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Elucidation of Lignin Structure through Degradative Methods: Comparison of Modified DFRC and Thioacidolysis

KEVIN M. HOLTMAN, HOU-MIN CHANG, HASAN JAMEEL, AND JOHN F. KADLA*

College of Natural Resources, North Carolina State University, Raleigh, North Carolina 27695-8005

Milled wood and milled wood lignin (MWL) samples were subjected to DFRC and thioacidolysis. Despite the fact that both methods selectively cleave aryl ether bonds, substantial differences in results were obtained. Lignin thioacidolysis gave total molar yields of degradation monomer products in the range of 3.5−7 mol % higher than DFRC. GPC analysis showed that the thioacidolysis-treated lignin was degraded to a lower average molecular weight than that treated by DFRC. Contrary to results reported for lignin model compounds, these results indicate that the DFRC method does not completely or efficiently degrade the lignin polymer. In fact, the DFRC-degraded lignin retained much of the characteristics of the original MWL. Elemental analysis revealed the presence of bromine in the DFRC-treated lignin, and two-dimensional $^{1}H-^{13}C$ HMQC NMR spectroscopy showed the presence of β-*O*-4 linkages in the DFRC-treated lignin. No β-*O*-4 interunit linkages were detected in the thioacidolysis-treated lignin. These results are consistent with the lower monomer yields and the higher average molecular weight of the DFRC-treated lignin and indicate inefficiency in the chemistry of the method, probably due to steric constraints of the polymeric nature of lignin.

KEYWORDS: Lignin thioacidolysis; derivatization followed by reductive cleavage (DFRC); HMQC NMR spectroscopy; milled wood lignin (MWL); GPC; *β*-*O*-4 linkages

INTRODUCTION

Lignin, arguably the second most abundant natural polymeric material on earth, is extremely complicated, and its structure has not yet been completely elucidated. Lignin in situ has no structural regularity. Unlike most natural polymers, which consist of a single intermonomeric linkage, lignin is a network polymer made up of many carbon-to-carbon and ether linkages. The tight physical binding and chemical linkages between lignin and cell wall polysaccharides also practically prevent its isolation in unaltered form. This makes it very difficult to use degradative or nondegradative methods for structural determination. As a result, our understanding of the structure of lignins, either in situ or in pulps, is formed as a sum of the information obtained from different fields of lignin research: studies concerning the elucidation of the mechanisms of lignin biosynthesis (*1*) and analytical data obtained in studies with isolated lignin specimens (*2*, *3*).

Most of the conclusions pertaining to the bonding pattern of native and residual lignins have been derived from degradative methods such as hydrogenolysis (*4*), acidolysis (*3*), and thioacidolysis (*5*−*7*). Through careful analyses of the low molecular weight compounds generated, a detailed picture of the original lignin can emerge. Unfortunately, these methods, which utilize gas chromatography (GC) to detect and quantify the degradation products, analyze only the monomeric or dimeric degradation products. They are confined to phenylpropanoid units that are linked via aryl ether linkages. Therefore, only noncondensed lignin structures are quantified. Nonetheless, a large amount of structural information can still be obtained.

Of the various degradative methods, thioacidolysis is proving to be one of the most widely utilized and effective diagnostic methods in the study of lignin structure. It is routinely used to estimate the amount and composition of uncondensed aryl ether structures. Thioacidolysis is an acid-catalyzed solvolysis reaction in dioxane−ethanethiol catalyzed by boron trifluoride etherate, which leads to the depolymerization of lignins. Specifically, thioacidolysis proceeds by either cleavage of α-ethers or substitution of α-hydroxyl groups by the thioethyl group, followed by β-aryl ether cleavage and ultimately the formation of trithioethyl monomeric products. However, thioacidolysis uses malodorous ethanethiol and is therefore not desirable for many researchers to use.

