STUDIES ON LIGNIN BIOSYNTHESIS AND BIODEGRADATION

by

RAMON A. RAZAL

DISSERTATION submitted to the Faculty of the
Virginia Polytechnic Institute and State University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy
in
Department of Wood Science and Forest Products

APPROVED:

Norman G. Lewis, Chair

Carole L. Cramer

Richard E. Ebel

Wolfgang G. Glasser

David G. I. Kingston

February 1990

Blacksburg, Virginia

STUDIES ON LIGNIN BIOSYNTHESIS AND BIODEGRADATION

by

RAMON A. RAZAL

Norman G. Lewis, Chair

Department of Wood Science and Forest Products

(ABSTRACT)

For the first time, the bonding patterns of specific carbon atoms in woody plant lignin have been identified *in situ*. This was accomplished by administering and incorporating into the lignin fraction of ***Leucaena leucocephala***, a tropical hardwood, ferulic acid enriched with ^{13}C at either the 1-, 2-, or 3-C atom of the side chain. The plants were grown hydroponically over extended periods of time (28 days) under aseptic conditions in media containing the ferulic acid precursor, and then the tissues were examined by solid-state ^{13}C NMR spectroscopy. Consequently, resonances due to the bonding patterns of the specific carbon atoms were determined. These resonances differ substantially from similarly labelled synthetic dehydrogenatively polymerized (DHP) lignin in both spectral profile and relative peak intensities.

Subsequent studies using phenylalanine as precursor showed that it was better translocated into the aerial portions of the plant, and that its uptake did not result in distortion of lignification in those tissues, both in amount and monomeric composition. Consequently, the difference spectra obtained by ^{13}C NMR analyses of phenylalanine-treated plants confirmed and extended the results obtained with ferulic acid. Evidence for the conversion of both precursors to the monolignols was shown by the difference spectra of [1-^{13}C]-precursor-fed tissues, where the dominant resonance at 61-63 ppm is consistent with substructures containing the hydroxymethyl functionality. The spectrum ob-

tained with roots administered [1-^{13}C] ferulic acid showed the presence of a minor resonance (170-174 ppm) attributable to carboxylic acids/esters. By allowing the plant to undergo further metabolism by growing in hydroponic media without the precursor, these signals disappeared from the resulting spectrum. The first direct evidence for the dominance of the β-O-4′ linkage of lignin *in situ* was shown by the appearance of the resonance at 83 ppm corresponding to this substructure in both [2-^{13}C] ferulic acid-treated roots and [2-^{13}C] phenylalanine-treated roots and stems. Evidence for the occurrence of α-O-carbohydrate or α-O-aryl linkage in intact plant tissues was obtained in the spectra of tissues administered [3-^{13}C] ferulic acid and [3-^{13}C] phenylalanine.

The effect of horseradish peroxidase/H_2O_2 in organic medium (dioxane/aqueous acetate buffer, pH 5, 95:5) on dehydrogenatively polymerized (DHP) lignin was reinvestigated. We found no evidence for vigorous depolymerization of DHP lignin under these conditions, contrary to claims made by Dordick, Marletta and Klibanov (1986, *Proc. Natl. Acad. Sci. USA* **83**:6255-6257). Furthermore, we did not detect ferulic acid as a degradation product following treatment of DHP lignin with HRP/H_2O_2.

Both coniferyl alcohol and DHP lignin were used in incubation experiments to determine effects of lignin peroxidase from the white-rot fungus ***Phanerochaete chrysosporium*** and H_2O_2 on these substrates. Gel filtration chromatography showed that polymeric materials of high molecular weights were the result of these treatments. Incubation of [1-^{13}C], [2-^{13}C] and [3-^{13}C] coniferyl alcohol with lignin peroxidase/H_2O_2 resulted in products similar to DHP lignins prepared by horseradish peroxidase/H_2O_2 with respect to occurrence of identical resonances in corresponding solution-state ^{13}C NMR spectra. Consequently, the role of polymerization of low molecular weight phenolics as a mechanism for detoxification was ascribed to these fungal peroxidases.

Acknowledgements

I would like to express my sincerest thanks to Dr. Norman G. Lewis, my major professor, for patiently directing my research. He was most supportive, not only professionally, but also personally, and this helped provide a stimulating research environment even when times were difficult. I would also like to thank the members of my committee, Drs. Carole L. Cramer, Richard E. Ebel, Wolfgang G. Glasser, and David G.I. Kingston for their invaluable suggestions and advice. The comments and suggestions of Prof. G.H.N. Towers from the University of British Columbia, who agreed to serve as external reviewer despite the very short time that was made available to him, are also most appreciated. I will be remiss if I do not acknowledge the guiding persistence of Dr. Etsuo Yamamoto who taught me how to become a more organized researcher; I have also learned most of my laboratory techniques from him.

This work would not have been possible without the help of Dr. Jan B. Wooten who skillfully obtained all of the solid-state ^{13}C NMR spectra through the facilities at the Philip Morris Research Center. I am extremely grateful for his assistance and interest in this work. The molecular weight distribution of the DHP lignin samples were deter-

mined in the laboratory of Dr. Simo Sarkanen (University of Minnesota), through his assistant, Matthew L. Iwen, and this is also gratefully acknowledged.

I would like to thank Dr. Geza Ifju and the staff of the Department of Wood Science and Forest Products for helping make my stay at Virginia Tech a pleasant one. I am particularly thankful to my co-workers in the Wood Chemistry/Biochemistry lab for their cooperation and moral support. The careful review and editing of this manuscript or portions thereof by Drs. Laurence Davin and Toshiaki Umezawa and by Ms. Ma. Estela J. Inciong are also gratefully acknowledged. I am also fortunate to have co-workers like Drs. Kali Dhara and Tom Piccariello, and the rest of Dr. N. G. Lewis' group, with whom informal discussions proved very fruitful and informative.

I also owe the Filipino community in Blacksburg many thanks for making this place just like home, and for the countless help that came in so many forms. I am indebted to the University of the Philippines for allowing me to go on leave to pursue a PhD degree. The encouragement, support and understanding of my relatives and friends in the Philippines, especially my cousin, Ate Auo and her husband and kids, are deeply appreciated. I am most thankful to the Lord for seeing me through all these times. Finally, I want to thank my Mama, who, for all her love and sacrifices, will never know that this work is dedicated to her.

Table of Contents

1.0 INTRODUCTION 1

2.0 OBJECTIVES 5

3.0 REVIEW OF LITERATURE 7

3.1 Biosynthesis and Structure of Lignin 7

3.1.1 Shikimate-chorismate pathway 7

3.1.2 The phenylpropanoid pathway 14

3.1.3 Biosynthesis of monolignols from activated cinnamic acids 18

3.1.4 The process of lignification 19

3.1.5 The determination of the structure of lignin 24

3.1.6 Applications of ^{13}C nuclear magnetic resonance (NMR) spectroscopy in lignin studies 28

3.2 Lignin Biodegradation 37

3.2.1 Microorganisms that degrade lignin 37

3.2.1.1 Bacteria. 38

3.2.1.2 Fungi. 39

3.2.2 Mechanisms for the "biodegradation" of lignin 40

3.2.3 Involvement of an alternative enzyme system in lignin biodegradation 45
3.2.4 Role of "lignin peroxidase" in lignin biodegradation 46

4.0 EXPERIMENTAL 49
4.1 General Methods 49
4.2 Examination of Lignin Structure *in situ* by Solid-state ^{13}C NMR Spectroscopy 52
4.2.1 Construction of an aseptic, hydroponic system for administering precursor 52
4.2.2 Preparation of plant material 56
4.2.3 Preparation of nutrient media 57
4.2.4 Plant growth 59
4.2.5 Incorporation of [2-^{14}C] ferulic acid (**30'**) into *Leucaena leucocephala* root tissue ... 60
4.2.6 Synthesis of [^{13}C]-labelled ferulic acid (**30a-c**) 63
4.2.7 Incorporation of [1-^{13}C] ferulic acid (**30a**) into *L. leucocephala* root tissue 65
4.2.8 Three-week [1-^{13}C] ferulic acid (**30a**) incorporation into *L. leucocephala*, followed by one-week growth in precursor-free media 65
4.2.9 Three-week incorporation of [1-^{13}C] ferulic acid (**30a**) into *L. leucocephala* root tissue 66
4.2.10 Incorporation of [2-^{13}C] ferulic acid (**30b**) into *L. leucocephala* root tissue 66
4.2.11 Incorporation of [3-^{13}C] ferulic acid (**30c**) into *L. leucocephala* root tissue 67
4.2.12 HPLC assay for ferulic acid (**30**) in the media 67
4.2.13 Incorporation of [U-^{14}C] L-phenylalanine (**7'**) into *L. leucocephala* root and stem tissues 68
4.2.14 Distribution of radioactivity in the roots and stems of [U-^{14}C] L-phenylalanine-fed *L. leucocephala* 69
4.2.15 Incorporation of ^{13}C-enriched L-phenylalanine (**7a-c**) into *L. leucocephala* root and stem tissues 71
4.2.16 Spectrophotometric assay for L-phenylalanine (**7**) in the media 72
4.2.17 Monitoring media for biological contamination 73
4.2.18 Acetyl bromide lignin determination [M-11] 75

4.2.19 Nitrobenzene oxidation 76
4.3 Lignin degradation by peroxidase in organic media 78
4.3.1 Purification of dioxane 78
4.3.2 Gel filtration chromatography 79
4.3.3 Fractionation of DHP lignin 80
4.3.4 Effect of horseradish peroxidase/H_2O_2 on DHP lignin 80
4.3.5 Enzyme assay 81
4.3.6 HPLC assay for ferulic acid (**30**) 81
4.4 Lignin peroxidase from *Phanerochaete chrysosporium*: Effects on coniferyl alcohol (**4**) and DHP lignin 82
4.4.1 Assay procedure for lignin peroxidase activity 82
4.4.2 Electrophoretic patterns of lignin peroxidase 83
4.4.3 Spectrophotometric assay for Mn^{+2}-dependent enzyme activity in lignin peroxidase. 87
4.4.4 Preparation of coniferyl alcohol (**4**) for incubation with lignin peroxidase 88
4.4.5 Treatment of coniferyl alcohol (**4**) with lignin peroxidase 89
4.4.6 Incubation of coniferyl alcohol (**4**) in 25 mM sodium tartrate buffer, pH 4.0, without lignin peroxidase 91
4.4.7 Effect on "DHP lignin" by further treatment with lignin peroxidase 92
4.4.8 Determination of molecular weight distribution of "DHP lignins" formed by lignin peroxidase 93

5.0 RESULTS AND DISCUSSION 94
5.1 Examination of Lignin Structure *in situ* by Solid-state ^{13}C NMR Spectroscopy 94
5.1.1 Development of a hydroponic system for administering lignin precursor to *L. leucocephala* 97
5.1.2 Incorporation of [2-^{14}C] ferulic acid (**30'**) into *L. leucocephala* 99
5.1.3 Incorporation of natural abundance ferulic acid (**30**) into *L. leucocephala* 103
5.1.4 Incorporation of [1-^{13}C] ferulic acid (**30a**) into *L. leucocephala* root tissue 106

5.1.5 Three-week [1-^{13}C] ferulic acid (**30a**) incorporation into *L. leucocephala*, followed by one-week growth in precursor-free media 111
5.1.6 Incorporation of [2-^{13}C] ferulic acid (**30b**) into *L. leucocephala* root tissue 114
5.1.7 Incorporation of [3-^{13}C] ferulic acid (**30c**) into *L. leucocephala* root tissue 116
5.1.8 Effect of ferulic acid (**30**) uptake on lignin metabolism in *L. leucocephala* 119
5.1.9 Incorporation of [U-^{14}C] phenylalanine (**7'**) into *L. leucocephala* 120
5.1.10 Effect of phenylalanine (**7**) on lignification and monomeric composition 126
5.1.11 Incorporation of [1-^{13}C] phenylalanine (**7a**) into *L. leucocephala* roots and stems 128
5.1.12 Incorporation of [2-^{13}C] phenylalanine (**7b**) into *L. leucocephala* roots and stems 131
5.1.13 Incorporation of [3-^{13}C] phenylalanine (**7c**) into *L. leucocephala* roots and stems 134
5.2 Lignin Biodegradation 136
5.2.1 Lignin degradation by peroxidase in organic media 138
5.2.2 Lignin peroxidase from *P. chrysosporium*: Effects on coniferyl alcohol (**4**) and DHP lignin 143
5.2.3 Electrophoretic patterns of the lignin peroxidase preparation 144
5.2.4 Spectrophotometric assay to determine Mn^{+2}-dependent enzyme activity in the lignin peroxidase preparation 147
5.2.5 Effect of lignin peroxidase treatment on coniferyl alcohol (**4**) 147
5.2.5.1 Molecular weight distribution of DHP lignins formed from coniferyl alcohol (**4**) by the action of lignin peroxidase. 149
5.2.5.2 DHP lignins from [1-^{13}C] coniferyl alcohol (**4a**). 152
5.2.5.3 DHP lignins from [2-^{13}C] coniferyl alcohol (**4b**). 154
5.2.5.4 DHP lignins from [3-^{13}C] coniferyl alcohol (**4c**). 154

6.0 CONCLUSIONS 158
6.1 Examination of Lignin Structure *in situ* by Solid-state ^{13}C NMR Spectroscopy 158
6.2 Lignin Biodegradation 160

BIBLIOGRAPHY 163

VITA 175

List of Illustrations

Figure 1. The shikimic acid pathway. From phosphoenol pyruvate (**9**) and erythrose-4-phosphate (**10**) to chorismic acid (**17**). 8

Figure 2. Biosynthesis of phenylalanine (**7**) and tyrosine (**8**) from chorismic acid (**17**); formation of p-coumaric acid (**23**). 9

Figure 3. The phenylpropanoid pathway, and biosynthesis of monolignols (**4-6**) . 17

Figure 4. Dehydrogenative polymerization of monolignols (**4-6**) into lignin. 22

Figure 5. Modified scheme for the catalytic cycle of lignin peroxidase. 44

Figure 6. Hydroponic unit for the culture of *L. leucocephala* 53

Figure 7. Standard curve for PAL assay. 74

Figure 8. Schematic of experimental protocol for administering labelled precursors to *L. leucocephala*. 100

Figure 9. Distribution of radioactivity in *L. leucocephala* administered [2-^{14}C] ferulic acid (**30'**). 102

Figure 10. Solid-state ^{13}C NMR spectra of *L. leucocephala* root tissue (natural abundance) 105

Figure 11. Dominant bonding patterns in lignin. 107

Figure 12. Solid-state ^{13}C NMR spectra of DHP lignin from coniferyl alcohol (**4**) 108

Figure 13. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots administered [1-^{13}C] ferulic acid (**30a**) 110

Figure 14. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots in experiments to determine turnover of ferulic acid (**30**) 113

Figure 15. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots administered [2-^{13}C] ferulic acid (**30b**) 115

Figure 16. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots administered [3-^{13}C] ferulic acid (**30c**) 117

Figure 17. Solid-state ^{13}C NMR spectra of *L. leucocephala* (a) stems and (b) roots administered [1-^{13}C] phenylalanine (**7a**). 130

Figure 18. Solid-state ^{13}C NMR spectra of *L. leucocephala* (a) stems and (b) roots administered [2-^{13}C] phenylalanine (**7b**). 132

Figure 19. Solid-state ^{13}C NMR spectra of *L. leucocephala* (a) stems and (b) roots administered [3-^{13}C] phenylalanine (**7c**). 135

Figure 20. Enzyme activity of horseradish peroxidase during incubation of DHP lignin in organic media. 140

Figure 21. Gel filtration, using 0.1M LiCl in DMF as eluant, of DHP lignin before and after incubation with horseradish peroxidase 141

Figure 22. Gel filtration, using dioxane as eluant, of DHP lignin before and after incubation with horseradish peroxidase 142

Figure 23. Protein and activity staining of lignin peroxidase preparation. 145

Figure 24. MW distribution of DHP lignins obtained from incubation of coniferyl alcohol (**4**) with lignin peroxidase/H_2O_2. 150

Figure 25. Effect of further treatment with lignin peroxidase/H_2O_2 on MW distribution of DHP lignin. 151

Figure 26. ^{13}C NMR solution spectra of DHP lignins from [1-^{13}C] coniferyl alcohol (**4a**) 153

Figure 27. ^{13}C NMR solution spectra of DHP lignins from [2-^{13}C] coniferyl alcohol (**4b**) 155

Figure 28. ^{13}C NMR solution spectra of DHP lignins from [3-^{13}C] coniferyl alcohol (**4c**) 156

List of Tables

Table 1. Composition of stock solutions and final concentration of modified Hoagland's media. 58

Table 2. Total radioactivity in the different organs of *L. leucocephala* administered [2-^{14}C] ferulic acid (**30'**). 101

Table 3. Lignin content and monomeric composition of *L. leucocephala* grown in soil and in hydroponic media containing ferulic acid (**30**). 121

Table 4. Incorporation of [2-^{14}C] ferulic acid (**30'**) and [U-^{14}C] phenylalanine (**7'**) into the lignified tissue of *L. leucocephala* 123

Table 5. Distribution of radioactivity in *L. leucocephala* administered [U-^{14}C] phenylalanine (**7'**). 125

Table 6. Lignin content and monomeric composition of *L. leucocephala* grown in soil and in hydroponic media containing phenylalanine (**7**). 127

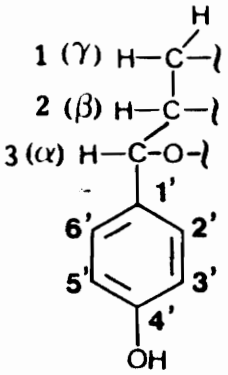
Table 7. Spectrophotometric assay for Mn^{+2}-dependent peroxidase activity in the enzyme preparation. 148

1.0 INTRODUCTION

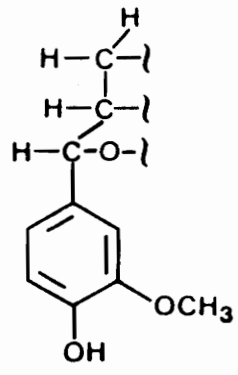
The term "lignins", as used in this work, pertains to the biomolecules that are polymers of phenylpropanoid units, linked to each other in a variety of ways, and constituting essentially all the non-carbohydrate portion of the cell walls and middle lamellae of terrestrial vascular plants. As naturally-occurring, renewable organic substances, they are generally regarded to rank only next to cellulose in abundance. They can be found not only in the wood, but also in the bark, cork, fruits, leaves and buds of vascular plants [55].

Lignins serve many purposes in plants. As the "cementing substances" in plant tissue, they impart rigidity to the plant cell walls thereby allowing them to withstand compressive stresses [60,75]. The presence of lignin in plants imparts resistance to fungal attack [60]. Due to their complex structure, lignins are practically impermeable to moisture which serves the plant well in physiological conduction of the sap and in preventing dehydration and leaching of water-soluble nutrients [60,75].

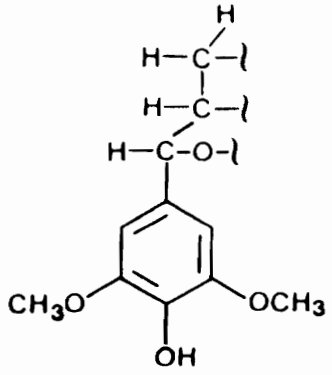
The structure and composition of native lignins depend on the plant species, on the tissue in which they are formed, and even on their morphological location within the plant cell wall [56,175]. Based on typical composition, lignins are generally classified as softwood, hardwood, or grass lignins. Softwood lignins are often comprised predominantly of guaiacylpropanoid units (**2**) and small amounts of *p*-hydroxyphenylpropanoid units (**1**) [142], although there are some exceptions to this trend (e.g., *Tetraclinis articulata* and *Podocarpus nerrifolius*) [115]. Hardwood lignins are found in angiosperms other than monocots, and usually consist of guaiacyl-, syringyl- (**3**), and *p*-hydroxyphenylpropanoid units, where guaiacyl- and syringylpropanoid units dominate and are generally present in almost equal amounts. The dicot *Erythrina crista-galli* is a notable exception [100]. Chemical analyses of its lignin indicated an unusually low syringyl content, as further evidenced by a negative Mäule test, a histochemical staining procedure specific for syringyl units. Finally, the guaiacyl/syringyl/*p*-hydroxyphenyl ratio of lignins in grasses varies from species to species, with *p*-hydroxyphenylpropanoid units predominating in some cases, and with ~10% of the lignin consisting of *p*-hydroxycinnamic acid moieties occurring in free or esterified forms [76,130].



(1)



(2)



(3)

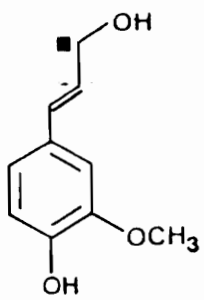
Hence, it is obvious that a rigid categorization of lignins according to taxonomic groups is not possible, since this would exclude lignins in some plants whose compositions essentially do not fall under these classifications.

While there is some agreement on the physiological roles lignins play in the plant, their structures within the plant cell wall are still poorly understood. To date, there is no single method to isolate lignins in their native, or intact, state. Consequently, lignin preparations are identified according to the procedure used in obtaining them, as well as by the type of plant material from which they are derived, e.g., softwood, hardwood, etc. Further, because of their importance in turnover of organic carbon, there has been considerable interest in defining the biochemical route operative in the biodegradation of lignins.

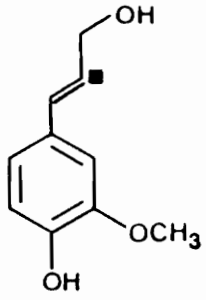
This study has two parts: The first is to characterize lignin in intact plant tissue. When the structure of lignin *in situ* has been ascertained, then the efficacy of the different methods used in isolating lignins can be compared. For purposes of studying lignin biosynthesis and structure, the method of choice for isolating lignin is that which results in least modification, if at all, of native lignin. To investigate the biogenesis and structure of lignin *in situ*, a fast-growing tropical hardwood, *Leucaena leucocephala* was selected. This species is widely utilized for reforestation in developing countries in Asia and Central America [77]. Using this plant, methodology was developed for determining the bonding patterns of specific carbons of the monomeric units that make up lignin *in vivo*. This involved administering to the plant lignin precursors ^{13}C-specifically labelled at different positions in the propyl side chain. For the most part, these are the sites mainly involved in the major inter-unit linkages in the lignin polymer. Following

uptake of precursors by the plant, lignins in the intact plant tissue/organ were then analyzed by solid state ^{13}C nuclear magnetic resonance (NMR) spectroscopy.

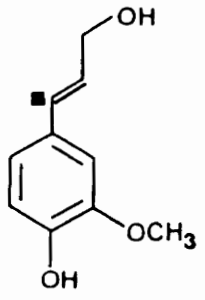
The second part of this study is aimed at establishing the role of extracellular peroxidases secreted by white-rot fungi in the biodegradation of lignin. These enzymes, together with horseradish peroxidase preparations [28], have been reported to be the catalytic agents in the decomposition of lignin [52,184]. In the first instance, lignin degradation experiments reported by Dordick, Marletta and Klibanov [28] using horseradish peroxidases (HRP)/H_2O_2 in organic media were reinvestigated, with the main exception that "degradation" products were separated by a gel permeation chromatographic system that partially suppresses lignin association [21]. Second, the effects of lignin peroxidases isolated from *Phanerochaete chrysosporium* on coniferyl alcohol (**4**) and a dehydrogenatively polymerized (DHP) lignin preparation were investigated. This was carried out by separately incubating [1-^{13}C]-, [2-^{13}C]-, and [3-^{13}C]-coniferyl alcohols (**4a-c**) with a preparation of lignin peroxidase and by treating a DHP lignin preparation with the enzyme in a similar manner. The molecular weight profiles of the resulting products were determined by gel filtration chromatography. In addition, solution-state ^{13}C NMR spectroscopy was employed to determine bonding patterns of the ^{13}C specifically-labelled side-chain carbon atoms in those products. Hopefully, the results will provide us with a better understanding of the mechanisms occurring during the depolymerization of lignins.



(4a) ■ = ^{13}C



(4b)



(4c)

2.0 OBJECTIVES

1. Main objective: To determine the bonding patterns of specific carbon atoms of lignin *in situ* in hardwoods.

   - Specific objectives:

   a. To develop a methodology for administering specifically-labelled lignin precursors to growing *Leucaena leucocephala* seedlings.

   b. To ascertain that such precursors are taken up intact, and that the lignin formed is comparable with that of plants grown "normally".

   c. To determine the bonding environments of ^{13}C-enriched carbon atoms of lignin *in situ* by solid-state ^{13}C NMR spectroscopy.

2. Main objectives: a) To further define the role of extracellular peroxidases excreted by lignin-biodegrading white-rot fungi; and b) If shown capable of degrading lignins, to determine their effects on lignin in plant tissue.

- Specific objectives:

a. To reassess the claim made by Dordick, Marletta and Klibanov [28] that horseradish peroxidases vigorously degrade lignin in organic solvents.

b. To determine the effects of extracellular peroxidases isolated from *Phanerochaete chrysosporium* on both coniferyl alcohol (**4**) and DHP lignin.

3.0 REVIEW OF LITERATURE

3.1 Biosynthesis and Structure of Lignin

3.1.1 Shikimate-chorismate pathway

The ultimate precursors in the biogenesis of lignin, namely *p*-coumaryl (**5**), coniferyl (**4**) and sinapyl (**6**) alcohols are derived from the aromatic amino acid(s) phenylalanine (**7**) (and tyrosine (**8**) in grasses), which are formed by the shikimate-chorismate pathway (Figures 1 and 2). The two most widely-studied metabolites in this pathway are shikimic acid (**14**), first discovered in the oriental plant *shikimi-no-ki* (*Illicium reliogosum*) by Eykmann in 1885 [71] and chorismic acid (**17**), a branchpoint for at least five metabolic routes leading to a variety of secondary plant products [45]. The biosynthetic sequence of this pathway was first elucidated using *Escherichia coli* and other bacteria [24], although consequently, all the enzymes that catalyze the respective transformations in the pathway have also been isolated from higher plants.

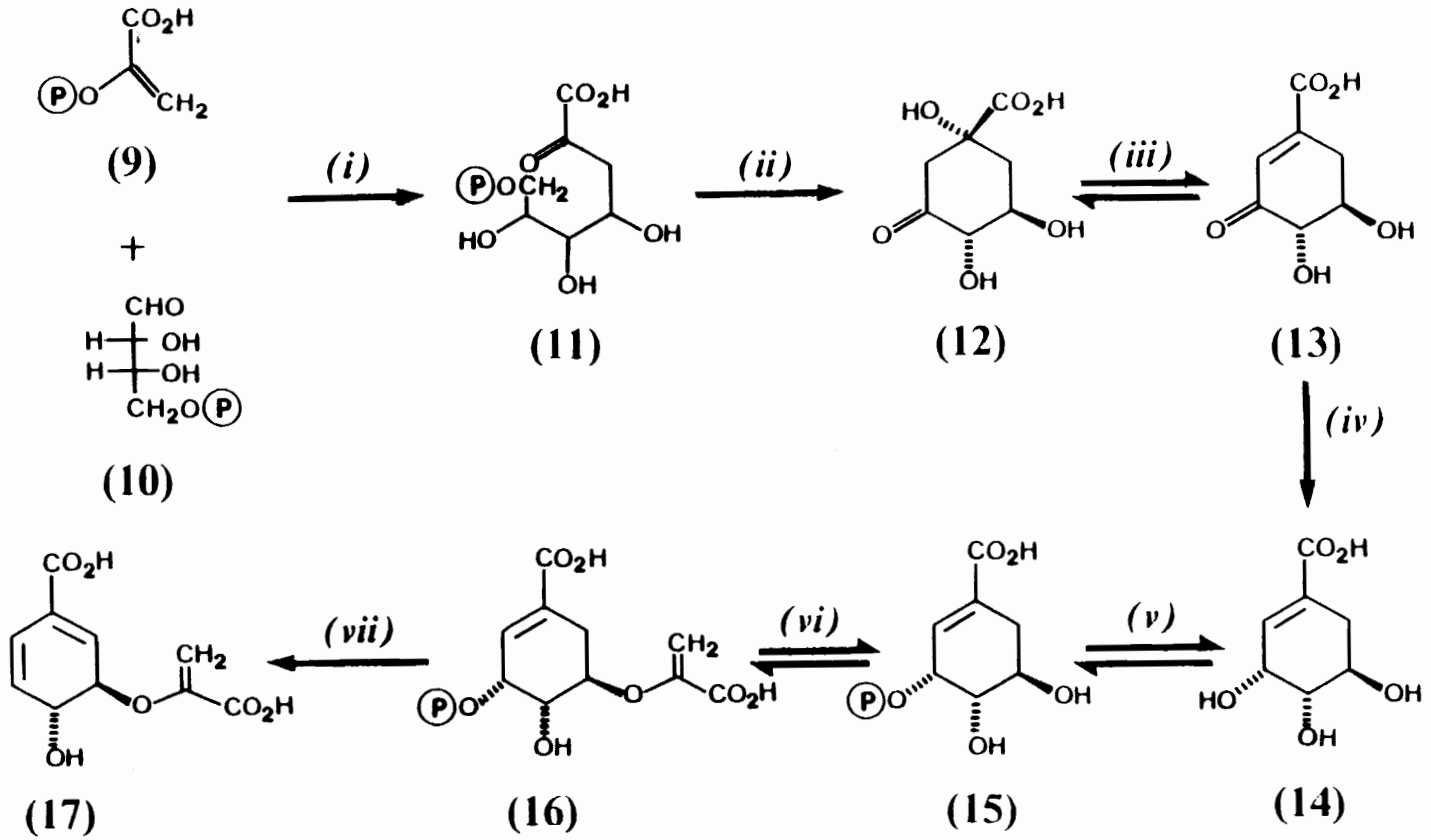


Figure 1. The shikimic acid pathway. From phosphoenol pyruvate (9) and erythrose-4-phosphate (10) to chorismic acid (17).
Enzymes: i) phospho-2-keto-3-deoxyheptonate synthase, ii) 3-dehydroquinate synthase, iii) 3-dehydroquinate dehydratase, iv) shikimate dehydrogenase, v) shikimate kinase, vi) 3-phospho-5-enol-pyruvylshikimate synthase, vii) chorismate synthase.

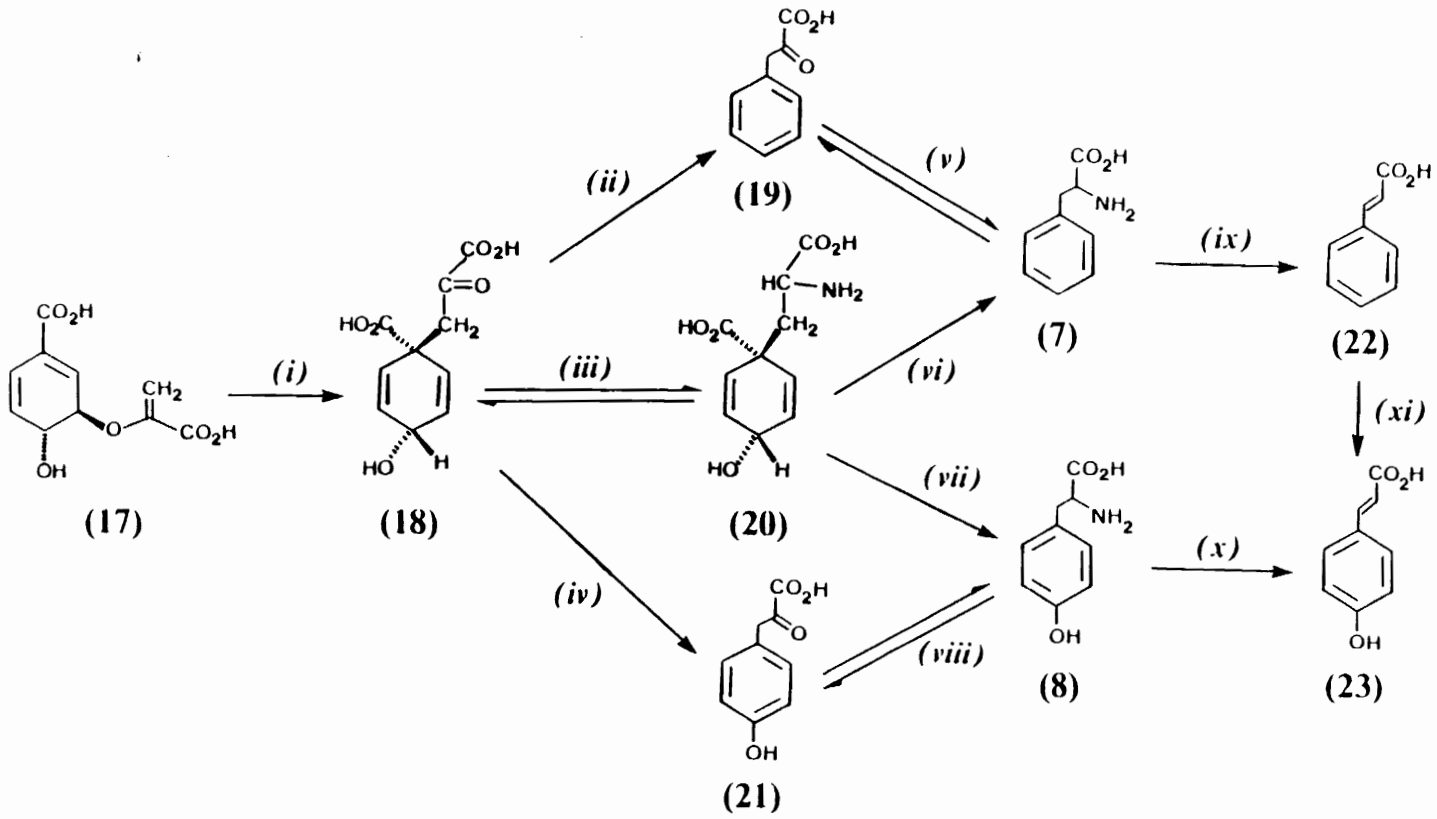


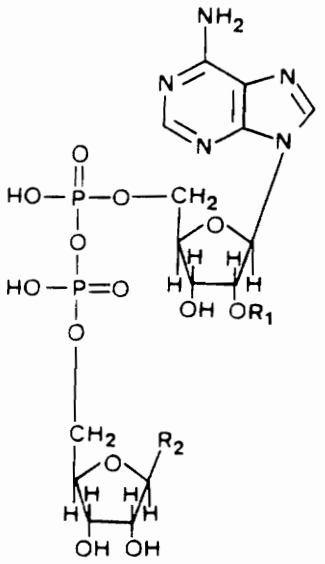
Figure 2. Biosynthesis of phenylalanine (**7**) and tyrosine (**8**) from chorismic acid (**17**); formation of *p*-coumaric acid (**23**).
Enzymes: i) chorismate mutase, ii) prephenate dehydratase, iii) prephenate aminotransferase, iv) prephenate dehydrogenase, v) phenylalanine aminotransferase, vi) arogenate dehydratase, vii) arogenate dehydrogenase, viii) tyrosine aminotransferase, ix) phenylalanine ammonia lyase, x) tyrosine ammonia lyase, xi) cinnamate-4-hydroxylase.

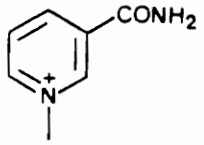
The first step in the biosynthetic route to the aromatic amino acids is an aldol-like condensation between phosphoenol pyruvate, PEP, (**9**) and D-erythrose-4-phosphate (**10**) giving 3-deoxy-D-arabinoheptulosonic acid-7-phosphate, DAHP (**11**). The enzyme responsible for this reaction is phospho-2-keto-3-deoxy-heptonate synthase (DAHP synthase), which in higher plants, has been characterized from cauliflower (*Brassica oleracea L. var. botrytis* Mill) [78] and carrots (*Daucus carrota*) [178]. The mechanism postulated for this reaction involves initial nucleophilic attack by H_2O at C-2 of PEP (**9**), subsequent addition of erythrose-4-phosphate (**10**) at C-3, and elimination of orthophospate from C-2 [25].

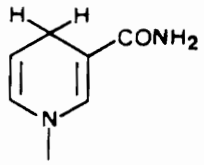
The second step involves cyclization of DAHP (**11**) to 3-dehydroquinic acid, 3-DHQ (**12**), in an intramolecular reaction that possibly involves an enolic intermediate, resulting in elimination of the remaining phosphate group and oxidation at C-3. These transformations are mediated by 3-dehydroquinate synthase, a nicotinamide adenine dinucleotide, NAD^+ (**24a**) and Co^{+2}-requiring enzyme. In higher plants, 3-DHQ synthase has been found in *Sorghum bicolor* [155] and *Phaseolus mungo* [194] seedlings.

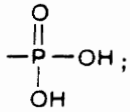
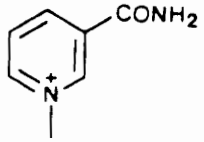
The dehydration of **12**, resulting from the *syn*-elimination of the elements of water from carbon atoms 1 and 2, is a reversible reaction which, in a forward direction, leads to the formation of 3-dehydroshikimic acid (**13**). Partially-purified enzyme preparations from different organs of *Zea mays* [10] showed the presence of 3-DHQ dehydratase, which functions as the catalyst for the dehydroquinate-dehydroshikimate interconversion.

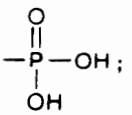
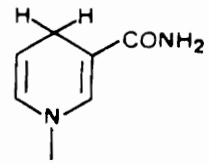
The next step is a reduction by shikimate dehydrogenase, which requires the reduced form of nicotinamide adenine dinucleotide phosphate, NADPH (**25b**), as a co-factor. Nucleophilic attack of the carbonyl functionality by a hydride ion from NADPH



(24a) $R_1 = H$; $R_2 =$ 

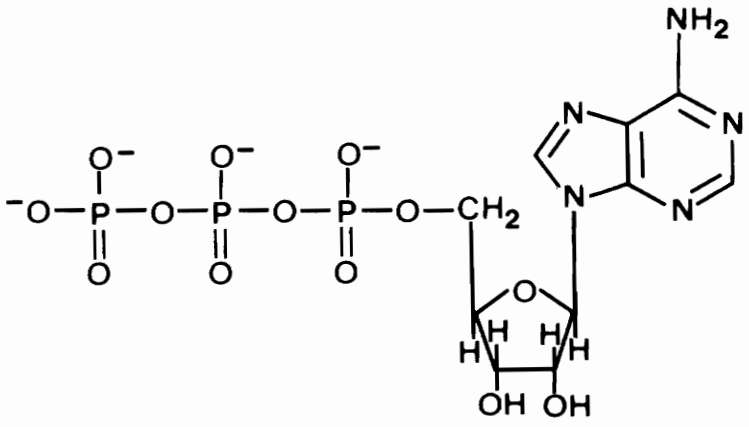
(24b) $R_1 = H$; $R_2 =$ 

(25a) $R =$  ; $R_2 =$ 

(25b) $R =$  ; $R_2 =$ 

produces shikimic acid (**14**). Spinach leaves and peas [6] were among the first higher plants in which the presence of shikimate dehydrogenase was demonstrated.

Shikimic acid (**14**) is then reversibly phosphorylated to give shikimate-3-phosphate (**15**). This reaction involves shikimate kinase, which transfers a phosphoryl group from adenosine triphosphate, ATP (**26**), to the hydroxyl at C-3. Shikimate kinase activity has been reported in cell-free preparations of etiolated shoots and green stems of *Sorghum bicolor* [11] and *Phaseolus mungo* [96], thereby completing the scheme for an intact biochemical machinery for shikimate biogenesis in higher plants.



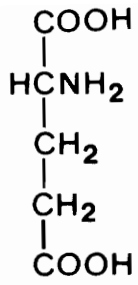
(26)

Following shikimate-3-phosphate (**15**) formation is the addition of a second molecule of PEP (**9**) to form 5-enol-pyruvylshikimate-3-phosphate (**16**). A reversible addition-elimination mechanism has been proposed for this reaction, catalyzed in both microbial organisms and higher plants by 3-phospho-5-enolpyruvylshikimate synthase [26,45,128].

The next step is chorismic acid (**17**) formation, which is thought to proceed via a *trans* 1,4 elimination of the elements of phosphoric acid [45]. This reaction occurs via the mediation of the enzyme chorismate synthase [70], which, in higher plants, was recently detected in tissue extracts and chloroplast preparations of pea (*Pisum sativum L.*) [129].

The metabolic route to the aromatic amino acids (Figure 2) proceeds with the repositioning of the 3-carbon moiety in **17** via a concerted intramolecular mechanism involving migration of sigma (σ) bonds with accompanying pi (π) bond shifts, yielding prephenic acid (**18**). The enzyme, chorismate mutase, which is responsible for this reaction is very well-studied, having been found in a variety of prokaryotes [26] and higher plants [23,174]. It is also of immense biochemical interest because its isoenzymic forms and their cellular compartmentation could provide possible clues for the regulation and control of the pathways that branch out from chorismic acid (**17**).

Tyrosine (**8**) formation was initially thought to occur via *p*-hydroxyphenylpyruvic acid (**21**), formed from decarboxylation and dehydrogenation of prephenic acid (**18**), in a reaction catalyzed by $NADP^+$-requiring prephenate dehydrogenase [50]. Alternatively, phenylalanine (**7**) formation was thought to proceed via phenylpyruvic acid (**19**) as a result of decarboxylation and dehydration of **18** by prephenate dehydratase [50]. Subsequent transamination with an amine donor, e.g. L-glutamic acid (**27**), of both **21** and **19** would yield tyrosine (**8**) and phenylalanine (**7**), respectively. Both conversions were presumably catalyzed by aromatic amino acid aminotransferases which lacked substrate specificity [193].



(27)

More recently, arogenic acid (**20**) has been proposed as an intermediate in tyrosine (**8**) and phenylalanine (**7**) biosynthesis in a number of herbaceous and graminaceous plants [15,20,44]. It now appears to be the only pathway for phenylalanine/tyrosine biogenesis in these organisms. The reaction takes place via a transamination mechanism between prephenic acid (**18**) and an amine donor (e.g., L-glutamic acid, **27**), and is mediated by prephenate aminotransferase [173]. Subsequent decarboxylation of arogenic acid (**20**), when accompanied by dehydration (catalyzed by arogenate dehydratase), results in phenylalanine (**7**) [84]. On the other hand, when decarboxylation is accompanied by dehydrogenation (via the $NADP^+$-requiring enzyme, arogenate dehydrogenase), tyrosine (**8**) is formed. In both cases, ring aromatization is obtained.

3.1.2 The phenylpropanoid pathway

Part of the metabolic route leading to the formation of monolignols belongs to the phenylpropanoid pathway, which includes the biogenetic steps towards activated

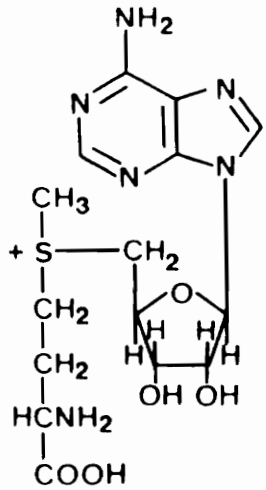
cinnamic acids (**33-35**), known intermediates for a variety of plant phenolics. The prevailing notion in plant biochemistry today is that *E*-monolignols (**4-6**) are formed in the plant principally for lignin biosynthesis, although their involvement in the biogenesis of lignans and suberin should not be discounted.

In all plants, phenylalanine (**7**) is deaminated by phenylalanine ammonia lyase (PAL) giving *trans*-cinnamic acid (**22**), which is also an intermediate in the biosynthesis of coumarins [12]. In grasses, tyrosine ammonia lyase (TAL) activity is found associated with PAL, and TAL deaminates L-tyrosine (**8**) to give *trans-p*-coumaric acid (**23**). In both deamination reactions, the *pro*-S hydrogen is abstracted from the alpha (α) carbon of the amino acid, leaving a *trans* double bond between the α-β carbons [32,79]. PAL is an enzyme inducible by light and by pathological factors, and studies relating to the response of this enzyme to such stimuli, as well as to the regulation of its activity, have been critically reviewed [16,81].

When TAL activity is absent, *trans-p*-coumaric acid (**23**) is formed from *trans*-cinnamic acid (**22**) via a hydroxylation step catalyzed by cinnamate-4-hydroxylase. This reaction is believed to occur via a NIH (National Institutes of Health) shift [55], a mechanism involving hydride migration from the carbon undergoing oxidation to an adjacent (*ortho*) carbon atom. A microsomal preparation of cinnamate-4-hydroxylase from 7-day old parsley suspension culture cells was shown to be stereospecific for *trans*-cinnamic acid (**22**) [149].

p-Coumarate (**23**) can either be further substituted in the aromatic ring or it can be activated (via hydroxycinnamyl CoA ligase). The product resulting from the latter reaction is an intermediate common to both flavonoid and lignin biosynthesis, and may also be involved in the formation of ester-bound *p*-coumaric acids.

The conversion of *p*-coumaric acid (**23**) to ferulic (**30**) and sinapic (**32**) acids is shown in Figure 3. First, *p*-coumaric acid (**23**) is hydroxylated by a phenolase, affording caffeic acid (**29**). A purified form of this enzyme has been isolated from the leaves of spinach beet (*Beta vulgaris*) [190]. In the presence of S-adenosyl methionine, SAM (**28**), an *O*-methyltransferase (OMT) catalyzes the methylation of caffeic acid (**29**) at the 3-position to give ferulic acid (**30**). An OMT preparation from Japanese black pine (*Pinus thunbergii*) seedlings was shown to specifically methylate lignin precursors at a position *meta* to the three-carbon aliphatic side chain [171]. 5-Hydroxyferulic acid (**31**) or 3,4,5-trihydroxycinnamic acid (**39**) are poor substrates for this gymnosperm-derived enzyme. On the other hand, OMT preparations from bamboos [172] and more recently, from differentiating xylem of aspen [14], catalyze the methylation of both caffeic (**29**) and 5-hydroxyferulic (**31**) acids. These observations are consistent with the occurrence of guaiacyl and syringyl units in angiosperms, and the predominance of guaiacyl units in gymnosperms, suggesting a possible role for the enzyme in determining the monomeric composition of lignins in plants [98]. By electrophoresis, it appeared that only one enzyme form in bamboo was responsible for the methylation of caffeic (**29**) and 5-hydroxyferulic (**31**) acids to ferulic (**30**) and sinapic (**32**) acids, respectively [172].



