ISOLATION AND CHARACTERIZATION OF ACTIVELY
ANABOLIZED DILIGNOL RHAPNOSIDES IN
THE LEAVES OF WESTERN RED CEDAR (THUJA PlicATA DONN)

by

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We accept this thesis as conforming to the
required standard.

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ABSTRACT

Three dilignol rhamnosides were isolated in 0.15 to 0.40% yield from the ethyl acetate solubles of a methyl alcohol extract of western red cedar (Thuja plicata Donn) leaves using silicic acid and Sephadex LH-20 pressure column chromatography.

One of the three dilignol rhamnosides was characterized as 1-(3'-methoxy-4'-hydroxyphenyl)-2-O-1''-[2''-hydroxy-4''-(propane-3''-α-L-rhamnoside)phenyl]-propane-1,3 diol, using NMR and chemical degradation in conjunction with mass spectral techniques on the compound and its derivatives. The other dilignol rhamnosides were not completely characterized, but were shown to be chemically related to the dilignol identified. Based upon NMR, chemical and mass spectral data, the uncharacterized dilignol rhamnosides are speculated to contain phenylcoumaran and guaiacyl benzodioxane structures.

The characterized dilignol rhamnoside represents the first reported occurrence of a free dilignol glycoside in plant tissues. The unusual α-L-rhamnose moiety of the dilignol occurs in a previously unreported linkage to the n-propyl hydroxyl group uncommon in lignin. The rhamnoside also displays the previously unreported guaiacylglycerol-catechol-β-aryl ether structure rather than the commonly observed guaiacylglycerol-guaiacyl-β-aryl ether structure.
A new combustion-absorption technique was developed and validated which allows high efficiency evaluation of low activity radioactive products separated on thin layer cellulose chromatography plates. The technique was applied to an analysis of the anabolic products of an infusion feeding of U-\(^{14}\)C-L-phenylalanine to western red cedar leaves. Facile imbibition of U-\(^{14}\)C-L-phenylalanine occurs within ten hours. Maximum incorporation of 0.30% and 0.40% of the available radioactivity occurs in the characterized dilignol rhamnoside, and its suspected phenylcoumaran homolog respectively, at the three to five hour period of the infusion feeding. The incorporation results indicate the participation of the dilignol rhamnosides in aromatic metabolism in the leaves of western red cedar. This feeding experiment is preliminary to future detailed biosynthetic studies in the leaf tissue.

The combustion-absorption technique is limited to combustible sample weights of 7 mg.
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INTRODUCTION

Lignin has generally been defined as a tridimensional phenylpropanoid polymer existing in plant tissues primarily for structural support. The polymeric nature of this substance suggests its multi-stage development over a wide range of phenylpropanoid metabolizing tissues. The lignin represents as much as 30% of the constituents of coniferous plant stems, and this high content has initiated many investigations into the "where" and "how" of its formation.

The question of where lignin is formed has resulted in many macro and micro quantitative analyses in woody stems. These investigations of amount and location of lignin in the wood, bark, cambium, cell wall, and cell cytoplasm have produced evidence, but no clear answers, as to where the lignin polymer is formed. The variation in quantitative results suggests that the determination of where lignin is formed may depend upon the lignin definition.

The definition of lignin need not be confined to its polymeric form. It is possible to expand this definition to include those monomeric, dimeric and oligomeric
phenylpropanoid precursors of lignin (lignols) as lignin. The inclusion of lignols into the lignin definition allows the determination of where lignin is formed to be associated more specifically to that point where the lignols first appear. It can be further inferred that the initial point of lignin formation is coincident with the appearance of the monolignols. However, these same monolignols may serve as precursors to other non-lignin phenylpropanoid compounds. Therefore, the first true lignin character is dimeric (dilignol) in the present defined nature of the chemical character of lignin, i.e., a guaiacyl-β-aryl ether in most conifers.

Dilignols have been located in the cambial region (36) and are considered to be formed from aromatic precursors originating from translocated sugars (78). This transformation appears to be a localized biosynthetic mechanism associated with the vitality and specialization of the tissue. It may be further suggested that other highly active tissues display similar localized biosynthetic mechanisms.

This investigation examines the hypothesis that the leaves of western red cedar (Thuja plicata Donn) may serve as a metabolic tissue important in the formation of dilignols. Such a hypothesis necessarily requires the
development of techniques capable of isolating dilignols for characterization. Verification of the dilignols' participation in the metabolism of the active tissue may be achieved through radioactive tracer studies. The anticipated low levels of incorporated radioactivity associated with these tracer studies will require new techniques for their measurement.
Definitions

Three general classes of compounds are considered in this dissertation; lignin, lignol and lignan. The close relationship in nomenclature between these compounds requires their definition prior to the ensuing discussion.

Lignin: Lignin has never been specifically defined. It may be generally defined as a widely occurring tri-dimensional biopolymer primarily composed of phenylpropanoid units. This biopolymer is essentially insoluble in neutral solvents and exists within and bonded to plant cell walls for the primary purpose of structural support. All reference to lignin in this dissertation will be to naturally occurring lignin. Synthetically produced lignin will be indicated as such.

Lignols: Lignol is a specific term introduced by Freudenberg (35) to describe the "trapped" intermediates in the enzymatic dehydrogenation of coniferyl alcohol to form synthetic lignin. Monomeric products from coniferyl alcohol are designated "monolignols", dimeric products "dilignols", trimeric products "trilignols", etc. The terms dilignol, trilignol...oligolignol as used in this dissertation will include those dimeric, trimeric...
oligomeric phenylpropanol derivatives, both synthetic and natural, which exhibit alkyl-aryl ether and/or carbon-carbon bonds between the propanol chain of one unit and the aromatic nucleus of the next unit. Such derivatives are not restricted to dehydrogenation products of coniferyl alcohol. These lignols, as Freudenberg's, are optically inactive.

Lignan: This term defines a natural dimeric compound which is obtained through joining together two phenylpropanoid units in a carbon-carbon bond between the middle (β-β') carbons of their propyl side chains. Such a combination is formed stereo-specifically as an optically active lignan which commonly has the 2-L, 3-D configuration.

The Status of Lignin

Since its first recognition by Payen in 1833, lignin has been the subject of extensive chemical and biochemical investigations. The net result of these investigations has been a clearer (if incomplete) understanding of lignin formation and structure, coupled with frustration in attempts to achieve significant economic exploitation of lignin based products. To those involved in the chemical conversion of forest products, lignin represents a non-utilizable waste product adding to ever enlarging pollution. This lignin comprises 22-34% of most woods and will occur as waste, in excess of 50 million tons, from the world's chemical pulping installations in 1970 (48). Harkin (48)
feels that the limits of accomplishment in lignin research, as regulated by present methods, has been approached. He suggests that the wood based industries must improve their lignin performance through the application of the knowledge gained in structural elucidation of lignin to date.

The Location of Lignin

Lignin is found in the fiber cell walls, fruits, stones, roots, bast, pith, and cork cells of the majority of members of the plant kingdom, except some lower forms of plant life (i.e., fungi). The differing basic chemical nature of lignin has enabled a further division as: gymnospermous, dicotyledonous and monocotyledonous lignins. This division is based upon the predominant character of aromatic substitution in the phenylpropane monomers polymerized to the respective lignin. The typical gymnospermous (coniferous) lignin contains guaiacylpropane (3-methoxy-4-hydroxyphenylpropane) monomers, while the dicotyledonous (hardwoods) lignin contain syringylpropane (3,5-dimethoxy-4-hydroxyphenylpropane) monomers and guaiacylpropane. The monocotyledons (annual plants and grasses) exhibit the 4-hydroxyphenylpropane monomers as well. Coniferous lignin has received the greatest amount of attention owing to its greater homogeneity and longer history as a residue of the chemical pulping industry.

Distributional studies of lignification in woody
stems of Japanese red pine (*Pinus densiflora* Sieb. et Zucc.) (12) have revealed that there is an increase in lignin content vertically in the tree while the lignin content in the wood and bark increases centripetally with distance from the cambial layer. Similar investigations in eucalyptus (*Eucalyptus regnans* T. Muell.) by Stewart *et al.* (91), including mono- and polysaccharide determinations, are in agreement. The polysaccharide results indicate that more than half of the cellulose and most of the hemicellulose is laid down in the secondary wall before the lignin is deposited.

The sequence of deposition was later substantiated in an ultraviolet microscopic study of lignification during xylarly differentiation in Monterey pine (*Pinus radiata* D. Don) (101). This study determined that lignin appears initially deposited in the primary wall near the cell corners during the formation of the $S_1$ layer. Subsequently, during the formation of the $S_2$ layer, lignin is laid down along the intercellular layer and then successively in the tangential and radial walls. These results indicate that the majority of lignin appears in the $S_2$ layer during or after formation of the $S_3$ layer while lignin is continuously deposited in the intercellular and outer layers of the cell wall. Recent work by Fergus *et al.* (25) has examined the distribution of lignin across the cell walls
of early- and latewood cells in black spruce (Picea mariana Mill.) using ultraviolet microscopy and densitometric analysis. Their results show that in the earlywood cell wall, 72% of the total volume of lignin is in the secondary wall compared to 28% occurring in the middle lamella. In the latewood, 82% was found to occur in the secondary wall while 18% occurred in the middle lamella.

The Isolation of Lignin

The chemical isolation of lignin involves either the removal of the carbohydrate with severe acid treatment leaving the insoluble lignin, or solution of the lignin in a protic solvent and its subsequent precipitation (73). Both of these methods produce lignin which may or may not resemble the original lignin. However, the polymeric nature of the lignin and its environment seriously restrict other alternatives of isolation.

Physical and biological methods of isolation are also available to separate lignin from the carbohydrate matrix. The physical methods employ the grinding of wood in a ball mill with or without nonswelling solvents and oxygen (10, 16, 17). Biological methods utilize brown-rotting fungi to digest plant polysaccharides leaving the lignin more accessible to solvent extraction (83). Isolated cellulases have also been used to give high yields of
lignin which has been slightly altered and will dissolve in polar organic solvents (77). All isolation techniques are limited by their propensity to produce lignin which may be only partially representative of natural lignin.

The Characterization of Lignin

The characterization of lignin as it is presently understood has evolved from degradative and synthetic investigations of lignols. The diverse nature of lignin has severely limited degradation techniques which have been successfully applied to other natural polymers (e.g., hydrolysis of proteins). Partial breakdown of lignin has been accomplished, and the nature of the degradation products formed is dependent upon the severity of the conditions used.

Mild oxidation techniques applied to lignin (alkaline nitrobenzene or the oxides of copper, mercury, or silver in air) have produced substantial yields of aromatic aldehydes (9, 75, 98). Three major aromatic aldehydes are obtained in these degradations: vanillin (3-methoxy-4-hydroxybenzaldehyde), syringaldehyde (3,5 dimethoxy-4-hydroxybenzaldehyde), and p-hydroxybenzaldehyde. Mild oxidation of coniferous bark lignin has produced the forementioned aldehydes plus protocatechualdehyde (3,4-dihydroxybenzaldehyde) (50). Vanillin contains the guaiacyl nucleus which
is predominant in the coniferous species, while both vanillin and syringaldehyde are found in dicotyledonous lignin. The monocotyledonous lignin contain mainly the p-hydroxybenzaldehyde nucleus. The dimer dehydrodivanillin (I) has been obtained as a minor product of this oxidation suggesting the existence of some aryl-aryl bonds in lignin. Stronger oxidative techniques, using permanganate on methylated wood (30, 59) yield a larger number of aliphatic and methoxyl-substituted aromatic acids from the oxidized lignin. Freudenberg et al. (37, 39, 40, 41) used the permanganate oxidation technique to obtain valuable lignin structural information from the radioactively labelled oxidation products of wood containing radioactively labelled lignin.

Reductive and solvolysis methods applied to lignin (79) (such as reduction by catalytic hydrogenation or solvolysis with alkali metals in liquid ammonia) yield derivatives of propylcyclohexanol or propylphenol. Acid catalyzed-ethanolysis of lignin produces Hibbert's ketones (58, 74), which indicate the occurrence of α and/or β-ether
linkages in lignin. The recent hydrogenolysis of the lignin of Hondo spruce \textit{[Picea jezoensis (Sieb. and Zucc.)]} (63) resulted in the isolation of a carbon-carbon bonded dimer from the hydrogenolysis products which was identified as 1-(3'-methoxy-4'-hydroxyphenyl)-2(2"-hydroxy-3"-methoxy-5"-n-propyl)-n-propane (II). Hydrogenolysis of dehydrodii-soeugenol (III) under the same conditions gave quantitative cleavage of the phenylcoumaran ring to yield II.

II

III

Nimz (68), has successfully degraded lignin using acetyl bromide or acetic anhydride with boron trifluoride and thioacetic acid with boron trifluoride (72) to liberate lignol fragments. These fragments were shown to undergo cleavage of the phenylcoumaran ring during isolation (68).

A trimeric lignol (IV) incorporating a phenylcoumaran structure was considered to occur in "spruce" lignin based upon the identification of \(\omega\)-hydroxyguaiacylacetone (V), the substituted phenylcoumarone (VI), and
the substituted stilbene (VII) in the reaction products of the acidolysis of Bjorkman "spruce" lignin and related acidolysis products from model compounds (1).

The products (VII and VI) were considered to arise from compound IV via a coumaran ring opening and loss of formaldehyde to form VII, and the reaction of the intermediate in a reverse Prinz reaction to form the isolated product VI. A comparison of the high yield of the ω-hydroxyguaiacylacetylacetone (V) with the yield of the compounds obtained
in the acidolysis of $\beta$-aryl ether model compounds was considered conclusive evidence in support of the existence of arylglycerol $\beta$-aryl ether bonds in lignin.