In 1997, Lu and Ralph (*8*−*10*) introduced a new selective α,β-aryl ether cleavage protocol, derivatization followed by reductive cleavage (DFRC). The DFRC method uses mild conditions and no malodorous chemicals. DFRC consists of three simple steps: bromination of the benzylic position and concomitant acetylation of free hydroxyl groups by acetyl bromide; reductive cleavage of the β-aryl ether bonds via zinc metal coordination; and acetylation of newly generated phenolic hydroxyl groups for quantification by gas chromatography. However, as with thioacidolysis the amount of structural information obtainable from DFRC is limited to noncondensed

* Corresponding author [telephone (919) 513-2455; fax (919) 515-6302; e-mail jfkadla@ncsu.edu].

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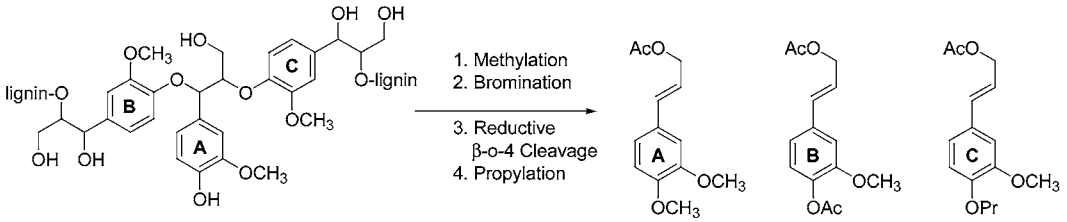

Figure 1. Modified DFRC method (unit A, from phenolic β-*O*-4 units; unit B, from α-*O*-4 units; unit C, from etherified β-*O*-4 units) (*12*).

structural moieties. Recently, Tohmura and Argyropoulos (*11*) and Ikeda et al. (*12*) have modified the DFRC protocol to further enhance the amount of structural information obtainable.

The DFRC method has been reported as being quantitative in the yields of low molecular weight products, with >92% yield of targeted products in model compound studies (*9*). By contrast, thioacidolysis monomer yields are reportedly much lower (*5*, *6*). However, both methods have recently been questioned as to the completeness of aryl ether cleavage and the quantitative recovery of the desired monomers (*12*). Therefore, a detailed investigation into the completeness of aryl ether cleavage and monomer yield from DFRC and thioacidolysis is warranted. In this study, vibratory-milled wood and milled wood lignin (MWL) prepared from loblolly pine were used as samples of native lignin and an isolated lignin, respectively. Both lignins were subjected to DFRC and thioacidolysis, and monomer yields were quantified by GC analysis. The resulting product mixtures were also analyzed by gel permeation chromatography (GPC) and $^1H-^{13}C$ 2D correlation NMR spectroscopy.

MATERIALS AND METHODS

Materials. *N*-Methyl-*N*-nitroso-*p*-toluenesulfonamide (Diazald), acetyl bromide, glacial acetic acid, zinc dust (50 mesh), ethanethiol, boron trifluoride etherate, sodium hydrogen carbonate, bis(timethylsilyl)-trifluoroacetamide (BSTFA), deuteriochloroform, propionic anhydride, and tetracosane were all purchased from Aldrich (Milwaukee, WI) and used as received. Diethyl ether, methanol, ethanol, potassium hydroxide, methylene chloride, acetic anhydride, and pyridine were purchased from Fisher Scientific and used as received. 1,4-Dioxane was purchased from Fisher Scientific (Suwanee, GA) and distilled over $NaBH_4$ and blanketed with argon before use.

MWL and vibratory-milled wood were produced from loblolly pine (*Pinus taeda* L.) sapwood. The sapwood was ground to pass a 20 mesh screen in a Wiley mill and Soxhlet extracted with ethanol/benzene (1:2, v/v) for 24 h, followed by ethanol for an additional 24 h. The milled wood (100 g) was ground for 1 week in a 1-gal porcelain jar under a nitrogen atmosphere using glass balls. This was followed by 48 h of vibratory milling under a nitrogen atmosphere in the presence of toluene in a vibratory ball mill (Siebtechnik GmbH, Mulheim, Germany) with stainless steel balls (vibratory-milled wood). MWL was then isolated from the vibratory-milled wood according to the method of Björkman (*13*).

