PROGRESS IN THE CHEMISTRY OF SHORTLEAF AND LOBLOLLY PINE BARK FLAVONOIDS

R. W. HEMINGWAY

Southern Forest Experiment Station,
USDA, Forest Service,
Pineville, Louisiana 71360

G. W. MC GRAW

Department of Chemistry,
Louisiana College,
Pineville, Louisiana 71360

SYNOPSIS

Southern pine barks are rich and abundant sources of flavonoids which have potential for a wide variety of uses, but little research has been published concerning their structure and reactions. To provide a basis for development of uses for southern pine bark, we have examined monomeric flavonoids, and water-soluble and alkali-soluble polyflavonoids. The composition and distribution of monomeric flavonoids did not differ between shortleaf and loblolly pine barks, but great specificity between tissues and substantial variation in B-ring hydroxylation between flavonoid classes were evident. Products obtained through thioglycolysis suggested that both the water- and alkali-soluble polyflavonoids were composed of (−) 2-3cis 3,3′,4′,5,7 hydroxyflavanol units that were linked C_4 to C_6 or C_8. Small proportions of the corresponding 2-3 trans isomers were also indicated. No evidence for 3,3′,4′,5,5′,7 or 3,4′,5,7 hydroxyflavanol units could be found in hot water- or alkali-soluble isolates. More complex structural features were suggested from the microanalytical and spectral properties of polyflavonoid isolates, however.

INTRODUCTION

The forest products industries of the southern United States harvest approximately 7 million dry tons of pine bark each year. This resource receives little utilization other than recovery of fuel values. Approximately 2 million dry tons (30–40% of bark dry weight) of potentially valuable polyflavonoids are burned annually. Conifer bark flavonoids have potential industrial application in manufacture of antioxidants, pigments, adhesives, clay dispersants, grouting agents, water-soluble heavy-metal fertilizers, ion exchange resins, and additives for boiler water, and drilling muds [1]. As witnessed by the recent interest in finding substitutes for phenol, the commercial

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potential for silvichemicals is improving greatly. Our inadequate knowledge of the structure and reactions of polyflavonoids in southern pine barks hampers development of silvichemicals from this forest resource. Substantial work has been done on the chemistry of polyflavonoids in western hemlock (*Tsuga heterophylla*), Douglas-fir (*Pseudotsuga menziesii*), and wattle (*Acacia* sp.) [1-3], but very little has been published on constituents of southern pine barks. Hergert [4] has examined the monomeric flavonoids of slash pine (*Pinus elliottii*), and conclusions from his study of polyflavonoids in southern pine barks are available in a review [5] and abstract [6]. Only two other studies [7, 8] devoted to polyflavonoids in southern pine barks have been published. In the present research the distribution of monomeric flavonoids was examined with emphasis on how they related to the more abundant polyflavonoids. Studies of the structure of water-soluble and alkali-soluble polyflavonoids were undertaken to assist in the development of products from southern pine barks.

EXPERIMENTAL

Distribution of Monomeric Flavonoids

Bark samples were collected from 4-foot-long bolts taken at the base, middle, and top (4-in. diameter inside the bark) of three 30-year-old trees each of shortleaf (*P. echinata* Mill.) and loblolly (*P. taeda* L.) pines growing on the Kisatchie National Forest in central Louisiana. Old outer bark was removed with a dull abrading tool, and new outer bark was collected by cutting along the cork cambium with a draw knife. Phloem was removed by scraping along the xylem cambium. New outer bark samples were hand-peeled to remove any adhering phloem, and all samples were frozen until needed for analysis. Bark was dried under vacuum at room temperature and ground to pass a 1-mm screen on a Wiley mill immediately prior to extraction of 20 g of each tissue with 30–60°C petroleum ether and then with methanol for 6 hr. Methanol-soluble material was concentrated to 2 ml and applied to 0.2 g columns of Polyamide-6 to remove interfering polyflavonoids prior to PC. Columns were eluted with 10 ml of methanol, the eluates were concentrated to 2 ml, and Whatman No. 1* papers were spotted with 20 or 40 μl of each extract. Sheets were developed overnight in the descending direction with n-butanol:acetic acid:water 6/1/2 parts by volume (BAW) and then for 4 hr with 6% acetic acid (6AA) in the second dimension. Papers were sprayed with either ferric chloride-potassium ferricyanide or diazotized sulfanilic acid after observation under ultraviolet light. Spot intensity was visually ranked in a scale of 0 to 5. Compounds were identified by comparison of their R_f values, color reactions, and ultraviolet spectral properties with those of authentic compounds (provided by Dr. W. E. Hillis, Division of Building Research, CSIRO, Melbourne, Australia).

Hot Water-Soluble Polyflavonoids

Composite samples of new outer bark from ten trees each of shortleaf and loblolly pines were extracted with 30–60°C petroleum ether and then with hot water for two 3-hr periods. Water extracts were combined, reduced in volume at 40°C under

* Mention of trade names in this paper is for information only, and does not imply endorsement by the U.S. Department of Agriculture.

vacuum, and freeze-dried to give yields of 8.3 and 13.3% water-solubles from loblolly and shortleaf new outer bark. Water-soluble extracts (10 g) were dissolved in water (20 ml) and poured into ethanol (200 ml) to precipitate a carbohydrate-rich fraction amounting to 3.5 and 4.9% of the ovendry weight of loblolly and shortleaf pine new outer bark. Ethanol-soluble materials were concentrated to near dryness, dissolved in water, and freeze-dried to obtain 4.8 and 8.1% of the ovendry weight of loblolly and shortleaf pine barks. Dialysis in Spectrapor No. 3 membranes (3500 M_w exclusion limit) gave retentions of only 28 and 35%. Although substantial amounts of water-soluble polyflavonoids were removed, these low yields (1.5 and 2.8% of loblolly and shortleaf dry bark weight) were accepted in the interests of obtaining fractions as free as possible of low-molecular-weight impurities.

