

A SOLUTION-STATE NMR APPROACH TO
ELUCIDATING PMDI-WOOD BONDING
MECHANISMS IN LOBLOLLY PINE

by

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A dissertation submitted in partial fulfillment of
the requirements for the degree of

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A dissertation entitled

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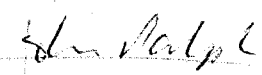
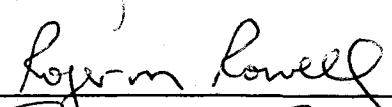
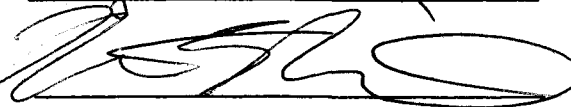
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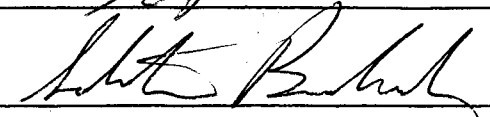
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ABSTRACT

Solution-state NMR spectroscopy is a powerful tool for unambiguously determining the existence or absence of covalent chemical bonds between wood components and adhesives. Finely ground wood cell wall material dissolves in a solvent system containing DMSO- d_6 and NMI- d_6 , keeping wood component polymers intact and in a near-native state. 2D NMR experiments, using ^{13}C - ^1H one-bond HSQC on non-derivatized cell wall material from loblolly pine reveal details about the major cell wall polymers. This technique can determine covalent bond formation between cell wall polymers and wood adhesives. Monofunctional model compounds of pMDI were reacted with loblolly pine under moisture-controlled conditions to derivatize the wood cell wall polymer hydroxyls. The reacted loblolly pine was ball-milled, dissolved, and characterized via 2D NMR experiments. NMR chemical shift data from carbamylated wood model compounds were then used to assign carbamate signals in the HSQC spectra from carbamylated wood polymers. The data obtained allow us to determine whether covalent bonds form between loblolly pine and pMDI at different adhesive concentrations and moisture levels. Results show that high concentrations of the pMDI model react with the loblolly pine to form quantifiable carbamate linkages essentially only with lignin sidechain units under dry conditions. No covalent bond formation was detected after using a miniature hot-press on MTW with pMDI models at 5% and 14% MC.

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LIST OF ABBREVIATIONS

ABES	automated bond evaluation system
BMW	ball-milled whole wood cell walls of loblolly pine
4-BPI	4-benzylphenyl isocyanate
1D	one-dimensional
2D	two-dimensional
DBTDL	dibutyltin dilaurate
DFRC	derivatization followed by reductive cleavage
DMAc	N,N-dimethylacetamide
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
ESEM	environmental scanning electron microscope
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
IPN	interpenetrating polymer network
MC	moisture content
Me4MeGlc	methyl 4-O-methyl- β -D-glucopyranoside
MTW	microtomed wood of loblolly pine
MWL	dioxane milled-wood lignin of loblolly pine
NMI	N-methylimidazole
pMDI	polymeric methylene diphenyl diisocyanate
NMR	nuclear magnetic resonance (spectroscopy)
OSB	oriented strand-board
PI	phenyl isocyanate
WPG	weight-percent gain

Chapter 1. Wood-pMDI bonding mechanisms

INTRODUCTION

The chemical versus mechanical interaction between wood and adhesives has been studied since 1929.¹ With the continued growth of the wood adhesive industry, and the increasing demand for novel formaldehyde-free adhesives, some fundamental questions about wood-adhesive interactions need to be addressed. In particular, “How does an adhesive chemically interact with wood under industrial conditions?” More specifically, (a) Which wood components, if any, are reacting with the adhesive? (b) What is the wood component order of reactivity? (c) Can the reacted components be quantified?

Defining Wood Adhesion

What is ‘adhesion’? Gent and Hamed² describe adhesion as “the state in which two surfaces are held together by interfacial forces, which may consist of valence forces, mechanical interlocking, or both.” There really is not a definition of adhesion that can describe all adhesive-substrate interactions. Therefore, in the field of adhesion, several models exist. **Table 1.1** below describes the various models.

Table 1.1. Existing models of adhesion.

Models			Description
Electrostatic			Between particles that bear opposite charge
Inter-diffusion			Self-welding or polymer chain entanglement
Mechanical Interlocking			Adhesive adheres by physically locking onto substrate; “lock and key” effect
Fundamental Forces	van der Waals	Dipole-dipole	Partial charges on a molecule are separated in space, creating a dipole moment; two dipoles can interact
		Dipole-induced dipole	Spherical, symmetrical charge distribution encounters a dipole, inducing the spherical electron distribution to separate into a dipole moment
		Instantaneous dipole	Spherical, symmetrical charge distribution, for an instant in time, has a partially unshielded nucleus on one side and excess electrons on the other, creating an attraction between atoms or molecules
	Hydrogen bonds		Adsorption forces; Hydrogen interacting with oxygen or nitrogen; either inter- or intra-molecular
	Ionic bonds		Salts, like NaCl
	Chemical bonds	Acid-base	Acid or base character of substrate may influence reactivity of adhesive toward substrate
		Covalent	Shared valence electrons

These models work well when describing fairly homogenous, non-porous materials like metals or plastics. However, applying these same models to explain wood bonding is insufficient. All of the described models (except ionic and electrostatic) are likely contributors to the adhesive bonding to wood. The ubiquitous van der Waals forces are always contributors to any molecular interaction and may constitute an important force between adhesives and wood. Hydrogen bonds are also important to the field of wood adhesion since they are omnipresent in wood and wood adhesives. However, if hydrogen bonds were the most crucial bonds between an adhesive and wood, why is it that some adhesives do not easily dissociate from wood under hydration? For example, when water is allowed access to the microstructure of the cell wall after an adhesive bond is cured, some wood-adhesive bonds can withstand the swelling forces induced by water molecules interrupting cell wall hydrogen bonding. One would expect, with this large

disruption of hydrogen bonding, that the wood-adhesive bond would disintegrate; however, this is not always the case when exterior-grade adhesives are used. Covalent bonds, as opposed to acid-base interactions, are what most organic chemists describe as ‘chemical bonds.’ They form one of the strongest bonds to atoms by sharing valence electrons. While covalent bonds are the most durable, their existence between exterior-grade adhesives and wood has been controversial. It is the covalent bond that will be the focus in this thesis.

From the above models, wood researchers have devised their own models to describe wood bonding. Three predominant models on how an adhesive adheres to wood are: (1) Interfacial adhesion, where an adhesive interacts only at the lumen surface of the cell wall, as seen in traditional adhesion models; (2) Lumen filling, where the adhesive fills the lumen and act as a mechanical interlock; and (3) Cell wall infiltration, where adhesive molecules are absorbed into the walls and bond covalently and crosslink with wood components.³ Interfacial adhesion is always important but is not always sufficient for providing durable bonds as evidenced by bonding studies with acetylated wood.⁴ Lumen filling or mechanical interlocking between the adhesive that has penetrated the lumina may be important, especially for highly porous woods with fine texture and uninhibited pits. Surface roughness and open anatomical features create more surface area for the adhesive to flow into and develop further points of contact for interlocking. If an adhesive has the capability of infiltrating the cell wall itself through microvoids or free-volume in the polymer matrix, there exists the possibility of reactions with various wood components.

Choosing an Adhesive for Study

Covalent bonds between an adhesive and wood should improve bond durability. However,

covalent bonds have never been unambiguously detected in an adhesive bondline. For an adhesive to form covalent bonds to wood, it must have the following characteristics: (1) exhibit strong wettability to wood; (2) be capable of infiltrating the cell wall; and (3) be highly reactive with wood polymer hydroxyls. Ideally, the reaction would be amenable to study using a monofunctional model compound of the adhesive so that chemical shift differences are distinct between unreacted and reacted wood polymers in solution-state NMR spectroscopy. See **Appendix A**, which describes my selection process to determine the best adhesive system to study.

An adhesive that has these characteristics is pMDI. The pMDI adhesive has been shown most likely to: (1) travel relatively long distances from the applied wood surface;⁵ (2) flow into microvoids if given access by a crack or fracture;⁶ (3) infiltrate the cell wall layers;^{7,8} and (4) chemically react with hydroxyls of polysaccharides (e.g., hemicelluloses) and lignin.^{7,9,10} Additional reasons for choosing pMDI for this study are that model compounds based upon pMDI are commercially available. Therefore, chemical shift changes in NMR spectra upon reaction with wood components can be studied with model compounds using 2D HSQC NMR.

Importance of pMDI adhesive

Isocyanate adhesives were first introduced during WWII to bond tire cords and rubber and were known to bond almost anything together. Their high cost and toxicity have given them many negative associations. However, the high reactivity and tremendous durability that can be achieved with low resin loading has kept isocyanates as key players in the wood composites and polyurethane industries. As old-growth forests became depleted, younger forest stands became the only viable source for harvesting trees to produce lumber. With these juvenile trees come

smaller diameter logs, more branches near the bole, and juvenile wood with a higher microfibril angle giving lower dimensional stability. OSB emerged to compete with the existing plywood markets to develop building products that retained mechanical properties (e.g., flexure strength and internal bonding) while increasing forest utilization. This new growth stimulated other structural composite markets, which developed laminated strand lumber (LSL) and I-beams with OSB webs.

The pMDI adhesive used for OSB composites has low volatility and the proper rheology for this application. Pre-polymerized MDI was named by the adhesive industry as *polymeric* MDI. However, the term “polymeric” is somewhat misleading; approximately half of the adhesive is difunctional or diisocyanate monomer, and the rest is oligomeric with a degree of polymerization less than 12. Typical number average and weight average molecular weights for pMDI are 255-280 g/mol and 470-550 g/mol.¹¹ Although the cost of pMDI per pound is higher than the common phenolic adhesives, several advantages offset the cost: (1) to achieve the same board properties as phenolic-based adhesive, less pMDI is needed (on a weight-basis); (2) lower hot-pressing temperatures may be used; (3) Faster pressing cycles are possible; (4) higher wood MCs (i.e., 14%) are not detrimental to the bond properties; (5) no solvent is necessary (i.e., no added water or volatile organics); (6) composite boards display lower thickness swelling; and (7) formaldehyde is not emitted in-service. Furthermore, through the development of pre-polymerized versions of MDI, “blocking” reactive sites on the MDI molecule, and emulsifying specific formulations, isocyanate adhesives have become customizable to almost any bonding application.

pMDI reactivity

Isocyanate adhesives have high reactivity with hydroxyls. The major reactions that can occur with pMDI and wood are between the isocyanate and free or bound water in the cells or the wood polymer hydroxyls. **Figure 1.1** shows the two main types of competing mechanisms expected during pMDI cure. Isocyanates react with water to form carbamic acid, which quickly releases CO₂ to form an aromatic amine. The reaction between the aromatic amine and other isocyanate molecules supercedes that of water or wood and a *urea* is formed. Further reaction of the urea with isocyanate may occur and *biuret* and *polyurea* will form. These urea-type reactions are important for forming the polymeric bridge between the two wood surfaces, but these reactions compete with the reaction of the isocyanate with hydroxyls on wood polymers. For example, when the ratio of the water concentration to isocyanate concentration is low, the isocyanate has the ability to react with wood polymer hydroxyls to form *urethanes* (referred to as *carbamates* when monofunctional isocyanates react with hydroxyls). The urethane may react with another isocyanate to form an *allophanate* linkage. The difficulty, because of their structural similarities as noted in Figure 1.1, in distinguishing between urea, biuret, urethane, and allophanate structures in wood-pMDI bondlines has been the focus of many studies.

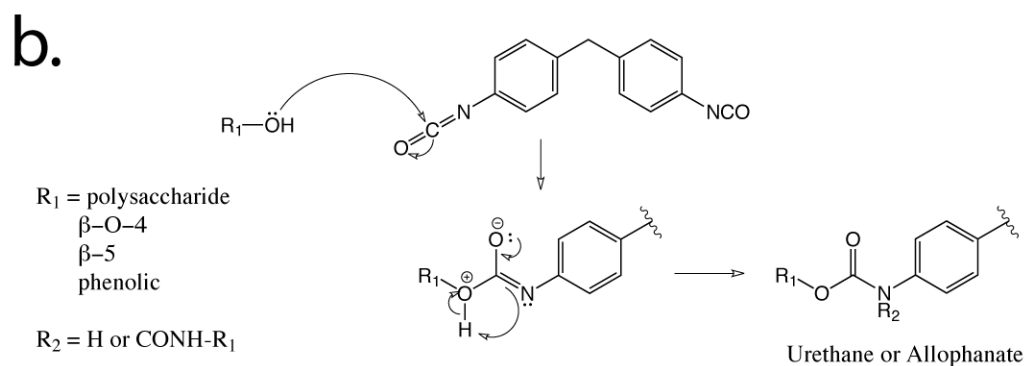
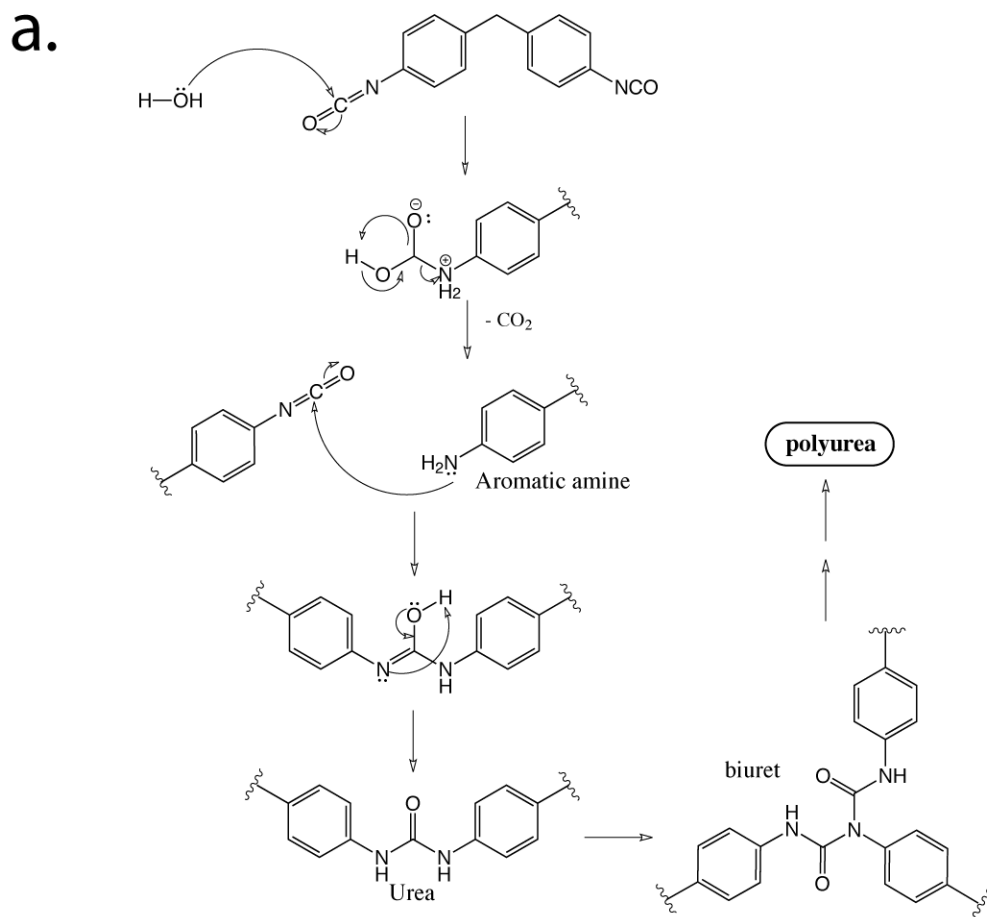


Figure 1.1. The two major competing reaction mechanisms during pMDI curing with: **(a)** water, where an aromatic amine (after the release of CO_2) reacts with isocyanate to form urea, biuret, and polyurea; and **(b)** wood, forming a urethane (referred to as a *carbamate* when the isocyanate is monofunctional) linkage and further reaction with isocyanate forms an allophanate linkage.

Along with the expected products formed during pMDI reactions with wood, more exotic mechanisms have been proposed to produce even more varied structures. Laser ionization mass spectroscopy of pMDI-wood samples produced mass fragments consistent with ring substituted condensation reactions between the aromatic ring of pMDI and methylol functionalities on the aromatic ring of lignin, shown in **Figure 1.2a**.¹² Electronic modeling studies have been performed supposedly supporting this hypothesis.¹³ This type of mechanism was shown to be plausible between pMDI adhesive and aromatic methylols (derived from nucleophilic addition between formaldehyde and phenol).¹⁴ However, since formaldehyde is not present during pMDI bonding with wood, aromatic methylols on lignin are not formed. Another plausible way the lignin aromatic ring may react with pMDI is at the C5 position through nucleophilic attack by an enolate onto the isocyanate, as shown in **Figure 1.2b**. In cases where isocyanate groups are limiting during wood-pMDI bonding (i.e., in later stages of pMDI cure when little free isocyanate is available), it is possible that the aromatic amine (formed with water) may react with carbonyls in wood (e.g., 4-*O*-methyl- α -D-glucuronic acid, coniferaldehyde) giving an amide, as shown in **Figure 1.2c**.

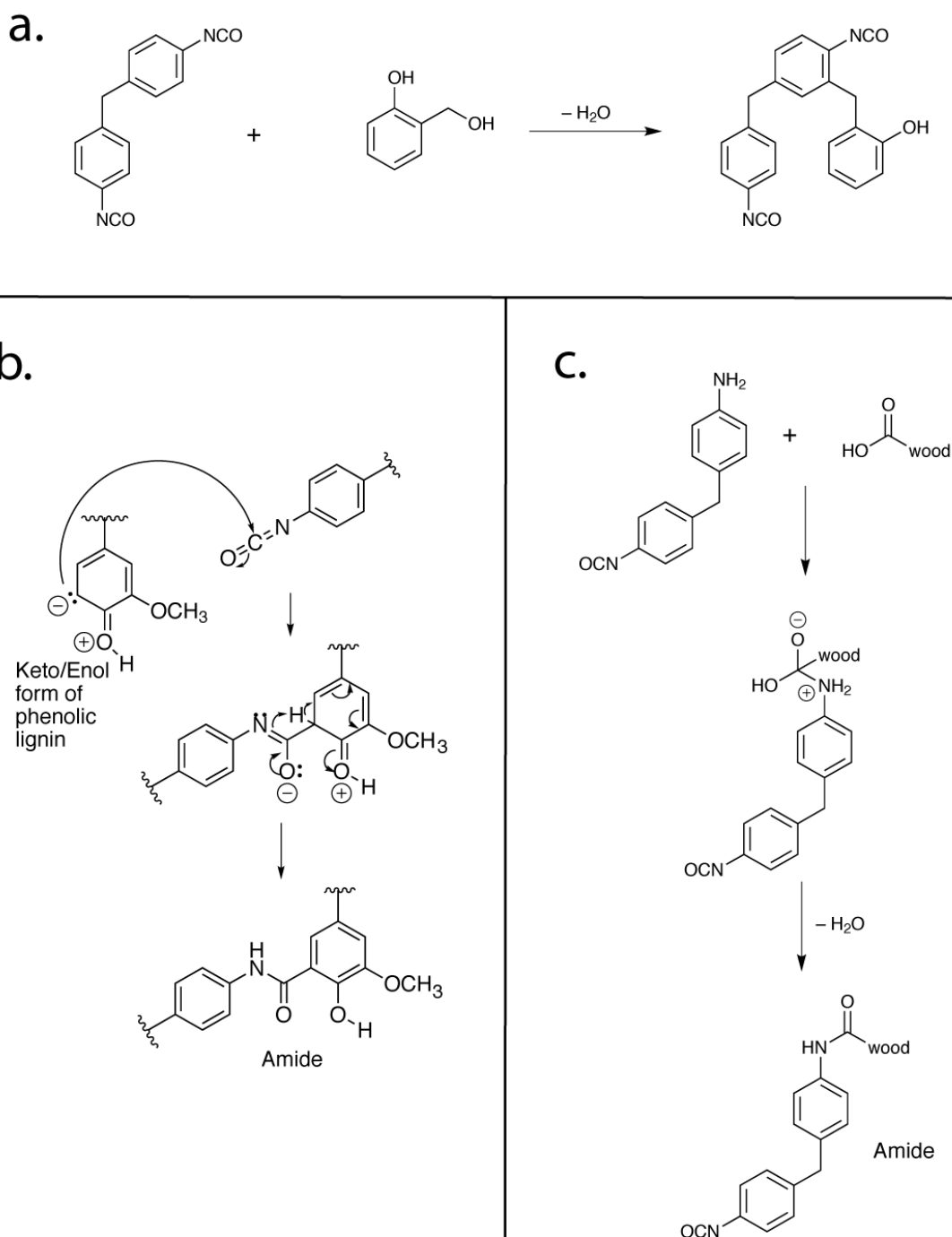


Figure 1.2. Other possible mechanisms during the curing of pMDI with wood: **(a)** any free formaldehyde can react with phenol to create a methylol that may condense with pMDI aromatic groups to form methylene crosslinks; **(b)** if C5 nucleophilic addition occurs with lignin aromatic groups forming an amide; and **(c)** if carboxylic acids from wood reacted with the aromatic amine of pMDI (i.e., from the reaction of water with isocyanates) to form an amide.

From the proposed mechanisms, it is prudent to hypothesize that pMDI adhesive forms covalent bonds with wood cell wall hydroxyls under normal bonding conditions. **Figure 1.3** shows four hypothetical schemes of how pMDI may interact with wood cell walls at the molecular level.¹⁵ In the schematic, the initial state 0 depicts two hydroxyl groups on the wood polymers (lignin, hemicellulose, & cellulose) that have a physical bond to these polymers. State 1 shows physical bonding (including hydrogen bonding) between a bulking polyurea and wood hydroxyl groups. For both physical and chemical bonding (e.g., urethane or allophanate) between a bulking polymer and a wood cell wall polymer, state 3 applies. State 4 depicts a chemical bond between a bulking polymer and a wood polymer in which the pMDI has two or more available isocyanate groups where pMDI creates a crosslink between either a wood polymer or another part of the adhesive. This research focused on the existence of state 3 and 4, in which a covalent bond is (or is not) formed between an isocyanate and a wood polymer.

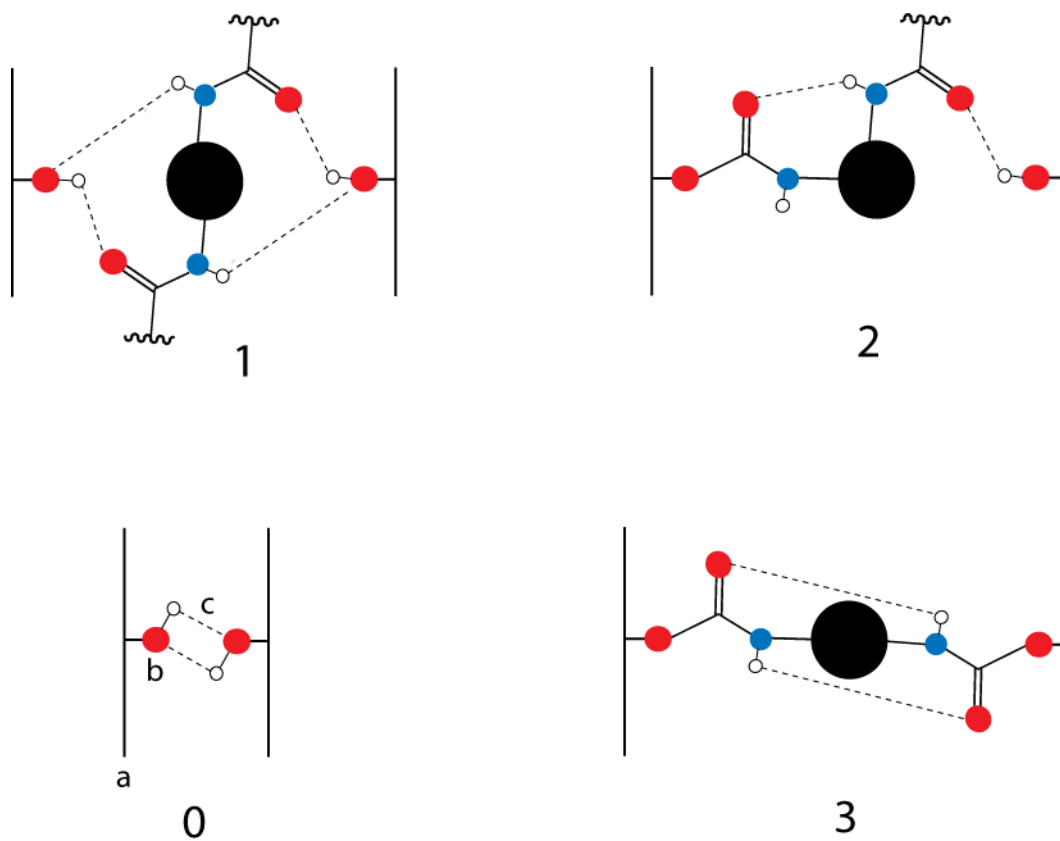


Figure 1.3. A model of the wood cell wall at the molecular level interacting with pMDI: (a) is a hypothetical polymer chain that is part of the cell wall; (b) is a hydroxyl group; (c) is a hydrogen bond. The red dots are oxygen atoms; empty circles are hydrogen atoms; blue dots are nitrogen atoms; and the black circles are bulky adhesive polymer. In the model: **(0)** is the initial untreated state showing physical bonding between wood cell wall components; **(1)** shows the bulky adhesive polymer without crosslinking, but with physical bonding to itself or wood; **(2)** shows a bulky adhesive polymer covalently bonded to a wood cell wall component with the possibility of physical bonding to itself or wood; **(3)** shows a bulky adhesive polymer covalently bonded across wood cell wall components to create a crosslink.

Characterizing pMDI reactivity

Wendler and Frazier found that when the wood is dry (MC <5%), pMDI reacts to form low molecular weight biuret structures.¹⁶ They found polyurea formation via the self-polymerization reaction of the diisocyanate with water to dominate as the wood moisture increased (>5%).¹⁶ Currently, the field of wood adhesion has no conclusive evidence showing that pMDI covalently bonds to wood. Distinguishing between urea, biuret, and urethane structures in wood-pMDI

bondlines is difficult because of the structural similarities of the various products. With solid-state NMR, urea versus urethane identification cannot be determined unambiguously due to signal overlap. For instance, ^{15}N -CPMAS,¹⁶ ^{15}N - ^1H HETCOR,¹⁷ or even double labeling¹⁰ (e.g., ^{15}N and ^{13}C) of pMDI cannot resolve the products from wood reactions; the experiments do not provide sufficient peak dispersion to distinguish wood component urethane reaction products from urea-type reaction products. Moreover, IR spectroscopy cannot determine the type of chemical bond formation with a wood component, only the presence of a functional group somewhere in the product.^{18,19}

Wood cell wall dissolution, a field of wood chemistry that is seeing increased interest, allows us to dissolve both the interface and interphase where adhesives interact with wood, and to use solution-state NMR to detect changes in chemical shifts due to reactions. High-resolution solution-state NMR spectroscopy is therefore a powerful tool for detecting covalent bonds between wood polymers and pMDI adhesive. As described in this thesis, using a non-degradative wood dissolution method,²⁰ finely ground wood cell walls dissolve in a solvent system containing DMSO- d_6 and NMI- d_6 , keeping wood polymers in a non-derivatized state.²¹ 2D NMR experiments, using ^{13}C - ^1H one-bond HSQC spectroscopy on whole cell walls reveal all of the ^{13}C - ^1H correlations of major cell wall polymers. This thesis discusses the research with pMDI model compounds (i.e., PI and 4-BPI) showing that HSQC spectroscopy can detect chemical shift changes upon reactions with wood cell wall polymers; reactions with lignin were shown to predominate.²²

Overall Objective

The objective of this research is to elucidate the bonding mechanism between pMDI and loblolly pine cell walls using high-resolution solution-state NMR. More specifically, this research will:

- Use solution-state NMR to structurally characterize wood cell wall polymers in a non-derivatized state through dissolution in perdeuterated DMSO and perdeuterated NMI.
- Use solution-state NMR to analyze and assign peaks in HSQC spectra of the (catalyzed) reaction products between a pMDI model (e.g., PI) and model wood components (e.g., BMW, MWL, lignin model compounds, holocellulose, cellobiose, and Me₄MeGlc).
- Determine if reaction with pMDI models (e.g., PI and 4-BPI) takes place with MTW in a good swelling solvent without catalysts.
- Determine if the reaction of a pMDI model (e.g. 4-BPI) takes place with MTW using typical industrial bonding conditions without solvent or catalysts.
- Quantify the pMDI reaction with MTW through the use of volume integration of specific ¹³C–¹H contours in the HSQC NMR spectra of reacted wood polymers.

Rationale and Significance

The cell wall is a sterically hindered environment compared to a more freely mobile environment encountered when using model compounds in solution. Therefore, these two environments must be approached differently for interpreting reactivity.

No adhesive bonding research reports using a pMDI model compound with monofunctionality that mimics its reactivity, steric mobility, and volatility in an application similar to industrial processing. Furthermore, no research using solution-state NMR to elucidate

adhesive bonding to wood has been reported. Non-degradative dissolution of wood allows characterization of individual cell wall components and gives remarkable peak dispersion and a wealth of chemical bonding data not obtainable with solid-state NMR. No prior research has been found on pMDI bonding to wood that links model compound reactions to solid (intact) wood reactions using industrially relevant conditions. Determining the degree of derivatization and sites of reaction of pMDI adhesive by analyzing chemical shifts of wood polymers with solution-state NMR has the potential to be more conclusive than prior attempts. For instance, trying to separate the urethane peak from other pMDI self-reaction products (urea, biuret, and polyurea) using ^{15}N solid-state NMR is obscured by signal overlap,^{10,16,17} but we anticipate that wood components derivatized by reactions with isocyanates, forming new covalent bonds, will be readily distinguishable in 2D ^{13}C - ^1H HSQC NMR.

This research is to better our understanding of what bonding mechanisms control pMDI bonding to wood. Since pMDI provides tremendous durability to wood composites, this adhesive is widely used in industry for making OSB and LSL, which drive the housing construction market for North America and abroad.

How will this impact the field of wood adhesion? Most thermosetting adhesives (e.g., pMDI, PF, MF) are thought to adhere through physical bonds (e.g., hydrogen bonds), not by covalent bonds. Thus, there is a drive towards understanding the durability mechanism of this adhesive. The techniques developed in this study can be used for studying chemical bond formation between wood adhesives and wood polymers. Moreover, the wood dissolution methods developed and described previously²⁰ have already been applied to brown-rot decayed wood where evidence of lignin sidechain cleavage has been found, overturning some long-standing theories on how brown-rot fungi attack wood. This recently published work, in which we utilized

emerging cell wall dissolution and NMR methods of benefit to the main study described in this thesis, is described fully in **Appendix B**.²³

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Chapter 2. Characterization of non-derivatized plant cell walls using high-resolution solution-state NMR spectroscopy[■]

SUMMARY

A plant cell wall dissolution system has been modified to use perdeuterated solvents to allow dissolution and high-resolution solution-state NMR of the whole cell wall without derivatization. Finely ground cell wall material dissolves in a solvent system containing DMSO-*d*₆ and NMI-*d*₆ in a ratio of 4:1 (v/v), keeping wood component structures mainly intact and in their near-native-state. 2D NMR experiments, using gradient-HSQC one-bond ¹³C–¹H correlation spectroscopy, on non-derivatized cell wall[☆] material from a representative gymnosperm *Pinus taeda* (loblolly pine) demonstrates the efficacy of the system. This chapter describes a method to synthesize NMI-*d*₆ with a high degree of perdeuteration, thus allowing cell wall dissolution and NMR characterization of non-derivatized polymer structures from plant cell walls, as well as carbamylated structures as described in the following chapters.

[■]This chapter can be found in the following publication, which includes the characterization of angiosperms *Populus tremuloides* and *Hibiscus cannabinus* described in **Appendix C**: Yelle, D.J.; Ralph, J.; Frihart, C.R. (2008). *Magnetic Resonance in Chemistry*, vol. 46, pages 508-517.

[☆] The term “cell wall” in this thesis includes the primary wall, middle lamella, and secondary wall layers (S1, S2, and S3).

INTRODUCTION

Component Isolation

Characterizing plant cell composition and structure requires a variety of approaches. Non-destructive spectroscopic methods that can be applied to the cell (or whole plant material), including solid-state NMR, IR, near IR, are often limited because of either poor resolution and/or poor structural determination. Degradative methods may be applied to the whole plant material, but study of individual components typically requires their separation. Improved solid-state NMR has been useful for examining the intact plants;¹⁻⁴ however, the decreased relaxation times and low peak dispersion with solid-state NMR limit its use for thorough characterization of intact polymers. As solution-state NMR instrumentation and experiments become more developed, higher sensitivity and resolution permit the detailed structural analysis of polymeric components in plants more effectively. Recently, two solvent systems were developed for cell wall dissolution to allow quite detailed structural analysis of the cell wall (following ball-milling), without component separation or degradation via high-resolution solution-state NMR.⁵

Secondary xylem is the anatomical tissue distinguishing a woody plant from a non-woody plant. It is the xylem cells, tracheids (gymnosperms) and libriform fibers and vessels (angiosperms) that biosynthesize lignified secondary cell wall layers. After the cell wall polysaccharides (cellulose and various hemicelluloses) are synthesized and assembled, *p*-hydroxycinnamyl alcohols are exported to the wall and are polymerized through radical coupling mechanisms to form a polymeric matrix (lignin),⁶ in which the polysaccharides are embedded. Thus, it is the lignin that acts as the mortar to support the polysaccharides. The plant cell wall, therefore, may be regarded as a highly complex nanocomposite.

