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Title: POLYPHENOLS FROM TREE BARKS: I. SYNTHESES OF DIARYLHEPTANOIDS; II. CHARACTERIZATION OF SOME DOUGLAS-FIR TANNINS

Abstract approved: Redacted for Privacy
Murray L. Laver

Oregonin, a natural diarylheptanoid xyloside, has been implicated in the stain forming phenomenon of red alder (Alnus rubra Bong.) wood and bark. This staining characteristic has long been a problem in the production of lumber and bleached paper. The structure deduced for the stain-forming precursor, oregonin, on the basis of partial synthesis has been further confirmed by the synthetic work described within. The synthetic product, 1,7-bis(3,4-dimethoxyphenyl)heptane-3-one-5-O-2, 3,4-tri-O-acetyl-β-D-xylopyranoside and the material derived from natural oregonin yielded identical nuclear magnetic resonance and mass spectral data.

Six new compounds are described and their spectral data ($^{13}C$ nmr, $^{1}H$ nmr, ir, ms) and physical constants are

included. These are 1,7-bis(3,4-diacetoxyphenyl)hepta-1,6-diene-3,5-dione; 1,7-bis(3,4-diacetoxyphenyl)heptane-3,5-dione; 1,7-bis(3,4-diacetoxyphenyl)heptane-3-one-5-ol; 1,7-bis(3,4-diacetoxyphenyl)heptane-3,5-diol; 1,7-bis(3,4-dibenzoylphenyl)hepta-1,6-diene-3,5-dione, and 1,7-bis(3,4-dimethoxyphenyl)heptane-3-one-5-*O*-2,3,4-tri-*O*-acetyl-β-*D*-xylopyranoside.

Other polyphenols in tree barks include the tannins. Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] inner and outer barks are rich in a complex mixture of polyphenols ranging from the simple (+)-catechin and (-)-epicatechin to highly polymeric substances. The water-soluble fraction of the acetone-water extract was investigated by two dimensional paper chromatography. The studies revealed the presence in the extract of a large number of individual compounds. The inner bark group included the flavan-3-ols (+)-catechin and (-)-epicatechin, the procyanidins B-1, B-2, B-3, B-4, C-1, and C-2, the flavan-3-oid dihydroquercetin glycoside, plus other phenolics. The outer bark group most notably contained the flavan-3-ols (+)-catechin and (-)-epicatechin plus dihydroquercetin, conidendrin and some highly polymeric phenols which remained at the origin of the chromatographs. Most of these compounds were present in the extracts in small amounts. They were identified by chromatographic $R_f$ values, color reactions, and by comparison with authentic compounds.

# Polyphenols from Tree Barks: I. Syntheses of Diarylheptanoids; II. Characterization of some Douglas-Fir Tannins

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# POLYPHENOLS FROM TREE BARKS: I. SYNTHESES OF DIARYLHEPTANOIDS: II. CHARACTERIZATION OF SOME DOUGLAS-FIR TANNINS

## INTRODUCTION

Polyphenols from tree barks are diversified in their overall chemical structures. They include the lignins, the hydrolyzable tannins, the condensed tannins, the diarylheptanoids, and related materials. However, these compounds have one feature in common, they possess hydroxyl groups attached directly to aromatic rings. Thus, they are closely related in chemical functionality. The work herein reported is concerned with two families of the polyphenols, the diarylheptanoids and the tannins. Although the families are related, for clarity of presentation, the work is reported in two parts: I. Syntheses of Diarylheptanoids; II. Characterization of some Douglas-fir Tannins.

The diarylheptanoids have recently been shown to be involved in the staining phenomenon of red alder (Alnus rubra Bong.) bark and wood (49,50). The bark becomes pinkish or reddish-brown in color when cut. The wood changes from a pale whitish color to orange or reddish-brown when cut. This staining characteristic is a problem in the production of red alder lumber and bleached paper (71). Karchesy, Laver, Barofsky, and Barofsky (49) and Karchesy (50) showed that a natural diarylheptanoid xyloside, 1,7-bis(3,4-dihydroxyphenyl)heptane-3-one-5-O-β-D-xylopyranoside (I), which they named oregonin, was

implicated as a precursor to the stain. The objective of the present work was to synthesize oregonin, or at least derivatives of oregonin which could be later converted to oregonin. The syntheses were deemed necessary to form the framework of a complete elucidation of the staining phenomenon.

The characterization of tannins, the second part of the work reported, involves polyphenols extracted from the inner bark and the outer bark of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco]. Douglas-fir is the principal commercial softwood of the pacific northwest, accounting for nearly three quarters of the conifer inventory. At advanced ages, Douglas-fir trees may attain diameters of 1.8 meters and heights of 75 meters (35). These trees typically are grown in a rotation of 50-80 years, and the timber is used for a substantial part of the pulp and paper, lumber, plywood, and particleboard produced in the United States. Large amounts of bark are harvested with the trees, most of which is burned for steam generation. However, Douglas-fir bark is a rich source of polyphenols and the petroleum shortage of 1973 renewed interest in the possibilities for using these polymers to replace phenol in wood adhesives (71). Also, recent studies have produced data to support the use of Douglas-fir bark to replace as much as 20 percent of the wood now used in high quality underlayment-grade particleboard (92). This use of bark is undoubtedly

enhanced by chemical reactions which occur between the polyphenols in the bark and the formaldehyde resins used in particleboard manufacture. However, more complete knowledge of the structures and properties of these polyphenols, most notably the tannins, is needed if advances in their use are to be made.

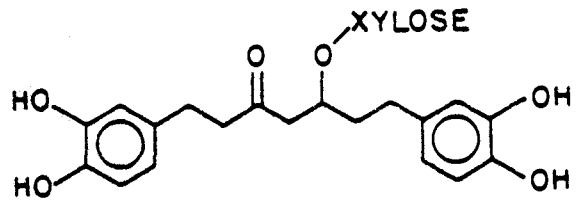
# I. SYNTHESES OF DIARYLHEPTANOIDS

## Historical Review

The first individuals to study the phenomenon of red alder stain were Kurth and Becker (58) in 1952, who assessed the ethyl acetate extract of red alder wood and bark. Finding that the extract content was higher in the bark they chose it as their source. An interesting sidenote to this is that Asakwa and coworkers (2,3) chose the buds of Alnus firma Sieb. et Zucc. (Betulaceae) to extract their diarylheptanoid, while Terazawa and Miyake (86) chose the buds, leaves, and inner bark of Shirakanba birch (Betula platyphylla) as the source of their fifteen extractives.

Although Kurth and Becker did not fully elucidate the structure or structures of the compound or compounds causing the stain they did characterize them as phenolic xylosides. A curious characteristic became evident when they tested the stain with acid, it had definite indicator properties. When testing the stain at pH 4.5 and below the color was yellow, but when the pH was raised to 4.8 a red color developed. Analagous to this characteristic, curcumin (II) the yellow pigmented diarylheptanoid isolated from Curcuma longa L. (90) shows indicator properties (4). When in solution at pH 4.6 and below, the color is yellow and as the pH is raised to 4.9 the color changes to orange-red.

( I )

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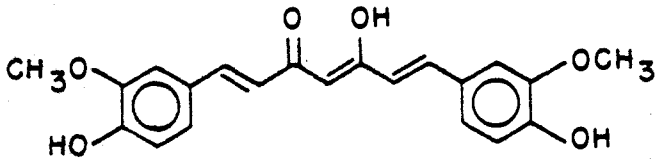


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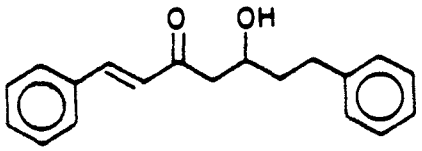


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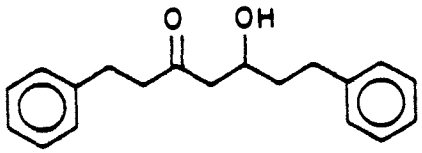


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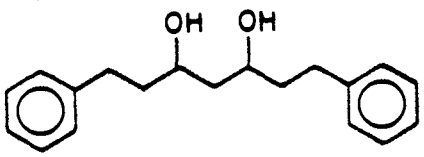


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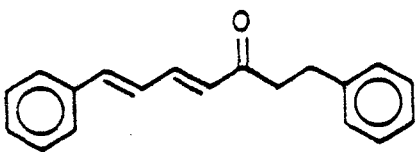
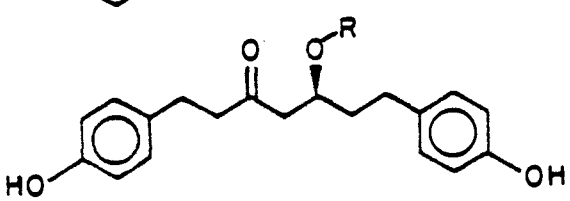
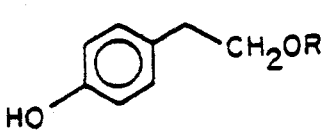


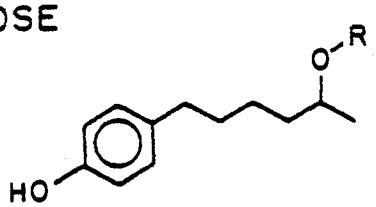

(VII) R = H

(VIII) R = GLUCOSE



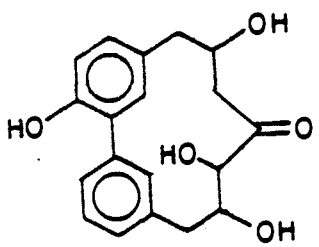

( IX ) R = H

( X ) R = GLUCOSE

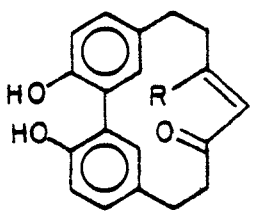



( XI ) R = H

( XII) R = GLUCOSE




(XIII)




( XIV) R = H

( XV ) R = OH $\Delta^{4,5}$ SAT

( XVI) R = H, 4,5-EPOXY

The causal factors of this reaction have not as yet been investigated.

After the work of Kurth and Becker (58), no subsequent investigation was undertaken until 1973 when Karchesy (50) chromatographed the ethyl acetate extract and observed a peculiar color reaction. One of the spots progressively developed the colors yellow→maroon→violet when sprayed with Roux's toluene-p-sulfonic acid reagent (77). This spot alone was characterized and the compound present was subsequently named oregonin (I). A serious study of the ethyl acetate extract should include the six other components indicated by Karchesy. The possibility of these being biosynthetic precursors is supported by the fact that others (86) have found precursors along with their sought after diarylheptanoids.

Many other species of alder have been investigated for the polyphenolic materials they contain. European black alder (20), which shows a similar staining phenomenon, was shown to contain a phenolic glycoside. The list of diarylheptanoids grows longer with the inclusion of seven compounds from the genus Alnus. Yashabushiketol (III), dihydroyashabushiketol (IV) from Alnus firma buds (2,3), the saturated diol (V) from Alnus fruticosa and Alnus manshurica leaves (89), and the ketone (VI) from Alnus pendula (85) are members of the diarylheptanoid group.

Alnus is not the only woody genus from which diarylheptanoids have been isolated. The birch genus, Betula, related by family (Betulaceae) to Alnus, has recently been investigated (86). Eight glycosides of phenolic compounds were isolated from Betula platyphylla. Two of them are the diarylheptanoids platyphyllanol (VII) and platyphylloside (VIII).

Several other compounds isolated such as tyrosol (IX), salidroside (X), betuligenol (XI) and betuloside (XII) may contribute to or be fragments of the biosynthetic product, platyphylloside (VIII).

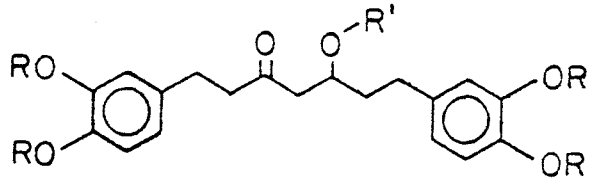
Meta-meta bridged biphenyls are implicated (9,24) in originating from diarylheptanoids. Asadanin (XIII) (94) from Ostrya japonica (Betulaceae) and the ketone (XIV) and its derivatives (XV) and (XVI) from Alnus japonica (65) are but a few of the meta-meta bridged biphenyls isolated from the Alnus genus. It is not known at this time whether the involvement of the above compounds are the cause of the staining phenomenon. However, the phenomenon could conceivably arise through the formation of these compounds.

The forest products industry has a keen interest in the chemistry of red alder aimed toward using the bark and wood as furnish for composite products such as particleboard and waferboard. This interest, kindled by the dwindling

availability of conventional sources of furnish, and the abundant supply of red alder (71) plus the fact that the wood and bark contain available phenolic components, has produced studies of the strength of these composite products. As it turns out 10 percent of red alder bark can be used as supplemental wood furnish in particleboard with acceptable strength properties (92). Waferboard has been produced by blending red alder flakes and phenolic resin with wax generating a board with strength properties far above those attained by other waferboard products (71). It has been suggested that pressing alder flakes (without resin) under pressure and temperature will produce a board which should have reasonable strength properties. This prospect should involve thermal degradation chemistry of the phenolics from red alder to completely envision these compounds as "intra adhesives" and why red alder is so very special in this regard.

## Results and Discussion

According to Karchesy (50), oregonin, the novel diarylheptanoid xyloside (I) is the major component of the ethyl acetate soluble portion of the methanol-water extract from red alder bark. This compound was isolated by thin-layer chromatography (tlc), and partially characterized by $^{1}H$ nuclear magnetic resonance (nmr), and mass spectrometry (ms). The structure was assigned on the basis of $^{1}H$ nmr, infrared (ir), ultraviolet (uv), and mass spectrometry of its tetra-O-methyl ether derivative (XVII), its tetra-O-methyl ether triacetate derivative (XVIII), and the total synthesis of the tetra-O-methyl ether derivatives (XIX) of its aglycon. The syntheses of XVIII and XX described in the present work provides additional evidence for the assigned structure.




(XVII) R = $CH_3$, R' = β-D-xylopyranoside

(XVIII) R = $CH_3$, R' = 2,3,4-tri-O-acetyl-β-D-xylopyranoside

(XIX) R = $CH_3$, R' = H

(XX) R = $OCOCH_3$, R' = H

As mentioned above, oregonin has been partially synthesized in the form of the tetra-O-methyl ether of the aglycon (XIX) (49,50). In the methylated form, however, without the sugar moiety, this compound does not show stain forming properties. Therefore, to make oregonin in its active, free phenolic form (I) it is necessary to: first, synthesize the diarylheptanoid structure, the aglycon; second, block the free phenolic hydroxyl sites; third, condense the correct glycosyl halide on the secondary alcohol; and finally remove the protecting groups without hydrolyzing the sugar. The aglycon, glycosyl halide and glycosylation syntheses are described in detail later in the text.

The first two requirements were solved by using protected diarylaldehydes and condensing them with acetyl acetone using Pabon's method for creating diarylheptanoids (67). The third requirement was resolved by using the Helferich modification of the Koenigs-Knorr glycosylation reaction. Finally, the choice of protecting groups was narrowed down to those easily removed with a base. This follows from the fact that acetals are hydrolyzed to hemiacetals when reacted with mineral acids, but are reasonably stable to bases. Acetate groups were chosen in the form of acetoxy for the aryl phenolic hydroxyl sites and the sugar hydroxyl sites, and benzoate, as an alternative

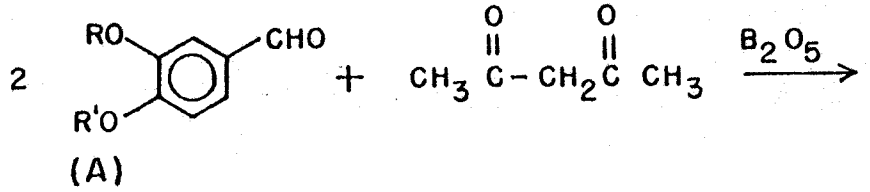
protecting group for the sugar moiety. These protecting groups are stable to the reaction conditions necessary to synthesize the diarylheptanoid portion and to the glycosylation reaction to put on the sugar portion, and yet they are easily removed with alcoholic base (alkoxide). Other protecting groups are available but due to time limitations, these were not tried.

When the synthesis of the tetraacetoxy aglycon (XX) was carried out, the overall yield was exasperatingly low. Thus, owing to a shortage of the tetraacetoxy aglycon (XX) the formal synthesis was carried out using the tetramethoxy derative (XIX). It's synthesis differed only with respect to starting material and increased overall yield. The glycosylation reaction of the tetramethoxy aglycon (XIX) with the glycosyl halide (XXXVIII) was then monitored as changes in reaction conditions were made to produce the maximum yield of product. When the glycosylation conditions are optimized and enough of the tetraacetoxy aglycon is synthesized, the reaction should be repeated. The seven acetate groups should then be removed with sodium methoxide or a modified procedure using an ion exchange resin IRA 400 ($OH^-$). In this way oregonin can be produced.

