old and the new medium was measured (medium exchanges are indicated by "Steps in Phenylalanine 11 Administration", see Table 14).

The medium was monitored for contamination by placing 100 μL aliquots of medium on Potato Dextrose Agar. Seedlings suffering from microbial contamination were discarded.

The plants grew quickly after being placed in the hydroponic medium. Two new leaves and stems emerged. The new leaves were the mature form, and the juvenile leaves died soon after the mature leaves emerged. Within 2 weeks, the leaves and stems stopped enlarging (final measurements: leaf, ~3 cm span; stems, ~6.5 and

~5.3 cm height). The growth of the roots could not be monitored since they were immersed in the medium contained in an opaque bottle.

After 64 days of administering [U-^{14}C-]L-phenylalanine 11, the plants were harvested. Each plant was wrapped in aluminum foil, frozen in liquid nitrogen, and lyophilized. The lyophilized plant material was very small (57.38 - 355.59 mg dry weight, see Table 15). Each plant was ground to a fine powder, and a portion of each plant (1 % dry weight) was counted by scintillation spectroscopy to detect carbon-14 activity (see Table 15). Authentic podophyllotoxin 6 (10 mg) was added to each sample as a carrier for isolating and purifying podophyllotoxin 6 produced by each plant. Without the carrier, endogenous podophyllotoxin 6 could not be isolated or purified with sufficient yield because the sample size would be too small.

Podophyllotoxin 6 carrier was ground with the pre-ground plant tissue, and the whole sample was subsequently extracted with acetone. Podophyllotoxin was isolated by preparatory thin layer chromatography, eluting sample with methylene chloride-methanol (100:5 v/v) (see Materials and Methods). The recovered podophyllotoxin 6 (13.64 mg) was converted to podophyllotoxin acetate 46. The podophyllotoxin acetate 46 was purified by crystallization from 95% ethanol (64 % recovery). Each sample was recrystallized to obtain constant specific activity (see Table 16). The absolute incorporation of [U-^{14}C-]L-phenylalanine 11 into podophyllotoxin was determined for each sample (see Table 17 and the Appendix). The plants took up 1.00 to 18.47 % of the [U-^{14}C-]L-phenylalanine 11 administered (25.5 mg L-phenylalanine 11; 2.47×10^7 dpm) over 64 days of culture. Based on the amount of carbon-14 radioactivity taken up by the plants, the maximum absolute incorporation of [U-^{11}C-]L-phenylalanine 11 was calculated to range from 3.30%.to 10.23% (data for Plant 4 is excluded from this discussion since the plant did not grow).

The purpose of this experiment was to determine if incorporation levels of L-phenylalanine 11 are sufficient for administering carbon-13 labeled L-phenylalanine 11. Since we observed that as much as 10.23% of administered [U-^{14}C-]L-phenylalanine 11 was incorporated into podophyllotoxin 6, then we should be able to observe incorporation of carbon-13 enriched L-phenylalanine 11 into podophyllotoxin 6 by carbon-13 NMR spectroscopy.

[1-^{13}C]-L-Phenylalanine Administration

To date, no carbon-13 incorporation has been reported in any lignan compound. The experiments were performed to develop a method to incorporate sufficient carbon-13 specifically enriched precursor to observe carbon-13 enrichment in the lignan podophyllotoxin 6 by carbon-13 NMR spectroscopy. This development is necessary to accumulate more data concerning the precursor relationship to lignans such as podophyllotoxin 6, so that we can better understand the stereospecific biosynthesis of lignans *in vivo*. The prerequisite experiment before administering a carbon-13 precursor is to demonstrate that a sufficient level of precursor does incorporate to a reasonable extent. In our previous experiment we showed that the radiolabeled precursor, L-phenylalanine 11, does convert into podophyllotoxin 6 at a significant level, with 10.23 % maximum absolute incorporation (an estimated 0.052 mg podophyllotoxin 6 biosynthesized from the administered precursor over the 64 day period). We therefore expected sufficient conversion of [1-^{13}C]-L-phenylalanine 11 into podophyllotoxin 6 to observe carbon-13 enhancement by carbon-13 NMR spectroscopy.

