HEARTWOOD EXTRACTIVES OF *MACLURA POMIFERA* AND THEIR ROLE IN DECAY RESISTANCE[1]

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ABSTRACT

A sample of ground heartwood of *Maclura pomifera* (Raf.) Schneid. was extracted sequentially with hexane, chloroform, and methanol. The dry methanol extract was partitioned between water and ether. The ether fraction was separated by liquid-liquid extraction with aqueous sodium bicarbonate and sodium carbonate solutions to differentiate the phenolics from organic acids and neutral materials. The phenolic components were separated by paper chromatography using benzene-acetic acid-water (125/72/3) as the developing solvent. The separated subfractions were examined quantitatively as well as qualitatively. The fungal toxicities of various extracts, fractions, and subfractions were determined by incorporating each material into nondurable ground wood or wood-base material and then inoculating the sample with one of several wood decay fungi. Fungal growth in wood was measured by respirometry or visual observation. The subfraction with the greatest inhibitory effect on wood decay fungi, as characterized by spectroscopic methods, appears to be a mixture of tetra- and pentahydroxystilbenes.

Keywords: Heartwood, decay resistance, extractives, *Maclura pomifera*.

The heartwood of osage-orange (*Maclura pomifera* (Raf.) Schneid.) has long been known as an exceptionally durable wood (USFPL 1961), based on its extremely long service life under conditions favorable to decay. According to the ASTM Scale (1980b), the heartwood can be rated in the highly resistant category. When exposed to decay fungi under controlled environmental conditions conducive to rot, the heartwood is considerably more durable than is its sapwood (Hart and Johnson 1970; Hart and Shrimpton 1979). Its decay resistance is reported to be due primarily to the presence of extractives, stilbenes, which inhibit fungal growth (Barnes and Gerber 1955; Wang et al. 1976). The wood extractives of osage-orange were located mainly in the heartwood and were more soluble in polar solvents such as methanol and ethanol-water than in nonpolar solvents (Wang et al. 1976). Decay resistance of the heartwood was removed or significantly reduced by extracting the wood with these polar solvents (Hart and Shrimpton 1979; Wang et al. 1976).

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Pinosylvin, a dihydroxystilbene, and its monomethyl-ether form occur in pine heartwood and are reported to be responsible for the durability of the heartwood (Erdtman 1955; Rudman 1963, 1965). Reservatrol, a trihydroxystilbene, may play a role in the durability of eucalypts (Hart and Hillis 1974). Thus, there are a number of reports suggesting that stilbenes increase the resistance of wood to deterioration (Scheffer and Cowling 1966). However, this hypothesis has recently been questioned (Hart and Shrimpton 1979; Loman 1970).

The study conducted by Barnes and Gerber (1955) did not provide information concerning the inhibitory values of the active compounds to decay fungi in wood or wood-base materials. Only the extent to which the compound (2,4,3′,5′-tetrahydroxystilbene) protected a liquid medium against the test fungi was determined. In addition, this research did not employ wood-inhabiting fungi or the decay fungi commonly recommended for such tests (ASTM 1980a, b). In view of these facts, many facets of potentially useful information concerning the durability of osage-orange heartwood remain to be developed.

The objectives of this study were to determine what factors are responsible for the natural decay resistance of osage-orange and to obtain more information on the chemical properties of this wood.

MATERIALS AND METHODS

The test wood came from an osage-orange tree, with a diameter inside bark of 15 cm, collected at W. K. Kellogg Forest, Augusta, Michigan, in September 1974. A portion of the stem, free from knots and defects, was selected for the test material. The bark was removed and the log was air-dried, cut into short logs and stored at 4 C. The logs were then cut into strips 1 cm by 1 cm, with heartwood and sapwood kept separate. These strips were ground in a Wiley mill to pass a 40-mesh screen and the ground wood was stored in plastic bags at 4 C.

Extraction and separation of extraneous components

Since the fungistatic extractive previously reported (Barnes and Gerber 1955; Wang et al. 1976) was of a phenolic nature, the chemical analysis focused on the isolation of phenolic substances. Several extractions were conducted (Fig. 1). Ground osage-orange heartwood (200 g) was extracted with each solvent for 10 days at room temperature in a glass column. The column was fitted at the bottom with a stopcock to control the flow of liquid, and the solvent exit was covered with a loose plug of glass wool.