(28)

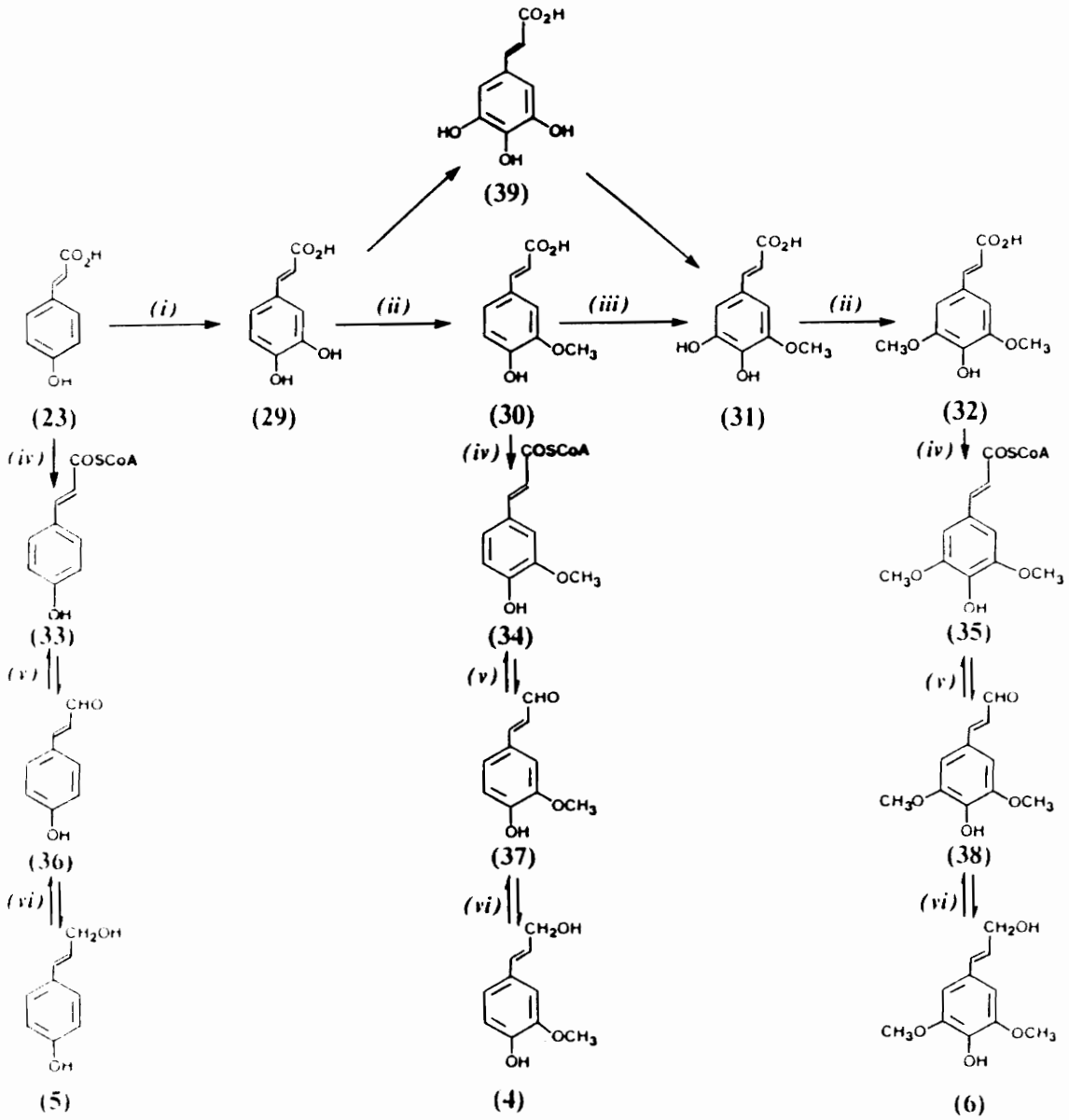


Figure 3. The phenylpropanoid pathway, and biosynthesis of monolignols (4-6): Enzymes: i) phenolase, ii) *O*-methyltransferase, iii) ferulate-5-hydroxylase; iv) hydroxycinnamate-CoA ligase, v) cinnamyl CoA reductase, vi) cinnamyl alcohol dehydrogenase.

It should be self-evident that two routes are possible for the biosynthesis of sinapic acid (**32**) (Figure 3). Its formation could proceed via 1] caffeic (**29**) → ferulic (**30**) → 5-hydroxyferulic (**31**) → sinapic (**32**) acid as discussed; or 2] caffeic (**29**) → 3,4,5-trihydroxycinnamic (**39**) → sinapic (**32**) acid. The likelihood that its biogenesis proceeds via 5-hydroxyferulic acid (**31**) is supported by the isolation of ferulic acid 5-hydroxylase which is preferentially localized in syringyl-rich sclerenchyma cells of poplar stems [57], and the demonstration of the presence of 5-hydroxyferulic acid (**31**) in cell walls of *Zea mays* and *Hordeum vulgare* [145]. Clearly, if sinapic acid (**32**) was formed exclusively by the first route, then the reactions involving ferulic acid 5-hydroxylase and *O*-methyltransferase are important control points for determining lignin composition in most angiosperms.

The reduction of the hydroxycinnamic acids (**23,30,32**) is initiated by an activation step mediated by hydroxycinnamate CoA ligase [63]. The reaction to form the CoA esters (**33-35**) is dependent upon ATP (**26**) and Mg^{+2}, as shown by incubating *p*-coumaric acid (**23**) in cell-free preparations from young stems of *Forsythia sp.* [177]. Interestingly, the sensitivity to light of these enzymes was demonstrated with cell-free preparations from cell suspension cultures of parsley [63]. It remains to be seen whether an increased response to light results in the overall stimulation of the biosynthesis of flavonoids, lignin or both.

3.1.3 Biosynthesis of monolignols from activated cinnamic acids

The cinnamoyl CoA esters (**33-35**) resulting from the ligase reaction are then reduced, in a two-step process, to the cinnamyl alcohols or monolignols (**4-6**) [61,121,191].

Cinnamoyl CoA reductases are responsible for the formation of the respective cinnamaldehydes (**36-38**), in a reversible reaction that requires NADPH (**25b**) as a co-factor. This reductase has been purified from poplar (*Populus* × *euramericana*) [165], together with cinnamyl alcohol dehydrogenase. Cinnamoyl CoA reductase from the stems of this plant showed decreasing affinity towards feruloyl CoA (**34**), sinapoyl CoA (**35**) and *p*-coumaroyl CoA (**33**) [165]. Cinnamyl alcohol dehydrogenase, on the other hand, mediates a reversible reaction which reduces the cinnamaldehydes (**36-38**) to their respective cinnamyl alcohols (**4-6**), and for which NADPH (**25b**) is also a requirement. For purified cinnamyl alcohol dehydrogenase, the best substrate was shown to be coniferaldehyde (**37**). By testing the **A** and **B** forms of NADP-[4-^{3}H(n)], it was determined that the dehydrogenase of *Forsythia suspensa* was of the class **A** type [125]. Recently, the strong substrate preference of cinnamyl alcohol dehydrogenase towards *trans*-coniferyl alcohol (**4**) rather than its *cis* analogue was demonstrated with crude preparations from beech bark [31,110]. This provided additional indirect proof that the *cis*/*trans* isomerization step occurs at the monolignol level. Finally, it may be of interest to mention experiments in which lignification in poplar tissues was apparently reduced by treatment with organic compounds shown to inhibit cinnamyl alcohol dehydrogenase activity [59].

3.1.4 The process of lignification

After nearly a century and a half of investigation, the process of lignification is only now beginning to be understood. Initially, and as a result of the work pioneered by Freudenberg [40], it became widely accepted that lignification in plants could be approximated by a reaction in which the monolignols (**4-6**) are enzymically dehydrogenated

to form mesomeric free radicals which initially couple to form, after rearomatization, dilignols and oligolignols. This reaction sequence, catalyzed by peroxidase, a hydrogen-peroxide requiring enzyme localized in the cell walls [68], continues until a large polymeric network results (Figure 4).

This resulted in a proposal that lignin formation was a statistically random process. Such an explanation accounted for the apparent complexity and lack of order in the structure of isolated lignins, and was favored by many lignin chemists in the 1950's through the 1970's. Since then, however, sophisticated techniques like autoradiography, high resolution electron microscopy and ^{13}C NMR spectroscopy have become available to investigators of lignin biochemistry and structure, such that the picture of native lignin that is now emerging is one of a macromolecule that is more regularly structured than was originally thought [56].

With regard to differences in monomeric composition among classes of plants (e.g., hardwoods vs. softwoods), it seems that the absence of some enzymes, like ferulic acid 5-hydroxylase in some gymnosperms, as well as the substrate specificity of *O*-methyltransferases, cinnamoyl CoA reductases and cinnamyl alcohol dehydrogenases (see discussion in the preceding section), play a significant part in determining which monomers end up in lignin that is deposited at a given lignification site. Likewise, different tissues and subcellular regions of the cell wall, even in the same plant, have different monomeric compositions, suggesting, among others, differences in enzyme localization during the different stages of lignification. Unfortunately, the paucity of information on the localization of the enzymes, the transport of the precursors into the lignifying site, and the mechanisms that are at play in determining which precursors are deposited at specific locations at a particular time or developmental phase impede our

progress towards a better understanding of these differences. In this regard, efforts now being expended to determine the subcellular composition of lignins are laudable. Along this line, Terashima's results indirectly suggest that different precursors are available to the lignifying tissue at different stages of lignification. For instance, they have found that in *Magnolia kobus* [182], guaiacyl moieties are incorporated into the cell corners and the middle lamella during the early stages of xylem differentiation, while the syringyl units are deposited towards the middle stage into the secondary wall. In a separate experiment in which labelled *p*-glucocoumaryl alcohol was used as precursor, it was found that *p*-hydroxyphenyl lignin was deposited mainly in the middle lamella, and only during the early phase of lignification [181].

Two methods of obtaining artificial lignins by *in vitro* synthesis - **Zulaufverfahren** and **Zutropverfahren**, result in what are called dehydrogenatively polymerized (DHP) lignins which can be distinguished by their bonding patterns [138]. **Zulaufverfahren** is a method in which the hydroxycinnamyl alcohol precursors are added to a reaction mixture containing peroxidase and hydrogen peroxide all at one time, resulting in bulk polymerization and considerable amounts of C-5 aryl-linked substructures [75,161]. On the other hand, by **Zutropverfahren**, the solutions of hydroxycinnamyl alcohol and hydrogen peroxide are delivered dropwise into the reaction mixture over an extended period of time, resulting in end-wise polymerization, and an increase in β-O-4′ coupling between the monomeric units. The dominance of β-O-4′ linkages over other modes of linkage has been demonstrated by chemical analysis following degradation of the polymeric product [75,161], but such dominance could not be observed by ^{13}C NMR spectroscopy [111].

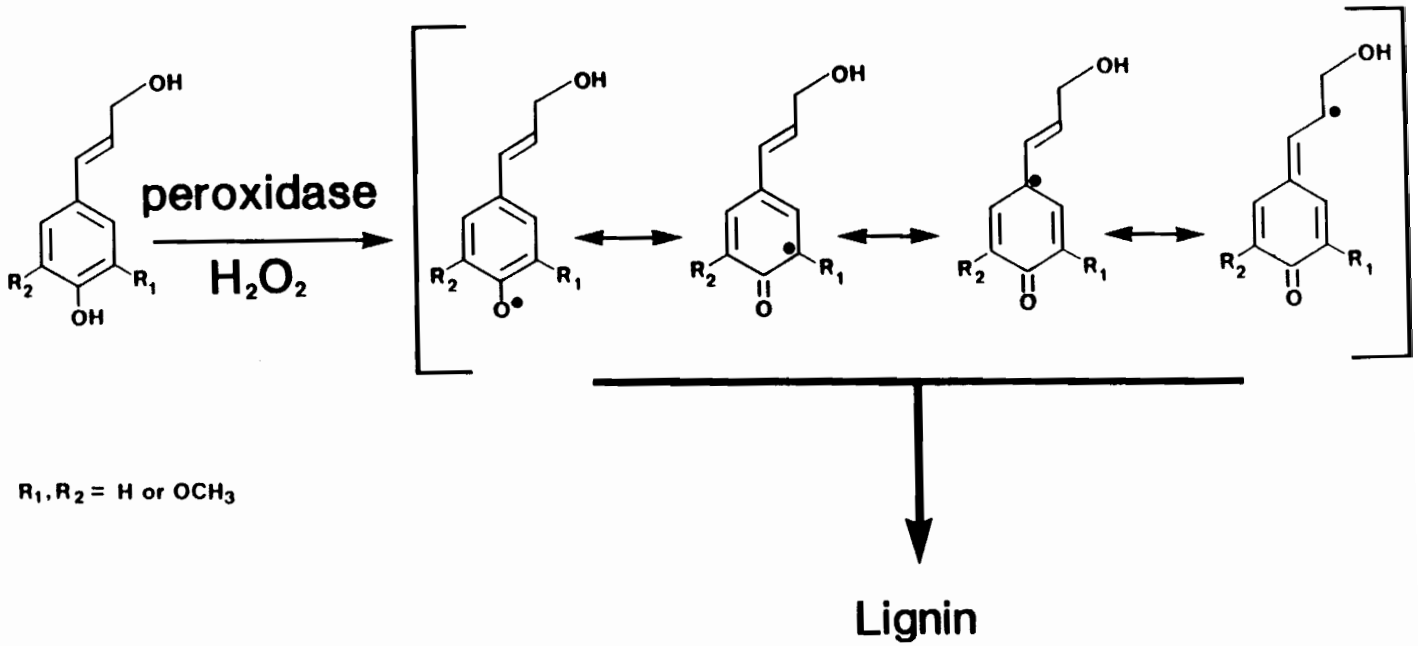


Figure 4. Dehydrogenative polymerization of monolignols (**4-6**).

Thus, in plants grown under "normal" conditions, it is reasonable to speculate that a steady flow of precursors to the site of lignification would result in coupling reactions that preferentially give rise to the β-O-4′ substructures. In *Thuja orientalis* and *Metasequoia glyptostroboides* compression wood (an abnormality that develops in lower parts of leaning branches or stems of softwoods, presumably as a response to gravity), the activities of four enzymes related to lignin biosynthesis (phenylalanine ammonia lyase, caffeate-3-*O*-methyltransferase, hydroxycinnamate CoA ligase and cinnamyl alcohol dehydrogenase) were higher than in the opposite, normal section of the stem [101]. These observations could partly explain the higher lignin content of compression wood than normal wood. However, the rationale for the high occurrence of *p*-hydroxyphenyl units, which results in low methoxyl content and high frequency of C-5 aryl-linked substructures in compression wood lignin, is not known. It has also been postulated that increased lignification in compression wood is triggered by high hormone levels, but the exact manner by which this is effected is not understood.

Some other factors appear to influence lignification. These include external factors such as light [58] and temperature [186,187], as well as internal influences such as pH variation [186] and Ca^{+2} concentration [192]. However, the precise mechanism by which these factors affect lignin formation has not been fully explained.

At this point, it is of interest to mention observations about lignification in tissues that are under stress, either by mechanical or chemical injury, by fungal attack, or by a combination of these agents. The common thread is that in stressed tissues, activities of enzymes of the phenylpropanoid pathway, like PAL and hydroxycinnamyl CoA ligase, increase, and consequently, phenolic compounds accumulate in the affected tissue [152,153]. From evidence obtained by histochemical staining and spectroscopic

techniques, it was concluded that lignin was among those phenolics whose synthesis was induced. It was also noted that the lignin formed was richer in *p*-hydroxyphenylpropane (1) content than that present in "normal" tissue. In future experiments, it will be of interest to determine how the inter-unit linkages between the monomers of lignin formed in the injured tissue differ from that of "normal" wood.

3.1.5 The determination of the structure of lignin

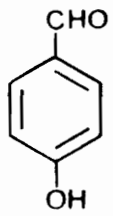
The structure of most natural plant products has been established following solvent extraction of plant material and standard purification and identification procedures. Unfortunately, this approach does not work for lignin, not only because it is insoluble in most solvents, but also because it is associated with the carbohydrates in the cell walls by what are now believed to be covalent linkages. More vigorous conditions for severing these covalent bonds and for breaking down the macromolecule (so it can diffuse out) are required, which alter the structure of lignin. In addition, the difficulty in obtaining identical lignin preparations from different plants, or between tissues of the same plant, is aggravated by the inherent variability in the size and composition of lignins from different origins [139]. Thus, there is no universal method for isolating lignin from plants nor can it be obtained in an unmodified form. Some of the current techniques used for isolating lignin from plants, and their advantages and drawbacks, have been summarized recently [141].

At least three strategies to establish the structure of lignins have been examined. The first, and possibly oldest strategy was to obtain lignin "preparations" from finely-ground plant tissue by extraction, generally with organic solvents, e.g. dioxane or ethanol,

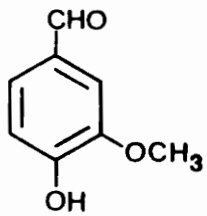
containing hydrolytic amounts of water or acid. The analytical techniques used to characterize lignin isolated from the plant tissue included the determination of functional groups and/or reactivity [83,126], and analyses of isolated lignin or its derivatives by spectroscopic methods like UV-, [9,54,80,127] IR-, mass-, and NMR spectroscopy [1,42,136]. In addition, the lignin preparation was subjected to chemical degradation, and then attempts to piece together the structure of lignin from the structures of the monolignols, dimers and oligomers obtained as degradation products were made [135]. This strategy suffers from three limitations: one, lack of completeness of the isolation procedure resulting in poor yields of lignin; two, modification of the structure of lignin not only by the extraction procedure itself, but also by the methods of degradation employed; and three, poor recovery of lignin fragments. The "weighting" of the degradation products, i.e., the determination of how much contribution each makes to the original lignin structure, could also be a source of error in arriving at an approximate structure of lignin.

The estimation of the monomeric composition of lignin has been almost routinely carried out by oxidative methods like nitrobenzene oxidation, permanganate oxidation of methylated samples, and more recently, thioacidolysis, of isolated lignins or the plant tissue itself [94,115]. The degradation products so obtained, and their derivatives, were then analyzed by gas chromatography or by high performance liquid chromatography, and in the case of radiolabelled materials, by autoradiography [183]. Analysis of the nitrobenzene oxidation products of lignin in softwoods established the predominance of the guaiacyl moiety in the lignin of these plants, while syringaldehyde (**42**) and vanillin (**41**), corresponding to syringyl and guaiacyl residues, respectively, were detected from those of hardwoods. These findings were corroborated by results obtained with the other methods. Simplicity and straightforwardness of the techniques appeal to many,

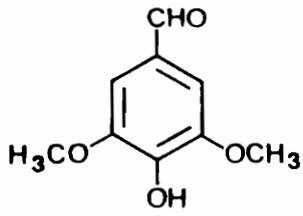
despite the disadvantage of obtaining degradation products only in qualitative yields. Analysis of monomeric composition by alkali nitrobenzene oxidation of hardwood lignin may also overestimate syringyl content since syringyl units are not involved in C-5 aryl-linked structures (position 5 in the aromatic ring of syringyl moiety is substituted with a methoxyl group) [162].



(40)



(41)



(42)

The second strategy involves simulation *in vitro* of the lignification process that occurs in the tissue, thereby obtaining a polymeric product which can then be characterized, again by degradative techniques, or by spectroscopic methods [111]. While artificial lignin prepared this way was originally thought to represent lignin *in situ*, among its drawbacks is that the natural lignification process being mimicked is, by itself, not fully understood. Thus, it is difficult to evaluate how closely the *in vitro* process approximates that which occurs in the tissue, especially in terms of the availability of peroxidase and the precursors, and in terms of the role that the cell wall matrix plays in the process (*vis a vis* formation of covalent linkages between lignin and the carbohydrates).

The third strategy, along which this work was carried out, involves determination of the structure of lignin without isolation from the tissue. A number of specialized methods have been developed and used towards this end, e.g., ultraviolet spectroscopy with or without transmission electron microscopy and energy dispersive X-ray techniques (TEM-EDXA) [38,39,156,157], autoradiography [183], and solid-state ^{13}C NMR spectroscopy [112,114,116,117]. Some of these techniques could only provide estimates of the relative amounts of the monolignols that comprise the lignin in the tissue and so only very little detailed information could be obtained. Also to some extent, mechanical/chemical treatments were needed to obtain specimens which lend themselves to some of these analyses. Despite the limitations mentioned above, there is no doubt that these strategies generated valuable information that has advanced our understanding of lignin structure and biosynthesis.

The development of a technique which now combines UV spectroscopy and transmission electron microscopy with energy dispersive X-ray analysis [156] of brominated wood evolved from interests in the cellular distribution of guaiacyl and syringyl units in the xylem of hardwoods. This technique was also claimed to have circumvented the problem of variations in syringyl to guaiacyl ratios depending upon the method used to isolate lignin. Lignins in the secondary walls of different cell types in white birch (*Betula papyrifera*) were shown to differ in monomeric composition. Vessels were found to be richer in guaiacyl units, while fibers and ray cells had higher syringyl contents. These results were supported by Hardell *et al.* [67] who determined by alkaline nitrobenzene oxidation the monomeric composition of fractionated xylem elements of *Betula verrucosa*, and by tracer experiments carried out by Terashima *et al.* [183] on poplar wood. Moreover, the monomeric units in the middle lamella containing the highest concentration of lignin, were found to be dependent upon the type of cells with which

they were associated. The biogenetic significance of these findings, in terms of monolignol transport and cellular control of lignification, have been discussed [51].

Another interesting development along the line of *in situ* lignin characterization is Raman spectroscopy [3]. Its application to lignin *in situ* is still very much in the exploratory phase, but it is intriguing that initial findings with lignin in the secondary wall of earlywood black spruce (*Picea mariana*) tissue, i.e., the preferential parallel orientation of the aromatic rings to the cell wall surface, were interpreted as indicative of a high degree of organization of the molecular structure of lignin *in situ*. It will be interesting to learn what further improvements in our understanding of lignin in plant tissue can be obtained from this technique.

3.1.6 Applications of ^{13}C nuclear magnetic resonance (NMR) spectroscopy in lignin studies

Like proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy can be used in determining the structure of organic compounds based on the ability of atomic nuclei, in this case, the carbon-13 isotope, to interact with electromagnetic radiation in the radio frequency (10^{6} to 10^{10} Hz) range. The interaction takes the form of a transfer of energy between the photons, or particles of radiation, and the ^{13}C nuclei oscillating in a uniform molecular motion at a frequency that matches that of the incident radiation. Absorption of radiation takes place under this condition, which is also referred to as resonance.

Of the three isotopes of carbon, ^{13}C is the one detectable by NMR because it is the isotope that possesses a nonzero nuclear spin ($I = ½$). Consequently, it has 2 spin states which separate, when placed in a magnetic field, according to energy differences determined by the direction of their respective magnetic moments. These nuclear magnetic moments precess at a frequency which is characteristic of the nucleus. Net absorption of radiation occurs at resonance, provided there is an excess of particles in the lower energy state than in the upper state. Such absorption is observed as a NMR signal, also more conveniently defined as a chemical shift (δ) and expressed as ppm.

For ^{13}C NMR spectroscopy, the chemical shift range is up to 250 ppm, over 20 times wider than ^{1}H NMR. In terms of resolution, this large range is often an advantage of ^{13}C NMR over ^{1}H NMR. The sensitivity of ^{13}C NMR, however, is poor compared to ^{1}H NMR because carbon-13 has a very low natural abundance (1.1%). In the past, this limitation was largely offset by using large sample size and/or long scan times. Improvements in data acquisition and enrichment, with ^{13}C, of specific carbons in the molecule of interest, have greatly enhanced the power of this technique.

^{13}C NMR spectroscopy is useful for proposing connectivities between carbon atoms in complex polymers, including lignin. It was first used by Ludeman and Nimz [119] to formulate proposed structures for beech and spruce lignins, which they originally developed from analyses of lignin degradation products [135]. Assignments in the ^{13}C NMR spectra of Bjorkman milled wood lignins (MWL) from these species were based on the chemical shifts obtained with dimeric and monomeric "lignin model compounds", mostly consisting of 4-hydroxyalkylbenzene derivatives containing substituents "typical" of structural units that make up the lignin macromolecule [120,135]. Subsequent applications of this technique were carried out on various DHP lignin preparations [138], and

its acetylated derivatives, the latter proving useful for estimating hydroxyl contents of lignins [137]. Thereafter, a report [43] came out describing the synthesis of DHP lignin from coniferyl alcohol (**4**) specifically-labelled at the benzylic (α) carbon, and its subsequent analysis by solution-state ^{13}C NMR spectroscopy. As a result, chemical shifts were observed and tentatively rationalized as due to the involvement of the alpha carbon (Cα) in bonding environments such as the *β-β′*, *β-5′*, *β*-O-4′ substructures, as well as a vinylic carbon in coniferyl alcohol (**4**) and cinnamaldehyde moieties.

Subsequent studies on lignin from various plants employed ^{13}C NMR spectroscopy for characterizing lignin isolates and in evaluating procedures for their isolation, for ascertaining the presence of lignin in intact plants or tissue cultures, and more ambitiously, for quantifying lignin contents in those tissues. This technique found an early application in the characterization of milled wood lignin isolated from soybean cell cultures [136]. In addition to guaiacyl resonances, signals for syringyl units (not detected by parallel thermofractography experiments on the isolated lignin preparation) and *p*-hydroxyphenyl groups were observed in the ^{13}C NMR spectrum, and on this basis, lignin from soybean cell cultures was classified as "hardwood lignin". However, similar studies with milled wood lignin from *Robinia pseudoacacia* [41] and *Rosa glauca* [154] callus cultures pointed to differences between intact plant lignin and callus lignin, not only in monomeric composition, but also in inter-unit linkages [41]. Previous observations regarding lignin heterogeneity in various morphological regions of the cell wall (see preceding section) gained some support with ^{13}C NMR spectroscopic results obtained for *Betula ermanii* [17] dioxane lignin and for *Populus trichocarpa* dioxane and cellulase-liberated lignins [103]. In these experiments, it was suggested that the respective isolation procedures employed resulted in distinct lignin fractions originating

from specific locations in the tissue (e.g., that cellulase-liberated lignin is derived from the cell wall, [103]).

In an effort to obtain ^{13}C-labelled lignin fractions suitable for ^{13}C NMR resolution enhancement and editing experiments, poplar cuttings were grown for three months in an environment with air containing 20% $^{13}CO_2$ [104]. Following isolation of the ^{13}C-enriched lignin fractions, solution- state ^{13}C NMR spectra were obtained, and comparisons between the enhanced spectra of the various fractions revealed differences which the authors ascribed to lignin heterogeneity. Apparently, it was assumed that all the carbon atoms in the lignin were uniformly enriched, although no mention was made of the efficiency of labelling.

^{13}C NMR spectroscopy was also employed to determine structural changes in lignin during chemical pulping processes and/or during its degradation by microbes. For instance, lignins obtained at various stages during the kraft pulping of spruce were analyzed by ^{13}C NMR spectroscopy, and among the more definitive results was the observed reduction, with time, in the amounts of intact β-aryl structures in the lignin [97]. Various lignin preparations from spruce were subjected to the lignin-degrading fungus, *Phanerochaete chrysosporium*, and based on the analysis by ^{13}C NMR of the degraded lignins, degradative mechanisms involving Cα-Cβ side chain cleavage, Cα-oxidation, aromatic ring cleavage, and possibly reduction, were proposed [19]. Similar mechanisms were noted with degradative studies of DHP lignin synthesized from specifically-enriched [^{13}C] coniferyl alcohol (**4**) incubated with the following organisms: *Pleurotus ostreatus*, *Chaetomium piluliforum* and *Nocardia autotrophica* [33].

In recent years, solid-state ^{13}C NMR spectroscopy has received considerable attention as a tool useful for the structural elucidation of lignin. The analysis of solid samples,

in general, arose 1) from the need to examine the molecular structure of samples which cannot be dissolved by any solvent; and 2) because of the need to preserve certain structural features which, otherwise could be lost or modified by sample dissolution [123].

Unlike solution-state ^{13}C NMR spectroscopy, spectral lines obtained by solid-state ^{13}C NMR are broader due to chemical shift anisotropy. This effect, described by Macomber [123] as originating from the "range of local environments for each set of nuclei and a range of orientations with respect to the applied magnetic field", and the slow relaxation time in solid samples, combine to give very low resolution in solid-state ^{13}C NMR spectroscopy. Fortunately, the method of cross polarization (**CP**) and magic angle spinning (**MAS**) have been developed to partially offset these limitations. Cross polarization (CP), based on the phenomenon where polarization of one set of nuclear spin states causes polarization in another set of nuclei [123], results in higher signal to noise (S/N) ratios and shorter experiment times. Magic angle spinning (MAS), on the other hand, "averages out" chemical shift anisotropy by spinning the sample about the magic angle (54^0 44'), which is the angle between the spinning axis and the magnetic field vector. Together, CP/MAS result in better resolved solid-state ^{13}C NMR spectral lines.

Cellulose was the first polymeric constituent of plant tissue studied by solid-state ^{13}C NMR [4,5,30], with results adding fuel to the debate regarding the true nature and origin of the crystalline structure of the various forms of native cellulose. When whole wood was analyzed by this technique [74,95,180], dominant resonances observed were due to the polysaccharides present, i.e., cellulose and hemicelluloses. Some discernible signals of solid wood indicative of lignin include those for the aromatic carbons

(~110-160 ppm) and that for the methoxyl substituent (~56 ppm). Chemical shifts for the aliphatic carbons in the propyl side chain (~50-90 ppm) , were masked by the signals for the carbon atoms of the carbohydrates. Despite signal overlaps, recent improvements in techniques/instrumentation are increasing the utility of solid-state ^{13}C NMR of solid wood *per se*. For instance, pulse sequence techniques provided useful information for estimating spatial relationships between the different chemical entities in plant cell walls [132].

The subject of lignin-carbohydrate linkages has also been examined using solid-state ^{13}C NMR spectroscopy. Their existence was indirectly proven by ^{13}C NMR spectra showing the presence of carbohydrate signals in a lignin preparation or of lignin signals in isolated carbohydrates [95]. Additional evidence for the lignin-carbohydrate species in plant tissues were obtained by employing relaxation techniques with ^{13}C CP/MAS NMR experiments on cellulase-treated *Picea glauca* pulp [48]. However, the exact nature of the linkages, e.g., which atoms were involved in the lignin-carbohydrate bond, were not described. Furthermore, because of the physicochemical treatment undergone by the *P. glauca* pulp prior to analysis, the possibility that some structural modifications of the wood cell wall components took place could not be ruled out.

Solid-state ^{13}C NMR studies of spruce lignin prepared by various isolation procedures (dioxane-, milled wood, cuoxam, and periodate lignins) confirmed the long-held observation that different methods do differ in the "changes" they impart upon the lignin preparation [8]. However, since the exact nature of lignin *in situ* is not known, there was no basis for determining the severity by which these isolation procedures affected native lignin structure.

The use of solid-state ^{13}C NMR spectroscopy was likewise explored for quantitative measurements [73], but it was obvious that additional technical improvements were needed to obtain more reliable quantitation of the different functionalities. Interestingly, delayed proton decoupling could be a step in that direction; using this technique, estimates of syringyl to guaiacyl (S/G) ratios obtained for seven hardwoods were comparable with results from methoxyl content/elemental composition determinations [124].

Aspen (*Populus* × *euramericana*) wood, grown under 10% and 20% $^{13}CO_2$ atmosphere, and lignin isolated from the wood by the MWL procedure, were analyzed by solid-state ^{13}C NMR spectroscopy with CP/MAS [7]. The solid-state ^{13}C NMR spectrum of the ^{13}C-enriched wood, by itself, did not reveal any significant differences from wood grown under natural conditions. Consequently, lignin in the enriched tissue had to be isolated in order to take advantage of the enrichment procedure in establishing connectivities between its carbon atoms. However, given the reservations about the procedures for isolating, without modification, lignin from plant tissues, it is apparent that more rational approaches are required to obtain more meaningful information from the solid-state ^{13}C NMR spectra of intact wood. Fortunately, one such approach had already been developed, which involved a procedure for selectively labelling specific carbon atoms of lignin, followed by analysis of the tissue by solid-state ^{13}C NMR spectroscopy [112,113,116].

DHP lignins, synthesized under optimized conditions from coniferyl alcohol specifically-enriched with ^{13}C at either the 1-, 2-, 3-, or 1,2-positions of the propyl side chain (**4a-c**), were analyzed by solid-state ^{13}C NMR spectroscopy [111]. Difference spectra of these DHP samples (obtained by subtracting from the enriched sample, resonances of the natural abundance sample) were compared with the corresponding

solution-state spectrum. As expected, the latter had better resolved peaks, in contrast with the broader peaks of the solid-state spectra. Peak assignments were made in accordance with literature values obtained for "lignin model compounds". It was encouraging to find that those peaks, albeit broad, corresponded to those in the solution-state spectra.

Additionally, the power of specifically-labelling carbon atoms in lignin was demonstrated with a clever experiment in which the [1-^{13}C]-enriched DHP lignin was blended with cellulose, in an attempt to simulate lignified plant tissue [111]. This clearly showed that similar labelling of specific carbon atoms of lignin in the plant at 1-2% incorporation rates, could lead to the determination of the dominant bonding patterns without *a priori* lignin isolation.

The first attempt to label specific carbon atoms of lignin in plant tissue with ^{13}C was successfully carried out with wheat (*Triticum aestivum*) [116]. In this experiment, wheat was grown for 24 days on agar medium containing either [1-^{13}C], [2-^{13}C], or [3-^{13}C] ferulic acid [**30a-c**] under aseptic conditions. The solid-state ^{13}C NMR spectra of the roots were taken, and after subtracting the natural abundance spectra, difference spectra showing the enhanced resonances corresponding to the bonding environments of the specifically-enriched carbon atoms were obtained. Comparison with the solid-state ^{13}C NMR spectra of ^{13}C-enriched DHP lignins showed marked differences in bonding patterns between wheat lignin *in situ* and artificial lignin. Subsequently, acetal lignin was isolated from the enriched tissues and its solution-state ^{13}C NMR spectra showed correspondence of the enhanced resonances with those of the lignin in the intact tissue [31]. The present work demonstrates the efficacy of this methodology with other plants.

Interestingly, significant differences in bonding patterns of lignin *in situ* among the plants investigated, as well as of DHP lignin, have been obtained [112-114].

Without doubt, probing the structure of lignin *in situ* could now be accomplished by solid-state ^{13}C NMR spectroscopy of tissues enriched with specifically-labelled precursors. Two applications immediately come to mind: First, it is now possible to monitor changes in the structure of lignin *in situ* during chemical pulping; and second, the action of degradative enzymes on lignin *in situ* could now be determined. The significance of experiments of this nature, in terms of providing valuable insights into the mechanisms of these processes, cannot be overemphasized.

Other polymeric plant constituents have been analyzed by solid-state ^{13}C NMR spectroscopy. These include cutin, the structural polymer of plant cuticle, and suberin, a protective polymer in bark and underground organs, both of which serve as barriers against moisture loss [46,176]. Solid-state ^{13}C NMR experiments with lime cuticles [176] and suberized potato cell walls [46] verified the polyester nature of these protective plant components, as well as the nature of the chemical bonds between the aromatic and the high molecular weight, aliphatic constituents of these polymers. The authors were optimistic that this procedure will be invaluable in gaining a better understanding of the biogenesis of these natural polymers.

3.2 *Lignin Biodegradation*

When plants die, their component organic matter, primarily consisting of polysaccharides and lignin, are cycled back into the available carbon pool of the biosphere as carbon dioxide (CO_2). Biotic factors, i.e., microorganisms, are presumably responsible for this transformation, while abiotic factors (e.g., heat, moisture and light) are assumed to play a secondary role.

Some biodegradative microorganisms are thought to be equipped with a biochemical machinery that enables them to attack and disintegrate the polymers that make up plant tissue cell walls and eventually metabolize the resulting fragments. Over the last two decades, some progress has been made in understanding the phenomenon involved in the biological degradation of plant organic matter. This has been especially true for the isolation and identification of enzymes/enzyme systems (e.g., cellulases) capable of using the polysaccharides in plants as substrates. The same cannot be said of the degradation of lignins, where the best candidate so far proposed, lignin peroxidase = ligninase, has produced conflicting results with respect to its net effect on lignins, since the first two reports of its isolation from the white-rot fungus, *Phanerochaete chrysosporium* [52,184].

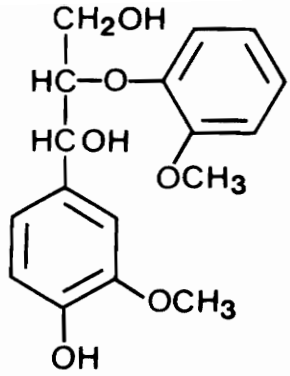
3.2.1 Microorganisms that degrade lignin

Two groups of microorganisms, bacteria and fungi, possess lignin degrading abilities [105,107] to varying extents. Fungi, particularly those responsible for white-rot in wood, are probably the more efficient lignin-degraders [93]. Bacteria, on the other hand, by

their sheer volume in the soil [18], may contribute substantially to the overall breakdown of lignin. However, this is disputed by some authors [91,107] who consider results of bacterial degradation of lignin minimal when compared with that of fungal degradation.

3.2.1.1 Bacteria.

A number of bacterial organisms [144,160] or their mixtures were tested for their ability to metabolize/degrade lignin model substrates and lignin in wood [134]. For example, investigations with *Pseudomonas paucimobilis* showed that guaiacylglycerol-β-guaiacyl ether (**43**) could be degraded by bacteria [159]. Following degradation experiments with mutant strains, a pathway involving formation of α-carbonyl structures prior to cleavage of β-ether linkage was proposed [158]. Another species, *Pseudomonas cepacia*, was also reported to degrade several dimeric, phenolic lignin models [144]. Recently, lignostilbene-α,β-dioxygenase was isolated from cell-free extracts of *Pseudomonas sp.* TMY 1009 which could account for the observed cleavage of interphenyl double bond of lignin-derived stilbenes [86]. By mixing bacterial cultures, the ability to degrade lignin model compounds was improved [148]. The difficulty of studying such a complex system is readily apparent, although it could be a more realistic representation of what is occurring in the natural environment. Recently, a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, was found to grow in medium supplemented with lignosulfonate, which the organism reportedly used as a primary source of sulfate [196]. The authors argued that for anaerobic organisms, degradation of substrate cannot always be measured by CO_2 production. This observation could apply to other lignin-decomposing systems which derive only part of their energy from carbon oxidation. Therefore, it is not usually wise to simply look at CO_2 evolution to assess lignin degrading abilities of these microorganisms.



(43)

3.2.1.2 Fungi.

Fungi that degrade wood are classified as either brown-rot, white-rot, or soft-rot fungi. Of these, the ones that do most extensive degradation on lignin in wood are the white-rots, which leave a pale, whitish residue, consisting largely of partially-degraded polysaccharide fraction. The breadth of lignin-degrading, white-rot fungi has been described in a number of review articles [22,105,107]. In recent years, owing in part to the reported discovery of extracellular, lignin-degrading peroxidases from *P. chrysosporium* [52,184], this organism has become the most widely-studied, with investigations ranging from optimizing physiological growth requirements to cloning the genes for the enzyme which, allegedly, comprise the bulk of its lignin-degrading system [93].

Among the fungi more recently shown to possess ligninolytic activities are *Lentinus edodes* [106], *Aspergillus fumigatus* [85], *Trichoderma harzianum* [88], *Chrysonilia sitophila* [29], *Phlebia radiata* [133] and *Coriolus versicolor* (= *Trametes versicolor* = *Polyporus*

versicolor) [27,34], with detection of lignin peroxidase activity only reported in the latter three. This is an important observation because it implies that lignin peroxidases may not be essential for degradation of lignin by these fungi. The extent of degradation of lignins by these species varies and is affected by the *in vitro* culture conditions of the fungus such as concentration of nutrients, presence or absence of other carbon sources, stationary versus shaken cultures, etc.

3.2.2 Mechanisms for the "biodegradation" of lignin

Because of their temporal and spatial correlation with ligninolytic activity in various fungi, the phenol oxidases - laccase and peroxidase, were initially viewed to be involved in lignin biodegradation. As a result, since the 1960's and the 1970's, enzymatic investigations of microbial degradation of lignins focussed on laccase [49,87,140] and peroxidase/H_2O_2 systems [13,49,87,168,195]. Experiments with phenolic lignin model substrates resulted in coupling reactions, while non-phenolic model compounds were hardly affected [49]. No depolymerization of DHP lignin treated with horseradish peroxidase and with laccase isolated from *C. versicolor* was observed [87]. Additionally, when milled wood lignin was used as substrate, polymerization apparently resulted [195]. It is, therefore, evident that early studies failed to demonstrate lignin degradation by HRP/H_2O_2 or laccase. Instead, coupling and polymerization reactions, opposite to those desired, were observed. This led to the conclusion that peroxidase and laccase, through the oxidative coupling reactions they catalyze, may precede the sequence of biochemical events resulting in the actual lignin breakdown by other (yet unknown) enzymes [49]. Indeed, both laccase and peroxidase have been ascribed the role of detoxifying low-molecular weight phenolic compounds, whose actions helped "maintain

metabolic balance around the fungi" [49]. As a result, interest in the lignin-degrading abilities of these enzymes waned.

For some time, the role of activated oxygen species in lignin biodegradation attracted the attention of several researchers [64]. Only the hydroxy (•OH) radicals (generated by Fenton's reagent) was shown to have a measurable effect on lignin depolymerization [99]. Recently, cytochemical staining and microscopic analysis of •OH-treated wood revealed patterns of degradation similar to those of fungal-treated wood [82]. It was suggested that •OH radicals could play a minor role in degradation, perhaps in propagating degradative reactions initiated by the enzyme system released by the microorganism.

In 1983, two independent groups of researchers [52,184] reported their findings, then heralded as the long-awaited breakthrough in the search for the elusive lignin-degrading enzyme. Tien and Kirk [184,185] claimed that a H_2O_2-requiring oxygenase from the extracellular fluid of *P. chrysosporium* cultures could degrade lignin substructure model compounds as well as spruce and birch "lignins". Glenn *et al.* [52], on the other hand, reported ~10% degradation of a [^{14}C]-ring labelled lignin by a concentrated extracellular fluid from 6-day old cultures of *P. chrysosporium* and a H_2O_2-generating system (glucose oxidase, 0.02 units mL^{-1}, plus glucose, 3 mM) at 37°.

These two papers were critically reviewed recently [115]. With reference to Tien and Kirk's report [184], questions regarding the appropriateness of a [^{14}C]-methyl iodide-methylated aqueous acetone extract from spruce wood as representative of lignin were raised. In addition, degradative data obtained for the incubation of the two "lignin" substrates (22% and 6% depolymerization rates for spruce and birch "lignins", respectively) were taken to task, citing a report by Faix *et al* [35] in which guaiacyl-syringyl lignin was more rapidly degraded by *P. chrysosporium* than guaiacyl lignin, exactly the

opposite of what was observed. In Glenn *et al*'s article [52], it was regrettable that the all-important gel filtration data showing the degradation of the ^{14}C-ring labelled lignin was not described. It was also noted [115] that since these two reports were published, no further substantiation of an enzyme preparation from *P. chrysosporium* capable of degrading the lignin polymer has been reported.

After the reported discovery of the "lignin-degrading" ability of the oxygenase which they called "ligninase", Tien and Kirk [185] quickly proceeded to purify the enzymes from the extracellular fluid of *P. chrysosporium* cultures with a series of steps which included ultrafiltration, dialysis and ion exchange chromatography. A single band, presumed to be the dominant H8 isozyme separated by HPLC in a subsequent work [92], was obtained by isolectric focussing (pI = 3.5) and sodium dodecyl sulfate-polyacrylalamide gel electrophoresis, SDS-PAGE (MW = 42,000). The enzyme was reported to be a glycoprotein with an unusually low pH optimum (pH 2.5-3.0) [185]. Gold *et al.* [53] proposed the name "diarylpropane oxygenase" for their own purified preparation from the white-rot fungus.

Next, the heme portion of "ligninase" was shown to resemble that of peroxidase, revealing the true nature of the enzyme [93]. The multiplicity of "lignin peroxidases" was also shown, with reports ranging from 3 [151] to 15 [109] distinguishable heme-containing species separated from the extracellular fluid of *P. chrysosporium.* Manganese peroxidase, another peroxide-dependent enzyme, was also found in the extracellular medium of ligninolytic cultures of *P. chrysosporium* [102]. Unlike "lignin peroxidase", this enzyme was reported to require Mn^{+2} to complete its catalytic cyle [146]; a role for generating H_2O_2 was also suggested.

Various monomeric and dimeric model compounds were incubated with the purified enzyme fraction (presumably the H8 isozyme detected in later studies) in the presence of H_2O_2 [185]. By determining the structures of the "major" products formed, reaction mechanisms including Cα-Cβ cleavage, benzylic alcohol oxidation, radical coupling of oxidized phenols, and hydroxylation of methylene groups, were proposed. H_2O_2 was definitely a requirement, but not as an oxygen source. Its involvement was limited to the formation of compound **I**, a 2-electron oxidized species formed in the catalytic cycle of the enzyme, and shown to be entirely analogous to that of horseradish peroxidase (Figure 5). Figure 5 is a modified pictorial summary of the catalytic cycle of lignin peroxidase proposed by Kirk [90]. Lignin *per se* has not been unambiguously shown to be a substrate for the enzyme. Compound **I** oxidizes aromatic substrates, resulting in the formation of compound **II** (the 1-electron oxidized form), which reverts to the native state in the presence of excess aromatic substrates.