Anthocyanidins were determined spectrophotometrically at 540–550 nm after refluxing isolates (100 mg) with 5% concentrated HCl in n-butanol (50 ml) for 2 hr and comparing the absorbance with standard cyanidin-chloride solutions [9]. Constituent anthocyanidins were determined by PC (2 N HCl:n-butanol upper phase [10] and BAW/6AA [11]).

Isolates (50 mg) in powdered KOH (1 g) were heated at 250°C for 1 hr in an N_2 purged muffle furnace, the products were acidified with 3 N HCl under N_2, and phenolics obtained by extraction into ethyl acetate were identified by comparison of R_f values and color reactions with authentic compounds by PC (BAW/6AA) or TLC (petroleum:chloroform:acetic acid 8/8/1). Plates and sheets were sprayed with diazotized sulfanilic acid after observation under ultraviolet light.

The freeze-dried powders were methylated repeatedly with diazomethane, extracted into chloroform from sodium carbonate, washed with water, and lyophilyzed from dioxane. Diazomethane methylated products were further methylated by heating for 4 hr with dimethylsulfate in acetone over potassium carbonate. Material that was soluble in acetone was extracted into chloroform from aqueous carbonate, washed with water, and separated into three fractions by chromatography on a silica gel column. One fraction was obtained by elution with chloroform, and two yellow bands were eluted with 5% methanol in chloroform. The three fractions were lyophilyzed from dioxane.

Methylated products were treated with mercaptoacetic acid, the thioglycolic acid derivatives were methylated with dimethylsulfate in acetone over potassium carbonate, and the acetone-soluble material was recovered by filtration [12]. TLC plates were developed 2–3 times [diethyl ether:hexane (1/1)], observed under ultraviolet light, sprayed with H_2SO_4-formalin, and heated to obtain red colors from the thioglycolates [13] (samples of methyl catechin and epicatechin thioglycolates were provided by Dr. J. J. Karchesy, Mrs. Patricia M. Loveland, and Dr. M. L. Laver, School of Forestry, Oregon State University, Corvallis, Oregon).

Two pairs of red reacting compounds (R_f 0.17–0.22 and 0.40–0.55) were isolated by preparative TLC and treated with $KMnO_4$ for 2 hr [14]. The MnO_2 was centrifuged down and the alkaline solution acidified with 3 N HCl. Materials extracted into diethyl ether were identified by comparison of R_f values, color reactions, and ultraviolet spectra with authentic p-methoxy- and 3,4-dimethoxybenzoic acids.

In addition, thioglycolic acid degradation products from methylated products were treated with diethylsulfate in acetone over potassium carbonate. The ethylated thioglycolates were isolated by preparative TLC, and ester exchange was performed by treating the ethyl esters overnight at room temperature with absolute methanol containing a few drops of sulfuric acid.

The major ethyl thioglycolate obtained from methylated polyflavonoids was treated with aniline-Mg-Br prepared according to Betts et al. [15]. Colored impurities were eluted from a silica gel column with petroleum ether:diethyl ether (3/2), and the acetanilide was eluted with diethyl ether. The acetanilide was further purified by preparative TLC, developing the plates with diethyl ether. A small amount was crystallized from benzene:hexane (3/1). Because of the low yields of thioglycolates obtained, spectral properties were determined on the chromatographically pure but amorphous material.

Cold Water Soluble Polyflavonoids

New outer bark from shortleaf pine was also extracted with water at room temperature to obtain 3.4% of the dry bark weight. One portion of the water extract was concentrated at 50°C under vacuum and poured into ethanol to precipitate a carbohydrate rich fraction (0.06%) and the ethanol soluble material (2.7%) was redissolved in water and dialyzed to retain a fraction amounting to 1.2% of the dry bark weight. Another portion of the water extract was freeze-dried directly and 50% of the extract or 1.7% of the dry bark weight was retained after dialysis.

The two polyflavonoid rich fractions were methylated with diazomethane in ether and ether-dioxane mixtures, treated with mercaptoacetic acid, and the thioglycolic acid derivatives were methylated with dimethylsulfate over potassium carbonate in acetone [12]. Yields of methyl thioglycolates obtained from: 1) cold water extracts which had not been exposed to heat; 2) cold water extracts which were concentrated at 50°C; and 3) hot water extracts were compared by visual observation of the spot intensity on TLC plates which were developed three times with diethyl ether:hexane (1/1).

The fraction obtained by cold water extraction which was not heated during concentration was used to prepare larger quantities of acetanilide derivatives of the thioglycolates by the procedures described above [12, 15].

Phenolic Acids

Shortleaf and loblolly new outer barks were exhaustively extracted with light petroleum ether, hot water, and ethanol to provide "extractive-free" bark. The extracted bark was treated with mercaptoacetic acid, soluble products were methylated with dimethylsulfate in acetone over potassium carbonate, and the acetone-soluble material was examined for thioglycolate derivatives by TLC as described above [12].

Samples of "extractive-free" bark of shortleaf pine were also treated with 0.5 N NaOH under N_2 at a bark to liquor ratio of 1 to 6 at room temperature for 1 hr. The extract was acidified by filtration from a separatory funnel into either acetic acid or HCl to avoid contacting air when at an alkaline pH. Precipitates were recovered by centrifugation, washed with water, and centrifuged down a second time. Acid-soluble material was dialyzed in Spectrapor #3 membranes and material retained was freeze dried. Total yields of products recovered ranged from 5–10% of the dry bark weight. Much higher yields could be obtained with more vigorous extraction conditions, but these were not used for structure analysis because of the relatively large carbonyl content [16] as well as problems with impurities.