Preliminary extractions with hexane and chloroform were conducted to remove most of the nonpolar extractives. After the completion of each extraction, the ground wood was removed from the column, dried at room temperature, and then replaced into the column for the next extraction. The extract was collected five times per day by draining the column to the level of the wood surface, and then refilling with fresh solvent. Each extract was dried in a rotary vacuum evaporator at less than 40 C, and the dry weight was recorded. The air-dried weights of ground wood before and after extraction were used for quantitative determinations.

To further fractionate the materials, several partitions were conducted (Fig. 2). The methanol extract was first partitioned between ether and water to separate those less polar substances from the highly polar materials. The air-dried meth-

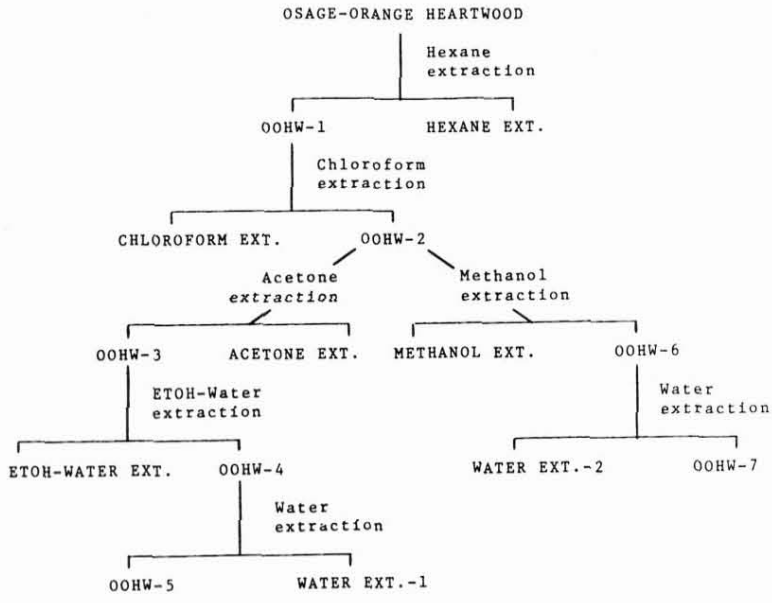


FIG. 1. Separation scheme for heartwood extractives of osage-orange.

anol extract was dissolved in ether and filtered to remove insoluble materials. The ether-insoluble materials were washed with distilled water to extract those materials soluble in water. The remaining water-insoluble substances were redissolved in methanol. The ether solution was then extracted with distilled water in a separatory funnel. The water phase was removed and washed with ether to extract entrained materials that were returned to the main ether solution.

The second and third partitions were accomplished by utilizing the weakly acidic character of phenolic substances (Browning 1967). These depend on the removal of strong acids by liquid-liquid extraction into an aqueous phase of sodium bicarbonate, a weak alkali, followed by extraction of the phenolics from the neutral materials by sodium carbonate, a strong aqueous alkali. Hence, the ether-soluble fraction was partitioned between ether and saturated aqueous sodium bicarbonate. The aqueous extract was washed with ether to remove entrained materials, and the washings were returned to the main ether fraction. The aqueous phase was then neutralized to pH 6.5 with hydrochloric acid, and the neutralized aqueous solution was extracted with ethyl acetate.

The third partition was the extraction of the remaining ether-soluble materials with saturated aqueous sodium carbonate. The procedures used were similar to those used during partitioning with sodium bicarbonate. Each fraction was dried in a rotary vacuum evaporator, and its weight was recorded.

Further separation and analysis were accomplished by using paper chromatography. Benzene-acetic acid-water (125/72/3) (Hillis and Ishikura 1968) gave good resolution of the components in the fraction F6 (Fig. 2).

The quantitative determinations were accomplished by weighing the various dry extracts and then calculating their percentages of the original wood based on the total air-dried weight of the wood from which each was obtained. The air-dried wood under the laboratory condition contained 8–9% moisture.