It is notable that the compound **I** form of "lignin peroxidase" can oxidize substrates with higher redox potential than the substrates of horseradish peroxidase [65]. Oxidation by compound **I** of non-phenolic substrates leads to abstraction of one electron from the aromatic nucleus producing an unstable radical cation [89]. It was then postulated that the resulting radical cation generated from non-phenolic substrates was an intermediate for a plethora of reactions which include Cα-Cβ cleavage, Cα-oxidation, intramolecular rearrangements, demethoxylation and aromatic ring cleavage. These reactions are no longer enzyme-mediated and proceed remote from the enzyme. To some extent, modes of side chain or ring cleavage reactions are governed by the nature of inter-unit linkages in dimeric models [37] and by the substitution pattern of the aromatic ring [188]. Umezawa [188] presented evidence for the formation of muconate, oxalates and cyclic carbonates of arylglycerol models as possible mechanisms for lignin peroxidase-catalyzed

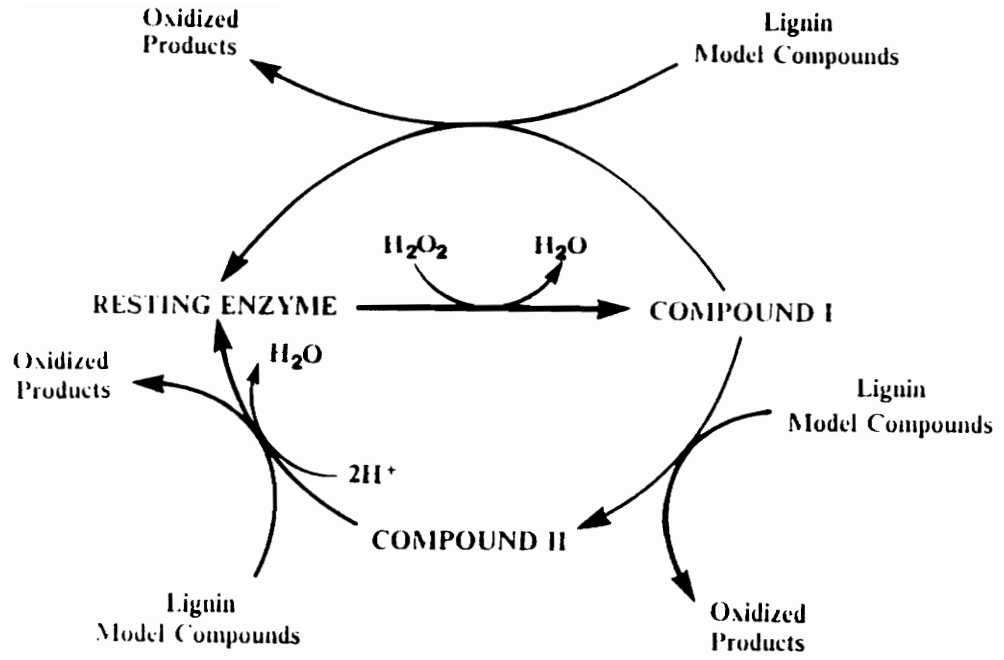
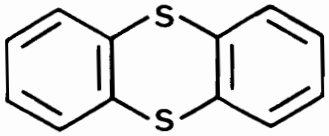


Figure 5. Modified scheme for the catalytic cycle of lignin peroxidase.

ring cleavage reactions. To my knowledge, the formation of radical cation intermediates in the degradation of the lignin polymer itself has not yet been demonstrated. In the case of phenolic substrates, it is noteworthy that products resulting from coupling reactions were observed, possibly via mechanisms involving phenoxy radical intermediates [93]. In fact, the single electron oxidation of phenolic moieties released by homolytic Cα-Cβ cleavage in arylglycerol β-aryl ether model compounds into phenoxy radicals is considered to be a more facile process than forming radical cation intermediates [163]. The consequent polymerization of these phenoxy radicals would be observed as the net effect of lignin peroxidase upon such systems.

Lignin peroxidases are able to catalyze the oxidation of a bewildering array of compounds, including polycyclic aromatic hydrocarbons and dibenzo[*p*]dioxins [65], thianthrene (44), a heterocyclic sulfur compound [169], and polychlorinated phenols [66].

As with the reactions involving lignin model compounds, a radical cation intermediate was proposed for non-phenolic systems. On the other hand, the formation of phenoxy radicals was not ruled out as a mechanism for the oxidation of the chlorophenols since dimeric products were observed [66]. The apparent lack of specificity of this enzyme precludes assignment of a specific biological function. For this reason, the use of "ligninase", (and even lignin peroxidase, 115) is discouraged (Report of the Lignin Research Workshop, University of Illinois, sponsored by the U.S. Department of Energy, Division of Energy Biosciences, [189]).

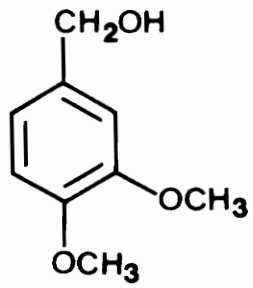


(44)

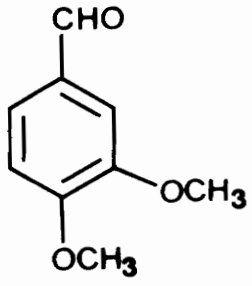
3.2.3 Involvement of an alternative enzyme system in lignin biodegradation

There is a growing body of evidence in the literature for an alternative system responsible for the biodegradation of lignin. It is now clear that isolated lignin peroxidases do not have the same effect on lignins as do intact fungal cultures [189]. Additionally, Leisola and co-workers [108] observed that cultures of *P. chrysosporium* with no detectable lignin peroxidase activity, degraded ^{14}C-labelled straw lignin to some extent. Addition of lignin peroxidase, or of both lignin peroxidase and veratryl alcohol (**45**), enhanced the lignin biodegradation rate [108] of the cultures. The authors also reported the observed reduction of veratraldehyde (**46**) to veratryl alcohol (**45**) by ligninolytic

cultures, pointing to the possibility of involvement of a reductive system. Degradation products indicative of reductive processes were also identified from incubations of ^{13}C-enriched DHP lignins with *Pleurotus ostreatus*, *C. piluliferum* and *N. autotrophica* [33] (see section on ^{13}C NMR applications in lignin studies). So far, of these microorganisms, only *P. ostreatus* was reported to possess lignin peroxidase activity [91], suggesting that a degradative system that does not contain "lignin peroxidase" may be involved in the others. Finally, following experiments in which quinones were reduced by ligninolytic cultures of *P. chrysosporium*, and subsequent partial isolation of the reductases involved, it was suggested that the presence of reductive degradation systems was partly responsible for tipping the degradation-depolymerization balance in favor of the latter [167]. However, cellobiose: quinone oxidoreductase (CBQase) capable of reducing phenoxy radicals had no effect on the rate of lignin degradation [143].



(45)



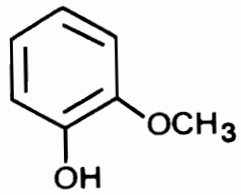
(46)

3.2.4 Role of "lignin peroxidase" in lignin biodegradation

"Lignin peroxidase" has been implicated as the central factor in the degradation of lignin by *P. chrysosporium*. As mentioned earlier, the "evidence" for this dates from 1983 when

the concentrated extracellular fluid of *P. chrysosporium* (presumably containing "lignin peroxidase" as major protein) was shown to partially depolymerize a methylated "lignin" preparation [184]. Observations in which the purified enzyme was shown to degrade "lignin model substrates" were taken as evidence to support that view and have since proliferated.

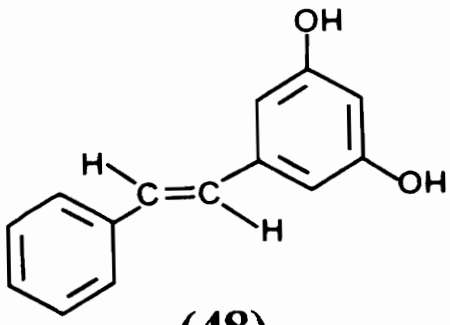
There are two important reasons for the controversy behind the exact role of "lignin peroxidase" in lignin degradation. First, the enzyme has only been shown to have degrading properties *in vitro*. Second, it was shown to cause low molecular weight phenolics (e.g. guaiacol, **47**) to polymerize, and for lignin and lignin derivatives to further increase their molecular size [143].



(47)

There are many indications that lignins attacked by fungi can undergo repolymerization. An early work [118] demonstrated that Na-lignosulfonates treated with immobilized peroxidases isolated from the lignin-degrading fungus, *T. versicolor*, undergo both depolymerization and repolymerization. The net effect observed was an increase in molecular weight [118]. More recently, in an experiment where alkali-isolated straw lignin, HCl-dioxane isolated straw lignin, and spruce milled wood lignin were separately treated with lignin peroxidase isolated from *P. chrysosporium*, all lignins were observed to undergo further polymerization [62]. With these observations, it becomes increas-

ingly appealing to ascribe the role of polymerizing low molecular weight phenolics released during fungal lignin degradation to these lignin peroxidases. Interestingly, "weak polymerization" was viewed to be one of the detoxification mechanisms by which fungi were able to destroy toxin-rich (e.g., pinosylvin, **48**) heartwoods of living trees [122]. In the present work, experiments were designed to determine what effects "lignin peroxidases" have on coniferyl alcohol (**4**), undoubtedly a "lignin model substrate". The results of this investigation may clarify confusions regarding the role of "lignin peroxidases" in the process of lignin biodegradation.



(48)

4.0 EXPERIMENTAL

4.1 General Methods

Thin-layer chromatography was conducted on either silica gel 60 F_{254} aluminum sheets (Merck, Darmstadt, FRG) or Kodak (Rochester, New York) silica gel sheets 13181. High performance liquid chromatography (HPLC) separations were carried out using either one of the two configurations described below: A Waters (Milford, MA) model 721 Programmable System Controller, a model 730 Data Module, 2 model 510 pumps, a WISP model 710B, with either a Lambda-Max model 481 spectrophotometer, or a Waters 990 Photodiode Array detector interfaced with a NEC APC IV personal computer. When the photodiode array detector was used, chromatograms were plotted on a Waters 990 plotter and UV spectra were printed using a NEC Pinwriter CP6.

For all HPLC separations, a Waters Nova-Pak C_{18} (3 μm C_{18}-coated silica particles, 3.9 mm × 15 cm) steel column was used with in-line column protection employing a Waters pre-column filter and guard column packed with Waters Bondapak C_{18}/Corasil

(37 - 50 μm). Aqueous (organic-free water purified by Sybron Barnstead [Boston, MA] NANOpure II purification system) and organic (HPLC-grade) components of the mobile phase were filtered (0.22 or 0.45 μm membrane) and degassed using either a Millipore Durapore (Bedford, MA) filtering system or a Kontes (Vineland, NJ) Ultraware HPLC Mobile phase handling system.

Mass spectral analyses were carried out with a VG 7070 EHF mass spectrometer at 70 eV. Spectrophotometric assays of enzyme activities and ultraviolet spectral analyses were carried out with either a Varian (Palo Alto, CA) Cary 219 UV-visible spectrophotometer or a Perkin Elmer (Norwalk, CT) Lambda 6/PECSS system, which consisted of a Lambda 6 UV/Vis spectrophotometer, a Lambda accessory interface, an Epson Equity I+ personal computer and an Epson EX800 printer. Melting points were determined using a Mel-Temp apparatus (Laboratory Devices, Cambridge, MA).

An LKB Wallac Rackbeta Liquid Scintillation Counter (Turku 10, Finland) was used for liquid scintillation counting. Liquid samples or finely ground, solid radioactive materials were counted in either Aquasol-2 (NEN Research Products, Boston, MA), Ecoscint, or Ecolume (ICN Biomedicals, Inc., Irvine, CA) scintillation cocktails. Most solid samples were combusted in the Packard model B306 Tri-Carb Sample Oxidizer (Downers Grove, IL). The radioactive $^{14}CO_2$ generated by combustion was absorbed with Carbo-sorb and the scintillation cocktail used was Permafluor (Packard Instrument Company, Inc., Downers Grove, IL). Counting efficiencies were determined from quenching curves obtained using either [^{14}C] toluene (ICN Biomedicals, 7.03×10^3 Bq/mL determined on October 14, 1985) or [^{14}C] hexadecane (Amersham, FRG, 1.443×10^4 Bq/mL determined on July 1, 1988).

Solution-state carbon-13 nuclear magnetic resonance (^{13}C-NMR) spectra were recorded with a Bruker WP 270 SY spectrometer. Ferulic acid (**30**), coniferyl alcohol (**4**) and DHP lignin samples were dissolved in deuterated acetone (99.9 atom % D), deuterated chloroform (99.8 atom % D), and deuterated dimethyl sulfoxide (99.9 atom % D), respectively. All deuterated NMR solvents were purchased from MSD Isotopes, Montreal, Canada and were used as internal reference for the chemical shifts of the respective samples analyzed. ^{13}C cross-polarization (CP)-magic angle spinning (MAS) NMR spectra of solid samples were obtained at 50 MHz on a Varian XL-200 spectrometer equipped with a DOTY Scientific MAS Probe. In the CP experiment, the Hartman-Hahn contact time was 1 msec and the γHz field was approximately 50 kHz. Chemical shifts were referenced to the aromatic signal of hexamethyl benzene (38.56 ppm) from an independently obtained spectrum taken under identical conditions. Samples (30 - 80 mg) were packed into fused alumina cylindrical rotors and spun at speeds in excess of 4 kHz. When the sample quantity was low, chromatographic silica gel was added to pack the rotors. Each spectrum was the result of overnight data acquisitions at a pulse repetition rate of 2 seconds and acquisition times of 60 msec with a spectral window of 17 kHz. No spinning side band suppression was used [116].

Prior to use, solvents and reagents were redistilled or recrystallized. ^{13}C-enriched compounds ([1,3-$^{13}C_2$]- and [2-^{13}C] malonic acid, and [1-^{13}C]-, [2-^{13}C]- and [3-^{13}C] phenylalanine, **7a-c**) were purchased from MSD Isotopes, Montreal, Canada. [2-^{14}C] Ferulic acid (**30'**) was previously prepared in our laboratory by Dr. K.P. Dhara. [1-^{13}C]-, [2-^{13}C], and [3-^{13}C] Coniferyl alcohols (**4a-c**) were prepared in our laboratory by Dr. T. Piccariello.

Leucaena leucocephala seeds were received as gifts from Dr. J.L. Brewbaker of the University of Hawaii (3190 Maile Way, Honolulu, HI 96822). Lignin peroxidase from *Phanerochaete chrysosporium* ATCC 34541 was generously provided by Dr. Matti S. A. Leisola of the Finnish Sugar Co. (Kantvik, Finland). Protein (5 mg mL^{-1}) in the preparation was dissolved in sodium acetate buffer (0.4 M, pH 6.4 at 4°) containing veratryl alcohol (**45**) (10 mM) as an enzyme stabilizer.

4.2 Examination of Lignin Structure in situ by Solid-state ^{13}C NMR Spectroscopy

4.2.1 Construction of an aseptic, hydroponic system for administering precursor

Figure 6 shows a schematic representation of the hydroponic unit designed and built for administering natural abundance, radio- or stable-isotope labelled precursors to the growing *L. leucocephala* seedlings under aseptic conditions. The three-level stand was made of 9.5 mm plywood and was designed with a front (middle level) platform on which the culturing chambers stood, while the bottom and top levels (towards the back) held waste and water reservoirs, respectively. The top surface of the box was made of transparent, fiberglass board in order not to interfere with plant illumination.

The culturing chamber was constructed from a 2-quart Mason (Thomas Scientific) glass jar, containing a 125-mL polypropylene (Nalgene) media jar. The media jar was secured

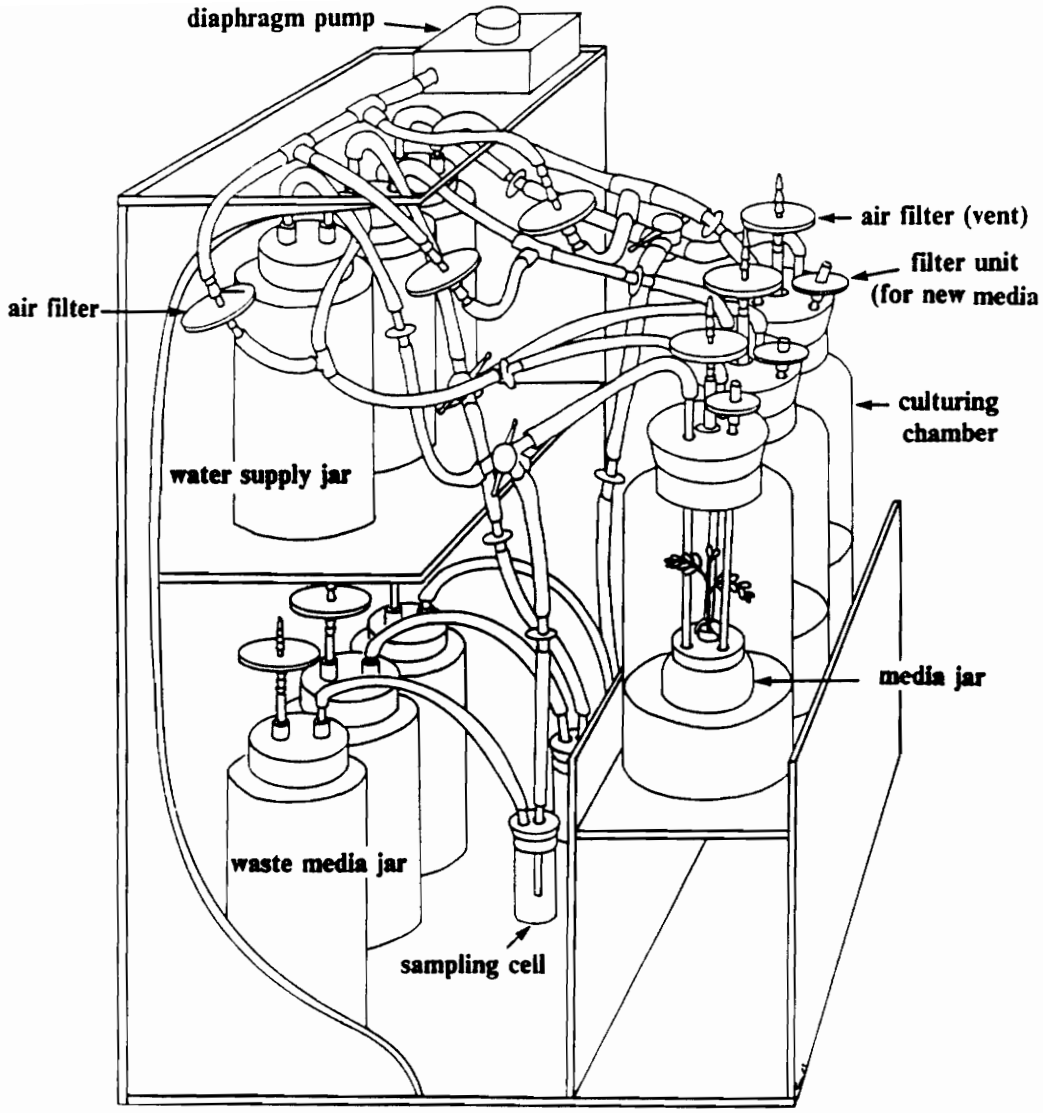


Figure 6. Hydroponic unit for the culture of *L. leucocephala*

at the bottom of the culturing chamber with cellulose sponge having a hole (5 cm) through which the media jar was wedged. The culturing chamber was covered with a No. 13 rubber stopper through which four holes were bored large enough to accommodate Teflon tubing (6.4 mm outer diameter [OD] × 1.2 mm wall thickness [WT]) for (1) aeration, (2) watering, (3) venting, and (4) introduction of fresh, sterile media (4.8 mm OD × 0.8 mm WT), respectively. The end of the aeration tubing submerged in the culture medium was sealed by heating, and slits, 0.5 - 1 cm long, were made with a surgical blade (No. 23, Miltex, Kai, Japan) on the same end for aeration. This end of the tubing was also lightly packed with glass wool to regulate air bubbling.

Water supply and waste media jars were 1 L polypropylene Mason jars (Nalgene) capped with polypropylene fluid-transfer closures (Nalgene). A polypropylene bottle (30 mL, Nalgene), connected to each culturing chamber, was used for obtaining sample aliquots before old media was discarded into the waste jar. The sampling jar was covered with a No. 4 silicone stopper (Thomas) through which two 4.8 mm diameter-holes were bored, one tubing for media coming from the culturing chamber, the other for expulsion of used media into the waste jar.

For aeration, a Whisper 500 aquarium air pump (Willinger Bros., Inc., Englewood, NJ) was connected, the air from which was filtered with Millex-FG_{50} filters (50 mm, 0.2 μm, Millipore). Each culture jar was vented by attaching a Millex filter to the tubing in the center of the stopper, effectively sealing the system from biological contaminants in the surrounding air.

Autoclavable plastic (Nalgene) tubing (4.8 or 6.4 mm internal diameter [ID] × 1.6 mm WT) was used to connect the different components of the system. A 3-way, polypropylene stopcock with a Teflon plug (2 mm bore, Nalgene), connected tubing

from the culture, water supply and sampling jars, respectively. This facilitated both independent watering and media exchange. Polypropylene reducing connectors, (5 mm × 6 mm tubing ID, Cole Parmer, Chicago, IL) were used to connect tubing between the pump air filter to the culture chamber, the water supply jar to the 3-way stopcock, and the 3-way stopcock to the sampling jar. Polypropylene T connectors (4.8 mm, Nalgene) were used to direct air flow from the pump to the three culture chambers, and also at the junction of the aeration tubing (towards the culture chamber) and the air inlet tubing to the water supply jar. When plants were being aerated, the tubing connecting the water supply jar was closed by clamping in order to direct air flow towards the culture jar. When the fluid level in the culture jars was low, the aeration tubing going to the culture jar was clamped, diverting the air into the water supply jar, thereby causing water to flow into the culture chamber. The 3-way stopcock would then be turned to cause water to flow in that direction.

All parts of the hydroponic unit were autoclaved prior to use. The water supply jar was already filled three-fourth's full of water (~750 mL) at the time it was autoclaved to minimize water transfer and handling during assembly. The hydroponic system was assembled under a laminar flow hood, and all tubing connections were securely wired.

Fresh, sterile Hoagland's media containing the precursor was added with the use of a 60-ml Becton-Dickinson (BD) syringe (Rutherford, NJ). The media was introduced into the media jar by passing it through a nylon, sterile filter unit (0.45 μm, Fisher) attached to the media tubing bored through the rubber stopper. After media exchange, a piece of aluminum foil was sprayed with 70% ethanol, flamed, and used to cover the filter unit.

To replace old media with fresh, the air filter (vent) was capped with its female Luer slip, causing pressure to build up inside the culture chamber. This pressure displaced the media out of the culture chamber into both sampling and waste jars. Sampling of old media was executed by inserting a sterile BD needle (23G TW), attached to a sterile BD syringe (3 mL), through the silicone stopper, with which 1-2 mL media volumes were drawn. Fresh, sterile media would then be added to the culture chamber as described.

The construction of the hydroponic unit therefore allowed the following: aeration of individual plants to prevent cross contamination between culturing chambers, replacement of media lost by evaporation with fresh, sterile distilled water from the water supply jars, aseptic media exchange at regular intervals, and media sampling for monitoring microbial contamination and precursor concentration.

4.2.2 Preparation of plant material

Leucaena leucocephala seeds (K29 variety, University of Hawaii) were washed with EtOH:H_2O (70:30) for 3 min, and scarified by soaking in concentrated sulfuric acid (~50 mL) for 13 to 15 min under a laminar flow hood. The acid was removed by successive washings with sterile distilled water (7 × 60 mL). Scarified seeds were then individually transferred to sterile petri dishes (100 × 15 mm) coated inside with potato dextrose agar, PDA, (Difco Laboratories, Detroit, MI) under aseptic conditions. PDA plates were prepared as follows: Dehydrated PDA (39 g) was weighed and suspended in distilled, deionized water (1 L) in an Erlenmeyer flask (2 L). The suspension was heated to boiling until dissolution was complete. Portions (500 mL) of the heated suspension were dispensed into Erlenmeyer flasks (1 L), and subsequently sterilized in an autoclave

for 20 min at 121°. The medium was cooled to 45-50°, and then aliquots (about 15 and 8 mL, respectively) were dispensed into sterile, disposable petri dishes (100 × 15 mm for germinating seeds, and 60 × 15 mm for contamination tests).

The petri dishes were sealed with parafilm, and the seeds were allowed to germinate in a growth chamber (16h day at 25°, 8h night at 19° cycle) for 10 to 14 days. When the cotyledons were fully expanded (i.e., the cotyledons were spread open at about a 90° angle with respect to the stem), the seedlings were assumed ready for transfer into the hydroponic unit under the laminar flow hood. The PDA plates with the germinated seeds were examined for microbial growth, possibly originating from seed-borne microorganisms, and only those which were not contaminated were selected.

4.2.3 Preparation of nutrient media

Modified Hoagland's medium [2] was prepared from stock solutions as shown (Table 1). Full strength media consisted of macronutrient solution (100 mL), calcium nitrate solution (100 mL), micronutrient solution (1 mL) and Na,Fe-EDTA solution (1 mL) which were combined and brought to about 1 L volume with distilled, deionized water in a beaker (1 L). The solution was first adjusted to pH 5.9 by dropwise addition of 0.1M KOH, and then made up in a volumetric flask (1 L) with distilled, deionized water. This is defined as full-strength Hoagland's medium. Aliquots (130 mL) of the prepared medium were dispensed into Erlenmeyer flasks (250 mL) and covered with two layers of aluminum foil and autoclaved for 21 min at 121°. As required, precursors (natural abundance, radio-labelled, and stable isotope-labelled ferulic acid, **30** or phenylalanine, **7**) were filter-sterilized (0.22 μm, Millex-GV filter unit, Millipore) and added to the media

Table 1. Composition of stock solutions and final concentration of modified Hoagland's media.

Stock No.	Components	Final Concentration in medium (mM)	Stock Concentration	Weight per liter of stock solution (g)
	Macronutrients			
1	$NH_4H_2PO_4$	1	10X	1.15
	KNO_3	6		6.07
	$MgSO_4$	2		2.41
2	$Ca(NO_3)_2.4H_2O$ (prepared separately to prevent precipitation)	4	10X	9.45
	Micronutrients			
3	H_3BO_3	0.0496	1000X	2.86
	$MnCl_2.4H_2O$	0.0091		1.81
	$ZnSO_4.7H_2O$	0.000765		0.22
	$CuSO_4.5H_2O$	0.00032		0.08
	$Na_2MoO_4.2H_2O$	0.0001		0.024
4	Na,Fe-EDTA	0.0245	1000X	9.88

to give ~0.175 mM concentration. For each plant, aliquots (1-2 mL) from both fresh and old media were taken during weekly media exchange. These aliquots were used to test for biological contamination, monitoring precursor concentration, and ^{14}C content when radio-labelled precursors were used.

4.2.4 Plant growth

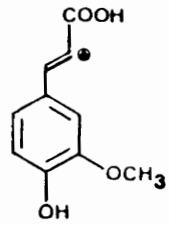
Seedlings were carefully transferred into the hydroponic unit (3 plants per unit) under the laminar flow hood. Fresh, sterile media, containing the appropriate precursor was added, and then the hydroponic unit was placed in a growth chamber under a 16h-day (25°) and 8h-night (19°) cycle. Light intensity in the growth chamber ranged from 300 to 580 μEinstein $m^{-2}sec^{-1}$. On the first week of plant growth, one-tenth strength nutrient concentration was used. Full strength nutrient concentration was used in subsequent media exchanges, which were carried out weekly for an additional three weeks. Growth rate was monitored by estimating plant heights (in cm) on a weekly basis.

L. leucocephala plants were also soil-grown inside the growth chamber under identical heat and lighting conditions as required.

4.2.5 Incorporation of [2-^{14}C] ferulic acid (30′) into *Leucaena leucocephala* root tissue

L. leucocephala seeds were germinated under aseptic conditions as described. Three uncontaminated seedlings were transferred from PDA plates, under the laminar flow hood, to the aseptic hydroponic unit.

A stock solution of [2-^{14}C] ferulic acid, **30′** (140 mg, 4.57×10^5 dpm mg^{-1}) was prepared as follows: natural abundance ferulic acid (**30**) (88.49 mg, 0.46 mmol) was combined with [2-^{14}C] ferulic acid (**30′**) (51.56 mg, 0.27 mmol, 1.24×10^6 dpm mg^{-1}) and dissolved in methanol (2.7 mL) in an Erlenmeyer flask (25 mL). With the use of an Eppendorf micropipettor, aliquots (3 mL) were dispensed into individual vials (4 mL), so that each vial contained 15.56 mg of ferulic acid (total activity per vial = 7.11×10^6 dpm), sufficient for the weekly precursor requirement of one hydroponic unit (3 plants).



(30′)

• = ^{14}C

The vials containing the ferulic acid (**30′**) solution were loosely capped and placed inside a desiccator which was attached to a water aspirator to remove the methanol under reduced pressure. The vials were then sealed and stored at -4° until required. Each time a media exchange was carried out, a vial containing the ferulic acid (**30′**) was used as described below.

Freshly-prepared, NH_4OH solution (550 μL, 0.2 N) was pipetted into the vial containing radio-labelled ferulic acid (**30**), and each vial warmed to 40-50° in a water bath until dissolution occurred. The ferulic acid solution was transferred (with a Pasteur pipette) to a sterile, disposable BD syringe (3 mL) containing a sterile filter attachment (0.22 μm, Millex-GV, Millipore), and filter-sterilized into a sterile vial (20 mL) in a laminar flow hood. An aliquot (10 μL) was removed for scintillation counting using an Eppendorf micropipettor equipped with a sterile pipet tip. Aliquots (150 μL, $\sim 1.94 \times 10^6$ dpm) were dispensed into Erlenmeyer flasks (250 mL) containing the sterilized Hoagland's media (~125 mL).

After media exchange and sampling, the hydroponic unit was transferred to a growth chamber using the growth conditions previously described. The media was replaced on a weekly basis with a freshly-prepared, sterilized solution containing filter-sterilized [2-^{14}C] ferulic acid (**30'**) as before. During each media exchange, aliquots (1-2 mL) were taken from old and new media, portions of which were analyzed for ferulic acid (**30**) content by HPLC, biological contamination and total radioactivity. In the latter case, two aliquots (500 μL) were dispensed into scintillation vials (20 mL) containing 10 mL of either Aquasol or Ecolume.

After four weeks growth, plants were harvested. At the time of harvest, aliquots of the media were removed, as previously described, under a laminar flow hood. Each *L. leucocephala* plant was removed by lifting the rubber stopper covering the culturing chamber, thereby pulling the media jar up, from which the plant was carefully removed with forceps. Each plant was then transferred to a beaker (250 mL) containing distilled water (~100 mL) to wash root tissue. The whole plant was next copiously washed with

distilled water, after which excess water was shaken off the plant, and its fresh weight determined.

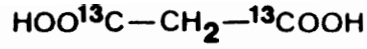
Subsequently, each plant was separated into leaflet, rachis, stem, root and cotyledon sections by means of stainless steel scissors and tweezers. While separating the leaflets from the rachis, the tissue was immersed in cold water in a glass petri dish (100 × 15 mm) to prevent dehydration. Each tissue was wrapped in aluminum foil, frozen with liquid nitrogen, and lyophilized. The dried plant material was shredded (stainless steel scissors). Root and stem sections were individually transferred to Whatman cellulose extraction thimbles (10 mm internal diameter [ID] × 50 mm length) and extracted for 12 h with benzene/ethanol (16 mL, 2.5:1, v/v) in a micro-Soxhlet extraction apparatus (Ace-Glass Inc., Vineland, NJ). The benzene/ethanol extract was recovered in a graduated cylinder (25 mL, TC), its total volume recorded, and an aliquot (500 μL) removed for scintillation counting.

The resulting benzene/ethanol extractive-free tissue was stripped of any remaining solvent under reduced pressure in a desiccator. The total weight of this dried material was obtained. Carefully-weighed portions (1-3 mg) were transferred to combusto-cones (Packard Instrument Company, Inc.) and oxidized in a Model B306 Tri-Carb Sample Oxidizer. The $^{14}CO_2$ generated was absorbed with Carbo-sorb and collected in scintillation vials (20 mL). These vials were counted using Permafluor (Packard Instrument Company, Inc.) as scintillation cocktail.

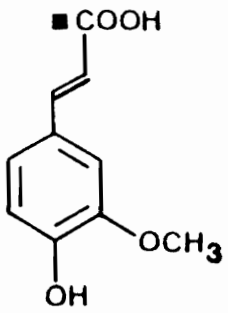
4.2.6 Synthesis of [^{13}C]-labelled ferulic acid (30a-c)

a) [1-^{13}C] Ferulic acid (**30a**):

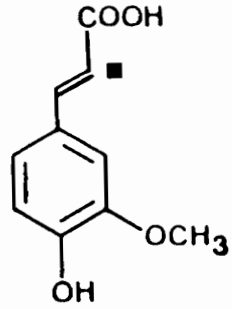
Malonic acid-[1,3-$^{13}C_2$] (**49**) (0.5 g, 4.8 mmol, 99 atom % ^{13}C, MSD Isotopes, Montreal, Canada) and vanillin (0.705 g, 4.64 mmol, Aldrich) were added to a 2-neck, round bottom flask (50 mL) containing dry redistilled pyridine (7.5 mL, Fisher). Two to three drops each of aniline (redistilled) and piperidine (Fisher) were then added and the contents placed under a nitrogen atmosphere. Following stirring for 22 h in an oil bath at 55 $\pm$ 2°, the solution was cooled to room temperature. The solvent was then removed under reduced pressure to afford an oily residue which when placed under high vacuum (0.5 torr) became solid (~500 mg). The solid residue was dissolved in glacial acetic acid (5 mL) by warming (~70°) in a hot water bath. More glacial acetic acid (~2 mL) was added, and the solution was transferred to an Erlenmeyer flask (125 mL). The round bottom flask was subsequently rinsed with glacial acetic acid (2 × 1 mL), and the washings were added to the Erlenmeyer flask. Distilled water (9 mL) was added while slowly swirling the flask, which was then allowed to stand at room temperature. [1-^{13}C] Ferulic acid (**30a**) crystals were collected by filtration in a Buchner funnel, washed with ice-cold distilled water (~ 50 mL), then recrystallized from water, to yield white needle-like crystals (419 mg, 45% yield; mp = 173-174°, lit. mp = 174°); MS (m/z): 195 (M^+, 100%), 180 (M^+-CH_3, 22%), 77 (M^++2H,-OH,-OCH_3,-CH=C$H^{13}CO_2H$, 20%), 99 atom % ^{13}C; ^{13}C NMR [$(CD_3)_2CO$] δ: 168.3 ppm.



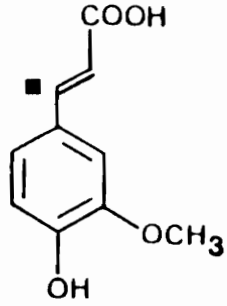
(49)



(30a)



(30b)



(30c)

■ = **13C**

b) [2-^{13}C] Ferulic acid (**30b**):

[2-^{13}C] Ferulic acid (**30b**) was prepared from malonic acid-[2-^{13}C] (99 atom % ^{13}C, MSD Isotopes) and vanillin (Aldrich), as described above. MS (m/z): 195 (M^+, 100%), 180 (M^+-CH_3, 22%), 134 (M^+ + H,-OH,-COOH, 21%), 99 atom % ^{13}C; ^{13}C NMR [$(CD_3)_2CO$] δ: 116.2 ppm.

c) [3-^{13}C] Ferulic acid (**30c**):

[3-^{13}C] Ferulic acid (**30c**) was prepared as above from malonic acid (Fisher) and [^{13}C-carbonyl] vanillin (99 atom % ^{13}C, described by Newman *et al* [131]). MS (m/z): 195 (M^+, 100%), 94 (M^+ + 2H,-OCH_3,-^{13}CH = CH-COOH, 59%), 99 atom % ^{13}C; ^{13}C NMR [$(CD_3)_2CO$] δ: 146.0 ppm.

4.2.7 Incorporation of [1-^{13}C] ferulic acid (30a) into *L. leucocephala* root tissue

L. leucocephala seedlings (3 per unit) were grown hydroponically under aseptic conditions in Hoagland's media containing 0.175 mM [1-^{13}C] ferulic acid (**30a**), as previously described. Plants were allowed to grow for a total of four weeks, with both media and precursor exchanged weekly, following which they were harvested. At seven-day intervals, samples (1-2 mL) of both fresh and old media were individually analyzed for biological contamination and ferulic acid (**30'**) content. After harvesting, plants were separated into component tissues (roots, stems, etc.) as described for previous [2-^{14}C] ferulic acid (**30'**) incubations. Each tissue (roots, stems, etc.) was individually wrapped in aluminum foil, frozen with liquid nitrogen, lyophilized and weighed. The dried root tissues were combined, shredded (with a pair of stainless steel scissors) and packed into scintillation vials (20 mL). Solid-state ^{13}C NMR analysis was carried out on *L. leucocephala* root tissue (83.5 mg) previously administered [1-^{13}C] ferulic acid (**30a**). A difference spectrum was obtained by subtracting from the spectrum of [1-^{13}C] ferulic acid-fed root tissue, that of control root tissue (i.e. from roots of plants incubated with natural abundance ferulic acid, **30**, or those grown hydroponically without precursor).

4.2.8 Three-week [1-^{13}C] ferulic acid (30a) incorporation into *L. leucocephala*, followed by one-week growth in precursor-free media

L. leucocephala plants were grown as described above, in hydroponic media containing 0.175 mM [1-^{13}C] ferulic acid (**30a**). After 3 weeks of growth and 2 media exchanges,

old media was replaced with fresh Hoagland's media containing no ferulic acid (**30**). The plants were then allowed to grow for an additional 7 days, following which they were harvested, separated into parts, freeze-dried and the roots (94.7 mg) analyzed by solid-state ^{13}C NMR.

4.2.9 Three-week incorporation of [1-^{13}C] ferulic acid (30a) into *L. leucocephala* root tissue

For purposes of comparison, three *L. leucocephala* plants were grown in media with 0.175 mM [1-^{13}C] ferulic acid (**30a**) and harvested after three weeks of hydroponic growth and weekly media exchange. Plants were then separated into tissues, freeze-dried, and the shredded roots (41.7 mg) analyzed by solid-state ^{13}C NMR.

4.2.10 Incorporation of [2-^{13}C] ferulic acid (30b) into *L. leucocephala* root tissue

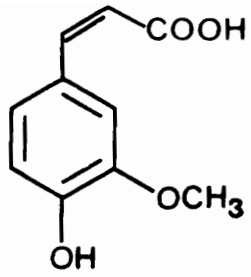
L. leucocephala seedlings were hydroponically-grown in Hoagland's media containing 0.175 mM [2-^{13}C] ferulic acid (**30b**) as described above. After four weeks of weekly media exchange, the plants were harvested, separated into tissues, and freeze-dried. The roots were again combined (32.4 mg), shredded (stainless steel scissors) and packed for analysis by solid-state ^{13}C NMR analysis. The resulting difference spectrum for this tissue was obtained as previously described.

4.2.11 Incorporation of [3-^{13}C] ferulic acid (30c) into *L. leucocephala* root tissue

L. leucocephala seedlings were administered [3-^{13}C] ferulic acid (**30c**) by growing them hydroponically as before. Thus, precursor and media application, as well as harvesting and preparation of the root tissue for solid-state ^{13}C NMR analysis were the same as previously described. The combined weight of root tissue used to obtain the difference spectrum for [^{13}C] ferulic acid fed roots was 59.7 mg.

4.2.12 HPLC assay for ferulic acid (30) in the media

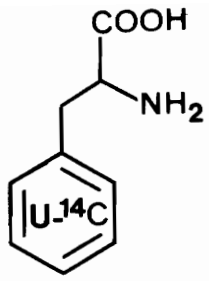
Distilled deionized water (1.3 mL) was added to aliquots (0.5 mL) of the hydroponic media containing ferulic acid (**30**). This solution was transferred to a disposable BD syringe (3 mL) equipped with a 0.45 μm ACRO LC3A filter assembly (Gelman Sciences, Ann Arbor, MI). Following filtration, 10-μL volumes were applied to a reverse-phase Nova-Pak C_{18} column and then eluted with 5% AcOH in H_2O: methanol (85:15, v/v), at a flow rate of 0.6 mL min^{-1}. Only *E*-ferulic acid (**30**) was detected in fresh media (retention time = 17.1 min), while both *E*- (**30**) and *Z*- (**30d**) ferulic acid were observed in aged (7 days) media (retention time of the *Z*-isomer = 20.8 min). (Retention times were a function of column age). Ferulic acid (**30**) concentrations in the media were determined by comparing integrated areas of chromatograms for media samples against a standard curve prepared from solutions of ferulic acid (**30**) of known concentrations.



(30d)

4.2.13 Incorporation of [U-^{14}C] L-phenylalanine (7′) into *L. leucocephala* root and stem tissues

L. leucocephala seedlings (3 per unit) were grown hydroponically (2 units) under aseptic conditions as previously described, in Hoagland's media containing 0.175 mM [U-^{14}C] L-phenylalanine (7′). For each hydroponic unit, the precursor solution was prepared as follows: Natural abundance L-phenylalanine (7) (10.87 mg, 0.066 mmol, Sigma) was added to an Erlenmeyer flask (25 mL) containing Hoagland's media (3.1 mL). With the use of an air-tight, Hamilton syringe (50 μL, Reno, NV), 28 μL of [U-^{14}C] L-phenylalanine (7) in HCl (0.01N) was drawn from a sealed vial (specific activity = 1.5 $\times$ 10^{10} Bq mmol^{-1}, ICN Biomedicals) and delivered to the Erlenmeyer flask. The resulting solution (21.2 mM, 1.051 $\times$ 10^5 Bq) was filter-sterilized to a sterile scintillation vial (20 mL) in a laminar flow hood. An aliquot (10 μL) was removed for scintillation counting by means of an Eppendorf micropipettor equipped with a sterile pipet tip. Aliquots (950 μL) were dispensed into individual flasks (250 mL) containing freshly-prepared, sterile Hoagland's media (125 mL) to give a 0.175 mM phenylalanine concentration and an average total activity of 3.98 $\times$ 10^4 Bq ($\pm$ 3.55 $\times$ 10^3 Bq).



(7′)

Once again, media samples (1-2 mL) were taken on a weekly basis, portions of which were analyzed for microbial contamination, total radioactivity and phenylalanine concentration using the phenylalanine ammonia lyase (PAL) assay.

Plants were harvested after four weeks of hydroponic culture. The procedure used for separating the plant into component tissues, to extraction of the roots and stems with benzene/ethanol (2.5:1, v/v) and determination of radioactivity in the benzene/ethanol soluble and insoluble portions were as for [2-^{14}C] ferulic acid-fed plants.

4.2.14 Distribution of radioactivity in the roots and stems of [U-^{14}C] L-phenylalanine-fed *L. leucocephala*

Dried roots or stems (~10 mg) were weighed and transferred to a mortar (90 mm OD) pre-chilled with liquid nitrogen. The tissue was ground into a fine powder using a chilled pestle while simultaneously covering the mortar with a piece of aluminum foil to prevent spread of radioactivity by sputtering. β-Mercaptoethanol (10 mM, 3 mL) was then added and grinding was continued for six min. If the β-mercaptoethanol solution froze,

grinding was continued for six min after thawing. Total grinding time, including that for thawing, was 9-10 min.

The resulting homogenate was transferred, with a Pasteur pipette, to a Corex glass centrifuge tube (15 mL). The mortar was rinsed with β-mercaptoethanol (10 mM, 3 × 2 mL), and the washings transferred to the centrifuge tube. The sample was centrifuged in a Sorvall SS-34 rotor (5 min, 480 × *g*, 10°) in a Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge (Sorvall, Inc., Newton, CT).

After centrifugation, the supernatant ("aqueous soluble" fraction) was decanted into a graduated cylinder (25 mL). The pellet in the centrifuge tube was resuspended with distilled water (2 mL) and poured onto a tared, Whatman glass microfibre filter GF/A (4.25 cm) in a Buchner funnel with a filtering flask attachment. The centrifuge tube was rinsed with distilled water (3 mL) and the washings poured onto the glass filter. The filtrate so obtained was combined with the "aqueous soluble" fraction in the graduated cylinder. The volume of this fraction was recorded, and two aliquots (500 μL each) were removed for radioisotopic scintillation counting.

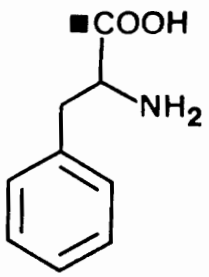
The residue on the glass filter was washed, first with 80% ethanol (3 × 2 mL), then methanol (3 × 2 mL), and finally, diethyl ether (3 × 3 mL). The organic filtrates ("organic soluble" fraction) were combined, transferred to a round bottom flask (25 mL) and dried under reduced pressure. The organic solubles were reconstituted in methanol (2 mL) with two-500 μL aliquots removed for scintillation counting.

The organic solvent-washed residue on the glass filter was transferred to a desiccator and dried *in vacuo* for at least an hour using a water aspirator. The resulting dried material on the glass filter was weighed and transferred to a scintillation vial for counting.

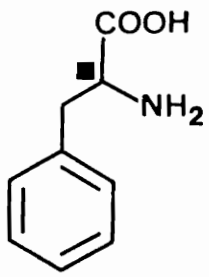
4.2.15 Incorporation of ^{13}C-enriched L-phenylalanine (7a-c) into *L. leucocephala* root and stem tissues

a) [1-^{13}C] Phenylalanine (**7a**) as precursor:

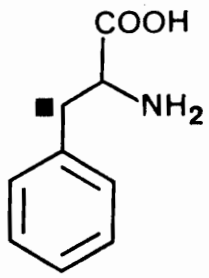
The procedure for the aseptic, hydroponic culture of *L. leucocephala* in media containing [1-^{13}C] phenylalanine (**7a**) was the same as that for [U-^{14}C] L-phenylalanine (**7'**) incorporation experiment except that media aliquots were not removed for scintillation counting. Thus, [1-^{13}C] phenylalanine (**7a**) (99 atom % ^{13}C) was administered to *L. leucocephala* plants grown hydroponically in aseptic units as shown in Figure 6. Solid-state ^{13}C NMR difference spectra were obtained from the enriched roots (80.0 mg) and stems (63.6 mg).



(7a)



(7b)



(7c)

■ = ^{13}C

b) [2-^{13}C]-Phenylalanine (**7b**) as precursor:

L. leucocephala seedlings were hydroponically-grown in Hoagland's media containing 0.175 mM [2-^{13}C] phenylalanine (**7b**) (99 atom % ^{13}C) as described above. After four weeks of media exchange, the plants were harvested, separated into tissues, and freeze-

dried. Roots (90.1 mg) and stems (73.8 mg) were separately combined, shredded (stainless steel scissors) and packed for analysis by solid-state ^{13}C NMR. The resulting difference spectra for both tissues were obtained as previously described.

c) [3-^{13}C] Phenylalanine (**7c**) as precursor:

L. leucocephala seedlings were administered [3-^{13}C] phenylalanine (**7c**) (99 atom % ^{13}C) by growing them hydroponically as above. Precursor and media application, as well as harvesting and preparation of the stem and root tissues for solid-state ^{13}C NMR analysis were the same as previously described. The combined weight of stem tissue used to obtain the difference spectrum was 71.8 mg, while the corresponding weight of the root tissue was 75.5 mg.

