Monitoring Biosynthesis of Wheat Cell-Wall Phenylpropanoids in Situ

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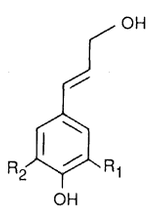
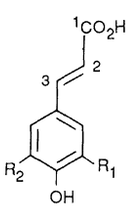
Lignins and suberins are complex plant cell-wall macromolecules that are composed mainly of phenylpropanoid residues derived from L-phenylalanine. Lignins and suberins are considered to be covalently linked to carbohydrates and to lipids, respectively. The bonding of these important structural materials within cell walls has never been established. By feeding specifically labeled [^{13}C]ferulic acid over extended durations to seedlings of *Triticum aestivum* L. and by using solid-state carbon-13 nuclear magnetic resonance techniques, the major resonances due to specific carbons in the propanoid side chains of these cell-wall polymers have been identified in situ. The signals were found to differ significantly from those of synthetic lignins, which have usually been considered to be good approximations of natural lignin structure.

MANY MAJOR CLASSES OF SECONDARY compounds in vascular plants are derived from the phenylpropanoid or cinnamate pathway, which originates in the deamination of L-phenylalanine and in some species of L-tyrosine as well. The most abundant metabolites of this pathway are lignins, which are complex polymers that are associated with cell-wall polysaccharides. Lignins are thought to be formed by the random coupling of free-radical intermediates that are derived from phenylpropanoid alcohols or monolignols (*1*). In gymnosperms these are (*E*)-*p*-coumaryl alcohol **1** and (*E*)-coniferyl alcohol **2**, whereas in angiosperms (*E*)-sinapyl alcohol **3** is also involved. Although there has been much research on the temporal and spatial deposition of this material in the cell wall, the final steps in the biosynthesis are not well understood (*2*). The polymerization step is thought to lack stereochemical control; much evidence indicates that covalent linkages are largely confined to the propanoid side chain (*3*). There is evidence for some preferred orientation of the aromatic rings of lignin in the secondary wall of black spruce wood (*Picea mariana*) (*4*).



1 R_1, R_2 = H
2 R_1 = OCH_3, R_2 = H
3 R_1, R_2 = OCH_3



4 R_1, R_2 = H
5 R_1 = OCH_3, R_2 = H
6 R_1, R_2 = OCH_3

The ratio of monomers, **1** through **3** varies not only with plant species (*5*) but also in their different organs (*6*). In addition to lignin, the cell walls of grasses and of certain other groups of plants contain appreciable quantities of hydroxycinnamic acids, **4** through **6**, that have been esterified to polysaccharides. These bound acids are thought to be involved in lignin biosynthesis but there is no strong evidence for this (*7*). Another widely distributed but poorly understood complex polymer is suberin, which provides a physical barrier to moisture loss in plants as well as a defensive shield against pathogens (*8*). It is a matrix of covalently linked aliphatic and aromatic domains with associated embedded waxes in these domains.

It has been difficult to determine the in situ bonding arrangements of lignins, suberin, and cell-wall esterified hydroxycinnamic acids. Solid-state ^{13}C nuclear magnetic resonance (NMR) (*9*) has a serious drawback because the important ^{13}C aliphatic resonances that correspond to interunit bonding between lignin or suberin monomers are masked by carbohydrate resonances. However, by feeding living plants with ^{13}C-labeled lignin precursors over extended periods we could enhance the signals from the newly formed bonding patterns of phenylpropanoids in situ to detectable levels.

