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LABELLING OF A LIGNIN FROM SUSPENSION CULTURES OF *PICEA ABIES*

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Key Word Index—*Picea abies*; Pinaceae; Norway spruce; cell cultures; lignin; ^{13}C-enrichment; 3D NMR spectra.

Abstract—The lignin released by suspension cultures of *Picea abies* into the culture medium was labelled with ^{13}C by adding uniformly-labelled glucose to the culture. The successful labelling (the lignin contained *ca* 20% ^{13}C) greatly facilitates the acquisition of detailed and reliable structural information using NMR and makes it possible to carry out three dimensional HMQC-HOHAHA experiments. These spectral data were compared with similar data from lignin that had been isolated from cell walls of intact pine (*Pinus sylvestris*) and from a polymer that had been prepared *in vitro* by oxidation of labelled coniferyl alcohol. Differences in chemical structure have been found that are of importance for the elucidation of the chemical processes involved in the deposition of lignin in the walls of lignifying tissues.

INTRODUCTION

Cell suspension cultures of *picea abies* have been found under certain conditions to release polymeric material into the culture medium [1, 2]. This material, that has been identified as almost pure lignin (RSCL), can yield useful information on the mechanism of lignin assembly and on the interaction of the lignifying system with the wall matrix. We have found [1] that the lignin is a highly branched polymer with a large proportion of C-C bonds. This results in low yields of thioacidolysis products and makes it necessary to look for other methods of elucidating the side-chain structure of this lignin. High field NMR is a powerful method for the study of the chemical structure of such biopolymers. When using ^{13}C NMR spectroscopy, the attainment of sufficient signal-to-noise ratios is hampered by the low natural abundance of ^{13}C . ^{13}C-enrichment greatly facilitates the acquisition of signals and makes it possible to obtain reliable assignments to chemical structures by using combinations of ^{1}H and ^{13}C NMR spectra. Previously, specific ^{13}C-labelling has been used for establishing certain types of chemical bonding in lignin [3]. In the present paper, we describe a labelling experiment where *P. abies* suspension cultures were uniformly labelled by adding ^{13}C-labelled glucose to the culture medium. The uniform labelling makes it possible to use 3D NMR spectroscopic methods and, thus, to dramatically improve the reliability of the assignments of the chemical substructures in the lignins. The use of 3D HMQC-HOHAHA allows indisputable assignments of side-chain connectivities in the lignin side-chains. We have compared the data with similar data from a dehydrogenation polymer from β and γ ^{13}C-labelled coniferyl alcohol and with 2D data from an unlabelled milled wood lignin (MWL) from pine. We can, among other things, show that the *cis*-isomer of the recently discovered dibenzodioxocin structure is visible in the RSCL preparations but not in MWL. ^{13}C-labelling and 3D NMR spectroscopy can yield important information regarding the chemical processes involved in the lignification of cell walls in vascular plants.

RESULTS AND DISCUSSION

The main advantage of using NMR for the elucidation of the chemical substructure in lignin preparations lies in the fact that it is a non-destructive method and allows the inspection of the whole structure in one experiment. The structural assignments obtained in NMR analysis of lignins rely largely on data obtained from synthetic model compounds i.e. low M_r compounds containing structural features expected to occur in lignin. We have recently published such assignments, together with the relevant

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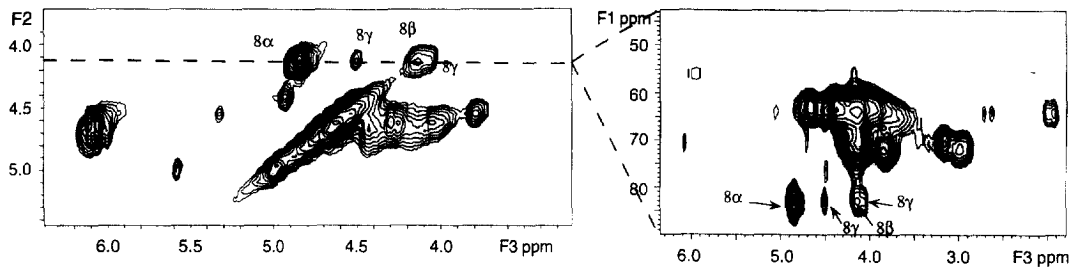


Fig. 1. Two dimensional slices of a 3D HMQC-HOHAHA spectrum of ^{13}C-enriched RSCL. The correlations for structure **8** (*trans*-dibenzodioxocin) are marked: a) Expansion of an F2F3 slice at carbon chemical shift 82 ppm, b) Expansion of an F1F3 slice at 4.10 ppm.

model compound data for several lignin preparations [4]. Two-dimensional experiments, such as proton-carbon correlation using inverse detection (HMQC) and proton-proton correlations (TOCSY) increase the reliability of structural assignments, especially in cases with a large number of overlapping signals, as in lignin preparations. Even these methods may become inadequate when looking for previously unknown structures and in cases where there is strong overlap of signals in the 2D spectra, making the full assignment open to errors. Recently, more dimensions have been introduced to solve complicated spectra of biological macromolecules, such as proteins or nucleic acids [5]. The experiments need long measuring times, as more evolution times must be incremented. Also, the low natural abundance of ^{13}C nuclei makes it necessary to use enriched samples.