Component isolation remains a valuable method to obtain relatively pure fractions of

individual wall polymers or polymer classes. Isolating lignin, cellulose, and hemicelluloses from wood has been long known to be a difficult task, since none of these components can be removed without either significant chemical or mechanical modification of the cell wall. Many attempts have been made to mill the cell wall to extract lignin and polysaccharides, but the intimate nanoscale domains make this difficult; the crystalline nature of cellulose and the interwoven nature of the lignin-hemicelluloses matrix are difficult to separate. Separation of the lignin from the polysaccharides is important for characterizing lignin, purifying the cellulose for paper, and to improve biomass conversion processes. Björkman was one of the first to isolate up to 50% of a 'native' lignin from ball-milled spruce via extraction with aqueous dioxane to give what is called MWL.⁷ The carbohydrate-free yield in practice is usually less than 30% of the total lignin, and is highly dependent on milling efficiency and the type of plant. Treating BMW with a cellulolytic enzyme that served to hydrolyze the polysaccharides allowed the isolation of a cellulolytic enzyme lignin,⁸ which retained essentially all the lignin, but also a substantial fraction of the polysaccharides.

Isolation of polysaccharide components from plant cell walls typically involves the degradation and removal of lignin to obtain a holocellulose. Sodium chlorite in acid solution on cell wall material retains hemicelluloses almost quantitatively.⁹⁻¹¹ Complete delignification, without significant loss of polysaccharides, is not obtainable by these methods. Specific hemicelluloses in the cell wall must be gently extracted and purified so as to maintain as natural a state as possible. A neutral solvent, such as DMSO, is useful for extracting holocellulose to obtain a xylan fraction with little structural change. Alkaline (KOH or NaOH) solutions containing sodium borate allow extraction of galactoglucomannans and glucomannans. However, alkali facilitates deacetylation of xylans and mannans.¹² To extract the naturally

acetylated mannans, thermo-mechanical pulp of spruce (2% consistency) may be suspended in distilled water with stirring at 60 °C,¹³ while spruce holocellulose may be extracted with boiling water to obtain the same.¹⁴ Further isolation of holocellulose components involves alkaline extraction, but the remaining α -cellulose steadfastly retains some mannan and xylan.

Methods that involve ball-milling are the basis of several cell wall characterization procedures, but ball-milling itself breaks bonds and therefore causes structural changes. Knowing the effect that ball-milling has on plant cell walls is important because milling the cell wall destroys anatomical information that once existed. It has been found that the intensity (ball-mill frequency) of milling has a larger influence on the particle size than prolonging of the milling time and that the cell corners and middle lamella are the most resistant layers to milling whereas the S1 and S2 layers are clearly fibrillated early in the milling process.^{15,16}

A Historical Perspective of Plant Cell Wall Dissolution

A thorough understanding of how wood cell wall dissolution evolved cannot be completely appreciated without knowledge of past developments in cellulosic solvents. Why start with cellulose? Cellulose has high natural crystallinity, which renders it perhaps the most stable and inaccessible cell wall component. To study the molecular properties of a plant cell wall, cellulose dissolution is necessary. I will focus this discussion on those systems thought to minimize degradation of the cellulose polymer. Cellulose itself is insoluble in typical organic solvents. Schweizer in 1857 was one of the first to develop a cellulose solvent based on metal complexes with cellulose chains; cuoxam combines copper and ammonium hydroxide to produce cupritetramine complexes where Cu^{2+} ions doubly deprotonate and chelate with cellulose hydroxyls.¹⁷ Jayme developed a more powerful complex with cobalt ethylene diamine (as well as

other similar complexes with Ni, Cd, Cu, Zn and Fe).¹⁸ The cadmium complex, cadoxene, and the iron tartratic sodium complex, EWNN, proved best to allow determination of molecular weight distributions of cellulose solutions. With improved modifications, the latter method is still in use today.

Other systems were developed based on non-aqueous solvents and were incorporated directly with bases, such as: (1) DMAc and lithium chloride (~10:1 w/w at 130 °C);¹⁹ (2) DMSO and N-methylmorpholine-N-oxide (1:1 w/w, 80 °C);²⁰ (3) DMSO and methylamine (6:1 w/w, 25 °C);²¹ (4) DMSO and hydrazine (1:9 w/w, >80 °C);²² and (5) DMSO with chloral and triethyl amine, which lead to the formation of hemiacetals allowing measurement of degree of polymerization via viscosimetric methods.²³

In some cases, non-aqueous solvents and reactants were used to partially or fully derivatize the cellulose through homogenous reactions. Such solvents include: (1) dimethylformamide (DMF) or DMAc with N₂O₄ or NOCl to form a cellulose nitrite;²⁴ (2) DMSO with paraformaldehyde to form monomethylol cellulose;²⁵ and (3) Triethyl amine, urea, and DMSO with phthalic anhydride to form cellulose phthalate esters;²⁶

Molten salt hydrates have also been used in the past, but more recent focus has been on recyclable ionic liquids. Historically, salts such as ZnCl₂•4H₂O,²⁷ LiClO₄•3H₂O and LiI•2H₂O,²⁸ and LiSCN•2H₂O²⁹ were used for viscometric investigations of cellulose. More sophisticated molten salt mixtures were recently developed, such as LiClO₄•3H₂O/MgCl₂•6H₂O, LiClO₄•3H₂O/Mg(ClO₄)₂/H₂O, LiCl/ZnCl₂/H₂O, and LiSCN/KSCN/NaSCN/H₂O, to allow more complete dissolution.³⁰ Other molten salts, now termed ‘ionic liquids’ emerged to increase the dissolution power of the system, to facilitate recyclability of the solvent, and to allow for more complete *in situ* derivatization of the cellulose. However, for the purposes of dissolving the

whole plant cell wall, dissolution temperatures beyond 50 °C begin to approach the thermal transition points of lignin, thus increasing the probability of degradation. Specific ionic liquids do have the capability of dissolving plant cell wall material,³¹⁻³³ but the lack of degradative assessments of these systems currently limit their application in structural studies. These ionic liquids have the similarity of an imidazolium ion, namely, 1-butyl-(2,3)-(di)methylimidazolium chloride, 1-allyl-3-methylimidazolium chloride, and 3,3'-ethane 1,2-diylbis(1-methyl-1*H*-imidazol-3-ium) dichloride.

Dissolution by all the solvent systems described above must always begin through some form of disruption of the microfibrillar structure to cause intracrystalline swelling, breaking of existing intramolecular hydrogen bonding, and solvation of the cellulose polymer in the form of its disintegrated chains. Philipp and others found that in dissolving cellulose using metal complexes, the swelling occurred throughout the entire thickness of the fiber, while with non-aqueous solvents with amines, swelling began at the surface of fibers in a more gradual process. Depending on the nature of the system, some hydrolytic degradation may occur, particularly via hydronium ions, which may swell the lattice, or via the salt cations that hydrate significantly to disrupt intramolecular hydrogen bonding.³⁴

Two systems that were shown to be effective and non-degradative in dissolving BMW have been described.⁵ DMSO with tetrabutylammonium fluoride,³⁵ or DMSO with NMI fully dissolves plant cell walls at room temperature. It is currently not well understood how these solvents act to facilitate dissolution. We hypothesize that NMI has the capability of disrupting the strong hydrogen bonding interactions between cellulose and other components to allow DMSO (a good swelling solvent for woody plants) further access to the cell wall structure. *In situ* acetylation in this solvent mixture can be performed to a high degree of derivatization so that

the resulting acetylated cell wall material, after precipitation and filtration, dissolves in CDCl_3 for NMR experiments. The cellulose, albeit after the degree of polymerization is dramatically decreased by ball-milling, from the DMSO/NMI process appears to remain intact. This non-degradative dissolution (followed by acetylation) allows characterization of the BMW to a much higher resolution than is possible with solid-state NMR.

^{13}C - ^1H NMR of the Whole Cell Wall

Acetylated BMW material from various wood and cereal crop species have been shown to fully dissolve.⁵ **Figure 2.1** shows typical 1D NMR spectra of loblolly pine. When running a 2D ^{13}C - ^1H HSQC NMR experiment, a ^{13}C atom correlates directly with a ^1H that is one-bond away, showing that cell wall components separate out in a “contour map,” as shown in **Figure 2.2** of loblolly pine. The black contours are those of the cellulose aliphatic correlations, which are surrounded by those from other unidentified polysaccharide components including the hemicelluloses. However, the anomeric C-H of cellulose and mannose can be seen as the large peaks in orange at 4.4/100 ppm and 4.6/98 ppm, respectively. The aromatic lignin region (magenta) shows all the aromatic ring C-H correlations found in the various guaiacyl (gymnosperm) units. Most of the lignin sidechain units have well resolved contours as well. The β -aryl ether (cyan) unit $\text{A}\alpha$ and $\text{A}\beta$ correlations are well resolved; however the $\text{A}\gamma$ is hidden under the black polysaccharide region. Phenylcoumaran (β -5) units in green have well resolved $\text{B}\alpha$, $\text{B}\beta$, and $\text{B}\gamma$ correlations. Other lignin units like resinol C (β - β) and dibenzodioxocin D (5-5/ β -O-4) are present in the spectrum at lower contour levels due to their lower abundance; the $\text{C}\alpha$ and $\text{C}\beta$ correlations can be seen at the level plotted in Figure 2.2.

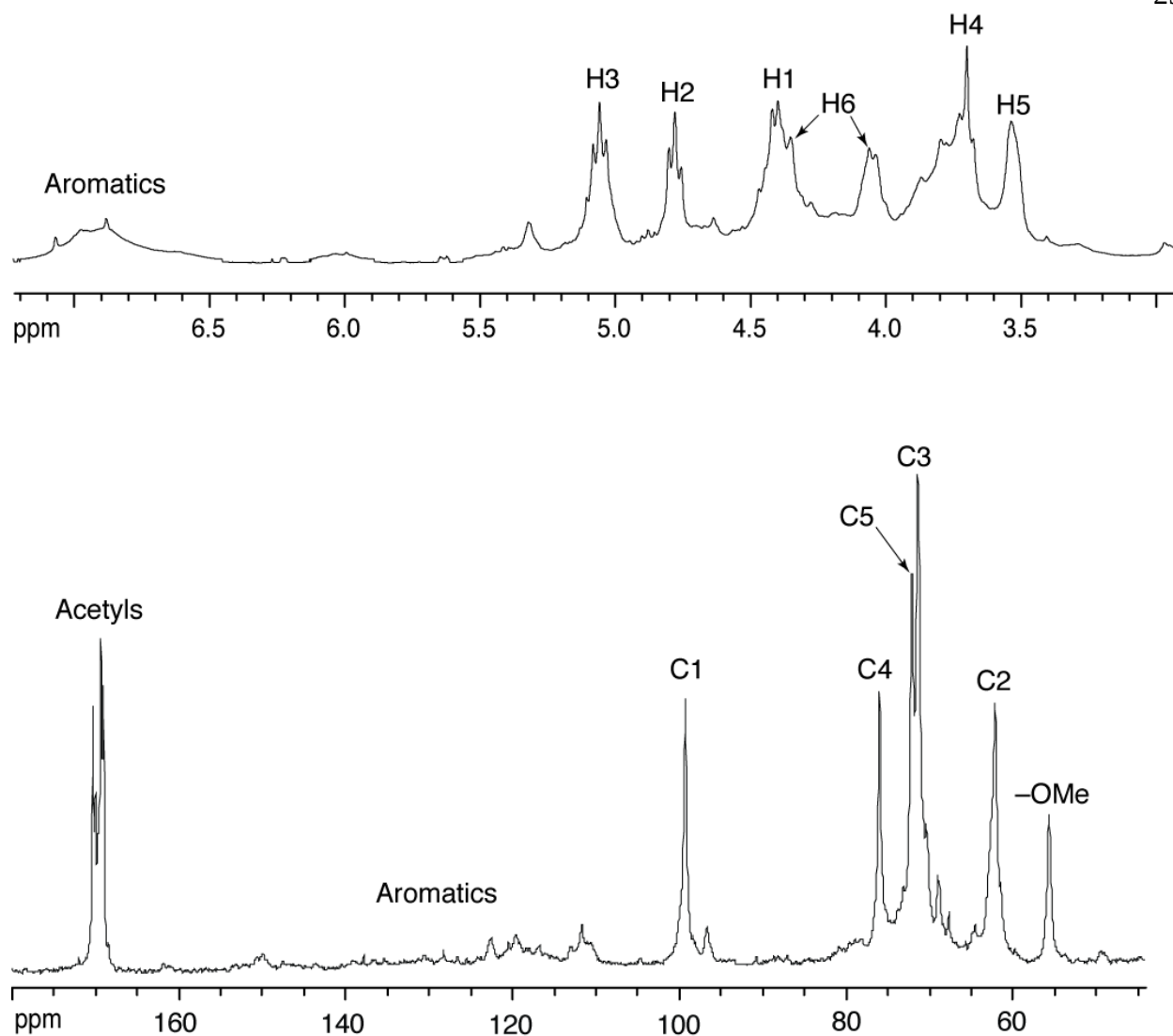


Figure 2.1. 1D NMR spectra of acetylated whole cell walls of loblolly pine: (top) ^1H NMR spectrum, (bottom) ^{13}C NMR spectrum. Tall peaks in spectra correspond to cellulose and acetyl carbonyl groups. Low intensity peaks are from lignin aromatic groups.

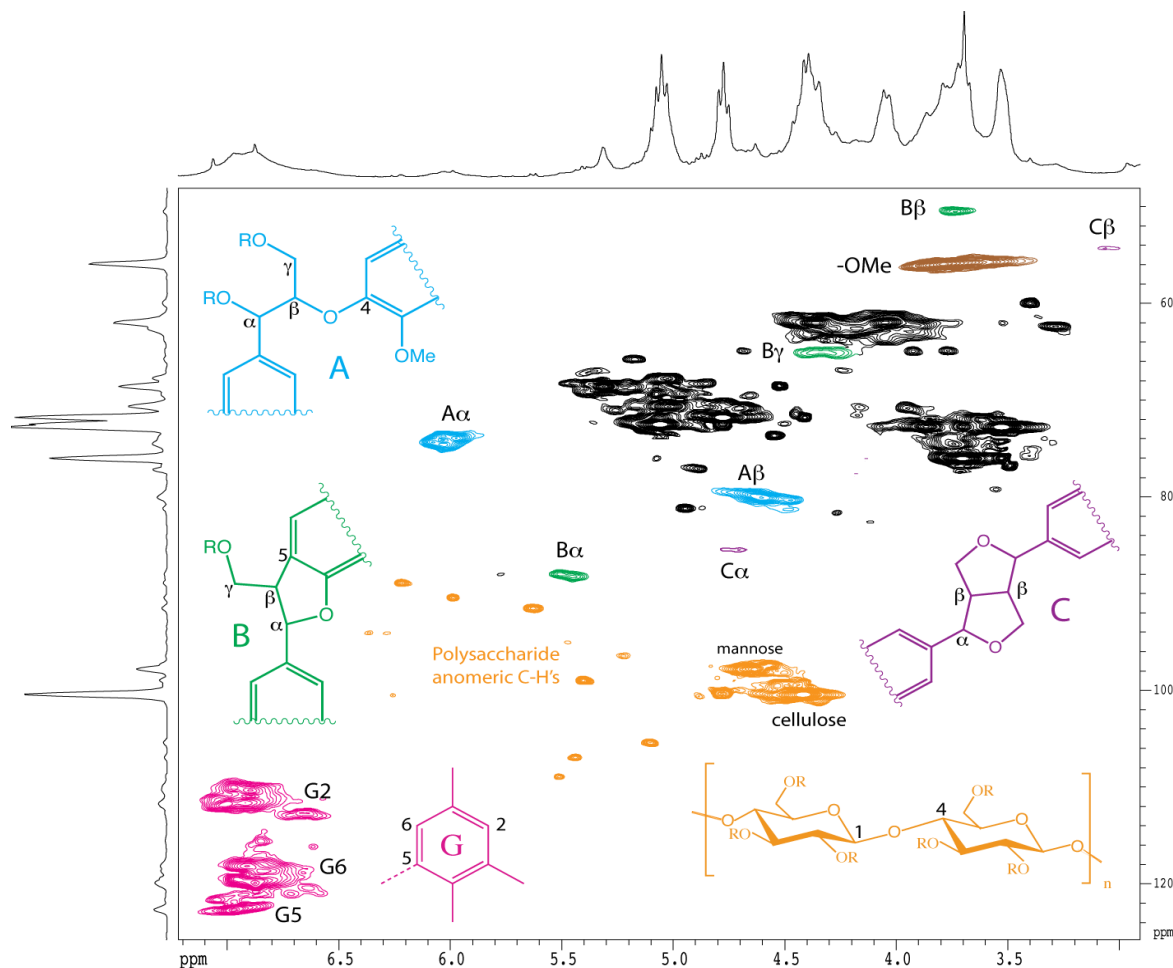


Figure 2.2. 2D HSQC NMR spectrum of acetylated whole cell walls of loblolly pine. Color-coded contours correspond to the structures shown; black contours are mainly from cellulose aliphatics.

A more demanding experiment for AcCW material, and polymeric material in general, is the long-range ^{13}C – ^1H correlation experiment called HMBC. This experiment is able to correlate a proton to a carbon that is 2 or 3 bonds away. It is a powerful experiment in that the connectivity of structural units can be identified. The challenge is that a delay of 80-100 milliseconds is needed to allow for long-range coupling interactions to evolve. If the sample relaxes too quickly, as occurs with polymeric or metal contaminated samples, the signal intensity is diminished. One can remove metal contamination through EDTA washing or use non-metallic ball-milling

devices to obtain largely metal-free BMW. Both techniques can allow successful an HMBC experiments to be performed, revealing whether β -ether units in hardwoods are syringyl and/or guaiacyl,³⁶ for example.

The cellulose component of the cell wall is observed through examining Figure 2.2 more closely. Decreasing the contour level of the whole spectrum, most readily accomplished on an NMR data-station, shows all 6 carbons of the cellulose acetate directly correlated to their respective protons. The strong intensity of these correlations allows facile determination of derivatization of cellulose.

To learn more about the reactivity or degradation of various cell wall components, it is desired to dissolve the cell wall, without derivatization, in suitable NMR solvents. The DMSO/NMI system will dissolve the cell wall. Logically, then, utilizing both perdeuterated DMSO and NMI should allow direct NMR analysis of non-derivatized walls. Characterizing the structure of the non-derivatized plant cell wall via HSQC ^{13}C - ^1H experiments depicts the plant cell wall polymers in a fairly native-state. Although it is well recognized that ball-milling already causes depolymerization of cell wall polymers, an advantage of non-derivatization is that the native polymeric structures often obscured by traditional derivatization practices can be studied. For example, natural acetates found on softwood hemicelluloses like galactoglucomannan contain acetyl side groups off their main chain. These natural acetates are masked in NMR spectra by the current practice of acetylating the cell wall.

Our approach is to leave the plant cell wall material in a minimally altered state so as to characterize all components without requiring extensive component extraction or isolation techniques. By doing so, we leave the cell wall component polymers essentially unchanged, thus enabling the characterization of specific cell wall structures not seen before through NMR.

Objective

In this chapter, I describe the following:

1. Synthesis of perdeuterated NMI
2. Dissolution of the plant cell wall in a minimally altered state
3. Determination of chemical shifts of various polymeric plant cell wall structures via solution-state NMR techniques.

The use of these techniques enables the characterization of specific cell wall components that may be partially reacted during derivatization or biological degradation, and allows the approximate quantification of natural acetates found in lignin and hemicelluloses.

MATERIALS AND METHODS

Chemicals

Ruthenium (III) chloride hydrate (35-40% Ru), tributyl phosphite (94%), and methanol- d_4 (99.8% D) were supplied by Acros Organics (Geel, Belgium). Imidazole (recrystallized), 1,4-dioxane (98%), palladium on activated carbon (10% Pd), D_2O (99.9% D), chloroform- d (99.8% D), and DMSO- d_6 (99.5% D) were supplied by Aldrich Chemical Company (Milwaukee, WI).

Synthesis of N-methyl- d_3 -imidazole

This synthesis is derived from prior published syntheses.^{37,38} Briefly, imidazole (10.89 g, 160 mmol), tributylphosphite (2.30 g, 9.2 mmol), ruthenium chloride hydrate (0.64 g, 3.1 mmol), and methanol- d_4 (20 g, 555 mmol) were sequentially added to dioxane (300 mL) in a 2 liter stainless steel Parr reactor vessel. The vessel was sequentially sealed, purged with nitrogen, stirred, heated to 200 °C, and pressurized with nitrogen to 44 bar. These conditions were maintained for 19

hours, then the vessel was cooled to 25 °C and depressurized before disassembly. The reaction mixture was decanted from the spent catalyst and rotary evaporated to recover the methanol- d_4 and to give a crude yellow liquid. The product was vacuum distilled (with assistance of a microcapillary) to recover N-methyl- d_3 -imidazole as a colorless liquid (9.01 g, 66.2%). GC/MS: m/z (EI) 88 (28.2), 87 (96.0), 86 (148.8), 85 (M^+ , 100.0). NMR ($CDCl_3$): δ_H 6.82 (H-4, s), 6.98 (H-5, s), 7.36 (H-2, s); δ_C 119.83 (C-5), 129.11, 128.96 (C-4, d), 137.56 (C-2), 32.39 (CD_3 , septet, $^1J_{^{13}C-^2H} = 21$ Hz).

Deuterium exchange of N-methyl- d_3 -imidazole to give N-methylimidazole- d_6

This exchange is derived from a combination of prior published methods.^{38,39} Briefly, dry palladium catalyst (1 g Pd/C) was well stirred for 1 hr in a double-necked flask purged with nitrogen followed by capping one neck of the flask with a 230 mm round balloon full of hydrogen. A hose, connected to the second neck of the flask, allowed hydrogen to flow through the flask and through a water bath at a rate of ~1 bubble per second via a needle valve. N-Methyl- d_3 -imidazole (5.03 g, 57.4 mmol) was dissolved in 25 g of D_2O , added to the reduced catalyst, degassed with three freeze-thaw cycles under slight vacuum, heated with stirring to 100 °C and held for 2 h. The product was filtered through a bed of celite and vacuum distilled to give N-methyl- d_3 -imidazole- d (4.25 g, 82%). In a sealed pressure tube, N-methyl- d_3 -imidazole- d (584 mg, 6.8 mmol) was dissolved in D_2O (20 g) and 63 mg of Pd/C was added. The tube was purged with hydrogen (as previously described), sealed, heated with stirring to 160 °C and held for 24 h. After cooling, the reaction mixture was diluted with H_2O (20 ml), filtered through a 0.2 μm nylon membrane, washed with H_2O (3×10 ml), and vacuum distilled to give NMI- d_6 (456 mg,

76%). GC/MS: m/z (EI) 89 (6.65) 88 (M^+ , 100.0), 87 (29.9), 86 (17.6). NMR: δ_C /ppm ($CDCl_3$), 119.03, 119.50, 119.96 (C-5, triplet), 128.42, 128.88, 128.34 (C-4, triplet), 136.82, 137.32, 137.82 (C-2, triplet), 32.39 (CD_3 , septet, $^1J_{^{13}C-^2H} = 21$ Hz).

GC/MS of imidazoles

All measurements were carried out with a Varian (Walnut Creek, CA) 4000 ion trap mass spectrometer coupled to a Varian 3800 gas chromatograph that was equipped with a CTC Analytics Combi-Pal autosampler (Zwingen, Switzerland). A Siltek-passivated single-gooseneck liner (Restek, Bellefonte, PA) and an Rxi-1ms (30 mm long $\times$ 0.25 mm inner diameter, 0.25 μ m film thickness, Restek, Bellefonte, PA) were used. Split (200:1) injections (1 μ l) were performed. Operating conditions: injector temperature, 275 $^{\circ}C$; carrier gas, helium at 1.0 ml/min; oven temperature program, 40-125 $^{\circ}C$ at 15 $^{\circ}C$ /min. The mass spectrometer was operated in positive electron ionization mode (70 eV) over a range of m/z 40-125 at the following temperatures: source, 170 $^{\circ}C$; manifold, 40 $^{\circ}C$; transfer line, 275 $^{\circ}C$.

Plant cell wall preparation for NMR

BMW was prepared from *Pinus taeda* sapwood. Sequentially, 5 g of plant material was chopped into approximately 2 $\times$ 10 mm pieces, placed into a Retsch (Newtown, PA) MM 301 mixer mill (equipped with two 50 ml stainless steel jars, each with one 25 mm stainless steel ball bearing at -196 $^{\circ}C$) for 2 minutes at 30 Hz. The wood was not extracted for reasons we describe in the results and discussion. Then, 2 g of pulverized plant material was loaded into a Retsch PM 100 planetary ball-mill (equipped with a 50 ml ZrO_2 jar and ten 10 mm ZrO_2 ball bearings) and

milled for 7 h (600 rpm, 10 min. pause every 20 min.); jar temperature remained at <50 °C.

Plant cell wall dissolution

To a 5 ml flask, BMW material (60 mg) was added, followed by DMSO- d_6 (400 μ l) and NMI- d_6 (100 μ l) and stirred at room temperature to give a concentration of 120 mg/ml. A clear solution formed in approximately 3 hours, depending on the sample, but stirring was usually continued overnight. An NMR tube pipette (178 mm) was used to transfer the viscous solution directly to a 5 mm NMR tube.

NMR, general

For imidazoles in $CDCl_3$, NMR spectra were acquired at 27 °C on a Bruker DRX-360 instrument (^1H 360.13 MHz, ^{13}C 90.56 MHz) fitted with a 5 mm ^1H /broadband gradient probe with inverse geometry (proton coils closest to the sample). Proton and carbon spectra were referenced to internal tetramethylsilane (TMS) at 0.0 ppm. For plant cell wall samples, NMR spectra were acquired at 300 K on a Bruker DMX-500 (^1H 500.13 MHz, ^{13}C 125.76 MHz) instrument equipped with a sensitive cryogenically cooled 5 mm TXI $^1H/^{13}C/^{15}N$ gradient probe with inverse geometry. The central solvent peak was used as an internal reference for plant cell walls, DMSO (δ_C 39.5, δ_H 2.49 ppm). All processing and numerical integration calculations, utilizing single specimen data, were conducted using Bruker Biospin's TopSpin v. 2.0 software. Performing such integrations on NMR data from polymeric materials is sensitive to crosspeak overlap; all integrations were therefore measured only on intense well-resolved crosspeaks.

Routine 1D ^1H and ^{13}C NMR spectra

^1H and ^{13}C spectra were recorded with 32K data points for a spectral width of 3,094 Hz (8.6 ppm) and 64K data points for a spectral width of 20,000 Hz (220 ppm), respectively. Exponential multiplication (LB = 0.3 for ^1H , 2.0 for ^{13}C) and one level of zero-filling was performed prior to Fourier transformation.

2D HSQC spectra

The standard Bruker pulse implementation (invietgssi) of the gradient-selected sensitivity-improved inverse (^1H -detected) HSQC experiment⁴⁰ was used for acquiring the 2D spectra. The phase-sensitive HSQC spectra were determined with an acquisition time of 183 ms using an F_2 spectral width of 3,501 Hz (7 ppm) in 1280 data points using 88 transients (16 dummy scans) for each of 400 t_1 increments of the F_1 spectral width of 16,349 Hz (130 ppm) (F_1 “acquisition time” of 12.2 ms). Processing used a Gaussian function for F_2 (LB = -0.18, GB = 0.005) and a cosine squared function for F_1 prior to 2D Fourier transformation. The resulting data matrix was 1024×1024 points. ^{13}C Decoupling during acquisition was performed by GARP composite pulses from the high-power proton decoupling channel.

NMR assignments

All polysaccharide ^1H and ^{13}C chemical shift assignments for the plant species described here were assigned using previous literature on *Populus tremula*,⁴¹ *Linum usitatissimum*,⁴² and *Picea abies*.^{14,43,44} Lignin assignments were confirmed with the NMR Database of Lignin and Cell Wall Model Compounds.⁴⁵ Assignment of several polysaccharide crosspeaks were not attempted here, but research towards more complete assignments is underway.

RESULTS AND DISCUSSION

Preparing the plant cell walls

The steps in preparing the plant material for dissolution are crucial for acquiring quality NMR spectra. The existing dissolution method⁵ provided the basis for this study that involved decreasing the number of steps towards dissolution. The new method is designed to decrease material losses, eliminate the acetylation and subsequent isolation steps, decrease the sample preparation time, and be able to analyze milligram scale quantities. In particular, if an EDTA (chelating) wash step is deemed necessary to remove the paramagnetic iron from the material (to increase relaxation behavior for high-resolution spectra), a ~10% weight loss of cell wall material can result. Also, we wanted to eliminate the extraction steps, which remove low molecular weight compounds from the cell wall. The major reason for analyzing the cell wall in a non-extracted state is for the purposes of better understanding the reactivity of all plant components (including low molecular compounds in the cell lumina) towards derivatization, in a project currently underway. **Figure 2.3** illustrates the process we used for dissolving the plant cell walls, beginning with chopped plant material and ending with ball-milled micron-sized agglomerates for NMR analysis. Through ESEM imaging of several samples, the mixer-milled material gave an estimated particle size of 100 μm and the BMW was estimated to be $< 5 \mu\text{m}$ prior to dissolution.

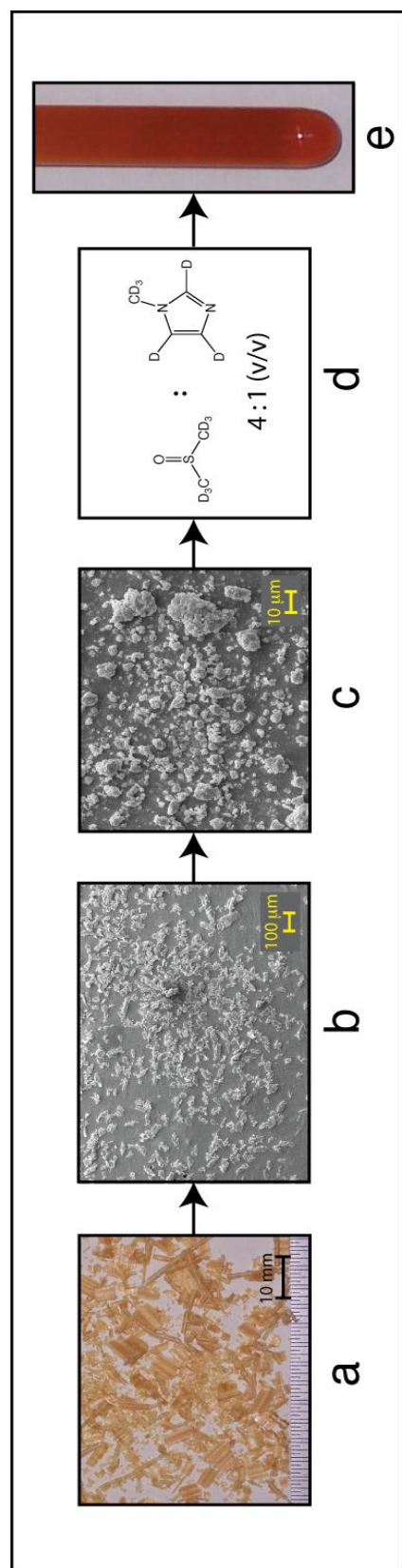


Figure 2.3. A diagram illustrating the non-derivatized dissolution of plant cell walls of *Pinus taeda*: **(a)**, shavings produced via conventional jointer; **(b)**, cryogenically mixer-milled shavings; **(c)**, planetary ball-milled particles from the mixer-mill; **(d)**, dissolution method using DMSO- d_6 and NMI- d_6 ; **(e)**, 5 mm NMR tube of pine cell walls in solution.

Synthesis of NMI-d₆

The dissolution method requires synthesis of the perdeuterated NMI by N-methylation with deuterated methanol followed by exchange of the hydrogens at the 2, 4, and 5 positions on the ring with deuteriums. The N-alkylation route we pursued was based on a ruthenium-trialkylphosphite complex to alkylate acidic NH groups of azoles.³⁷ A modification of this procedure uses methanol-d₄, which has been shown to give N-alkylation of imidazole in 49% yield.³⁸ Alkylation by this method in our lab gave an isolated yield of 66% after vacuum distillation. GC/MS analysis of the product showed complete (–CD₃) N-methylation with a distribution of products with the ring protons exchanged, as well. For example, the expected N-methyl-d₃-imidazole (27%) was found in addition to N-methyl-d₃-imidazole-d (40%), N-methyl-d₃-imidazole-d₂ (26%), and N-methylimidazole-d₆ (7%).

After N-methylation, further exchange of the protons in the N-methyl-d₃-imidazole was accomplished. Imidazoles are much less prone to isotope exchange than their corresponding imidazolium salts.^{46,47} Previous researchers used D₂O with the heterogeneous catalyst palladium (10%) on activated carbon to exchange all ring protons to give a yield of 91%.³⁸ In our several attempts with this method, the only position that exchanged to a high degree was H(2) with very little H(4/5) exchange. Since palladium catalysts seem to be quite variable in their activity with this method, our result prompted the search for a more consistent ring proton exchange method for H(4/5). Other researchers described a method of deuterium exchange of heterocyclic compounds with D₂O and heterogeneous Pd/C under 2.5 atm of hydrogen at 160 °C.³⁹ Although they did not study NMI, most heterocycles studied displayed almost quantitative deuterium incorporation at protonated aromatic ring positions. Utilizing a somewhat modified procedure, we achieved quantitative exchange of the H(4/5) protons, but little H(2) exchange. Therefore, to

obtain high deuterium exchange of NMI ring protons, we incorporated modifications of both procedures, to give an overall yield of ~62% of the fully deuterated material. **Figure 2.4** shows the ^1H spectra at the various stages of perdeuteration of NMI.

