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

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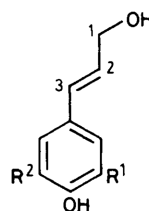
Solid state ^{13}C n.m.r. analysis of lignin *in situ*, in the root tissue of the woody angiosperm, *Leucaena leucocephala*, and specifically labelled at a carbon site thought to be involved in the majority of inter-unit linkages, revealed significant bonding differences when compared to an artificial lignin preparation generally viewed more or less to represent native lignin structure.

Lignin is a generic term used to describe the complex phenylpropanoid polymers in vascular plant tissue, and which functions in a number of structural and defensive roles. Being frequently referred to as the second most abundant naturally occurring organic substance,[1] it is most often represented as a random free-radical induced, dehydrogenative polymer derived exclusively from the three *E*-monolignols (**1**)—(**3**).[2] The monomer (**1**)—(**3**) ratios of lignin in plants are species dependent, *i.e.* softwood lignin is derived from *E*-coniferyl (**2**) and *E*-coumaryl (**1**) alcohols, whereas in hardwoods and grasses *E*-sinapyl alcohol (**3**) is also involved.[3]

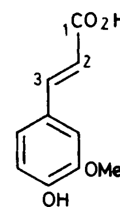
Surprisingly, native lignin structure itself has never been



(**1**) $R^1 = R^2 = H$

(**2**) $R^1 = OMe, R^2 = H$

(**3**) $R^1 = R^2 = OMe$



(**4**)

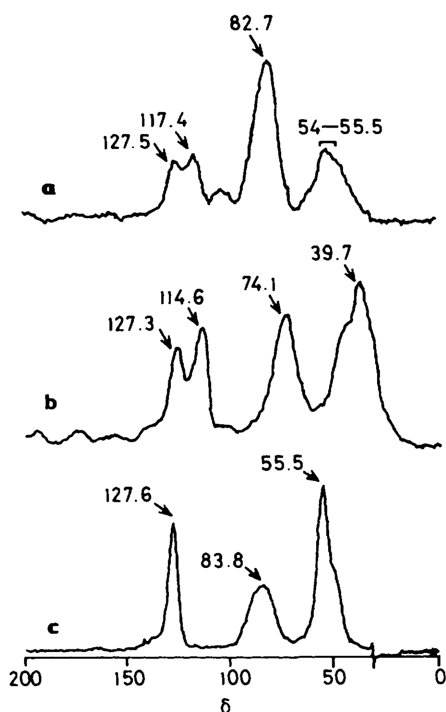


Figure 1. ^{13}C N.m.r. solid state difference spectra of (a) *L. Leucocephala* root tissue, (b) *T. aestivum* L root tissue, previously administered [2-^{13}C]ferulic acid (**4**), and (c) a [2-^{13}C]DHP lignin polymer from coniferyl alcohol (**2**). Each spectrum only shows enhanced resonances. Cross-polarisation magic angle spinning spectra were obtained at 50 MHz on a Varian XL-200 Spectrometer equipped with a Doty Scientific MAS probe.

