

The DFRC Method for Lignin Analysis. 6. A Simple Modification for Identifying Natural Acetates on Lignins

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By modifying the derivatization followed by reductive cleavage (DFRC) procedure to be completely acetate-free, the presence and regiochemistry of natural acetates on lignins can be determined. Derivatization is conducted with propionyl bromide in propionic acid instead of acetyl bromide in acetic acid; reductive cleavage uses zinc in propionic acid instead of in acetic acid, and the final derivatization step uses propionic anhydride instead of acetic anhydride. Applying the modified procedure to lignins or cell walls from kenaf bast fibers proved that their lignins are highly γ -acetylated, mainly on syringyl units, supporting results of previous NMR work. Application to isolated hardwood lignins or cell walls confirmed reported low-level acetylation and elucidated its γ -regiochemistry. The DFRC method therefore provides a convenient screen for many types of lignin acylation.

Keywords: Lignin; acylation; acetylation; DFRC; lignin acetates; lignin esters; kenaf; Tainung kenaf; aspen

INTRODUCTION

Some lignins have long been known to be naturally acylated by various acids (Smith, 1955), although the biochemistry associated with such acylation remains unresolved. All grasses (both C₃- and C₄-grasses) are *p*-coumaroylated (Monties and Lapierre, 1981; Ralph and Helm, 1993); some hardwood lignins, notably in willow (*Salix*) and aspen (*Populus*), are *p*-hydroxybenzoylated (Nakano et al., 1961; Landucci et al., 1992); and acetates have been implicated in many hardwood lignins (Sarkanen et al., 1967).

Sites of acylation (regiochemistry) become important as the position of attachment of the acyl group suggests its biochemical incorporation pathway (Ralph and Helm, 1993). Attachment at the α -position of the lignin side chain implicates attack by the free acid on quinone methide lignin intermediates in a purely chemical reaction; specific acylation of lignin oligomer/polymer α -OH's following radical-coupling steps is not ruled out but is generally considered less likely. Attachment at the γ -position suggests that the traditional hydroxycinnamyl alcohol lignin monomers **1** are first preacylated and that these acylated monolignols **2** are then incorporated (by traditional radical-coupling mechanisms) into polymeric lignins (Figure 1, pathway a) (Shimada et al., 1971; Nakamura and Higuchi, 1976, 1978a,b; Ralph and Helm, 1993; Ralph et al., 1994). The alternative possibility that acylation occurs following lignification (Figure 1, pathway b), via a transferase enzyme and activated acid, cannot be ruled out. However, the observation (Ralph et al., 1994) that *p*-coumarates on grass lignins are on many types of units (both isomers of β -O-4 units, β -5 units, and even

cinnamyl alcohol end-groups) suggests that enzyme-assisted acylation of the lignin polymer (pathway b) is unlikely—the enzyme would have to be remarkably nonspecific. Regiochemical determinations in lignins are complicated by acyl migration known to occur with acetates (Helm and Ralph, 1993b) and uronates (Li and Helm, 1995) but not *p*-coumarates (Helm and Ralph, 1993a). *p*-Coumarates on grasses are exclusively at the γ -positions of lignin side chains (Ralph et al., 1994; Crestini and Argyropoulos, 1997). *p*-Hydroxybenzoates were originally thought to be partially at the α -position in *Populus* (Nakano et al., 1961) and bamboo (Shimada et al., 1971; Nakamura and Higuchi, 1978a,b), but they may also be (exclusively) at the γ -position (Ralph, unpublished results, 1998). The small amounts of *p*-hydroxybenzoates released by hydrogenolysis of *Populus* lignins (Nakano et al., 1961) and attributed to benzylic esters may easily have come from benzoates on the minor allylic cinnamyl alcohol end-groups—*p*-coumarates were found on such end-groups in maize lignins (Ralph et al., 1994). Recent NMR studies showed that *p*-hydroxybenzoates on willow lignins are free-phenolic (Landucci et al., 1992), analogous to *p*-coumarates.

