HEARTWOOD EXTRACTIVES OF MACLURA POMIFERA AND THEIR ROLE IN DECAY RESISTANCE

DISSERTATION FOR THE DUAL DEGREE OF PH. D.

MICHIGAN STATE UNIVERSITY

SHIH-CHI WANG

1977

MICHIGAN STATE UNIVERSITY LIBRARIES
3 1293 00080 7887

LIBRARY
Michigan State
University

This is to certify that the

thesis entitled

Heartwood Extractives of Maclura pomifera
and Their Role in Decay Resistance

presented by

Shih-chi Wang

has been accepted towards fulfillment
of the requirements for

Dual Ph.D. degree in Botany and Plant
Pathology
and Forestry

Major professor

Date 5/20/77

O-7639

AUG 01 2002
05 03 04

ABSTRACT

HEARTWOOD EXTRACTIVES OF *MACLURA POMIFERA*

AND THEIR ROLE IN DECAY RESISTANCE

By

Shih-chi Wang

The heartwood of osage-orange (*Maclura pomifera* (Raf.) Schneid.) is considered one of the most durable woods. Hence, the mechanism of decay resistance and some of the chemical properties of this wood were investigated.

Samples of ground heartwood were extracted with hexane, ether, chloroform, *t*-butanol, ethanol-benzene, acetone, methanol, ethanol-water or distilled water. Polar solvents removed the largest amount of extraneous material. The decay resistance of the extracted ground wood was determined by the oxygen consumption method. Decay resistance decreased significantly following extraction with polar solvents, particularly methanol and ethanol-water.

Extracted wood blocks were exposed to decay fungi to investigate the structural mechanism of decay resistance. After exhaustive extraction, the weight losses of heartwood blocks increased from 0.4% to 16.2%, from 0.6% to 37.0%, from 0.2% to 2.5%, from 0.4% to 7.0% for *Poria placenta*, *Coriolus versivolor*, *Gloeophyllum trabeum*, and *Lentinus lepideus* respectively. These results revealed that the mechanism of decay resistance of *Maclura pomifera* was mainly chemical.

However, the limited decay caused by Gloeophyllum trabeum after exhaustive extraction suggested that a structural mechanism may be partially involved.

To isolate and identify the active component in the wood, ground heartwood was extracted sequentially with hexane, chloroform and methanol. The dry methanol extract was partitioned between water and ether to separate the water-soluble materials from those soluable in ether. Further separation was accomplished by liquid-liquid extraction of the ether fraction 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 components were examined quantitatively as well as qualitatively. The fungal toxicities of various fractions and compounds were determined by incorporating each material into non-durable 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 compound with the greatest inhibitory effect on wood decay fungi, as determined by spectroscopic methods , appears to be a pentahydroxystilbene.

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

By

Shih-chi Wang

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the dual degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

Department of Forestry

1977

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. John H. Hart for his enthusiastic support, assistance and encouragement throughout the period of my study at the Michigan State University.

Thanks are extended to Dr. Eldon A. Behr for his counsel and guidance for this work.

I highly appreciate the contributions of other members of my guidance committee: Dr. Everett S. Beneke, Dr. James W. Hanover and Dr. Otto Suchsland.

Acknowledgments are also extended to Dr. Jacob B. Huffman, Dr. Robert A. Schmidt and Dr. Ramon C. Littell at the University of Florida for establishing my training and attitudes on Wood Technology, Forest Pathology and Statistical Methods during early years of my career. Their inputs are gratefully remembered.

I also wish to express deep gratitude to my parents, Mr. and Mrs. Yun-lan Wang, for their encouragement during many years of my schooling and for their unfailing support of high goals.

Thanks are also due to Dr. William H. Reusch and Mr. K. P. Subrahamanian for their assistance with the spectroscopic analysis.

TABLE OF CONTENTS

	Page
LIST OF TABLES AND FIGURES	iv
INTRODUCTION	1
MATERIALS AND METHODS	5
Determinations of Natural Resistance of the Wood and Its Mechanism	5
Extraction and Separation of the Extraneous Components in the Wood	8
Investigation of the Fungistatic Properties of Each Fraction and Subfraction of the Extractives	12
Examination of the Chemical Nature of the Fungistatic Component	14
RESULTS AND DISCUSSION	16
The Natural Decay Resistance of Wood	16
The Decay Resistance Mechanism: Chemical vs Structural	19
Extraction, Separation and Bioassay of the Extraneous Components in the Heartwood	23
Fractionation and Bioassay of the Methanol Extract	26
Chemical Nature of the Fungistatic Component	35
CONCLUSIONS	42
LITERATURE CITED	43
APPENDIX	46

LIST OF TABLES AND FIGURES

Page

Table

1. Weight losses (mean of 8 determinations) of the heartwood blocks of *Maclura pomifera* caused by *Gloeophyllum trabeum*, *Poria placenta*, *Lentinus lepideus* and *Coriolus versicolor* 17

2. Weight losses (mean of 6 determinations) of the heartwood and sapwood of *Maclura pomifera* caused by *Gloeophyllum trabeum*, *Poria placenta*, *Lentinus lepideus* and *Coriolus versicolor* 18

3. Solubilities of the wood of *Maclura pomifera* in various solvents (mean of 6 determinations) 20

4. Oxygen consumption, µl/Hr (average of 5-hour testing), and growth rate of *Coriolus versicolor* and *Gloeophyllum trabeum* on ground heartwood of *Maclura pomifera* extracted with various solvents.. 21

5. Weight loses of exhaustively extracted blocks of *Maclura pomifera* caused by *Poria placenta*, *Gloeophyllum trabeum*, *Coriolus versicolor* and *Lentinus lepideus* 22

6. Effect on each heartwood extract of *Maclura pomifera* on *Coriolus versicolor* and *Gloeophyllum trabeum* as determined in ground aspen wood by the oxygen consumption method 24

7. The calculated percentage (air-dried weight basis) of each fraction of the methanol extract in the heartwood of *Maclura pomifera* 27

8. 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 30

Page

Table

9. The calculated percentage (air-dried weight basis) of each component of F6 in the heartwood of Maclura pomifera 33

10. Effect of each component of the fraction F6 on wood decay fungi as evaluated by fungal growth on treated cellulose paper 34

11. The color reactions of the component F6-A 36

Figure

1. Separation scheme for heartwood extractives of Maclura pomifera 9

2. Separation scheme for the methanol extract 10

3. Diagrammatic representation of the paper chromatographic separation of the fraction F6 from the heartwood extractives of Maclura pomifera 32

4. NMR spectrum of the component F6-A from the heartwood extractives of Maclura pomifera 37

5. Mass spectrum of the component F6-A from the heartwood extractives of Maclura pomifera 38

INTRODUCTION

The principal components of the wood cell wall — polysaccharides and lignin — are essentially similar in all species of wood. In addition to these structural components, all woods contain smaller amounts of minor or extraneous components which are much more diverse in their chemical nature. This diversity often accounts for the difference in chemical features among various wood species. These extraneous components are termed extractives because they can, to a large extent, be extracted from the wood with neutral solvents, without destroying the structure of the wood. They include many different classes of organic compounds, ranging in complexity from relatively simple molecules, such as phenols and sugars, to highly complex coloring matters, i.e. tannins, resin, etc. These components are of considerable importance because a number of technically important properties of wood are dependent upon the presence of extractives in the wood. One of the most important of these properties is the wood's natural durability. Wood extractives and their significance have been reviewed by Hillis et al (17).

Factors contributing to decay resistance of wood have been studied over a period of years and the presence of certain

fugistatic extractives in heartwood is known to be the major mechanism for the decay resistance of wood. Hawley and co-workers (1924) were the first to demonstrate that the presence of extractives in heartwood is toxic to wood-destroying fungi (15). Since then a large number of heartwood constituents have been tested *in vitro* for their funcicidal or insecticidal properties. Most of the active compounds have been found to be of a phenolic nature. This chemical mechanism of wood durability — the presence of fungistatic extractives — is by far the most commonly accepted theory for the decay resistance of wood (26). Nevertheless, there is some evidence (22) that wood structure is also a factor contributing to the decay resistance.

The heartwood of osage-orange (*Maclura pomifera* (Raf.) Schneid.) has long been known as an exceptionally durable wood (31), based on its extremely long service life under conditions favorable to decay. When exposed to decay fungi under controlled environmental conditions conducive to rot, it is also very durable (13). However, little is known of the mechanism of ecay resistance of osage-orange heartwood. The only work done on relating the chemistry with the durability of this species is that by Barnes and Gerber (6). They reported the presence of 2, 4, 3', 5'-tetrahydroxystilbene in the heartwood and *in vitro* tests revealed that it had antifungal properties.