4.2.16 Spectrophotometric assay for L-phenylalanine (7) in the media

From a stock solution of phenylalanine in Hoagland's media (50 mg L^{-1}, 0.30 mM), aliquots were removed to prepare a series of diluted phenylalanine (**7**) solutions in Hoagland's media. The assay was carried out by pipetting to a cuvette (1.5 mL) the following: 1.05 mL of 0.05 M Tris-HCl buffer (pH 8.75 at 30°), 15 μL of standard L-phenylalanine (**7**) solution, and 20 μL of phenylalanine ammonia lyase, PAL, (0.04 unit, Sigma Grade I). (One unit will deaminate 1 μmol L-phenylalanine (**7**) to cinnamic acid (**22**) and ammonia per min at 30° and pH 8.75). The cuvette was inverted 3 times to ensure mixing, immediately placed in a spectrophotometer at 30°, and the change in absorbance was measured at 290 nm for 1 min. The slope was calculated, and the resulting value plotted against L-phenylalalanine (**7**) concentration, giving a standard

curve (activity versus [Phe]). A standard curve was prepared for every batch of media sample analyzed, a typical example of which is given in Figure 7.

Media (fresh and aged), were analyzed for phenylalanine content (7) by pipetting into a 1.5 mL cuvette the following: 1.05 mL of 0.05 M Tris-HCl buffer (pH 8.75 at 30°), 15 μL of the sample media containing L-phenylalanine (7), and 20 μL of PAL as described above. The reaction rate was observed as above, and then the slope (change in absorbance per min) was calculated. The concentration of L-phenylalanine (7) in the media analyzed was determined by interpolating from the standard curve the corresponding L-phenylalanine (7) concentration for the observed activity.

4.2.17 Monitoring media for biological contamination

Media aliquots (~0.5 mL) taken weekly from each *L. leucocephala* plant hydroponic media were applied, under a laminar flow hood, onto disposable petri dishes (60 x 15 mm) containing cured, sterile potato dextrose agar (PDA, Difco). The petri dishes were then sealed with Parafilm and stored in cupboards at room temperature. The petri dishes were checked daily and visually observed for microbial growth. Plants growing in contaminated media were discarded during the next media exchange following discovery of contamination (i.e., when growth of microbial colonies was observed in the corresponding PDA plates).

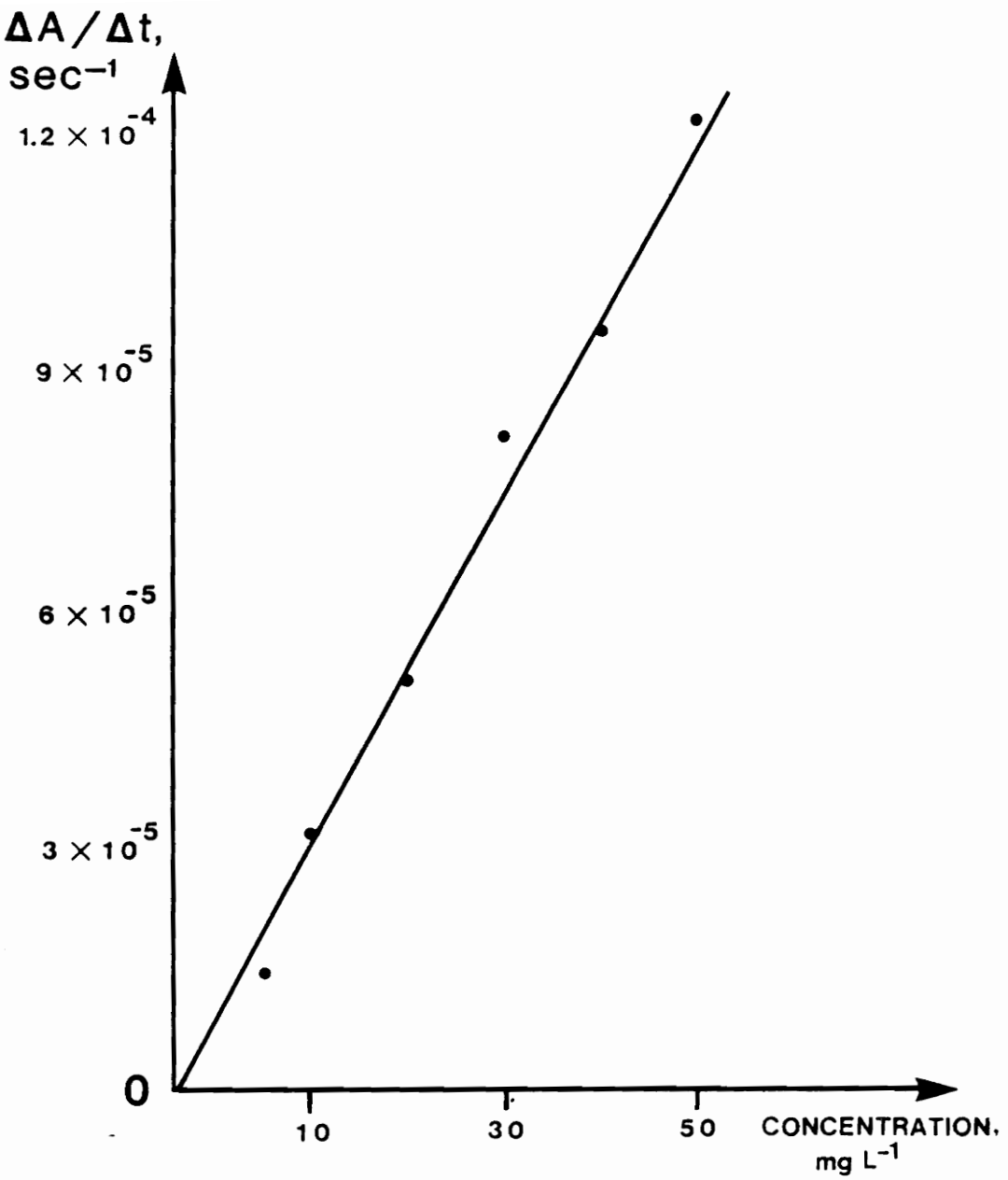


Figure 7. Standard curve for PAL assay.

4.2.18 Acetyl bromide lignin determination [127]

Tissues previously extracted with benzene/ethanol (2.5:1, v/v) (1-3 mg) were weighed and transferred into test tubes (18 × 150 mm). A solution (5 mL) of acetyl bromide (Aldrich) in glacial acetic acid (Fisher) (25%, v/v) was added to each test tube, and then each was capped with solid glass spheres (17 mm diameter). Tubes were heated for 30 min in a 70° water bath (Precision Scientific Company, Chicago, IL) with continuous shaking. The test tubes were cooled to room temperature and the contents transferred to a volumetric flask (100 mL) containing 2N NaOH (4.5 mL) and glacial acetic acid (25 mL). A small amount of glacial acetic acid (~3 mL) was used to rinse each test tube, and the washing was transferred to the volumetric flask. Hydroxylamine hydrochloride (Sigma) solution (7.5 M, 0.5 mL) was added to the volumetric flask followed by glacial acetic acid which was added to a final volume of 100 mL. The flask was shaken vigorously and allowed to stand for at least an hour at room temperature. The blank experiment (no tissue) was conducted identically.

With the use of a UV spectrophotometer, the absorbance of the solution at 280 nm was determined. Absorbance was converted into lignin content (% g/g dry matter) using the equation given below [127]:

$$\% \text{ Lignin} = 5.12A - 0.74$$

$$\text{where } A = (A_s - A_b)/c$$

A_s = sample absorption

A_b = blank absorption

c = dry organic matter concentration, g L^{-1}.

4.2.19 Nitrobenzene oxidation

Freeze-dried plant tissues were weighed and transferred to vials (20 mL) for storage at -20°. When needed, tissues were allowed to warm to room temperature and shredded with sharp stainless steel scissors. Tissues were transferred to tared Whatman cellulose extraction thimbles (10 mm ID × 50 mm length) and reweighed. Each thimble was placed inside a micro-Soxhlet extraction apparatus (Ace Glass Inc., Vineland, NJ) and extracted successively with benzene/ethanol (2.5:1, v/v), ethanol (95%), and distilled, deionized water. The extraction flask contained 16 mL of solvent, and 1 to 3 boileezer (Fisher) chips. (Glass beads were added when extracting with water). Extraction with each solvent was carried out for 12 hours. Tissues were air-dried between extractions.

Extractive-free tissues (air-dried) were transferred to a mortar (70 mm OD) and ground to a fine powder using a pestle. About 10 mg of the resulting ground tissue were weighed and transferred to a stainless steel vessel (10 mm ID, ~1.5 mL capacity). NaOH (150 μL, 2N) and distilled nitrobenzene (15 μL, Fisher) were then added to each vessel. Before each vessel was sealed, the atmosphere was continually exchanged with nitrogen.

Each vessel was submerged in a silicon oil bath (Fisher Hi-Temp Bath Model 160) pre-heated to 165 $\pm$ 3°. The vessels were rotated and shaken for 130 min, removed from the oil bath, and cooled with cold running water.

The lid of each vessel was carefully removed. Using a Pasteur pipette, the contents were transferred to a 10-mL test tube (13 × 100 mm) containing distilled, deionized, organic-free water (250 μL). Each vessel was rinsed with distilled water (3 × 250 μL), with each washing transferred to the test tube.

Nitrobenzene was removed from the mixture by extraction (by sonication in a Branson ultrasonic cleaner, Shelton, CT) with diethyl ether (3 mL). The ether layer was removed using a Pasteur pipette. The aqueous soluble mixtures remaining were then reextracted with ether (2 × 3 mL), and the ether extracts were subsequently discarded.

The aqueous layer was next acidified to pH 3.0 by dropwise addition of HCl (5N). (The pH was checked using pH indicator strips [Color pHAst, pH 0-6, EM Science, Cherry Hill, NJ]). The resulting acidic mixture was extracted (by sonication) with diethyl ether (3 mL) containing 1 mM veratraldehyde (**46**) as internal standard. The ether layer was removed with a Pasteur pipette and transferred to an Erlenmeyer flask (50 mL). Extraction with diethyl ether (2 × 3 mL) was repeated and the ether extracts were combined and dried (Na_2SO_4). The resulting ether solution (including Na_2SO_4 diethyl ether washings, 2 × 3 mL) was evaporated to dryness under reduced pressure in a round bottom flask (25 mL).

The flask was rinsed with HPLC-grade methanol (3 × 1 mL). The combined methanol solution was filtered through an ACRO LC3S filter assembly (Gelman Sciences, Ann Arbor, MI) attached to a 3-cc disposable BD syringe, in preparation for HPLC analysis.

Separation of the oxidation products on Waters Nova-Pak C_{18} column was achieved using a linear gradient of two solvent systems: A - (H_2O/MeOH/CH_3COOH, 95:5:0.1) and B - (MeOH/CH_3COOH, 99:1). The gradient was run for 25 min from 100% A (0%

B) to 60% A (40% B) at a flow rate of 1 ml min^{-1}. An aliquot (10 μL) of the sample was applied to the column with the components of interest detected at 280 nm. Integrated sample peak areas were compared with those of authentic standards of **p**-hydroxybenzaldehyde (**40**), vanillin (**41**) and syringaldehyde (**42**). Efficiency of sample recovery was determined by comparison of peak areas for veratraldehyde (**46**) used as internal standard with pure veratraldehyde (**46**) samples.

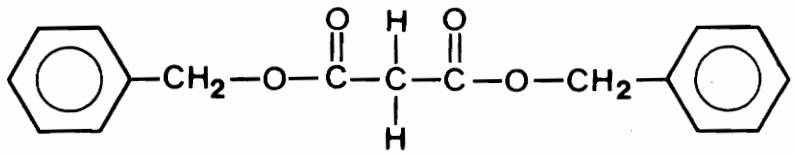
4.3 Lignin degradation by peroxidase in organic media

4.3.1 Purification of dioxane

A mixture of dioxane (3 L) plus concentrated HCl (37.5 mL) in distilled water (300 mL) was refluxed for 12-18 h under dry N_2 in a 5-L round bottom flask. The solution was cooled to room temperature and excess solid KOH slowly added with stirring. The dioxane was decanted from the resulting thick, dark aqueous layer and stored over KOH (150 g KOH per L dioxane). The dioxane was then placed in a 5-L round bottom flask containing Na metal (15-22 g), and again under N_2, its temperature was raised until refluxing began. This was maintained for 12 h before redistilling to afford pure, dry, peroxide-free dioxane.

4.3.2 Gel filtration chromatography

A low-pressure liquid chromatography system consisting of Sephadex LH-20 packed in an SR 10/50 column (Pharmacia, Uppsala, Sweden) was used for analysis. Sephadex gel (8-11 g) was swollen in the liquid phase (0.1M LiCl in redistilled N,N-dimethylformamide or anhydrous dioxane) for 3 h before packing and equilibrated with six column volumes of eluant. Each column was calibrated by applying the following standards of known molecular weight: polystyrenes with MW of 2000 and 800 (Pressure Chemical, Pittsburgh, PA) and MW of 1000 (Polymer Laboratory, Church Stretton, England) and dibenzyl malonate (**50**) MW 284 (Aldrich). The eluate was monitored at 280 nm using a LKB Bromma 2158 Uvicord SD detector. Elution was either with eluant A (0.1 M LiCl in redistilled N,N-dimethylformamide) at a flow rate of 0.23-0.24 mL min^{-1} or eluant B (anhydrous dioxane) at a flow rate of 0.18-0.19 mL min^{-1}. Solvent flow through the column was regulated by using a Pharmacia P-3 peristaltic pump.



(50)

4.3.3 Fractionation of DHP lignin

Dehydrogenatively polymerized (DHP) lignin prepared from coniferyl alcohol (**4**) as described [111], was used. To obtain uniformly high molecular weight fractions of dioxane-soluble material, 300-μL aliquots of DHP lignin (12.5 mg mL^{-1} dimethyl formamide) were passed through the Sephadex LH-20 column using anhydrous dioxane as eluant. Fractions eluted at the void volume, V_0, (MW>2000) were collected and evaporated to dryness under reduced pressure. From one representative fractionation of DHP lignin in DMF (12.5 mg mL^{-1}, 300 μL), 3.26 mg of high MW DHP was recovered.

4.3.4 Effect of horseradish peroxidase/H_2O_2 on DHP lignin

High molecular weight DHP lignin was incubated with Sigma type II horseradish peroxidase as follows: DHP lignin (0.3 mg) dissolved in dioxane (950 μL) was placed in a glass vial 20 mL). A solution of H_2O_2 (10 μL, 200 mM in 10 mM sodium acetate buffer, pH 5) and a solution (40 μL) of horseradish peroxidase in the same buffer (12.5 mg mL^{-1}) were added. Attempts to dissolve horseradish peroxidase directly in 95:5 dioxane/aqueous buffer failed. A control mixture without peroxidase was likewise prepared.

Incubations were carried out in a Precision low-temperature incubator model 818 (Precision Scientific Company, Chicago, IL) at 20 $\pm$ 0.5°, and vials containing the incubation mixture were agitated on a Lab-Line (Melrose Park, IL) orbit shaker (model 3520) at 250 rpm. After incubation periods of 0, 24, and 96 h, two 10-μL aliquots were

removed and diluted with 990 μL of acetate buffer in preparation for assay of residual enzymatic activity. The remainder of the incubation mixture was diluted with 2 volumes of dimethylformamide, stirred for 45-60 min and centrifuged at 7710 $\times$ g for 20 min to precipitate the enzyme. Aliquots (100 μL) were then removed from the supernatant and subjected to Sephadex LH-20 column (1 $\times$ 46.6 cm) chromatography as described.

4.3.5 Enzyme assay

The initial specific activity of the Sigma horseradish peroxidase type II was 150.4 purpurogallin units mg^{-1} (as determined by the Sigma enzyme assay procedure). Prior to and during incubations, the enzyme activity was determined using guaiacol (**47**) as substrate [150]. The assay mixture consisted of potassium phosphate buffer (0.1M, pH 7.0 at 25°), 50 μL guaiacol (20.1 mM) (**47**) (Fluka Chemika, Switzerland) solution, and enzyme. The reaction was initiated by addition of H_2O_2 (30 μL, 12.3 mM). The total volume of the reaction mixture was 3.18 mL. Activity was monitored at 436 nm. Enzyme activity was calculated using an extinction coefficient (436 nm) of 6.39 cm^2 μmol^{-1} for guaiacol (**47**) [150]. One unit of enzyme activity was defined as 1 μmol of guaiacol (**47**) consumed (oxidized) per min.

4.3.6 HPLC assay for ferulic acid (30)

Aliquots of the incubation mixture were assayed for their ferulic acid (**30**) content using the HPLC procedure described for monitoring ferulic acid in Hoagland's media (See section 4.2.12).

4.4 Lignin peroxidase from Phanerochaete chrysosporium: Effects on coniferyl alcohol (4) and DHP lignin

4.4.1 Assay procedure for lignin peroxidase activity

This assay procedure for lignin peroxidase activity was developed by the Finnish Sugar Co. Ltd (personal communication with Jari Puhakka, 1989). One unit of activity is defined as the amount of veratryl alcohol (**45**) (μmol) oxidized by lignin peroxidase to veratraldehyde (**46**) in 1 minute at 30° and pH 3.0. For calculating enzyme activity, an extinction coefficient of 9300 M^{-1} cm^{-1} of veratraldehyde (**46**) at 310 nm was used.

Sodium tartrate buffer (0.33 M, pH 3.0 at room temperature) and a stock solution of 1.0 M veratryl alcohol (**45**) were prepared. Each time an assay was performed, an aliquot (30 μL) was removed from the veratryl alcohol (**45**) stock and transferred to a volumetric flask (10 mL) containing 9 mL of sodium tartrate buffer. The resulting solution was adjusted to final volume with distilled, deionized water to give the veratryl alcohol (**45**) substrate (3 mM, 10 mL).

The following components were pipetted into a cuvette (3.5 mL): enzyme solution diluted with distilled water (2 mL, ~0.25-0.30 units), veratryl alcohol (**45**) substrate (1 mL), and freshly prepared solution of 54 mM H_2O_2 (15.4 μL). The cuvette was inserted into the cell holder of a UV spectrophotometer which was equilibrated at 30°. Absorbance

at 310 nm was read for 1 min. Slope was calculated from 20 to 32 seconds into the reaction.

When guaiacol (**47**) was used as substrate, the reaction was carried out in 0.33 M sodium tartrate buffer at pH 4.0, and the change in absorbance measured at 436 nm. The substrate solution consisted of guaiacol (1.32 mM) in sodium tartrate buffer. In this assay, 1 unit of enzyme activity was defined as 1 μmol of guaiacol (**47**) oxidized by lignin peroxidase per minute at 30° at pH 4.0. For calculating enzyme activity, an extinction coefficient of 6390 M^{-1} cm^{-1} was used.

4.4.2 Electrophoretic patterns of lignin peroxidase

a. Preparation for isoelectric focussing. Using a nitrocellulose membrane and a collodion apparatus (Schleicher and Schuell, Inc., Keene, NH), 1 mL of the lignin peroxidase solution was dialyzed overnight against 20 mM acetic acid (with 3 × ~500 mL solvent changes). The solution was then concentrated, under reduced pressure, in the nitrocellulose membrane (Grade: UH 100/25, MW cut-off = 25,000; cap.= 8 mL) for almost 8 h to a final concentration of 1 mg mL^{-1}.

b. Isoelectrofocussing. The concentrated lignin peroxidase was separated by isoelectric focussing on a pre-made 5% polyacryalamide gel (Ampholine PAGPlate, LKB Bromma) containing 2.4% ampholine for a pH range of 3.5 - 9.5. The flat bed electrophoresis equipment was connected to a circulating water bath at 10°. Kerosene was used as the insulating fluid between the metal plate, the template and the plastic gel support. The template was first laid on top of the metal plate. Kerosene was carefully applied between

the metal plate and the template to make sure that no air bubbles were trapped between. The PAGPlate was cut with a surgical blade to a length of 243 mm so that its length corresponded to that of the electrodes and was placed on top of the template. Electrode strips were saturated with H_3PO_4 (1 M) for the anode and NaOH (1 M) for the cathode and laid carefully onto the edge of the PAGPlate as marked on the template.

Sample application pieces were positioned (using forceps) 5 mm apart along lane 5 (or about 3.3 cm from the anode) of the PAGPlate. Using an Eppendorf micropipettor, 15 μL of the enzyme solution was delivered onto the sample application pieces. LKB pI markers (pI range 2.4 - 5.65) were also applied (5 μL of 1:3 marker in distilled water) on sample application pieces at selected positions along the length of the plate.

During the first 30 min, the gel was focussed at a constant voltage of 600 V. After 30 min, the sample application pieces were removed with forceps, and then electrofocussing was continued for 1.5 h at 1100 V.

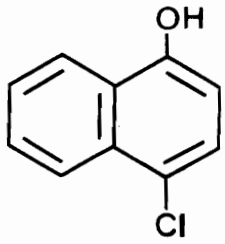
The electrode strips were then removed and the PAGPlate was placed on a glass plate. With the use of a surgical blade, the PAGPLate was divided into 5 sections for visualization by protein and activity staining.

c. Protein staining. The following solutions were prepared: Fixing solution: Trichloroacetic acid (11.5 g, Sigma) and sulfosalicylic acid (3.45g, Fisher) were added to distilled water and made up to volume in a volumetric flask (100 mL). Destaining solution: Ethanol (250 mL) and glacial acetic acid (80 mL) were mixed and diluted with distilled water in a volumetric flask (1 L). Staining solution: Coomassie Brilliant Blue R-250 (0.230 g, Bio-Rad) was dissolved in 200 mL destaining solution, then filtered. Preserving solution: Glycerol (10 mL) was added to 100 mL of the destaining solution.

The kerosene on the plastic support of the PAGPlate was removed by wiping the undersurface with several sheets of paper towel. The portion of the gel cut for protein staining was lowered into the fixing solution for 0.5 to 1 h to precipitate the protein and to allow the ampholine to diffuse out. The plate was placed in destaining solution for 5 min, and then stained for at least an hour with the staining solution at room temperature. Destaining was carried out with several changes of destaining solution until the gel background was clear. The gel was placed in the preserving solution for at least an hour, and then placed on top of a glass plate (20 × 20 cm) to dry. Finally, the gel was covered with a plastic sheet and cut to size for storage.

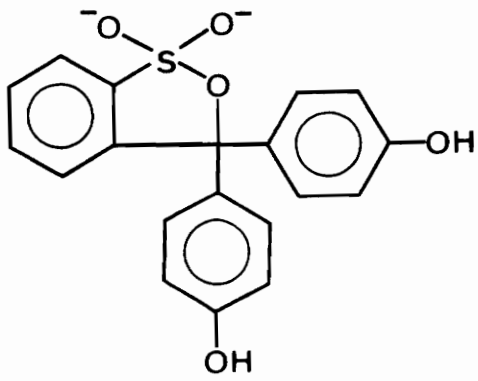
d. Guaiacol (47) staining. Activity staining with guaiacol (**47**) was carried out in a solution (50 mL) containing sodium tartrate buffer (50 mM, pH 4.0 at room temperature), guaiacol (**47**), 12 mM (Fluka) and H_2O_2 (2.5 mM). The bands stained brown with oxidized guaiacol within 3 min. The gel was left to dry on a glass plate, then covered with a plastic sheet and stored.

e. 4-Chloro-1-naphthol (51) staining. Activity staining with 4-chloro-1-naphthol (**51**) was carried out in a solution (50 mL) containing tartrate buffer (50 mM, pH 3.0 at room temperature), 4-chloro-1-naphthol (**51**), 0.45 mM (Bio-Rad) and H_2O_2 (50 μM). The solution of 4-chloro-1-naphthol (**51**) was prepared as follows: 4-Chloro-1-naphthol, **51** (60 mg), was dissolved with methanol (20 mL). Tris-buffered saline solution (100 mL, 20 mM, in 500 mM NaCl, pH 7.5 at room temperature) was added for a total volume of ~120 mL. Staining to pale blue was evident after 5 to 10 minutes. The gel was dried and stored as described above.



(51)

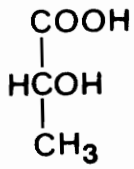
f. Phenol red (52) staining. Assays were carried out using phenol red (**52**) as substrate in solutions with or without Mn^{+2}. Two mixtures (50 mL each) were prepared consisting of sodium succinate buffer (50 mM, pH 4.5 at room temperature), 0.01% phenol red (**52**) and H_2O_2 (50 μM). $MnSO_4$ (100 μM) was added to one of the mixtures. Separate gels were then placed into each solution and an intense yellow color appeared within 5 min. The gels were dried and stored as before.



(52)

4.4.3 Spectrophotometric assay for Mn^{+2}-dependent enzyme activity in lignin peroxidase.

Two 1-mL reaction mixtures consisting of 0.01% phenol red, (**52**) (Sigma), 25 mM lactate (**53**) (JT Baker Chem. Co.), 0.1% chicken egg albumin (Sigma) and H_2O_2 (100 μM) were prepared. $MnSO_4$ (30 μL, 3.34 mM) was added to one of the mixtures to obtain a final concentration of 100 μM Mn^{+2}. An equal volume of distilled, deionized water was added to the other mixture. Na succinate buffer (1.0 mL, 20 mM, pH 4.5 at 30°) was then added to each mixture. The mixture was transferred to a cuvette, and enzyme solution (10 μL, with an activity equivalent to 0.08 μmol of guaiacol consumed per minute at 30°) was then added. For 0 min reaction time, NaOH (40 μL, 2N) was added immediately to the mixture in the cuvette, inverted 3 times, and the absorbance determined with a UV spectrophotometer at 610 nm. Otherwise, the reaction was allowed to proceed for 5 minutes at 30°, then terminated with addition of NaOH (40 μL, 2N). The absorbance at 610 nm was immediately determined.



(53)

In separate experiments, the following components were removed, one at a time, from the reaction mixture: lactate (**53**), H_2O_2 and enzyme. A separate mixture containing only the enzyme and the buffer was also prepared. In all cases, absorbance at 610 nm were determined using a UV spectrophotometer, at both 0 and 5 min reaction times. Reaction was terminated by addition of NaOH (40 μL, 2N) as above.

4.4.4 Preparation of coniferyl alcohol (4) for incubation with lignin peroxidase

a) [1-^{13}C] Coniferyl alcohol (**4a**):

Unlabelled coniferyl alcohol (**4**) (40.2 mg), was mixed with [1-^{13}C] coniferyl alcohol (**4a**) (25.0 mg), to give coniferyl alcohol enriched with ^{13}C at C-1 to the extent of 24 atom %. The mass and NMR spectral data for the resulting material is given as follows: (MS m/z): 181 (M^+, 40%), 180 (89%), 137 ($M^+ + H$,-$CH^{13}CH_2OH$, 100%), 24 atom % ^{13}C; ^{13}C NMR [$CDCl_3$] δ: 63.8 ppm.

b) [2-^{13}C] Coniferyl alcohol (**4b**):

Spectral data for [2-^{13}C] coniferyl alcohol (**4b**) is as follows: MS (m/z): 181 (M^+, 70%), 180 (3%), 137 ($M^+ + H$,-$^{13}CHCH_2OH$, 100%), 96 atom % ^{13}C; ^{13}C NMR ($CDCl_3$) δ: 126.5 ppm. This was used without dilution with unlabelled coniferyl alcohol (**4**).

c) [3-^{13}C] Coniferyl alcohol (**4c**):

Spectral data for [3-^{13}C] coniferyl alcohol (**4c**) is as follows: MS (m/z): 181 (M^+, 70%), 180 (23%), 138 ($M^+ + H$,-$CHCH_2OH$, 100%), 74 atom % ^{13}C; ^{13}C NMR ($CDCl_3$) δ: 131.7 ppm. This was used without dilution with unlabelled coniferyl alcohol (**4**).

4.4.5 Treatment of coniferyl alcohol (4) with lignin peroxidase

a) [1-^{13}C] Coniferyl alcohol (**4a**)

Lignin peroxidase (7.1 mL, 54 to 60 guaiacol (**47**) units, with veratryl alcohol (11.96 mg, 0.07 mmol) present as stabilizer) was added to a three-neck round bottom flask (250 mL) containing a magnetic stir bar (7.9 × 25 mm) and equipped with two polyethylene capillary inlet tubes and a nitrogen inlet. Sodium tartrate buffer (56.9 mL, 25 mM, pH 3.0 at room temperature) was added to the flask in order to bring the pH of the mixture to 4.0.

Two solutions, designated as **A** and **B**, were prepared as follows: [1-^{13}C] coniferyl alcohol, **4a** (30.4 mg, 0.17 mmol), was dissolved in sodium tartrate buffer (25 mL, 25 mM, pH 4.0, room temperature) to give solution **A**. Solution **B** was prepared by adding 30% H_2O_2 (22.7 μL, 0.20 mmol) to 25 mL tartrate buffer (25 mM, pH 4.0 at room temperature).

These two solutions were delivered simultaneously into the flask containing lignin peroxidase through the two polyethylene capillary tubing by means of LKB Bromma model 2232 or 2132 Microperpex peristaltic pump. Flow rate was 0.52 mL h^{-1} for a total reaction time of 48 h. The reaction was carried out at 25° and the mixture constantly stirred under N_2.

Aliquots (~0.08 guaiacol units) were removed to determine enzyme activity at time 0, 24 and 48 h. After 48 h, the mixture was transferred to four Corex centrifuge tubes (30 mL) and centrifuged for 2 h at 9,750 × *g* at 10° using a Sorvall SS-34 rotor. After

centrifugation, the supernatant was decanted into the three-neck round bottom flask. A solution of H_2O_2 (25 mL, 8.0 mM in 25 mM tartrate buffer, pH 4.0 at room temperature) was added over a 24 h period with the use of a peristaltic pump (flow rate = 1.04 mL h^{-1}).

The pellets in the centrifuge were combined, resuspended in distilled water (~10-15 mL), and centrifuged at 9,750 × *g* for an additional 2 h. The supernatant was discarded, and a small amount of fresh, distilled water (~0.5-1.0 mL) was added. The suspension was transferred to a tared vial (20 mL), frozen with liquid nitrogen, and lyophilized. The resulting dry, light-beige to reddish brown product was weighed (7.5 mg, 24.8% yield) and aliquots were removed for solution-state ^{13}C NMR analysis and molecular weight determination by gel permeation chromatography. The product of the second round of synthesis was obtained as described above (23.7 mg, 78.2% yield). An aliquot from the final reaction mixture was also removed to assay for residual enzyme activity.

b) [2-^{13}C] Coniferyl alcohol (**4b**):

Solution **A** was prepared by dissolving [2-^{13}C] coniferyl alcohol (**4b**), (31.1 mg, 0.17 mmol), in tartrate buffer (25 mL, 25 mM, pH 4.0 at room temperature). The solution of H_2O_2 (solution **B**) was prepared as described above. These two solutions were delivered simultaneously into a flask containing lignin peroxidase (as previously described) over a 48 h period with a peristaltic pump (flow rate = 0.52 mL h^{-1}). The reaction vessel was maintained under a nitrogen atmosphere. Aliquots were also removed to determine enzyme activity. After 48 h, the reaction mixture was centrifuged as above and the resulting pellet washed as before. The weight of the material obtained after freeze-drying was 16.92 mg (54.3% yield). Portions were removed for solution-state ^{13}C NMR and gel filtration chromatography analyses.

The product of the second round of synthesis was obtained as described above (17.64 mg, 56.6% yield). An aliquot from the final reaction mixture was also removed to assay for residual enzyme activity.

c) [3-^{13}C] Coniferyl alcohol (**4c**):

Solution **A** was prepared by dissolving [3-^{13}C] coniferyl alcohol (**4c**), (32.4 mg, 0.18 mmol), in sodium tartrate buffer (25 mL, 25 mM, pH 4.0 at room temperature). A solution of H_2O_2 (solution **B**) was also prepared. These two solutions were delivered simultaneously into a flask containing lignin peroxidase (as previously described) over a 48 h period with a peristaltic pump (flow rate = 0.52 mL h^{-1}). The reaction vessel was maintained under a nitrogen atmosphere. Aliquots were removed to determine enzyme activity. Instead of stopping the reaction after 48 h, H_2O_2 (25 mL, 8.0 mM, in 25 mM tartrate buffer, pH 4.0 at RT) was delivered to the flask for another 8.3 h (flow rate = 3 mL h^{-1}). The reaction mixture was centrifuged as above and yielded 33.0 mg of product (quantitative yield). Portions were removed for solution-state ^{13}C NMR and gel filtration chromatography analyses.

4.4.6 Incubation of coniferyl alcohol (4) in 25 mM sodium tartrate buffer, pH 4.0, without lignin peroxidase

A blank reaction mixture (containing no lignin peroxidase) was likewise set up in a three-neck round bottom flask containing 10 mM veratryl alcohol (**45**) in 0.4 M sodium acetate buffer (7.1 mL, pH 6.4) and sodium tartrate buffer (56.9 mL, 25 mM, pH 3.0). Coniferyl alcohol, **4** (30.66 mg, 0.17 mmol), was dissolved in sodium tartrate buffer (25

mL, 25 mM, pH 4.0) as in solution **A** above. Likewise, a fresh solution of H_2O_2 (designated as solution **B**), (25 mL, 8 mM) was prepared and simultaneously with the coniferyl alcohol (**4**) solution, delivered over a 48 h period, into the round bottom flask with constant stirring and under an inert environment (N_2).

No precipitate was observed in the reaction mixture. An aliquot of the reaction mixture was removed after 48 h for HPLC analysis using a Nova-Pak C_{18} reverse-phase column. The aliquot was filtered through an Acro LC3A filter assembly (0.45 μm), 10 μL of which was removed for chromatographic separation by isocratic elution with MeOH:H_2O (15:85) at a flow rate of 1.3 mL min^{-1} and using a detection wavelength of 262 nm. Comparison with the chromatogram of a standard solution of coniferyl alcohol (**4**) (2.56 mM) showed that the coniferyl alcohol (**4**) remained unchanged in the reaction mixture. The retention time for coniferyl alcohol (**4**) under this condition was 12.8 min.

4.4.7 Effect on "DHP lignin" by further treatment with lignin peroxidase

[2-^{13}C] DHP lignin (10 mg), synthesized from [2-^{13}C] coniferyl alcohol (**4b**), was added to a round bottom flask (250 mL) containing ~18 guaiacol units of lignin peroxidase (from stock solution described previously) and sodium tartrate buffer (19.0 mL, 25 mM, pH 3.0). Hydrogen peroxide solution (0.13 mmol H_2O_2 in 16.7 mL of sodium tartrate buffer, 25 mM, pH 4.0) was added directly to the flask. The reaction mixture was stirred for 5 h under N_2. H_2O_2 (0.13 mmol) dissolved in distilled dioxane (10 mL) was added to the flask and the incubation was continued for another 15 h. The dioxane was then removed under reduced pressure. The resulting aqueous suspension was centrifuged for

2 h at 9,750 × *g* and 10°. The resulting pellet was resuspended in distilled water and centrifuged for another 2 h at 9,750 × *g*. After discarding the supernatant, a small amount of fresh, distilled water was added to the pellet and the resulting suspension freeze-dried.

4.4.8 Determination of molecular weight distribution of "DHP lignins" formed by lignin peroxidase

Labelled and unlabelled reaction products obtained from incubation of coniferyl alcohol (**4**) with lignin peroxidase were dissolved, at concentrations ranging between 3 and 5 g L^{-1}, in carbonate-free aqueous NaOH (1 mL, 0.1 N) and applied to a Sephadex G100 column (2.5 × 100 cm). Elution with carbonate-free aqueous NaOH (0.1 N) at a flow rate of 30 mL h^{-1} was monitored at 280 nm with a double-beam ISCO model V^4 detector at the column outlet. The column dispersion for the species with molecular weight around 500 was less than 8% of the relative column retention volume. The raw data were digitized and transformed to elution profiles of absorbance (A_{280}) *vs.* relative retention volume, V_R. Calibration was achieved by correlation with paucidisperse fractions from a dissociated kraft lignin preparation, the molecular weight distribution of which had been exhaustively characterized through ultracentrifuge sedimentation equilibrium measurements [47]. The effective incubation times represent the time elapsed between dissolution and appearance of the profile.

5.0 RESULTS AND DISCUSSION

5.1 Examination of Lignin Structure in situ by Solid-state ^{13}C NMR Spectroscopy

One of the most challenging problems which have confounded lignin chemists over the years is the determination of the native structure of lignin [147]. Lignin in plant tissue is presently depicted by structural schemes developed from characterization studies of isolated or artificially-prepared lignins. However, none of the known isolation procedures thus far employed can remove lignin from the cell wall matrix without destroying the integrity of the polymer, while the properties of synthetic lignins are influenced by the conditions used to make them. Consequently, research efforts have been directed in recent years (see Review of Literature) towards methodologies that would allow examination of the structure of lignin *in situ*, thereby avoiding the physicochemical alterations that the macromolecule undergoes during the process of isolation.

^{13}C specific labelling of selected carbon atoms of lignin *in situ*, followed by solid-state ^{13}C NMR spectroscopy of the enriched tissue, provide structural information not attainable with the other techniques. In fact, this has already been achieved for *T. aestivum*, a grass species, where ^{13}C-enrichment of specific carbon atoms of the propanoid side-chain and subsequent ^{13}C NMR spectroscopy of the root tissue led to the identification of the major bonding environments of these carbon atoms [116].

Labelling of the phenylpropanoids in the roots of *T. aestivum* was accomplished by administering ^{13}C-specifically-labelled ferulic acid (**30a-c**) to individual plants through their roots over extended periods of time. It should be noted that *T. aestivum* seedlings were grown in agar medium containing the precursor [116]. One of the problems associated with methods employing solid media is the means of ensuring perpetual availability of precursors (or nutrients) to the growing cultures. This is usually circumvented by subculturing in fresh medium thereby making available fresh precursor (or nutrient) for growth of cultures over long periods of time. This was one of the major considerations in the development of the system employed in the present work, where subculturing is obviously not a practical procedure. Consequently, the hydroponic unit shown in Figure 6 was constructed, which, among other features, allowed replacing old media with fresh at desired frequency, thereby ensuring an uninterrupted supply of precursor to the plant for its entire growth period.

In developing methodology for this study, ferulic acid (**30**) was initially used as lignin precursor. Subsequently, ^{13}C specifically-labelled phenylalanine (**7a-b**) was employed. These phenylpropanoid compounds are two of the more commonly used precursors for radio- (and stable-) isotope labelling studies of lignin. Ferulic acid (**30**) is quite often used because, depending upon the species investigated, it can be converted into coniferyl

(**4**) and sinapyl (**6**) alcohols. Its use is also favored because of its biochemical proximity to the final product of interest - lignin; i.e., only a few other components of the plant tissue (e.g., suberins and lignans) will be labelled. The availability of specifically-labelled ferulic acid is not a limiting factor for its use because it can be easily obtained by condensation of appropriately-labelled reactants, malonic acid and vanillin (**41**) (see Experimental section).

[U-^{14}C] Phenylalanine (**7'**) is another widely used precursor for radio-labelling studies of lignin, as it is readily available from a number of commercial suppliers. Phenylalanine (**7**) is relatively non-toxic to the plant at dosages used for efficient incorporation. Unlike ferulic acid (**30**), phenylalanine (**7**) suffers from the disadvantage that it can be incorporated into other components in the tissue, e.g., polyflavonoids and proteins, in addition to lignins, suberins and lignans. Consequently, in the present study, it was necessary to demonstrate that lignin was the major metabolite derived from phenylalanine (**7**) incorporation. This was required since whole plant tissues were to be examined without any prior extraction.

Other practical considerations which must be met by precursors for biosynthetic studies are as follows [31]: 1) chemical stability while being administered to the plant; 2) intactness of incorporation (i.e., no scrambling of label); and 3) sufficiently high incorporation levels to allow detection by solid-state ^{13}C NMR spectroscopy of the tissue. It is also very important that the precursor not perturb the normal metabolic and physiological functions of the plant.

5.1.1 Development of a hydroponic system for administering lignin precursor to *L. leucocephala*

The fast-growing tropical hardwood, *L. leucocephala*, was chosen because its rapid growth could provide sufficient plant tissue needed for solid-state ^{13}C NMR spectroscopy and other chemical analyses within reasonable lengths of time. The overall growth period was twenty eight (28) days, which is many times longer than the duration of most ^{14}C feeding experiments performed in earlier studies on the biosynthesis of lignin. Longer durations are needed to ensure adequate lignin formation which would allow enhancement to detectable levels of resonances in the ^{13}C NMR spectrum. Administering precursors to intact plants minimized physiological responses to wounding, which may be induced if plant cuttings were employed.

In the early development stages of an aseptic hydroponic system, *L. leucocephala* seedlings were grown in glass jars (90 mm ID × 170 mm height). These were provided with Teflon supports with a hole in the center through which the stem was wedged, thereby enabling the roots to come in contact with the nutrient solution. The jars were sealed with autoclavable plastic bags and agitated with a rotary shaker inside a growth chamber (16h-day 25°, 8h-night 19° cycle). Media exchange and sampling for ferulic acid content and biological contamination were carried out on a weekly basis.

One of the main problems encountered with this prototype was the high biological contamination rates of the cultures. The procedure of media exchange (which involved removal of the plastic cover to replace old media with fresh), although done in a laminar flow hood, still resulted in considerable microbial contamination. Sterile conditions were required for growth, since it was determined by HPLC that microbial contam-

ination of nutrient media containing ferulic acid (**30**) resulted in the rapid conversion of the precursor to other metabolites. Even without biological contamination, the system was not suitable for growing plants indefinitely due to the lack of fresh air and adequate venting, resulting in suppression of plant growth. For our experiments, it was necessary to grow plants under conditions which do not adversely affect normal metabolic processes. It became readily apparent that the preliminary design was not acceptable for our purposes.

The development of the hydroponic system described in the experimental section, and shown in Figure 6, minimized the problems associated with the earlier protocol. With the new design, old media could be replaced without opening up any part of the system which might lead to exposure to microbial contaminants. Additionally, by continued sterile aeration, adequate supply of fresh, sterile air, was assured. A system for sterile venting was also provided.

Consequently, *L. leucocephala* seedlings grown using the improved system were visually healthier than those grown in an enclosed jar with shaking. The leaves were greener and each plant was two to four times the size and biomass of those grown by shaking. No significant differences in plant growth rates were observed between those grown in media with ferulic acid, **30** (or phenylalanine, **7**) and in media without precursor. Additionally, no differences were visually observed between the hydroponically-grown plants and those soil-grown inside a growth chamber under similar conditions. However, for future experiments where longer durations of growth may be required, the system can lend itself to some minor modifications (e.g., changing size and configuration of growth chamber) and some other improvements with respect to handling and operation.

5.1.2 Incorporation of [2-^{14}C] ferulic acid (30′) into *L. leucocephala*

Preliminary studies were carried out by administering [2-^{14}C] ferulic acid (**30′**) to *L. leucocephala* to determine incorporation rates and to ensure that no molecular scrambling (in the media) of isotopic label would occur. Following administration of this radio-labelled precursor to *L. leucocephala* grown hydroponically for four weeks under aseptic conditions, seedlings were harvested, separated into leaf, root, stem, and rachis sections, and individually freeze-dried. A summary of the experimental protocol is given in Figure 8. The media was analyzed for biological contamination, which were visually observed in PDA plates onto which aged media aliquots were applied. Plants were immediately discarded if biological contamination was detected. As described in the experimental section, media samples containing [2-^{14}C] ferulic acid (**30′**) were also analyzed by HPLC and scintillation counting to determine the extent of precursor uptake for every seven-day period. Lyophilized individual plant parts, on the other hand, were subjected to benzene/ethanol (2.5:1, v/v) extraction for 12 h, with aliquots of the organic solubles removed for determination of radioactivity, while portions of the insoluble residue were removed, dried, ground in a mortar and pestle, and then transferred to a scintillation vial for counting. Typical quantitative results after administration of [2-^{14}C] ferulic acid (**30′**) to *L. leucocephala* for four weeks are shown in Table 2 and in Figure 9. Highest incorporation rates were observed in the roots and accounted for as much as 80% of the total radioactivity. The remaining radioactivity was distributed among the leaves, stems, and rachis, in decreasing order.

With ferulic acid (**30**) as precursor, very little translocation had occurred into the aerial portion of the plants. A recent report [170], however, showed uptake of ferulic acid (**30**) into the stems and other aerial parts of hydroponically-grown, 18-day old cucumber

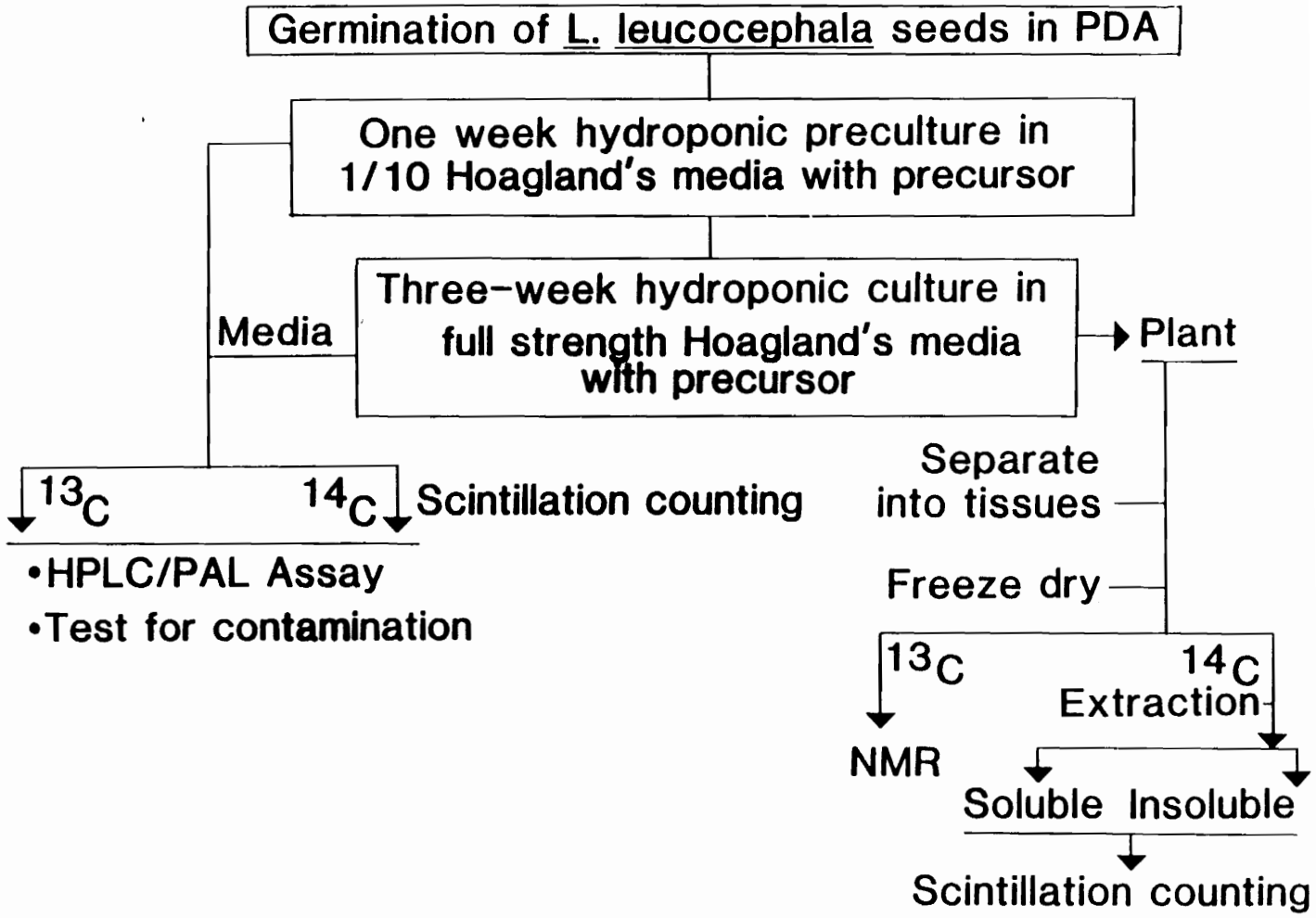


Figure 8. Schematic of experimental protocol for administering labelled precursors to *L. leucocephala*.

