
5 NMR of Lignins

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INTRODUCTION

NMR IN LIGNIN RESEARCH

NMR spectroscopy has enormously facilitated investigations into structural aspects of complex lignin polymers. Principally due to its high resolution and the high dispersion of chemical shifts, ^{13}C NMR has played a major role in the qualitative and quantitative understanding of lignin structure. Now modern two- and three-dimensional correlative methods are powerful tools for identifying structural units.

Nuclear Magnetic Resonance (NMR) spectroscopy has developed far beyond the simple acquisition of a proton or carbon spectrum. Today there are literally thousands of “NMR experiments” and variants that can be run to target the type of information sought. Fortunately, these experiments fall naturally into a handful of classes of useful experiments. Two-dimensional (2D) NMR, where more than a single NMR parameter is presented in a spectrum, is common and has been applied to the elucidation of lignin structure and bonding. Three-dimensional (3D) NMR has more recently been applied to problems in lignin chemistry. NMR hardware has become increasingly sophisticated and recent advances have improved both the sensitivity and the quality of data that can be acquired. Spectrometers operating at a 1000 MHz proton frequency are commercially available. The increase in magnetic field strength provided by such spectrometers offers enormously improved sensitivity and spectral dispersion over the 60 MHz instruments that were available at the time Sarkanen and Ludwig edited their famous book on Lignins [1].

This chapter will consider the basic aspects and findings of several forms of NMR spectroscopy, including separate discussions of proton, carbon, heteronuclear, and multidimensional NMR. Enhanced focus will be on ^{13}C NMR, because of its qualitative and quantitative importance, followed by NMR’s contributions to our understanding of lignin structure by topic rather than by the type of NMR experiment. The more recent availability of lignin-biosynthetic-pathway mutant and transgenic plants has greatly aided the NMR analysis of perturbed and normal lignins, providing deeper insights into the lignification process. Solution-state NMR analysis of lignins, without the need for isolation from the other cell wall components, is also covered.

SENSITIVITY OF NMR SPECTROSCOPY

Compared to other spectral techniques, NMR may be orders of magnitude less sensitive. Ultraviolet and infrared spectroscopy involve transitions from an extensively populated ground state to an excited state that is not occupied under normal conditions because the energy levels are sufficiently far apart. Relatively large energies are involved. In NMR, the “ground state” and excited energy levels are so close in energy that thermal equilibrium assures that both states are essentially equally populated. In low-field spectrometers, for example, there may be only one in a million protons more in the lower energy state than in the high energy state. It is only the population difference that contributes to the NMR signal. This factor alone explains a sensitivity several orders of magnitude lower than other spectral techniques. The low energy involved, however, provides one striking advantage to NMR. Since the RF excitation frequency only acts on nuclear spins, and since the energy involved is too low to cause significant other physical or chemical changes in the molecule, NMR is the only spectroscopy that does not “visibly” perturb the system being measured. In other words, the structure and three-dimensional shape of a compound in solution being “viewed” by NMR is exactly the same as the structure when it is not being viewed. Other techniques are invasive; they perturb the system they are measuring.

Several nuclei of interest suffer from low natural abundance: protons are essentially 100% ^1H ($\sim 0.015\%$ ^2H); phosphorus is 100% ^{31}P ; the major carbon isotope, ^{12}C , is not NMR-active, while the ^{13}C isotope is only present in 1.11% relative abundance. Consequently, ^{13}C NMR at natural abundance loses another two orders of magnitude in sensitivity. Also, the energy levels are even closer in ^{13}C than in ^1H (roughly a factor of $1/4$), resulting in an even smaller population difference between the levels and causing yet another decrease in sensitivity. Sample sizes typically in the milligram range are required to obtain good spectra in reasonable times. Despite this apparently abominable sensitivity, the information content of NMR spectra is significantly higher than for other spectral techniques. NMR alone can often fully identify compounds. Structure and bonding patterns are readily elucidated even in complex molecules.

Considerable gains in sensitivity have been realized through other approaches. A 2-dimensional(2D) ^{13}C – ^1H correlation spectrum, for example, can now be acquired much more quickly than a 1-dimensional (1D) ^{13}C spectrum [2]. Although a relatively insensitive spectroscopic method, NMR still provides an amazingly detailed picture of lignin structure from a combination of experiments, if sufficient sample (typically 10–100mg) is available.

EVOLUTION OF NMR METHODS

Proton and FT-NMR

Proton NMR was the first type of NMR spectroscopy to become available to the researcher. The high natural abundance of the NMR-active ^1H -nucleus ($\sim 100\%$) and its relatively high sensitivity allowed early low-field continuous-wave (CW) spectrometers, with little in the way of signal-averaging capabilities, to successfully produce spectra from milligram quantities of small organic molecules. Such instruments,

however, were far from ideal for studying a polymer such as lignin. One problem is to get a significant weight of material to dissolve in the small volume required for NMR (typically 0.5 ml). Even if this were accomplished, researchers were then faced with lignin's complexity, which meant that the concentration of any given structural type is rather low. Some kind of signal averaging was required to improve the signal-to-noise ratio of such spectra. Before the implementation of Fourier Transform NMR (FT-NMR, described below), this could only be accomplished by acquiring and adding frequency-domain data directly. A typical ^1H NMR scan on an early CW instrument might take up to 5 minutes. With the right accessories, a second scan could then be recorded and added to the first, and so on. The signal-to-noise improvement from such scan-averaging comes from the differing signal and noise properties; coherent signals from the sample will grow in proportion to the number of scans (NS), whereas the noncoherent noise grows only as $\text{NS}^{1/2}$. Therefore, the signal to noise of the spectrum grows as $\text{NS}/\text{NS}^{1/2} = \text{NS}^{1/2}$. Thus, the signal-to-noise ratio of a spectrum will be doubled if four scans are averaged; a 10-fold increase in signal-to-noise will require averaging 100 scans. With scans requiring as long as 5 minutes, the amount of signal averaging possible was limited.

The advent and implementation of Fourier Transform NMR (FT-NMR) allowed essentially unlimited signal averaging by digitally acquiring time-domain Free Induction Decay (FID) data at a far higher rate than could be accomplished by acquiring and adding frequency-domain data directly. In FT-NMR, the entire frequency response (amplitude vs time) is acquired following the application of a sharp, narrow duration ($\sim 10\ \mu\text{s}$) pulse. Once the sample has relaxed back toward its equilibrium state, the sample can be pulsed again, and the response acquired again, adding it to the former. Two aspects make this method superior. First, the experiment is ready to be repeated after typically just a few seconds (rather than the 5 minutes on the CW instrument). This means that acquiring even a hundred thousand scans becomes feasible. Secondly, the required frequency data can be extracted from the amplitude vs time response by a Fourier transformation. The Fourier transform itself was initially a computationally intensive task that could tie up a minicomputer for hours; however, advances in computing power have been such that ID transform times are now subsecond. Another innovation was required to provide the stability required to allow accumulation of scans over an extended time. Independent RF circuitry was used to monitor the deuterium NMR signal supplied via the sample's solvent, allowing the instrument's electronics to track and compensate for any "drift" of the field within the sample.

Carbon NMR

The carbon skeleton of any organic molecule is a key feature; thus, ^{13}C NMR becomes an important diagnostic spectroscopic method. Fourier-transform-NMR was the enabling technology that allowed ^{13}C -NMR to be viable on real samples [3,4]. Due to the low sensitivity associated with the ^{13}C -nucleus, researchers needed rapid and extensive signal averaging. Once the sensitivity aspects could be overcome, ^{13}C NMR had some advantages over ^1H NMR. Primarily, the chemical shift range is significantly larger, $\sim 200\ \text{ppm}$ vs $\sim 10\ \text{ppm}$ for protons in typical organic molecules. Dispersion in ^{13}C NMR is considerable because the line widths are comparatively

narrow; that is, it is relatively rare for two unrelated carbons in a small molecule or in a mixture to have identical chemical shifts. However, the complex nature of lignins leads to broad signals and to considerable overlap. A significant feature of ^{13}C -NMR is that chemical shifts are almost entirely dictated by through-bond, rather than through-space, interactions, except in cases of steric compression, where carbons are forced to occupy positions within the Van der Waals radii of other atoms [5,6]. The result is that a carbon chemical shift can reasonably be predicted by adding up contributions from its bonding environment [3,4,7]. For lignins, the chemical shifts in good model compounds are, therefore, the same as their counterparts in the polymer. In practice, side-chain carbon chemical shifts can be rather well modeled by dimeric lignin models such as guaiacylglycerol- β -guaiacyl ether, even though the “B-ring” (with no side-chain) is not well modeled [8,9]. Modeling side-chain carbon shifts in etherified units by using simple 4- O -Me derivatives is often satisfactory. The aromatic carbons can be much more difficult to model. Obviously, free-phenolic β -ether units can be modeled successfully using the A-ring of the same β -aryl ether model. However, to successfully model an etherified unit, it is necessary to use the internal unit of a trimeric model compound [10,11]. Data from β -aryl ether homopolymers are now also available [12]. Detailed analysis and assignment of model compound data provides good insight into the interpretation of the NMR signals of polymeric lignins, as will be explained below.

Another consideration was crucial to the development of the ^{13}C NMR method. Two NMR-active nuclei will normally interact by coupling interactions. The interactions of two ^{13}C nuclei can be neglected in most experiments, due to the low abundance of ^{13}C isotopes; most ^{13}C nuclei will find themselves within range of only ^{12}C nuclei that are not NMR active and that will, therefore, not couple. However, essentially all protons in a molecule are ^1H isotopes. Therefore, a ^{13}C nucleus will interact with any ^1H within range—usually within three bonds of the carbon. Such short- and long-range coupling interactions give rise to rather complex ^{13}C NMR spectra. This is the primary reason why, from the outset, protons are usually “decoupled” in ^{13}C NMR. Initially, ^1H -decoupling was via broadband irradiation of proton frequencies, but is now more efficiently accomplished via pulse sequences such as WALTZ [13,14] and MLEV [15]. The result is that all signals in ^{13}C NMR spectra of normal organic molecules (with normal isotopic distributions) are a series of single, sharp signals (peaks)—one peak for each (magnetically distinct) carbon. Another advantage of decoupling is that the carbon signal intensity is enhanced by irradiating attached protons; this is typically referred to as the nuclear Overhauser enhancement, or nOe [16]. Enhancements of up to a factor of 3 provide much needed sensitivity improvements for this insensitive nucleus. A disadvantage is that unequal enhancements produce peaks with areas that are not proportional to the number of carbons contributing to the peak. Quantitative (integrable) ^{13}C -NMR spectra can be acquired under special conditions, as will be covered in the Quantitative ^{13}C NMR of Lignins section.

Pulsed NMR Experiments

The power of NMR methods rose exponentially with the introduction of NMR pulse sequences. The first experiments simply pulsed the system and directly measured

the response (a so-called single-pulse experiment). Later, pulse sequences strung together a series of pulses, each characterized by (a) the angle through which they flip the nuclear spin of the applied nucleus, and (b) the specific delays between the pulses and before the eventual acquisition of the signal. The intricacies of pulse-programming and the details of how such pulse experiments work are well beyond this review; a number of texts deal with these aspects [17–24]. The scope of such experiments and the ingenuity with which they were developed seemed unlimited; hundreds of experiments became available. One of the earliest experiments, the Attached Proton Test or APT experiment [25], allowed spectra to be acquired that distinguished the number of protons attached to each carbon, an extraordinarily useful feature. What followed were increasingly more sophisticated experiments, with increasingly bizarre acronyms such as INEPT, Insensitive Nuclei Enhanced by Polarization Transfer [26]. Unfortunately, not all of the new acronyms were relevant or accurate; some were proposed before researchers elucidated the mechanisms by which the experiments worked. The resulting jungle of fanciful, to silly acronyms remains. It is normal today to use the acronym and reference the experiment, but not to expand the acronym.

2D (and nD) NMR

Two-dimensional NMR resulted naturally from pulse programming concepts [20]. The basic idea is that a second dimension could be simulated by acquiring a series of spectra in which a delay period in the pulse sequence was incrementally increased. In what seemed at first like pure magic, it became possible, for example, to correlate a carbon's chemical shift in one dimension with the proton chemical shift of its attached proton in a second dimension. Again, the ingenuity for designing pulse programs to tease out the data of interest was boundless, and development of new experiments has continued unabated until the present.

A particular advantage of the ^{13}C – ^1H correlative 2D NMR methods for lignin (described in more detail below) is the virtual dispersion that can be realized. Many of the protons in lignin from different units severely overlap, but their carbons are often distinct (or vice versa). The ability of experiments to separate overlapping peaks from one type of nucleus (e.g., ^1H) by correlating it with separated peaks from another nucleus (e.g., ^{13}C) produces spectra far better resolved than exhibited in 1D NMR. The initial impediments to 2D NMR were mainly associated with data size and computational difficulties, but advances in computing have obviated any such concerns.

Correlations anticipated in various homonuclear (^1H – ^1H) and heteronuclear (^{13}C – ^1H) 2D NMR experiments are conceptualized in Figure 5.1. A hypothetical model compound (the chemical shifts are not accurate and are for illustrative purposes only) with three aromatic protons and four side-chain protons on its three side-chain carbons is used to illustrate the information available from each experiment. A set of five experiments, in addition to the standard 1D proton and carbon spectra, are useful for characterizing any model compound or lignin. The correlation spectroscopy (**COSY**) experiment correlates directly coupled protons (Figure 5.1a).

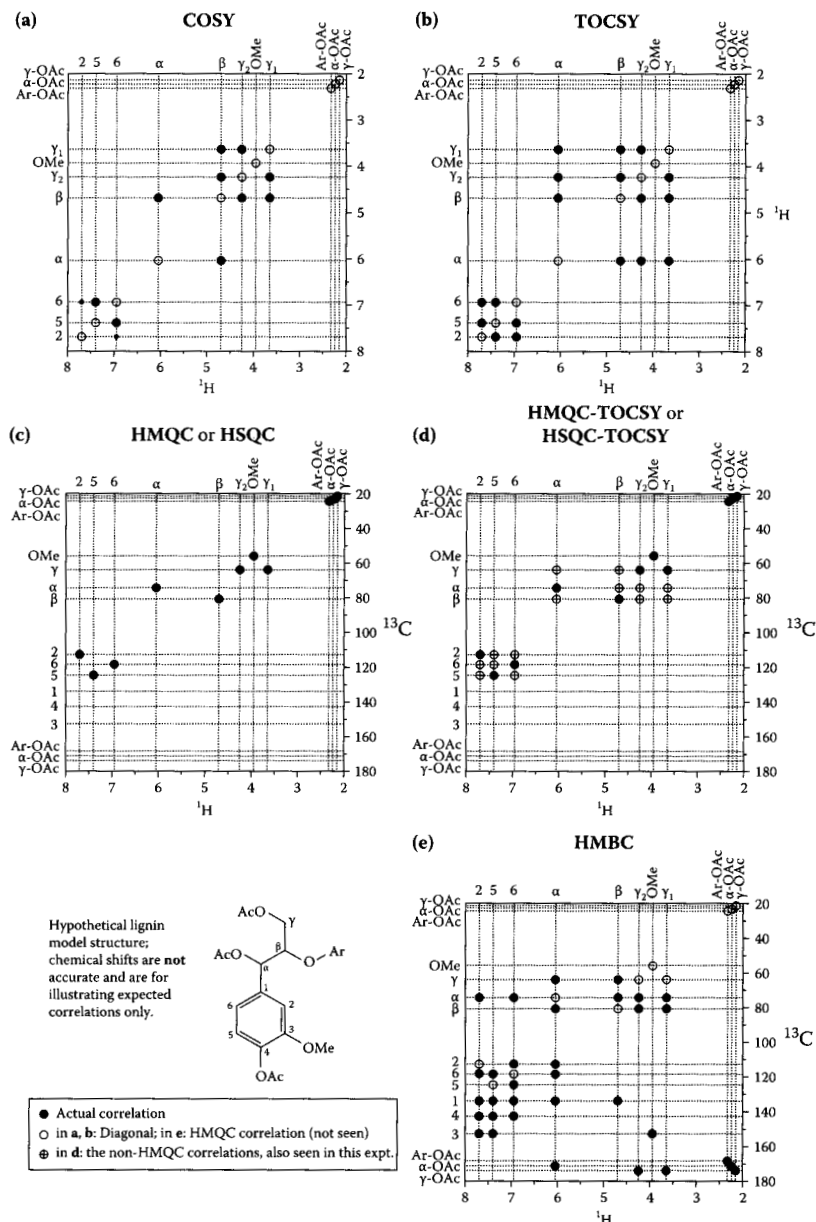


FIGURE 5.1 Conceptual representation of the correlation contours resulting from various homonuclear (^1H – ^1H) a) COSY and b) TOCSY and heteronuclear (^{13}C – ^1H) c) HMQC or HSQC, d) HMQC-TOCSY or HSQC-TOCSY, e) HMBC) 2D NMR experiments. A hypothetical lignin model compound (the chemical shifts are not accurate and are for illustrative purposes only) with 3 aromatic protons and 4 side-chain protons on its 3 side-chain carbons is used (See text for details.)

Thus, H_α correlates with H_β , which also correlates with the two H_γ -protons, etc. A weak H_2 – H_6 correlation is shown; the coupling constant is ~ 2 Hz for this long-range correlation. In the TOCSY experiment (Figure 5.1b), a proton correlates with all other protons in its coupling network. Thus, all side-chain protons, for example, correlate with each other. The HMQC or HSQC experiment simply correlates each carbon with its directly attached proton (Figure 5.1c). Thus, H_α correlates with C_α , etc. The HMQC-TOCSY or HSQC-TOCSY experiment extends the HMQC by correlating a carbon not only with its attached proton but all other protons in the same coupling network (Figure 5.1d). Thus, C_α correlates with H_α , H_β , and the two H_γ -protons. As a corollary, H_α correlates with the three side-chain carbons C_α , C_β and C_γ . The heteronuclear multiple-bond correlation (HMBC) experiment correlates protons and carbons that are related by long-range coupling interactions over 2–3 bonds, providing extremely valuable connectivity information (Figure 5.1e). Thus, for example, H_α correlates with all carbons within 3 bonds; C_β , C_γ , C_1 , C_2 , C_6 , and the α -acetate carbonyl. It is quickly apparent that carbon assignments can be made unambiguously from HMBC data. For example, C_1 is the only aromatic carbon able to correlate with H_β , C_3 is the only carbon that can correlate with the methoxyl protons, and α - and γ -acetate carbonyls are distinguished by their correlations with α - vs γ -protons.

In the following section, which briefly describes the various experiments, spectra from various synthetic and isolated lignins will be used for illustration; synthetic lignins have the advantage of containing all of the major lignin unit types, without the complications of carbohydrates.

Homonuclear Correlation Spectroscopy, COSY TOCSY, and INADEQUATE Classes

Proton-proton 2D experiments are an extraordinary improvement over 1D proton spectra (see Figure 5.7e later in the Broadness of Proton Spectra section). Broadness per se is not a significant deterrent in 2D spectra, which are taken at much lower resolution than 1D spectra. Homonuclear COSY experiments correlate protons that are coupled to each other with reasonable coupling constants, typically >2 Hz, as conceptualized in Figure 5.1a. Normally, these are vicinal protons (protons on adjacent carbons) and geminal protons (protons on the same carbon). The side-chain carbons of the common units in lignin all bear protons, so correlations are evident from H_α to H_β to the H_γ protons for each side-chain unit [2,27,28]. As with most other experiments, a gradient-edited COSY experiment allows acquisition of spectra without extensive phase cycling, so that even a single scan per increment is sufficient. For lignins, acquiring several scans per increment improves signal-to-noise, so remains worthwhile, particularly for identifying minor components. Multiple-quantum-filtered variants are useful to prevent strong (single-quantum) correlations from the methoxyl and acetate methyl groups from dominating the spectrum. An example of COSY experiment for a pine compression wood acetylated milled wood lignin (MWL) is shown in Figure 5.2a. Long-range coupling is typically under 2 Hz. The long-range variant of the COSY experiment can enhance correlations from protons associated by small coupling constants (down to -0.1 Hz), particularly in the case of low-molecular mass lignin models and lignans [29].

Typical synthetic dehydrogenation polymers (DHPs), such as the one giving the data in Figure 5.2b, have an overabundance of units arising from dimerization of monomers—resinols C and cinnamyl alcohol end-groups **X1**, and also noncyclic α -aryl ethers **A2**. Resinols and cinnamyl alcohol end-groups show up at lower contour levels. One of two types of the minor dihydroconiferyl alcohol (DHCA) units **X5** is clearly evident in the pine lignin, Figure 5.2c; the other higher δ component seen in Figure 5.2b arises from 5-coupling of such units and can be seen in lignins with increased incorporation of DHCA.*

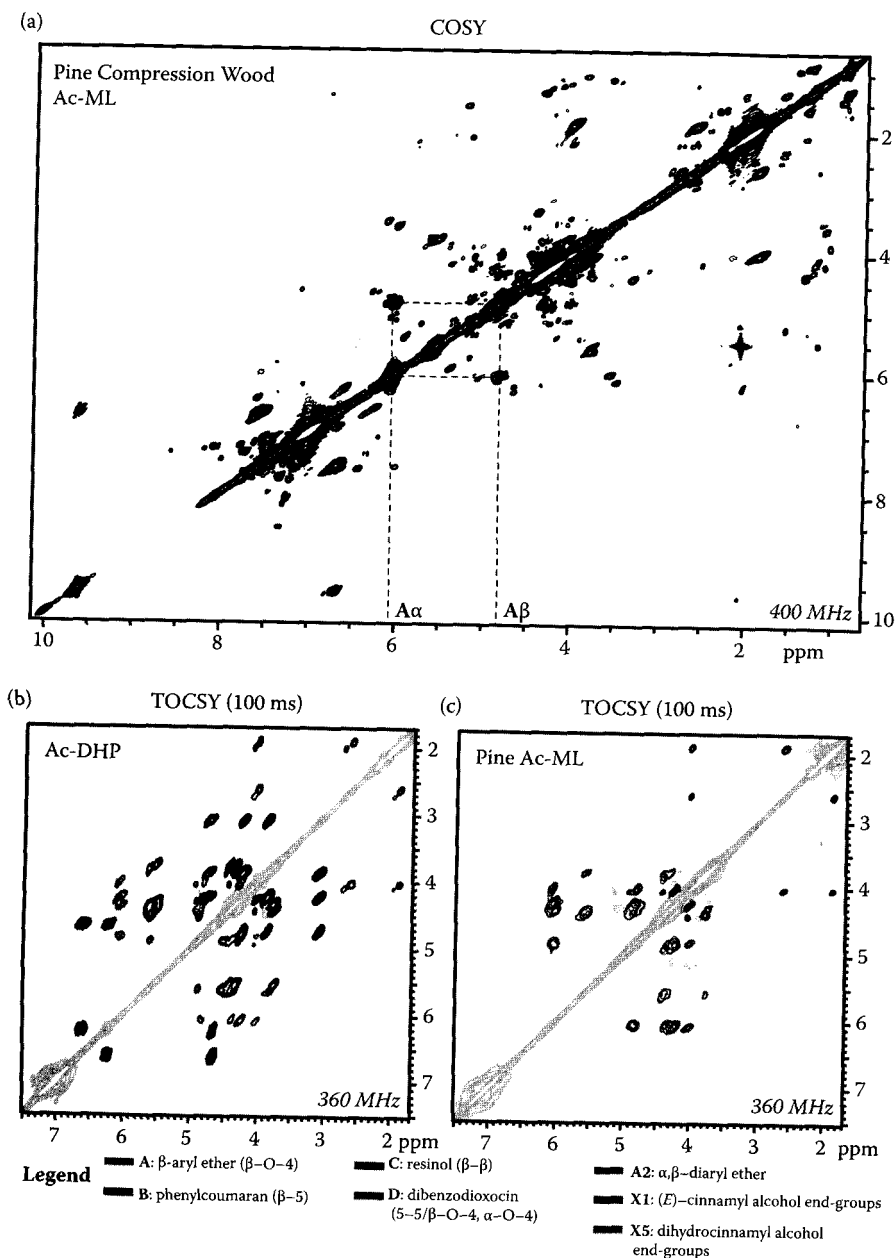
Although COSY type experiments are useful, the extra correlations provided by TOCSY experiments (Figure 5.1b) are particularly valuable [2,30,31]. Figures 5.2b and 5.2c show example TOCSY spectra for a synthetic lignin (DHP) and an isolated softwood lignin, both acetylated. Unlike typical DHP spectra, the spectra of isolated softwood lignins confirm the low abundance of products from monolignol coupling, having only minor contours corresponding to end-groups **X5** and resinols C. They also have a virtual absence of α,β -diethers **A2**. One type of DHCA product **X5** appears in the pine lignin, but two different products **X5** are in the DHP, which was synthesized from coniferyl alcohol containing ~2% DHCA. The second product comes from 5–5-coupling of at least one, and probably two, DHCA units. This component becomes significant in a pine mutant down-regulated in cinnamyl alcohol dehydrogenase (CAD), which is described later in the CAD-Deficient Pine: Incorporation of Dihydroconiferyl Alcohol and Guaiacylpropane-1,3-diol section and in Figure 5.12.

In TOCSY spectra, the mixing time determines the relative intensity of the various contours; mixing times of 60–120ms are typically used. A short mixing time (10–30ms) yields essentially COSY-type spectra, where protons correlate only with their directly coupled neighbors. The TOCSY (also called HOHOHA) experiments have some advantages over COSY and long-range COSY experiments for lignins [30]. During the mixing time, relaxation is less significant and TOCSY spectra are less sensitive to the initial state of the magnetization, which means that pulsing can be faster; full 2D TOCSY experiments can be acquired in as little as 2 minutes. Model compound data agreed well with corresponding data in lignins, obtained by TOCSY experiments [31]. TOCSY spectra have been reported often [2,27,30–40] and have been valuable in finding minor structures in lignins [34,41]. The older relayed coherence transfer experiments (where magnetization is transferred sequentially from one proton to the next coupled proton) provided similar data, but is seldom used today; these experiments were useful for assignments in the eight isomers of trimeric lignin β -ether models [11].

INADEQUATE experiments [42–44] correlate two neighboring ^{13}C -resonances and operate through direct ^{13}C – ^{13}C coupling. They are consequently low-sensitivity experiments at natural ^{13}C -abundance (1.1%). Uniform ^{13}C -labeling at the 10–15% level provides an enormous sensitivity increase, while keeping carbon 1D spectra relatively free of homonuclear coupling peaks and minimizing long-range-coupling effects. Many versions of the experiment exist, including more sensitive implementations with INEPT-type transfer and with gradients [45,46]. However, some of these implementations only provide connectivity for protonated carbons. A version

* see later in Figure 5.12

providing symmetric COSY-like correlations [44] was used in the only previously published lignin spectra of this type [2,47–50]. A series of papers [47–49] illustrated the connectivity available for the major units in a uniformly ^{13}C -enriched poplar lignin [51]. INADEQUATE-type experiments provide diagnostic connectivity information on the carbon skeleton of lignins; however, connectivity



information can be more easily obtained by using long-range C–H correlations in the more sensitive HMBC experiment (Long-Range C–H Correlation, HMBC Class section).

J-Resolved Experiments

Chemical shifts in the acquired dimension and proton-proton coupling in the second dimension can be separated using “J-resolved experiments.” Refocusing of relaxation effects from magnetic field inhomogeneity is an asset when seeking small (long-range) coupling constants in model compounds; however, J-resolved experiments are not widely used because of second-order artifacts that arise from tightly coupled protons. Higher magnetic fields can often simplify these spectra; tight coupling, where two distinct coupled protons have chemical shifts that differ by less than $\sim 10J$ (where J is the coupling constant in Hz), becomes less frequent. Two protons which are, for example, only 20 Hz apart at 200 MHz are 75 Hz apart at 750 MHz. For lignins, J-resolved experiments have revealed useful information. The first-order α -protons in β -ether units of acetylated lignins allow *threo*- and *erythro*-isomers to be distinguished based on a combination of their chemical shifts and coupling constants [28]. Both etherified and free-phenolic *threo*-isomers can also be resolved [2]. In CDCl_3 , there is further resolution of etherified vs nonetherified β -ether units [28].

Through-Space Interactions, NOESY and ROESY Class

NOESY or ROESY spectra correlate protons that are close to each other in space (rather than through-bond). The magnitude of the interaction falls off with the 6th power of the distance between the two protons, so such interactions are diagnostic for interproton distances of less than about 5 Å. Consequently, they are preeminent experiments for deducing 3D solution-state conformations for proteins, where the NOESY data provide sufficient numbers of distance constraints to allow constrained molecular modeling to produce essentially unambiguous 3D structures. However, lignin correlations are few and limited to intraunit responses, providing little conformational insight; this is largely due to the absence of regularly repeating macrostructures in lignins and perhaps to lignin’s rather linear nature, with little chain folding. The major correlations seen are (a) between the methoxy protons and the aromatic 2-proton in guaiacyl units or the 2/6-protons in syringyl units and (b) between side-chain protons ($\text{H}\alpha$, $\text{H}\beta$ and $\text{H}\gamma$) and aromatic protons (H_2 and H_6) of the same unit [30,33]. Perhaps the limited correlations in NOESY/ROESY spectra provide the best evidence that MWLs and other solvent-extractable lignins have little in the way of structural regularity, a conclusion also supported by the complexity of

FIGURE 5.2 (See color insert following page 336.) (Opposite) COSY and TOCSY spectra. (a) COSY spectrum (gradient, double quantum filtered) from a pine compression wood acetylated milled wood lignin in acetone- d_6 , at 400 MHz. Methoxyl and acetone (solvent) artifacts are severely suppressed by using the double quantum filtered variant shown here. (b) TOCSY spectrum, using a 100 ms mixing period, from an acetylated synthetic lignin (DHP, made from coniferyl alcohol containing a few percent of dihydroconiferyl alcohol, DHCA); and (c) an acetylated isolated lignin from a softwood (pine), in acetone- d_6 , at 360 MHz. Contour assignment coloring scheme follows that to be established in Figure 5.3.

1D ^{13}C -NMR spectra of lignins. Useful interunit correlations are even not seen in a highly syringyl-rich lignin, where long arrays of β -O-4-coupled units exist, such as in birch [33] or the bast fibers of kenaf [37]. On low molecular mass lignin models and lignans, the NOESY experiment is, of course, valuable for identifying isomers and conformational insight. For example, isomers of the various 8-O-coupling products from ferulates and coniferyl alcohol can be nicely distinguished by NOESY and long-range COSY experiments [29].

Short-Range C-H Correlation, HMQC and HMQC-TOCSY Class

By far the most useful experiments are those providing correlations between protons and carbons in two dimensions. Extra apparent resolution is gained, which exceeds anything that can be achieved in 1D spectra with today's field strengths. Overlapping protons that are attached to carbons with different shifts are pulled apart by those carbon shift differences; overlapping carbons may be distinguished by their attachment to protons with different chemical shifts [2].

So great are the advantages of the so-called inverse-detected experiments that little use is now made of the older normal-mode experiments, except on older instruments. The former acquires data in the proton dimension, while the latter the carbon dimension. Satisfactory HMQC (or HSQC) spectra can be run in as little as 4 minutes with gradient selection [2], or about 15 minutes without. Longer experiments are still recommended for detailed work, since longer acquisitions obviously still give valuable signal-to-noise improvements and consequent interpretability enhancements.

HSQC or HMQC Spectra Figure 5.3a shows a gradient-HSQC [52–54] spectrum of an acetylated synthetic lignin, with interpretation of major structural units; the structures, structure numbers, and color coding established here are used throughout this chapter. An HSQC contour implies that a proton at the proton frequency of the contour (x-axis) is directly attached to a carbon at the carbon frequency of the contour (y-axis) (Figure 5.1c). In all isolated lignins, such as those in Figures 5.3c and 5.3e, β -ether structures **A** dominate. The availability of an assigned spectrum, e.g., the clean synthetic lignin spectrum in Figure 5.3a, allows major structures in other spectra to be rapidly assigned. HMQC or HSQC spectra of lignins have been reviewed [2] and well reported [27,30,32–34,38–40,50,55–77,78–113].

The HMQC/HSQC experiments have been valuable in assigning major structures and have been indispensable in identifying new and minor units. The clear identification of dibenzodioxocins **D** as major new structures in lignins has been a significant finding [114–116]. In acetylated lignins, dibenzodioxocins are readily identified by unique and often well-resolved correlations in HMQC/HSQC spectra, Figures 5.3a and 5.3c (and later in Figure 5.10). Evidence provided by 2D NMR is far more diagnostic than 1D data, purely because of the simultaneous constraints that are revealed in the data. Thus, the observation that there is a proton at 4.9 ppm directly attached to a carbon at 84.4 ppm and a proton at 4.1 ppm attached to a carbon at 82.5 ppm is more revealing than just observing two new carbons at 84.4 and 82.5 ppm in a 1D spectrum. Also, dibenzodioxocins are not readily observed in 1D ^{13}C NMR spectra due to spectral overlap and their disperse (broad signal) nature.