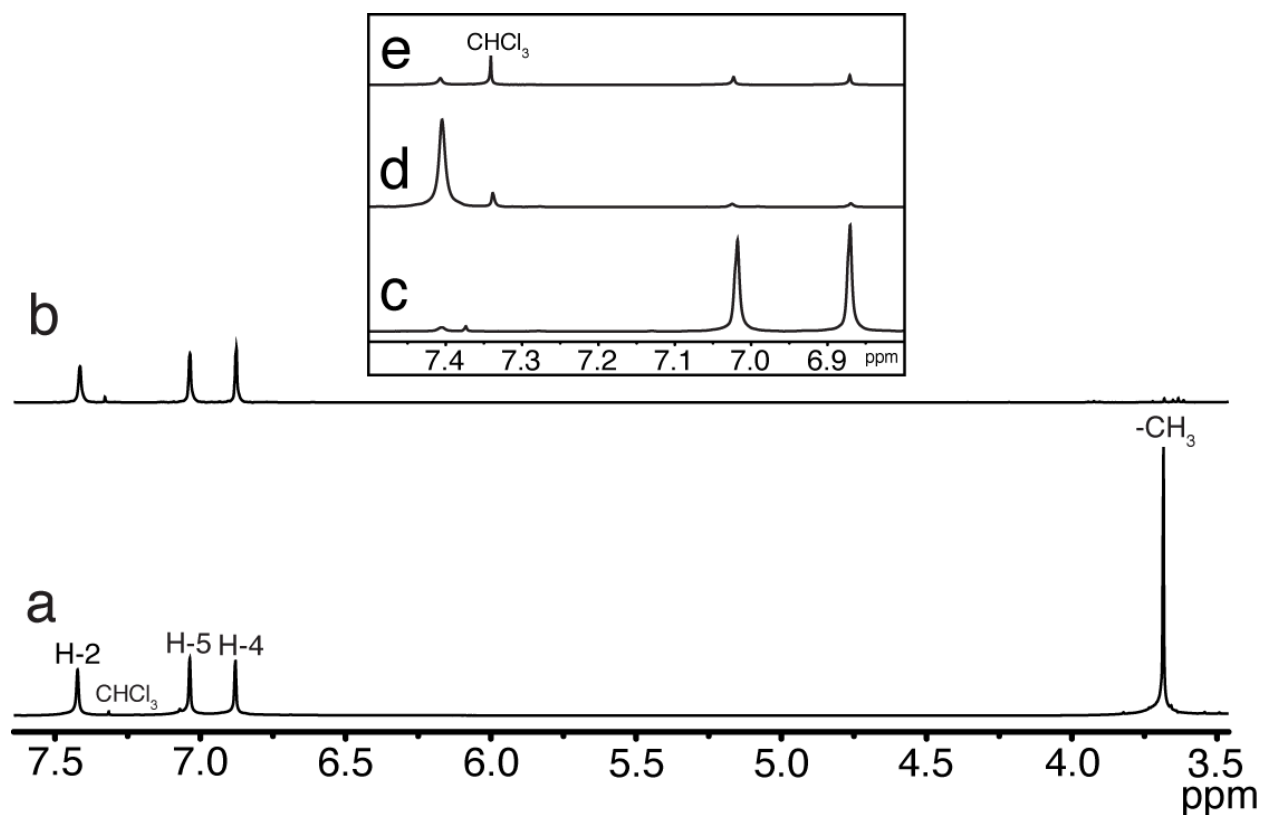


Figure 2.4. Proton spectra of various stages in NMI deuteration: (a) NMI; (b) N-methyl- d_3 -imidazole; (c) H-2 deuteration; (d) H-4 and H-5 deuteration; e, full ring deuteration.

Plant cell wall dissolution

In the system previously described,⁵ the DMSO:NMI ratio was 2:1 (v/v) with plant cell wall material at a concentration of 40 mg/ml. The solution becomes completely clear in a matter of 3 hours or less, suggesting dissolution. For dissolution of plant cell walls to be directly placed into a 5 mm NMR tube, this would mean that only 20 mg of plant cell wall material would be dissolved in 0.5 ml. Particularly for 1D ^{13}C NMR, analyzing less than 60 mg of plant cell walls

in 0.5 ml of solvent requires a very high number of scans (i.e., days rather than hours), especially if the cell wall component of interest is lignin, typically only ~20% of the cell wall. We found that the use of DMSO- d_6 and NMI- d_6 in a ratio of 4:1 (v/v), to give a cell wall concentration of 120 mg/ml, was sufficient for dissolution. Clarity of the solution at this concentration is not as high, and the solution is also quite viscous; however, such solutions are still quite suitable for 2D NMR.⁴⁸ Spectra reacquired on identical samples after several months were identical and showed no degradation.

¹³C-¹H NMR of the plant cell wall

The spectra described in the following figures contain color-coded contours, which correspond to their respective colored structure shown in the **Key 1** below. **Figure 2.5** shows HSQC one-bond ¹³C-¹H correlation spectra the gymnosperm *Pinus taeda* (loblolly pine). All major cell wall components are depicted, including polysaccharides (with anomeric regions differentially colored) and lignin (side-chain and aromatic) regions. The enhanced peak dispersion and sensitivity using a cryogenically cooled sample probe allows for the potential to fully assign the polymeric structures in the whole cell wall. In the aromatic region, one of the major differences between angiosperms and gymnosperms is immediately evident; angiosperms have both syringyl and guaiacyl units, while gymnosperms only have guaiacyl units. It is evident that most of the polysaccharide anomeric correlations are fairly well resolved, allowing for partial characterization of the H-1/C-1 crosspeaks of glucan (cellulose), xylan, mannan, galactan, and arabinan. Here, we focus on assignments of lignin side-chain units, naturally acetylated polysaccharides, and polysaccharide anomeric.

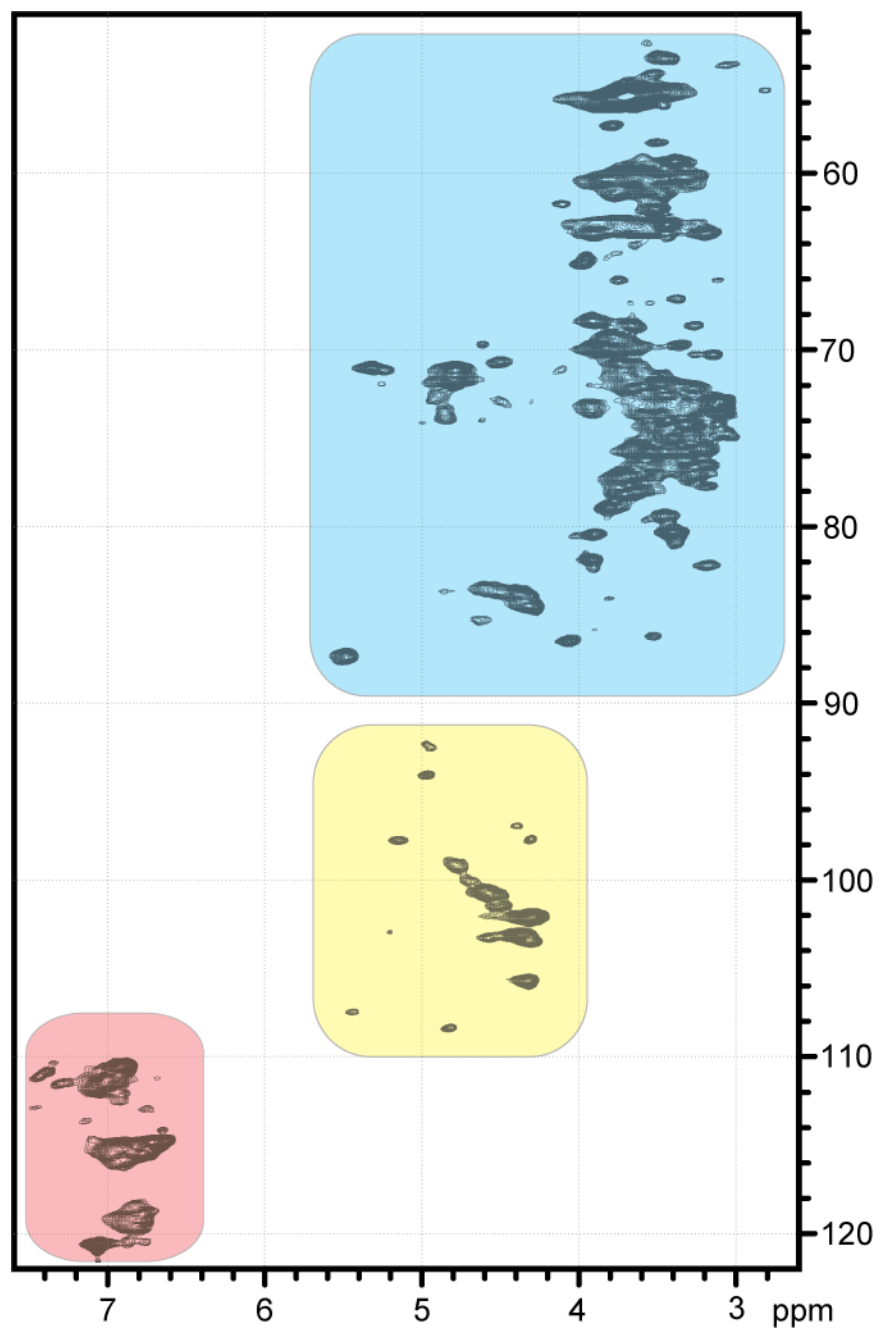
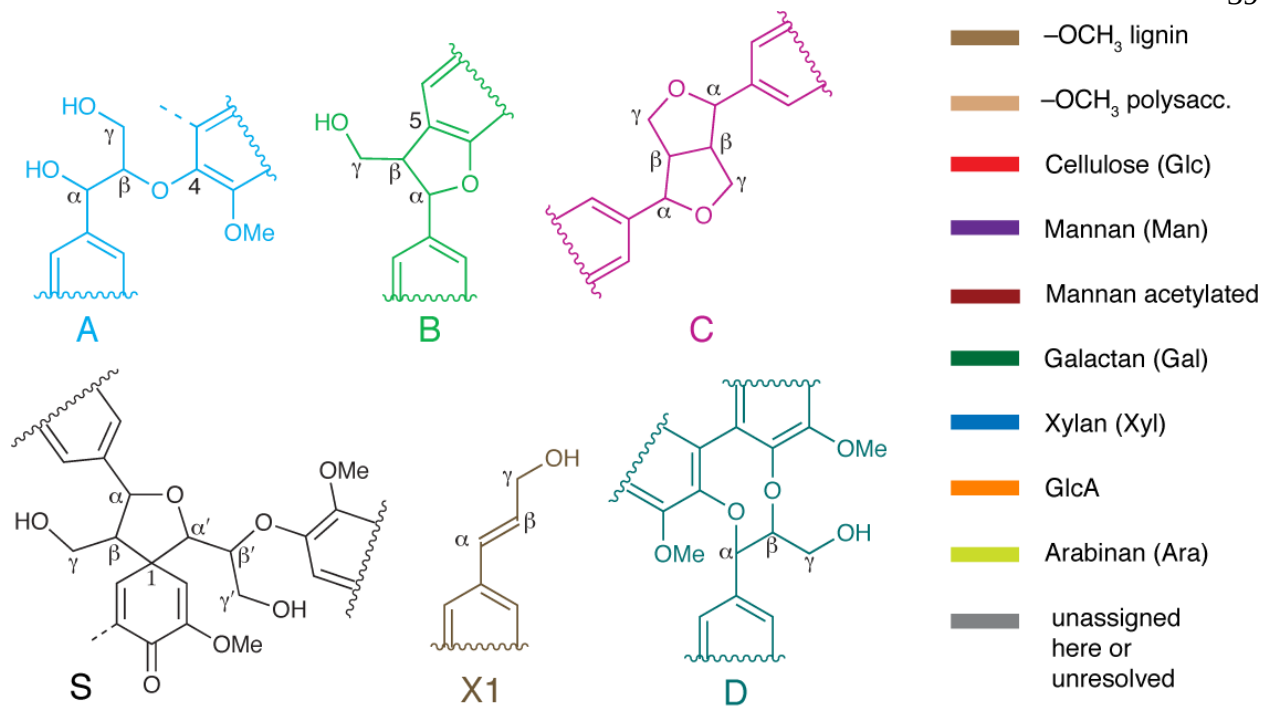


Figure 2.5. HSQC spectra from non-derivatized loblolly pine cell walls at 500 MHz with cryogenic probe. Spectra show three regions: blue, sidechain aliphatics; yellow, polysaccharide anomers; red, lignin aromatics.



Key 1. A key to the structures in the HSQC spectra for figures to follow in this chapter. All contour colors can be matched to their respective structure: (A) β-aryl ether in cyan, (B) phenylcoumaran in light green, (C) resinol in raspberry, (D) dibenzodioxocin in sea green, (S) spirodienone/β-1 in black, (X1) cinnamyl alcohol endgroups in mocha, guaiacyl in dark blue, syringyl in magenta. Other structures include: lignin methoxyl in brown, polysaccharide methoxyl in tan, cellulose in red, mannan in purple, H-2/C-2 & H-3/C-3 correlations for the acetylated structure of β-D-Manp¹ in maroon, xylan in blue, 4-O-methyl-α-D-glucuronic acid in orange, arabinan in chartreuse, galactan in dark green, and unassigned or unresolved contours in gray.

i. Lignin side-chain units

Figure 2.6a shows the remarkably well-resolved lignin side-chain correlations. Depicted are the major β-aryl ether units (A), phenylcoumaran units (B), resinol units (C), dibenzodioxocin units (D), cinnamyl alcohol endgroups (X1), and the lignin methoxyls (–OMe). The β-aryl ether units are well dispersed. For example, the β-aryl ether linkage (Aβ_G) has its Aβ correlation at 4.37/84.0 ppm.⁴⁵ Pine, having exclusively guaiacyl units, displays strong phenylcoumaran correlations (Bα, 5.48/87.4 ppm; Bβ, 3.46/53.5 ppm).⁴⁵ Resinol correlations (Cα, 4.64/85.3 ppm;

$C\beta$, 3.05/53.9 ppm; $C\gamma_1$, 4.20/71.5 ppm)⁴⁵ are also quite visible. Generally, $C\gamma$ correlations are only visible in isolated MWLs; the $C\gamma_2$ correlation remains masked by polysaccharides. The dibenzodioxocin correlations $D\alpha$ and $D\beta$ in pine are shown here to reside at 4.86/83.6 ppm and 3.90/85.8 ppm.⁴⁵ The cinnamyl alcohol endgroup correlation $X1\gamma$ is shown at 4.14/61.7 ppm.⁴⁵ There is some suspicion that another structure may overlap with this correlation. Spirodienone (β -1) structures were detected in the spectra, but because of the low quantity of such linkages in lignin the spectrum showed these only at low contour intensity. Spirodienone correlations $S\alpha$, $S\beta$, and $S\beta'$ (not shown) were found at 5.14/81.4 ppm, 2.81/60.3 ppm, and 4.18/79.8 ppm.^{49,50} Lignin methoxyls with its three ^{13}C - ^1H correlations typically produce a strong and broad contour⁴⁵

ii. Naturally acetylated structures

Loblolly pine shows a large contour centered at 1.99/20.9 ppm corresponding to the acetate methyls. We integrated these acetate contour regions and compared them to those of the methoxyl correlations. The values obtained show that for pine the ratio of methoxyl to acetate was 1:0.1. For pine, natural acetates are expected to be on the mannans (~10% of loblolly pine hemicelluloses),⁵¹ in particular galactoglucomannan, the principal hemicellulosic component in gymnosperms. The C-2 and C-3 positions of the mannan chain are partially substituted with *O*-acetyl groups at a frequency of about 1 out of 3-4 hexose units.⁵² The acetylated mannan (2-*O*-Ac- β -D-Manp and 3-*O*-Ac- β -D-Manp) H-2/C-2 and H-3/C-3 correlations (shown in Figure 2.6a) are evidenced at 5.33/71.0 ppm and 4.85/73.8 ppm.^{43,44} Previous researchers have shown that the ratio of 2-*O*-Ac- β -D-Manp : 3-*O*-Ac- β -D-Manp in Norway spruce is not equal, with the 2-*O*-Ac-

β -D-Manp slightly predominating (e.g., 2.2:1, 1.7:1, and 1.1:1).^{14,43,53} Here, through volume integration of the above contours, we report a ratio of 1.5:1 for loblolly pine.

iii. Polysaccharide anomeric

In **Figure 2.6b**, we show the anomeric region for all three species, tentatively assigning several anomeric, including D-glucan, D-xylan, D-mannan, and D-galactan as the major contours. The HSQC spectrum shows internal anomeric correlations of the (1 $\rightarrow$ 4)-linked β -D-glucopyranosyl (β -D-Glcp^I) at 4.38/103.1 ppm,⁴²⁻⁴⁴ as well as the (1 $\rightarrow$ 4)-linked β -D-xylopyranosyl (β -D-Xylp^I) at 4.32/102.1 ppm⁴¹ and (1 $\rightarrow$ 4)-linked β -D-mannopyranosyl (β -D-Manp^I) at 4.57/100.7 ppm.^{43,44} The pine depicts anomeric correlations of the terminal α -D-galactopyranosyl (α -D-Galp^T), (1 $\rightarrow$ 6)-linked to β -D-Manp, at 4.84/99.0 ppm.^{43,44} The pine spectrum also shows anomeric correlations of a terminal β -D-galactopyranosyl (β -D-Galp^T), (1 $\rightarrow$ 6)-linked to β -D-Manp, at 4.32/105.7 ppm.^{43,44} Acetylated mannan structures show their respective anomeric correlations at 4.78/99.1 ppm (2-O-Ac- β -D-Manp) and 4.69/100.0 ppm (3-O-Ac- β -D-Manp).⁴³ Arabinoglucuronoxylan, 5-10% of the cell wall weight in gymnosperms, is a framework of (1 $\rightarrow$ 4)-linked β -D-Xylp^I units that are substituted at C-2 by 4-O-methyl- α -D-glucuronic acid (4-O-MeGlcA) groups in a frequency of two residues per ten β -D-Xylp^I units.⁵² The 4-O-MeGlcA anomeric correlations for pine are depicted at 5.15/97.8 ppm. The arabinoglucuronoxylan is also substituted with terminal (1 $\rightarrow$ 3)-linked α -L-arabinofuranosyl (α -L-Araf^T) units in a frequency of 1.3 residues per ten β -D-Xylp^I units.⁵² Thus, the α -L-Araf anomeric correlations for pine are depicted at 4.83/108.4 ppm.^{42,43,54} Reducing ends of the polysaccharides can also be seen in the spectra. The reducing α and β ends of D-Glcp residues

are shown at 4.94/92.7 ppm and 4.40/96.9 ppm.^{43,44} For pine, the reducing α end of D-Manp and the reducing α and β ends of D-Galp residues are shown at 4.97/94.0 ppm,^{43,44} 4.98/92.8 ppm and 4.31/97.7 ppm.⁴⁴

The new ^1H and ^{13}C chemical shifts presented in this chapter for non-derivatized and naturally acetylated plant cell wall polymer structures from loblolly pine are summarized in **Table 2.1**, along with references.

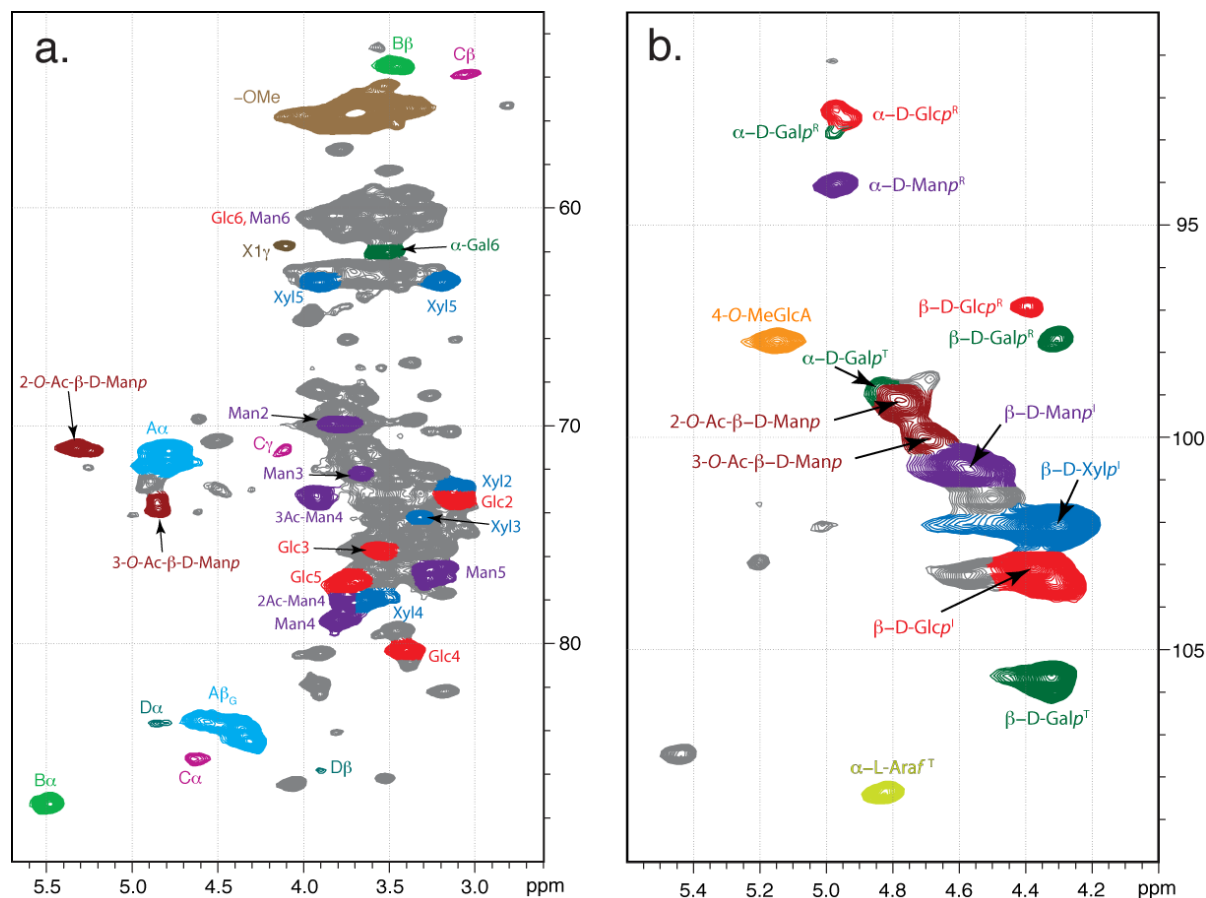


Figure 2.6. Aliphatic (a) and anomeric (b) regions of the whole plant cell wall HSQC spectra at 500 MHz. Aβ_G is guaiacyl coupled to a guaiacyl β-aryl ether. 2-O-Ac-β-D-Manp, H-2/C-2 correlation for the acetylated structure of β-D-Manp^I; 3-O-Ac-β-D-Manp, H-3/C-3 correlation for the acetylated structure of β-D-Manp^I;⁴³ Tentative assignments for anomeric: β-D-Glcp^I, internal β-D-glucopyranosyl units (cellulose);⁴²⁻⁴⁴ β-D-Manp^I, internal β-D-mannopyranosyl units;^{43,44} β-D-Xylp^I, internal β-D-xylopyranosyl units;⁴¹ α-D-Galp^T, terminal α-D-galactopyranosyl units;^{43,44} β-D-Galp^T, terminal β-D-galactopyranosyl units;⁴⁴ α-L-Araf^T, terminal α-L-arabinofuranosyl units;^{42,43,54} 2-O-Ac-β-D-Manp, anomeric correlation for the acetylated H-2/C-2 structure of β-D-Manp^I; 3-O-Ac-β-D-Manp, anomeric correlation for the acetylated H-3/C-3 structure of β-D-Manp^I;⁴³ 4-O-MeGlcA, 4-O-methyl-α-D-glucuronic acid;⁴¹ α and β-D-Glcp^R, reducing ends of glucopyranosyl;^{43,44} β-D-Manp^R, reducing end of mannopyranosyl;⁴³ α and β-D-Galp^R, reducing ends of galactopyranosyl;⁴⁴ α and β-D-Xylp^R, reducing ends of xylopyranosyl units.⁴¹ See **Key 1** for color-coded structure labels.

Table 2.1. Chemical shifts of native wood cell wall polymer structures in loblolly pine.

Structure or Position	¹ H (δ , ppm)	¹³ C (δ , ppm)	Reference for Assignments
Non-derivatized lignin structures in MWL and BMW (DMSO- <i>d</i> ₆)			
A α	4.80	71.46	ref. 45
A β	4.36	83.89	ref. 45
A γ	3.49, 3.67	59.87	ref. 45
B α	5.47	87.09	ref. 45
B β	3.47	53.39	ref. 45
B γ	3.91	63.24	ref. 45
C α	4.63	84.95	ref. 45
C β	3.05	53.63	ref. 45
C γ	3.78, 4.11	71.00	ref. 45
phenolic G4	–	148.34	ref. 45
–OCH ₃	3.70	55.61	ref. 45
Non-derivatized polysaccharide aliphatics in holocellulose and BMW (DMSO- <i>d</i> ₆)			
Glc2	3.18	72.95	refs. 42, 43, 44
Glc3	3.59	75.38	refs. 42, 43, 44
Glc4	3.44	80.13	refs. 42, 43, 44
Glc5	3.73	76.94	refs. 42, 43, 44
Glc6	3.79, 3.40	60.07	refs. 42, 43, 44
Man2	3.86	69.65	refs. 43, 44
Man3	3.71	71.76	refs. 43, 44
Man4	3.83	78.67	refs. 43, 44
Man5	3.31	76.46	refs. 43, 44
Man6	3.67, 3.56	60.13	refs. 43, 44
2-O-AcMan2	5.36	70.68	ref. 43
3-O-AcMan3	4.89	73.43	ref. 43
2-O-AcMan4	3.77	77.81	ref. 43
3-O-AcMan4	3.96	72.89	ref. 43
Xyl2	3.16	72.62	ref. 41
Xyl3	3.36	73.97	ref. 41
Xyl4	3.70	77.75	ref. 41
Xyl5	3.93, 3.22	63.07	ref. 41
α -Gal6	3.56	61.72	ref. 43
GlcA-CH ₃	3.47	60.90	refs. 41, 43
Ac-CH ₃	2.02	20.61	refs. 41, 42, 44
Non-derivatized polysaccharide anomers (DMSO- <i>d</i> ₆)			
Glc	4.42	102.90	refs. 42, 43, 44
α -Glc ^R	5.00	92.15	refs. 42, 43, 44
β -Glc ^R	4.44	96.73	refs. 42, 43, 44
Man	4.61	100.48	refs. 43, 44

α -Man ^R	5.03	93.70	refs. 43, 44
Xyl	4.34	101.81	ref. 41
β -Xyl ^R	4.43	97.66	ref. 41
α -Xyl ^R	5.04	93.88	ref. 41
α -Gal	4.88	98.46	refs. 43, 44
β -Gal	4.36	105.40	ref. 43
α -Gal ^R	5.05	92.58	ref. 43
β -Gal ^R	4.34	97.50	ref. 43
Ara	4.86	108.12	refs. 42, 43, 54
GlcA	5.20	97.47	ref. 41

CONCLUSIONS

The methodologies developed here help in characterizing the structures of several native-state plant cell wall components. After developing an effective synthesis of perdeuterated NMI to a high degree of deuterium incorporation, we described an approach to dissolve BMW in DMSO- d_6 and NMI- d_6 and characterize the majority of the plant cell wall components via high-resolution solution-state 2D NMR. Directly dissolving BMW cell wall material in non-degradative solvents and running NMR of these samples allows characterization without derivatization and loss of material. The HSQC one-bond ^{13}C - ^1H correlation spectra of loblolly pine depict all major plant cell wall components with excellent resolution, dispersion, and in their near-native-state. Through these spectra, we assigned correlations to naturally acetylated mannan structures found in galactoglucomannan. The well-dispersed contours in the polysaccharide aliphatic and anomeric regions of the HSQC spectra allowed us to tentatively assign several correlations to specific structures in hemicelluloses. Through contour integration techniques, we estimated the ratios of polymer structures in the cell wall. We assume that utilizing adiabatic sequences to remove J-dependence, and possibly applying determined response factors, will allow more accurate quantification in the future. More research needs to be

done to define polysaccharide chemical shifts; most chemical shift data obtained for assignments were derived from oligosaccharides dissolved in D_2O , not $DMSO-d_6/NMI-d_6$. However, this research broadens the utility of the DMSO/NMI method to better understand plant cell wall chemistry, which may be applied to various fields. We are currently employing these methods to investigate chemical modification, microbiological decay mechanisms, and genetically engineered plants and trees.

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Chapter 3. Characterizing model pMDI reactions with loblolly pine using solution-state NMR spectroscopy. I. Catalyzed reactions with wood models and wood polymers

SUMMARY

Reactions between wood model compounds and a pMDI model compound (PI) were characterized using solution-state NMR spectroscopy. To compare the reactivity of the wood model compounds to wood cell wall polymers, finely ground loblolly pine cell wall material, MWL, holocellulose, cellobiose, and Me4MeGlc were derivatized with the pMDI model compound. One-bond ^{13}C – ^1H HSQC experiments on derivatized and dissolved BMW revealed which wood cell wall polymers (and their respective hydroxyls) reacted with the pMDI model to form carbamates. The chemical shifts of the derivatized wood model compounds corresponded precisely to those in derivatized BMW chemical shifts. These preliminary model and cell wall experiments facilitate the next phase of the research – the study of pMDI model compound reactions occurring within intact wood cell walls under conditions similar to those used industrially for OSB production.

INTRODUCTION

Derivatization of lignocellulosics has commonly been used to modify their chemical, physical, and biological degradation properties. The types of chemical modification reactions include: acetylation, carbamylation, and formylation, as well as the modifications that occur by treatments with propylene oxide, treatment with phenol formaldehyde, treatment with hydroxymethylated resorcinol, and even impregnation with polyethylene glycol, or heat treatments, etc. Modification through carbamylation (i.e., the derivatization of wood using isocyanates) has been shown to decrease water absorption and produce modified wood with increased mechanical strength.¹⁻³ Isocyanates, particularly those produced with the wood composite adhesive pMDI, are known to impart considerable moisture durability, reducing thickness swelling and providing high flexural strength to the composite. The mechanism of isocyanate reaction with wood has been investigated; it is thought to react with wood polymers almost exclusively.⁴ However, isocyanates have also been shown to react competitively with water and, thus, self-condense to form urea-type products, which are capable of intermolecular hydrogen bonding.⁵ Therefore, under ambient conditions, isocyanates have the capability of forming carbamate bonds (i.e., via wood reactions) and urea bonds (i.e., via water reactions) within cell lumina and cell walls.

Homogeneous carbamylation of wood cell wall polymers

Cellulose carbamoylation has become the standard derivatization method for determining the molecular weight distribution of cellulose. As these carbamates are soluble in many organic solvents and do not form aggregates in solution, molar mass can be accurately measured using size exclusion chromatography or viscometry. Notably, these characterization methods allow for

monitoring polysaccharide degradation during sulfite, kraft, or other wood pulping processes. Characterizing the chemistry of these cellulose carbamates using solution-state NMR has been performed to some extent, but has been limited to 1D ^1H and ^{13}C experiments. The characterization of the carbamylated whole plant cell wall has not been studied. Characterizing all carbamylated wood cell wall polymers would provide a dataset for using NMR as a powerful tool for analyzing cell wall chemistry and should enhance our understanding wood polymer reactivity with isocyanates.

Many homogenous carbamylation methods for cellulose exist, but several give urea-type byproducts that mask proper characterization of the desired carbamate.⁶⁻⁸ NMI and pyridine, although a very effective solvent/catalyst system for acetylations with acetic anhydride,⁹⁻¹¹ were found to catalyze depolymerization of cotton linters during carbamylation reactions at temperatures around 60 °C.^{8,12} Mormann and Michel described a method that separates the byproducts from cellulose carbamates during precipitation of the homogeneously reacted product.¹³ Using DMAc as the solvent and DBTDL as the catalyst at 60 °C, full derivatization of cellulose can be obtained.

Lignin carbamylation has seen much less consideration compared to polysaccharides. However, studies performed with various types of lignin-based polymers were reviewed.¹⁴ For example, lignin extracted from wood using hydrochloric acid or sulfuric acid was reacted in dioxane with 1,6-hexamethylene diisocyanate and/or tolylene diisocyanate using triethylamine as a catalyst at 100 °C and characterized using IR spectroscopy.^{15,16} Lignin model compound reactions with isocyanates have been performed to a limited degree and the studies that exist used lignin models that were not good representations of the major lignin linkages in wood. One study reacted 2,4-tolylene diisocyanate with isoeugenol, ethylveratrylcarbinol, and

ethylguaiacylcarbinol at OH:NCO ratios of 1:1 and 1:2 in dioxane with ferric acetylacetonate (FeAA) as the catalyst.¹⁷ Using IR, they found that the phenolic hydroxyl of all compounds reacted exclusively at 100 °C while compounds with a benzylic hydroxyl reacted both at 40 °C and 100 °C, suggesting that the benzylic hydroxyl is more reactive than the phenolic hydroxyl under identical conditions. Another study reacted hexamethylene diisocyanate with simple lignin model compounds (i.e. isoeugenol, phenol, parahydroxy-benzaldehyde, vanillin, and guaiacol) without catalysts at room temperature or 100 °C and reported quantitative yields for all derivatizations.¹⁸ Their reaction products were characterized using IR and ¹H NMR spectroscopy, showing the N-H chemical shift of the urethane to be between 5.00 and 5.75 ppm; however, urea products would also have N-H chemical shifts in this range. Producing polyurethanes from lignin-based polymers has also seen some attention,^{19,20} but such processes have not shown commercial viability.