Two dimensional representations of the resulting three dimensional HMQC-TOCSY spectrum can either be planes specified by carbon chemical shifts, which will show TOCSY correlations such as illustrated in Fig. 1(a), or planes specified by proton chemical shifts which will comprise HMQC correlations [Fig. 1(b)]. To illustrate the interpretation of such 3D data, we shall demonstrate the assignment of chemical shifts for the dibenzodioxocin structure **8**. In the 2D HMQC spectrum of RSCL [Fig. 2(a)], the β-proton to carbon correlation is clearly visible at 4.14/82.5

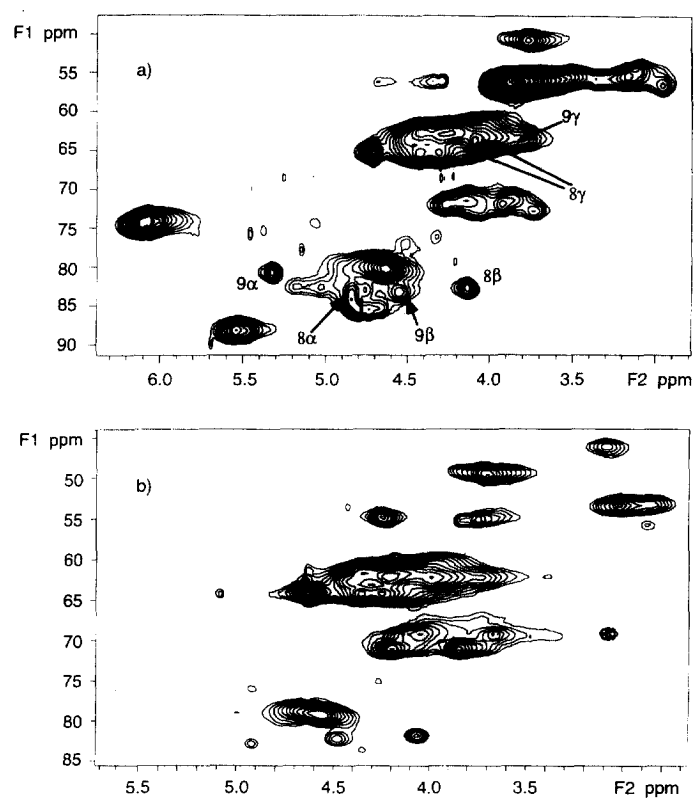


Fig. 2. Expansion of side-chain area of a 2D HMQC spectrum of ^{13}C-enriched RSCL (2a) and a β,γ-^{13}C-labelled DHP (2b). The correlations for the dibenzodioxocin structure **8** (*trans*) and **9** (*cis*) are marked.

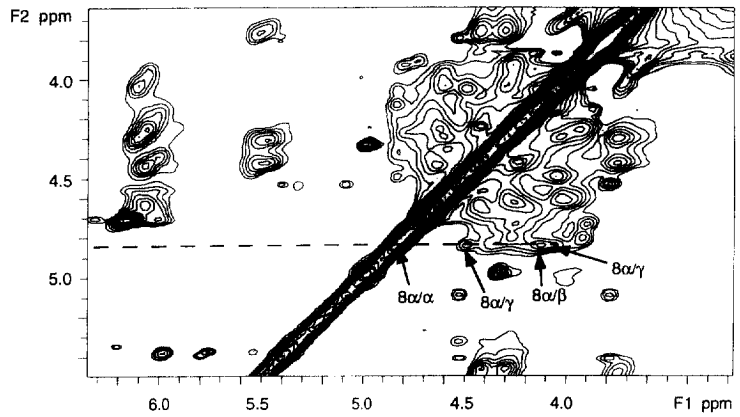


Fig. 3. Expansion of a 2D HOHAHA spectrum of ^{13}C-enriched RSCL. Autocorrelations can be seen on the diagonal and the correlations to other protons of the same spin-system on a horizontal line. The correlations of structure **8** (*trans*-dibenzodioxocin) are marked.