Table 2. Total radioactivity (dpm) in the different organs of *L. leucocephala* administered [2-^{14}C] ferulic acid (30′).

Organ	Plant No. 1		Plant No. 2	
	B/E[a] Extractable	Insoluble Residue	B/E Extractable	Insoluble Residue
Roots	6934 (9.07)[b]	54320 (71.04)	3572 (3.71)	71814 (74.55)
Stems	688 (0.90)	1023 (1.34)	465 (0.48)	1277 (1.33)
Leaflets	4932 (6.45)	8325 (10.89)	3518 (3.65)	14268 (14.81)
Rachis	181 (0.24)	59 (0.08)	428 (0.44)	983 (1.02)

[a] B/E = Benzene/Ethanol (2.5:1, v/v).
[b] Numbers in parentheses represent radioactivity as per cent of total found in the plant.

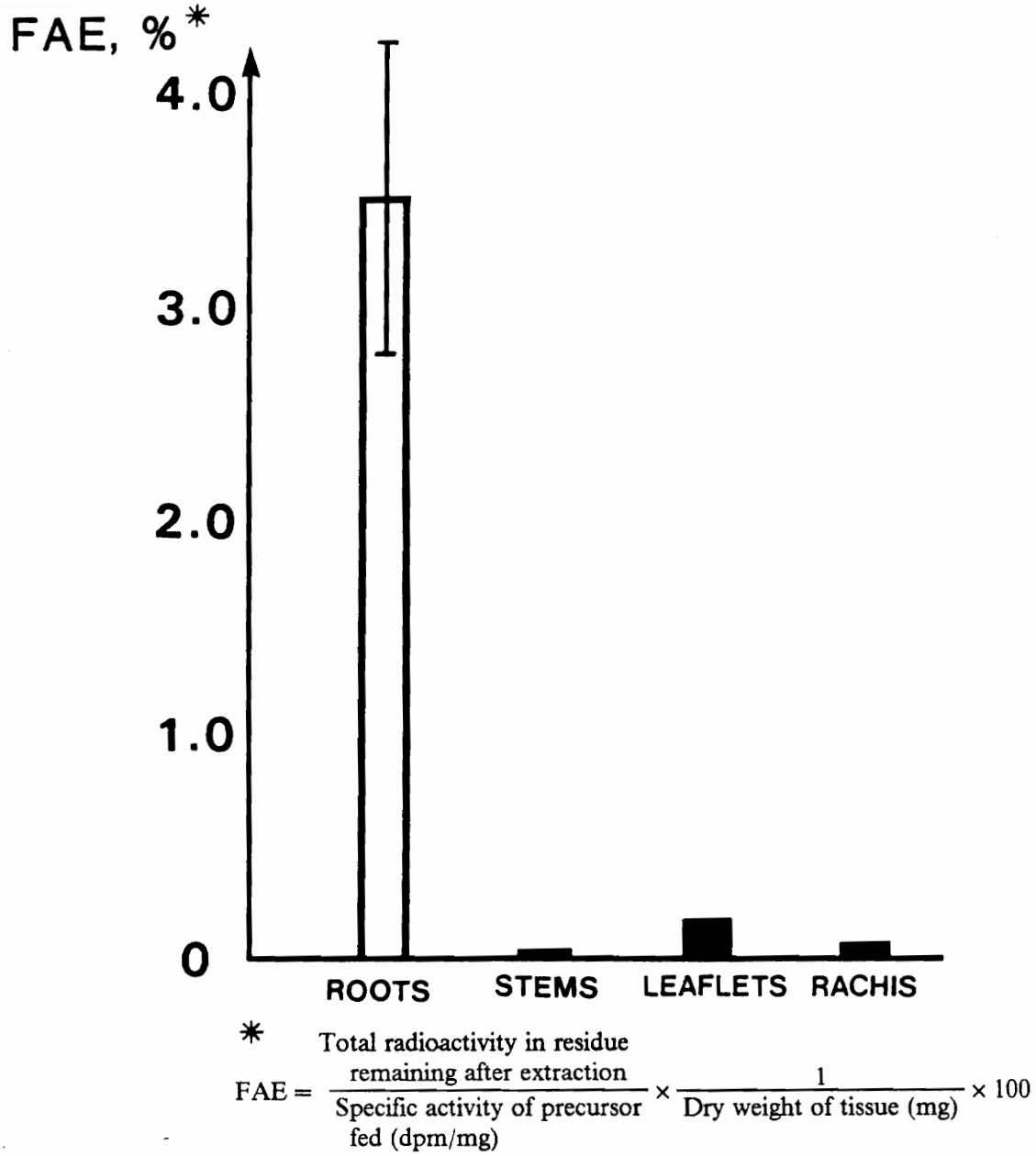


Figure 9. Distribution of radioactivity in *L. leucocephala* administered [2-^{14}C] ferulic acid (30′).

(*Cucumis sativus*) exposed to [U-ring-^{14}C] ferulic acid for up to 5 hours. Incorporation rates ranged from 1.6 to 17.9%, depending upon concentration of ferulic acid (**30**) in the media and age of the seedling. In these experiments, the effects of microbial breakdown of ferulic acid (**30**) must be considered since the plants were not grown under aseptic conditions. In our experiments, high levels of radioactivity were observed in the leaves of *L. leucocephala* plants grown in biologically-contaminated nutrient media. This suggests that conversion of [U-^{14}C] ferulic acid (**30'**) to other metabolites occurred, with concomitant scrambling of isotopic label, when microbes were present.

Radioactivity in the benzene-ethanol soluble fraction of the root tissue may be attributed to [2-^{14}C] ferulic acid (**30'**) or to some reduced aldehyde or monolignol derivatives that diffused through the root epidermis, and then to the cell lumina of actively differentiating and/or lignifying cells. In these plants, the soluble fraction accounted for only 5-12% of the total radioactivity in the roots. The greater portion (> 70%) of the radioactivity taken up was in the insoluble (i.e., lignified) plant residue, and this was presumed to be incorporated into the lignin polymer in the cell walls.

5.1.3 Incorporation of natural abundance ferulic acid (30) into *L. leucocephala*

L. leucocephala seedlings were also grown hydroponically in either precursor-free media or in media containing natural abundance ferulic acid (**30**). After harvest, the roots were dried, shredded, and then analyzed by solid-state ^{13}C NMR spectroscopy without prior extraction. The solid-state ^{13}C NMR spectra of the root tissues are shown in Figures 10a-b. The spectra are essentially identical; the signals for the aliphatic inter-unit link-

ages of lignin are buried under the signals for the carbon atoms of the carbohydrates which make up the bulk of the tissue. No enhancement of lignin signals was noticeable in the root tissue administered with natural abundance ferulic acid (**30**). The similarity between the spectral profiles for both plant materials suggest very little deleterious effect on overall plant metabolism by uptake of ferulic acid (**30**). (Later in this discussion, results obtained from the nitrobenzene oxidation of these tissues will be presented, where differences in monomeric composition between the ferulic acid (**30**) precursor-treated and untreated plants were found). Consequently, either spectrum was used in obtaining the difference spectrum of *L. leucocephala* root tissues enriched with ^{13}C-ferulic acid at specific positions of the propyl side chain. A ^{13}C NMR difference spectrum results from subtraction of ^{13}C NMR natural abundance spectrum from the ^{13}C NMR spectrum of corresponding enriched sample, and contains resonances due to the bonding environments of selectively ^{13}C enriched carbon atoms.

In the analyses of the solid-state ^{13}C NMR spectra of *L. leucocephala* tissues enriched with ^{13}C-labelled precursors, reference will be made to Figure 11 showing five bonding environments or substructures believed to be predominant in the lignin macromolecule. These bonding environments were established from structural determinations involving isolated and dehydrogenatively polymerized (DHP) lignin preparations and a whole series of lignin "model compound" studies [43,111,138]. Substructure **A** represents hydroxymethyl-containing moieties linked to the rest of the lignin polymer by means of a phenolic ether linkage. Substructure **B** is the so-called β-O-4′ linkage, presumed by many authors to be the dominant inter-unit linkage between the monomers in lignin (36). Substructure **C** corresponds to either pinoresinol (R = H) or syringaresinol (R = OCH_3) substructures, while substructures **D** and **E** are known to be the phenylcoumaran (β-5′) and β-aryl (β-1′) linked substructures, respectively. Note that the

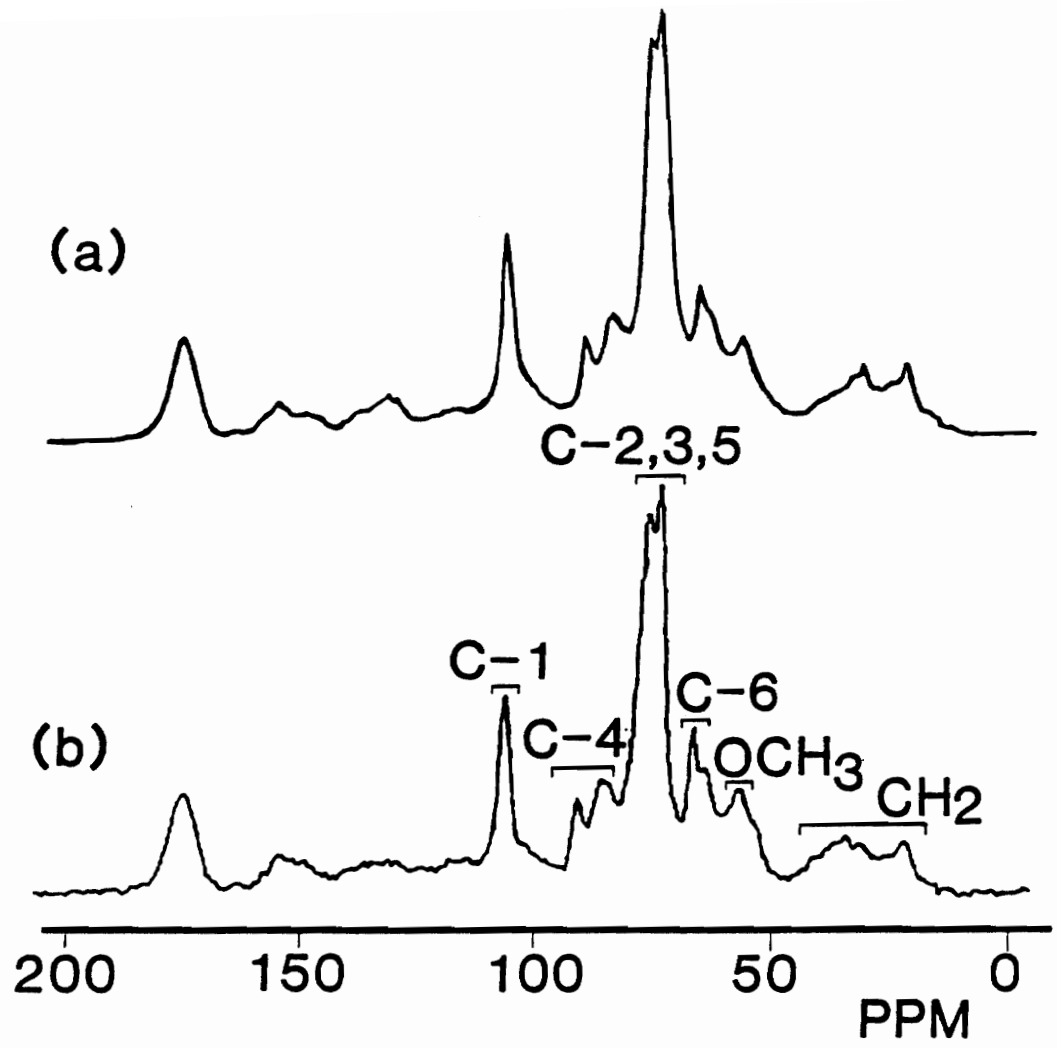


Figure 10. Solid-state ^{13}C NMR spectra of *L. leucocephala* root tissue (natural abundance): Difference spectra of *L. leucocephala* hydroponically-grown in media (a) without precursor; (b) with natural abundance ferulic acid (30).

inter-unit linkages in each of the four dimeric substructures (**B-E**) involve at least a carbon atom of the propyl side chain. It has been suggested that most of the inter-unit linkages in lignin involve the propyl side chain and that substructures **B** and **D** alone account for > 60% of the inter-unit linkages in the lignin macromolecule [36]. The proposed dominant involvement of the propyl side-chain in these linkages justifies the use, in the present experiment, of precursors specifically-labelled at a carbon atom in the propyl side chain.

For purposes of comparison, the solid-state ^{13}C NMR spectra of DHP lignin prepared from coniferyl alcohol specifically-labelled at the 1-, 2-, and 3-positions (**4a-c**) are shown in Figures 12a-c, respectively [111]. The following discussion will emphasize similarities and differences between these spectra and those obtained from ^{13}C-enriched *L. leucocephala* tissues.

5.1.4 Incorporation of [1-^{13}C] ferulic acid (30a) into *L. leucocephala* root tissue

The solid-state ^{13}C NMR difference and enriched spectra of *L. leucocephala* roots grown for four weeks in media containing [1-^{13}C] ferulic acid (**30a**), 99 atom % ^{13}C, are shown in Figures 13a-b, respectively. The difference spectrum (Figure 13a) was obtained by subtracting the natural abundance spectrum (Figure 12) from the root tissue treated with the ^{13}C-labelled precursor (Figure 13b). Enhancement of resonances attributable to the bonding environments of C-1 was obtained. The strongest enhancements are at 63 ppm (with a small shoulder at 72 ppm) and at 170-174 ppm. The signal at 63 ppm corresponds to a hydroxymethyl (-CH_2OH) functionality present in the tissue as integral

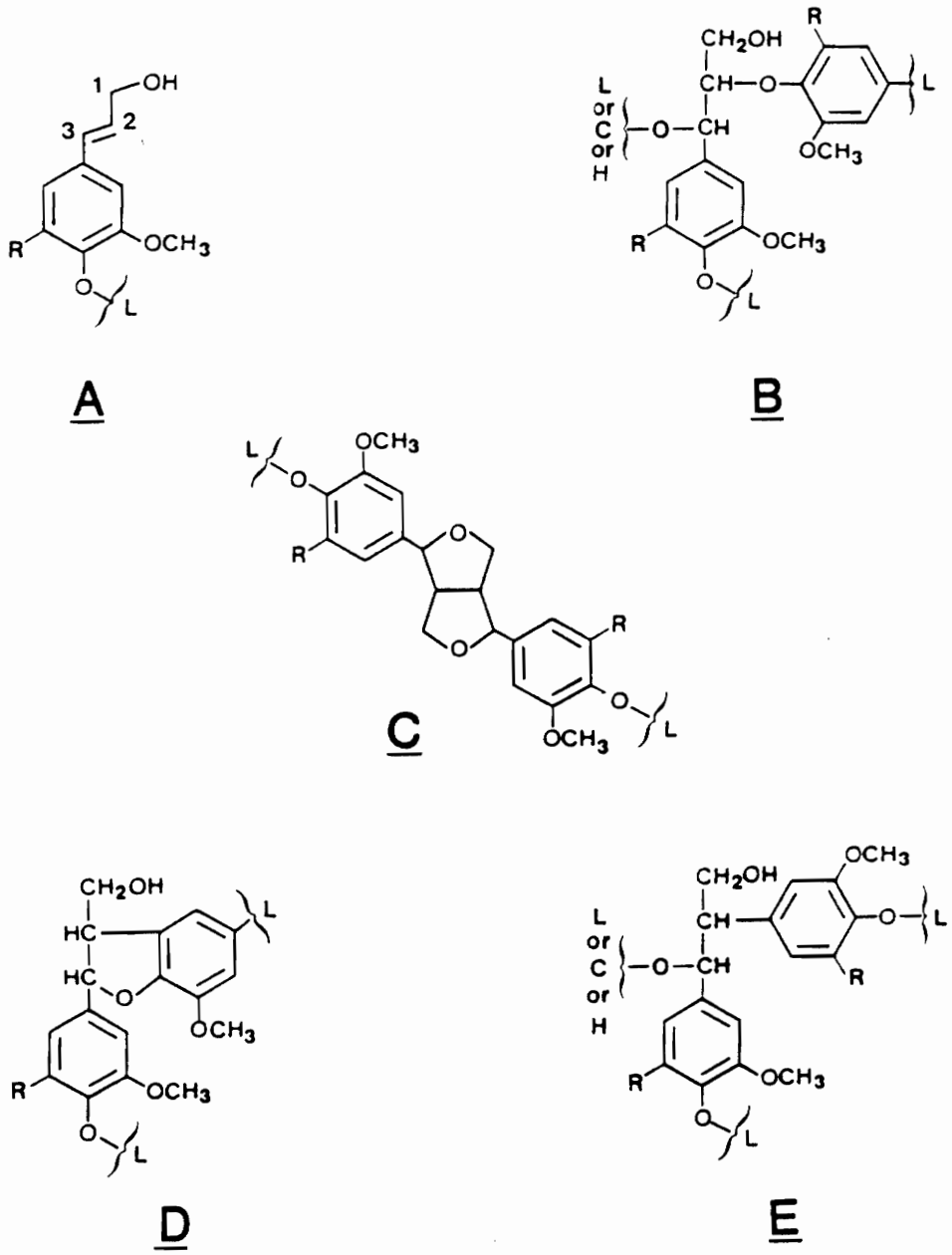


Figure 11. Dominant bonding patterns in lignin.

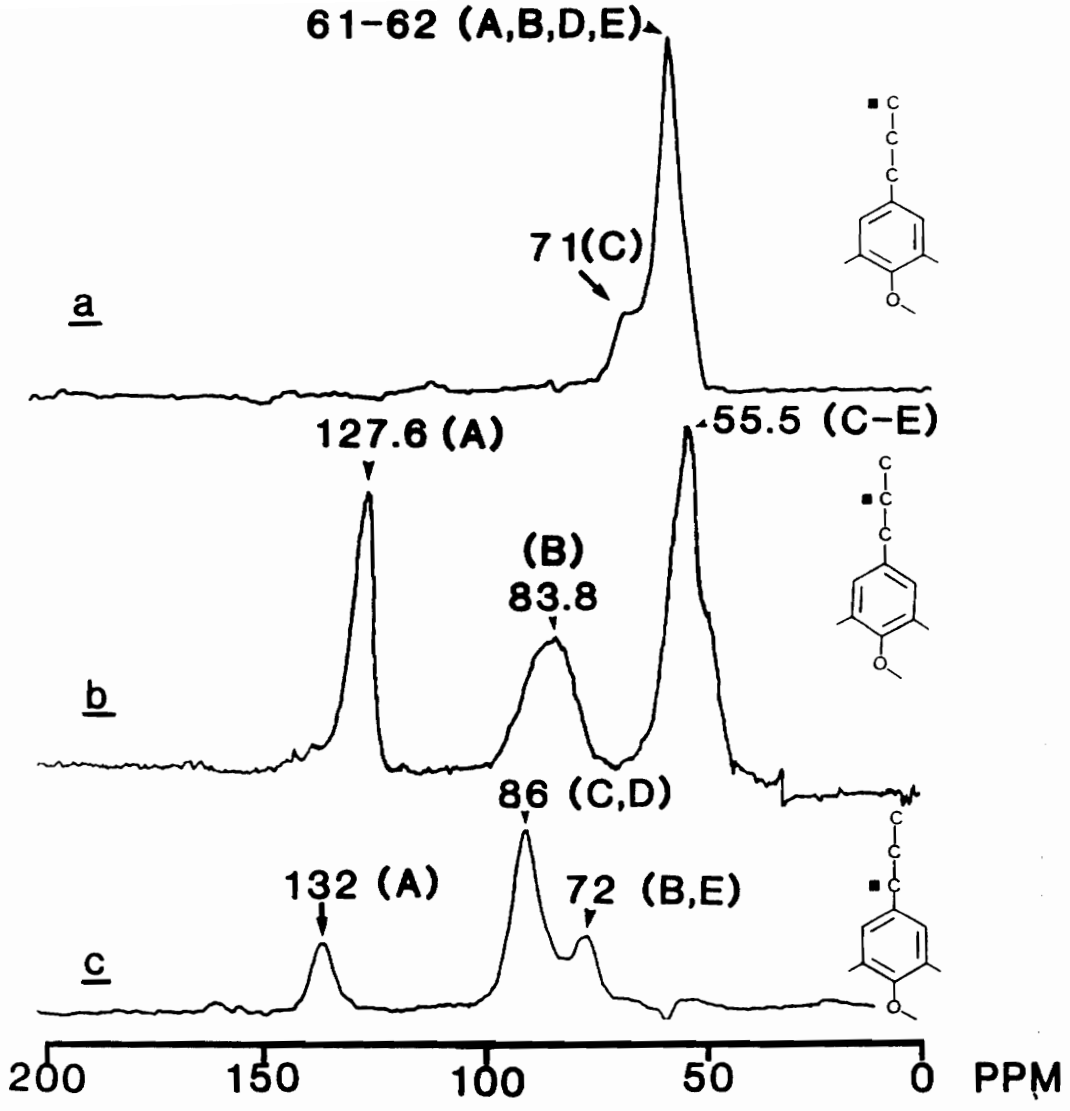


Figure 12. Solid-state ^{13}C NMR spectra of DHP lignin from coniferyl alcohol (4): Difference spectra of DHP lignin from (a) [1-^{13}C] coniferyl alcohol (**4a**), (b) [2-^{13}C] coniferyl alcohol (**4b**) and (c) [3-^{13}C] coniferyl alcohol (**4c**).

part of the lignin macromolecule; this suggests that the exogenously-supplied precursor was reduced to hydroxymethyl-containing derivatives. This observation is consistent with our present understanding of the biochemistry of lignin precursor formation in vascular plants. The metabolism of phenylalanine-derived hydroxycinnamic acids - *p*-coumaric (**23**), ferulic (**30**) and sinapic (**32**) acids, involves successive enzymatic reductions to give rise to the respective monolignols - *p*-coumaryl (**5**), coniferyl (**4**) and sinapyl (**6**) alcohols (Figure 3). These cinnamyl alcohols in turn serve as substrates for wall-bound peroxidases which catalyze the final polymerization step. Undoubtedly, we have shown that the exogenously-supplied ferulic acid (**30**) went through this metabolic process to form the appropriate alcohols, which were ultimately incorporated into the lignin of the tissue.

The resonance at 63 ppm is consistent with substructures **A, B, D** and **E**, since resonances for appropriate model compound substructures are obtained in the 60-63 ppm range of the solution-state spectrum (137). At present, due to severe line broadening in the solid-state spectrum, these resonances do not separate into individual signals. At best, it can only be claimed that the resonance at 63 ppm corresponds to the coincident peaks for these hydroxymethyl-containing substructures. As a result, the relative importance of each substructure in the lignin macromolecule cannot be determined. The shoulder at 72 ppm suggests the presence of pinoresinol-like substructures (**C**, R = H) or syringaresinol (**C**, R = OCH_3) moieties in the lignified tissue. Note that in a previous report, the β-β' substructure in the milled-wood lignin of *Populus* × *euramericana*, another hardwood, was determined by 2D-INADEQUATE ^{13}C NMR spectroscopy to consist exclusively of the syringaresinol type [7]. The signal enhancements at 170.3 and 174.1 ppm correspond to carboxylic acids in either esterified or carboxylic acid-unbound forms.

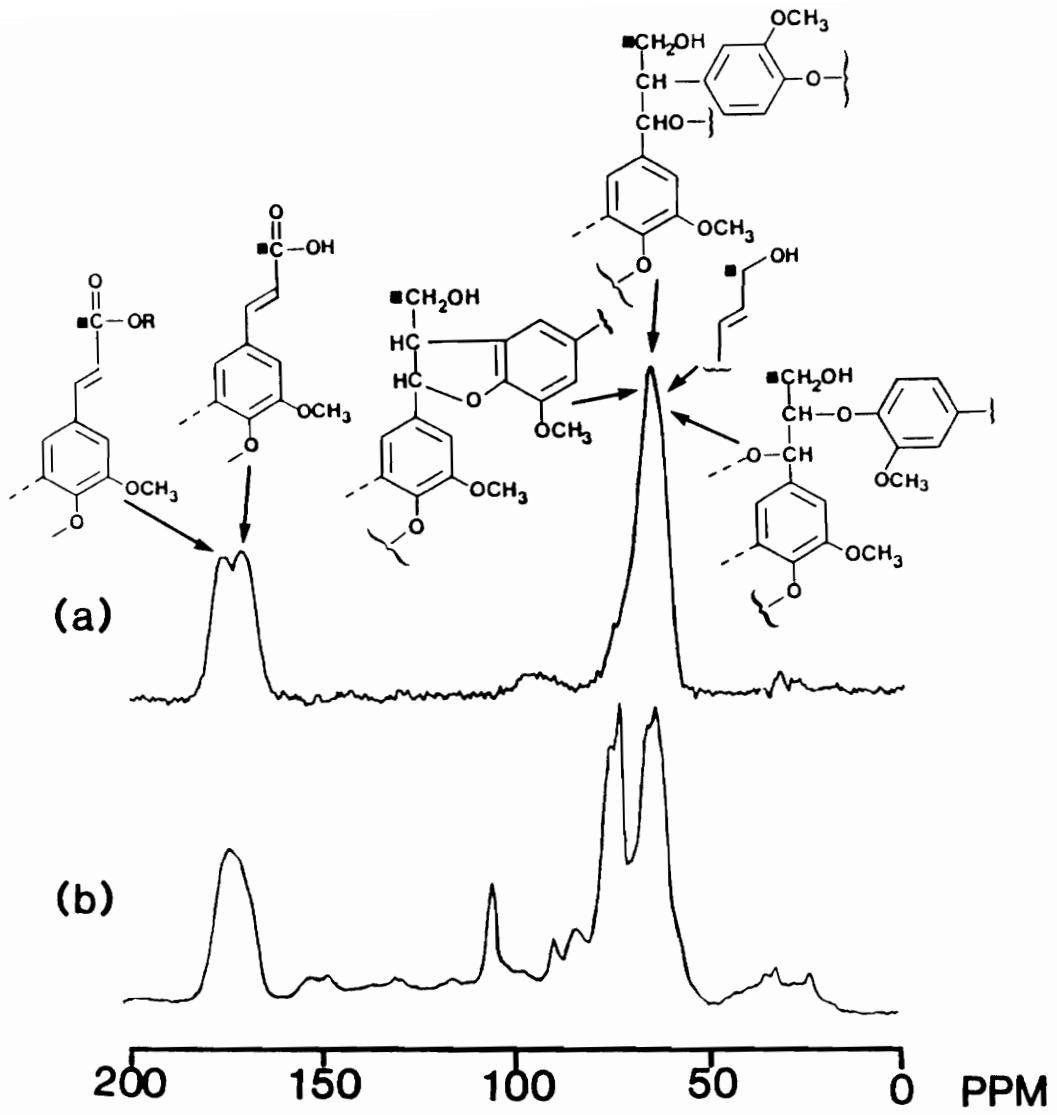


Figure 13. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots administered [1-^{13}C] ferulic acid (30a): (a) Difference spectrum, and (b) Enhanced spectrum.

Upon comparison of this spectrum with the solid-state ^{13}C NMR spectrum of a DHP polymer obtained from [1-^{13}C] coniferyl alcohol (**4a**), a resemblance of the upfield signals (δ 55-75) in both spectra was observed due to overlapping resonances of hydroxymethyl-containing substructures **A, B, D** and **E**. Consequently, no judgment on the relative frequencies of these bonding environments in the two lignins could be made. One striking difference observed between the spectra of DHP lignin and that of the lignified tissue is the presence of resonances due to ferulic acid moieties in the latter. There are at least 2 possibilities which could explain the occurrence of these signals in the spectrum of the lignified tissue: 1) they are due to ferulic (**30**) and/or sinapic (**32**) acid moieties or related metabolites occurring as an integral part of lignin *in situ*; or 2) they are due to ferulic acid (**30**) precursor or some modified derivatives (e.g., CoA esters) not incorporated into lignin (and possibly awaiting metabolism and incorporation into lignin). This second possibility could be determined by analysis of plant tissues initially grown in media with the enriched precursor, followed by growth in precursor-free media. If the first case holds, then corresponding signal intensities for ferulic acid (**30**) would not be diminished by further metabolism. Otherwise, the opposite would suggest that turnover of ferulic acid (**30**) had occurred.

5.1.5 Three-week [1-^{13}C] ferulic acid (30a) incorporation into *L. leucocephala*, followed by one-week growth in precursor-free media

In order to determine the turnover of exogenously-supplied ferulic acid (**30**) or its derivatives in lignifying root cells, *L. leucocephala* plants were initially grown hydroponically under aseptic conditions as described, in nutrient media containing [1-^{13}C] ferulic acid (**30a**). On the fourth week, the plants were allowed to continue

growth in media without the precursor. The plants were harvested, freeze-dried and shredded, and the solid-state ^{13}C NMR recorded.

In a separate experiment, *L. leucocephala* seedlings were hydroponically grown in media containing [1-^{13}C] ferulic acid (**30a**), and then harvested after three weeks. The solid-state ^{13}C NMR spectrum of the resulting freeze-dried root tissue was obtained. The results for these two experiments are shown in Figure 14.

The solid-state ^{13}C NMR spectra of three-week (Figure 14b) and four-week (Figure 13) old *L. leucocephala* root tissues are essentially the same. However, for the root tissue of four-week old plants from whose media [1-^{13}C] ferulic acid (**30a**) was withdrawn on the last week, a conspicuous reduction in the intensity of the signals at δ 170-174 was observed (Figure 14a). These results are interpreted as follows: On the first three weeks of plant growth in media containing [1-^{13}C] ferulic acid (**30a**), the plants were not able to utilize all of the exogenously supplied precursors. However, during the fourth week of hydroponic growth in precursor-free media, the remaining precursors were metabolized and subsequently deposited in the cell walls as lignin. Thus, the small resonances for carboxylic acids (or their esters) observed in the solid-state ^{13}C NMR spectrum of *L. leucocephala* root tissue administered [1-^{13}C] ferulic acid (**30a**) were due to ferulic acid (**30**) or some related intermediate(s) *en route* to lignification.

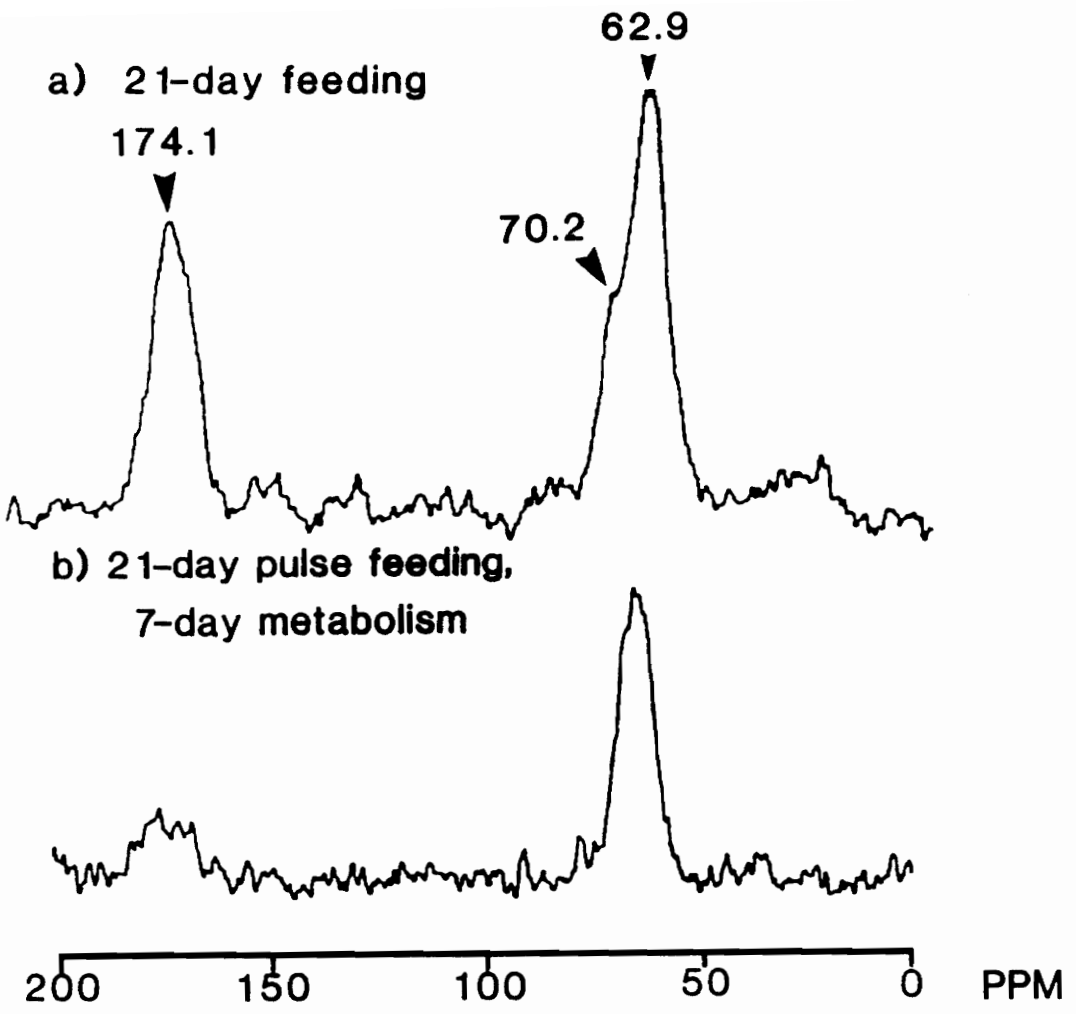


Figure 14. **Solid-state ^{13}C NMR spectra of *L. leucocephala* roots in experiments to determine turnover of ferulic acid (30):** Difference spectrum of *L. leucocephala* roots grown (a) for three weeks in media containing ferulic acid (30), and (b) for three weeks in media containing ferulic acid (30) followed by one more week of growth in precursor-free media.

5.1.6 Incorporation of [2-^{13}C] ferulic acid (30b) into *L. leucocephala* root tissue

The difference and enhanced spectra of [2-^{13}C] ferulic acid-fed *L. leucocephala* root tissue are shown in Figures 15a-b, respectively. The most dominant resonance in the difference spectrum (Figure 15a) is at 82.7 ppm. This signal corresponds to a C-2 bonding environment shown in substructure **B**, more commonly described as the β-O-4′ linkage. While this has long been thought to constitute the most prevalent bonding pattern in lignin [36,75], this is the first direct evidence for its predominance *in vivo*.

The other signals are assigned as follows: The broad but less intense resonance at 54 ppm corresponds to substructures **C** to **E**, while that at 127.5 ppm is due to substructure **A** (i.e., coniferyl (**4**) or sinapyl (**6**) alcohol bound to the rest of the lignin polymer via a phenolic ether linkage). The small resonance at δ 117.4 can be attributed to the presence of free or covalently-bound hydroxycinnamic acid moieties, the intensity of which would be expected to collapse if plants were allowed to metabolize the precursor further.

Comparison of the solid-state ^{13}C NMR spectrum of the [2-^{13}C] ferulic acid-enriched lignified tissue (Figure 15) with that for a DHP lignin formed from [2-^{13}C] coniferyl alcohol (**4b**) (Figure 12b) shows that the intensities of the corresponding resonances are essentially inverted. It was earlier noted that the resonance with the highest intensity (δ 82.7) in the lignified tissue corresponds to the β-O-4′ substructure (**B**). In the DHP polymer, however, the intensity of this signal was dwarfed by the resonances at δ 127.6 and at δ 55.5 corresponding to substructure **A** and the coincident resonances of substructures **C-E**, respectively. The latter resonances, however, were not as prominent in the lignified tissue. These discrepancies suggest that the relative abundance of the actual

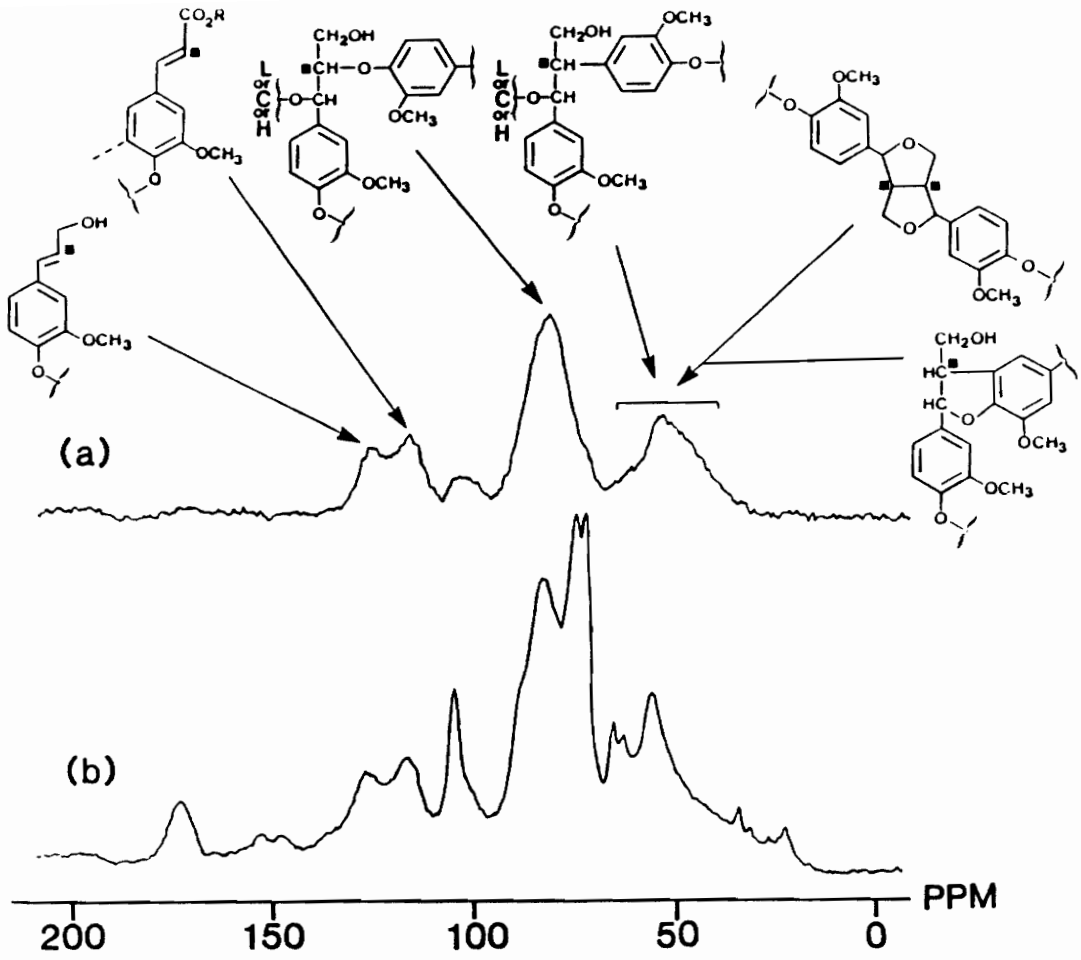


Figure 15. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots administered [2-^{13}C] ferulic acid (30b): (a) Difference spectrum, and (b) Enhanced spectrum.

bonding patterns of lignin *in situ* differs from that of DHP lignin. Thus, artificially-prepared DHP lignin polymer may not be an adequate model for representing lignin structure *in situ*. This is not surprising since *in vitro* synthesis of DHP lignin fails to take into account temporal and spatial deposition of particular monomers, the regulation of polymerization in the plant, and the fact that the natural lignification process occurs within a carbohydrate matrix.

5.1.7 Incorporation of [3-^{13}C] ferulic acid (30c) into *L. leucocephala* root tissue

The ^{13}C difference and enhanced spectra of [3-^{13}C] ferulic acid (**30c**) enriched *L. leucocephala* root samples are shown in Figures 16a-b, respectively. The resonances observed in the difference spectrum support the prevalence of the β-O-4′ linkage for lignin *in vivo*, a conclusion made on the basis of the spectrum obtained from the [2-^{13}C] ferulic acid (**30b**) feeding experiments. Thus, the most intense resonance (74.7 ppm) was assigned to a benzylic carbon in a bonding arrangement in which the adjacent carbon (Cβ) is involved in a β-O-4′ linkage (as in substructure **B**) [179].

In addition, the involvement of the benzylic carbon in the formation of an ether bond with either a carbohydrate or another aryl group (which could be part of the lignin) was also observed. This is evident in the presence of the large resonance at 83.2 ppm, previously shown to be the signal at which an α-O-carbohydrate (or an α-O-aryl) functionality in lignin-carbohydrate model systems appears [166,179]. This result is significant in the light of the controversial issue of the existence of chemically-labile "lignin-carbohydrate complexes" formed during plant cell wall development. This peak

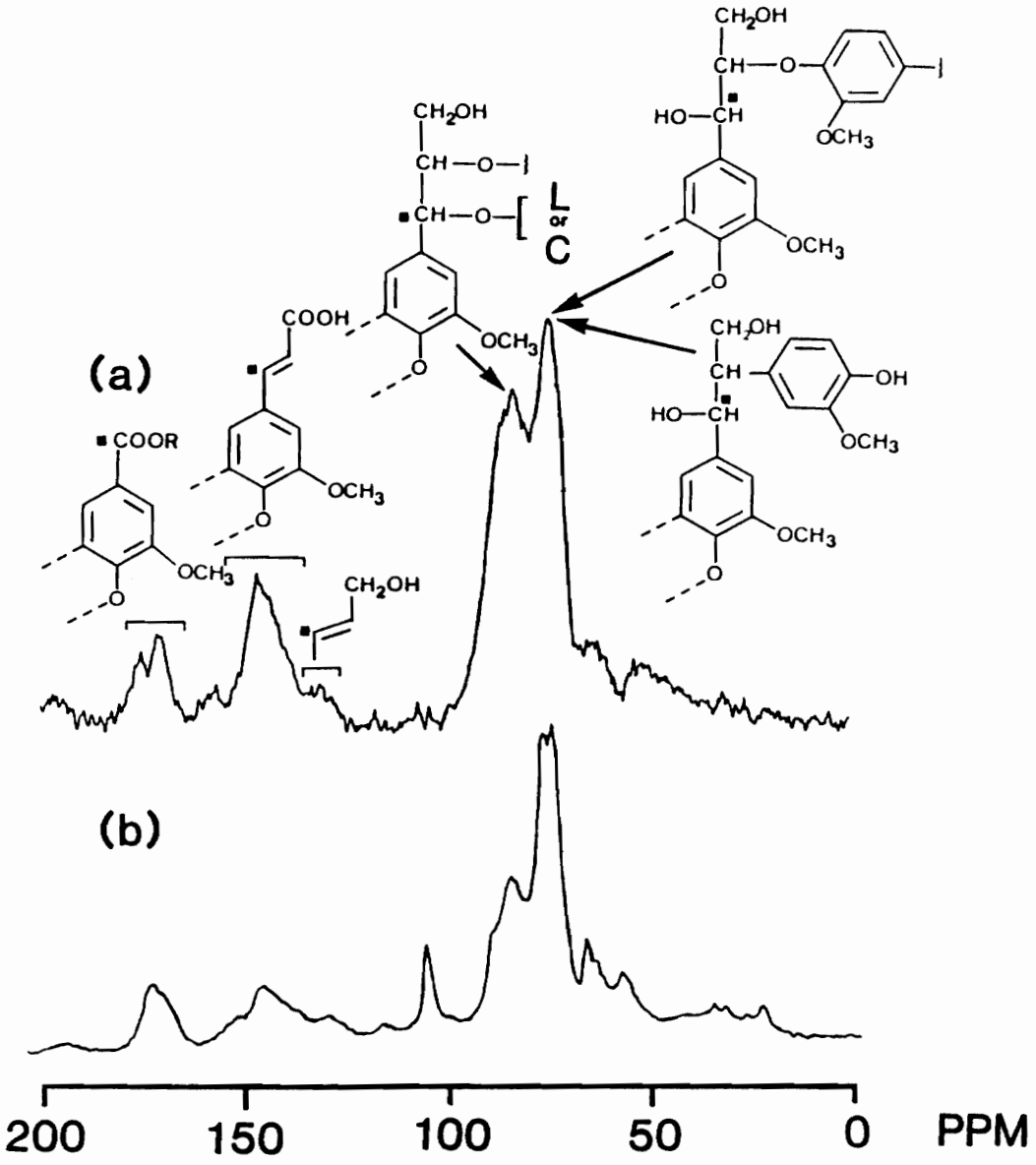
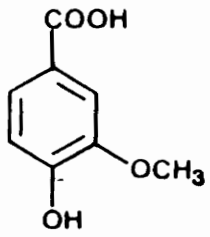
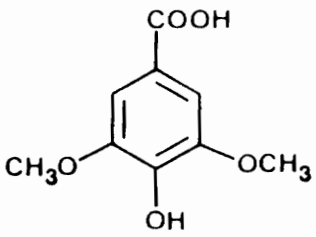


Figure 16. Solid-state ^{13}C NMR spectra of *L. leucocephala* roots administered [3-^{13}C] ferulic acid (30c): (a) Difference spectrum, and (b) Enhanced spectrum.