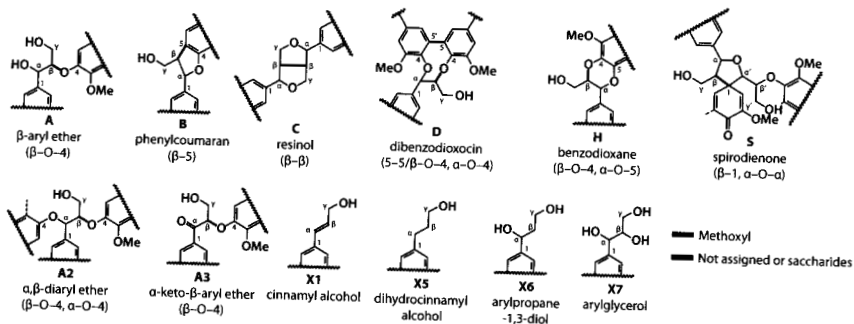
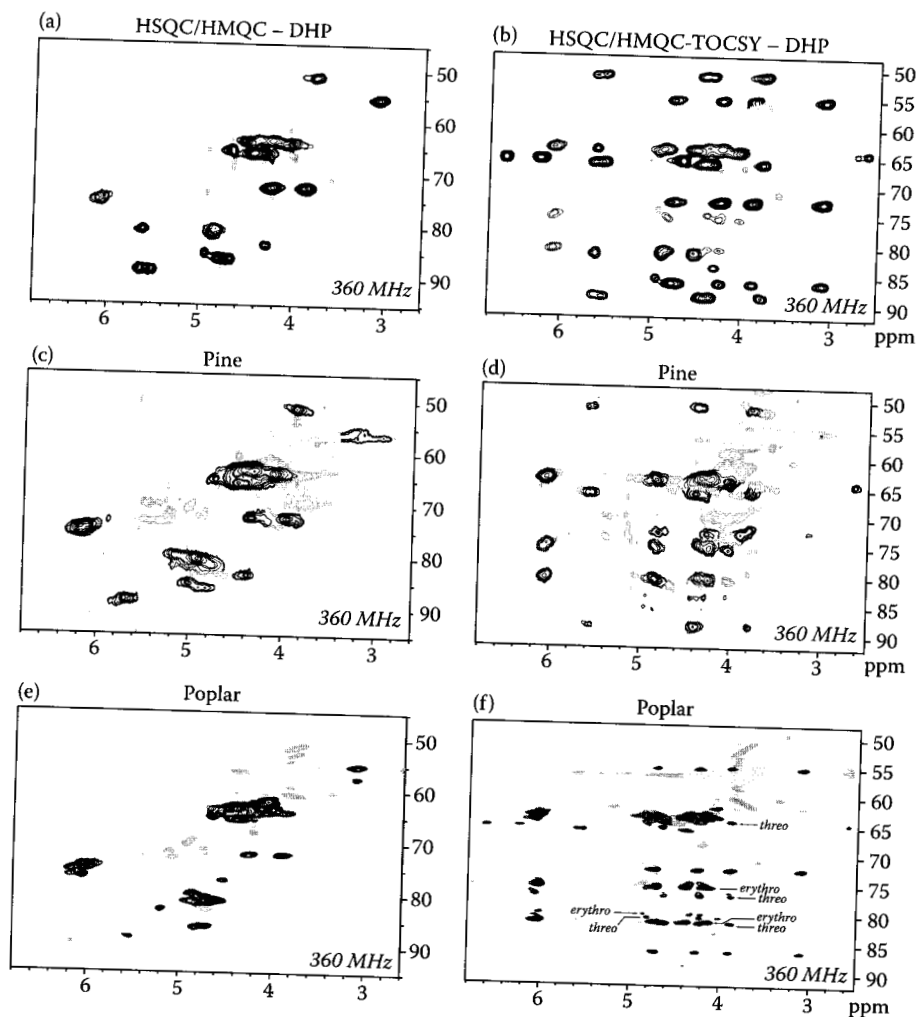
Although 2D correlative spectra have the advantage over 1D of providing at least two pieces of simultaneous data, overlap and confusion can still occur in 2D. One pitfall in HMQC and HSQC spectra is found with structures **A2** (α , β -diaryl ethers) and **A3** (α -keto- β -aryl ethers). The α -proton/carbon contour in **A2** is located in almost exactly the same region ($\sim 56/81$ ppm) as the β -proton/carbon contour in **A3** [117]. Keto-units **A3** occur mainly in syringyl-rich isolated lignins, and may be produced during ball-milling and lignin isolation [1,78]; however, synthetic lignins that have not been ball-milled also contain such oxidized units. The two components are best distinguished in HMBC spectra (see Long-Range C–H Correlation, HMBC Class section), where guaiacyl α -ethers signals for H-**A2**_a (5.6 ppm) correlate with carbons γ (63.5), β (81.5), 1 (137.5), 2 (112.3), 6 (120.0), and 4 (147.5) of the next unit [2]. In contrast, in units **A3**, H-**A3**_a (5.6 ppm) correlates with the γ carbons (64.5) and, diagnostically, the α -carbonyl (194.8). One should confirm structural assignments from all obtainable data. None of the spectra in Figure 5.3 reveals any **A3** units, but these can be seen later in Figures 5.10f, 5.10g, 5.18e, and 5.18g.

The 2D HSQC pulse experiment contains more pulses and delays than its HMQC counterpart and requires more time during the pulse sequence before the actual signal acquisition. It can, therefore, be a less sensitive experiment for rapidly relaxing samples, but offers some advantages, including artifact reduction (although it is sensitive to the 180° pulse accuracy, which should be measured on each sample). Early versions were also reduced in sensitivity by a factor of two by selection of only one of the two coherence pathways [118,119]. More recent “sensitivity enhanced” or “sensitivity improved” HSQC schemes select both pathways and have become the standard experiments [52,53]. Phasing of HSQC spectra can be a problem. The older gradient-HMQC experiment may sometimes be preferable for rapidly relaxing samples. A useful discussion of the issues in HMQC vs HSQC pulse sequences has been published [120].

Multiplicity editing in 2D NMR is possible via INEPT- or DEPT-HMQC [121] experiments. Choosing the editing pulse or delay, as in 1D experiments, can yield spectra with CH and CH₃ contours positive and CH₂ contours negative [2,74]. Useful and potentially cleaner spectra can be obtained this way; however, these sequences have been little utilized in lignin work to date. They are experiencing renewed interest with the adoption of the latest shaped-pulse variants.

Improving the uniformity of pulse excitation has become more important, particularly with the expansion in use of cryogenically cooled probes and high-field instruments. Specialty shaped pulses, such as adiabatic pulses, provide significant improvements. Such pulses appear to be complex in terms of their rather extreme phase ramping or amplitude modulation, but are designed to produce broad and uniform excitation. Peaks near the edges of the acquisition windows in spectra acquired over wide sweep widths may be significantly more intense in adiabatic-pulse versions of HSQC spectra than in the normal (hard, rectangular pulse) versions, especially at high field (see Recent Advances in NMR: Inverse Detection, Gradients, and Cryoprobes section).

HMQC-TOCSY or HSQC-TOCSY Spectra Although less sensitive than their HMQC/HSQC counterparts (because of the extra correlations for each proton and



carbon), 2D HMQC-TOCSY spectra [122], or its relative, HSQC-TOCSY [118], enjoy deservedly more widespread use. They provide a useful redundancy in information and a clear identification of coupling networks (see Figure 5.1d) [2,33,35,37–39,41,57]. Assignments in HMQC/HSQC spectra, e.g., the synthetic lignin spectrum in Figure 5.3a, must be made with good knowledge of both carbon and proton chemical shifts for each structural type. In contrast, in HMQC-TOCSY spectra (Figure 5.3b), just knowledge of rough proton shifts allows for a rapid identification of units; this is because of the useful redundancy in the data. Knowing that acetylated β -ether units have α -protons at ~ 6 ppm, β -protons at ~ 5 ppm, and γ -protons between 4 and 4.6 ppm, allows us to readily assign the blue contours β -ether structures **A**, for example. It is further easy to see which carbons are in the same structure by simply looking for matching sets of correlations in the proton dimension. The DHP spectrum is particularly useful because the major inter-unit structures are present in a single sample. Due to the differences in proton coupling constants and the ability to transfer magnetization around the coupling network, the proton correlations for each carbon (or vice versa) will not all have the same intensity, and some may even be absent. It is, however, easy to recognize that all the blue peaks (β -ether structure **A**) belong together, as do all the green peaks (phenylcoumaran structure **B**), etc. The simple interpretability of the side-chain region of a 2D HMQC/HSQC-TOCSY spectrum and the valuable redundancy of correlation peaks make this experiment very useful.

As can be seen from the higher resolution Poplar spectra in Figures 5.3e and 5.3f, we can distinguish nicely between *erythro*- and *threo*-isomers of the dominant β -ether units in particular regions of the spectra. The *threo*-units always have one higher-field (lower δ) γ -proton; additionally, various carbon shifts are partially resolved. Although HSQC-TOCSY spectra are not quantitative, it remains apparent that hardwood lignins, with a substantial syringyl component, heavily favor the *erythro*- β -ether **A** isomers (see again later in Figure 5.18g vs 5.18f).

The HMQC-TOCSY experiment was the key to rapid identification of (a) dihydroconiferyl alcohol units **X5** and arylpropane diol units **X6** present in a mutant pine lignin (see later in Figure 5.12) [38], (b) tyramine units in genetically altered tobacco lignins [69,71], and (c) the tracing out of spirodienone side-chains [57], as noted in later sections.

HSQC or HSQC-TOCSY Difference Spectra In cases where there is an obvious control sample and one with some perturbation, difference spectra can be

FIGURE 5.3 (See color insert following page 336.) (Opposite) Heteronuclear 2D correlation NMR spectra (HSQC/HMQC, HSQC/HMQC-TOCSY, side-chain regions) of (a–b) an acetylated synthetic lignin derived from coniferyl alcohol, and acetylated isolated lignins from (c–d) a softwood (loblolly pine) and (e–f) a hardwood (poplar). Prominent structures **A–D**, **S**, **X** are colored for ready identification. The left-hand spectra are HSQC/HMQCs; the right-hand spectra are HSQC/HMQC-TOCSYs with 80–100 ms TOCSY mixing times. The reason why the poplar spectra, Figures 5.3e and 5.3f, are more highly resolved is because they were more recently acquired at higher resolution in F_1 (the carbon dimension) and a single level of forward linear prediction was used in processing. This figure establishes the color coding for lignin units in all figures in this chapter (unless otherwise noted).

generated by electronically subtracting two spectra. Just as 1D difference spectra have tremendous value in delineating assignments [123–126], 2D difference spectra are now being similarly used. The first example is from synthetic lignins generated from specifically labeled coniferyl alcohol [111,127].

The 2D HSQC and HSQC-TOCSY difference spectra in Figure 5.4 illustrate how strikingly clean these spectra are and indicate how powerful this method may prove in the future. The spectra are unedited (apart from colorization of the contours) difference spectra from Terashima's DHPs [111], showing just the major units at this time. Delving into details from lower contour levels is the obvious next step to look into minor structures. Similar experiments on lignins or solubilized whole cell wall Ginkgo plant material, fed with labeled precursors, have been reported [111]. The difference spectra were readily generated using Bruker processing software. The quality is presumably dependent on machine stability and having samples of similar quality. Obviously all spectra do need to be run under the same conditions on a single instrument (or at least on instruments with the same field strength). Difference spectra have also now been used on isolated lignins and whole-cell-wall samples [97,99,104]. They can be particularly valuable for visualizing minor components, such as the *bis*-8-O-4-ethers that arise from the elevated incorporation of ferulic acid into lignins in plants deficient in cinnamoyl-CoA reductase (CCR) [97,104].

Long-Range C–H Correlation, HMBC Class

HMQC/HSQC-type spectra are valuable for their direct attachment information and because of the apparent extra dispersion they provide over 1D spectra. Long-range ^{13}C - ^1H correlation experiments provide enormously valuable connectivity data (Figure 5.1e). Two- and 3-bond ^{13}C - ^1H coupling constants are in the 2–15 Hz range, and HMBC [128] experiments are typically set with coupling evolution times of 60–120 ms, corresponding to coupling constants of 4–8 Hz. It is possible to miss correlations because they may be twice the set value; the response is a sine curve peaking at 1/25. In some circumstances it may be appropriate to run spectra with different long-range coupling delays to allow for all correlations to become visible; however, as with the HSQC-TOCSY experiment, the useful redundancy of data often makes this unnecessary. If a given correlation from a particular structure is required, the experiment can be easily optimized for that correlation by appropriately choosing the coupling evolution delay as $0.5/J_{\text{LRCH}}$, using the long-range coupling constant of interest. For example, 110 ms corresponding to the measured $^3J_{\text{C-O-C-H}}$ of 4.5 Hz was chosen to observe ferulate arabinosyl moiety attachments [129]. What must be kept in mind with polymeric lignin samples, however, is that the longer this delay, the less overall intensity the spectrum will have due to relaxation losses. Obviously, if the (proton) FID from the lignin is reduced to the noise level in 100 ms, no signal will be produced in an HMBC experiment incorporating a 100 ms delay. Some isolated lignins, especially those prepared by steel ball-milling, can have very short relaxation times that can often be improved by EDTA washing the lignin to remove metals [56]. Acetylated samples can readily be EDTA-washed in an extraction step, and this is frequently worthwhile [71].

Applications of HMBC experiments to lignins have been documented [2,27,32,35,36,55–57,60,64,70,71,74,106,129–132]. The enormous power of this

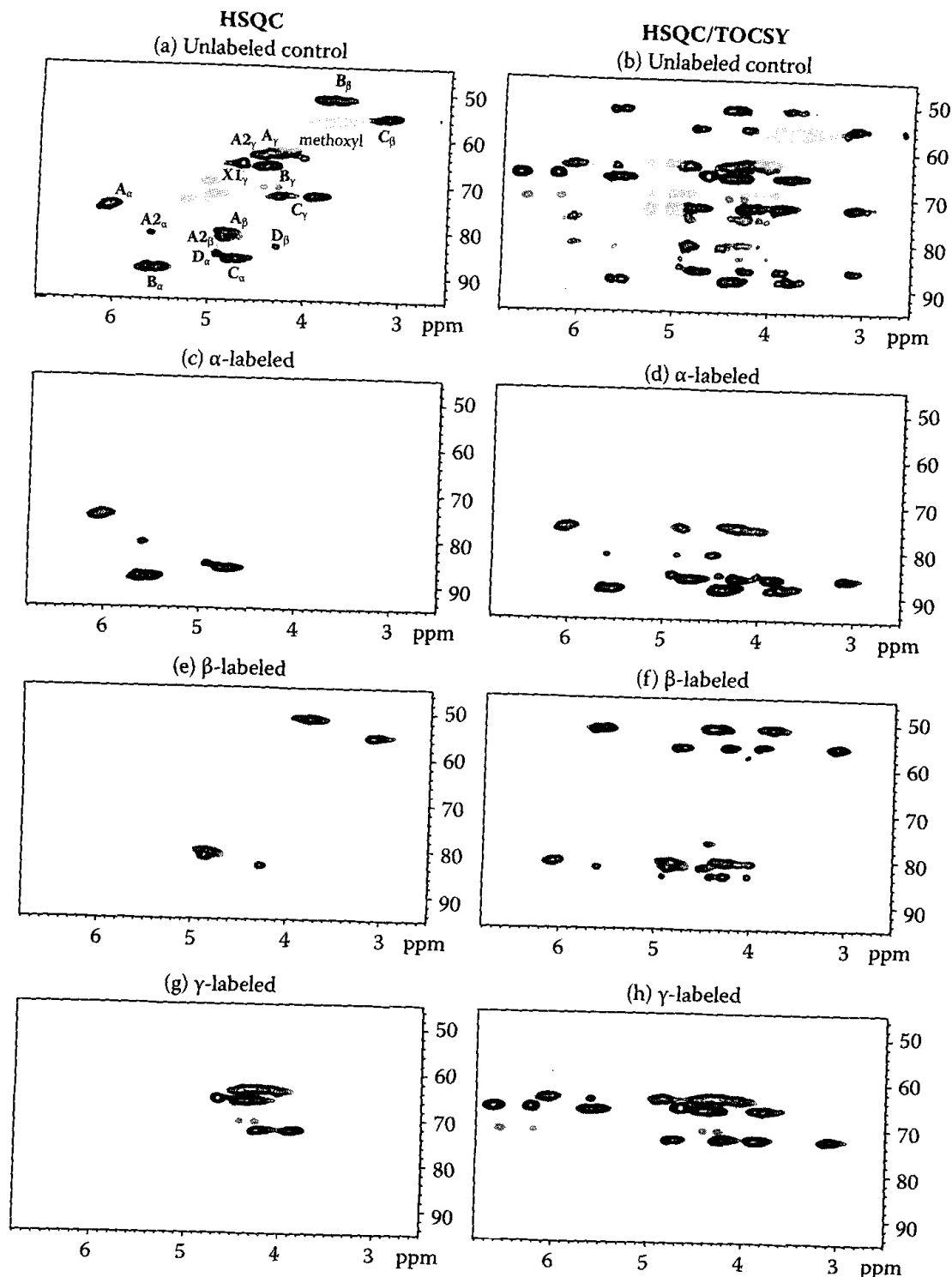
experiment can be seen in diverse applications that show unambiguously, for example, how *p*-coumarates are attached to γ -positions of lignins (Naturally Acylated Lignins section) [56], and how ferulates are responsible for lignin-polysaccharide cross-linking in grasses (Ferulates in Lignins; Cell Wall Cross-Linking in Grasses by Ferulates section) [55,129,130,133]. One of the most useful applications of HMBC experiments is in delineating the monolignol incorporation into lignins and the consequent distribution of syringyl, guaiacyl, and *p*-hydroxyphenyl (and other) structures (Figure 5.5) (see also the Lignins in Monolignol-Biosynthetic-Pathway Mutant or Transgenic Plants section and later in Figures 5.17, 5.20, and 5.21). The α -protons correlate with carbons β and γ of the side-chain and carbons 1, 2 and 6 of the aromatic rings; all of these carbons are within 3-bonds of the α -proton. What makes these correlation spectra diagnostic is that the equivalent syringyl S2/S6 carbons, resonating at ~ 105 ppm, are well separated from their guaiacyl counterparts (for which G2 and G6 are different, at -112 and -120 ppm), and from *p*-hydroxyphenyl H2/H6 carbons at -129 ppm. Consequently, the partial HMBC spectra from, for example, the β -ether **A** α -protons gives an immediate impression of the distribution of such units in the lignin. Hardwoods (e.g., Figure 5.5d) obviously differ from softwoods (e.g., 5.5b). More compelling examples derive from the analysis of perturbations caused in compression wood (5.5c) and in the various mutants and transgenics (5.5a, 5.5g, 5.5h, and 5.5j) discussed in more detail in the Lignins in Monolignol-Biosynthetic-Pathway Mutant or Transgenic Plants section. The experiment can even be run on the whole cell wall component, without first isolating the lignin (Figure 5.5e) [64]. The information rivals that from the diagnostic ^{13}C – ^{13}C -correlated INADEQUATE experiments; however, HMBC spectra are obtained with significantly higher sensitivity and allow correlations to be made over several bonds (and therefore through oxygen and nitrogen, for example), which the INADEQUATE experiments cannot.

Quantification in 2D NMR

Attempts have been made to utilize 2D NMR experiments for quantification, due to their improved signal dispersion over 1D experiments. The most logical experiments to utilize in a quantitative or semiquantitative fashion are those that depend only on one type of coupling interaction, and for which the sensitivity to that coupling constant is relatively low. The best candidate is, therefore, the (short-range) ^{13}C – ^1H correlation experiments, HMQC or HSQC. A general method to limit the dependence of signal intensity on the 1-bond coupling constant, and on signal offset (from the center of the spectrum), has been described [72]. It has the advantage of being largely structure-independent, but is less sensitive than the modern “sensitivity-improved HSQC experiment. Guidelines have been provided for acquiring lignin spectra for quantification using integrals from quantitative 1D ^{13}C NMR spectra as “internal standards” for deriving relative response factors between the various types of units [95]. The semiquantification available from the volume-integration of cross-peak contours in HMQC/HSQC spectra can be strikingly useful, especially for comparative purposes. Such integration is illustrated in the Lignins in Monolignol-Biosynthetic-Pathway Mutant or Transgenic Plants section.

For aromatic carbon-2 (or carbons-216 in symmetrical *p*-hydroxyphenyl or syringyl units) ^{13}C – ^1H correlations, essentially no correction appears necessary to

achieve quantification [60]. Quantification correction factors, like response factors in GC, can be determined for side-chain correlations most efficiently via model compounds that contain at least two types of structural units [60]. However, the authenticity and portability of such methods have not been determined. It should be emphasized that not all 2D spectra are equally quantitative. HSQC/HMQC may



be quantitative, particularly if a small spectral window is acquired (to minimize off-resonance effects). However, HMBC experiments, which have correlation contour integrals that depend on a variety of coupling constants, are far from quantitative; some 2- or 3-bond ^{13}C – ^1H correlations may not be evident at all, i.e., have essentially zero integral. This will happen, for example, if the actual long-range coupling constant J_{LR} is twice that corresponding to the chosen long-range coupling evolution period; for example, if the long-range coupling delay is chosen to be 100 ms (corresponding to an optimal J_{LR} of 5 Hz), a C–H pair having a coupling constant of 10 Hz will peak at 50 ms and be a null at 100 ms. Similarly COSY-type correlations depend on ^1H – ^1H coupling constants. TOCSY integrals have a complex dependence on all the coupling constants in the proton network and on the mixing time.

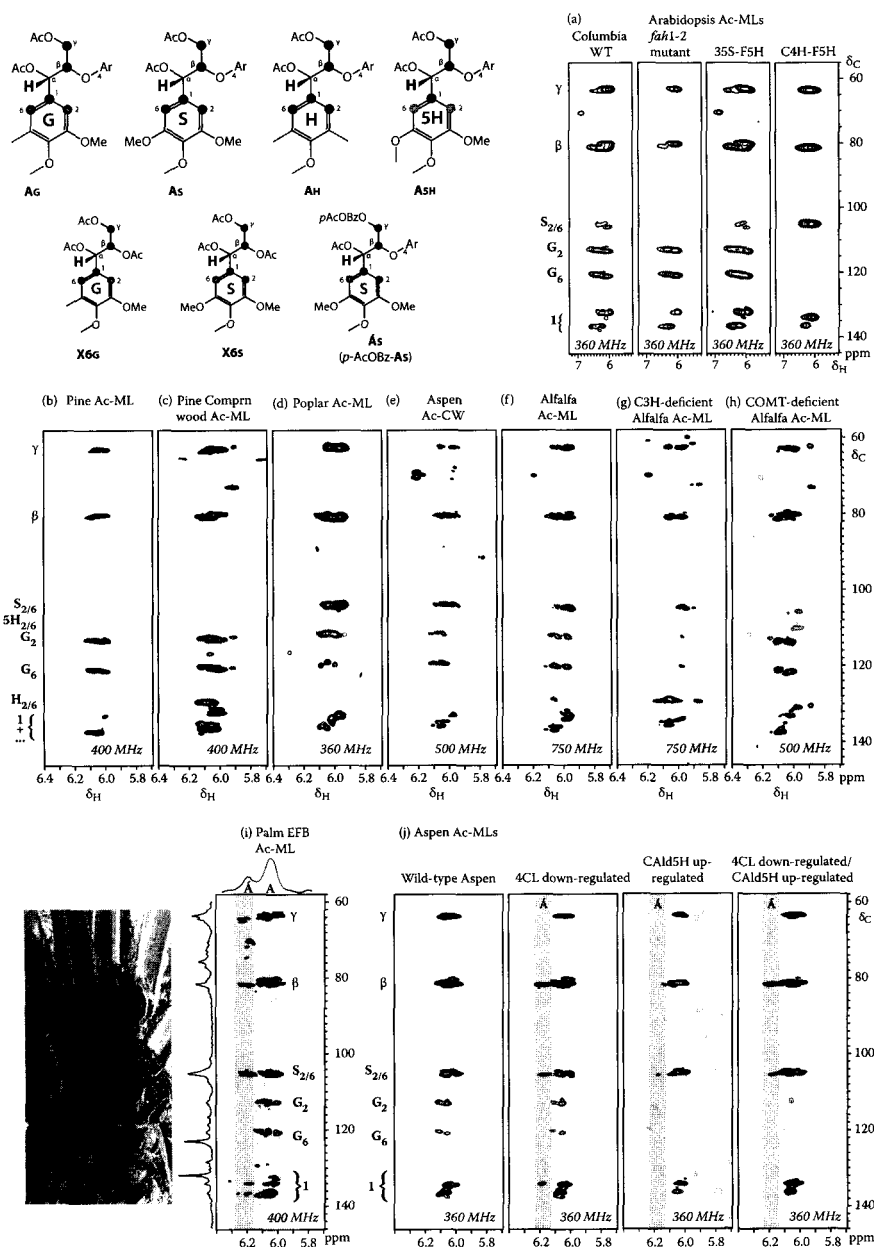
3D Experiments for Lignins

NMR is not limited to one and two dimensions. Three-dimensional experiments are now commonplace, and 4D, 5D, and higher-D experiments have been applied to labeled proteins. Much of the value of these experiments comes from the further dispersion realized by correlating over the additional dimensions. This is particularly valuable in proteins where ^{13}C , ^1H , and ^{15}N dimensions are available. The expectation that 3D experiments necessarily are more time demanding overlooks the fact that signal-to-noise is gained on the *total number of scans in the entire experiment*. Lignins do not contain nitrogen; however, acquiring 3D spectra with one carbon and two proton dimensions is useful. The 3D HMQC-TOCSY experiment (with one ^{13}C and two ^1H axes) was first applied with some success using uniformly ^{13}C -enriched samples [2,50,77,78,134]. The increased complexity and data size, and the reduced resolution compared to 2D experiments are offset by the ability to “isolate” spin systems on their own planes. Although labeled materials facilitate 3D (as well as 1D and 2D) experiments, valuable spectra from unlabeled materials are readily obtained. For example, the spectra shown later in Figure 5.19f were acquired from unenriched samples in 24 h at 750 MHz. At 360 MHz, 48 h produces very satisfactory 3D spectra. Figure 5.6 is particularly impressive because it is on ~70 mg of a whole cell wall sample that is only ~30% lignin, but was acquired using a 500 MHz cryoprobe system in 36 h [61].

The two experiments used with lignin to date are variations of 3D HSQC-TOCSY and the reverse TOCSY-HSQC experiments (or their HMQC analogs). The

FIGURE 5.4 (See color insert following page 336.) (Opposite) HSQC and HSQC-TOCSY spectra and difference-spectra of acetylated DHPs (400 MHz, acetone- d_6) from specifically ^{13}C -enriched coniferyl alcohols (Adapted from Terashima, N., Akiyama, T., Ralph, S.A., Evtuguin, D., Pascoal Neto, C., Parkås, J., Paulsson, M., Westermarck, U., and Ralph, J., *Holzforschung*, **63**, 379–384, 2009). (a–b) unenriched control; (c–h) 2D Difference spectra obtained by subtraction of the spectrum of the unenriched control from the spectra of DHPs specifically ^{13}C -enriched at side-chain carbon, (c–d) $\text{C}\alpha$, (e–f) $\text{C}\beta$, (g–h) $\text{C}\gamma$. Note that there are two components in these DHPs that are different from usual DHPs and may have been a result of the preparation mode (Adapted from Terashima, N., Atalla, R.H., Ralph, S.A., Landucci, L.L., Lapiere, C., and Monties, B., *Holzforschung*, 50(1), 9–14, 1996); one is a glucose or glucoside component (light green), the other appears to be perhaps a γ -ether (light pink). Contour assignment coloring scheme otherwise follows that established in Figure 5.3.

former provides planes of 2D HSQC-TOCSY spectra at given proton frequencies, while the latter gives particularly clean HSQC spectra at planes corresponding to any of the protons of the coupling network; other planes may also be useful—see caption to Figure 5.6. Both experimental types have been illustrated and reviewed [2,50,61,68,77,78,134]. Researchers in this field love the ready interpretability of



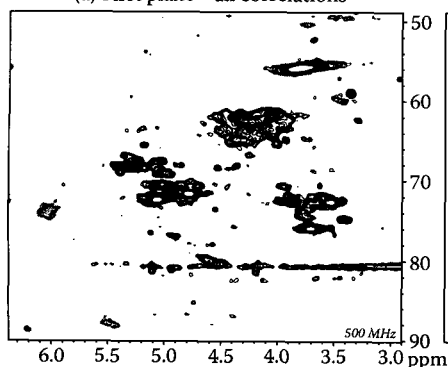
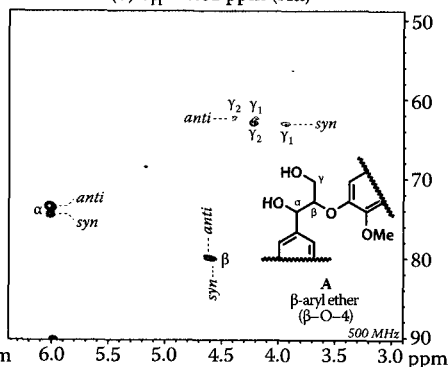
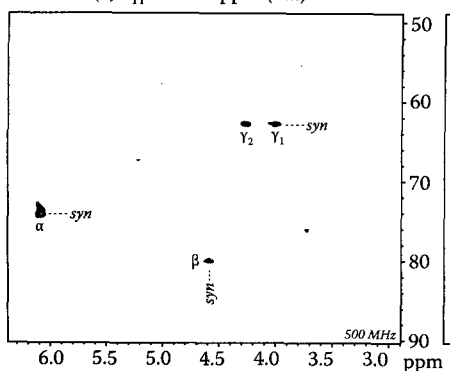
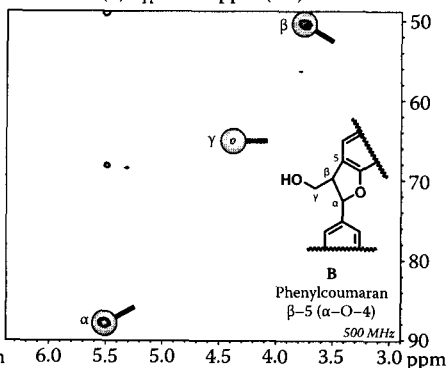
the 3D HSQC-TOCSY experiment (for the same reason that 2D HSQC-TOCSY is valuable — the appearance of the whole spin system is pleasing). The 3D TOCSY-HSQC experiment is, however, more sensitive, since only HSQC correlations are seen after magnetization is first transferred to the entire proton system (before the HSQC-part of the experiment). It is possible to observe HSQC spectral planes that are purely from a single substructure in lignin, even at natural ^{13}C abundance [2,68,135] (see Figures 5.19e and 5.19f, the COMT-Deficiency: Benzodioxanes from Incorporation of 5-Hydroxyconiferyl Alcohol section), and even when the sample is not an isolated lignin but the entire cell wall component (following ball milling and cell wall dissolution) (Figure 5.6) [61].

FIGURE 5.5 (See color insert following page 336.) (Opposite) Gradient-selected 2D HMBC subspectra showing a-proton correlations in β -aryl ether units **A** (and glycerols **X7**), illustrating their value in delineating H:G:S differences (and more). (a) Spectra from wild-type arabadopsis, the *fah1-2* mutant deficient in ferulate 5-hydroxylase (F5H, see caption to Figure 5.16), and two transgenics upregulated in F5H, the C4H-F5H one spectacularly so (Marita, J., Ralph, J., Hatfield, R.D., and Chapple, C., *Proc. Natl. Acad. Sci.*, 96(22), 12328–12332, 1999), all in acetone- d_6 . The wild-type and 35S-F5Hlignins are guaiacyl-rich syringyl/guaiacyl copolymers. In the *fah1-2*mutant, syringyl units **As** are essentially absent. When F5H is expressed under the control of the C4H-promoter, the lignin that is deposited is comprised almost exclusively of syringyl units **As**. (b–h) Selection of similar HMBC regions of acetylated samples (in acetone- d_6 , unless otherwise indicated) for H:G:S elucidation: (b) pine ML, showing only **AG** and no **As** (nor **AH** at this contour level); (c) Pine compression wood ML (from Ian Suckling and Bernadette Nanayakkara, SCION) showing increase **AH** level; (d) Poplar ML, a typical S/G lignin; (e) An Aspen dissolved whole cell wall sample, in CDCl_3 , showing that valuable HMBC spectra can be acquired without the need for lignin isolation; (f) a control alfalfa ML in CDCl_3 , a typical G-rich S/G lignin, but with minor **AH** levels; (g) a heavily C3H-down-regulated alfalfa ML, in CDCl_3 , with ~65% **AH** units, and showing that β -ethers are derived from addition to the growing polymer of coniferyl, sinapyl, and especially *p*-coumaryl alcohol monomers; (h) a COMT-deficient alfalfa, showing an almost complete absence of S- β -ethers**As**, and the appearance of novel 5-hydroxyguaiacyl β -ethers **AsH**, derived from lignification using 5-hydroxyconiferyl alcohol. Note that glycerols **X7** are most prominently seen at the lower proton chemical shift (~5.9 ppm) in c, g, and h. (i) Lignin isolated from the empty fruit bunches of oil palm (Sun, R. C., Mott, L., and Bolton, J., *Journal of Agri. Food Chem.*, 46(2), 718–723, 1998) in which normal β -ethers **A** are S/G but those naturally acylated with γ -*p*-hydroxybenzoate groups, in which the proton chemical shifts are higher (~6.2ppm) are S-only **AS**. (j) Aspen acetylated lignins from a study in which the interactions between down-regulation of 4CL and up-regulation of F5H to successfully produce reduced-lignin transgenics with higher S:G ratios were explored (Li, L., Zhou, Y., Cheng, X., Sun, J., Marita, J.M., Ralph, J., and Chiang, V.L., *Proc. Natl. Acad. Sci.*, 100(8), 4939–4944, 2003). The 4CL-down-regulated transgenic had a lower lignin content with similar structure and S:G as the wild-type, but a higher level of *p*-hydroxybenzoates in units **AS**. (F5H- or) CAlD5H-upregulated aspen, like the C4H-F5H alfalfa in (a), was extremely S-rich. The doubly manipulated transgenic in which 4CL was down-regulated and F5H was up-regulated showed that the effects were essentially additive—the lignin level was lower, the **As** content high (indicating a high flux toward syringyl lignin), and the *p*-hydroxybenzoate on lignin level was elevated as indicated by **AS**- β -ethers. Contour assignment coloring scheme is for S:5H:G:H delineation and does not follow that established in Figure 5.3; the colors are the same as those used for similar purposes in Figures 5.16a through c and 5.17.