TABLE I
Distribution of Methanol-Soluble Extractives in Shortleaf and Loblolly Pine Bark Tissues

	Methanol solubility, % of OD wt.		
	Phloem	New outer bark	Old outer bark
		SHORTLEAF	
Top	17.8	17.2	7.1
Middle	19.7	12.7	
Base	21.7	8.6	2.9
		LOBLOLLY	
Top	28.0	15.2	8.9
Middle	30.2	10.1	3.7
Base	20.5	9.3	3.8

Isolates were methylated either with diazomethane in a series of dioxane-water solvents [8] or with dimethylsulfate under N_2. Methylated products extractable into chloroform from base were separated on a silica column where a major product was eluted with 5% methanol in chloroform. This fraction was lyophilyzed from dioxane.

Ultraviolet and visible spectra were determined with a Beckman DK-2 and IR spectra (KBr disks) were determined with a Perkin-Elmer 457 or a Beckman IR-18. PMR spectra were determined in either $CDCl_3$ or D_6-acetone with a Varian T-60 by the staff of the Improved Chemical Utilization of Wood Project, Forest Products Laboratory, Forest Service, USDA. ORD spectra were determined in $CHCl_3$ with a Jasco J-20 by Dr. W. L. Mattice, Dept. of Biochemistry, and mass spectra were determined by Mrs. Paula Moses, Dept. of Chemistry, Louisiana State University, Baton Rouge, Louisiana. Elemental analyses were done by Galbraith Laboratories, Knoxville, Tennessee.

RESULTS

Distribution of Monomeric Flavonoids

Amounts of methanol-soluble material varied greatly with sampling position in the tree (Table I). Although the methanol solubility of phloem was high, only small proportions of flavonoids were present. Both new and old outer bark contained less methanol-soluble material at the base than at the top of the tree, and there was substantially less methanol-soluble material in old outer bark than in new outer bark at the same height in the tree. This distribution suggested that methanol solubility decreased with increasing age of the outer bark. Flavonoid composition did not differ between loblolly and shortleaf sampled at the same position in the stem. Greatest changes were associated with radial location, i.e., phloem, newly formed outer bark, and old outer bark (Table II).

TABLE II
Distribution of Flavonoids in Shortleaf and Loblolly Pine Barks

	Phloem	New outer Bark	Old outer Bark
Afzelechin			
Catechin	+++	++++	
Epicatechin			
Gallocatechin			
Leuco-proanthocyanidins	++–+++(2)[b]	++(4)	
Dihydrokaempferol			
Dihydroquercetin		+++–+++	++
Dihydroquercetin glucoside	+–+++		
Dihydromyrisetin		++–+++	++
Kaempferol			
Quercetin		++	++–+++
Myricetin		+++	+++–++++

[a] – indicates not yet detected; if present they are in small proportions; + = small amount; ++ = small-moderate; +++ = moderate; ++++ = moderate-large; +++++ = large.
[b] Number of major spots.

Flavonoids in the phloem were dominated by catechin (Fig. 1) (V, R_1R_2 = OH, R_3 = H) and two leuco- (IV, R_1R_2 = OH, R_3 = H) or proanthocyanidins, both of which gave cyanidin after treatment with Hcl:n-butanol. Phloem also contained moderate amounts of a glucoside of dihydroquercetin (II, R_1R_2OH, R_3 = H), but glycosides of other flavonoids have not as yet been found. Neither flavanon-3-ol nor flavon-3-ol aglycones were generally observed. Occasionally small amounts of myricetin (III, $R_1R_2R_3$ = OH) were detected, but they may have resulted from a wound reaction or contamination from small amounts of new outer bark.

When compared with phloem extracts, there were many more flavonoids in new outer bark. Both the number and amounts of leuco- or proanthocyanidins were larger in new outer bark than in phloem and they were major constituents even at the base of the tree, where the average tissue was comparatively old. Catechin remained a major constituent of new outer bark. Neither afzelechin (V, R_1R_3 = H, R_2 = OH) nor gallocatechin (V, $R_1R_2R_3$ = OH) have been found. Dihydroquercetin and dihydromyricetin (II, $R_1R_2R_3$ = OH) were present in large amounts and approximately equal proportions. We have not found substantial amounts of either dihydrokaempferol (II, R_2 = OH, R_1R_3 = H) or pinobanksin (II, $R_1R_2R_3$ = H), both of which have been reported in slash pine bark [4]. Their concentration must be low compared to dihydroquercetin and dihydromyricetin. The glucoside of dihydroquercetin that was observed in the phloem was not detected in new outer bark. Concentrations of quercetin (III, R_1R_2 = OH, R_3 = H) and myricetin were lower than their corresponding dihydro-analogues, and there was generally more myricetin than

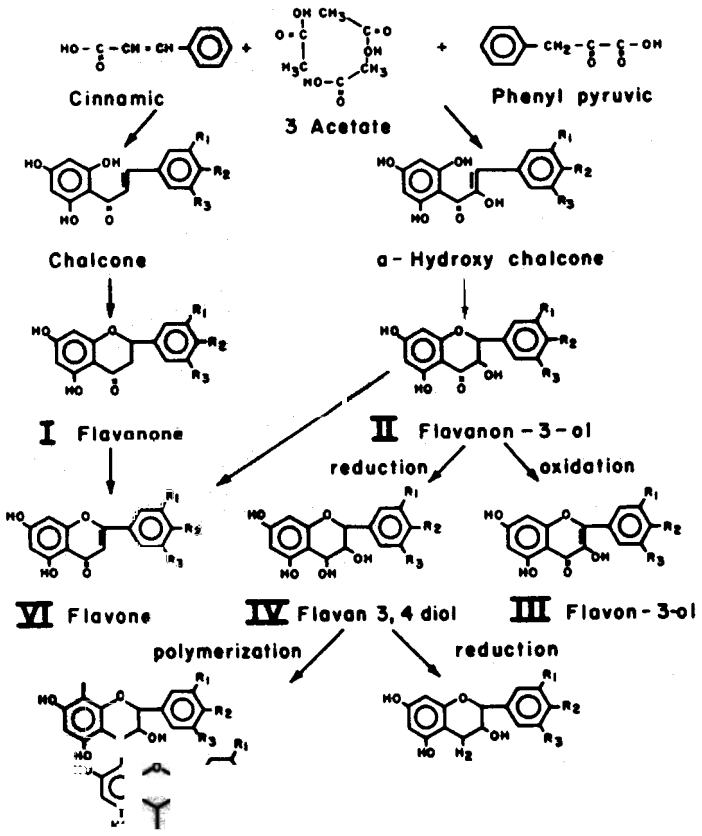

FIG. 1. Flavonoid biosynthesis (after Roux [17]).

quercetin. Kaempferol (III, R_2 = OH, R_1R_3 = H) and pinocembrin (I, $R_1R_2R_3$ = H) have not yet been isolated. They may occur in low concentrations.