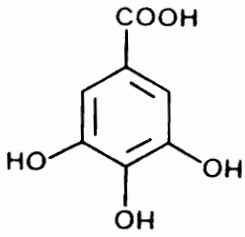
unequivocably lends support to the existence of such complexes, occurring at Cα of certain monolignol residues in the lignified tissue of a woody angiosperm. The likelihood of small amounts of Cα-Cβ cleavages, leading to the formation of vanillic (**54**) and syringic (**55**) acids is suggested by the occurrence of signals at 170.1 and 174.3 ppm in their esterified and free acid forms, respectively. Radiolabelling studies for gallic acid (**56**) biogenesis indicated the possibility of a dual pathway for the synthesis of this plant phenolic, with the minor pathway entailing Cα-Cβ cleavage of phenylpropanoid intermediates [72]. Thus, the phenylpropanoid bond cleavage observed in the present study (and with previous labelling experiments of *T. aestivum*, [116]) is not unusual. Further evidence for the presence of ferulic acid (**30**) in free or unbound form and for hydroxycinnamyl alcohol moieties as previously noted with the [1-^{13}C] and [2-^{13}C] ferulic acid (**30a,b**)-enriched root tissues was shown by the presence of small resonances at δ 146.5 and δ 129-137, respectively. An explanation for the occurrence of free or bound carboxylic acid moieties was presented earlier, when the ^{13}C NMR spectral data for the root tissue administered [1-^{13}C] ferulic acid (**30a**) was discussed.



(54)



(55)



(56)

It is difficult to correlate the solid-state ^{13}C NMR spectrum of the lignified root tissue with that obtained for DHP lignin synthesized from [3-^{13}C] coniferyl alcohol (**4c**). There were 3 resonances observed in the latter (Figure 12c). The most dominant signal is at 132 ppm due to substructure **A**, and two smaller ones at 86 ppm (due to substructures **C** and **D**) and at 72 ppm (due to substructures **B** and **E**). None of these signals precisely matched those observed in the ^{13}C-enriched tissue. The results of these comparisons again suggest differences in bonding patterns and their relative frequencies between DHP lignin preparations and lignin in the plant tissue, further emphasizing the need for re-evaluating the use of DHP lignin to represent lignin *in situ*.

5.1.8 Effect of ferulic acid (30) uptake on lignin metabolism in *L. leucocephala*

We have shown that enriching specific carbon atoms of lignin in intact woody plant tissue with ^{13}C followed by solid-state ^{13}C NMR spectroscopic examination of the plant material allowed direct examination of the bonding environments of carbon atoms involved in inter-unit linkages in the lignin macromolecule. Consequently, tentative assignments were made for the enhanced resonances observed in the difference spectra of the various root specimens. This is the first time this has been accomplished for woody plant material.

It is important to show that the ^{13}C NMR spectral resonances obtained correspond to lignin whose formation (i.e., amount and composition) was essentially unaffected by uptake of ferulic acid (**30**). Thus, lignin content determination as well as nitrobenzene oxidation of hydroponically-grown *L. leucocephala* tissues were carried out to assess the

effects of precursor treatment on the lignification process in these plants. These experiments were performed on tissues successively extracted with benzene/ethanol (2.5:1, v/v), ethanol and distilled water using a micro-Soxhlet apparatus.

Table 3 shows the results obtained for lignin determination by the acetyl bromide method (127) and for the monomeric composition as determined by nitrobenzene oxidation. For purposes of comparison, data for soil-grown plants was also presented. There appears to be no significant difference in the lignin content between the roots of soil- and hydroponically-obtained plants grown in the presence of ferulic acid (**30**). However, a substantial difference in the lignin content of the stems is evident. With respect to monomeric composition, it appears that uptake of ferulic acid (**30**) resulted in a slightly higher guaiacyl content and lower *p*-hydroxyphenyl content in the roots, but not in the stems.

5.1.9 Incorporation of [U-^{14}C] phenylalanine (7′) into *L. leucocephala*

It should now be apparent that one of the limitations of ferulic acid (**30**) as a precursor is its inability to be translocated into all parts of the plants, particularly the stems. No enhancement of the bonding environments of the side-chain carbon atoms of lignin in stems was observed, due primarily to low or negligible translocation of the precursor from the roots. In addition, slight effects on lignification and some perturbation of monomeric composition of lignin formed in *L. leucocephala* administered with ferulic acid (**30**) were observed.

Tracer experiments with [U-^{14}C] phenylalanine (**7′**) were performed to determine if it was a better precursor not only in terms of translocation into the various portions of the

Table 3. Lignin content and monomeric composition of *L. leucocephala* grown in soil and in hydroponic media containing ferulic acid (**30**).

	Soil-Grown				Hydroponically-Grown with Ferulic Acid (**30**)			
	Roots		Stems		Roots		Stems	
	1[a]	2[a]	1	2	1	2	1	2
Lignin Content (%) [b]	15.51	17.24	13.27	16.00	16.93	16.70	9.42	10.15
Molar Ratio[c]								
[H]/[V][d]	0.144	0.138	0.111	0.111	0.063	0.063	0.103	0.109
[S]/[V]	0.561	0.573	0.875	0.898	0.486	0.481	0.971	0.979
[H]/[V]/[S]	8:59:33	8:58:33	6:50:44	6:50:45	4:65:31	4:65:31	5:48:47	5:48:47

[a] Numbers 1 and 2 refer to sample used for analysis.
[b] Determined by acetyl bromide method, expressed as % of dry tissue.
[c] Determined by nitrobenzene oxidation.
[d] H = *p*-hydroxybenzaldehyde (**40**); V = vanillin (**41**); S = syringaldehyde (**42**).

plant, particularly the aerial parts of hydroponically-grown *L. leucocephala*, but also in terms of further avoiding distortions of the lignification process and monomeric composition as a result of precursor uptake. Thus, four-week old *L. leucocephala* seedlings grown in the presence of [U-^{14}C] phenylalanine (**7′**) were harvested, separated into component tissues, and freeze-dried. The dried roots and stems were extracted with benzene-ethanol (2.5:1, v/v), and radioactivity in the soluble and insoluble fractions determined. The results for [U-^{14}C] phenylalanine-fed plants, as well as for those simultaneously grown in the presence of [2-^{14}C] ferulic acid (**30′**), are compared in Table 4.

Both [2-^{14}C] ferulic acid (**30′**) and [U-^{14}C] phenylalanine (**7′**) were incorporated into the root sections of the plants at comparable rates. It should therefore be expected that the administration of ^{13}C-specifically labelled phenylalanine (**7a-c**) would lead to enhancements of bonding environments of specific carbon atoms in the lignin formed in the roots similar to those observed with ferulic acid (**30**). One important distinction between the two precursors as far as distribution of radioactivity in the roots is concerned, is the higher proportion of benzene/ethanol soluble radioactivity with phenylalanine (2× that of [2-^{14}C] ferulic acid-treated plants). This soluble radioactivity accounts for as much as 21% of the total radioactivity in the phenylalanine-treated root tissue, compared to only ~13% in those treated with [2-^{14}C] ferulic acid (**30′**). This result proves that our apprehensions regarding the use of phenylalanine (**7**) as a precursor were not unfounded. We speculated that since phenylalanine is an intermediate for the biosynthesis of a greater number of solvent-extractable components in plants, then plants so administered could utilize the phenylalanine (**7**) precursors for (poly)flavonoids and proteins, in addition to lignans and suberins, which can be derived from either precursor.

Table 4. Incorporation of [2-^{14}C] ferulic acid (**30′**) and [U-^{14}C] phenylalanine (**7′**) into the lignified tissue of *L. leucocephala*.

Organ	Fraction	Radioactivity in Ferulic Acid (**30**) Equivalents[a]			
		[2-^{14}C] Ferulic Acid (**30′**)		[U-^{14}C] Phenylalanine (**7′**)[b]	
		1[c]	2[c]	1	2
Roots	B/E[d] Extractable	0.67	0.52	1.05	1.02
	Insoluble Residue	3.69	4.14	3.87	3.76
Stems	B/E Extractable	0.01	0.01	0.07	0.12
	Insoluble Residue	0.04	0.02	1.79	2.26

[a] Ferulic acid equivalents (FAE) is calculated as follows:

$$\text{FAE} = \frac{\text{Total radioactivity in residue remaining after extraction}}{\text{Specific activity of precursor fed (dpm/mg)}} \times \frac{1}{\text{Dry weight of tissue (mg)}} \times 100$$

[b] Corrected for formula weight difference between ferulic acid (**30**) and phenylalanine (**7**).
[c] Numbers 1 and 2 refer to sample used for analysis.
[d] B/E = Benzene/ethanol (2.5:1, v/v).

Only [U-^{14}C] phenylalanine (7′) was efficiently translocated into the aerial plant portion (Table 4). Furthermore, following benzene/ethanol extraction of the stems from phenylalanine-treated plants to determine distribution of radioactivity between soluble and insoluble fractions, it was determined that only ~4.5% of the observed radioactivity was solvent extractable. Extraction with benzene/ethanol (2.5:1, v/v) is commonly used as a first step in the preparation of extractive-free plant tissues. It is generally useful for removing low-molecular weight, non-polar constituents, leaving behind polar and/or high molecular weight components like tannins and proteins, which are virtually insoluble in this solvent. Because phenylalanine (7) can be metabolized into these components, it was necessary to perform a more rigorous extraction procedure that could remove them from *L. leucocephala* plant tissues. In this way, their contribution to (or interference with) the resulting solid-state ^{13}C NMR spectra of [^{13}C] phenylalanine-enriched tissues could be assessed. For this purpose, extraction of [U-^{14}C] phenylalanine-fed tissues was carried out by first grinding them in a mortar and pestle in an aqueous medium, followed by centrifugation of the homogenate to separate H_2O-soluble radioactivity from that of the solid residue, and finally, successive washings of the resulting pellet to remove organic-soluble materials (see Experimental section). The results in Table 5 show an increase in the proportion of radioactivity in the soluble fraction of both roots (~35%) and stems (~13%) relative to that revealed by merely extracting with benzene/ethanol. This clearly demonstrated that [U-^{14}C] phenylalanine (7′) was incorporated not only into high molecular weight lignins, but also to other, largely water-soluble, components like (poly)flavonoids and proteins including low molecular weight lignins, particularly in the roots (Table 5). Nevertheless, the greater portion of absorbed radioactivity still remained in the insoluble residue, especially in the stems. Thus, in this tissue, the contribution of soluble, phenylalanine-derived metabolites to the overall ^{13}C NMR signals should be minimal.

Table 5. Distribution of radioactivity in *L. leucocephala* administered [U-^{14}C] phenylalanine (7′).

Fraction	Radioactivity in Organ (%)[a]			
	Roots		Stems	
	1[b]	2[b]	1	2
Aqueous soluble	30.3	37.5	11.78	13.65
Organic soluble	2.00	1.12	0.70	0.76
Insoluble residue	67.68	61.34	87.52	85.59
Recovery	72.23	75.86	87.27	95.80

[a] As per cent of total radioactivity in tissue determined by combustion.
[b] Numbers 1 and 2 refer to sample used for analysis.

5.1.10 Effect of phenylalanine (7) on lignification and monomeric composition

The lignin content and monomeric composition of *L. leucocephala* administered natural abundance phenylalanine (**7**) were analyzed to determine whether uptake of phenylalanine (**7**) resulted in substantial distortion of lignin metabolism. Results obtained from acetyl bromide lignin determination and nitrobenzene oxidation of stem and root sections successively extracted with benzene/ethanol (2.5:1, v/v), ethanol, and distilled water are shown in Table 6. Side by side with these results are those obtained for soil-grown plants, again for purposes of comparison. The data suggest that the lignin content of the roots of *L. leucocephala* grown hydroponically in media containing phenylalanine (**7**) differs from that of soil-grown plants. To some extent, administration of this precursor through the roots resulted in interferences with the biosynthetic machinery for lignin and phenylpropanoid metabolism in this plant tissue. The stems, however, appear to be unaffected. As earlier noted, (see Table 5), one difference between these tissue sections from [U-^{14}C] phenylalanine-treated plants was the much larger amount of soluble radioactivity recovered in the roots compared to the stems.

The results obtained from the nitrobenzene oxidation of *L. leucocephala* root and stem sections are also shown in Table 6. As mentioned earlier, nitrobenzene oxidation provides a qualitative estimate of the monomeric components of lignin, and to some extent, is used to ascertain the presence of lignin in plant tissues. It is evident that additional differences between the use of ferulic acid (**30**) and phenylalanine (**7**) as precursors are underscored by the nitrobenzene oxidation data. It was earlier noted (Table 3) that treatment of *L. leucocephala* with ferulic acid (**30**) resulted in an almost 50% reduction

Table 6. Lignin content and monomeric composition of *L. leucocephala* grown in soil and in hydroponic media containing phenylalanine (7).

	Soil-Grown				Hydroponically-Grown with Phenylalanine (7)			
	Roots		Stems		Roots		Stems	
	1[a]	2[a]	1	2	1	2	1	2
Lignin Content (%) [b]	15.51	17.24	13.27	16.00	12.66	11.76	14.57	16.39
Molar Ratio[c]								
[H]/[V][d]	0.144	0.138	0.111	0.111	0.152	0.148	0.094	0.069
[S]/[V]	0.561	0.573	0.875	0.898	0.653	0.628	1.030	0.998
[H]/[V]/[S]	8:59:33	8:58:33	6:50:44	6:50:45	8:55:36	8:56:35	4:47:49	3:48:48

[a] Numbers 1 and 2 refer to sample used for analysis.
[b] Determined by acetyl bromide method, expressed as % of dry tissue.
[c] Determined by nitrobenzene oxidation.
[d] H = *p*-hydroxybenzaldehyde (40); V = vanillin (41); S = syringaldehyde (42).

of *p*-hydroxyphenyl moieties in the root tissue. On the other hand, phenylalanine (**7**), did not seem to have the same effect, in either the root or stem sections. Theoretically, these results appear logical since phenylalanine (**7**) is a precursor to all monolignols while ferulic acid (**30**) is not. In the stems of ferulic-acid treated plants (where ferulic acid, **30**, was not translocated) and those administered phenylalanine (**7**), composition of lignin was essentially identical with soil-grown plants. Thus, uptake of phenylalanine (**7**) into the stem did not cause any significant modification of the monomeric composition of lignin in this tissue.

The data in Tables 3 and 6 support the view of heterogeneity of lignin between tissues, even in the same plant. Whereas in the stems, guaiacyl moieties (indicated by vanillin, **41**) are present in almost the same amounts as syringyl moieties (indicated by syringaldehyde, **42**), guaiacyl and syringyl moieties are present in an almost 2:1 ratio in the roots. This suggests differences in lignin formed in the various plant tissues and further underscores the need for caution in classifying lignins simply by their taxonomic origins.

5.1.11 Incorporation of [1-^{13}C] phenylalanine (7a) into *L. leucocephala* roots and stems

Having resolved the difficulty with translocation of ferulic acid (**30**) into the aerial sections of *L. leucocephala* by using another precursor, phenylalanine (**7**), and having demonstrated that the latter was a more "natural" precursor in terms of its effect on lignification and monomeric composition, we turned our attention to ^{13}C-labelling experiments with specifically-labelled phenylalanine (**7a-c**). Hydroponically-grown *L.*

leucocephala plants were administered [1-^{13}C] phenylalanine (**7a**) for 28 days as before, harvested, separated into component tissues, dried, and the resulting lyophilized tissue analyzed by solid-state ^{13}C NMR spectroscopy. The difference spectra obtained by subtraction of corresponding natural abundance spectra from [1-^{13}C] phenylalanine-enriched stems and roots are shown in Figures 17a and b, respectively. The spectrum of the [1-^{13}C]-phenylalanine-fed roots was essentially similar to that obtained for the [1-^{13}C] ferulic acid-treated root tissue. Two sets of strong signals were observed, one at 63 ppm with a small shoulder at 72 ppm, and the other at 173.5 ppm. The resonance at 63 ppm was assigned to the hydroxymethyl-containing substructures **A, B, D** and **E** (Figure 11) as before, while the shoulder at 72 ppm was attributed to pinoresinol (**C**, R = H) or syringaresinol (**C**, R = OCH_3) substructures. These results further validate the findings obtained for [1-^{13}C] ferulic acid (**30a**), where the corresponding solid-state ^{13}C NMR spectrum (Figure 13) demonstrated that ferulic acid (**30**) precursor was metabolized into the appropriate monolignols, then subsequently polymerized into lignin. The resonance at 173.5 ppm may again be due to carboxylic acid/esters, or yet unconverted [1-^{13}C] phenylalanine (**7a**). The latter would be expected at δ 174.7, but probably cannot be distinguished from cinnamic acids (or their esters) by solid-state ^{13}C NMR. Remember that by allowing plants previously grown in media containing [1-^{13}C] ferulic acid (**30a**) to continue growth in media without precursor and to metabolize excess precursors, the intensity of the resonances due to carboxylic acid/esters was reduced to near zero.

Figure 17a shows the solid-state ^{13}C NMR spectrum obtained for the stem of *L. leucocephala* grown in the presence of [1-^{13}C] phenylalanine (**7a**). This is the first time that the bonding patterns of specific carbon atoms of lignin in the stem of a woody plant tissue have ever been directly examined. Note that similar analysis of the stems of ferulic

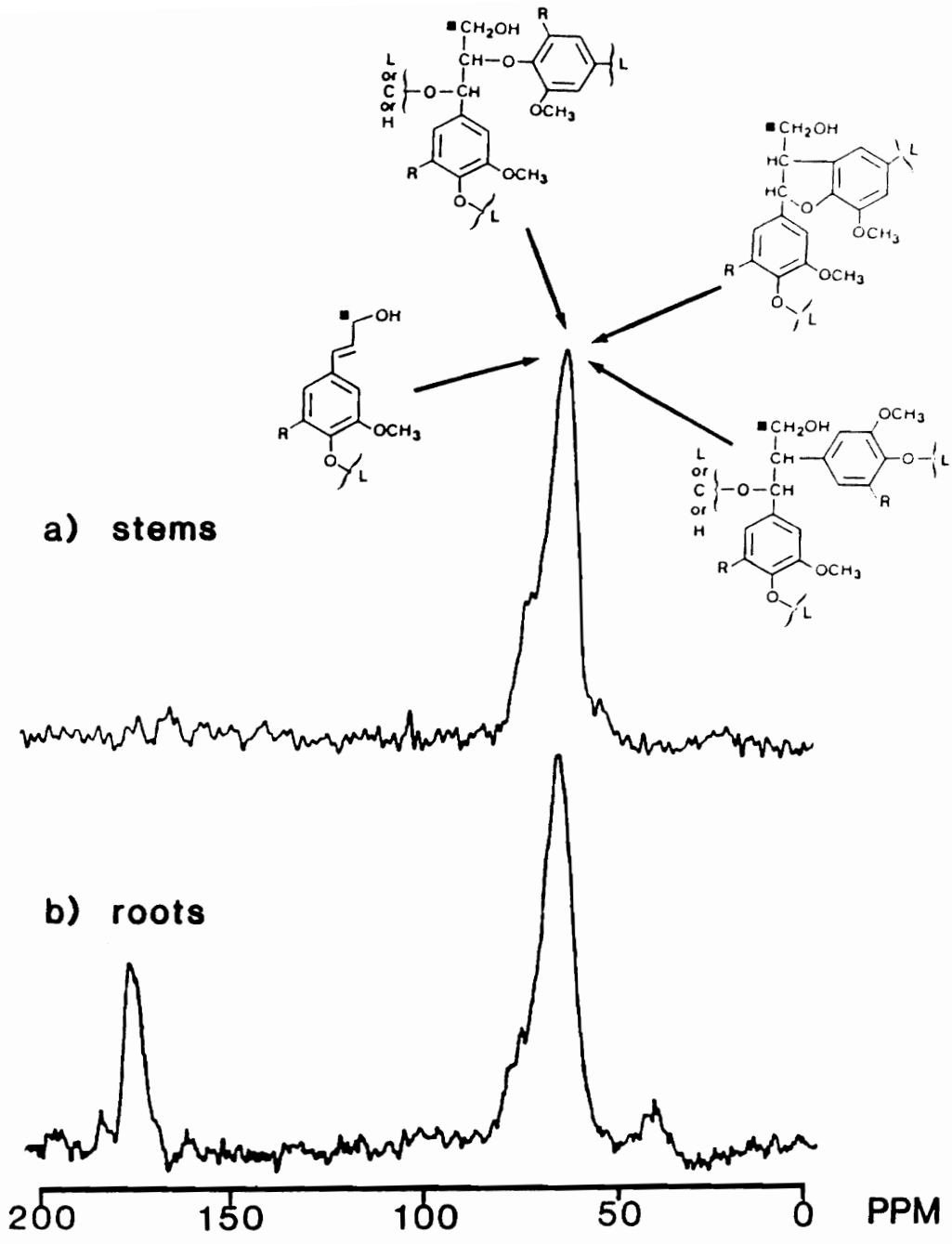


Figure 17. Solid-state ^{13}C NMR spectra of *L. leucocephala* (a) stems and (b) roots administered [1-^{13}C] phenylalanine (7a).

acid-treated plants did not result in enhancement of the solid-state ^{13}C NMR spectrum due to very low incorporation. It is significant that only one intense signal (δ 63) could be seen in the spectrum, with again a small shoulder at 72 ppm. The large resonance corresponds to substructures **A, B, D** and **E** which contain the hydroxymethyl functionality. It is also noteworthy that resonances corresponding to carboxylic acid/ester moieties (~170-174 ppm region) are absent in the stem spectrum. This large signal at 63 ppm, therefore, provides an unequivocal proof that the exogenously-supplied [1-^{13}C] phenylalanine (**7a**) was taken up by *L. leucocephala* through its roots, then efficiently translocated into the stems, where it was reduced to the monolignols (**4-6**) prior to incorporation into lignin.

5.1.12 Incorporation of [2-^{13}C] phenylalanine (7b) into *L. leucocephala* roots and stems

Results of tracer experiments with [U-^{14}C] phenylalanine (**7'**) and labelling studies with [1-^{13}C] phenylalanine (**7a**) unequivocably demonstrated that incorporation of phenylalanine (**7**) into the stem lignin of *L. leucocephala* had taken place. To complete the information for the bonding environments of C-2 and C-3 of lignin in the stem, *L. leucocephala* plants were separately grown in hydroponic units as described, in media containing either [2-^{13}C]- or [3-^{13}C] phenylalanine (**7b** or **7c**). As before, the resulting dried tissues were subjected to solid-state ^{13}C NMR spectroscopic analysis without prior extraction.

The difference spectra for stem and root tissues of *L. leucocephala* plants grown in media containing [2-^{13}C] phenylalanine (**7b**) are shown in Figures 18a-b, respectively. In the

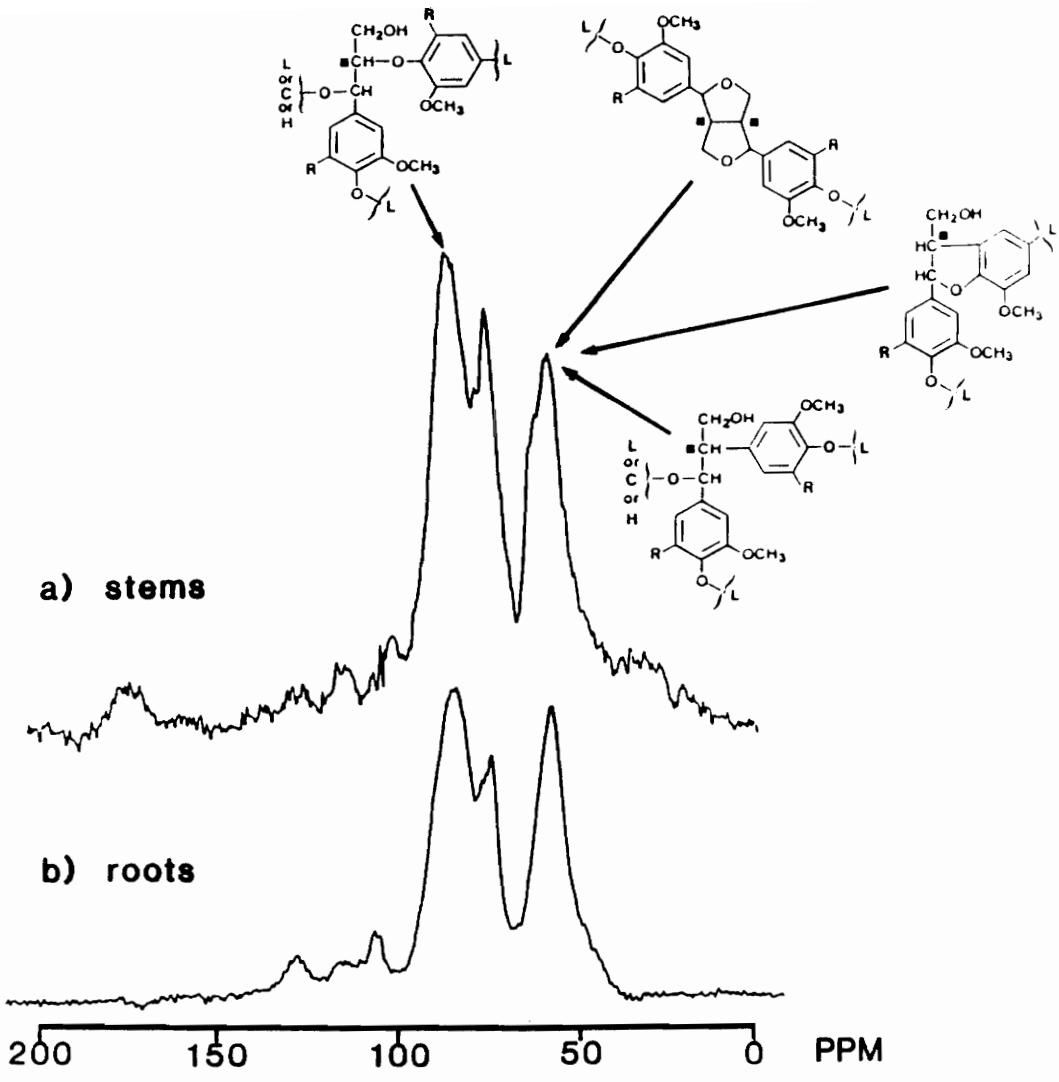


Figure 18. Solid-state ^{13}C NMR spectra of *L. leucocephala* (a) stems and (b) roots administered [2-^{13}C] phenylalanine (7b).

roots (Figure 18b), the most intense resonance is observed at 83 ppm which corresponds to substructure **B** (β-O-4 substructure). A shoulder, which is yet unassigned, is detected at 74.1 ppm. Finally, the resonance at 54-55 ppm is assigned to substructures **C, D** and **E**, and is present at an intensity almost as large as the signal at 83 ppm. This spectrum deviates significantly from that of the root tissue administered [2-^{13}C] ferulic acid (**30b**) (Figure 15), where the shoulder at 74.1 ppm is absent and the 54-55 ppm resonance occurs at a much lower intensity. In terms of relative intensities of the signals, and except for the presence of the yet unassigned peak at 74.1 ppm, the stem spectrum appears to be much more similar to that obtained for the roots administered [2-^{13}C] ferulic acid (**30b**). The most intense resonance is observed at 83 ppm with a shoulder at 74.1 ppm (this shoulder was absent in the difference spectrum of the root tissue administered [2-^{13}C] ferulic acid, **30b**). The dominance of the β-O-4′ linkage (δ 83) in the lignin of the stems is evident. The signal at 54-57 ppm, which was much reduced compared to that in the roots, was again attributed to the coincident signals for the bonding environments corresponding to substructures **C, D** and **E**. The resonance of [2-^{13}C] phenylalanine (**7b**) occurs at δ 57.1 and could have made significant contribution to the resonance observed in this region of the root spectrum. Furthermore, since phenylalanine (**7**) could undergo conversion to *p*-coumaryl alcohol (**5**), in addition to coniferyl (**4**) and sinapyl (**6**) alcohols, it would be interesting to probe if *p*-hydroxyphenylpropane linkages contributed to the intensity of the signal at 54-57 ppm. No assignment was made for the signal at 74.1 ppm. The small resonance at ~170 ppm is presumably due to incomplete subtraction, and is absent in the corresponding root spectrum. Interestingly, the resonance at 127.5 ppm observed in the spectrum of root tissues administered [2-^{13}C] ferulic acid (**30b**) (Figure 15) and which was attributed to phenolic ether-linked monolignols, was essentially absent in the stem spectrum (Figure 18a). In contrast, this resonance

made a significant contribution to the spectrum of [2-^{13}C] DHP lignin (Figure 12b) further emphasizing differences between lignin *in situ* and artificial lignin preparation.

5.1.13 Incorporation of [3-^{13}C] phenylalanine (7c) into *L. leucocephala* roots and stems

Figures 19a-b show the solid-state ^{13}C NMR spectra of *L. leucocephala* roots and stems, respectively, previously administered [3-^{13}C] phenylalanine (**7c**). The most intense resonance in the root spectrum is seen at 38 ppm; this is the first time it appeared in any of the spectra. This is followed in intensity by the signals at 74 and 83 ppm. The resonance at 38 ppm may be due to unconverted [3-^{13}C] phenylalanine (**7c**) which occurs at 37.2 ppm in the solution-state ^{13}C NMR spectrum. Partial support for this explanation is the high radioactivity (~34%) recovered in the aqueous extract of [U-^{14}C] phenylalanine-treated root tissues (Table 5). The absence of the signal at 38 ppm in the difference spectrum of [3-^{13}C] ferulic acid-treated roots (Figure 16) is just one of the remarkable differences between the spectra of the two root samples. Conversely, resonance due to hydroxycinnamic acid/ester moieties (δ 146) present in the difference spectrum of the [3-^{13}C] ferulic acid-treated roots, was absent in the roots of the [3-^{13}C] phenylalanine-treated root tissues. However, the relative intensities of the resonances at 74.7 and at 83 ppm were almost identical in each spectrum, while signals suggestive of Cα-Cβ cleavage (~170-174 ppm) were also present in both, indicating that these may be the only real signals attributable to lignin. Thus, assignments previously made for the resonance at 74.7 and 83.2 ppm with the [3-^{13}C] ferulic acid-treated root spectrum, which were attributed to C-3 in a bonding configuration as in substructure **B** (and possibly, with some contributions due to substructure **E**) and to Cα-O-aryl or

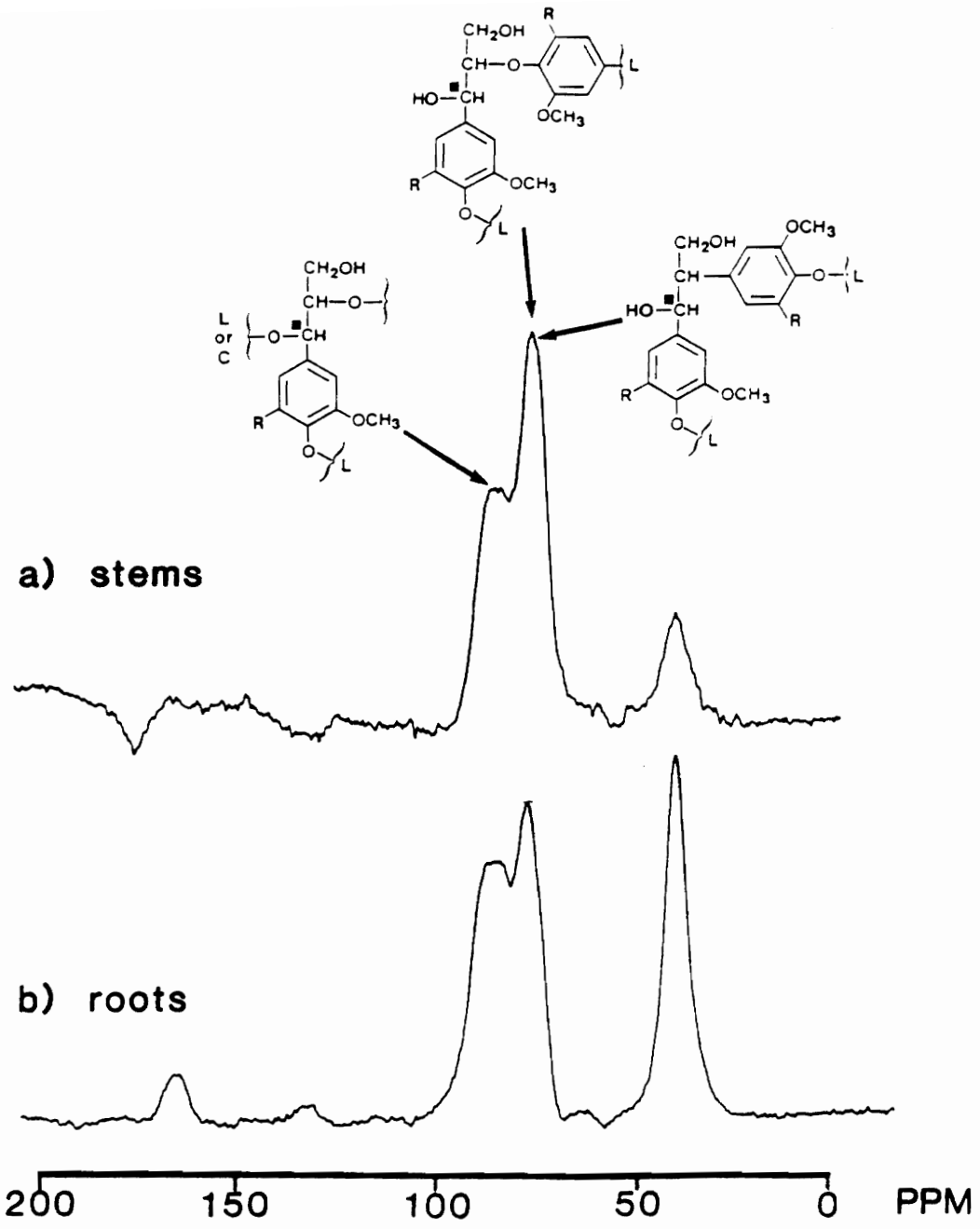


Figure 19. Solid-state ^{13}C NMR spectra of *L. leucocephala* (a) stems and (b) roots administered [3-^{13}C] phenylalanine (7c).

Cα-O-carbohydrate linked model systems, respectively, were extended in the observations with the [3-^{13}C] phenylalanine-treated root tissues.

In the difference spectrum for the stem tissues (Figure 19a), enrichments are evident at 83, 74 and at 38 ppm. The large resonance at 74 ppm is again assigned to a benzylic carbon involved in a bonding environment as in substructure **B**, which is in agreement with the assignment made for the [3-^{13}C] ferulic acid-fed root tissues. Resonances due to substructure **E** may have also contributed to the intensity of this peak. The strong resonance at 83 ppm is significant, as it provides evidence for the involvement of C-3 in an α-O-carbohydrate or α-O-aryl linkage in lignin of woody angiosperm stems. The presence of such linkage was first demonstrated by labelling and solid-state ^{13}C NMR experiments with the roots of *T. aestivum* [116] and is similar to the findings with [3-^{13}C] ferulic acid (**30c**). Notice that the resonance at ~38 ppm, which was most dominant in the root spectrum and which may be due to phenylalanine (**7**), was greatly diminished in the stem.

5.2 Lignin Biodegradation

In the first part of this study, we described the development of a novel procedure that enables us to determine the bonding environments of specific carbon atoms of lignin in woody plant tissue. Hence, dominant bonding arrangements in lignin *in situ* were identified with a degree of certitude never before made possible by examination of isolated lignin preparations. Importantly, the procedure can be used not only for investigating lignin structure and biosynthesis, but also would allow studies for improving our under-

standing of lignin utilization and biodegradation. It should now be possible to determine changes in the bonding environments of specific carbon atoms of lignin *in situ* during the process of degradation by ligninolytic organisms or by lignin-degrading enzymes derived from them.

In the second half of this study, we looked at two components, the media and the enzyme, of what could be a model lignin biodegrading system under which we could carry out biodegradative studies with our substrate - specifically ^{13}C-enriched lignin *in situ*. Dordick and co-workers [28] reported that "lignin-degrading" capabilities of horseradish peroxidase could be enhanced tremendously by performing the degradation reaction in organic solvents rather than in aqueous media. Evidence for this claim was obtained following experiments in which various lignin preparations were separately incubated for 96 h in an organic solvent (e.g., dioxane/aqueous acetate (10 mM, pH 5), 95%, v/v) containing a suspension of horseradish peroxidase and H_2O_2. As a control, lignins were suspended in aqueous acetate buffer (10 mM, pH 5.0) and subjected to enzymatic treatment as above. The respective reaction mixtures were then diluted with 2 volumes of dimethylformamide (DMF) to solubilize the lignin, and the resultant solution analyzed by gel filtration on a Sephadex LH-20 column. Allegedly, vigorous depolymerization occurred as evidenced by changes in the molecular weight profiles of lignin following incubation with horseradish peroxidase/H_2O_2. In addition, ferulic acid (**30**) was reportedly identified among the "degradation products" obtained from the incubation of a copolymer of coniferyl (**4**) and vanillyl alcohols.

The second component of the "model system" for biodegrading our lignin preparation is the enzyme to use. In 1983, Tien and Kirk [184] and Glenn and co-workers [52] independently reported that the concentrated extracellular fluid of *Phanerochaete*

chrysosporium, a white-rot fungus, contained an enzyme which could depolymerize lignin (see Review of Literature). In subsequent experiments, [53,185], purified forms of the enzyme, which were referred to as "ligninases" and later, as "lignin peroxidases", were obtained and shown to catalyze the degradation of lignin "model substrates". Other than the ability to effect degradation of low molecular weight, appropriately substituted, monomeric or dimeric phenylpropanoids, however, no further proof of lignin degrading ability by these enzymes was presented. One of the probable obstacles to gaining experimental support for the original observations is the availability of well-defined lignin substrates with which to study the biodegrading abilities of these enzymes. Clearly, ^{13}C-enriched lignin *in situ* provides an opportunity to rationally address this problem.

5.2.1 Lignin degradation by peroxidase in organic media

We repeated the experiments described by Dordick *et al.* [28] for the purpose of evaluating the viability of their procedure of depolymerizing lignin with horseradish peroxidase/H_2O_2 in organic media. This was necessary because earlier investigations failed to provide any evidence for the formation of low molecular weight products following incubation of "lignin model compounds" with horseradish peroxidase [e.g., 69]. Furthermore, polymeric lignin substrates were reported to undergo only internal rearrangement under the influence of horseradish peroxidase/H_2O_2, with no net depolymerization [69]. Thus, DHP lignin, prepared *in vitro* from coniferyl alcohol (**4**) using horseradish peroxidase and H_2O_2, was incubated for 96 h in dioxane/aqueous buffer containing horseradish peroxidase and H_2O_2 (as described in the Experimental section). Reaction mixtures were removed after 0, 24 and 96 h, and aliquots were obtained to assay for residual enzyme activity. Typical results of the enzymatic assay are

shown in Figure 20. Note that enzyme activity dropped within 24 h, but levelled off until 96 h of incubation. After this incubation, DMF was added to the remainder of the mixture to solubilize the lignin, and the resulting mixture centrifuged. The supernatant containing "degradation products" was then applied to a Sephadex LH-20 column, eluted first by using 0.1 M LiCl in DMF. The high ionic content (0.1 M LiCl) in organic solvents has been reported to partially suppress molecular association of lignin preparations during their passage through a Sephadex column [21,164]. The resulting gel filtration chromatograms of the incubation mixtures sampled at different times, as well as that of a control mixture, are shown in Figure 21. Except for a small reduction in the amount of UV absorbance in Figures 21b-c, no other differences were observed between these chromatograms and that of the control. The Sephadex LH-20 elution profiles are independent of incubation time (Figure 21); no evidence for the formation of low molecular weight products was obtained.

Dordick and co-workers [28] used dioxane as eluant in their chromatographic system for monitoring "changes" undergone by the lignin preparations following treatment with horseradish peroxidase. We decided to employ the same chromatographic procedure to see if the differences in elution profiles they observed were reproducible. The results obtained following chromatographic separation under these conditions for samples taken after 0, 24 and 96 h of incubation are shown in Figure 22. As predicted, except for the slight reduction in the UV absorbance, there were no significant differences in the profiles that would warrant an interpretation based on changes in molecular weight distribution, presumably as a result of extensive lignin degradation.

Further, after 96 h of incubation of DHP lignin with horseradish peroxidase/H_2O_2 as described, an aliquot was taken for HPLC analysis, the purpose of which was to detect

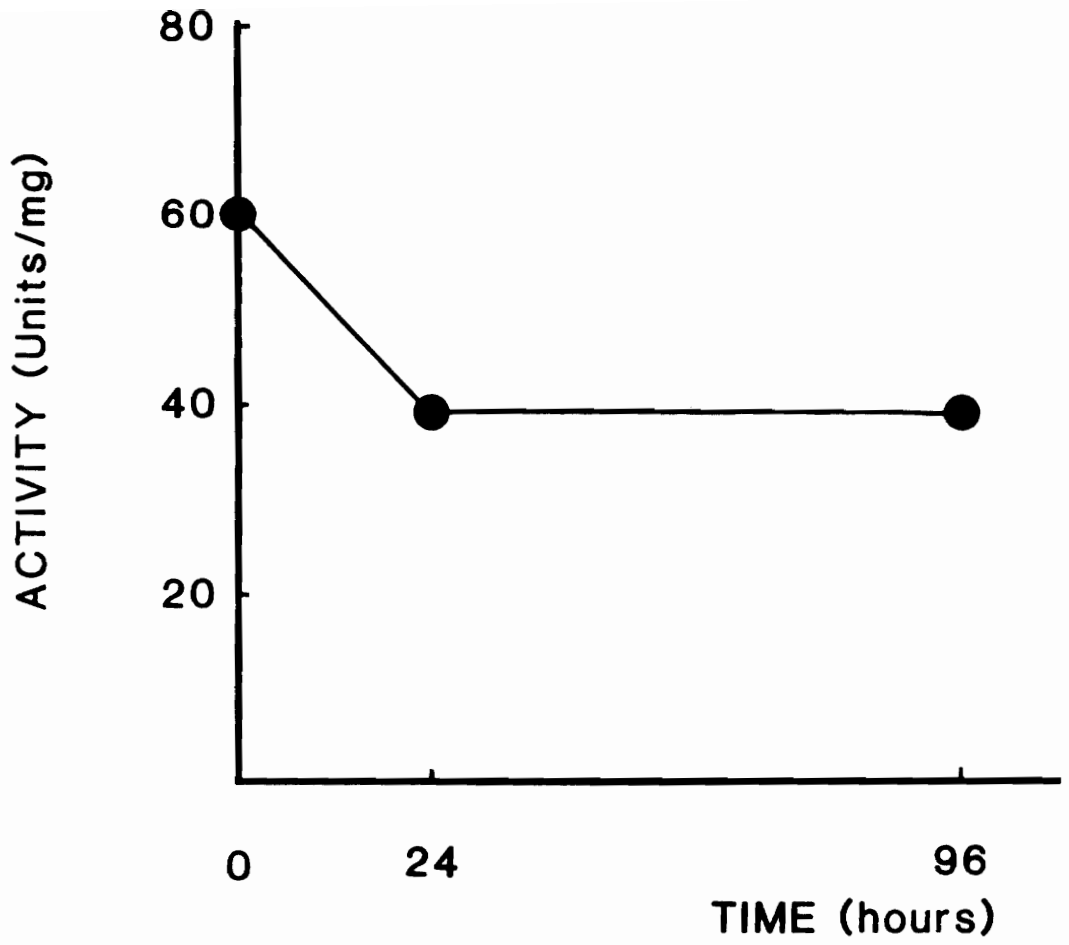


Figure 20. Enzyme activity of horseradish peroxidase during incubation of DHP lignin in organic media.

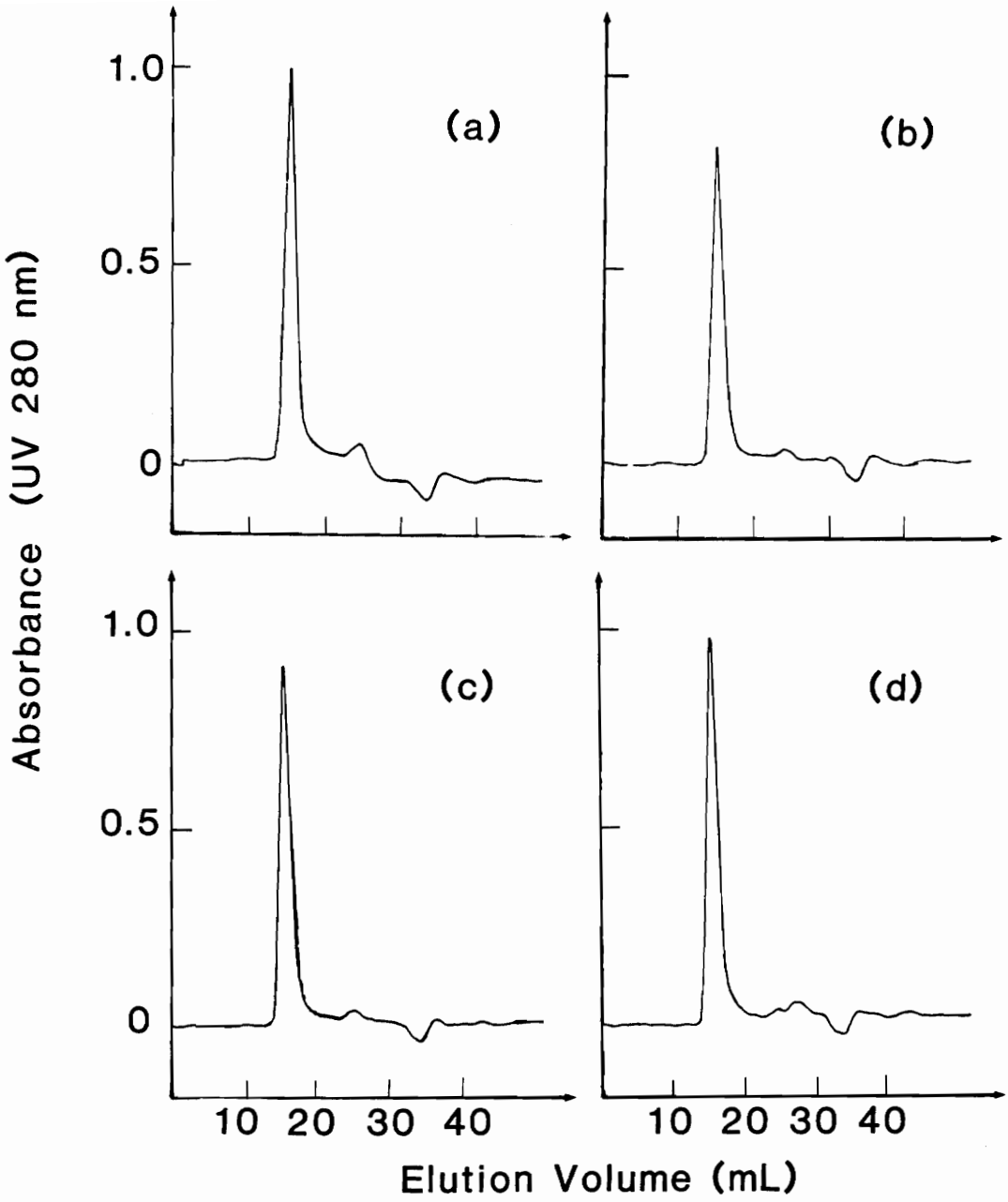


Figure 21. **Gel filtration, using 0.1M LiCl in DMF as eluant, of DHP lignin before and after incubation with horseradish peroxidase:** Incubated for (a) 0 h, (b) 24 h, (c) 96 h, and (d) control (96 h without peroxidase).