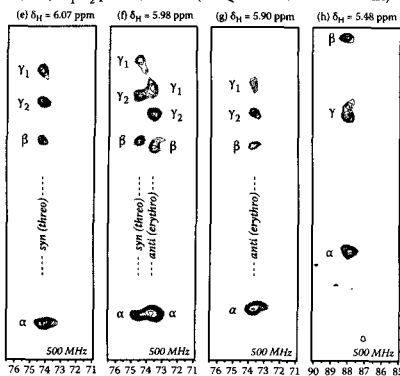
The 2D slices from the 3D TOCSY-HSQC spectrum (Figure 5.6), show how elegantly individual unit types in lignin can be “isolated.” Although these spectra are from a polysaccharide-dominated sample (as seen in Figure 5.6a), even isomers of lignin units may be resolved onto their own planes. Individual planes representing HSQC spectra at a given proton shift (Figures 5.6b through 5.6d), HSQC-TOCSY

3D TOCSY-HSQC: F_2 - F_3 planes, ^{13}C - ^1H (HSQC at a given ^1H shift)

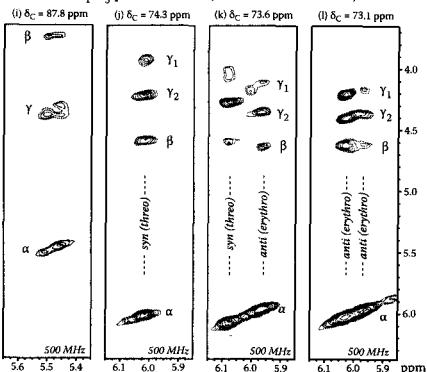
(a) First plane—all correlations

(b) $\delta_{\text{H}} = 6.01$ ppm ($\text{A}\alpha$)(c) $\delta_{\text{H}} = 6.095$ ppm ($\text{A}\alpha$)(d) $\delta_{\text{H}} = 5.50$ ppm ($\text{B}\alpha$)

(e–h) F_1 - F_2 planes, ^{13}C - ^1H (HSQC-TOCSY at a ^1H shift)



(i–l) F_1 - F_3 planes, ^1H - ^1H (TOCSY at a ^{13}C shift)



sub-spectra at a given proton shift (5.6e through h), and TOCSY subspectra at a given carbon shift (5.6i through l) can all be viewed. (It is actually also possible to project onto planes other than these three orthogonal ones defined by the acquisition axes, and useful information may be obtained from those.)

Frankly, the biggest “problem” with 3D NMR is not its acquisition or processing but its simplicity. To the uninitiated, seeing only a few spots on a plane seems unimpressive. The simplicity, however, belies its incredible power of isolating structural units, from a polymer as complex as lignin, in a sample as complex as the entire cell wall, on their own planes. How much easier would Freudenberg’s task have been with such a powerful tool?

Recent Advances in NMR: Inverse Detection, Gradients, and Cryoprobes

Recent advances in NMR technology have resulted in features so essential that it is now difficult to contemplate serious structural studies on complex molecules without them. “Inverse detection” came from a recognition that, no matter what nucleus you were interested in, physically acquiring data from the most sensitive nucleus in the system, usually proton, brought large sensitivity gains. Further developments allowed the more sensitive nucleus to be initially excited, with another significant gain in sensitivity. These gains are powers of the gyromagnetic ratios of the nuclei involved. For example, a polarization transfer experiment is γ_H/γ_X (~4 for carbon) times more sensitive than direct observation (without nOe). The enhancements are even larger for the lower- γ nuclei such as ^{15}N and ^{29}Si . A 2D ^{13}C - ^1H correlation experiment carried out by acquiring proton data (rather than ^{13}C -data, as was conventional) experiences an incredible $4^{3/2}$ or 31.6-fold gain in sensitivity over a direct observation experiment and $4^{3/2}$ or 7.9-fold gain over the polarization transfer experiment (in which the protons are excited initially, but carbon data is still acquired).

The time required to obtain a given signal to noise (S/N) is proportional to the square of the S/N. A 2D ^{13}C - ^1H correlation experiment that required 24 h. in the “normal mode” experiment would, therefore, take only 23 minutes using the inverse mode to acquire a spectrum with essentially the same sensitivity. Obviously, experiments that require a weekend to run in inverse mode cannot even be contemplated using the older scheme. Before these inverse experiments, it would have been impossible to study the minor (~1% of the lignin) ferulate cell wall cross-linking elements in grass lignins. This operating method alone allows 2D ^{13}C - ^1H experiments to be acquired far more quickly than a 1D ^{13}C NMR spectrum. For many samples (and for proteins in particular) the carbon data are therefore obtained without ever acquiring an actual 1D ^{13}C NMR spectrum. Significant difficulties with such experiments have

FIGURE 5.6 (See color insert following page 336.) (Opposite) Slices from a 3D NMR spectrum of acetylated pine ball-milled cell walls (Lu, F. and Ralph, J., *Plant J.*, 35(4), 535–544, 2003), illustrating the spectacular lignin substructural editing possible in this complex sample (Ralph, J. and Lu, F., *Org. Biomol. Chem.*, 2(19), 2714–2715, 2004). The 3D spectrum was acquired with the acquisition restricted to the lignin side-chain region. (a–d) F_2 - F_3 (HSQC) slices at a given ^1H shift; (e–h) F_1 - F_2 (HSQC-TOCSY) slices at a given ^1H shift; (i–l) F_1 - F_2 (TOCSY) slices at a given ^{13}C shift. Contours in figure d are artificially enlarged to enhance their visibility. Partial substructures of β -ether units **A** and phenylcoumaran units **B** are given with conventional side-chain labeling.

been overcome by improvements in hardware and clever pulse programming and phase cycling. An example will serve to illustrate the issues involved.

To acquire a “normal mode” ^{13}C – ^1H correlation spectrum, researchers traditionally acquired, with proton decoupling, a set of 1D ^{13}C NMR spectra. Proton decoupling was already well established with the advent of 1D ^{13}C NMR. If it is desired to acquire proton spectra instead, we need a method to detect only those protons that are attached to ^{13}C -carbons, not ^{12}C -carbons. Therefore, some way of getting rid of the 99% unwanted signals had to be devised. This was initially via phase cycling—ensuring that the pulse sequence produced additive in-phase signals for the desired ^{13}C – ^1H pairs, but opposite and canceling signals (in alternate scans, for example) for ^{12}C – ^1H pairs. Also, instead of decoupling protons from ^{13}C , the instrument was now faced with the problem of decoupling ^{13}C s from protons. Unfortunately, the large ^{13}C -spectral width required huge power levels in conventional decoupling schemes, sufficient to easily boil the sample. The advent of more efficient decoupling schemes such as GARP-decoupling [136], and the hardware to implement them were the keys to making such experiments routinely available. Again, the enormous improvement in sensitivity for these “inverse-detected” experiments over their traditional “normal-mode” counterparts, and the rapid evolution of NMR instrumentation to confront the rigors of ever more complex and more variable pulse sequences, has allowed these “inverse” experiments to almost completely replace the original ones. The term inverse is being phased out, since it has now become the “normal” mode.

The latest innovation to completely alter the way spectra are acquired is in the application of large pulsed field gradients during an NMR pulse sequence [137]. For years, NMR spectroscopists sought the strongest and most uniform stable magnetic fields possible in which to place their samples. Then theoretical specialists began advocating strategic use of “homospoil” pulses (to temporarily wreck the homogeneity of the magnetic field) and field gradients (where the field is ramped rapidly). Again, these methods have become accepted in “normal” experiments due to the beautiful way they can clean up spectra (by “coherence selection”) and reduce artifacts without requiring phase cycling. Long-range ^{13}C – ^1H correlation experiments have particularly benefited from the application of pulsed field gradients [118,138]. For lignins, the use of gradients and inverse detection allows acquisition of strikingly good 2D spectra that allow substantive interpretation in as little as 4 minutes [2].

The move to all-digital instrument components and digital oversampling (as used in CD players, for example) added further benefits. Commercial systems now offer cryogenic probes, which improve signal-to-noise (S/N) by reducing the operating temperature of the coil and the preamplifier. The increase in the S/N ratio by a factor of 4, as compared to conventional probes, leads to a possible reduction in experiment time of 16-fold or a reduction in required sample concentration by a factor of 4. An NMR system with a cryoprobe may have sensitivity exceeding a traditional system with a much higher-field magnet.

Finally, as we alluded to above (HSQC or HMQC Spectra section), advances in pulse-shaping hardware and software have led to pulse-shaped variants of many pulse programs becoming the standard [139,140]. For example, the more energy-efficient adiabatic-pulse variants of HSQC-type experiments are recommended as standard experiments on newer high-field instruments, particularly those equipped

with cryogenically cooled probes. They offer the advantage of a wide inversion bandwidth meaning minimized ^{13}C -pulse offset effects, lower power, and a wider decoupling range using CHIRP or WURST decoupling [140-142].

PROTON AND CARBON NMR

Arranging lignin studies under NMR-specific headings (such as ^1H , ^{13}C , 2D etc) leads to considerable redundancy (with the exception of multinuclear NMR, which is treated in its own section). Consequently, after a brief introduction to proton and carbon NMR of lignins, we provide the arguably more important contributions of NMR to various aspects of lignin investigation in the following sections. Where possible, the techniques used are delineated.

INTRODUCTION TO PROTON NMR OF LIGNINS

Broadness of Proton Spectra

NMR practitioners know that increasing molecular weight broadens resonances due to reduced relaxation times. But lignins appear broader and more featureless than, for example, comparably sized proteins (Figure 5.7e vs 5.7f). It is easily recognized that much of this broadness comes from the huge range of environments in the lignin polymer. Since isolated lignins are polydisperse and have no regularly repeating macrostructures, each unit finds itself in a unique magnetic environment. But complexity in lignin extends far beyond the complexities caused by irregular bonding of one unit to the next. Even a β -O-4-ether homopolymer is a stereochemical nightmare [11]. A simple trimeric β -ether model compound (Figures 5.7c and 5.7d), has four optical centers and therefore $2^4 = 16$ optical isomers or $2^4/2 = 8$ physically different (and NMR-different) isomers. A pure random β -ether hexamer, such as might be isolated, especially from syringyl-rich lignins, has 10 optical centers and therefore $2^{10} = 1024$ optical isomers and half that number, 512 of physically distinct isomers. (In general a molecule with n optical centers has 2^n optical isomers and $2^n/2 = 2^{n-1}$ real isomers.) In reality, although β -aryl ether units in guaiacyl lignins are essentially random ($\sim 50:50$ *threo:erythro*), such units in syringyl lignins are mainly ($\sim 75:25$) *erythro*-isomers [143,144]. Also, units such as phenylcoumarans and resinols are found as only single isomers in nature. Therefore, the stereochemical complexity in oligomers is reduced, but still becomes overwhelming with increasing molecular size. What is often overlooked by those unfamiliar with basic chemical stereochemistry is that, for example, a phenylcoumaran-phenylcoumaran trimeric unit still has two physically distinct isomers, even though there is only a single isomer of each dimeric moiety.

The stereochemical complexity causes significant broadening due to the overlapping of NMR signals, as illustrated in Figure 5.7e. Even in a trimer, with 8 real (NMR-distinct) isomers [11], the α -, β -, and γ -protons become complex (Figures 5.7c and 5.7d). The lines in these spectra were sharpened for presentation and assignment purposes by careful Gaussian resolution-enhancing apodization of the data. Even so, broadening is present due to the increased molecular weight. Obviously, spectra of the trimer will be markedly broader than those of the dimer. It is not, therefore, surprising that proton spectra of lignins (Figure 5.7e), are so

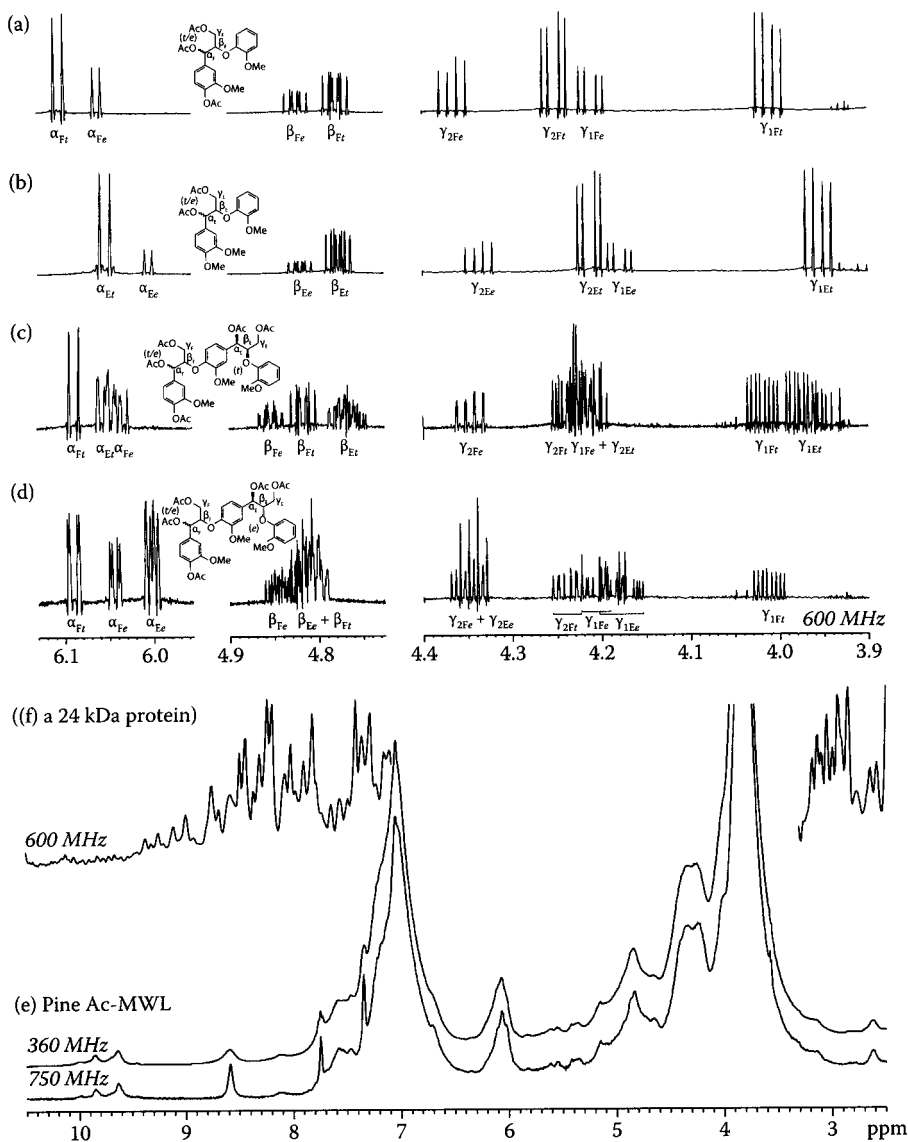


FIGURE 5.7 1D spectra of side-chain regions of β -ether dimers and trimers, and lignin showing how lignins are broadened by its stereochemical complexity. Figures a to d are 600 MHz resolution-enhanced spectra of acetylated β -ether models: (a) a free-phenolic dimer; (b) an etherified dimer; (c) 4 of the 8 isomers of a trimer (etherified dimeric unit is *threo*); (d) the other 4 of the 8 isomers of a trimer (etherified dimeric unit is *erythro*). (e) Acetylated pine isolated lignin, at 360 and 750 MHz. (f) A section of a 24 kDa protein at 600 MHz showing how much more resolved and detailed the proton NMR spectrum of even a much higher molecular mass protein is due to its being a single molecular structure (Cornilescu, Gr., Cornilescu, C.C., Zhao, Q., Frederick, R.O., Peterson, F.C., Thao, S., and Markley, J.L., *J. Biomol. NMR*, 29(3), 387–390, 2004).

apparently featureless, especially when compared with spectra of even much larger polymers with a single structure, e.g., the protein in Figure 5.7f [410]. Careful interpretation of lignin ^1H NMR spectra can reveal important details and, fortunately, the broadness is not an overbearing deterrent to producing valuable 2D correlations, as will be demonstrated.

ID Proton NMR Studies of Isolated and Synthetic lignins

A comprehensive review of proton NMR of lignin model compounds, synthetic, isolated, and degraded lignins and their derivatives prior to 1968 was made in Ludwig's chapter 8 [145] in *Lignins: Occurrence, Formation, Structure and Reactions* [1]. Much of the work reported stemmed from the seminal studies by Ludwig, Nist, and McCarthy published in 1964 [146,147], and the 1968 review by Lenz [148].

More recently, Lundquist has provided systematic and comprehensive studies on lignins by proton NMR at magnetic field strengths ranging up to 500 MHz. Formyl groups in isolated lignins were found at a level of $\sim 7\%$ [126]. They were primarily due to coniferaldehyde ($\sim 4\%$) and vanillin end-groups. The aldehyde signals completely disappeared following borohydride reduction, as was elegantly shown in the first application of difference spectroscopy reported in lignin literature. Birch [149] and spruce [150] lignins, purified to low carbohydrate level, were examined in considerable detail with the help of spectra from model compounds [151]. Carbohydrates, present either as contaminants or linked to lignins, were primarily xylans in birch; evidence for benzyl ester linkages was presented [152,153].

Comparisons were made between synthetic coniferyl alcohol dehydrogenation polymer (DHP) lignins and MWL from spruce [154]. MWLs contained more aldehydes, possibly attributable to oxidative lignin aging reactions. DHPs contained more β -5- and β - β -units as well as cinnamyl alcohol end-groups and less β -O-4 units; the DHPs had a consequently lower hydroxyl content. Differences in interunit distributions between DHPs and isolated lignins have come to be cited as a justification for a theory of carefully controlled lignification [155]. However, the result is simply explained by the relatively large amount of dimerization that occurs in DHPs compared with the endwise polymerization that occurs during lignification when the coniferyl alcohol supply or the radical generation capacity is more limited, as reviewed [107]. These ideas, and the notion of "bulk" vs "endwise" polymerization were lucidly discussed at the end of chapter 4 [156] in the original *Lignins* book [I], and in Adler's 1977 review [157]. Enhancing cross-linking of a monolignol with a dilignol (model), using only simple methods to limit diffusion, elegantly demonstrated that such simple explanations were all that were required to explain high β -ether levels [158].

Compositional differences between various fractions of isolated lignins, in studies also using ^{13}C -NMR, suggested that differences in monomeric composition relate to in situ heterogeneity of poplar lignins [159]. Proton NMR was applied to an examination of the ratios of *threo*- and *erythro*-isomers of β -aryl ether units in softwoods and hardwoods, using a set of guaiacyl-guaiacyl, guaiacyl-syringyl, and syringyl-syringyl model compounds [160]. The study extended earlier proton [150,151] and ^{13}C NMR work [161–163] that showed that the two isomers were present in roughly

equal amounts in softwood guaiacyl lignins, but that *erythro*-isomers predominated in hardwood syringyl-guaiacyl lignins.

The solvent DMSO has particular value in NMR. This is due to its being a poor facilitator of proton exchange that, therefore, limits the normal broadening seen in the resonances of exchangeable protons, such as in hydroxyl groups. The various phenolic hydroxyl groups in lignin are well dispersed in this solvent (8.1–9.3 ppm) and can be quantified [123,124]. Aldehydes from 9.3–10.3 ppm (as in other solvents) are also quite well resolved. In acetylated lignins, the various phenolic and aliphatic acetates have acetate methyl protons that are reasonably well resolved. Lignin is sufficiently complex that absolute quantification is difficult, but reasonable quantification of various units and functional groups is possible.

Proton 1D NMR continues to be extremely useful for lignin structural studies, as reviewed more recently [164]. Its role is being augmented by 2D methods.

Quantification in 1D Proton NMR

Proton NMR spectra are inherently quantitative, providing the sample is allowed to relax sufficiently between scans (as is normally the case, since proton T_1 -relaxation times in polymeric lignins are typically well under 1 s). The sensitivity is considerably higher than in ^{13}C NMR. Some groups, such as methoxyls and acetate methyl groups in acetylated lignins, receive enhancements due to the multiplicity of identical protons. Proton NMR and quantitative aspects were extensively treated in the previous treatise on lignins [145] and will not be reviewed here. The emphasis on ^{13}C NMR quantification in the Quantitative ^{13}C NMR of Lignins section of this chapter is a reflection more of the need to understand the issues of such quantification, and should not be interpreted as implying any superiority over ^1H NMR quantification. In both cases, the inability to fully resolve resonances of interest provides the most significant quantification problems.

INTRODUCTION TO ^{13}C NMR OF LIGNINS

The introduction of ^{13}C NMR opened up a powerful new spectroscopic method for the analysis of complex polymers, such as lignins. Its value lay mainly in qualitative aspects; unlike ^1H NMR, ^{13}C NMR requires special time-consuming methods for quantification (see Quantitative ^{13}C NMR of Lignins section). A series of seminal papers established the necessary model compound database and led to signal assignments to synthetic and isolated lignins from a variety of plants [8,9,161,162,165–170]. Most of the assignments made in subsequent papers on lignin ^{13}C NMR spectra are based directly on the data in this series, which were, in turn, based on numerous monomeric and dimeric model compounds. The most important paper in this era, published by Nimz et al., is a listing of about 40 ^{13}C chemical shift assignments of MWLs isolated from maple, beech, oak, cherry, compression wood, bamboo, wheat straw, a GSH-DHP, and an H-DHP (these terms are defined below) [166]. It is worth noting, however, that assignments in many subsequent papers are often not fully authenticated and there is still considerable overlap. Suspected structures must have all of their peaks present. For

some structures it is difficult to unambiguously identify diagnostic peaks that are separated sufficiently from those of other structures. The previous limitations of the model compounds are perhaps the main reason why typically only 35–50 chemical shift assignments were made in subsequent reports prior to the recent characterization of G-, GS-, and S-DHPs, in which over 100 assignments were made [171]. Work continues on improving the accuracy and reliability of assignments, based primarily on the data of model compounds that more accurately represent structural entities in lignin.

In a ^{13}C NMR spectrum of underivatized lignin, almost all signals from side-chain carbons are found in a rather narrow chemical shift range (52–90 ppm) and there are few signals that can be attributed to single lignin structures. Carbon-13 NMR signals are broadened by the structural and stereochemical complexity noted in the Broadness of Proton Spectra section. Without complete signal resolution, ^{13}C NMR suffers from many of the same problems as ^1H NMR.

Researchers use a variety of conditions for acquiring lignin (and model compound) NMR data. Underivatized lignins are usually run in either DMSO- d_6 or acetone- d_6 /D $_2\text{O}$ (typically ~9:1) solvent. In both cases, chemical shifts are somewhat sensitive to solute concentrations, temperature, and, in the latter case, the exact make-up of the solvent. Tables of NMR data in these solvents appear in several references [171–173]. Acetylated lignins provide more ideal samples. They are quite soluble in common organic solvents and solute interactions are less severe. Nevertheless, there are significant differences between samples run in CDCl_3 vs acetone- d_6 , and attempts to relate data between the two solvents have only been partially successful. From extensive solvent effect studies of lignin model compounds, researchers have concluded that acetone- d_6 is a better solvent choice if the intent is to correlate model chemical shifts with lignin chemical shifts [174].

In an attempt to address the issues of solvent on chemical shift, researchers have established a database of lignin model compounds in which they attempted to run all compounds in three solvents, acetone- d_6 , CDCl_3 , and DMSO- d_6 [175]. However, the massive undertaking of unequivocally assigning spectral peaks has really only been met for samples in acetone- d_6 , the solvent upon which our groups have standardized for acetylated lignins. Others, with their own databases of chemical shifts, have standardized on CDCl_3 or DMSO. Each solvent system offers its own small advantages (and personal preferences); a convergence to one solvent is not likely in the near future. The electronic model compound database is updated regularly and is freely available [175].

Since ^{13}C NMR of lignins has been reviewed a number of times [176,177], we will not provide particularly much detail here. A comprehensive table of chemical shift assignments for fully acetylated lignins was recently compiled by comparing ^{13}C NMR data from numerous G, GS, and S trimeric model compounds with data from DHPs and lignins isolated from softwoods and hardwoods [10,178,179]. Table 5.1 indicates the authors' best attempt to reliably assign peaks in representative acetylated softwood and hardwood isolated lignins. The assignments are based mainly on dimeric and trimeric model compounds and synthetic lignins, aided by 2D studies.

TABLE 5.1

¹³C-NMR Chemical Shift Assignments for Acetylated lignins in Acetone-d₆

Chemical Shift		Assignment ^a
From	To	
20.2	20.3	AcMe on phenolic OH of S-rings
20.5	20.6	AcMe on Bβ in S-b-S-glycerol
20.6	20.7	AcMe on primary (γ) OH
20.8	20.9	AcMe on phenolic OH of G-rings & on benzylic OH (α)
29.83		Center signal of acetone-d ₆ multiplet (solvent and internal standard)
50.7	51.1	β in EG-bl-GF
51.1	51.4	β in G-c-G
55.3	55.5	β in G-r-G, G-r-S, S-r-S
56.3	56.5	OMe
62.8	63.3	γ in G-b-G(e), S-b-G(e), *G-c-G-glycerol
63.2	63.6	γ in G-b-S(e), S-b-S(e), G-a-G
63.4	63.8	γ in G-b-G(t), S-b-G(t)*
64.2	64.5	γ in G-b-S(t), S-b-S(t) γ in dibenzodioxocin moiety
65.0	65.2	γ in EG-bl-GF
65.2	65.5	γ in C-alc & S-alc end-groups
65.9	66.1	γ in G-c-G
72.4	72.7	γ in G-r-G, G-r-S, S-r-S
72.9	73.4	Bβ in G-c-G-glycerol, S-b-S-glycerol
74.4	74.7	α in G-b-G(e)
14.9	75.4	α in G-b-S(e)
75.2	75.7	α in G-b-G(t), S-b-S(e)
75.7	15.8	α in EG-bl-GF
16.3	76.1	α in G-b-S(t)
16.5	77.0	α in S-b-S(t)
79.9	80.4	β in G-b-G(e), S-b-G(e)*
80.3	80.7	β in G-b-G(t); S-b-S(e)
80.6	81.0	a in G-a-G
81.0	81.4	β in G-b-S(e), S-b-S(e)
81.4	81.8	β in G-b-S(t), S-b-S(t), G-a-G
83.3	83.6	β in dibenzodioxocin moiety
85.3	85.5	α in dibenzodioxocin moiety
86.1	86.3	α in G-r-G
86.3	86.	α in S-r-S
88.3	88.6	α in G-c-G
88.6	88.9	α in S-c-G
103.0	103.3	2,6F in S-r-S
103.4	103.6	2,6F in G-r-S
103.6	103.9	2,6E in S-r-S, S-c-G
104.5	105.0	2,6E,F in S-b-S(e)

TABLE 5.1**¹³C-NMR Chemical Shift Assignments for Acetylated Lignins in Acetone-d₆ (Continued)**

Chemical Shift		Assignment ^a
From	To	
105.2	105.5	2,6E in S-b-S(<i>t</i>)
106.6	107.3	2,6 in S-rings with αC=O
111.0	111.3	2F in G-c-G, G-r-G, G-r-S
111.3	111.7	2E in G-c-G, G-r-GC-alc, vanillin
111.7	112.0	2F in G-55-G
112.2	112.4	2F in G-b-S; B2 in G-c-C-alc
112.4	112.5	2 in EG-b1-GF
112.5	113.1	2E,F in G-b-G, G-c-G; 2E in G-b-S, cinnamaldehydes; 2F in G-a-G
114.2	114.4	2F in EG-b1-GF
115.7	115.9	5E in vanillin
116.2	116.4	B6 in G-c-C-alc
118.2	118.4	5E in cinnamaldehydes
118.5	118.6	6F in G-r-GG-r-S
118.6	118.7	6F in G-c-G
118.5	119.1	5E in G-b-G, G-b-S, G-r-G, EG-b1-GF
119.0	119.2	5E in C-alc; 6E in G-c-G
119.3	119.5	5E in G-c-G, 6E in G-r-G
119.9	120.1	6F in G-b-S
120.1	120.7	6E,F in G-b-G; 6E in G-b-SEG-b1-GFC-alc; 6F in G-a-G, in dibenzodioxocin moiety
121.5	121.7	6F in G-55-G
122.1	122.3	BE in C-alc; 6F in EG-b1-GF
122.4	122.6	5F in G-r-S, in dibenzodioxocin moiety
122.7	122.9	3,5E in <i>p</i> -OH-benzoate
123.0	123.2	5F in EG-b1-GF
123.2	123.4	5F in G-b-G, G-b-S, G-a-G; BE in C-alc
123.6	123.8	5F in G-c-G, G-r-G; 6E in cinnamaldehydes
124.0	124.2	BE in S-alc
125.9	126.1	6E in vanillin
127.8	128.0	1F in <i>p</i> -OH-benzoate
128.2	128.4	BE in C-ald
128.7	128.9	4F in S-r-S
128.9	129.3	B5 in G-c-G
129.3	129.5	4F in S-b-S
129.9	130.1	1E in cinnamaldehydes
131.0	131.2	B1 in G-c-G-glycerol
131.5	131.7	5F in dibenzodioxocin moiety; B1 in G-c-C-alc; 2,6F in <i>p</i> -OH-benzoate

(Continued)

TABLE 5.1

¹³C-NMR Chemical Shift Assignments for Acetylated Lignins in Acetone-d₆ (Continued)

Chemical Shift		Assignment [#]
From	To	
131.7	132.0	5F in G-55-G
132.0	132.2	1E in vanillin
132.2	132.6	1E in C-alc
132.6	133.0	1E in G-b-G
133.0	133.3	B5 in dibenzodioxocino moiety
133.3	133.4	1E in S-alc
133.8	134.4	1E in S-b-S
134.0	134.2	αE in C-alc
134.3	134.5	αE in S-alc
134.5	134.7	Bα in G-c-C-alc
134.7	134.9	1 in EG-bl-GF
135.5	135.7	4 in ES-r-S
135.8	136.0	1 in FG-55-G
136.0	136.6	1 in FG-b-G; 1F & 4E in S-b-S; 4 in ES-c-G;
136.6	136.8	1 in EG-r-G
137.0	137.3	1 in FG-b-S; 1 in EG-c-G
137.5	137.7	1 in FG-a-G
137.1	137.9	B1 in EG-bl-GF
138.0	138.2	1F in dibenzodioxocino moiety
138.3	138.5	4 in FG-55-G
138.5	138.9	1 in ES-r-S
140.0	140.2	4 in FG-r-G, EG-bl-GF
140.5	140.7	4F in G-b-S, G-a-G
140.7	141.0	4F in G-b-G, in dibenzodioxocino moiety; 1,4F in G-c-G
141.2	141.4	1 in FS-r-S
141.7	142.0	1 in FG-r-G
145.0	145.4	B3 in G-c-G
147.0	147.6	4 in EG-r-G
147.8	148.0	4 in EG-bl-GF
148.0	148.5	4E in G-b-G, C-alc
149.2	149.4	B4 in G-c-C-alc, S-c-C-alc
150.9	151.0	4E in C-ald
151.4	151.7	3E in G-b-G, G-b-S, vanillin, EG-bl-GF
151.8	152.2	3E in C-alc, cinnamaldehydes, G-r-G, G-c-G; 3F in G-b-G, G-b-S, G-a-G, EG-bl-GF in dibenzodioxocino moiety; 4E in G-b-S
152.3	152.7	3F in G-r-G, G-c-G, G-55-G
152.8	153.3	3,5F in S-b-S, S-r-S; αE in C-ald
153.4	153.6	4E in vanillin
153.7	154.1	3,5E in S-b-S-S-r-S
155.6	155.8	4F in <i>p</i> -OH-benzoate

TABLE 5.1

¹³C-NMR Chemical Shift Assignments for Acetylated Lignins in Acetone-d₆ (Continued)

Chemical Shift		Assignment [#]
From	To	
165.5	166.5	Benzoate C=O in <i>p</i> -OH-benzoate
168.2	168.3	Phenolic AcC=O on S-rings with a C=O
168.4	168.7	Phenolic AcC=O on S-rings
168.9	169.0	Phenolic AcC=O on G-rings
169.2	169.4	Phenolic AcC=O on <i>p</i> -OH-benzoate entities
169.9	170.0	Benzylic (α) AcC=O in G- & S-rings
170.2	170.4	BB AcC=O in G-c-G-glycerol, S-b-S-glycerol
170.6	170.8	Primary (γ) AcC=O in G-b-GG-b-S, G-a-GG-c-G-glycerol
170.9	171.0	Primary (γ) AcC=O in G-c-G
180.5	180.9	<i>p</i> -Quinone C=O in G-rings
191.1	191.5	C=O in vanillin
193.8	194.2	C=O in cinnamaldehydes

Legend: G = guaiacyl, S = syringyl, C = coniferyl, S = sinapyl, V = vanillyl, E = etherified phenolic, F = free phenolic, a = α-O-4, b = β-O-4, r = resinol (B-B), c = coumaran (B-5), bl = β-1, 55 = 5-5 (biphenyl), (e) *erythro*, (t) = *threo*, alc = alcohol, ald = aldehyde. [#]All assignments refer to the A-ring of the dimer fragment unless otherwise noted. [#]Shifts are from model compound S-b-Gonly and were not found in lignins or DHPs.