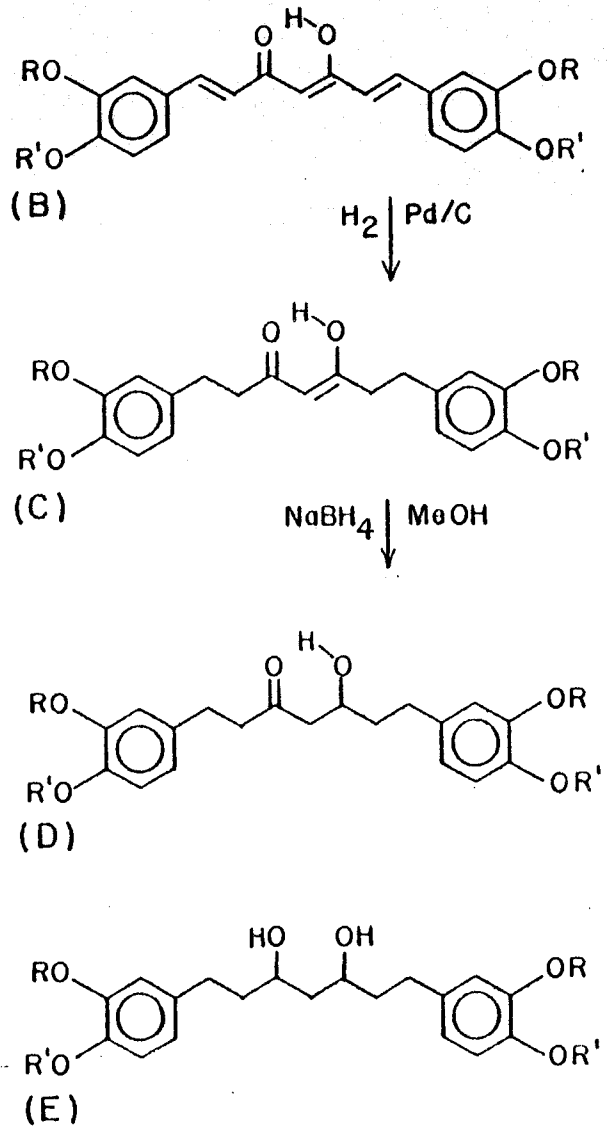
In post analysis the following alternative synthesis can be imagined. First, create the 2,3,4-tri-O-benzyl-α-D-xylopyranosyl bromide; second, synthesize 1,7-bis(3,4-

dibenzylphenyl)hepta-1,6-diene-3,5-dione, reduce the double bonds with Wilkenson's catalyst $[(Ph_3P)_3RhCl]H_2$, then one of the ketone groups with a bulky hydride reducing reagent [selectride]. These reactions would yield 1,7-bis(3,4-dibenzylphenyl)heptane-3-one-5-ol. After the glycosylation reaction using the Helferich modification of the Koenigs-Knorr reaction, the removal of the benzyl groups with hydrogen ($H_2$) and palladium on carbon (Pd/C) would give the free phenolic form in a reductive atmosphere thereby preventing the oxidative coupling usually associated with phenolic compounds like oregonin. Crystalization of the xyloside using standard procedures under this reductive atmosphere could yield oregonin in its crystalline form.

Syntheses of Aglycons.--Pabon's method (67) of condensing two moles of an arylaldehyde with one mole of acetyl acetone complexed with boric anhydride to yield an unsaturated diarylheptanoid (Scheme 1) was used to synthesize XXV, XXVI, II, and XXVII. Catalytic hydrogenation ($H_2$ and 10% Pd/C) of XXV, XXVI, and II gave the saturated diketones XXVIII, XXIX, and XXX respectively. All of these in chloroform existed totally in the enolic form, as shown by the $^1H$ nmr, and $^{13}C$ nmr spectra. Billman, Sojka, and Taylor (11) have shown that $^{13}C$ nmr can be used for observing keto-enol tautomerism. It compares very well with ir and $^1H$ nmr methods of determining percent enol by comparing




| R<br>R' | O<br>-CCH3<br>O<br>-CCH3 | -CH3<br>-CH3 | -CH3<br>-H | -BENZ<br>-BENZ |
|---|---|---|---|---|
| A | XXI | XXII | XXIII | XXIV |
| B | XXV | XXVI | II | XXVII |
| C | XXVIII | XXIX | XXX | |
| D | XX | XXXI | XXXII | |
| E | XXXIII | XXXIV | XXXV | |




Scheme 1. Syntheses of diarylheptanoids.

the integrated peak intensities of the carbon atoms associated with the diketone. Treatment of XXVIII, XXIX, and XXX with sodium borohydride (1.5 ketone group equivalents) in methanol at room temperature gave the ketols XX, XXI, and XXXII plus the diols XXXIII, XXXIV, and XXXV respectively. The diols are presumably a meso and a (±) pair. The diols, which migrated very close to each other, were collected separately from preparative tlc.

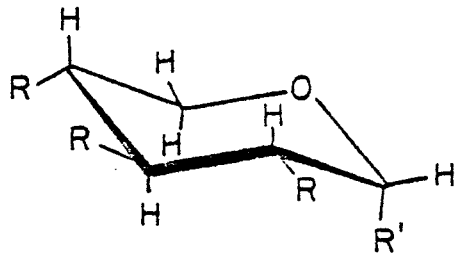
Spectral and combustion analyses of selected materials satisfied structures XX, XXXI, and XXXII. The synthetic product XVIII and Karchesy's (50) naturally derived oregonin gave identical spectra.

Syntheses of Glycosyl Halides.--The acylated 1-halo sugars widely used in glycoside syntheses are usually obtained as the thermodynamically more stable form having an axial (α) halogen on C-1 (25). The halides of the β-(D or L) series are very unstable but can be prepared in kinetically controlled reactions. They are characterized by their marked tendency to invert to the stable form, especially in the presence of hydroxylic compounds. This is attributed to the anomeric or double bond no bond effect, that is a destabilization arising from electrostatic interaction between the parallel p orbitals of oxygen and halogen (72).

The steric course of the glycosylation reaction of peracylated haloses is, however, determined not by the axial

or equatorial orientation of the halogen but by its interaction with the neighboring substituent on C-2 of the halose. Glycosylation with 1,2-cis-haloses generally proceed with inversion at C-1 to give 1,2-trans-glycosides (45), whereas the 1,2-trans-haloses (the β-form for xylose derivatives), afford 1,2-trans-glycosides. Therefore, the aglycon XX to give the desired product XVII was prepared by modified procedures each having yields greater than 90 percent. D-(+)-Xylopyranose (XXXVI) was treated with benzoyl chloride in pyridine and chloroform to yield the α-tetra benzoate (XXXIX). This material was then dissolved in ethylene dichloride and reacted with hydrogen bromide in glacial acetic acid to yield 2,3,4-tri-O-benzoyl-α-D-xylopyranosyl bromide (XL).

The 2,3,4-tri-O-acetyl-α-D-xylopyranosyl bromide (XXXVIII) was synthesized by first treating D-(+)-xylopyranose with a mixture of acetic anhydride and pyridine (19). The resulting α-tetraacetate was solubilized in acetic anhydride and dry hydrogen bromide gas was bubbled through to yield the desired 2,3,4-tri-O-acetyl-α-D-xylopyranosyl bromide (XXXVIII) (63).




(XXXVI ) R', R = OH
(XXXVII ) R', R = ACETATE
(XXXVIII) R' = Br, R = ACETATE
(XXXIX ) R', R = BENZOATE
(XL ) R' = Br, R = BENZOATE

Glycosylation.--The number of glycosides that have been prepared by the Koenigs-Knorr synthesis (54) is extensive. Many reviews (26,15,47) dealing with the synthesis have appeared in Advances in Carbohydrate Chemistry, as well as a review of O-acylglycosyl halides (37) and one covering mechanisms of replacement reactions (61). Excellent reviews of the problems of O-glycoside synthesis (70) and many specific reactions dealing with xyloside formation abound in the literature (56,46,63,88). The reaction consists, in theory, of allowing an O-acyl glycosyl halide to react with an alcohol, giving a substituted glycoside plus the hydrogen halide. As discussed earlier (15), 1,2-cis-acylglycosyl halides react with alcohols under these conditions with inversion of configuration (Walden inversion) at the anomeric center of the glycosyl-halide

to form 1,2-*trans*-glycosides. Preferential formation of *trans*-glycosides from *cis*-haloses with Walden inversion would be most readily explained by assuming an $S_N2$ mechanism (25).

The presence of the liberated hydrogen halide is detrimental because it tends to reverse glycoside formation, and may also effect partial deacylation of the substituted glycoside (45). To overcome these effects, an acid acceptor, commonly silver carbonate or silver oxide (66), is employed to remove the acid. A second reaction then takes place, resulting in the formation of silver halide, carbon dioxide, and water.

When the Koenigs-Knorr reaction is carried out using simple liquid aglycons, the alcohol also serves as the solvent for the halide and is frequently present in large excess. In these cases, the halide is rapidly converted into the glycoside, and the water formed in the secondary reaction between the liberated hydrogen halide and silver carbonate is seldom cause for concern.

However, secondary hydroxyl groups react sluggishly with *O*-acylglycosyl halides and completion of the reaction may require several hours. Consequently, water is being generated within the reaction medium long before the halide is completely converted into the glycoside. Anhydrous calcium chloride (38) or calcium sulfate (Drierite) (72)

have been used to remove the water which would cause hydrolysis of unconsumed halide. While this reaction is well regarded (63), in some cases (86), the yields and anomeric specificity lack strict control. By the use of modifications of the Koenigs-Knorr reaction strict control with increased yields can be attained.

A significant improvement in the Koenigs-Knorr synthesis was made by Helferich and Wedemeyer (42) who, in 1944, introduced mercuric cyanide as an acid acceptor. Subsequently, Helferich and coworkers (39,40), employing nitromethane as the solvent, showed that the use of mercuric cyanide led to excellent yields in the synthesis of glycosides from solid aglycons and, further, that it was not necessary to use a large excess of the aglycon. Although potential side reactions can occur (41), these are relatively insignificant (95). On the basis of limited evidence (61) this procedure may be dependent on the particular aglycon or halide or both. The prospects of this reaction giving the glycoside with high yield and anomeric purity compared to unmodified Koenigs-Knorr was brought out by Kovac (56) with specific regards to xyloside formation. He tried both the modified and unmodified Koenigs-Knorr reactions and found that the yields were favored by the modified reaction.

Glycosylations of alcohols in the presence of Lewis acids are not without precedent. Studies (48) have shown

that in the presence of catalytic amounts of stannic chloride, 1,2-ortho-esters undergo rapid transformation into the corresponding glycosides. Lewis acid glycosylations have been extended successful to the preparation of several complex glycosides of high anomeric purity and good yields ca. 80-90%. Hanession and Banoub (33) contend that under these mild conditions (the O-acylglycosyl halide is reacted with equal molar amounts of alcohol and stannic chloride in dichloromethane at 0°) anomerization reactions are minimized.

In this laboratory both the Helferich and Lewis acid modifications were tried. The Lewis acid modification gave minimal results. The anomeric purity of the reaction of XXXI with XXXVIII was high but the yields were very low (35% and 27% on two attempts).

Therefore, based on the fact that the substrate, a secondary alcohol, reacted very fast the Helferich modification was attempted with excellent results. The glycosyl halide XXXVIII and the aglycon XXXI were condensed under the conditions of the Helferich modification of the Koenigs-Knorr synthesis using a 50% excess of the amount of the bromide XXXVIII, calculated for the total substitution of the hydroxyl group in XXXI. The yield of this reaction was 68% of the theoretical. According to $^{13}C$ nmr, and $^{1}H$ nmr the anomeric purity was very high, ca. 90%-β linkage.

In the syntheses of complex natural glycosides, conformational analysis is not a trivial matter. In studies of structural determinations and $^{13}C$ nmr spectral signal assignments of natural plant glycosides, it has been found that the $^{13}C$ nmr signal shifts corresponding to the change from aglycon alcohol and pyranose into the glycopyranosides are characteristic of the chemical and steric environments of the hydroxy group in which the glycosidation takes place (81). This discovery has become important and useful for determining the glycosidation position in an aglycon moiety.

Among the several glycosidation shifts, the shift difference between signals due to two carbons β to the glycosidic oxygen atom (Figure 1) in chiral secondary alcoholic glycosides appeared to be the most practical to use for determining the absolute configuration of the secondary hydroxyl group in a chiral secondary alcohol.

Using the general rules and tables gleaned from Seo, Tomita, Tori, and Yoshimura's work (81) the absolute configuration of 1,7-bis(3,4-dimethoxyphenyl)heptane-3-one-5-O-2,3,4-tri-O-acetyl-β-D-xylopyranoside (XVIII) can be ascertained. The synthesized derivative, 1,7-bis(3,4-dimethoxyphenyl)heptane-3-one-5-O-2,3,4-tri-O-acetyl-β-D-xylopyranoside (XVIII) will then be compared to the derivative from the natural product to determine similarity of configuration.

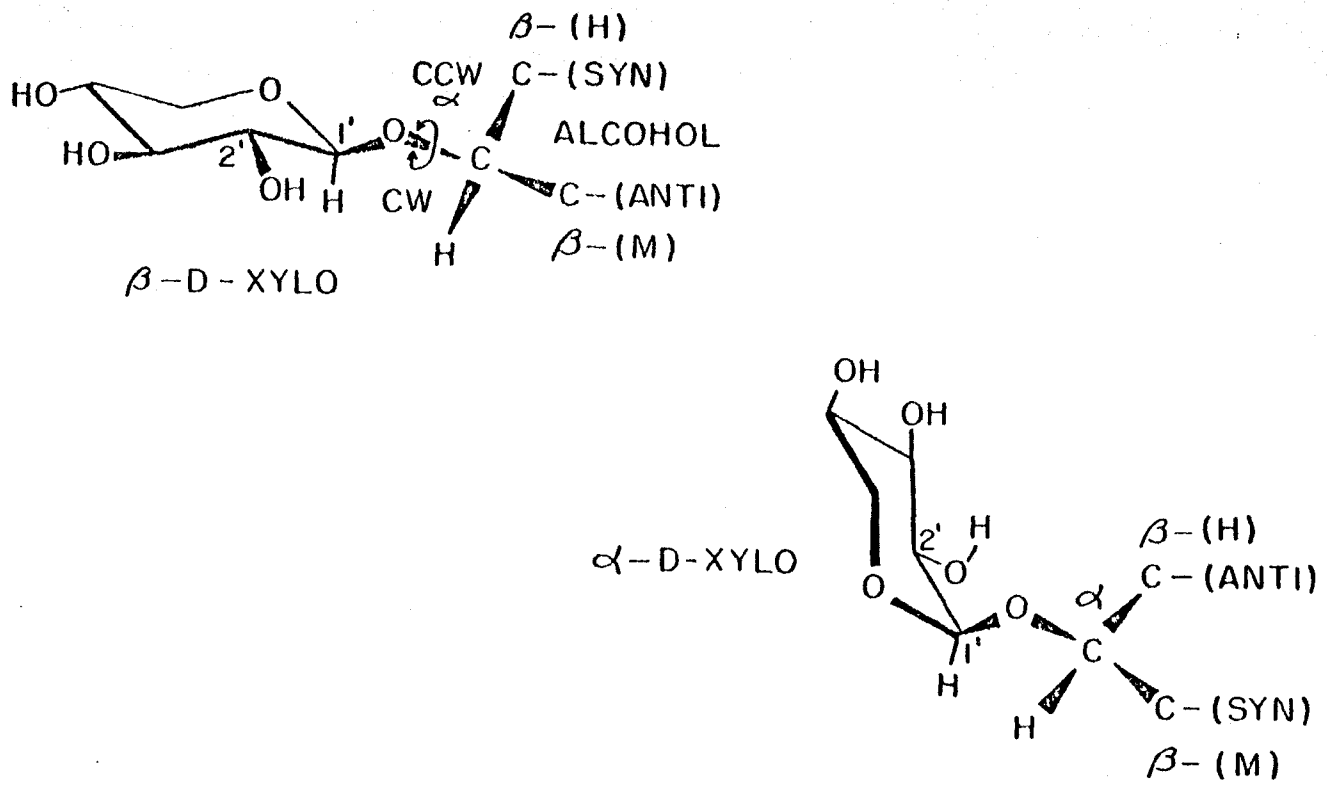



Figure 1. Conformation around xylopyranoside linkages.

The synthesized 1,7-bis(3,4-dimethoxyphenyl)heptane-3-one-5-O-2,3,4-tri-O-acetyl-β-D-xylopyranoside (XVIII) is identical in its nmr and ms data to the same derivative prepared from the natural source (49,50). The $^{1}H$ nmr spectrum shows that the protons on C-2 and C-4 of the xylopyranose ring resonate at δ 5.01-4.78 as a multiplet. The proton on C-3 of the xylopyranose ring resonates as a triplet at δ 5.18. A doublet at δ 4.54 ($J$ = 7 Hz) is due to the anomeric proton in the β configuration. Two quartets, δ 4.10 and 3.36 are due to the two protons on the C-5 of the xylopyranose ring. Three singlets [(δ 2.03, 2.09, 2.11)(9 H)] integrate for nine acetyl protons. This portion of the nmr spectrum of the triacetate derivative (XVIII) closely resembles the spectrum of β-D-xylopyranose tetraacetate (XXXVII). The relative position of the anomeric proton differs due to different substituents at C-1.

The proton signals due to the aglycon portion of XVIII are: a multiplet at δ 6.88-6.64 for 6 aromatic protons; a one proton multiplet at δ 4.10 for $-CH_2-\underline{C}HOR_2-CH_2-$; two singlets for twelve methoxyl protons δ 3.89, 3.82; an eight proton multiplet δ 2.43-2.9 due to the benzylic and α-keto-$CH_2$-protons; and a two proton multiplet at δ 1.81 due to $-CHOR-\underline{C}H_2-CH_2-$.

