OCCURRENCE AND STRUCTURAL CHARACTERISTICS OF HIGHLY ACYLATED (ACETYLATED AND/OR *p*-COUMAROYLATED) NATIVE LIGNINS FROM DIVERSE HERBACEOUS PLANTS

José C. del Río[1], Jorge Rencoret[1], Gisela Marques[1], Ana Gutiérrez[1], David Ibarra[2], J. Ignacio Santos[2], Jesús Jiménez-Barbero[2] and Ángel T. Martínez[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; E-mail: delrio@irnase.csic.es

ABSTRACT

We have studied the occurrence of native acylated (with acetates and/or *p*-coumarates) lignin in a large set of vascular plants, including both angiosperms and gymnosperms by DFRC and HSQC-NMR. Acylated lignin units were found in all angiosperms, including mono- and eudicotyledons, but were absent in the gymnosperms analyzed. Acylation occurred exclusively at the γ-carbon of the lignin-side chain and predominantly on syringyl units. In some plants (e.g. sisal, kenaf, abaca, curaua or hornbeam), lignin acylation occurred at a very high extent, up to 80 %. The structure of these highly-acylated lignins was characterized by a very high syringyl/guaiacyl ratio, a predominance of β-O-4´ linkages (up to 94% of all linkages) and a very low proportion of β-β´ linkages, which indeed are completely absent in the lignins from abaca and curaua. In all cases, acylation appears to occur at the monomer stage and sinapyl and coniferyl acetates have been demonstrated to behave as real lignin monomers participating in lignification.

I. INTRODUCTION

Lignins are complex natural biomacromolecules characteristics of vascular plants. The lignin polymer results from the random oxidative coupling of *p*-hydroxycinnamyl alcohols (monolignols) mediated by peroxidases and/or laccases (Ralph et al., 2004). 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 becoming increasingly clear that other monolignols also participate in coupling reactions to give rise to the lignin macromolecule. This is the case of γ-acylated lignins which are being discovered in many plants (Ralph, 1996; del Río et al., 2004,2007). Different grass lignins are partially *p*-coumaroylated and some hardwood lignins, such as poplar, aspen or willow, are *p*-hydroxybenzoylated. In this work, we study the occurrence and structure of some naturally-acylated lignins occurring in several vascular plants by a combination of spectroscopic (2D-NMR) and degradative techniques, including modified DFRC (derivatization followed by reductive cleavage).

II. EXPERIMENTAL

Samples. The plant samples selected for this study consist of both woody and nonwoody angiosperms and gymnosperms (**Table 1**). Plant materials were ground to sawdust using a knife mill. MWL was extracted from finely ball-milled (150 h) plant material, free of extractives and hot water soluble material, using dioxane-water (9:1, v/v), followed by evaporation of the solvent, and purified as described (Bjorkman, 1956).

DFRC´. A modification of the standard DFRC method by using propionyl instead of acetyl reagents (DFRC´) was used (Ralph and Lu, 1998; del Río et al., 2007). Lignins were stirred for two hours at 50 ºC with propionyl bromide in propionic acid (8:92, v/v). 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 powered Zn was added. After 40 min stirring at room temperature, the mixture was transferred into a separatory funnel with dichloromethane and saturated ammonium chloride. The aqueous phase was adjusted to pH < 3 and the organic layer was separated. The dichloromethane fraction was dried and the residue subsequently propionylated with propionic anhydride and pyridine. The propionylated lignin degradation compounds were collected after evaporation of the solvents, and subsequently analyzed by GC/MS. The GC/MS analyses were performed in a Varian Saturn 2000 using a 12m x 0.25 mm i.d., 0.1 μm, DB-5HT length capillary column. The oven was heated from 50 (held 0.2 min) to 100 ºC at 30 ºC/min, then raised to 300 ºC at 5ºC/min, and held for 5 min at the final temperature. The injector and transfer line were kept at 300 ºC.

