ON THE OCCURRENCE OF ACYLATED NATIVE LIGNINS IN VASCULAR PLANTS

del Río, J.C.[1*], Rencoret, J.[1], Marques, G.[1], Gutiérrez, A.[1], Ibarra, D.[2], Jiménez-Barbero, J.[2], Martínez, Á.T.[2]

[1] Instituto de Recursos Naturales y Agrobiología de Sevilla, CSIC, P.O. Box 1052, E-41080-Seville, Spain
[2] Centro de Investigaciones Biológicas, CSIC, Ramiro de Maeztu 9, E-28040 Madrid, Spain

ABSTRACT

Lignin acylation seems to be widespread among angiosperms and to occur at monolignol level. Monolignol acylation is discussed here as a mechanism contributing to regulate the structure of lignin.

BACKGROUND

The lignin polymer results from the random oxidative coupling of *p*-hydroxycinnamyl monolignols [1,2]. The three primary monolignols are *p*-coumaryl, coniferyl and sinapyl alcohols, which produce, respectively, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) phenylpropanoid units when incorporated into the lignin polymer.

However, it is now accepted that other monomers also participate in coupling reactions giving rise to the lignin macromolecule. Some lignins are known to be naturally acetylated at the γ-carbon of the side chain, and preferentially over S-units [3-5]. Recent studies have provided strong evidence that sinapyl acetate is implicated as a monomer in lignification and that the naturally acetylated lignin derives not from acetylation of the lignin polymer but from polymerization of pre-acetylated monolignols [4,5]. Other acids (*p*-coumaric and *p*-hydroxybenzoic acids) are also found naturally acylating lignin [2, 6-8].

In this work, we investigate the occurrence and extent of lignin acylation in a wide set of vascular plants. For this purpose, we use the DFRC degradation method, which cleaves α- and β-aryl ether bonds but leaves γ-esters intact, allowing therefore the analysis of γ-acylated lignin [9]. The original DFRC degradation method, however, does not allow the analysis of native acetylated lignin because the degradation products are acetylated during the degradation procedure, but with appropriate modification by substituting acetylating reagents with propionylating reagents, DFRC´, it is also possible to obtain information about the occurrence of native lignin acetylation [10]. In addition, a more detailed study was performed on selected highly acylated lignins with the purpose of correlating the type and extent of acylation with the structure of lignins. For this purpose, we used 2D-NMR spectroscopy that provides information of the structure of the whole lignin and direct determination of the different lignin moieties and inter-unit linkages.

EXPERIMENTAL

Samples. A wide set of plant samples (see text) was selected for this study. Milled-wood lignin (MWL) was extracted from finely ball-milled plant material, free of extractives and hot water soluble material, using dioxane-water (9:1, v/v), and purified as described [11].

DFRC (derivatization followed by reductive cleavage). A modification of the standard DFRC method by using propionyl instead of acetyl reagents (DFRC´) was used [10]. Lignins (10 mg) were stirred for two hours at 50ºC with propionyl bromide in propionic acid (8:92). The solvents and excess of bromide were removed by rotary evaporation. The products were then dissolved in dioxane/propionic acid/water (5:4:1, v/v/v), and 50 mg Zn was added. After 40 min stirring, the mixture was transferred into a separatory funnel with dichloromethane and saturated ammonium chloride. The pH of the aqueous phase was adjusted to less than 3, the mixture vigorously mixed and the organic layer separated. The water phase was extracted twice more with dichloromethane. The combined dichloromethane fraction was evaporated and the residue propionylated with propionic anhydride and pyridine. The propionylated lignin degradation products were collected after rotary evaporation of the solvents, and subsequently analyzed by GC/MS.