Field desorption mass spectrometry (FD-MS) yielded m/e values consistent with the desired compound 1,7-bis (3,4-dimethoxyphenyl)heptane-3-one-5-O-2,3,4-tri-O-acetyl-β-D-xylopyranoside (XVIII) (Figure 2). The calculated molecular weight of XVIII is 660.2769 for $C_{34}H_{44}O_{13}$. The highest mass peak from the FD-MS spectrum was 661 (low resolution mass analyzer) which represents the molecular ion plus one ($M^+ + 1$). This peak was clearly the protonated molecular ion peak. The majority of ions produced by FD-MS become protonated before they reach the mass analyzer and so the mass numbers are nearly always one mass unit more than the chemical formulae would indicate. The 661 peak was many times larger than any of the other peaks in the spectrum. The relative intensity of the various peaks in FD-MS are not necessarily linear as to fragment quantity as they are in electron impact mass spectrometry and so the heights of the peaks shown in Figure 2 are not quantitative.

Although fragmentation by FD-MS is not usually extensive, there were some key fragments detected in the spectrum (Figure 2). The m/e 403 peak is from the protonated aglycon ($C_{23}H_{30}O_6$, mol wt 402.2034) which resulted from removal of the xylose triacetate moiety. The m/e 385 peak resulted from the dehydration of the aglycon through loss of HOH as a β-hydroxy ketone. The m/e 201.5

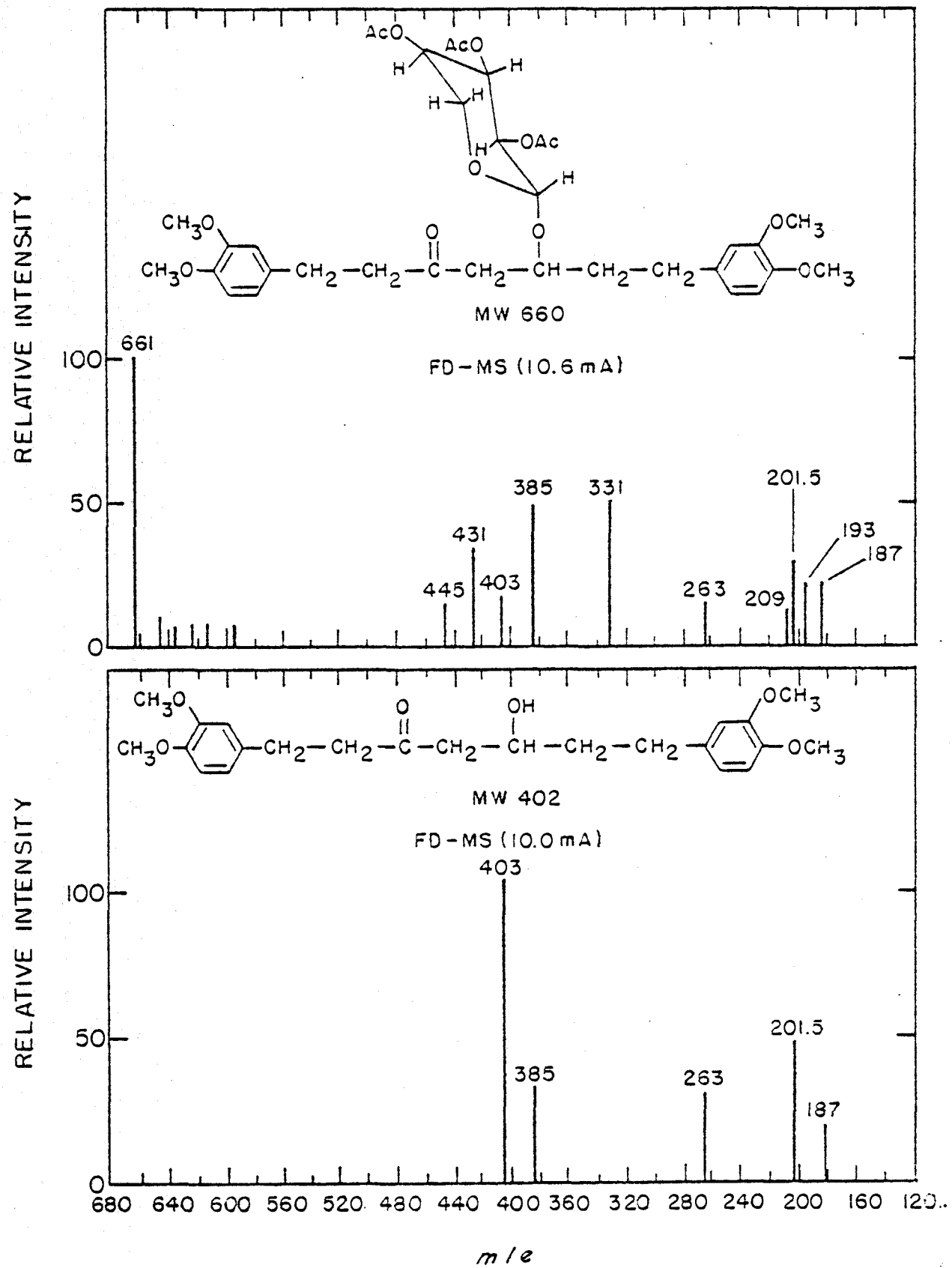


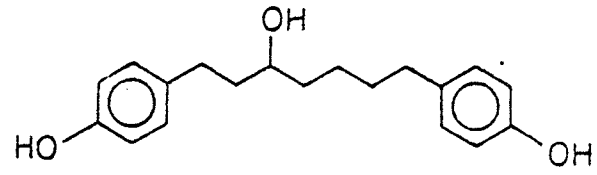

Figure 2. Field desorption mass spectra of tetra-O-methyloregonin triacetate and the tetra-O-methylaglycon of oregonin.

peak was from a doubly charged aglycon ion. The remaining peaks in Figure 2 were not interpreted.

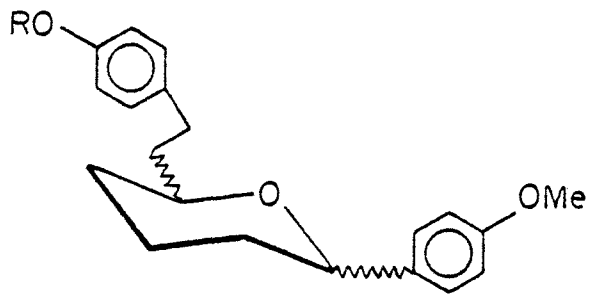
The aglycon itself was analyzed separately and its FD-MS spectrum is shown in the bottom part of Figure 2. The m/e peaks 403, 385 and 201.5 are from the same fragments as described for compound XVIII and reinforce the structure assigned to XVIII. Several other peaks were also the same and support the interpretation that XVIII contains the aglycon.

There were no peaks which could be readily assigned to the xylose triacetate portion of XVIII. This is not too unusual because often one part of a molecule becomes a positively charged fragment which is detected, and the other part becomes a negatively charged fragment which, in the mass analyzer used, would not be detected.

Renewed interest in diarylheptanoids during the early seventies was evoked by the discovery of relatives sharing the 1,7-diaryl skeleton. They apparently form a group biogenetically related to curcumin (II) (24). Many have been found in the Alnus spp. (Betulaceae) (III), (IV), (V), (VI) (see historical), and centrolobol (XLI), centrolobin (XLII), and di-O-methylcentrolobin (XLIII) have been isolated from the Centrolobium spp. (Leguminosae) (1,17).




(XLI)




(XLII) R = H

(XLIII) R = Me

It has been noted that meta-meta-bridged biphenyls may occur through an oxidative coupling reaction with suitable diarylheptanoids (76). Laccase and/or peroxidase may catalize such a reaction (75). Another group of compounds related to those mentioned previously are the polyhydroxy derivatives of perinaphthenone (30). One of these, haemocorin occurs as a glycoside which yields, on hydrolysis, a purple-red aglycon (59). The three groups mentioned above, diarylheptanoids, m,m-bridged-biphenyls and perinaphthenones are believed to have similar biosynthetic pathways involving the combination of phenylalanine, tyrosine and acetic acid (24). However, biosynthetic work done on curcumin was not able to support these conclusions (75). Obviously much more work needs to be accomplished before tying the three groups together.

Possible Fates of Oregonin.--Karchesy (50) has shown the involvement of oregonin in the staining phenomenon by treating it with acid, generating a red-orange tar and also by reacting oregonin with peroxidase and hydrogen peroxide thereby generating a transient red-orange color. In the presence of peroxidase, oxidative coupling is an overwhelming possibility.

It has been suggested that there is a possibility of quinone methide formation. This would ensue from the reaction of oregonin (I), or the aglycon of oregonin (XLIV), with an enzyme, e.g. peroxidase (Figure 3). In nature there are many examples of quinone methide compounds (ca. 500). These compounds possess varied yellow to red colorations depending on the extent of conjugation. Representative structures are shown below (XLV, XLVI, XLVII, XLVIII, II) (the numbers in parenthesis are literature references).

These compounds are all from plant sources with woody habit of growth. Brazilin (XLV) and haematoxylin (XLVI) are the red dying principles of red wood and logwood respectively. And while the quinonoid triterpene pristimerin (XLVII) is an orange wood pigment, obtusaquinone (XLVIII) is a red pigment isolated from the heart wood of Dalbergia obtusa Lecomte. Again we have curcumin (II), the orange-red coloring matter of Curcuma longa L. Their

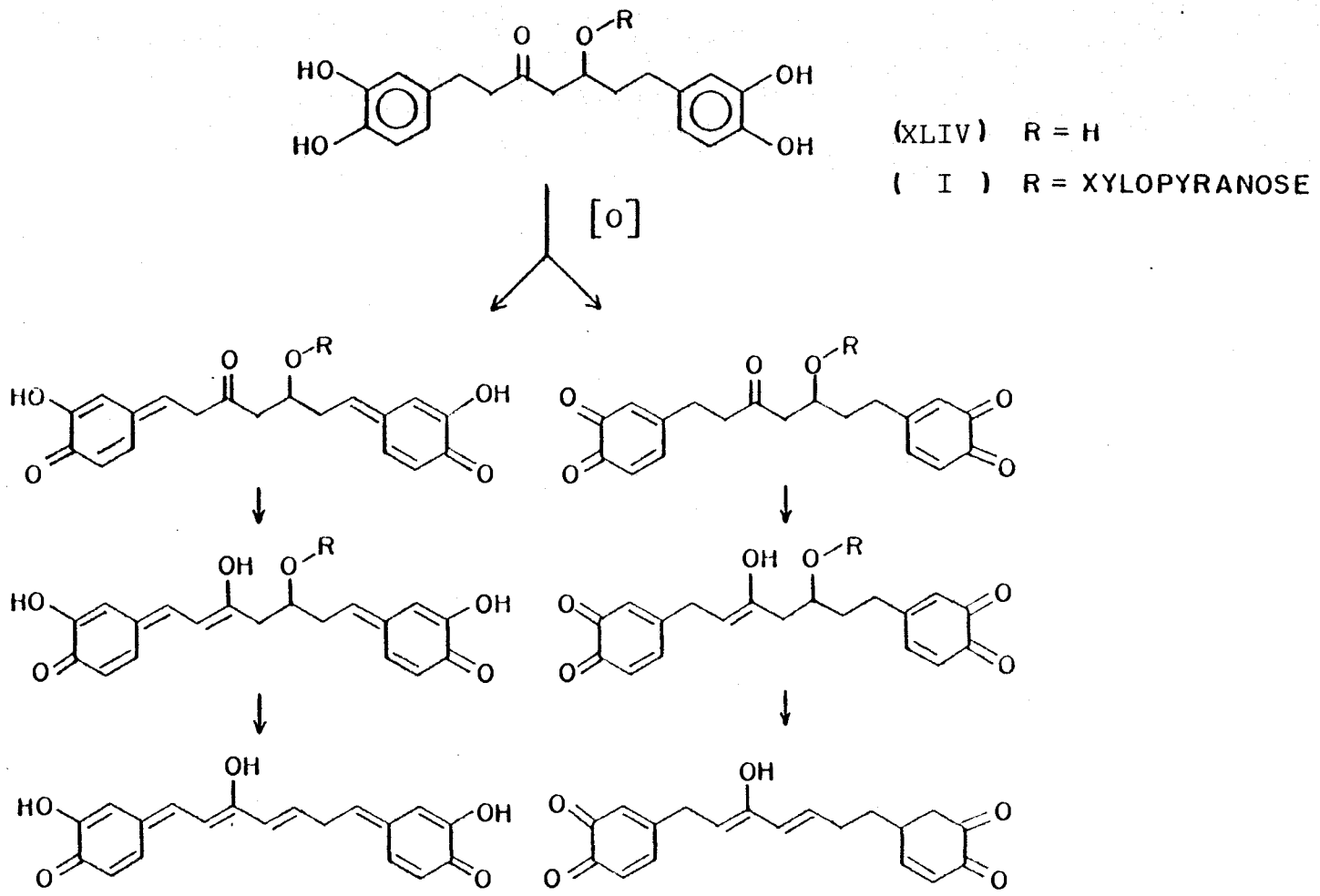
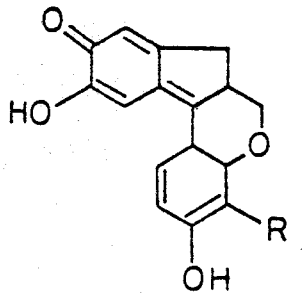
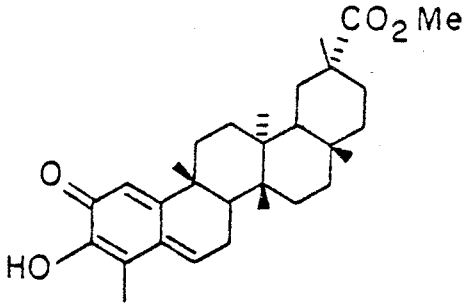



Figure 3. Chromophor development.

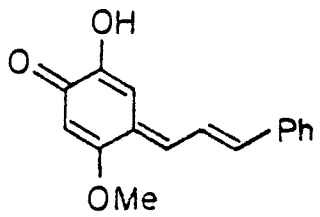
absorptions indeed dictate further investigation of these types of chromophoric compounds.



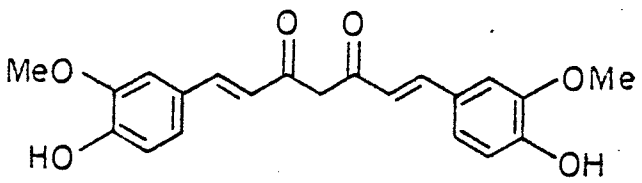

(XLV) R = H $\lambda$ MAX 412 ( 73 )
(XLVI) R = OH $\lambda$ MAX 430 (20,73)




(XLVII) $\lambda$ MAX 423 ( 34 )




(XLVIII) $\lambda$ MAX 399 ( 32 )




( II ) $\lambda$ MAX 420 (16,64)

The oxidative state structure, ortho-quinone, is another possibility for consideration. This chromophoric moiety, like the quinone methide, could only be the start of conjugation, however, since alone the absorption would not give the needed coloration of the stain (Figure 3).

Another possibility is the formation of a 9-phenyl-perinaphthenone (Figure 4) through, the loss of the xylose moiety, dehydration to an α-β unsaturated ketone (XLIX),

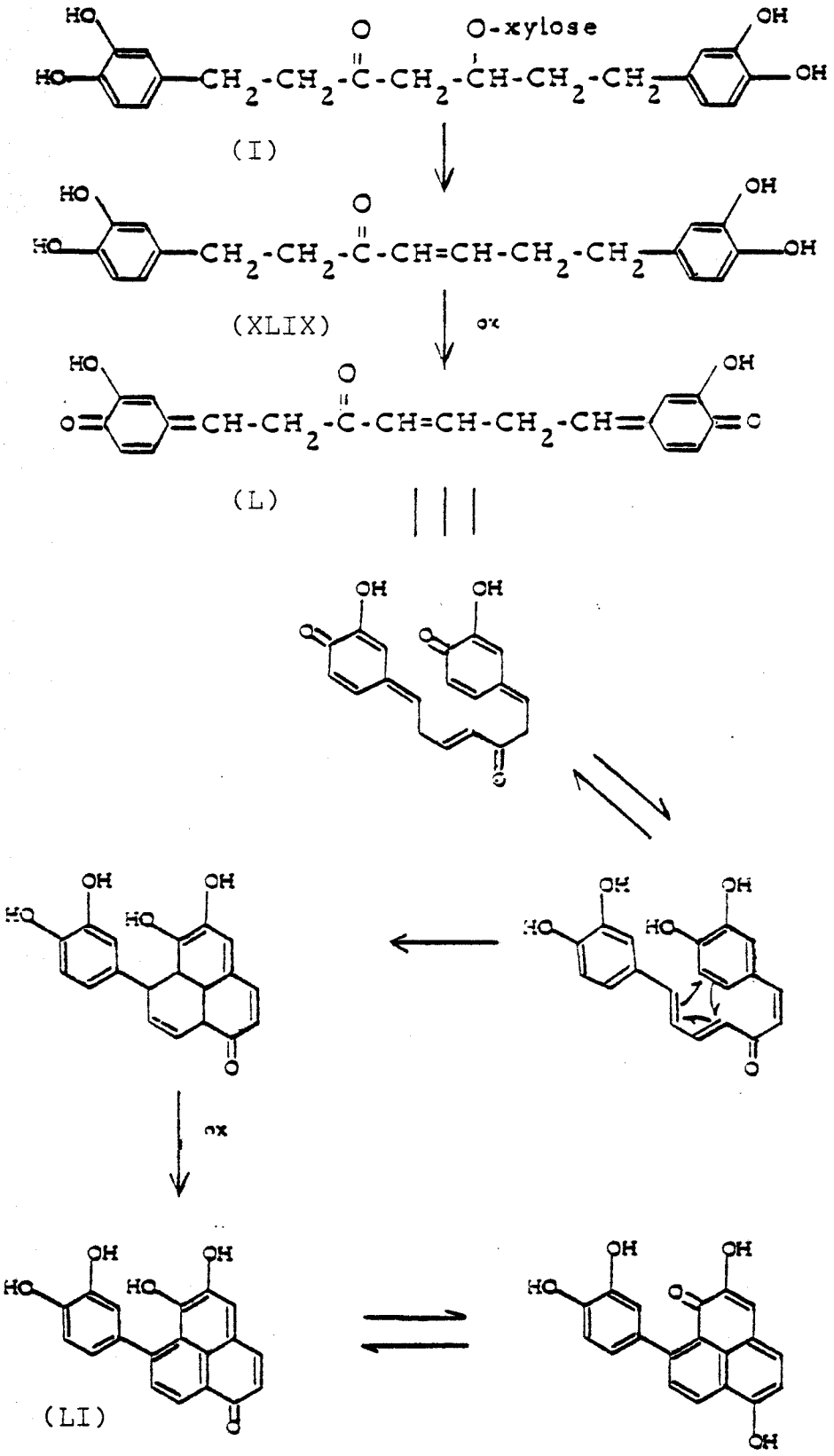


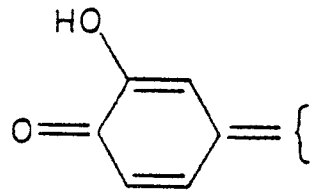

Figure 4. Formation of a 9-phenylperinaphthenone [from Karchesy (50)].