The objective was to demonstrate that the ^{13}C specifically enriched [1-^{13}C]-L-phenylalanine 11 converts into podophyllotoxin 6 where the specific

carbon-13 enrichment is located at the C-9 and C-9′ of the lactone ring (see Figure 25 for carbon atom notation) as evidenced by carbon-13 NMR spectroscopy. Therefore, we administered [1-^{13}C]-L-phenylalanine 11 (99.0 atom %, 0.5 mM) to *P. peltatum* and *P. hexandrum* seedlings to demonstrate that peak enhancement at C-9 and C-9′of podophyllotoxin 6 (δ 173.6 and 71.3 ppm respectively for the acetate derivative) can be observed by carbon-13 NMR spectroscopy.

Sterile seedlings of *P. peltatum* and *P. hexandrum* were obtained by aseptically excising embryos directly from seeds (see Materials and Methods). The seeds were treated differently from the *P. peltatum* used for the previous [U-^{14}C-]L-phenylalanine 11 hydroponic experiment. Since stratification via placing the imbibed seeds on Potato Dextrose Agar for two months prior to embryo excision did not appear to have any impact on seed germination, this period was omitted from this carbon-13 experiment. Seeds were disinfected and the embryos were excised without an incubation period. The embryos were then placed in petri dishes containing MS medium. The embryos had developed to seedlings within 2 months. Each seedling was composed of a juvenile leaf (~0.5-2 cm) and radicle (~0.6-2 cm). *P. peltatum* seedlings were aseptically transferred to sterile hydroponic units while *P. hexandrum* seedlings were held back for future use.

The hydroponic units were maintained in an environmental chamber with 16 hours illumination (24°C) and 8 hours dark (20°C) for 3 months. Hoagland's medium was supplemented with [1-^{13}C]-L-phenylalanine 11 (0.5 mM L-Phe). The medium was exchanged every two weeks, and microbial contamination was monitored by incubating 100 μL aliquots of the medium on Potato Dextrose Agar. If contamination was detected, the plant was discarded. Otherwise, the plants were maintained for 3 months.

Within the first three weeks of initiating the hydroponic units, little visible growth of the seedlings was observed. Indeed, approximately half of the plants developed small callus and embryoids on their leaves. It is speculated that this resulted from a physiological response from an embryo excised prior to a stratification period. The seedlings that developed these symptoms were harvested. *P. hexandrum* seedlings replaced the *P. peltatum* seedlings that were harvested. The initial juvenile leaf and radicle size of the *P. hexandrum* seedlings were ~1 cm and ~4 cm, respectively. The *P. hexandrum* failed to grow over the 8 week period, and there was no ^{13}C enhancement of podophyllotoxin 6 as evidenced by carbon-13 NMR spectroscopy.

After three months, the final size of the *P. peltatum* plants were: leaves, 1.2-3.0 cm; stems 2-4 cm; and root 0.6-5.0 cm with dry weight ranging 48.5-64.3 mg. Three *P. peltatum* plantlets were combined to serve as a single sample (final weight 176.8 mg). Since only trace amounts of podophyllotoxin 6 was formed in the three plants (approximately 0.1 mg of endogenous podophyllotoxin 6), authentic podophyllotoxin 6 (5.6 mg) was added as carrier. The resulting podophyllotoxin 6 was then isolated and purified from the plant tissue.

The whole sample was ground to a fine powder in a mortar and was subsequently extracted with acetone. Podophyllotoxin 6 was purified from the acetone extract by thin layer chromatography, (see Materials and Methods). The isolated podophyllotoxin 6 was acetylated and crystallized once from 95% ethanol, (5.5 mg; a 89% recovery based on podophyllotoxin 6 added). The purified podophyllotoxin acetate 46 was analyzed by ^{13}C NMR spectroscopy.

Because the sample was diluted with unlabeled carrier, a system had to be developed to see the carbon-13 enrichment of podophyllotoxin 6 (in the acetate form) using carbon-13 NMR spectroscopy. First, the sample of podophyllotoxin acetate 46 (5.5 mg) was taken up in deuterated chloroform (250 μL) with TMS added as a stand-

ard for the NMR spectrum. This sample was scanned 21,972 times. Unlabeled podophyllotoxin acetate 46 (5.5 mg) was treated in the same manner and scanned 21,737 times under identical conditions to obtain the carbon-13 natural abundance spectrum. The natural abundance spectrum of podophyllotoxin acetate 46 was subtracted from the carbon-13 enriched podophyllotoxin acetate spectrum. By this method we were able to see the only carbon signals that were carbon-13 enriched (see Figure 26). As predicted, carbon-13 enhancement was observed only for C-9 and C-9′ (δ 173.6 and 71.3 ppm respectively, see Figure 26 and Figure 27).