The GC/MS analyses were performed with a Varian model Star 3800 GC equipped with an ion trap detector (Varian model Saturn 4000) using a medium-length (15 m) capillary column (DB-5HT, 5 m × 0.25 mm I.D., 0.1 μm film thickness). The oven was heated from 120 (1 min) to 330 ºC at 6 ºC/min, and held for 4 min at the final temperature. The injector was programmed from 60ºC to 350ºC at a rate of 200ºC/min and held until the end of the analysis. The transfer line was kept at 300 ºC. Quantification of the released individual monomers was performed using tetracosane as external standard.

NMR spectroscopy

NMR spectra were recorded at 25 ºC on a Bruker AVANCE 500 MHz equipped with a z-gradient triple resonance probe. Around 40 mg of lignin were dissolved in 0.75 mL of deuterated dimethylsulfoxide (DMSO-d_6) and 2D-NMR spectra were recorded in HSQC (heteronuclear single quantum correlation) experiments. The $^1J_{CH}$ used was 140 Hz. The *J*-coupling evolution delay was set to 3.2 ms. The central solvent peak was used as an internal reference (δ_C 39.5; δ_H 2.50 ppm). HSQC cross-signals were assigned by comparing with the literature [2,7,8]. A semiquantitative analysis of the intensities of the HSQC cross-signal intensities was performed. In the aliphatic oxygenated region, inter-unit linkages were estimated from C_α-H_α correlations and the relative abundance of side-chains involved in inter-unit linkages were calculated. In the aromatic region, $C_{2,6}$-$H_{2,6}$ correlations from S units and C_2-H_2 plus C_6-H_6 correlations from G units were used to estimate the S/G ratio of lignin, and *p*-coumaric acid content was estimated from its $C_{2,6}$-$H_{2,6}$ correlation signal. Lignin acylation was estimated from the intensities of C_γ-H_γ correlations in acylated and non-acylated side-chains.

RESULTS AND DISCUSSION

Degradation Followed by Reductive Cleavage (DFRC and DFRC´)

The chromatogram of the DFRC´ products released from a representative lignin (sisal) is shown in **Figure 1**. All the analyzed lignins released the *cis* and *trans* isomers of guaiacyl (*c*-G and *t*-G) and syringyl (*c*-S and *t*-S) lignin monomers (as their propionylated derivatives) arising from normal γ-OH units in lignin. In addition, the presence of originally γ-acetylated guaiacyl (*c*-G_{ac} and *t*-G_{ac}) and syringyl (*c*-S_{ac} and *t*-S_{ac}) lignin units could also be clearly observed in the chromatograms of all of the selected lignins indicating that acetylation occurred exclusively at the γ-carbon of the lignin side-chain.

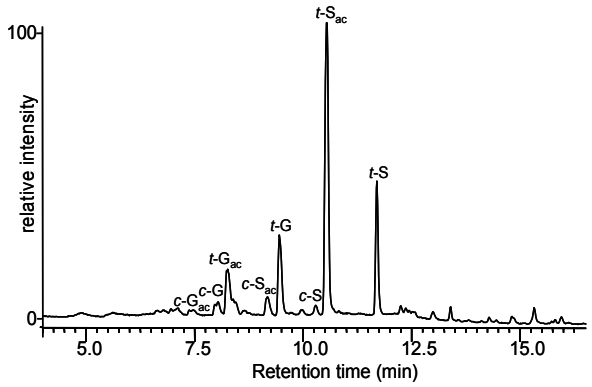

Figure 1: Chromatogram of the DFRC´ degradation products of MWL from sisal. *c*-G, *t*-G, *c*-S and *t*-S are the normal *cis*- and *trans*-guaiacyl and syringyl monomers. *c*-G_{ac}, *t*-G_{ac}, *c*-S_{ac} and *t*-S_{ac} are the originally acetylated *cis*- and *trans*-guaiacyl and syringyl monomers, respectively.

DFRC´ analysis also indicated that *p*-coumarate groups are attached to the γ-carbon in some lignins (abaca, curaua and bamboo), and again, predominantly on syringyl units. Trace amounts of the respective coniferyl *p*-coumarate could also de detected in these lignins.