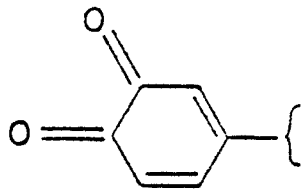
oxidation (L), a Diels-Alder reaction and then further oxidation which would yield LI (50). The following table (Table 1) is a summary of the above possibilities.

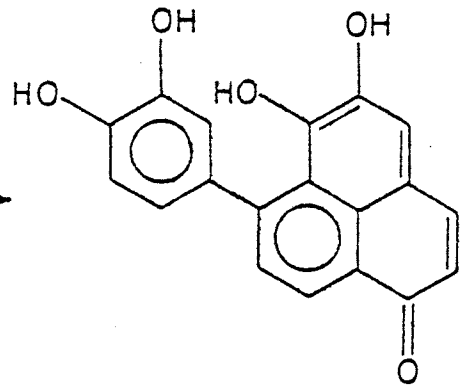
In order to test the hypotheses of quinone methide and/or ortho-quinone formation, reduced curcumin 1,7-bis(4-hydroxy-3-methoxyphenyl)heptane-3-one-5-ol (XXXII) was synthesized in this laboratory. This compound would only allow the quinone methide to form upon selective oxidation. In studies of quinone methides Buchran, Findlay and Turner (14) found that when guaiacylacetone (LII) was treated with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ) in aqueous dioxane an oxidized "intractable" mixture formed. When the reaction was done in anhydrous dioxane a polymerized product resulted. The above reactions thus indicate the formation of the extended quinone (LIII), which simply polymerized through oxidative coupling.

A reaction of DDQ with reduced curcumin may very well reveal a chromophoric moiety. Then comparing the spectral data of the oxidized compounds with the staining chromophor [$\lambda_{max}$ 433 (MeOH), 478 (phosphate buffer pH 6)] (50) this should tell us if we are on to the right idea. Further oxidation with silver oxide or an enzyme, e.g. peroxidase should further conjugate the chromophore (Figure 3). This we also should check against the spectral data of "Oregon Orange" (50).

Table 1. Possible pathways of color formation.

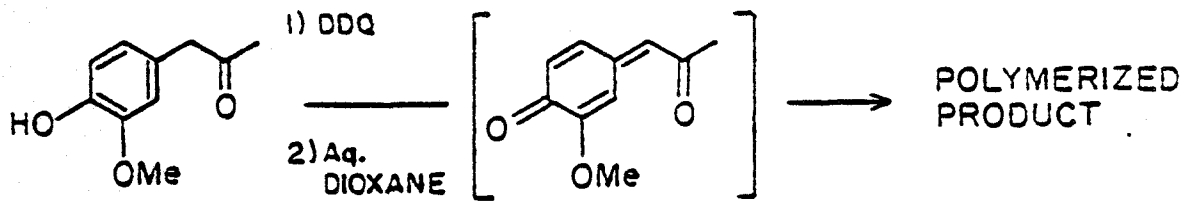
A. QUINONE METHIDE ⟶ 

B. ORTHOQUINONE ⟶ 

C. 9-PHENYLPERINAPTHENONE ⟶ 

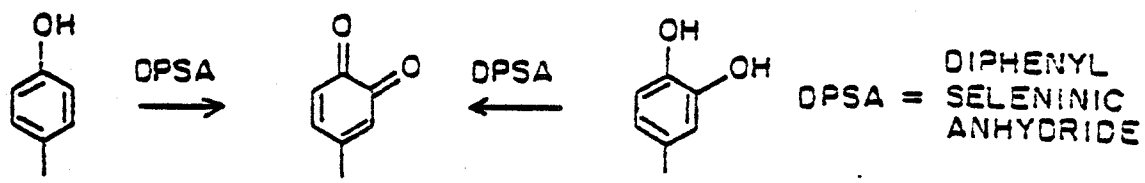
D. POLYMERIZATION

E. COMBINATIONS

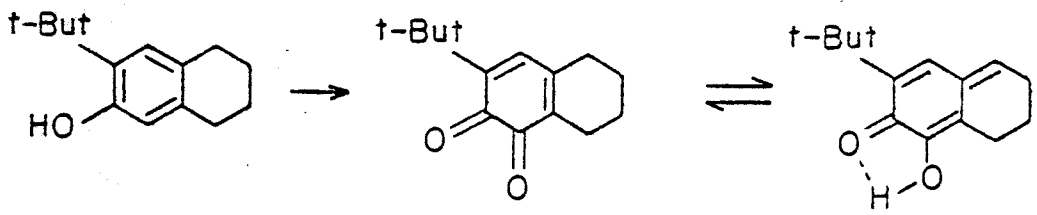



( LII ) ( LIII)

To create the ortho-quinone, 1,7-bis(3,4-dibenzyloxyphenyl)hepta-1,6-diene-3,5-dione (XXVII) was synthesized in this laboratory. The benzyl ether groups were to be removed with hydrogen ($H_2$) and platinum on carbon (Pt/C) and the resulting 1,7-bis(3,4-dihydroxyphenyl)hepta-1,6-diene-3,5-dione was to be treated with an oxidizing agent to yield the ortho-quinone. Barton, Brewster, Levy and Rosenfeld (5) have used the mild phenol oxidizing agent, diphenylseleninic anhydride (DPSA), to create ortho-quinones. The initial study was done on mono-hydroxy aryl compounds and the resulting ortho-quinones were stable. Further studies with this mild agent on dihydroxy aryl compounds (catechols) showed that they too were oxidized to the ortho-quinones.



Findlay and Turner (27) also studied ortho-quinones but they used Fremy's salt to oxidize LIV. An unstable orange oil was produced, which appeared from the spectral data to be an ortho-quinone which would be LV. In air and under nitrogen the oil decomposed. Spectral data showed that decomposition was via the tautomeric hydroxyquinone methide which would be (LVI). The above reaction could indeed be analogous to the staining phenomenon of red alder.




(LIV) (LV) (LVI)

If the dihydroxy compound LVII, under the influence of enzymes released during injury, goes directly to the ortho-quinone LVIII, the reaction sequence shown in Figure 5 might occur. In the first steps the xylose moiety of oregonin may or may not be cleaved but under possible acidic conditions resulting from injury the xylose function would surely be cleaved leaving the fully conjugated chromophore LIX. The above reactions could cause the pattern needed for the staining chromophore. Clearly, having confirmed the structure of oregonin now provides the basis for further studies into the mechanisms of the staining reaction.

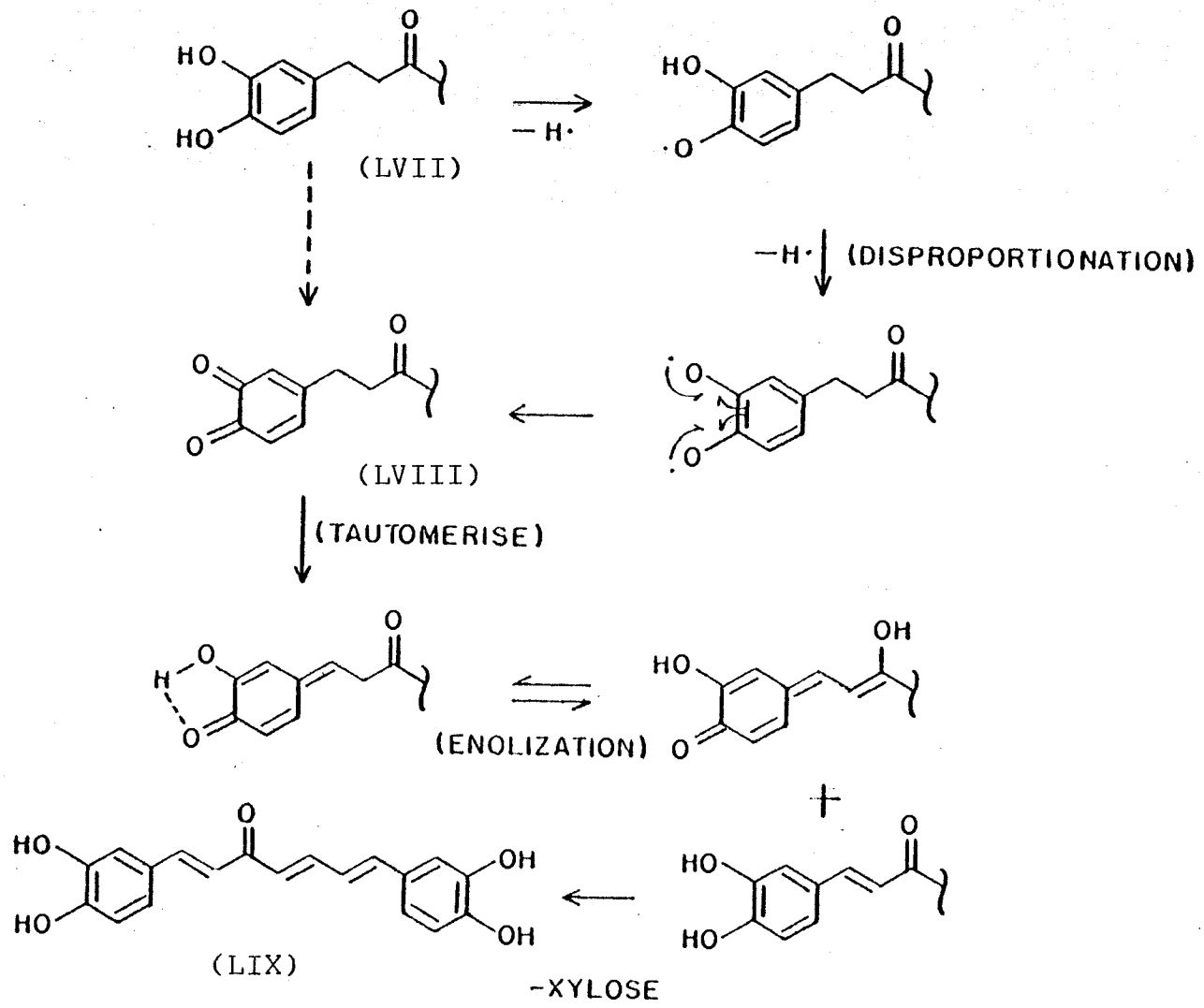



Figure 5. Possible formation of a chromophore.

Considering biogenetic possibilities (24) the third prospect in the scheme is that the stain forms from the loss of the glycoside function with subsequent formation of a 9-phenylperinaphthenone (LI) as mentioned by Karchesy (50) and discussed earlier in this work. Therefore, in consideration for future work a scheme for the synthesis of the 9-phenylperinaphthenone (LI) is outlined in Figure 6.

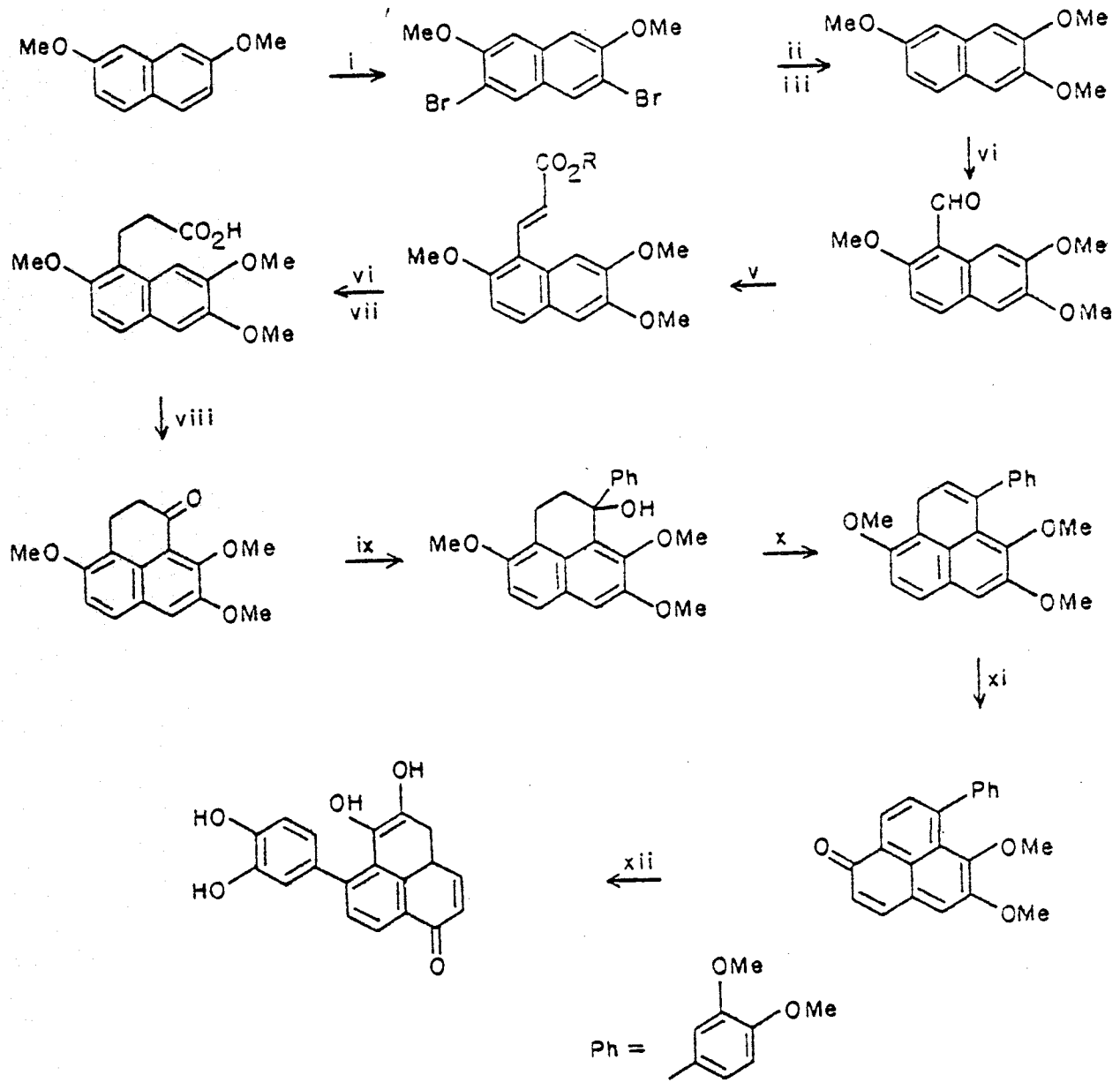



Figure 6. Synthesis of proposed 9-phenylperinaphthenone thought to be derived from oregonin. Reagents: (i) $Br_2$, HOAc, Sn; (ii) n-BuLi-$Et_2O$, $CH_3BH_2$, $H_2O$, $H_2O_2$, $CH_3OH$; (iii) $CH_2N_2$-$Et_2O$; (iv) $POCl_5$, DMF, toluene, NaOAc; (v) $Ph_3PCH_2CO_2Et$, benzene; (vi) $H_2$-Pd/C; (vii) KOH, $H_2O$, EtOH; (viii) poly $H_3PO_4$; (ix) PhMgBr, $Et_2O$; (x) HCl, $H_2O$; (xi) DDQ; (vii) $BBr_3$, $Et_2O$.