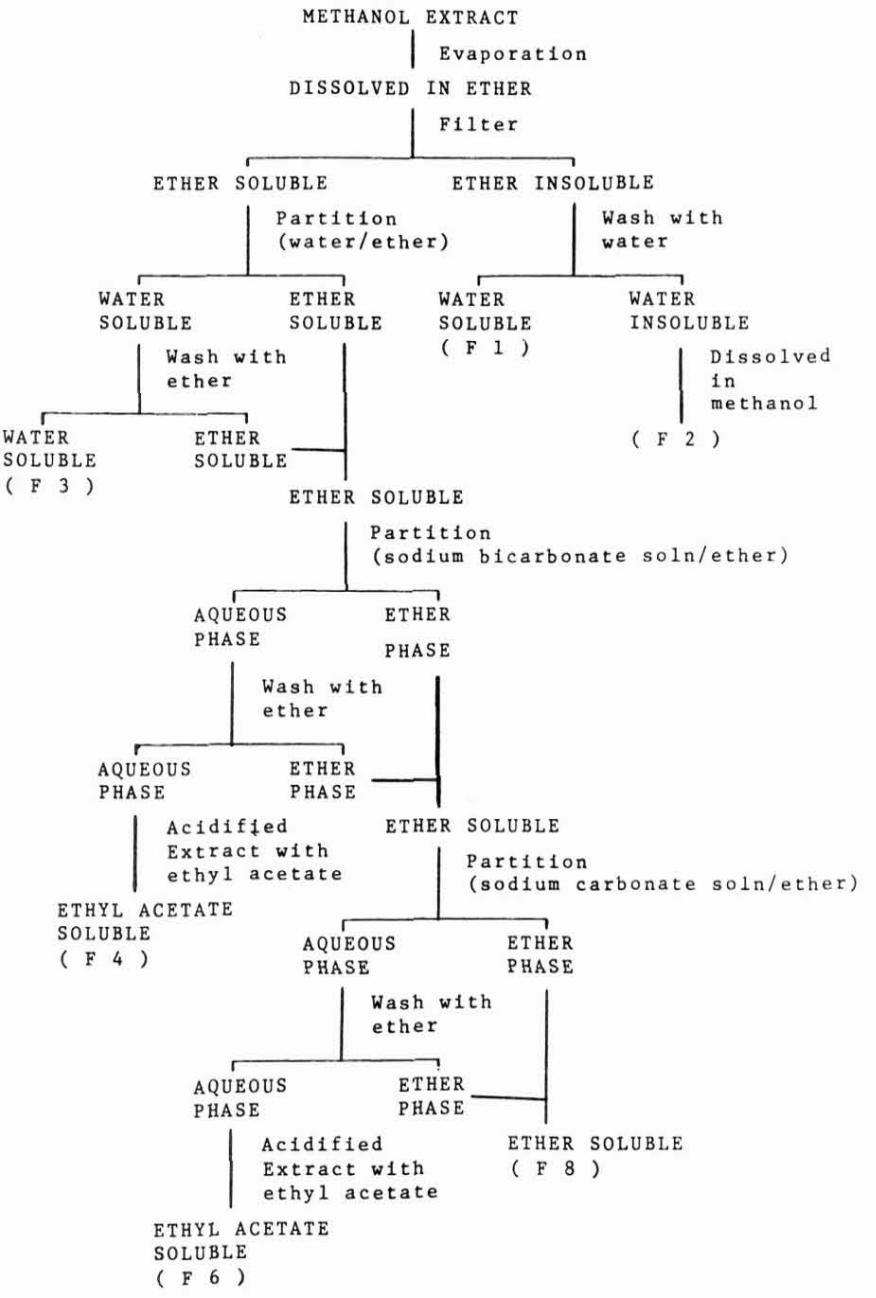


FIG. 2. Separation scheme for methanol extract (from Fig. 1).

Fungistatic properties of each fraction

To identify the components responsible for decay resistance, a 10-ml aliquot of each fraction containing the amount of that material extractable from 10 g of osage-orange heartwood was used to impregnate a 10-g sample of ground aspen

sapwood. Samples, including treated woods and untreated controls, were then exposed to *Coriolus versicolor* (L.) Quel. or *Gloeophyllum trabeum* (Pers. ex Fr.) Murr. The procedures of fungistatic testing were similar to those as previously described for the woodmeal-respirometry technique (Wang et al. 1980).