NMR spectroscopy. NMR spectra were recorded at 25 ºC in 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-d6) and HSQC-NMR spectra was recorded. The spectral widths for the HSQC were

5000 Hz and 13200 Hz for the ^{1}H- and ^{13}C-dimensions, respectively. The number of collected complex points was 2048 for ^{1}H-dimension with a recycle delay of 5 s. The number of transients for the HSQC spectra was 64, and 256 time increments were always recorded in ^{13}C-dimension. The *J*-coupling evolution delay was set to 3.2 ms in HSQC. Squared cosine-bell apodization function was applied in both dimensions. Prior to Fourier transform the data matrixes were zero filled up to 1024 points in the 13C-dimension. Residual DMSO (from DMSO-d6) was used as reference for chemical shifts. Signal volume integration was used to calculate the S/G ratio (from $S_{2,6}$ and G_2+G_6 cross-signals) and percentage of side-chains involved in different inter-unit linkages (from C_α-H_α correlations). The acylation percentage was obtained by integrating the signals from C_γ-H_γ correlations in acylated and non-acylated lignin structures.

III. RESULTS AND DISCUSSION

DFRC´. The selected MWL samples were analyzed by DFRC´. All the analyzed lignins released the *cis* and *trans* isomers of G and S lignin monomers (as their propionylated derivatives) in different proportions, arising from normal (γ-OH) units in lignin. In addition, the presence of originally γ-acetylated G and S lignin units could also be clearly observed in the chromatograms of most of the analyzed lignins. Acylation with *p*-coumarate was also observed in the MWL of bamboo, and especially, abaca and curaua. Acylation occurred exclusively at the γ-carbon of the lignin side-chain. The results from the DFRC´ analysis of the MWL selected for this study, namely the molar yields of the released monomers, the S/G ratios and the percentages of naturally acetylated guaiacyl (%Gac) and syringyl (%Sac) lignin moieties, are presented in **Table 1**. Naturally acetylated lignin units were found to occur in all angiosperms analyzed, including both mono- and eudicotyledons. However, no traces of acetylated or *p*-coumaroylated lignin units could be found in the MWL of the gymnosperms (pine and spruce) studied here. The data also indicated that in most lignin samples acylation occurred predominantly on S units. The occurrence of naturally acetylated lignin units seems to be widespread among angiosperms and restricted only to this group of vascular plants, being particularly abundant in S-rich lignins. Especially important is the high extent of lignin acetylation observed in the MWL from the herbaceous plants sisal, kenaf, abaca and 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, dimeric compounds derived from the β-β´ homo- and cross-coupling of sinapyl acetate and sinapyl alcohol were observed in the lignins of sisal and kenaf, indicating that in these lignins sinapyl alcohol is preacetylated and behaves as a real monolignol participating in post-coupling reactions. However, in abaca and curaua lignins, no traces of any type of β-β´ linkages could be detected after DFRC´.

Table 1. Abundance (Molar Yields) of the DFRC´ Degradation Monomers of the MWL Isolated from the Different Plants Selected for This Study, S/G Ratios and Relative Abundances of Acetylated Lignin Moieties.

		Monomers (μmol/g lignin)						
Species	Name	G	G_{ac}	S	S_{ac}	S/G	$\%S_{ac}$ [a]	$\%G_{ac}$ [b]
Angiosperms								
Monocotyledons								
Agave sisalana	Sisal	122	124	108	378	2.0	77.7	50.4
Cocos nucifera	Coir	819	5	174	14	0.2	7.4	0.6
Bambusa sp.	Bamboo	256	13	280	4	1.1[c]	1.2[c]	4.8
Musa textilis	Abaca	50	3	21	131	5.1[c]	92.4[c]	5.6
Ananas erectifolius	Curaua	250	252	515	595	2.6[c]	60.5[c]	49.9
Eudicotyledons								
Fagus sylvatica	Beech	126	2	165	20	1.4	10.8	1.6
Carpinus betulus	Hornbeam	146	4	230	185	2.8	44.6	2.7
Cannabis sativa	Hemp	286	2	177	2	0.6	1.1	0.7
Hibiscus cannabinus	Kenaf	390	38	543	780	3.1	59.0	8.9
Corchorus capsularis	Jute	299	1	336	23	1.2	6.4	0.3
Populus tremula	Aspen	651	5	662	8	1.0	1.2	0.8
Eucalyptus globulus	Eucalypt	154	8	275	3	2.3	1.1	4.9
Gymnosperms								
Picea abies	Spruce	520	0	0	0	0.0	-	0.0
Pinus sylvestris	Pine	402	0	0	0	0.0	-	0.0