LIGNIN MODEL STUDIES

Low molecular weight model compounds, particularly dimers and trimers, are at the heart of NMR studies on lignins. Nearly all studies that attempt to assign new structures to lignin spectra incorporate model syntheses to provide key NMR data and for structural authentication; these are, therefore, dealt with under the lignin NMR sections. To accumulate much of the required data in a single repository, researchers have established an NMR database of lignin model (and related) compounds, which continues to be regularly updated [175]. A great deal of literature is associated with the painstaking synthesis, structural authentication, and spectral interpretation of a range of lignin model compounds.

POLYMERIC LIGNIN MODELS (DHPs)

Synthetic polymers can be made from monolignols using single-electron oxidants under a variety of conditions. Perhaps the most biomimetic approach is using a peroxidase and hydrogen peroxide. The polymerization in bulk solution does not mimic all aspects of lignification, and the polymers produced can vary considerably from those in plants [154,166,169,177,180–186]. Even when considerable efforts are made to limit the rate at which radicals encounter each other [156,158,187–189],

dimerization reactions may be more prevalent than in lignification, where the predominant reaction is cross-coupling of the monolignol (radical) with the growing polymer (radical) [158]. Nevertheless, the similar reaction chemistry provides all of the major products observed in lignins; thus, DHPs occupy a place of enormous value in structural and spectroscopic studies.

Utility of DHPs

Dehydrogenation polymers (DHPs) of *p*-hydroxycinnamyl alcohols have been used as oligomeric/polymeric models for the lignin structure for decades. There are many reasons for using DHPs rather than natural isolated lignins, such as a MWL. A DHP is free of extraneous wood components, such as extractives, tannins, and proteins. It can be difficult to remove all of the carbohydrate material from isolated lignins, especially because a fraction is thought to be chemically bound to the lignin. All of the major subunit types are represented in DHPs, although the linkage type distribution is quite different from native or isolated lignins. Another significant advantage of DHPs is that they are relatively simple to label isotopically in specific positions by using appropriately labeled *p*-hydroxycinnamyl alcohols [111,180,190–192] (see also the HSQC or HSQC-TOCSY Difference Spectra section and Figure 5.4). The use of isotopically labeled DHPs is important in the study of enzymatic delignification (pulp- ing) and bleaching, and in microbial degradation studies. Use of a DHP prepared from [**a**-¹³C]-coniferyl alcohol facilitated the interpretation of the ¹³C NMR spectra of the product resulting from the oxidation of the labeled DHP by a lignin peroxidase [193]. Labeled DHPs were essential in studies of lignin/polysaccharide complexes, which are important in forage digestibility research [36,133].

It is also possible to incorporate into DHPs minor structures (labeled or unlabeled) that are known to be present in natural lignins but are not formed from *p*-hydroxycinnamyl alcohols during dehydropolymerization. For example, *p*-hydroxybenzoic acid with a ¹⁴C-labeled carboxyl carbon was incorporated into a DHP for the purpose of determining the nature of this unit on lignins and its fate during alkaline hydrolysis [194]. Incorporation of strategically ¹³C-labeled ferulates and diferulates into synthetic lignins and the application of various 2D experiments, primarily HMBC, provided the range of ferulate incorporation pathways [55,129,133]. Synthetic DHPs incorporating coniferyl *p*-coumarate and coniferyl *p*-hydroxybenzoate were used to elucidate mechanisms of lignin acylation [195]. Synthetic lignins from coniferaldehyde were used to model lignins in plants deficient in CAD [65,196]. Synthetic lignins incorporating small amounts of ¹³C-labeled ferulic acid were used to provide confirmatory evidence for the incorporation of ferulic acid into lignin in transgenic plants deficient in cinnamoyl-CoA reductase (CCR) [97,104].

Experiments that utilize labeled DHPs supplement the more difficult and time-consuming experiments involving the labeling of natural lignins by feeding labeled precursors to the plant [197–202]. Nevertheless, DHPs are, like lignins, not homogeneous compounds and their structures are not completely determinable. Some of the problems in lignin structural elucidation are also evidenced with DHPs.

An extension of the DHP methods is to prepare lignins within preformed cell walls [203,204]. The walls may have their own peroxidases and other features, such as ferulates that may act as lignin nucleation sites in grass walls [130]. The

lignins produced in this manner appear to be much closer in composition/structure to natural lignins, presumably due to the slow diffusion of monomer (radicals) into the lignifying zone [203]. Again, minor or novel monomers can be incorporated into lignins [112]. In this way, a case was made for incorporating coniferyl (or sinapyl) ferulate into lignins to improve delignification and enzymatic degradability of walls [113].

Enzyme-Initiated Dehydropolymerization

DHPs prepared from coniferyl alcohol are the most common and give guaiacyl DHPs (G-DHP) that are intended as models of lignins isolated from softwoods. Polymers prepared from a mixture of coniferyl and sinapyl alcohols (GS-DHP) are used as models of hardwood lignins, and those prepared from a mixture of all three alcohols (GSH-DHPs) are generally considered as models for grass lignins and some wood lignins with significant contents of *p*-hydroxyphenyl moieties [166]. DHPs derived from pure sinapyl [205] and pure *p*-coumaryl alcohol [206] have been prepared even though it was suspected that neither one relates to any natural polymer. In fact, lignins in certain monolignol-biosynthetic-pathway mutants or transgenics can come remarkably close (Lignins in Monolignol-Biosynthetic-Pathway Mutant or Transgenic Plants section). A DHP's main utility is as a mechanistic and characterization tool to obtain data on minor structural entities.

The key step in both natural lignification processes and conventional laboratory dehydrogenations is the enzyme-initiated generation of phenoxy radicals of the *p*-hydroxycinnamyl alcohols. This simple oxidation leads to a complex variety of nonenzymatic processes that includes radical coupling reactions, nucleophilic additions to quinone methides, rearrangements, side-chain oxidations, and side-chain eliminations to generate the complex lignin macromolecule [207]. Peroxidase and/or a phenol oxidase (such as laccase) have been postulated to be involved in lignification [208]. A horseradish peroxidase/hydrogen peroxide system is commonly used in laboratory dehydrogenations [205,209].

Guaiacyl DHPs were also prepared from coniferin by the action of β -glucosidase and peroxidase, with hydrogen peroxide generated *in situ* through the action of oxygen and glucose oxidase on the glucose liberated from the coniferin [188]. Examination of these DHPs by ^{13}C NMR spectroscopy indicated a closer approximation to the structure of a softwood lignin than DHPs prepared in the conventional manner from coniferyl alcohol.

A number of studies addressed the impact of reaction conditions, including the presence of various saccharides on the types and stereochemistries of various lignin units [143,210–212]. The potential for production of novel β – γ -linkages from sinapyl alcohol in the presence of pectins was illustrated [211]. *Pinus taeda* suspensions fed with labeled phenylalanine demonstrated that lignins resembling native lignins could be produced *in situ* [213]. In addition, DHPs synthesized within primary cell walls of suspension-cultured maize, using native peroxidases within the walls, but exogenously supplied H_2O_2 and monolignols, produced lignins that were remarkably similar to maize lignins, as determined by ^{13}C NMR as well as degradative methods [203]. An extracellular lignin produced by suspension cultures of *Picea abies* had intermediate character [186].

ADHPs synthesized using NMR-invisible methoxyl groups, prepared from coniferyl alcohol synthesized via $^{12}\text{CD}_3\text{I}$ (^{13}C -depleted, trideuterated) allowed NMR spectra to be acquired without the complication of the overwhelming methoxyl group that can produce artifacts particularly in 2D spectra run without the use of gradient-edited pulse sequences [2,74]. That DHP is used for 2D illustrations here. The possibility of incorporation of *cis*-coniferyl alcohol into lignins was examined via a DHP [214]. Unfortunately, NMR was not used to characterize the nature of residual cinnamyl alcohol end-groups or the stereochemistries of the resulting units. A more recent study showed that the *cis*-double bonds remained in the product and that most of the units were produced as the same isomers; however, a number of currently unidentified units were also produced [39]. No evidence for *cis*-cinnamyl alcohol side-chains has been found in isolated lignins to date, suggesting that *cis*-hydroxycinnamyl alcohols are not significantly present in the cell wall during lignification.

Dehydropolymerization Initiated by Metal Oxidants

Dehydrodimerizations and polymerizations have alternatively been carried out using metallic single-electron oxidants in a variety of solvents. This nonenzymatic approach to synthesis of DHPs can result in poly(lignols) that, in some aspects (such as β -ether frequency), have a closer resemblance to corresponding MWLs than conventionally prepared DHPs [179,215]. Using metal one-electron oxidants (iron, manganese, and copper salts) is more flexible, in that the reaction conditions are not constrained within the temperature, solvent, and pH limits required by the enzyme. Other examples of metal salt-initiated polymerizations of *p*-hydroxycinnamyl alcohols reported in the literature are the preparation of DHPs using ferric chloride in various organic-aqueous solvents [216–218]. However, no ^{13}C NMR data were obtained. β -Ether coupling of sinapyl alcohol was shown to be favored by increasing the dioxane content or acidity of the reaction medium [218].

Comparisons of DHPs with Natural Lignins

In general, synthetic lignins have excessive monolignol-monomer coupling resulting in too frequent β - β -units (**B**, resinols) and unsaturated cinnamyl alcohol side-chains **X1** (from β -5- or β -O-4-dehydrodimerization). Attempts to improve the resemblance have met with variable success. Brunow found that low pH favors β -ether formation [219]. The closer resemblance of a typical Mn(III)-derived coniferyl alcohol guaiacyl G-DHP to a guaiacyl MWL isolated from spruce wood is seen in the ^{13}C NMR spectrum of the side-chain region (Figure 5.8). In contrast, in the spectrum of the conventional enzymatic G-DHP, signals due to β -5- **B** and β - β -units **C** are much more intense than in the MWL or the Mn(III)-derived DHP. A feature in common with both of the DHPs is the presence of coniferyl alcohol end units **X1**. This unsaturation is less prominent in a MWL. A comparison of a similarly prepared guaiacyl/syringyl GS-DHP and a conventional DHP with a MWL isolated from elm wood is shown in Figure 5.9. As with the G-DHP, the overabundance of the β -5- **B** and β - β -units **C** and unsaturated end-groups **X1** is apparent in the conventional DHP. The Mn(III)-derived GS-DHP has an even greater resemblance to the GS-MWL because, unlike the corresponding G-DHPs, unsaturated end units **X1** are almost absent.

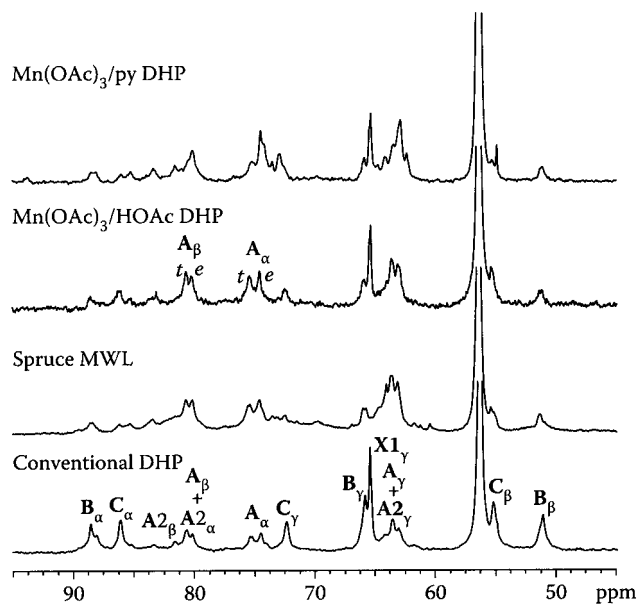


FIGURE 5.8 Comparison of the side-chain regions of 1D ^{13}C NMR spectra of various acetylated G-DHPs with a softwood lignin. Peak assignment follows that established in Figure 5.3 (Landucci, L.L., *J. Wood Chem. Technol.*, 20(3), 243–264(2000)).

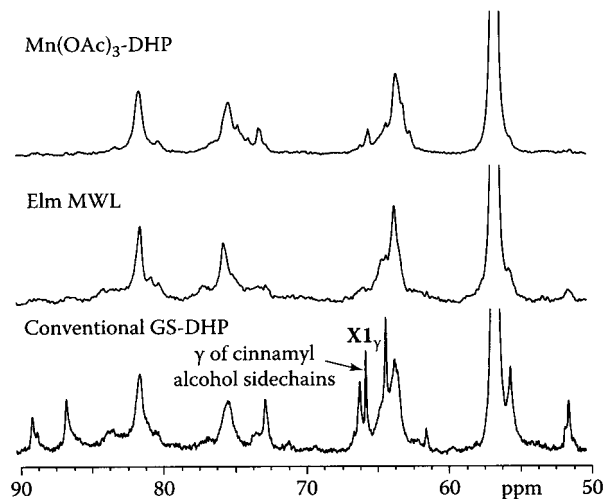


FIGURE 5.9 Comparison of the side-chain regions of 1D ^{13}C NMR spectra of two acetylated GS-DHPs with a hardwood lignin (Landucci, L.L., *J. Wood Chem. Technol.*, 20(3), 243–264(2000)).

NATURAL LIGNINS

A requirement of solution-state NMR is that samples must be soluble in a suitable organic solvent. Lignins isolated following solvent extraction (usually in 96:4 dioxane:water) of ball-milled plant cell wall materials provide perhaps the best, and most frequently used, materials of this type [220–222]. Unfortunately, only a fraction of the cell wall lignin is represented this way. MWL yields from woods are typically around 15–20% [221], although higher yields are easily obtained for some samples, particularly if crude polysaccharidases are used following ball milling and prior to lignin extraction. Milling time affects both the yield and the β -ether frequency in the product [223]. Poplar lignin was recently obtained in ~70% yield in NMR-suitable form this way [224]. In Lapierre's method, where no lyophilizing is done during the isolation, lignins from immature poplar are recovered essentially quantitatively [225,226]. These lignins are soluble in DMSO, but resolubilization is difficult following lyophilization. Some grasses have more soluble lignins; an isolated mature maize lignin was 67% of the total lignin [56]. Isolated lignins are typically dissolved in acetone- d_6 : D_2O (~9:1) or DMSO- d_6 for NMR studies of underivatized lignins. More commonly, the isolated lignins are acetylated, which leads to good solubility in acetone- d_6 or $CDCl_3$, and less-viscous solutions. For NMR experiments that involve long delays in the pulse sequences, such as in long-range ^{13}C - H correlation experiments (e.g., HMBC), treating lignins with metal chelators can be beneficial [2,56].

Selectively labeling lignins by feeding specifically labeled precursors is an approach toward unambiguously assigning NMR resonances. This has been particularly successful with ginkgo, in which cut stems continue to grow for a month or more when the cut section is immersed in water. Exogenously administering coniferin does not appear to perturb lignification and provides a "natural selective enrichment" [50].

Quality lignins can also be isolated via mild acidolysis, including in conjunction with cellulolytic treatments [227–232].

A relatively recent development, aided by the high sensitivity of modern NMR instruments equipped with cryogenic probes, is to obtain lignin data from spectra run on the entire cell wall component; i.e., without lignin fractionation/isolation [61,64,98,100]. Ball-milled cell walls can be dissolved or simply swelled in various solvents to yield strikingly high quality spectra [102,103].

Elucidation of Lignin Substructures

NMR provides unparalleled methods for elucidating the structures of organic molecules. It is difficult to envision determining reaction mechanisms via model compound studies, for example, without the use of NMR.

Although lignin is complex, its derivation from only a few monomers suggests that the number of structures to be found in lignin should be relatively small, and should have all easily succumbed to identification. It was, therefore, a surprise that, as recently as 1995, a major new structure common to all lignins was discovered [114–116]. Since then, other pathways during lignification have also been revealed by less prevalent structures. More mysteries abound, as many peaks in 1D ^{13}C (or contours in 2D NMR) spectra of lignins remain unidentified. Not all of these can necessarily be attributed to *in vivo* lignins; structural changes certainly occur during

lignin isolation. Nevertheless, reliably assigning all of the peaks or contours in lignin spectra remains a formidable challenge. Delineating the changes to lignin during various treatments, such as chemical pulping, requires as complete a characterization of the original lignin as possible.

Early proton and ^{13}C NMR spectra indicated that the already well-known major lignin structural units could be identified diagnostically from their side-chain proton and carbon shifts [9,146–149]. These included β -O-4**A**, β -5-**B** (or phenylcoumaran), β - β -**C** (or resinol) units, as well as cinnamyl alcohol **X1** and cinnamaldehyde and benzaldehyde end-units. The major units were also revealed in suspension-cultured *Pinus taeda* walls by solid-state NMR following feeding of side-chain-labeled precursors [213]. Two types of units, α -aryl ethers **A2** (actually α,β -diaryl ethers) and β -1-units (see next section) were not readily observed (see below). Since aromatic shifts are more difficult to assign, and are severely overlapped in both proton and carbon NMR, researchers have more trouble detecting biphenyl (5–5-) and biaryl ether (4–O–5) units. A great number of papers and reviews have dealt with establishing the nature and content of these standard units. Salient features are noted under the headings below.

β -O-4-ethers (β -aryl Ethers, **A**)

By virtue of its importance in lignins and in pulping mechanisms, the β -ether unit **A** has been the most studied. Various methods for assigning *threo*- and *erythro*-isomers in models and in lignins have appeared based on ^1H , ^{13}C , ^{19}F , and ^{31}P NMR [8,151,233–235]. Enormous numbers of isomers must be dealt with as molecules become more complex [107,236]. Although there are only two isomers of the popular β -ether dimeric model guaiacylglycerol- β -guaiacyl ether, there are eight isomers of the analogous trimer (Figure 5.7). Many of the proton chemical shifts are resolvable at high magnetic field, e.g., 600 MHz [11]. Fortunately, the isomers cluster into groups for *threo*- and *erythro*-isomers of each unit type, etherified or free-phenolic, and syringyl or guaiacyl. The various combinations of S and G units have been studied using a series of dimers [237], and more recently using all of the possible S/G trimers, for which detailed NMR data have been presented [10].

Isomer Distributions Lignin β -guaiacyl ether units **A** are essentially 50:50 *erythro*:*threo* [143]. Syringyl lignins have a much higher proportion of *erythro*-isomers [238], as beautifully evident in NMR spectra (see for example Figures 5.3f, 5.18f, and 5.18g) [2,70,144,239]. This is because, under a wide range of aqueous conditions, water adds to β -guaiacyl quinone methides essentially randomly, whereas water addition to β -syringyl quinone methides favors *erythro*-isomers by about 75:25 [143,144]. Natural lignins are analogous in this regard to synthetic lignins and to β -O-4-dimers produced from the monolignols via peroxidase in aqueous systems. The product distribution is logically a result of simple chemical control [236], an observation that is inconsistent with recent proposals advocating absolute structural control of the polymer produced during lignification [155].

Syringyl and guaiacyl *threo*- and *erythro*- β -ether protons and carbons overlap somewhat. The *threo*-isomers always have a higher-field γ -proton, which makes them particularly easy to identify in 2D spectra, especially TOCSY and HMQC-TOCSY.

Figure 5.3f shows an example 2D HMQC-TOCSY spectrum of a syringyl-rich poplar acetylated MWL, in which the predominance of the *erythro*-isomers is clearly revealed, particularly in the correlation with C- α (~75 ppm). The C α -C β correlation clusters for *erythro*- and *threo*-isomers and for syringyl and guaiacyl units are beautifully disperse in a 2D INADEQUATE spectrum [50]. A plot of model shifts for all 16 of the β -ether combinations (*erythro*/*threo*, syringyl/guaiacyl, and etherified/free-phenolic), correlated well with major features of the contour complex, on the same spectrum.

Chemical cross-coupling propensities of monolignols with guaiacyl and syringyl units in lignin are becoming available [10,158,210]. Sinapyl alcohol does not appear to readily cross-couple with guaiacyl units *in vitro*. Thus, in Table 5.1, shifts from model compound S-b-G (b = β -O-4) are listed, but such structures have not been authenticated in lignins or DHPs. The presence or absence of hetero-dimeric entities (G-b-S or S-b-G)_n in natural lignins could not be confirmed by ¹³C NMR spectroscopy because the side-chain signals from both *erythro*- (*e*-) and *threo*- (*t*-) GS entities overlap the corresponding signals from *e*- and *t*-S-b-S entities; signals from *e*-S-b-G entities overlap those from *e*-G-b-G entities; and signals from *t*-S-b-G entities overlap those from both *e*-S-b-S (α -carbon) and *t*-G-b-G (β - and γ -carbons). The difficulty of preparing the S-b-G entity in trimers by oxidative coupling of sinapyl alcohol with G-b-G or G-b-S dimers suggests that S-b-G-units might not be found in the polymer. However, there is some evidence for such structures; all four combinations, including G-b-S of β -ether dimers can be released by incomplete thioacidolysis [240]. In contrast, the ease of preparing the G-b-S entity in trimers by coupling of coniferyl alcohol with S-b-G or S-b-S dimers suggests that the S-b-G-entity is likely in lignin. Unfortunately the INADEQUATE C α -C β contours noted above cannot unambiguously resolve this issue, although it appears to reveal S-b-G ethers [50]. Revealing such details of the lignin structure requires extensive use of carefully synthesized model compounds, for which authentic NMR data are available in several solvents.

β -5-Units (Phenylcoumarans, Arylcoumarans, B)

Phenylcoumarans **B** are typically recognized as the second most abundant inter-unit linkage type in lignins; they will, obviously, be at low to negligible levels in syringyl-rich lignins. Although several arylcoumaran natural products were reported as having a *cis*-ring, it is likely that most such products in nature are in fact *trans* [241,242]. The most extensive model compound data is from Miyakoshi and Chen [243,244], although these authors used mainly γ -methyl models that do not model lignins well; substituent effects were also examined earlier [245]. More appropriate model data is found in several references [8,34,175,241]. Model data derives primarily from synthetic schemes by Nakatsubo [246] or Brunow [247]. A clarification of the stereochemistry of various model compounds was addressed [248]. Trimeric compounds containing phenylcoumaran units were described [249–251].

β - β -Units (Resinols, C and Other Products)

Resinols **C** are distinguished from the more prominent units in lignin by their formation mechanism. They are generally thought to result from monomer–monomer

coupling, although the dearth of releasable (i.e., etherified) pinoresinol units in softwood lignins has led to suggestions that they may be formed during chain extension rather than just dehydrodimerization [252, 253]; however, no compelling evidence has been presented. Coupling of monolignols is relatively rare in lignification, where monomer-polymer coupling is the principal manner in which polymerization occurs. Since monolignols strongly prefer to dehydrodimerize than to cross-couple, the paucity of these structures (and of the cinnamyl alcohol end-groups) led researchers to conclude long ago that monomer supply (or radical supply) is carefully limited during lignification to encourage “endwise polymerization” [1,157]. Their levels are about 2–3% in softwoods and up to about 8% in syringyl-rich angiosperms. This difference is possibly due to the higher stability and longer lifetimes of the sinapyl alcohol radicals, and the dimerization favoring β – β -coupling by about 95% for sinapyl alcohol vs about 40% for coniferyl alcohol in aqueous systems with peroxidase [217,254]. The high β – β -coupling propensity of sinapyl alcohol in dimerization reactions also explains why lower amounts of unsaturated side-chains occur in syringyl-rich lignins.

Resinol structures **C** are readily seen in 1D and 2D NMR spectra because of the relative insensitivity of the side-chain proton and carbon chemical shifts to the limited ring substitution that can occur. Those derived from *p*-coumaryl alcohol, however, have a significantly higher α -proton shift [60]. Resinols must be judiciously quantified since, in 2D HMQC or HSQC spectra, the contours for the resinol unit are twice as intense as for any other (unsymmetrical) unit; two coniferyl alcohol monolignols, for example, yield one pinoresinol unit with two of each type of carbon/proton whereas β –O–4-coupling results in only a single β –O–4-unit (with only one set of β –O–4-carbons and protons contributing). Resinol structures **C** are readily seen in the spectra of Figures 5.2, 5.3, 5.4, 5.8, 5.9, 5.10, 5.17, 5.18, 5.19, and 5.24. Only one isomer has been detected in lignins that have not been subjected to acidolytic conditions [255]; *epi*-pinoresinol, for example, has not been verified in lignins.

Crossed resinols are apparently possible, as revealed by degradative analyses such as thioacidolysis [256,257], and as discovered in low-molecular weight oligomers during metabolic profiling [258]. However, NMR has not been used to address this issue in the polymer. The β – β -cross product of ferulate with both sinapyl and coniferyl alcohols has been validated in vivo. HMBC spectra of a ryegrass lignin convincingly revealed both syringyl and guaiacyl cross-products [130].

A recent finding was unanticipated. In spruce isolated lignin it is possible to detect a putative β – β -coupled α –O– α -tetrahydrofuran that appears must result from addition of water to the intermediary quinone methide [259]. A similar structure is found in Kenaf, when trapping of the quinone methide by the γ -OH is prevented due to its natural acylation [260]—see Naturally Acylated Lignins section and later in Figure 5.11. The whole range of such structures bearing γ -acetate, γ -*p*-coumarate, and γ -*p*-hydroxybenzoate groups has been rather unambiguously identified by NMR [101]. Analogs have also been found in ferulate coupling in grasses and cereal grains [261–263]. However, β – β -structures, other than the resinols **C**, were not expected monolignol coupling products, since it has always been assumed that internal trapping of the quinone methide by the γ -OH would be rapid and complete. This data suggests that lignification may be consistent with low pH conditions where the

protonation-dependent addition of water is more rapid; the paucity of acyclic α -aryl ethers in lignins has been noted to be consistent with low-pH conditions [264].

5-5-Units (Biphenyls)

Biphenyls were difficult to authenticate and quantify by NMR, since only severely overlapped aromatic resonances were thought to be involved; the side-chains were assumed to be insignificantly different from their counterparts in non-5-5-coupled structures [177]. In fact, the side-chains of the biphenyl units themselves remain difficult to assign, but units coupled to them may be highly diagnostic, since 5-5-units are frequently involved in cyclic structures, dibenzodioxocins **D** (see next section). Prior assignments of 5-5-units as fully vs partially etherified need to be reexamined. Some model data are available [175,265,266] and substituent effects have been examined [267]. Trimers containing biphenyl units have recently had NMR data reported [265]. Unfortunately the β -O-4-dimer that were 5-5-linked in tetrameric models [268], and the trimers linked in hexameric models [269] did not have full NMR assignments.

Dibenzodioxocins D The elucidation of dibenzodioxocins **D** in lignins has been an exciting recent development [114,115]. It provides a new pathway for 5-5-linked units to be incorporated into cyclic ether structures. Despite being ethers, such structures cannot necessarily be fully released by solvolytic methods. NMR was crucial to the identification of the novel units and to the unambiguous demonstration of their presence, at significant levels, in isolated lignins [2,76] and whole-cell-wall preparations [64]. A survey of plants reveals that dibenzodioxocins are present in all lignin classes (from softwoods and hardwoods as well as grasses and legumes).

Dibenzodioxocins **D** show up easily in HMQC or HSQC spectra, where the $C\alpha-H\alpha$ and $C\beta-H\beta$ correlations are unique and well-resolved (Figure 5.10). Due to diverse proton-proton coupling constants, the full side-chain correlation matrix does not always show up well in HSQC-TOCSY experiments with typical (80–100 ms) TOCSY mixing times. Figure 5.10a shows an HSQC-TOCSY spectrum of a guaiacyl DHP acquired using a 125 ms TOCSY mixing time, which emphasizes the full **D** coupling array. The peaks or correlations can be difficult to find in unacetylated samples, yet readily show up in acetylated samples. The reason appears to be that the chemical shifts are more variable in unacetylated samples, smearing out the required correlation peaks. They sharpen a little in DMSO. The contours in 2D spectra are also easier to find than significant peaks in 1D ^{13}C NMR spectra. Obviously, since 5-5-units arise only from guaiacyl units (and typically minor *p*-hydroxyphenyl, but not syringyl units), dibenzodioxocin levels can be very low in syringyl-rich lignins from angiosperms (hardwoods, grasses, or legumes). At lower contour levels, such as those shown in the spectra from ryegrass (Figure 5.10f) or aspen (Figure 5.10g), they can almost always be detected. An exception is in the strikingly guaiacyl-depleted lignins in an *Arabidopsis* mutant upregulated in ferulate 5-hydroxylase (see later in Figure 5.18e), **F5H (or Cald-5H)** section.

An array of dibenzodioxocins with chemical shifts differing from those seen previously in normal guaiacyl-syringyl lignins are found in the *p*-hydroxyphenyl-rich lignins from C3H-down-regulated alfalfa plants [60], as seen later in Figure 5.16e. Synthesis of a range of dibenzodioxocins incorporating *p*-hydroxyphenyl units is required to fully assign these data.

4–0–5-Units (Biaryl Ethers)

Biaryl ethers are also difficult to identify and quantify in lignins. Due to the severe overlap of aromatic resonances, they are not readily identified in syringyl–guaiacyl lignins. Few appropriate model compounds are available for this unit; simple models have been described [270–272], and more continue to appear in the lignin model compound database [175]. One interesting conundrum is that the anticipated aromatic correlations for 4–0–5-linked units cannot be detected in softwood lignins [273], despite there being purportedly some 4% of such units [157]. The ^{13}C – ^1H correlations, which should be readily evident in softwood spectra, have not, to date, been found, either in isolated lignins or in whole-cell-wall samples.

α -Aryl Ethers (or α , β -Diaryl Ethers) **A2**

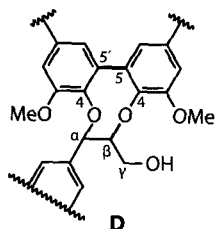
Such structures **A2**, easily visible in spectra of synthetic lignins (e.g., Figures 5.2b, 5.3a, 5.3b, 5.4, 5.8, 5.10a, 5.10b, and 5.10e), are difficult to detect in most isolated lignins, despite prior estimates that such units were in the 6–8% range [157]. A significant proportion of such estimates can presumably now be attributed to dibenzodioxocins **D**. In studies on pine lignins, noncyclic α -ethers **A2** were concluded to be less than 0.3% of their isolated lignins [40]. It is likely that some of the assignments reported in the literature, from the carbon at -81 ppm, are actually attributable to α -keto- β -ethers **A3** (see α -Keto- β -Ethers **A3** section).

One notable exception to the paucity of noncyclic α -aryl ethers in isolated lignins may occur in tobacco [71]. A tobacco lignin isolate was more similar to a synthetic lignin than any other isolated lignin reported to date. It had significant amounts of β – β -units (resinols **C**) and a substantial α -ether **A2** component characteristic of a bulk polymer. It appears that lignification is not controlled by slow diffusion-limited monomer supply as seen in other plants that have distinctly endwise lignins. Lignins from suspension-cultured spruce also appear more similar to synthetic DHPs, presumably because the lignins are formed outside the cell wall [274].

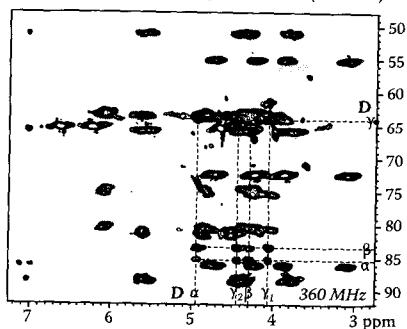
Diarylpropanes (β -1-Units)

Although β -1-units feature prominently in products from various acidolytic treatments, they have been extraordinarily difficult to find by NMR of lignins isolated by dioxane:water extraction, but have been reported [28,30,34,59,134,275,276]. In part this was because of overlapping signals in 1D and 2D experiments. Ede et al. initially estimated that the level was $<2\%$ in pine [28]. It was later found that β -1-units were more efficiently extracted into acetone–water fractions, or by direct derivatization and extraction into acetic anhydride [276]. TOCSY spectra clearly authenticated β -1-units for the first time. The structure is rather convincingly revealed in spectra of an acetylated poplar MWL [50,78,134]. It is likely that this acidic extraction converted other β -1-precursors in lignins to the conventionally described β -1-products, as discussed further below. The most extensive model data is from Miyakoshi and Chen [243,244]. Other data derives primarily from reports on the synthesis of such model compounds [250,277,278].