This is the first report of an intact incorporation of a carbon-13 specifically enriched precursor into any lignan. The incorporation is significant for future lignan biosynthesis studies because now experiments using precursors specifically enriched with carbon-13, deuterium, or a combination of carbon-13 and deuterium can be employed to show the precise incorporation through carbon-13 NMR spectroscopy.

Concluding Remarks

Carbon-13 specifically enriched L-phenylalanine 11 administered to actively growing *P. peltatum* can be incorporated intact into the lignan podophyllotoxin 6 at sufficient levels to observe specific carbon-13 enrichment by carbon-13 NMR spectroscopy. We have shown that *P. peltatum* plants grown in an aseptic hydroponic environment can be administered carbon-13 precursors over a long period of time, and intact incorporation of the precursor is observed into podophyllotoxin 6. More work still must be done to attain better growth of *P. peltatum* and *P. hexandrum* plants in the *in vitro* system designed in our experiments. Overcoming this problem will allow larger lignan samples to be extracted from the plant tissue, without diluting the sample with unlabeled carrier.

Table 9. Murashige and Skoog Medium

Compound	mg/L
$(NH_4)NO_3$	1650.0
KNO_3	1900.0
$CaCl_2$-$2H_2O$	440.0
$MgSO_4$-$6H_2O$	370.0
KH_2PO_4	170.0
$FeSO_4$-$7H_2O$	27.8
Na_2EDTA	33.6
$MnSO_4$-$4H_2O$	22.3
$ZnSO_4$-$4H_2O$	8.6
H_3BO_3	6.2
KI	0.83
Na_2MoO_4-$2H_2O$	0.25
$CuSO_4$-$5H_2O$	0.025
$CoCl_2$-$6H_2O$	0.025
myo-inositol	100.0
nicotinic acid	1.0
pyridoxine-HCl	1.0
thiamine-HCl	10.0
sucrose	30.0 g/L
Bacto agar	7.0 g/L

