THE POLYOXYPHENOLS OF WESTERN RED CEDAR (THUJA PLICATA DONN.)

I. ISOLATION AND PRELIMINARY CHARACTERIZATION OF PLICATIC ACID[1]

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ABSTRACT

An amorphous, optically active, heat and light sensitive, polyoxyphenolic acid ($pK_a = 3$) has been isolated from the mixture of polyoxyphenols present in the aqueous extractive of western red cedar. Analyses of crystalline derivatives are consistent with a molecular formula of $C_{20}H_{22}O_{10}$. Color tests, spectra, methylation, and alkaline nitrobenzene oxidation results indicate it to be a propylphenol dimer, a lignan acid, in which one aromatic ring is 3-methoxy-4-hydroxyphenyl and the other is 3,4-dihydroxy-5-methoxyphenyl. Acetylation results and lactone formation require the presence of three alcoholic hydroxyls, one of which is in the γ position to the carboxyl.

In a previous paper (1), the presence in the acetone extractive of western red cedar of several water-soluble polyoxyphenols was reported. This phenolic mixture constituted 4 to 5% of wood samples having acetone solubilities of 10 to 14%. These substances were also extracted with water and accounted for the main portion (85–95%) of the hot-water extractive of butt outer heartwood which was found to have hot-water solubilities ranging from 10 to 23% (2). This indicated high content of natural phenolic substances together with the availability of large volumes of western red cedar wood as mill and forest residue in British Columbia prompted a detailed investigation of the chemistry of the phenolic constituents.

Paper chromatography of the aqueous extractive, a brown resin, showed the presence of at least seven different phenols and arabinose. By steaming the mixture during aqueous extraction of the wood, the steam-volatile tropolones, thujic acid and methyl thujate, were virtually eliminated. The arabinose and other non-phenols in the extractive were removed by treatment with lead acetate solution and regeneration of the phenols from the precipitated lead salts. Attempts to fractionate the regenerated phenols with a variety of solvents were made, and the separation efficiency was checked by means of paper chromatography of the fractions. In this manner it was established that a major component ($R_f = 0.9$, carbon dioxide – water) was very soluble in water and, compared with the others, practically insoluble in ethyl acetate. Thus, thorough extraction of the phenolic mixture with ethyl acetate gave a residue which was paper chromatographically pure in respect to carbohydrates and other phenols. This product was an amorphous brown powder which gave positive tests for pyrocatechol, methoxyl, and carboxyl groups. In view of its acidity and source, this substance was termed plicatic acid.

Additional quantities of plicatic acid were isolated simply by extracting it from the mixture of phenols in methyl ethyl ketone with sodium bicarbonate solution. The yield was 40% of the aqueous extractive, but since the product was brown in color and failed to crystallize or give crystalline methyl ethers with diazomethane, milder methods of isolation not involving the use of acids and alkalis were investigated.

Column chromatography with a variety of packings including cellulose, magnesol–

[1]*Manuscript received June 15, 1959.*
Contribution from the Vancouver Laboratory, Forest Products Laboratories of Canada, a Division of the Forestry Branch, Department of Northern Affairs and National Resources. Paper presented at the 133rd National Meeting, American Chemical Society, San Francisco, California, April 13–18, 1958.

celite, alumina, and ion-exchange resins was examined. The low solubility of the aqueous extractive in most solvents limited the loading of most columns to very small quantities. Also, although weakly basic ion-exchange resins separated plicatic acid from the other phenols, acid was required to elute the plicatic. The most successful column separation of the phenols was obtained with a weakly acid (carboxylic) cation-exchange resin which is also useful in the separation of flavonoids (3). Elution with water of a column loaded with the aqueous extractive yielded plicatic acid and the phenols, R_f .7 and .5 (carbon dioxide – water), in that order with some overlapping. Subsequent graded elution with water–ethanol gave the phenols of R_f .52 and .03 and finally a mixture of the remainder, R_f .03, .06, and .1. Paper chromatography showed that the first plicatic acid eluted from the column was contaminated with arabinose. Subsequent fractions showing no contamination were combined and lyophilized to provide amorphous plicatic acid in small quantities (1 g or less).