ppm. The other proton to carbon correlations of the spin system (the α-and the two γ-protons) are in crowded areas of the spectrum (*ca* 4.9 and 4 ppm). In an ordinary 2D TOCSY (Fig. 3), it is also difficult to locate the signals in this crowded area; signals from a minor component, such as **8**, easily become obscured. The 3D HMQC-TOCSY experiment solves this problem. A TOCSY type (i.e. F2F3 plane) slice made close to 82.5 ppm in the ^{13}C (F1) dimension gives Fig. 1(a), where one can easily see the auto correlation of the β-proton signal at the diagonal, as in a typical 2D TOCSY, and the other proton correlations of the same spin system in a horizontal line, without the interference caused by proton signals from other spin systems. One can now check the carbon signal corresponding to the β-proton using HMQC (i.e. F1F3) slices. In a slice selected near 4.13 ppm, the correlations close to this shift value are projected in the F1F3 plane [Fig. 1(b)]. The slice shows the auto-correlation at 4.13, with the corresponding carbon chemical shift at 82.5 ppm. That this is indeed the β-carbon signal of structure **8** can be deduced from the other proton signals of the same spin system (α and two γ's) that lie in a line at the same carbon (82.5 ppm) level, and from the fact that corresponding signals can be found in spectra of model compounds. The chemical shifts of the other carbons (α and γ) can be checked in the same manner by taking an F1F3 slice at the shift value for each of the α and γ protons. The signal for the α-carbon, for instance, can be located by taking the slice near 4.8 ppm. In this fashion, the side-chain structures for a complicated structure like the lignin molecule can be mapped out. The 3D experiment allows unambiguous assignment of what carbon and proton signals belong to the same spin-system. This makes structural assignments much more reliable than conventional NMR spectral assignments that rely on single proton and carbon signals obtained from model compounds.

Isotopic-labelling not only makes assignments unambiguous but also gives enhanced sensitivity that makes minor structures more visible. The assignments for the side-chain structures in the acetylated RSCL, as well as corresponding data for a β,γ C-13 enriched dehydrogenation polymer from coniferyl alcohol (DHP) and a sample of milled wood lignin MWL (unlabelled) from pine (*Pinus sylvestris*) (data from ref. [4]) are shown in Table 1. The assignments are based on 3D data from the RSCL and the DHP experiments. The general pattern of structural units of the cell culture lignin is typical for a conifer lignin. The dominant structures are the arylglycerol β-aryl ether (**1**), the phenyl coumaran (**2**), the dibenzodioxocin (**8** and **9**) and the pinoresinol structures (**3**). In this respect, all three samples are similar. However, the RSCL is formed in an extracellular process, MWL is extracted from the cell walls of the wood and the DHP is made *in vitro*. Hence, the structural differences that can be detected are of interest for our understanding of the lignification process.

A feature of the HMQC from RSCL that has not been visible in HMQC spectra of softwood MWLs is a prominent correlation at 5.32/80.5 ppm that we have identified as the Hβ/Cβ correlation of the *cis*-isomer (**9**) of the dibenzodioxocin structure **8**. The α- and the γ-correlations of this spin-system were identified from the 3D data. Both dibenzodioxocin isomers have been isolated from oxidative coupling experiments with model compounds [6] and have also been detected in the labelled DHP. In spectra of pine MWL, only the *trans*-form has been detected. Since the *trans*-form is thermodynamically more stable (Karhunen, P. unpublished results) a possible explanation for the absence of the *cis*-form in wood is that some *cis* to *trans* isomerization occurs during ageing of lignin.

It is of particular interest to note the absence of some minor structural features that have been observed in MWLs. In softwood MWLs, weak signals have been observed for structure **4** (the 1,2-diarylpropane-1,3-diol, or β-1). We have suggested earlier [7] that **4** is not uniformly distributed in lignins and that they seem to accumulate in low M_r fractions.

Table 1. ^{1}H and ^{13}C assignments for spin-systems of uniformly labelled RSCL, β,γ-labelled DHP and unlabelled pine MWL lignin