## Experimental

General Methods.--Melting points were determined on a Thomas-Houver micro capillary apparatus. Optical rotations were measured in 1-dm, semimicro tubes with a Perkin-Elmer No. 141 polarimeter. Infrared spectra were recorded with a Beckman spectrophotometer, Model IR-20A. $^{1}H$ nmr spectra were recorded at 60 MHz with a Varian EM-360, 80 MHz with a Varian FT-80T and at 100 MHz with a Varian HA-100 instrument. $^{13}C$ nmr spectra were recorded at 23.7 MHz on a Varian FT-80T spectrometer. The solvents used were chloroform-d, pyridine-$d_5$ and acetone-$d_6$ each containing up to 2% of tetramethylsilane (TMS) as internal standard.

Mass spectra (70 eV) were recorded, at low resolution, with a Varian MATCH-7 (single focus, electron impact with a source-temperature of 175°), and a Finnigan-4023 (quadrupole, chemical ionization with methane gas and a source temperature of 300°), and at high resolution with a CEC-21-110B (double focusing-electron impact). The field desorption mass spectra were recorded on a Hitachi Perkin-Elmer RMU-6E mass spectrometer converted to field desorption operation. The accelerating voltages were +2 kV applied to the anode (emitter) and -7 kV applied to the cathode. Emitter heating currents were varied between 0 and 16 mA. The instrument is housed at the Oregon Graduate Center, Beaverton, Oregon. Karchesy (50) gave a good description

of the operation and principles of field desorption mass spectrometry.

Evaporations were conducted in vacuo with a rotary evaporator with the bath temperature kept below 40°. Ethyl acetate and 1,2-dichloroethane were dried with calcium chloride, decanted, and distilled from phosphorus pentaoxide. Chloroform was washed six times with water then distilled from calcium chloride. Acetonitrite was dried with calcium hydride decanted and distilled. All of these solvents were stored over 4Å molecular sieve.

Chromatographic methods.--Thin-layer chromatography (tlc) was performed on precoated plates of Silica Gel 60F-254, 0.25 mm thick (E. Merck AG, Darmstadt, Germany). The plates supplied were cut to a length of 6 cm before use, but otherwise were used without pretreatment. All proportions of solvent are v/v: solvent A benzene/acetone 9:1; solvent B benzene/acetone 6:1. Preparative-layer chromatography (plc) was performed on precoated Silica Gel 60F-254, PLC plates, 2 mm thick (Merck). The spray reagent was sulfuric acid/methanol 1:5, and after spraying the plates were heated to 100°. Iodine crystalls were also used as a detection system. When the plates were developed more than once, they were dried in air between each development. Elution of the desired spots from plc plates

was achieved by eluting the silica, which was placed in a 250-ml chromatography column, with the appropriate solvent.

Syntheses.--3,4-Diacetoxybenzaldehyde (XXI):--3,4-Dihydroxybenzaldehyde (13.8 g, 0.1 mole) was dissolved in a 1.0 N aqueous potassium hydroxide solution (200.0 ml) in a 1000-ml separatory funnel. While cooling under cold water a solution of diethyl ether (400 ml) containing acetic anhydride (20.4 g, 2 moles) was added to the separatory funnel and shaken. As soon as the dark color of the water layer disappeared, the water layer was removed. The diethyl ether layer was shaken with sufficient 1.0 N aqueous potassium bicarbonate solution to neutralize the mixture. The aqueous phase was again removed and the diethyl ether layer was dried with anhydrous sodium sulfate. The diethyl ether was decanted and evaporated *in vacuo* at room temperature. The remaining slightly yellow oil crystallized with scraping. The crystals, or sometimes the uncrystallized oil, was dissolved in 95% ethanol (200 ml), decolorized with bone charcoal and filtered. The ethanolic solution was mixed with hot water (60°, 500 ml). The resulting solution was stored at 0° until white crystals formed (approximately 1 week): m.p. 54° [lit. 54° (68)], $C_{11}H_{10}O_5$ requires: C, 59.45 H, 4.53 found: C, 59.47 H, 4.57, $\nu_{max}^{KBr}$ 1764, 1680, 1590, 1475, 1410, 1350, 1243, 1164, 1080, 994 $cm^{-1}$, $\delta(CDCl_3)$ 2.25 (6H, s), 7.35-7.65 (3H, m), 9.9

(1H, s), $M^+$ 220.053 ($C_{11}H_{10}O_5$ requires 220.053), $^{13}C$ ($CDCl_3$) ppm from TMS: 20.33, 20.43, 124.05, 124.12, 127.82, 134.79, 142.86, 147.01, 167.28, 167.62, 189.79.

1,7-Bis(3,4-diacetoxyphenyl)hepta-1,6-diene-3,5-dione (XXV):--3,4-Diacetoxybenzaldehyde (4.44 g, 0.02 mole) and tributylborate [9.6 g (11.25 ml), 0.04 mole] were dissolved in freshly dried ethyl acetate (10 ml). Acetyl acetone (1.0 g, 0.01 mole) and boric anhydride (0.5 g, 0.007 mole) were mixed into a paste and the paste was added to the ethyl acetate solution. The reaction mixture was stirred while butylamine (0.2 ml) was added dropwise over a fifteen minute period. Stirring was continued for 4 hr and the mixture was let stand overnight.

Aqueous hydrochloric acid (0.4 N, 15 ml) was added and the mixture was stirred and then warmed at 60° for 60 min. The layers were separated and the aqueous layer extracted (three times) with ethyl acetate (100 ml). The ethyl acetate layers were combined and washed free of acid. The solution was evaporated to about 10 ml and about 5 ml of methanol was added. Light yellow plates formed on standing (0.8 g). The mother liquor was not used for further recrystallizations. Recrystallization from ethyl acetate-methanol (3:1, v/v) gave yellow needles: m.p. 193-195°, $C_{27}H_{24}O_{10}$ requires: C, 63.77 H, 4.75 found: C, 63.05 H, 4.69, $\nu_{max}^{KBr}$ 1750, 1610, 1540, 1500, 1485, 1400, 1350,

1265, 1234, 1190, 1185, 990, 950, 880, $cm^{-1}$, $\delta$ ($CDCl_3$) 2.3 (12 H, s), 5.81 (1 H, s), 6.56 (1H, d, $\underline{J}$ = 16 Hz), 7.18-7.48 (6H, m), 7.60 (1H, d, $\underline{J}$ = 16 Hz), 15.31 (1H, br), $^{13}C$ in ppm from TMS ($CDCl_3$): 20.55, 102.01, 122.68, 123.98, 125.32, 126.41, 134.03, 138.80, 138.85, 142.75, 143.57, 167.79, 167.89, 183.02.

1,7-Bis(3,4-diacetoxyphenyl)heptane-3,5-dione (XXVIII):-- 1,7-Bis(3,4-diacetoxyphenyl)hepta-1,6-diene-3,5-dione (0.05 g) was dissolved in ethyl acetate (10 ml) and 10% palladium on charcoal (10 mg) was added. The metallic green reaction mixture was stirred under 1 atmosphere of hydrogen overnight. The reaction mixture was filtered through Celite on a sintered glass funnel and the filtrate was evaporated to give 0.053 g of a clear oil.

1,7-Bis(3,4-diacetoxyphenyl)heptane-3-one-5-ol (XX):-- 1,7-Bis(3,4-diacetoxyphenyl)heptane-3,5-dione (0.1 g) was dissolved in methanol (5 ml) and stirred while 10 mg of sodium borohydride in 2 ml of methanol was added dropwise. The reaction mixture was allowed to stand for 30 min. Water (10 ml) was added and the reaction mixture was warmed on a hot plate for 5 min and then evaporated to about 5 ml under reduced pressure. Diethyl ether (25 ml), was added and the layers separated. The aqueous layer was extracted twice more (25 ml each) with diethyl ether and the

combined diethyl ether solutions were dried (anhydrous sodium sulfate) and reduced in volume. They were then applied to preparative silica tlc plates.

Four spots were resolved: solvent A, $R_f$ 0.52, 0.45, 0.19, 0.10. Collection of the material at $R_f$ 0.45 and elution with acetone-chloroform-ethyl acetate gave 0.03 g of a white crystalline material. Recrystallization from methanol gave white plates: m.p. 153-156°, $C_{27}H_{30}O_{10}$ requires: C, 63.30 H, 5.84 found: C, 63.18 H, 5.63, $M^+$ 514.3 ($C_{27}H_{30}O_{10}$ requires 514.5).

3,4-Dibenzyloxybenzaldehyde (XXIV):--A mixture of protocatechinic aldehyde (25.0 g), of benzyl bromide (50.0 ml), dry butanol (50.0 ml) and anhydrous potassium carbonate (60.0 g) were added to a 3-necked 300-ml round-bottomed flask. This mixture was refluxed with vigorous stirring for 24 hr. The mixture, now dark brown, was poured and scraped into water and extracted with ethyl acetate (2 x 100 ml) and diethyl ether (2 x 100 ml). The organic layer was washed several times with saturated sodium chloride solution then with 5% aqueous sodium hydroxide solution, until the basic wash water was slightly colored. Removal of the solvent left a viscous dark brown oil that soon solidified. This residue was extracted repeatedly with boiling petroleum ether (60-80° b.p.). Chilling of these extracts caused precipitation of 3,4-dibenzyloxybenzaldehyde.

These crystals were dissolved in acetone and water was added until cloudy. The beaker was sealed with parafilm and placed in the refrigerator at 5°. Light tan colored crystals formed: m.p. 92-93°, $C_{21}H_{18}O_3$ requires: C, 79.2 H, 5.70 found: C, 79.1 H, 5.67, $\nu_{max}^{KBr}$ 1868, 1810, 1672, 1583, 1510, 1421, 1384, 1345, 1263, 1240, 1160, 1130, 1021, 930, 898, 850, 814, 750, 730, 686, 654, 620, $\delta$ [$(CD_3)_2CO$] 2.95 (1H, s), 5.25 (4H, d, $\underline{J}$ = 4 Hz), 7.42 (13H, m), 9.84 (1H, s), $M^+$ 318.125 ($C_{21}H_{18}O_3$ requires 318.126). $^{13}C$ in ppm from TMS [$(CD_3)_2CO$]: 71.5, 71.7, 113.9, 114.5, 126.12, 128.3, 128.45, 128.65, 129.2, 129.5, 129.6, 131.65, 137.88, 138.11, 150.26, 155.32, 191.25, 206.28.

1,7-Bis(3,4-dibenzyloxyphenyl)hepta-1,6-diene-3,5-dione (XXVII):--3,4-Dibenzyloxybenzaldehyde (2.0 g, 6.3 mmole), tributyl borate (2.9 g, 3.38 ml, 12.6 mmole), and the reaction product of acetyl acetone (0.32 g, 3.15 mmole) and boric anhydride (0.15 g, 2.21 mmole) were dissolved in ethyl acetate (3.15 ml). Butylamine (0.063 ml) was added over a period of 40 min while stirring. Stirring was continued for 4 hr.

Hydrochloric acid (0.4 N, 15 ml) was added and the mixture was stirred and then warmed at 60° for 60 min. The layers were separated and the aqueous layer extracted (three times) with 100-ml portions of petroleum ether (60-80° b.p.). The petroleum ether layers were combined with

the original ethyl acetate layer and washed free of acid with water. The organic solution was evaporated to about 15 ml and about 5 ml of water was added. Light tan colored needles formed on standing: m.p. 167-170°, $C_{47}H_{32}O_6$ requires: C, 79.8 H, 6.56, found: C, 79.5 H, 6.41, $M^+$ 706.317 ($C_{47}H_{32}O_6$ requires 706.329).

3,4-Dimethoxybenzaldehyde (XXII):--Recrystallization of commercially available material was as follows: dimethoxybenzaldehyde (50.0 g) was heated with technical petroleum ether (b.p. 60-80°) (200 ml) until boiling. After about 50 ml of the petroleum ether had evaporated the flask was removed from the hot plate and allowed to cool. After the solution had separated and cleared, the upper layer was separated into a beaker. White needles formed within one hour, m.p. 43-44°.

1,7-Bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione (XXVI):--3,4-Dimethoxybenzaldehyde (33.2 g, 0.2 moles) and tributylborate (92.0 g, 105.0 ml, 0.4 mole) were dissolved in 100.0 ml of freshly dried ethyl acetate. Acetyl acetone (10.0 g, 0.1 mole) and boric anhydride (5.0 g, 0.14 mole) were mixed into a paste and the paste was added to the ethyl acetate solution. The reaction mixture was stirred while 2.0 ml of butylamine was added dropwise over a period of 15 min. Stirring was continued for 4 hr and the mixture

was allowed to stand overnight. The solution color was a bright deep orange and a curd precipitate formed. Hydrochloric acid (0.4 N, 150.0 ml) at 60° was added and the mixture was stirred for 1 hr. The mixture was poured into a 1000-ml separatory funnel and the layers separated. The yellow precipitate was recovered and washed with ethyl acetate (3 x 100 ml). The combined ethyl acetate layers were washed with water until free of acid and evaporated to about 150 ml. Methanol (100.0 ml) was added and, after 3 hr in the refrigerator, orange crystals formed. The crystals were recovered by filtration, washed with cold methanol until the wash liquid was yellow, and dried, to yield 25 g (62%) of light orange needles, m.p. 130-131° (50), $C_{23}H_{24}O_6$ requires C, 69.67, H, 6.11 found C, 69.55, H, 6.09 $\lambda_{max}^{EtOH}$ 262, 420 nm, $\nu_{max}^{KBr}$ 1632, 1590, 1513, 1467, 1446, 1425, 1137, 1025 $cm^{-1}$, $M^+$ 396.159 ($C_{23}H_{24}O_6$ requires 396.157), δ ($CDCl_3$) 15.86 (<1$H_1$ br, enolic OH), 7.60, (2H, d, $\underline{J}$ = 16 Hz), 7.21-6.81 (6H, m), 6.49 (2H, d, $\underline{J}$ = 16 Hz), 5.81 (0.9 H, s), 3.91 (6H, s), 3.80 (6H, s), 3.49 (0.2H, s).

<u>1,7-Bis(3,4-dimethoxyphenyl)heptane-3,5-dione (XXIX)</u>:-- 1,7-Bis(3,4-dimethoxyphenyl)hepta-1,6-diene-3,5-dione (2.0 g) was dissolved in 50.0 ml of ethyl acetate and 70.0 mg of 10% palladium on charcoal was added. The metallic green reaction mixture was stirred under 1 atmosphere of

hydrogen overnight. The reaction mixture was filtered by means of a sintered glass funnel containing a pad of Celite and the filtrate was evaporated to give 2.05 g of crystalline material. Recrystallization from methanol gave white plates, m.p. 68-69° (50) $C_{23}H_{28}O_6$ requires C, 68.97, H, 7.05 found C, 68.96 H, 7.00, $\lambda_{max}^{EtOH}$ 228, 280 $\nu_{max}^{KBr}$ 1612, 1598, 1515, 1468, 1457, 1444, 1157, 1141, 1028 $cm^{-1}$, $M^+$ 400.187 ($C_{23}H_{28}O_6$ requires 400.189), $\delta$ ($CDCl_3$) 15.48 (0.8H, br), 6.87-6.63 (6H, m), 5.44 (0.8H, s), 3.85 (12H, s), 3.06 (0.4H, s), 3.05-2.46 (8H, m). $^{13}C$ in ppm from TMS (pyridine-$d_5$) 29.5, 31.4, 45.3, 56.4, 56.5, 67.2, 100.1, 104.8, 105.1, 105.2, 121.2, 121.3, 123.5, 150.1, 200.7.

1,7-Bis(3,4-dimethoxyphenyl)heptane-3-one-5-ol (XXXI):-- 1,7-Bis(3,4-dimethoxyphenyl)heptane-3,5-dione (2.0 g) was dissolved in methanol (200.0 ml) and stirred while 80.0 mg of sodium borohydride in 10.0 ml of methanol was added dropwise. The reaction mixture was allowed to stand for 35 min. Water (40.0 ml) was added and the reaction mixture was warmed on a hot plate for 5 min followed by evaporation under reduced pressure to about 100 ml in volume. Diethyl ether (100 ml) was added and the layers separated. The aqueous layer was extracted with diethyl ether (2 x 100 ml) and the combined diethyl ether solutions were dried (anhydrous sodium sulfate) and reduced in volume. They were then applied to preparative silica plc plates. Four

spots were resolved; solvent A $R_f$ 0.63, 0.35, 0.20, 0.15. Collection of the material at $R_f$ 0.35 and elution with acetone-chloroform gave 0.6 g of a white crystalline material. Recrystallization from methanol gave white plates, m.p. 99-100° [lit (50) 99-100°] $C_{23}H_{30}O_6$ requires C, 68.62 H, 7.52 found C, 68.58 H, 7.50 $M^+$ 402.206 $C_{23}H_{30}O_6$ requires 402.204; FD-MS m/e major peaks, 403 (largest), 385, 263, 201.5, 187. $^{13}C$ in ppm from TMS (pyridine-$d_5$) 30.0, 32.4, 40.6, 45.0, 51.6, 55.9, 56.8, 56.9, 67.9, 114.0, 114.2, 114.5, 114.6, 121.3, 121.5, 123.8, 123.9, 135.0, 136.0, 150.4, 209.4.