Feland wheat (*Triticum aestivum* L.), which was grown for 21 days on agar medium and under aseptic conditions, had the highest content of lignin (10%) in its root tissue; its solid-state ^{13}C NMR spectrum is shown in Fig. 1A (*10*). According to the work of Atalla *et al.* (*11*) and Earl and VanderHart (*12*) with isolated cellulose, the dominant carbohydrate resonances can be assigned as follows: positions C-2, C-3, and C-5, 74 and 72 ppm; C-1, 105 ppm, C-4, 89 ppm (with a shoulder at 83 ppm); and C-6, 64 ppm (with a high-field shoulder). Unfortunately, these signals mask the weaker resonances that correspond to the important aliphatic linkages of lignin, suberin, and related molecules. The only noncellulosic aliphatic signals are at 56 and 20 to 45 ppm. The former resonance corresponds to aromatic methoxyl groups, and the latter are presumably due to aliphatic methylenic functionalities. At lower field (>110 ppm) the resonances observed are mainly due to aromatic, olefinic, and carboxylic functionalities.

To identify the resonances due to specific carbons in lignin and other phenylpropanoid substances in plant tissue, we developed methodology for the continual uptake of specifically labeled ^{13}C phenylpropanoid precursors into intact plants over extended periods (weeks or months). Optimized precursor feeding conditions were initially determined with radioactively labeled [2-^{14}C]ferulic acid **5** incorporated through the roots to the extent of 10% over a 21-day period. We repeated this experiment with specifically labeled forms of ferulic acid that contained 90 to 99 atomic percent ^{13}C at C-1 (**5a**), C-2 (**5b**), and C-3 (**5c**).

After uptake of [2-^{13}C]ferulic acid over this time period the solid-state ^{13}C NMR spectrum for wheat roots (from about 20 to 30 plants) was recorded (Fig. 1B) and this spectrum was subtracted from that of a control (Fig. 1A) to obtain a difference spectrum (Fig. 1C). Each experiment was individually repeated three times; in each case the same results were obtained. In Fig. 1C, ^{13}C-enrichments are evident at 127.3, 114.6, 74.1, and 39.7 ppm. We assign the resonance at 114.6 ppm to C-2 of **5b** in free or bound form [we cannot expect to distinguish between these possibilities since the signal for C-2 of ferulic acid esterified to a trisaccharide from bagasse is shifted upfield by only 0.3 ppm (*13*)]. The broad signal at 127.3 ppm can be attributed to C-2 of coniferyl and perhaps sinapyl alcohols (*14*). Hydroxycinnamyl alcohol moieties have not been unambiguously detected previously as such in intact plant tissue. The exact nature of the chemical bonding patterns of these three alcohols to other plant constituents remains to be established. These alcohols may not only be linked to lignin through their phenolic groups but may also be ester-linked to other constituents, since the diacetate of coniferyl alcohol has a C-2 resonance at 123 ppm (*15*).

A considerable portion of the presumed

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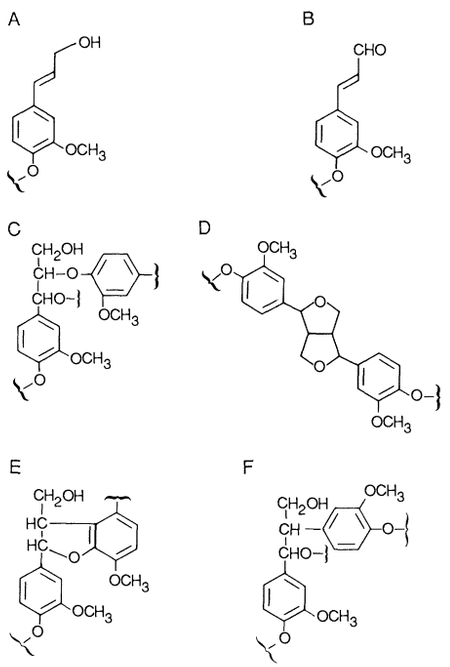
dominant bonding patterns of lignin (substructures A through F) purportedly involve the C-2 of the monolignols, **1** through **3**, according to ^{13}C NMR analysis of model compounds (*16, 17*), isolated lignins (*16*), and "synthetic lignin" preparations (*14, 17, 18*). Synthetic lignins, which are thought to approximate lignin, are formed by the random in vitro dehydrogenative polymerization (DHP) of the monolignols, **1** through **3** (*18*). For DHP lignin obtained from [2-^{13}C]coniferyl alcohol there are two dominant resonances at 127.6 ppm (substructure A) and 53 to 54 ppm (substructures D through F), and a less intense resonance at 83 to 84 ppm (substructure C) (*14*).