The desire to obtain lignol fragments from lignin has led to the application of mild hydrolysis techniques. Nimz (69) concluded that it was necessary to eliminate acidic and alkaline hydrolytic conditions, in order to prevent condensation reactions of the very reactive benzyl hydroxyl and benzyl ether groups of lignin. He percolated "spruce" wood in water at $100^\circ$C, and observed that 10% of the lignin went into solution. These results were in agreement with those of Freudenberg et al. (33), who observed that mild hydrolysis conditions would cleave benzyl aryl ether groups in "spruce" lignin. Nimz (69) considered the dissolved lignin to represent the exposed portion of lignin polymer which was probably bound to the "core" lignin by benzyl-aryl ether bonds. Monomeric, dimeric, and oligomeric phenolic degradation products were obtained and their structures elucidated (VIII to XV). Two dimeric dehydrogenation products of coniferyl alcohol (XVI) had been previously obtained in a mild methyl alcohol-hydrochloric acid hydrolysis of "spruce" lignin (31). These were $D,L$-pinoresinol (XIII) and dehydrodiconiferyl alcohol (XI). The trimeric (XIV) and tetrameric (XV) lignols, isolated and characterized by Nimz (69) had
glycerol β-aryl ether and glycerol β-alkyl-aryl linkages. Guaiacylglycerol (VIII) and the guaiacylglycerol-β-0-4'-coniferylalcohol (IX) occurred in racemic mixtures. Compounds X and XIV have also been isolated and identified in the dioxane:water hydrolysis of a Hondo spruce wood meal (81). Nimz (70) isolated and identified the trimeric (XIV) and tetrameric (XV) lignols in the water percolation products of "pine" wood.

The mild, acidic hydrolysis products have furnished convincing evidence on the structure of lignin. It is realized that these hydrolysis products represent the identification of only the low molecular weight peripheral groups of lignin. The core of the lignin should contain similar groupings whose hydrolyzable ether bonds are less accessible and consequently not cleaved. Substantiation of this lignin character can be found in the work of Forss et al. (28), who proposed a basic polymeric guaiacylpropane repeating unit for lignin. This basic unit was thought to be internally bound by alkyl-aryl ether and aryl-aryl, alkyl bonds and externally by alkyl-aryl ether bonds only. It was the external alkyl ether bonds which were selectively hydrolyzed under mild acidic conditions. Assurance of the reliability of these mild acidic hydrolysis results was strengthened by the characterization of similar products in studies of alkaline hydrolysis of lignin (100).
The Biosynthesis of Lignin

The structure of lignin has been clarified by simulated biosynthesis, and verified through chemical degradation (35). This approach is contrary to normal chemical characterization which would require the reverse. The unorthodox procedure results from the nature and environment of the lignin, and the difficulties it imposes upon orderly degradative analysis. These difficulties fostered the concept of "trapping" artificially biosynthesized lignin intermediates during the growth of the biopolymer under simulated natural conditions. However, such an approach required the prior clarification of aromatic biosynthesis and the isolation and identification of chemical and enzymatic intermediates necessary to the biosynthetic production of lignin.

The extensive use of radioactive tracers, enzymatic and chromatographic techniques have been the primary factors in the elucidation of the biosynthesis of aromatic compounds from atmospheric carbon dioxide. Two recent reviews (13, 35) examine the role of aromatic biosynthesis in lignin formation. These reviews and more recent work of specific interest are summarized below.

The conversion of carbon dioxide to carbohydrate via photosynthesis is well understood, and is presented
in a text examining plant biochemistry (8). The carbohydrate precursors known to initiate biosynthetic formation of aromatic compounds originate from carbohydrate metabolism (EMP pathway or pentose phosphate shunt) or photosynthesized CO₂. The conversion of the carbohydrate precursors (phosphoenol-pyruvate and D-erythrose-4-phosphate) to the aromatic amino acids phenylalanine and tyrosine is also well understood. This pathway (Figure 1) was elucidated in nutritional studies with the bacteria E. coli and has been reviewed (88). Chorismic acid was the last intermediate to be identified (42, 43) and serves as the branch point to the formation of prephenic acid or anthranilic acid in the aromatization sequence. Although this pathway was determined in bacteria, subsequent studies (3, 4, 5) have shown the pathway to be applicable to higher plants as well.

Brown and Neish (14, 15) noted that phenylalanine and, to a limited extent tyrosine, were good precursors to lignin in numerous plants indicating the conversion of shikimic acid-derived aromatic amino acids into lignin. In 1961, Koukol and Conn (55) were able to isolate and characterize the enzyme, phenylalanine ammonia-lyase, which catalyzed the deamination of phenylalanine to cinnamic acid. The concurrent isolation of tyrosine ammonia-lyase in grass (66) established the similar deamination of
tyrosine to p-hydroxycinnamic acid. With the establishment of the transformation of the aromatic amino acid to cinnamic acid derivative, it became necessary to prove that these cinnamic acid derivatives could be further metabolized to eventually form lignin. Smith and Neish (87) claimed to
demonstrate the irreversibility of cinnamic acid formation when they showed that all of the carbon atoms of labelled cinnamic acid were incorporated into "spruce" and "aspen" twig lignin rather than being incorporated into cinnamic acid precursors. In a tabulation of tracer experiments utilizing cinnamic, p-coumaric, caffeic, ferulic, and sinapic acids by Neish (35), it was demonstrated that all of these cinnamic acid derivatives were good precursors to lignin, with sinapic acid being an effective precursor in those species (angiosperms) with syringyl lignin. Verification of the cinnamic acid metabolism route to lignin utilized these ring substituted cinnamic acid derivatives, yet the mechanism of ring hydroxylation and/or methoxylation had only been inferred because of the limited data available regarding hydroxylation and methylation.

Although it had been demonstrated in 1954 (18) that methionine serves as a methyl group donor in lignin formation, the origin of the required hydroxylated substrate and methylating enzyme were not determined until later. In 1965, cinnamic acid hydroxylase was discovered in spinach acetone powders (64) which was capable of forming p-hydroxy-cinnamic acid from cinnamic acid. Recently (99), a purified phenolase from spinach leaf has been shown to catalyze the production of caffeic acid from p-coumaric acid. Methylating enzymes have now been found which are capable of
methylating caffeic acid in the presence of S-adenosylmethionine in grasses and woody shrubs (26, 27). Higuchi, et al. (51) have also described an enzyme preparation (S-adenosylmethionine:catechol-0-methyltransferase) which selectively methylated the meta-hydroxyl of caffeic and 5-hydroxyferulic acids in bamboo shoots. Little evidence is available regarding further hydroxylation and methylation of the guaiacyl nucleus leading to the formation of the syringyl nucleus. It is evident, however, that metabolic sequences lead to the substituted cinnamic acids previously considered as good precursors to lignin. Such a sequence may be considered as: phenylalanine → cinnamic acid → p-coumaric acid → caffeic acid → ferulic acid → sinapic acid.

Coniferin (4-O-coniferyl alcohol β-D-glucoside) has been shown to be an excellent precursor to lignin in conifers (38) suggesting reduction of a cinnamic acid carboxyl function prior to lignification. Experiments in tobacco leaves (89) have established the biosynthetic pathway to the formation of the quinic acid ester of caffeic acid (chlorogenic acid) from cinnamic acid, concluding as well that the chlorogenic acid does not take part in polyphenol or lignin biosynthesis. El-Basyouni and co-workers (23, 24) demonstrated the occurrence of metabolically active intermediates (acetone and alcohol insoluble), derived
from phenylalanine, which were readily incorporated into the lignin of wheat and barley plants. These intermediates were better lignin precursors than soluble analogs and produced hydroxycinnamic acids upon hydrolysis. Bland and Logan (11) found, in lignification studies on *Eucalyptus* spp. shoots, that hydroxylated cinnamic acids were converted to glucose esters prior to their incorporation into lignin. Higuchi and Brown (49), studying wheat plant lignification, concluded that coniferin was not an obligatory precursor to lignin formation, based upon the observations that: (a) coniferyl alcohol could dilute the lignification intermediate formed from ferulic acid, and (b) that coniferaldehyde and coniferyl alcohol were obtained in ferulic acid-\(^{14}C\) feedings rather than coniferin. These data suggest the importance of esterified cinnamic acid intermediates in lignin formation. In summary, the biosynthetic pathway to lignin from \(\text{CO}_2\) may be considered to be: \(\text{CO}_2\) \text{photosynthesis} -> carbohydrates -> shikimic acid pathway -> aromatic amino acids' deamination, hydroxylation, methylation -> substituted cinnamic acids -> esterification -> insoluble esters of cinnamic acid -> reduction -> alkyl ethers of cinnamic alcohols -> polymerization -> lignin.

The concluding step in the above scheme has been the basis for formation studies on artificial lignin. This
concept of determining lignin structure is now almost synonymous with Freudenberg and the Heidelberg school of lignin research. Freudenberg, after Klason, used coniferyl alcohol as a substrate in conjunction with an enzyme preparation from a common field mushroom *Psalliota campestris* to produce, at neutral pH, a polymerized substance which closely resembled lignin (34). In the last 20 years of work on this enzymatically produced polymer, intermediates of the condensation reactions have been isolated and identified thereby revealing the mechanism of lignification and the structure of this lignin. Freudenberg's most recent comprehensive review of his work and related work traces this evolution (35) which culminated with schematic formulae for lignin based on the compiled results.

The process of polymerization of p-hydroxycinnamyl alcohols to lignin is now considered to proceed via the phenoxide forms which give metastable free radicals with four principal mesomers (Figure 2). Harkin (48) has classified the reaction with which these free radicals combine to form stabilized forms by the following five mechanisms at least:

1. Molecular growth by free-radical pairing to form unstable quinone methides.

2. Intramolecular rearrangements of some quinone methides to form phenoxides (phenols) that may undergo renewed oxidation to free radicals.
3. Stabilization of some quinone methides by addition of electrophiles (plus protons) to reform phenoxides (phenols) also capable of further oxidation.

4. Incidental side chain oxidations due to free-radical transfers and subsequent disproportionations.

5. Stabilization of some quinone methides by side-chain elimination, a type of disproportionation.

Harkin (48) illustrates the formation of "lignin" products via the five processes, through combinations of radicals (Figure 2). Process 1 (e.g. $R_a + R_b$, $R_b + R_c$, $R_b + R_c$, $R_c + R_c$) followed by Process 2 will produce the dilignol structures such as guaiacylglycerol-β-O-4'-coniferyl alcohol (IX) and pinoresinol (XIII).
Process 3 introduces water to form benzyl alcohols and probably is involved in the covalent lignin-carbohydrate bond by condensation with carbohydrates. This process also may result in the branching of lignin by the formation of noncyclic benzyl-aryl ethers after condensation with unoxidized phenoxides. Process 4 gives rise to carbonyl functions in alkyl side chains, while Process 5 leads to structures such as diarylpropane diols, diphenyl ethers, esters, dioxepins, and arylglyceric acid or arylglyceraldehyde ethers. Therefore, most of the isolated components of the synthetic lignin, and the degradation products of lignin, can be explained in terms of free radical combinations and subsequent rearrangements.

Recently, Connors et al. (19) formed three dilignols from the dimerization of coniferaldehyde in the presence of peroxidase enzyme and hydrogen peroxide in
aqueous solution. The products were 2,3-diformyl-1,4-di-5-guaia
cylbuta-1,3-diene (XVII), α-(4-β-formylvinyl-2-methoxyphenoxy)
coniferylaldehyde (XVIII), α-(5-β-formylvinyl-2-hydroxy-3-methoxy-
phenyl) coniferylaldehyde (XIX).

These dimers can be explained via the free radicals of
Figure 2. The aldehydic side chains of two coniferyl-
aldehydes, comprising the α, β-enone system rearranges
at the quinone methide stage to form a stable phenol,
which through the loss of the acidic proton from the car-
bon α to the carbonyl, subsequently rearomatizes with the
final protonation of the phenoxy anion to yield the
dimeric products. At this point it is important to con-
sider what free precursors (monomeric, dimeric, etc.) have
been isolated unchanged from lignifying systems to further substantiate the conclusions drawn from degradative and biosynthetic results.

Aromatic Constituents Related to Lignin

Several investigations of actively metabolizing plant tissues have considered aromatic constituents. The cambial sap of "spruce", deactivated with formaldehyde, has been shown to contain the β-D-glucosides of 4-O-sinapyl and 4-O-p-coumaryl alcohol (32). Coniferin, various sugars, small amounts of coniferyl alcohol, quinic acid, and unknown substances of high molecular weight which gave yellow or orange-yellow color reactions with diazotized sulfanilic acid, have also been shown to be present in deactivated "spruce" sap (36). The "spruce" sap, without formaldehyde treatment, was also shown to contain: shikimic acid, protocatechuic acid, D,L-pinoresinol (XIII), guaiacylglycerol β-coniferyl ether (IX), and dehydrodi­

\[ \text{IX} \]

\[ \text{XI} \]

\[ \text{XIII} \]
In an examination of the cambium and sapwood of western hemlock (Tsuga heterophylla (Raf.) Sarg.) for lignin precursors, Goldschmid and Hergert (46) found many compounds including several suspected lignan glycosides whose structures were not elucidated. Among the compounds detected in the sapwood were: conidendrin (XX), hydroxymatairesinol (XXI), oxomatairesinol (XXII), pinoresinol (XIII), dehydrodiconiferyl alcohol (IX), and several suspected lignan glycosides.

XX

XXI

XXII

Barton (6) has very recently studied western hemlock sapwood and reported the occurrence of liovil (XXIII) and a novel dilignol 2-(α-hydroxy-vanillyl)-5-ω-hydroxypropyl)-7-methoxy coumaran (XXIV). This compound (XXIV) exhibits a unique β-γ-linkage, and was also thought to occur as an
Studies on leaf tissue have mainly been concerned with carbohydrate content. However, in a study of low molecular weight aromatic compounds in the leaves of Scots pine \((\text{Pinus sylvestris L.})\) (\text{97}), two glycosides of guaiacylglycerol were identified. The two glycosides were \(\alpha\)-threo-\(\beta\)-\(D\)-glucoguaiacylglycerol (XXV) and \(\beta\)-threo-\(\beta\)-\(D\)-glucoguaiacylglycerol (XXVI).
In a study of the extractives of the leaves of tamarack (*Larix laricina* (Du Roi) K. Koch), Niemann (67) found the β-glucosides of vanillic and p-coumaric acids and the α-glucoside of p-hydroxybenzoic acid. Takahashi et al. (96) have examined the polyphenolics in the leaves of one hundred conifers noting primarily the occurrence of flavonoids. A recent incorporation study (80) using the leaves of Douglas fir (*Pseudotsuga menzesii* (Mirb.) Franco) showed incorporation of the T-glucovanillin into lignin whereas T-ferulic acid was not incorporated. A recent examination (52) of phenylalanine ammonia-lyase (PAL) activity in eucalyptus (*Eucalyptus* *sieberti* L. Johnson and *E. sideroxylon* A. Cunn et Wools) leaves indicated that glucose may serve as a better precursor than phenylalanine in the formation of polyphenols such as the stilbenes and flavanoids. It was speculated that PAL activity may be more directly related to lignification than polyphenol biosynthesis. When phenylalanine was fed to the leaves, p-coumarylquinic acid and chlorogenic acid were the first metabolic products to be formed while glucose feeding produced catechin and the glycosides of stilbenes and flavonoids first.