The recently developed derivatization followed by reductive cleavage (DFRC) method is a degradation procedure that produces analyzable monomers **5** and dimers by cleaving α - and β -ethers in lignins **3** (Lu and Ralph, 1997a–c, 1998a–c; Peng et al., 1998a,b; Ralph et al., 1998). Although the mechanism is completely different, it is conceptually similar to methods such as acidolysis (Lundquist, 1992) and thioacidolysis (Lapierre, 1993). One advantage of the method is that γ -ester groups on lignins **4** remain intact (Lu and Ralph, 1998c). The method has allowed us to confirm that *p*-coumarate groups are at the γ -positions of grass lignins, predominantly on syringyl units of the grasses examined (Lu and Ralph, 1998c,d). Without modification, however,

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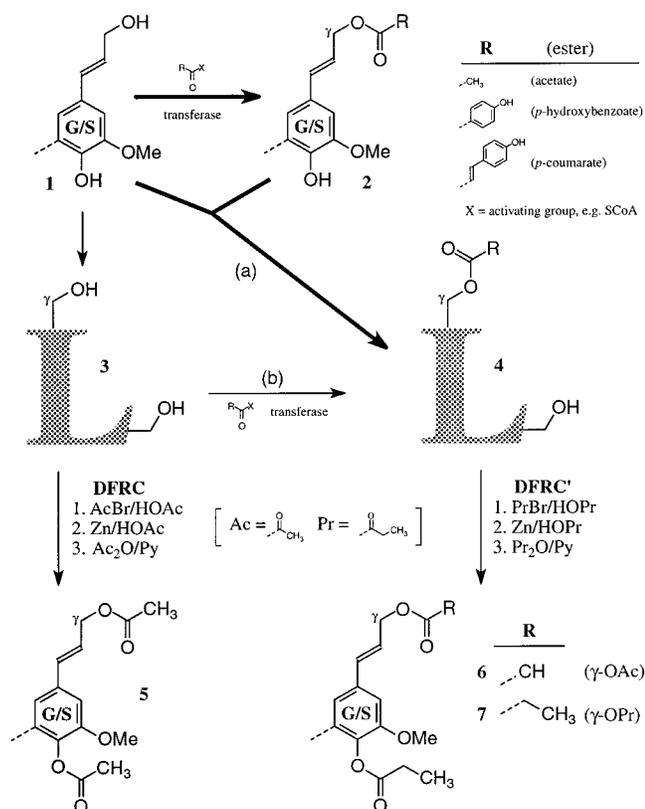


Figure 1. Formation and degradation pathways for acylated lignins **4**. Monolignols **1** can be acylated by suitable activated acids (acetic, *p*-hydroxybenzoic, *p*-coumaric, and others are known in nature) via transferase enzymes to monolignol esters **2**, presumably before diffusing into the cell wall. Esters **2**, along with monolignols **1**, can then be incorporated via radical coupling reactions into lignins **4** with partial γ -acylation (pathway a). Alternatively, normal lignins **3** can be subsequently acylated (pathway b). The normal DFRC protocol cleaves α - and β -ethers (in unacylated lignins **3**) releasing, following acetylation, quantifiable monomers **5**. Lignin γ -esters (such as in **4**) are not cleaved during the normal DFRC protocol, and the method can be used for identifying *p*-coumarate, *p*-hydroxybenzoate (and presumably other) esters (not specifically shown in this figure). A modification to the DFRC protocol, substituting all acetate-based reagents with their propionate analogues (DFRC' protocol), allows determination of acetates that are naturally on lignin γ -positions. Thus, normal lignin β -ether units can release the 4, γ -dipropoxy monomers **7**, whereas units originally bearing γ -acetates will release 4-propoxy- γ -acetoxy monomers **6**. Releasable units originally bearing acetates can then be readily distinguished from normal (unacylated) units by GC.

any information about acetate groups is lost because the DFRC method utilizes various acetate-containing reagents in its protocol. Information on natural lignin acetates has also been scarce from NMR studies, in which lignins are frequently acetylated for improved NMR properties or purified using acetic acid (Björkman, 1956; Lundquist et al., 1992) when acetylation artifacts might arise.