For *T. aestivum*, the signals at higher field that correspond to the important DHP lignin substructures C (83 to 84 ppm) and D through F (53 to 54 ppm) are essentially absent. Instead, a large broad signal centered at 74.1 ppm that we could not assign is observed. Both this and the large resonance at 39.7 ppm due to C-2 methylenic functionalities were unexpected based on the previous model studies.

The solid-state ^{13}C NMR spectra obtained from wheat roots after uptake of [3-^{13}C]ferulic acid **5c** is shown in Fig. 1D. Enrichments are evident in the difference spectrum (Fig. 1E) at 195, 165.5, 146.5, 137.13, 83, 73, and 40 to 56 ppm. However, for DHP lignin produced from [3-^{13}C]coniferyl alcohol there is a strong resonance at 86 ppm (substructures D and E) with two weaker signals at 132 ppm (substructure A) and 72 ppm (substructures C and F) (*14*). For *T. aestivum* the small enhancement at 195 ppm is direct evidence for the existence of C-3 carbonylic functionalities. The other small resonance at 165.5 ppm is attributed to esterified vanillic acid formed by cleavage of the double bond at C-2 after uptake of the acid **5c**. Such bond cleavage of ferulic acid is well documented (*19*), for example, in the biogenesis of hydrolyzable tannins.

The resonance at 146.5 ppm corresponds to [3-^{13}C]ferulic acid either in free or bound form as previously discussed. Resonances at 137.13 and 128.7 ppm are due to hydroxycinnamyl alcohol moieties (**2** or **3**) in various bound chemical environments; the larger resonance at 137.13 ppm is due to [3-^{13}C]coniferyl alcohol, either in its ester- or phenolic-bonded forms. At higher field, resonances occur at 40 to 56, 73, and 83 ppm. The small resonances from 40 to 56 ppm are due to methylenic functionalities at C-3. This was also unexpected. By comparison with DHP lignin models (*14*), the resonance at 73 ppm would be due to substructures C and F. However, our incorporation data with [2-^{13}C]ferulic acid did not support that bonding pattern as such in the intact plant. This point must be resolved. Simple model compound studies show that bonding of the C-3 carbon [to either carbohydrate (*20*), phenolic, or hydroxycinnamic acid (*21*) moieties] yield resonances between 80 to 82 ppm, so that the strong resonance at 83 ppm is direct evidence for such chemically labile bonding in intact plants.

The solid-state ^{13}C NMR spectra for that of *T. aestivum* roots previously administered [1-^{13}C]ferulic acid **5a** are shown in Fig. 1F. There are strong resonances in the difference spectrum (Fig. 1G) at 174.9 and 169.6 ppm and weaker ones at 60 to 72 ppm. DHP lignin from [1-^{13}C]coniferyl alcohol shows an intense signal at 62 ppm (substructures A, C, E, and F) with a shoulder at 71 ppm