DFRC Procedure. The DFRC and modified DFRC procedures were identical to those reported in the literature (*9*, *12*).

Thioacidolysis Procedure. The thioacidolysis and modified thioacidolysis procedures were performed as described elsewhere (*5*, *14*).

Gas Chromatography Analysis. Monomeric products produced from the DFRC and thioacidolysis treatments were quantitatively determined by GC (Hewlett-Packard 6890). The analytical column was a 30 m × 0.32 mm i.d. HP-1 (Hewlett-Packard). The carrier gas was helium with a flow rate of 2.0 mL/min. The GC conditions were as follows: injection temperature, 220 °C with a split ratio of 10:1; FID detector temperature, 310 °C; column temperature, 100 °C, which was held for 1 min, raised at 3 °C/min to 240 °C, held for 1 min, raised at 30 °C/min to 300 °C, and held for 5 min. The amounts of individual monomers were determined using response factors derived from pure compounds relative to tetracosane as the internal standard (*12*, *15*).

Sample Preparation. Prior to GPC, NMR, and elemental analyses, reaction solvents were removed and the samples exhaustively dried. Specifically, the solvent was evaporated at 40 °C and 20 mTorr reduced pressure until the presence of any solvent (e.g., acetic anhydride or pyridine in the case of DFRC) could no longer be detected. The samples were then placed in a vacuum desiccator and dried at 40 °C for 72 h. The samples were then ground to a fine powder and stored in a drying pistol over P_2O_5 and refluxing chloroform until analyses were performed.

Gel Permeation Chromatography. GPC analyses were performed on a Waters HPLC system at ambient conditions using two μ-Styragel columns (HR-4 and 5E) connected in series. THF was the eluent (0.5 mL/min), and fractions were monitored using refractive index (Waters refractometer model 410) and UV absorbance at 280 nm (Waters UV spectrometer model 484). All lignins were analyzed after excess reagent was removed by evaporation. The derivatized lignins and the DFRC and thioacidolysis product mixtures were dissolved in THF at a concentration of 1 mg/mL, and 120 μL of this solution was then injected into the HPLC. Molecular weight determinations were made using polystyrene as a calibration standard.

^1H—^{13}C 2D Correlation NMR Spectroscopy. Spectra of the lignin and DFRC and thioacidolysis degradation product mixtures were recorded on an Avance 500 MHz spectrometer (Bruker, Billerica, MA) using a narrow-bore magnet (Oxford, U.K.) in $CDCl_3$; the chloroform-*d* peak at 77.25/7.265 was used as internal reference. About 30 mg of dry lignin or dried material after DFRC or thioacidolysis degradation was accurately weighed and dissolved in 0.75 mL of $CDCl_3$. The system was controlled by the SGI INDY host workstation, and the data were processed with XWIN NMR. The instrument was equipped with three frequency channels with waveform memory and amplitude shaping, three channel gradient control units (GRASP III), and one variable temperature unit, as well as one unit for precooling and temperature stabilization. All measurements have been carried out with a 5 mm i.d. ^1H/BB ($^{109}Ag-^{31}P$) triple-axis gradient ID500-5EB probe (Nalorac Cryogenic Corp., Martinez, CA). The operational frequency for ^1H nucleus was 500.128 MHz, and conditions for analysis included a 90° pulse width of 10 μ and a 1.5 s pulse delay (d_1).

Bromine Analysis. Bromine analysis was performed by Complete Analysis Laboratories, Inc., Parsippany, NJ.

RESULTS AND DISCUSSION

Comparison of Monomer Yields from Thioacidolysis and DFRC Treatment of Milled Wood and MWL. Recently, we demonstrated that the DFRC method could be modified to provide additional structural information through the determination of three different structural monomeric products originating in lignin (*12*). In this protocol, the free phenolic hydroxyl groups in lignin are methylated by diazomethane followed by the same acetyl bromide treatment and zinc reduction steps as in the original DFRC procedure. In the final step, propionic anhydride, instead of acetic anhydride, is used to esterify the newly generated phenolic hydroxyl groups. Thus, the modified DFRC method allows the quantitative determination of three different monomeric units in lignin; the uncondensed phenolic β-*O*-4 (unit A), the uncondensed α-*O*-4 (unit B), and the uncondensed etherified β-*O*-4 (unit C) structures (**Figure 1**).