Since final separation of the extractives was conducted by paper chromatography, collection of sufficient material to impregnate a sample of ground aspen sapwood was difficult. Hence, the fraction containing the fungistatic extractive was streaked on Whatman 3 MM chromatographic paper, and eluted with benzene-acetic acid-water (125/72/3) in one direction. The developed paper was cut into strips corresponding to individual subfractions, and the impregnated paper was used as an alternative substrate to evaluate fungistatic properties. Several chromatograms were run and analyzed quantitatively until the amount of each separated subfraction of F6 retained in the developed paper strips was equivalent to the concentration of that subfraction in the wood. The paper strips were cut into approximately 5-cm squares, placed in a Petri dish, wetted with an optimum amount of distilled water as determined by the preliminary tests, and sterilized in an autoclave. A piece of fungal inoculum (15-mm-diameter, 5-mm-thick malt agar disc) of the same age was placed on the center of each test paper. Dishes were then incubated for three weeks at a relative humidity of 95–100% and 25 C. After incubation, fungal growth was evaluated by visual examination. Controls used were untreated paper and paper impregnated with 5% pentachlorophenol solution.

Chemical nature of the fungistatic components

The location of fluorescent compounds was determined on developed chromatograms, both before and after exposure to the fumes of concentrated ammonium hydroxide. Several chromogenic sprays, suggested for phenolic substances by Browning (1967), were used to detect wood extractives on the developed chromatogram. The resulting colors and the R_f values were recorded for semi-qualitative determinations.

The active subfraction was further characterized by nuclear magnetic resonance (NMR) and mass spectroscopic analysis. A chromatographic paper-strip containing the active subfraction was eluted with acetone to isolate fungistatic components. The acetone was removed in a rotary vacuum evaporator at less than 40 C, and any residual acetone was allowed to evaporate in a hood. The dry compound was dissolved in deuteron acetone and transferred into a capillary tube for NMR spectroscopic analysis. For mass spectroscopic analysis, a concentrated acetone solution of the active subfraction was used. The spectra were examined and compared with several reference spectra of similar compounds.

RESULTS AND DISCUSSION

Extraction, separation, and bioassay of the extraneous components

The total amount of extraneous chemicals obtained from the ground heartwood of osage-orange by successive extraction with four solvents of increasing polarity (hexane, chloroform, methanol, and water) comprised 1.5%, 0.5%, 16.2%, and 4.8% of the air-dried wood, respectively.

The fungal toxicity of each extract shown in Fig. 1, as determined by the woodmeal-respirometry technique is presented in Table 1. The acetone, ethanol-

TABLE 1. *Effect of heartwood extracts of osage-orange on growth of* Coriolus versicolor *and* Gloeophyllum trabeum *as determined in ground aspen wood by the oxygen consumption method.*[a]

Extract	*C. versicolor*		*G. trabeum*	
	Oxygen consumed[b] (μl/h)	Visual exam[c]	Oxygen consumed[b] (μl/h)	Visual exam[c]
Hexane extract	40.7	3.0	80.0	3.4
Hexane[d]	33.0	3.3	68.6	3.9
Chloroform extract	52.2	2.8	75.9	3.2
Chloroform[d]	46.3	3.5	68.4	3.2
Acetone extract	14.4[g]	1.0	0.7[g]	1.0
Acetone[d]	43.5	3.8	90.9	3.8
Ethanol-water extract	19.3	2.0	7.9	1.9
Ethanol-water[d]	26.9	3.5	52.8	3.6
Water extract-1	76.1	3.8	58.3	4.8
OOHW-5	93.3	4.5	122.6	5.0
Methanol extract	10.5[h]	1.0	1.8[h]	1.0
Methanol[d]	28.0	4.5	76.6	4.8
Water extract-2	40.6	3.3	41.5	4.5
OOHW-7	72.1	4.3	104.6	5.0
Aspen control	40.5	3.3	94.2	4.4
OOHW[e]	1.5	1.0	2.7	1.0
OOSW[f]	211.1	5.0	178.7	3.6

[a] Various extracts refer to Fig. 1 and the concentration of each extract in each ground aspen sample is equivalent to its concentration in the wood.
[b] Average of 5-hour testing at 30 C, means of three determinations.
[c] Each integer (1, 2, 3, 4, 5) represents coverage of the wood surface of 0–20% (1) to 80–100% (5); means of four estimations.
[d] Solvent only.
[e] Ground heartwood of osage-orange.
[f] Ground sapwood of osage-orange.
[g] Values for the acetone extract are significantly different from the values for acetone or aspen controls at 0.05 level.
[h] Values for the methanol extract are significantly different from the values for methanol or aspen controls at 0.05 level.

water, and methanol extracts contained fungitoxic materials because they, when applied to ground aspen wood, decreased the fungal activity as indicated by the reduced oxygen consumption. Among them, methanol extract had the greatest inhibitory effect. The hexane, chloroform, and both water extracts of fractions OOHW-4 and OOHW-6 (Fig. 1) did not inhibit the growth of either fungus in the ground aspen wood.