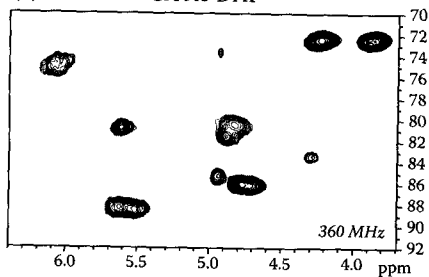
The occurrence of dienone structures was suggested as an explanation for the discrepancy between degradation and NMR spectral studies [275,279–281]. Mild acidolysis of methylated wood produced β -1-dimers with an unmethylated B-ring; the monomethylated β -1-dimers were always methylated in the A ring and the B-ring was free,

dibenzodioxocin (5-5/ β -O-4, α -O-4)

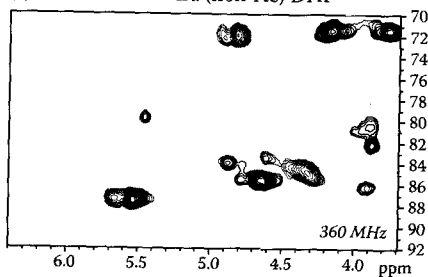
(a) CA Ac-DHP HMQC-TOCSY (125 ms)



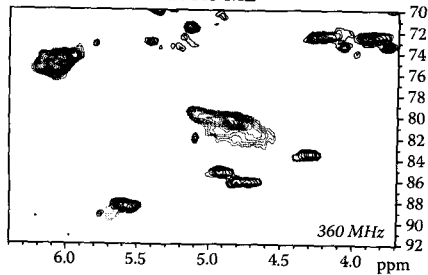
(b) CA Ac-DHP



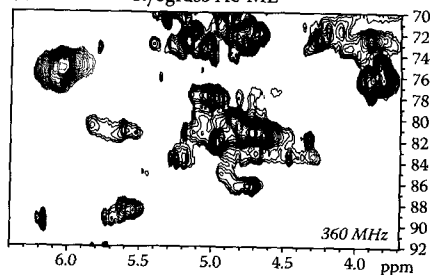
(e) CA/diFA-Ara (non-Ac) DHP



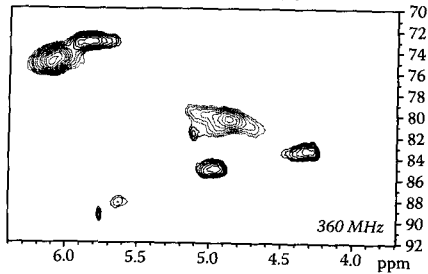
(c) Pine Ac-ML



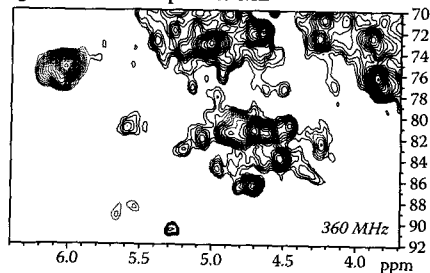
(f) Ryegrass Ac-ML



(d) CAD-null Pine Ac-ML



(g) Aspen Ac-ML



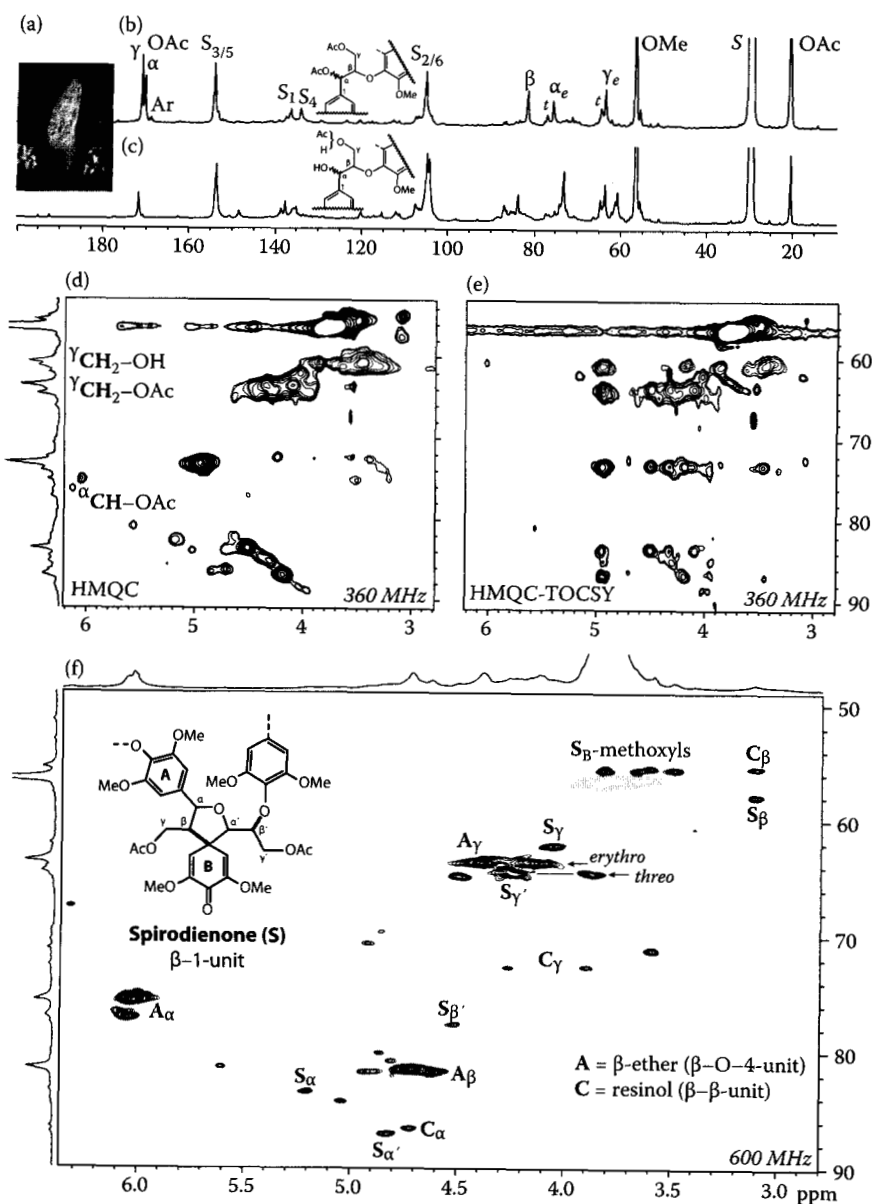
suggesting that it is not actually in the form of a phenolic-OH in lignin [282]. In addition to the dienones postulated from water addition to the quinone methide [279,280], spirodienones **S** were proposed as logical intermediates [283]; sinapate esters can form isolable analogs upon oxidative coupling. NMR evidence for spirodienones was found in poplar lignins [78]. Carbonyl peaks at -182 ppm units have been detected [59]; although small (and most readily seen in quantitative spectra, presumably because of their long T_1 -relaxation times), these carbons had long-range ^{13}C - ^1H correlations reasonable for spiro-hexadienone structures. Further compelling NMR evidence for spirodienones in lignins from spruce, aspen, poplar kenaf, alfalfa, and even various fruits, has now been reported [57,59,60,103,104,106,107]. High-syringyl lignins appear to contain elevated amounts of such units (see the Poplar lignin in Figure 5.3e, for example). Remarkable amounts of syringyl spirodienones **S** appear in Kenaf bast fiber lignins, where the amount is comparable to that of the traditional resinol unit **C** (Figure 5.11f) [57].

The isolation of arylisochromans, following DFRC degradation, and the observation by NMR of trace amounts in isolated lignins, also suggests that dienone β -1-coupling products are formed during lignification [41]. Aryl isochromans can be identified in some softwood isolated lignins by their diagnostic HMQC or HSQC correlation at δ_{C} 41.3, δ_{H} 3.60 (acetylated units, in acetone). The complete side-chain of the crucial unit is seen in TOCSY spectra [41,284]. Whether the structures are present as such in native lignins is not yet clear, but, even if not, the internal trapping of a β -1-quinonemethide intermediate is presumably operating *in vivo*. Compounds assigned as having β -6-linkages resulting from degradative procedures [256,285–287] should be carefully reexamined to determine if they are in fact their β -1/ α -6 arylisochroman isomers (that are difficult to authenticate from mass spectrometry alone) and, therefore, also result from β -1-coupled units.

Revealingly, no β -1-coupling products are detectable in the highly *p*-hydroxyphenyl-rich lignins in C3H-deficient alfalfa, yet spirodienones are readily detected in the

FIGURE 5.10 (See color insert following page 336.) (Opposite) Dibenzodioxocins **D** are elegantly revealed in HMQC or HSQC spectra, where the $\text{C}\alpha$ - $\text{H}\alpha$ and $\text{C}\beta$ - $\text{H}\beta$ correlations, red, are unique and readily identified. (b–g) 360 MHz HSQC spectra representing various classes of lignins: (b) from the acetylated “methoxy-less” DHP (Ralph, J., Zhang, Y., and Ede, R.M., *J. Chem. Soc., Perkin Trans., 1* (16), 2609–2613, 1998); (c) a *Pinus taeda* acetylated milled wood lignin; (d) from the CAD-deficient *Pinus taeda* mutant (Ralph, J., MacKay, J.J., Hatfield, R.D., O’Malley, D.M., Whetten, R.W., and Sederoff, R.R., *Science.*, 277, 235–239, 1997) — note the enhanced dibenzodioxocin levels due to the high levels of 5–5-linked DHCA units from the incorporation of dihydroconiferyl alcohol DHCA into this lignin; (e) a nonacetylated (the reason for the different peaks of structure **D**!) synthetic DHP derived from coniferyl alcohol and 5% [^{13}C]5–5-diFA-Ara, a 5–5-coupled dimer of a ferulate-arabinofuranoside ester (Quideau, S. and Ralph, J., *J. Chem. Soc., Perkin Trans., 1*(16), 2351–2358, 1997); (f) a ryegrass acetylated lignin known to contain diferulates and therefore likely to contain dibenzodioxocins derived from 5–5-coupled diferulate (Ralph, J., Grabber, J.H., and Hatfield, R.D., *Carbohydr. Res.*, 275(1), 167–178, 1995); (g) an acetylated aspen lignin. Due to diverse proton-proton coupling constants, the full side-chain correlation matrix does not always show up well in HSQC-TOCSY experiments with typical (80–100 ms) TOCSY mixing times, e.g., Figures 5.3b, 5.3d. Figure 5.10a shows an HSQC-TOCSY acquired using a 125 ms TOCSY mixing time, which beautifully emphasizes the full **D** coupling array. Contour assignment coloring scheme follows that established in Figure 5.3.

wild-type control [60]. It is logically concluded that *p*-coumaryl alcohol does not favor β -1-coupling and/or that coniferyl and sinapyl alcohol do not favor cross-coupling with *p*-hydroxyphenyl- β -ethers, although this remains to be validated. Wild-type control alfalfa was useful for demonstrating that spirodienones **S** cleave to the traditional β -1-products (and the glyderaldehyde 2-aryl ether) upon acidic treatment; mild acidolysis of the residue from the milled lignin preparation produced a further NMR-analyzable fraction that had no spirodienones, but the conventional β -1-unit was evident [60].



It appears that the product of β -1-coupling in lignins is the spirodienone **S** and that other β -1-products are likely the result of isolation. Thus NMR spectroscopy has again proven to be a revealing and diagnostic structural tool, with ramifications for the biosynthesis of lignin.

Cinnamyl Alcohol End-Groups X1

Cinnamyl alcohol end-groups **X1** result almost exclusively from monolignol dimerization reactions and are consequently relatively minor in lignins (which are formed primarily from endwise coupling of monolignols with the growing lignin polymer). Due to the relative invariance of particularly the γ -proton and carbon signals, they are, however, readily identified in proton [149,150] and carbon NMR spectra [288]. For the same reason, they stand out strongly in 2D spectra, particularly HMQC/HSQC and their TOCSY variants (see Figures 5.2b, 5.3, 5.4, 5.8, 5.9, 5.10a, 5.16d, 5.16e, 5.18, and 5.19a through 5.19c).

The possibility of (*Z*)-cinnamyl alcohols being involved in lignification has been addressed [39,214]. Synthetic lignins prepared using (*Z*)-coniferyl alcohol retained much of the (*Z*)-geometry in the cinnamyl alcohol side-chains [39]. No evidence of such (*Z*)-cinnamyl alcohol end-groups in lignin has been observed, however.

Aldehydes

Formyl groups were easily identified in proton [126,149,150] and carbon NMR spectra [9]. Benzaldehyde and cinnamaldehyde units can be distinguished; they have chemical shift differences and the coupling with other side-chain protons allows their proton networks to be traced in COSY spectra (for example, see the correlations of protons at δ 9.6 in Figure 5.2a). A further discussion of aldehyde structures in lignins appears later in a section (CAD-Deficiency) that deals with the elevated incorporation of aldehydes into lignins in CAD-deficient plants.

Dihydrocinnamyl Alcohols X5 and Derivatives

Softwood isolated lignins contain small amounts of dihydrocinnamyl alcohol units **X5** [27,289,290], most readily seen in TOCSY and HMQC-TOCSY spectra [2,38,66], e.g., Figures 5.2, 5.3, and 5.12. The low level coupling is predominantly via cross-coupling with normal lignin units (Figure 5.2c). In cross-coupling with coniferyl

FIGURE 5.11 (See color insert following page 336.) (Opposite) Spectra from *Tainung* Kenaf isolated lignin. (a) The lignin was isolated from the bast fibers, not the core; (b) 1D ^{13}C -NMR spectrum of acetylated kenaf lignin shows it to be particularly syringyl-rich; (c) 1D ^{13}C -NMR spectrum of unacetylated kenaf shows that the lignin bears its own natural acetates ((a-c) Ralph, J., *J. Nat. Prod.*, 59(4), 341-342, 1996); (d) a gradient HMQC spectrum of unacetylated kenaf lignin shows clear acylation at the γ -position; a low amount of α -acylation comes from natural acetyl group migration; (e) a gradient HMQC-TOCSY (100 ms mixing time) spectrum again shows the clearly acetylated γ -position ((d-e) Ralph, J., Marita, J.M., Ralph, S.A., Hatfield, R.D., Lu, F., Ede, R.M., Peng, J., Quideau, S., Helm, R.F., Grabber, J.H., Kim, H., Jimenez-Monteon, G., Zhang, Y., Jung, H.-J.G., Landucci, L.L., MacKay, J.J., Sederoff, R.R., Chapple, C., and Boudet, A.M., *Advances in Lignocellulosics Characterization*, TAPPI Press, Atlanta, GA, 1999); (f) 600 MHz HSQC spectrum showing how elegantly β -1-spirodienone structures **S** are revealed (Zhang, L., Gellerstedt, G., Ralph, J., and Lu, F., *J. Wood Chem. Technol.*, 26(1), 65-79, 2006). Colors in spectra (b-e) do not relate to those in other figures.

alcohol, β -O-4 and β -5-products will obviously be the most preponderant. A phenylcoumaran dimer with a propanol side-chain was released following dioxane-water hydrolysis of preextracted *Quercus mongolica* wood [291]. It was optically inactive, suggesting that it arose from lignin and not from lignans; the aglycone of an analogous glucoside isolated from Scotch pine (*Pinus sylvestrus*), for example, was optically active [292].

The derivation of the unsaturated side-chain is not clear. An NADPH-dependent enzyme has been reported to produce the β -5-compound from the coniferyl alcohol dimer β -5-dehydrodiconiferyl alcohol [293]. However, the lignin product is racemic. DHCA units occur at high levels in pine mutant trees that are deficient in CAD (see section CAD-Deficiency, Figure 5.2b) [294]. It is likely that DHCA is directly incorporated at low levels during softwood lignification, rather than arising from some post-lignification transformation. Coniferyl alcohol is apparently reduced to DHCA by the NADPH-dependent enzyme. The CAD-deficient mutant tree appears to have reduced capability of producing coniferyl alcohol, suggesting that DHCA may be formed by an alternative reduction pathway from coniferaldehyde [38].

When DHCA levels become elevated, such as in a CAD-deficient pine mutant (CAD-Deficiency section), or in synthetic lignins made from coniferyl alcohol containing a few percent of DHCA (Figure 5.2b), significant homo-dimerization can also occur, producing mainly 5-5-structures and elevated dibenzodioxocin levels (Figure 5.12d) [38]. These 5-coupled DHCA-derived units **X5** have higher side-chain proton chemical shifts and can be distinguished in TOCSY spectra (Figure 5.2b, for example). The presence of DHCA monomers during peroxidase- H_2O_2 -promoted lignification also leads to other products in lignins [135]. Following two sequential formal $H\cdot$ abstractions (or, more likely, radical disproportionation), DHCA monomers will generate quinone methides that will produce guaiacylpropane-1,3-diol (GPD) upon re-aromatization by water addition (Figure 5.12d). Derived units **X6** also appear in lignins; their tell-tale propan-1,3-diol side-chains have been identified in pine MWLs, along with oxidized analogs that may have resulted during lignin isolation [135]. Figure 5.12 shows the identification of three structures derived from DHCA in the lignin of a CAD-deficient pine mutant. It is important to note, however, that these structures also show up at lower levels in lignins from normal pine (see, for example, the **X6 Ca-Ha** correlation in Figure 5.12c, and the DHCA **X5** proton coupling network identified in the TOCSY spectrum of pine lignin in Figure 5.2c). The appearance of these structures in lignins provides compelling evidence that DHCA is a minor monomer in lignification. Figure 5.12 is discussed in more detail in CAD-Deficiency section.

Arylglycerols **X7**

Glycerol side-chains can result from oxidizing conditions and have been detected in enzymatic dehydrogenation products [60,295], but their presence in natural lignins remains in question [296]. A dimeric phenylcoumaran structure, containing an arylglycerol side-chain, is produced upon oxidation of coniferyl alcohol with Ag_2O (compound #262 in the "Database of Lignin and Cell Wall Model Compounds" [175]). The guaiacyl and syringyl monomers are also in the database (compounds #240, #272). The ^{13}C NMR assignments appropriate for lignins are given in Table 5.1. Considerable amounts have been found in DHPs made from *p*-coumaryl alcohol [60]; glycerol units **X7** show

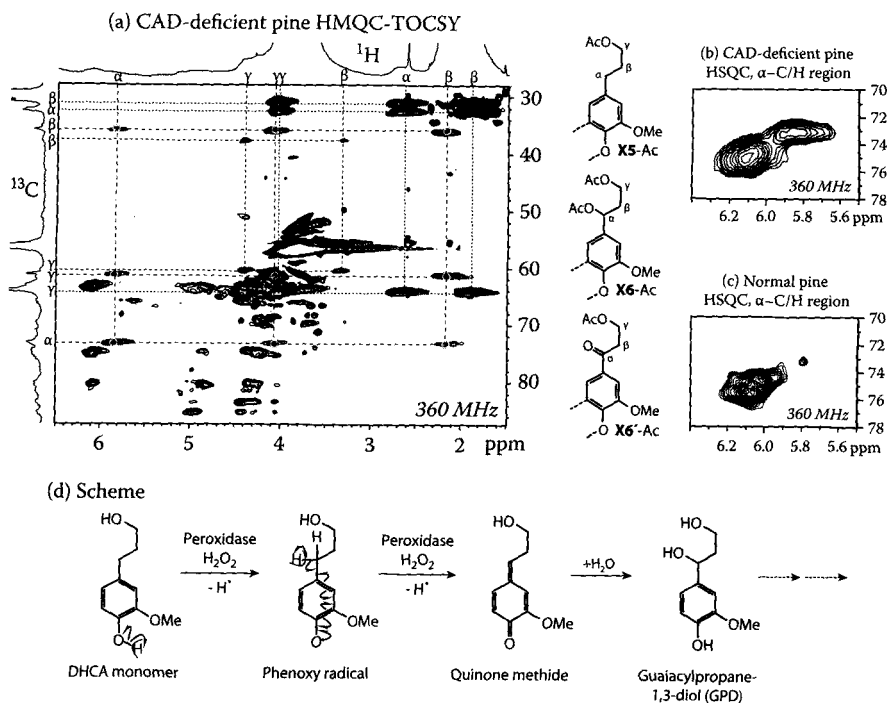


FIGURE 5.12 (See color insert following page 336.) Dihydroconiferyl alcohol and its derived products in pine lignins (Ralph, J., Kim, H., Peng, J., and Lu, F., *Org. Lett.*, 1(2), 323–326, 1999). (a) Partial 2D HMQC-TOCSY NMR spectra showing dihydroconiferyl alcohol (DHCA) units **X5**, derived guaiacylpropane-1,3-diol (GPD) units **X6**, and the benzylic ketone **X6'** derived from GPD units in an acetylated lignin isolated from a CAD-deficient pine mutant (Ralph, J., MacKay, J.J., Hatfield, R.D., O'Malley, D.M., Whetten, R.W., and Sederoff, R.R., *Science*, 277,235–239,1997). Data from the acetylated model 4-benzyloxy-3-methoxyphenylpropane-1,3-diol are at the center of the yellow circles. Note also that dibenzodioxocin correlations are strong in this sample; see Figure 5.10d and caption. (b–c) The β -ether A and GPD **X6** **Ca-Ha** correlations in HSQC spectra from acetylated isolated lignins from the CAD-deficient pine and normal pine; GPD structures **X6** (along with DHCA units **X5**) are present at low levels in normal softwoods (Ralph, J., Kim, H., Peng, J., and Lu, F., *Org. Lett.*, 1(2), 323–326, 1999). (d) Scheme showing the generation of GPD monomer from DHCA monomer. Contour assignment coloring scheme, except for structure **X6'**, follows that established in Figure 5.3.

up readily in lignins derived from high levels of *o*-coumaryl alcohol [60], including in pine compression wood lignins—see Figures 5.5c, 5.5g, 5.16d, 5.16e, and 5.17. They are also elevated in lignins derived from the novel monomer 5-hydroxyconiferyl alcohol [63]—see Figures 5.5h and 5.20. Arylglycerols are now readily identified in “normal lignins”—see for example the HSQC spectra from alfalfa (Figure 5.16d) [60].

α -Keto-*P*-Ethers **A3**

Mainly found in high-syringyl isolated lignins, α -keto- β -ethers (structures **A3**) are thought to arise during isolation steps, presumably during ball milling; however, they are also found in synthetic lignins that have not been subjected to milling. Syringyl

units are more prone to such oxidation. α -Keto- β -ethers **A3** have been found, for example, in aspen (Figure 5.10g) and arabidopsis (Figure 5.18e and 5.18g) lignins [70,117]. They are most readily identified in 1D ^{13}C NMR spectra by their shifted S2/6 peaks at ~ 107 ppm, and in 2D HSQC spectra by their C β –H β correlation peak at $\sim 81/5.6$. It should be noted that the C α –H α peak of α , β -diaryl ethers **A2** also occurs in this region. α -Keto- β -ethers **A3** can, however, be readily distinguished by correlating this contour with its α -carbonyl carbon (at 194.8 in acetylated samples in acetone- d_6) in HMBC spectra [70].

Quinone Methides

Although quinone methides are not structural units found in lignins themselves, they are important intermediates in lignification and in pulping reactions. They were initially considered to be too unstable to be subject to NMR but are in fact stable for considerable periods. The first relevant model spectra, both proton and carbon, were published in 1983 [297]. Guaiacylquinone methides were shown to exist as a mixture of orientational isomers, in an approximately 70:30 ratio; the isomers derive from the relative orientation of the side-chain with respect to the methoxyl side of the ring. Further studies utilized 2D NMR methods to complete or improve assignments [298] and even provided evidence for differences in steric compression and reactivity between the two guaiacyl isomers [299]. An unexpectedly stable quinone methide resulting from β – β -coupling of sinapyl *p*-hydroxybenzoate was authenticated by NMR [300]. Further aspects of lignin quinone methides appear in a recent review [301].

Hydroxyl Groups

Hydroxyl groups in lignins are characteristic. Various types of phenols are recognized in proton spectra run in DMSO [123]. DMSO is a solvent that does not favor proton exchange; phenolic hydroxyl groups are therefore relatively sharp. In ^{13}C NMR, the acetate carbonyl resonances for phenolic, primary (γ) and secondary benzylic (α) acetates are resolved and can be used for quantifying such units [185,302]. Derivatizing with ^{13}C -labeled acetate (via [$1-^{13}\text{C}$]-acetyl chloride) allows for rapid quantification, via ^{13}C NMR, of hydroxyl environments in lignins [303].

Guaiacyl MWLs

Guaiacyl lignins appear to be remarkably similar to one another, regardless of their origin. For example, Figure 5.13 compares the ^{13}C signals from aromatic carbons from MWLs obtained from three different genera, *Ginkgo*, *Pinus*, and *Picea*. The similarity of this "fingerprint" region illustrates the uniformity amongst guaiacyl MWLs. The only differences generally observed between guaiacyl MWLs isolated from different wood species are in the side-chain region of the NMR spectra. These differences are mainly due to varying amounts of carbohydrates that are not separated from the lignins during isolation. The apparent relative homogeneity of guaiacyl lignin structure even across the cell wall, was indicated by a ^{13}C NMR comparison of lignin isolated from the middle lamella and secondary wall of Norway spruce (*Picea abies*) [304].

A study in which significant differences were found amongst guaiacyl lignins was the ^{13}C NMR comparison of MWLs isolated from fossil woods from three different

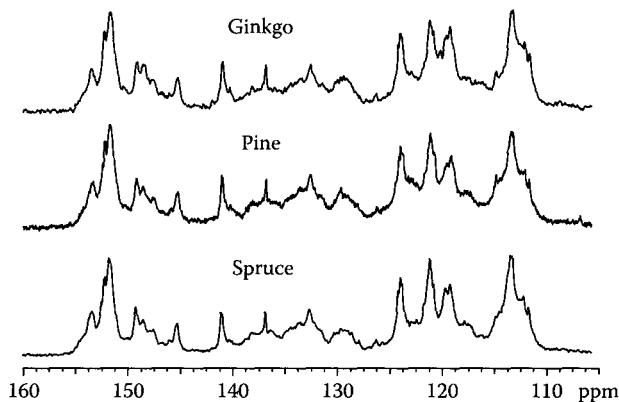


FIGURE 5.13 Comparison of the aromatic regions of 1D ^{13}C NMR spectra of three acetylated softwood lignins (Landucci L.L., *J. Wood Chem Technol.*, 20(3), 243-264, 2000)

locations in the Canadian Arctic [305]. The approximately 50-million-year-old lignins were found to be more condensed, demethylated, and side-chain-degraded than recent lignin from pine. The differences amongst the three fossil MWLs were likely due to varying degrees of degradation during the long burial time, and not because of initial differences.

The first published application of a 2D proton-carbon correlation of lignin [306] confirmed that loblolly pine contained no detectable (by NMR) syringyl content. The weak signals (at about 105 ppm) previously assigned to syringyl units by others were found to be due to residual carbohydrates. Low syringyl contents have been reported for loblolly pine (0.015%), radiata pine (0.001%), and ginkgo (0.060%) as measured by pyrolysis-gas chromatography/mass spectrometry or of the syringaldehyde formed upon nitrobenzene oxidation [307]. Generally, substructures of abundance of less than about 0.5% in lignins are not observable by ^{13}C NMR [308]. Contamination artifacts perhaps cannot be ruled out in the above studies; our investigations, using high-sensitivity GC-MS spectra from DFRC degradation products of pine, failed to reveal even traces of syringyl monomers from loblolly pine.

Guaiacyl/Syringyl MWLs

In contrast to the similarity of G-lignins, GS-lignins are frequently dissimilar. For example, the ^{13}C NMR spectra (aromatic region) of GS lignins isolated from willow, beech, birch, elm, and sweetgum are shown in Figure 5.14. One common difference between hardwood MWLs is the relative amount of guaiacyl and syringyl units. This varies from very guaiacyl-rich lignins, such as those from elms and poplars, to syringyl-rich lignins such as those from birch and sweetgum. In addition, hardwood lignins are rather heterogeneous, so lignin isolated from various parts of the wood cell can have different proportions of G and S units [159,306]. Due to the unique chemical shift of the syringyl signals, especially those from the 2,6-carbon on the aromatic rings, it is generally simple to determine the relative abundances of G and

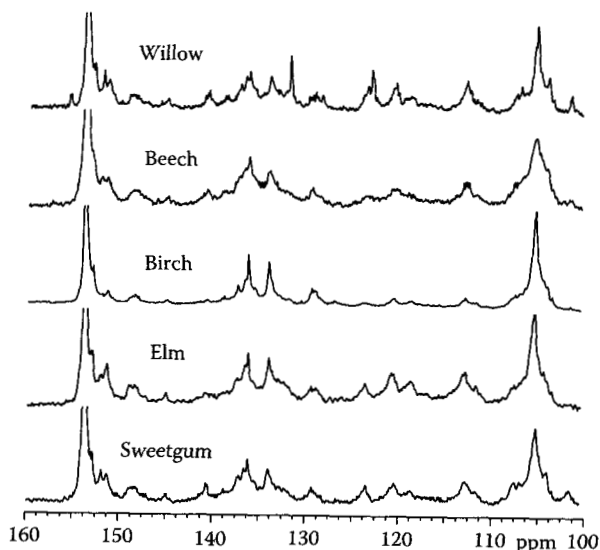


FIGURE 5.14 Comparison of the aromatic regions of 1D ^{13}C NMR spectra of five acetylated hardwood lignins (Landucci, L.L., *J. Wood Chem. Technol.*, 20(3), 243–264, 2000).

S units by quantitative ^{13}C NMR spectroscopy (Quantitative ^{13}C NMR of Lignins section) or from 2D HSQC spectra (Quantification in 2D NMR section). Other differences between GS lignins are frequently the presence of “unique” entities, such as incorporated hydroxycinnamates (Ferulates in Lignins; Cell Wall Cross-Linking in Grasses by Ferulates section), and γ -acylated units (*p*-Hydroxybenzoates in Various Hardwoods section) [309].

A study similar to one mentioned in the Guaiacyl MWLs section on ancient wood has also been conducted. However, the wood in this case was much younger (–6600 years) and could be identified as the hardwood species *Bischofia polycarpa*. [310]. Similar to the ancient guaiacyl lignin, the ancient guaiacyl/syringyl MWL had a higher degree of condensation, a lower methoxyl content and side-chain degradation, but not nearly to the same extent as the much older guaiacyl MWLs.

Naturally Acylated Lignins

Esters of various types are found in certain lignins. Hardwood lignins contain acetate groups [311,312], and all grasses contain varying levels of *p*-coumarates [36,56,313–320], and some plants contain *p*-hydroxybenzoates on lignin [194,309,321]. NMR is not only able to confirm such lignin acylation, but provides unambiguous regiochemical elucidation; the regiochemistry (or site of attachment) provides insights into the biochemistry of such acylation.

Acetates

Kenaf bast fiber lignins (Figure 5.11a), contain striking amounts of acetate [37,312]; over 50% of the units appear to be acetylated. Figures 5.11b through 5.11e show the

1D and 2D spectra identifying such acetylation and establishing that the acetates are primarily at the γ -side-chain position. Acetates will migrate from the γ - to the α -position [322], so strict regiochemistry is not expected. Experiments based on the DFRC (Derivatization Followed by Reductive Cleavage) degradative method confirm the regiochemistry and further reveal that acetates are almost entirely on syringyl units [312].

Such products implicate the biosynthesis of sinapyl γ -acetate as a precursor of Kenaf lignin (see Novel Mono-tetrahydrofurans Provide Evidence that Acylated Monolignols are True Lignin Precursors section) [101,260]. The functional role of such high levels of acetates in kenaf lignins awaits explanation. Although present at much lower levels, except in palms where they may be substantial [323], acetates on the γ -position have been confirmed in aspen by a modified DFRC analysis [312]. Detailed NMR studies elucidating the nature of these minor acetate levels have not been carried out.

p-Coumarates on Grass Lignins

All grasses contain lignins that are partially acylated by *p*-coumarate. The *p*-coumarate units remain free-phenolic (as most easily revealed by the anticipated shifts of resonances when the lignins are acetylated), and do not participate to a significant extent in the radical coupling reactions occurring during lignification. The functional role has not been fully elucidated, although some hypotheses are emerging [324–326]. The elucidation of attachment regiochemistry by NMR is elegant and unambiguous [2,56]. The application of HMQC experiments on maize lignins established that lignin γ -units were acylated and that α -acylation was undetectable. Subsequent HMBC experiments confirmed that it was *p*-coumarate that was acylating the lignin γ -positions. Experiments with other grasses, derived from either C_3 and C_4 biosynthetic pathways, revealed the same information—that acylation is entirely at the γ -position [2,75,76]. The additional finding of *p*-coumarates on many types of lignin units (both isomers of β -ethers, phenylcoumarans, and even cinnamyl alcohol end-groups) suggests that such acylation results from incorporation into lignin of pre-acylated monolignols [56]. More conclusive evidence is the recent discovery of nonresinol β - β -tetrahydrofuran units (see Novel Mono-tetrahydrofurans Provide Evidence that Acylated Monolignols are True Lignin Precursors section).

p-Hydroxybenzoates in Various Hardwoods

Aspen, poplar, and the willows all have significant amounts of *p*-hydroxybenzoates on their lignins [194,309,321]. *p*-Hydroxybenzoates have been found in significant quantities in lignins from oil palm empty fruit bunches [327,409]. Like *p*-coumarates, the *p*-hydroxybenzoates remain as free-phenolic pendant groups and appear as sharp resonances due to their increased mobility compared with the polymer backbone (see CCR-Deficiency section). The ^{13}C NMR chemical shifts of the *p*-hydroxybenzoate units are, therefore, predictably different in underivatized vs acetylated lignins [224,309]. Again, NMR experiments indicate that it is the lignin γ -positions that are acylated [323]. Furthermore, the HMBC experiments again show that it is syringyl units that are acylated by *p*-hydroxybenzoate; acylated guaiacyl units cannot be detected (Figure 5.5i); the γ -*p*-hydroxylated β -ethers **Å** conveniently have a higher α -proton chemical shift, allowing the S:G distribution on such units to be determined.

Novel Mono-tetrahydrofurans Provide Evidence that Acylated Monolignols are True Lignin Precursors

There is one lignification pathway that is significantly altered by preacylation of the monolignols [101,260]. That is the pathway in which the γ -OH on the monolignol becomes involved in postcoupling reactions, i.e., the pathway normally leading to β - β -coupled(resinol) units. With the γ -position acetylated, β - β -coupling or cross-coupling can still presumably occur, but the rearomatization reactions following the radical coupling step can no longer be driven by the internal attack of the γ -OH on the quinone methide intermediates—the γ -acetylation prevents such a reaction. The acyl group remains attached in nonresinol β - β -coupling products; such products could not have arisen from postcoupling acetylation reactions. Therefore, finding these nonresinol syringyl β - β -structures in lignins establishes beyond reasonable doubt that lignin acylation arises through incorporation of preacylated sinapyl alcohol, as a lignin precursor, via radical coupling mechanisms [101,260].