Whereas DFRC is a flexible method due to its three distinctly separate steps, thioacidolysis does not have this utility. Thio-

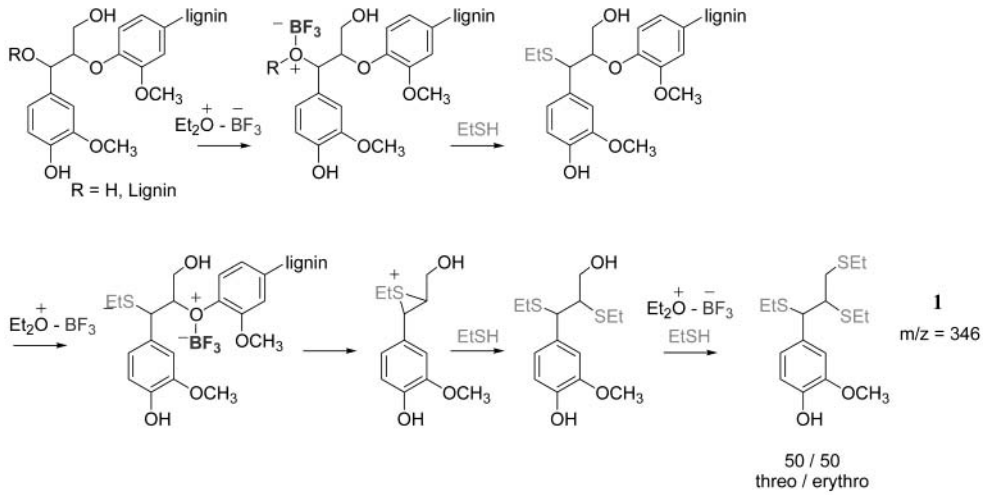

Figure 2. Proposed mechanism of lignin thioacidolysis (*7*).

Table 1. Total Yields and Unit Composition Data for Modified DFRC and Thioacidolysis Analysis of Vibratory-Milled Wood and MWL

	modified DFRC				modified thioacidolysis		
	unit A, %	unit B, %	unit C, %	total yield, μmol/g	unit A, %	units B + C, %	total yield, μmol/g
milled wood	26.7	7.8	65.5	813	21.8	78.2	1198
MWL	41.3	7.7	51.0	797	33.4	66.6	995

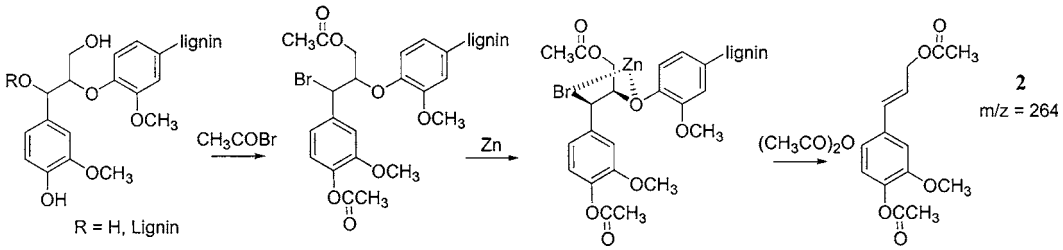

Figure 3. Proposed mechanism of DFRC degradation of lignin (*22*).

acidolysis follows a pathway similar to kraft pulping in which the thiol group displaces the α-hydroxy or α-ether group and the *β*-aryl ether to form an episulfide-type intermediate (**Figure 2**). Because these reactions all proceed during the single thioacidolysis step, such derivatizations as in the modified DFRC method cannot be performed. Therefore, thioacidolysis can differentiate only between phenolic hydroxyl groups (unit A) and aryl ethers, which will be referred to as units B + C.