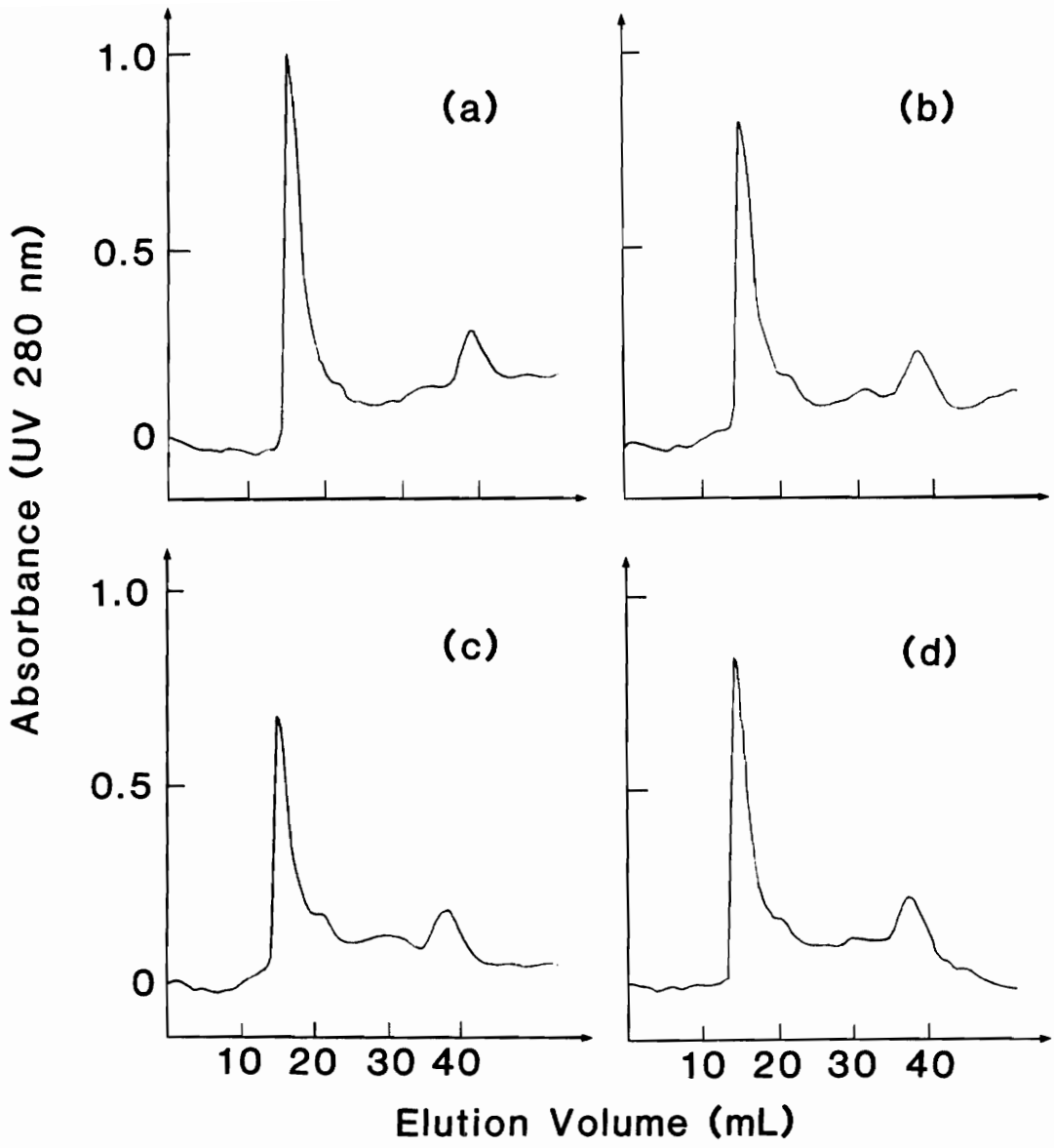


Figure 22. Gel filtration, using dioxane as eluant, of DHP lignin before and after incubation with horseradish peroxidase: Incubated for (a) 0 h, (b) 24 h, (c) 96 h, and (d) control (96 h without peroxidase).

the presence of ferulic acid (**30**), claimed to be a major product (~16%) resulting from the "degradation" of lignin with horseradish peroxidase. However, no ferulic acid (**30**) was detected in our incubation mixtures, suggesting that ferulic acid (**30**) was not formed under the conditions described. This is in agreement with other results obtained following solution and solid-state ^{13}C NMR analyses of DHP lignin prepared by treating [1-^{13}C] coniferyl alcohol (**4a**) with horseradish peroxidase/H_2O_2. The ^{13}C NMR spectra recorded for the resulting DHP samples showed no evidence for the formation of allylic carboxylic functionalities (as in ferulic acid, **30**) as a consequence of this treatment [111].

5.2.2 Lignin peroxidase from *P. chrysosporium*: Effects on coniferyl alcohol (4) and DHP lignin

The "lignin"-degrading ability of the putative lignin-degrading enzyme, "ligninase" or "lignin peroxidase", has been based on results obtained from incubation of low molecular weight, non-phenolic "model substrates" with lignin peroxidase. For the most part, phenolic substrates have been avoided, and the few phenolic structures used underwent coupling reactions. Given the phenolic nature of lignins, the lignin-degrading capabilities of these enzymes were therefore suspect. It is not known how ligninolytic organisms contend with phenolic materials released during lignin biodegradation. A plausible scenario is for these phenolics to undergo polymerization under the influence of an oxidative enzyme, for example fungal peroxidases, resulting in high molecular weight species disabled from diffusing through the hyphal cell walls. Consequently, these peroxidases modify/detoxify the polymeric products by introducing functional groups at appropriate sites in the molecule, the end result of which is for them to become more susceptible to degradation by other enzymes released by the microorganism.

Intact fungal cultures of *P. chrysosporium* have been reported to effect lignin biodegradation, and such ability has been attributed to extracellular "lignin peroxidases" purified from the fungus. The experiments described in this section were carried out to test this hypothesis. Although it is known that coniferyl alcohol (**4**) is a precursor for lignin formation, it is surprising that it has never been used as a substrate for degradative studies with the peroxidases of *P. chrysosporium*. This is the first report of such an attempt. In addition, the products resulting from the incubation of coniferyl alcohol (**4**) with lignin peroxidase were subjected to further treatment with the enzyme to determine if depolymerization would be the net effect.

5.2.3 Electrophoretic patterns of the lignin peroxidase preparation

We needed to determine whether the semi-purified preparation of lignin peroxidase that was provided by Dr. M.S.A. Leisola was contaminated with Mn^{+2}-dependent peroxidases. In addition, we wanted to determine the number of isozymes of lignin peroxidases present, and by subsequent activity staining with selected substrates, determine their multiplicity. These experiments would be helpful in discounting the involvement of enzymatic species other than lignin peroxidase for the effects observed when coniferyl alcohol (**4**) was incubated with the enzyme preparation.

The results obtained from the isoelectrofocusing experiments and subsequent staining procedures are shown in Figures 23a-e. Coomassie blue staining (Figure 23a) showed 10 distinct bands within the pH range investigated.

Other portions of the gel were used for various staining procedures reported to be specific for certain enzyme activities. 4-Chloro-1-naphthol (**51**) has been used to stain for

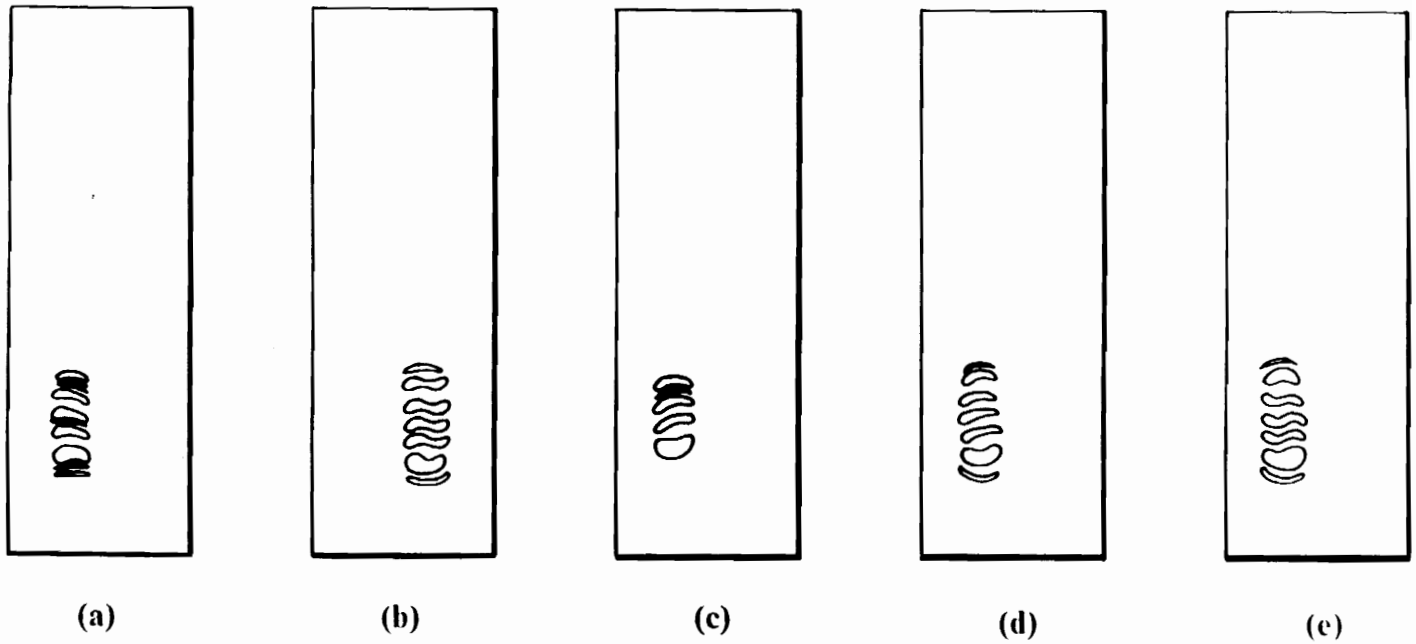


Figure 23. Protein and activity staining of lignin peroxidase preparation following isoelectric focussing.
a) Protein staining; b) 4-Chloro-1-naphthol (51) staining; c) Guaiacol (47) staining;
d) Phenol red (52) staining with Mn^{+2}; e) Phenol red (52) staining without Mn^{+2}.

lignin peroxidase isoenzymes in the presence of H_2O_2. Seven bands were observed following this treatment (Figure 24b). Guaiacol (**47**) staining was carried out to identify isozyme bands responsible for activity towards guaiacol (**47**), a substrate widely used in spectrophotometric assays to determine peroxidase activity. After ~3 min of immersion in the mixture containing guaiacol (**47**) and H_2O_2, five bands (Figure 24c) distinctly changed their color to brown, and coincided with five of those that stained with 4-chloro-1-naphthol (**51**) (Figure 24b).

Staining with phenol red (**52**) was carried out to determine if a Mn^{+2}-dependent peroxidase was present in the enzyme preparation. Two assay mixtures were prepared, one with Mn^{+2} (as $MnSO_4$) (Figure 24d), another without (Figure 24e). Both resultant gels, following separate immersions in the two mixtures, showed only the presence of seven bands which stained yellow in both cases. These indicate that there are no Mn^{+2}-dependent enzymes in the preparation of lignin peroxidase. Incidentally, the location of these bands coincided with those which stained with 4-chloro-1-naphthol (**51**) (Figure 24b) and with the five that stained with guaiacol (**47**) (Figure 24c).

Thus, enzymes with multiple specificity were present in the enzyme preparation. The bands responsible for staining with guaiacol (**47**) are likely to effect polymerization rather than depolymerization of phenolic substrates. It was also significant that these bands corresponded to the lignin peroxidase isoenzymes (i.e, those which turned pale blue when stained with 4-chloro-1-naphthol, **51**). The result suggests that lignin peroxidase could very well be involved in reactions which result in net polymerization of phenolic substrates.

5.2.4 Spectrophotometric assay to determine Mn^{+2}-dependent enzyme activity in the lignin peroxidase preparation

This spectrophotometric procedure of Kuwahara *et al.* [102] was used to further ascertain the absence of a Mn^{+2}-dependent enzyme activity in the lignin peroxidase preparation. The conversion of phenol red (**52**) to product(s) that absorb(s) at 610 nm in a basic medium was followed and the results shown in Table 7. Kuwahara *et al.* characterized manganese peroxidase as an enzyme with an absolute dependence for lactate (**53**) and Mn^{+2} [102]. However, the absence of either lactate or Mn^{+2} did not have an effect on the reactions with phenol red (**52**). This supports the electrophoretic data, and confirms the suggestion that lignin peroxidases are solely responsible for the observed reactions in subsequent incubations of coniferyl alcohol (**4**) with the preparation of lignin peroxidase.

5.2.5 Effect of lignin peroxidase treatment on coniferyl alcohol (4)

Coniferyl alcohol (**4a-c**) specifically-labelled with ^{13}C at either the 1-, 2-, or 3 position of the propyl side chain, was incubated with lignin peroxidase as described in the experimental section. The first treatment was carried out for 48 h, following which the incubation mixture was centrifuged to separate the pellet which was then washed and lyophilized, and the supernatant of which was further incubated for another 24 h with addition of a fresh solution of H_2O_2. Aliquots of the dried pellets were removed for analysis of molecular weight distribution by gel filtration and for characterization by solution state ^{13}C NMR spectroscopy. The results are described below:

Table 7. Spectrophotometric assay for Mn^{+2}-dependent peroxidase activity in the enzyme preparation.

Assay Condition	$A_{610\ nm}$		$\Delta A_{610\ nm}$
	t = 0 min	t = 5 min	
a) Complete	0.151	1.493	1.342
b) -Enzyme	0.141	0.148	0.007
c) -Lactate	0.146	1.283	1.137
d) -$MnSO_4$	0.138	1.528	1.390
e) -H_2O_2	0.158	0.158	0.000
f) Enzyme + buffer	0.004	0.004	0.000

5.2.5.1 Molecular weight distribution of DHP lignins formed from coniferyl alcohol (4) by the action of lignin peroxidase.

By gel filtration chromatography on a Sephadex G100 column eluted with NaOH (0.1 N), the molecular weight distribution of the products resulting from the incubation of coniferyl alcohol with lignin peroxidase/H_2O_2 was obtained. The elution profiles of samples subjected to various treatments (as described in the legends to the chromatograms) are shown in Figures 24 and 25. In each chromatogram, the elution profile for blue dextran (excluded from the column), is included for reference purposes.

Incubation of coniferyl alcohol (**4**) with lignin peroxidase/H_2O_2 resulted in a high molecular weight product. In all cases, an upper bound near 20,000 for the apparent molecular weight distribution (MWD) of the DHP's was reported. In Figure 24, profile 1 represents the MWD of DHP from unlabelled coniferyl alcohol (**4**) obtained after incubation for 48 h with lignin peroxidase. Profile 2 represents the MWD of the DHP formed as a product following incubation of the supernatant resulting from the initial 48 h incubation of [1-^{13}C] coniferyl alcohol (**4c**) with lignin peroxidase. Incubation of an aliquot of this material in NaOH (0.1 N) for 29.3 h had no effect on the molecular weight distribution (Profile 2d).

Since lignin peroxidase has been reported to be active in lignin degradation, we next incubated [2-^{13}C] DHP products with additional lignin peroxidase. Figure 25 shows the effect of treating polymeric DHP to further incubation with lignin peroxidase/H_2O_2. Profiles 1 and 2 correspond to DHP from [2-^{13}C] coniferyl alcohol (**2b**) prior to and after further treatment, respectively. Apparently, the treatment resulted in formation of an even higher molecular weight polymer as evidenced by the increase in proportion of

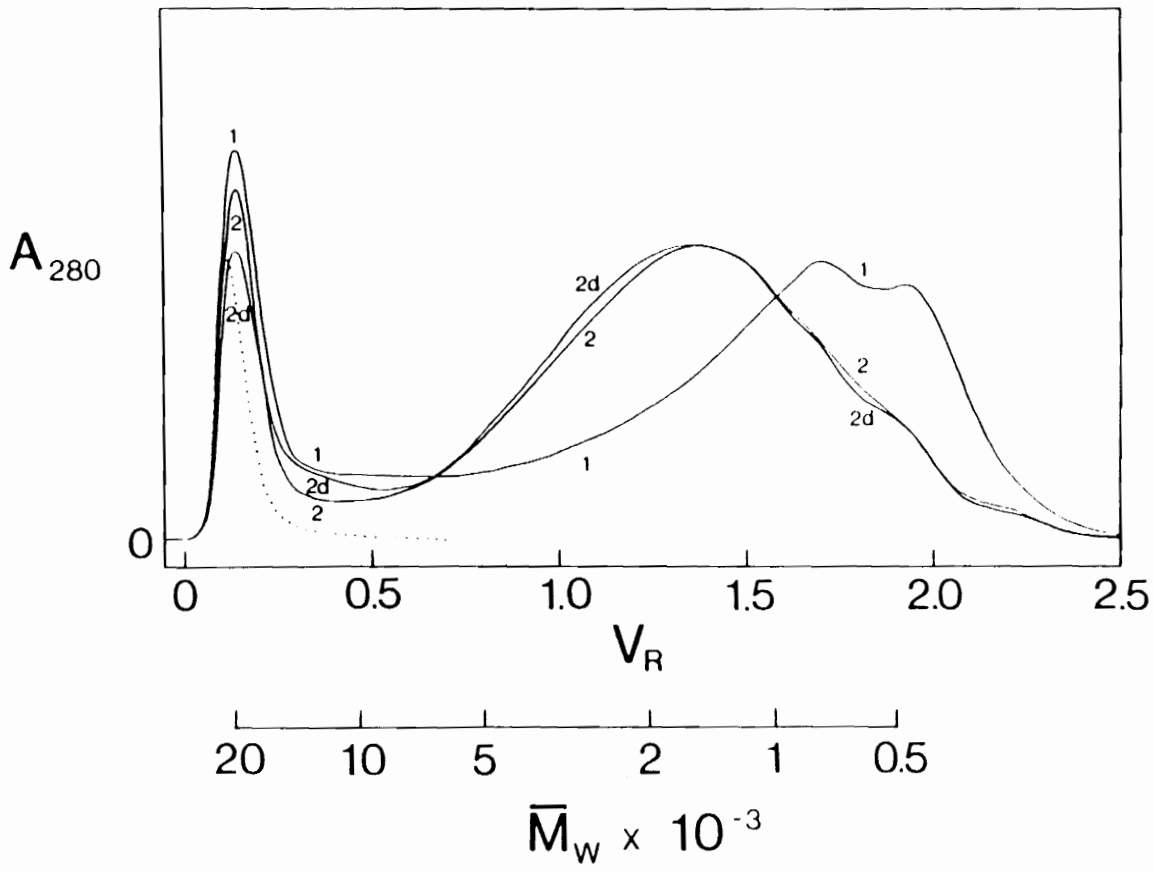


Figure 24. MW distribution of DHP lignins obtained from incubation of coniferyl alcohol (**4**) with lignin peroxidase/H_2O_2.

Profile 1. DHP from unlabelled coniferyl alcohol (**4**) after first treatment with lignin peroxidase/H_2O_2.

Profile 2. DHP from [1-^{13}C] coniferyl alcohol (**4a**) after second treatment with lignin peroxidase/H_2O_2; incubated for 6.3 h in NaOH (0.1 N) prior to gel filtration.

Profile 2d. DHP from [1-^{13}C] coniferyl alcohol (**4a**) after second treatment with lignin peroxidase/H_2O_2; incubated for 29.3 h in NaOH (0.1N) prior to gel filtration.

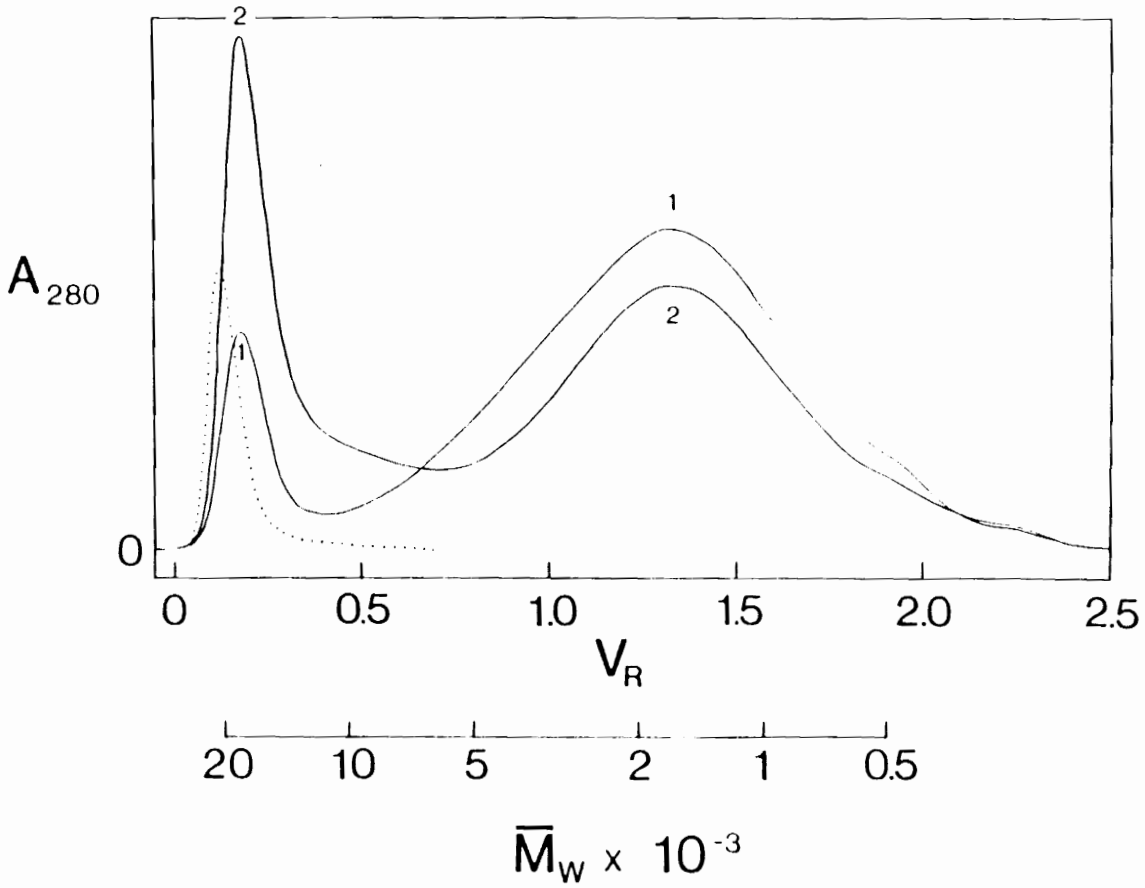


Figure 25. Effect of further treatment with lignin peroxidase/H_2O_2 on MW distribution of DHP lignin.
Profile 1. DHP from [2-^{13}C] coniferyl alcohol (4b) prior to further treatment with lignin peroxidase/H_2O_2.
Profile 2. DHP from [2-^{13}C] coniferyl alcohol (4b) after further treatment with lignin peroxidase/H_2O_2.

products eluted near the column exclusion volume. This observation corroborates earlier reported findings in which further polymerization of the various lignin preparations was the net result of treatment with lignin peroxidase [62]. With either coniferyl alcohol (**4**) or DHP lignin polymer, no direct evidence was obtained suggesting a direct degradative role for the enzyme *in vitro*.

*5.2.5.2 DHP lignins from [1-^{13}C] coniferyl alcohol (**4a**).*

The solution state ^{13}C NMR spectrum of the reaction product obtained from incubation of [1-^{13}C] coniferyl alcohol (**4a**) with lignin peroxidase/H_2O_2 is shown in Figure 26a. For purposes of comparison, the corresponding spectrum obtained for a DHP lignin [111] polymerized from [1-^{13}C] coniferyl alcohol (**4a**) with horseradish peroxidase/H_2O_2 is included (Figure 26b). The two spectra are similar in the sense that no new signals are evident, although the relative intensities differ as expected. In both cases, the only resonances observed were at 60.0, 61.6, 62.6-62.9, and 70.9 ppm which correspond to enrichments presumably due to substructures **B, A, E** or **D**, and **C** (Figure 11), respectively. One minor difference is the absence of a signal at 193.9 ppm (an aldehyde), which is observed in the spectrum of the DHP polymer prepared with horseradish peroxidase (Figure 26b), due to oxidation, on standing, in the solvent [111]. Thus, treatment of [1-^{13}C] coniferyl alcohol with lignin peroxidase in the presence of H_2O_2 results in a NMR spectrum closely resembling that of the corresponding DHP lignin catalyzed by horseradish peroxidase/H_2O_2 system.

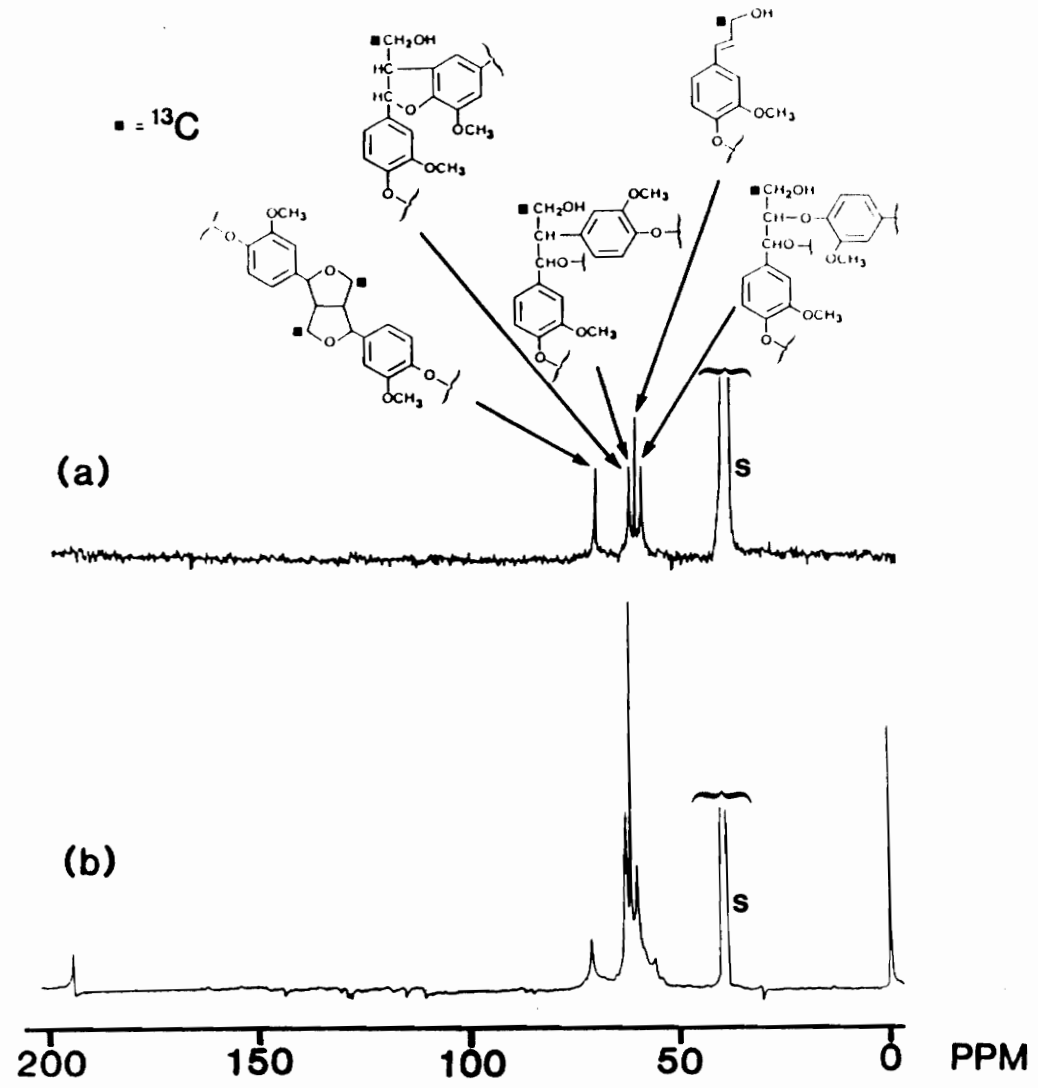


Figure 26. ^{13}C NMR solution spectra of DHP lignins from [1-^{13}C] coniferyl alcohol (4a): Incubated with (a) lignin peroxidase/H_2O_2 and (b) horseradish peroxidase/H_2O_2; S = solvent.

*5.2.5.3 DHP lignins from [2-^{13}C] coniferyl alcohol (**4b**).*

Figures 27a-b show the solution-state ^{13}C NMR spectra for DHP lignins resulting from the incubation of [2-^{13}C] coniferyl alcohol (**4b**) with lignin peroxidase/H_2O_2 and horseradish peroxidase/H_2O_2, respectively. Again, a remarkable resemblance between the two spectra was noted. Enhancements at δ 53 (substructures **D-E**), δ 83-84 (substructure **B**) and at δ 128 (substructure **A**) can clearly be identified in both spectra. Additionally, no resonance suggesting oxidation of the adjacent C-1 to either an aldehyde or to a carboxylic functionality was observed in either spectra.

*5.2.5.4 DHP lignins from [3-^{13}C] coniferyl alcohol (**4c**).*

Finally, the solution-state ^{13}C NMR spectrum of the product(s) obtained from incubation of [3-^{13}C] coniferyl alcohol (**2c**) with lignin peroxidase/H_2O_2 is shown in Figure 28a. The corresponding spectrum obtained for DHP synthesized from [3-^{13}C] coniferyl alcohol (**4c**) with horseradish peroxidase/H_2O_2 is shown in Figure 28b. Inspection of the two spectra again reveals substantial similarities. In both cases, resonances at 70.8, 71.5, 84.9, 86.9, and 128.5-128.9 ppm, which are assigned to bonding patterns as in substructures **B, E, C, D** and **A**, respectively, were enhanced, indicating once again, the strong similarities between both preparations. At this point, it is crucial to mention that no peak indicative of Cα-oxidation was seen in the spectrum for the dehydrogenative product obtained from the incubation of [3-^{13}C] coniferyl alcohol with lignin peroxidase. This is significant because based on "model compound" studies, Cα-oxidation is one of the major mechanistic pathways proposed for the enzymatic action of lignin peroxidase

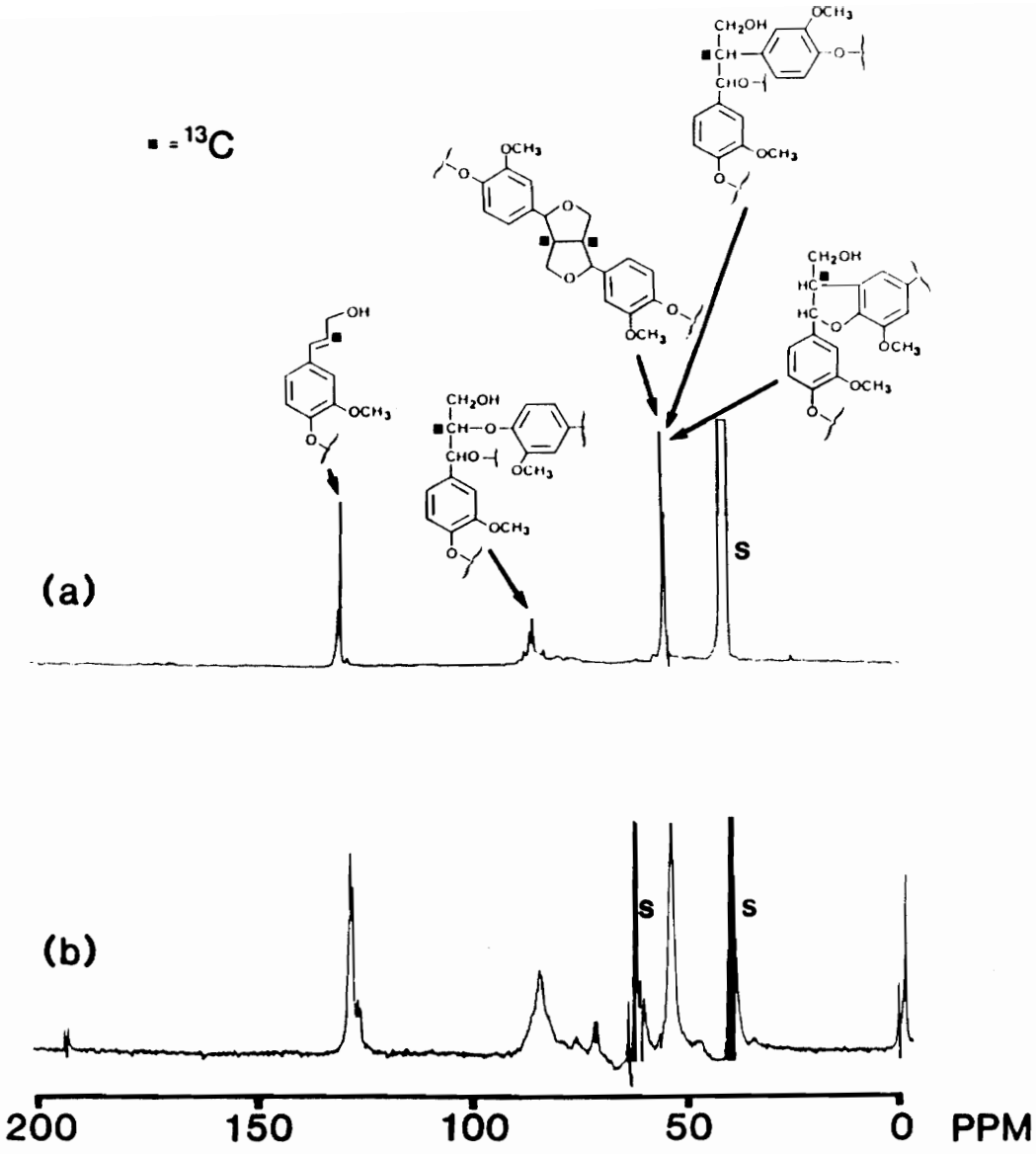


Figure 27. ^{13}C NMR solution spectra of DHP lignins from [2-^{13}C] coniferyl alcohol (4b): Incubated with (a) lignin peroxidase/H_2O_2 and (b) horseradish peroxidase/H_2O_2. The latter was obtained by subtraction of [1-^{13}C] DHP resonances from those of [1,2-^{13}C] DHP; S = solvent.

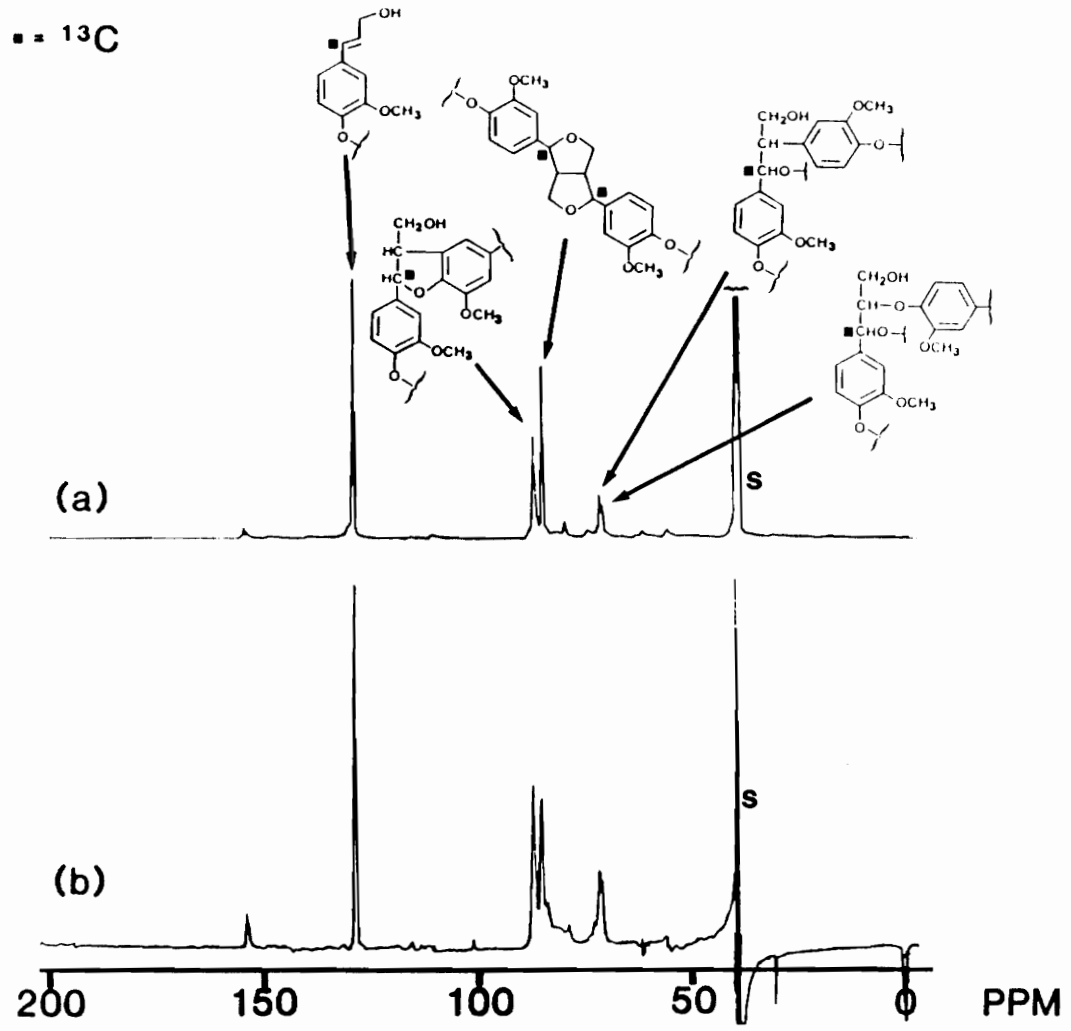
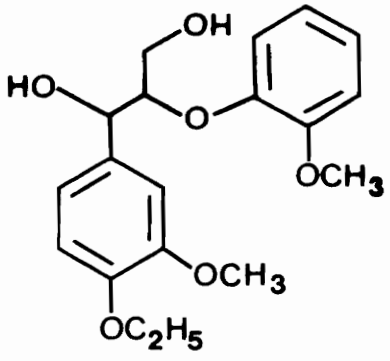


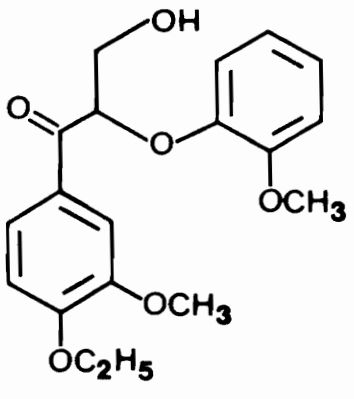
Figure 28. ^{13}C NMR solution spectra of DHP lignins from [3-^{13}C] coniferyl alcohol (4c): Incubated with (a) lignin peroxidase/H_2O_2 and (b) horseradish peroxidase/H_2O_2; S = solvent.

in its "breakdown" of lignins [184]. Herein also lies one of the sources of confusion regarding the role of lignin peroxidases in degrading polymeric lignins.

It was shown by Fenn and Kirk [37] that chemical oxidation of the benzylic hydroxyl in 1-(3-methoxy-4-ethoxyphenyl)-2-(*o*-methoxyphenoxy)propane-1,3-diol (**57**) to a carbonyl group (**58**), markedly retarded the metabolism of the non-phenolic β-O-4 model compound by fungal cultures of *P. chrysosporium*. On the other hand, selective introduction of α-carbonyl group to a spruce lignin preparation enhanced its rate of depolymerization by *P. chrysosporium*. Sarkanen *et al* [163] have attributed such an enhancement to the observed increase in the rate of nucleophilic displacement of the aroxy group from the β-position effected by the presence of a carbonyl group at the α-position. The foregoing raised the possibility that in intact cultures of *P. chrysosporium* which engender the breakdown of polymeric lignin, a different enzyme may be responsible for degrading the lignin previously modified by "lignin peroxidase". Our *in vitro* experiments were not carried out long enough to provide evidence for the occurrence of modifications in the substrates used other than the polymerization of coniferyl alcohol (**4**) and the further increase in the molecular weight of DHP lignin.



(57)



(58)

6.0 CONCLUSIONS

6.1 Examination of Lignin Structure in situ by Solid-state ^{13}C NMR Spectroscopy

This research study brings us much closer to elucidating the structure of lignin *in situ* without isolation from the tissue. For many years, this appeared to be an insurmountable problem. We have successfully developed a methodology for labelling specific carbon atoms of lignin in woody plant tissue which can be analyzed by solid-state ^{13}C NMR spectroscopy without any prior extraction. As a result, the bonding patterns of specific carbon atoms of lignin in the stems and roots of woody plants have been directly examined. Note that in the first instance, we judiciously chose to label carbon atoms in the side chain, because these are the ones involved in majority of the inter-unit linkages in the lignin macromolecule.

Ferulic acid (**30**) was initially used as precursor, but it was superseded by phenylalanine (**7**) which overcame the translocation problem (**30**) and minimized the distortions in monomeric composition and lignification as a result of precursor uptake. Both precursors were shown to be taken up from the hydroponic media through the roots, metabolized into monolignols, and then subsequently incorporated into lignin. Through this mechanism, ^{13}C enrichment of specific carbon atoms of lignin in the plant tissue was achieved, giving rise to resonance enhancements in the solid-state ^{13}C NMR spectra and making possible, assignment of bonding substructures to the resulting difference spectra consistent with previous lignin "model compound" studies.

Evidence for the conversion of precursors to the monolignols and subsequent incorporation into lignin was obtained from the difference spectra of the [1-^{13}C] ferulic acid-treated roots and the [1-^{13}C] phenylalanine-treated roots and stems, where a resonance (63 ppm) due to bonding substructures consisting of hydroxymethyl functionality was observed. The first direct evidence for the dominance of the β-O-4′ linkage in lignin *in situ* was obtained from the results of the solid-state ^{13}C NMR analyses of the roots administered [2-^{13}C] ferulic acid (**30b**) and of both the roots and stems of plants administered [2-^{13}C] phenylalanine (**7b**). Support for the presence of the chemically-labile lignin carbohydrate complexes in plant tissue, the first direct evidence for which was demonstrated with similar studies involving *T. aestivum*, was obtained in the root tissues treated with [3-^{13}] ferulic acid (**30c**) and [3-^{13}C] phenylalanine (**7c**) and stems administered [3-^{13}C] phenylalanine (**7c**). Minor resonances due to carboxylic acids/esters in the roots ([1-^{13}C] ferulic acid and [1-^{13}C] phenylalanine) disappear if plants were allowed to further metabolize them. These resonances were conspicuously absent in the stem spectrum. Phenolic-ether linked monolignols are relatively unimportant in the stems (i.e., signals coincident with this substructure were absent in the [2-^{13}C] phenylalanine-treated

stem spectrum). Resonances for phenylcoumaran and β-aryl substructures appear to be ubiquitous, but their relative magnitudes were substantially less than that of the β-O-4′ substructure. Furthermore, because of overlap of the resonances for substructures containing the hydroxymethyl functionality, it was not possible to determine the relative frequencies of these substructures in the lignin macromolecule.

Upon comparison of the ^{13}C enriched lignin in intact *L. leucocephala* plant tissue with the corresponding DHP lignin artificially prepared from ^{13}C specifically-labelled coniferyl alcohols (**4a-c**) with horseradish peroxidase (HRP)/H_2O_2, differences in the nature of the bonding arrangements and their relative frequencies were observed. These structural differences highlight the disparities between the natural lignification process occurring within the carbohydrate matrix of living plant tissues and that which occurs *in vitro*. Thus, lignin structure *in situ* cannot be adequately represented by DHP lignin.

6.2 Lignin Biodegradation

The occurrence of lignin as the second most abundant natural polymer underscores its importance in the carbon cycle. Although the study of the mechanisms involved in lignin biodegradative processes has been the focus of investigations by several researchers in many parts of the world, no unifying theme as to what constitutes depolymerization of lignins by microbial enzyme systems had emerged. Partly, the problem stems from the fact that unravelling the nature of lignin as a substrate is, in itself, a dilemma.

Our ^{13}C-labelling and solid-state ^{13}C NMR studies with lignin *in situ* present an opportunity to rationally address the issue. Consequently, based on claims made by some investigators, we conceived a "model system" that could be used to monitor changes in the bonding patterns of specific carbon atoms of lignin *in situ* during its degradation. First, we investigated the effects of horseradish peroxidase (HRP)/H_2O_2 in organic media (dioxane/acetate buffer, pH 5, 95:5) on the depolymerization of synthetic lignin. Following separation of the degradation products obtained by incubating DHP lignin with HRP/H_2O_2 in a Sephadex LH-20 column using an eluant (LiCl in DMF, 0.1 N) that partially suppresses association of lignins, we obtained no evidence to support the claim of vigorous depolymerization [28]. Further, subjecting DHP lignin to this treatment did not result in the formation of ferulic acid (**30**) as a degradation product. Thus, horseradish peroxidase did not cause depolymerization of lignins in organic media.