The formation of the lignans is another important dimerization reaction of monomeric phenylpropane
precursors. No specific evidence is available regarding the biosynthesis of lignans. Neish (65) has postulated that the lignans are probably formed through a reductive coupling of cinnamyl alcohols through the β carbon atoms of the propylene side chain. Such a coupling is suggested to be specifically enzyme controlled, thereby producing the common 2-L, 3-D configuration of the lignans. The specific configuration and the lack of triphenylpropeynoid lignans, precludes the quinone methide pathway since the polymerization of coniferyl alcohol via free radical reactions, gives optically inactive products. The route of formation of lignans and lignin, however, may be closely related biosynthetically since they both arise from cinnamyl alcohol coupling reactions.

AROMATICs OF WESTERN RED CEDAR

The study of lignans and tropolones has been extensive in western red cedar (Thuja plicata Donn). Characterization of these components has played a primary role in the utilization of the species (62). The tropolones (2-hydroxy-2,4,6-cycloheptatrien-1-ones) are non-benzenoid aromatics which are steam volatile. Those which have been isolated and characterized from western red cedar wood, according to Barton and MacDonald (7), are: α-thujaplicin (XXVII), β-thujaplicin (XXVIII), γ-thujaplicin (XXIX), β-thujaplicinol (XXX), β-dolabrin (XXXI), together with
similar compounds such as nezukone (XXXII), methyl thujate (XXXIV), and thujic acid (XXXIII). The lignans which have been isolated and characterized in western red cedar are either derivatives of β,γ-dibenzylbutane: thujaplicatin (XXXVa), dihydroxythujaplicatin (XXXVb), thujaplicatin methyl ether (XXXVIa), hydroxythujaplicatin methyl ether (XXVIb), dihydroxythujaplicatin methyl ether (XXVIc), or of tetrahydronaphthalene: plicatic acid (XXXVII), plicatin (XXXVIII), plicatinapthol (XXIX), and plicatinapthalene (XL).

The large number of lignans in western red cedar has generated interest into their biosynthesis and site of formation. In a chromatographic study of the lignans of western red cedar related to the sapwood-heartwood transformation, Swan et al. (95) concluded that the major portion of the lignans (in western red cedar) are formed in situ at the sapwood-heartwood boundary. The transformation through hydroxylation was considered to continue into the heartwood for many years by the route: thujaplicatin → dihydroxythujaplicatin → plicatin → plicatic acid. In a later addition to this proposal, Swan and Jiang (94) considered that plicatinapthol was derived from plicatin through dehydration and hydroxylation with the transformation extending well into the newly formed heartwood. Hydroxylation of the tropone nezukone to form the thujaplicins
was observed to occur wholly at the sapwood-heartwood boundary. Mono- and dihydroxylated thujaplicatin methyl ether derivatives were considered to be formed in a manner similar to the formation of plicatin. No recognized intermediates which could define the transformation of phenylpropane to a lignan have been isolated. Swan (95) suggests that the search for such unknown intermediates must extend to the sapwood and cambial areas by utilizing in vitro or in vivo radioactive tracer experiments.

Metabolism and Lignin Formation

This discussion would be incomplete without an examination of the total consecutive utilization of nutrients leading to lignin formation in the living plant. Coniferous leaf photosynthesis studies using $^{14}$CO$_2$ have shown radioactivity incorporation into sucrose, raffinose, D-glucose, and D-fructose with the majority of activity occurring in sucrose (85, 86). A recent study of photoassimilation of $^{14}$CO$_2$ in the branches of red pine (Pinus resinosa Ait) (78) revealed the distribution of photosynthate predominantly up the main stem to the apical growth region. The radioactivity assimilating from the lower worlds moved down the stem and could be detected in the roots. Autoradiography of internodal sections revealed the concentration of lignin precursors (shikimic acid, quinic acid) in newly differentiating tissue.
Preferential labeling of lignin over cellulose, suggested that lignin biosynthesis competed successfully for new photosynthate during growth. The data indicates the translocation of carbohydrates (particularly sucrose) from the leaves to the cambial tissues for further metabolism. Stewart (90), in a summary of evidence relating to the sequential formation of secondary tissues in trees, notes that the cambium converts disaccharides and oligosaccharides to monosaccharides for further metabolism by the differentiating cell. The bulk of the available converted carbohydrate is incorporated into the primary wall in the biosynthesis of polysaccharides controlled by the protoplast. The shikimic acid pathway is probably initiated at this stage.

During the cytoplasmic stages of polysaccharide synthesis, the aromatic amino acids produced by the shikimic acid pathway, participate in aminotransferase and aminohydrolase reactions to produce the hydroxyaromatic acids. These in turn are converted to esterified precursors of the polyphenols and lignin, as previously described. These esterified precursors may then be excreted into the cell vacuole, excreted into the translocatory systems of the rays, or incorporated into the cell wall. In the last instance, the glycosides are hydrolyzed, and converted to lignin through the action of an oxidoreductase. As the
fiber enters senescence, the concentration of carbohydrates decreases causing a breakdown of the cytoplasmic membrane, and a consequent blending of vacuole components with those of the general cytoplasm. During this stage much of the phenolic excretory constituents diffuse through the cell wall. It is this change in biological activity associated with senescence which is probably responsible for more than 50% of the lignin being deposited after most of the cellulose has been laid down.

**Literature Summary and Observations**

It is apparent from the literature that the process of lignin formation has been extensively studied from synthetic, biosynthetic, and degradative viewpoints. The composite view of lignin formation involves a carbohydrate to aromatic monomer to polymer relationship which corresponds to a leaf to cambium to cell locational sequence in woody stems. The nature of intermediates at all early stages allows their efficient transport and eventual transformation to lignin.

Most studies of lignin formation in woody stems have been concerned with cambial or sapwood regions with several important monomeric intermediates located and characterized. Considerably fewer free dilignols to oligolignols have been located or characterized in all
plant systems, although dilignols have been shown as effective precursors to lignin formation in synthetic studies.

The leaf-branch relationship provides a promising area for the location of stable intermediates related to the biosynthesis of polyaromatic compounds. The study of actively metabolizing leaf tissues offers the added advantage of ease of experimental manipulation in radioactive and enzymatic studies in comparison to woody tissues. Development of analytical techniques capable of accelerated analysis of test results are necessary, in order to obtain experimental data consistent with the metabolic capability of the tissue. It is remarkable that so little research has been done on the aromatic constituents of coniferous plant leaves. The leaves of western red cedar offer particular potential for lignin studies, since the species contains a higher than average amount of both lignin and lignans.
MATERIALS AND METHODS

Collection of Leaves

Leaf samples were obtained from low level branches of five or more randomly selected western red cedar (Thuja plicata Donn) trees on the University of British Columbia campus. Collections were made in January 1969, April 1969, June 1969, October 1969, and June 1970. The first three collection dates provided samples for the development of isolation techniques. The third collection was used for feeding experiments and the isolation of glycosides. The final collection served to repeat the isolation procedures and to develop improvements. The leaves were cut from the trees taking care to avoid branch material, cones or flowers. The samples were immediately placed in a plastic bag, sealed, and transported to the laboratory.

Leaf Extraction

Following the removal of leaf samples for \(^{14}\)C feeding experiments and moisture determination, the remaining leaves were chopped in a large Waring blender without solvent. The chopped leaves were weighed and placed in a large Soxhlet extractor for extraction with methyl alcohol over a 72 hour period. A single extraction was decided
upon because the high percentage of moisture in the leaves prevented true sequential extraction in a pilot attempt.

The methyl alcohol extract which was obtained was worked up according to Figure 3. The ethyl acetate extract obtained contained leaf phenolic glycosides without interfering chlorophylls.

**Chromatography**

**Thin layer cellulose chromatography**

Thin layer cellulose plates were prepared using 25 grams of Avicel microcrystalline cellulose (FMC Corp. Div. of American Viscose Co.), in 85 ml of water followed by homogenization of fifteen seconds in a Waring blender. The resultant slurry (thickness 0.25 mm) was applied to five 8" x 8" glass plates in a Shandon thin layer applicator. The plates were allowed to air-dry overnight.

Four solvent systems were used in thin layer cellulose chromatography: (1) n-butanol: chloroform: acetic acid: water (4:1:1:1) (BCAW); (2) lower layer chloroform: acetic acid: water (2:3:1.5) (CAW); (3) 2% aqueous acetic acid (2%); and (4) isopropanol:ammonia:water (20:1:2) (IAW). The first two solvents were considered as the reference system for the study. Thin layer cellulose chromatography of sugar residues used ethyl acetate: pyridine: water (12:5:4) as the developing solvent.
Western Red Cedar Leaves
- Waring blender
  Chopped leaves

Leaf samples for $^{14}$C feeding study
- Extraction with methyl alcohol
  72 hours

Discard leaves

Methyl alcohol solubles
- Filter through celite

Methyl alcohol filtrate
1) Evaporate to dryness
2) Wash with 1 liter chloroform

Chloroform insolubles
1) Add 1.5 liters and celite to form thick slurry
2) Filter through celite

Chloroform washings-discarded

Water insolubles-discarded

Water solubles
1) Concentrate on evaporator
2) Extract with ethyl ether

Ethyl ether solubles
0.8% - not analyzed

Water solubles

Extract with ethyl acetate

Ethyl acetate solubles 3.9%

Figure 3. Scheme for the extraction and separation of components from western red cedar leaves.
Detection of the compounds on the cellulose plates primarily utilized diazotized sulfanilic acid. Other sprays, including diazotized p-nitroaniline and Barton's reagent \((\text{FeCl}_3, K_3 \text{Fe(CN)}_6)\) were used to detect phenolics, while sugar residues were located with p-anisidine hydrochloride.

**Thin layer silica gel chromatography**

Silica gel plates were prepared using a slurry of 35 g of Merck Silica Gel G in 70 ml of water. This slurry was applied at a thickness of 0.25 mm or 0.50 mm (for preparative chromatography) on five 8" x 8" glass plates using a Shandon applicator. The plates were then placed in a 130°C oven for 1¾ hours for activation. Plates not used immediately were stored in a 70°C oven to prevent deactivation.

Two solvents were used for thin layer silica plates: (1) chloroform:methyl alcohol (4:1) (CM), and (2) benzene:ethyl alcohol (9:1) (BE). A spray of concentrated sulfuric acid, concentrated nitric acid (1:1) followed by heating was used to detect compounds on the plates. Alternatively, diazotized sulfanilic acid was also used as a detection reagent.

**Column chromatography**

Column chromatography was the most important means of isolating the phenolic glycosides in this investigation.
Silicic acid (Fisher) and Sephadex LH-20 (Pharmacia) were the two major chromatographic media used. The selection of LH-20 as a gel-filtration medium resulted from prior chromatographic experiments (79) and with pilot studies on the ethyl acetate extract. Earlier trials, using Sephadex G-10 and G-25, provided excellent results in the separation of the then unknown dilignol glycosides from other mono- and polyphenolic glycosides. These gels were eluted with water or water-methanol and the desired glycosides were reclaimed by ethyl acetate extraction. Sephadex LH-20 was used with comparable results using organic solvent elutions. The solvent system which proved the most effective on LH-20 was chloroform: ethyl alcohol (4:1) (CE). Cellulose column chromatography was also examined and although it provided separation of the glycosides, the separation was much less definitive and again required ethyl acetate extraction of the water eluant.

Deactivated silicic acid was used as the other chromatographic medium with either CM or BE as eluants. These solvents are coincident with those used on silica gel plates and the separation seen on the plates was generally reproducible on the silicic acid columns. This medium was used primarily in the final stages of separation of the phenolic glycosides.
In an effort to mitigate the often long and laborious task of column chromatography, newer methods of pressure chromatography (47) and ultraviolet monitoring were used. The schematic diagram (Figure 4) depicts the chromatographic apparatus used in the investigation.

![Schematic diagram of pressure column chromatography system]

Figure 4. A schematic representation of the pressure column chromatography system.

The pump was a Milton-Roy Model 196-47 capable of delivering 240 ml/hr at a discharge pressure of 1000 psi. This pump was used to apply pressure flow to the chromatographic columns at 50 to 100 psi. The ultraviolet recording meter was a Gilson Model UV 280 IF coupled to a Leeds Northrup strip recorder. Column No. 1 had an LH-20
bed dimension of 76 cm x 2.5 cm with CE as the eluant. Column No. 2 had a silicic acid packing (60 cm x 1 cm) and either CM or BE were used as eluants. Flow rates were adjustable in the LH-20 column for 0-4 ml/min, while the silicic acid had a maximum flow rate of 1 ml/min due to pressure limitations of its connection. These flow rates represent as much as 10 times that available with gravity feed on the same columns.

**Derivative Preparations**

**Acetylation**

Acetic anhydride: pyridine (1:1) was used to acetylate free hydroxyl groups. The acetylation mixture and compound were allowed to stand overnight at room temperature and the following morning they were warmed on a steam bath (100°C) for 1 hour. The excess reagent was removed from the mixture under vacuum.

**O-methylation**

Methylation of phenolic hydroxyl groups utilized excess diazomethane in methyl alcohol. The compound to be methylated was dissolved in minimal methyl alcohol, cooled to about -10°C for 1 hour after which cold diazomethane was added. The mixture was then returned to -10°C overnight. The following morning the residual methylating agents were removed under vacuum.
Dimethyl sulfate-potassium carbonate was used in conjunction with methyl iodide-silver oxide in an attempt to fully methylate one of the isolated glycosides. Dimethyl sulfate (50% excess) and potassium carbonate (50% excess) were added in portions to an anhydrous acetone solution of the glycoside. This solution was refluxed under nitrogen for 5 hours with the exclusion of moist air. The resultant mixture was cooled and filtered. The inorganic salts were washed thrice with acetone. The acetone solution was evaporated to dryness in a rotary evaporator and redissolved in dimethylformamide. Methyl iodide (3 equivalents per OH group) and silver oxide (2 equivalents per OH group) were added at room temperature and the mixture left overnight. The following morning water was added to the mixture and the methylated derivative was extracted from the aqueous solution with chloroform. Stronger methylation conditions were required to complete the total methylation procedure. These conditions utilized dimethyl sulfate-sodium hydroxide in a procedure identical to that mentioned above without the subsequent use of methyl iodide/silver oxide.