Consideration of the high solubility of plicatic acid in water and very poor solubility in ethyl acetate together with the poor solubility of the other phenols in water and their good solubility in ethyl acetate led to isolations on a larger scale by a six-stage countercurrent distribution of the aqueous extractive between these two solvents. This procedure provided almost equal quantities of chromatographically pure plicatic acid and plicatic acid contaminated with 5–6% of arabinose and traces of other sugars in a combined yield of 40% on the aqueous extractive.

As obtained by the column or countercurrent distribution technique, the plicatic acid was a very light tan-colored, amorphous, optically active powder, very soluble in water, soluble in absolute acetone and ethanol, and very slightly soluble in ethyl acetate and ether.

The color reaction with aqueous ferric chloride was pinkish purple shifting to blue-green at neutrality and thence to deep red in sodium carbonate solution. This indication of the presence of adjacent phenolic hydroxyls was confirmed by positive tests with ferrous sulphate, ammonium molybdate, titanium chloride, and bromine solutions. A test for three vicinal phenolic hydroxyls with ferrous sulphate – potassium tartrate solution was negative, but the chlorine – sodium sulphite test for pyrogallol derivatives was positive. Alkaline nitrobenzene oxidation gave a 4% yield of vanillin. A test for methylenedioxy groups was negative.

Solutions of plicatic acid were unstable to sunlight, rapidly turning dark red-brown in color. Heating the solid or an aqueous solution caused a gradual conversion to another phenol of R_f 0.7 (carbon dioxide – water) having an infrared absorption characteristic of a γ-lactone. This substance, according to paper chromatography, was identical with one of the other phenols occurring with plicatic acid in the cedar extractive. Acidification of an alkaline solution regenerated plicatic acid.

Elementary analyses and molecular weight determinations indicated an empirical formula for plicatic acid of $C_{20}H_{24}O_{10}$. For two methoxyl groups and one carboxyl group per molecule, this requires a methoxyl content of 14.6% and a neutral equivalent of 424. The best values found were 13.7% and 432 respectively. The heating (65–100° C) necessary for moisture removal prior to analyses also caused some decomposition making analytical results on plicatic acid itself subject to question.

Methylation of plicatic acid with dimethyl sulphate and alkali in aqueous acetone solution followed by diazomethane in dioxane gave a crystalline product which could also be obtained in poorer yield directly from plicatic with diazomethane. Analytical values for carbon, hydrogen, and methoxyl content and molecular weight fitted an empirical

formula of $C_{24}H_{30}O_{10}$. This corresponds to the methyl ester of the trimethyl ether of a plicatic acid containing two fewer hydrogens per molecule than the formula above. Since dehydrogenation during diazomethane methylation is unlikely and the analytical values for plicatic are based on amorphous heat-labile material, the $C_{20}H_{22}O_{10}$ formula for plicatic acid is favored.

The methyl ester of the trimethyl ether effervesced when heated above its melting point, evolving methanol and yielding a crystalline product which analyzed as the trimethyl ether lactone, $C_{23}H_{26}O_9$. The lactone character was confirmed by the appearance of a strong band in the infrared absorption spectrum at 1784 cm^{-1}, the region assignable to the carbonyl group of five-membered lactones (4) and disappearance of the band at 1716 cm^{-1} assignable to the carbonyl group in the ester (5). The infrared absorption spectrum of the lactone also indicated additional hydroxyl group content.

Acetylation of plicatic acid in acetic anhydride and pyridine gave an amorphous product analyzing for methoxyl content as a hexaacetate.

The neutralization curve for plicatic acid disclosed it to be a strong acid, $pK_a = 3$, comparable to orthohydroxybenzoic acids. However, in acids of this type having strong intramolecular hydrogen bonding the absorption band in the infrared spectrum assignable to the carbonyl occurs below 1680 cm^{-1} (5), whereas for plicatic acid the band assignable to the carbonyl is at 1713 cm^{-1}, a frequency normal for monobasic aliphatic acids.

