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Cryoprobe 3D NMR of acetylated ball-milled pine cell walls

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Received 17th August 2004, Accepted 2nd September 2004
First published as an Advance Article on the web 13th September 2004

3D NMR of solubilized ball-milled pine cell walls reveals striking details of lignin units, right down to differentiating stereoisomers in the polymer. Such 3D spectral editing therefore overcomes the need to isolate wall components to study their structures.

A major impediment to structural elucidation in the complex lignin biopolymer has been the requirement to first isolate the lignin, in relatively pure form, before NMR studies. The problem is that lignin is a complex hydroxyphenylpropanoid polymer[1–3] that is an integral part of the plant cell wall, polymerizing in after wall polysaccharides have been laid down. Its extraction is non-trivial. Björkman devised a solvent system to fairly selectively extract lignins from finely divided (ball-milled) wood,[4] and further purification steps are possible.[5,6] NMR of such lignin isolates, particularly with modern 2D and 3D methods augmenting traditional 1D approaches, provides outstanding levels of detail that allows substantive structural insights into the complex structures.[7] The methods have been enormously valuable in delineating pathways in lignin biosynthesis and, more recently, in understanding structural changes that occur when lignin biosynthetic pathway genes are up- and down-regulated in transgenic plants.[8] However, a recurring problem is that isolated lignins represent only a fraction, typically 15% in softwoods, rarely over 50% in hardwoods, and sometimes below 10% in valuable model plants such as tobacco and arabidopsis. Questions of structural partitioning and of true representation therefore arise.

We have recently revealed a method to overcome some of the limitations associated with component isolation.[9] The method involves dissolving ball-milled wood (and plant cell walls in general) in one of two solvent systems—DMSO–*N*-methylimidazole or DMSO–tetrabutylammonium fluoride. DMSO–*N*-methylimidazole is favored for NMR studies on lignins since it allows trivial *in situ* acetylation. Following acetylation, essentially the entire cell wall component becomes soluble in $CDCl_3$ for NMR.[9] High-resolution studies require an EDTA-washing step to remove metal contaminants that degrade the spectral quality and may reduce relaxation times to the point that complex experiments become ineffective. This step loses typically 10% of the total sample; we have not yet determined which components (other than the metals) may be lost. The result, nevertheless, is that a large fraction of the cell wall, and the lignin component of interest, can now be subjected to the usual array of structurally revealing NMR experiments. The problem of course is that the solution now contains overwhelming amounts of components that may not be of interest (for example, the polysaccharides, when it is lignin that is being studied). Also the components of interest are present in reduced total amounts, since it is only possible to dissolve about 60–100 mg of cell wall *vs.* 60–100 mg of pure lignin in the 0.4–0.5 ml volume required in a 5 mm NMR tube. If the sample is only 20% lignin, then there is at least 5-fold less lignin in the tube. Newly commercialized cryoprobe technology overcomes much of the latter limitation, delivering about a 5-fold sensitivity enhancement. The dispersing power of 2D NMR methods are particularly useful for revealing at least some correlations from the components of interest in the congested spectra.[9]

Here we reveal that 3D NMR on the whole cell wall fraction provides strikingly valuable editing, allowing details as fine as lignin unit stereochemistry to be "isolated" from the cell wall mélange. Such methods therefore open the door to detailed NMR analysis of cell walls without the problems associated with the laborious component isolation.

Fig. 1 demonstrates the extraordinary detail revealed in strikingly clean slices from a 3D TOCSY-HSQC experiment.† The first F_2–F_3 plane (Fig. 1a) is essentially a 2D HSQC ^{13}C–^{1}H spectrum. Amongst the overwhelming polysaccharide correlations, a relatively intense lignin methoxyl is seen (brown) along with three nicely resolved correlations from the two most prominent unit types in softwood lignins, the β-aryl ether units (cyan) and the phenylcoumaran or β-5-units (green). Other correlations of interest are in regions that are too congested. However, often at least one (^{1}H or ^{13}C) resonance of a unit is separated from others, allowing 2D planes through that resonance to isolate out that unit. And the nature of the TOCSY-HSQC experiment is such that the detail from the entire sidechain proton or carbon coupling network becomes associated with that plane. Thus, Fig. 1b shows a 2D F_2–F_3 (HSQC) slice at 6.01 ppm in the proton spectrum. Both *syn*- and *anti*- (or *threo*- and *erythro*-) isomers of prominent β-ether units have α-proton resonances at this frequency. Consequently, the 2D slice is a beautifully clean HSQC spectrum of these two components. The *syn*-isomer is distinguished particularly by its lower chemical shift γ-proton pair; obviously such details are lost in the 2D spectrum. Moving slightly down to 6.095 ppm provides a slice that is almost purely of a *syn*-isomer, Fig. 1c. Note that this is clearly not exactly the same unit as in the slice in Fig. 1b—the proton chemical shifts are different. Screening database shifts[11] indicates that the *syn*-β-ether in Fig. 1b is etherified, whereas the one in Fig. 1c is unetherified (*i.e.* free-phenolic in the original cell wall preparation and the phenol is acetylated in this acetylated sample). This level of detail is going to require far more extensive and efficient databasing of model compounds and lignins to allow detailed assignments, an enormous process that is beginning now. The slice at 5.50 ppm (through the phenylcoumaran α-proton, Fig. 1d) is not quite as clean but clearly reveals the phenylcoumaran sidechain correlations.