**Hydrogenolysis**

It was necessary to catalytically hydrogenate the benzyl alcohol group of one of the isolated glycosides.
This hydrogenolysis was done with a 20 mg sample in approximately 5 ml of ethyl alcohol in a test tube to which 50-70 mg of catalyst [(PdCl$_4$-BaSO$_4$) Adler and Marton (2)] was added. The test tube was then placed in a Parr low pressure hydrogenation apparatus. The sample was first evacuated and then hydrogen was introduced to a pressure of 40 psi. The mixture was shaken for 15 min and the sample removed and filtered.

Degradative Techniques

Alkaline nitrobenzene oxidation

Alkaline nitrobenzene oxidation was used to determine the basic aromatic nuclei and the manner of linkage of phenylpropane units in the major occurring glycoside. The procedure of Stone and Blundell (92) with the improvements of Pepper and Siddequeullah (76) was used. A 10 mg sample was oxidized 2.5 hours at 160°C in the presence of nitrobenzene and sodium hydroxide. The resultant mixture was extracted with ether, acidified with hydrochloric acid and extracted with ether to obtain the aromatic aldehydes.

Qualitative thin layer cellulose was run on the final products using CAW. The compounds were located on the thin layer cellulose chromatography plates using diazotized sulfanilic acid (DSA).
Ethanolysis

Ethanolysis of Compound A or fully methylated Compound A was run on a 10 mg sample contained in one ml of 2% hydrochloric acid-ethyl alcohol. The sample tube was sealed under nitrogen and heated for one hour at 100°C. The ethanolysis mixture was cooled and neutralized with sodium bicarbonate and run on thin layer cellulose in n-butanol saturated with aqueous ammonia or on thin layer silica gel (BE) for the methylated derivative. Comparative standard compounds were run on the chromatogram to determine what fragments were formed in the reaction.

Periodate oxidation

Periodate oxidation was applied to a methylated derivative (aromatic hydroxyls) of one of the compounds isolated from the ethyl acetate extract of the western red cedar leaves. This oxidation was run according to the procedure of Dyer (21). The methylated derivative (39.7 mg) was dissolved in 1 ml of ethyl alcohol, and water was added to a total volume of 5 ml. This solution was added to 10 ml of 0.0693 M periodate solution and the solution was brought to 25 ml and stored at 0°C. A 5 ml aliquot was withdrawn after 1 and 2 hours. Sodium bicarbonate, excess sodium arsenite and starch indicator were added and the solution was titrated with 0.0534 M iodine solution.
to the starch end point. Determination of molar uptake was based on a prior determination of iodine required to titrate the arsenite available in solution.

**Hydrolysis**

Hydrolysis of the non-methylated glycosides was attempted with 2% aqueous oxalic acid. The oxalic acid solution was added to an aqueous solution of the glycoside and the mixture refluxed for 2 hours. Following the reflux time, the mixture was cooled and thrice extracted with chloroform. The chloroform extract was evaporated to dryness and chromatographed to determine the degree of hydrolysis. Several of the compounds were difficult to hydrolyze and required re-hydrolysis or hydrolysis under conditions used for the methylated derivatives.

Hydrolysis of the methylated derivatives was run in 6% hydrochloric acid in methanol. The hydrolysis solution was added to the glycoside and refluxed for 2 hours. The cooled solution was then extracted with chloroform: water and the chloroform solubles were analyzed chromatographically for the aglycone, while the water solubles were deionized with a strong quaternary amine exchange resin and chromatographically compared to standard sugars.

**Liquid Scintillation Counting of Low Activity Chromatographic Samples**

A new method for determining levels of radioactivity
of chromatographically separated compounds was developed in this study. Because the method is a new technique, it was necessary to test its validity and to compare it to present methods which it would potentially replace. The proven technique could then be applied in a pilot feeding study in the leaves of western red cedar.

Table 1 denotes the sample preparations used in validation of the method. The samples for the experiment were taken from two cellulose plates of a five plate preparation as previously described. On these plates, 1 cm diameter circles were inscribed through the cellulose layer to the glass. The radioactive samples were prepared by applying 10 μl of U\(^{14}\)C-L-phenylalanine solution to the 1 cm circles (five replicates). Other non-radioactive compounds and chromogenic sprays were then applied to the samples. Those samples which were to be combusted were lifted from the plates by applying a 3% w/v solution of cellulose nitrate (Parlodion-Mallinkrodt) in ethyl alcohol: ether (1:1) dropwise to just cover each circle. The cellulose nitrate solution also contained 3 ml/l black marking ink. As the circles dried, they could be lifted cleanly from the glass without leaving a residue. The samples were then mounted on wire stands and placed in scintillation vials ready for combustion.
### Table 1

Sample preparation and results of liquid scintillation counting comparison of scraping and combustion methods applied to samples from thin layer cellulose plates.

<table>
<thead>
<tr>
<th>Compound added</th>
<th>Amount added (µg)</th>
<th>Spray</th>
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Just prior to combustion, 1 ml of chilled CO$_2$ absorbant [ethanolamine: ethylene glycol monomethyl ether (1:2)] was placed in the bottom of each vial. Stopcock grease was spread thinly about the lip of the vial and the vial was then flushed with oxygen for 5 seconds. The cap was quickly tightened on the vial and the sample was combusted using an external focused infrared light. Immediately after combustion the sample was shaken and cooled at $-10^\circ$C for 10 minutes. Thirteen ml of scintillation cocktail [5.5 g PPO in toluene: Cellosolve (ethylene glycol monomethyl-ether) (3:1)] was then added and the sample was ready for scintillation counting.

Those samples requiring scraping were removed from the plate as cleanly as possible using a scalpel. The scraped sample was placed in a scintillation vial to which 14 ml of Liquifluor (New England Nuclear Corp.) was added. The vials were shaken vigorously prior to counting. All samples were counted (2 x 5 min) in a Packard 1200 scintillation counter with a calibrated external standard. The results of this experiment are included in Table 1. A separate experiment was run to determine the limits of spot size upon combustion efficiency.
Leaf $^{14}$C Feeding

A portion of the leaves collected in the field were immediately prepared for a $^{14}$C feeding experiment. The purpose of this experiment was twofold: (1) to further substantiate the applicability of the newly developed combustion technique and (2) to determine the relative anabolic activity of isolated dilignols as a preliminary step to kinetic studies in the leaves of western red cedar.

The collected leaves were cut with scissors into pieces averaging about $\frac{1}{8}$ inch in length. These cut leaves were carefully inspected to eliminate woody material. A portion of the leaves available for the feeding experiment were immediately weighed and placed in a $105^\circ$C oven for 18 hours to determine oven-dry weight. The remaining leaves were weighed into four 2 g portions and placed in four petri dishes containing approximately 2 $\mu$g/ml of $^{14}$C-L-phenylalanine in 6 ml of sterilized water.

Three 10 $\mu$l samples of the feeding solution were withdrawn from each petri dish immediately after introduction of the samples. These samples were placed in counting vials containing Liquifluor (New England Nuclear Corp.) and were subjected to scintillation counting to determine the amount of labeled phenylalanine available to the leaves at the beginning of the feeding period. The dishes were then
covered and illuminated by two 250 watt incandescent bulbs at a distance of approximately 3 feet. The four dishes represented feeding times of 1, 3, 5 and 10 hours. At the end of each time period, 3 more 10 μl samples were withdrawn from the individual feeding solutions and counted to determine the percent uptake of the labelled solution with time. Upon completion of each feeding the leaves were filtered from the labelled solution and washed with distilled water. The washed leaves were then placed in individual micro-soxhlets for extraction with methyl alcohol. Extraction and workup according to the scheme depicted in Figure 3 yielded a gross ethyl acetate extract.

The ethyl acetate extract was taken to dryness under vacuum and transferred in ethyl alcohol to four 2 ml volumetric flasks. Based upon earlier chromatographic evidence, it was assumed at this point that the compounds of interest would be present in the ethyl acetate extract. Three 60 μl replicates of each feeding solution (representing the four time periods) were applied to separate thin layer cellulose plates and developed two dimensionally with BCAW in the first direction followed by air-drying and development in the second direction with CAW. The plates were then dried and sprayed with diazotized sulfanilic acid. The compounds designated as A and B on the schematic chromatogram (Figure 5)
were then stripped from the plate and counted according to the method previously described. Chromatographic spots with irregular shape and size required careful subdivision with a razor blade following removal from the plate surface. This subdivision insured total combustion of the spots, with the count rates of the individual sections being summed to obtain the representative count rate for each spot. The absorbed radioactivity was counted using a Packard 1200 scintillation counter and the observed count rates were related to the original $^{14}$C uptake to determine per cent incorporation in each compound related to feeding time.

Spectral Techniques

**Ultraviolet and infrared**

Ultraviolet spectra were obtained for column chromatographically purified samples or methyl alcohol eluants of spots scraped from cellulose plates. The spectra were run in methyl alcohol in a Beckman DK-2 recording spectrophotometer using appropriate blanks. Spectral shifts of the samples were determined in the presence of 0.1 N sodium methoxide. Ultraviolet analysis was also used in the determination of phenolic hydroxyl according to the method of Goldschmid (45). The method involves the determination of difference spectra obtained for the shift of the phenoxide
Figure 5: A schematic chromatogram of the clarified ethyl acetate extract from western red cedar leaves. (Spray -DSA).
ion formed with the addition of 0.1 N sodium methoxide. The extent of change of maxima was compared to a eugenol standard to determine free phenolic hydroxyl content. Infrared spectra were obtained from samples in potassium bromide pellets on a Perkin-Elmer 521 infrared spectrophotometer.

**Nuclear magnetic resonance**

All nuclear magnetic resonance (NMR) spectra were obtained on a Varian HA-100, 100 mHz NMR spectrophotometer. The samples were prepared in deuterochloroform or deuterated acetone. Tetramethylsilane was added as an internal standard and lock signal, at τ = 10. Normal sample dilution was approximately 10 mg/300 μl of solvent. For samples of less than 5 mg a micro-cell was used with the dilution of 1-5 mg/30 μl of solvent. Nuclear magnetic double resonance (NMDR) experiments were applied to derivatives to determine coupled signals.

**Mass spectroscopy**

Mass spectra were obtained for purified acetate derivatives of dilignol rhamnosides. These spectra were run by Morgan-Schaffer Corporation, Montreal, Quebec. High amplification spectra of high molecular weight fragments were included.
RESULTS

Isolation of Compounds

Yields of 1.47 g of ethyl ether solubles and 23.8 g of ethyl acetate solubles were obtained from 1.5 kg (58.6% moisture) of fresh western red cedar leaves treated according to Figure 3. These extract weights represent yields of 0.76% (ethyl ether) and 3.86% (ethyl acetate) by weight of the oven-dry leaves.

Chromatographic examination of the gross ethyl acetate extract revealed a wide range of polyphenolics including flavonoids. Several compounds which gave an orange, red-orange reaction with diazotized sulfanilic acid (DSA) ran near the solvent front when developed in 2% aqueous acetic acid on thin layer cellulose plates. This reaction with DSA is indicative of an alpha hydroxy guaiacyl nucleus (46). Two dimensional chromatography using BCAW in the first direction and CAW in the second direction, with thin layer cellulose plates, resulted in good separation of these compounds (Figure 5, compounds A to I) with a wide range of Rf values in CAW. The Rf values in CAW are inversely related to the number of free hydroxyl groups of the chromatographed compounds (22).
Consideration of the two dimensional chromatographs sprayed with DSA indicated that Compound A occurred in the highest yield and gave the very distinct orange color reaction. Several other compounds also gave an orange color reaction (B, E, I, J) but occurred in lower yield. Some compounds also gave a red or red-orange reaction with DSA (C, D, K, F, G, H, L). Other compounds (0 to T) also reacted with DSA but were not investigated beyond chromatographic detection.

Compounds A, B and C—Isolation

The gross ethyl acetate extract was found to fractionate on LH-20 with chloroform: ethyl alcohol (4:1) as the eluting solvent. Pilot studies also determined that a preparative LH-20 separation of the extract was advisable prior to a quantitative run. Therefore, the crude ethyl acetate extract was dissolved in the eluting solvent, divided into two fractions (approximately 25 ml each and applied separately to a LH-20 column (3.5 cm x 40 cm). Fifty ml fractions were taken and monitored one dimensionally on thin layer silica gel plates (CM). The compounds under study were obtained in the first 500 ml of eluant. The schematic chromatogram (Figure 5) reveals the subfraction now devoid of almost all of the undesirable flavonoid and related compounds. The column was stripped using chloroform: methyl alcohol (3:7), reswollen to near the original
bed level, and the second half of the extract was applied and run, again being monitored by thin layer silica gel plates. The elution volume remained constant.

The clarified extract was now ready for application to the quantitative LH-20 column. It was concentrated in the vacuum evaporator (estimated weight 10 to 15 g), divided into 4 equal portions and applied under pressure at a flow rate of 2 ml/minute, and 10 ml fractions were taken. The eluant was monitored using the Gilson UV monitor and silica plates. The elution curve of a typical run is shown in Figure 6.

![Elution curve](image)

**Figure 6.** Typical elution curve of clarified ethyl acetate extract from western red cedar leaves as run on LH-20 (CHCl₃:EtOH(4:1)).
Thin layer chromatography revealed that the 220 to 280 ml fraction contained Compounds B, C, D as major constituents while Compounds A and J were the major constituents of the 420 to 480 ml fraction. A fraction appearing near 100 ml appeared to be carbohydrate or a cyclitol and was not investigated further. The fractions from the four runs were separated according to the two major elution peaks and concentrated to a small volume (5 to 10 ml). These fractions were now applied to a preparative silica gel column (3.5 cm x 40 cm) and eluted with CM. Ten ml fractions were taken up to a total elution volume of 500 ml. The desired compounds were eluted from this column with approximately 250 ml solvent. The column served two main purposes: first, any high molecular non-phenolic compounds were eliminated either by failing to run on the column or by running near the front; second, some primary separation of the compounds B, C, D and A and J prior to application to the semi-micro quantitative silica column was achieved.