Stilbenes are diphenylethylenes which can be extracted with acetone or alcohol. Generally they occur free in wood

and are only substituted by hydroxyl or methoxyl groups. All of them exhibit an intense blue fluorescence under ultraviolet light (18). Pinosylvin, a dihydroxy-stilbene, and its monmethyl either occur in the pines, and are of special significance in connection with the durability of pine heartwood (10, 24, 25). Reservatrol, a trihydroxy-stilbene, may play a role in the durability of eucalypts (14). Thus, there are a number of reports suggesting that stilbenes increase the resistance of wood to deterioration (26). However, this hypothesis has recently been questioned (14, 20).

A recent study (14) has emphasized the difficulties in relating the results of in vitro testing of wood extractives and their pure components with the durability of the wood from which the extractives were obtained. The study conducted by Barnes and Gerber (6) did not provide information concerning the fungistatic values of the active compounds in wood or wood-base materials. Their in vitro testing determined only the extent that the compound protected the liquid medium against the test fungi. In addition, their research did not employ wood-inhabiting fungi or the decay fungi commonly recommended for such tests (3, 4). 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 extend our present knowledge of the decay resistance of osage-orange heartwood,

to determine what factors are responsible for the natural decay resistance and to obtain more information on the chemical properties of this wood.

MATERIALS AND METHODS

The test wood came from an osage-orange tree (Maclura pomifera), collected at W. K. Kellogg Forest, Augusta, Michigan, in September 1974, with a diameter inside bark of 15 cm. A portion of the stem, free from knots or defects, was selected for the test material. The bark was removed and the log was air-dried, cut into short logs and stored in a cold room. To prepare samples for decay tests, the heartwood was cut into blocks 2.5 x 2.5 x 0.9 cm (longitudinal) and the sapwood into blocks 2.5 x 0.5 (radial) x 2.5 cm. The remainder of the log was cut into 1 cm^2 strips, with heartwood and sapwood kept separate. These strips were ground in a Wiley Mill with a 40-mesh screen and the ground wood stored in plastic bags at $4^{\circ}C$.

Determinations of Natural Decay Resistance of the Wood and Its Mechanism

Using the standard soil-block or agar-block method (4), the wood blocks were exposed to a decay fungus — Poria placenta Murr (Madison 698), Coriolus versicolor L. ex Fr. (Madison 697), Gloeophyllum trabeum Pers. ex Fr. (Madison 617) or Lentinus lepideus Fr. (Madison 534). The test blocks were weighed before and after exposure, and the loss

in weight was used to determine the amount of decay. The test was terminated when 60% weight loss was obtained in non-durable aspen sapwood blocks.

To determine the possibility of the presence of fungistatic extractives in the heartwood, samples of the ground wood were extracted individually in Soxhlet apparati for eight hours with hexane, ether, chloroform, t butanol, ethanol-benzene (1/2, v/v), acetone, methanol, ethanol-water (1/1, v/v) or distilled water. After extraction, the solubility of the wood in each solvent was determined. The procedures used for the extraction and solubility tests were similar to those described in ASTM-D 1107 or ASTM-D 1108 (1, 2).

The decay resistance of the extracted samples was determined by respirometry (7, 11, 28, 29, 30). Petri dishes with a thin layer of malt-agar were inoculated with C. versicolor or G. trabeum. After the fungal mycelium covered the agar plate, a 25 ml sample, previously sterilized, was evenly spread over the agar surface. Unextracted osage-orange heartwood and unextracted aspen sapwood were used as decay-resistant and decay-susceptible controls, respectively. Samples were incubated for three weeks at 25°C and 90-95% relative humidity. After incubation, fungal growth was evaluated by visual examination, and rated on a scale from 1 to 5 with each integer representing coverage of the wood surface of 0-20% to 80-100%, respectively. Incubated ground wood was

then removed from the plate and tightly packed in a vial to obtain a 2 ml volume. These samples were transferred to Warburg flasks and changes in manometer levels were recorded hourly for five hours. Differences in oxygen consumption were used for estimating the rate of fungal growth in each sample which was used to evaluate the loss of decay resistance of the extracted ground wood.

Decay resistance due to wood structure was tested by measuring the decay resistance of exhaustively extracted blocks. To accomplish this objective, the extractives were removed from the wood without destroying its structure. Blocks of osage-orange heartwood were successively extracted in Soxhlet apparati with ethanol-benzene (1/2, v/v), ethanol-water (1/1, v/v), acetone and distilled water for 66 hours, 72 hours, 24 hours and 72 hours respectively. Three transverse canals were drilled on each side of the test block to facilitate extraction of soluable materials. Blocks were weighed before and after extraction to measure the amount of material removed. The extracted wood blocks were then tested (4) for decay resistance against P. placenta, C. versicolor, G. trabeum and L. lepideus. Sapwood blocks extracted in the same manner were used as controls.

Extraction and Separation of the Extraneous Components in the Wood

Since the fungistatic extractive previously reported (6) was of a phenolic nature, the chemical analysis was focused on the isolation of phenolic substances. Several extractions were conducted as shown in Figure 1. Ground osage-orange heartwood (200 g) was extracted with each solvent for ten 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 replacing fresh solvent. Each extract was reduced in a roatary vacuum evaporator at less than 40^{o}C, air-dried at room temperature, and the dry weight recorded. The air-dry weights of ground wood before and after extraction were used for quantitative determinations.

To fractionate the extraneous materials, several partitions were conducted as shown in Figure 2. The methanol extract was first partitioned between ether and water to separate

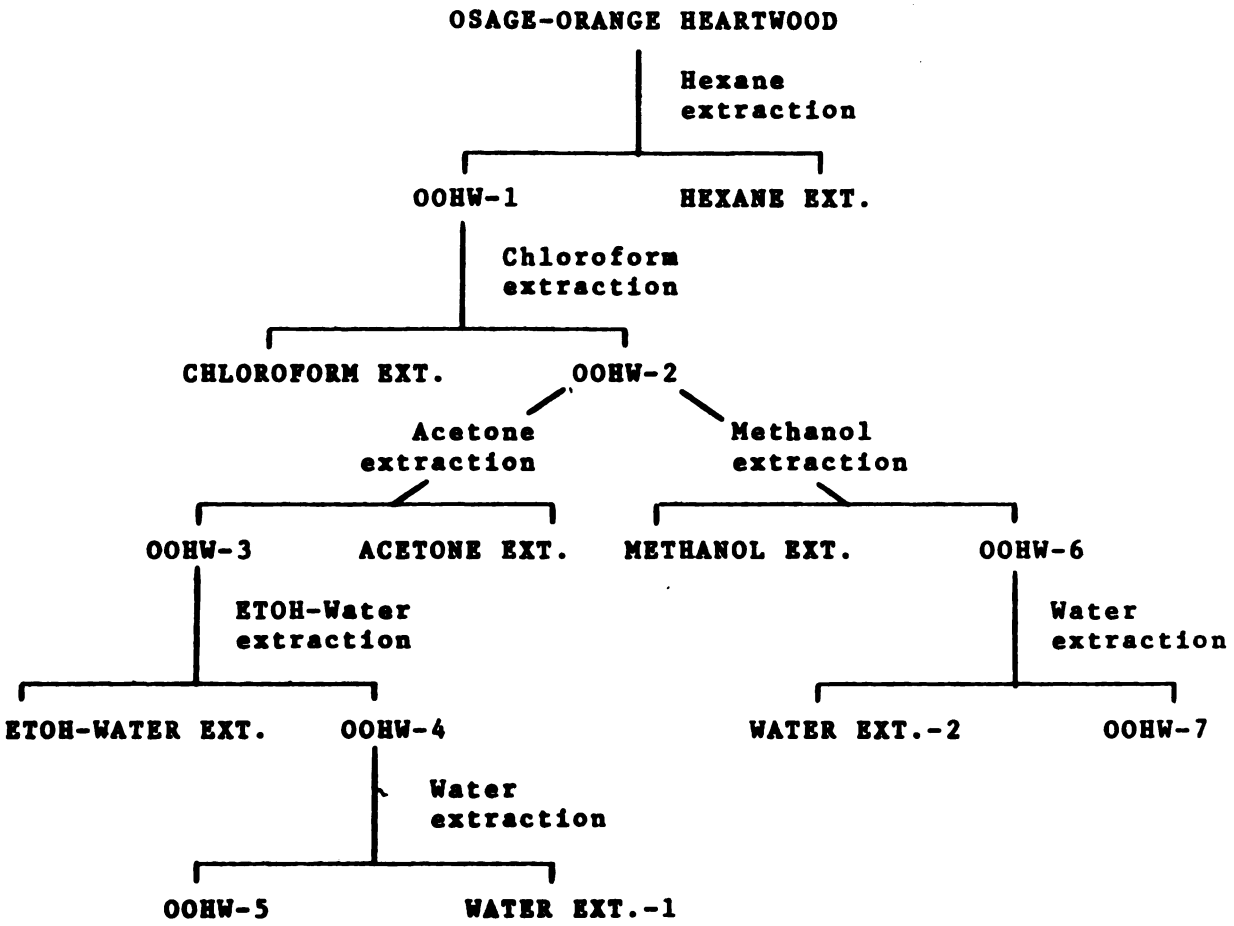


Figure 1. Separation scheme for heartwood extractives of Maclura pomifera.