Old outer bark from the lower portion of the stem contained small proportions of monomeric flavonoids. Both leuco- or proanthocyanidins and catechin were absent. Only small proportions of dihydroquercetin and dihydromyricetin were found. Myricetin and quercetin were major constituents of the old outer bark, and we generally found more myricetin than quercetin. Old outer bark samples from the top of the stem had a flavonoid composition similar to new outer bark. Flavonoid composition like methanol solubility appeared to change in relation to the age of the outer bark. As the tissue aged, there was a shift in dominant flavonoid class from leuco- or proanthocyanidins and catechin to flavanon-3-ols and finally to the flavon-3-ols.

Hot Water Soluble Polyflavonoids

Shortleaf pine contained more water-soluble polyflavonoids than did loblolly, but since degradation products of their polyflavonoids did not differ importantly, the following discussion applies to materials obtained from both species (Table III).

TABLE III
Water Soluble Materials in Shortleaf and Loblolly Pine New Outer Bark

	% of O.D. wt.	
	Shortleaf	Loblolly
Total hot water soluble	13.3	8.3
Ethanol insoluble		
Crude	4.9	3.5
Dialyzed	3.9	2.7
Ethanol soluble		
Crude	8.1	4.8
Dialyzed	2.8	1.5

Hydrolysis with 72% H_2SO_4 gave less than 1% of the isolates as reducing sugars and 70% as acid insolubles. These materials gave high yields of anthocyanidins (10–13%) compared with yields obtained by Roux and Bill [9] from model compounds. Cyanidin was the only product identified. Spots at R_f values corresponding with pelargonin or delphinidin were not detected. After alkaline fusion, however, 3,4-dihydroxybenzoic acid, p-hydroxybenzoic acid and phloroglucinol were obtained in approximate ratios of 4:1:1, respectively. Only traces of catechol were observed. We did not find either gallic acid or pyrogallol, both of which should have been readily detected if present.

Treatment of the diazomethane-methylated isolates with mercaptoacetic acid followed by methylation gave two pairs of products on TLC plates [12, 13]. The lower pair was at R_f values identical with authentic methyl epicatechin and catechin thioglycolates. Permanganate oxidation of isolates from this R_f region gave 3,4-dimethoxybenzoic acid as a major product. The suspected 2-3 cis isomer occurred at about five times the concentration of the 2-3 trans isomer, judged from relative spot intensity. The pair of compounds at higher R_f value occurred at approximately one-fourth the concentration of the epicatechin and catechin thioglycolates. The ratio of the lower to higher R_f member of this pair was also about 5:1. Permanganate oxidation of material obtained from this R_f zone gave some p-methoxybenzoic acid. Multiple development of TLC plates gave some streaking from the origin, but a distinct band which might be a 3′,4′,5′ B-ring hydroxylated derivative was not observed.

When the thioglycolic acid derivatives were ethylated, the two pairs of red spots were observed at R_f values slightly higher than those of the methylated compounds. Ester exchange in methanol containing a small amount of H_2SO_4 gave products at R_f values identical with the methylated derivatives, suggesting that ethylation of thioglycolic acid derivatives did not introduce aromatic ethoxyl groups. Several unsuccessful attempts were made to purify and crystallize the ethyl ester of the major product. Severe instability, possibly because of hydrolysis of the ester, was apparent. Betts et al. [15] report a melting point of only 47–50°C for the methyl epicatechin thioglycolate so the major product was treated with aniline·Mg·Br to obtain a more stable derivative. The UV spectra of the product (MeOH) showed λ_{max} 205 nm with shoulders at 233 and 275 nm. The IR spectra (KBr) showed amide bands at 1680,

3350, and 1625 cm^{-1} and a strong aromatic C—N stretch at 1330 cm^{-1}. The PMR spectra ($CDCl_3$) showed: t 0.90 (1H,s,NH); 2.35-2.70 (5H,m,aniline Ph); 2.85-3.05 (3H,m,B-ring); 3.75-3.90 (2H,q,A-ring); 4.50 (1H,s,H_2); 5.70 (1H,d,H_4); 5.85 (1H,m,H_3); 6.10, 6.25, 6.35 (6H,3H,3H, each s, aromatic OCH_3); and 6.45-6.50 (2H,m,SCH_2). The hydroxyl region was too broad for interpretation. Aliphatic impurities were evident at t 9.0, but an aromatic ethoxyl was not present. The ORD spectra was a strongly levorotary plain dispersion curve, but was only about 70% of the rotation at each wavelength reported by Betts et al. [15]. A small amount of material was crystallized from benzene:hexane (3:1) which had a melting point of 160-162°C (lit. 162-163°C, [15]).

Because of the low yields of thioglycolates obtained from the methylated hot water-soluble polyflavonoids, cold water-soluble polyflavonoids were used to prepare larger quantities of anilide derivatives. As in degradation of the hot water-soluble materials, a compound with TLC R_f values and color reactions identical with authentic methyl epicatechin thioglycolate was the major product obtained from the cold water-soluble polyflavonoids regardless of the method used to concentrate the extract. Smaller amounts of a product with TLC properties identical with those of authentic methyl catechin thioglycolate were present in addition to a pair of red reacting compounds at much higher R_f values.