In the F_1–F_2 plane, slices at a given proton chemical shift now provide an HSQC-TOCSY ^{1}H–^{13}C spectrum correlating the attached carbon with all of the protons in that sidechain coupling network. Thus at a β-ether α-proton shift of 6.07 ppm, Fig. 1e, the α-carbon (at 74.2 ppm) correlates with the α-, β-, and

† The 3D spectrum in Fig. 1 was acquired on a 500 MHz Bruker DMX-500 equipped with an inverse gradient $^{1}H/^{13}C/^{15}N$ cryoprobe using a 3D HSQC-TOCSY experiment utilized previously.[7,10] Acquisition conditions were: 1k × 128 × 64 datapoints taken over sweep widths of 3.6 (^{1}H, F_3), 60 (^{13}C, F_2), and 3.6 (^{1}H, F_1) ppm, total experiment time 41 h, Bruker pulse program mleviietf3gs3d. Processing used matched Gaussian (LB = −1, GB = 0.005) apodization in F_3 and squared sine-bell in F_2 and F_1, and processed with 1k × 256 × 128 datapoints with forward linear prediction in F_2 and F_1.

DOI: 10.1039/b412633e

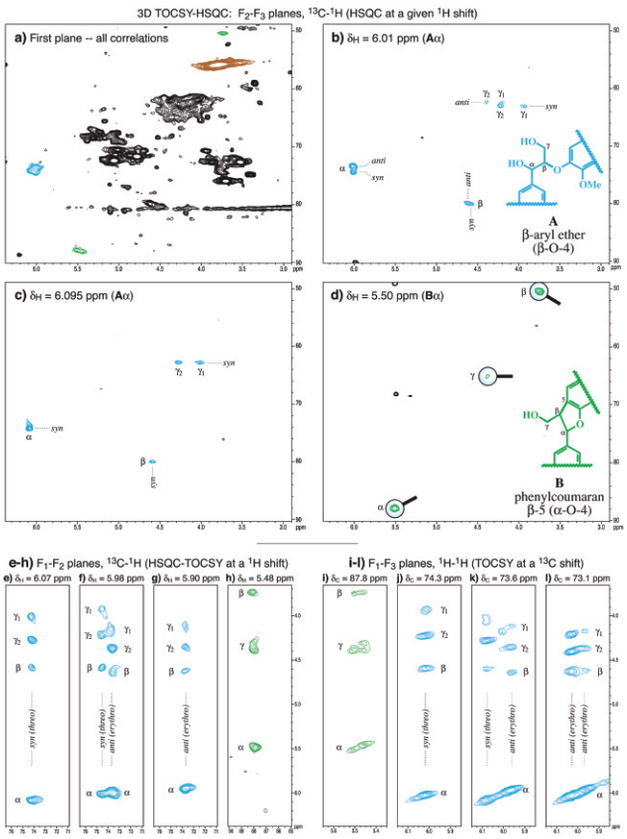

Fig. 1 Slices from a 3D NMR spectrum of acetylated pine ball-milled cell walls, illustrating the spectacular lignin substructural editing possible in this complex sample. The 3D spectrum was acquired with the acquisition restricted to the lignin sidechain region. a–d) F_2–F_3 (HSQC) slices at a given ^{1}H shift; e–h) F_1–F_2 (HSQC-TOCSY) slices at a given ^{1}H 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 sidechain labeling.

γ-protons in that unit's sidechain; this unit appears to be a *syn*-isomer and is likely unetherified. At 5.98 ppm, Fig. 1f, is evident a mixture of a different *syn*-isomer (etherified) and an *anti*-isomer (likely unetherified), resolved by their different α-carbons at 74.6 and 73.7 ppm. At 5.90 ppm, Fig. 1g, an essentially pure *anti*-isomer (unetherified) is isolated. The plane at 5.48 ppm, Fig. 1h, reveals two slightly different phenylcoumaran units, one likely etherified and the other unetherified, with similar α-carbon chemical shifts (~88 ppm).

The F_1–F_3 planes, Figs. 1i–l, have a similar appearance but provide quite different information and illustrate another way of extracting data and "purifying" spectra out of the 3D. These are actually ^{1}H–^{1}H TOCSY spectra on planes at a given carbon shift. The plane through a phenylcoumaran α-carbon at 87.9 ppm again shows two types of phenylcoumaran sidechains differing in their proton shifts, and being resolved by their differing α-proton shifts (~5.51 and 5.46 ppm, suggesting unetherified *vs* etherified units, respectively). Slices through various α-carbons in the β-ether units again nicely resolve various *syn*- and *anti*-isomers.

In looking at the beautiful spectra in these slices, it is easy to forget that they came from the entire cell wall fraction. The striking detail now demands a significantly improved method to unequivocally assign these resonances. Revealing more minor components in the same manner appears to be beyond this currently acquired spectrum, but the incredible detail and the efficient isolation of individual units of interest by the 3D method suggests that laborious component isolation may not be needed to reveal significant details of lignin structure. As such, solubilization followed by NMR should allow considerably more rapid profiling of structural details in plants, including the novel lignins in new mutants and transgenics, than is possible when component isolation is required. The NMR method is far from a screening approach, but when structural detail is required to understand biochemical pathways, it has no rival.

Acknowledgements

We gratefully acknowledge partial funding through the DOE Energy Biosciences program (#DE-AI02-00ER15067). NMR experiments on the Bruker DMX-500 cryoprobe system were carried out at the National Magnetic Resonance Facility at Madison with support from the NIH Biomedical Technology Program (RR02301) and additional equipment funding from the University of Wisconsin, NSF Academic Infrastructure Program (BIR-9214394), NIH Shared Instrumentation Program (RR02781, RR08438), NSF Biological Instrumentation Program (DMB-8415048), and the U.S. Department of Agriculture.

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