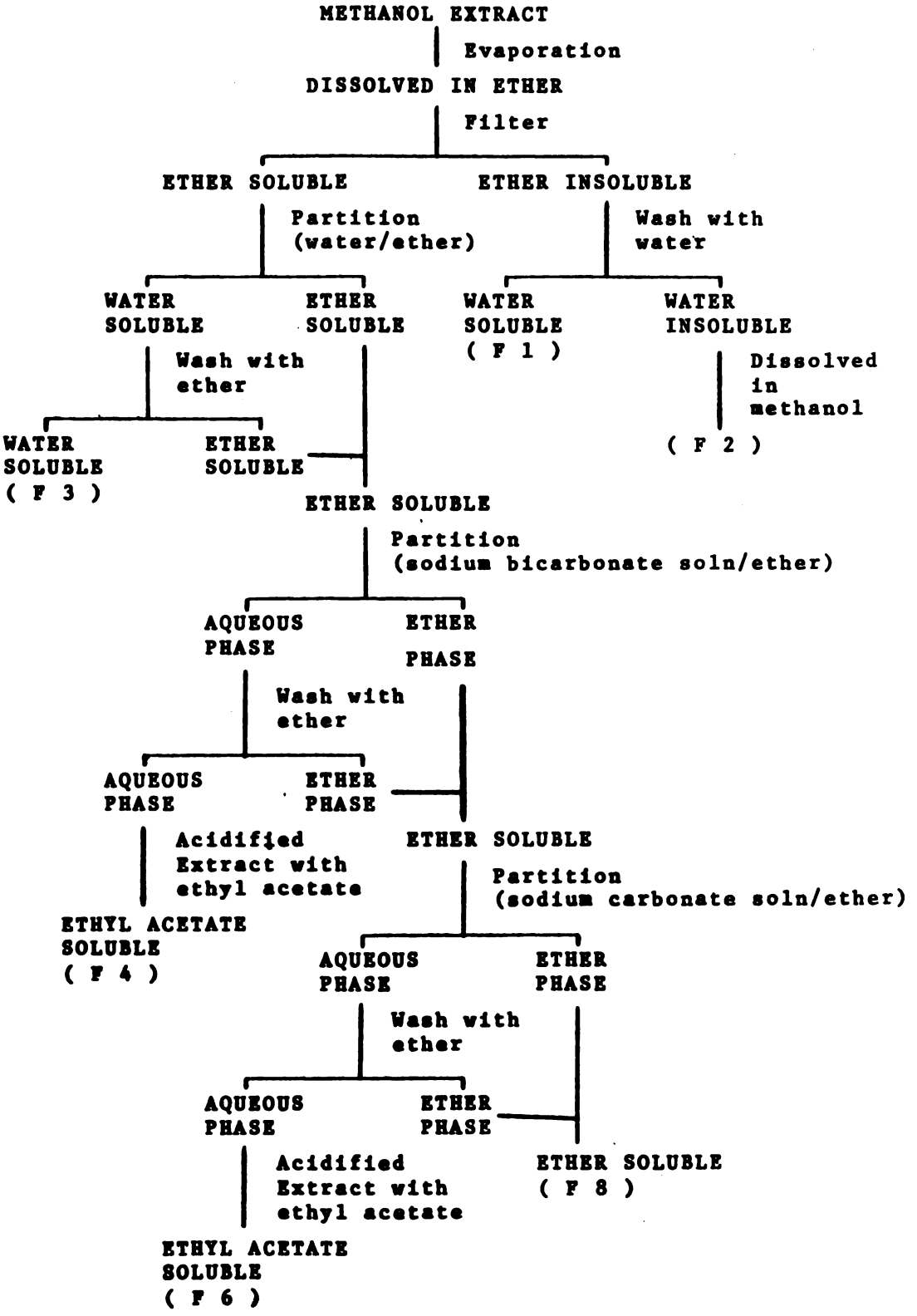


Figure 2. Separation scheme for the methanol extract (see Figure 1.).

those less polar substances from the highly polar materials. The air-dried methanol 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-soluble 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 which were returned to the main ether solution.

The second and the third partitions were accomplished by utilizing the weakly acidic character of phenolic substances (9). These depend on the removal of strong acids by liquid-liquid extraction into an aqueous phase of a weak alkali (sodium bicarbonate) followed by extraction of the phenolics from the neutral materials by a strong aqueous alkali (sodium carbonate). Thus, the ether-soluble fraction was next partitioned between ether and saturated aqueous sodium bicarbonate. The aqueous extract was washed with ether to remove entrained materials and the washings 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 recorded for quantitative determinations.

Further separation and analysis were accomplished by using paper chromatography. Benzene-acetic acid-water (125/72/3) (18) gave good resolution of the components in F4, F6, and F8 fractions. Toluene-acetic acid-water (4/1/5) (9) separated the components in F1, F2, and F3 fractions.

The quantitative determinations were accomplished by weighing the various dry extracts and then calculating their percentage of the original wood based on the total air-dried weight of the wood from which each was obtained.

Investigation of the Fungistatic Properties of Each Fraction and Subfraction of the Extractives

To identify the component responsible for the decay resistance, a 10 ml aliquot of each fraction of subfraction 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. The ground aspen sapwood and the extractive solution were mixed until the solvent was completely evaporated. To obtain even impregnation, excess solvent was added and periodically stirred. The treated samples and the untreated controls — aspen sapwood,

and sapwood and heartwood of osage-orange, were sterilized in an autoclave. All samples were then exposed to Coriolus versicolor or Gloeophyllum trabeum and fungal growth was measured by respirometry as previously described.

Since final separation of the extractives was conducted by paper chromatography, collection of enough of a compound 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 compounds and the impregnated paper was used as a substrate to evaluate fungistatic properties. The use of cellulose paper as a test substrate has the advantage that the paper is a wood-base material. Coriolus versicolor, a white-rot fungus, can readily degrade cellulose paper without other wood constituents (16).

The amount of the extractive in the paper was equivalent to the concentration of that chemical in wood. The paper strips were cut approximately 5 cm^2, placed in a Petri dish, wetted with distilled water, and sterilized in an autoclave. An equal amount of even-aged fungal inoculum 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.

Examination of the Chemical Nature of the Fungistatic Component

The location of fluorescent compounds was determined on developed chromatograms, both before and after exposure to the funes of concentrated ammonium hydroxide. A large number of color reactions with chromogenic sprays are available for the detection of wood extractives by applying these reagents to the developed chromatogram. Many of these reactions exhibit considerable selectivity, and are of value in examination of mixtures and estimation of possible structures. The spray reagents used were similar to those suggested for phenolic substances by Browning (9). The resulting colors and the R_f values were recorded for semiqualitative determinations.

The active compound was further characterized by NMR and mass spectroscopic analysis. The fungistatic fraction was separated into individual components by paper chromatography. Components were eluted from the paper with acetone. A 2 g portion of anhydrous sodium sulphate was added to the solution which was stirred, allowed to settle for ten minutes and filtered. The acetone was removed in a rotary vacuum evaporator at less than 40°C and any residual acetone 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, the acetone was not completely removed because the solvent used was acetone. The spectra were examined and compared with several reference spectra of similar compounds.

RESULTS AND DISCUSSION

The Natural Decay Resistance of Wood

The weight losses caused by various decay fungi are summarized in Table 1 and Table 2. The data revealed that the heartwood was very decay resistant to the test fungi and that the sapwood was not resistant. According to the ASTM scale (4), the heartwood can be rated in the highly resistant category. These results are similar to those obtained by Hart and Johnson (13).