The fractions containing the compounds were concentrated in a rotary evaporator and applied in 2 ml portions to column No. 2 under pressure. The column was eluted under constant pressure at a rate of 0.75 ml/min and 4 to 5 ml fractions were collected. The compounds eluted from the column in the 35 to 50 ml range. Pure fractions were determined by thin layer silica gel chromatography and set
aside. Those fractions not containing desirable compounds were discarded and the remaining fractions were recombined for other runs. By these procedures, chromatographically pure fractions of Compounds A, B and C were obtained.

The yield of Compound A (based on the oven-dry weight of leaves) was 0.15%. It was estimated that the yield of all compounds giving distinctive color reactions with DSA was approximately 0.5% of the weight of oven-dry leaves.

Compounds A, B and C - Properties

Table No. 1 presents the chromatographic, chromogenic, spectral and physical properties of Compounds A, B, and C in tabular form. In considering chromatographic properties, both Rf values and chromogenic spray reactions are included. It should be noted that the chromatographic solvent designated BCAW was used in lieu of the more usual BAW [butanol, acetic acid, water (4:1:5)] because it gave less streaking while duplicating the Rf values of the classic solvent.

In the determination of an alkaline difference curve, a eugenol standard was run to check the procedure and reaffirm the calculated $\Delta e_{\text{max}}$ value of $4.1 \times 10^3$ as being representative of one free aromatic hydroxyl group. The major peaks of the compound's infrared spectra are presented in Table 2. The NMR and mass spectra obtained for the compounds and their derivatives are represented in Figures 7 to 9 and Figures 11 to 18. Figure 10 is a specific representation of mass spectral fragmentation associated with the acetate derivative of Compound A.
| TABLE 2 |
|---|---|---|---|---|---|---|---|---|
| PROPERTIES OF COMPOUNDS A, B AND C |
| CHROMATOGRAPHIC PROPERTIES | CHROMOGENIC PROPERTIES | SPECTRAL PROPERTIES |
| --- | --- | --- | --- | --- | --- | --- | --- |
| ON CELLULOSE | SILICA | ON CELLULOSE | SILICA | UV | INFRARED BANDS cm⁻¹ | ACETATE DERIVATIVE | MASS, CALC., L.I., (uncorr.) |
| BCAW | CAW | 2% | IAW | BE | CM | DSA | B | pNA | S | ACID | BASE | Δε | BAND | Str | BAND | Str | Str | MM/H | Emp. Form | %C | %H |
| COMPOUND A | 0.63 | 0.50 | 0.76 | 0.71 | 0.14 | 0.32 | b x D | (+) | R-D | (-) | 417 | Y-D | 279nm | 289.5nm | 804 | 3400 | e | 1207 | e | 10 | 78677477 | 58.08 | 6.32 |
| | | | | | | | | | | | | | | | | | | 39H | 10 | 57.98 | 5.84 |
| COMPOUND B | 0.72 | 0.95 | 0.61 | 0.87 | 0.23 | 0.51 | b x D | (+) | 0 | (-) | Y | 280.5nm | 288nm | 240nm | 3400 | e | 1120 | m | 78674215 | 32.08 | 6.32 |
| | | | | | | | | | | | | | | | | | 39H | 10 | 79.78 | 5.84 |
| COMPOUND C | 0.73 | 0.84 | 0.70 | 0.20 | 0.48 | R | (+) | Y | (-) | abs. 0 | 281nm | 281nm | 251nm | 3270 | e | 1230 | m | 3270 | e | 1230 | m | 78674215 | 32.08 |
| | | | | | | | | | | | | | | | | | 39H | 10 | 79.78 | 5.84 |
| BCAW = butanol, chloroform, acetic acid, water (4:1:1:1) |
| CAW = chloroform, acetic acid, water (2:1:1:1.3) |
| IAW = isopropanol, ammonia, water (20:1:1:2) |
| 2% = 2% aqueous acetic acid |
| BE = benzene, ethanol (9:1) |
| CM = chloroform, methanol (4:1) |
| DSA = diazotized sulfinic acid |
| B = Barton's reagent |
| S = Schrezeader's reagent |
| pNA = p-nitroaniline |
| * not determined |
Figures 9, 13, 18 are bar graph representations of the mass spectral fragmentation of the acetates of Compounds A, B and C. For convenience, the figures have been divided into two parts so that the high mass end of the spectrum is in magnified proportion.

Those mass fragments which appear as primary decomposition products have been labeled with their molecular ion number. Metastable peaks have not been noted on these spectra. In all three mass spectra the m/e 43 ion was the 100% ion.

Compounds A, B and C - Derivatives

Methylation

Aromatic-hydroxyl methylation of Compound A used diazomethane in methyl alcohol. Thin layer silica gel chromatography (CM) of the methylation reaction mixture showed the presence of two compounds (Rf 0.45, 0.55). The lower Rf compound displayed a positive reaction to Barton's reagent (indicative of an aromatic hydroxyl), while the high Rf compound showed no reaction. The methylated reaction mixture was separated on the silica gel pressure column using CM as the eluant. The separated low Rf compound was collected and subjected to re-methylation with diazomethane. Thin layer chromatography of the products from this reaction showed further formation of the Rf 0.55 compound.
Based upon the chromatographic behaviour and color reactions, the Rf 0.55 compound was considered the fully aromatic hydroxyl methylated derivative of Compound A, while the lower Rf (0.45) compound was considered to be a partially methylated derivative of Compound A.

Complete (all hydroxyls) methylation of Compound A required more stringent conditions. Methylation was first attempted using dimethyl sulfate with sodium carbonate followed by methyl iodide/silver oxide in dimethyl formamide. These conditions were chosen to minimize rearrangements which might occur because of the highly reactive benzyl hydroxyl group in Compound A. Thin layer chromatography showed methylation to be incomplete for these conditions even after two attempts. It was therefore necessary to apply the stronger methylation conditions on the partially methylated product using dimethyl sulfate with sodium hydroxide. Verification of the complete methylation was obtained by a negative reaction of the methylated product to Barton's reagent and the lack of infrared absorbance in the 2700 nm to 2900 nm range.

**Acetylation**

Acetate derivatives of these three compounds were formed according to the procedure previously outlined. Triturated acetates were obtained through scratching of the
acetylated mixture under petroleum ether (65-110°C) and cooling at 5°C.

Hydrogenolysis

Catalytic hydrogenolysis was attempted on Compound A using a catalyst (2) highly reactive in the reduction of benzyl hydroxyl groups. The reaction was run with the compound dissolved in ethyl alcohol for 10 minutes and the product was chromatographically examined to determine the degree of hydrogenolysis. The 10 minute hydrogenolysis resulted in the apparent conversion of Compound A to a second compound (Rf 0.51 in CHCl₃:MeOH), Compound B, in about 50% yield. When isolated, this compound gave a negative Gierer test for the benzyl hydroxyl group.

Compounds A, B and C - Degradative Studies

Hydrolysis

Hydrolysis of Compounds A, B and C was attempted using 2% aqueous oxalic acid or 6% hydrochloric acid in methanol. Hydrolysis of Compounds A and B was generally considered unsuccessful because of the formation of several by-products; however hydrolysis of Compound C resulted in a low yield of a single product, the putative aglycone of the parent glycoside. An examination of the hydrolysis products of Compound B revealed the presence of Compound C,
indicating a chemical relationship existing between the two compounds under conditions of hydrolysis.

The primary presence of L-rhamnose and minor amounts of L-arabinose and D-xylose were determined by chromatography of the hydrolysate of initially separated compounds. Enzymatic hydrolysis was also applied to Compound A. Chromatography revealed the probable formation of some aglycone in the enzyme hydrolysis but in yield insufficient for effective study.

**Ethanolysis**

Compound A was subjected to ethanolysis as previously described. The resulting products were neutralized with sodium bicarbonate and run on thin layer cellulose in butyl alcohol saturated with ammonia. Standard compounds placed on the plate included: 1-guaiacylpropan-1,2-one, 1-ethoxy-1-guaiacylpropan-2-one, 1-guaiacyl-2-ethoxypropan-1-one, and 1-guaiacylpropan-2-one. When sprayed with DSA, the ethanolysis products of Compound A showed 2 spots, one at Rf 0.92 which displayed a red-orange color, and a second spot Rf 0.20 which displayed a lemon yellow color. The high Rf compound exhibited essentially the same Rf value as 1-ethoxy-1-guaiacylpropan-2-one or 1-guaiacylpropan-2-one, with a color reaction similar to the former. The DSA color reaction of the spot occurring at Rf 0.20 is considered typical of p-hydroxyl benzyl groups (46).
The fully methylated derivative of Compound A was also subjected to ethanolation. The resulting ethanolation product was neutralized with sodium bicarbonate and chromatographed in comparison to dihydroconiferyl alcohol on thin layer silica plates in BE. One of the major constituents of the ethanolation was shown to run at exactly the same Rf value as the dihydroconiferyl alcohol standard and displayed an identical color reaction with DSA.

**Alkaline nitrobenzene oxidation**

Compound A was subjected to alkaline nitrobenzene oxidation according to the method previously described. The results of this oxidation showed two major spots when chromatographed in CAW (Rf 0.95 and Rf 0.50). The high Rf compound gave an orange color when sprayed with DSA, while the lower Rf compound gave a less distinct color reaction (off white to cream). Chromatography of the alkaline nitrobenzene product, in comparison to the standards vanillin and protocatechualdehyde, showed identical color reactions and Rf values.

**Periodate oxidation**

The partially methylated derivative of Compound A (phenolic hydroxyls only) was subjected to periodate oxidation according to the procedure of Dyer (21). The reaction mixture was titrated for excess arsenite after one and two hours. Consumption of periodate showed 2 moles consumed at
the end of one and two hours. Therefore, the reaction was considered complete. The reaction mixture was acidified and extracted with chloroform. The chloroform extract was concentrated to dryness and taken up in methyl alcohol. An ultraviolet spectrum of the product, showed a peak at 280 nm which gave no shift upon the addition of 0.1 N sodium methoxide.

Liquid Scintillation Technique

This study includes a preliminary investigation of the infusion of U-\textsuperscript{14}C-L-phenylalanine into the leaves of western red cedar. The investigation was concerned particularly with the formation of dilignol glycosides in leaves floated in an aqueous solution of the radioactive phenylalanine. The feeding method and the chemical properties of the dilignol glycosides established that two primary factors would govern the measurement of incorporated radioactivity. First, low levels of incorporation (near 1 per cent) could be expected. Second, those compounds incorporating radioactivity could be chromatographically located specifically through autoradiography or generally with chromogenic spray reagents.

The experimental design required evaluation of incorporation on chromatographically separated samples. Four methods were available for analysis of the chromatographically separated radioactive compounds: (a) autoradiography (densi-
metric analysis) (b) chromatographic strip counting (thin window Geiger-Meuller counter), (c) combustion-absorption (liquid scintillation analysis), (d) suspension (liquid scintillation analysis).

The anticipated low levels of activity effectively eliminated autoradiography and strip counting. Autoradiography would require excessive time, while strip counting was of too low efficiency for measuring low activity. The phenolic nature of the compounds to be studied, and their required location with chromogenic spray reagents, could severely limit liquid scintillation analysis of suspended low activity samples. Many phenolic compounds and their colored products (from various chromatographic spray reagents) are recognized to be effective chemical quenchers during liquid scintillation analysis. It has also been shown by Houx (53) that scraped suspended silica gel samples adversely affect the recorded external standard ratio regardless of the chemical quenching agent present.

Of the available alternatives, combustion-absorption of chromatographic samples seemed the most plausible because it would effectively eliminate chemical and physical quenching, while nearly 100 per cent of the $^{14}$C label would be available for liquid scintillation analysis (50). The limiting factor to a combustion technique was the efficient combustion of chromatographically separated samples. The combustion
would be most feasible if associated with paper, or thin layer cellulose chromatography. Silica gel was not considered because it does not burn under normal combustion conditions. Thin layer cellulose chromatography offered the most suitable medium to produce small sized chromatographic spots which could be combusted.

Radioactive sample preparations from thin layer cellulose plates in the past had paralleled those from thin layer silica plates. These preparations involve scraping of the chromatographic spot from the plate and subsequently counting the sample as a suspension. Although the scraped sample can be burned, a cleaner, more efficient method of preparation was desirable. The method used in this study allows the clean removal of the chromatographic spots from the plate after spraying. These prepared samples are then combusted directly in the scintillation vial in the presence of a CO₂ absorbent, and then counted.