The HSQC NMR spectra of the Kenaf lignin in Figure 5.15 (left column) show that all diagnostic side-chain ($^{13}\text{C}_\alpha\text{--H}_\alpha$, $^{13}\text{C}_\beta\text{--H}_\beta$, $^{13}\text{C}_\gamma\text{--H}_\gamma$) correlations corresponding to the novel β - β -structures are observed and identified by comparison with the synthesized model compounds (right column).

Further evidence in the case of *p*-hydroxybenzoates comes from the discovery of a novel β - β -coupling product bearing a *p*-hydroxybenzoate substituent in actively lignifying poplar xylem [258]. It was identified by NMR as a cross-coupling product between sinapyl alcohol and sinapyl *p*-hydroxybenzoate, demonstrating that sinapyl *p*-hydroxybenzoate is the “monomer” in the coupling reaction *in planta* [258,300].

Ferulates in Lignins; Cell Wall Cross-linking in Grasses by Ferulates

All grasses also contain ferulate that is intimately incorporated into the cell wall, as has been reviewed [133]. Polysaccharide OH groups (primarily the C5-OH of arabinosyl units in arabinoxylans) are acylated by (activated) ferulic acid to form ester bonds; dehydrodimerization of two ferulate esters can result in polysaccharide-polysaccharide cross-linking [133,272]. Both ferulates and ferulate dehydrodimers, which are bound to polysaccharides, can “attach” to lignins via radical coupling reactions with mono- or oligolignols, resulting polysaccharide-lignin cross-linking [55,129,133,203]. The radical nature of the coupling is indicated by the type of ferulate-lignin bonding that is present. Bonding to ferulate can occur, in the typical combinatorial sense, to the C β side-chain carbon, or the aromatic 5- or 4-O-positions and to analogous carbons on lignin, as has been elegantly revealed by NMR studies [129,130,133].

A synthetic lignin from coniferyl alcohol was made in the presence of ~5% of a labeled ferulate model; the single carbonyl resonance in the model produced some five different environments in the DHP [129]. Coupling modes were readily identified using inverse-detected long-range $^{13}\text{C}\text{--}^1\text{H}$ correlation (HMBC) spectra of the synthetic lignin, along with model compounds for the various possibilities [129]. In particular, there were products in which the 8-position of ferulate (analogous to the β -position of monolignols) was involved in radical coupling reactions to produce 8-5-, 8-O-4- and 8- β -structures [129].

With the database of cross-coupling products from the synthetic lignin studies, researchers attempted to look for these components in real lignins. Unfortunately, the

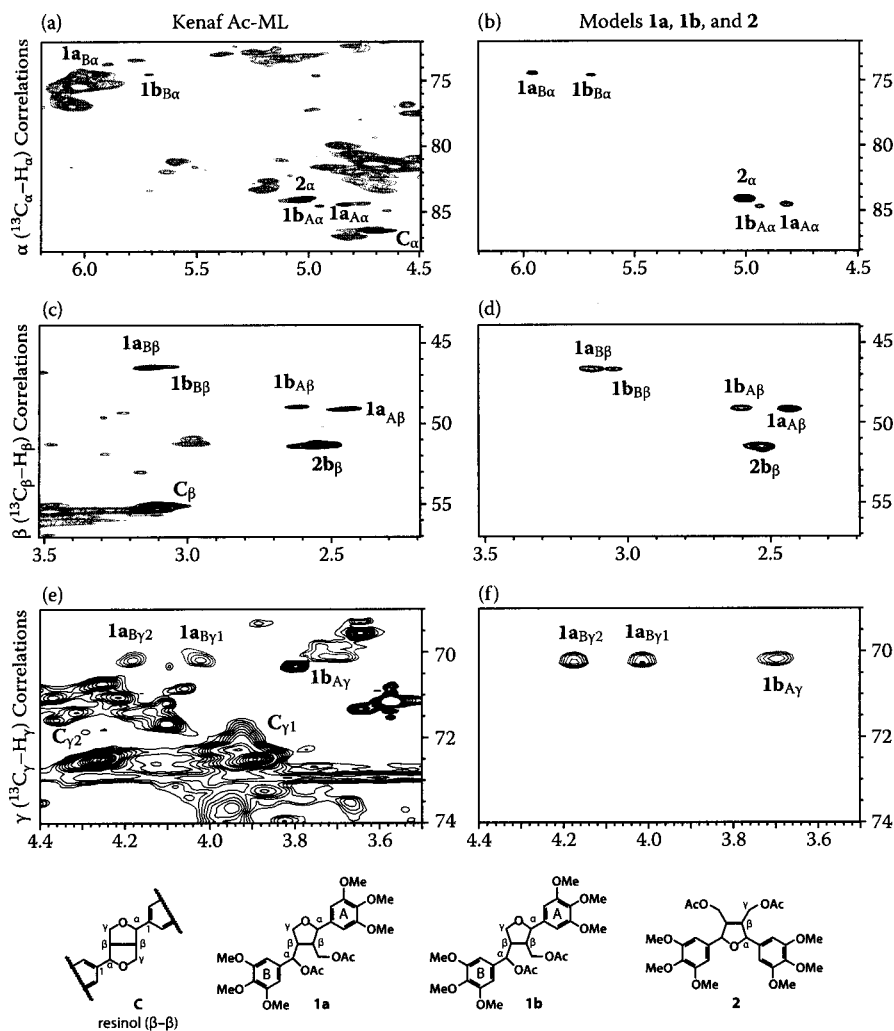


FIGURE 5.15 (See color insert following page 336.) HSQC NMR spectra showing the (a) α - ($^{13}\text{C}_\alpha$ - $^1\text{H}_\alpha$), (b) β - and (c) γ -correlations of novel β - β -coupled structures produced from γ -acetylated sinapyl alcohol in Kenaf Ac-ML. (d-f) HSQC NMRs of methylated and acetylated model compounds (right column; individual of compounds **1a**, **1b**, and **2** are overlaid) are compared with MLs (left column). There are two isomers of **1**; stereochemical assignments have not yet been made. Only the color of contours **C** follows that established in figure 5.3.

levels are so low (a few percent of the lignin), and the number of products so diverse, that spectra cannot easily show these products. However, NMR has sufficient dispersion and resolution to find the products if the signal to noise can be raised. Therefore, plants were grown in ^{13}C -enriched ($\sim 15\%$) CO_2 to provide uniformly labeled material [130]. Enrichment of ^{13}C in a sample to 15% provides a 15-fold sensitivity gain in

^{13}C NMR (and any 2D method that involves carbon), or a savings of $15^2 = 225$ in time to the same signal-to-noise. The incorporated ferulates were readily discerned.

Ferulate-monolignol 8- β -cross-products were readily identified in ryegrass lignin, providing compelling evidence that ferulates cross-couple with normal monolignols, both sinapyl and coniferyl alcohols, during lignification in grasses. The evidence that both monolignols reacted with ferulate was unexpected, since ferulates were suggested to be involved in early wall development, whereas syringyl units resulted later [319,328,329]. The NMR spectral data further suggested that ferulate in the wall reacts with monolignols and not preformed oligomers, suggesting that ferulates therefore act as nucleation sites for lignification in grasses [130,133].

Lignins in Monolignol-Biosynthetic-Pathway Mutant Or Transgenic Plants

Recent advances in genetic engineering have allowed researchers to perturb the monolignol biosynthetic pathway [330–333], allowing the abundance of normally minor components to be substantially enhanced and, therefore, structurally analyzed [38,60,62,63,65–69,71,135,226,239,334–339]. This approach provides valuable insights into the control of lignification and into the remarkable biochemical flexibility of the lignification system [294,330]. Studies to date indicate that up- or down-regulating any of the enzymes of the major pathways can affect the course and/or extent of lignification.

Down-Regulation of Early Pathway Enzymes

General Lignin Down-Regulation General down-regulation of the whole lignification pathway can be achieved by targeting enzymes early in the monolignol biosynthetic scheme. Chiang's group obtained aspen with significantly lower lignin contents by down regulation of an early CoA Ligase, Pt-4CL1 [117,239]. Such down-regulation had little effect (a) on the syringyl/guaiacyl ratio, as determined by examination of the HMBC spectra, shown in Figure 5.14j, and (b) on alteration of lignin structure. However, the *p*-hydroxybenzoate level on β -ether units was elevated. Meyermans et al. obtained a lesser reduction in lignin levels in aspen by downregulating the early *O*-methyl transferase, CCoA-OMT (CCOMT) [224], in poplar. A similar lignin reduction was found in antisense-CCOMT tobacco [340] and alfalfa [63,341]. Despite having significantly lower than normal lignin levels, the plants in these cases produce essentially normal lignins, as delineated by NMR [63,68,117]; the levels of *p*-hydroxybenzoates on the aspen/poplar lignins increased, however, suggesting that the pathways to the monolignol esters might not be fully coupled to general monolignol synthesis [342]. In tobacco transgenics down-regulated in both CCR and CAD, a general down-regulation of lignification was also observed. Again, the lignins were comparatively normal, showing only small indications of the previously substantial changes noted when each gene, singly, was down-regulated [69,343]. Down-regulation of PAL, the first committed enzyme in the phenylpropanoid pathway, also results in lower lignin levels [344].

Down-Regulation of C3H; Inhibition of Both G and S Lignin Formation Richly detailed NMR spectra revealed both striking and subtle structural differences between the syringyl/guaiacyl lignins in normal wild-type alfalfa vs the *p*-hydroxyphenyl-rich

lignins in the heavily C3H-down-regulated plants [60,345]. The alfalfa transgenics became the first plants available in sufficient quantities for lignin isolation and NMR; a stunted *Arabidopsis* mutant with no G- or S-lignin detectable via nitrobenzene oxidation [346] was not available in sufficient quantities for early studies.

The anticipated effect of C3H-deficiency, an enhancement of the relative level of *p*-hydroxyphenyl (H) units in the lignin, was compellingly demonstrated in the aromatic profiles revealed by HMBC spectra (Figures 5.5f and 5.5g), and HSQC NMR spectra (Figures 5.16b and 5.16c). Wild-type plants have syringyl/guaiacyl lignins with only low levels of H-units (Figures 5.5f and 5.16b). A severely down-regulated line (Figures 5.5g and 5.16c) was G- and S-depleted and strikingly H-rich. Reasonable quantification, gained by volume-integrating the contours in the HSQC spectra (Figures 5.16b and 5.16c), indicated that the H-content went from ~1% in the wild-type to ~65% in the transgenic (Table 5.2) [60].

High-field HSQC spectra of the side-chain regions were more revealing regarding the manner in which the monomeric units are assembled. The most immediately noticeable feature from Figure 5.16d and 5.16e is that the minor, but important, spirodienones **S**, resulting from β -1-coupling reactions, are absent in the C3H-deficient lignin (Figure 5.16e). It seems logical from the absence of β -1-coupling products that *p*-coumaryl alcohol simply does not efficiently 0-1-couple with H-units in the lignin, nor probably with G- or S-units. As has been discovered with other units, this is likely due to a simple chemical incompatibility. The second striking difference is the multitude of dibenzodioxocins **D** that appear in the C3H-deficient lignins due to the coupling of the full range of hydroxycinnamyl alcohols with traditional guaiacyl-guaiacyl 5-5-biphenyl units, as well as the biphenyls formed from *p*-hydroxyphenyl units. Less readily seen before quantification (Table 5.2), is that β -ether units **A** were lower in the transgenic (~56%. vs ~75% of the units quantified); this was compensated for by the two other major units, phenylcoumarans **B** and resinols **C**, each of which nearly doubled in relative proportion. The higher resinol concentration suggested that more monomer-monomer coupling reactions occurred during the lignification in the H-rich plants. Although still quite low, the relative dibenzodioxocin **D** level was about double that in wild-type plants. Glycerol structures **X7** were also at significantly higher (~9-fold) levels.

Significant details regarding lignification via enhanced levels of *p*-coumaryl alcohol came from analysis of the HMBC spectra (Figure 5.17). The data from these spectra allow a determination of which monomers are involved in forming each type of interunit linkage. From Figure 5.17, for example, essentially all phenylcoumarans **B** were revealed to be formed by coupling reactions involving *p*-coumaryl alcohol; this is the kind of information that is required to understand the cross-coupling propensity of *p*-coumaryl alcohol. The spectra, unfortunately, do not reveal whether the coupling was with a guaiacyl or another *p*-hydroxyphenyl unit (it obviously could not have been a syringyl unit, since the 5-position is not available for coupling).

Similarly, the change in resinols **C** from being almost entirely derived from sinapyl alcohol in the wild-type to being derived from all three monomers, and *p*-coumaryl alcohol in particular, in the transgenic plants, is striking. It is logical that sinapyl alcohol monomers find themselves only rarely able to dimerize (with other sinapyl alcohol monomers) in this sinapyl-alcohol-depleted plant, so presumably β - β -cross-couple with coniferyl, and possibly *p*-coumaryl alcohol; studies are

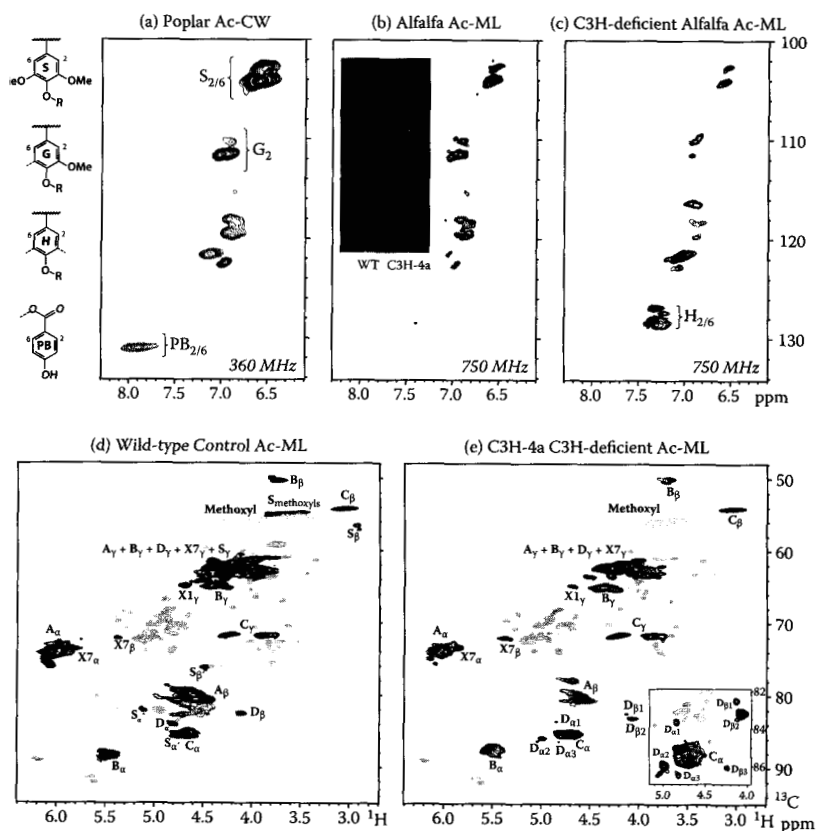


FIGURE 5.16 (See color insert following page 336.) HSQC spectra of acetylated lignins, in CDCl₃, with different H:G:S ratios. (a–c) Aromatic regions of acetylated isolated lignins showing quantifiable H:G:S levels in (a) Poplar (which also shows how γ -*p*-hydroxybenzoates can be evidenced in this spectral region); (b) Alfalfa wild-type control, a guaiacyl-rich S/G lignin with only traces of H-units (~2%); (c) a C3H-deficient alfalfa, extraordinarily enriched in H-units (~65%) from the typically minor monolignol *p*-coumaryl alcohol (Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P.F., Marita, J.M., Ralph, S.A., Reddy, M.S.S., Chen, E., and Dixon, R.A., *J. Biol. Chem.*, 281(13), 8843–8853 2006). (d–e) Side-chain regions from the alfalfa lignins: (d) Wild-type control, (e) C3H-deficient. C3H-deficiency, and the incorporation of higher levels of *p*-coumaryl alcohol into the lignin, produces significant changes in the distribution of interunit linkage types. The absence of spirodienone S units in the transgenic reveals that *p*-coumaryl alcohol does not apparently favor β –1 cross-coupling reactions. Several types of new dibenzodioxocins **D** are more readily seen at the lower contour levels in the more highly resolved partial spectrum in the inset. Note that the contour levels used to display the two spectra were chosen to highlight the structural similarities and differences; with no internally invariant peaks, interpretation of apparent visual quantitative differences needs to be cautious. Photographic inset shows WT and C3H-deficient transgenic plants at the WT flowering stage (Reddy, M.S.S., Chen, F., Shadle, G.L., Jackson, L., Aljoe, H., and Dixon, R.A., *Proc. Natl. Acad. Sci.*, 102(46), 16573–16578 2005). Semi-quantitative data derived from corrected volume integrals are given in Table 5.2. Contour assignment coloring scheme in d–e follows that established in Figure 5.3; contour assignment coloring for S:G:P:PB delineation is the same as used for similar purposes in Figures 5.5 and 5.17.

TABLE 5.2

S:G:H Measurement and Linkage Data from Corrected Volume Integrals for Contours in the Side-Chain Region of ^{13}C - ^1H Correlation Spectra of Acetylated Isolated Stem Lignins from Control (Wild-Type) and C3H-Deficient Alfalfa

Lignin↓ Unit→	A	B	C	D	S	X1	X7		S	G	H
Alfalfa											
WT*	75	9	9	1.1	0.6	4.8	0.5		41	58	1
C3H-antisense*	56	18	16	2.6	—	2.9	4.6		18	17	65

Source: From Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P.F., Marita, J.M., Ralph, S.A., Reddy, M.S.S., Chen, F., and Dixon, R.A., *J. Biol. Chem.*, 281(13), 8843–8853, 2006.

Note: **A** = β -ether (β -O-4), **B** = phenylcoumaran (β -5), **C** = resinol (β - β), **D** = dibenzodioxocin (β -O-4/ α -O-4), **S** = spirodienone (β -1); **X5** = cinnamyl alcohol end-group; **X7** = arylglycerol end-group. These percentages are corrected ratios, via responses from model dimers, from the volume integrals of contours (Figures 5.16d and 5.16e), arise from units **A-D**, **S**, **X1** and **X7**, and are therefore better attempts at true ratios. **S:G:H** values are from uncorrected volume integrals of the S2/6, G2, and H2/6 correlations from HSQC spectra (Figures 5.16b and 5.16c). More details, and the quantification of other lignin fractions, have been reported.

required to determine which of these cross-coupling reactions are chemically feasible, i.e., if sinapyl alcohol and *p*-coumaryl alcohol, for example, are compatible for radical cross-coupling. Examination of thioacidolysis or DFRC dimers may eventually shed light on the occurrence of mixed resinols; mixed dimers can be elucidated by GC-MS [257].

Although it is less clear since dibenzodioxocins **D** did not show useful correlations in the wild-type lignins, it appears that the coupling in the C3H-deficient plant also involved coupling of *p*-coumaryl alcohol with the dibenzodioxocin-precursor 5-5-end-unit. The crucial β -aryl ether units derive from all three monolignols, illustrating that *p*-coumaryl alcohol is able to function in the most important endwise-coupling reactions that allow polymer growth.

Down-Regulation of Later Pathway Enzymes; Lignin Compositional and Structural Change

Down-regulating enzymes later in the pathway frequently did not significantly alter the quantity of “lignin” produced, but caused more extensive structural alterations.

Syringyl Lignin Pathways Two key enzymes (beyond the coniferyl alcohol pathway) are required for the production of sinapyl alcohol, which is the essential monolignol for production of syringyl units in lignins. Ferulate 5-hydroxylase, FH5, now perhaps more appropriately called CALD-5H to reflect coniferaldehyde as the preferred substrate [347,348], effects the 5-hydroxylation. Caffeate *O*-methyltransferase,

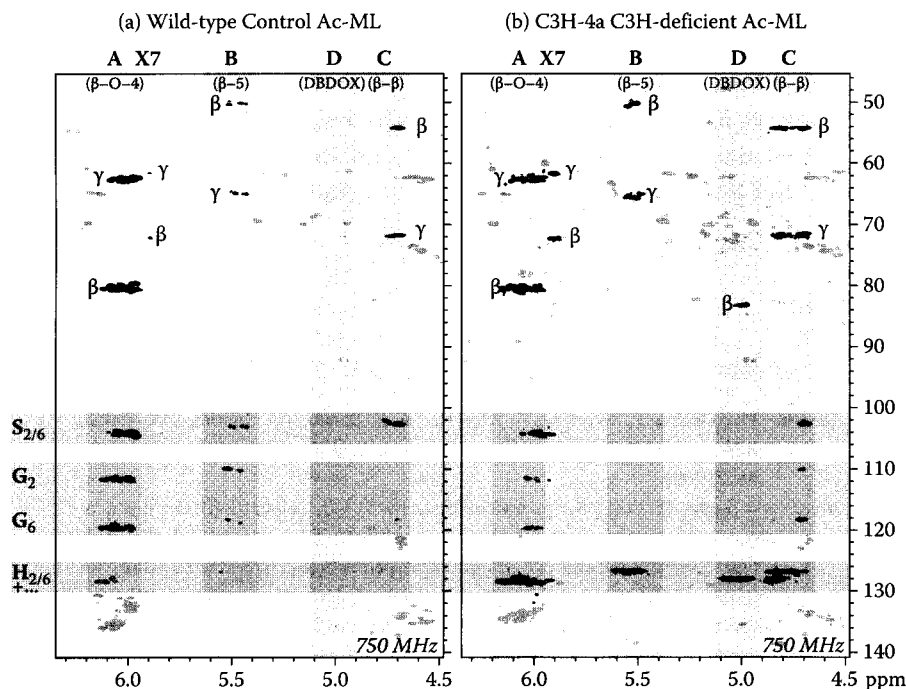


FIGURE 5.17 (See color insert following page 336.) Partial HMBC spectra of lignins, in CDCl_3 , isolated from alfalfa, (a) the wild-type control and (b) a C3H-deficient line (Ralph, J., Akiyama, T., Kim, H., Lu, F., Schatz, P.F., Marita, J.M., Ralph, S.A., Reddy, M.S.S., Chen, F., and Dixon, R.A., *J. Biol. Chem.*, 281(13), 8843–8853 2006). These spectra allow a determination of the monolignol involved in forming each type of structural unit (see text). The correlations highlighted are from α -protons to the carbons within three bonds, most diagnostically those to the 2- and 6-positions on the aromatic rings of *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units. Contour assignment coloring scheme does not follow that established in Figure 5.3 (except for the subunit designations along the top); contour assignment coloring for S:G:H delineation is the same as used for similar purposes in Figures 5.5 and 5.16a through c.

COMT, for which the preferred substrate now appears to be 5-hydroxyconiferaldehyde [342], catalyzes the methylation of the new 5-OH.

F5H (or Cald5H) The most striking examples of compositional flexibility in the two normally predominant monolignols came from the F5H (ferulate 5-hydroxylase) *Arabidopsis* mutants and transgenics produced by Chapple's group. An F5H-deficient mutant, derived from chemical mutagenesis [349] had (essentially) no syringyl component in its lignin, as shown by NMR [70] and other methods [349]. When a suitably promoted *F5H* gene was introduced into the F5H-deficient mutant, the researchers observed that up-regulation of F5H proved to be strikingly effective at diverting the monolignol pool beyond coniferyl alcohol. With an approximately 3% guaiacyl component, these plants' lignins have a far lower guaiacyl contents than any plants reported to date [70].

The incredible compositional change, from extremely guaiacyl-rich to essentially guaiacyl-depleted, is dramatically illustrated in the NMR spectra given in Figures 5.18 and 5.5a [2,70]. Figure 5.5a shows the HMBC correlations between the α -protons of the major β -aryl ether units in lignins from wild-type control, the down-regulated mutant, and a C4H-promoted up-regulated transgenic. The control contains guaiacyl and syringyl (β -ether) units. The mutant has no detectable syringyl component. The lignin in the most highly up-regulated C4H-F5H transgenic is extraordinarily guaiacyl-deficient; only weak guaiacyl peaks can be discerned at lower levels. In fact, the syringyl:guaiacyl ratio in this transgenic is higher than has been recorded in any plant to date; but, as shown below and in Figure 5.18e, the methylation of 5-hydroxyguaiacyl precursors cannot apparently keep pace and 5-hydroxyconiferyl alcohol becomes a significant component of the lignin from this transgenic [66].

Similarly remarkable compositional changes were effected in Aspen by manipulation of the analogous gene and the enzyme, now called CAld5H to reflect the preferred substrate specificity for coniferaldehyde by the enzyme [347,348]. The HMBC spectra in Figures 5.5j show that CAld5H up-regulation is again strikingly effective at ramping up the monolignol pool to sinapyl alcohol, producing high-syringyl lignins. The F5H-upregulated poplars had up to -94.5% S, and had a consequently dramatic benefit on pulping efficiency [350,351].

COMT-Deficiency: Benzodioxanes from Incorporation of 5-Hydroxyconiferyl Alcohol COMT is one of two enzymes required to 5-methoxylate guaiacyl monomeric units to produce sinapyl alcohol and eventually produce syringyl units in angiosperm lignins. If COMT is down-regulated, 5-hydroxyconiferyl aldehyde might build up and be expected to be reduced to 5-hydroxyconiferyl alcohol if the next enzyme, CAD, is sufficiently nonspecific. In fact, it appears that 5-hydroxyconiferyl alcohol is indeed formed, shipped out to the wall, and incorporated into lignin analogously to other lignin monomers (although it produces some novel structures in the final lignin).

Again, NMR provides compelling evidence that benzodioxane structures are produced in lignins that incorporate 5-hydroxyconiferyl alcohol. Figure 5.19b shows the side-chain region of an HMQC spectrum from an (acetylated) isolated lignin from a COMT-deficient gene-silenced poplar (*Populus tremula* $\times$ *Populus alba*) described recently [336]. The degree of COMT suppression was higher than in other trials using anti-sense suppression [226,334]. As with the dibenzodioxocins **D** [114,115], the benzodioxanes **H** are readily apparent in short-range ^{13}C - ^1H correlation spectra (HMQC or HSQC) of acetylated isolated lignins. Well separated contours at $\delta_{\text{C}}/\delta_{\text{H}}$ of 76.8/4.98 (a), and 75.9/4.39 (B) are diagnostic for the benzodioxanes **H**; the γ -correlations overlap with those in other lignin units. Anticipated shifts occur following acetylation; in the unacetylated lignins (not shown), the correlations are centered at $\delta_{\text{C}}/\delta_{\text{H}}$ of 76.5/4.87 (a), and 78.9/4.06 (B). As seen in Figures 5.19b and 5.19d, the side-chain correlations are consistent with those in a model compound for the trans-benzodioxane, synthesized by biomimetic cross-coupling reactions between coniferyl alcohol and a 5-hydroxyguaiacyl unit [67]. The α -proton shift deviated the most, presumably since this is an acetylated (and therefore originally free-phenolic) model, rather than a phenol-etherified structure, which would correspond to most of the units in the lignin polymer. Lignins from COMT antisense poplars also contain benzodioxane units [67].

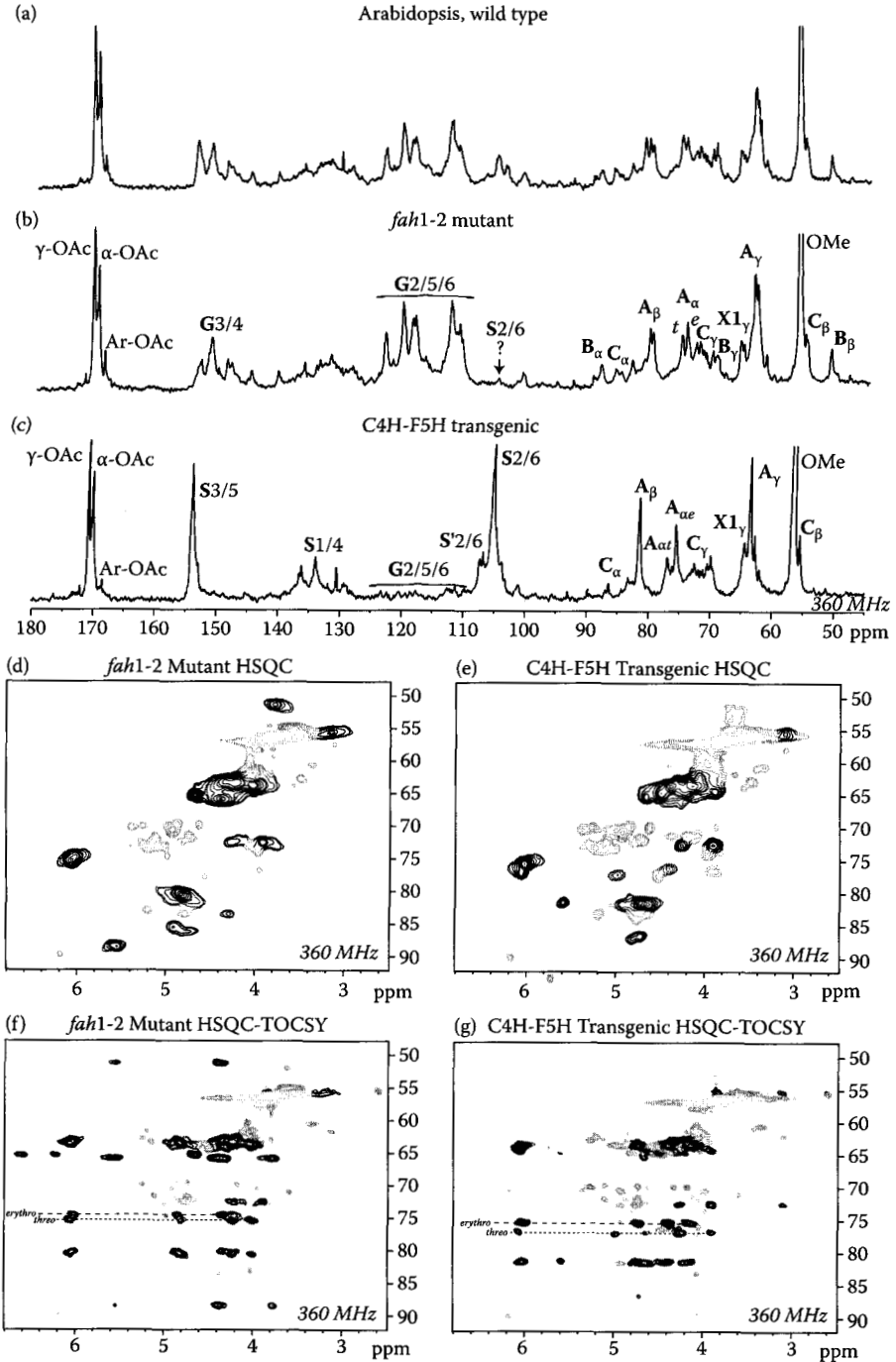
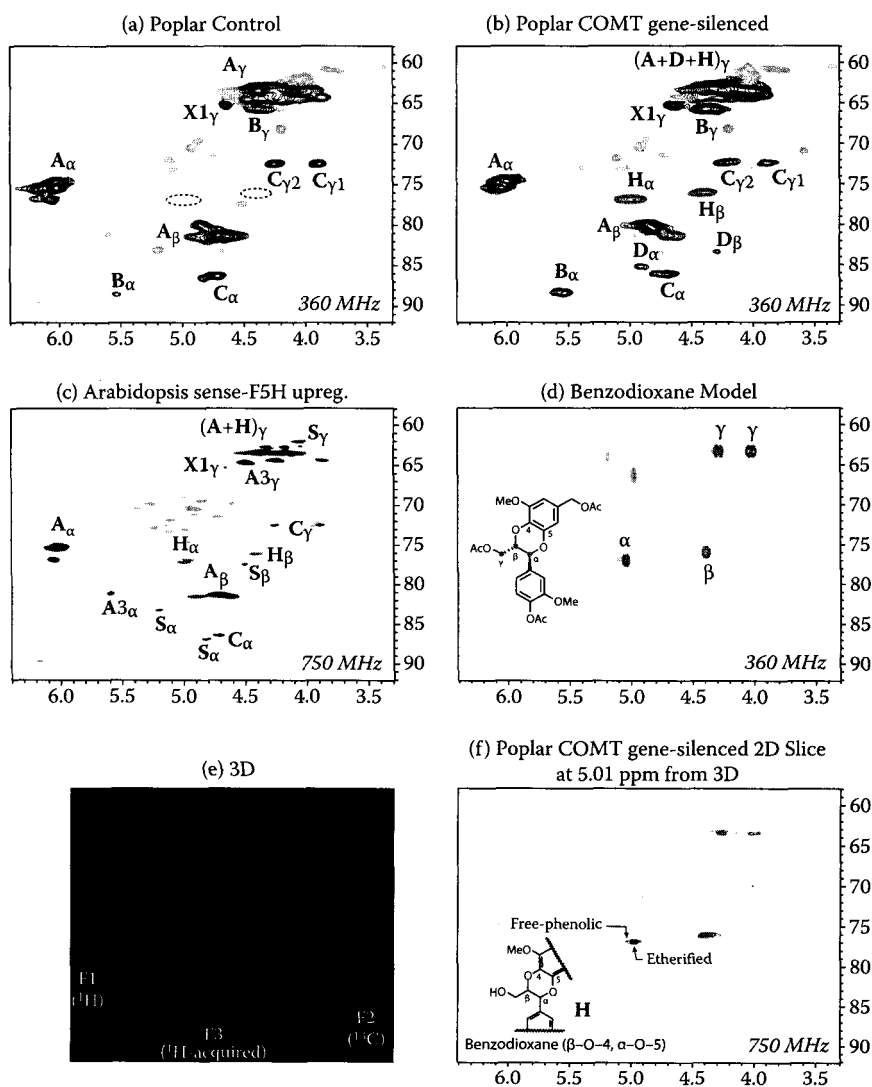


Figure 5.19e shows the first 3D spectrum of a natural ^{13}C -abundance lignin. It is a 3D gradient-selected TOCSY-HSQC taken on a 750 MHz instrument. In less than 24 h, the 3D experiment provided ample sensitivity to authenticate all of the major units in the natural ^{13}C -abundance sense-suppressed COMT transgenic poplar lignin. The F_2 - F_3 slice of the new benzodioxane structure H is spectacularly clean with its α -, β -, and γ -correlations fully resolved (Figure 5.19f). In the 3D experiment, the γ -correlations of the benzodioxane in the F_2 - F_3 plane are nicely resolved and isolated from the plane at its α -proton frequency (5.01 ppm), as well as at the β -proton frequency (-4.47 ppm; not shown) along the F_1 axis (Figure 5.18f). Obtaining a “pure” slice, leaves little ambiguity between correlations of different structures. For example, the γ_1 - and γ_2 -correlations of the benzodioxane units that overlap with the γ_1 - and γ_2 -correlations of the β -aryl ether A units and the dibenzodioxocin D units in the 2D spectra are unique to their respective 3D slices. This was the first reported “isolation” of the side-chain ^{13}C - ^1H coupling network of a new lignin component using 3D NMR experiments at natural abundance. Again, the data in this slice agree well with those from a benzodioxane model compound [67].