Treatment of either the ethyl or methyl esters of the major thioglycolic acid derivative with aniline·Mg·Br gave a product with UV spectra (λ_{max} 205, 233sh, 275sh) identical with that obtained from the hot water-soluble polyflavonoids. After precipitation from n-hexane, the white amorphous powders gave IR spectra with much reduced absorbtion in the aliphatic CH stretch region (2965 and 2850 cm^{-1}) compared with the material obtained from the hot water-soluble polyflavonoids which had not been precipitated from hexane. The PMR spectra ($CDCl_3$) of acetanilides obtained from both the ethyl and methyl esters were very similar to that obtained from the derivative obtained from the hot water-soluble materials, except that much less aliphatic impurity was evident at t 9.0. The H_2 signal at t 4.55 with $J < 1.0$ Hz and H_4 at 5.80 with $J \simeq 2.0 - 3.0$ Hz suggested a 2-3 cis, 3-4 trans configuration [15,31]. An aromatic ethoxyl was not present in material which was ethylated after treatment with mercaptoacetic acid. Needle shaped crystals (mp 114-115°C) were obtained from benzene:hexane (3:1) with the acetanilide derivative of both the methylated and ethylated thioglycolates from the cold water soluble polyflavonoids. Despite the great similarity in the PMR spectra of products obtained from hot and cold water-soluble polyflavonoids, different crystal types were found. Elemental analysis showed C = 65.7, H = 5.84 and N = 2.49% (calculated for $C_{27}H_{29},NO_7S$: C = 63.4, H = 5.67 and N = 2.74). The high carbon and hydrogen contents suggested that approximately 1 mole of benzene for every 2 moles of the acetanilide derivative may have been included in the crystal lattice. Mass spectra obtained at a sample temperature of 80°C were typical of benzene: (m/e; 78, 50, 51, 52, and 39), but significant amounts of hexane were not detected. At higher temperatures (170°C), the mass spectra were as expected for the suspected acetanilide derivative: [m/e; (511, 1%); (344, 63%); (327, 96%); (190, 96%); and (167, 100%)]. The ORD spectra ($CHCl_3$) of these needle shaped crystals showed: [α] (589 nm,—63°); 578 nm,—67°); (546 nm,—73°); 436 nm,—122°), and (365 nm,—188°); values which are approximately 85-90% of those reported by Betts et al. [15]. The optical rotations would be expected to be approximately 90% of those reported by Betts et al. [15] if benzene were present in

amounts suggested by the elemental analysis and mass spectra (recrystallization from methanol-water gave prisms, mp 161–162°C).

The product obtained in second highest yield was the lower member of the high R_f pair (R_f 0.43 compared with 0.17 and 0.22 for authentic methyl epicatechin and catechin thioglycolates respectively). Permanganate oxidation of material obtained from this R_f zone after degradation of hot water soluble polyflavonoids gave some p-methoxybenzoic acid but it was not obtained from these larger isolates prepared from the cold water soluble polyflavonoids. A small amount of 3,4-dimethoxybenzoic acid was present. The methyl ester was treated with aniline·Mg·Br and the product, after purification by preparative TLC, was precipitated from n-hexane. The UV spectrum was essentially identical with that of the acetanilide derivative described above. The IR spectra differed from that of the major product primarily in a greatly reduced OH absorption (3450 cm^{-1}) and sharp additional bands at 1100 and 1080 cm^{-1}, suggesting etherification of the aliphatic hydroxyl. The PMR spectrum ($CDCl_3$) showed: t 0.90 (1H,s,NH); 2.30–2.65 (5H,m,aniline PH); 2.85–3.10 (3H,m,B-ring); 3.80–3.95 (2H,q,A-ring); 4.55 (1H,s,H_2); 5.65–5.75 (1H,d,H_4); 6.10,6.20,6.30 (6H,3H,3H, each s, aromatic OCH_3); 6.50 (2H,s,SCH_2) and 6.80 (3H,s,aliphatic OCH_3). The H_3 signal was no longer evident at t 5.90 and splitting of the SCH_2 protons was reduced so that it appeared as a singlet. The IR and PMR spectra strongly suggest that this product is an artifact produced by methylation of the aliphatic hydroxyl at C_3. Yields obtained when the thioglycolic acid derivatives were methylated with dimethylsulfate or ethylated with diethysulfate were remarkably consistent considering the large number of times the reaction was repeated and the wide range in proportions of reagents.

The compound obtained in third highest yield had R_f values and color reactions indicative of a catechin derivative but we have not as yet obtained sufficient amounts of derivatives of this product to establish its identity.

Although the above degradation products suggest that the polymer is composed of a (−) 2-3 cis,3,3′,4′,5,7-hydroxyflavanol linked through C_4 to C_6 or C_8, examination of the methylated polymers directly rather than through their degradation products suggested that more complex structures may also be present. It should be recognized that yields of degradation products obtained from these polymers were generally low. Material methylated with diazomethane had a lower methoxyl content (23.1%) than when it was further methylated with dimethylsulfate (26.4–32.5%). All methoxyl contents were significantly lower than calculated values (35.9%) for a model of a 3,3′,4′,5,7 pentahydroxyflavanol linked through C_4—C_6 or C_8 as indicated by the degradation products. Incomplete methylation of the diazomethane-methylated material was indicated by the relatively low absorbance in the aliphatic CH (2930 cm^{-1}) and high hydroxyl (3450 cm^{-1}) in relation with the aromatic skeletal vibration (1590 cm^{-1}). Methylation with dimethylsulfate did not greatly increase the carbonyl absorption (1750 cm^{-1}). Contrary to expected results, the first fraction eluted from silica columns with chloroform had the lowest methoxyl content (26.4%) of the three dimethylsulfate methylated isolates. The high hydrogen content (7.1 versus 5.8% calculated) and the strong aliphatic CH absorption in the IR spectra indicated that this fraction contained considerable aliphatic impurity. In comparison with the chloroform eluates, the first band eluted with 5% methanol in chloroform had a slightly higher methoxyl content (28.2%) and carbon and hydrogen contents of 60.0 and 6.31%. The intensity of the hydroxyl and carbonyl relative to aromatic absorption compared favorably with material eluted with chloroform. The second fraction eluted with 5%

methanol in chloroform contained the most methoxyl (32.5%), but also had a very low carbon content (55.7%), and the IR spectra indicated substantially more carbonyl and hydroxyl than the other fractions. Despite the major differences between these isolates, yields of ethyl thioglycolate derivatives from all four materials were similar.