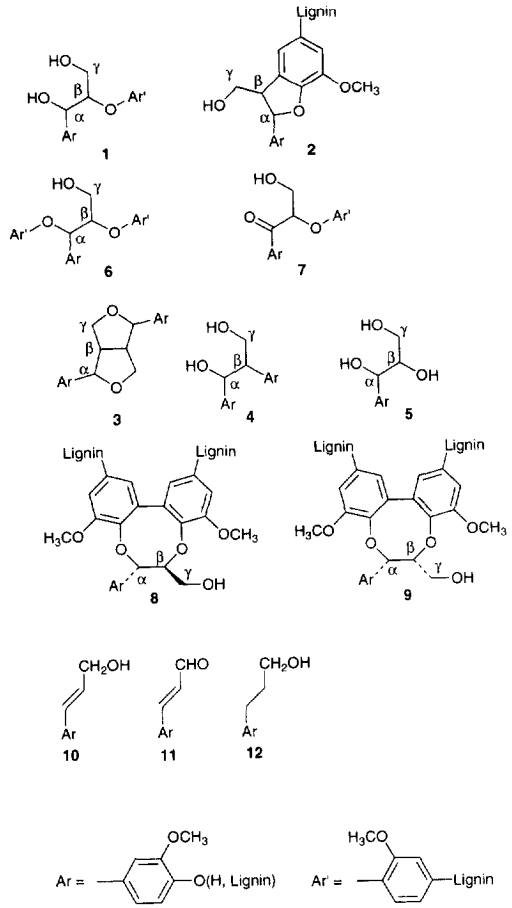
	$^{1}H\alpha/^{13}C\alpha$	$^{1}H\beta/^{13}C\beta$	$^{1}H\gamma/^{13}C\gamma$	RSCL	DHP	MWL
1	5.88–6.19/74.1	4.63/80.5	4.01, 4.42	+	+	+
β-O-4			/62.4			
2	5.53/88.3	3.78/50.8	4.29–4.31, 4.43	+	+	+
β-5			/65.1			
3	4.73–4.75/85.1	3.09/54.6	4.26, 3.92/72.3	+	+	+
β-β			4.14, 3.85/71.6			
4	6.00/75.1	3.35/49.5	4.10, 4.26/70.2	–	–	(+)
β-1						
5	5.92/	5.41/72.5	3.81, 4.24/	+	+	+
Arylglycerol						
6	5.45/80.2–80.7	4.69–4.75	4.13–4.70	–	–	–
α-O-4		/80.7–82.0	/63.4–66.2			
7		5.42/81.5	4.56/65.3	–	–	–
α-carbonyl						
8	4.84/82.1–83.7	4.13/82.9	4.54, 4.05/63.8	+	+	+
trans-dibenzodiox						
9	5.33/80.6	4.54/83.0	3.79/63.3	+	+	–
cis-dibenzodiox						
10	6.58/134.1	6.17/121.4	4.71/65.2	+	+	+
conif. alcohol						
11	6.58/127.6	7.41/152.7	9.67/193.8	(+)	+	+
conif. aldehyde						
12	1.95/30.2	2.65/31.7	4.10/63.5	+	+	+
dihydroconif. alcohol						
13*	2.56/46.4	2.85/33.6	3.75, 4.08/72.6	+	–	?
14*	2.94/56.2	4.00/61.5	5.01, 5.17/83.5	+	+	+?

(+) only a very small correlation observable.
* unknown compounds.

No signals from diarylpropane units were detected in RSCL. Thioacidolysis of the RSCL [1] does, however, yield significant amounts of a dimeric degradation product that originates from **4**. The explanation for this apparent contradiction may be that some of the β-1 structures are formed from some unknown precursor in lignin during the acid treatment. Another structure that could not be detected in RSCL in spite of C-13 enrichment is the non-cyclic benzyl aryl ether structure **6**. The absence of **6** is consistent with earlier findings concerning softwood lignins [7]. Traces of such structures have been observed in a C-13 enriched hardwood MWL [8] but no spectrometric evidence has been found so far for the existence of such bonds in softwood lignins.

In the HMQC spectrum of MWL from pine and birch, a weak signal at 3.53–3.66/68.7 was found to match with the signals from a model compound where structure **1** was etherified in the γ-position (J. Sipilä, unpublished results). No such signal was found in the labelled RSCL. The fact that the RSCL is formed outside the cell wall and contains no carbohydrates may have some significance when looking for an explanation for this difference between cell wall lignin and cell culture lignin. The coniferyl alcohol side-chains (**10**) are more abundant in both RSCL and DHP than in MWL. This indicates a similarity in the formation mechanisms for RSCL and DHP. The α-carbonyl structure **7**, which has been detected in both aspen and in birch MWL [8] is not found in RSCL nor in the pine MWL. Since this structure represents an oxidation product, this probably reflects the easier oxidation of syringyl compared to guaiacyl structures. Dihydroconiferyl alcohol side-chains **12** are present both in DHP and in RSCL. The occurrence of side-chains with a lower degree of oxidation has been observed in spruce lignin [9] by NMR and, recently [10], such dimers have been found in lignifying tissues. The occurrence of substructures with reduced side-chains indicates that lignin formation is not entirely an oxidative process. There are some spin-systems that have not yet been assigned for lack of appropriate model compounds: two such systems have been added to Table 1.