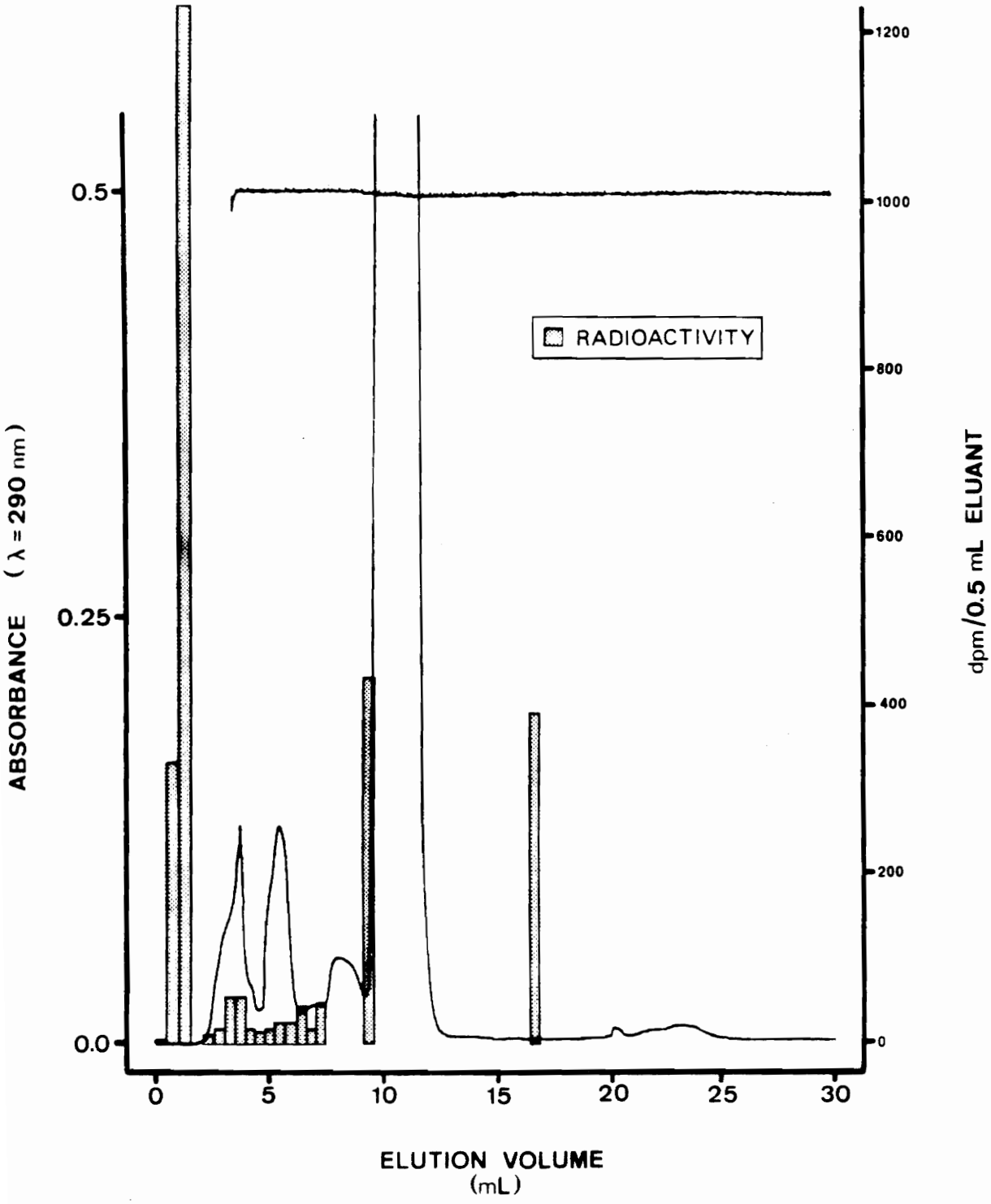


Figure 23. Radioactivity (^{14}C) corresponding to the HPLC chromatogram of Podophyllotoxin 6 isolated from P. peltatum Callus.

Table 10. Incorporation into Podophyllotoxin 6 after Administering [U-^{14}C-]L-Phenylalanine 11 to P. peltatum Callus.

Isolation Step	Fraction Containing Podophyllotoxin 6 (mg)	Specific Activity (dpm/mg) X10^3
EtOH Extract	148.75	20.8
TLC region of podophyllotoxin 6	21.11	2.50
Podophyllotoxin 6 purified by HPLC	0.027	0

The average dry callus weight before extraction was 520.72 mg; total [U-^{14}C]L-phenylalanine 11 taken up by the callus was 6.82X10^6 dpm. Data are averages of two samples; each sample was counted twice. Disintegrations per minute (dpm) are corrected for background.

Table 11. Incorporation into Podophyllotoxin after Administering [U-^{14}C-]L-Phenylalanine 11 to P. peltatum Rhizomes.

Isolation Step	Fraction Containing Podophyllotoxin 6 (mg)	Specific Activity dpm/mg X10^3
EtOH extract	71.36	15.1
TLC region of podophyllotoxin 6	15.95	0.316
Podophyllotoxin 6 purified by HPLC	0.028‡	0

The average dry weight of rhizomes are 372.58 mg; the total [U-^{14}C]L-phenylalanine 11 taken up by the rhizomes was 2.25X10^6 dpm/mg during a 96 hour incubation period. Data are averages of two samples; each sample was counted twice. Disintegrations per minute (dpm) are corrected for background. ‡Amount of material applied to column was 1.425 mg with 450 total dpm.

Table 12. Incorporation into Podophyllotoxin after Administering [U-^{14}C-]L-Phenylalanine to P. peltatum Rhizomes for 24 h.

Repeated Recrystallizations	Dry Wt. (mg)	dpm/mg	% Total Incorporation
First crystallization	15.50	402.48	-
Second crystallization	12.05	47.86	-
Third crystallization	9.71	3.31	0.005

Data are average of two samples; each sample was counted twice. Disintegrations per minute (dpm) are corrected for background.