The mechanism by which thioacidolysis and DFRC degrade various lignin structures has been thoroughly investigated using specific lignin model compounds (*7*, *16*). In both thioacidolysis and DFRC the predominant *β*-aryl ether structures in lignins are selectively degraded as depicted in **Figures 2** and **3**.

Each method releases monomeric products from an uncondensed portion of the lignin (i.e., a monomer linked by a *β*-*O*-4 linkage through both the *β*- and the 4-positions) or a *β*-*O*-4-linked monomer end group. Therefore, it is expected that the yield of monomeric degradation products detected would be comparable. However, **Table 1** shows that the modified thioacidolysis gives much higher monomer yields than the modified DFRC: 813 versus 1198 μmol/g, respectively, for the vibratory-milled wood and 995 versus 797 μmol/g, respectively, for the MWL. These results are in good agreement with literature values (*8*−*10*, *17*, *18*) and clearly indicate that the DFRC method is inefficient at cleaving *β*-aryl ether linkages.

According to the reaction mechanism proposed for DFRC degradation of *β*-*O*-4 linkages (**Figure 3**), a cis orientation of the bromine and *β*-*O*-4 ether oxygen is required. The inability to obtain such geometry would preclude the reductive cleavage of the *β*-*O*-4 ether linkage, and the α-brominated structure would persist. In fact, Iiyama and Wallis (*19*) reported a 9% bromine content in the acetyl bromide dissolution of wood pulp (*Pinus radiata*). Likewise, Ralph et al. (*20*) showed that cinnamyl model compounds on DFRC treatment gave rise to brominated aryl propanes. Bromine analysis of the DFRC degraded lignin revealed a 3 mol % bromine content.

Another possible explanation for the ineffective cleavage of *β*-*O*-4 linkages by the DFRC method may be due to hydrolysis of the α-bromide on etherified phenylpropanoid units prior to reductive cleavage (*21*). Prolonged exposure of the lignin to AcBr or the presence of water can facilitate the elimination of the α-bromo group and the replacement with an α-OAc group.

Comparison of the yields of phenolic hydroxyl groups (unit A) obtained from the modified DFRC and thioacidolysis reveals that the unit A composition from the DFRC method is higher than that from thioacidolysis for either the vibratory-milled wood or the MWL. The percent increases for both samples are essentially the same, that is, 18% for the milled wood and 19% for the MWL. This percent increase may be explained by the fact that there are *β*-*O*-4 linkages remaining in the DFRC degradation product. These linkages are likely of the etherified *β*-*O*-4 type (unit C), rather than end groups (unit A). Therefore, complete cleavage would result in a higher relative unit C composition and a lower relative unit A composition.

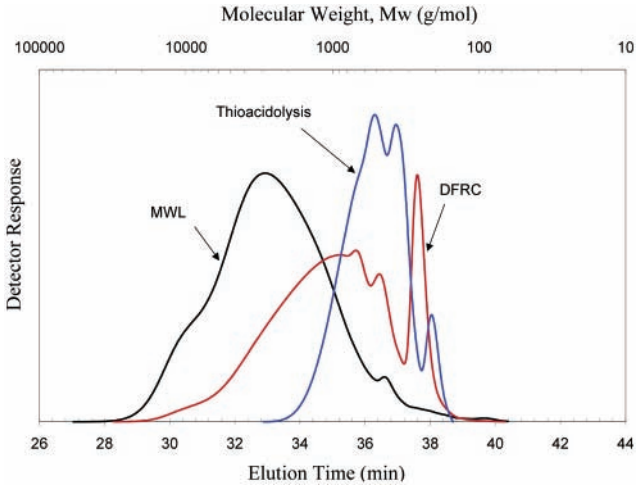

Figure 4. GPC chromatographs of MWL before and after thioacidolysis and DFRC treatment.