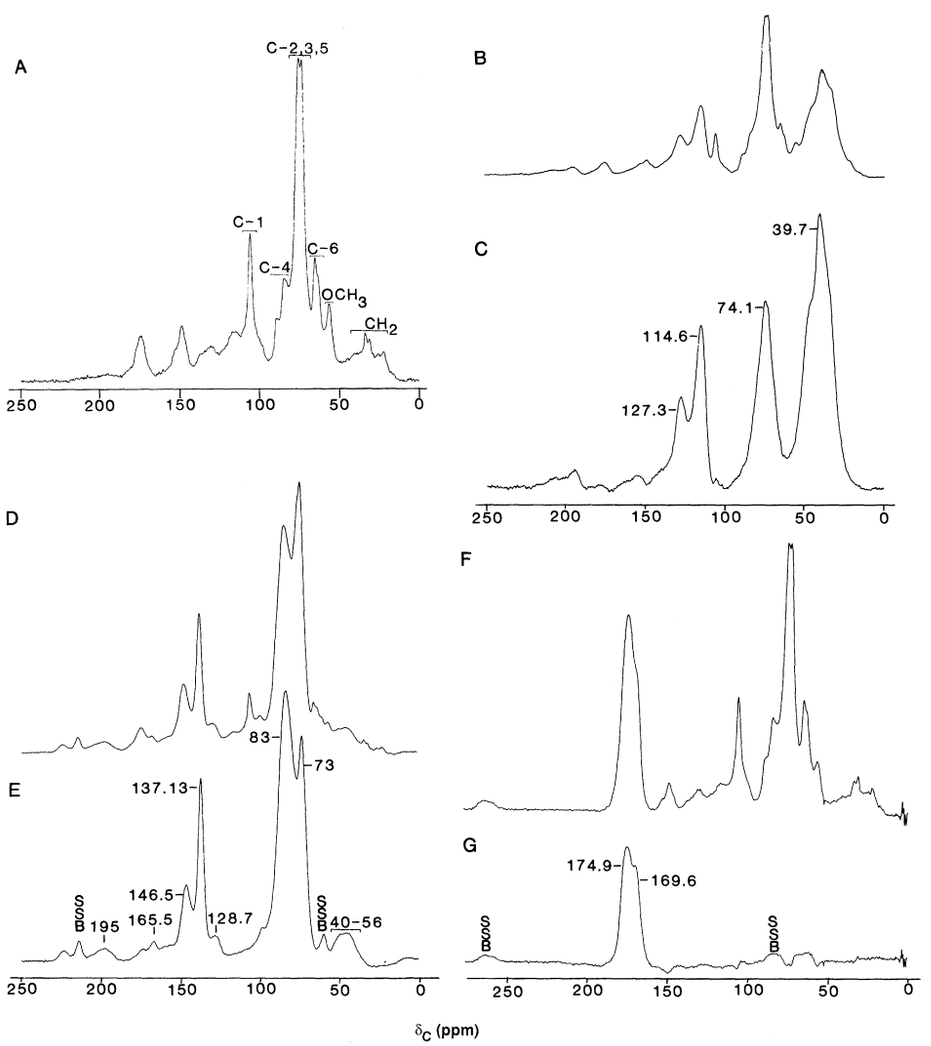


Fig. 1. (**A**) Solid-state ^{13}C NMR spectrum of *T. aestivum* roots (natural abundance). (**B** through **G**) Typical solid-state ^{13}C NMR spectra of *T. aestivum* roots that were administered: [2-^{13}C]ferulic acid (B and C); [3-^{13}C]ferulic acid (D and E); and [1-^{13}C]ferulic acid (F and G). For each pair of spectra the latter is a difference spectrum formed by subtracting the enhanced spectrum from the control shown in (A). Spinning side bands are denoted as SSB.

(substructure D) (*14*). For *T. aestivum*, the resonance at 174.9 ppm corresponds to bonded (esterified) ferulic (or sinapic) acid and the signal at 169.6 ppm corresponds to either free ferulic acid (or sinapic) acid. We made this assignment because the carbonyl resonance shifts downfield by 7 ppm when ferulic acid is ester-bonded to a trisaccharide (*13*). The smaller resonances at 60 to 72 ppm can be attributed to a hydroxymethylenic functionality in substructures A and C through F.

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Synthetic Peptide Immunoassay Distinguishes HIV Type 1 and HIV Type 2 Infections

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Efforts to solve the epidemiologic puzzle of AIDS in Africa are complicated by the presence of multiple human retroviruses. Simple serologic tests that unambiguously distinguish among infections by these retroviruses are essential. To that end, a partially conserved immunoreactive epitope was identified in the transmembrane glycoproteins of human immunodeficiency viruses (HIV) types 1 and 2. Synthetic peptides derived from these conserved domains were used in sensitive and specific immunoassays that detect antibodies in sera from patients infected with HIV-1 or HIV-2. By making single amino acid substitutions in the HIV-1 peptide, it was possible to demonstrate HIV-1 strain-specific antibody responses to this epitope. Such custom-designed peptides synthesized from this domain are likely to detect newly discovered HIV types, define infection with specific HIV strains, and allow detection of group-common antibodies.