There was a difference between the soil-block method and the agar-block method in the evaluations of decay caused by C. versicolor and P. placenta (Table 1). The difference was particularly pronounced with C. versicolor, a white-rot fungus, which produced more decay when the agar-block method was employed. The decay caused by C. versicolor might be limited by some environmental factor in the soil-block method, e.g., this method might not have allowed adequate wetting of samples for optimum fungal growth. Hence subsequent decay tests were conducted by the agar-block method which resulted in a higher moisture content of test wood.

Table 1. Weight losses (mean of 8 determinations) of the heartwood blocks of *Maclura pomifera* caused by *Gloeophyllum trabeum*, *Poria placenta*, *Lentinus lepideus* and *Coriolus versicolor*.

Fungus	Test Method	Weeks of Decay	Heartwood % Wt Loss[a]	Aspen % Wt Loss[b]
G. trabeum	Soil-block	11	0.2	51.6
P. placenta	Soil-block	9	0.5	64.8[e]
L. lepideus	Soil-block	16	0.4	47.3
C. versicolor	Soil-block	16	0.1	35.5[c]
G. trabeum	Agar-block	11	0.2	48.2
P. placenta	Agar-block	9	0.4	43.5[f]
L. lepideus	Agar-block	16	0.4	50.1
C. versicolor	Agar-block	16	0.6	68.0[d]

a is significantly different from b at 0.05 level (T test).

c is significantly different from d at 0.05 level (T test).

e is significantly different from f at 0.05 level (T test).

Table 2. Weight losses (mean of 6 determinations) of the heartwood and sapwood of Maclura pomifera caused by Gloeophyllum trabeum, Poria placenta, Lentinus lepideus and Coriolus versicolor.

Fungus	% Wt. Loss in 8 Weeks[a]	
	Heartwood[c]	Sapwood[d]
G. trabeum	0.2	38.9
P. placenta	0.3	32.3
L. lepideus	0.4	35.1
C. versicolor	0.4	37.5

[a]Measured by Agar-block method.

c is significantly different from d at 0.05 level (T test).

The Decay Resistance Mechanism: Chemical vs Structural

The decay resistance of wood is commonly believed to be due to the presence of extractives which inhibit fungal growth activity. To examine the amount of various extractives and their effects on the decay resistance, samples of ground heartwood were extracted (1, 2) with various solvents and the extracted samples were bioassayed. The extractives of M. pomifera were mainly located in the heartwood (Table 3) and they were more soluble in the more polar solvents such as methanol and ethanol-water. The results of the bioassays accomplished by respirometry (Table 4) revealed that the decay resistance of the ground heartwood was removed or significantly reduced by extracting the wood with a polar solvent, particularly methanol or ethanol-water. These results indicated that certain extractives in the heartwood were inhibitory to wood-destroying fungi and those extractives were most soluble in methanol or ethanol-water (1/1, v/v).

A structural mechanism has also been suggested (22) for the durability of wood. The weight losses caused by various decay fungi of heartwood blocks following exhaustive extraction (Table 5) enabled the effect of wood structure on decay resistance to be evaluated. By comparing these weight losses with those obtained with the agar-block method (Table 1), it can be seen that decay increased from 0.4% to

Table 3. Solubilities of the wood of Maclura pomifera in various solvents (mean of 6 determinations).

Solvent	Solubility %	
	Heartwood	Sapwood
Hexane	2.1	2.1
Ether	2.4	1.3
Chloroform	0.7	0.1
t Butanol	5.6[a]	2.6[b]
Ethanol-Benzene	19.6[c]	6.3[d]
Acetone	14.7[e]	3.7[f]
Methanol	26.7[g]	7.3[h]
Ethanol-Water	30.9[i]	9.0[j]
Water	16.7[k]	10.7[l]

a, c, e, g, i, k are significantly different from b, d, f, h, j, l respectively at 0.05 level (T test).

Table 4. Oxygen consumption, µl/Hr (average of 5-hour testing), and growth rate of Coriolus versicolor and Gloeophyllum trabeum on ground heartwood of Maclura pomifera extracted with various solvents.

Extracting Solvent	C. versicolor		G. trabeum	
	O_2 consumpt[a] (µl/Hr)	Growth Rate (1---5)[b]	O_2 consumpt[a] (µl/Hr)	Growth Rate (1---5)[b]
None	2.5[c]	1[d]	3.7[c]	1[d]
Hexane	2.0	1	3.4	1
Ether	6.2	1.3	3.3	1.1
Chloroform	4.2	1.3	3.7	1.4
t-Butanol	15.0[e]	1.3	2.1	1.5
Ethanol-Benzene	81.2[e]	4.3	136.0[f]	4.9
Acetone	35.1[e]	3.3	18.5[f]	3.1
Methanol	90.2[e]	5	154.4[f]	5
Ethanol-Water	94.3[e]	5	167.4[f]	5
Water	22.5[e]	2.1	44.0[f]	3.6
Aspen Control	40.0	4	100.6	4

[a]Test temperature: 30°C.

[b]Each integer represents coverage of the wood surface of 2-20% of 80-100%.

[c]Mean of 3 determinations.

[d]Average of 4 estimations.

[e]Values are significantly different from the value for unextracted samples (2.5) at 0.05 level (T test).

[f]Values are significantly different from the value for unextracted samples (3.7) at 0.05 level (T test).

Table 5. Weight losses of exhaustively extracted blocks of *Maclura pomifera* caused by *Poria placenta*, *Gloeophyllum trabeum*, *Coriolus versicolor* and *Lentinus lepideus*.

Fungus	Weeks of Decay	% Weight Loss[a]	
		Heartwood[b]	Sapwood[c]
P. placenta	9	16.2[d]	48.5[d]
G. trabeum	11	2.5	24.4
C. versicolor	16	37.0	78.3
L. lepideus	16	7.0	55.8

[a]Determined by the Agar-block method.

[b]Average % extractive removed: 25.

[c]Average % extractive removed: 5.

[d]Mean of 6 determinations.

Decay resistance of extracted heartwood blocks are significantly different from those of unextracted heartwood blocks (Table 1) at 0.05 level (T test).

to 16.2%, from 0.6% to 37.0%, from 0.2% to 2.5%, from 0.4% to 7.0% (on oven-dried weight basis) for P. placenta, C. versicolor, G. trabeum and L. lepideus, respectively. The limited increase in decay caused by G. trabeum after exhaustive extraction suggests that a structural mechanism of decay resistance may be partially involved for this fungus.

Extraction, Separation and Bioassay of the Extraneous Components in the Heartwood

The total amount of various extracts obtained from the ground heartwood of M. pomifera by successive extraction with four solvents of increasing polarity (hexane, chloroform, methanol and distilled water) was 23% (on air-dried weight basis). The hexane, chloroform, methanol and water extracts comprised 1.5%, 0.5%, 16.2% and 4.8%, respectively.

The fungal toxicity of each extract, as determined by the oxygen consumption method using ground aspen wood, is presented in Table 6. The data revealed that fungistatic extracts obtained from the first extraction series (hexane, chloroform, acetone, ethanol-water, and water) were the acetone and ethanol-water extracts. In the second extraction series (hexane, chloroform, methanol, and water), only the methanol extract contained toxic materials. The hexane, chloroform, and both water extracts of OOHW-4 and OOHW-6

Table 6. Effect on each heartwood extract of Maclura pomifera on Coriolus versicolor and Gloeophyllum trabeum as determined in ground aspen wood by the oxygen consumption method[a].

Extract	C. versicolor		G. trabeum	
	O_2 consumpt[b] (µl/Hr)	Visual exam (1---5)[c]	O_2 consumpt[b] (µl/Hr)	Visual exam (1---5)[c]
Hexane Ext	40.7[f]	3[g]	80.0[f]	3.4[g]
Hexane[h]	33.0	3.3	68.6	3.9
Chloroform Ext	52.2	2.8	75.9	3.2
Chloroform[h]	46.3	3.5	68.4	3.2
Acetone Ext	14.4	1	0.7	1
Acetone[h]	43.5	3.8	90.9	3.8
Ethanol-Water Ext	19.3	2	7.9	1.9
Ethanol-Water[h]	26.9	3.5	52.8	3.6
Water Ext - 1	76.1	3.8	58.3	4.8
OOHW-5	93.3	4.5	122.6	5
Methanol Ext	10.5	1	1.8	1
Methanol[h]	28.0	4.5	76.6	4.8
Water Ext - 2	40.6	3.3	41.5	4.5
OOHW-7	72.1	4.3	104.6	5
Aspen Control	40.5	3.3	94.2	4.4
OOHW[d]	1.5	1	2.7	1
OOSW[e]	211.1	5	178.7	3.6

[a]Various extracts refer to Figure 1 and the retention of treating chemicals in each ground aspen sample is equivalent to their concentration in the wood.