In the characterization of carbamylated wood cell wall polymers, there is a lack of structural confirmation of the derivatization reactions with isocyanates. Establishing that an isocyanate has unambiguously formed a carbamate to a wood polymer hydroxyl is crucial to characterizing isocyanate reactivity with wood. To that end, full derivatization of wood polymer hydroxyls is desired through the use of BMW to increase the accessibility of the pMDI model towards reaction. Furthermore, the adhesive bonding mechanism between pMDI and wood cannot be discerned without structural confirmation of carbamate and/or urea formation; discerning which mechanism(s) are involved during pMDI-wood bonding remains a formidable challenge, but proving whether or not covalent bonding is a probable contributing mechanism is certainly possible.

Objective

The objective of this study is to characterize carbamylated wood cell wall polymers using high-resolution solution-state NMR spectroscopy using the following approaches:

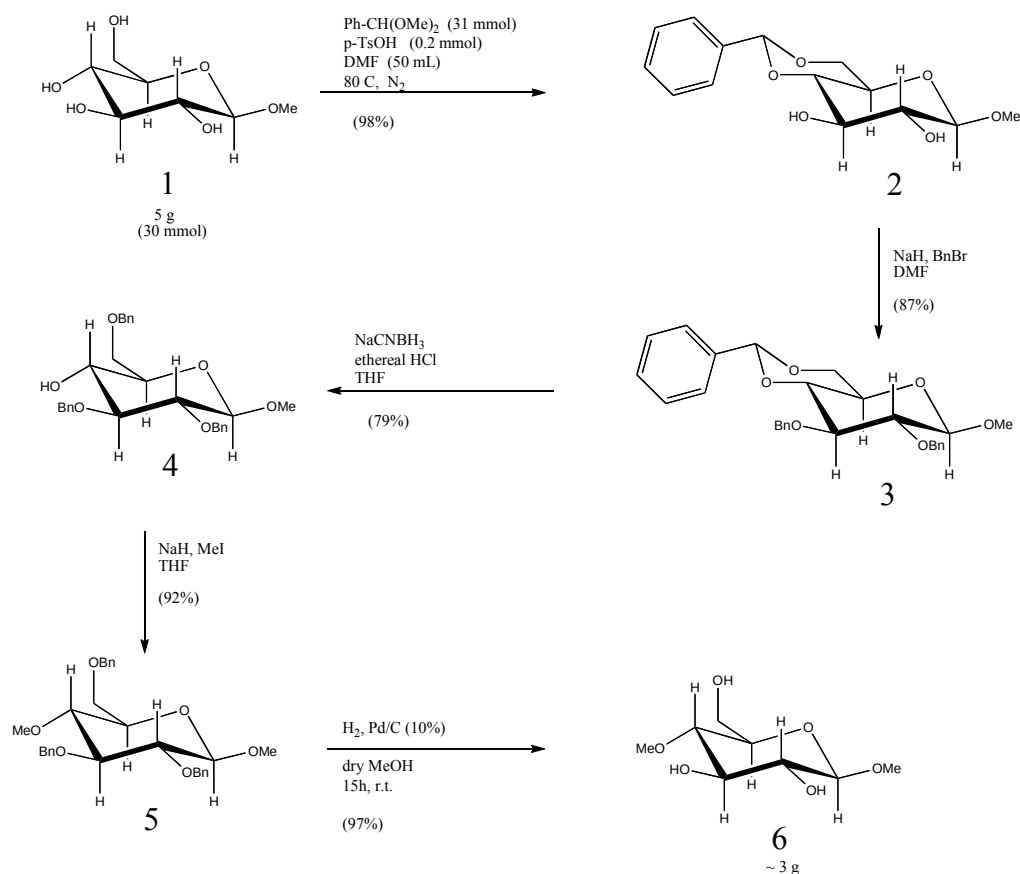
1. Synthesize wood model compounds (e.g., lignin dimeric β -aryl ether, phenylcoumaran, and pinoresinol dimer models, and Me₄MeGlc as a cellulose model).
2. React the wood model compounds with a pMDI model (e.g., PI) using DBTDL as catalyst to form wood model carbamates.
3. Homogeneously react isolated wood polymers (e.g., loblolly pine holocellulose, MWL, and BMW) with PI using DBTDL as catalyst to form wood polymer carbamates.
4. Dissolve the carbamylated products and characterize them using 2D solution-state NMR experiments to provide a chemical shift database for wood cell wall carbamates.

MATERIALS AND METHODS

Materials

The pMDI model (PI), DMAc (distilled over 3Å molecular sieves), the catalysts ferric acetoacetate (FeAA) and DBTDL, dioxane, and toluene were obtained from Aldrich Chemical Co. (Milwaukee, WI) and used as supplied. BMW (400 mg) was prepared using a Retsch PM100 planetary ball-mill with ZrO₂ ball bearings and vessel at 600 rpm milling for 4 hours. MWL was prepared from BMW according to Björkman²¹ via 96:4 dioxane:water extraction. Holocellulose from loblolly pine was prepared according to previously described methods using sodium chlorite.²² The lignin model dimers (e.g., β -aryl ether, phenylcoumaran, and pinoresinol dimers) were prepared by standard methods.²³ DMSO-*d*₆ (99%), guaiacol, and D(+)-cellobiose (98%) were supplied by Acros Organics (Geel, Belgium). NMI-*d*₆ was synthesized as described in

Chapter 2. The model Me4MeGlc is a good model for the internal units of cellulose and, with its highly accessible hydroxyl groups, regioselectivity experiments are more facile. Me4MeGlc was synthesized by methods similar to those described previously,^{24,25} as shown in **Scheme 1** below.



Scheme 1. Reaction steps used to synthesize the cellulose model **6**, Me4MeGlc.

Reactions with phenyl isocyanate

All reactions were carried out under N_2 using an appropriate solvent with 0.02 M DBTDL as catalyst. The wood models and polymers were dried in a vacuum oven at 50°C overnight before all reactions. The reaction times varied from 2 h for wood models (reactions monitored

for completeness by TLC using ethyl acetate:chloroform, (1:1) to 12 h for wood polymers.

Work-up of the reacted wood models were performed as follows: (1) Dry methanol was added to quench excess isocyanate; (2) Saturated NH_4Cl (5 mL) was added to render the solution slightly acidic and, due to the high salt concentration, allow improved partitioning of the products into organic solvent; (3) The reaction mixture was extracted with ethyl acetate (3×3 mL); (4) The separated organic (EtOAc) layer was dried over MgSO_4 ; (5) Solvents were removed by rotary evaporation at reduced pressure. Wood polymer reactions were considered complete when the reacted wood polymers went fully into solution. At that point, excess PI was removed by vacuum distillation (to about half of the original volume) and DMAc was added to bring the solution back to its original volume. Carbamylated wood polymers were then precipitated by adding the solutions dropwise to a stirred $\text{MeOH}/\text{H}_2\text{O}$ mixture ($v/v = 5:1$), the precipitate filtered onto a 0.2 μm nylon membrane, and washed with further $\text{MeOH}/\text{H}_2\text{O}$, dried in a vacuum oven at 50 $^\circ\text{C}$, and analyzed via NMR. The filtrate, containing methyl carbamate and diphenyl urea, was isolated via vacuum distillation of the filtrate solvents, recrystallized, further purified using preparative TLC with ethyl acetate:chloroform (1:1, v/v), and their structures confirmed via ^1H and ^{13}C NMR.

Solution-state NMR

The lignin model compounds were dissolved in acetone- d_6 (500 μL) and cellulose model compounds were dissolved in $\text{DMSO}-d_6$ (500 μL) and a suite of 1D and 2D experiments were acquired on a Bruker (Rheinstetten, Germany) DPX-250 (62.9 MHz for the ^{13}C) at 25 $^\circ\text{C}$.

Assignments were confirmed using the Lignin NMR Database²⁶ and HMBC experiments. For the wood polymers, one-bond $^{13}\text{C}-^1\text{H}$ correlation (HSQC) NMR spectra were acquired at 55 $^\circ\text{C}$ on a Bruker DMX-500 instrument equipped with a cryogenically cooled 5-mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$

gradient probe with inverse geometry (proton closest to the sample). Samples (approximately 90 mg each) were added to 5 mm diameter NMR tubes and dissolved in 500 μ l of DMSO- d_6 and NMI- d_6 was added only when analyzing carbamylated holocellulose and carbamylated BMW. The central DMSO solvent peak was used as an internal reference ($\delta_C = 39.5$ ppm, $\delta_H = 2.49$ ppm). HSQC experiments had the following parameters: sweep width, 6.4-2.8 ppm in F_2 (^1H) using 718 data points (acquisition time, 200 ms) and 110-40 ppm in F_1 (^{13}C) using 256 increments (F_1 “acquisition time” 14.5 ms). The number of scans was 200 with a 1-s interscan delay, the d_{24} delay was set to 1.72 ms ($\sim 0.25/J$, where J is an average 145 Hz ^{13}C - ^1H coupling constant), and the total acquisition time was 18 h. Processing used typical matched Gaussian apodization in F_2 and a squared cosine-bell in F_1 .

RESULTS AND DISCUSSION

Model reactions

Table 3.1 shows the reaction conditions and yields for the wood model reactions. The carbamylated cellobiose dissolved readily in DMSO- d_6 at room temperature for NMR experiments. **Figure 3.1a** shows the ^{13}C and **Figure 3.1b** the HSQC spectrum for the cellobiose carbamate overlaid with unreacted cellobiose. One of the important features to point out in the ^{13}C spectrum is the region around 152-153 ppm, which corresponds to the carbamate carbonyl carbon. In particular, the ^{13}C NMR spectrum shows several (more than 8) peaks in this region, meaning that there are possibly other types of carbonyls present (possibly urea-type). However, this also means that the carbamates have formed between the cellobiose and the isocyanate. Long-range correlation HMBC (not shown here), which correlates a carbon and a proton that is 2 or 3 bonds away, shows 8 peaks in the carbamate carbonyl region (152.5 ppm) that show

correlation to protons at 6.3, 6.05, 5.95, 5.6, 5.35, 5.0, 4.95, and 4.9 ppm on the proton spectra.

These are all cellobiose ring protons, thus confirming full derivatization of the cellobiose hydroxyls.

Table 3.1. Selected reaction conditions for wood model compounds with PI.

Model Compound		Molar Equivalents PhNCO:Model	Solvent	Catalyst	Temp (°C)	Molar Yield	
Name	Structure					%	position
β -aryl ether, A1 (etherified)		2:1	Dioxane	FeAA	40	10	γ
		2:1	DMAc	DBTDL	40	80	γ
						9	α
β -aryl ether, A2 (free-phenolic)		3:1	DMAc	DBTDL	40	63	$\gamma + \alpha$
		3:1	Toluene	DBTDL	40	92	$\gamma + \alpha +$ phenolic
Phenylcoumaran, B		3:1	DMAc	DBTDL	40	13	$\gamma + \gamma'$
		3:1	Toluene	DBTDL	40	44	$\gamma + \gamma' +$ phenolic
Pinoresinol, C		2:1	Toluene	DBTDL	40	56	phenolic
Guaiacol		1:1	Toluene	DBTDL	40	90	phenolic
D(+)-Cellobiose		8:1	DMAc	DBTDL	60	86	C2, C2', C3, C3', C4, C4', C6, C6'
Methyl 4-O- methyl- β -D- glucopyranoside		1:1	DMAc	DBTDL	60	27	C2
		2:1	DMAc	DBTDL	60	36	C2
						12	C2 + C6

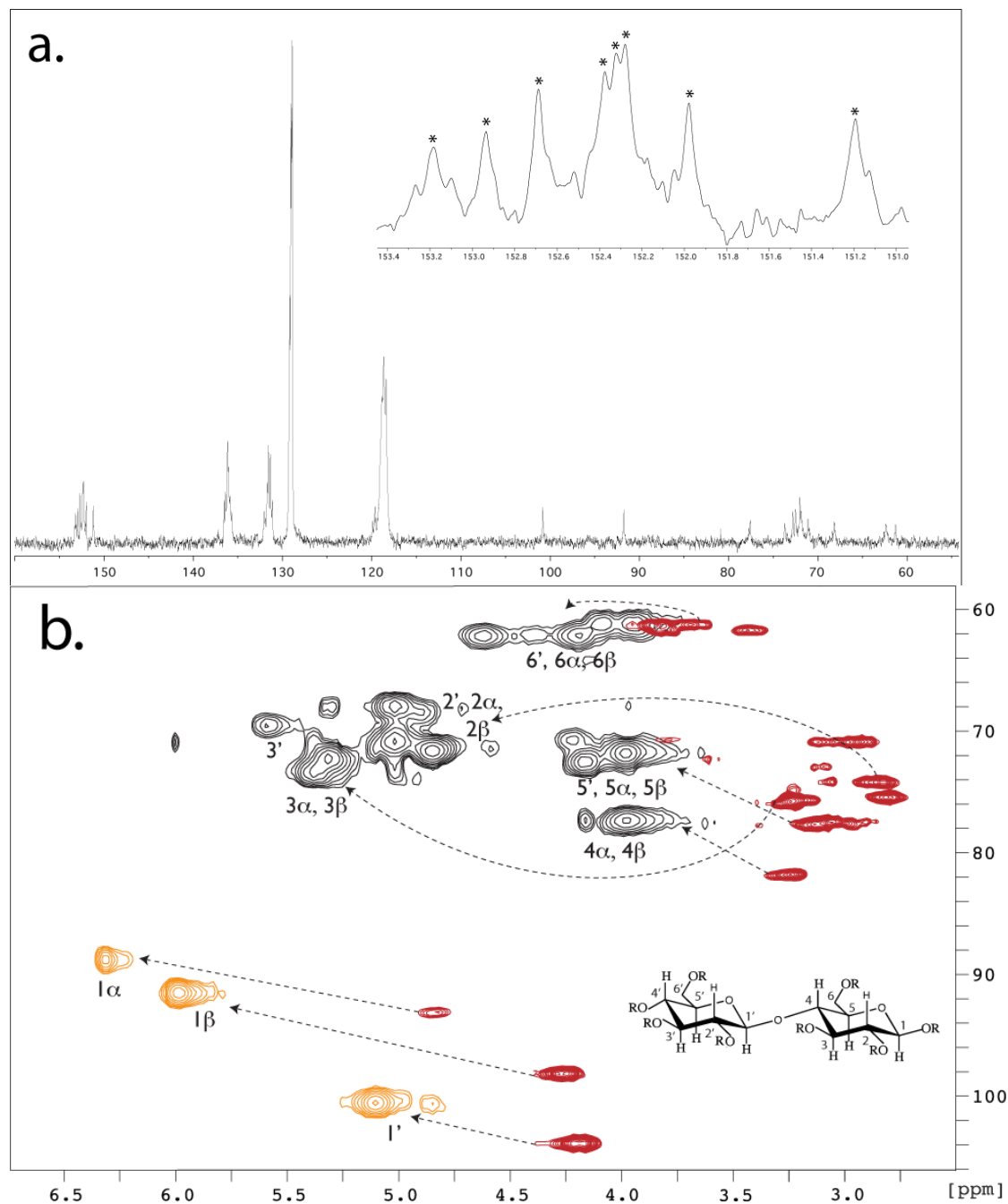


Figure 3.1. NMR spectra of cellobiose carbamate: **(a)** ^{13}C spectrum with expansion of the eight carbamate carbonyl peaks denoted with asterisks; **(b)** 2D HSQC spectrum of D-(+)-cellobiose fully reacted with PI. Red contours denote the unreacted cellobiose spectrum overlaid to show differences in chemical shifts between derivatized & unreacted cellobiose hydroxyls. Dashed arrows denote the important changes in chemical shifts upon derivatization. Orange contours denote anomeric correlations from derivatized cellobiose.

In reactions of Me₄MeGlc with PI shown in **Figure 3.2**, it was instructive to determine the regioselectivity of derivatization at each hydroxyl group. When using a 1:1 (NCO:model) equivalents ratio, the ¹³C and HSQC of the product showed that the C2 position reacts first. When the NCO:OH was increased to a 2:1 equivalents ratio, the HSQC revealed that both the C2 and C6 positions reacted, showing that C6 reacts second. Therefore, it follows that the order of reactivity found with this Sn-catalyzed model system is C2>C6>C3. It is quite unexpected that a secondary hydroxyl group would react preferentially to a primary hydroxyl group since the primary hydroxyl is known to be kinetically favored. This abnormal reactivity has been observed previously with carbohydrates.²⁷ Stereochemical or acidity considerations may be playing a role. It has also been found that the presence of strong intramolecular hydrogen bonding between primary carbohydrate hydroxyls prevent hydrogen abstraction.²⁸ Migration of a carbamyl group from 6-OH to 2-OH is another possibility due to the more thermodynamically stable carbocation at C2;²⁹ however, more research is needed to elucidate these mechanisms.

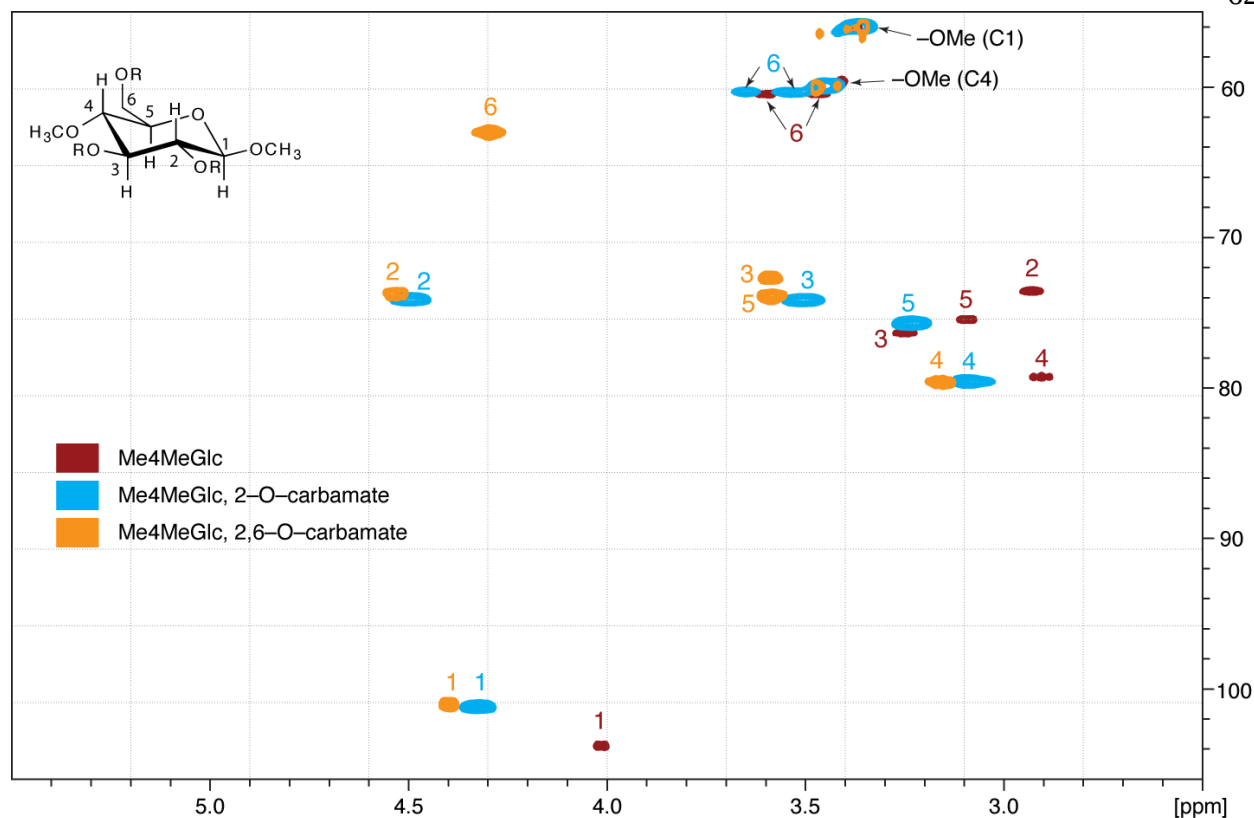


Figure 3.2. 2D HSQC NMR spectra of Me4MeGlc reacted with PI. Red contours denote the unreacted Me4MeGlc spectrum overlaid with the 2-OH (blue) and 2,6-OH (orange) derivatives to show differences in chemical shifts between derivatized & unreacted hydroxyls.

Prior literature on the carbamylation of lignin model compounds, as discussed in the introduction, were starting blocks for my lignin model reactions; however, the lack of experimental detail in these studies made repeatability challenging. Using the conditions described in Table 3.1 initial reactions between PI and etherified β-ether model **A1** used dioxane as the solvent and FeAA as the catalyst at 40 °C. The reaction products showed that the γ-carbamate was obtained in only 10% yield, which was unexpectedly low. Increasing the amount of isocyanate and bringing the temperature to 100 °C, free-phenolic β-ether model **A2** was expected to fully derivatize at the γ-, α-, and phenolic positions as described previously.¹⁷ This reaction resulted in five products that were shown by NMR to be degraded fragments of the

model compound along with some starting material. These results compelled me to explore other known carbamylation systems, such as DMAc as the solvent with DBTDL as the catalyst, a system that had been used for derivatizing cellulose. Changing the solvent and catalyst to this system enabled model **A1** to react predominantly at the γ -, but also at the α -position, yielding 80% of the γ -carbamate (66% *threo*, 14% *erythro*) and 9% of the α -carbamate model. **Figure 3.3** shows HSQC spectra of these products overlaid with those from the unreacted control. Further experiments utilized model **A2** to attempt sidechain and phenolic derivatization. Using DMAc and DBTDL once again with 3 equivalents of PI to 1 equivalent of **A2**, γ - and α -hydroxyls were derivatized, leaving the phenolic position unreacted, as was confirmed through ^{13}C NMR where the aromatic C4 chemical shift showed no indication of phenol (4-OH) carbamylation. Further attempts to derivatize the phenolic position utilized more equivalents of PI to **A2**, but these forceful conditions were not successful.

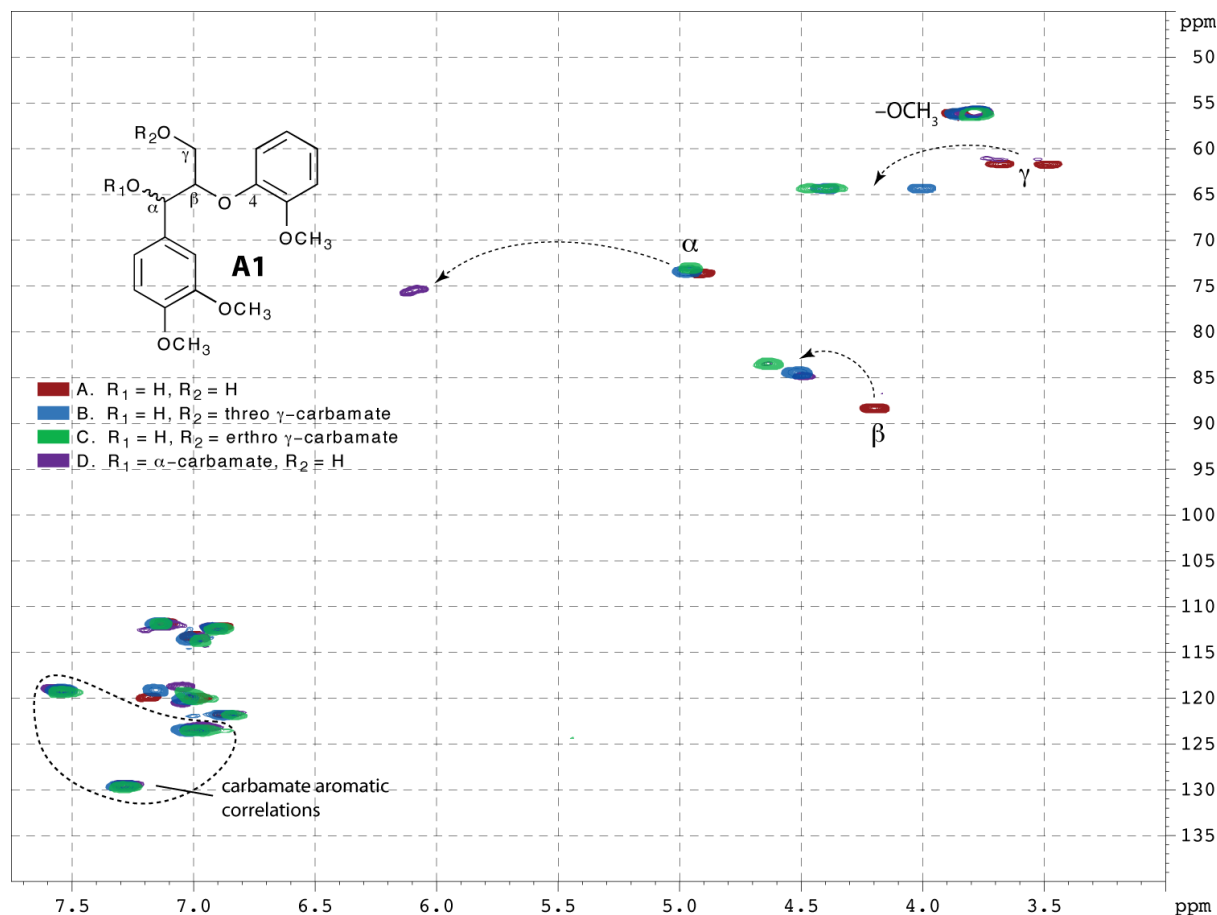


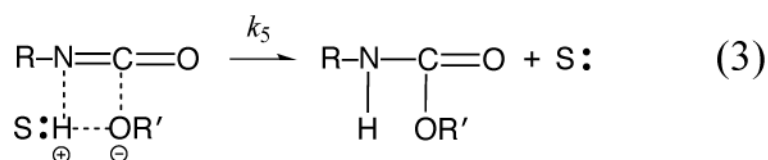
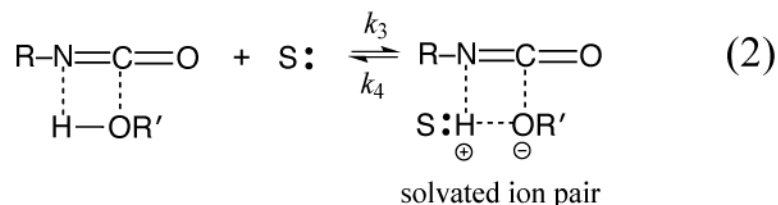
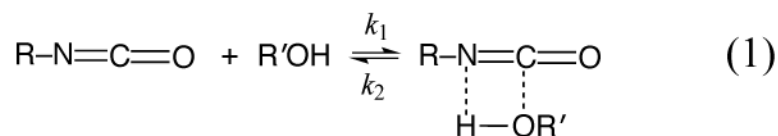
Figure 3.3. Overlay of HSQC NMR spectra of β-aryl ether lignin model compound **A1** derivatized with PI at 2:1 (equivalents) as shown in Table 3.1. Structures **A-D** are denoted by the color scheme shown. Dashed arrows denote the important changes in chemical shifts upon derivatization.

The phenolic position of lignin model compounds has therefore been shown to be the least reactive under the conditions employed above. Further review of several isocyanate-alcohol articles indicated that the kinetics and mechanisms of these reactions are not fully understood. Researchers have found that isocyanate-alcohol reactions are highly dependant on hydrogen bonding complexes between the alcohol, PI, and aprotic solvents, and electron donor number³⁰ of the aprotic solvent, giving rise to catalysis and inhibition effects.³¹⁻³⁵

Solvents having higher polarity (calculated from their dielectric constants) and capable of

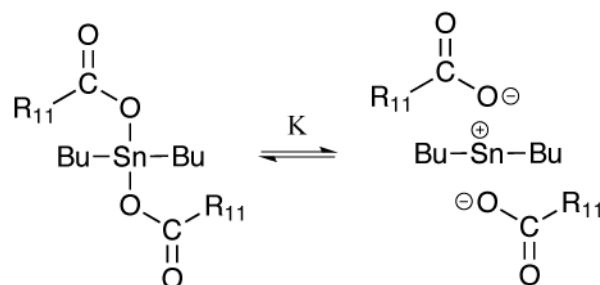
forming hydrogen bonds with the alcohol appeared to result in a lower reaction rate through the increased formation of solvent-alcohol complexes. Polar aprotic solvents like dioxane, having strong hydrogen bonding power and a low DN value[☆] give a strong inhibition effect rather than a catalytic effect and lowers the concentration of the desired isocyanate-alcohol complex. Solvents such as DMAc and DMSO, having strong hydrogen bonding capabilities and high DN values, give a strong catalytic effect.³² The proposed mechanisms for these observations are that: (1) the alcohol forms a hydrogen-bonding complex with the isocyanate, and (2) the non-associated species of the alcohol and aprotic solvent can solvate the acidic hydrogen in the complex to form an ion-pair, which allows the urethane reaction to occur more easily. The solvation power is dependent on the DN values of the solvent and alcohol. **Scheme 2** below shows this proposed mechanism.³² The higher the DN value of the electron donor solvent, the greater the tendency of step 2 to proceed to the right.

[☆] DN, or donor number, is a qualitative measure of Lewis basicity and measures the ability of a solvent to solvate cations and Lewis acids.



Scheme 2. Ion-pair mechanism of a non-catalyzed isocyanate reaction with alcohols, where S: is a non-associated species of an electron donor solvent and k is the rate constant of the reaction.

The dioxane inhibition effect may be the reason for such low yields in these model reactions, but the DMAc catalytic effect is not so clear given the difficulty of derivatizing the phenolic position of the **A2** model. While the mechanism of non-catalyzed reactions with isocyanate-alcohol systems are well understood, tin-catalyzed reactions remain unclear. It seems that other factors besides the strong hydrogen bonding and high DN value of DMAc in conjunction with the DBTDL catalyst gives a competing inhibitory effect in phenolic derivatization with these lignin model compounds. Studies on the catalytic behavior of DBTDL in dimethylformamide, a polar aprotic solvent like DMAc, showed that DBTDL disassociates into a catalytically active species, shown in **Scheme 3** below.



Scheme 3. Dissociation mechanism of DBTDL, where K is the dissociation constant of the catalyst and R₁₁ is a long-chain hydrocarbon (11 units long).

It is possible therefore that DMAc interferes with DBTDL dissociation and/or that DMAc in combination with DBTDL decreases the concentration of the isocyanate-alcohol complex in reactions with compound A2. The phenolic group in lignin model compounds has a DN value around 11 kcal/mol and according to the ion-pair mechanism (scheme 2) the phenolic position would therefore give an inhibitory effect.

It was decided then to choose an aprotic solvent with low hydrogen bonding capability to derivatize the phenolic position. Toluene, having a hydrogen bonding index of 1.5 (compared to 9.7 for dioxane and 12.3 for DMAc), has been used in industrial processes with DBTDL/alcohol systems to produce carbamates and polyurethanes and kinetic studies have shown isocyanate-alcohol complexes to be stable with these systems.³⁶ To simplify the system, I used guaiacol to study phenolic derivatization. With toluene as the solvent and reacting guaiacol with 1 equivalent of PI at 40 °C with DBTDL as the catalyst, the yield of the guaiacol carbamate was almost quantitative. **Figure 3.4** shows ¹³C NMR spectra of the guaiacol and guaiacol carbamate with a structure showing chemical shift assignments. The phenolic position derivatization is confirmed by the distinct chemical shift of the phenolic quaternary carbon on the guaiacol (148.3 ppm) versus that of the derivatized phenolic quaternary carbon (140.8 ppm).