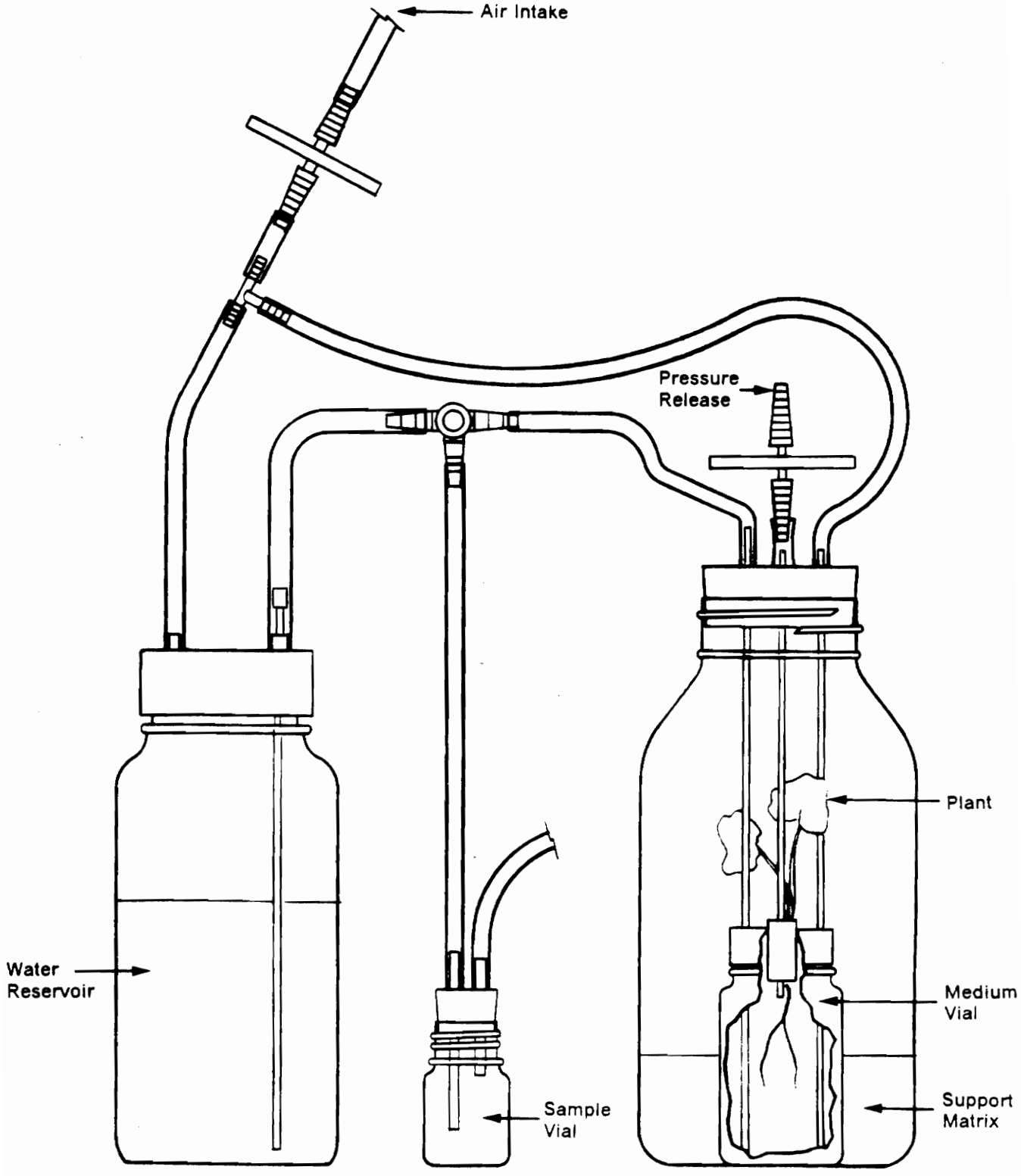


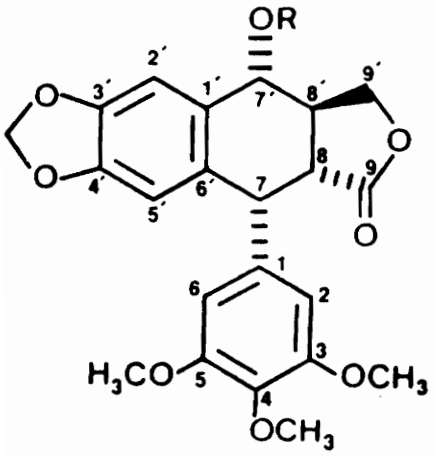
Figure 24. Hydroponic Unit

Table 13. Hoagland's Medium

Compound	mg/L
$NH_4H_2PO_4$	115.000
KNO_3	7.000
$Ca(NO_3)_2$-$4H_2O$	945.000
$MgSO_4$	241.000
H_3BO_3	2.860
$MnCl_2$-$4H_2O$	1.810
$ZnSO_4$-$7H_2O$	0.220
$CuSO_4$-$5H_2O$	0.080
Na_2MoO_4-$2H_2O$	0.024
Na_2Fe-EDTA	0.988