The percentages of naturally acetylated guaiacyl (%G_{ac}) and syringyl (%S_{ac}) moieties for the lignins analyzed are presented in **Table 1**. Naturally acetylated lignin units were found to occur in all angiosperms analyzed while they were absent in the two gymnosperms (pine and spruce). In all cases, acetate and *p*-coumarate groups are preferentially attached to syringyl units. Interestingly, a high extent of acetylation was observed in the lignin of several herbaceous plants (kenaf, sisal, abaca, curaua) and in the hardwoods hornbeam and, in a minor extent, beech, all of them characterized by high S/G ratios.

On the other hand, the question to whether acylated lignin derives from polymerization of acylated monolignols or from acylation of the lignin polymer has recently been addressed and sinapyl acetate has been demonstrated to behave as a monomer in lignification participating in coupling reactions [4,5,8,12].

TABLE 1: RELATIVE ABUNDANCES OF ACETYLATED LIGNIN MOIETIES IN THE SELECTED LIGNIN SMAPLES

Species	Name	%Sac	%Gac
Angiosperms			
Agave sisalana	Sisal	77.7	50.4
Cocos nucifera	Coir	7.4	0.6
Bambusa sp.	Bamboo	1.2[a]	4.8[a]
Musa textilis	Abaca	80.3[a]	5.6[a]
Ananas erectifolius	Curaua	61[a]	51[a]
Fagus sylvatica	Beech	10.8	1.6
Carpinus betulus	Hornbeam	44.6	2.7
Cannabis sativa	Hemp	1.1	0.7
Hibiscus cannabinus	Kenaf	59.0	8.9
Corchorus capsularis	Jute	6.4	0.3
Populus tremula	Aspen	1.2	0.8
Eucalyptus globulus	Eucalypt	1.1	4.9
Gymnosperms			
Picea abies	Spruce	-	0.0
Pinus sylvestris	Pine	-	0.0

[a]Some amounts of γ-*p*-coumaroylated were found and were included in the estimation %S_{ac} and %G_{ac}.

HSQC-NMR

The lignins having a higher extent of acylation (sisal, kenaf, abaca and curaua) were studied by 2D-NMR. The spectra of two representative lignins (sisal and abaca) are shown in **Figure 2**, and the main substructures found are depicted in **Figure 3**.

The spectra show intense signals corresponding to acylated γ-carbon (δ_C/δ_H 63.5/3.83 and 4.30 ppm) together with signals from normal hydroxylated γ-carbon (δ_C/δ_H 60.2/3.30 and 3.70 ppm). The spectra confirms that these lignins are extensively acylated and that acylation occurs exclusively at the γ-position of the lignin side-chain. An estimation of the percentage of γ-acylation was calculated by integration of the signals corresponding to the hydroxylated and acylated γ-carbon and ranged from 58% in kenaf bast lignin up to 80% in abaca lignin (**Table 2**).

The side-chain region of the spectra gives additional information about the inter-unit linkages in these lignins. β-O-4´ aryl ether substructures (**A**,**A´**,**A´´**) were highly predominant in all the lignins analyzed although other substructures were also observed. Small signals corresponding to spirodienone substructures (**D**) were observed in both spectra. Phenylcoumaran (**B**) and resinol (**C**) substructures were found in very small proportions in the spectra of sisal but were absent in the spectrum of abaca. The relative abundances of the main inter-unit linkages present in the highly acylated lignins selected for this study (sisal, kenaf, abaca and curaua) are shown in **Table 2**. All these highly acetylated lignins share a common characteristic, a strikingly high proportion of β-O-4´ ether linkages (up to 94% of all linkages) and a very low proportion of condensed linkages (i.e. β-1´, β-5´ and β-β´). Some of these condensed linkages (β-5´ and β-β´) are even absent in some lignins (abaca and curaua).