The high acidity of plicatic acid indicated it would displace acetic acid from its salts. Treatment of an alcoholic solution with alcoholic potassium acetate precipitated a crystalline monopotassium salt, $C_{20}H_{21}O_{10}K$, in high yield. Therefore the possibility of isolating and purifying plicatic acid directly from the aqueous extractive by precipitation as the potassium salt is now being examined.

The analytical and molecular weight results on plicatic acid and its crystalline derivatives indicate it to be a propylphenol dimer of the lignan type and, pending completion of degradative studies, some deductions may be made as to the nature of the aromatic nuclei. The formation of some vanillin by oxidation indicates one aromatic ring to be 3-methoxy-4-hydroxyphenyl. The presence of two additional phenolic hydroxyls adjacent to one another is shown by the methylation data and the color tests. This together with the positive test for pyrogallol derivatives indicates a vicinal arrangement of these two phenolic hydroxyls with the other methoxyl on the other aromatic ring. In addition to the three phenolic hydroxyls and one carboxylic hydroxyl which, with two methoxyls, account for seven of the 10 oxygens required by the empirical formula, $C_{20}H_{22}O_{10}$, there are three alcoholic hydroxyls as shown by formation of a hexaacetate. One of these is used in γ-lactone formation by the methyl ester of the trimethyl ether, and the other two account for the hydroxyl band remaining in the infrared absorption spectrum of the lactone. Thus the formula for plicatic acid can be provisionally written: $C_6H_3(OH)(OCH_3).C_5H_4(OH)_3(COOH).C_6H_2(OH)_2(OCH_3)$, and it is reasonable to conclude that it is a highly hydroxylated lignan acid. Structural investigations are continuing.

While no lignan acids have been isolated from plant material heretofore, many of the lactones have (6), and it is significant that the infrared absorption spectrum of the lactone of methylated plicatic acid is similar to those published for the podophyllotoxin series (4).

The presence in plicatic acid of unetherified neighboring phenolic hydroxyls as in another lignan, nordihydroguaiaretic acid, a well-known commercial antioxidant, justifies its testing as an antioxidant. Similarly, biological testing of it and its lactone derivatives is in order in view of the fact that many lactones have marked physiological activity and some are valuable medicinals (7).

EXPERIMENTAL

Evaporations were conducted under reduced pressure at 40° C. Melting points were determined on a Fisher–Johns apparatus and are uncorrected. Carbon and hydrogen analyses were by Clark Microanalytical Laboratory, Urbana, Illinois. Infrared absorption spectra were determined on a Baird Atomic KM-1 spectrophotometer. Solvent mixtures for paper chromatography were carbon dioxide – water and butanol – acetic acid – water (40–10–50) for phenols and the latter and butanol–pyridine–water (10–3–3) for sugars.

Preparation of the Aqueous Extractive of Western Red Cedar

Straw-colored shingle mill spalts were ground in a Wiley mill to pass a 20-mesh screen. The wood meal (1.49 kg moisture-free basis, TAPPI hot-water solubility, 14.5%) was leached with four batches of hot water in an earthenware crock. The temperature was maintained at 100° C and agitation achieved by passing steam into the mixture. Results were as follows:

Extraction time (hours)	Volume of extract (liters)	Solids content (g)
2	14.3	84.4
1	7.7	27.1
1	7.0	13.4
1/2	9.3	11.8
Totals	38.3	136.7

After the solution was cooled, the combined extract was decanted from settled solids (10 g) and evaporated at reduced pressure to a viscous brown syrup, (168 g, 25% moisture). Yield based on the wood, 8.5% or 59% of the TAPPI hot-water solubility. Paper chromatograms (carbon dioxide – water) showed spots for phenols at R_f 0, .03, .07, .12, .16, .50, .70, .90. Color tests for thujaplicins were negative. Paper chromatograms showed arabinose and traces of other sugars.