[a] $\%S_{ac}$ is the percentage of acetylated S units respect to the total S units. [b] $\%G_{ac}$ is the percentage of acetylated G units respect to the total G units. [c]Some amounts of γ-*p*-coumaroylated S units were found (27, 124 and 195 μmol/g lignin for bamboo, abaca and curaua, respectively) and were included in the estimation of total S units for calculation of S/G and $\%S_{ac}$.

HSQC-NMR spectra of highly acetylated lignins. The structure of the highest acylated MWL (sisal, kenaf, abaca and curaua) was characterized in detail by 2D-NMR. The HSQC spectra (side-chain and aromatic regions) of two selected MWL (from sisal and abaca) are shown in **Figure 1**. The spectra clearly show the presence of intense signals corresponding to acylated γ-carbon, together with the presence of signals from normal hydroxylated γ-carbon. The HSQC spectra indicate 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 from the HSQC spectra (**Table 2**), and ranged from 58% in kenaf bast lignin up to 80% in abaca lignin. All the spectra showed prominent signals corresponding to β-O-4´ aryl ether linkages (structures **A**, **A'** and **A''**), which were highly predominant in all the lignins analyzed here. Small signals corresponding to spirodienone substructures (**D**) can be observed in sisal, kenaf, abaca and curaua lignins. Phenylcoumaran substructures (**C**) were also found, although in very small proportions in sisal, kenaf and curaua, but were absent in abaca. Finally, resinol (substructures **B**) were clearly observed in kenaf, and in very small traces in sisal, but were completely absent in abaca and curaua lignins, in agreement with DFRC´ results. The relative abundances of the main inter-unit linkages in these highly acylated MWL, calculated from the HSQC, are shown in **Table 2**. The main cross-signals in the aromatic region of the HSQC spectra (**Figure 2**) correspond to the aromatic rings of the different S and G lignin units, and therefore S/G ratios were calculated (**Table 2**). Prominent signals corresponding to *p*-coumarate structures were observed in the lignins of abaca and curaua, corresponding to the *p*-coumarate units in structure **A''**. In general, the structure of all these highly-acylated lignins is characterized by having a very high S/G ratio, a strong predominance of β-O-4´ linkages (over 90% of all linkages) and a very low proportion of other lignin substructures such as resinols (β-β´), spirodienones, phenylcoumarans, and dibenzodioxocins (several of them being absent from some of the lignins), resulting in highly-linear lignin polymers.

Table 2. Structural characteristics (percentage of γ-acylation, relative abundance of the main inter-unit linkages, and S/G ratio) 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 involved)				
β-O-4'	89	84	94	94
β-1' (spirodienone)	5	6	6	4
β-5'	2	2	0	2
β-β´ (syringaresinol)	4	8	0	0
S/G ratio	3.9	5.6	8.7	4.9

IV. CONCLUSIONS

The structure of the MWL isolated from the herbaceous plants sisal, kenaf, abaca and curaua has been elucidated by 2D-NMR and DFRC techniques. The analyses indicated that the lignins from these plants are extensively acylated at the γ-carbon of the lignin side-chain (with either acetate and/or *p*-coumarate groups) and preferentially over S moieties. The structure of these highly acylated lignins can be essentially regarded as S units linked mostly through β-O-4´ ether bonds, where the γ-carbons of the side-chains are extensively acylated. The lignin polymer is therefore extremely linear and unbranched, making these lignins unique. The study of highly acylated lignins will significantly contribute to redefine the structure of lignin and to complete the lignin biosynthetic pathways.