The need for an independent method to determine acetates on native lignin follows from our discovery of high levels of acetate on kenaf bast fiber lignins (Ralph, 1996) and a general disbelief of the results by other researchers. DFRC provides unequivocal new evidence that kenaf bast fiber lignins are indeed highly γ -acetylated (in nature) and that such acetylation is disproportionately high on syringyl units.

Two DFRC modifications avoid acetates in all steps of the protocol. One uses trideuterioacetate reagents and

looks for isotopically normal acetate in the monomeric products by GC/MS. Apart from the expense and inconvenience of preparing such isotopically labeled reagents, the requirement for GC/MS determinations of labeled versus unlabeled monomers is not simple enough to be carried out routinely. The second method, in which all acetate-based reagents are replaced by their propionate analogues, is therefore the method chosen here. It is then a simple matter to differentiate fully propionylated monomers **7** (which come from normal unacylated lignin units) from any γ -acetylated monomers **6**, which must come from γ -acetylated units in the native lignin (Figure 1).

EXPERIMENTAL PROCEDURES

General. All reagents were from Aldrich (Milwaukee, WI) and used as supplied. Solvents (AR grades) were from Mallinckrodt (Paris, KY).

Cell Wall and Lignin Isolations. Cell walls were isolated simply by solvent-extracting ground (2 mm, Wiley mill) lyophilized plant-stem material. The kenaf was from the bast fiber fraction only because its core contains lignin of a different composition (Seca et al., 1998). Solvent extraction was sequentially with water, methanol, acetone, and chloroform. Lignin isolations were from ball-milled material following described procedures (Ralph et al., 1994; Ralph, 1996). No purification steps involving acetic acid were used, and no steps in the extractions or isolations involved any acetyl-containing reagents.

Modified DFRC (DFRC') Procedure. Lignin (5 mg) was stirred overnight at room temperature with propionyl bromide in propionic acid (1:2, 3 mL). Alternatively, the solvent-extracted cell wall sample (30 mg) was stirred for 16 h at 50 °C with 5 mL of the same reagent. The solvents and excess bromide were removed at 50 °C under a stream of nitrogen and then at high vacuum (~50 mTorr). The product was then dissolved in 8 mL of dioxane/propionic acid/water (1:1:0.1), and 100 mg of powdered Zn was added. After 30 min of stirring, the product was worked up as usual (Ralph et al., 1994; Ralph, 1996). Propionylation with propionic anhydride in pyridine completed the procedure. Analysis was by GC and/or GC/MS as described previously. Absolute yields were not calculated because response factors for the variously acetylated and propionylated monomers were not determined. Monomer yields from normal DFRC on these materials were approximately 1260 $\mu\text{mol/g}$ for kenaf lignin and 900 $\mu\text{mol/g}$ for aspen lignin.

RESULTS AND DISCUSSION

Propionyl bromide in propionic acid readily derivatized and dissolved lignin samples but was a little less efficient than the normal acetyl bromide system for whole-cell-wall samples; some finely divided insoluble material remained. Use of fresh propionyl bromide was crucial: reagent from an old bottle of unknown purity and composition did not dissolve even isolated lignin samples.

The GC spectra are easy to interpret, particularly when aided by MS. Figure 2a shows the monomeric products **6** and **7** that resulted from the modified DFRC procedure applied to isolated lignin from kenaf bast fiber (Ralph, 1996); this lignin was never subjected to acetyl reagents in any form. Compounds **7** arise from normal (γ -OH) units in lignins, whereas compounds **6** (γ -OAc) arise from originally γ -acetylated units in lignins **4** ($R = \text{CH}_3$). The predominant 4-propoxysyringyl- γ -acetate peak **S-6** proves that syringyl units were highly acetylated in the isolated lignin. The same procedure applied to whole cell walls from kenaf bast fiber produced the