1,7-Bis(3,4-dimethoxyphenyl)heptane-3,5-diol (XXXIV):-- Material corresponding to $R_f$ 0.20 from above was removed from preparative plc plates and were eluted from the silica with acetone. Evaporation of the acetone gave 0.5 g of a viscous oil which crystallized on standing. Recrystallization from 70% methanol-water gave white plates, m.p. 85-88°, $C_{23}H_{32}O_6$ requires C, 68.28 H, 7.98 found C, 68.23 H, 7.78, $\lambda_{max}^{EtOH}$ 229, 280 $\nu_{max}$ ($CHCl_3$) 3624, 3500, 1593, 1510, 1464, 1453, 1442, 1418, 1253, 1240, 1027, 851 $cm^{-1}$, $\delta$ ($CDCl_3$) 15.48 (1H, br), 6.87-6.63 (6H, m), 4.02 (2H, m), 3.85 (12H, s), 2.92 (2H, s), 2.84 (4H, m), 1.77 (6H, m), $M^+$ 400.187 ($C_{23}H_{32}O_6$ requires 400.189).

1,7-Bis(3,4-dimethoxylphenyl)heptane-3,5-diol (XXXIV):-- Material corresponding to $R_f$ 0.15 from above was removed

from preparative plc plates and were eluted from the silica with acetone. Evaporation of the acetone gave 0.4 g of a viscous oil which crystallized on standing. Recrystallization from 70% methanol water gave white plates, m.p. 96-98° $C_{23}H_{32}O_6$ requires C, 68.28 H, 7.98, found C, 68.23 H, 7.80, $\lambda_{max}^{EtOH}$ 229, 280, $M^+$ 400.187, ($C_{23}H_{28}O_6$ requires 400.189.

1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (XXIII):--4-Hydroxy-3-methoxybenzaldehyde (6.08 g, 0.04 mole) and tributyl borate (2.55 g, 0.011 mole) were dissolved in 100.0 ml of dry ethyl acetate. The compound formed from 10.0 g of acetylacetone (0.1 mole) and 5.0 g of boric anhydride (0.07 mole) was added and the mixture stirred for 5 min. While stirring, 0.5 ml of butylamine were added dropwise every 10 min (total of 2.0 ml). Stirring was continued for 4 hr, after which time the mixture was allowed to stand overnight.

The next day 100.0 ml of 0.4 N hydrochloric acid at 60° was added and the mixture was stirred for 60 min. The layers were separated, and the aqueous layer was extracted three times with 50.0 ml portions of ethyl acetate. The combined ethyl acetate layers were washed with water until free of acid and evaporated to about 70 ml. Ethanol (50.0 ml) was added and, after 3 hr in the refrigerator, the crystals which had formed were recovered by filtration, washed with cold methanol until the wash liquid was yellow, and dried: yield 5.20 g (62%) orange-crystals, m.p. 184° $C_{21}H_{20}O_6$ requires C, 68.48 H, 5.44 found C, 68.40 H, 5.39,

$\lambda_{max}^{EtOH}$ 268, 430, δ [$(CD_3)_2CO$] 7.58 (2H, d, J = 16 Hz), 7.30 (2H, d, J = 2Hz), 7.6 (2H, dd, J = 2Hz and 8 Hz), 6.86 (2H, d, J = 8Hz), 6.66 (2H, d, J = 16 Hz), 5.96 (1H, s), 3.91 (6H, s), $M^+$ 3.68.125 $C_{21}H_{20}O_6$ requires 3.68.126.

1,7-Bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-dione (XXX):--1,7-Bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (0.6 g) was dissolved in ethyl acetate (20.0 ml) and 10% palladium on charcoal (50.0 mg) was added. The metallic green reaction mixture was stirred under 1 atmosphere of hydrogen for 2 days. The reaction mixture was filtered through Celite on a sintered glass funnel and the filtrate was evaporated to give 0.61 g of a white material. The solid was dissolved in methanol (50.0 ml) and reduced in volume *in vacuo* (bath temp 40°) until the solution became cloudy. This solution was placed in the refrigerator at 5° for 3 days. White crystals (0.55 g) formed: m.p. 94-95° [lit 95-96° (75), 96° (94)], ($C_{21}H_{24}O_6$ requires C, 67.74 H, 6.45 found C, 67.53 H, 6.40, δ ($CDCl_3$) 6.81 (2H, d, J 8Hz, 10, 10'-Hz), 6.66 (2H, s), 6.61 (2H, d, J 8 Hz), 5.54 (2H, ArOH), 5.40 (2H, s), 3.83 (6H, s) and 3.0-2.4 (8H, m), $M^+$ 372.0 $C_{21}H_{24}O_6$ requires 372.0.

1,7-Bis(4-hydroxy-3-methoxyphenyl)heptane-3-one-5-ol (XXXII):--1,7-Bis(3-methoxy-4-hydroxyphenyl)heptane-3,5-dione (0.2 g) was dissolved in methanol (10.0 ml) and

stirred while sodium borohydride (30.0 mg) in methanol (4.0 ml) was added dropwise over a fifteen minute period. The reaction mixture was allowed to stand for 30 min. Water (10.0 ml) was added and the reaction mixture was warmed on a hot plate for 5 min followed by evaporation to about 5 ml under reduced pressure. Diethyl ether (25.0 ml) was added and the layers separated. The aqueous layer was extracted twice more (10 ml each) with diethyl ether and the combined diethyl ether solutions were dried (anhydrous sodium sulfate) and reduced in volume. The solution was applied to preparative silica plc plates. Four spots were resolved; Solvent A $R_f$ 0.55, 0.47, 0.32, 0.10. Collection of the material at $R_f$ 0.47 and elution with acetone-benzene 6:1 gave 0.10 g of a white crystalline material. Recrystallization from methanol gave white plates, m.p. 80°. [lit 78-80° (75)] $C_{21}H_{26}O_6$ requires: C, 67.4; H, 6.95 found C, 67.3; H, 6.8, $M^+$ 374. $\delta$ ($CDCl_3$) 6.79 (2H, d, $\underline{J}$ 8 Hz), 6.65 (2H, s), 6.61 (2H, d, $\underline{J}$ = 8 Hz), 5.72 (2H, ArOH), 4.02 (2H, m), 3.81 (6H, s), 3.0-2.4 (8H, m) 1.68 (2H, m).

<u>1,7-Bis(4-hydroxy-3-methoxyphenyl)heptane-3,5-diol (XXXV)</u>:-- Material corresponding to $R_f$ 0.32 + 0.10 from above was removed from preparative plc plates and were eluted from the silica with acetone. Evaporation of the acetone gave 0.6 g of a viscous oil which crystallized on standing.

Recrystallization from 70% methanol gave a mixture of diastereomers, m.p. 75°.

2,3,4-Tri-O-acetyl-α-D-xylopyranosyl bromide (XXXVIII):--

Crystalline 1,2,3,4-tetraacetyl-β-D-xylopyranose (19) (10.0 g) was placed in a 100-ml round-bottomed flask and acetic anhydride (15.0 ml) was added. The flask was immersed in an ice-bath and anhydrous hydrogen bromide was passed into the mixture until the acetate had all dissolved. To the solution was added 10 g more of the tetraacetate and hydrogen bromide passed in until the solution was saturated at 0°. A calcium chloride drying tube was attached to the flask and it was allowed to stand for 3 hr in a hood at room temperature. Reduced pressure was applied from an aspirator to the flask which was immersed in a water-bath. The temperature of the water-bath was gradually raised to 45°. When the hydrogen bromide had been removed the material in the flask crystallized. The crystals were mixed well with absolute diethyl ether (50.0 ml) and cooled in a freezing bath. After fifteen minutes the crystals were removed by filtration and washed with a little cold absolute diethyl ether. The product was recrystallized by dissolving in the minimum amount of hot chloroform, adding four times the volume of absolute diethyl ether, and cooling in the refrigerator for 4 hr. A yield of 90% was

obtained with m.p. 101° [lit 98-99° (19), 102° (46)], $[\alpha]_D^{25}$ = + 211.1.

2,3,4-Tri-O-benzoyl-α-D-xylopyranosyl bromide (XL):--

Fifteen grams of crude α-D-xylopyranose tetrabenzoate (28) (m.p. 118-120°) was dissolved in ethylene dichloride (30.0 ml) in an Erlenmeyer flask. The solution was cooled in an ice-bath, treated with 30% hydrogen bromide (30.0 ml) in glacial acetic acid and allowed to stand tightly stoppered at room temperature for 2 hr. The solution was poured into a funnel containing ice which rested in the top of a 500-ml separatory funnel that contained ice water. The flask was rinsed with ethylene dichloride and poured through the funnel. The organic layer was then shaken with several portions of a saturated aqueous solution of sodium bicarbonate until no further effervescence occurred. The organic layer was washed with water, dried over anhydrous magnesium sulfate, and filtered. The 1,2-dichloroethane was removed under reduced pressure on a rotary evaporator. The solid which remained was dissolved in benzene (40.0 ml) at 40° and n-hexane (40.0 ml) was added. This solution was placed in the refrigerator for 3 days. Colorless needles formed: m.p. 136-137 [Lit 136-137 (19)] $[\alpha]_D^{25}$ + 118.5° in $CHCl_3$ c 2.15.

1,7-Bis(3,4-dimethoxyphenyl)heptane-3-one-5-O-2,3,4-tri-O-acetyl-β-D-xylopyranoside (XVIII):--A three-necked 25-ml round-bottomed flask was flamed with anhydrous argon gas passing through. To the mixture, 1,7-bis(3,4-dimethoxyphenyl)heptane-3-one-5-ol (0.072 g, 0.177 mmole), mercuric cyanide (0.034 g, 0.133 mmole) and calcium sulfate (0.05 g, dried at 500° for 5 hr then cooled over phosphorus pentoxide) in dry acetonitrile, was added 2,3,4-tri-O-acetyl-α-D-xylopyranosyl bromide (0.354 mmole, 0.142 g). After 2 hr of stirring at room temperature, with the exclusion of moisture (calcium chloride drying tube), sodium bicarbonate (0.02 g) was added and the mixture stirred for another 15 min. The mixture was then filtered, the solid washed with benzene and the filtrate concentrated. The residue was then partitioned between benzene and an aqueous 1 M solution of potassium bromide. The benzene layer was washed with water and concentrated. The concentrate was then applied to plc plates: solvent $R_f$ 0.20, 0.30, 0.41, 0.47, 0.72, collection of the material at 0.30 and elution with benzene-acetone 6:1 v/v gave 82 mg, 68% yield of a yellow oil, FD-MS m/e major peaks, 661 (largest), 445, 431, 403, 385, 331, 263, 209, 201.5, 193, 187. $[\alpha]_D^{25}$-6.9°. (c. 0.081 $CHCl_3$).

## Summary and Conclusions

The most likely precursor to the stain in red alder is considered to be a phenolic xyloside. The diaryl-heptanoid xyloside, oregonin (I) was found to be the major constituent of the red alder bark extract. Oregonin has been implicated in the formation of the stain by its ability to change color (orange-red) under the influence of enzymes and hydrolysis conditions. The structure elucidation of oregonin was confirmed with the synthesis of tetra-O-methyloregonin triacetate. Spectral data (ms, $^{1}H$, $^{13}C$ nmr) of the synthesized compound showed all similarities to the product derived from nature by Karchesy (50).

# II. CHARACTERIZATION OF SOME DOUGLAS-FIR TANNINS

## Historical Review

Previous work on Douglas-fir bark polyflavanoids concentrated on polyphenols from whole bark (21,31) and newly formed inner bark. Results of the studies (21) on whole bark suggested polymers dominated by (+)-catechin and (-)-epicatechin units linked by C-4 to C-8 (or C-6) bonds. However, as yet no one has separately studied inner and outer bark condensed tannins.

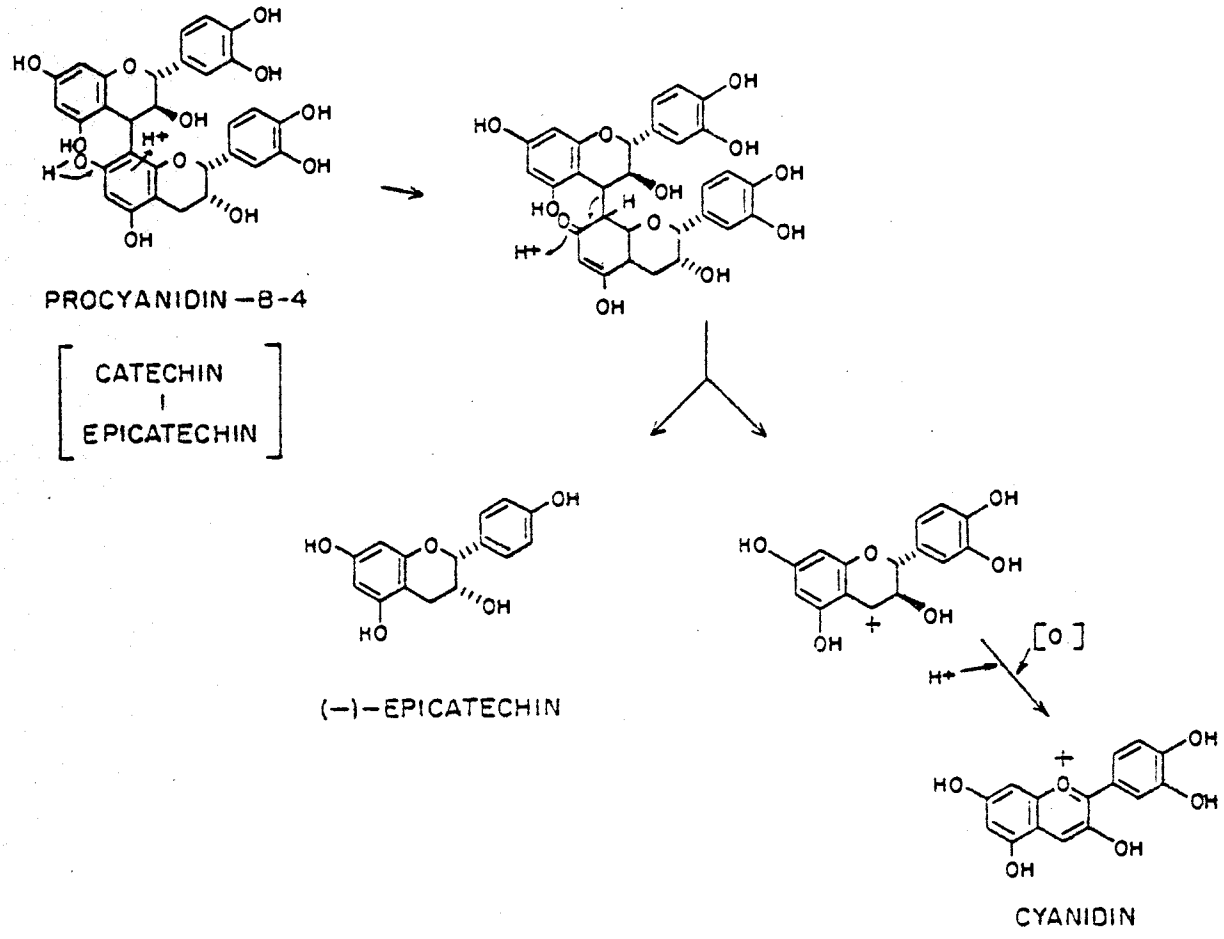
It has been suggested (52) that during the conversion of inner to outer bark, several processes could cause differences in the chemistry of polymers from the two tissues. Outer-bark polymers of structures that differ from those found in the inner bark may be formed during death of phloem parenchyma, or by the cork cambium in much the same manner as occurs in heartwood formation. In addition, secondary changes such as oxidation and further polymerization may occur during the many years that polyflavanoids are stored in the outer bark. All of these processes have complicated the problems of isolation and elucidation of the structures of the polymers in the outer bark. A thorough study of the polyflavanoids should include a characterization of the differences in the polymers of outer and inner bark. Although past years have seen a

considerable amount of interest centered on the chemical composition of conifer barks it was believed that additional knowledge concerning the structure of polyflavanoids would be valuable in aiding the possibility for using these polymers to replace phenol in wood adhesives.

## Results and Discussion

The purpose of this work was to characterize the procyanidins (tannins) of inner and outer Douglas-fir bark. Procyanidins are so named because they are chemically degradable to give the pigment cyanidin (Scheme 2). Many workers have shown the presence of complex mixtures of polyphenolic compounds in the tissues of plants with a woody habit of growth. Hergert (43) studied the chemical composition of tannins and polyphenols from conifer wood and bark by descending paper chromatography but failed to identify the tannins. Thompson, Jacques, Haslam, and Tanner (87), as a part of a program of biosynthetic studies, elaborated earlier surveys of Robinson and Robinson (73,74) and Bate-Smith and Lerner (8), and then correlated procyanidin distribution with that of (+)-catechin and (-)-epicatechin in the living tissues of twenty-nine plant species. The analyses were carried out in a number of cases by isolation and comparison with authentic samples, but more generally by two-dimensional paper chromatography.

In their studies, Thompson, Jacques, Haslam, and Turner (87) found many examples which showed one of the diastereomeric flavan-3-ols, (+)-catechin (LX) or (-)-epicatechin (LXI), (Figure 7) clearly in dominance and occasionally to the apparent exclusion of the other isomer. The procyanidins show a similar chromatographic pattern.




Scheme 2. Acid catalyzed degradation of procyanidin B-4.