The unextracted sapwoods of osage-orange and both the extracted woods,

TABLE 2. *The calculated percentage (air-dried weight basis) of each fraction of the methanol extract*[a] *in the heartwood of osage-orange.*

Extractive fraction[b]	Amount in wood[c] (%)
F1	2.1
F2	2.2
F3	1.3
F4	2.5
F6	7.4
F8	0.7

[a] Methanol extract explained in Fig. 1.
[b] Fraction codes explained in Fig. 2.
[c] Theoretically a maximum value.

TABLE 3. *Effect of each fraction of the methanol extract on the growth of* Coriolus versicolor *and* Gloeophyllum trabeum *as measured in ground aspen wood by respirometry.*[a]

	C. versicolor		*G. trabeum*	
Extract fraction	Oxygen consumed[b] (μl/h)	Visual exam[c]	Oxygen consumed[b] (μl/h)	Visual exam[c]
F1	31.4	4.4	28.3	2.3
F2	27.8	3.4	11.9	2.0
F3	33.5	4.9	50.9	4.0
F4	46.3	4.5	18.4	2.0
F6	11.6[e]	1.3	6.2[e]	2.0
F8	41.5	4.5	37.0	2.8
Ether[d]	32.9	4.5	55.1	5.0
Ethyl acetate[d]	25.5	4.5	60.8	5.0
Aspen control	40.2	3.3	91.4	3.8

[a] Various fractions shown in Fig. 2. The concentration of each fraction in each ground aspen sample is equivalent to its concentration in the wood.
[b] Average of 5-hour testing at 30 C; means of three determinations.
[c] Each integer (1, 2, 3, 4, 5) represents coverage of the wood surface of 0–20% (1) to 80–100% (5); average of four estimations.
[d] Solvent only.
[e] F6 is significantly different from the solvents or aspen control at the 0.05 level.

OOHW-5 and OOHW-7 (Fig. 1), were more susceptible to fungal growth than the aspen controls. Thus, aspen wood may have contained certain materials that were slightly inhibitory to fungal growth.

Fractionation and bioassay of methanol extract

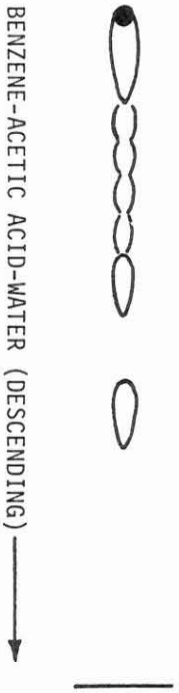
Since the methanol extract was the most fungitoxic, this material was fractionated (Fig. 2). The amount of each fraction (% air-dried weight basis) in the heartwood was determined (Table 2).

Evaluation of the fungistatic properties of each fraction (Fig. 2) of the methanol extractives showed that fraction F6 was the only fraction toxic to *C. versicolor* (Table 3). Fractions F2, F4, and F6 reduced the growth of *G. trabeum* but F6, based on the oxygen consumption data (Table 3), was the most inhibitory.

The seven subfractions in fraction F6 were separated by paper chromatography, and the amount of each in the heartwood (% air-dried weight basis) is given in Fig. 3. The fungistatic properties of each subfraction in F6 were evaluated by the rate of fungal growth as shown in Table 4, F6-A being the most inhibitory. Use of chromatographic paper as bioassay substrate allowed for the testing of very small quantities of a compound on a cellulose base.

Chemical nature of the fungistatic component

The developed chromatogram of the active fraction F6 (Fig. 2), with the R_f values and the color of each subfraction under ultraviolet illumination, is shown in Fig. 3. After the developed chromatogram was sprayed with ferric chloride-potassium ferricyanide, the fungistatic subfraction, F6-A, gave an instantaneous deep blue spot, which is the positive reaction for phenolic substances (Browning 1967). The color reactions with diazotized p-nitroaniline (yellow brown), bisdiazotized benzidine (dark rust), diazotized sulfanilic acid (dark tan) and the color under ultraviolet were similar to those obtained from known stilbenes (Hillis and



	Rfx100	COLOR UNDER UV	PERCENT IN AIR-DRIED HEARTWOOD
F6-A	6	Bright Blue	2.4
F6-B	15	Rust	0.8
F6-C	20	Blue	0.5
F6-D	25	Green-Yellow	0.6
F6-E	32	Tan	0.4
F6-F	40	Sky Blue	1.3
F6-G	59	Violet Blue	1.4

FIG. 3. Diagrammatic representation of the paper chromatographic separation of the fraction F6 (see Fig. 2) from the heartwood extractives of osage-orange.