We then studied the ability of lignin peroxidases from *P. chrysosporium* for biodegrading lignins. We used one of the phenolic precursors of lignin, coniferyl alcohol (**4**), as a substrate. We found that treatment of coniferyl alcohol (**4**) with lignin peroxidase/H_2O_2 resulted in a polymeric product. Further increase in molecular size was obtained by extending the treatment with lignin peroxidase/H_2O_2 to the resulting dehydrogenatively polymerized (DHP) lignin. In addition, by using side chain ^{13}C specifically-labelled coniferyl alcohols (**4a-c**) as substrates, we demonstrated that the polymeric products had bonding patterns arguably identical with the corresponding DHP lignins obtained from a horseradish peroxidase (HRP)/H_2O_2 system. It can therefore be concluded that lignin peroxidase(s) is(are) not directly responsible for lignin biodegradation. Instead, we obtained evidence which agrees with earlier proposals that the primary role of fungal peroxidases is that of detoxification, and that one of the

mechanisms by which they perform this role is by polymerizing low molecular weight phenolics to non-diffusible, high molecular weight "degradation" products.

BIBLIOGRAPHY

1. Aoyama, M. and A. Sakakibara. **1979**. Hydrolysis of lignin with dioxane-water. XVII. Isolation of three dilignols from hardwood lignin. *Mokuzai Gakkaishi* **25**(2):149-157.

2. Asher, C.J. and D.G. Edwards. **1983**. Modern solution culture techniques. Chapter 1.3 in "Encyclopedia of Plant Physiology" New Ser. Vol. 15A Inorganic Plant Nutrition (Eds. Lauchli, A. and R. Bieleski) Springer-Verlag, Berlin pp. 94-119.

3. Atalla, R.H. and U.P. Agarwal. **1985**. Raman microprobe evidence for lignin orientation in the cell walls of native woody tissue. *Science* **227**:636-638.

4. Atalla, R.H., J.C. Gast, D.W. Sindorf, V.J. Bartuska and G.E. Maciel. **1980**. ^{13}C NMR spectra of cellulose polymorphs. *J. Amer. Chem. Soc.* **102**:3249-3251.

5. Atalla, R.H. and D.L. VanderHart. **1984**. Native cellulose: A composite of two distinct crystalline forms. *Science* **223**:283-284.

6. Balinsky, D. and D.D. Davies. **1961**. Aromatic biosynthesis in higher plants. 1. Preparation and properties of dehydroshikimate reductase. *Biochem. J.* **80**:292-296.

7. Bardet, M., D. Gagnaire, R. Nardin, D. Robert and M. Vincendon. **1986**. Use of ^{13}C enriched wood for structural NMR investigation of wood and wood components, cellulose and lignin, in solid and in solution. *Holzforsch.* **40**(Suppl.):17-24.

8. Bartuska, V.J., G.E. Maciel, H.I. Bolker and B.I. Fleming. **1980**. Structural studies of lignin isolation procedures by ^{13}C NMR. *Holzforsch.* **34**:214-217.

9. Bolker, H.I. and N.G. Somerville. **1962**. Ultraviolet spectroscopic studies of lignins in the solid state I. Isolated lignin preparations. *TAPPI* **45**(10):826-829.

10. Boudet, A. M., R. Lecussan and A. Boudet. **1975**. *Mise en evidence et proprietes de deux formes de la 5-deshydroquinate hydro-lyase chez les vegetaux superieurs. Planta* **124**:67-75.

11. Bowen, J.R. and T. Kosuge. **1977**. The formation of shikimate-3-phosphate in cell-free preparations of *Sorghum*. *Phytochemistry* **16**:881-884.

12. Brown, S.A. **1981**. Comarins. Chapter 10 in "The Biochemistry of Plants" Vol. 7. Secondary Plant Products. (Ed. Conn, E.E.) Academic Press, New York pp. 269-300.

13. Brunow, G., H. Wallin and V. Sundman. **1978.** A comparison of the effects of a white-rot fungus and H_2O_2-horseradish peroxidase on a lignosulfonate. *Holzforsch.* **32**(6):189-192.

14. Bugos, R.C., V.L. Chiang and W.H. Campbell. **1989.** Isolation of *O*-methyltransferase associated with lignin biosynthesis in aspen. *TAPPI Intl. Symp. Wood and Pulping Chem.* pp. 345-347.

15. Byng, G., R. Whitaker, C. Flick and R.A. Jensen. **1981.** Enzymology of L-tyrosine biosynthesis in corn (*Zea mays*). *Phytochemistry* **20**(6):1289-1292.

16. Camm, E.L. and G.H.N. Towers. **1973.** Phenylalanine ammonia lyase. *Phytochemistry* **12**:961-973.

17. Cho, N. S., J.Y. Lee, G. Meshitsuka and J. Nakano. **1980.** On the characteristics of hardwood compound middle lamella lignin. *Mokuzai Gakkaishi* **26**(8):527-533.

18. Christman, R.F. and R.T. Oglesby. **1971.** Microbiological degradation and the formation of humus. Chapter 18 in "Lignins: Occurrence, Formation, Structure and Reactions". (Eds. Sarkanen, K.V. and C.H. Ludwig) Wiley-Interscience, New York pp.769-795.

19. Chua, M.G.S., C.-L. Chen, H.-M. Chang and T.K. Kirk. **1982.** ^{13}C NMR spectroscopic study of spruce lignin degraded by *Phanerochaete chrysosporium*. *Holzforsch.* **36**(4):165-172.

20. Connelly, J.A. and E.E. Conn. **1986.** Tyrosine biosynthesis in *Sorghum bicolor*: Isolation and regulatory properties of arogenate dehydrogenase. *Z. Naturforsch.* **41c**:69-78.

21. Connors, W.J., S. Sarkanen and J.L. McCarthy. **1980.** Gel chromatography and association complexes of lignin. *Holzforsch.* **34**(3):80-85.

22. Crawford, R.L. **1981.** "Lignin Biodegradation and Transformation". John Wiley & Sons, New York 154 pp.

23. d'Amato, T.A., R.J. Ganson, C.G. Gaines and R.A. Jensen. **1984.** Subcellular localization of chorismate-mutase isoenzymes in protoplasts from mesophyll and suspension-cultured cells of *Nicotiana silvestris*. *Planta* **162**:104-108.

24. Davis, B.D. **1955.** Intermediates in amino acid biosynthesis. In "Advances in Enzymology" Vol. 16 (Ed. Nord, F.F.) Interscience Publishers, Inc., New York pp. 287-289.

25. DeLeo, A.B., J. Dayan and D.B. Sprinson. **1972.** Purification and kinetics of tyrosine-sensitive 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthetase from *Salmonella*. *J. Biol. Chem.* **248**(7):2344-2353.

26. Dewick, P.M. **1986.** The biosynthesis of shikimate metabolites. *Natural Product Reports* **3**:565-585.

27. Dodson, P.J., C.S. Evans, P.J. Harvey and J.M. Palmer. **1987.** Production and properties of an extracellular peroxidase from *Coriolus versicolor* which catalyzes $C\alpha$-$C\beta$ cleavage in a lignin model compound. *FEMS Microbiol. Lett.* **42**:17-22.

28. Dordick, J.S., M.A. Marletta and A.M. Klibanov. **1986.** Peroxidases depolymerize lignin in organic media but not in water. *Proc. Natl. Acad. Sci. USA* **83**:6255-6257.

29. Duran, N., I. Ferrer and J. Rodriguez. **1987.** Ligninases from *Chrysonilia sitophila* (TFB-27441 strain). *Applied Biochem. Biotechnol.* **16**:157-167.

30. Earl, W.L. and D.L. VanderHart. **1980.** High resolution, magic angle sample spinning ^{13}C NMR of solid cellulose I. *J. Amer. Chem. Soc.* **102**:3251-3252.

31. Eberhardt, T.L. **1988.** Studies on lignin biosynthesis and structure. Master's thesis. Virginia Polytechnic Institute and State University, Blacksburg, VA.

32. Ellis, B.E., M.H. Zenk, G.W. Kirby, J. Michael and H.G. Floss. **1973.** Steric course of the tyrosine ammonia-lyase reaction. *Phytochemistry* **12**:1057-1058.

33. Ellwardt, V.P.-C., K. Haider and L. Ernst. **1981.** *Untersuchungen des mikrobiellen Ligninabbaues durch ^{13}C-NMR- Spektroskopie an spezifisch ^{13}C-angereichertem DHP-Lignin aus Coniferylalkohol. Holzforsch.* **35**(3):103-109.

34. Evans, C.S. and J.M. Palmer. **1983.** Ligninolytic activity of *Coriolus versicolor. J. Gen. Microbiol.* **129**:2103-2108.

35. Faix, O., M.D. Mozuch and T.K. Kirk. **1985.** Degradation of gymnosperm (guaiacyl) *vs.* angiosperm (syringyl/guaiacyl) lignins by *Phanerochaete chrysosporium. Holzforsch.* **39**(4):203-208.

36. Fengel, D. and G. Wegener. **1983.** "Wood: Chemistry, Ultrastructure, Reactions". Walter de Gruyter & Co., Berlin 613 pp.

37. Fenn, P. and T.K. Kirk. **1984.** Effects of Cα-oxidation in the fungal metabolism of lignin. *J. Wood Chem. Technol.* **4**(2):131-148.

38. Fergus, B.J. and D.A.I. Goring. **1970.** The location of guaiacyl and syringyl lignins in birch xylem tissue. *Holzforsch.* **24**(4):113-117.

39. Fergus,B.J. and D.A.I. Goring. **1970.** The distribution of lignin in birch wood as determined by ultraviolet microscopy. *ibid.* 118-124.

40. Freudenberg, K. **1965.** Lignin: Its constitution and formation from *p*-hydroxycinnamyl alcohols. *Science* **148**:595-600.

41. Fukuda, T. **1983.** Studies on tissue culture of tree cambium IX. Characterization of callus lignin from black locust. *Mokuzai Gakkaishi* **29**(1):74-81.

42. Fukuda, T., R.L. Mott and C. Harada. **1988.** Studies on tissue culture of tree cambium XI. Characterization of lignin in suspension-cultured cells of loblolly pine. *Mokuzai Gakkaishi* **34**(2):149-154.

43. Gagnaire, D. and D. Robert. **1977.** A polymer model of lignin (D.H.P.) ^{13}C selectively labelled at the benzylic positions: Synthesis and NMR study. *Makromol. Chem.* **178**:1477-1495.

44. Gaines, C.G., G.S. Byng, R.J. Whitaker and R.A. Jensen. **1982.** L-Tyrosine regulation and biosynthesis via arogenate dehydrogenase in suspension-cultured cells of *Nicotiana silvestris* Speg. et Comes. *Planta* **156**:233-240.

45. Ganem, B. **1978.** From glucose to aromatics: Recent developments in natural products of the shikimic acid pathway. *Tetrahedron* **34**:3353-3383.

46. Garbow, J.R., L.M. Ferrantelo and R.E. Stark. **1989.** ^{13}C nuclear magnetic resonance study of suberized potato cell wall. *Plant Physiol.* **90**:783-787.

47. Garver, T.M. Jr. and S. Sarkanen. **1986.** Kraft lignins: The legacy of native structural characteristics I. The verdict from ^{1}H NMR spectra of highly purified paucidisperse fractions of discrete kraft lignin components. *Holzforsch.* **40**(suppl.):93-100.

48. Gerasimowicz, W.V., K.B. Hicks and P.E. Pfeffer. **1984.** Evidence for the existence of associated lignin-carbohydrate polymers as revealed by carbon-13 CPMAS solid-state NMR spectroscopy. *Macromolecules* **17**:2597-2603.

49. Gierer, J. and A.E. Opara. **1973.** Studies on the enzymatic degradation of lignin: The action of peroxidase and laccase on monomeric and dimeric model comounds. *Acta Chem. Scand.* **27**:2909-2922.

50. Gilchrist, D.G. and T. Kosuge. **1980.** Aromatic amino acid biosynthesis and its regulation. Chater 13 in "The Biochemistry of Plants" Vol. 5. Amino acid and Derivatives (Ed. Miflin, B.J.) Academic Press, New York. pp. 507-531.

51. Glasser, W.G. **1985.** Lignin. Chapter 3 in "Fundamentals of Thermo Chemical Biomass Conversion" (Eds. Overend, R.P., T.A. Milne and L.K. Mudge) Elsevier Applied Science pp. 61-76.

52. Glenn, J.K., M.A. Morgan, M.B. Mayfield, M. Kuwahara and M.H. Gold. **1983.** An extracellular H_2O_2-requiring enzyme preparation involved in lignin biodegradation by the white rot Basidiomycete *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **114**(3):1077-1083.

53. Gold, M.H., M. Kuwahara, A.A. Chiu, and J.K. Glenn. **1984.** Purification and characterization of an extracellular H_2O_2-requiring diarylpropane oxygenase from the white rot Basidiomycete, *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **234**(2):353-362.

54. Goldschmid, O. **1954.** Determination of phenolic hydroxyl content of lignin preparations by ultraviolet spectrophotometry. *Anal. Chem.* **26**(9):1421-1423.

55. Goodwin, T.W. and E.I. Mercer. **1983.** "Introduction to Plant Biochemistry" (**2d** edition) Pergamon Press, Oxford 677 pp.

56. Goring, D.A.I. **1989.** The Lignin Paradigm. Chapter 1 in "Lignin: Properties and Materials" (Eds. Glasser, W.G. and S. Sarkanen) ACS Symp. Ser. No. 397 pp. 2-10.

57. Grand, C. **1984.** Ferulic acid 5-hydroxylase: a new cytochrome P-450-dependent enzyme from higher plant microsomes involved in lignin synthesis. *FEBS Lett.* **169**(1):7-11.

58. Grand, C., R. Ranjeva, A.M. Boudet and G. Alibert. **1979.** Photoregulation of the incorporation of guaiacyl units into lignins. *Planta* **146**:281-286.

59. Grand, C., F. Sarni, and A.M. Boudet. **1985.** Inhibition of cinnamyl-alcohol-dehydrogenase activity and lignin synthesis in poplar (*Populus* × *euramericana* Dode) tissues by two organic compounds. *Planta* **163**:232-237.

60. Grisebach, H. **1981.** Lignins. Chapter 15 in "The Biochemistry of Plants" Vol. 7. Secondary Plant Products (Ed. Conn, E.E.) Academic Press, New York pp. 457-478.

61. Gross, G.G. and W. Kreiten. **1975.** Reduction of coenzyme A thioesters of cinnamic acids with an enzyme preparation from lignifying tissue of *Forsythia. FEBS Lett.* **54**(2):259-262.

62. Haemmerli, S.D., M.S.A. Leisola and A. Fiechter. **1986.** Polymerisation of lignins by ligninases from *Phanerochaete chrysosporium. FEMS Microbiol. Lett.* **35**:33-36.

63. Hahlbrock, K. and H. Grisebach. **1970.** Formation of coenzyme A esters of cinnamic acids with an enzyme preparation from cell suspension cultures of parsley. *FEBS Lett.* **11**(1):62-64.

64. Hall, P.L. **1980.** Enzymatic transformations of lignin: 2. *Enzyme Microb. Technol.* **2**:170-176.

65. Hammel, K.E., B. Kalyanaraman and T.K. Kirk. **1986.** Oxidation of polycyclic aromatic hydrocarbons and dibenzo[*p*]-dioxins by *Phanerochaete chrysosporium* ligninase. *J. Biol. Chem.* **261**(36):16948-16952.

66. Hammel, K.E. and P.J. Tardone. **1988.** The oxidative 4-dechlorination of polychrorinated phenols is catalyzed by extracellular fungal lignin peroxidases. *Biochem.* **27**:6563-6568.

67. Hardell, H.-L., G.J. Leary, M. Stoll and U. Westermark. **1980.** Variations in lignin structure in defined morphological parts of birch. *Svensk Papperstidn.* No.3, 71-74.

68. Harkin, J.M. and J.R. Obst. **1973.** Lignification in trees: Indication of exclusive peroxidase participation. *Science* **180**:296-298.

69. Hartenstein, R., E.F. Neuhauser and R.M. Mulligan. **1977.** Mechanism of action of lignins and lignin model compounds with horseradish peroxidase. *Phytochemistry* **16**:1855-1857.

70. Hasan, N. and E.W. Nester. **1978.** Purification and properties of chorismate synthase from *Bacillus subtilis*. *J. Biol. Chem.* **253**(14):4993-4998.

71. Haslam, E. **1979.** Shikimic acid metabolites. Chapter 30.3 in "Comprehensive Organic Chemistry - The Synthesis and Reactions of Organic Compounds" Vol. 5 "Biological Compounds" (Ed. Haslam, E.) Pergamon Press, Oxford pp. 1167-1205.

72. Haslam, E. **1985.** "Metabolites and Metabolism". Clarendon Press, Oxford 161 pp.

73. Hatfield, G.R., G.E. Maciel, O. Erbatur and G. Erbatur. **1987.** Qualitative and quantitative analysis of solid lignin samples by carbon-13 nuclear magnetic resonance spectrometry. *Anal. Chem.* **59**(1):172-179.

74. Haw, J.F., G.E. Maciel and H.A. Schroeder. **1984.** Carbon-13 nuclear magnetic resonance spectrometric study of wood and wood pulping with cross polarization and magic-angle spinning. *Anal. Chem.* **56**:1323-1329.

75. Higuchi, T. **1985.** Biosynthesis of Lignin. Chapter 7 in "Biosynthesis and Biodegradation of Wood Components" (Ed. Higuchi, T.) Academic Press, Inc. Orlando, FL pp. 141-160.

76. Higuchi, T., Y. Ito and I. Kawamura. **1967.** *p*-Hydroxyphenylpropane component of grass lignin and role of tyrosine-ammonia lyase in its formation. *Phytochemistry* **6**:875-881.

77. Hubbell, J.G. **1987.** Mike Benge and his marvelous tree. *Reader's Digest* (August) **131**(784):103-107.

78. Huisman, O.C. and T. Kosuge. **1974.** Regulation of aromatic amino acid biosynthesis in higher plants. II. 3-Deoxy-*arabino*-heptulosonic acid 7-phospate synthetase from cauliflower. *J. Biol. Chem.* **249**(21):6842-6848.

79. Ife, R. and E. Haslam. **1971.** The shikimate pathway. Part III. The stereochemical course of L-phenylalanine ammonia lyase reaction. *J. Chem. Soc.* (C) 2818-2821.

80. Johnson, D.B., W.E. Moore and L.C. Zank. **1961.** The spectrophotometric determination of lignin in small wood samples. *TAPPI* **44**(11):793-798.

81. Jones, D.H. **1984.** Phenylalanine ammonia-lyase: Regulation of its induction, and its role in plant development. *Phytochemistry* **23**(7):1349-1359.

82. Joseleau, J.-P. and K. Ruel. **1989.** Enzyme excretion during wood cell wall degradation by *Phanerochaete chrysosporium*. Chapter 32 in "Plant Cell Wall Polymers: Biogenesis and Biodegradation" (Eds. Lewis, N.G. and M.G. Paice) ACS Symp. Ser. No. 399, pp. 443-453.

83. Jouin, D., M.-T. Tollier and B. Monties. **1988.** Lignification of oak wood. I. Lignin determinations in sapwood and heartwood. *Cellulose Chem. Technol.* **22**:231-243.

84. Jung, E., L.O. Zamir and R.A. Jensen. **1986.** Chloroplasts of higher plants synthesize L-phenylalanine via L-arogenate. *Proc. Natl. Acad. Sci. USA* **83**:7231-7235.

85. Kadam, K.L. and S.W. Drew. **1986.** Study of a lignin biotransformation by *Aspergillus fumigatus* and white-rot fungi using ^{14}C-labeled and unlabeled kraft lignins. *Biotech. Bioeng.* **28**:394-404.

86. Kamoda, S., N. Habu, M. Samejima and T. Yoshimoto. **1989.** Purification and some properties of lignostilbene-α,β-dioxygenase responsible for the Cα-Cβ cleavage of a diarylpropane type lignin model compound from *Pseudomonas sp.* TMY 1009. *Agric. Biol. Chem.* **53**(10):2756-2761.

87. Kaplan, D.L. **1979.** Reactivity of different oxidases with lignins and lignin model compounds. *Phytochemistry* **18**:1917-1919.

88. Kern, H. **1983.** Transformation of lignosulfonates by *Trichoderma harzianum. Holzforsch.* **37**:109-115.

89. Kersten, P.J., M. Tien, B. Kalyanaraman and T.K. Kirk. **1985.** The ligninase of *Phanerochaete chrysosporium* generates cation radicals from methoxybenzenes. *J. Biol. Chem.* **260**(5):2609-2612.

90. Kirk, T.K. **1987.** Lignin-degrading enzymes. *Phil. Trans. R. Soc. Lond. A* **321**:461-474.

91. Kirk, T.K. **1988.** Biochemistry of lignin degradation by *Phanerochaete chrysosporium.* In "Biochemistry and Genetics of Cellulose Degradation". Proceedings of the Federation of the Microbiological Societies Symp. No. 43; (Eds. Aubert, J.-P., P. Beguin, and J. Millet). Academic Press, Inc. pp. 315-332.

92. Kirk, T.K., S. Croan, M. Tien, K.E. Murtagh and R.L. Farrell. **1986.** Production of multiple ligninases by *Phanerochaete chrysosporium*: Effect of selected growth conditions and use of a mutant strain. *Enzyme Microb. Technol.* **8**:27-32.

93. Kirk, T.K. and R.L. Farrell. **1987.** Enzymatic "combustion": The microbial degradation of lignin. *Annu. Rev. Microbiol.* **41**:465-505.

94. Kirk, T.K. and J.R. Obst. **1988.** Lignin Determination. In "Methods in Enzymology" Vol. 161 (Eds. Wood, W.A. and S.T. Kellogg) Academic Press, Inc. San Diego, CA pp. 87-101.

95. Kolodziejski, W., J.S. Frye and G.E. Maciel. **1982.** Carbon-13 nuclear magnetic resonance spectrometry with cross polarization and magic-angle spinning for analysis of lodgepole pine wood. *Anal. Chem.* **54**:1419-1424.

96. Koshiba, T. **1979.** Organization of enzymes in the shikimate pathway of *Phaseolus mungo* seedlings. *Plant Cell Physiol.* **20**(3):667-670.

97. Kringstad, K.P. and R. Morck. **1983.** ^{13}C-NMR spectra of kraft lignins. *Holzforsch.* **37**(5):237-244.

98. Kuroda, H., M. Shimada and T. Higuchi. **1975.** Purification and properties of *O*-methyltransferase involved in the biosynthesis of gymnosperm lignin. *Phytochemistry* **14**:1759-1763.

99. Kutsuki, H. and M.H. Gold. **1982.** Generation of hydroxyl radical and its involvement in lignin degradation by *Phanerochaete chrysosporium. Biochem. Biophys. Res. Commun.* **109**(2):320-327.

100. Kutsuki, H. and T. Higuchi. **1978.** The formation of lignin of *Erythrina crista-galli. Mokuzai Gakkaishi* **24**(9):625-631.

101. Kutsuki, H. and T. Higuchi. **1981.** Activities of some enzymes of lignin formation in reaction wood of *Thuja orientalis, Metasequioa glyptostroboides* and *Robinia pseudoacacia. Planta* **152**:365-368.

102. Kuwahara, M., J.K. Glenn, M.A. Morgan and M.H. Gold. **1984.** Separation and characterization of two-extracellular H_2O_2-dependent oxidases from ligninolytic cultures of *Phanerochaete chrysosporium. FEBS Lett.* **169**(2):247-250.

103. Lapierre, C., J.Y. Lallemand and B. Monties. **1982.** Evidence of poplar lignin heterogeneity by combination of ^{13}C and ^{1}H NMR spectroscopy. *Holzforsch.* **36**(6):275-282.

104. Lapierre, C., B. Monties, E. Guittet and J.Y. Lallemand. **1984.** Photosynthetically ^{13}C-labelled poplar lignins: ^{13}C NMR experiments. *Holzforsch.* **38**(6):333-342.

105. Lawson, L.R.,Jr. and C.N. Still. **1957.** The biological decomposition of lignin - literature survey. *TAPPI* **40**(9):56A-80A.

106. Leatham, G.F. **1986.** The ligninolytic activities of *Lentinus edodes* and *Phanerochaete chrysosporium. Applied Microbiol. Biotechnol.* **24**:51-58.

107. Leisola, M.S.A. and A. Fiechter. **1985.** New trends in lignin biodegradation. *Adv. Biotechnol. Processes* **5**:59-89.

108. Leisola, M.S.A., S.D. Haemmerli, R. Waldner, H.E. Schoemaker, H.W.H. Schmidt and A. Fiechter. **1988.** Metabolism of a lignin model compound, 3,4-dimethoxybenzyl alcohol by *Phanerochaete chrysosporium. Cellulose Chem. Technol.* **22**:267-277.

109. Leisola, M.S.A., B. Kozulic, F. Meussdoerffer and A. Fiechter. **1987.** Homology among multiple extracellular peroxidases from *Phanerochaete chrysosporium. J. Biol. Chem.* **262**(1):419-424.

110. Lewis, N.G., P. Dubelsten, T.L. Eberhardt, E. Yamamoto and G.H.N. Towers. **1987.** The *E/Z* isomerization step in the biosynthesis of *Z*-coniferyl alcohol in *Fagus grandifolia. Phytochemistry* **26**(10):2729-2734.

111. Lewis, N.G., J. Newman, G. Just and J. Ripmeister. **1987.** Determination of bonding patterns of ^{13}C specifically enriched dehydrogenatively polymerized lignin in solution and solid state. *Macromolecules* **20**:1752-1756.

112. Lewis, N.G., R.A. Razal, K.P. Dhara, E. Yamamoto, G.H. Bokelmann and J.B. Wooten. **1988.** Incorporation of [2-^{13}C] ferulic acid, a lignin precursor, into *Leucaena leucocephala* and its analysis by solid state ^{13}C N.M.R. spectroscopy. *J. Chem. Soc., Chem. Commun.*, 1626-1628.

113. Lewis, N.G., R.A. Razal, E. Yamamoto, G.H. Bokelmanm and J.B. Wooten. **1989.** ^{13}C specific labeling of lignin in intact plants. Chapter 12 in "Plant Cell Wall Polymers: Biogenesis and Biodegradation" (Eds. Lewis, N.G. and M.G. Paice) ACS Symp. Ser. No. 399, pp. 169-181.

114. Lewis, N.G., R.A. Razal, E. Yamamoto and J.B. Wooten. **1989.** Lignin bonding patterns in woody plants. *TAPPI Intl. Symp. Wood and Pulping Chem.* pp. 349-357.

115. Lewis, N.G. and E. Yamamoto. **1990.** Lignins: Occurrence, Biogenesis and Biodegradation. *Annu. Reviews Plant Physiol. Plant Mol. Biol.* **41**:455-97.

116. Lewis, N.G., E. Yamamoto, J.B. Wooten, G. Just, H. Ohashi and G.H.N. Towers. **1987.** Monitoring biosynthesis of wheat cell-wall phenylpropanoids *in situ. Science* **237**:1344-1346.

117. Lewis, N.G., E. Yamamoto, J.B. Wooten, G. Just and G.H.N. Towers. **1987.** Unravelling the bonding patterns of lignin *in situ* in living plants. *TAPPI Intl. Symp. Wood and Pulping Chem.* pp. 245-247.

118. Lobarzewski, J., J. Trojanowski and M. Wojtas-Wasilewska. **1982.** The effects of fungal peroxidase on Na-lignosulfonates. *Holzforsch.* **36**:173-176.

119. Ludemann, H.-D. and H. Nimz. **1973.** Carbon-13 nuclear magnetic resonance spectra of lignins. *Biochem. Biophys. Res. Commun.* **52**(4):1162-1169.

120. Ludemann, H.-D., and H. Nimz. **1974.** *^{13}C-Kernresonanzspektren von Ligninen, I Chemische Verschiebungen bei monomeren und dimeren Modellsubstanzen. Die Makromolekulare Chemie* **175**:2393-2407.

121. Luderitz, T. and H. Grisebach. **1981.** Enzymic synthesis of lignin precursors: Comparison of cinnamoyl-CoA reductase and cinnamyl alcohol:NADP$^+$ dehydrogenase from spruce (*Picea abies L.*) and soybean (*Glycine max L.*). *Eur. J. Biochem.* **119**:115-124.

122. Lyr, H. **1962.** Detoxification of heartwood toxins and chlorophenols by higher fungi. *Nature* **195**:289-290.

123. Macomber, R.S. **1988.** "NMR Spectroscopy: Basic Principles and Applications". Harcourt Brace Jovanovich. Orlando, FL 209 pp.

124. Manders, W.F. **1987.** Solid-state ^{13}C NMR determination of the syringyl/guaiacyl ratio in hardwoods. *Holzforsch.* **41**(1):13-18.

125. Mansell, R.L., G.G. Gross, J. Stockigt, H. Franke and M.H. Zenk. **1974.** Purification and properties of cinnamyl alcohol dehydrogenase from higher plants involved in lignin biosynthesis. *Phytochemistry* **13**:2427-2435.

126. Mansson, P. **1983.** Quantitative determination of phenolic and total hydroxyl groups in lignins. *Holzforsch.* **37**:143-146.

127. Morrison, I.M. **1972.** Improvements in the acetyl bromide technique to determine lignin and digestibility and its application to legumes. *J. Sci. Fd. Agric.* **23**:1463-1469.

128. Mousdale, D.M. and J.R. Coggins. **1984.** Purification and properties of 5-enolpyruvylshikimate 3-phosphate synthase from seedlings of *Pisum sativum* L. *Planta* **160**:78-83.

129. Mousdale, D.M. and J.R. Coggins. **1986.** Detection and subcellular localization of a higher plant chorismate synthase. *FEBS Lett.* **205**(2):328-332.

130. Nakamura, Y. and T. Higuchi, **1976.** Ester linkage of *p*-coumaric acid in bamboo lignin. *Holzforsch.* **30**:187-191.

131. Newman, J., R.N. Rej, G. Just and N.G. Lewis. **1986.** Syntheses of (1,2-^{13}C), (1-^{13}C) and (3-^{13}C) coniferyl alcohol. *Holzforsch.* **40**:369-373.

132. Newman, R.H., G.J. Leary and K.R. Morgan. **1989.** Nuclear spin diffusion studies of spatial relationships between chemical components of plant cell walls. *TAPPI Intl. Symp. Wood and Pulping Chem.* pp. 221-223.

133. Niku-Paavola, M.-L. **1987.** Ligninolytic enzymes of the white rot fungus *Phlebia radiata. TAPPI Intl. Symp. Wood and Pulping Chem.* pp. 301-303.

134. Nillson. T. and D. Holt. **1983.** Bacterial attack occurring in the S_2 layer of wood fibres. *Holzforsch.* **37**(2):107-108.

135. Nimz, H. **1974.** Beech lignin - proposal of a constitutional scheme. *Angew. Chem. Internat. edit.* **13**(5):313-321.

136. Nimz, H., J. Ebel and H. Grisebach. **1975.** On the structure of lignin from soybean cell suspension cultures. *Z. Naturforsch.* **30**:442-444.

137. Nimz, V.H.H. and H.-D. Ludemann. **1976.** *Kohlenstoff-13-NMR-Spektren von Ligninen, 6.) Lignin- und DHP-Acetate. Holzforsch.* **30**(2):33-40.

138. Nimz, H., I. Mogharab and H.-D. Ludemann. **1974.** *^{13}C-Kernresonanzspektren von Ligninen, 3) Vergleich von Fichtenlignin mit Kunstlichem Lignin nach Freudenberg. Die Makromolekulare Chemie* **175**:2563-2575.

139. Nimz, H.H., D. Robert, O. Faix and M. Nemr. **1981.** Carbon-13 NMR spectra of lignins, 8. Structural differences between lignins of hardwoods, softwoods, grasses and compression wood. *Holzforsch.* **35**:16-26.

140. Noguchi, A., M. Shimada and T. Higuchi. **1980.** Studies on lignin biodegradation. I. Possible role of non-specific oxidation of lignin by laccase. *Holzforsch.* **34**(3):86-89.

141. Obst, J.R., and T.K. Kirk. **1988.** Isolation of Lignin. In "Methods in Enzymology" Vol. 161 (Eds. Wood, W.A. and S.T. Kellogg) Academic Press, Inc. San Diego, CA pp. 3-12.

142. Obst, J.R. and L.L. Landucci. **1986.** The syringyl content of softwood lignin. *J. Wood Chem. Technol.* **6**(3):311-327.

143. Odier, E., M.D. Mozuch, B. Kalyanaraman and T.K. Kirk. **1988.** Ligninase-mediated phenoxy radical formation and polymerization unaffected by cellobiose: quinone oxidoreductase. *Biochimie* **70**:847-852.

144. Odier, E. and C. Rolando. **1985.** Catabolism of aryglycerol-β-aryl ethers lignin model compounds by *Pseudomonas cepacia* 122. *Biochimie* **67**:191-197.

145. Ohashi, H., E. Yamamoto, N.G. Lewis and G.H.N. Towers. **1987.** 5-Hydroxyferulic acid in *Zea mays* and *Hordeum vulgare* cell walls. *Phytochemistry* **26**(7):1915-1916.

146. Paszczynski, A., V.-B. Huynh and R.L. Crawford. **1986.** Comparison of ligninase-I and peroxidase-M2 from the white-rot fungus *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **244**(2):750-765.

147. Pearl, I.A. **1967.** "The Chemistry of Lignin". M. Dekker, New York 339 pp.

148. Pellinen, J., J. Jokela and M.S. Salkinoja-Salonen. **1987.** Degradability of different lignins by bacteria. *Holzforsch.* **41**(5):271-276.

149. Pfandler, R., D. Scheel, H. Sandermann, Jr. and H. Grisebach. **1977.** Stereospecificity of plant microsomal cinnamic acid 4-hydroxylase. *Arch. Biochem. Biophys.* **178**:315-316.

150. Putter, J. **1974.** Peroxidases. In "Methods of Enzymatic Analysis" (Ed. Bergmeyer, H.U.) Verlag Chemie, Weinheim and Academic Press, London, pp. 685-690.

151. Renganathan, V., K. Miki and M.H. Gold. **1985.** Multiple molecular forms of diarylpropane oxygenase, an H_2O_2-requiring, lignin degrading enzyme from *Phanerochaete chrysosporium. Arch. Biochem. Biophys.* **241**(1):304-314.

152. Rhodes, J. M. and L.S.C. Wooltorton. **1978.** The biosynthesis of phenolic compounds in wounded plant storage tissues. In "Biochemistry of Wounded Plant Tissues" (Ed. Kahl, G.) Walter de Gruyter, Berlin pp. 243-286.

153. Ride, J.P. **1975.** Lignification in wounded wheat leaves in response to fungi and its possible role in resistance. *Physiol. Plant Pathology* **5**:125-134.

154. Robert, D., A. Mollard and F. Barnoud. **1989.** ^{13}C NMR qualitative and quantitative study of lignin structure synthesized in *Rosa glauca* calluses. *Plant Physiol. Biochem.* **27**(2):297-304.

155. Saijo, R. and T. Kosuge. **1978.** The conversion of 3-deoxyarabinoheptulosonate 7-phosphate to 3-dehydroquinate by sorghum seedling preparations. *Phytochemistry* **17**:223-225.

156. Saka, S. and D.A.I. Goring. **1988.** The distribution of lignin in white birch wood as determined by bromination with TEM-EDXA. *Holzforsch.* **42**(3):149-153.

157. Saka, S., R.J. Thomas, and J.S. Gratzl. **1978.** Lignin distribution: Determination by energy-dispersive analysis of X rays. *TAPPI* **61**(1):73-76.

158. Samejima, M., S.-I. Abe, Y. Saburi and T. Yoshimoto. **1988.** Metabolism of lignin model compounds with β-aryl ether linkage by *Pseudomonas sp.* TMY 1009 and its mutants. *Cellulose Chem. Technol.* **22**:279-286.

159. Samejima, M., Y. Saburi, T. Yoshimoto, T. Fukuzumi and T. Nakazawa. **1985.** Catabolic pathway of guaiacylglycerol-β-guaiacyl ether by *Pseudomonas sp.* TMY 1009. *Mokuzai Gakkaishi* **31**(11):956-958.

160. Samejima, M., N. Tatarazako, T. Arakawa, Y. Saburi and T. Yoshimoto. **1987.** Metabolism of 3,4-dimethoxycinnamic acid and ferulic acid by mutant strains derived from *Pseudomonas sp* TMY 1009. *Mokuzai Gakkaishi* **33**(9):728-734.

161. Sarkanen, K.V. **1971.** Precursors and their polymerization. Chapter 4 in "Lignins: Occurrence, Formation, Structure and Reactions" (Eds. Sarkanen, K.V. and C.H. Ludwig) Wiley-Interscience, New York pp. 95-163.

162. Sarkanen, K.V. and H.L. Hergert. **1971.** Classification and Distribution. Chapter 3 in "Lignins: Occurrence, Formation, Structure and Reactions" (Eds. Sarkanen, K.V. and C.H. Ludwig) Wiley-Interscience, New York pp. 43-94.

163. Sarkanen, S., R.A. Razal, T. Piccariello, E. Yamamoto, M.S. Leisola and N.G. Lewis. **1990.** Lignin peroxidase: Towards a clarification of its role *in vivo.* (Submitted).

164. Sarkanen, S., D.C. Teller, J. Hall and J.L. McCarthy. **1981.** Lignin. 18. Associative effects among organosolv lignin components. *Macromolecules* **14**:426-434.

165. Sarni, F., C. Grand and A.M. Boudet. **1984.** Purification and properties of cinnamoyl-CoA reductase and cinnamyl alcohol dehydrogenase from poplar stems (*Populus* × *euramericana*). *Eur. J. Biochem.* **139**:259-265.

166. Scalbert, A., B. Monties, C. Rolando and A. Sierra-Escudero. **1986.** Formation of ether linkage between phenolic acids and graminae lignin: A possible mechanism involving quinone methides. *Holzforsch.* **40**(3):191-195.

167. Schoemaker, H.E., E.M. Meijer, M.S.A. Leisola, S.D. Haemmerli, R. Waldner, D. Sanglard and H.W.H. Schmidt. **1989.** Oxidation and reduction in lignin biodegradation. Chapter 33 in "Plant Cell Wall Polymers: Biogenesis and Biodegradation" (Eds. Lewis, N.G. and M.G. Paice) ACS Symp. Ser. No. 399, pp. 454-471.

168. Schreiber, W. **1975.** Degradation of 3-hydroxyflavone by horseradish peroxidase. *Biochem. Biophys. Res. Commun.* **63**(2):509-514.

169. Schreiner, R.P., S. E. Stevens, Jr. and M. Tien. **1988.** Oxidation of thianthrene by the ligninase of *Phanerochaete chrysosporium. Applied Environ. Microbiol.* **54**(7):1858-1860.

170. Shan, J.R. and U. Blum. **1987.** The utilization of exogenously supplied ferulic acid in lignin biosynthesis. *Phytochemistry* **26**(11):2977-2982.

171. Shimada, M., H. Fushiki and T. Higuchi. **1972.** *O*-Methyltransferase activity from Japanese black pine. *Phytochemistry* **11**:2657-2662.

172. Shimada, M., H. Kuroda and T. Higuchi. **1973.** Evidence for the formation of methoxyl group of ferulic and sinapic acids in *Bambusa* by the same *O*-methyltransferase. *Phytochemistry* **12**:2873-2875.

173. Siehl, D.L., J.A. Connelly and E.E. Conn. **1986.** Tyrosine biosynthesis in *Sorghum bicolor*: Characteristics of prephenate aminotransferase. *Z. Naturforsch.* **41**c:79-86.

174. Singh, B.K., J.A. Connelly and E.E. Conn. **1985.** Chorismate mutase isoenzymes from *Sorghum bicolor*: Purification and properties. *Arch. Biochem. Biophys.* **243**(2):374-384.

175. Sjostrom, E. **1981.** "Wood Chemistry Fundamentals and Applications". Academic Press, New York. 223 pp.

176. Stark, R.E., T. Zlotnik-Mazori, L.M. Ferrantello and J.R. Garbow. **1989.** Molecular structure and dynamics of intact plant polyesters: Solid-state NMR studies. Chapter 16 in "Plant Cell Wall Polymers: Biogenesis and Biodegradation" (Eds. Lewis, N.G. and M.G. Paice) ACS Symp. Ser. No. 399, pp. 214-229.

177. Stockigt, J., R.L. Mansell, G.G. Gross and M.H. Zenk. **1973.** Enzymic reduction of *p*-coumaric acid via *p*-coumaroyl-CoA to *p*-coumaryl alcohol by a cell-free system from *Forsythia sp. Z. Pflanzenphysiol.* **70**:305-307.

178. Suzich, J.A., J.F.D. Dean and K.M. Herrmann. **1985.** 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase from carrot root (*Daucus carota*) is a hysteretic enzyme. *Plant Physiol.* **79**:765-770.

179. Taneda, H., S. Hosoya, J. Nakano and H.-m. Chang. **1985.** Behavior of lignin-hemicellulose linkages in chemical pulping. *TAPPI Intl. Symp. Wood and PUlping Chem. Poster Presentations* pp. 117-118.

180. Taylor, M.G., Y. Deslandes, T. Bluhm, R.H. Marchessault, M. Vincendon and J. Saint-Germain. **1983.** Solid state ^{13}C NMR characterization of wood. *TAPPI Journal* **66**(6):92-94.

181. Terashima, N. and K. Fukushima. **1989.** Biogenesis and structure of macromolecular lignin in the cell wall of tree xylem as studied by microautoradiography. Chapter 11 in "Plant Cell Wall Polymers: Biogenesis and Biodegradation" (Eds. Lewis, N.G. and M.G. Paice) ACS Symp. Ser. No. 399, pp. 160-168.

182. Terashima, N., K. Fukushima and K. Takabe. **1986.** Heterogeneity in formation of lignin. VIII. An autoradiographic study on the formation of guaiacyl and syringyl lignin in *Magnolia kobus* DC. *Holzforsch.* **40**(suppl.):101-105.

183. Terashima, N., K. Fukushima, S. Tsuchiya and K. Takabe. **1986.** Heterogeneity in formation of lignin. VII. An autoradiographic study on the formation of guaiacyl and syringyl lignin in poplar. *J. Wood Chem. Technol.* **6**(4):495-504.

184. Tien, M. and T.K. Kirk. **1983.** Lignin-degrading enzyme from the hymenomycete *Phanerochaete chrysosporium* Burds. *Science* **221**:661-663.

185. Tien, M. and T.K. Kirk. **1984.** Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterization and catalytic properties of a unique H_2O_2-requiring oxygenase. *Proc. Natl. Acad. Sci. USA.* **81**:2280-2284.

186. Tomimura, Y., T. Yokoi and N. Terashima. **1979.** Heterogeneity in formation of lignin. IV. Various factors which influence the degree of condensation at position 5 of guaiacyl ring. *Mokuzai Gakkaishi* **25**(11):743-748.

187. Tomimura, Y., T. Yokoi and N. Terashima. **1980.** Heterogeneity in formation of lignin. V. Degree of condensation in guaiacyl nucleus. *Mokuzai Gakkaishi* **26**(1):37-42.

188. Umezawa, T. **1988.** Mechanisms for chemical reactions involved in lignin biodegradation by *Phanerochaete chrysosporium. Wood Research* No. 75:21-79.

189. U.S. Dept. of Energy, Office of Energy Research. Div. of Energy Biosciences. **1988.** Summary report of a workshop on lignin research: Needs and opportunities. Univ. of Illinois, Apr. 18-19, 1988.

190. Vaughan, P.F.T., R. Eason, J.Y. Paton and G.A. Ritchie. **1975.** Molecular weight and amino acid composition of purified spinach beet phenolase. *Phytochemistry* **14**:2383-2386.

191. Wengenmayer, H., J. Ebel and H. Grisebach. **1976.** Enzymic synthesis of lignin precursors. 1. Purification and properties of cinnamoyl-CoA: NADPH reductase from cell suspension cultures of soybean (*Glycine max*). *Eur. J. Biochem.* **65**:529-536.

192. Westermark, U., H.-L. Hardell and T. Iversen. **1986.** The content of protein and pectin in the lignified middle lamella/primary wall from spruce fibers. *Holzforsch.* **40**(2):65-68.

193. Wightman, F. and J.C. Forest. **1978.** Properties of plant aminotransferases. *Phytochemistry* **17**:1455-1471.

194. Yamamoto, E. **1977.** Alicyclic acid metabolism in plants. 10. Partial purification and some properties of 3-dehydroquinate synthase from *Phaseolus mungo* seedlings. *Plant & Cell Physiol.* **18**:995-1007.

195. Young, M. and C. Steelink. **1973.** Peroxidase catalyzed oxidation of naturally-occurring phenols and hardwood lignins. *Phytochemistry* **12**:2851-2861.

196. Ziomek, E. and R.E. Williams. **1989.** Modification of lignins by growing cells of the sulfate-reducing anaerobe *Desulfovibrio desulfuricans. Applied Environ. Microbiol.* **55**(9):2262-2266.

VITA

Ramon A. Razal was born on August 31, 1956 in Guinobatan, Albay, Philippines. He was raised by his mother, Margarita, in this small town until they moved to the Tagalog region in 1973 when he pursued the Bachelor of Science in Forest Products Engineering degree at the University of the Philippines at Los Banos (UPLB). He finished his bachelor's degree (*cum laude*) in 1977, and the Master's degree in Wood Science and Technology from the same university in 1983. He was a recipient of the Florencio Tamesis award for scholastic excellence and leadership at the time of his graduation from college. He was hired as instructor in the Department of Wood Science and Technology at UPLB immediately after finishing college, where he taught until he left for the University of Melbourne, Australia in 1985. He came to Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, Virginia in the same year on an assistantship to pursue his Ph.D.

He received the best student paper award in June, 1988 for his presentation "Lignin Bonding Patterns in Woody Plants" at the Phytochemical Society meeting at the University of Iowa, Iowa City. In 1989, the A.B. Massey award for outstanding performance as a graduate student was presented to him by the School of Forestry and Wildlife Resources at Virginia Tech.

He completed all the requirements for the Ph.D. degree in Wood Science and Forest Products at Virginia Tech in February, 1990.