Table 6, continued

[b]Test temperature: 30°C. µl/Hr: Average of 5-hour testing.

[c]Each integer represents coverage of the wood surface of 0-20% to 80-100%.

[d]Ground heartwood of M. pomifera.

[e]Ground sapwood of M. pomifera.

[f]Mean of 3 determinations.

[g]Average of 4 estimations.

[h]Solvent only.

Value for acetone extract is significantly different from the value for acetone or aspen control at 0.05 level (T test).

Value for methanol extract is significantly different from the value for methanol or aspen control at 0.05 level (T test).

did not inhibit the growth of either fungus in the ground aspen wood.

The unextracted sapwood of M. pomifera and both the extracted woods, OOHW-5 and OOHW-7 (see Figure 1), were more susceptible to fungal growth than the aspen controls. For both fungi, the difference was particularly pronounced with the unextracted sapwoods. This suggested that some components in the sapwood promoted the growth of both fungi. In addition, aspen wood may have contained certain materials that were slightly inhibitory to fungal growth.

Fractionation and Bioassay of the Methanol Extract

Since the methanol extractives contained the fungitoxic compounds, this material was fractionated as shown in Figure 2. The amount of each fraction (% air-dried weight basis) in the heartwood was determined (Table 7).

Obtaining of a reliable quantitative information depends greatly on the extraction and partition performed. The nature of each extract was primarily determined by the solvent used. Since solubility characteristics of most organic compounds are determined chiefly by their polarity. Non-polar solvents or weakly polar compounds dissolve in non-polar or weak polar solvents; highly polar compounds dissolve in highly polar solvents (21). This rule of thumb, "like dissolves like", was the basic principle used for selecting

Table 7. The calculated percentage (air-dried weight basis) of each fraction of the methanol extract in the heartwood of *Maclura pomifera*[a].

Extractive Fraction[b]	% in Wood[c]
F1	2.1
F2	2.2
F3	1.3
F4	2.5
F6	7.4
F8	0.7

[a]Methanol extract refers to Figure 1.

[b]Fraction codes refer to Figure 2.

[c]Theoretically maximum value.

solvent and designing sequence of extractions. Thus, hexane and chloroform were used to remove non-polar compounds and methanol or water were used to extract polar or ionic compounds. The majority compounds in the methanol extract were phenolic compounds which were detectable by chromatography (9). Owing to the instability of some extractives particularly at high temperature, hot extraction was not suitable for the isolation of active compounds. To avoid possible decomposition of the active components, the highly efficient Soxhlet extraction was not employed.

Partitions with solvents follow the so-called distribution law which states that at a given temperature, a solute distributes itself between two immiscible solvents in such a way that an equilibrium is reached (12). The equilibrium ratio of the two concentrations of a given solute in two given solvents at a given temperature is a constant (distribution coefficient). The effectiveness of liquid-liquid extraction at given temperatures is evaluated by the following formula (5):

$$W_1 = W_o\left(\frac{V_1}{KV_2 + V_1}\right)^n$$

Where: W_o = weight of original solute.

W_1 = weight of solute remaining in solvent 1 after n extractions

V_1 = volume of solvent 1.

V_2 = volume of solvent 2 (extracting solvent) used in each extraction.

K = distribution coefficient of the solute.

n = number of extractions.

The most efficient way to minimize W_1 is to increase n, the number of extractions. As it can be seen from the above formula, the extraction is never complete, but it can be made nearly so by repeated extractions with fresh solvent. For this reason, each liquid extraction conducted in this experiment was repeated with fresh solvent for five times to enable a satisfactory separation.

The selection of developing solvents was essential to achieve a successful separation in paper chromatograph. Many solvent systems can be found in the literature to deal with the majority of commonly occurring compounds. The solvent system for optimum separation of components depends on the nature of these components and the complexity of the mixture. In examination of materials for which conditions have not been well established, trial of several developers may be necessary. Solvents of many compositions have been tried for separating components in each extractive fraction. Benzene-acetic acid-water (125/72/3) (18) was selected because it gave a good separation of components in F4, F6, and F8.

Evaluation of the fungistatic properties of each fraction of the methanol extractives showed that fraction F6 was the only fraction toxic to C. versicolor (Table 8). Fractions

Table 8. 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].

Extractive Fraction	C. versicolor		G. trabeum	
	O_2 consumpt[b] (µl/Hr)	Visual exam (1---5)[c]	O_2 consumpt[b] (µl/Hr)	Visual exam (1---5)[c]
F1	31.4[d]	4.4[e]	28.3[d]	2.3[c]
F2	27.8	3.4	11.9	2
F3	33.5	4.9	50.9	4
F4	46.3	4.5	18.4	2
F6	11.6	1.3	6.2	2
F8	41.5	4.5	37.0	2.8
Ether[f]	32.9	4.5	55.1	5
Ethyl Acetate[f]	25.5	4.5	60.8	5
Aspen Control	40.2	3.3	91.4	3.8

[a]Various fractions refer to Figure 2 and the retention of treating chemicals in each ground aspen sample is equivalent to their concentration in the wood.

[b]Test temperature: 30°C. µl/Hr: Average of 5-hour testing.

[c]Each integer represents coverage of the wood surface of o-20% to 80-100%.

[d]Mean of 3 determinations.

[e]Average of 4 estimations.

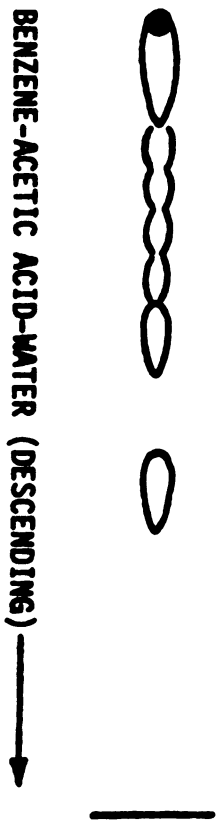
[f]Solvent only.

F6 is significantly different from the solvents or aspen control at 0.05 level (T test).

F2, F4 and F6 reduced the growth of G. trabeum but F6, based on the oxygen consumption data (Table 8), was the most inhibitous.

Separation of components in fraction F6 was obtained by paper chromatography (Figure 3). The amount of each of the seven components in F6 in the heartwood (% air-dried weight basis) is given in Table 9. The fungistatic properties of each component in F6 were evaluated by the rate of fungal growth on treated cellulose paper (Table 10). Cellulose paper, a wood-base material, was used to substitute for the ground aspen wood as the substrate of this bioassay. High humidity was very critical to the growth of these fungi on the cellulose paper. Several tests were necessary to find out the optimum conditions required.

Many studies on the fungal toxicity of wood extractives have been conducted by tests utilizing agar or nutrient solution. The main objection of using agar or nutrient solutions to test the toxicity is that the substrate is not wood (8), because it determines the extent that the chemical protects agar or nutrient solutions against fungi, and no data are obtained as to its protective effect for wood or wood component. Hence, the advantage of the paper bioassay is that it allows for the testing of very small quantities of a compound on a cellulose base. This may help to minimize the dangers of misinterpretation regarding the causes of decay resistance of wood.



	Rfx100	COLOR UNDER UV
F6-A	6	Bright Blue
F6-B	15	Rust
F6-C	20	Blue
F6-D	25	Green-Yellow
F6-E	32	Tan
F6-F	40	Sky Blue
F6-G	59	Violet Blue

Figure 3. Diagrammatic representation of the paper chromatographic separation of the fraction F6 from the heartwood extractives of Maclura pomifera.

Table 9. The calculated percentage (air-dried weight basis) of each component of F6 in the heartwood of *Maclura pomifera*[a].

Component	% in Wood[b]
F6-A	2.4
F6-B	0.8
F6-C	0.5
F6-D	0.6
F6-E	0.4
F6-F	1.3
F6-G	1.4

[a]Various components refer to Figure 3.

[b]Theoretically maximum value.

Table 10. Effect of each component of the fraction F6 on wood decay fungi as evaluated by fungal growth on treated cellulose paper[a].