In order to substantiate the technique, an experiment was designed to establish: (1) the comparative efficiency of the cellulose nitrate combustion versus the scraping-suspension method, (2) changes in efficiency due to chemical quenching for the scraped and combusted samples, (3) the efficiency changes as a result of chromogenic spray reactions for the two methods, and (4) sample size limitations in combustion.
Table 1 denotes the samples prepared to test the proposals and the counting results obtained from five replications of each sample. Quercetin (3, 5, 7, 3',4' pentahydroxyflavone) was used as a phenolic quenching agent because of its extensive conjugation, color, and availability. Pinosylvin (3,5 dihydroxystilbene) was chosen as a chemical quenching agent because it is able to absorb ultraviolet radiation and fluoresce. The choice of Barton's reagent and bis-diazo-treated benzidine as sprays was primarily because of their common use in detecting phenolic compounds. However, the sprays cause color reactions via different mechanisms. Barton's reagent depends upon chelation of phenols with ferric iron to produce a distinctive blue complex; bis-diazotized benzidine depends upon azo-coupling to phenolic compounds. The data of Table 1 is graphically recorded in Figures 19 and 20. Figure 21 graphically presents the results of the separate determination of chromatographic spot size on counting efficiency of combusted samples.

\[ ^{14}\text{C Feeding} \]

The analysis of scintillation results obtained in the leaf feeding experiment are summarized in Table 3. Figure 22 represents the preliminary autoradiographic verification of U-\(^{14}\text{C-L-phenylalanine incorporation into Compounds A and B in western red cedar leaves. The radioactivity uptake by the leaves during the later infusion study is shown in Figure 23. The relative incorporation of radioactive phenylalanine into Compounds A and B is depicted graphically in Figure 24. The results repre-
<table>
<thead>
<tr>
<th>Feeding time hr.</th>
<th>Activity in leaves dpm</th>
<th>Inc.,%</th>
<th>Activity in Compound A dpm</th>
<th>Inc.,%</th>
<th>Activity in Compound B dpm</th>
<th>Inc.,%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.22x10^6</td>
<td>16.1</td>
<td>1865</td>
<td>0.15</td>
<td>3063</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>1.34x10^6</td>
<td>20.3</td>
<td>3970</td>
<td>0.30</td>
<td>5370</td>
<td>0.40</td>
</tr>
<tr>
<td>5</td>
<td>1.78x10^6</td>
<td>23.5</td>
<td>5340</td>
<td>0.30</td>
<td>7040</td>
<td>0.39</td>
</tr>
<tr>
<td>10</td>
<td>2.82x10^6</td>
<td>37.1</td>
<td>5035</td>
<td>0.18</td>
<td>6600</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Table 3. Uptake of U-^{14}C-L-phenylalanine and its incorporation into Compounds A and B in the leaves of western red cedar.
sent the average values of three runs with no statistical variance being calculated.
DISCUSSION

Structural Studies of the Dilignols

Compound A

Compound A was obtained as an amorphous solid from the column chromatographic separation of the ethyl acetate extract as previously described. Attempts to crystallize the compound from organic solvents were unsuccessful and preparation of a derivative was initiated. The preparation of an acetate derivative also yielded an amorphous solid which failed to crystallize from organic solvents. A triturated solid was obtained by scraping under petroleum ether (65°C-110°C) followed by cooling at 5°C. The petroleum ether was allowed to evaporate in a vacuum dessicator leaving the triturated acetate derivative with a melting point of 35°C-38°C (uncorrected). Attempts at recrystallization of this derivative were unsuccessful. Compound A was later shown, by NMR, to be a mixture of erythro- and threo-isomers which accounts for the low melting point range of its acetate derivative. There was no question of the purity of Compound A since thin layer chromatography (silica and cellulose), in several solvent systems, of Compound A and its acetate derivative showed a single spot.
Mass spectral determination of the acetate derivative of Compound A revealed a molecular weight of 804 with a calculated empirical formula (from M+1/M ratio) of C_{39}H_{48}O_{18}, supported by carbon and hydrogen analyses. Sixteen degrees of unsaturation, indicative of at least two substituted benzene rings, are calculated from the empirical formula. An ultraviolet \Delta \epsilon value of 8.04 \times 10^3 for Compound A showed the existence of two phenolic hydroxyl groups. Infrared data substantiated the existence of aromatic nuclei, absence of carbonyl, and a large number of hydroxyl groups. The 18 oxygen atoms of the formula for Compound A acetate were assigned (on the basis of NMR and mass spectral data) to two phenolic acetates, one benzylic acetate, four aliphatic acetates, one methoxyl group, and three ether or hemiacetal oxygens.

On the basis of the following results, Compound A was considered to be 1-(3'-methoxy-4'-hydroxyphenyl)-2-O-1"-[2"-hydroxy-4"-(propane-3"'-O-\alpha-L-rhamnoside)phenyl]-propane-1,3 diol (XLI).

![Diagram of Compound A](image)

XLI, R=H
XLIIa, R=Ac
Chemical characterization.

Compound A reacts to produce a bright orange color when sprayed with DSA, which is characteristic of an α-hydroxyguaiacyl compound (46). A positive test with Gierer's reagent (44) further substantiates the existence of a benzyl hydroxyl group. Schroeder's reaction (82), for the detection of o-dihydroxy groups, was negative. Since alkaline nitrobenzene oxidation of Compound A (to determine the aromatic substitution pattern) showed the presence of vanillin and protocatechualdehyde (establishing the existence of guaiacyl and catechol nuclei), the test with Schroeder's reagent showed that one of the hydroxyl groups on the catechol nucleus must be etherified.

Hydrolysis of the compound with 2% aqueous oxalic acid showed several phenolic products. The presence of L-rhamnose in the molecule was conclusively established by thin layer cellulose chromatography of the hydrolysate. The occurrence of this uncommon sugar bound through an unusual aliphatic glycoside linkage will be further substantiated later in this discussion.

An empirical formula of $\text{C}_{25}\text{H}_{34}\text{O}_{11}$ is indicated for Compound A from the formula obtained for the acetate derivative. This discussion has accounted for ten oxygen atoms (four on the benzene rings, one benzylic hydroxyl,
five from the \( \text{L-rhamnose} \). There remains the placement of one aliphatic hydroxyl group and a description of its relationship to the other aliphatic carbon and oxygen atoms. These features were delineated in the following two experiments.

The first experiment substantiated Compound A as a guaiacylglycerol-\( \beta \)-aryl ether through ethanolysis. Comparison of the products of ethanolysis with standard Hibbert's ketones showed the presence of \( 1 \)-guaiacyl-2-ethoxypropan-1-one and \( 1 \)-guaiacylpropan-2-one. These products were considered proof of the guaiacyl glycerol-\( \beta \)-aryl ether nucleus for Compound A. The glycerol side chain contains two isomeric carbons and is responsible for the mixture of erythro- and threo-isomers previously mentioned. Another ethanolysis product showed a color reaction and \( R_f \) values indicative of catechol derivatives.

The second experiment was an ethanolysis of the fully methylated\( [(\text{CH}_3)_2\text{SO}_4/\text{CH}_3\text{I}] \) derivative of Compound A. This ethanolysis yielded dihydroconiferyl alcohol (XLII) as a major product. The free aromatic hydroxyl group of dihydroconiferyl alcohol established the unusual \( 4''\)-n-propan-3''-ol sidechain and the 2-4'' alkyl-aryl ether linkage in Compound A (XLI). The free aliphatic hydroxyl group of XLII established the point of attachment of the \( \text{L-rhamnose} \) in the molecule. The \( n \)-propyl side chain accounted for the remaining aliphatic
carbon atoms. Another product from this ethanolation showed a color reaction and Rf values indicative of veratryl derivatives.

Proof that the arrangement of catechol and L-rhamnose groups were as shown (XLI), and not interchanged, or that the substitution was as a β-aryl ether rather than γ- etc. was obtained from the following experiment. Periodate oxidation of diazomethane methylated (aromatic hydroxyls) Compound A showed an uptake of exactly two moles. This two mole consumption of periodate is solely from the rhamnose moiety and further establishes the absence of any other vicinal diols in Compound A. Lack of a bathochromic shift of the ultraviolet maxima of the oxidized product in base was evidence that the glycosidic bond did not occur through an aromatic hydroxyl.

Only the structure shown for Compound A (XLI) explains the forementioned data. Further verification was achieved by NMR and mass spectral analyses.
NMR spectra of Compound A and its acetate.

NMR spectroscopic techniques confirmed the basic structure of Compound A as determined in degradative studies. The NMR spectrum of Compound A was obtained in deuteroacetone and is presented in Figure 7. The NMR of the acetate derivative of Compound A was obtained in deuterochloroform and is shown in Figure 8. NMR spectral analysis was primarily done on the acetate derivative of Compound A (Figure 8). The ensuing discussion will therefore refer to this spectrum and the NMR results of the unacetylated compound will be treated separately.

The total proton integral of Figure 8 is 47 to 49 protons. Six of these protons are seen to resonate at $\tau = 3.04-3.30$ and are representative of the aromatic protons of Compound A. These protons must be representative of two aromatic rings since the spectra also indicates a three proton methoxyl resonance ($\tau = 6.22$). Two aromatic hydroxyls are considered to occur in Compound A, based upon the two non-equivalent aromatic acetate resonances at $\tau = 7.73$ and 7.77. This slightly different shielding of these acetate resonances show the phenolic hydroxyls of Compound A to be non-equivalent.
Figure 7. The NMR spectrum of Compound A.
Figure 8. The NMR spectrum of the acetate derivative of Compound A.
Five aliphatic hydroxyls are indicated for Compound A by the five acetate resonances occurring in the \( \tau = 7.9 - 8.4 \) region of Figure 8. The anomic proton of rhamnose is seen to occur as a singlet to low field (\( \tau = 5.36 \)) as a result of its association to the \( \alpha - L \)-hemiacetal bonded \( C_1 \) (61). The high field three proton doublet at \( \tau = 8.83 \) originates from the methyl group of the rhamnose coupling with the \( C_5 \) proton (\( J = 6.5 \text{Hz} \)). The \( C_2-C_4 \) protons are shifted to lower field (\( \tau = 4.6 - 5.2 \)) in the acetate derivative of Compound A and are specifically located and described as: \( H_2, \tau = 4.79 \) (\( J_{2.3} = (\text{co.}) 1 \text{Hz} \)); \( H_3, \tau = 4.77 \) (\( J_{2.3} = 1.5 \text{Hz} \), \( J_{3.4} = (\text{co.}) 10 \text{Hz} \)); \( H_4, \tau = 5.0 \) (\( J_{4.5} = 4.5 \text{Hz} \)). The \( C_5 \) proton is not shifted through acetylation and its chemical shift was determined to be \( \tau = 6.21 \) by nuclear magnetic double resonance experiments (NMDR). Application of NMDR to the \( H_5 \) resonance resulted in a collapse of the high field doublet to a singlet. Two resonances at \( \tau = 4.00 \) (integrated value, one proton) can be assigned to the benzyl proton of a glycerol grouping. These doublets (\( \tau = 3.97, J_{\alpha \beta} = 7.0 \text{Hz} \); \( \tau = 4.05, J_{\alpha \beta} = 4.0 \text{Hz} \)) can be attributed to the occurrence of a diastereomeric pair of \( \beta \)-guaiacylglycerol isomers of Compound A (60). Since the \( \beta \) proton is associated with an ether bonded carbon, it should appear in
the region $\tau = 4.5 - 6.0$ (54). Using NMDR techniques, the $\tau = 4.5 - 6.0$ region was scanned while monitoring the low field doublet for any changes. In this way, the $\beta$ proton resonance was located at $\tau = 5.38$, hidden beneath the anomeric proton resonance. NMDR of the $\beta$ proton caused the $\alpha$ proton doublets to collapse to two singlets ($\tau = 3.97, 4.05$). This irradiation of the $\beta$ proton also resulted in clarification of the multiplets in the region $\tau = 5.6 - 6.1$ indicating the $\gamma$ protons resonance associated with the multiplet in this range. The chemical shift in this resonance is characteristic of an esterified hydroxymethylene (54).

The occurrence of the two proton triplet at $\tau = 7.37$ ($J = 3.7$ Hz) (Figures 7 and 8) and the two proton multiplet at $\tau = 8.18$ (Figure 7) are characteristic of the $\alpha'$ and $\beta'$ protons of an n-propyl chain. When the resonance occurring under the aliphatic acetate envelope at $\tau = 8.18$ was irradiated in an NMDR experiment, the triplet at $\tau = 7.37$ collapsed to a singlet thereby establishing it as the $\alpha'$ proton resonance and the $\tau = 8.18$ resonance as the $\beta'$ resonance. Irradiation of the $\beta'$ resonance clarified the multiplet in the range $6.30 \tau$ to $7.75 \tau$ to a distinct AB quartet centered at $\tau = 6.5$ with $\tau_1 = 6.35, \tau_2 = 6.65$ ($J_{(\text{gem})} = 10.0$ Hz). This resonance is representative of the $\gamma'$ protons, and the chemical shift of the AB quartet suggests
that they are methylene protons associated with an ether bond rather than an ester bond (occurrence in \( \tau = 5.5 - 6.0 \) range). The AB nature of the spin-spin coupling of the geminal protons of the methylene group indicates non-equivalence and suggests restricted rotation due to the bulky \( \beta\)-rhamnose moiety at the \( \beta' - \gamma' \) carbon-carbon bond.

Examination of the NMR spectrum of unacetylated Compound A reveals several features including: a total integral of 30 to 35 protons, a more definitive six proton aromatic resonance (\( \tau = 2.9 - 3.61 \)), two aromatic hydroxy resonances (\( \tau = 1.30, 2.55 \)), a twelve to fourteen proton multiplet in the \( \tau = 6.1 - 6.8 \) region which includes the rhamnose hydroxyl groups. Other specific features include: benzyl proton resonances \( \tau = 5.07 \), the methine proton resonance (as determined by NMDR) \( \tau = 6.00 \), and the two proton resonance multiplet of the \( \beta' \) protons at \( \tau = 8.18 \) without interfering acetate resonances. Methylation of Compound A produces a nine proton resonance at \( \tau = 6.21 \) representative of three methoxyl groups or two free aromatic hydroxyl groups in Compound A.

The chemical shift data and nature of the NMR spectrum of the acetate derivative of Compound A is in agreement with similar data recorded for 1-(3,4-dimethoxy)
-2-(2'-methoxyphenoxy)-propane-1,3 diol acetate (XLIII) reported by Ludwig et al. (60).

\[
\text{XLIII}
\]

The discussion of the NMR spectrum of compound XLIII relates the character of the \( \alpha \), \( \beta \), and \( \gamma \) resonances in relation to the existence of two diastereoisomeric forms. These characteristics are in close agreement for resonances recorded for the acetate of Compound A. On the basis of this comparison and on the degradative and NMR data compiled for Compound A, the compound is considered to be characterized as an isomeric mixture of 1-(3'-methoxy-4'-hydroxyphenyl)-2-0-1'-[2"'-hydroxy-4"'-(-propane-3"'-0-\( \alpha \)-L-rhamnoside)phenyl]-propane-1,3 diol (XLI).

Mass spectrum of Compound A acetate.

The mass spectrum of the acetate of Compound A is shown in Figure 9. The parent ion is shown to occur at m/e=804. Ex-
Figure 9. The mass spectrum of the acetate derivative of Compound A.
amination of the high mass end of the spectrum reveals six transitions which may be associated with acetate decomposition. Two of the transitions are accompanied by metastable ions: \( 804 \xrightarrow{m^* = 722.2} 762 + \text{CH}_2=\text{C}=0 \) and \( 762 \xrightarrow{m^* = 521} 702 + \text{CH}_3\text{C}=\text{O} \). The other transitions for which metastables are not present are: \( 702 \xrightarrow{} 642 + \text{CH}_3\text{C}=\text{O} \), \( 642 \xrightarrow{} 600 + \text{CH}_2=\text{C}=0 \), \( 702 \xrightarrow{} 660 + \text{CH}_2=\text{C}=0 \), and \( 660 \xrightarrow{} 600 + \text{CH}_3\text{C}=\text{O} \). These acetate degradations indicate the occurrence of at least two aromatic acetates and two aliphatic acetates.