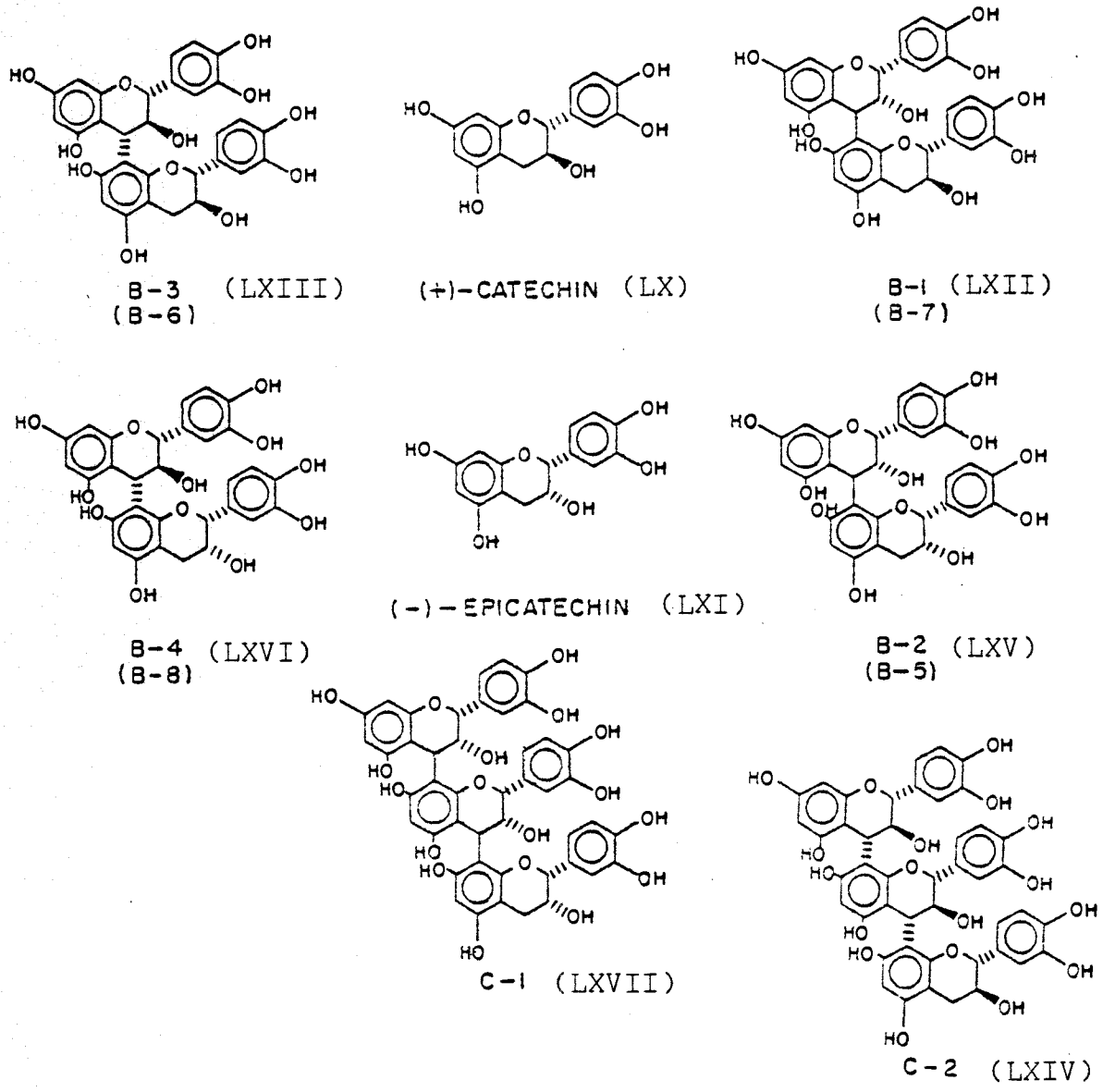



Figure 7. Bark procyanidins.

When there is a predominance of (+)-catechin there is also the presence of the dimers B-1 (LXII) and B-3 (LXIII) and the trimer C-2 (LXIV). Analogously when (-)-epicatechin predominates so do the dimers B-1 (LXII), B-2 (LXV) and B-4 (LXVI) and the trimer C-1 (LXVII). The most frequently recognised pattern was the co-occurrance of (-)-epicatechin with B-1, B-2 and C-1. In no case, the study showed, was (+)-catechin or (-)-epicatechin found without the simultaneous detection of one or more of the procyanidin dimers.

Several procyanidin oligomers have been described (91) and Weinges and his colleagues have isolated (91,87) as their acetates, four dimeric procyanidins B-1 (LXII), B-2 (LXV), B-3 (LXIII), and B-4 (LXVI), (Figure 7). The structures proposed for the dimers B-1, B-2, B-3, and B-4 were based on $^{1}H$ nmr and mass spectral data for the acetate and methyl ether derivatives. However, due to some interesting questions of biosynthesis and possible physiological functions, Thompson, Jacques, Haslam and Tanner (87) isolated and characterized the dimers, B-1, B-2, B-3, and B-4 in their free phenolic forms. They were then characterized by analysis, paper chromatography, $^{1}H$ nmr, and optical rotatory dispersion and circular dichroism measurements. The dimers B-5, B-6, B-7, and B-8 (linked C-4 to C-6) (Figure 7) were established by Thompson,

Jacques, Haslam and Tanner (87) as isomers of B-2, B-3, B-1 and B-4 respectively. The most valuable piece of information generated from the literature was the $R_f$ values of the procyanidins. With the observations above, the $R_f$ values, and the model chromatogram of the plant procyanidins (Figure 8) the characterization of bark procyanidins became systematic.

Traditionally, two-dimensional paper chromatography has been employed in the preliminary qualitative analyses of condensed tannins and associated compounds (6). Numerous solvent systems are employed, most of which are based on solvent pairs which effect separation in one direction by partitioning effects and in the other by adsorption effects. Acetic acid (aqueous 6%) followed by butan-2-ol-acetic acid-water (14:1:5 v/v) is usually the most diagnastic for procyanidins (87). Detection of phenolic compounds on chromatograms is commonly achieved by various spray reagents and with ultraviolet light. The use of specific spray reagents which identify certain functional groups by means of color reactions often provides considerable structural information (55). The bis-diazotised benzidine reagent affords information regarding the A nucleus when it consists of either phloroglucinol or resorcinol nuclei. It is also useful for confirming evidence derived from use of the Gibbs reagent (0.5% 2,6-dibromobenzoquinone-4-chloroimide

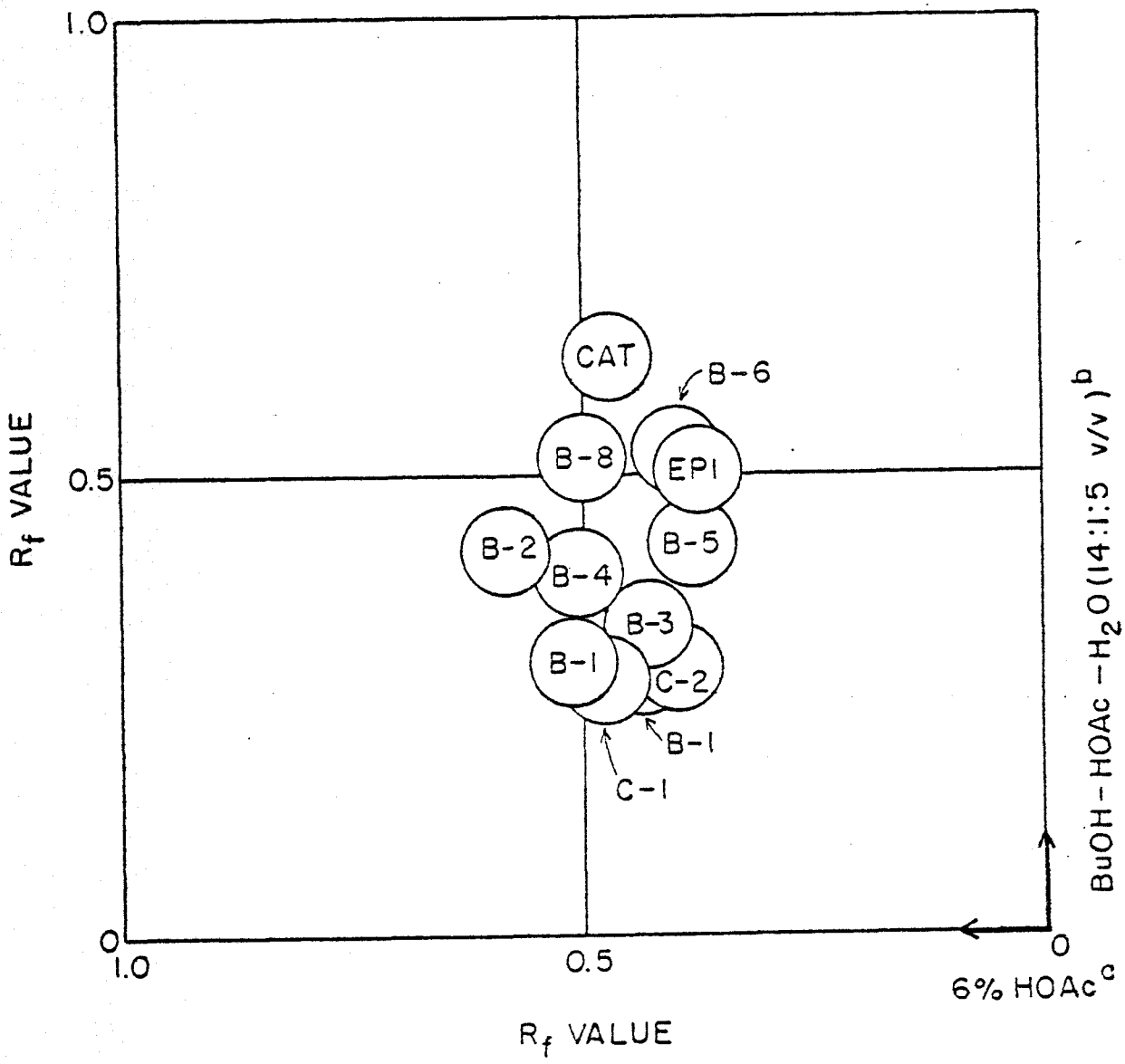



Figure 8. Model chromatogram of plant procyanidins from literature $R_f$ values.

[a]Solvent used in the first direction.

[b]Solvent used in the second direction.

in acetone followed by saturated aqueous sodium bicarbonate) (87), giving claret-maroon colors with phloroglucinol-containing catechins and flavan-3,4-diols but not necessarily with phloroglucinol-containing 2,3-dihydroflavanols. The benzidine reagent is of great diagnostic value and especially sensitive in its color reactions, giving colors which are the sum total of the colorations of the A and B phenolic moieties. It also reflects the presence of strong hydrogen bondage between 5-hydroxyl and 4-carbonyl groups when these are present (79). Where the nucleus contains meta-hydroxy substituents, for example, the 5,7-dihydroxy group, the deep claret-maroon color developed is identical to that of the meta-dihydroxyphenol, resorcinol. This intense coloration presumably overshadows the pale yellow or deeper yellow of catechol and pyrogallol B nuclei (79).

The $R_f$ values generated from the literature compared to those obtained from my chromatographic analyses (Table 2) have shown the expected results for the inner bark (Figure 9). The flavan-3-ols (+)-catechin (LX) and (-)-epicatechin (LXI) dominate the polyphenolics. The combination of phloroglucinol (A nucleus) and catechol (B nucleus) in the catechins, e.g. (+)-catechin and (-)-epicatechin gives a claret-maroon color. The procyanidin dimers B-1 (LXII), B-2 (LXV), B-3 (LXIII) and B-4 (LXVI) (Figure 7), and the trimers C-1 (LXVII) and C-2 (LXIV) are less pronounced but

Table 2. Comparison of authentic procyanidins with Douglas-fir bark procyanidin $R_f$ values.

| Procyanidin | $R_f(C)^{a,c}$ | $R_f(D)^{b,c}$ | $R_f(C)^{a,d}$ | $R_f(D)^{b,d}$ |
|---|---|---|---|---|
| (+)-Catechin | 0.47 | 0.51 | 0.53 | 0.63 |
| (-)-Epicatechin | 0.37 | 0.50 | 0.45-39 | 0.55-52 |
| B-1 | 0.51 | 0.30 | 0.61 | 0.29 |
| B-2 | 0.58 | 0.42 | 0.64 | 0.40 |
| B-3 | 0.43 | 0.34 | 0.49 | 0.42 |
| B-4 | 0.50 | 0.40 | 0.59 | 0.46 |
| B-5 | 0.38 | 0.43 | --- | --- |
| B-6 | 0.46 | 0.52 | --- | --- |
| B-7 | 0.43 | 0.29 | --- | --- |
| B-8 | 0.50 | 0.52 | --- | --- |
| C-1 | 0.48 | 0.28 | 0.53 | 0.22 |
| C-2 | 0.40 | 0.29 | 0.49 | 0.29 |

[a]Solvent system (C) is 6% aqueous acetic acid.

[b]Solvent system (D) is butan-2-ol-acetic acid-water (14:1:5 v/v).

[c]$R_f$ values from the literature.

[d]$R_f$ values from Douglas-fir bark obtained in this work.

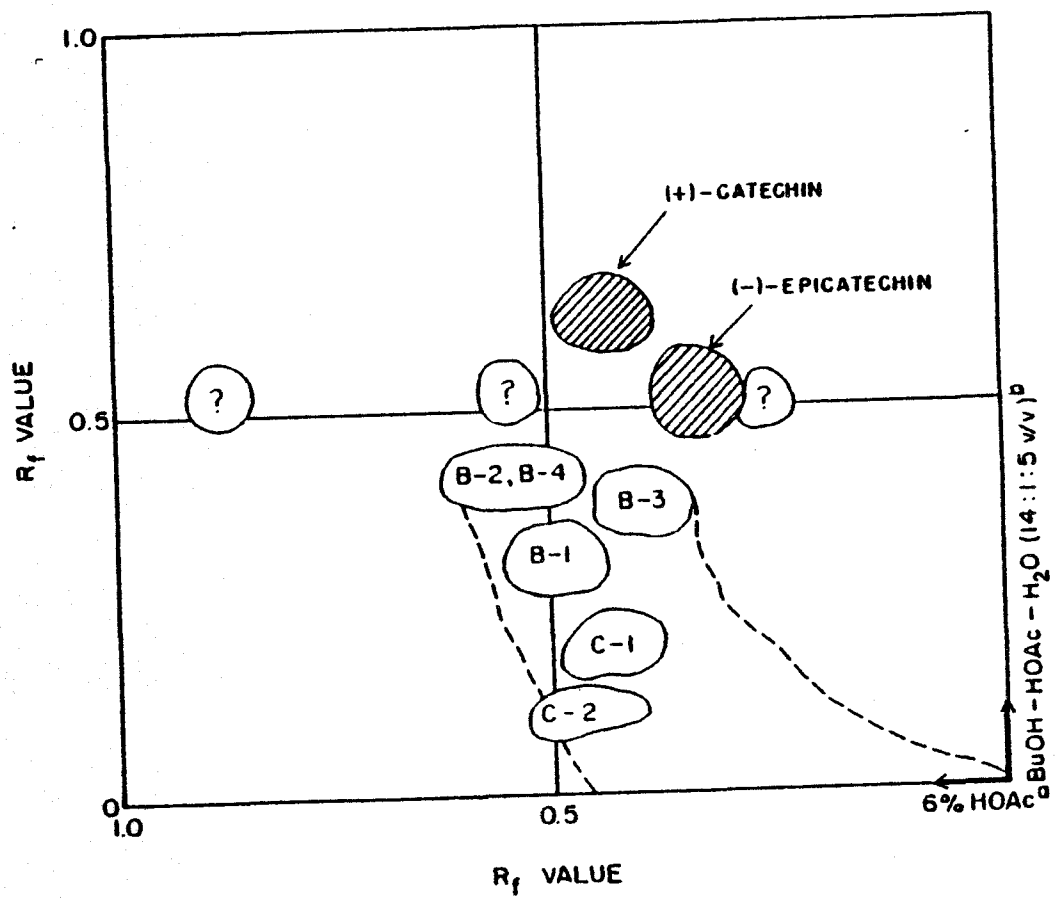


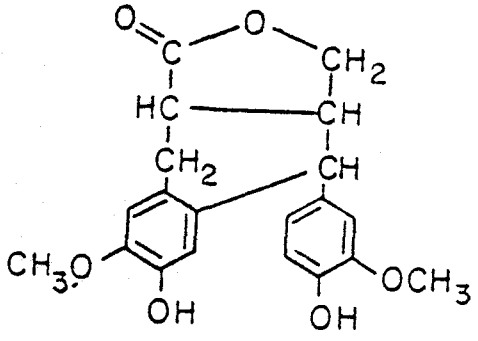

Figure 9. Paper chromatogram of inner bark extract.

[a]Solvent used in the first direction

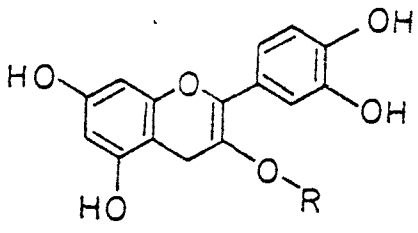
[b]Solvent used in the second direction

are still separable for visual analysis. Again the combinations of phloroglucinol (A nucleus) and catechol (B nucleus) gives a distinctive claret-maroon color but presumably due to polymerization the colors are quite light in shade (Table 3). The Gibbs reagent gives a distinctive mauve-purple coloration for the procyanidin compounds. Dihydroquercetin glycoside (LXVIII), plus some other unidentified phenolics which fluoresce under long-wave ultraviolet light, are present in low concentrations.