Fungus	Control[b]	% Fungal Growth[c] Based on Untreated Control						
		Pentachlorophenol	A	B	C	D+E	F	G
C. versicolor	100	0	22	85	100	100	95	100
P. placenta	100	0	23	95	100	93	100	100
G. trabeum	100	0	27	88	100	100	100	100

[a]Various components refer to Figure 3 and the retention of treating chemical in each sample is equivalent to its concentration in the wood.

[b]Control sample was previously eluted with solvent.

[c]Average of 4 estimations.

A is significantly different from the control at 0.05 level (T test).

Chemical Nature of the Fungistatic Component

The developed chromatogram of the active fraction-F6, with the R_f values and the color of each component under ultraviolet illumination is shown in Figure 3. The important color reactions with chromogenic sprays are given in Table 11. The NMR and mass spectra of the fungistatic component, F6-A, are presented in Figure 4 and Figure 5 respectively.

After spraying the developed chromatogram with ferric chloride-potassium ferricyanide, the fungistatic component, F6-A, gave an instantaneous deep blue spot which is the positive reaction for phenolic substances (9). The color reactions with diazotized p-nitroaniline, biodianzotized benzidine, diazotized sulfanilic acid and the color under ultraviolet were similar to those obtained from known stilbenes (18). These color reactions suggest that F6-A is a phenolic compound, possibly a stilbene.

F6-A was further characterized by NMR spectroscopic analysis and the spectrum was translated into a moleular structure by comparing it with the spectra of several known stilbenes including the previous reported 2, 4, 3', 5'-tetrahydroxystilbene (6). Since the reactions of F6-A with chemical indicators suggested it to be a phenolic compound, an Ar-OH group was suspected. A strong singlet at δ 7.0

Table 11. The color reactions of the component F6-A[a].

Reagent	Color
Ferric chloride-potassium ferricyanide	Deep Blue
Diazotized p-nitroaniline	Yellow Brown
Diazotized sulfanilic acid	Dark Tan
Bisdiazotized benzidine	Dark Rust
Vanillin-toluene-p-sulfonic acid	Violet Red
Ferric ammonium sulfate	---
Sodium molybdate	---

[a]Code F6-A refers to Figure 3.

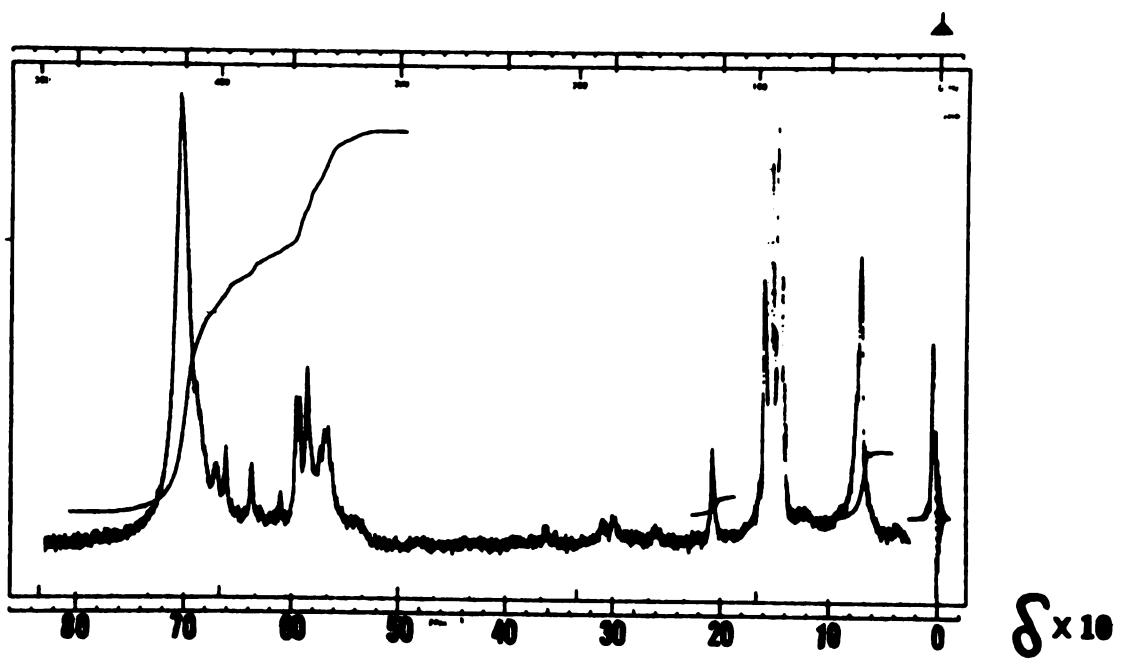


Figure 4. NMR spectrum of the component F6-A from the heartwood extractives of Maclura pomifera.

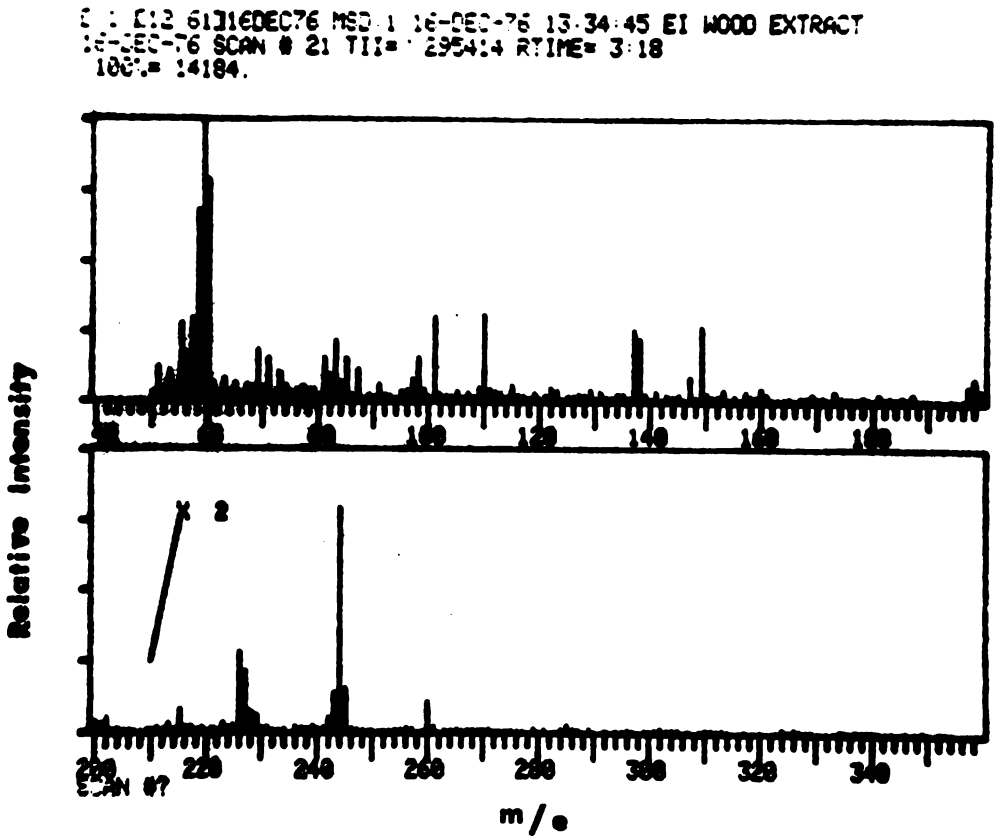
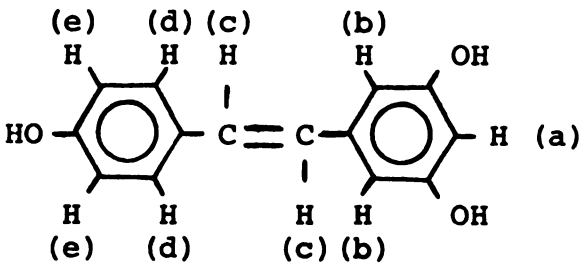


Figure 5. Mass spectrum of the component F6-A from the heartwood extractives of Maclura pomifera.

was identified to be the signal for phenolic protons and this identification was confirmed by a D_2O test-elimination of this peak by shaking with D_2O (27). The possibility of a -CH=CH- group was confirmed by the olefinic proton absorption in the NMR spectrum at about δ 6.0. The spectrum of authentic 3, 4', 5-trihydroxystilbene gave a similar absorption pattern. Thus, the doublet at δ 5.9 was translated into the absorption of olefinic protons. The remaining peaks down field are rather complex and are considered to be the aromatic signals. The peaks at about δ 1.5 were identified as the solvent signals. The peaks at δ 2.1 and δ 0.7 were considered to be the signals of impurities.