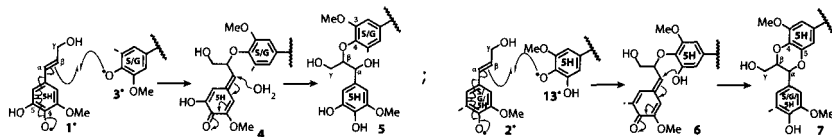
Another detail revealed regarding benzodioxane units H in the 3D and 2D spectra is the degree of etherification. In acetylated lignins, units that were free-phenolic in lignin become phenol-acetylated, whereas those that were originally etherified remain so. Phenol acetylation causes H_a in H units to move to a lower field (higher ppm). Thus, unetherified units have H_a at 5.04 ppm, whereas etherified units are at 4.96 ppm. The 2D slice for the 3D experiment shown in Figure 5.19f has only a trace of correlations for the acetylated component. Other slices reveal slightly more. It appears that the benzodioxane units H are substantially etherified and, therefore, have been fully integrated into the polymer by further monolignol coupling reactions during lignification.

A reasonable quantification of units H (and other units) can be accomplished by measuring volume integrals in the 2D spectra [95], particularly if the similar Ca - H_a correlations are used. As can be seen in Table 5.3, the 5-hydroxyconiferyl alcohol-derived benzodioxane H units can become the second most abundant

FIGURE 5.18 (See color insert following page 336.) (Opposite) Spectra from wild-type *Arabidopsis*, a mutant deficient in ferulate 5-hydroxylase (F5H), and a transgenic upregulated in F5H (Marita, J., Ralph, J., Hatfield, R.D., and Chapple, C., *Proc. Natl. Acad. Sci.*, 96(22), 12328–12332, 1999). In the *fahl*-2 mutant, syringyl units are almost completely absent (Marita, J., Ralph, J., Hatfield, R.D., and Chapple, C., *Proc. Natl. Acad. Sci.*, 96(22), 12328–12332, 1999; Meyer, K., Shirley, A.M., Cusumano, J.C., Bell-Lelong, D.A., and Chapple, C., *Proc. Natl. Acad. Sci.*, 95(12), 6619–6623, 1998). When F5H is expressed under the control of the C4H-promoter, the lignin that is deposited is comprised almost exclusively of syringyl units. G = guaiacyl, S = syringyl. (a–c) 1D ^{13}C NMR spectra of *Arabidopsis* lignins from (a) the wild type, (b) the *fahl*-2 mutant, and (c) the C4H-F5H transgenic. Major peaks are shown with their assignments; G is used to represent a general guaiacyl unit, S a general syringyl unit; *e* = erythro-isomer, *t* = threo-isomer; oligosaccharides are present in these lignins. (d–g) Gradient-selected 2D HSQC spectra of *Arabidopsis* lignins from d, the *fahl*-2 mutant, and e, the C4H-F5H upregulated transgenic, as well as higher F_1 -resolution (carbon dimension) gradient-selected 2D HSQC-TOCSY spectra from (f) the *fahl*-2 mutant and (g) the C4H-F5H transgenic. Contour assignment coloring scheme follows that established in Figure 5.3.



(g) Scheme for 5-hydroxyconiferyl alcohol incorporation into lignin, and benzodioxane formation



interunit type in transgenics and mutants with low COMT levels. Since the lignins analyzed by NMR represent 65% of the total lignin in the poplar transgenic, it is logical that the benzodioxane structures would remain a significant component, even if the lignins were drastically partitioned by the isolation process. The total β -ether frequency (normal β -ether **A** plus dibenzodioxocins **D** plus benzodioxanes **H**) in the

transgenic is around 77%, lower than in the control because of the higher guaiacyl content. However, there are a few units not covered by these percentages, since they have no resonances in the aliphatic side-chain region of the NMR spectra (cinnamaldehyde end-groups and β -1-structures). The COMT-deficient brown-midrib-3 mutant of maize contains very significant levels of benzodioxanes **H** [352]. Although the data are limited at present, it appears that 5-hydroxyconiferyl alcohol may quantitatively make up for the sinapyl alcohol deficiency.

The incorporation of 5-hydroxyconiferyl alcohol into lignins has been documented several times [353–356]. Benzodioxanes have been proposed to be in lignins previously [336,353,355], and analogous structures occur in lignans [357,358]; however, until NMR spectra were obtained, the occurrence of benzodioxanes in lignins had not been observed directly. The likely mechanism for formation of the novel benzodioxane units is shown in Figure 5.19g, as described in the caption. Since etherified benzodioxanes don't cleave under alkaline pulping conditions, pulping efficiency would, therefore, likely be reduced. Recent pulping trials with COMT-deficient poplars confirm a lower pulping efficiency [336].

FIGURE 5.19 (See color insert following page 336.) (Opposite) Partial spectra from gradient HMQC NMR experiments highlighting new peaks for benzodioxane units **H**. Acetylated lignins were from (a) a control poplar, (b) a COMT-down-regulated poplar transgenic (Ralph, J., Lapierre, C., Marita, J., Kim, H., Lu, F., Hatfield, R.D., Ralph, S.A., Chapple, C., Franke, R., Hemm, M.R., Van Doorselaere, J., Sederoff, R.R., O'Malley, D.M., Scott, J.T., MacKay, J.J., Yahiaoui, N., Boudet, A.-M., Pean, M., Pilate, G., Jouanin, L., and Boerjan, W., *Phytochem.*, 57(6), 993–1003, 2001; Marita, J.M., Ralph, J., Lapierre, C., Jouanin, L., and Boerjan, W., *J. Chem. Soc., Perkin Trans.*, 1(22), 2939–2945, 2001), (c) an F5H-upregulated Arabidopsis (cf. Figure 5.18d, but acquired at higher field and with higher resolution in the F₂ (carbon) dimension) (Ralph, J., Lapierre, C., Marita, J., Kim, H., Lu, F., Hatfield, R.D., Ralph, S.A., Chapple, C., Franke, R., Hemm, M.R., Van Doorselaere, J., Sederoff, R.R., O'Malley, D.M., Scott, J.T., MacKay, J.J., Yahiaoui, N., Boudet, A.-M., Pean, M., Pilate, G., Jouanin, L., and Boerjan, W., *Phytochem.*, 57(6), 993–1003, 2001), and (d) similar correlations from a benzodioxane model compound (Ralph, J., Lapierre, C., Lu, F., Marita, J.M., Pilate, G., Van Doorselaere, J., Boerjan, W., and Jouanin, L., *J. Agr. Food Chem.*, 49(1), 86–91, 2001). (e–f) The first natural ¹³C-abundance lignin 3D NMR spectrum (Marita, J.M., Ralph, J., Lapierre, C., Jouanin, L., and Boerjan, W., *J. Chem. Soc., Perkin Trans.*, 1(22), 2939–2945, 2001). (e) A 3D gradient-selected TOCSY-HSQC spectrum (70 ms TOCSY) of a natural ¹³C-abundance lignin from the sense-suppressed COMT transgenic; (f) 2D F₂-F₃ slices for the structural unit **H**. (g) Scheme for production of benzodioxanes **7** in lignins via incorporation of 5-hydroxyconiferyl alcohol **1** into a guaiacyl lignin (Ralph, J., Lapierre, C., Marita, J., Kim, H., Lu, F., Hatfield, R.D., Ralph, S.A., Chapple, C., Franke, R., Hemm, M.R., Van Doorselaere, J., Sederoff, R.R., O'Malley, D.M., Scott, J.T., MacKay, J.J., Yahiaoui, N., Boudet, A.-M., Pean, M., Pilate, G., Jouanin, L., and Boerjan, W., *Phytochem.*, 57(6), 993–1003, 2001). Cross-coupling of 5-hydroxyconiferyl alcohol **1**, via its radical **1**, with a guaiacyl lignin unit **3G**, via its radical **3G**, produces a quinone methide intermediate **4** which rearomatizes by water addition to give the p-ether structure **5** (possessing a 5-hydroxyguaiacyl end-unit). This unit is capable of further incorporation into the lignin polymer via radical coupling reactions of radical **5**. Reaction with the monolignol coniferyl alcohol **2G**, via its radical **2G**, produces a quinone methide intermediate **6**. This time, however, quinone methide **6** can be internally trapped by the 5-OH phenol, forming a new 5–Oa-bond, and creating the benzodioxane ring system in **7**. Compound numbers relate only to this figure. Contour assignment coloring scheme follows that established in Figure 5.3.

TABLE 5.3

Volume Integrals for Acetylated Lignin Units							
Lignin↓ Unit→	A	B	C	D	H	X5	Σ(β-ethers)
Poplar							
WT	8	8	3	7	0	0	88
COMT-silenced	53	13	5	6	18	5	78
COMT-antisense	65	12	5	3	10	4	78
Maize							
WT	86	7	0	0	0	6	86
bm3-mutant	60	7	0	0	2	5	85
Arabidopsis							
WT	6	7	1	3	6	6	73
F5H-sense	81	<1	6	0	10	4	91
Alfalfa							
WT	81	8	6	1	0	3	83
COMT-antisense	4	4	8	3	4	3	85
CCOMT-antisense	88	5	6	0	<1	2	88

Source: Marita, J.M., Vermerris, W., Ralph, J., and Hatfield, R.D., *J. Agr. Food Chem.*, 51(5), 1313–1321, 2003; Marita, J.M., Ralph, J., Hatfield, R.D., Guo, D., Chen, F., and Dixon, R.A., *Phytochem.*, 62(1), 53–65, 2003; Marita, J.M., Ralph, J., Lapierre, C., Jouanin, L., and Boerjan, W., *J. Chem. Soc., Perkin Trans.*, 1 (22), 2939–2945, 2001; Marita, J., Ralph, J., Hatfield, R.D., and Chapple, C., *Proc. Natl. Acad. Sci.*, 96(22), 12328–12332, 1999; Marita, J.M., *Lignin in Lignin-Biosynthetic-Pathway Mutants and Transgenics: Structural Studies*. Ph.D., U. Wisconsin-Madison (University Microfilms International #3012586), 2001.

Note: Uncorrected Volume Integral Ratios of Contours in the Side-Chain Region of ¹³C–H Correlation Spectra of Acetylated Isolated Lignins.
A = β-ether (β-O-4), B = phenylcoumaran (β-5). C = resinol (β-β), D = dibenzodioxocin (β-O-4/α-O-4/5-5), H = benzodioxane (β-O-4/α-O-5); the last column is the sum of all β-O-4 components, Σ(β-ethers) = A + D + H. *Note:* percentages are *not* traditional interunit linkage percentages since no account is taken of units that do not have specific contours in the side-chain region of their HSQC spectra (e.g., 5–5,4–O–5β-1 units). These percentages are uncorrected ratios of the volume integrals of contours arising from units A–D, H, and X and are therefore for comparative purposes only, not true quantification.

The levels of benzodioxanes in a lignin isolated from COMT-down-regulated alfalfa were higher than in other samples analyzed; however, partitioning of this unit into the soluble fraction was evident, implying that it was not necessarily higher in the whole lignin [63]. Spectral (HMBC) evidence elegantly reveals the presence of sequential benzodioxane units (Figure 5.20), indicating that 5-hydroxyconiferyl alcohol, along with the traditional monolignols, could β–O–4-couple to the 5-hydroxyguaiacyl groups in these lignins.

There is another sample in which benzodioxanes are evident, and interesting variant in the COMT-deficiency class. *Arabidopsis* transgenics with sense-upregulated F5H have previously been shown to have only a minor guaiacyl component [70,349], as noted above. Contours not immediately identified in the 2D NMR spectra of their lignins [70] later were shown to result from benzodioxane structures (Figure 5.18e) [66]. The observations here imply that, whereas syringyl production was enormously up-regulated in these transgenics, the methylation could apparently not keep pace with the accelerated production of 5-hydroxy units (e.g., 5-hydroxyconiferaldehyde and 5-hydroxyconiferyl alcohol). The results is a significant incorporation of 5-hydroxyconiferyl alcohol into the monolignol pool for the most heavily F5H-upregulated transgenics, ~10% as measured from contour volumes in the HMQC spectrum in Figure 5.18e and Table 5.3.

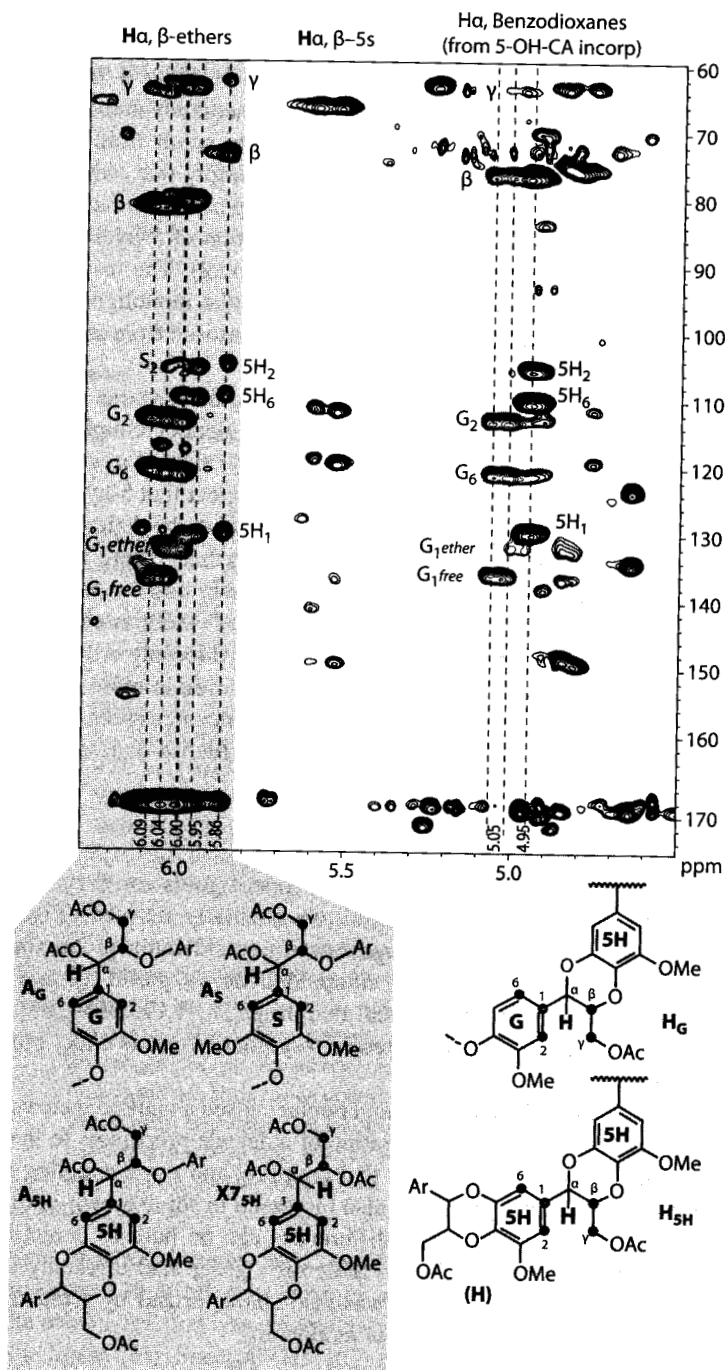
Evidence from degradative and NMR methods indicates that coniferyl and sinapyl alcohols, as well as further 5-hydroxyconiferyl alcohol, couple with the new terminus created by incorporation of 5 hydroxyconiferyl alcohol monomers into the growing polymer [63,66,336]. It has, therefore become clear that 5-hydroxyconiferyl alcohol can be used as a lignin monomer by plants to offset the deficiency in sinapyl alcohol monomers [107,236,359]. The recent statement that "There is, however, no known precedent for the free interchange of monomeric units in any biopolymer assembly, then or now, and no biochemical evidence..." [360] is clearly incorrect [107]. In fact, plants and other organisms have long used loosely ordered chemistry to enhance their viability and resistance [361]. Indeed, even in more highly ordered polysaccharide biosynthesis, monomer substitution can occur, as seen in the substitution of α -L-fucose by α -L-galactose xyloglucans in a fucose-deficient *Arabidopsis* mutant [362].

CAD-Deficiency

CAD is the last enzyme on the pathway to the monolignols coniferyl and sinapyl alcohols **2G** and **2S**, from which lignins are normally derived (Figure 5.21). When CAD is down-regulated, the hydroxycinnamaldehyde precursors to the monolignols, coniferaldehyde **1G** and sinapaldehyde **1S**, build up and may be incorporated into the polymer by radical coupling [38,71,196,363-368]. Indeed, coniferaldehyde was readily incorporated into synthetic lignins under biomimetic conditions [196].

Down-regulation of CAD in tobacco and poplar caused a marked increase in aldehyde components [71,363,369]; incorporation of aldehyde precursors to the monolignols into synthetic lignins established that coupling and cross-coupling reactions were possible [65,196]. Aldehydes were also increased in some brown-midrib maize and sorghum mutants [370], although differences can be hard to detect by NMR [365]. A pine CAD mutant identified in Sederhoff's group had markedly increased aldehyde levels [38]. This latter lignin was also characterized by a rather massive incorporation of dihydroconiferyl alcohol monomer.

CAD-Deficient Angiosperms The availability of a ^{13}C -enriched lignin from a CAD-deficient tobacco transgenic [71] and a series of model compounds [335] allows NMR methods to be used to ascertain hydroxycinnamaldehyde in vivo



cross-coupling propensities. Data revealing such details of plant lignification are rarely obtained.

Radical cross-coupling of monolignols **2** (Figure 5.21), with the growing lignin oligomer/polymer **3** is the major reaction occurring during lignification. Thus, the hydroxycinnamyl alcohol **2** (primarily at its β -position) couples with phenolic units **3** (at the 4-O- or 5-position for guaiacyl units **3G**, and almost exclusively at the 4-O-position for syringyl units **3S**) to form a chain-extended oligomer/polymer, also with the general formula **3**. Figure 5.21 (right) shows a selected region of a long-range ^{13}C - ^1H correlation (gradient-HMBC) spectrum from a CAD-deficient tobacco lignin, with peaks in the ^{13}C -projection and the resultant contours in the HMBC spectrum colored to match structures on the left for easy identification. In fact, the aldehyde structures in the lignins likely have been further incorporated into the polymer by primarily 4-O-coupling with the next monolignol, so they are not strictly the phenolic compounds **4** shown. Available coupling sites are indicated in Figure 5.21 by the dashed arrows.

Correlation of the aldehyde carbonyl carbons in cross-products **4** to the H7-protons 3-bonds away identifies the type of lignin units involved (Figure 5.21). Protons H7 resonate at ~ 7.3 ppm for hydroxycinnamaldehydes (either **1G** or **1S**) coupled 8-O-4 to guaiacyl units **3G** (compounds **4GG** and **4SG**, $\delta_{\text{C}} = 188.1$ ppm); whereas, they resonate considerably upfield, at ~ 6.7 ppm when coupled 8-O-4 to syringyl units **3S** (compounds **4GS** and **4SS**, $\delta_{\text{C}} = 186.8$ ppm). Correlations from these H7 protons into the ring identify the hydroxycinnamaldehyde involved in the coupling; 3-bond correlations identify equivalent S2/6 carbons derived from sinapaldehyde **1S** units at higher field than the nonequivalent G2 and G6 carbons from units derived from coniferaldehyde **1G**. Therefore, for 8-O-4 cross-coupled units **4** in lignins, the G/S nature of both the hydroxycinnamaldehyde component (coupled 8-) and the lignin unit (coupled 4-O-) are diagnostically revealed. Other aromatic protons in the complex lignin polymer resonate in the H7 regions; so, correlations that are not of interest here will result. An absence of correlations is, therefore, more diagnostic, revealing the absence of a component.

Of the four possible aldehyde 8-O-4 incorporation products **4** (Figure 5.21), only three can be detected in the antisense-CAD tobacco lignin by NMR methods. Product **4GG** is conspicuously absent. The data imply that sinapaldehyde **1S** cross-couples 8-O-4 with both guaiacyl and syringyl units (to form cross-coupled structures **4SG** and **4SS**), but that coniferaldehyde **1G** cross-couples 8-O-4 *only* with syringyl units **3S** and not with guaiacyl units **3G**. Thus cross-coupling product **4GS** is

FIGURE 5.20 (See color insert following page 336.) (Opposite) Partial 2D HMBC of lignin isolated from the COMT-deficient alfalfa transgenic (Marita, J.M., Ralph, J., Hatfield, R.D., Guo, D., Chen, F., and Dixon, R.A., *Phytochem.*, 62(1), 53-65, 2003), run on a 500 MHz cryoprobe instrument. The left columns reveal that β -ethers **A** are largely guaiacyl **AG** as expected from the low syringyl content, and as seen by the low level of syringyl β -ether **AS** component. 5-Hydroxyguaiacyl β -ethers **A5H** are clearly evident, as are new 5-hydroxyguaiacyl glycerol structures **X75H**. The right columns reveal which monolignols have coupled to the new 5-hydroxyguaiacyl units. In addition to the expected coniferyl alcohol addition, giving **HG** units, it is clear that "chains" of benzodioxane units may occur, as seen by the **H5H** units, which demonstrate the presence of two sequential benzodioxanes.

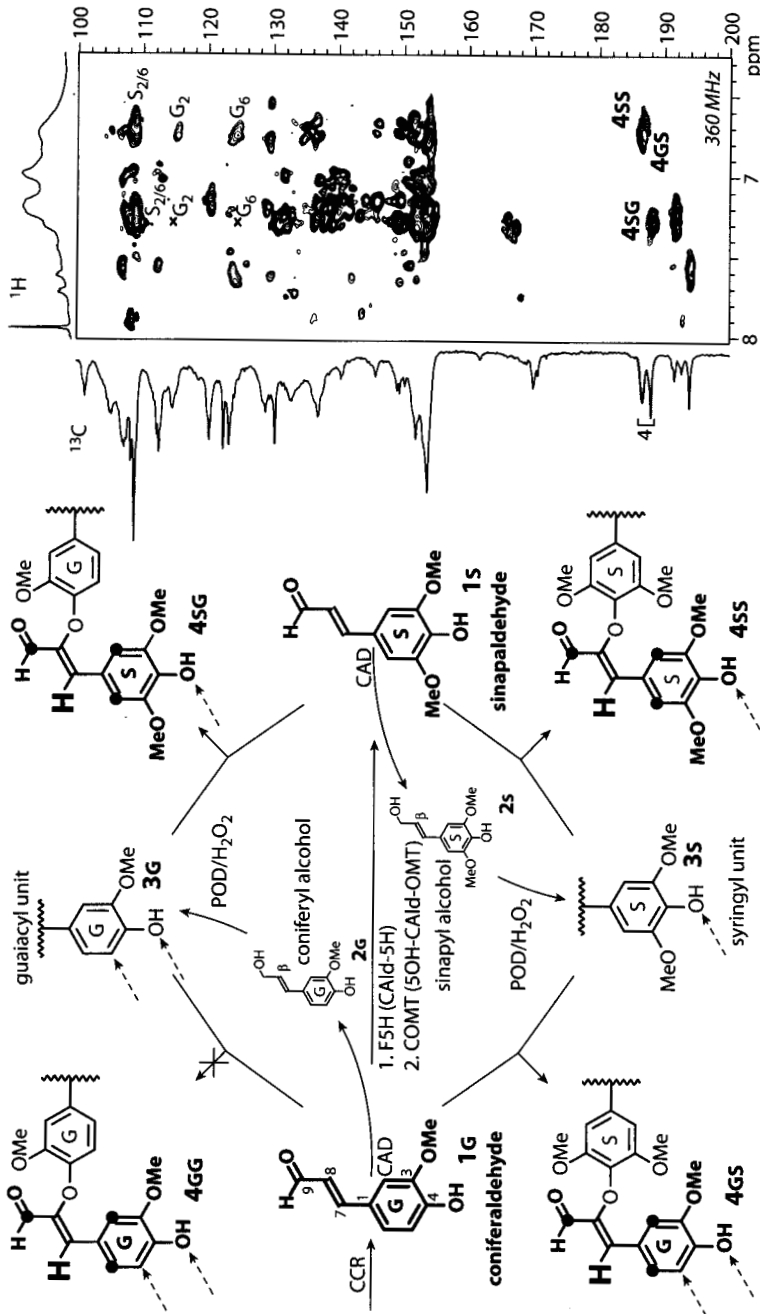


FIGURE 5.21 (See color insert following page 336.) (Opposite) Cross-coupling of hydroxycinnamaldehydes **1G** and **1s** with guaiacyl and syringyl lignin units **3G** and **3s** can potentially produce four 8-O-4-cross-coupled structures **4**. Only three of the four cross-products (**4Gs**, **4sS**, and **4ss** but not **4cc**) are significantly represented, as determined by analysis of the gradient-HMBC partial spectrum to the right. The HMBC section shows, in a lignin isolated from CAD-deficient tobacco stems, correlations from the aldehyde carbonyl carbons (185–195 ppm) to the H7 protons within 3 bonds in structures **4** that have been further incorporated into lignin at positions indicated by the dashed arrows. The H7 protons diagnostically correlate with the 2- and 6-carbons of the cinnamaldehyde ring; the G2 and G6 carbons for guaiacyl units, and the higher-field equivalent S2/6 (syringyl) carbons are identified and colored correspondingly. Colored dots on the structures indicate the carbons correlated by $^3J_{C-H}$ to the H7-protons (which are shown in a larger font size) shown in the HMBC spectrum. The aldehydes at ~188.1 ppm, with their corresponding H7s at ~7.3 ppm result from hydroxycinnamaldehydes **1** 8-O-4-coupled to guaiacyl units **3G** in lignin; correlations from H7 indicate, however, that sinapaldehyde **1s**, but not coniferaldehyde **1G**, couples with guaiacyl units **3G**—the green X's show where correlations are present in a model for **4GG**. The aldehydes at ~186 ppm, with their corresponding H7s at ~6.7 ppm result from hydroxycinnamaldehydes **1** 8-O-4-coupled to syringyl unit **3s** in lignin; correlations from H7 indicate that sinapaldehyde **1s** and coniferaldehyde **1G** each couple with syringyl units **3s**. S = syringyl, G = guaiacyl, CCR = cinnamoyl-CoA reductase, CAD = cinnamyl alcohol dehydrogenase, F5H = ferulate 5-hydroxylase, which has recently also been termed Cald5H = cpmoferadeide 5-hydroxylase, to reflect the preferred in vivo substrate (Osakabe, K., Tsao, C.C., Li, L., Popko J.L., Umezawa, T., Carraway, D.T., Smeltzer, R.H., Joshi C.P., and Chaing, V.L., *Proc. Natl. Acad. Sci.*, 96(16), 8955–8960, 1999; Humphreys J.M., Hemm, M.R., and Chappel C., *Proc. Natl. Acad. Sci.* 96 (18) 10045–10050, 1999), COMT caffeoyl *O*-methyl transferase (for which the preferred substrate appears to be 5-hydroxyconiferaldehyde (Li, L., Popko J.L., Umezawa, T., and Chiang, V.L., *J. Biol. Chem.*, 275(9), 6537–6545, 2000), POD = peroxidase. Convention for compound numbering: e.g., **4Gs** = a guaiacyl aldehyde (coniferaldehyde **1G**) coupled 8-O-4 to a syringyl lignin unit (**3s**). Compound numbers and coloring relate only to this figure.

readily detected, whereas **4GG** cannot be detected even when spectra are viewed at close to the baseplane noise level. This cross-coupling propensity is presumably the reason why 8–O–4-coupled structures are not found to be prominent in CAD-deficient softwood lignins [38].

These observations require no departure from the existing theory of radical coupling of phenols into polymeric lignins, since they appear to reflect simple chemical coupling propensities [236]. Attempts to prepare compounds modeling **4GG** by biomimetically cross-coupling coniferaldehyde **1G** (at the 8-position) with coniferyl alcohol **2G** or with a simple guaiacyl model 1-(4-hydroxy-3-methoxy)-ethanol (at the 4–O-position) also failed to produce 8–O–4-coupled cross-products. Apparently the same factors that promote cross-coupling in vitro apply in vivo, suggesting that these radical coupling reactions and those involved in “normal” lignification are likely to be under simple chemical control in the plant [236,294]. In the cross-products, the chemical shifts match those in model compounds synthesized by traditional means [335]. Also available from the HMBC spectrum in Figure 5.21 is some information regarding the incorporation of the hydroxycinnamaldehydes **1** and their derived hydroxybenzaldehydes at ring 4–O-positions, as described elsewhere [335]. The novel coniferaldehyde and sinapaldehyde 8–O–4-cross-coupling structures in lignins have been validated by thioacidolysis marker compounds diagnostically derived from them [371,372].

CAD-Deficient Pine: Incorporation of Dihydroconiferyl Alcohol and Guaiacylpropane-1,3-diol Preliminary NMR observations [38] on the novel lignins from a CAD-deficient pine (*Pinus taeda*) mutant were being strengthened as more diverse evidence accumulated [373,374]. The plant, despite a reduction in CAD levels to less than 1% of normal levels [375], appeared to be producing normal levels of lignin (Klason lignin values were almost identical). The wood was highly colored and stained strongly with phloroglucinol, suggesting a higher aldehyde component. In addition, NMR studies on isolated lignins indicated that aldehydes and DHCA were substantial components [2,38].

New aldehyde components were originally misidentified [38], but corrected in subsequent publications [2,71,135]. A book chapter on NMR details how the incorrect assignment was made [2]. The aldehyde components all stem from incorporation into the lignin of more significant quantities of coniferaldehyde (and its product vanillin) than in normal pine. The aldehyde levels seen in normal lignins could possibly result from postlignification oxidation, but the levels seen in this and other CAD-deficient plants suggest that incorporation of hydroxycinnamaldehydes (and hydroxybenzaldehydes) into lignins is a normal process that becomes more significant when the pathway to the final monolignols is down-regulated.

The CAD-deficient pine lignin had another feature that may or may not be attributable directly to the CAD-deficiency. Since this was a natural mutant, other unknown enzymes may have also been impacted by the mutation(s); however, this is unlikely because the genetic determinant of the modified wood colocalized with the coding sequence for the enzyme CAD [375]. Substantial levels of an unexpected unit derived from dihydroconiferyl alcohol (DHCA, Figure 5.12) were identified in the mutant's lignin [2,38,294]. Although found at lower levels in normal plant lignins [2,27,77,290], DHCA's derivation is not completely clear. Claims that the unit could

only arise as a modified metabolic product following dimerization of traditional lignin monomers [376,377] were incorrect; there is ample evidence that DHCA is formed as a monomer in the CAD-deficient pine and that the monomer is incorporated into the lignin via radical coupling processes [2,38,294,374]. Evidence from NMR indicates that about half of the DHCA units in the soluble lignin fractions were involved in 5–5-coupled structures [2,38]; coniferyl alcohol has never been shown to 5–5-dimerize. Dissolution of the (dioxane-water insoluble) residual lignin in acetyl or propionyl bromide led to acylated and α -brominated lignin and caused some other structural changes; but, other than acylation, dissolution did not affect the saturated arylpropanol side-chain. Experiments employing HMQC and COSY NMR then unambiguously determined that DHCA was a component of that residual lignin, although quantification was not possible [2].

More recently, GPDs were also identified in lignins isolated from the CAD-deficient pine mutant (Figure 5.12). This helped to unravel the nature of weak correlations often seen in pine lignin ^{13}C – ^1H correlation NMR spectra (Figure 5.12b); smaller amounts of GPDs are now recognized to be present in lignins from normal pines and presumably other softwoods. The structures in the complex lignin polymers are readily proven by NMR of isolated lignins.

Figure 5.12a shows a subplot of the side-chain region from a 2D HMQC-TOCSY NMR experiment on an acetylated lignin isolated from the mutant pine, highlighting the new (acetylated) guaiacylpropane-1,3-diols **X6**, along with the DHCA **X5** units. Data from a model compound, 1,3-diacetoxy-1-(4-*O*-benzyl-3-methoxyphenyl)-propane [135], are at the center of the yellow circles and obviously match well.

Invariably, traces of the novel components found in lignins from transgenic or mutant plants, can also be found in lignins from control plants. Figure 5.12b shows the α -C/H region of an HSQC spectrum of the mutant's lignin, where the β -aryl ether units (blue) and the strong new GPD unit **X6** (red) appear most cleanly. Figure 5.12c shows the same region in lignin from a normal pine control. Although the GPD peak is weak, it is diagnostic and, with other correlations evident (not shown), well authenticated.