ALTHOUGH THE ACQUIRED IMMUNE deficiency syndrome (AIDS) is now recognized as an epidemic of global dimensions, Africa has the highest prevalence of this disease (*1*). Infection with human immunodeficiency virus type 1 (HIV-1; also known as HTLV-III, LAV-1, and ARV), the retrovirus first shown to cause AIDS (*2*), is widespread in Central Africa (*3*). Serologic surveys have also confirmed the presence of HIV-1 in East Africa (*4*) and West Africa (*5, 6*), although the number of AIDS cases in these regions is thus far much smaller than in Central Africa.

Recently another pathogenic human retrovirus, termed HIV type 2 (formerly LAV-2), was recovered from West African patients with AIDS (*7*). HIV-2 infection is associated with an immunodeficiency syndrome clinically indistinguishable from that caused by the prototype AIDS virus, HIV-1 (*8, 9*). HIV-2 is related to but distinct from HIV-1 and from simian immunodeficiency virus (SIV or STLV-III), the etiologic agent of simian AIDS (*7*). Extensive serologic cross-reactivity exists between HIV-2 and SIV, whereas cross-reactivity between HIV-2 and HIV-1 is limited to core antigens (*7, 9*). Furthermore, numerous serologic surveys, in which a retroviral isolate termed HTLV-IV was used as antigen, have confirmed human infection in several West African countries (*5, 6, 10*). However, HTLV-IV is not genetically distinguishable from SIV (*11*) or antigenically distinguishable from HIV-2 (*7, 10*). The precise taxonomic relationships among these viruses have not yet been established.

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Table 1. Sequences of synthetic peptides derived from transmembrane glycoproteins of HIV-1 and HIV-2. The amino acid position numbers for HIV-1 are based on the sequence published by Wain-Hobson *et al.* (*21*); those for HIV-2 are based on the sequence published by Guyader *et al.* (*20*). Residues in the HIV-1 peptides that differ from the sequence of the prototype isolate (peptide 2) are underlined. The boxed areas show residues conserved between HIV-1 and HIV-2.

Peptide number	Isolate name	Source	Amino acid sequence*	Reference
			598 609	
1	HIV-1 (Z-3)	Zaire	Leu-Gly-Leu-Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile-Cys	(14)
2	HIV-1 (LAV_{BRU}; $HTLV\text{-}III_B$; $HTLV\text{-}III_{RF}$; WMJ-1; ARV-2)	France; United States	Leu-Gly-Ile-Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile-Cys	(21,29)
3	HIV-1 (LAV-ELI)	Zaire	Leu-Gly-Ile-Trp-Gly-Cys-Ser-Gly-Lys-His-Ile-Cys	(19)
4	HIV-1 (LAV-MAL)	Zaire	Leu-Gly-Met-Trp-Gly-Cys-Ser-Gly-Lys-His-Ile-Cys	(19)
			592 603	
5	$HIV\text{-}2_{ROD}$	Cape Verde Islands	Leu-Asn-Ser-Trp-Gly-Cys-Ala-Phe-Arg-Gln-Val-Cys	(20)

*The three-letter and single-letter abbreviations for the amino acids are: Ala (A), alanine; Arg (R), arginine; Asn (N), asparagine; Asp (D), aspartic acid; Cys (C), cysteine; Gln (Q), glutamine; Glu (E), glutamic acid; Gly (G), glycine; His (H), histidine; Ile (I), isoleucine; Leu (L), leucine; Lys (K), lysine; Met (M), methionine; Phe (F), phenylalanine; Pro (P), proline; Ser (S), serine; Thr (T), threonine; Trp (W), tryptophan; Tyr (Y), tyrosine; and Val (V), valine.