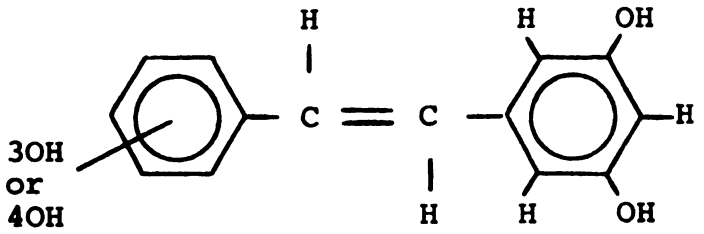
After a close examination of the nature of the chemical shifts and splitting in the NMR spectrum of 3, 4', 5-trihydroxystilbene, the following information was obtained:

Kinds of proton	Chemical shift δ
a	5.6
b	5.8 - 5.9
c	6.3
d	6.6 & 6.8
e	6.1 & 6.2

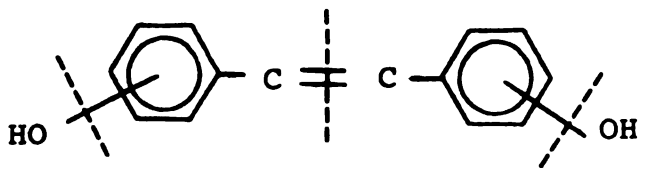


This information suggests that F6-A is a trans-hydroxystilbene containing a resorcinol group. The integration indicates that the phenolic peak contains five or six protons. Hence

the likely structure for F6-A from information discussed so far is:



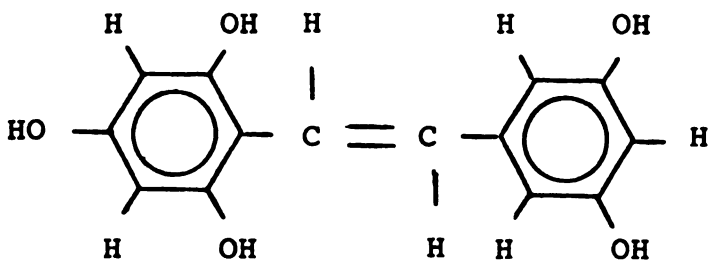
The parent peak at m/e 260 in the mass sepctrum of F6-A suggests a molecular weight of 260. This would indicate that F6-A is a pentahydroxystilbene. Pentahydroxystilbene is the most fully substituted natural stilbene so far encountered. The previous report concerning the pentahydroxystilbene in wood was that of 3, 4, 5, 3', 5'-pentahydroxystilbene which was found in the ether extract of Vouacapoua macropetala (19). The fragmentation was generally similar to those of authentic stilbenes with the following pattern:



Thus, presence of a fragment peak at m/e 138 is an additional evidence in favor of a pentahydroxystilbene. No further

information was gained from the mass spectrum for characterization.

After being sprayed with vanillin-toluene-p-sulfonic acid, flavonoid substances containing phloroglucinol nuclei turned a strong violet-red (23). The reaction of F6-A with vanillin-toluene-p-sulfonic acid was a strong violet-red similar to that produced by authentic phloroglucinol. These results suggest that F6-A contains a phloroglucinol group. Hence, the molecular structure of F6-A, based on the information available, appears to be:



However, additional information is needed before the exact location of the various hydroxy groups on the rings is firmly established.

CONCLUSIONS

1. The heartwood of Maclura pomifera has a very high decay resistance.

2. The chemical responsible for the decay resistance appears to be a pentahydroxystilbene.

LITERATURE CITED

LITERATURE CITED

1. A.S.T.M. 1975. Alcohol-benzene solubility of wood. Standard D-1107-56, A.S.T.M., Philadelphia.

2. A.S.T.M. 1975. Ether solubility of wood. Standard D-1108-56, A.S.T.M., Philadelphia.

3. A.S.T.M. 1975. Standard method of testing wood preservatives by laboratory soil-block cultures. Standard D-1413-61, A.S.T.M., Philadelphia.

4. A.S.T.M. 1975. Standard method for accelerated laboratory test of natural decay resistance of wood. Standard D-2017-63, A.S.T.M., Philadelphia.

5. AYRES, G. H. 1958. Quantitative chemical analysis. Harper and Brothers, New York, 726 p.

6. BARNES, R. A. and N. N. GERBER. 1955. The antifungal agent from osage-orange wood. J. Am. Chem. Soc. 7: 3259-3262.

7. BEHR, E. A. 1972. Development of respirometry as a method for evaluating wood preservatives. Forest Prod. J. 22(4): 26-31.

8. BEHR, E. A. 1973. Decay test methods. In wood deterioration and its prevention by preservative treatment, Vol. 1, Chap. 6, 217-246 (Nicholas, D. D., Editor), Syracuse Univ. Press, Syracuse, 390 p.

9. BROWNING, B. L. 1967. Methods of wood chemistry, Vol. 1, Interscience, New York. 384 p.

10. ERDTMAN, H. 1955. The chemistry of heartwood constituents of conifers and their taxonomic importance. Intern. Congr. Pure Appl. Chem., 14: 156-180.

11. HALABISKY, D. D. and G. IFJU. 1968. Use of respirometry for fast and accurate evaluation of wood preservatives. Proc., A.W.P.A. 64: 215-223.

12. HAMILTON, L. F. and S. G. SIMPSON. 1964. Quantitative chemical analysis. 12th Ed. Macmillan, New York, 576 p.

13. HART, J. H. and K. C. JOHNSON. 1970. Production of decay resistance sapwood in response to injury. Wood Science and Technology 4: 267-272.

14. HART, J. H. and W. E. HILLIS. 1974. Inhibition of wood-rotting fungi by stilbenes and other polyphenols in Eucalyptus sideroxylon. Phytopath. 64: 939-948.

15. HAWLEY, L. F., L. C. FLECK and C. A. RICHARDS. 1924. The relation between durability and chemical composition in wood. Industrial and Engineering Chemistry 16: 699-700.

16. HIGHLEY, T. L. 1975. Can wood-rot fungi degrade cellulose without other wood constituents? Forest Prod. J. 25(7): 38-39.

17. HILLIS, W. E. 1962. Wood extractives and their significance to the pulp and paper industries. Academic Press, New York and London, 513 p.

18. HILLIS, W. E. and ISHIKURA. 1968. The chromatographic and spectral properties of stilbene derivatives. J. Chromatog. 32: 323-336.

19. KING, E. E., T. J. KING, D. H. GODSON and L. C. MANNING. 1956. The chemistry of extractives from hardwoods. Pt. XXVIII. The occurrence of 3, 4, 3',5'-tetrahydroxy and 3,4,5,3',5'-pentahydroxyl-stilbene in Vouacapoua species. J. Chem. Soc. 1956: 4477-4480.

20. LOMAN, A. A. 1970. Bioassays of fungi isolated from Pinus contorta var. latifolia with pinosylvin, pinosylvin monomethyl ether, pinobanksin, and pinochembrin. Can. J. Bot. 48: 1303-1308.

21. MORRISON, R. T. and R. N. BOYD. 1973. Organic chemistry. 3rd Ed. Allyn and Bacon, Boston, 1258 p.

22. PETERSON, C. A. and E. B. COWLING. 1964. Decay resistance of extractive-free coniferous woods to white-rot fungi. Phytopath. 54: 542-547.

23. ROUX, D. G. and A. E. MAIHS. 1960. Selective spray reagents for the identification and estimation of flavonoid compounds associated with condensed tannins. J. Chromatog. 4: 65-74.

24. RUDMAN, P. 1963. The cause of natural durability in timber. Pt. XI. Some tests on the fungi toxicity of wood extractives and related compounds. Holzforschung 17: 54-57.

25. RUDMAN, P. 1965. The cause of natural durability in timber. Pt. XVIII. Further notes on the fungi toxicity of wood extractives. Holzforschung 19: 57-58.

26. SCHEFFER, T. C. and E. B. COWLING. 1966. Natural resistance of wood to microbial deterioration. Am. Rev. Phytopath. 4: 147-170.

27. SILVERSTEIN, R. M., G. C. BASSLER and T. C. MORRILL. 1974. Spectrometric identification of organic compounds. 3rd Ed. John Wiley and Sons, New York, 340 p.