Ishikura 1968). These color reactions suggest that F6-A contains phenolic compounds, possibly stilbenes.

Characterization of F6-A was also based on the NMR spectroscopic analysis (Fig. 4). The spectrum was translated into a molecular structure by comparing it with the spectra given by several known stilbenes including *cis*-stilbene, *trans*-stilbene, p-hydroxystilbene, 3,4′,5-trihydroxystilbene, and 2,4,3′,5′-tetrahydroxystilbene (Fig. 5). Since the reactions of F6-A with chemical indicators sug-

TABLE 4. *Effect of each subfraction of F6 on wood decay fungi as evaluated by fungal growth on treated cellulose paper.*[a]

Fungus	Untreated control[b]	Fungal growth (% of untreated control)[c]						
		Pentachlorophenol	A	B	C	D+E	F	G
Coriolus versicolor	100	0	22	85	100	100	95	100
Poria placenta	100	0	23	95	100	93	100	100
Gloeophyllum trabeum	100	0	27	88	100	100	100	100

[a] Various subfractions refer to Fig. 3 and the concentration of each subfraction in the paper was equivalent to its concentration in the heartwood.
[b] Control sample was previously eluted with solvent.
[c] Average of four estimations; subfraction A is significantly different from the control at 0.05 level.

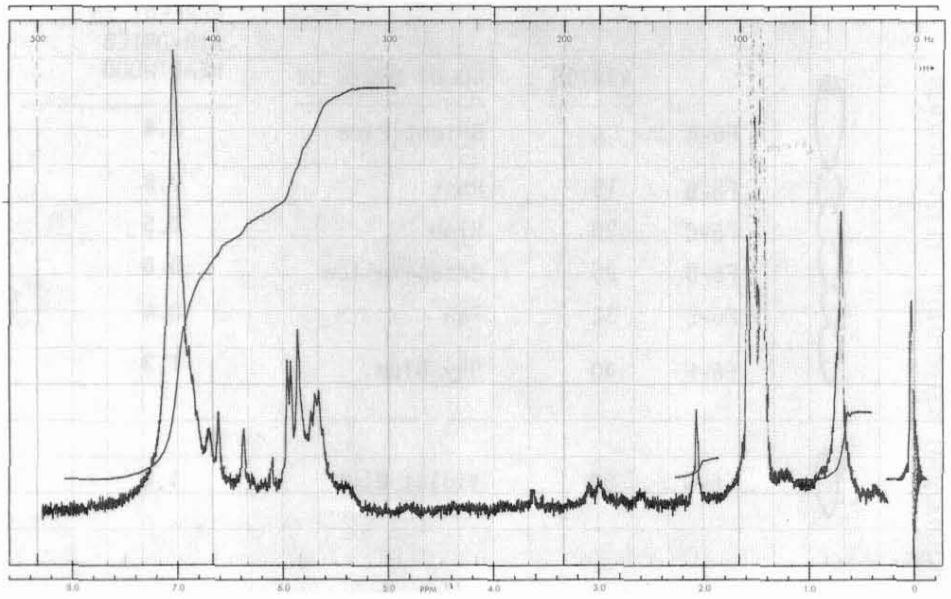


FIG. 4. NMR spectrum of component F6-A from the heartwood extractives of osage-orange. 60 MHz NMR spectrum was obtained under the following test conditions: sweep offset, 0 Hz; spectrum amplitude, 25; integral amplitude, 25 × 3; spinning rate, 40 RPS; sweep time, 250 sec; sweep width, 500 Hz; filter, 1; RF power level, 0.1; solvent, CD_3COCD_3.

gested it to be a phenolic compound, an aromatic-OH group was suspected. A strong singlet at δ7.0 was identified to be the signal for phenolic protons and this identification was confirmed by a D_2O exchange (Silverstein et al. 1974). This signal appears to be in accord with the phenolic peaks shown in the spectra given by the authentic hydroxystilbenes (Fig. 5—III, IV, V). The possibility of a CH=CH group was confirmed by the olefinic proton resonance in the NMR spectrum at about δ5.9. The spectra of 3,4′,5-trihydroxystilbene and 2,4,3′,5′-tetrahydroxystilbene (Fig. 5—IV and V) gave similar chemical shift for olefinic protons. The remaining peaks down field are the aromatic signals, but they have not been assigned. The peaks at about δ1.5 were identified as the solvent signals. The peaks at δ2.1 and δ0.7 were considered to be the signals of impurities.