All the isolates contained low carbon contents compared with calculated values (66.7%). After correction for the low methoxyl contents, a carbohydrate content of 10–33% would be required to explain the low carbon content. PMR signals in the τ 6.2–6.8 region suggest small proportions of carbohydrates but they were not large enough to account for the low carbon content. The polyflavonoids must have more complex structures than indicated by the degradation products.

Phenolic Acids

The proportions of methyl-thioglycolates obtained from shortleaf and loblolly "extractive-free" barks did not differ. As in the degradation of the water soluble polyflavonoids, a pair of compounds was obtained with properties identical with epicatechin and catechin thioglycolates in ratios of about 5 to 1.

If extraction conditions were mild, sodium hydroxide-soluble fractions amounting to 5–10% of the dry "extractive-free" bark could be obtained without encountering substantial carbonyl contents. In all fractions, carbonyl appeared only as a shoulder on the aromatic band. Treatment of the alkali-soluble extracts with acetic acid yielded higher proportions of soluble/precipitated phenolics than did HCl acidification. Both acetic acid-soluble and -insoluble fractions showed more OH absorption in their IR spectra and substantial impurities in the τ 6.2–7.0 region of the PMR spectra after methylation than did the corresponding HCl acidified materials.

After methylation with dimethylsulfate, the HCl-insoluble material had a methoxyl content of 28.3%, and the PMR spectra integrated as three B-ring protons (τ 3.0–3.1), one A-ring proton (τ 3.8–4.0), and 15 protons in the methoxyl region (τ 5.5–6.1). Peak broadening was too severe to detect protons of the C-ring and the peak at τ 6.0 was very broad on the upfield side; so, although high, the integration of this peak was not too far removed from a calculated value of 12 protons. The methylated HCl-soluble fraction had a methoxyl content of 33.4%, but the PMR spectra showed substantial impurity as a sharp singlet at τ 6.6 and a broad band centering at τ 6.4.

DISCUSSION

Distribution of Monomeric Flavonoids

Both short leaf and loblolly pines belong to the Australes group of the Diploxylon subgenera, so it is not surprising that no substantial differences in the composition of flavonoids were found between these species. This is not to suggest that there was little specificity in the biosynthesis of flavonoids in southern pines. As evidenced in other classes of compounds, variations in flavonoid composition between tissues are great. The flavonoids in bark differed from those in heartwood [18] leaves [19], and pollen [20], all of which are different from each other in flavonoid composition.

There was great specificity in B-ring hydroxylation patterns of different classes of flavonoids in the barks. Flavon-3-ols contained more 3′,4′,5′, than 3′,4′ hydroxy

substitution, and the 4′ hydroxyflavon-3-ol, if present, was in low concentrations. Flavanon-3-ols were evenly divided between 3′,4′ and 3′,4′,5′ hydroxy substituted compounds. The leuco- or proanthocyanidins and flavan-3-ols were dominated by a 3′,4′ hydroxy substitution. Another important feature of the distribution of monomers was persistence of leuco- and/or proanthocyanidins in new outer bark tissues even at the base of the tree where the tissue was relatively old. The dominant flavonoid class gradually shifted from leuco- or proanthocyanidins and catechin to flavanon-3-ols and finally to flavon-3-ols as the outer bark became older. This suggested a comparatively slow polymerization process and may explain the apparent heterogenity of the polymers in both molecular weight and structure.

Water-Soluble Polyflavonoids

Many structures have been verified or proposed for polyflavonoids in plants (Fig. 2). A structure found by Freudenberg et al. [21] after acid catalyzed condensation of catechin was favored by Erman and Lyness [8] in their evaluation of phenolic acids in slash pine bark (Fig. 2, VII). Hathway [22-24] and Hathway and Seakins [25] observed orthoquinone condensation to biphenyl structures when studying polyphenol oxidase enzymes with catechin. They proposed biphenyl structures for bark polyflavonoids of *Quercus pedunculata, Q. sessiliflora* (VIII). Hergert [6] originally proposed C_3 and C_4—O—C_7 ethers resulting from dehydration of leucoanthocyanidins as the dominant linkage in western hemlock bark (IX). A similar structure was proposed by Betts et al. [26] for polyflavonoids in heather (*Calluna vulgaris*), but this was prior to the observation by Sears and Casebier [27] that thioglycolic acid cleaved not only benzylic ethers but also the C_4—C_6 or C_8 linkage of model polyflavonoids. More recent work by Hergert [1] and Sears and Casebier [12] has favored a C_4—C_8 linkage (X). Roux and coworkers [3,28,29] found that a C_4—C_6 linkage predominated in crystalline leucofisetinidins from *Acacia mearnsii* (XI). Weinges et al. [30] and Thompson et al. [31] have demonstrated preponderance of C_4—C_8 linkage in 3,3′,4′,5,7-hydroxy bi- and triflavanols isolated from a wide variety of plants. Karchesy [32] recently demonstrated that the condensed tannin in *Alnus rubra* was a C_4—C_6 or C_8 linked 3,3′,4′,5,7-pentahydroxypolyflavonoid. Porter [33], studying the dimeric and trimeric procyanidins in *Pinus radiata* phloem, also found the C_4—C_8 linkage.