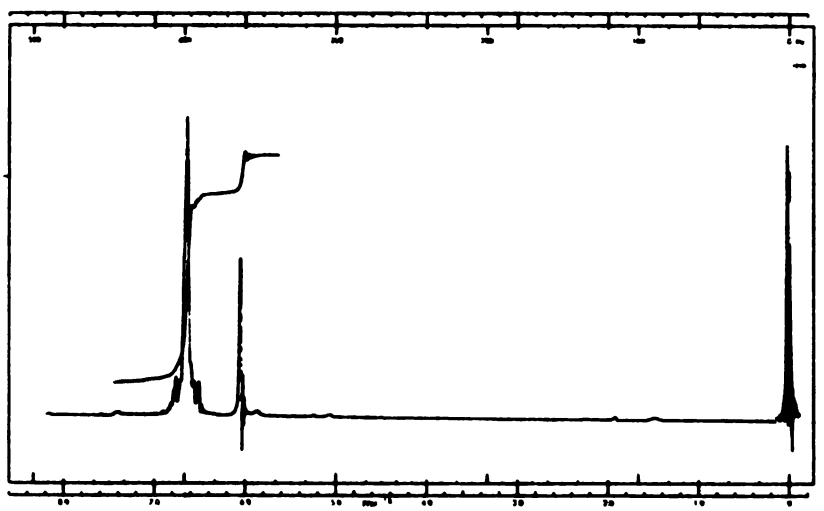
28. SMITH, R. S. 1969. Wood preservative toxicity evaluation using wood weight loss and fungal respiration methods. Wood Science 2(1): 44-63.

29. SMITH, R. S. 1975. Automatic respiration analysis of the fungitoxic potential of wood preservatives, including an oxathin. Forest Prod. J. 25(1): 48-53.

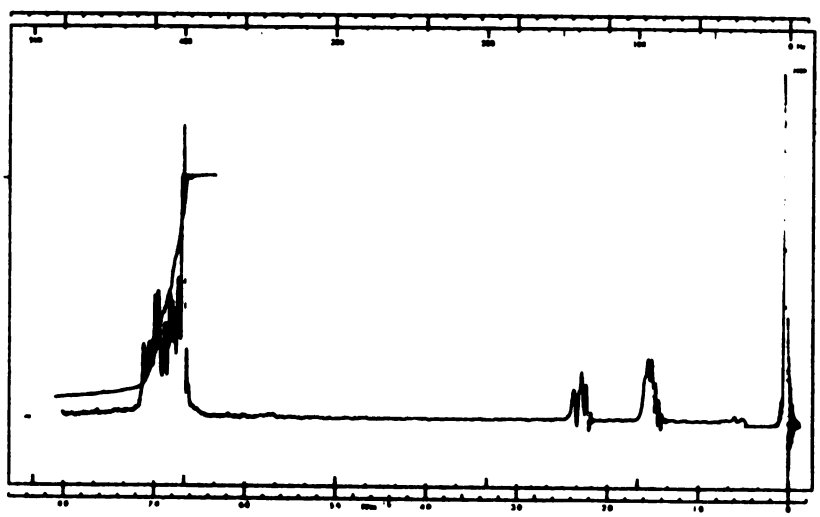
30. TOOLE, E. R. 1975. Oxygen utilization by decay fungi for the evaluation of wood preservatives. Forest Prod. J. 25(7): 46-68.

31. U. S. Forest Products Laboratory. 1961. Comparative decay resistance of heartwood of different native species when used under conditions that favor decay. Tech. Note No. 229. Revised May 1961.

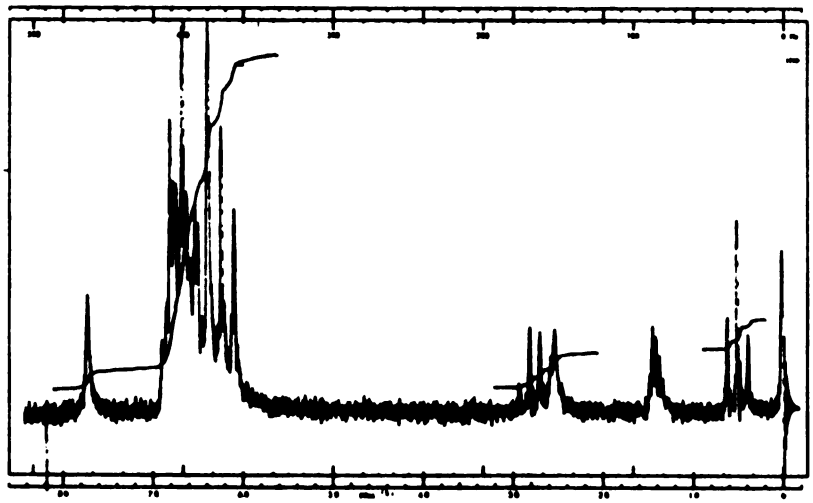
APPENDIX



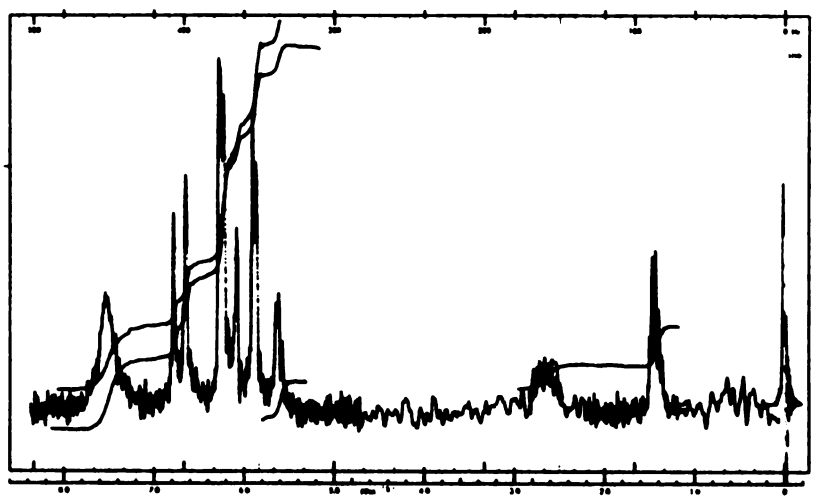
Appendix A. NMR spectrum of cis stilbene (Sample was purchased from Pfaltz and Bauer, Inc. Flushing, N. Y.).



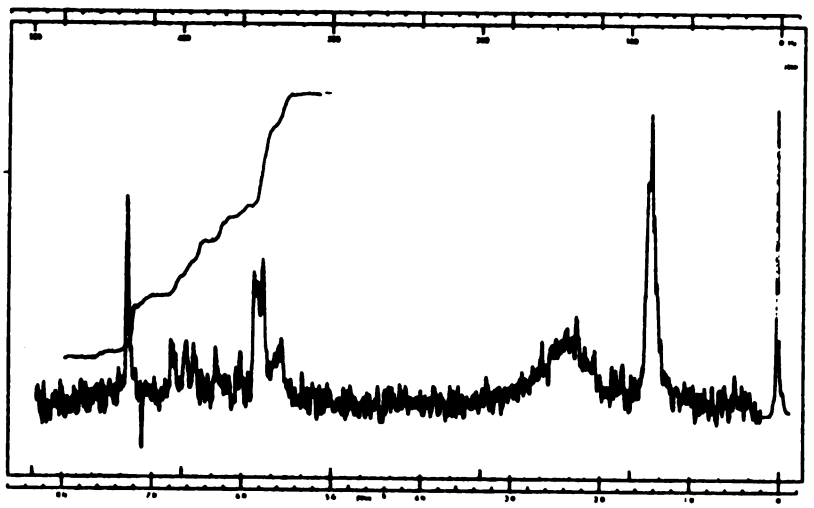
Appendix B. NMR spectrum of trans stilbene (Sample was purchased from Eastman Kodak Co. Rochester, N. Y.).



Appendix C. NMR spectrum of p-hydroxystilbene (Sample was purchased from Pfaltz and Bauer, Inc. Flushing, N. Y.)



Appendix D. NMR spectrum of 3,4',5-trihydroxystilbene (Sample furnished courtesy Dr. W. E. Hillis, Forest Products Laboratory, Division of Applied Chemistry, CRISO, South Melbourne, Australia).



Appendix E. NMR spectrum of 3,5,2',4'-tetrahydroxy-stilbene (Sample furnished courtesy Dr. W. E. Hillis, Forest Products Laboratory, Division of Applied Chemistry, CRISO, South Melbourne, Australia).

MICHIGAN STATE UNIV. LIBRARIES
31293000807887