The GPD monomeric units are derived from DHCA monomer **5** via the action of peroxidase and hydrogen peroxide (Figure 5.12d). The mechanism, via a vinylogous quinone methide, involves two H-radical abstractions. Abstraction from a benzylic CH_2 to produce quinone methides from phenoxy radicals has been noted previously [378]. When DHCA is subjected to peroxidase- H_2O_2 , we found monomeric GPD **2**, as well as the range of homo- and crossed-dimers involving DHCA and GPD.

Also evident in the HMQC-TOCSY spectrum (Figure 5.12a), are (acetylated) ketones **X6'** (magenta contours). Products of benzylic alcohol oxidation are seen in various isolated lignins, notably from syringyl(3,5-dimethoxy-4-hydroxy-phenyl) units; they may arise during lignin isolation (particularly in the ball-milling step). Ketones **X6'** provide additional confirmatory evidence for the GPD structures **X6** described above.

In summary, a previously unidentified unit present in small quantities in normal lignins was identified by NMR as a major component of the hydroxyphenylpropanoid polymeric component of a pine mutant deficient in CAD. The guaiacylpropane-1,3-diol units arise from conversion of dihydroconiferyl alcohol monomers by radical reactions and also incorporate into lignins as monomers. Like DHCA, however, GPD does not possess an unsaturated side-chain and is, thus, limited to coupling on the ring 4–0-, 5–,

and possibly 1-positions, and necessarily forming terminal units in lignin (except via 5-O-4-coupling). High MW polymers cannot be created without the incorporation of authentic hydroxycinnamyl alcohols (or other cinnamyl derivatives) as well. The CAD-deficient pine lignin is more soluble in alkali and has reduced molecular weight [373].

CCR-Deficiency

Tobacco plants down-regulated in CCR, the prior enzyme to CAD on the pathway, caused a reduction in lignin levels, but at the expense of structural integrity and plant vigor [379]. Such "lignins" incorporated significant amounts of tyramine ferulate, a known wound-response product in tobacco [71]. Hydroxycinnamoyl amide structures were established by NMR experiments, which provided preliminary evidence that the ferulate moiety incorporated into the polymer (similarly to its incorporation into lignins in grasses).

Criticisms [380] that the tyramine units are not part of the polymer, because they are sharp peaks (when the remaining peaks are broader), are invalid [236]. The lignins were MW-fractionated, but that aside, the comment belies a concept worth stating regarding NMR of various lignin polymers. Higher MW compounds or polymers do indeed yield generally broader peaks than smaller molecules. But mobile components on those polymers act far more like smaller molecules and appear sharper. This is the case for not only the tyramine units in these samples [71], but also for *p*-coumarates on grass lignins, and *p*-hydroxybenzoates on aspen, poplar, willow, and palm lignins (Naturally Acylated Lignins section). Such units are all free-phenolic (i.e., uncoupled) terminal appendages on the polymer that are free to rotate more rapidly than the bulk polymer. Rapid rotation in solution is associated with long relaxation times and sharp peaks. Also, in all these cases, the carbons and protons are relatively invariant, being rather remote from the nearest structural differences. The comment [380] that the tyramine peaks have to be from low-MW components of the sample can be dismissed when it is realized that the ferulate moieties to which they are attached would also then be sharp; in fact, it is hard to find them since they are radically coupled in the typical variety of ways in a complex set of structures embedded fully into the polymer [71]. Broad peaks come from the high MW, but more importantly from the many environments in which units find themselves (Broadness of Proton Spectra section).

A more general response, and particularly in plants that do not normally make tyramine ferulate (such as poplar and arabidopsis) appears to be the direct incorporation of ferulic acid itself into lignins. In sharp contrast with claims in another report [381], we readily detected thioacidolysis marker compounds for 4-O-etherified ferulic acid, as well as for a novel *bis*-(β -O-4-ether) that derives from ferulic acid radical coupling reactions, in natural grasses and in CCR-deficient plant materials; decarboxylation truncates the sidechain providing a pathway for a second β -O-4-coupling reaction [97,104]. Both types of structures are evidenced in NMR spectra [97,104].

Conclusions Regarding NMR Studies of

Lignin-Biosynthetic-Pathway Mutants and Transgenics

Perturbing lignification is an ideal way to increase or reduce components entering lignification. The value in structurally assessing the resultant lignins has been a tremendously enhanced understanding of lignins' structure and the lignification

process itself. With the variety of useful experiments available, NMR is uniquely suited to delineating often novel new structures in complex polymers. Also, NMR studies have shown that manipulating specific lignin-biosynthetic-pathway genes can produce profound alterations in plant lignins. Although the lignins in mutant and transgenic plants may appear to be strikingly different from normal lignins, they often represent merely broad compositional shifts; most of the novel units that have been found to date appear to be (often previously unidentified) minor units in lignins from "normal" plants. A salient observation is that the process of lignification appears to be flexible enough to readily incorporate phenolic phenylpropanoids other than the traditional monolignols. The incorporation of hydroxycinnamaldehyde monomers, as well as 5-hydroxyconiferyl alcohol, also implies that the plant is apparently sending these products of incomplete monolignol biosynthesis out to the cell wall for incorporation. Such data require no alterations in the current model for lignification, since a solely chemical polymerization process remains the best theory [107,236,338].

The resultant modified lignins in some transgenics evidently have properties sufficient to accommodate the water transport and mechanical strengthening roles of lignin and to allow the plant to be viable, if not vigorous. Whether such plants will be able to confront the rigors of a natural environment replete with a variety of pathogens remains to be determined. However, the plant's flexible approach toward lignification, i.e., polymerizing monolignol precursors and derivatives along with the traditional monolignols, is a testament to their flexible strategy; in a single generation, these plants have circumvented genetic obstacles to remain viable. The recognition that novel units can incorporate into lignin provides significantly expanded opportunities for engineering the composition and consequent properties of lignin for improved utilization of valuable plant resources in processes ranging from natural ruminant digestion to industrial chemical pulping.

Pulping Mechanisms, and Lignins from Pulp and Pulping Liquors

Anthraquinone Pulping

In the early '80s, ^{13}C NMR was just coming on stream to aid in the elucidation of the mechanism of action of a newly discovered novel organic catalyst for alkaline pulping, anthraquinone. Both ^{13}C and ^1H NMR of isolated adducts between lignin model quinone methides and anthrahydroquinone suggested that carbon-carbon bonding had taken place between the α -position of the quinone methide and C-10 of the anthracenyl system [382-384]. Interesting shielding effects in proton NMR spectra, and hindered rotation occurring on the NMR timescale suggested a 3D conformation of the adducts that was compellingly verified by a subsequent X-ray crystal structure [385]. Preparation of adducts between anthranol and isolated lignins confirmed that similar structures, and even the same isomers, could be produced in the polymer [386].

Soda Pulping

The application of ^{13}C NMR techniques to the characterization of soda lignin confirmed that formaldehyde was released from the γ -hydroxymethyl groups of C9 units during pulping via reverse aldol reactions with subsequent formation of vinyl ether structures in the lignin matrix [387]. Furthermore, in the same study, it was confirmed

by means of specific ^{13}C labeling that the released formaldehyde condensed with lignin fragments to form diarylmethylene structures, which was counterproductive to delignification.

Kraft Pulping

Comprehensive ^{13}C studies of underivatized kraft lignins in DMSO [388] and acetylated kraft lignins in acetone- d_6 [389], along with over 100 corresponding model compounds, provided a solid foundation for signal assignments. The NMR spectra of lignins isolated from kraft pulping liquors are much more complex than the corresponding spectra of MWLs because of all of the new structures formed, which are not originally present in the lignin. Main characteristics of lignins isolated during kraft pulping are a degraded side-chain, more unsaturation due to stilbenes and vinyl ethers, many new signals in the 25–50 ppm region due to fully reduced side-chains, and a much higher phenolic content.

Oxygen/Peracid Treatments

Researchers used ^{13}C NMR spectroscopy to examine products from MWLs from rice straw, Japanese beech (*Fagus crenata* Bl.) and Yezo spruce (*Picea jezoensis* Carr.) following oxygen pulping treatment [390] and from spruce MWL and a G-DHP following peracetic acid treatment [391].

Other Lignin Treatments

Spruce MWL was depolymerized with dry hydrogen iodide and then analyzed by ^{13}C NMR to reveal that ether bonds were cleaved and substitution of α -hydroxyl and α -alkoxy groups by iodine occurred [392]. Light-induced degradation of lignin was studied using spruce MWL impregnated on handsheets prepared from cotton linters. Results of NMR examination showed that α -ether, β -ether, and α - β -bond cleavage were the main reactions [393]. Lignins isolated from steam-exploded aspen [394], *Pinus radiata* wood [395], *Eucalyptus* [396], and beech [397] were studied by ^{13}C NMR spectroscopy. Extensive β -aryl ether cleavage, high phenolic content, and an extensively modified propanoid side-chain were common features in this type of lignin.

Lignin Side-Chain Proton/Carbon Assignment Table for 2D and 3D Spectra

In 2D and 3D NMR spectra, resonances are spread over a considerable volume; the contours are not sharp. However, the reliability of the assignments improve by utilizing at least two chemical shift parameters (e.g., the chemical shifts of a carbon and its attached proton) to define a contour as belonging to a given structural unit. Table 5.4 provides, for many of the units to be found in lignins, side-chain ^{13}C and ^1H chemical shifts that can be used to assign 2D and 3D spectra. All shifts are from acetylated lignins in the favored solvent, acetone- d_6 , unless noted.

QUANTITATIVE ^{13}C NMR OF LIGNINS

THE NUCLEAR OVERHAUSER EFFECT AND T_1 RELAXATION

Both conventional and somewhat unconventional techniques have been used for liquid-state ^{13}C NMR quantification of lignins. The two main problems that must be

TABLE 5.4

2D Correlation Chemical Shifts of Acetylated Lignins in Acetone- d_6

Structure	Type	H _α	H _β	H _{γ2}	H _{γ1}	C _α	C _β	C _γ
A	<i>Ge</i>	6.03	4.84	4.33	4.22	74.5	80.2	63.2
	<i>Gr</i>	6.07	4.82	4.23	4.01	75.3	80.4	63.3
	<i>Se</i>	6.03	4.73	4.39	4.15	75.4	81.4	63.4
	<i>St</i>	6.08	4.63	4.24	3.90	76.8	81.6	64.3
B	<i>G</i>	5.66	3.77		4.36	88.4	51.1	65.8
C	<i>G/S</i>	4.8	3.1	4.26	3.90	86.5	55.2	72.4
D	<i>G/S-GG</i>	4.94	4.30	4.45	4.07	85.1	83.3	64.0
	<i>G/S-GG^c</i>	4.81	4.11	-	-	84.0	82.5	-
H	<i>S/G</i>	4.98	4.39	4.28	4.02	76.8	75.9	63.4
S	<i>SA</i>	5.20	3.10		4.06	83.2	57.6	61.9
	<i>SB</i>	4.82	4.50	4.30	4.18	86.9	77.4	64.7
A2	<i>G</i>	5.61	4.89	4.56	4.45	80.9	81.5	63.5
A3	<i>S</i>	-	5.59		4.48	194.8	81.3	64.7
X1	<i>G</i>	6.63	6.23		4.65	134.2	122	65.3
X5a	<i>G</i>	2.62	1.90		4.04	32.1	30.9	64.0
X5b	<i>G</i>	2.73	1.89		4.11	32.2	30.9	64.1
X6	<i>G</i>	5.83	2.18		4.10	73.1	35.9	61.2
X6'	<i>G</i>	-	3.33		4.41	198.3	37.6	60.4
X7	<i>G</i>	5.91	~5.4		~4.1	~74	72.9	61.2
	<i>S^c</i>	5.88	5.37		-	73.5	72.2	61.5
	<i>P^c</i>	5.84	5.38		-	73.7	72.2	62.2
	<i>SH</i>	5.86					73.7	62.2
Benzaldehyde	<i>G/S</i>	9.86	-		-	191.6	-	-
Cinamaldehyde	<i>G/S</i>	7.60	6.70		9.68	153.1	129.8	194.0
Ald-b-G	<i>S</i>	7.31	-	-	9.57	136.1	149.8	188.3
Ald-b-S	<i>G</i>	6.71	-	9.32	126.7	152.1	187.0	
Ald-b-S	<i>S</i>	6.69	-	9.32	126.8	152.1	187.0	

Note: Data is presented to show how 2D correlation chemical shifts of acetylated lignins in acetone- d_6 (unless denoted with a superscript c) from various 2D spectra (author-generated from various in-house sources) can be used as a guide for assigning COSY, TOCSY, HMQC/HSQC, HMQC/HSQC-TOCSY, HMBC, etc. spectra. the author-generated data include components that may be easily seen only in various lignin-biosynthetic-pathway mutants and transgenics.

A = β-O-4, **B** = β-5, **C** = β-β, **D** = dibenzodioxocin, **A2** = α,β-di-ether, **A3** = α-keto-β-ether, **H** = benzodioxanes (α,β-ethers from 5-hydroxyguaiacyl units), **S** = spirodienones (β-1, note that shifts are given for the A- and B-ring moieties; see Figure 5.11). **X1** = cinnamyl alcohol, **X5** = dihydrocinnamyl alcohol (**X5a** = 5-β and 4-O-β-linked units, **X5b** = 5-5-linked units), **X6** = arylpropanediol, **X6'** = benzylic-oxidized arylpropanediol, **X7** = arylglycerol. See structures in Figures 5.3 and 5.12. **Ald-b-G/S** = the cinnamaldehyde 8-O-4G/S products, c.f. structures **4** in Figure 5.21. Data from sample in CDCl₃. Similar data tables for acetylated lignins in CDCl₃ have been provided (Kilpeläinen, I., Sipilä, J., Brunow, G., Lundquist, K., and Ede, R.M., *J. Agr. Food Chem.*, 42(12), 2790–2794, 1994; Ämmälähti, E., Brunow, G., Bardet, M., Robert, D., and Kilpeläinen, I., *J. Agr. Food Chem.*, 46(12), 5113–5117, 1998). Chemical shifts in italics are from model data (where data from actual lignins overlaps or is not clear).

dealt with when making the transition from qualitative to quantitative ^{13}C NMR are the nuclear Overhauser enhancement, or nOe and the difference in relaxation rates of carbon nuclei [16].

The nOe is observed as a change in intensity of the signal of one nucleus when another nearby nucleus is perturbed in some way. One of the most important manifestations of the nOe is the carbon signal enhancement that results upon irradiating the proton nuclei, such as is done during decoupling. Although the nOe can result in a threefold increase in S/N, it is a complication in quantitative NMR, because the enhancement is generally not equal for all types of carbon signals. Therefore, reliable quantification over the full spectral range of carbon signals required that the nOe be minimized or eliminated. This is accomplished by the classical inverse-gated experiment in which the proton decoupling is gated off during the interpulse delay. In conventional proton decoupled qualitative ^{13}C NMR experiments the nOe builds up during this delay. The major disadvantage of eliminating this irradiation and consequent nOe is the lower resultant sensitivity.

The second problem in quantification is the relatively slow relaxation rate of carbon nuclei. This problem is simply addressed by providing a relatively long delay between scans so the carbon nuclei are completely relaxed. The major relaxation process is the spin-lattice relaxation defined by the time constant T_1 and complete relaxation requires a delay of at least $5 \times T_1$ seconds [398]. Fortunately, because lignin is a large molecule and tumbles relatively slowly in solution, the carbon nuclei relax very rapidly. This is a disadvantage when attempting to resolve close peaks, or in some experiments (such as 2D long-range coupling, HMBc), when longer signal persistence is desired; however, rapid relaxation is an advantage in quantification. The T_1 relaxation times can be determined by use of the inversion recovery pulse sequence or by the progressive saturation method although the latter is more demanding and subject to systematic errors [165,398].

The T_1 ranges of selected carbon types in various lignins are listed in Table 5.5 [308]. The data in Table 5.5 provide sufficient information to estimate the delay times required for valid quantification for almost any lignin type. It should be readily

TABLE 5.5
Spin-Lattice Relaxation Times (T_1)

T_1 Ranges of Selected Carbon Types (seconds)			
Lignin sample ^a	Methoxyl	Ternary	Quaternary
Cottonwood MWL (CU)	0.79	0.11–0.18	1.1–2.8
Poplar SE lignin (PU)	0.67	0.18–0.21	1.6–2.1
Acet. Poplar SE lignin (PA)	0.76	0.21–0.34	1.3–2.5
Indulin (IU)	0.82	0.16–0.23	1.3–2.8
Organosolv lignin (RU)	0.87	0.23–0.26	1.3–3.5

Source: Landucci, L.L., *Holzforschung*, 45(6), 425–432, 1991.

^a Standard samples supplied for Round-Robin Lignin Analysis at IEA Symposium, New Orleans, 1991.

TABLE 5.6
Effect of Temperature on Spin-Lattice Relaxation Times (T_1)
of Selected Signals from Aspen MWL in DMSO- d_6

Signal (ppm)	T_1 (seconds)		
	30°C	50°C	70°C
169.5	1.52	2.19	2.23
169.2	1.35	2.30	2.88
131.3	—	0.21	0.23
106.7	0.09	0.13	0.15
104.3	0.14	0.15	0.15
63.2	0.06	0.08	0.09
55.9	0.48	0.76	0.74
20.9	0.55	0.76	0.79

Source: Landucci, L.L., *Holzforschung*, 45(6), 425–432, 1991.

apparent that slow relaxation of the quaternary carbons is what limits the scan repetition rate of the inverse-gated experiment. The minimum delay to be used for these lignins is based on the quaternary carbon relaxation at ambient temperature when a 90° tim angle is used. For example, the 5T1 delay for poplar steam-exploded lignin should be $5 \times 2.1 = \sim 11$ s, whereas with the organosolv lignin it should be $5 \times 3.5 = \sim 18$ s. With the increasing popularity of the use of DMSO as a lignin solvent, it is a common practice to heat NMR samples to 50° or more during data acquisition. This is acceptable for qualitative NMR, because sharper signals and greater resolution are obtained, especially if the sample solution is relatively concentrated. However, sharper signals translate to slower relaxation, so it is disadvantageous to heat samples during quantification. On the contrary, is more signal is desired over a given period of time, cooling the sample would be advantageous, because the more viscous the sample solution is, the faster the relaxation will be.

The effect of temperature on relaxation rate of selected signals from aspen MWL in DMSO has been reported and is shown in Table 5.6 [399]. As indicated from the T_1 values, heating the sample from 30° to 70° doubles the required relaxation delay, if valid quantification is desired over the entire spectral range. This viscosity effect is much more noticeable in DMSO than in acetone or chloroform.

Finally, even when all these parameters are taken into account, spectral baselines are never flat. The situation has improved considerably as instruments have gone fully digital and taken advantage of efficient digital filtering, but setting the baseline remains a significant source of error.

PRECISION AND ACCURACY

The concepts of precision and accuracy are sometimes misunderstood as they relate to lignin analysis. The most important of these concepts is precision, as this is a prerequisite to accuracy. Simply defined, precision is the ability to reproduce a result. If

this cannot be done, then accuracy is simply unobtainable. In the lignin literature, replications of identical experiments are seldom reported due to the excessive instrument time required for such studies. However, it has been shown that with large amounts (>500 mg) of lignin and multiple replications on older (predigital) instruments, precisions of $\pm 1\%$ are possible [400]. More realistic values with smaller amounts of lignin (100–500mg) are generally in the range off 10 to f 25%. Only after the desired precision is obtained can the accuracy be improved. Accuracy generally depends on a set of assumptions, because, unfortunately, true values in lignin analysis are unknown. A comprehensive examination of the effects of various operations during NMR quantification on precision has been reported [400]. No systematic study using digital instrumentation, nor the recently available cryogenically cooled probes (in which both ^1H and ^{13}C coils are now cooled to reduce electronic noise), has been reported.

APPROACHES TO QUANTIFICATION

Signal Cluster Analysis

Shown in Figure 5.22 is a quantitative spectrum of a willow (*Salix*) MWL with various spectral regions highlighted [309]. These regions generally correspond to integral limits that are predefined and remain constant throughout a particular study. For high-precision work, this step is essential. Information obtained by measuring these signal clusters results in values of useful ratios, as listed in Table 5.7. The accuracy of these ratios generally depends upon certain assumptions. For example, the OMe/Aryl ratio assumes no unsaturation in the side-chain of C9 units. This ratio has been determined for a large number of MWL types by use of the inverse-gated experiment [306]. If unsaturation is present and the signal(s) position is known, a correction factor can be determined, as was reported in the quantitative analysis of a guaiacyl DHP [171]. In the case of the aliphatic carbon/C6 aryl ratio of lignin carbons, an additional assumption of a carbohydrate-free sample is made. With acetylated samples, the ratio of primary to secondary to phenolic hydroxyl groups can easily be determined with relatively high precision by quantifying the signals from the acetate carboxyl groups [185,401]. A

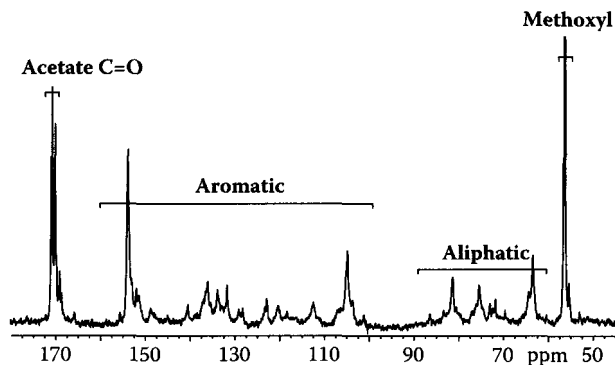


FIGURE 5.22 Quantitative ^{13}C NMR spectrum of a willow lignin with highlighted spectral regions (Landucci, L.L., *Holzforschung*, 39(6), 355–359, 1985).

TABLE 5.7**Useful Ratios for lignin Characterization^a**

Ratio	Assumptions
Methoxyl/Aryl(C6)	No unsaturation in side-chain
Aliphatic/Aromatic(C6)	No unsaturation in side-chain No carbohydrate impurity
Pri/Sec/Phenolic OH (acetylated samples)	No carbohydrate impurity
Ternary/Quaternary (aromatic region)	None, but a spectral editing experiment such as DEPT is needed to determine CH/C boundary.

Values for Selected Round-Robin Samples

Lignin sample ^b	Methoxy/Aryl(C6)	Alip/Aryl(C6)	Tern/Quat
Cottonwood MWL	1.4	2.7	2.4/3.6
Poplar SE lignin	1.2	1.2	2.1/3.9
Acet. Poplar SE lignin	1.2	1.3	2.1/3.9
Indulin	0.7	0.8	2.8/3.2
Organosolv lignin	1.0	0.7	2.4/3.6

Source: Landucci, L.L., *IEA Presymposium on Analysis of Wood, Annual Plant Lignins*, New Orleans, LA., 1991.

^a Area of aryl region (95–160ppm) is set to 6.0 units.

^b Standard samples supplied for Round-Robin Lignin Analysis at IEA Symposium, New Orleans, 1991.

modification of this type of quantification using ¹³C-labeled acetyl chloride for derivatization was reported [303]. Determination of the hydroxyl distribution in lignin also requires carbohydrate-free samples, since secondary hydroxyls from carbohydrates contribute to the central signal of the primary, secondary, and phenolic trio. However, it is possible to quantify carbohydrates, such as xylans, to some extent from the anomeric carbon (C1) that appears at about 100ppm. Then a correction can be made [309].

There are two common approaches to quantification, each of which has advantages and disadvantages. The first approach is to set the aromatic + aliphatic regions (minus the methoxyl group) to nine carbons. This approach is unaffected by unsaturation in the side-chain. Disadvantages are that it is not valid for highly degraded lignins such as kraft lignin and not valid if carbohydrates are present. The second approach is based on the assumption that there are six aromatic carbons per C9 unit and that the signals all appear in the region between 100 and 160ppm. Advantages of this approach are that it can be applied to a lignin material that is suspected of having considerable side-chain degradation—that is, one in which a C9 unit is unrealistic. Also, this approach is not seriously affected by the presence of carbohydrates. A disadvantage is that it is not valid if there is extensive aliphatic unsaturation in the side-chain such as that from vinyl ethers and stilbenes. Therefore, this approach would not be a good one for a kraft lignin.

Application of both of these approaches to a pine MWL is illustrated in Table 5.8 with some selected values [402]. Clearly, there is no significant difference in the two

TABLE 5.8
¹³C NMR Derived Values for Pine Wood Ac-MWL

Functional Unit	Approach 1		Approach 2	
	(content/monomeric unit)		unit)	
Methoxyl	0.93		0.93	
Acetoxy Me	1.86		1.86	
Acetoxy C=O				
primary	0.85		0.86	
secondary	0.61		0.61	
phenolic	0.35		0.35	
Aromatic	5.97		6.00	
Aliphatic				
60–100 ppm	2.78		2.79	
35–55 ppm	0.24		0.24	
Total	3.02		3.03	

Source: Netzel, D.A., *Anal. Chem.*, 59, 1775–1779, 1987.

TABLE 5.9
¹³C NMR Derived Values for Fossil Wood Ac-MWLs

MWL => Functional Unit	GDH-80		Resolute		Grattan	
	Approach		Approach		Approach	
	1	2	1	2	1	2
Methoxyl	0.88	0.73	0.91	0.80	0.73	0.71
Acetoxy Me	1.52	1.27	0.96	0.85	1.37	1.20
Acetoxy C=O						
primary	0.52	0.43	0.46	0.41	0.51	0.45
secondary	0.35	0.29	0.12	0.10	0.26	0.23
phenolic	0.58	0.48	0.38	0.34	0.55	0.48
Aromatic	7.17	6.00	6.77	6.00	6.85	6.00
Aliphatic						
60–100 ppm	1.50	1.25	1.84	1.62	1.63	1.43
35–55 ppm	0.33	0.28	0.41	0.37	0.49	0.43
Total	1.83	1.53	2.25	1.86	2.12	1.86

Source: Netzel, D.A., *Anal. Chem.*, 59, 1775–1779, 1987.

columns because, for a relatively intact lignin with a low carbohydrate content, both approaches are valid. A contrasting situation is illustrated in Table 5.9 where the same two approaches are applied to three different MWLs that were isolated from wood that was found in tertiary fossil forests in the Canadian Arctic [402]. These lignins are thought to be between 25 and 65 million years old. Visually comparing these MWLs to

the recent pine MWL on the top indicates extensive side-chain degradation and more aromatic character than the pine lignin. Also, it is interesting that the Resolute lignin was almost completely lacking in secondary acetate carbonyls and, with the other two lignins, this functionality was less predominant than in the recent pine MWL. The values generated from the two approaches are listed in Table 5.9. Clearly, they are very different. It is not really clear which approach is better with these unusual samples. If the degradation mechanisms involved appreciable condensation and aromatization then approach 1, which assumes a C9 entity might not be a bad assumption, because it would simply mean a shift of the aliphatic carbons into the aromatic pool.

Use of Internal Standards

Theoretically, the best approach to quantification would be the use of an internal standard because no assumptions concerning lignin structure need be made. However, this is usually not practical for lignin analysis because standards, which are generally simple and low molecular weight substances, have long relaxation rates. Therefore, the $5T_1$ requirement for the pulse delay would be unrealistically long. However, for specific applications in which only protonated carbons in the internal standard are utilized, the reliable “spiking” method can be useful. As an example, a vinyl ether model compound was added and used to quantify such structures in a lignin isolated from a soda cook [404]. Using this type of quantitative experiment, with conditions based on the protonated carbons of the model compound, the researchers were able to generate a fairly accurate value of the content of this functionality in the lignin. In this case, the long relaxation rate of quaternary carbons in the low MW compound was not a problem because the quaternary carbon signals were not needed for the quantification. Comparison of one protonated carbon in the model with the corresponding protonated carbon in the polymer required a delay of 5–10s, sufficient for complete relaxation of the β -carbon in the model.

Quantification by Curve Fitting

Comparison of an experimental inverse-gated spectrum with a simulated spectrum (of the signals of interest) generated by a curve fitting program can provide more precise quantification. Advantages of this method are that there is no noise in the simulated spectrum and individual signals, rather than signal clusters, can be quantified. In fact, this is the only practical method to quantify individual signals in crowded regions of a spectrum, since signal overlap is generally not a problem. An example of this technique, schematically illustrated in Figure 5.23, is the quantification of the *p*-acetoxybenzoate group in a willow MWL. Based on the OMe/aryl ratio, the abundance of this entity was determined to be 0.04–0.06/C9 units in a series of six similar willow MWLs [309]. With this method it is desirable to simulate at least two or three signals of the entity of interest, so as to provide an internal check of the method. In this case, the 1/1/2 ratio of the C = O/C4/C2,6 provided such a check. This application involved the relatively discrete and sharp resonances from a substituent on lignin side-chains. Unfortunately, the backbone lignin units themselves (e.g., β -5, β -O-4) comprise differently populated substructures. The signals from a particular lignin structure can, therefore, not be expected to be symmetric. Curve fitting methods must therefore be applied with caution.

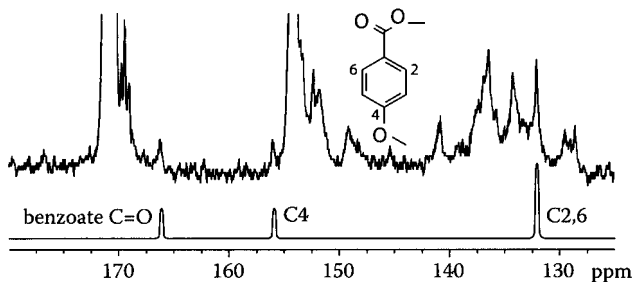


FIGURE 5.23 Quantification of *p*-acetoxybenzoate groups in acetylated willow ML (Landucci, L. L., Deka, G. C., and Roy, D. N., *Holzforschung*, 46(6), 505–511, 1992).

SEMIQUANTITATIVE METHODS

Comparison of the Same Signal(s) from Two or More Spectra

One technique is simply to compare signal intensities or areas of a particular signal or signals among different spectra. With this technique the same sample concentration, and acquisition and processing parameters must be maintained from sample to sample. The primary advantage is that the time-consuming inverse-gated technique is not necessary as identical carbon nuclei are being compared. Also, the signal enhancement feature of the nOe can be utilized. This technique is most frequently used when following the fate of a particular entity during some reaction or treatment of a given lignin.

Comparison of Signals from Protonated Carbons of Similar Type

Another technique that does not require an inverse-gated spectrum is illustrated in Figure 5.24 with the measurement of relative signal areas of the protonated carbons in the side-chain region of a guaiacylDHP [215]. All of the signals were assigned by comparison with authentic dimeric and trimeric lignin models. This spectrum indicates that this DHP is relatively simple compared to a guaiacyl lignin and contains only the four major unit types shown here. The accuracy of this method depends upon the similarity of the relaxation behavior of these protonated carbons. This technique has also been applied to the determination of syringyl/guaiacyl ratio in poplar lignin [405].

Spectral Editing Experiments

The DEPT experiment detects only protonated carbons and depends upon polarization transfer or energy transfer from protons to adjacent carbons [17]. The efficiency of this transfer process depends largely upon a delay in the pulse sequence that is related to the proton-carbon coupling constant, but is relatively insensitive to coupling constant variation [406,407]. Therefore, the process can only be perfect, or quantitative, for one coupling constant. Since coupling constants for the various proton/carbon pairs can vary widely in lignin, as in most other materials, an average coupling constant must be used. For best results, it has been demonstrated by investigators in coal research that relatively long relaxation delays must be used [403]. Therefore, much of the advantage of the DEPT sequence disappears. However, in

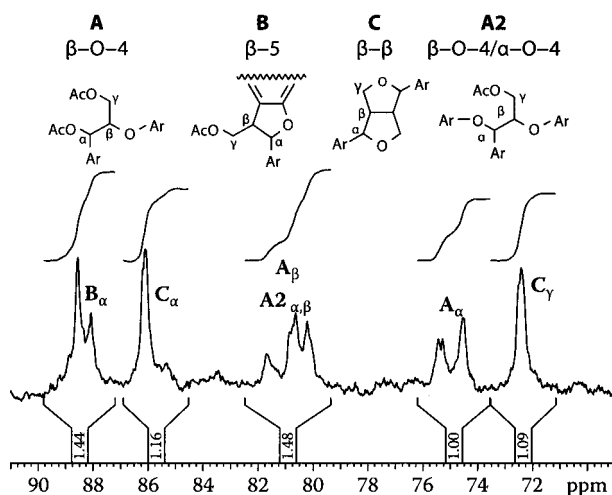


FIGURE 5.24 Integration of signals in the side-chain region of a guaiacyl DHP. Peak assignment follows that established in Figure 5.3. (Landucci, L.L., *Holzforschung*, 39(6), 355-359, 1985).

spite of all of these limitations, it has been shown to be a viable method for approximating the ternary/quaternary ratio in kraft lignins, which are notoriously difficult materials because of their appreciable unsaturated side-chain content in the aromatic region and extensive loss of carbon from the side-chain [408].

CONCLUSION

In summary, true quantification in lignins is difficult due to signal overlap and other factors. The best quantification method remains the relatively tedious inverse-gated technique, along with an internal standard substance. There are other options, but all are imperfect to some degree. With substances like lignins there are few guidelines and there are no absolutes; so, any value obtained from a nonquantitative technique must be compared to a corresponding value obtained from an inverse-gated experiment to determine its quantitative validity. Fortunately, most of the semiquantitative methods that have been described are adequate when the researcher wants to follow changes in structure over time during a particular treatment provided that the desired precision is maintained.

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