The degradation products we obtained from water-soluble polyflavonoids of both shortleaf and loblolly pine barks indicated that 3,3′,4′,5,7-hydroxyflavanols were linked C_4—C_6 or C_8 in a substantial portion of the polymer. Structures such as VII or VIII would not be consistent with the good yields of anthocyanidins or the alkaline fusion and thioglycolate products obtained. If ether linkages such as IX were dominant, we would not have obtained 3-hydroxy-3′,4′,5,7-tetramethoxyflavan-4-yl-thio) acetanilide after ethylation of the thioglycolic acid degradation products. An aromatic ethoxyl would have been observed.

The thioglycolic acid degradation products we obtained from the water-soluble polyflavonoids were predominantly of the 2-3 cis configuration. We found catechin and not epicatechin, however, as a major constituent of the monomeric flavonoids as did Hergert [4], studying *Pinus elliottii*, and Markham and Porter [34], studying *Pinus radiata*. Hergert [5] reported that the 2-3 trans, 3-4 cis flavan-3,4-diol was the predominant leucoanthocyanidin in longleaf pine (*Pinus palustris*) phloem.

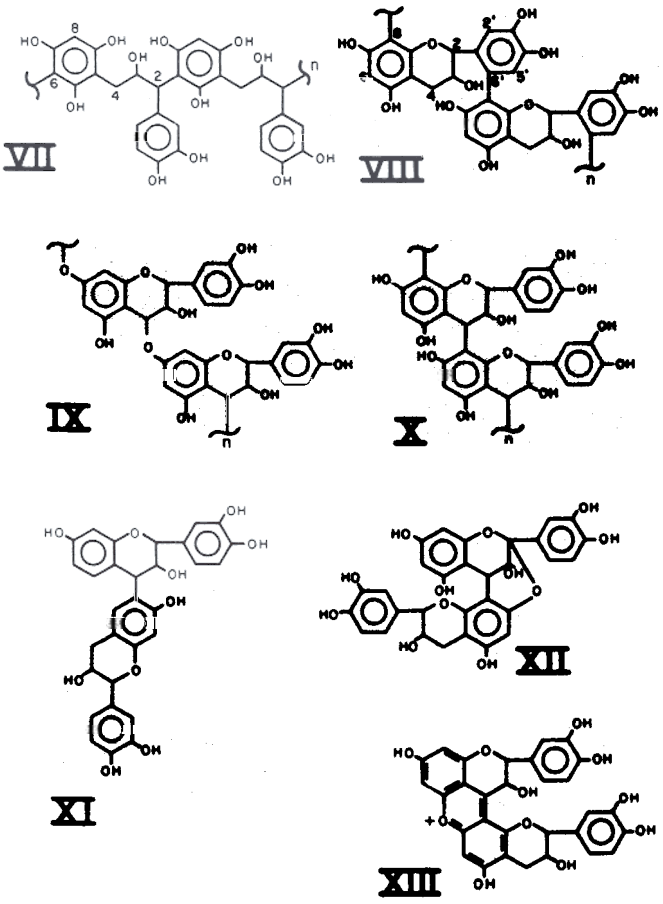

FIG. 2. Polyflavonoid structures proposed or verified.

Porter [33] found that the 2-3 trans isomers predominated in the procyanidins of *P. radiata* phloem.

Sears and Casebier [27] and Betts et al. [15] show that the 2-3 stereochemistry is preserved in reaction with thioglycolic acid. Since the H_2 proton appears at τ 4.50–4.55 (J<Hlz) in the 2-3 cis isomer and at τ 5.10 (J 6.3–9.1 Hz) in the 2-3 trans isomer [12], the PMR spectra of the two isomers are clearly different. Although the spectra for the ethyl ester at the lowest R_f value showed impurities, a singlet was evident at τ 4.55, but no signal was evident at τ 5.00–5.20. The H_2 signal of the acetanilide derivative was also a sharp singlet at τ 4.50–4.55 which was similar to the assignment found for the 2-3 cis,3-4 trans isomer synthesized by Betts et al. [15].

Since the 2-3 cis configuration can be deduced from the PMR spectrum, only four isomers remain possible of which one (XIV) was synthesized and its ORD spectrum reported by Betts et al. [15] (Fig. 3). Isomers XIV and XV would arise from the naturally occurring (−) epicatechin structure while isomers XVI and XVII would arise from epimerized (+) catechins. Since the optical rotation of the acetanilide obtained from water-soluble polyflavonoids in pine bark was strongly levorotary, isomer XVII can be eliminated as it is the mirror image of XIV which has been synthesized and shown to be strongly levorotary [15]. Isomer XV seems unlikely as Betts

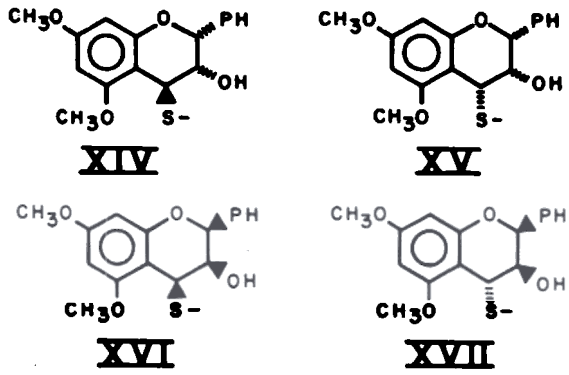

FIG. 3. Configuration of isomeric forms of 2-3 cis (3-hydroxy,3',4',5,7-tetramethoxyflavan-4-yl-thio) acetanilide.