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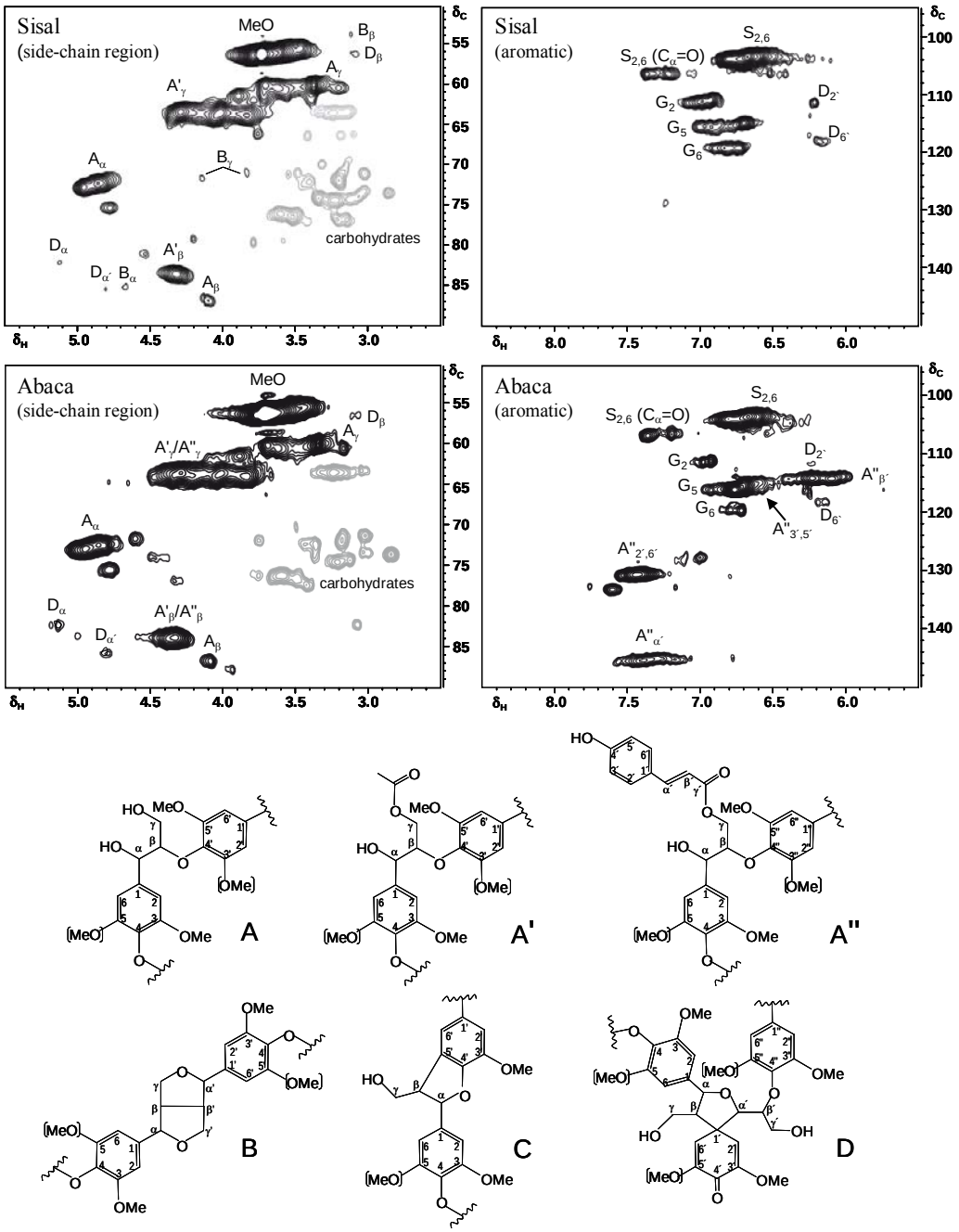

Figure 1.- Expanded side-chain region, δ_C/δ_H 50-90/2.5-5.5 ppm, and aromatic region, δ_C/δ_H 95-150/5.5-8.5 ppm, of the HSQC spectra of the MWL from sisal and abaca. Main lignin structures (**A**-**C**) are also shown.

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