Validation of the occurrence of Compound A as a rhamnoside can be seen in the occurrence of an \( m/e = 273 \) ion for the rhamnose triacetate fragment. This fragment can in turn decompose along a pathway similar to that proposed by Pearl and Darling (74) for glucoside acetates. A proposed decomposition path of the rhamnose triacetate is depicted in Figure 10. The prominent ions 273, 213, 153, 111, and 109 are in agreement with the proposed pathway.
Figure 10. Proposed mass spectral fragmentation pathway for rhamnose triacetate.

Several other features of Compound A can be illustrated through probable formulae related to major ions of the mass spectrum. Some possibilities are illustrated on the following page.

Several characteristics of Compound A are novel and require comment. First, this compound constitutes the first isolation and characterization of a free dilignol glycoside. Second, the compound exists as an unusual
rhamnoside rather than much more commonly observed glucosides. Third, the rhamnoside is connected to the compound through a previously unreported aliphatic hydroxyl position rather than the expected aromatic hydroxyl position. Fourth, the existence of a saturated propyl side chain is unreported among dilignols isolated using hydrolytic conditions and is biosynthetically unusual. Finally, the existence of a catechol group is unreported among dilignols previously isolated and characterized.
The complete analysis of the analytical and spectral data available for Compound A has enabled its positive characterization as described. Such data are incomplete for Compounds B and C which therefore cannot be as completely characterized. However, speculative conclusions may be made regarding the structures of these compounds based upon the available data and their observed chemical relationship to Compound A. The following discussions include such speculations.

**Compound B**

Compound B was isolated, from the column chromatographic separations previously described, as an amorphous solid which failed to crystallize from organic solvents. A triturated acetate derivative was obtained by scratching under petroleum ether (65°-110°C). The melting point of the acetate derivative of Compound B was 34° - 43°C (uncorrected).

Mass spectral determination of molecular weight was not clearly defined because of the inability to count mass units at the high mass end of the spectrum. However, a molecular weight of 744 or 786 was indicated by interpolation. No meaningful empirical formula could be calculated. Difference spectra were not obtained for this compound because of its apparent existence as a mixture. The infrared spectrum of Compound B was essentially identical with
that of Compound A, showing an aromatic nature, no carbonyl, and a high degree of hydroxylation.

Compound B shows an orange reaction with DSA while displaying negative reactions with Gierer's (44) and Schroeder's reagent (82). The compound therefore contains neither an o-dihydroxyphenyl group nor a benzyl hydroxyl group. The low yield of Compound B prevented its analysis using ethanolysis or alkaline nitrobenzene degradation techniques. Chromatography of the 2% oxalic acid hydrolysis products showed a mixture of phenolic compounds (including Compound C) and a major occurrence of L-rhamnose. Compound B was also shown to be formed in 50% yield under conditions of catalytic reduction of Compound A. Other low yield products, in addition to unreacted Compound A, were noted in the chromatographic examination of the reaction products.

Examination of the NMR and mass spectral data of Compound B in comparison to that of Compound A, and the observed chemical relationship between the compounds, led to the speculative phenylcoumaran structure XLIV for Compound B.
NMR spectra of Compound B and its acetate.

The NMR spectra of Compound B and its acetate derivative were obtained as with Compound A and are depicted in Figures 11 and 12. The similarity of these spectra to those for Compound A is evident and only the distinguishing differences will be discussed.

The total integrals of 28 to 32 and 45 to 47 protons for Figures 11 and 12, respectively, can only be considered as approximations because of the known occurrence of Compound B as a mixture. The aromatic proton region of these spectra integrates for five to six protons. The high field rhamnose methyl resonance shows a five proton multiplet in Figure 11 and clarifies somewhat in Figure 12 to show the expected doublet. The methoxyl resonance at $\tau = 6.20$ integrates to three protons but shows three distinct peaks indicating the existence of a methoxyl varying between three aromatic hydroxyl sites. The doublet associated with the $\alpha$ proton is centered at 5.21 $\tau$ and integrates to less than one proton, while the total integral (5.00 - 5.40 $\tau$), including the anomeric singlet, integrates to two protons. These observations are in agreement with the occurrence of this compound as a molecular and/or isomeric mixture even though it appears chromatographically pure. The NMR spectrum of the diazomethane methylated derivative of this compound indicated six methoxyl protons or at least one free aromatic hydroxyl. The methylated
Figure 11. The NMR spectrum of Compound B.
Figure 12. The NMR spectrum of the acetate derivative of Compound B.
product also shows secondary splitting of the low field benzyl proton doublet, further suggesting an isomeric mixture of erythro- and threo-configuration.

The NMR of the acetate derivative shows one aromatic acetate (τ = 7.72) and a low field resonance (τ = 2.25) which may be a free hydroxyl group. Such a hydroxyl resonance in the acetate derivative suggests a strongly hydrogen bonded or sterically hindered hydroxyl which is not efficiently acetylated. It is however, more probable that the low field resonance is the result of an impurity. The acetate derivative (Figure 12) also revealed no down-field shift of the benzyl proton doublet on acetylation (τ = 5.05). The spectrum presents only four aliphatic acetate resonances (τ = 7.8 – 8.2) suggesting that the benzyl carbon atom is involved in an ether linkage causing the shift of the α proton resonance to lower field. NMDR experiments on the acetate established the appearance of the β and γ proton resonances at 5.83 τ and 5.48 τ, respectively. These resonance locations represent opposite chemical shift values to those observed for the acetate of Compound A. Such changes in chemical shift are noted for dehydrodiconiferylalcohol triacetate (XLV) (see page 90) in comparison to 1-(3,4-dimethoxyphenyl)-2-(2′-methoxyphenoxy)-propane-1,3 diol diacetate (XLII) as described by Ludwig et al. (60).
Mass spectrum of Compound B acetate.

The mass spectrum of the acetate derivative of Compound B is shown in Figure 13. The occurrence of potential parent ions at m/e = 702, 744 and 786 indicates the existence of the compound in a molecular mixture. Such a mixture severely restricts the analysis of the spectrum for meaningful ions, however, the m/e = 744 ion would be in agreement with the molecular weight of the speculated structure for Compound B. The previously mentioned ions associated with rhamnose triacetate decomposition are also present in the spectrum. The general character of the spectrum is in agreement with the spectrum of the phenylcoumaran dehydrodiconiferyl alcohol as reported by Kovacik and Skamla (56).

The probable existence of more than one compound (and threo- and erythro- isomers) makes exact analysis of Compound B difficult. However, it would appear that the benzyl carbon atom is either ether or alkyl-aryl linked to a second phenyl nucleus, thereby eliminating the α hydroxyl and the associated downfield shift of the benzyl proton after acetylation. Also, the β carbon is bonded in such a manner as to cause an upfield shift of its resonance in comparison to the same resonance in Compound A. The nature of the substituent bonded to the
Figure 13. The mass spectrum of the acetate derivative of Compound B.
β carbon atom may cause the downfield shift of the γ proton in comparison to the same resonance in Compound A. Based upon the nuclear magnetic resonance data in comparison to that of Ludwig et al. (60) and Compound A, and the observed chemical relationship between Compound B and Compound A, a phenylcoumaran (XLIV) is considered as the most probable structure.

The acceptability of structure XLIV for Compound B may be strengthened by the observation of Nimz (71) that an α, β-phenoxy migration can occur for α-hydroxy-guaiacyl-β-aryl ether compounds under mild acidic conditions. Nimz showed the conversion of XLVI to XLVII in water (100°C) in seven days. The catalytic hydrogenolysis conditions under which Compound A was formed at 50% yield from Compound B may be comparative to Nimz' conditions leading to the formation of a quinone methide ion capable of rearrangement to yield compound XLIV. Biosynthetic formation
of the naturally occurring Compound B may result from Compound A by a similar biosynthetic reaction, or it may be formed as a separate entity prior or concurrent to the formation of Compound A via the quinone methide pathway.

**Compound C**

Compound C, as isolated from the chromatographic column, was an amorphous solid which failed to crystallize from organic solvents. A triturated acetate derivative was obtained through scraping under petroleum ether (65° - 110°C), melting point 39° - 43°C (uncorrected). The molecular weight of the acetate derivative was determined to be 702 from the molecular ion of its mass spectrum. A calculated empirical formula of \( \text{C}_{35}\text{H}_{42}\text{O}_{15} \) was obtained from the M + 1/M ratio and 15 degrees of unsaturation were calculated from the empirical formula. The compound reacted with DSA to produce a red color and tests for o-dihydroxy and benzyl hydroxyl groups were negative. No alkaline ultraviolet spectrum was obtained because of the low yield of this compound.

Hydrolysis of Compound C was successful in producing a low yield of a chromatographically pure aglycone and \( \text{L}-\text{rhamnose} \) was established as the glycoside. Low yields of the compound prevented the application of further degradative techniques. The infrared spectrum of this compound,
was again similar to Compounds A and B. Based on the following examination of NMR and mass spectral data in relation to the chemical relationship of Compound C to Compounds A and B, Compound C is considered to be a guaiacyl benzodioxane dilignol possessing either structure XLVIII or XLIX.

NMR spectra of Compound C and its derivatives.

The NMR spectra of Compound C and its acetate derivative were obtained as for Compounds A and B and displayed the same general characteristics (Figures 14 and 15). The total approximate proton integrals for Figures 14 and 15 respectively, are 29-33 and 40-43. Important features include: six aromatic protons, no shift of the benzyl proton (\( \tau = 5.14 \)) in the acetate derivative, similarity of chemical shifts of the \( \beta \) and
Figure 14. The NMR spectrum of Compound C.
Figure 15. The NMR spectrum of the acetate derivative of Compound C.
γ resonances (τ = 5.91, 6.37, Figure 15) to those of the acetate of Compound B (Figure 13) rather than the acetate of Compound A, one aromatic acetate resonance (τ = 7.72), and four aliphatic acetate resonances (τ = 7.82 – 8.30). These characteristics show Compound C to be similar to Compound B with an ether or an alkylaryl bond to the α carbon of the guaiacylglycerol group. However, this compound contains one less aromatic hydroxyl group.

Examination of the NMR spectra of the aglycone of Compound C and its acetate derivative (Figures 16,17) offers further evidence for the structure of Compound C. Both of these spectra show six aromatic protons in the τ = 3.00 – 3.40 range. The α proton is shown to occur at τ = 5.14 in Figure 16 and does not shift after acetylation (Figure 17). NMDR established the occurrence of the β and γ protons at 5.92 τ and 6.39 τ respectively. Such chemical shifts are indicative of the association of these protons with electron-withdrawing groups bonded to both the β and γ carbon atoms. NMDR experiments on the α and β protons in the acetate NMR spectrum revealed no shifts after acetylation while the γ protons have been shifted to lower field (τ = 5.65).

NMDR experiments established the chemical shifts of the α', and β', and γ' protons to occur at τ = 7.41, 8.15 and 6.37 respectively. The acetylation of the
Figure 16. The NMR spectrum of the aglycone of Compound C.
Figure 17. The NMR spectrum of the acetate derivative of the aglycone of Compound C.
aglycone produces a shift of the γ' proton to 6.08 τ while the α' and β' protons show no change. This evidence is proof of the existence of the n-propanol side chain in the aglycone of Compound C. The existence of only one aromatic acetate in both the glycoside acetate and the aglycone acetate spectra offers evidence of the existence of the glycosidic linkage through this aliphatic hydroxyl. An AB quartet similar to that reported for the acetate derivatives of Compounds A and B, is observed upon irradiation of the resonance at 8.18 τ in the NMR spectra of the acetate of Compound C. These observations are considered proof of the existence of the rhamnosidic linkage through the n-propanol side chain as reported for Compounds A and B.

Mass spectrum of Compound C acetate.

Figure 18 shows the mass spectrum of the acetate derivative of Compound C. The molecular ion (M = 702) and acetate decomposition pathways are consistent with the speculated structure. Molecular ion transitions 702 → 660, 660 → 600, 642 → 600, and 702 → 642, are observed and substantiated by the existence of metastable ions at 621, 546, 562, and 587, respectively. This acetate decomposition indicates one aromatic acetate and one
Figure 18. The mass spectrum of the acetate derivative of Compound C.
aliphatic acetate. The mass spectrum reveals the ions previously mentioned for the rhamnose triacetate and its decomposition products. Prominent ions are also seen at m/e = 149, 222. These ions may originate from the cleavage of the dioxane ring of the speculated structure of Compound C, followed by loss of acetate. Two possible molecular ion structures are presented below which may be representative of the m/e = 222 and 149 ions.

In considering the transformation of Compounds A and B to Compound C by hydrolysis and the related NMR data of Compounds A and B, Compound C is speculated to be a guaiacyl benzodioxane derivative (XLVIII or XLIX).
These structures may be speculated to be chemically derived from Compound A from an intermediary quinone methide form of Compound B originating according to the rearrangement proposed by Nimz (71), which may subsequently react along three distinct pathways. First, in the quinone methide stage, the free catechol hydroxyl group may react with the resonance-stabilized benzyl carbon atom to form structure XLIX. Second, the 1,2 phenoxy shift of Nimz (71) may occur, with the subsequent formation of alkyl-aryl ring closure to form the phenylcoumaran XLIV. Finally, instead of phenylcoumaran formation, rotation may occur about the newly formed α ether bond to place the catechol hydroxyl in close proximity to the β carbonium ion (formed in the phenoxy transfer) with subsequent formation of structure XLVIII. The characterization of Compound A and the speculation of Compounds B and C implies the pathway of Nimz.
as plausible in the A to B to C interrelationship both chemically and biosynthetically.

It should again be noted that the presentation of structural formulae for Compounds B and C are speculative and are based solely on spectral data. However, the general relationship of A to B to C can strengthen the observations based upon the known structure of A. Further research will establish if this is a probable link of lignin to lignan biosynthesis in western red cedar. Further research will also establish the exact nature of the α and β bondings in Compounds B and C. The isolation of other compounds giving the positive DSA reaction in the ethyl acetate extract will add valuable information to the elucidation of these structures. It is of interest to note that the three dilignol rhamnosides exhibit a "missing" methoxyl group, common among the western red cedar heartwood lignans (e.g., XXXVIII).
Lignin Biosynthesis

The results of this investigation lead to speculation into the role of the isolated compounds in lignin biosynthesis. The glycosidic nature of the isolated dilignols suggests the possibility of their involvement in a translocatory process to a site of active lignin formation with their subsequent hydrolysis, and polymerization into the lignin molecule. The noted occurrence of catechol groupings in bark lignins (50) further suggests one possible destination of the dilignol glycosides. However, translocation much beyond local environs, of aromatic (particularly dimeric) lignin precursors is unlikely. Thus, the isolated compound would be destined to incorporation into leaf or branch lignin.