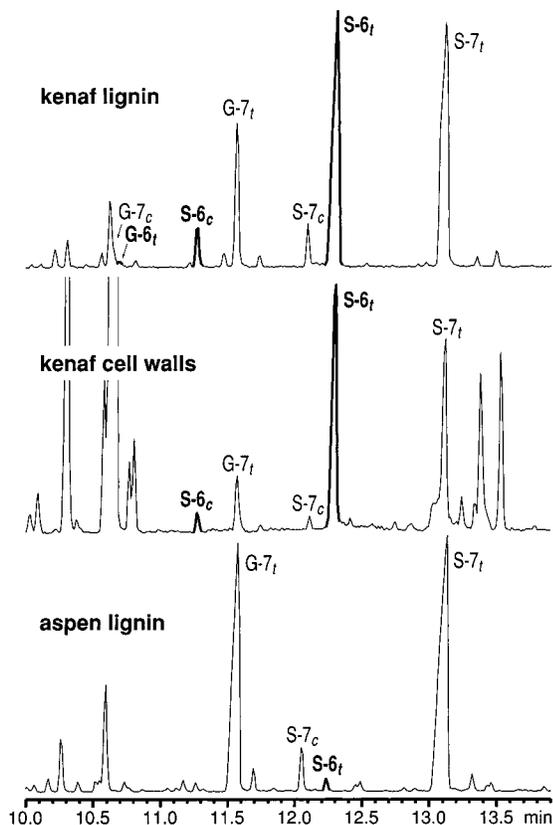


Figure 2. GC/MS total ion chromatograms of monomers from the modified DFRC procedure applied to (a, top) isolated Tainung kenaf lignin, (b, middle) Tainung kenaf whole-cell walls, and (c, bottom) isolated aspen lignin. Components **S-6** and **G-6** (bold peaks) were from acetylated units in the original lignin; **S-7** and **G-7** were from normal (unacetylated) units. *c* = cis; *t* = trans. The peak labeled **S-6_t** in (c) contains a significant coeluting component that was not identified. GC/MS is therefore crucial for compound authentication.

chromatogram in Figure 1b. This sample had been extracted only with toluene/ethanol to remove extractives. Obviously, the unisolated cell wall lignin also had syringyl units that were heavily acetylated. In both cases, only minor amounts of acetate were on guaiacyl units, **G-6**. Although the guaiacyl component of kenaf lignins is strikingly low, the preference for acetylation of syringyl components suggests a specific enzymatic process (although chemical acetylation may also be selective). Presumably this is the acetylation of sinapyl alcohol **S-1** via a transferase and activated (e.g., S-CoA) acetic acid. Strictly speaking, these observations apply to uncondensed structures because we are looking at only monomeric DFRC products here, but if lignification is through preacetylated hydroxycinnamyl alcohols, the products are likely to be representative of the whole lignin.

The modified DFRC procedure also allowed detection of minor acetate components in hardwood lignins. Thus, for example, the chromatogram from isolated aspen lignin also showed a small 4-propoxysinapyl- γ -acetate **S-6** peak; the labeled peak actually contains two components, so the compound really is quite minor but definitely present as confirmed by MS. The analogous guaiacyl **G-6** peak could not be detected. The methods applied here do not exclude acetylation at other positions in hardwoods, but apparently, as in kenaf, a similarly selective transferase enzyme exists in aspen

for acetylating sinapyl alcohol prior to its export to the wall for lignification.

CONCLUSIONS

Modification of the DFRC protocol by use of propionate analogues of normal reagents and solvents allows acetates in lignins to be unambiguously detected and confirms their presence at the γ -positions of lignin side chains. Kenaf bast fiber lignin is naturally highly acetylated as originally reported (Ralph, 1996), overwhelmingly on syringyl units; releasable sinapyl monomers were >50% acetylated in Tainung kenaf (reported here) and in three other varieties (Ralph, 1998, unpublished data). Supporting literature observations (Sarkanen et al., 1967), selected hardwood lignins were only slightly γ -acetylated, again predominantly on syringyl units. The results suggest relatively specific acetylation of sinapyl alcohol prior to lignification. The modified DFRC protocol is a powerful new method for analyzing naturally acetylated lignins and pinpointing acetylation sites. It will allow researchers to screen hardwoods and other plant materials for the presence of acetates on lignins, although it is recommended that GC/MS (or LC/MS) be used for product authentication. The propionyl modification described provides a useful complement to the acetyl DFRC method and other lignin analytical methods.

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