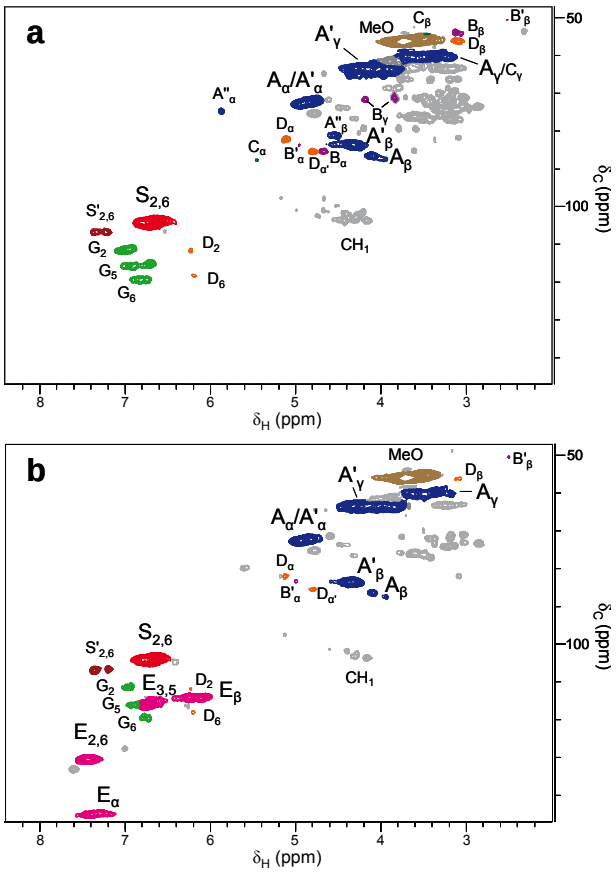

Figure 2: HSQC spectra of the lignins from (a) sisal, and (b) abaca. Figure 3 for the main lignin structures (A-D) identified.

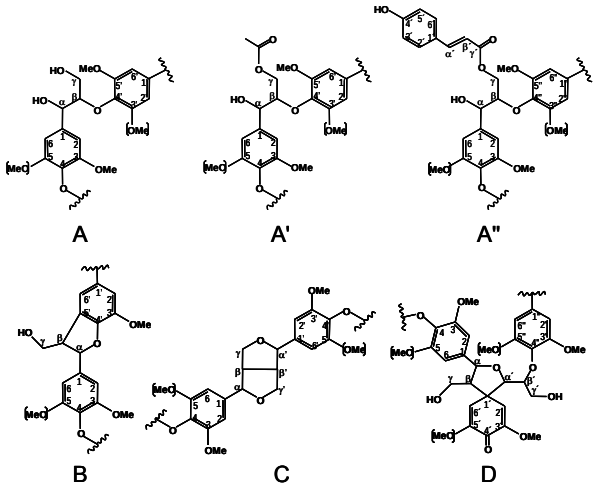

Figure 3: Main structures present in the highly acylated lignins studied: (A) β–O-4´ aryl ether linkages; (A') β–O-4´ aryl ether linkages with acetylated γ-carbon; (A'') β–O-4´ aryl ether linkages with *p*-coumaroylated γ-carbon; (B) phenylcoumaran structures; (C) resinol structures; and (D) spirodienone structures

The main cross-signals in the aromatic region of the HSQC spectra correspond to the aromatic rings of the different lignin units. Signals from syringyl- (S) and guaiacyl- (G) lignin units can be observed in all spectra. No signals for *p*-hydroxyphenyl (H) lignin units could be detected in the HSQC spectra of these lignins. An estimation of the relative proportions of the S and G-lignin units in the HSQC spectra revealed that all the lignins selected for this study present a very high S/G ratio, ranging from 3.9 in sisal to 8.7 in abaca (**Table 2**). Prominent signals corresponding to *p*-coumarate structures were observed in the lignins of abaca and curaua, in agreement with the results observed by DFRC.