Effect of Thioacidolysis and DFRC on the Molecular Weight Distribution of Lignin. Figure 4 shows the GPC chromatograms of the MWL before and after thioacidolysis and DFRC treatment. Comparison of the chromatographs of the two treatments reveals substantial differences. Thioacidolysis has much more completely degraded the lignin than DFRC. As can be seen in **Figure 4**, thioacidolysis degraded the lignin polymer to a significant extent, and it no longer has any resemblance to the initial MWL molecular weight profile. The GPC chromatogram shows the presence of three sharp peaks, which calibrated against polystyrene standards are below an average relative molecular mass of 700 Da. The first peak is centered near 650 Da and is probably composed of dimeric material. The second is centered near 350 Da and probably represents **1** (*m*/*z* 346), the uncondensed monomer released from aryl ether cleavage. The final peak is centered near 250 Da and represents other monomeric material. This observation implies that thioacidolysis caused significant depolymerization of the lignin and that a significant number, if not all, of the *β*-*O*-4 interunit linkages have been cleaved. Again, as with the DFRC protocol, incomplete cleavage could occur in lignin thioacidolysis if the method is not carefully optimized (*22*).

By comparison, the DFRC-treated MWL exhibits a GPC trace that maintains much of the characteristics of the original MWL curve (**Figure 4**). Like the thioacidolysis-treated MWL, the DFRC-treated MWL has two peaks that are representative of low relative molecular weight moieties, monomers and dimers. However, a large amount of high relative molecular weight highly polydispersed material similar to the initial MWL is still present. Probably due to incomplete *β*-aryl ether cleavage, these findings are consistent with and support the lower monomer yields detected by GC analyses from DFRC degradation relative to thioacidolysis. In addition, the lower relative incidence of monomers from etherified *β*-*O*-4 linkages (unit C) verifies that the decrease in yield is attributable to internally located moieties that remain uncleaved and result in the observed higher degree of polymerization. It should be noted that the relative intensities of the monomeric peaks by no means imply concentration. The UV absorbance will be dependent on the compound structure, with compounds having double bonds (**2**) having stronger absorbance than those without (**1**) or the higher relative molecular weight fractions.

^{1}H—^{13}C HMQC NMR Analysis of Thioacidolysis- and DFRC-Degraded Lignins. To minimize the number of structural differences in the analyzed lignins and ease the interpretation of the NMR spectroscopic data, the standard DFRC protocol was used. **Figure 5** show the HMQC spectra for the MWL and DFRC- and thioacidolysis-degraded lignins.

The aliphatic oxygenated region of the HMQC spectra of the DFRC-treated MWL (**Figure 5b**) exhibits cross signals of δ_C/δ_H 80.0/4.63 and δ_C/δ_H 73.9/6.0 corresponding to C_β/H_β and C_α/H_α respectively, for *β*-*O*-4, **3** (**Figure 5**), structures. The intensity is substantially lower than the corresponding signal in the spectrum of the original MWL (**Figure 5a**). By comparison, these signals are not observed in the HMQC spectra of the thioacidolysis lignin (**Figure 5c**). This is consistent with the results obtained from GC and GPC analyses and confirms that complete *β*-aryl ether cleavage occurs as a result of thioacidolysis, but is incomplete in the DFRC procedure. In thioacidolysis the lignin macromolecule undergoes a series of thioethylation reactions (**Figure 2**). Under the acidic reaction conditions used the lignin becomes completely solublized. This facilitates better accessibility of the reagents to the lignin and as a result more complete lignin degradation. Because DFRC fully solubilizes the lignin, it is not clear, however, as to why the DFRC method does not completely cleave all of the *β*-*O*-4 linkages. This may be due to accessibility issues, inefficiency in the chemistry of the method, or the existence of lignin units that are less susceptible to cleavage by DFRC.