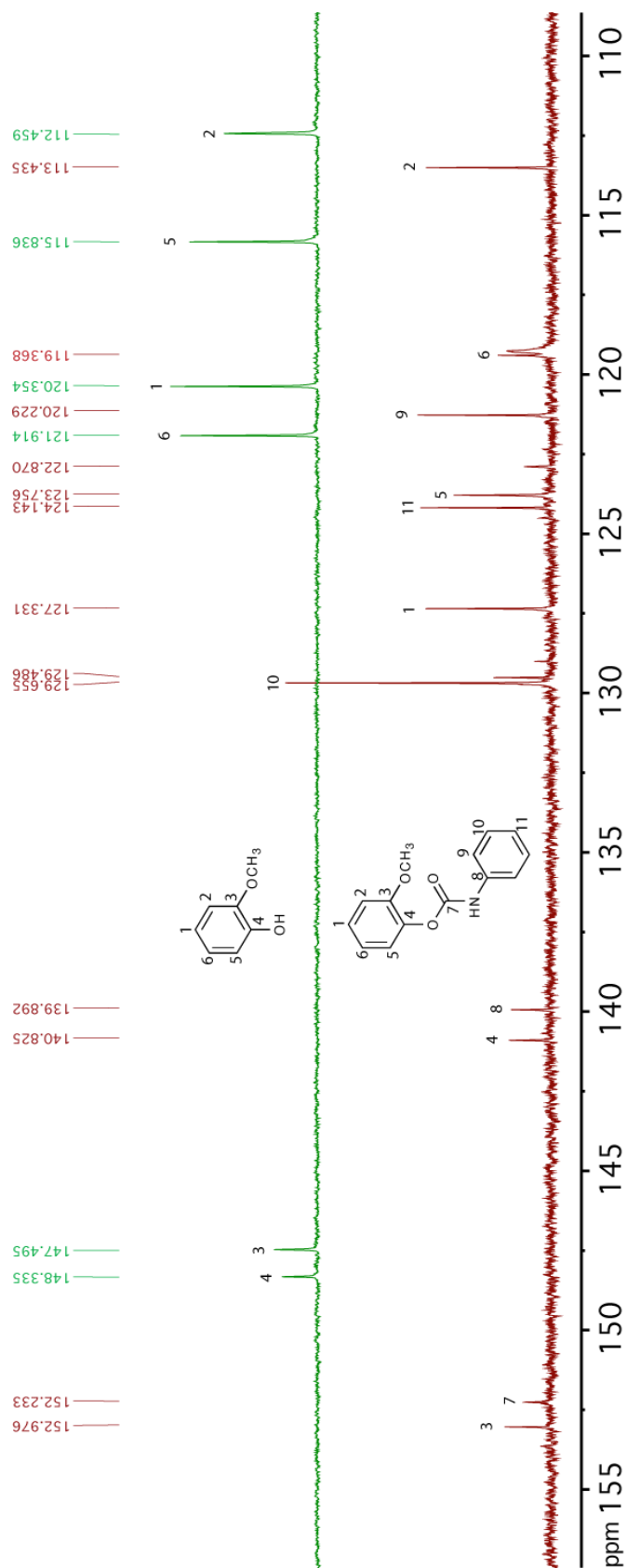


Figure 3.4. ^{13}C NMR spectra of guaiacol, and guaiacol derivatized with phenyl isocyanate as shown in Table 3.1. The unreacted guaiacol (green) and derivatized guaiacol (red) spectra are stacked with labeled peaks.

Further reactions with the lignin model **A2** were performed using the above conditions with 3 equivalents of PI to 1 equivalent of **A2** to obtain the fully derivatized β -aryl ether carbamate in high yield, confirmed with ^{13}C , HSQC, and HMBC NMR. **Figure 3.5** shows the HSQC spectrum of this carbamate. The use of toluene, rather than DMAc, for the solvent conveniently allowed formation of the desired **A2** carbamate, which crystallized as the reaction progressed. The phenylcoumaran **B** carbamylation reaction was performed initially with DMAc as solvent. This reaction resulted in low yield of the γ -carbamate. When toluene was used as the solvent, the reaction gave the γ - and phenolic carbamate in fair yield (44%); the product's structure was confirmed using ^{13}C , HSQC, HMBC NMR. **Figure 3.6** shows the HSQC spectrum of the phenylcoumaran carbamate, overlaid on the source phenylcoumaran. The pinoresinol **C** carbamylation reaction yielded two products which were difficult to separate. The diphenolic carbamate gave a fair yield (56%); its structure was confirmed by the presence its carbamate carbonyl signal (152.3 ppm) in ^{13}C NMR spectra. **Figure 3.7** shows the HSQC spectrum of the pinoresinol dicarbamate. With the model compound HSQC spectra acquired for the reacted cellobiose, Me₄MeGlc, and lignin model compounds (e.g., **A1**, **A2**, **B**, and **C**), these chemical shift data will be used in confirming assignments of reacted MWL, holocellulose, and BMW, as discussed in the following paragraphs.

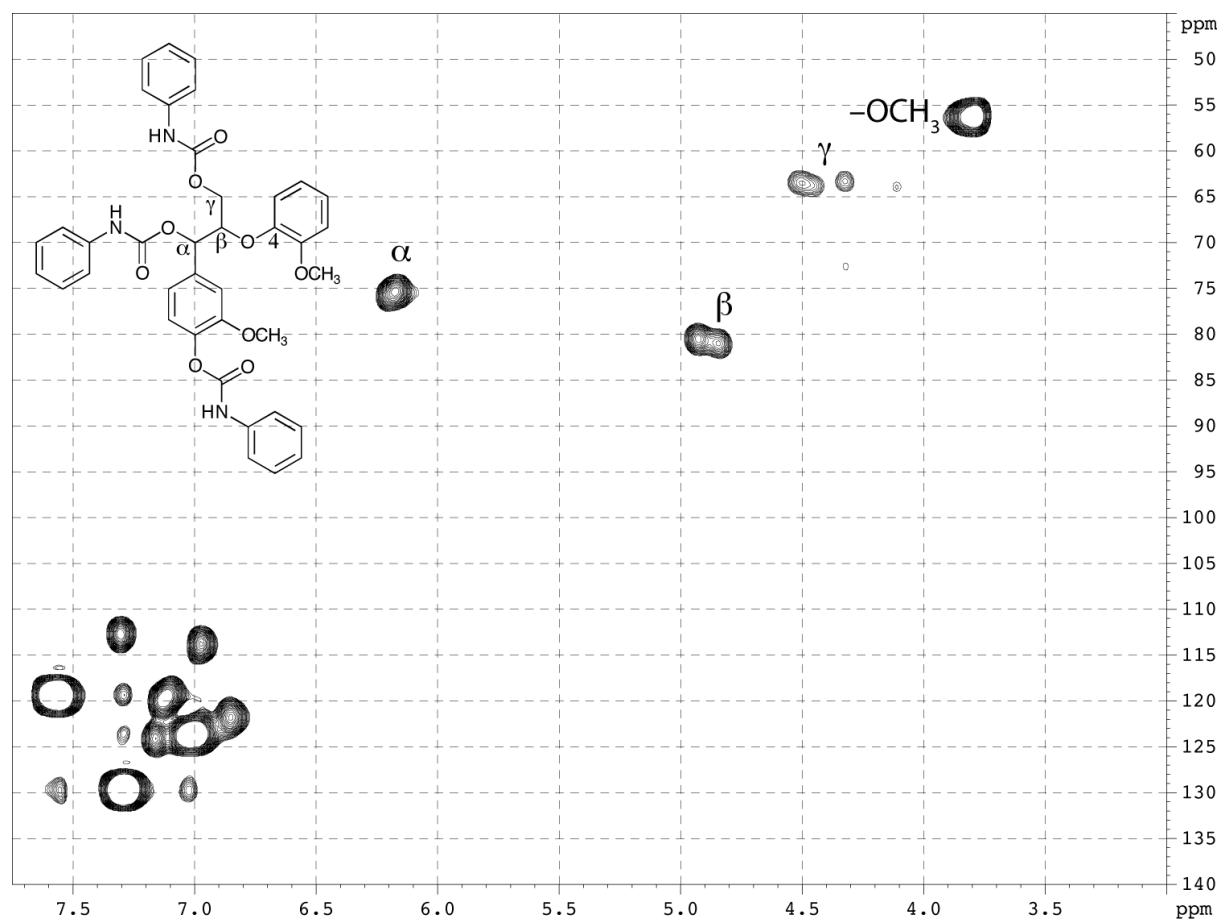


Figure 3.5. HSQC NMR spectra of β -aryl ether lignin model compound **A2** derivatized in toluene with PI at 3:1 (equivalents) as shown in Table 3.1.

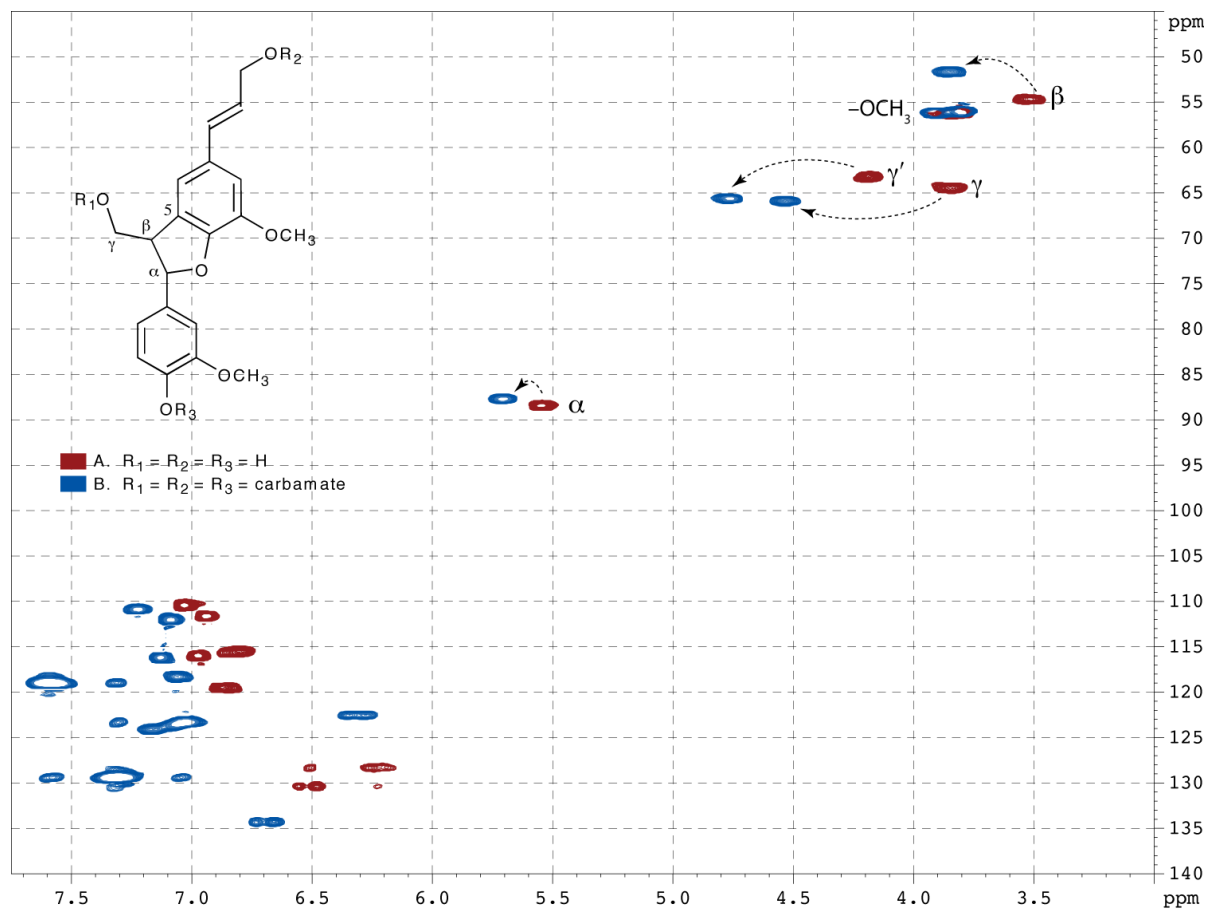


Figure 3.6. Overlay of HSQC NMR spectra of phenylcoumaran lignin model compound **B** derivatized with PI at 3:1 (equivalents) as shown in Table 3.1. Structures **A** (unreacted) & **B** (derivatized) are denoted by the color scheme shown. Dashed arrows indicate important chemical shifts.

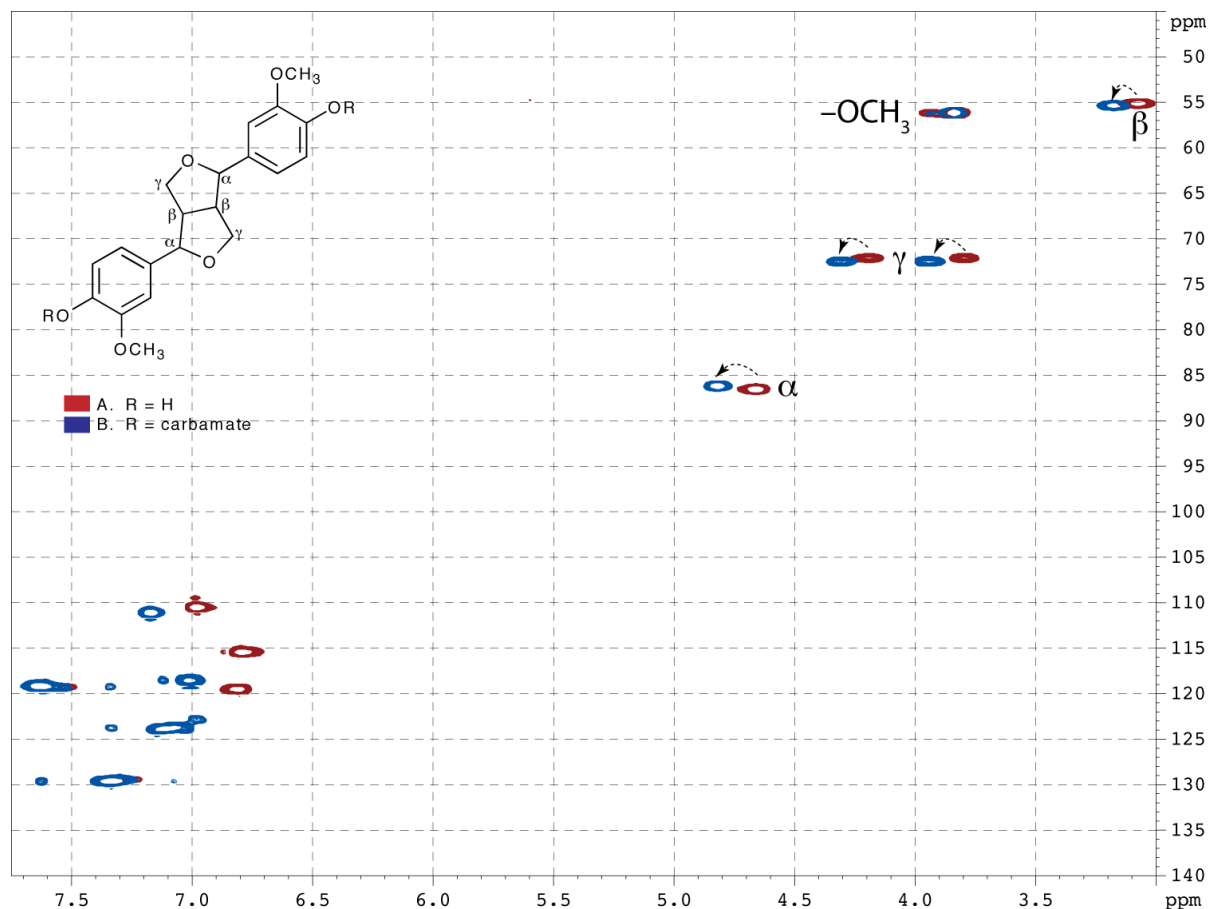


Figure 3.7. Overlay of HSQC NMR spectra of pinoresinol lignin model compound **C** reacted with PI at 2:1 (equivalents) as shown in Table 3.1. Structures A (unreacted) & B (derivatized) are denoted by the color scheme shown. Dashed arrows denote the important changes in chemical shifts upon derivatization.

With wood polymer reactions, the swelling solvent DMAc was necessary to facilitate homogeneity of the reaction solution during carbamylation; DBTDL was used as the catalyst. Two key steps in isolating carbamylated wood polymers were to: (1) remove as much moisture from the initial wood polymers as possible before reaction, and (2) after the reaction to remove excess isocyanate at low temperature. Step 2 was accomplished with a miniature vacuum (5 mbar) distillation apparatus with a short distance between the heated flask and the receiving flask

(immersed in a dry-ice slurry). Once isocyanate was removed, the product was dissolved again in DMAc and precipitated in dry methanol to afford the desired carbamylated product.

Table 3.2 shows the wood polymers that were derivatized with PI with a goal to achieve complete derivatization of available hydroxyls. The WPG of the BMW, MWL, and holocellulose polymers show that other products besides polymer carbamates had formed. The BMW, MWL, and holocellulose carbamates were isolated quantitatively based on theoretical molar calculations described in Table 3.2. The molar quantities of methyl carbamate recovered from the reactions were derived from quenching excess isocyanate with methanol to halt the reaction. Even though the wood polymers and reagents were dried to remove moisture from the system, some moisture was present during the reactions, as reflected in the recovery of some diphenyl urea in the products shown in Table 3.2. The amount of diphenyl urea in the product can give an estimate of the water level in the starting wood polymer. That is, for every mole of water in the reactant solution, one mole of diphenyl urea is produced; assuming an excess of PI is present in the solution. For example, for the BMW, MWL, and holocellulose the MC of the initial reactions were estimated to be 1.8, 1.4, and 2.1%, respectively; the calculated MCs (measured via the oven-dry method) for all wood polymers before reactions were approximately 2%.

Table 3.2. Reaction conditions for wood polymers with PI.

Polymer	Stoichiometry NCO:OH (mmol)	Solvent	Catalyst	Temp. (°C)	WPG (%)		Products recovered (mmol)		
					Theo.	Actual	polymer carbamate	methyl carbamate	diphenyl urea
Ball-milled loblolly pine	2:1 ^a	DMAc	DBTDL	60	127	280	0.97	0.95	0.10
Milled wood lignin (loblolly pine)	2:1 ^b	DMAc	DBTDL	40	85	184	0.65	0.62	0.08
Holocellulose (loblolly pine)	2:1 ^c	DMAc	DBTDL	60	135	261	0.91	0.39	0.06

a. The stoichiometry was such that full derivatization of the isocyanate with wood might occur. Reactivity and selectivity in wood may be different than with model compounds. These ratios are based on data of % acetyl content of BMW on which I obtained 40.6% WPG after acetylation (djyB82). Since the hydroxyl groups are converted to *O*-acetyl groups by acetylation on a 1:1 molar basis, the reactive hydroxyl content of the BMW is equal to the acetyl content of the acetylated BMW. This can be calculated by knowing the MW of an acetyl = 43.05 mg/mmol.

Therefore, for 100 mg of BMW I estimate there to be 0.94 mmol of –OH groups, or 9.4 mmol/g.

b. Following the same approach as described in (a), I obtained 27.0% WPG after acetylation of the MWL (djyA125). Therefore, I calculated that for 100 mg of loblolly pine MWL, there are 0.63 mmol of –OH groups, or 6.3 mmol/g. Månsson calculated a value of 6.8 mmol/g for total hydroxyls in MWL.

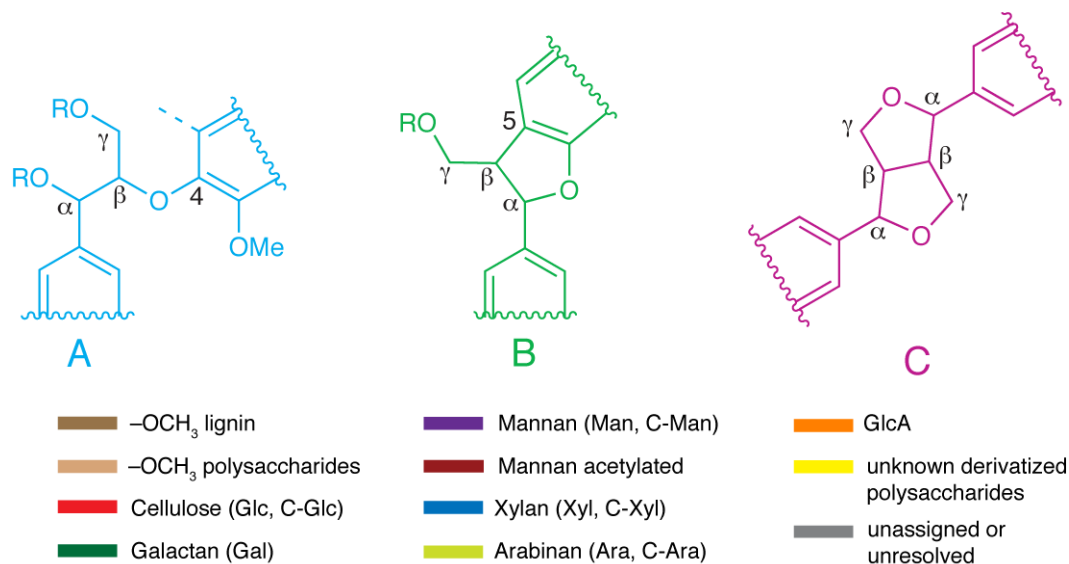
c. Following the same approach as described in (a), I obtained 43.2% WPG after acetylation of the holocellulose (djyC152). Therefore, I calculated that for 100 mg of holocellulose, there are 1.00 mmol of –OH groups, or 10.0 mmol/g.

Wood polymer NMR spectra

Dissolution of the carbamylated wood polymer samples in DMSO-*d*₆ was a key factor in obtaining NMR spectra with well-dispersed peaks. For the cellulosic wood polymers (i.e., carbamylated BMW and carbamylated holocellulose), dissolution only occurred once heated to 50 °C. The carbamylated MWL, on the other hand, dissolved readily at room temperature.

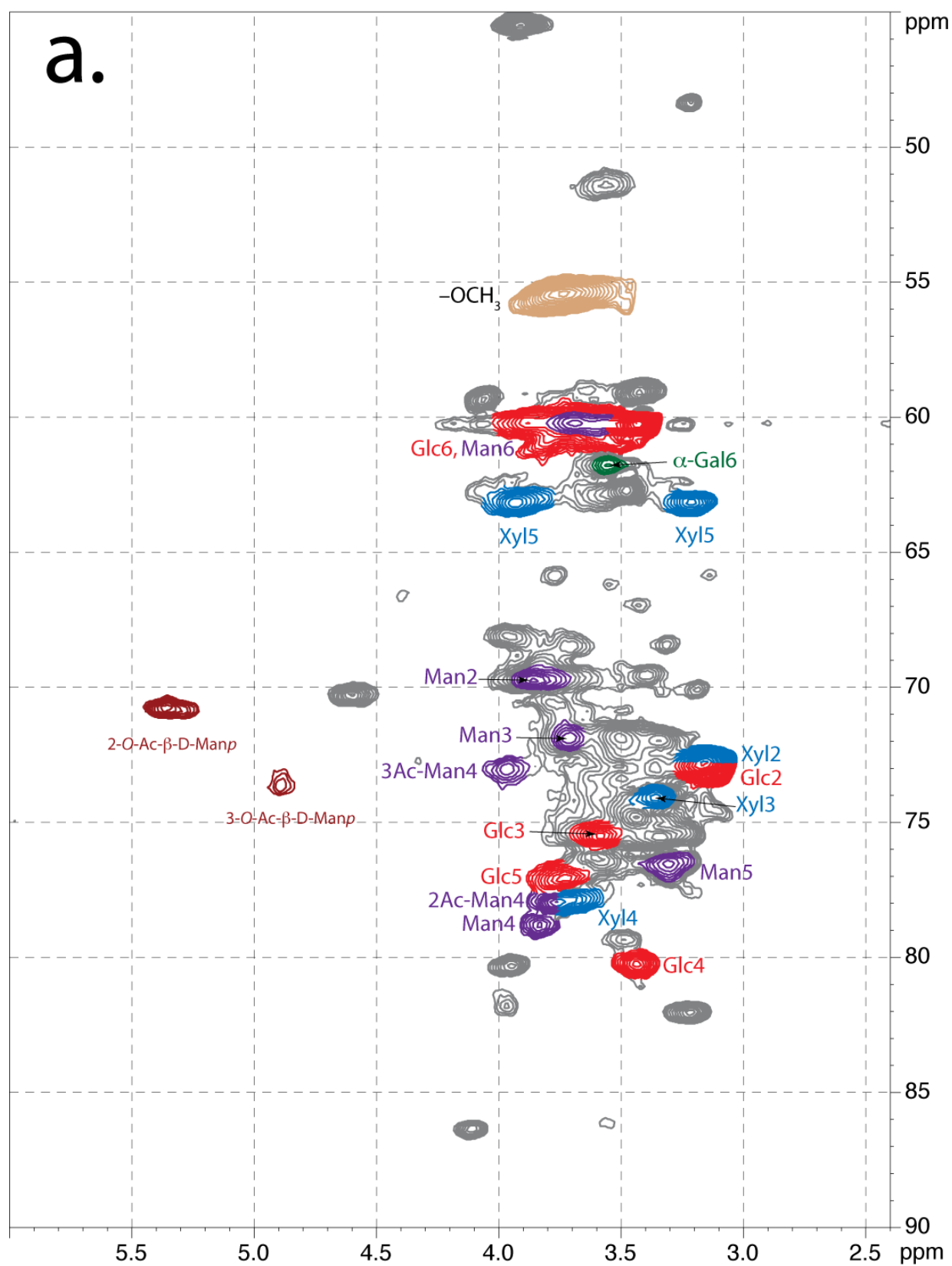
For all HSQC spectra to follow in this chapter and chapter 4, a key to structures, **Key 2**, is shown before the following figures. **Figure 3.8** shows HSQC spectra from loblolly pine holocellulose. Figures 3.7a and 3.7c show that native mannan is partially acetylated at the H2/C2 and H3/C3 positions. From the carbamylated holocellulose spectra, Figures 3.8b, 3.8d, it is evident that only partial derivatization has occurred. The H6/C6 correlations, and the large H2/C2 and H3/C3 correlations at 4.6/72 ppm and 5.0/73 ppm, are consistent with derivatized and

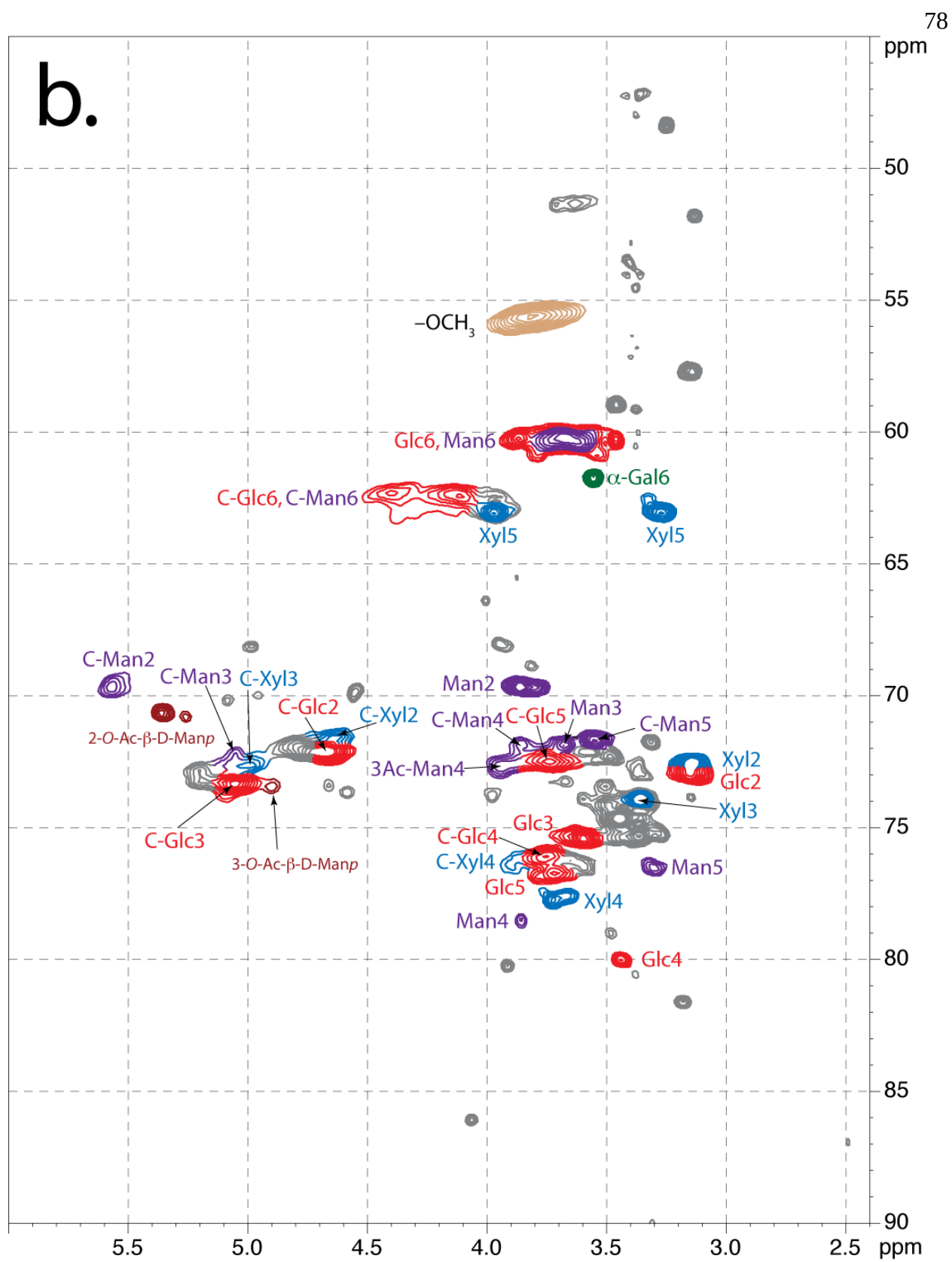
non-derivatized cellulose (glucan, Glc)¹³ and xylan (Xyl).³⁷ However, there is also a significant amount of unreacted Glc and Xyl as evidenced by the H2/C2 correlation at 3.1/72.5ppm. Some anomeric (i.e., H1/C1) correlations shown in Figure 3.8d are also consistent with derivatized and unreacted Glc, indicating that obtaining complete derivatization along the main chain of polysaccharides, even under catalyzed conditions, is not a straightforward process. Evidence of derivatized and unreacted Glc reducing end-groups indicates that complete derivatization may not even occur at the (presumably more accessible) ends of the cellulose chains. According to volume integration of the Glc reducing end-groups (β -C-Glc^R & α -C-Glc^R vs. β -Glc^R & α -Glc^R), 73% of the Glc end-groups were derivatized in the carbamylated holocellulose.

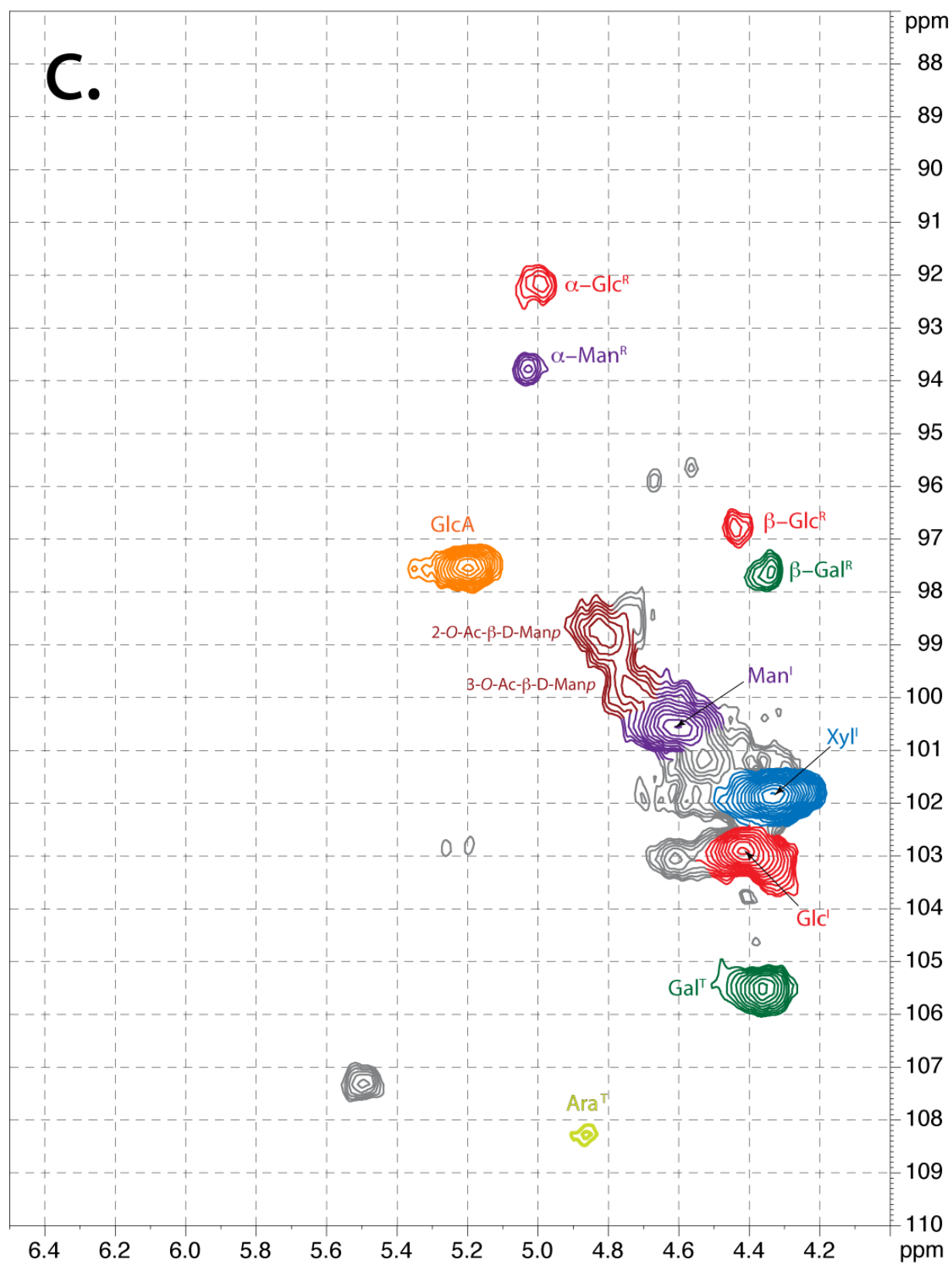


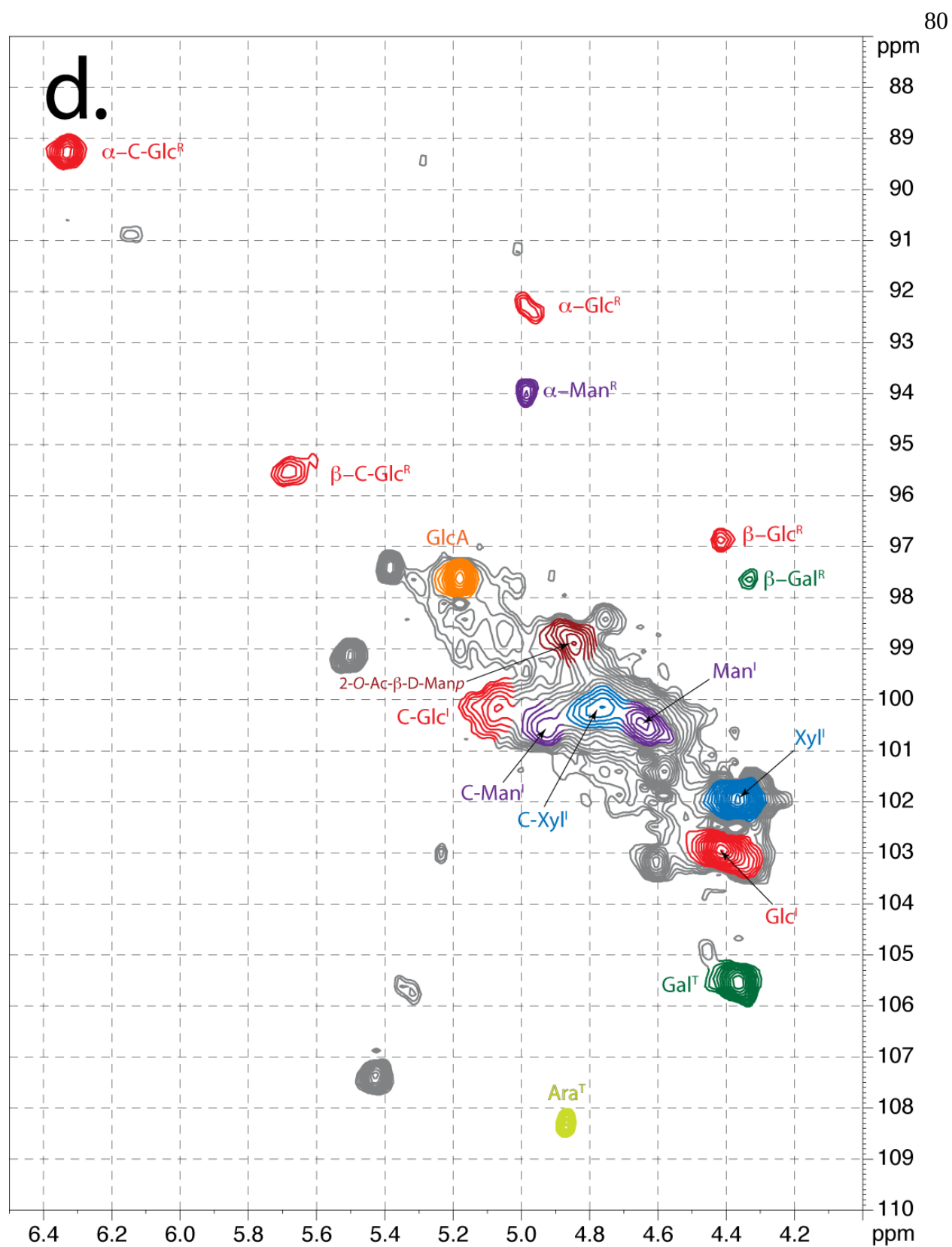
Key 2. A key to the structures found in the wood polymer HSQC spectra for Chapters 3 and 4. All contour colors can be matched to their respective structure: (A) β -aryl ether in cyan, (B) phenylcoumaran in light green, (C) resinol in raspberry, lignin methoxyl in brown, polysaccharide methoxyl in tan, β -D-Glcp units in red, β -D-Galp units in forest green, β -D-Manp units in purple, acetylated β -D-Manp units in maroon, β -D-Xylp units in blue, α -L-Araf units in chartreuse, 4-O-methyl- α -D-glucuronic acid units in orange, modified polysaccharide structures currently unknown in yellow, and other unresolved structures in gray.