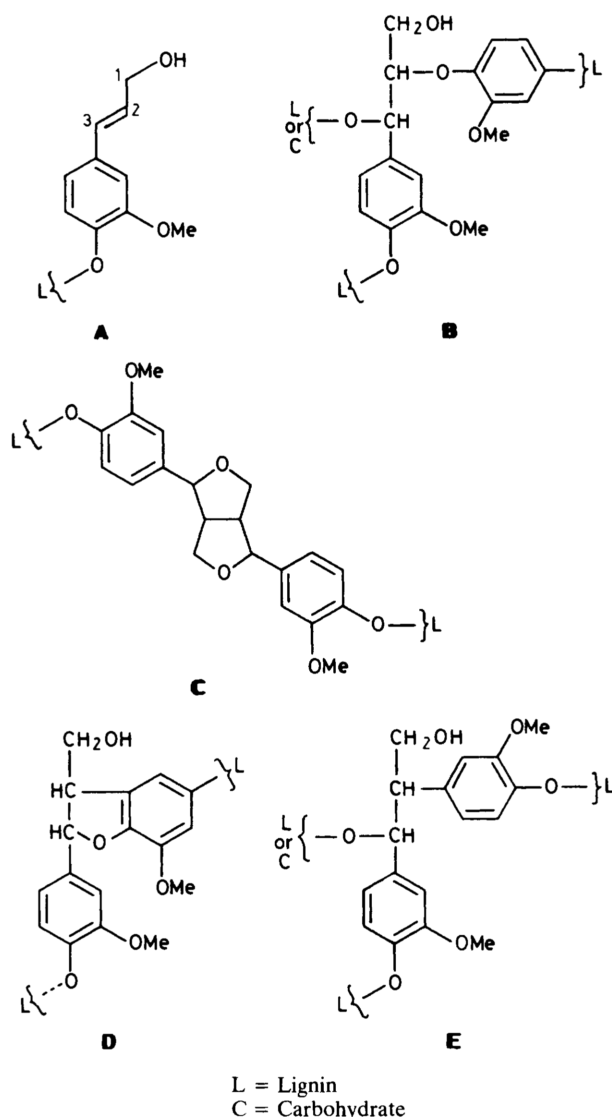


Figure 2. Main bonding substructures in lignin from coniferyl alcohol (**2**).

determined since no known method exists for its isolation in its native (unaltered) form. This is because isolated lignins have undergone substantial structural modifications, which are both irreversible and poorly understood, during their removal from plant tissue. While this is particularly true for lignin derivatives released *via* chemical means,[4] even the effects of supposedly mild delignification treatments, such as those encountered in the isolation of milled wood and enzyme-liberated lignin preparations, also need to be clarified. Currently depictions of lignin 'structure' are based upon structural information obtained from the analysis of *isolated* lignin-derived materials,[5] lignin model compounds[5,6] and artificial synthetic dehydrogenative polymers (DHPs),[1,2,7] generally considered to be representative of native lignin structure. These synthetic lignins can be produced *in vitro* from *E*-monolignols (**1**)—(**3**) by the action of horse-radish peroxidase and H_2O_2. It can thus be logically argued that none of these 'lignin' preparations or isolates adequately represents lignin structure *in situ*. We have developed methodology to obtain precise information about lignin bonding patterns *in situ*, thereby overcoming these prior limitations.

Figure 1a reveals the first example of the bonding environments of lignified root tissue from woody plant seedlings of *Leucaena leucocephala*, following continuous uptake of [2-^{13}C]ferulic acid (**4**) over a 28-day period and subsequent analysis of the resulting freeze-dried root tissue by solid state ^{13}C n.m.r. spectroscopy. All plants were grown hydroponically under aseptic conditions, and the tissues examined had lignin contents of 16.82% as evidenced by acetyl bromide lignin determinations.[8] No significant differences in the lignin contents of these plants were observed from that of plants grown in soil. It is important to note that the labelled ferulate (**4**) was administered *via* uptake from the hydroponic media, the extent of which was determined every seven days by h.p.l.c. analyses. To ensure that no molecular scrambling of the isotopic label had occurred, [2-^{14}C]ferulic acid (**4**) was also administered; the radioactivity in the hydroponic media only corresponded to ferulic acid (**4**), as evidenced by scintillation counting and h.p.l.c. analyses.