Table 14. Stepwise Administration of [U-^{14}C]L-Phenylalanine 11 into P. peltatum Seedlings during growth (Plant 1)

Steps in Phenylalanine Administration	Time of culture (days)	dpm measured by scint. X10^6	L-Phe (PAL assay) (mg)	Specific Activity (dpm/mg) X10^6
1	0 14	8.67 7.56	8.26 6.44	1.05 1.17
2	14 38	7.86 6.33	8.18 6.43	0.96 0.98
3	38 64	8.21 6.55	9.06 7.10	0.91 0.92



Where: R = H ; **Podophyllotoxin 6**

R = $-\overset{O}{\overset{\|}{C}}CH_3$; **Podophyllotoxin Acetate 46**

Figure 25. Carbon Atom Notation Used for Podophyllotoxin 6 and Podophyllotoxin Acetate 46.

Table 15. ^{14}C Incorporation into P. peltatum Seedlings.

Plant Number	Final Plant Dry Wt. (mg)	Total Activity Taken Up $X10^5$ dpm	% Activity Taken up by Plant
1	88.61	4.66	1.88
2	57.38	5.82	2.35
3	355.59	4.57	18.47
4	19.50	2.48	1.00

Total radioactivity administered to plants over the 64 day period was $2.47X10^7$ dpm. Disintegrations per minute (dpm) are corrected for background.

Table 16. Constant Specific Activity (dpm/mg) for Podophyllotoxin Acetate 46 by Recrystallization.

Plant Number	First Crystallization $X10^3$ dpm/mg	Second Crystallization $X10^3$ dpm/mg	Third Crystallization $X10^3$ dpm/mg
1	3.28	4.16	4.13
2	1.99	2.04	1.94
3	25.2	25.4	--
4	0	--	--

Table 17. Absolute Incorporation of [U-^{14}C-]L-Phenylalanine 11 into Podophyllotoxin 6 Formed by P. peltatum Plants

Plant Number	Total Activity of Podophyllotoxin X10^5 dpm	Total Activity Taken Up by Plant (X10^5 dpm)	% Absolute Incorporation
1	0.477	4.66	10.23
2	0.192	5.82	3.30
3	2.851	45.7	6.24
4	0	2.48	0

