Monolignol acylation and lignin structure in some nonwoody plants: A 2D NMR study

Ángel T. Martínez [a,*], Jorge Rencoret [b], Gisela Marques [b], Ana Gutiérrez [b], David Ibarra [a,1], Jesús Jiménez-Barbero [a], José C. del Río [b]

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

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ABSTRACT

Lignins from three nonwoody angiosperms were analyzed by 2D NMR revealing important differences in their molecular structures. The *Musa textilis* milled-wood-lignin (MWL), with a syringyl-to-guaiacyl (S/G) ratio of 9, was strongly acylated (near 85 of side-chains) at the γ-carbon by both acetates and *p*-coumarates, as estimated from ^{1}H–^{13}C correlations in C_γ-esterified and C_γ–OH units. The *p*-coumarate $H_{3,5}$–$C_{3,5}$ correlation signal was completely displaced by acetylation, and disappeared after alkali treatment, indicating that *p*-coumaric acid was esterified maintaining its free phenolic group. By contrast, the *Cannabis sativa* MWL (S/G ~0.8) was free of acylating groups, and the *Agave sisalana* MWL (S/G ~4) showed high acylation degree (near 80) but exclusively with acetates. Extensive C_γ-acylation results in the absence (in *M. textilis* lignin) or low abundance (4 in *A. sisalana* lignin) of β–β′ resinol linkages, which require free C_γ-OH to form the double tetrahydrofuran ring. However, minor signals revealed unusual acylated β–β′ structures confirming that acylation is produced at the monolignol level, in agreement with chromatographic identification of γ-acetylated sinapyl alcohol among the plant extractives. In contrast, resinol substructures involved 22 side-chains in the *C. sativa* MWL. The ratio between β–β′ and β-O-4′ side-chains in these and other MWL varied from 0.32 in *C. sativa* MWL to 0.02 in *M. textilis* MWL, and was inversely correlated with the degree of acylation. The opposite was observed for the S/G ratio that was directly correlated with the acylation degree. Monolignol acylation is discussed as a mechanism potentially involved in the control of lignin structure.

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1. Introduction

Lignin is generally considered as formed by the dehydrogenative polymerization of three *p*-hydroxycinnamyl alcohol precursors (Fig. 1): *p*-coumaryl (**1**), coniferyl (**2**) and sinapyl (**3**) alcohols (Boerjan et al., 2003; Fengel and Wegener, 1984; Higuchi, 1997). Each of these monolignols gives rise to a different type of lignin unit called H (*p*-hydroxyphenyl), G (guaiacyl) and S (syringyl) units, respectively. The dehydrogenation reaction is initiated by one-electron oxidation of the phenolic monolignols to their phenoxy radicals by plant cell-wall peroxidases and maybe also laccases. Then, it proceeds by radical coupling between specific positions of two phenoxy radicals, corresponding to their resonant forms, and gives rise to different carbon-carbon and ether linkages. The resulting quinone methides rearomatize by water addition or by reaction with neighbor nucleophiles forming the different dilignols (including β-O-4′, phenylcoumaran and resinols, among others). Polymerization progress requires new activation of both a monolignol molecule and the growing lignin polymer for subsequent radical coupling, which is mainly produced by an end-wise reaction. However, chain branching can also be produced due to the existence of some 5-O-4′ or 5–5′ linkages leaving a free phenolic unit that can be oxidized again giving rise to a second (e.g. 4-O-β″) inter-unit linkage.

Recently, a relatively large number of other phenolic compounds (alcohols, aldehydes, acids, esters and amides) have been reported to act as lignin precursors, to illustrate the structural "plasticity" of the polymer and the adaptability of the lignification mechanisms in plants (Boerjan et al., 2003; Ralph, 2007). However, several of these compounds (such as *p*-hydroxycinnamaldehydes, ferulic acid or 5-hydroxyconiferyl alcohol) only provide a significant contribution to lignin in transgenic plants with modified monolignol biosynthesis (Ralph, 2007; Vanholme et al., 2008) and are minor lignin precursors in normal plants (del Río et al., 2007c). On the other hand, some phenolic compounds with saturated or no side-chains, e.g. dihydrocinnamyl alcohol, sporadically
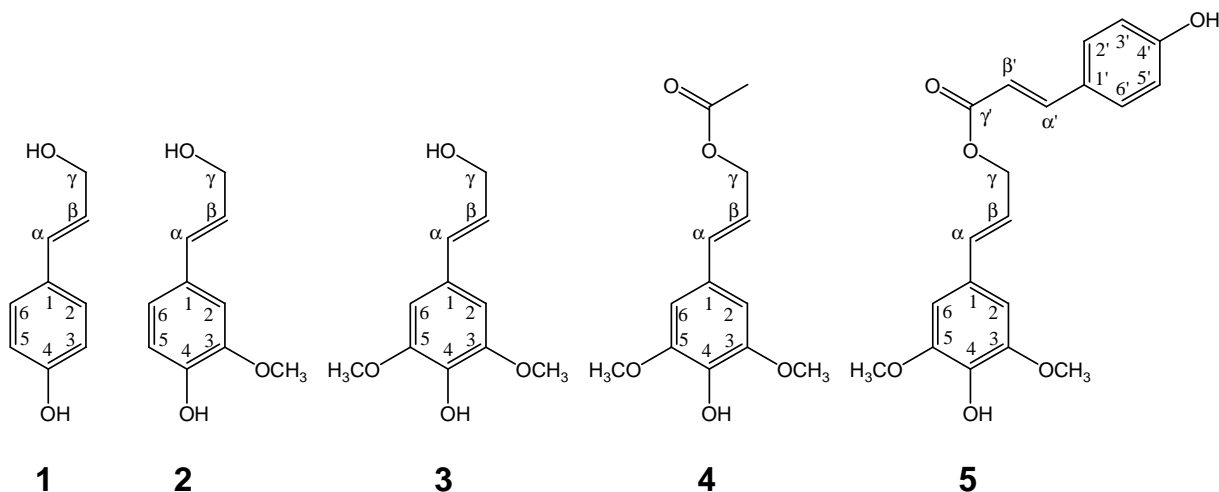
Fig. 1. Classical and acylated monolignols acting as precursors of nonwoody lignins: (1) *p*-coumaryl alcohol; (2) coniferyl alcohol; (3) sinapyl alcohol; (4) sinapyl acetate; and (5) sinapyl *p*-coumarate.