The outer bark, as mentioned before, contains (in decreasing observable quantities) dihydroquercetin (LXVIX), conidendrin (LXX), (+)-catechin (LX), and (-)-epicatechin (LXI) (Table 4, Figure 10).




(LXX )




(LXVIX) R = H

(LXVIII) R = GLUCOSE

The outer bark is so highly polymerized that very little procyanidin remains detectable. This is the reason many workers shun the outer bark in favor of the inner bark

Table 3. Paper chromatographic analyses of the inner bark extract.

| | $R_f(C)^a$ | $R_f(D)^b$ | Spray Indicator | | |
|---|---|---|---|---|---|
| | | | (i)[c] | (ii)[d] | U.V. |
| ( 1) | 0.57 | 0.22 | --- | orange-brown | --- |
| ( 2) | 0.61 | 0.29 | mauve-purple | brown-orange | --- |
| ( 3) | 0.49 | 0.38 | mauve-purple | orange-tan | --- |
| ( 4) | 0.64 | 0.40 | mauve-purple | brown-claret | --- |
| ( 5) | 0.35 | 0.55 | --- | light pink | --- |
| ( 6) | 0.45 | 0.55 | mauve-purple | red-brown-orange | --- |
| ( 7) | 0.89 | 0.55 | --- | --- | yellow |
| ( 8) | 0.63 | 0.57 | --- | pink | --- |
| ( 9) | 0.53 | 0.63 | mauve-purple | red-brown-orange | --- |
| (10) | 0.54 | 0.14 | --- | yellow-orange | --- |

[a]Solvent system (C) is 6% aqueous acetic acid.

[b]Solvent system (D) is butan-2-ol-acetic acid-water (14:1:5 v/v).

[c]Indicator (i) is Gibbs reagent.

[d]Indicator (ii) is bis-diazotised benzidine reagent.

Table 4. Paper chromatographic analyses of the outer bark extract.

| | $R_f(C)^a$ | $R_f(D)^b$ | Spray Indicator | | |
|---|---|---|---|---|---|
| | | | (i)[c] | (ii)[d] | U.V. |
| (1) | 0.45 | 0.55 | light mauve-purple | yellow-brown | ---- |
| (2) | 0.53 | 0.63 | light mauve-purple | yellow-brown | ---- |
| (3) | 0.54 | 0.71 | mauve-purple | red-brown | ---- |
| (4) | 0.25 | 0.76 | --- | light red-brown | ---- |
| (5) | 0.61 | 0.74 | --- | --- | Blue |
| (6) | 0.12 | 0.91 | mauve-purple | red-brown | ---- |

[a]Solvent system (C) is 6% aqueous acetic acid.

[b]Solvent system (D) is butan-2-ol-acetic acid-water (14:1:5 v/v).

[c]Indicator (i) is Gibbs reagent.

[d]Indicator (ii) is bis-diazotised benzidine reagent.

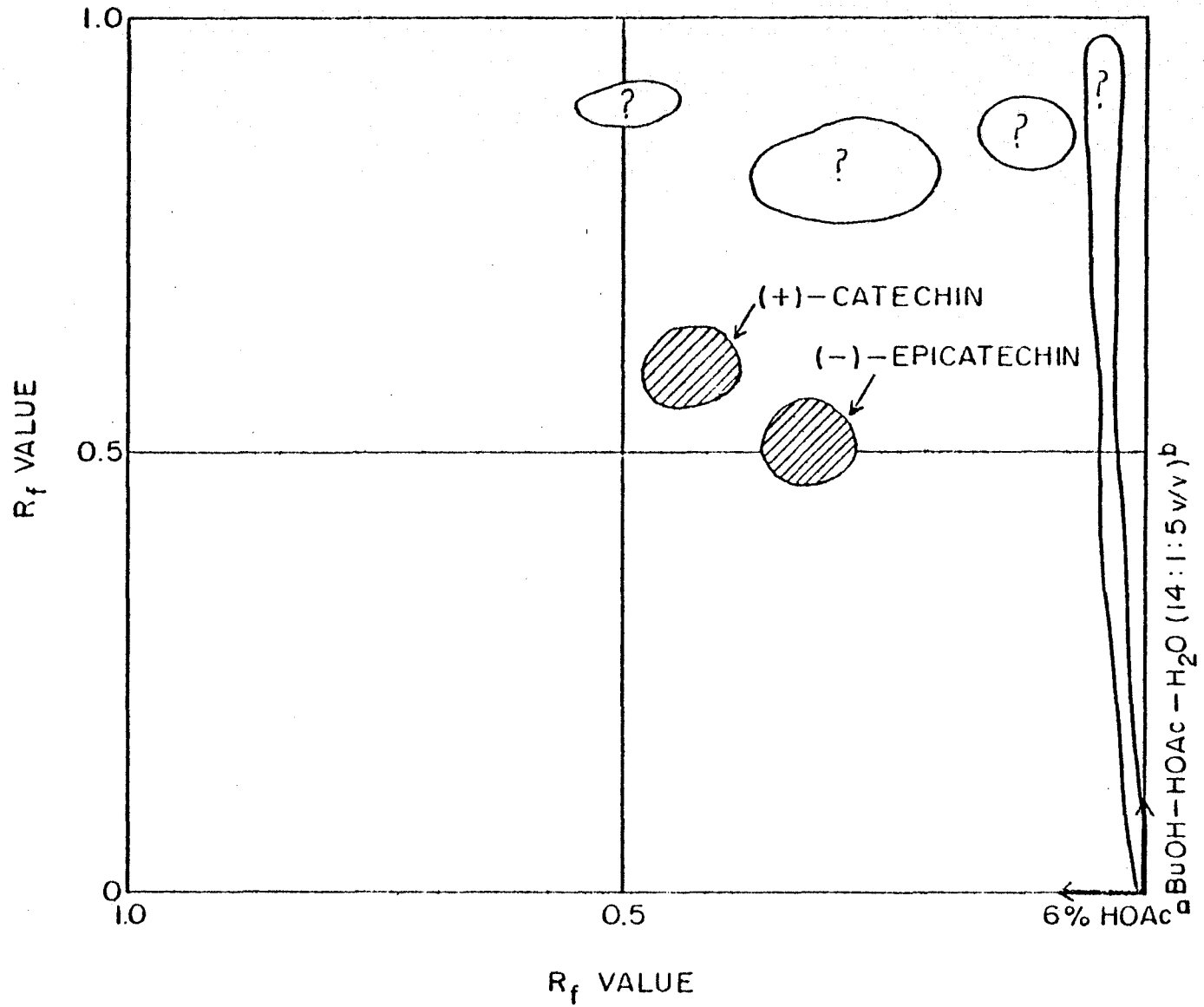



Figure 10. Paper chromatogram of outer bark extract.
[a]Solvent used in the first direction.
[b]Solvent used in the second direction.

where they can inspect the "...loot in the garbage bin of plant metabolism" (36) without bumping their heads in the dark.

The more traditional column packing for the isolation of polyphenols (Sephadex LH-20) (87) was not used due to the time required for separation. The elution pattern from Sephadex LH-20 in ethanol, however, follows the order: (i) flavan-3-ols [(+)-catechin, (-)-epicatechin]; (ii) procyanidin dimers [B-2→B-1→A-1, A-2, B-5, B-8→B-3, B-4→B-6, B-7]; and (iii) procyanidin oligomers [C-1, C-2, D-1 and D-2]. Instead of Sephadex, a dry packed cellulose column (62) was used to separate the water-soluble tannins of inner and outer bark. However, the CF 11 cellulose packing did not allow for adequate resolution of the mixture (Figure 11) for isolation purposes. Enough qualitative information was acquired, however, from the two-dimensional paper chromatograms of the eluate (Figures 9, 10) to allow the characterization of Douglas-fir procyanidins. A number of high-pressure liquid chromatographic techniques for the separation and quantitation of naturally occurring phenolic compounds have been developed (93). The technique offers selectivity, resolution, speed and sensitivity (minimum detectable amounts below 30 µg) far superior to the classical techniques. A system of this kind could be used to detect polyphenolic compounds in specific cell types micromanipu-

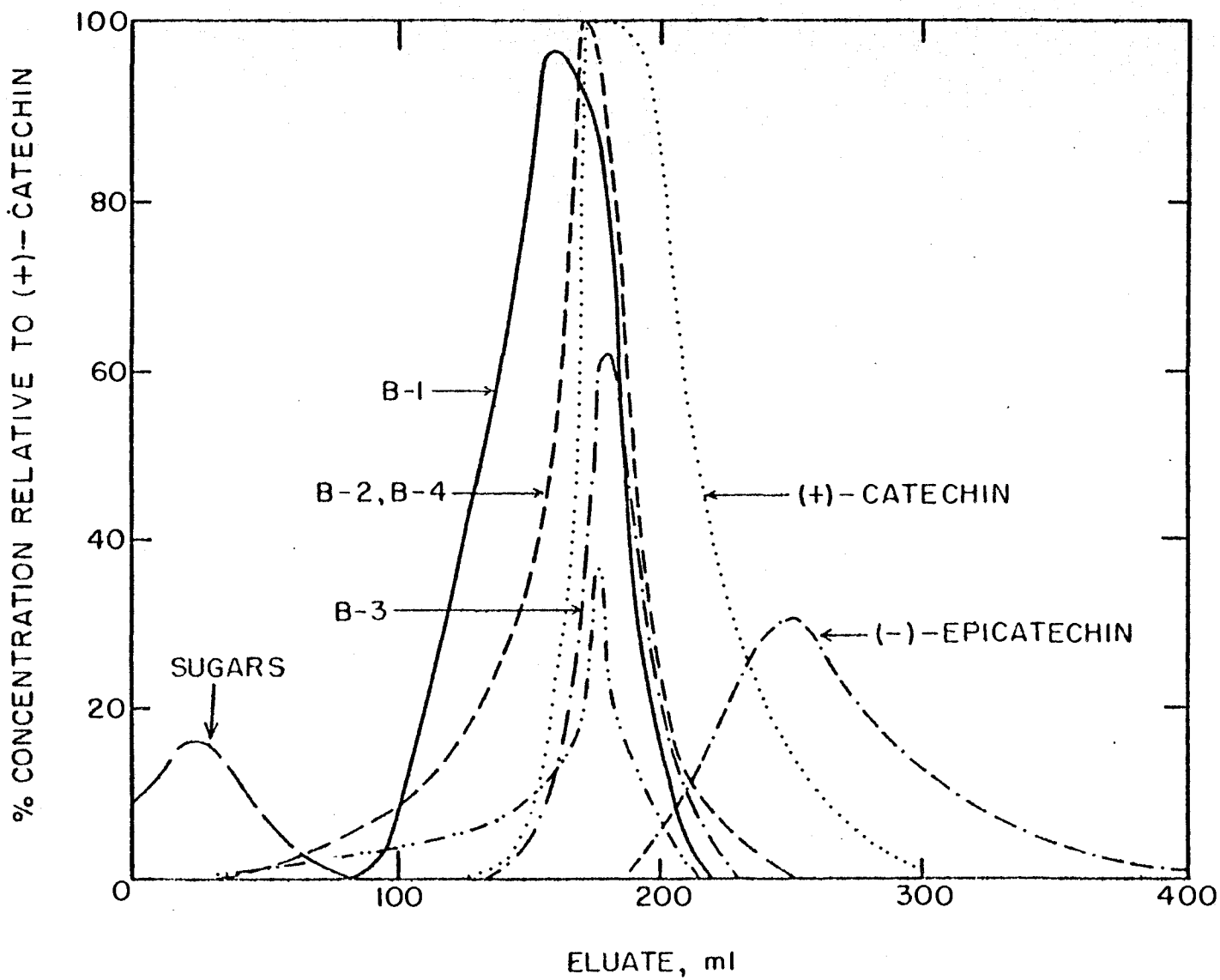



Figure 11. Elution of inner bark procyanidins from a column of Whatman cellulose powder CF 11.

lated from the inner and outer bark. This could give us clues as to where the conversion of the monomers (+)-catechin and (-)-epicatechin to the dimers and oligomers occur.

## Experimental

General Methods.--Ascending two-dimensional paper chromatographic analyses were carried out at 25°±2° with Whatman No. 1 paper (22.9 cm x 22.9 cm) in the following solvent systems: (C) 6% aqueous acetic acid (v/v); (D) butan-2-ol-acetic acid-water (14:1:5 v/v). The procyanidins were detected by illumination with ultraviolet (uv) light and by the use of the following spray reagents: (i) 2,6-dibromobenzoquinone-4-chloroimide (0.5% v/v in acetone) (Gibbs reagent), followed by sodium bicarbonate (saturated aqueous solution) (spots appeared mauve-purple on a white background); (ii) bis-diazotised benzidine, (a) benzidine (5 g) was stirred with concentrated hydrochloric acid (14 ml) and the suspension was dissolved in water (980 ml), (b) 10% sodium nitrite. Two parts of (b) were added to three parts of (a) and the reagent was used immediately after mixing (spots appeared claret-maroon). Chromatograms sprayed with the reagents were allowed 1-3 min for full development of colors, and immediately washed for 20 min in running tap water to avoid a yellow background due to excess benzidine reagent (be careful--the paper is easily torn during this procedure). The chromatograms were hung and air dried.

Carbohydrates were analyzed by descending paper chromatography carried out at 25°±2° with Whatman No. 1

paper (48 cm x 19.2 cm) irrigated with solvent system (E) ethyl acetate-pyridine-water (8:2:1 v/v). The sugars were detected with uv light and by the use of (iii) aniline phthalate spray reagent [0.93 g aniline (freshly distilled) and 1.66 g o-phthalic acid were dissolved in 100 ml of n-butanol saturated with water]. After irrigating (three times) the papers were air dried and sprayed with the aforementioned reagent. The chromatograms were placed in an oven at 105°C for 10 min. Pentoses were brown maroon while hexoses were light brown in color. The chromatograms were placed under long-wave uv and inspected for sugars. The sugars showed up as a light bluish-green fluorescence.

Collection of Barks.--Douglas-fir bark was collected from a freshly cut tree taken from McDonald Forest, Benton County, Oregon on April 21, 1980. The tree was approximately 124 annual rings old and 850 cm in diameter at breast height. The outer bark was removed from the tree with a hatchet and the inner bark was stripped from the tree by hand. The inner and outer barks were separated into plastic bags and air-dried in the laboratory.

Extraction of Barks.--A typical procedure for both inner and outer bark is described. The air-dried bark (1.0 Kg) was macerated then soaked at room temperature for 2 days in 5-liter Erlynmeyer flasks with 3 liters of acetone-water

(70:30 v/v) solution. The extraction of the bark was repeated three times, each time with fresh solvent. The acetone-water solution was decanted from the flasks and the acetone was removed under reduced pressure on a rotary evaporator. The remaining aqueous suspension was extracted with diethyl ether (3 times, 500 ml), then filtered through glass wool to remove the sticky, red solids which remained.

The filtrate was extracted with ethyl acetate (3 times, 500 ml) and the aqueous layer was reduced in volume under reduced pressure with a bath temperature of 60°. The freeze drying of this solution yielded 90 g (9.05% of the original inner bark) and 34 g (3.4% of the original outer bark) of a spongy, tan-colored tannin-material.

Chromatographic Analyses.--Two-dimensional paper chromotography of the inner bark extract with (a) 6% acetic acid and then (b) butan-2-ol-acetic acid-water (14:1:5 v/v) revealed ten spots (Figure 9) which are recorded with selected color reactions in Table 3. Single applications of $2.0 \times 10^{-3}$ g of extract solids, redissolved in acetone, were applied. The water solubles were examined paper chromatographically using ethyl acetate-pyridine-water (8:2:1 v/v) as developing solvent and uv and aniline phthalate as indicators. The quantities were estimated by visual comparison with known concentrations of standard

sugars. The sugars and their visual amounts are reported in Table 5.

Isolation of Bark Procyanidins.--Bark material (1-2 Kg) was treated as previously described to give the crude phenolic extract (10-20 g). The crude water-soluble tannin (3 g) was dissolved in 40 ml of hot water and applied directly to 150 g of cellulose (Whatman CF 11) packed in a nylon tube to make a column 3.25 by 80 cm and then eluted with cold water. The eluate was fractionated (100 x 10 ml) and analyzed by two-dimensional paper chromatography. The elution pattern from cellulose in cold water is shown in Figure 11.

Table 5. Visually observed quantities of monosaccahrides in Douglas-fir bark extract.

| Sugar | Inner Bark | Outer Bark |
|---|---|---|
| Mannose | light tan | very light tan |
| Glucose | tan | light tan |
| Galactose | light tan | very light tan |
| Arabinose | --- | --- |
| Xylose | trace (visible under uv) | trace |

## Summary and Conclusions

Douglas-fir tannins have been investigated previously with the establishment of procyanidins consisting in part of epicatechin and catechin units linked from position C-4 of one to an undetermined position of another. The sequence of units was, however, not determined. In this investigation the previous studies were supported plus additional information regarding the sequencing of the monomeric units. It was found, through the use of two-dimensional paper chromatography, that the distinguishable procyanidins in Douglas-fir bark are B-1→B-4 which are linked C-4 to C-8. None of the procyanidin dimers B-5→B-8 were observed which means there are no dimers with linkages of C-4 to C-6 present in Douglas-fir barks.

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