One possibility for the observed difference is that dibenzodioxocin structures, **6**, which contain both α-aryl and *β*-aryl ether linkages through the phenolic groups of a biphenyl (5-5′) condensed lignin structure, are some how stable to DFRC (*11*). The DFRC mechanism (**Figure 3**) proceeds through a five-membered Zn-coordination step, depending on the stereochemistry, *erythro* versus *threo*; the lignin macromolecular structure about this region may be too rigid to allow this coordination. Thus, the bromination and resulting α-aryl ether cleavage step may occur, but the *β*-aryl ether reductive cleavage will be prevented, leaving the unreacted *β*-*O*-4 linkage intact. **Figure 5a** clearly shows the existence of **6** in the original MWL; however, the cross signals δ_C/δ_H 84.5/4.84 and δ_C/δ_H 82.6/4.13 corresponding to C_α/H_α and C_β/H_β, respectively, are absent in the DFRC-degraded material (**Figure 5b**). Although during DFRC degradation α-bromination will result in ring-opening (cleavage of the α-*O*-4 linkage), the typical *β*-*O*-4-linked structure (δ_C/δ_H 80.0/4.63 and δ_C/δ_H 73.9/6.0 corresponding to C_β/H_β and C_α/H_α, respectively) and the ability to differentiate the dibenzodioxocin structure will be lost. In fact, it has been shown that rearrangement reactions seen in model compound studies under DFRC conditions do not occur in MWL as a result of steric factors, which imposed rotational restrictions within the lignin macromolecule (*23*).

In addition to 5-5′ linkages, lignins contain other interunit carbon−carbon bonds: *β*-5, *β*−*β*′, *β*-1, etc. Of these subunits, the *β*-5 (**4**) and *β*-*β*′ (**5**) structures contain α-aryl ether linkages that one would expect to be cleaved during DFRC (**Figure 3**). The cleavage of these bonds would not release a monomeric unit detectable by GC, but incomplete cleavage would be detectable using the HMQC correlation signals. Lignin moieties such as **5** show cross signals δ_C/δ_H 85.4/4.78/δ_C/δ_H 85.1/4.67 for the respective C_α/H_α and δ_C/δ_H 71.8/4.27/δ_C/δ_H 71.9/3.93 for the respective C_γ/H_γ correlations. The HMQC spectrum of the DFRC degradation products clearly indicates that these ether linkages have been cleaved.

The HMQC spectra for **4** has cross signals δ_C/δ_H 88.2/5.50 and δ_C/δ_H 50.4/3.77 for the C_α/H_α and C_β/H_β correlations, respectively (*24*). Although the intensities of these correlations are clearly reduced in the DFRC-reacted MWL (**Figure 5b**)