In earlier work [1], we have concluded from thioacidolysis experiments that RSCL with its high proportion of *p*-hydroxyphenyl units represents an 'early' stage of lignification [11]. The comparatively low yields of low M_r thioacidolysis products and the large amount of phenolic hydroxyls in RSCL compared with MWLs from softwood, indicate a polymer structure with a large proportion of interunit C-C bonds. The 3D NMR spectra do not allow accurate quantitative deductions but comparison of the correlation intensities for the α-carbons in pine MWL and in the RSCL indicates that the RSCL does contain a higher



proportion of condensed units (5-5 and β-5 units) than MWL.

The released cell culture lignin has been formed by the cells, presumably by using the same enzyme apparatus that is used for depositing lignin in the cell wall. The fact that this lignin is structurally different from that isolated from the cell wall gives us clues to the importance of the cell wall matrix in the lignification process. More data are needed, especially from uniformly labelled softwood lignins, before more definite conclusions can be drawn.

EXPERIMENTAL

Suspension cultures. Pieces of green callus ($\approx$ 0.05 g) of *P. abies* (L.) Karsten (embryo origin) were transferred from solid nutrient medium (combination 2 without organic N_2, cf ref. [12]) to Erlenmeyer flasks (50 ml) containing 25 ml of special liquid medium [13]. The medium contained T macro mineral, (NH_4^+ : NO_3^-, 1:2) and SS-micro nutrients [12]. It was supplemented with myo-inositol (100 mg l^{-1}), and 25 mM sucrose (BDH), which was autoclaved separately, and sterile-filtered 0.25 mM arginine and 0.5 mM glutamine (final concn). Kinetin (2.5 μM) and 2,4-D (0.5 μM) were used as growth regulators and the medium contained vitamins according to ref. [14]. Cultures were incubated in continuous light (70 μE m^{-2} s^{-1}) in an orbital shaker (100 rpm) at 25° with 1% CO_2 supplementation (cf ref. [13]). After 3 days in culture when the first lignin could be observed in the liquid medium a sterile-filtered aq. soln of D[U-^{13}C] glucose (25 mM final concn, 99% ^{13}C, Cambridge Isotope Laboratories) was added into the nutrient medium. Five days later, the resulting lignin was harvested by centrifugation from the decanted milky nutrient medium. The pellet was washed with distilled H_2O several times and freeze-dried for chemical analyses (cf. ref. [13]).

Preparation of [β,γ-^{13}C] *DHP.* Coniferyl alcohol (β,γ-^{13}C labelled) was synthesized according to the procedure of ref. [15]. Firstly, two different solns were prepd, a soln of 0.54 g labelled coniferyl alcohol (3 mmol) in 5 ml of Me_2CO and a soln of 0.36 ml 9 M H_2O_2 in 5 ml of citrate-Pi buffer (pH 3.5). Using a syringe pump, both of the solns were then added simultaneously during 5 hr to a stirred soln of 15 mg HRP in 60 ml Me_2CO—pH 3.5 buffer (1:5), at room temp. and protected from light. Stirring was continued overnight. The ppt. was sepd by filtration and a dioxane-soluble fr. was isolated (0.13 g) [16]. From the supernatant, an additional 0.13 g of lower *M*, DHP was obtained by extraction with EtOAc.

NMR. All NMR spectra were measured using a Varian Unity 500 spectrometer (11.7 T). Samples were acetylated and dissolved in $CDCl_3$. Chemical shifts were referenced to int. TMS (0 ppm). The inverse detected ^{1}H-^{13}C correlation spectra (HMQC) [17] and the homonuclear Hartman-Hahn (HOHAHA) [18] spectra were measured as described earlier [8]. Three dimensional ^{1}H detected ^{1}H-^{13}C correlation-homonuclear Hartman-Hahn spectra (HMQC-HOHAHA) were measured as described in ref. [19]. The spectral width was set to 6 kHz in F2 and F3 and 25 kHz in F1; 32 transients in 70 time increments in F1 and F2 were collected using the hypercomplex method. The polarization transfer delay between ^{1}H and ^{13}C was set to assumed 140 Hz, a relaxation delay of 0.5 seconds and spin-lock lengths of 30, 60 and 100 ms were used to obtain the spectra. The time domain data of F1 were appended to 124 points using forward linear prediction. The spectra were processed using $\Pi/2$ shifted squared sinebell functions in all dimensions prior to Fourier transformation.

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