Assuming that translocation is limited, the dilignols may maintain one of two positions regarding their role in lignin biosynthesis. First, the dilignols isolated may be representative of a defined class of extractive components which may undergo further minor structural alteration but will exist eventually as free dilignols. Second, the dilignols may serve as true intermediates to lignin formation.

The occurrence of the dilignol rhamnosides as a distinct class may find some substantiation in their high yield in leaf tissues, and their unusual chemical
structures (compared to other dilignols).

The high yield in leaves may be compared to that of the flavonoid glycosides in the same tissue. Such an analogy would place the dilignol rhamnosides as end products of phenolic anabolism with secondary physiological functions. One such physiological role might be the participation of the compounds in protection mechanisms in the leaves. It is possible that injury or disease reactions may occur in the leaves which would catalyze the cleavage of the unusual rhamnosidic linkage of the dilignol rhamnoside to render the aglycone available for participation in detoxification or sealing-off activities.

The n-propyl side chain of the dilignol rhamnosides is unusual in lignin and phenylpropane biosynthesis. Active precursors to lignin and polyphenolics are considered to require phenylpropene character to allow the oxidative or reductive coupling reaction essential in the formation of lignin and polyphenolics. Participation of the dilignol rhamnosides in wood lignification would require methylation of the free catechol hydroxyl grouping and oxidation of the propyl side chain prior to further polymerization to lignin in the accepted sense. While methylating enzymes are available, the oxidative step is highly improbable.
The foregoing discussion would seem to preclude the participation of the observed dilignol rhamnosides in wood lignin formation. However, the assumed non-translocatory nature of the compounds and their unusual structural character suggest their possible participation in the less intensively studied formation of bark or leaf lignin.

Degradative examination of bark lignin has established the occurrence of catechol groupings in the lignin structure (50). A specific study of western red cedar bark using NMR (93) has shown a lower methoxyl content and more aliphatic character than wood lignin. Such evidence leads to speculation that the dilignol rhamnosides of this study may act as precursors to lignin different in character than that observed in the wood. Such lignin may contain aryl-aryl bonded lignin polymers, which originate from coupling ortho to the n-propyl-side chain of dilignols activated by the free catechol hydroxyl grouping. If the dilignol rhamnosides do participate in bark lignin formation, they could originate in the inner phloem, in contrast to the origin of wood lignin precursors in the cambium. This discussion is highly speculative. The bark tissues must be specifically examined for the participation of the dilignols in bark lignin formation.

Evidence regarding the nature and formation of
Scintillation-Chromatography Technique

The validation procedure in establishing combustion as a superior analytical technique in the measurement of low activity, chromatographically separated compounds is described in a previous section. The results are tabulated in Table 1 and are graphically depicted in Figures 19, 20 and 21. Examination of the data yielded the following results.

A comparison of Figures 19 and 20 reveals a much smaller effect of external component upon activity ratio in the combusted samples. The scraped-suspended samples show significant effects of both external component and spray reagent upon the recorded activity ratio.

The largest effect on the activity ratio appears when the chromogenic spray bis-diazotized benzidine was used as a detecting reagent. When only 2 μg of quercetin were present on the chromatographic spots, subsequent detection with this spray, followed by careful scraping from the chromatographic plate, produced a reduction of over 20 per cent in detectable activity. In contrast, combustion and scintillation counting of that same sample
Figure 19. The effect of an external quenching compound on the determination of activity of scraped chromatographic samples.
Figure 20. The effect of an external quenching compound on the determination of activity of combusted chromatographic samples.
Figure 21. The effect of chromatographic spot size on liquid scintillation counting of combustion samples.
shows a loss of less than 10 per cent in the detectable activity. When quercetin was detected with Barton's reagent, followed by scraping and suspension in the scintillation solution, the reduction in activity ranged from 7 to 28 per cent. Comparative results for the combustion technique show losses of less than 5 per cent. Figures 19 and 20 show a vivid comparison between the scraped-suspension and combustion results for pinosylvin without spraying. The sample shows an increase in activity of 15-20 per cent when analyzed as a scraped sample. This increase must be the result of chemoluminescence, which the scintillation counter cannot distinguish from true radioactivity. Analysis of the results of combustion for this same sample shows reductions in activity ratio of less than 5%. Chemoluminescence may also explain the increase in activity noted for 2 μg of quercetin.

In general, the combustion technique minimizes the loss of detectable radioactivity due to the external influences of chemical and physical quenching.

Figure 21 is a summary of data related to the effect of spot size upon counting efficiency for the combustion technique. It can be seen from the figure that an increase in spot size from 0.6 cm² to 2 cm² resulted in a reduction of counting efficiency from 82% to 75%. Total
weight data for the spot removed from the chromatographic plate showed that a maximum of 7 mg per spot could be combusted efficiently and the optimum weight of chromatographic spots was in the range of 4 to 5 mg. Such a chromatographic spot weight was representative of a spot approximately 1 cm$^2$ including the addition of the cellulose nitrate solution.

The combustion of the chromatographic spots removed from thin layer cellulose plates offers a substantial advantage in counting low activity samples compared to the scraping-suspension method. The sample to be combusted is removed from the plate cleanly, easily handled, and minimizes losses during sample transfer. In comparison, scraped samples involve tedious isolation of a particulate sample which can easily be scattered during transfer. The scraped samples often exhibit electrostatic properties which further hamper handling.

Thus, the combustion technique offers the ability to count phenolic compounds, which can only be detected using chromogenic spray reagents, while limiting experimental error associated with sample preparation, handling, and counting.

This combustion technique also provides an advantage in terms of experimental design. It enables radioactive precursor inhibition into metabolizing tissues, fol-
allowed by extraction, chromatographic isolation and counting of labelled chromatographic spots in a matter of days. When autoradiography is used in a similar sequence, the results would not be available for a month or two for low activity samples. Other techniques, such as thin window counting of chromatographic strips, or sample isolation to constant specific activity, are either limited by the level of activity present or long experimental workup.

The low activity combustion technique was limited to thin layer cellulose chromatographic plates and may have limitations associated with chromogenic sprays not investigated. Although the method is applied only to cellulose plates, it is possible that it may find future applications on other chromatographic supports which can be combusted (e.g., polyamide, dextrose). The combustion technique is not applicable to thin layer silica gel chromatography because it is non-combustible. However, the cellulose nitrate solution is useful in removing chromatographic spots from the silica gel plate prior to suspension counting.

Precursor Feeding Study

The metabolic activity of leaves makes them a very promising tissue for the study of incorporation of known aromatic precursors. Phenylalanine represents a well established aromatic precursor which, when administered to
actively metabolizing tissues, will participate in the formation of aromatic compounds with a minimum reversion to the glycolytic pathway. It is for these reasons that phenylalanine was chosen for aromatic incorporation studies in the leaves of western red cedar.

Administration of lignin precursors into actively metabolizing tissues generally falls into two separate classes: (1) infusion, and (2) implantation. Kratzl (57) has shown that the infusion of radioactive lignin precursors into the stem produces higher radioactive incorporation into lignin than the implantation of the same precursors into the cambial area. Implantation techniques are considered to initiate secondary wound reactions which subsequently alter the pathway to the formation of aromatic compounds. Freudenberg (29) has noted the formation of radioactive wood lignin using the technique of infusion of known lignin precursors through the leaves of coniferous species. It is evident therefore, that infusion feeding methods allow the efficient uptake of lignin precursors which may be altered in the leaf tissue prior to the ultimate formation of lignin in the wood. These observations suggested the closer examination of western red cedar leaves for lignin precursors which would show active metabolism of radioactively labeled aromatic precursors during infusion feeding.
Preliminary infusion feeding experiments established that the leaves of western red cedar were able to assimilate radioactive phenylalanine to form radioactive phenolic compounds. Autoradiography (Figure 22) of the gross ethyl acetate extract of the preliminary feeding experiment showed incorporation of the label in a four hour feeding time. Four major spots occurring between Rf 0.1 and 0.45 on the chromatogram displayed an orange color reaction when sprayed with DSA. The incorporation of U-14C-L-phenylalanine into two of these compounds (designated A and B) was later examined in a ten hour feeding experiment utilizing the validated thin layer chromatographic combustion technique. Diazotized sulfanilic acid (DSA) was used as a detecting reagent in place of bis-diazotized benzidine in the leaf feeding experiment since it gave more distinct color reactions and somewhat higher counting efficiency after combustion (Table 1). The use of DSA did entail some problems in the spot removal from the chromatographic plate, making it necessary that the cellulose layer be of consistent thickness. It was also necessary to allow adequate time for the DSA spray to dry prior to removal of the chromatographic spots with cellulose nitrate. The spots representative of Compounds A and B in the ten hour leaf feeding experiment required division to smaller size prior to combustion. The results of the combusted samples in the ten hour feeding experiment are tabulated in Table 3 and
Figure 23. Autoradiogram of the gross ethyl acetate solubles from U-14C-L-phenylalanine fed western red cedar leaves (solvent-BE).
Table 3 reveals that floating cut western red cedar leaves in a radio-active solution of phenylalanine is an efficient method of administering the aromatic precursor. The per cent incorporation of the labeled precursor with time is shown in Figure 23 which shows 16 per cent uptake in hour 1, with continuous uptake of the labeled precursor to a level of 37 per cent after 10 hours. This rate of uptake should effectively minimize the destruction of the aromatic precursor by micro-organisms which might be present in the leaves. This rapid rate should also optimize the introduction of the aromatic precursor into the metabolizing areas during a period of normal metabolism.

The level of activity incorporation into Compounds A and B during 1, 3, 5, and 10 hour feeding periods is depicted in Table 3 and Figure 24. Incorporation levels of 0.15% to 0.40% were observed for radioactive phenylalanine into Compounds A and B. Examination of the data indicate that Compound B incorporates a larger portion of the available radioactivity than does Compound A. Both compounds showed a reduction in activity after 5 hours feeding. This reduction in activity after 5 hours may be associated with further metabolism of the compounds. Subsequent experiments are needed to clarify this point.
Figure 23. Uptake of \( ^{14}\text{C}-\text{L-phenylalanine} \) by western red cedar leaves.

Figure 24. Incorporation of \( ^{14}\text{C}-\text{L-phenylalanine} \) into Compounds A and B in the leaves of western red cedar.
The results of this feeding experiment revealed two important points. First, it is apparent that phenylalanine is actively incorporated into dilignol rhamnosides in the leaves of western red cedar. Second, the level of incorporation into these precursors is such that future kinetic studies may biosynthetically relate the dilignol glycosides and subsequently explain the formation of lignin precursors in the leaves of western red cedar.
CONCLUSION

The ethyl acetate soluble portion of methyl alcohol extracts of fresh western red cedar leaves was examined. The purpose of the investigation was to isolate and characterize metabolically active dilignols, which may serve as precursors in lignin formation. Three such dilignol rhamnosides have been isolated through the application of pressure chromatography and gel filtration techniques. The rapid metabolic formation of these dilignol rhamnosides was shown through their incorporation of $^{14}C$-L-phenylanine in a leaf infusion feeding experiment.

Of the three dilignols isolated, the dilignol rhamnoside 1-(3'-methoxy-4'-hydroxyphenyl)-2-O-1'-(2'-hydroxy-4'-(propane-3''-O-α-L-rhamnoside)phenyl)-propane-1,3 diol was obtained in high yield (0.15%), and was characterized successfully by chemical degradation in conjunction with NMR and mass spectral techniques.

The other two dilignol rhamnosides have been isolated in lower yields (less than 0.05% each), their structures were not completely characterized. Based upon accumulated chemical, NMR, and mass spectral data, however, the compounds were found to be closely related homologs.
of the characterized dilignol rhamnoside. This relationship appeared to be associated with reactions of the reactive benzylhydroxyl and catechol groups. Speculative phenylcoumaran and guaiacyl benzodioxane structures satisfied much of the evidence collected for these two compounds. Chromatographic evidence suggested the existence of several other similar compounds. A total yield for all such components in the leaves of western red cedar may be as high as 0.5%.

The characterized dilignol rhamnoside possessed several novel structural characteristics in relation to previously reported dilignols including: (1) the first reported occurrence of a free dilignol glycoside, (2) the unusual occurrence of the glycoside as an α-L-rhamnoside, (3) the previously unreported existence in a potential lignin precursor of the glycosidic linkage through an aliphatic hydroxyl, (4) the existence of the dilignol as a previously unreported guaiacylglycerol-catechol-β-aryl ether rather than the common guaiacylglycerol-guaiacyl-β-aryl ether and, (5) the unusual saturated propyl side chain in a compound of lignin character.

A specific radioactive infusion study revealed that the characterized dilignol rhamnoside, and its suspected phenylcoumaran homolog, incorporated 0.3% and 0.4% U-¹⁴C-L-phenylalanine within a ten hour feeding period.
The degree of incorporation into these compounds was obtained through the application of a newly developed technique for the efficient counting of low radioactivity samples, separated chromatographically on thin layer cellulose plates. A separate validation of the techniques showed its superiority to comparable methods of analysis for low activity samples.

The role of the dilignol rhamnosides in lignin biosynthesis was discussed. It was suggested that the dilignol glycosides of the leaves may be important bark and leaf lignin precursors, based upon their structures. The high yield of these compounds in the actively metabolizing leaf tissue indicated that they may also fulfill important physiological roles (e.g. disease protection, wound-sealing) in the leaf tissues.

Future Research

The discovery of the dilignol rhamnosides and their observed metabolic participation suggests many important future investigations. Five major proposals relating to future research with these compounds are as follows:

The characterization of the two remaining dilignol rhamnosides found in the leaves of western red cedar and the clarification of the chemical and biochemical relationship between them and Compound A.

The isolation and characterization of other dilignol and/or oligolignols which may be present in these leaves.
Further biosynthetic labelling studies with more precise precursors to ascertain specific pathways in the biogenesis of the dilignols and lignin.

Radiochemical kinetic studies utilizing the thin layer cellulose sample technique to associate the order of formation of the dilignols and their subsequent conversions.

The establishment of the character of "leaf" lignin in relation to other lignins.
LITERATURE CITED