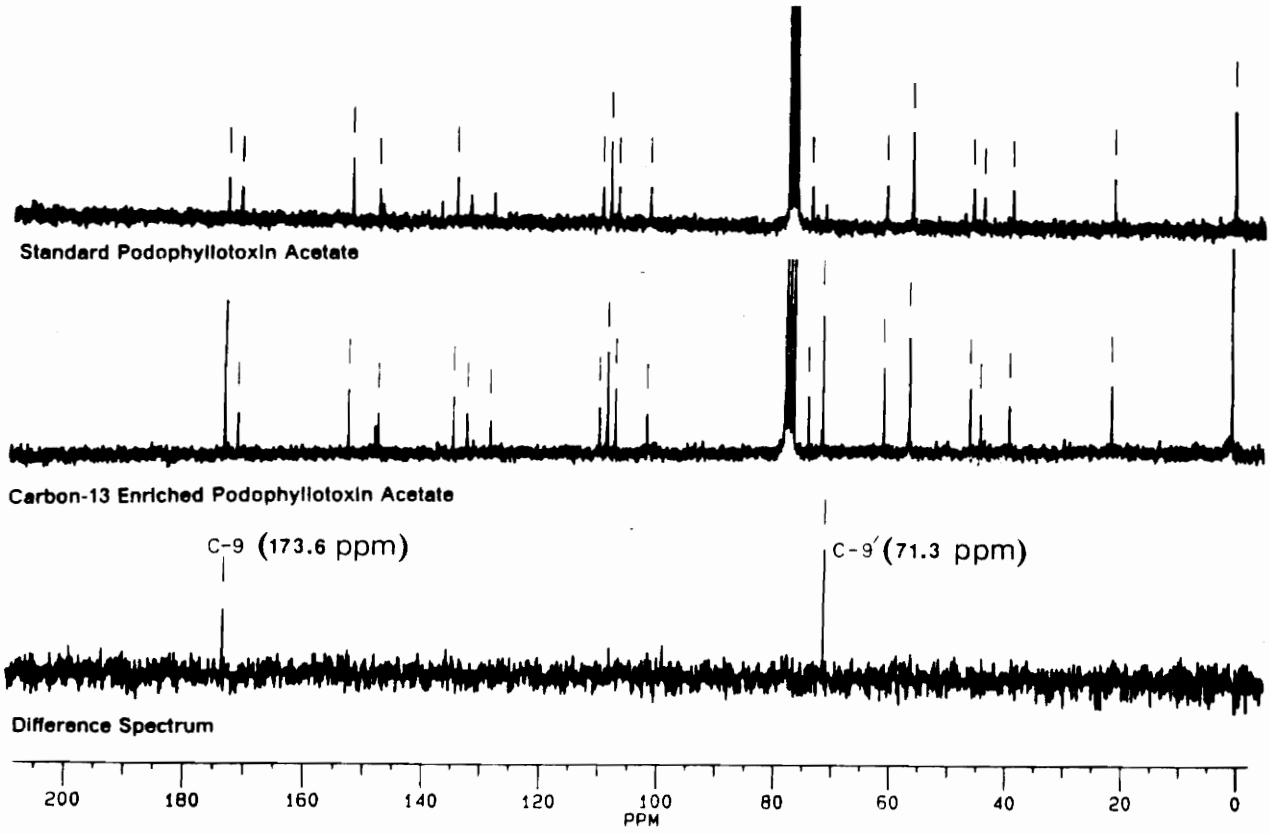


Figure 26. Carbon-13 NMR Spectra of Podophyllotoxin Acetate 46

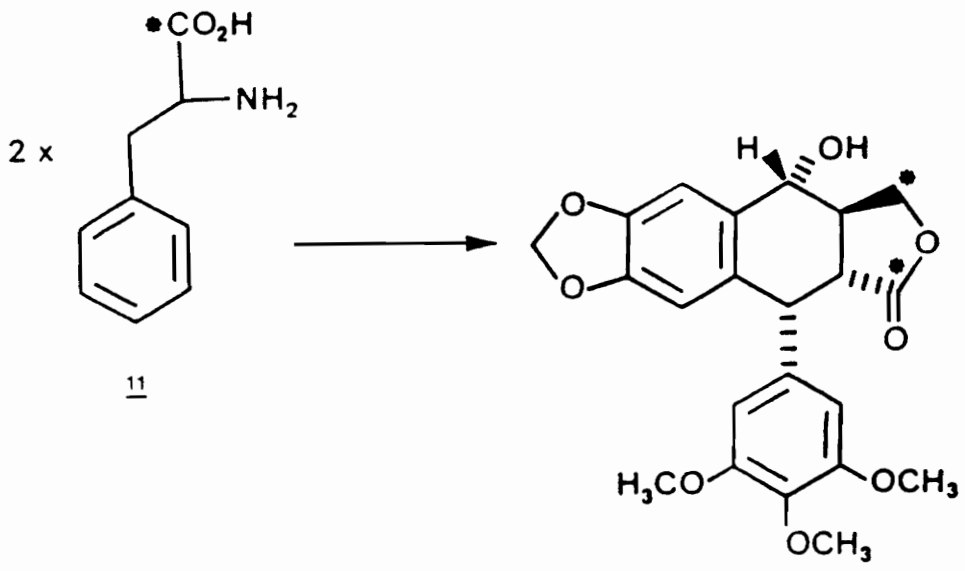


Figure 27. Location of Carbon-13 Enrichment in Podophyllotoxin 6 from [1-^{13}C]-L-Phenylalanine 11

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Appendix

Calculating Absolute Incorporation (%)

After determining the total activity* for podophyllotoxin acetate 46, corrections as outlined below were made to account for losses incurred throughout the experimental procedure, followed by the calculation for absolute incorporation.

1. $$\frac{\text{Weight of Podophyllotoxin Carrier}}{\text{Mol. wt. of Podophyllotoxin}} \times \text{Mol. wt. of Derivative} = \text{Weight of Derivative}$$

2. $$\frac{\text{Weight of Derivative}}{\text{Actual Recovered wt. of Derivative}} \times \text{Total Activity* of Isolated Derivative} = \text{Corrected Activity for Podophyllotoxin}$$

3. $$\frac{\text{Corrected Activity for Podophyllotoxin}}{\text{Total Activity Taken up by Plant}} \times 100 = \text{Absolute Incorporation into Podophyllotoxin}$$

*All cpm data were corrected for background and quenching as representative for all counting efficiencies.

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