Figure 3.8. HSQC spectra of loblolly pine holocellulose: **(a)** unreacted aliphatics; **(b)** partially derivatized aliphatics; **(c)** unreacted anomeric; **(d)** partially derivatized anomeric. Colored contours correspond to specific polysaccharides shown in the **Key 2**. Gray contours are currently unassigned. Polysaccharides labeled with a “C” (e.g., CGlc) represent carbamylated structures.



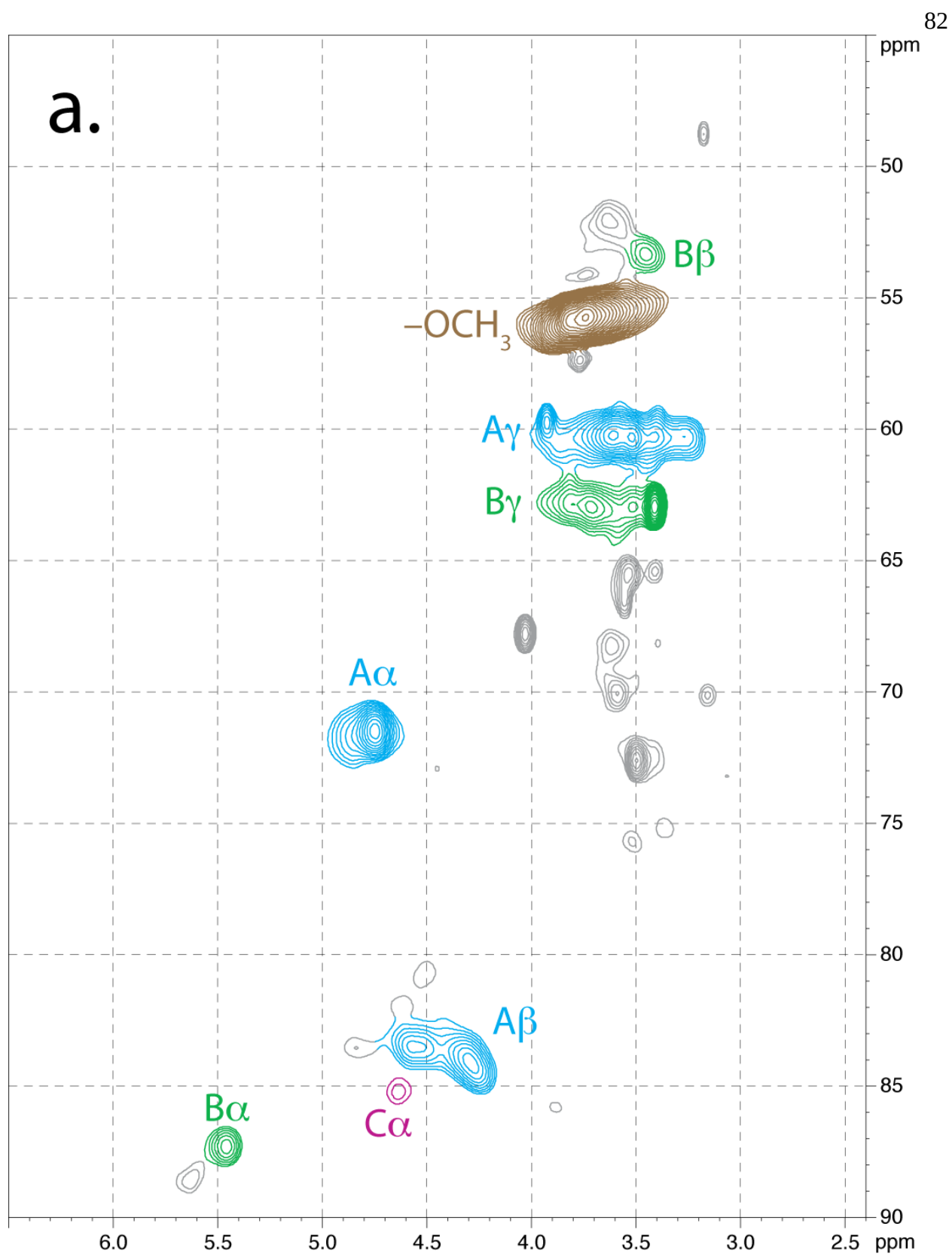






Figures 3.9 and **3.10** show HSQC spectra for derivatized and unreacted MWL and BMW, respectively. Both MWL and BMW spectra show clear evidence of complete lignin sidechain carbamylation. The β -aryl ether and phenylcoumaran correlations are consistent with those in carbamylated lignin model compounds, indicating complete sidechain derivatization under these catalyzed reaction conditions. To investigate phenolic derivatization, ^{13}C NMR spectra were acquired on the derivatized and unreacted MWL (spectra not shown). Signals at 152.55 ppm and 152.42 ppm of the carbamylated MWL ^{13}C NMR spectra confirms that phenolic derivatization has occurred; lignin sidechain carbamates fall between 153.4 ppm and 154.3 ppm. The major polysaccharide correlations in the BM spectra were found to be consistent with derivatized cellulose (H1/C1, H2/C2, H3/C3, H4/C4, H5/C5, and H6/C6) from the cellobiose carbamate and holocellulose carbamate chemical shift data.

Figure 3.9. HSQC spectra of MWL **(a)** and MWL derivatized with PI **(b)** under conditions shown in Table 3.2. Color-coded lignin structures shown in spectra correspond to **Key 2**; “C” in the contour labels correspond to a carbamylated structure.



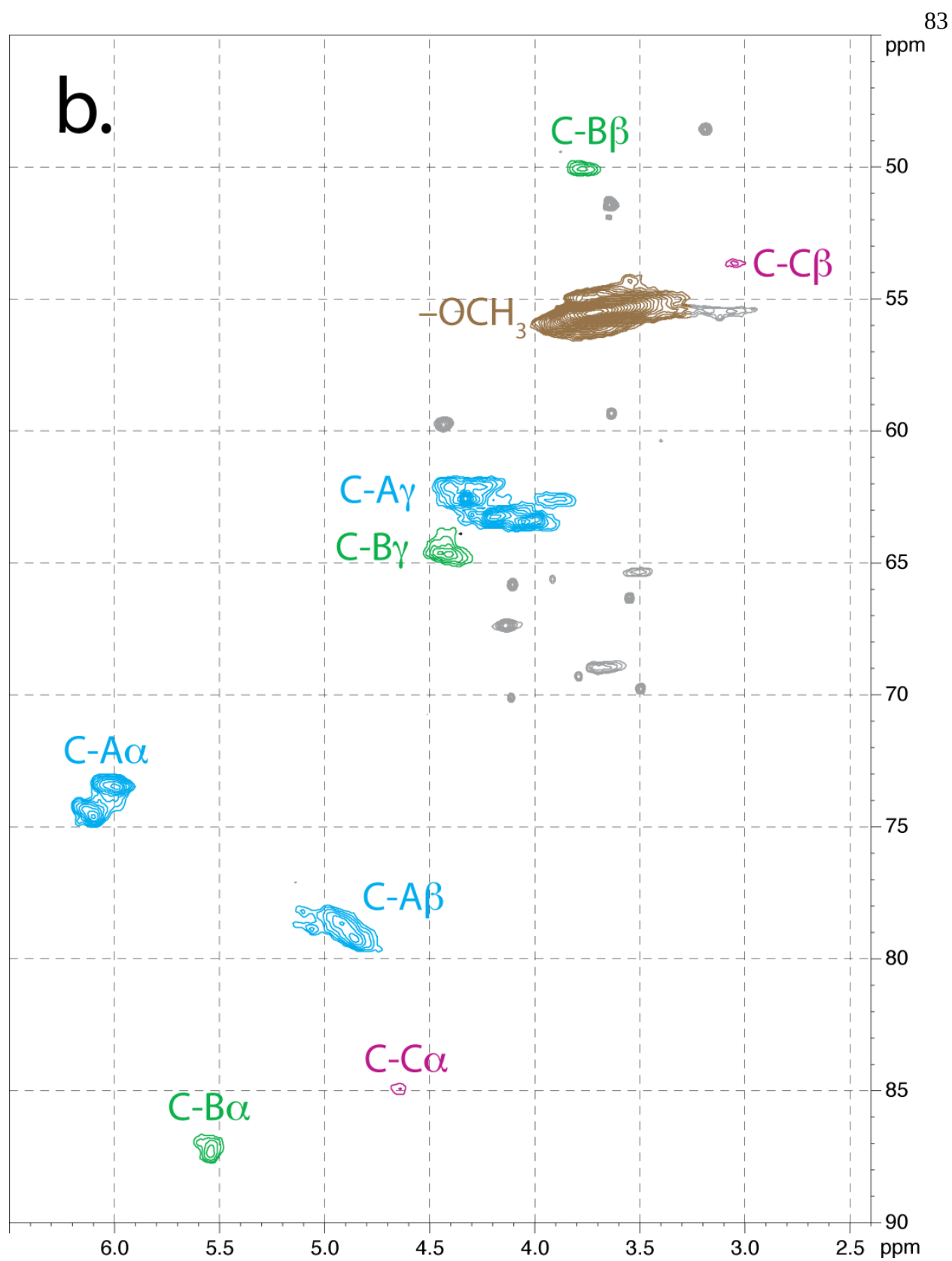
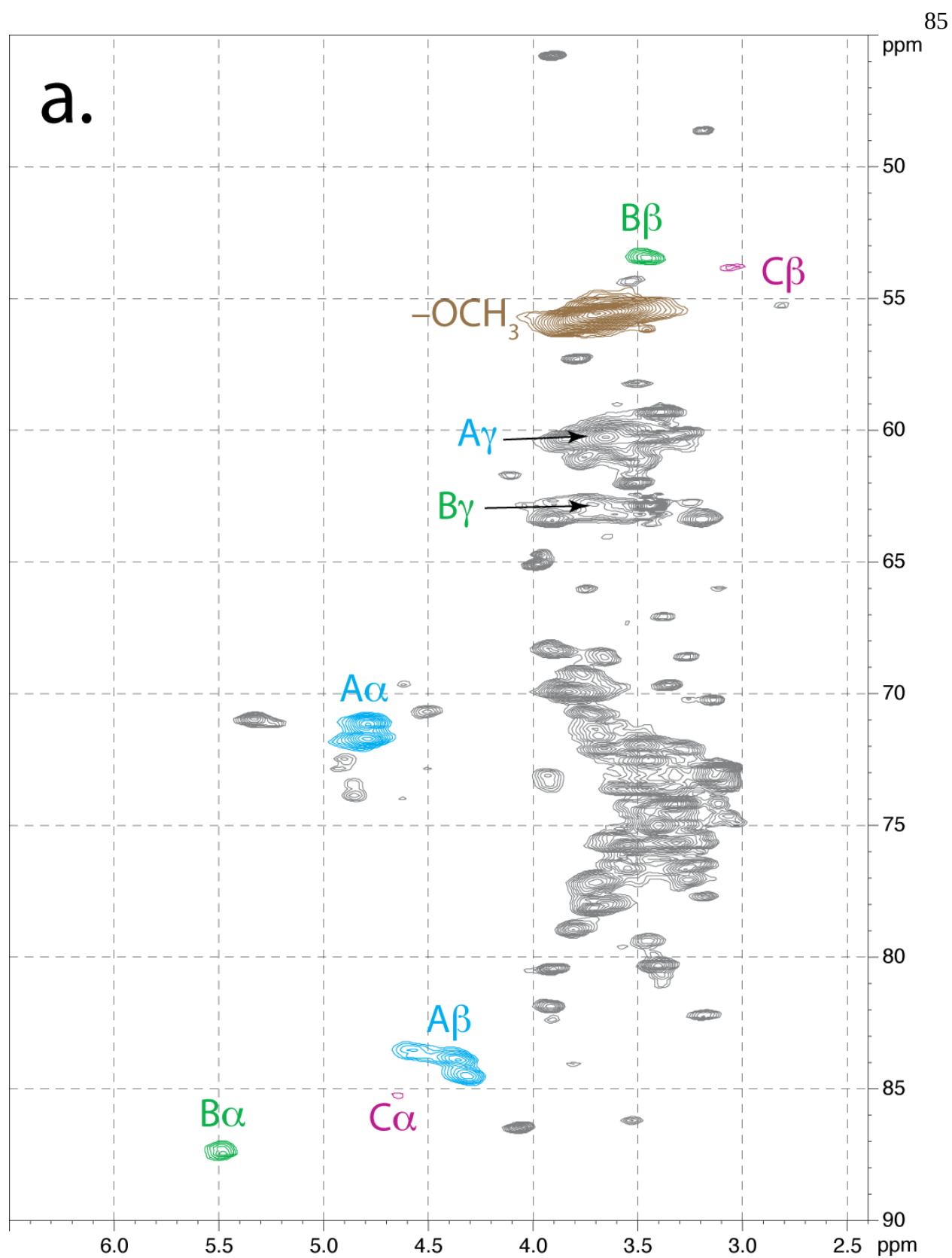


Figure 3.10. HSQC spectra of BMW **(a)** and BMW derivatized with PI **(b)** under conditions shown in Table 3.2. Color-coded structures shown in spectra correspond to **Key 2**; “C” in the contour labels correspond to a carbamylated structure. Some polysaccharides are shown as grey contours and are not labeled here. The A γ correlations are overlapped with cellulose and mannan H6/C6 correlations and the B γ correlations are overlapped with the xylan H5/C5 correlations, noted by black arrows.



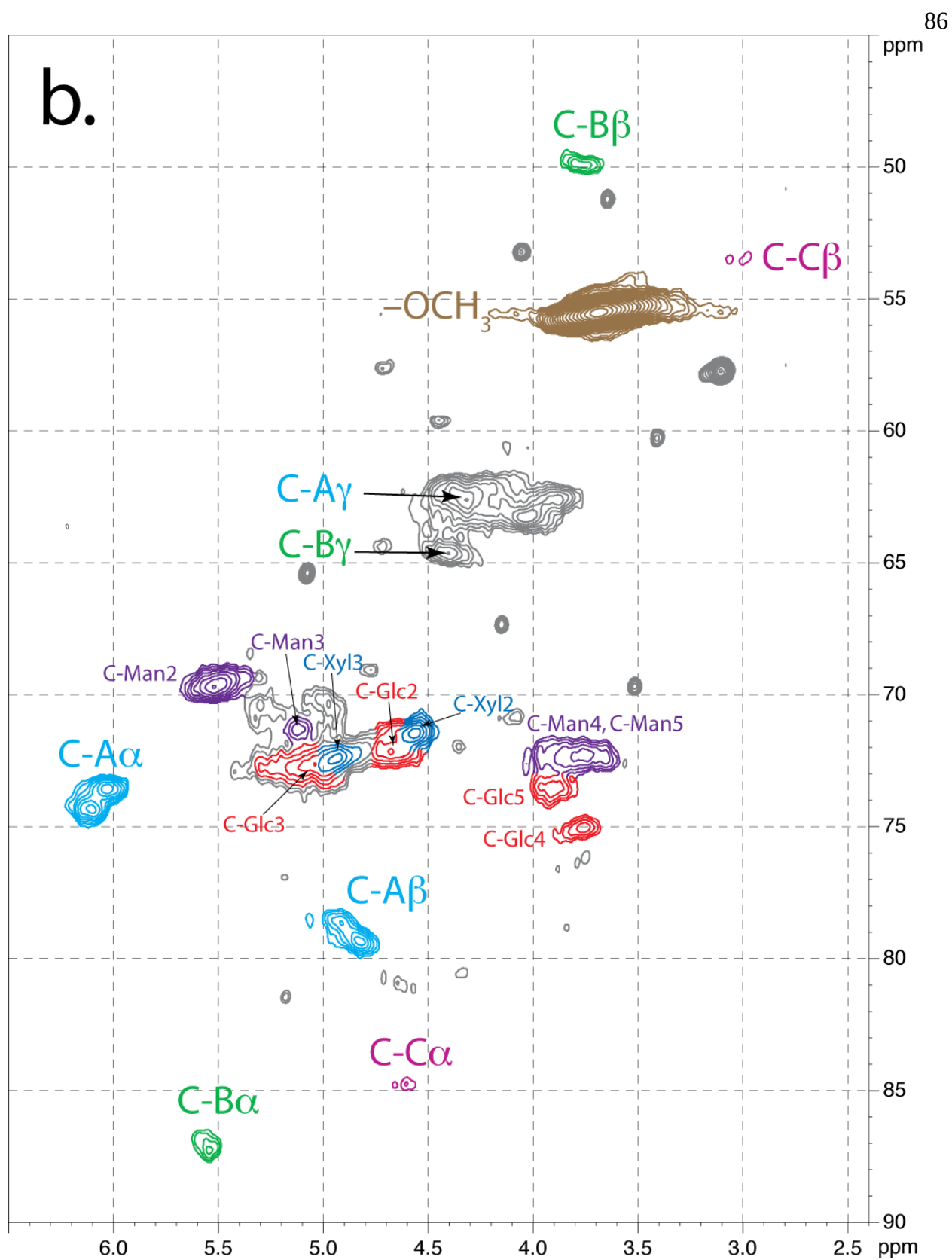


Table 3.3 summarizes the chemical shift assignments for the derivatized and unreacted wood models along with the assignments for the derivatized and unreacted wood polymers. It is evident from the HSQC spectra of derivatized and unreacted wood polymers that the chemical shifts are dramatically different and distinct for each hydroxylated H/C position. From Table 3.3, the polymer chemical shifts are shown to be comparable to those of the corresponding models, indicating that the model compound chemical shift data can be used to identify derivatized structures in wood polymers.

Table 3.3. Chemical shifts for wood model compounds and wood cell wall polymers in loblolly pine after derivatization with PI.

Structure or Position	^1H (δ , ppm)	^{13}C (δ , ppm)	Reference for Assignments
Derivatized lignin model compound A1 α -derivative (acetone- d_6)			
<u>Aα</u>	6.12	75.73	^1H , ^{13}C , HSQC
A β	4.50	84.87	^1H , ^{13}C , HSQC
A γ	3.71, 3.55	61.13	^1H , ^{13}C , HSQC
–NH–CO–	8.84	153.34	^1H , ^{13}C
Derivatized lignin model compound A1 γ -derivative (acetone- d_6)			
A α	4.97	73.27	^1H , ^{13}C , HSQC
A β	4.52	84.36	^1H , ^{13}C , HSQC
<u>Aγ</u>	4.40, 4.01	64.23	^1H , ^{13}C , HSQC
–NH–CO–	8.78	154.05	^1H , ^{13}C
Derivatized lignin model compound A2 $\gamma + \alpha +$ phenolic-derivative (acetone- d_6)			
<u>Aα</u>	6.18	75.08	^1H , ^{13}C , HSQC
A β	4.92	80.26	^1H , ^{13}C , HSQC
<u>Aγ</u>	4.49, 4.11	63.10	^1H , ^{13}C , HSQC
<u>phenolic G4</u>	–	140.70	^{13}C
–NH–CO–	8.91, 8.88, 9.16	153.33, 154.10, 152.30	^1H , ^{13}C , HMBC
Derivatized lignin model compound B (acetone- d_6)			
B α	5.69	87.84	^1H , ^{13}C , HSQC
B β	3.84	51.78	^1H , ^{13}C , HSQC
<u>Bγ</u>	4.52	65.97	^1H , ^{13}C , HSQC
<u>Bγ'</u>	4.75	65.76	^1H , ^{13}C , HSQC
<u>phenolic G4</u>	–	140.91	^{13}C
–NH–CO–	8.88, 8.68, 9.17	154.23, 154.29, 152.14	^1H , ^{13}C , HMBC

Derivatized lignin model compound C (acetone- d_6)			
Ca	4.80	86.11	^1H , ^{13}C , HSQC
C β	3.16	55.32	^1H , ^{13}C , HSQC
C γ	4.29, 3.93	72.43	^1H , ^{13}C , HSQC
phenolic G4	–	140.86	^{13}C
–NH–CO–	9.16	152.27	^1H , ^{13}C
Derivatized lignin structures in MWL and BMW (DMSO- d_6)			
A α	6.14	74.46	^1H , ^{13}C , HSQC
A β	4.86	79.22	^1H , ^{13}C , HSQC
A γ	4.42, 4.06	63.20	^1H , ^{13}C , HSQC
B α	5.54	87.26	^1H , ^{13}C , HSQC
B β	3.77	50.11	^1H , ^{13}C , HSQC
B γ	4.43	64.66	^1H , ^{13}C , HSQC
Ca	4.65	84.90	^1H , ^{13}C , HSQC
C β	3.05	53.65	^1H , ^{13}C , HSQC
C γ	4.13, 3.76	71.01	^1H , ^{13}C , HSQC
phenolic G4	–	nd	^{13}C
–NH–CO–	8.63, 8.67, 8.70, 8.76, 8.94, 9.18	152.43, 152.55, 153.44, 153.65, 153.92, 154.26	^1H , ^{13}C , HMBC
Derivatized D(+)-cellobiose (DMSO- d_6)			
H1, C1	5.08	100.91	^1H , ^{13}C , HSQC
β -reducing end	5.96	91.87	^1H , ^{13}C , HSQC
α -reducing end	6.30	89.11	^1H , ^{13}C , HSQC
H2, C2	4.83	72.10	^1H , ^{13}C , HSQC
H3, C3	5.29	72.70	^1H , ^{13}C , HSQC
H4, C4	3.98	77.63	^1H , ^{13}C , HSQC
H5, C5	3.97	72.11	^1H , ^{13}C , HSQC
H6, C6	4.62, 4.20	62.50	^1H , ^{13}C , HSQC
–NH–CO–	9.21, 9.40, 9.45, 9.50, 9.58, 9.60, 9.75, 9.84	151.20, 151.99, 152.29, 152.35, 152.39, 152.68, 152.95, 153.19	^1H , ^{13}C , HMBC
Derivatized methyl 4-O-methyl- β -D-glucopyranoside, 2-OH derivative (DMSO- d_6)			
H1, C1	4.32	100.81	^1H , ^{13}C , HSQC
H2, C2	4.49	73.77	^1H , ^{13}C , HSQC
H3, C3	3.50	73.77	^1H , ^{13}C , HSQC
H4, C4	3.07	79.16	^1H , ^{13}C , HSQC
H5, C5	3.23	75.32	^1H , ^{13}C , HSQC
H6, C6	3.64, 3.54	59.97	^1H , ^{13}C , HSQC
–NH–CO–	9.64	152.75	^1H , ^{13}C , HMBC
Derivatized methyl 4-O-methyl- β -D-glucopyranoside, 2,6-OH derivative (DMSO- d_6)			
H1, C1	4.40	100.93	^1H , ^{13}C , HSQC
H2, C2	4.55	73.78	^1H , ^{13}C , HSQC
H3, C3	3.58	72.55	^1H , ^{13}C , HSQC
H4, C4	3.15	79.50	^1H , ^{13}C , HSQC

H5, C5	3.59	73.86	^1H , ^{13}C , HSQC
H6, C6	4.30	62.98	^1H , ^{13}C , HSQC
-NH-CO-	9.66, 9.78	152.73, 154.10	^1H , ^{13}C , HMBC
Derivatized polysaccharide aliphatics in holocellulose and BMW (DMSO- d_6)			
Glc2	4.66	72.28	^1H , ^{13}C , Fig. 3.1, ref. 13
Glc3	5.04	73.51	^1H , ^{13}C , Fig. 3.1, ref. 13
Glc4	3.74	76.21	^1H , ^{13}C , Fig. 3.1, ref. 13
Glc5	3.75	72.50	^1H , ^{13}C , Fig. 3.1, ref. 13
Glc6	4.38, 4.10	62.55	^1H , ^{13}C , Fig. 3.1, ref. 13
Man2	5.55	69.8	–
Man3	5.03	72.33	–
Man4	3.84	72.11	–
Man5	3.53	71.77	–
Man6 ^a	–	–	–
Xyl2	4.62	71.71	ref. 37
Xyl3	4.98	72.77	ref. 37
Xyl4	3.88	76.6	ref. 37
Xyl5	3.95, 3.25	63.21	ref. 37
-NH-CO- (Glc)	8.77, 8.88, 9.10	152.00, 152.40, 153.5	^1H , ^{13}C , ref. 13
Derivatized polysaccharide anomers in holocellulose and BMW (DMSO- d_6)			
Glc	5.09	100.02	^1H , ^{13}C , Fig. 3.1, ref. 13
β -Glc ^R	5.65	95.33	Fig. 3.1
α -Glc ^R	6.30	89.06	Fig. 3.1
Man	4.92	100.06	–
Xyl	4.76	100.02	ref. 37

a. overlapping with Glc6 contours

CONCLUSIONS

Reactions between the pMDI model compound, PI, and wood was analyzed to elucidate whether covalent bonds form between isocyanates and the wood cell wall polymers cellulose, hemicelluloses, and lignin. It was found that lignin model reactions in DMAc with DBTDL as catalyst were able to derivatize sidechain hydroxyls, but not able to derivatize the phenolic position; however, toluene/DBTDL systems, under identical molar ratios of NCO:Model, derivatized the phenolic position in good yields. Reactions with lignin and polysaccharide model compounds produce carbamate derivatives that display characteristic chemical shifts in solution-state 2D NMR. These model chemical shifts, and the shift differences between derivatized and non-derivatized components, were used to identify the degree and regiochemistry of derivatization of wood polymer hydroxyls for each cell wall component. Dissolution of carbamylated cellulosic wood polymers at 55 °C allowed for adequate peak dispersion in HSQC spectra. 2D ^{13}C – ^1H correlation NMR spectroscopy therefore provides a useful method to characterize derivatized and non-derivatized plant cell wall polymers.

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Chapter 4. Characterizing model pMDI reactions with loblolly pine using solution-state NMR spectroscopy. II. Non-catalyzed reactions with the wood cell wall

SUMMARY

Covalent bond formation between wood and pMDI has been proposed as a significant contributor to wood-pMDI bondline durability. However, covalent bonds in pMDI-wood bondlines have not been identified unambiguously. Solution-state NMR provides a powerful tool for determining the existence or absence of covalent bonds between wood components and adhesives. Through a logical simplification of a non-degradative wood dissolution method, finely ground wood cell wall material dissolves in a solvent system containing DMSO- d_6 and NMI- d_6 , keeping wood component polymers intact and in a near-native state. 2D NMR experiments, using ^{13}C - ^1H one-bond HSQC experiments on non-derivatized cell wall material reveal the major cell wall components in a way that allows the whole cell wall to be structurally analyzed via high-resolution solution-state NMR. We applied this technique to loblolly pine that was reacted with pMDI model compounds under controlled moisture and temperature conditions. Using NMR we then determined chemical shift data for carbamates formed between the pMDI model and loblolly pine. The results show that: (a) isocyanate reactivity is highly dependent on isocyanate concentration and MC, but under dry conditions isocyanates will form carbamates in loblolly pine preferentially with β -O-4-linked and β -5-linked lignin side-chain hydroxyls in a swelling solvent, (b) PI is more capable of derivatization in the cell wall than the bulkier 4-BPI, (c) under reaction conditions with high concentrations of PI and at 5% and 14% MC, detectable carbamates were found only on γ -hydroxyls of β -O-4-linked units, and (d) under typical

industrial OSB bonding conditions using a hot-press at 5% and 14% MC, no carbamates were detected.

INTRODUCTION

Since their conception in the late 1960s, isocyanate adhesives, used for OSB and particleboard production, have been touted as the only adhesive that chemically reacts with wood cell wall polymers. It is true that heterogeneous carbamylation of wood and isolated cell wall polymers has seen considerable amounts of study, in which derivatization of the wood hydroxyls is the main objective. Researchers, using thickness swelling data, have concluded that isocyanate groups must form primary valences (i.e., urethane linkages) with hydroxyls in lignin, hemicelluloses, and even to some extent with cellulose microfibrils in wood.¹ Methyl isocyanate was shown to derivatize southern pine wood blocks, which produced WPGs of up to 50% using non-catalyzed treatments at 120 °C and 150 psi, and which showed a resulting effective resistance to brown-rot decay.² Others found that, when *n*-butyl isocyanate was reacted, catalyzed with pyridine, with Scots pine at 100 °C and WPG was measured as a function of time, lignin reacted approximately ten times faster than holocellulose.³ IR spectroscopy has been used extensively to characterize urethane linkages in cellulose after various isocyanates were reacted under catalyzed⁴ and uncatalyzed⁵⁻⁷ conditions at MCs of < 19%.

In the industrial OSB manufacturing process, pMDI is typically used for the core adhesive and phenol formaldehyde on the faces. Bonding pMDI is shown to tolerate higher MCs (> 6%) and relatively fast press speeds (e.g., 4 mm/s) when steam-injection is incorporated; this allows for continuous pressing where mats can be injected with steam to accelerate heat transfer and curing, thus increasing modulus and internal bond properties of the final panel. Normal bonding

conditions for pMDI include: solid wood (veneer, strands, etc.) at an MC of 5-10 %; no catalyst; no solvent; temperatures of curing between 150-190 °C; resin level between 2 – 6% based on oven-dry weight of wood; curing time between 60 and 180 seconds; pressures of approximately 40 bar during pressing between platens.