Isolation of Plicatic Acid from Aqueous Extractive

(*a*) *By Sodium Bicarbonate Extraction of Lead-precipitated Phenols*

Aqueous extractive (331 g, moisture-free basis) in water solution was treated with lead acetate solution (900 g in 2 l.) and the precipitated white lead salts (427 g) removed by filtration. A portion (273 g) of the lead salt was agitated with 2-butanone (2 l.) containing excess sulphuric acid (6 *N*). After filtration, the 2-butanone solution was extracted with 5% sodium bicarbonate solution (500 ml, 3×250 ml). The bicarbonate solution after back extraction with 2-butanone was saturated with sodium chloride, acidified with dilute sulphuric acid (12 *N*), and extracted with 2-butanone (2×500 ml, 2×100 ml). The 2-butanone solution was dried over sodium sulphate and evaporated to yield brown amorphous plicatic acid (119 g), which in paper chromatography showed no contamination with sugars or other phenols.

(*b*) *By Column Chromatography*

Aqueous extractive (2.6 g, moisture-free basis) in warm water (400 ml) was added to a column bed (5 cm × 30 cm) of Amberlite IRC-50 (H) (Rohm and Haas, Philadelphia, Penn.) at a rate of 1.5 ml/minute. The column was eluted at the same rate, first with water and then with graded ethanol–water using a fraction collector loaded with 15-ml tubes. The contents of each fifth tube were checked by paper chromatography. The water

eluted arabinose and phenols of R_f .9, .7, and .5 in that order with partial overlapping. The ethanol–water eluted phenols of R_f .52, .03, and a mixture of R_f .03, .06, and .1 in that order. Fractions which were pure by paper chromatography were combined and lyophilized. Total yields were estimated from the pure yields and paper chromatographic data on fractions showing overlapping as follows:

Tube No.	Eluant	R_f of phenols	Yield of pure material (mg)	Total yield estimated (mg)
0–40	H_2O	—	—	—
40–80	H_2O	.90	650	1250
65–140	H_2O	.70	—	200
80–200	H_2O	.50	215	525
200–320	5–20% EtOH	.52	110	225
310–330	20–25% EtOH	.03	30	100
330–340	25–30% EtOH	.03		
		.06	200	200
		.10		
	Totals		1105	2500

The plicatic acid (R_f 0.9) obtained was a light tan, amorphous powder.

(*c*) *By Countercurrent Distribution with Ethyl Acetate – Water*

Aqueous extractive solids (12 g) were added to a 1-liter separatory funnel containing water-saturated ethyl acetate (400 ml) and ethyl-acetate-saturated water (400 ml) and the mixture shaken 5 minutes before separation. The water layer was transferred to a second funnel and similarly treated with fresh ethyl acetate while the ethyl acetate layer was treated with fresh water in the first funnel. This procedure was repeated through six stages involving six funnels until the original water was in funnel six in contact with the last added ethyl acetate and the original ethyl acetate was in funnel one in contact with the last added water. The water layer in funnel six on lyophilization yielded plicatic acid (2.5 g) contaminated with 7–9% arabinose (as determined by quantitative paper chromatography). The combined water layers from funnels four and five with the ethyl acetate layers from five and six yielded paper chromatographically pure plicatic acid (2.3 g), R_f in carbon dioxide – water, 0.9; in butanol – acetic acid – water, 0.4, a light tan-colored powder; $[\alpha]_D^{21}$ −9.99 (water). Ultraviolet absorption in water: min. 256 mμ (log ϵ = 3.23); max. 281 mμ (log ϵ = 3.58); shoulder, 307 mμ (log ϵ = 2.42). Infrared absorption, ν_{max}^{KBr} in cm^{-1}: 3340, 1713, 1611, 1518, 1200, 1088. Calc. for $C_{20}H_{24}O_{10}$: C, 56.6; H, 5.71; OCH_3, 14.6; mol. wt. and neutral equivalent, 424.2; for $C_{20}H_{22}O_{10}$: C, 56.9; H, 5.26; OCH_3, 14.67; mol. wt. and neutral equivalent, 422.2. Found: C, 56.24; H, 5.84; OCH_3, 13.7; mol. wt. (Rast), 433; neutral equivalent, 432. Approximate pK_a from pH at half-neutralization, 3.0.

Color tests with aqueous solutions of plicatic acid were as follows: aqueous ferric chloride, pinkish purple shifting to blue and then red on addition of sodium carbonate; ammonium molybdate and acetic acid (8), reddish brown; with Folin–Denis reagent (9) blue; ferrous sulphate – sodium potassium tartrate at pH 5.6 (10), negative; titanium chloride, yellow; chlorine – sodium sulphite (11), red; Gaebel test for methylenedioxy groups (12), negative.