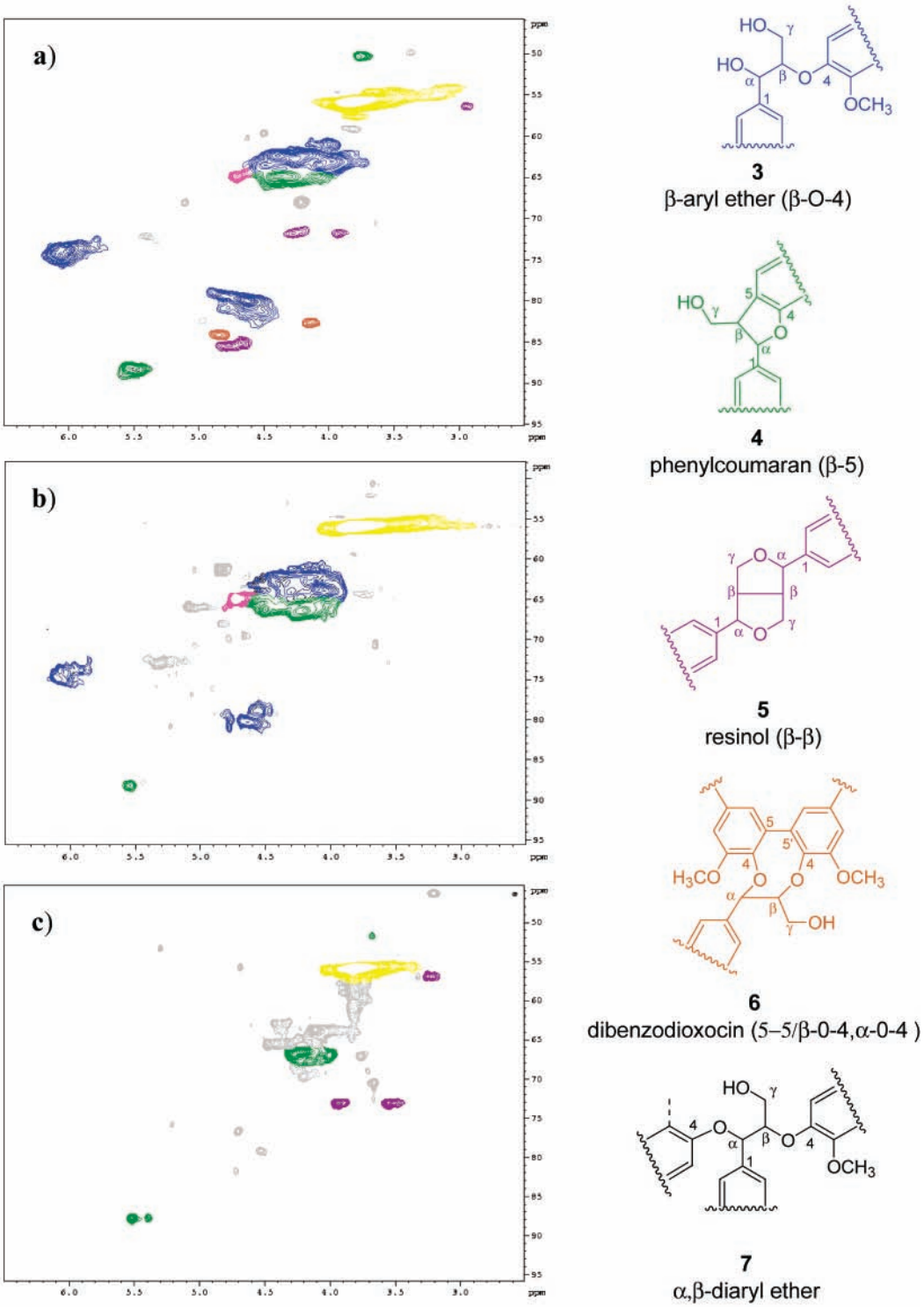

Figure 5. Expansion of the oxygenated aliphatic region of the ^{1}H–^{13}C HMQC spectrum of the (a) acetylated MWL, (b) acetylated DFRC degraded MWL, and (c) acetylated thioacidolysis degraded MWL.

relative to the original MWL (**Figure 5a**), it is apparent that *β*-5 structures remain in the DFRC degradation products. However, it is unclear as to whether this is the result of incomplete bromination or displacement of the bromide ion via an intramolecular etherification reaction.

Thioacidolysis of milled wood and MWL produced 7 and 3.5 mol %, respectively, higher monomer yields than DFRC. GPC analysis revealed the thioacidolysis-treated lignins were degraded to a lower average molecular weight than those degraded by DFRC. In fact, the DFRC-treated MWL retained much of the characteristics of the original lignin. Two-dimensional $^{1}H-^{13}C$ HMQC NMR spectroscopy showed the presence of *β-O*-4 linkages in the DFRC-treated lignin. No *β-O*-4 interunit linkages were detected in the thioacidolysis-treated lignin (**Figure 5c**). In addition, the DFRC-treated MWL had a ~3 mol % bromine content. Contrary to results reported for lignin model compounds, these findings indicate that the DFRC method does not completely or efficiently degrade the lignin polymer. The presence of elemental bromine within the lignin, combined with the existence of *β-O*-4 interunit linkages

and the high average relative molecular weight, suggests the DFRC treatment of the lignin may be affected by the rigid three-dimensional structure of the lignin macromolecule; restricted rotational mobility of certain C9 units precludes the formation of the necessary geometry to enable reductive cleavage of all of the β-*O*-4 interunit linkages.

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