It is important to note that the ^{13}C spectrum shown in Figure 1a is a difference spectrum, obtained by the subtraction of a natural abundance spectrum from that of carbon-13 enriched tissue, *i.e.* only resonances corresponding to a particular enriched carbon (C-2) are observed. The signal at δ 127.5 corresponds to hydroxycinnamyl alcohol moieties [*i.e.* monolignols (**2**) and (**3**)] bonded *via* phenolic linkages to the rest of the lignin polymer (see Figure 2, substructure **A**) proving that the acid (**4**) had been converted to the corresponding hydroxycinnamyl alcohol. The smaller resonance in Figure 1a at δ 117.4 can be attributed to covalently bound ferulic acid (**4**), since the free acid has a resonance at δ 114.5. Of particular interest, however, is the very intense resonance at δ 82.7

(Figure 1a) which can be assigned to substructure **B** (Figure 2) based upon previous model compound[5] and DHP lignin[1,7] studies. In this tissue, this is the most dominant signal in the spectrum obtained and therefore presumably reflects the most prevalent bonding environment. This particular substructure has long been proposed[4] as the most dominant bonding pattern in woody lignin plant species; this result now confirms that suggestion. Lastly, the broad but weaker signals centred at δ 54—55 are coincident with substructures **C—E**, again based upon earlier model compound studies.[5]

For comparative purposes, Figure 1b shows the ^{13}C n.m.r. difference spectrum previously obtained for root tissue from the graminaceous plant, wheat (*Triticum aestivum* L),[9] which had been administered [2-^{13}C]ferulic acid (**4**) as before. Clearly, there are significant differences between the bonding pattern of this lignified tissue and that observed for *L. leucocephala* (Figure 1a). Thus, while the small signal at δ 127 for *T. aestivum* (Figure 1b) can again be attributed to substructure **A** (Figure 2), this is the only resonance that is coincident with one of *L. leucocephala* tissue. The large resonance at δ 114.6 in Figure 1b corresponds to C-2 of ferulate (**4**). This enhanced signal at δ 114.6 was observed in both the solid state ^{13}C n.m.r. spectrum, as well as in the ^{13}C n.m.r. spectrum of its soluble acetal lignin isolate,[10] verifying the presence of hydroxycinnamic acid components in the lignin polymer for that tissue. Graminaceous plants, such as wheat, differ from woody gymnosperm and angiosperm species by having significant quantities of cell-wall bound hydroxycinnamic acids [*e.g.* (**4**)]. These acids have long been speculated to be incorporated into the lignin polymer[9] and these findings now provide good evidence for this.

In the solid state ^{13}C n.m.r. spectrum of wheat root tissue (Figure 1b), there is essentially no resonance at δ 82 corresponding to substructure **B** (Figure 2), and previously noted as the dominant signal in the *L. leucocephala* spectrum (Figure 1a). Instead, large, yet unassigned, aliphatic resonances were observed at δ 74.1 and 39.7 respectively, which do not correspond to previously described lignin bonding patterns. Thus, in the two lignified plant tissues investigated to date, both have different and unique bonding patterns for the phenylpropanoid polymers.

These spectra may be compared with that of a DHP polymer obtained from (2-^{13}C)coniferyl alcohol (**2**) (Figure 1c).[1,7] In this case, two large resonances were observed at δ 127.6 and 55.5 (with a shoulder at δ 49) as well as a broader, less intense, signal at δ 83.8. These have been assigned previously as substructures **A** (δ 127.6), **B** (δ 83.8), and **C—E** (δ 55.5) respectively.

Interestingly, while the DHP polymer (Figure 1c) and *L. leucocephala* (Figure 1a) have similar sets of resonances, the relative intensities of each signal in both spectra are essentially inverted. However, in the case of *T. aestivum*, there are no obvious similarities in bonding patterns when compared to the DHP polymer.

These findings thus demonstrate that our knowledge of the natural lignification process, and of its ultimate structure *in situ*, is inadequate. Clearly, neither tissue studied to date has structures adequately represented by the DHP polymer described. These results suggest that the generic description of lignin requires re-evaluation. The actual bonding patterns of lignin, and their relative abundances in different plant tissues, must be determined. In this way, a more accurate portrayal of lignin structure will be made possible.

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