In wood adhesion, the objective is to form strong and durable bonds to wood. The wood adhesives industry has formulated pMDI adhesives for OSB, particleboard, and laminated strand lumber to meet these objectives. Yet, researchers in the field of wood adhesion still do not fully understand the bonding mechanisms that lead to durable pMDI bonds to wood. Several studies have been conducted to investigate how pMDI cures and interacts with wood during adhesive application and hot-pressing of OSB.⁸⁻¹⁵ The bound moisture in the wood cell wall has dramatic effects on cure^{9,13} and, in some cases, the wood species will affect pMDI reaction kinetics.¹⁴

To characterize specific linkages in the cured pMDI-wood bondline researchers have used solid-state NMR spectroscopy. Using pMDI with a double-isotopic label (i.e., ¹⁵N & ¹³C) in the isocyanate allowed researchers to use 1D solid-state NMR spectroscopy to detect urethane crosslinks in the cured bondline.¹ However, urethane and polyurea signals are not well resolved by solid-state NMR; therefore, covalent bonds couldn't be identified unambiguously in the acquired spectra. 2D solid-state ¹⁵N–¹H correlation NMR experiments have also been conducted with wood reacted with pMDI adhesive. These experiments characterized different urea-type structures formed during the reaction of moisture in the wood when wood MCs were 7 and 14%; urethane formation was suggested to occur with lignin powder, but this was not confirmed with model studies. Furthermore, the unfortunate peak dispersion and resolution cannot not distinguish the urea ¹⁵N–¹H from the urethane ¹⁵N–¹H unambiguously.¹⁶

High-resolution solution-state NMR spectroscopy is an instrumental method capable of

characterizing and confirming detailed molecular structure through measurement of the interaction of radio-frequency electromagnetic radiation with nuclei immersed in a strong magnetic field. Organic chemists have used NMR methods since the early 1960s to confirm structures of relatively small molecules. With the development of larger magnets, cryogenically cooled probes, and sophisticated pulse programs, biopolymers from plant cell walls are amenable to detailed investigation. Using non-degradative and non-derivatized wood dissolution described in chapter 2, and 2D NMR experiments, using ^{13}C – ^1H one-bond HSQC spectroscopy on whole cell walls, structural details on the major cell wall polymers are revealed. This allows for determining whether and to what degree isocyanates have derivatized a wood cell wall polymer.

Objective

The objective of this study is to determine the reactivity of pMDI with loblolly pine under appropriate conditions. The approaches to achieve this objective are to:

1. Derivatize wood polymer hydroxyls to carbamates using PI and 4-BPI with varying isocyanate concentrations and wood MC.
2. Use solution-state NMR to detect carbamate formation and elucidate its regiochemistry.
3. Quantify carbamate formation using 2D volume integration of specific contours in HSQC spectra.

MATERIALS AND METHODS

Materials

All reagents were from Aldrich Chemical Company (Milwaukee, WI). PI and 4-BPI and distilled DMAc were dried over 3Å molecular sieves. NMI- d_6 was synthesized as described in

chapter 2 of this thesis.¹⁷ BMW was prepared using a Retsch (Newtown, PA) PM100 planetary ball-mill with ZrO₂ balls. Loblolly pine was microtomed using an Spencer Lens Company (Buffalo, NY) sled microtome (serial 3423) into tangential earlywood sections of 50 μ m and 300 μ m. The sections were refluxed in acetone to remove any acetone extractables, dried, and were equilibrated at 0%, 5%, and 14% MCs (i.e., in chambers with phosphorus pentoxide, saturated calcium bromide, and saturated sodium chloride) prior to reactions with pMDI models.

Reactions

Loblolly pine sections (50 μ m thick $\times$ 20 mm $\times$ 20 mm) were removed from their respective humidity controlled chambers and 8 sections per sample treatment were weighed (approximately 100 mg) and added to a dry 50 mL round-bottom flask. Reactions were carried out under the purge of N₂ using distilled DMAc as the solvent at 160 °C and refluxed for 2 h. An Automated Bond Evaluation System (ABES) miniature hot-press was used at 160 °C for 10 min. at ~150 psi to press-treat loblolly pine sections (300 μ m thick $\times$ 20 mm $\times$ 20 mm). After all reactions, dry methanol was added to quench the excess isocyanate. Then wood sections were extracted in refluxing acetone to extract the byproducts (e.g., diphenyl urea and methyl carbamate) and the treated wood sections were dried at 50 °C overnight using a vacuum oven. The weight gain of reacted wood and urea-type extractables were measured to 0.01 mg accuracy using a Mettler-Toledo (Columbus, OH) model XS105 analytical balance. Separation and purification of methyl carbamate and diphenyl urea was performed as described in Chapter 3.

Solution-state NMR

Approximately 50 mg of reacted BMW was dissolved directly in a 5-mm NMR tube with DMSO- d_6 and NMI- d_6 using sonication. NMR experiments (^1H , ^{13}C , HSQC) were acquired at 50 °C on a Bruker DMX-500 instrument equipped with cryogenically cooled 5-mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ gradient probe with inverse geometry (proton coils closest to the sample). The central solvent peak was used as an internal reference ($\delta_{\text{C}} = 39.5$ ppm, $\delta_{\text{H}} = 2.49$ ppm). Adiabatic HSQC experiments (pulse program = hsqcetgpsisp2.2)¹⁸ had the following parameters: sweep width, 9.0-1.0 ppm in F_2 (^1H) using 1698 data points (acquisition time, 170 ms) and 150-10 ppm in F_1 (^{13}C) using 420 increments (F_1 “acquisition time,” 11.93 ms). The number of scans was 32 with a 1-s interscan delay, the d_{24} delay was set to 0.89 ms ($\sim 0.125/J$, where J is an average 145 Hz ^{13}C - ^1H coupling constant), and the total acquisition time was 4 h 28 min. Processing used typical matched Gaussian apodization in F_2 and a squared cosine-bell in F_1 . Volume integration of HSQC contours was performed both on Bruker’s TopSpin 2.1 software and Bruker’s XwinNMR 3.0 software.

RESULTS AND DISCUSSION

Southern yellow pines have abrupt transition growth rings with large density differences between the earlywood and latewood. For example, in loblolly pine the specific gravity of earlywood and latewood are 0.29 and 0.63, based on unextracted green volume and oven-dry weight.¹⁹ The reason for the density difference stems from the diameter and thickness differences between the earlywood and latewood longitudinal tracheids. That is, for loblolly pine earlywood single-cell-wall thickness is on average 3.4 μm and latewood is 8.2 μm . For loblolly pine tracheid diameter, earlywood is on average 46.8 μm and latewood is 42.8 μm .²⁰ Therefore, void

volume is much lower in the latewood than the earlywood, which has substantial effects on penetrability of chemicals into the lumina and bordered pits and on the permeability of the cell wall layers.

Anatomical and ultrastructural features of loblolly pine are established in part by its cell wall polymer structure. **Table 4.1** shows the various chemical components of the loblolly pine sapwood used in this study as analyzed using high pH anion-exchange chromatography.²¹ Although not performed in this study, it has been known that loblolly pine earlywood contains more lignin than latewood (i.e., 28.1% in earlywood versus 26.8% in latewood)²² and earlywood has about 3% lower α -cellulose content than latewood.²³ Furthermore, a preliminary study using 2D NMR experiments on loblolly pine tree-rings show that the ratio of β -aryl ethers in latewood to that of earlywood can vary from year-to-year (Yelle and Kretschmann, 2009, unpublished). A unique analytical approach to quantifying releasable acetylated monolignols, named the DFRC method, found that in loblolly pine $\sim 690 \mu\text{mol/g}$ (based on Klason lignin) can be attributed to β -aryl ethers.²⁴

Table 4.1. Chemical composition of loblolly pine wood used in this study*

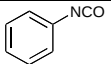
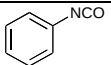
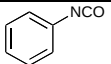
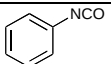
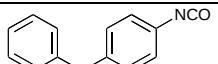
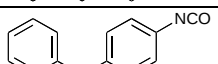
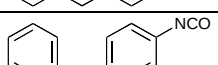
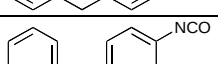
	Klason lignin (mg)	Glucose (mg)	Mannose (mg)	Xylose (mg)	Galactose (mg)	Arabinose (mg)	Rhamnose (mg)
Per gram of dry loblolly pine	301	409	103	55	35	10	1

* Extractives make up approximately 9% of this wood sample.

The reactivity of loblolly pine, with the physical and chemical characteristics above, towards pMDI was assessed. Given that earlywood is less dense and higher in lignin (and β -aryl ethers)

than latewood, and that pMDI is known to interact intimately with lignin and hemicelluloses in the cell wall,^{11,25,26} reacting pMDI with loblolly pine earlywood became the focus of this study. Microtoming the earlywood sections (tangentially) to 50 μm provides wood material that averages one-cell thickness, giving the isocyanate ample opportunity to react. Moreover, the DMAc, being an excellent wood swelling solvent,²⁷ increases the free volume between cell wall polymers. The reaction conditions for MTW are summarized in **Table 4.2** below.

Table 4.2. Reaction conditions, percent weight gain of derivatized wood, and extractable product recovered.

Sample	pMDI Model	Molar ratio of NCO:OH ^a	Percent moisture content	Solvent ^b or Hot press ^c	Percent weight gain of wood ^d	Extractable Products Recovered (mmol)	
						methyl carbamate	diphenyl urea
i.	control	na	0	Solvent	0.0	0	0
ii.		2:1	0	Solvent	31.7	1.04	0.10
iii.		1:1	0	Solvent	14.8	0.32	0.08
iv.		1:1	5	Solvent	12.9	0.18	0.31
v.		1:1	14	Solvent	6.7	0.06	0.83
vi.		1:1	0	Solvent	0.5	0.93	0.13
vii.		1:1	0	Press	3.9	—	—
viii.		1:1	5	Press	0.1	—	—
ix.		1:1	14	Press	-0.5	—	—

a. See Table 3.2 notes for a description of determining NCO:OH ratio for these reactions.

b. DMAc at 160 °C

c. 25 mm $\times$ 25 mm, 160 °C, 150 psi (1.03 MPa)

d. Based on dry weight of original wood

The weight gain of MTW reacted in DMAc is shown to decrease as available NCO groups decrease, as expected. The NCO concentration will decrease not only as the initial concentration is decreased (i.e., samples ii and iii), but also as MC in the wood increases (i.e., samples iii, iv, and v). When the 4-BPI is reacted in DMAc (sample vi), a dramatic decrease in weight gain is observed compared to when PI is used (sample iii). Since the functionality of the two models are not different, this result may be caused by a size effect of the bulkier 4-benzylphenyl group compared to just a phenyl group. For reactions using the hot-press, only 4-BPI was used since 160 °C is close to the boiling point of PI. No weight gains were detected after the acetone extraction for sample viii (5% MC) and sample ix (14% MC). However, just after hot-pressing, sample viii had a 27% weight gain, while sample ix had an 11% weight gain. Therefore, the combination of pressure and temperature used during pressing allowed for penetration of the 4-BPI into the cell lumina, but it is not clear whether the 4-BPI infiltrated the cell wall since acetone, being a non-swelling solvent in wood, was able to fully extract the reactant. The measurement of diphenyl urea in the reactant solution after each reaction was, as described in Chapter 3, used to determine the water level during each reaction.

Quantification of urethanes on lignin sidechains, as shown in **Table 4.3**, was performed using 2D NMR volume integration of the A β and B β contours. As discovered in Chapter 3, the gamma- ^{13}C - ^1H correlations from β -aryl ethers and phenylcoumarans of carbamylated BMW overlap with polysaccharide contours. Therefore, directly integrating these reacted gamma-C/H contours is not possible. However, from the chemical shift data obtained in Chapter 3 on partially reacted β -aryl ether and phenylcoumaran models (i.e., derivatized at the gamma-position only, Figure 3.4 and 3.6), the β -position contours show distinct separation in the

derivatized versus the unreacted models. These β -contours were used for quantifying gamma reactivity of lignin sidechains.

Table 4.3. Percent lignin sidechain derivatization in MTW with pMDI model compounds as determined by contour integration in HSQC NMR spectra.

Sample ^a	Percent <i>A</i> γ derivatized with model ^b	Percent <i>A</i> α derivatized with model ^b	Percent <i>B</i> γ derivatized with model ^b
i.	—	—	—
ii.	94	13	85
iii.	53	1	37
iv.	13	nd	12
v.	7	nd	3
vi.	4	nd	nd
vii.	4	nd	nd
viii.	nd	nd	nd
ix.	nd	nd	nd

a. See Table 4.2 for sample conditions

b. *A* γ , *A* α , and *B* γ structures are shown in **Key 2**

nd means none detected

The loblolly pine reactions in DMAc displayed high lignin sidechain reactivity with PI under 0% MC conditions. With a 2:1 (NCO:OH) molar ratio the β -O-4- and β -5- γ -hydroxyls are 94% and 85% reacted at 0% MC. Some polysaccharide reaction was evident only with the 2:1 (NCO:OH) molar ratio, possibly at the C2 hydroxyls and primary C6/C5 hydroxyls of hemicelluloses. At a 1:1 (NCO:OH) molar ratio, the β -O-4- and β -5- γ -hydroxyls are 53% and 37% reacted at 0% MC. As the MC increased to 5%, a 1:1 (NCO:OH) molar ratio showed 13% of the β -O-4- and 12% of the β -5- γ -hydroxyls had reacted. At an MC of 14% and a 1:1

(NCO:OH) molar ratio, 7% of the β -O-4- and 3% of the β -5- γ -hydroxyls had reacted. When MTW in DMAc was reacted with 4-BPI, reaction was substantially less effective, suggesting that the bulkier molecule had limited access to most lignin sidechains. Using 4-BPI at a 1:1 (NCO:OH) molar ratio, the β -O-4- γ -hydroxyls are only 4% reacted at 0% MC. When MTW (300 μ m thick) were bonded in the hot-press using 4-BPI with a 1:1 (NCO:OH) molar ratio, 4% of the β -O-4- γ -hydroxyls reacted at 0% MC; no urethane formation was detected with 2D NMR using the ABES press at 5% or 14% MC.

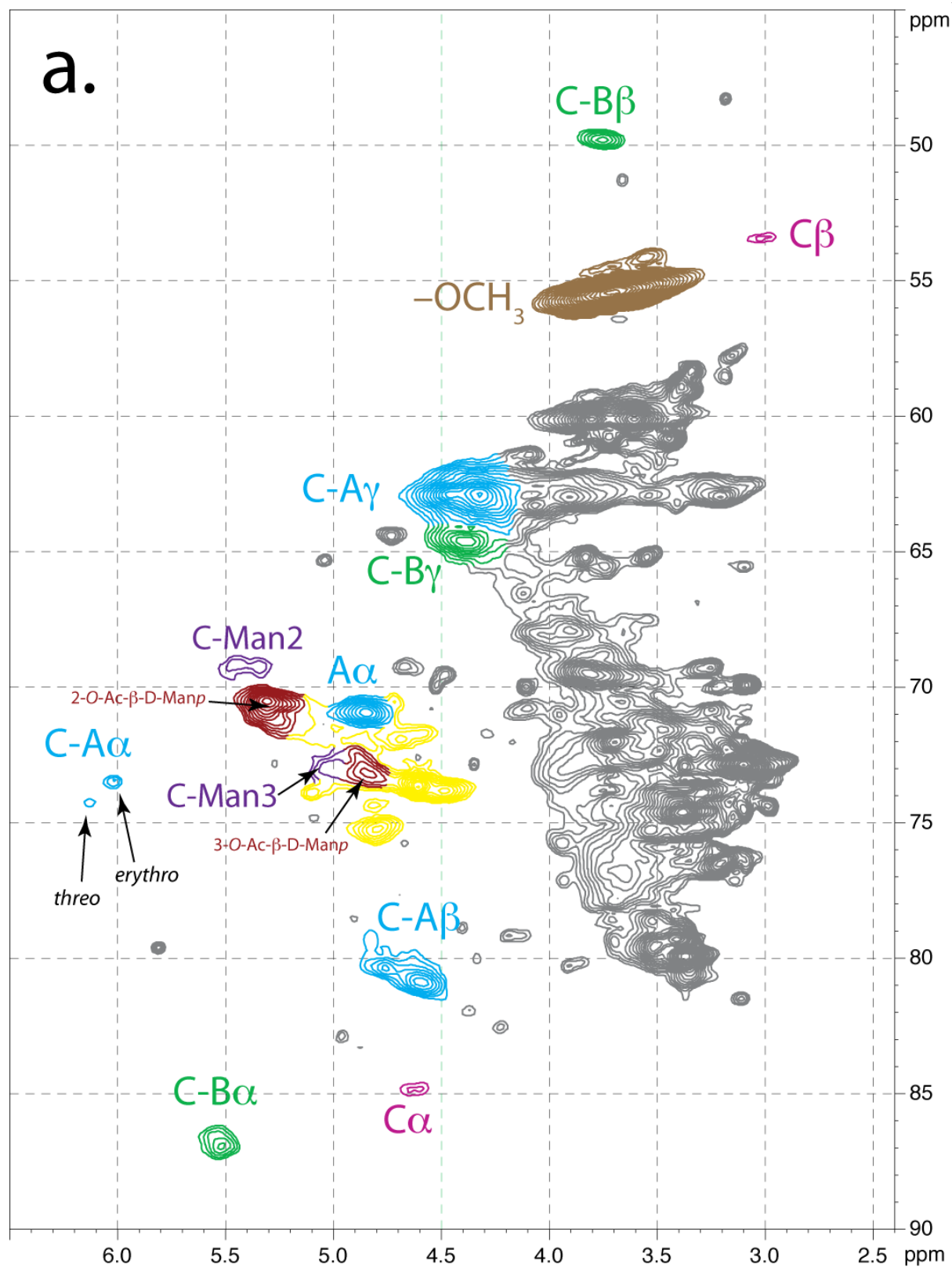
HSQC spectra from the derivatized loblolly pine sections, in particular for samples ii–vii, are shown in the following figures; samples viii and ix displayed no distinguishable changes in the HSQC spectra (see Table 4.2 for sample conditions). **Figure 4.1** shows the HSQC spectra of sample ii (2:1 molar ratio, 0% MC) in both the aliphatic and anomeric regions. In sample ii, a high level of lignin derivatization was observed along with some polysaccharide modification. For example, the β -aryl ether and phenylcoumaran gamma-C/H sidechains show chemical shifts consistent with the derivatized MWL and derivatized β -aryl ether model compounds described in Chapter 3. Also, the alpha position of β -aryl ethers show reactivity; the alpha-C/H contour of the derivatized β -aryl ether in sample ii shows both the *erythro* and *threo* stereoisomers of the carbamate derivative present, with a slight preference for the *erythro* stereoisomer. Hemicellulose reactivity is also evident from the spectra in Figure 4.1a. For example, the H2/C2 and H3/C3 contours of carbamylated mannan are shown in the spectra along with some unassigned contours in yellow. The correlation at 4.80/75.1 ppm is consistent with an α -L-arabinosyl unit H2/C2. In Figure 4.1b, the α -L-arabinosyl H1/C1 correlation has shifted downfield slightly. Also, the anomeric region shows no β -D-galactosyl units present, suggesting that galactan may have been derivatized.

Figure 4.2 shows the HSQC spectra of sample iii (1:1 molar ratio, 0% MC) in both the aliphatic and anomeric regions. Similarly to sample ii, sample iii shows strong preference for derivatization of the β -aryl ether and phenylcoumaran gamma-hydroxyls with some hemicelluloses derivatization depicted by yellow contours in Figure 4.2a. The anomeric region (Figure 4.2b) shows that the α -L-arabinosyl H1/C1 correlation is showing slight derivatization of C2, C3, or C5 hydroxyls; a small contour just downfield of the larger contour is present.

Figure 4.3 shows the HSQC spectra of sample iv (1:1 molar ratio, 5% MC) in both the aliphatic and anomeric regions. Again, the β -aryl ether and phenylcoumaran gamma-alcohols are derivatized with some hemicellulose derivatization depicted by yellow contours in Figure 4.3a; however, the derivatization is much less evident here because of the competition of the water in the wood for the isocyanate. As the MC was increased to 14% as in sample v (**Figure 4.4**), the derivatization of lignin sidechains decreased even further, showing that even when a high concentration of PI is present, water is kinetically dominant and self-condensation of the isocyanate with water will be favored over reaction with the wood hydroxyls.

Figures 4.5 and 4.6 show HSQC spectra of samples vi and vii where typical industrial bonding conditions are used with 4-BPI. Sample vi used DMAc at 160 °C and shows little derivatization of the β -aryl ether and no derivatization of phenylcoumaran or hemicelluloses. Sample vii used the same isocyanate molar ration as sample vi, but was hot-pressed as in industrial applications. Again, little derivatization of the β -aryl ether and no derivatization of phenylcoumaran or hemicelluloses was found.

Figure 4.1. HSQC spectra of sample ii (2:1 molar ratio, 0% MC). **(a)** aliphatic region, **(b)** anomeric region. Colored contours correspond to specific lignin and polysaccharide structures shown in **Key 2** (Chapter 3). Gray contours are not assigned here. Lignin and polysaccharides structures labeled with a “C” (e.g., C-A α) represent carbamylated structures.



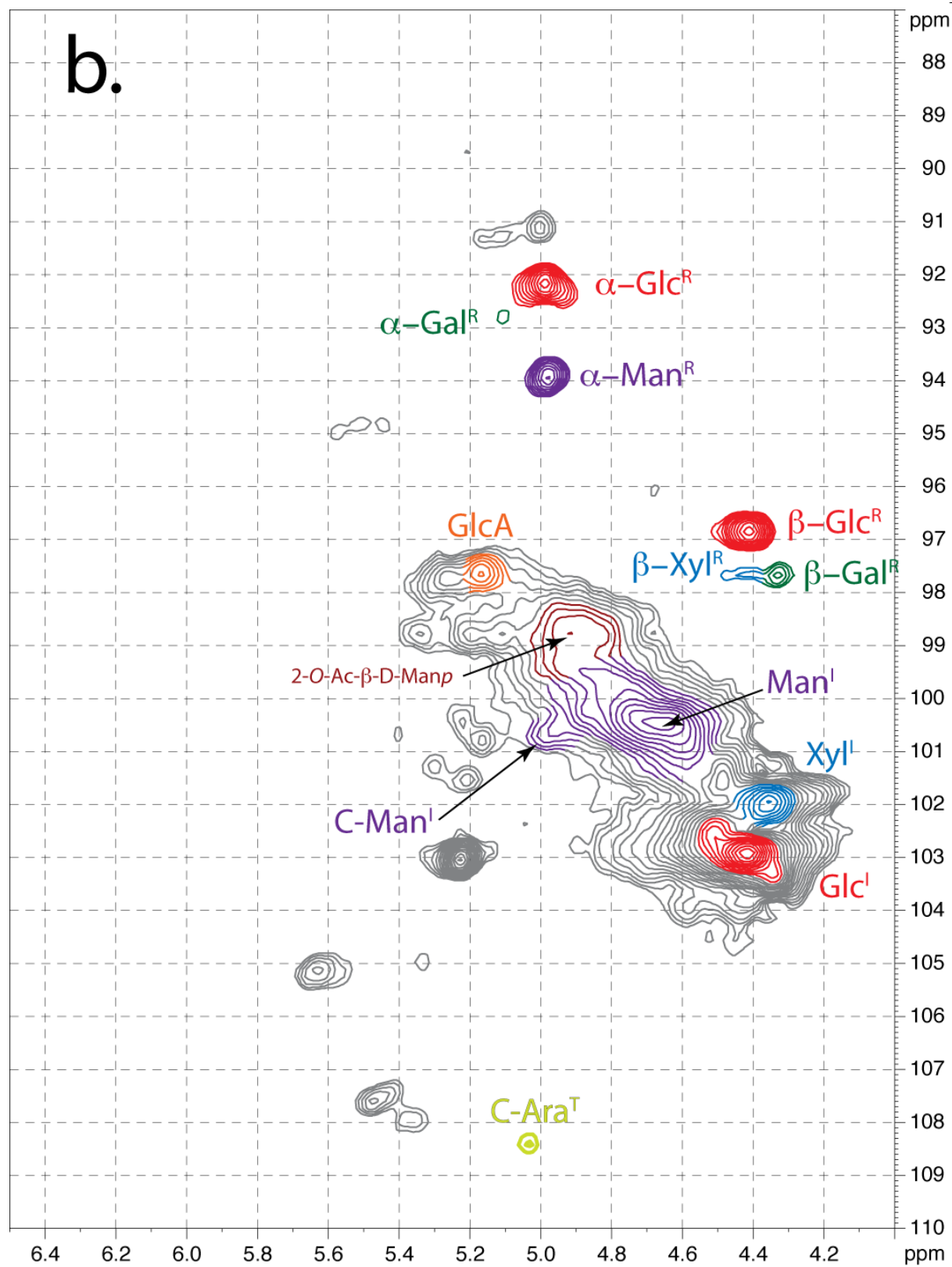
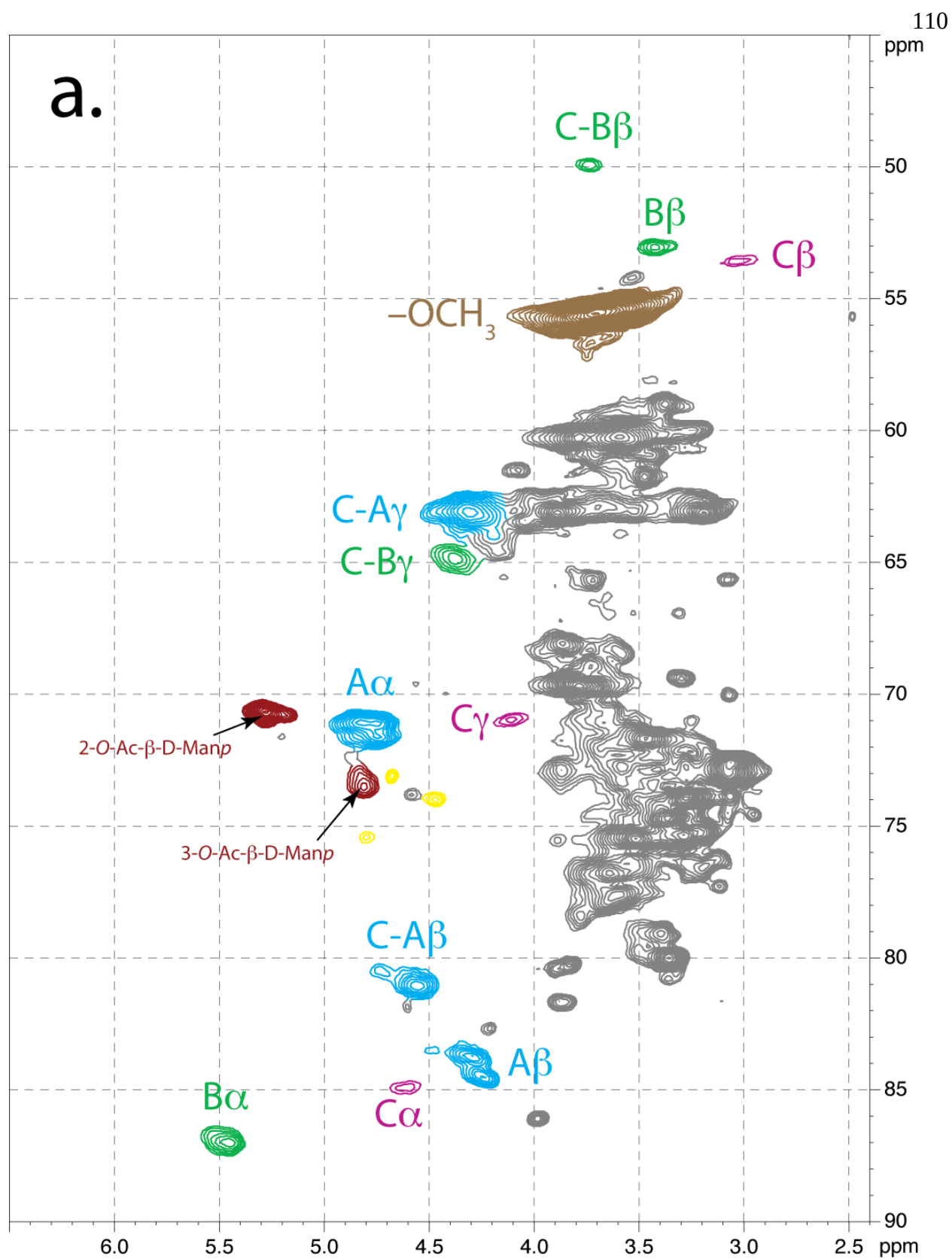


Figure 4.2. HSQC spectra of sample iii (1:1 molar ratio, 0% MC): (a) aliphatic region, (b) anomeric region. Colored contours correspond to specific lignin and polysaccharide structures shown in **Key 2** (Chapter 3). Gray contours are not assigned here. Lignin and polysaccharides structures labeled with a “C” (e.g., C-A γ) represent carbamylated structures.



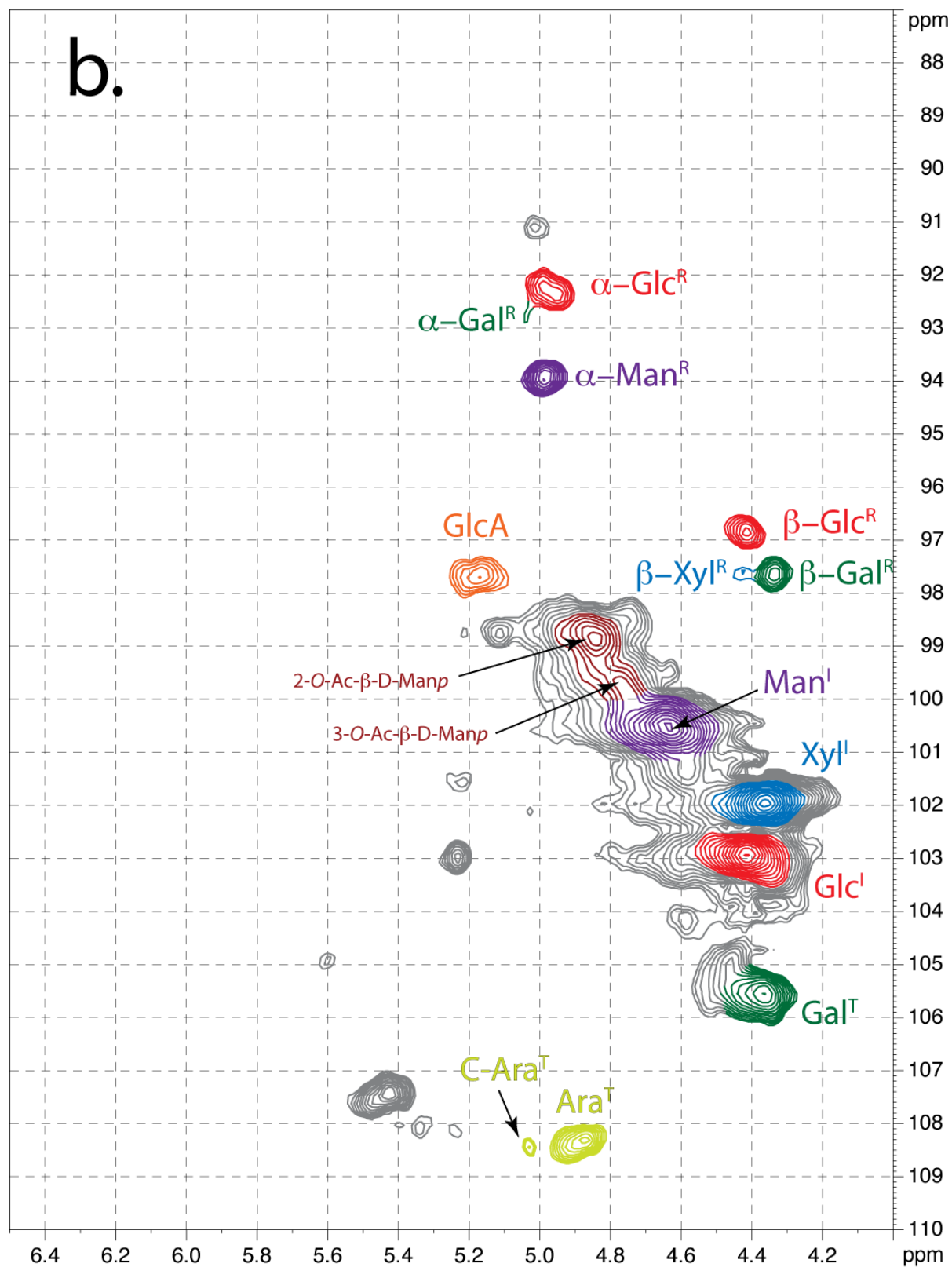
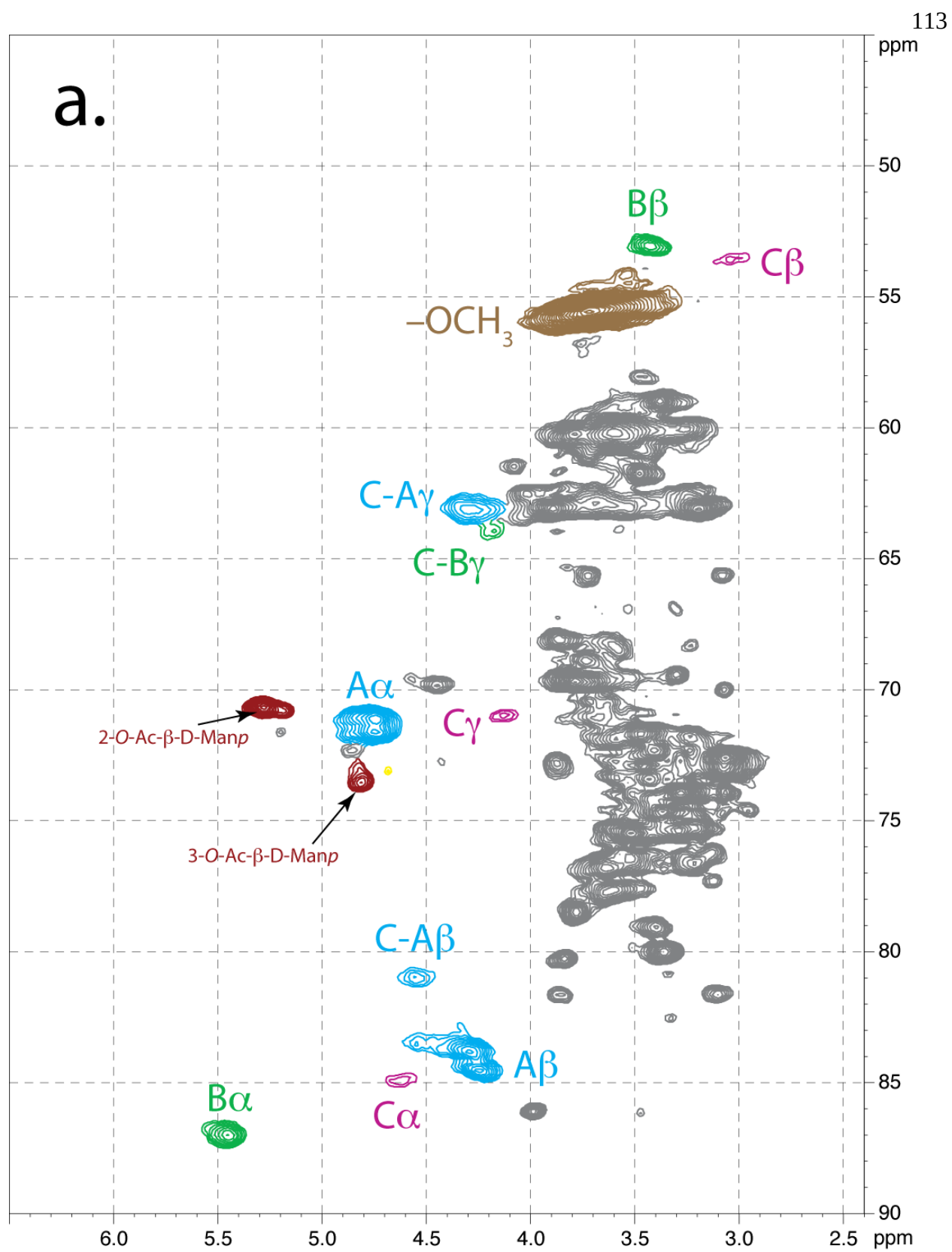


Figure 4.3. HSQC spectra of sample iv (1:1 molar ratio, 5% MC): (a) aliphatic region, (b) anomeric region. Colored contours correspond to specific lignin and polysaccharide structures shown in **Key 2** (Chapter 3). Gray contours are not assigned here. Lignin and polysaccharides structures labeled with a “C” (e.g., C-A γ) represent carbamylated structures.