incorporate to the lignin polymer as terminal units as confirmed by 2D NMR (Balakshin et al., 2005; Zhang et al., 2003).

A significant exception to the only sporadic contribution of "non-classic" monolignols has been found in several plants that seem to use acylated monolignols to synthesize highly-acylated lignins. Lignin from kenaf (*Hibiscus cannabinus* L.) bast fibers was one of the first examples including up to 60% acetylated S units (Ralph, 1996) derived from sinapyl acetate (Lu and Ralph, 2002) (Fig. 1, structure **4**). After a preliminary study using analytical pyrolysis (del Río et al., 2004), we recently showed by modified DFRC (derivatization followed by reductive cleavage) that lignin acetylation is widespread among nonwoody angiosperms (del Río et al., 2007b) with some lignins showing even higher extent of acetylation (up to 80% of S units) than kenaf lignin. Surprisingly, different degrees of lignin acetylation were also found in all the woody angiosperms analyzed, attaining up to 45% acetylation of S units in *Carpinus betulus* L. lignin, although it seemed to be absent from gymnosperm lignins. Acetic acid is not the only organic acid esterifying monolignols. Maize (*Zea mays* L.) lignin is *p*-coumaroylated, again predominantly at the S units (Lu and Ralph, 1999; Ralph et al., 1994) that would derive from sinapyl *p*-coumarate (Fig. 1, structure **5**), and the same has been suggested for *Musa textilis* Nee (abaca) lignin (del Río et al., 2007b). Moreover, *p*-hydroxybenzoylation has been reported in lignins from different origins (Landucci et al., 1992; Smith, 1955b; Sun et al., 1999).

The presence of acylating groups in some lignins is known for long time (Smith, 1955a), although only recently it has been shown that lignin acylation is widespread among angiosperms, and recognized that the esterification reaction occurred at the monolignol level (Lu and Ralph, 2002; Lu et al., 2004). However, little is known about the structural or functional role of the lignin acylation processes. The difficulties for recognizing the influence of monolignol acylation on lignin structure are related to the complexity of the lignin polymer and the difficulties for its accurate structural analysis, which are being overcome by the use of modern analytical techniques. Development of 2D (and 3D) NMR provided a powerful tool for lignin analysis, since signals overlapping in the ^{1}H and ^{13}C NMR spectra were resolved revealing both the aromatic units and the different inter-unit linkages present in lignin (Ralph et al., 1999). Signals related to the already known inter-unit linkages were already identified in the first 2D NMR spectra of lignins (Ede and Brunow, 1992; Fukagawa et al., 1991). Moreover, this technique enabled the discovery of dibenzodioxocins (Karhunen et al., 1995) and spirodienones (Zhang and Gellerstedt, 2001) as two new lignin sub-

structures. 2D NMR has been successfully applied to the structural characterization of lignins in wood and their modification in paper pulp manufacturing (Balakshin et al., 2001, 2003; Capanema et al., 2001; Chen et al., 2003; Ibarra et al., 2007a,b). However, studies on nonwoody lignins are comparatively scarce (Crestini and Argyropoulos, 1997; Galkin et al., 1997; Ralph et al., 1994; Sun et al., 2005). In the present paper, lignin preparations from three nonwoody angiosperms – *Agave sisalana* Perrine (sisal), *M. textilis*, and *Cannabis sativa* L., (hemp) were characterized by heteronuclear single quantum correlation (HSQC) 2D NMR. The results obtained suggested a possible role of monolignol acylation regulating the structure of lignin in angiosperm plants.

2. Results and discussion

2.1. Lignins analyzed

2D NMR was used to investigate lignin structure and acylation type and degree in three selected nonwoody angiosperms. Three lignin preparations were analyzed by NMR for each of the plant species investigated: milled-wood-lignin (MWL), *in vitro* acetylated MWL, and alkalilignin. MWL (Björkman, 1956) is often considered as the election preparation in lignin studies, in spite of its low yield and the existence of some modification during milling (Holtman et al., 2006). Moreover, lignin S/G ratio and acylation degree, similar to those described below after MWL isolation, have been found by 2D NMR of the whole plant material at the gel state (Rencoret et al., 2008b). Most NMR studies of lignins were carried out in acetylated samples to increase their solubility (Ralph et al., 1999). However, this prevented detection of natural acetylation, and explains why the widespread occurrence of acetylation in angiosperm lignins reported by del Río et al. (2007b) was not described earlier. In the present study, the HSQC spectra of lignins were acquired from both underivatized and *in vitro* acetylated MWL samples. The former revealed the natural acylation of lignin, whereas the latter informed on free hydroxyl groups and contributed to cross-signal assignment by comparison with the literature. Finally, alkalilignin samples obtained from plant fibers informed on the presence of alkali-labile bonds in lignins. The main cross-signals identified in the different regions of the HSQC spectra are discussed below, followed by a description of the different lignins analyzed, and a discussion on the possible influence of acylation on lignin structure and biosynthesis.