Based on the nature of chemical shifts and splitting shown in the NMR spectra of 3,4′,5-trihydroxystilbene and 2,4,3′,5′-tetrahydroxystilbene, the NMR spectrum of F6-A suggested that F6-A is a *trans*-hydroxystilbene containing a resorcinol group. Since the phenolic peak shown in Fig. 4 is stronger than that of tetrahydroxystilbene (Fig. 5—V), it seems likely that the F6-A may contain a component which has more than four hydroxyl groups.

Spraying F6-A with vanillin-toluene-p-sulfonic acid gave a strong violet-red similar to that produced by authentic phloroglucinol or flavonoid substances containing phloroglucinol nuclei (Roux and Maihs 1960). Thus, this color reaction would suggest that F6-A also contains a phloroglucinol group. Hence, it seems possible that F6-A contains a pentahydroxystilbene. Pentahydroxystilbene is the most fully substituted natural stilbene so far encountered. The previous report

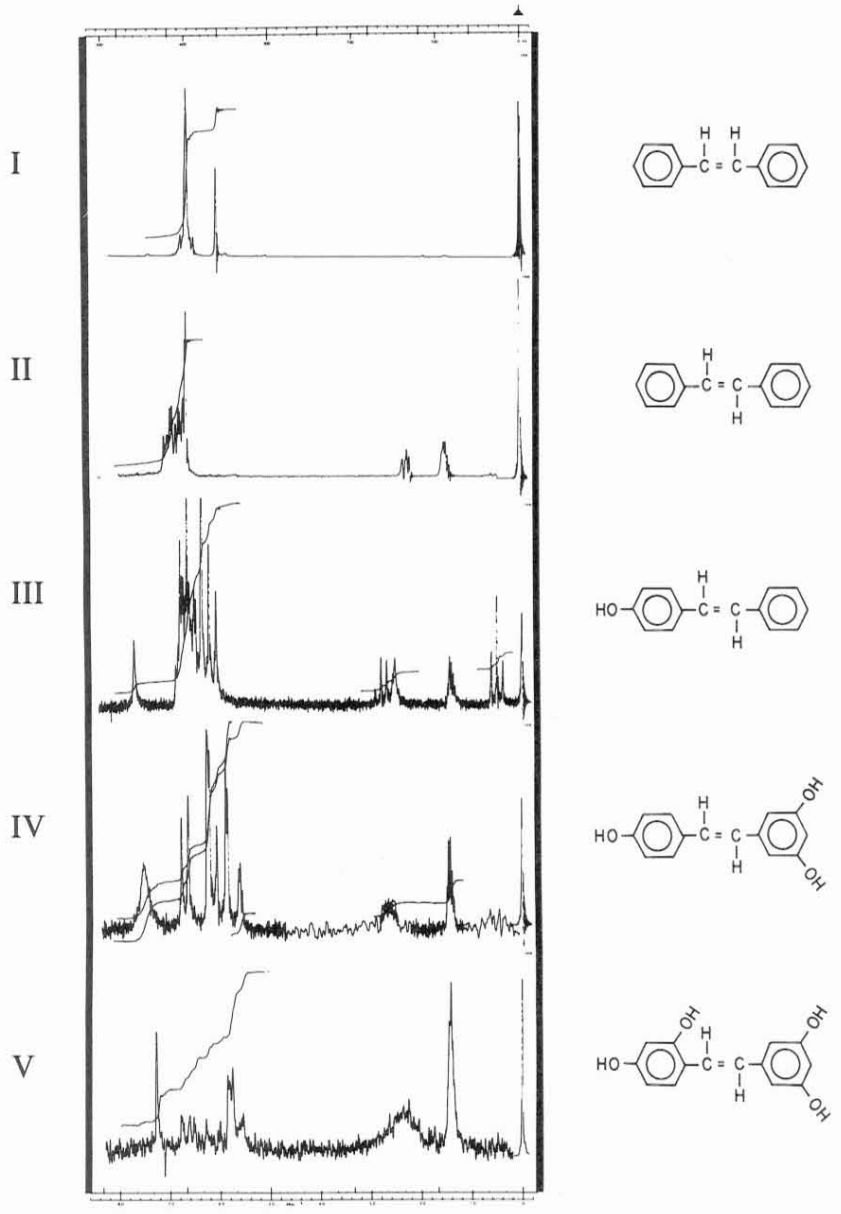


FIG. 5. NMR spectra of the authentic stilbenes.