TABLE 2: STRUCTURAL CHARACTERISTICS OBSERVED FROM THE HSQC SPECTRA OF THE SELECTED HIGHLY ACYLATED MWL

	sisal	kenaf	abaca	curaua
Percentage of γ-acylation	68	58	80	69
Linkage relative abundance (% of side-chains)				
β-O-4' structures	89	84	94	94
spirodienones	5	6	6	4
phenylcoumarans	2	2	0	2
resinols	4	8	0	0
S/G ratio	3.9	5.6	8.7	4.9

Correlation between acylation degree and lignin structure

To extend the possible correlations between acylation degree and differences in lignin composition and inter-unit linkages, additional information was collected from HSQC spectra of MWL from other plant species. **Figure 4** presents the S/G ratio and the relative amount of β-β' side-chains (with respect to β-*O*-4 chains) against the acylation degree in the following 8 plant species (from different botanical groups): *C. sativa* (Rosales); *Eucalyptus globulus* Labill. (eucalypt, Myrtales); *Corchorus capsularis* L. (jute, Malvales); *Cocos nucifera* L. (palm tree, Arecales), *Hibiscus cannabinus* L. (kenaf, Malvales), *A. sisalana* (Asparagales), *Ananas erectifolius* L.B. Smith (curaua, Poales) and *M. textilis* (Zingiberales). It is possible to see that the general tendency found in the three model lignins described here, is maintained in the other lignins, i.e. highly acylated lignins are often characterized by a high S/G ratio, and a low percentage of β-β' linkages, whereas the opposite often occurred in scarcely acylated lignins.

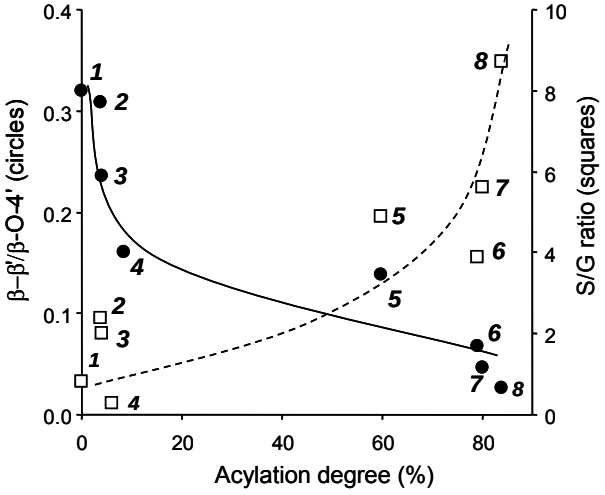

Figure 4: Differences in native lignin composition (S/G ratio, white squares and dashed tendency line) and inter-unit linkages (β-β'/β-*O*-4' ratio, black circles and continuous line) as a function of the acylation degree in MWL from 8 plant species analyzed by HSQC NMR: 1) *C. sativa*; 2) *E. globulus*; 3) *C. capsularis*; 4) *C. nucifera*; 5) *H. cannabinus*; 6) *A. sisalana*; 7) *A. erectifolius*; and 8) *M. textilis*.

Taking the above findings together, it is proposed that some angiosperms use monolignol acylation as a mechanism to regulate the structure of lignin [13]. In this way, acylation of monolignols would result in lower presence of resinols and other β-β' interunit linkages in S-rich lignins, promoting formation of a highly-etherified (β-*O*-4' linked) and more labile lignin polymer. In contrast, low (or null) acylation degrees would result in lignins with higher content of C-C interunit linkages, that will be more recalcitrant towards chemical and/or biological degradation.

CONCLUSIONS

We have shown that lignin γ-acetylation is widespread, and probably ubiquitous, among angiosperms, although at different extents, but is absent from conifers. Moreover, the lignins of many plants (e.g. the non-woody sisal, kenaf, abaca or the hardwood hornbeam) are particularly extensively acetylated (up to 80% of the S-lignin moieties). Monolignols acylation would result in lower presence of resinols and other β-β' interunit linkages in S-rich lignins, promoting formation of a highly-etherified (β-*O*-4' linked) lignin. The results obtained suggested a possible role of monolignol acylation regulating lignin structure in plants.

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