et al. [15] and Thompson et al. [31] report quite stereospecific reaction of (−) epicatechin models with mercaptoacetic acid and toluene-α-thiol such that 3-4 trans isomers are recovered exclusively. Such is not the case with (+) catechin models where the 3-4 cis isomer predominates but both 3-4 cis and trans isomers are obtained [15,31]. Isomer XVI would be obtained if there was: 1) epimerization of (+) catechin units through inversion of the B-ring; and 2) a 3-4 cis stereospecific reaction of the carbonium ion with mercaptoacetic acid. Some epimerization of a (+) catechin polymer might be expected based on studies of the epimerization of 2-3 trans flavan-3-ols [35,36], 2-3 trans,3-4 trans flavan-3,4-diols [37], and all trans biflavanols [29]; however, inversion of the B-ring would be expected to be far too limited to account for the high proportion of 2-3 cis isomers obtained from the pine bark. There were no significant differences in the ratio of 2-3 cis to 2-3 trans isomers obtained from polyflavonoids isolated by cold or hot water extraction. In addition, a stereospecific 3-4 cis reaction of the mercaptoacetic acid with a 2-3 cis carbonium ion seems unlikely. The most logical choice is the isomer XIV which was synthesized by Betts et al. [15]. While the first crystalline product obtained from the hot water-soluble polyflavonoids had a melting point (160–162°C) very close to that reported by Betts et al. [15] for isomer XIV (162–163°C), insufficient amounts were obtained to determine the ORD spectra on this crystalline product. When larger amounts were obtained, the crystal shape and melting point differed from the data reported by Betts et al. [15] because some benzene was obviously included in the crystal lattice (recrystallization from methanol-water gave prisms, mp 161–162°C). The ORD spectrum was only 85–90% of the rotation reported for XIV as would be expected from the amounts of benzene calculated to be present based on the elemental analyses.

A conclusion that the polyflavonoids of the outer bark are made up of (−) epicatechin units while (+) catechin was found as the dominant monomer and (+) catechin proanthocyanidins [33] are present in the phloem has very important implications from a biosynthesis viewpoint. While it is true that yields of thioglycolates were generally low, reaction conditions were varied over wide ranges in reagent concentrations and time periods of 1 to 16 hr with the same result. If the polymers of outer bark are formed via a self-condensation process, the (−) epicatechin monomer has definite advantages as the 3 hydroxyl—ether hydrogen bonding does not have to be forfeited to obtain a conformation in which the B-ring is equitorial and the spacing

around C_4 is adequate for addition of the bulky A-ring of the second C-15 unit. Because of its important implications on the biochemistry of outer bark polyflavonoids, further study of the stereochemistry of polyflavonoids in phloem and outer bark tissues should be done to resolve this question.

Phenolic Acids

The great similarity in proportions of thioglycolates obtained from the phenolic acid fractions and the water-soluble polyflavonoids indicated that the structures of these materials are similar. These results support Hergert's thesis [1] that differences in solubility are essentially due to differences in molecular weight and accessibility rather than in structure. While a significant proportion of the phenolic acids appeared to be 2-3 cis 3,3′,4′,5,7 pentahydroxyflavanols linked through C_4-C_6 or C_8, as in the water-soluble polyflavanoids, microanalysis and spectral properties indicated more complex structures. The methoxyl content of the best isolates (in terms of their PMR and IR spectra and C and H contents) of both phenolic acids and water-soluble polyflavanoids was 28.2–28.3% or about 80% of calculated values. The PMR spectra indicated 3 B-ring protons and 1 A-ring proton per flavanoid unit. The IR spectra did not show substantial carbonyl, so rearrangement products such as catechinic acid described by Sears et al. [16] would not appear to be a major cause of low methoxyl and carbon contents.

The water-soluble extracts contained a material, possibly unrelated to polyflavonoids, which gave substantial amounts of p-hydroxybenzoic acid after alkaline fusion. This material could account for a significant proportion of the low methoxyl content found for both water- and alkali-soluble polyflavonoids. Either structures such as the procyanidins A_1 and A_2 (XII) as described by Weinges and coworkers [30] or xanthylium linkages (XIII) as described by Jurd [38] would reduce the methoxyl and carbon content of methylated products and retain the A- to B-ring proton ratio found. Structures such as these cannot be discounted and may well be present in southern pine bark polyflavonoids.

CONCLUSIONS

1. The composition and distribution of monomeric flavonoids were similar in shortleaf and loblolly pine barks.
2. Monomeric phenolics were dominated by 3,3′,4′,5,7 and 3,3′,4′,5,5′,7 hydroxyflavonoids.
3. Variation in B-ring hydroxylation between classes of flavonoids was extensive: flavon-3-ols contained more 3′,4′,5′ than 3′,4′ hydroxy substitution, flavanon-3-ols were made up of nearly equal proportions of 3′,4′,5′ and 3′,4′ hydroxy compounds, while the flavan-3-ols and polyflavonoids were dominated by 3′,4′ hydroxy B-ring substitution. No evidence for 4′ or 3′,4′,5′ hydroxy substituted polyflavonoids was found.
4. Flavonoid composition changed abruptly from phloem to outer bark and within outer bark, varied with tissue age. Judged from the presence of leuco- or proanthocyanidins in new outer bark tissues even at the base of the tree, these compounds were apparently polymerized slowly.
5. While shortleaf pine barks contained more water-soluble polyflavonoids than did loblolly, polyflavonoid degradation products indicated similar structures.
6. Products obtained from both hot and cold water-soluble polyflavonoids suggested

that these polymers were composed of (−) 2-3 cis 3,3′,4′,5,7-hydroxyflavanols linked C_4 to C_6 or C_8. Small proportions of the 2-3 trans isomers were also indicated.

7. Thioglycolates obtained from "extractive-free" bark and spectral properties of phenolic acid isolates indicated that they were of similar structure to the water-soluble polyflavonoids.

8. Elemental analysis and spectral data obtained from the methylated polymers indicated that more complex structural features were present in addition to the C_4 to C_6 or C_8 linked epicatechin model suggested by degradation products.

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