concerning the pentahydroxystilbene in wood was that of 3,4,5,3′,5′-pentahydroxystilbene, which was found in the ether extract of *Vouacapoua macropetala* Sandwith (King et al. 1956).

The mass spectrum of F6-A (Fig. 6) has a parent peak at m/e 260, which would be evidence in favor of the presence of a pentahydroxystilbene. However, the mass spectrum also suggests that the F6-A is a mixture because a strong peak is shown at m/e 244. This peak, at m/e 244, may confirm the presence of previously

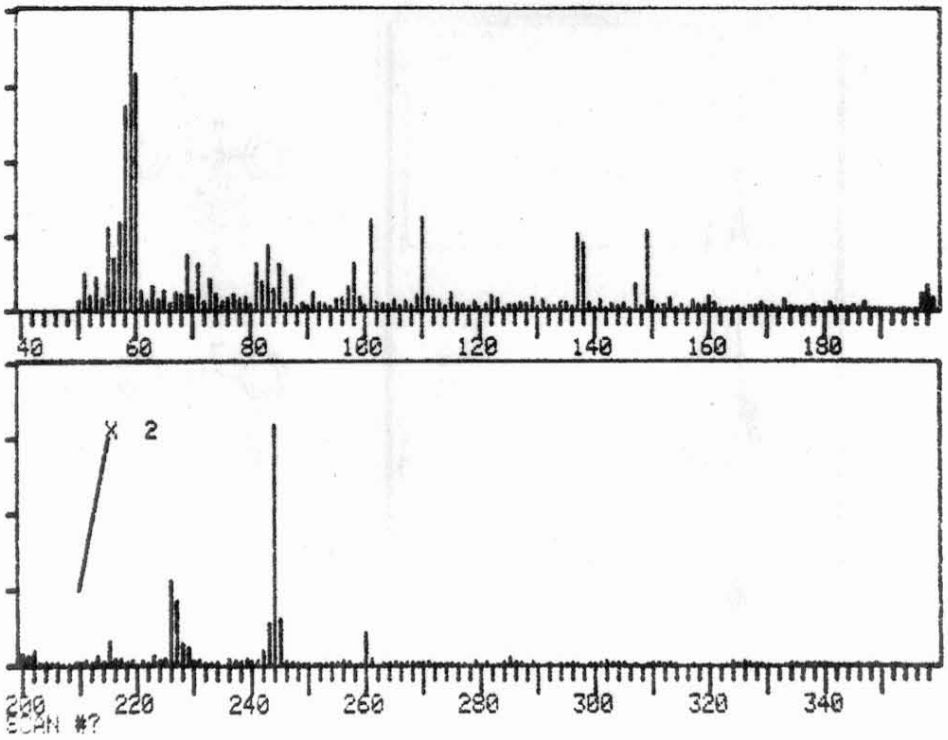


FIG. 6. Mass spectrum of component F6-A from the heartwood extractives of osage-orange.

reported tetrahydroxystilbene (Barnes and Gerber 1955). Hence, the data suggest that a small quantity of pentahydroxystilbene is coexistent with the tetrahydroxystilbene in the heartwood of osage-orange. The coexistence of 3,4,3,′,5′-tetrahydroxystilbene and 3,4,5,3′,5′-pentahydroxystilbene has been found in the heartwood of *Vouacapoua macropetala* Sandwith (King et al. 1956). Because 1) all examples so far recorded for phytochemical stilbenes are the derivatives of 3,5,-dihydroxystilbene, and 2) the reaction of the phloroglucinol test is positive, the hydroxy groups of the pentahydroxystilbene found in osage-orange heartwood are most likely attached on the 2,4,6,3′,5′ positions.

CONCLUSIONS

The major fraction of extractives responsible for the decay resistance of osage-orange (*M. pomifera* (Raf.) Schneid.) appears to be a mixture of tetra- and pentahydroxystilbenes with the tetrahydroxystilbene predominating.

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