A quantitative alkaline–nitrobenzene oxidation test, using the technique of Stone and Blundell (13) showed 4% vanillin formation.

Repeated attempts to obtain a crystalline acetate or benzoate of plicatic acid were

unsuccessful. The amorphous product of treatment with acetic anhydride and pyridine in benzene solution was precipitated in fractions by stepwise addition of ligroin. The last most soluble fraction had a methoxyl content of 9.1; calculated for a hexaacetate, $C_{32}H_{34}O_{16}$, 9.17.

Plicatic Acid Trimethyl Ether Methyl Ester

Plicatic acid (5 g) in a mixture of water (5 ml) and acetone (9 ml) was treated with dimethyl sulphate (4 ml) and 5 *N* sodium hydroxide dropwise at reflux for 2 hours while maintaining the pH at 8–9. The methylated plicatic precipitated as the white sodium salt during the latter stages of the reaction. The reaction mixture was acidified to pH 3, the acetone removed, and the mixture extracted with chloroform. The residue from evaporation of the chloroform solution was methylated in dioxane with excess diazomethane to yield a product which crystallized from benzene in 42% yield. Melting point after recrystallization from benzene 204–205° C $[\alpha]_D^{22}$ −29.9 ($CHCl_3$).

A methylation of plicatic acid in methanol directly with diazomethane gave the same product contaminated with large amounts of black ether-soluble tar which complicated the purification and reduced the yield of pure product to 23%.

The product, recrystallized from benzene, contained benzene of crystallization. Recrystallization from ethanol gave colorless crystals m.p. 203–204° C. Calc. for $C_{24}H_{30}O_{10}$: C, 60.24; H, 6.32; OCH_3, 38.9; mol. wt., 478.6. Found: C, 59.91; H, 6.02; OCH_3, 38.7; mol. wt. (Rast), 478. Infrared spectrum, ν_{max}^{KBr} in cm^{-1}: 3460, 1716, 1588, 1522, 1511, 1451, 1422, 1256, 1235, 1215, 1128, 1002, 861.

Plicatic Acid Trimethyl Ether Lactone

The trimethyl ether methyl ester on the melting point stage was observed to evolve a gas after melting and then recrystallize. The material evolved was condensed and found to be methanol and the solid, m.p. 252–255° C had an infrared absorption characteristic of a γ-lactone. Accordingly the ester (0.1 g) was heated under nitrogen at 220° C until evolution of gas had ceased and the material had resolidified. Recrystallization from ethanol gave colorless crystals, m.p. 257° C. Calc. for $C_{23}H_{26}O_9$: C, 61.93; H, 5.88; OCH_3, 34.72. Found: C, 61.82; H, 5.99; OCH_3, 34.66. Infrared spectrum, ν_{max}^{KBr} in cm^{-1}: 3450, 1784, 1593, 1518, 1465, 1425, 1322, 1255, 1220, 1126, 992, 862.

Potassium Plicatate

Plicatic acid (2 g) in anhydrous ethanol (50 ml) was treated with anhydrous potassium acetate (0.6 g) in ethanol (10 ml). The white salt which precipitated immediately was separated on the centrifuge and washed well with ethanol (70 ml) before drying under pressure at 65° C. Yield 1.5 g (68.5% of theory). The product was quite soluble in methanol and could be reprecipitated by addition of ethanol. Calc. for $C_{20}H_{21}O_{10}K$: C, 52.16; H, 4.60; OCH_3, 13.45; K as sulphated ash, 18.82. Found: C, 52.17; H, 5.11; OCH_3, 13.39; K as sulphated ash, 18.7. The infrared absorption spectrum was characteristic of the salt of an organic acid (5), ν_{max}^{KBr} in cm^{-1}: 3340, 1610 and 1600 (broad doublet), 1515, 1350–1320 (broad), 1205, 1090, 872.

ACKNOWLEDGMENT

The authors are indebted to Canadian Forest Products Limited for supplying cedar wood samples.

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