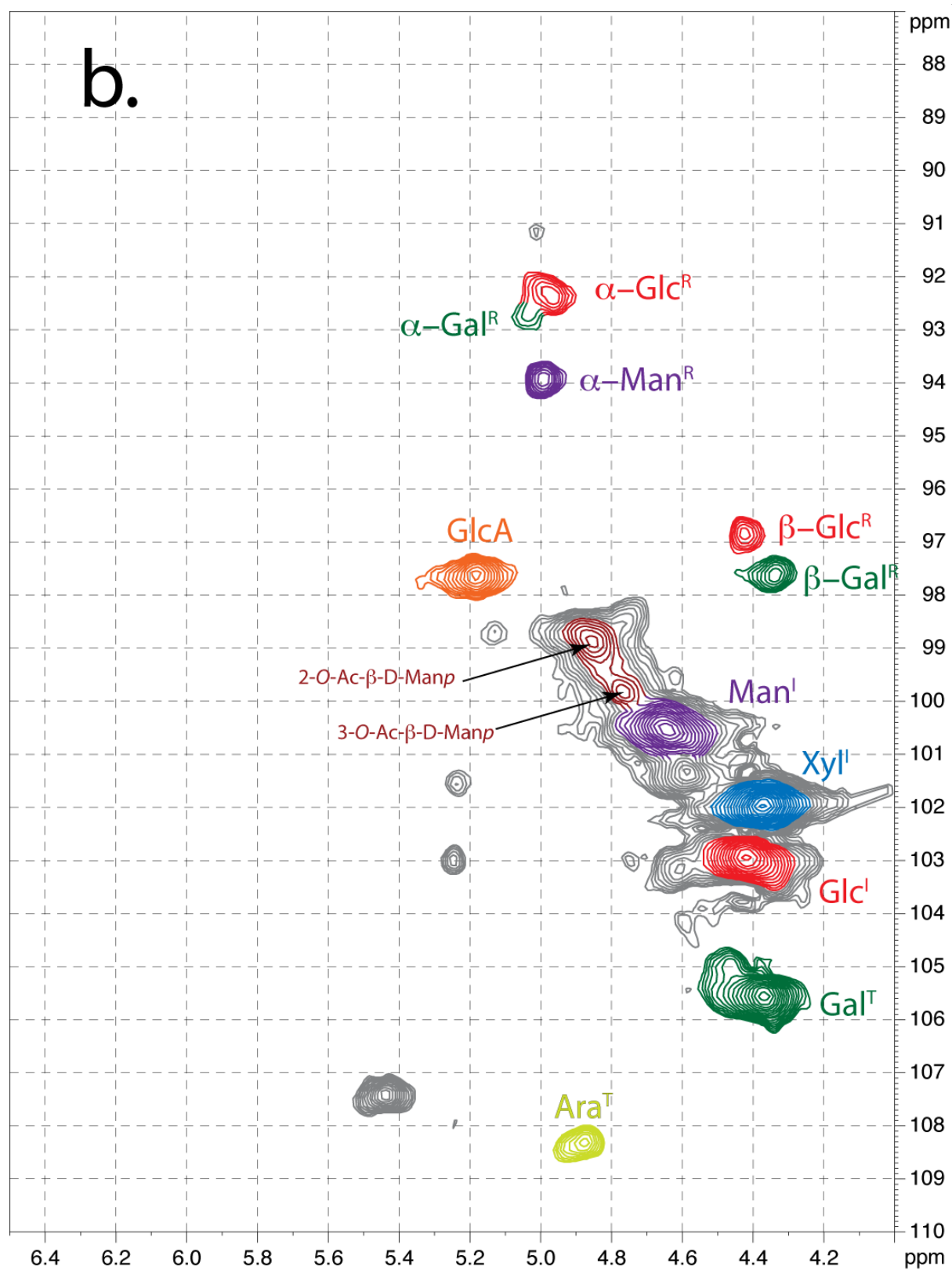


Figure 4.4. HSQC spectra of sample v (1:1 molar ratio, 14% MC). Colored contours correspond to specific lignin and polysaccharide structures shown in **Key 2** (Chapter 3). Gray contours are not assigned here. Lignin and polysaccharides structures labeled with a “C” (e.g., C-A γ) represent carbamylated structures.

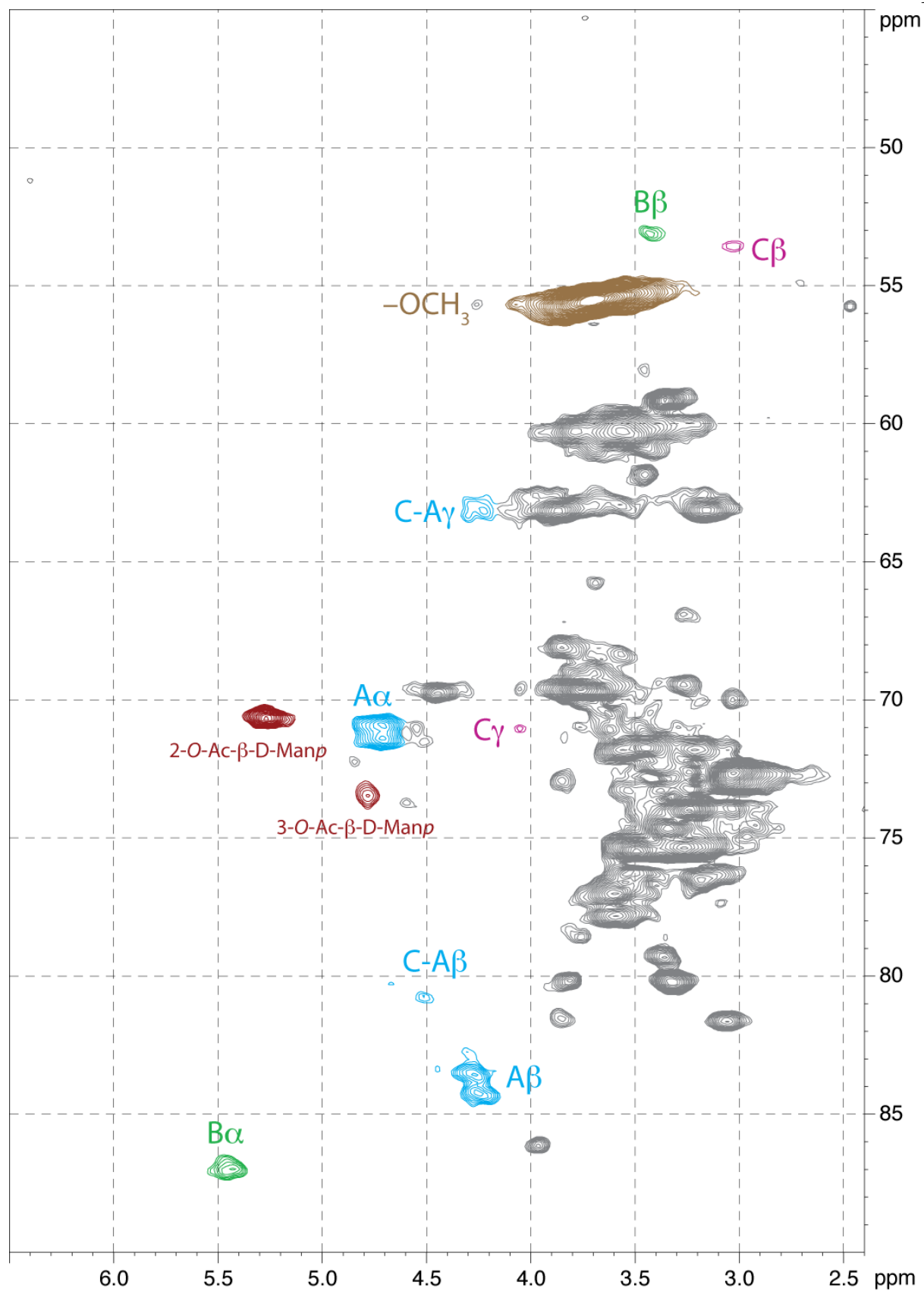


Figure 4.5. HSQC spectra of sample vi (1:1 molar ratio, 4-BPI, 0% MC, DMAc). Colored contours correspond to specific lignin and polysaccharide structures shown in **Key 2** (Chapter 3). Gray contours are not assigned here. Lignin and polysaccharides structures labeled with a “C” (e.g., C-A γ) represent carbamylated structures.

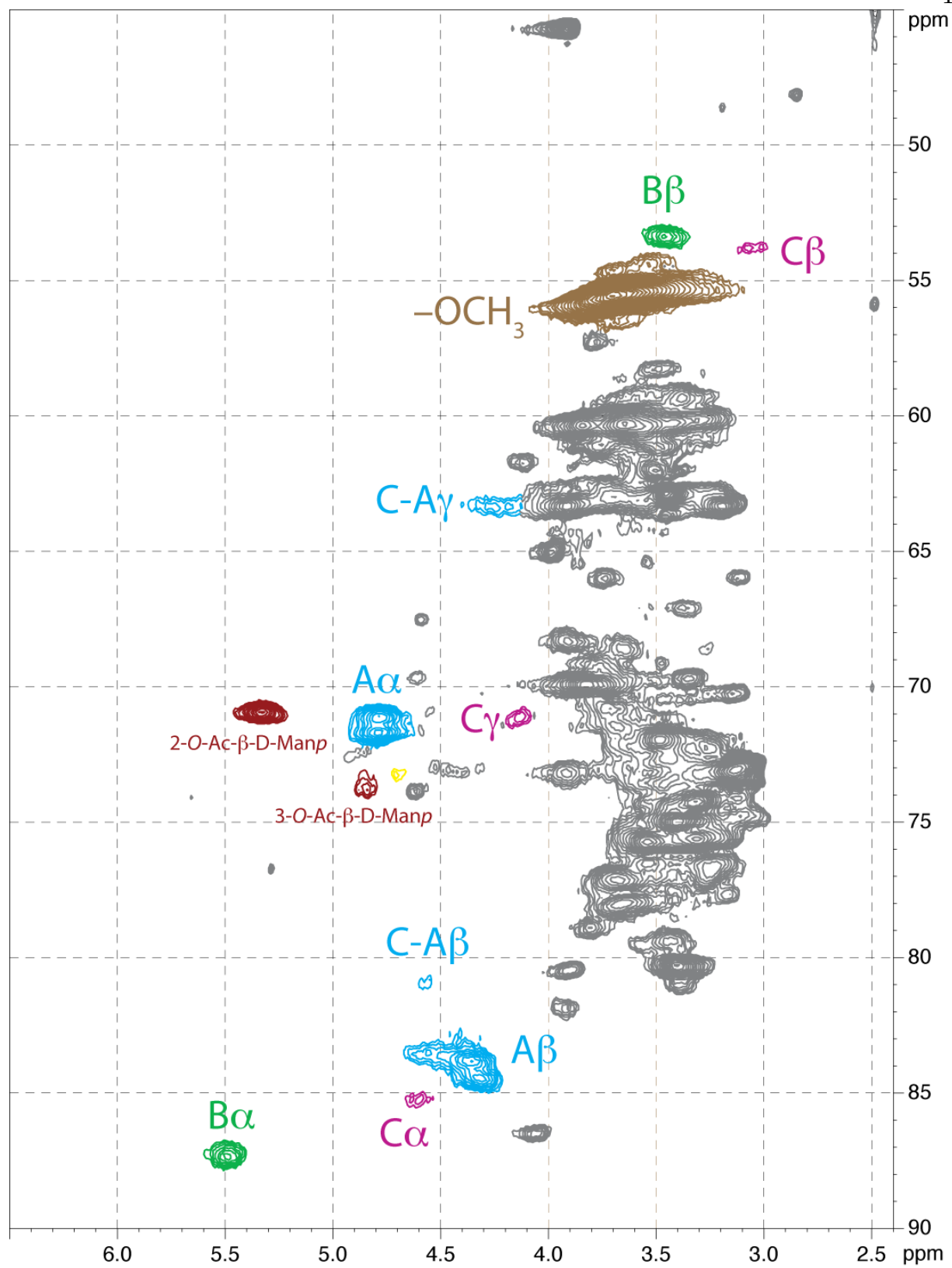
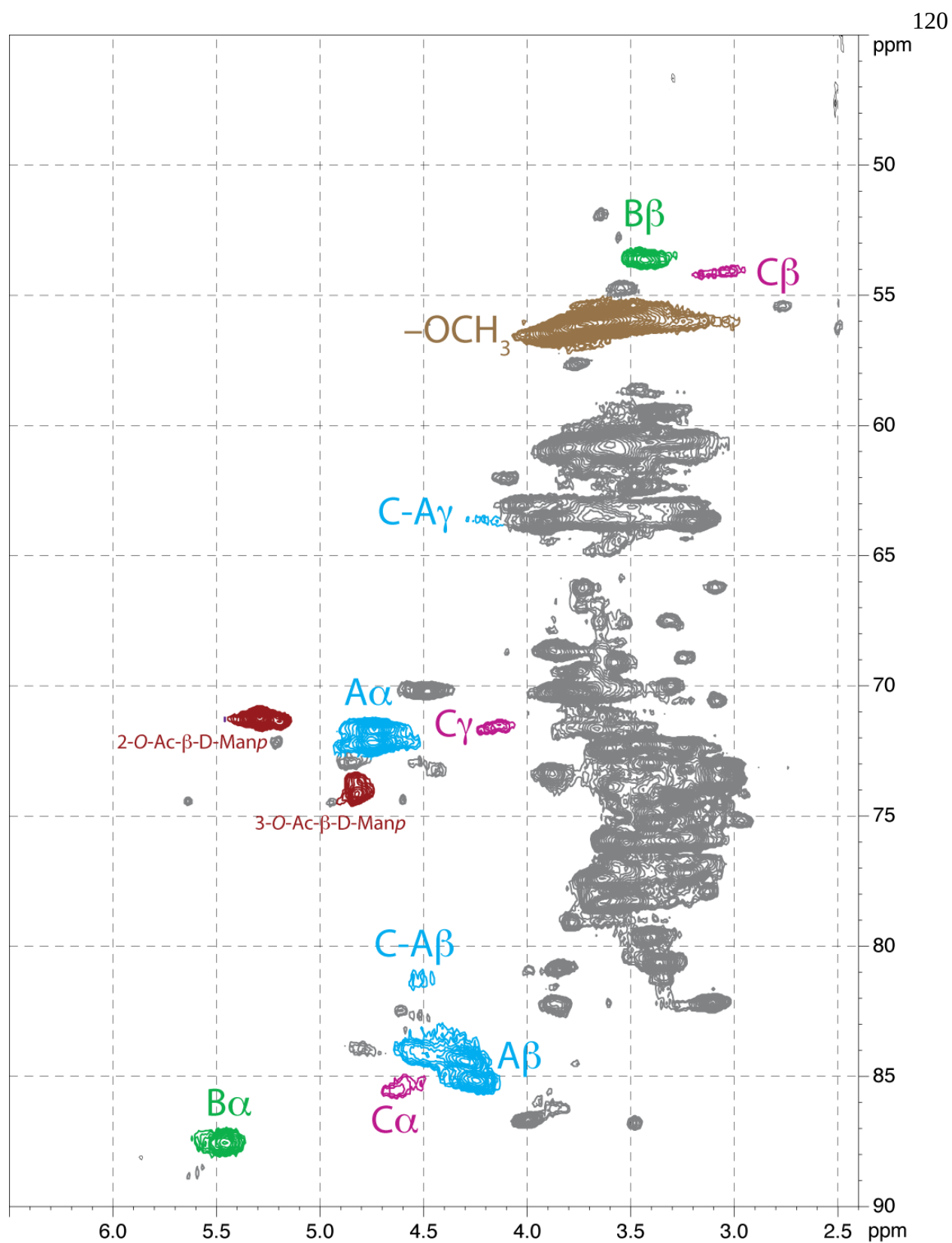


Figure 4.6. HSQC spectra of sample vii (1:1 molar ratio, 4-BPI, 0% MC, miniature hot-press). Colored contours correspond to specific lignin and polysaccharide structures shown in **Key 2** (Chapter 3). Gray contours are not assigned here. Lignin and polysaccharides structures labeled with a “C” (e.g., C-A γ) represent carbamylated structures.



CONCLUSIONS

This NMR method unambiguously answers the long-standing question of whether covalent bonds can form between wood cell wall polymers and pMDI. The data presented here showed that:

1. HSQC spectral data from lignin model compounds (β -O-4, β -5 & MWL) are directly relevant for assignment in spectra of BMW.
2. HSQC spectra from the reacted MTW, under dry conditions with high NCO:OH molar ratios in a swelling solvent, revealed that β -O-4 and β -5 lignin sidechains were the major wood polymer reactants, reacting first at their primary γ -hydroxyls, then secondary α -hydroxyls; minor reactivity was observed with mannan (and possibly arabinan) at their secondary hydroxyls (C2 & C3), and primary hydroxyls (C6 & C5).
3. As moisture increased from 0% to 5% to 14% MC and NCO:OH molar ratios decreased from 2:1 to 1:1, a dramatic decrease in lignin sidechain hydroxyl reactivity was observed.
4. Under identical reaction conditions, PI reacted with β -O-4- γ -hydroxyls 13 times more frequently than 4-BPI, which shows that molecular size of the infiltrating pMDI model controls carbamate formation to a large extent.
5. At 0% MC conditions using pressing parameters similar to industrial OSB, and with high isocyanate concentration, 4% of the β -O-4- γ -hydroxyls were derivatized with carbamates.
6. No carbamate formation was detected after using a miniature hot-press on MTW with 4-BPI at 5% and 14% MC, even under a high NCO:OH molar ratio.

Therefore, when loblolly pine is $\geq 5\%$ MC, it is not possible for wood polymers to react with pMDI under industrial OSB conditions.

An alternative hypothesis of pMDI-wood bonding

This research showed emphatically that covalent bonding is not a contributing mechanism for durable pMDI-wood bond formation. This conclusion is in agreement with previous research conducted on pMDI-treated particleboard where an IR probe (placed into the formed mat) monitored the chemistry of the curing board during hot-pressing.¹⁰ The boards were pressed with wood particles containing 6-7% MC at pMDI resin levels typically used in the OSB and particleboard industry. Urea products were the major product obtained under these conditions and no urethane (carbamate) was detected. Another study used 2D solid-state NMR (^{15}N – ^1H correlation) and found that urea and biuret were the predominate products from pMDI reactions with aspen and southern yellow pine.¹⁶ Furthermore, a recent study conducted on pMDI-bonded wood blocks at various MCs showed that the hot-pressed assemblies at 0% MC failed to bond 83% of the time, suggesting that under dry conditions not only is there poor network formation, but that urethane formation is not beneficial for bond strength.²⁸

It has been shown that low molecular weight oligomers of pMDI enter into the micrometer size voids of wood via capillary action.²⁹ It also is apparent that infiltration occurs down to the angstrom scale, thus influencing with cell wall polymers. Wood swelling caused by pMDI showed a maximum swelling of 0.48% using a thermomechanical analyzer; nearly half of this swelling occurs in 5-10 minutes.²⁵ This swelling by pMDI may lead to a subsequent curing process involving an interpenetrating polymer network (IPN) where the diisocyanate monomers first enter the microvoids in the cell wall, and secondly the polymer free volume (if accessible). This accessible void space in the wood cell wall has been estimated by several researchers and ranges from approximately 1.2 – 5 nm in diameter.^{30,31} However, this void space most certainly is variable between wood species, drying method employed, cell type, and cell wall layer.

It is believed that creating a nanoscale IPN will lead to improved adhesive bond stability due to resistance to hydrolysis throughout the lifetime of the bondline. If pMDI adhesive does enter the cell wall, the adhesive can react with bound water, promoting urea-type self-condensation reactions, which then promotes hydrogen bonding and van der Waals interactions. The pMDI may also be absorbed into the wood hemicelluloses/lignin matrices where polymer free volumes allow pMDI to intimately interact with polymer chains, giving pMDI the opportunity to solubilize wood polymers.

The proposed IPN^{11,25} of polyurea in the cell wall will depend not only on the time between application and cure, but also the temperature at which the bondline reaches during cure;⁸ during OSB hot-pressing, approximately 30% of the initial pMDI may be attributable to urea formation, depending on MC of the furnish.³²

Returning to the hypothetical schematic first described in Chapter 1, **Figure 4.7** shows a more detailed description of how pMDI may be interacting with wood on the cell lumina level, the cell wall level, the nanoscale level, and the molecular level. At the cell lumina level the lumina are coated with a thin film of pMDI with some of the lower molecular weight compounds infiltrating the cell walls. Then, at the cell wall level, pMDI begins to micro-finger itself into the S3/S2 layers, finding bound water to self-condense with for polyurea formation. Further infiltration is depicted at the nanoscale level where the pMDI enters the hemicelluloses/lignin matrices. Finally, at the molecular level the pMDI can form hydrogen bonds and van der Waals interactions with each other and/or wood polymer hydroxyls as shown in state 1.

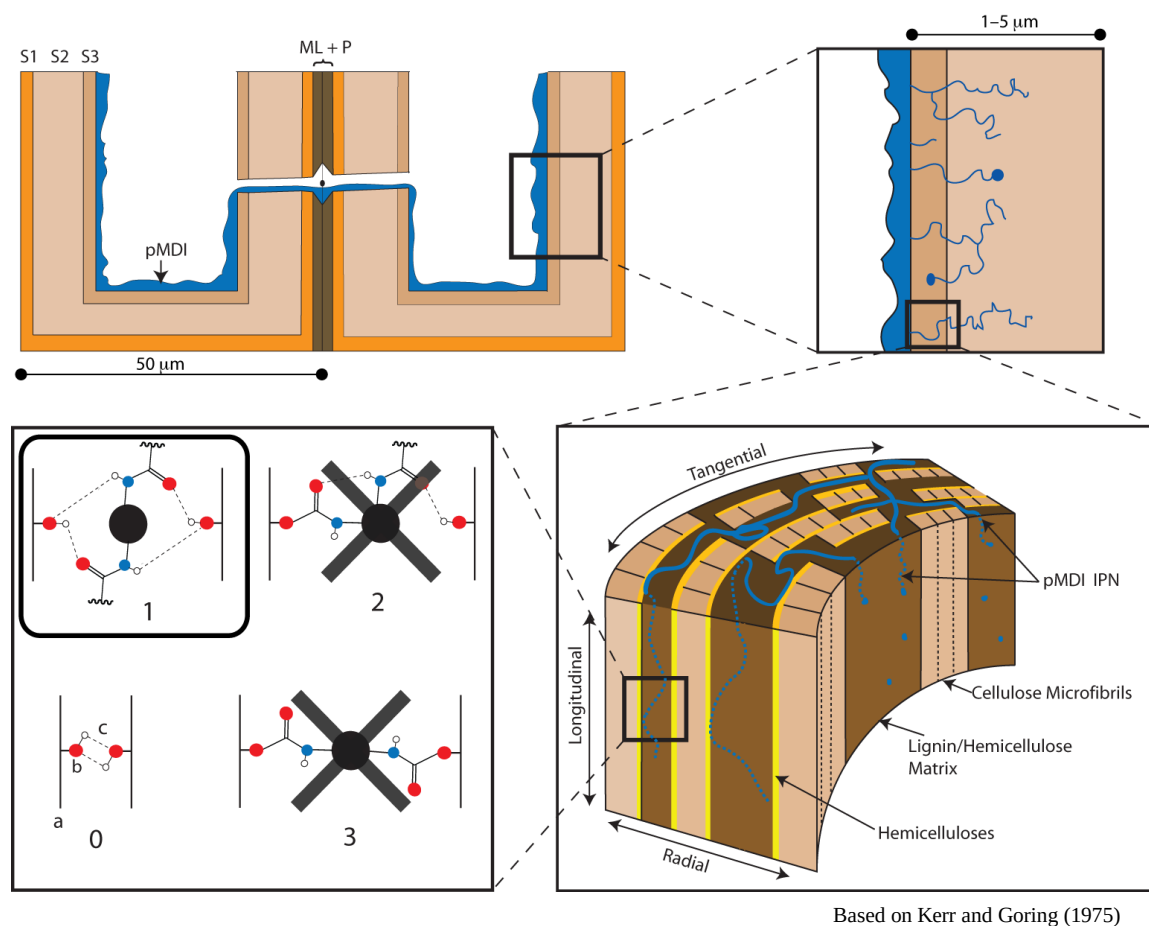


Figure 4.7. Hypothetical schemes for how pMDI may interact with wood cell walls of loblolly pine during OSB production. Starting with the upper left, pMDI wets the cell lumina and passes through bordered pits between longitudinal tracheids. At the microscale level (upper right) the pMDI infiltrates the microvoids of the secondary cell wall layers creating a size-exclusion effect as pMDI travels away from the cell lumen. Some pMDI may diffuse into the free-volume of the lignin/hemicelluloses matrix at the nanoscale level (lower right).³³ At the molecular level (lower left), hydrogen bonding and van Der Waals forces must play a major role in pMDI adhesion (state 1); note that state 2 and 3 have been deemed unlikely contributors to pMDI adhesion.

Future work

Chromatographic methods for quantifying wood polymer carbamates are currently in progress. These methods would enhance and supplement the characterization of the wood-pMDI reaction investigated in this thesis research. Further investigation into the reactivity of specific hemicelluloses must be made. In this thesis, mannan and arabinan were found to be the most reactive hemicelluloses in loblolly pine. Therefore, reacting a galactoglucomannan and an arabinoglucuronoxylan from loblolly pine with pMDI models will be studied. To investigate the strength properties of the wood-pMDI bondline in loblolly pine, work is in progress using nanoindentation to determine the effects IPNs have on the modulus of the S2 cell wall layer.

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Appendix A. Selection of an adhesive for study

Table 1.A. Criteria for selecting a wood adhesive for studying with solution-state NMR.

Adhesive	Reactivity with wood Functional group contact	Reactivity with itself Kinetics of homopolymerization	Dependence on moisture content of wood	Accessibility Physical ability to penetrate the wood	Wettability The distance it may travel in the wall is dependent on interfacial tensions and thermodynamics	Solubility Depends on wettability	pH dependency	Temperature	Ability to detect reaction using NMR	Ability to use model	Importance as a wood adhesive
pMDI	-OH of polysaccharide or β -O-4 or β -5, Phenolic -OH of aromatic lignin, C5 of guaiacyl?	Will only react with itself in the presence of water or heat or both to form urea and biuret structures. Reaction with water is very fast. If it is to react with wood the moisture content must be very low.	High. Will easily react with water molecules if it comes into contact with water before wood hydroxyls.	No swelling of the cell wall is expected due to hydrophobic nature. Travels long distances from surface. May penetrate microvoids if given access by a crack or fracture.	Adhesive will not fill lumen. Its hydrophobic nature is more likely to very slowly wet the hydrophilic polysaccharides. More likely to wet lignin and extractive components.	No solvent. Adhesive is much more hydrophobic, May dissolve lignin & extractives better than most adhesives.	Neutral	~190 °C	HSQC NMR can detect chemical shift changes with all wood components. New carbamates with wood currently modeling.	Model is readily available from Aldrich. Not expensive.	Important for the North American OSB industry to make durable products with little resin load. Expensive though.
RF (HMR)	C5 of guaiacyl, -OH of polysaccharide or β -O-4 or β -5 Schiff base of guaiacyl C5 if reacted with $H_2C=O$ Under acidic conditions the α -benzyl alcohol or α -benzyl ether of lignin will split off to form the benzyl cation, thus making the resorcinol a good nuc.	Very fast. Highly reactive with formaldehyde. Would need to be in close proximity to a wood hydroxyl very early to compete with another resorcinolic nucleophile	Low. However, swelling of wood will be great if MC is low, maybe creating microcracks	Water may facilitate swelling of microvoids to allow penetration into cell wall	Adhesive will not fill lumen, but diffuse into the wall as the H-bonds are disrupted. More apt to wet hemicelluloses	Water based adhesive, hemicelluloses	8.5 to 9 to form novolac	25 to 30 °C	HSQC spectra show that HMR does not overlap with the aromatic lignin. C5 of guaiacyl is easily detectable. New methylene & ether bonds to wood will need to be modeled.	Synthesis of 2,4-DMR is possible, but is somewhat lengthy. Model work will be difficult due to the high reactivity resorcinolic compounds at room temperature. May need to run low temperature reactions.	Has a more specialized niche for use with epoxies and possibly chemical modification to increase dimensional stability of surface
MF	C5 of guaiacyl, -OH of polysaccharide or β -O-4 or β -5, Phenolic -OH of aromatic lignin, Schiff base of guaiacyl C5 if reacted with $H_2C=O$	Reactivity with itself is slow and needs heat to catalyze. Has more time to penetrate and get in close proximity with wood components than most adhesives.	Low. However, swelling of wood may become significant if MC is low. During heating, too high of MC may cause microblows and hinder bond formation	Water may facilitate swelling of microvoids to allow penetration into cell wall	Adhesive may fill lumen and diffuse into the wall as the H-bonds are disrupted. More apt to wet hemicelluloses and branched lignin	Water based adhesive, but not that water-soluble. May dissolve hemicelluloses or lignin	Acidic, 3 to 5 for full methylol formation	~140 °C	Would need to use long-range $^1H/^15N$ 2D HMBC or use ^{15}N -enriched melamine or specifically label the amino-group nitrogen with ^{15}N . New methylene & ether bonds to wood will need to be modeled.	Model would probably need to be synthesized. Have several German papers. Not many recent papers on its blocking reactive sites of melamine. Can buy the hydroxymethylated model, but is expensive.	Important to replace or add to UF resins for the European market for exterior grade products. Melamine is expensive.
Epoxy	-OH of polysaccharide or β -O-4 or β -5	In the presence of a catalyst, homopolymerization is dominant	High. This adhesive can polymerize in the presence of water.	Prepolymer is very viscous. No swelling of cell wall is expected. Lumens may be filled easily, but not likely to travel into microvoids.	Its hydrophobic nature is more likely to wet extractives and lignin components	No solvent. Adhesive is mostly hydrophobic. May dissolve extractives better than most adhesives.	Neutral or more on the basic side since amines are used frequently for catalysts or hardeners	25 to 30 °C. however, rapidly catalyzed by heat	HSQC NMR can probably detect presence of a reaction. New ether bonds with wood need to be modeled.	Model is readily available from several epoxy resin companies. Can get free sample usually.	Important for gap-filling applications and to reinforce a polymers (FRPs) for bonding to wood products (Glu-lams)

Monofunctional Synthetic Adhesives

Polymeric material in solution diminishes the effectiveness of studying not only the short-range coupling interactions, but also the desirable long-range coupling interactions. Adhesives are polyfunctional, and thus, they react to form higher molecular weight polymers that are often heavily crosslinked. This necessitates a decrease of the extent of reaction, preferentially making it monofunctional and eliminating crosslinking to avoid forming insoluble adhesive polymers in the wood cell walls. The largest molecule it could make would then be a dimer, which is small enough to extract out of the wood using neutral solvents (e.g., acetone or toluene). Having less functionality, but similar reactivity, will give an advantage of higher solubility. Therefore, the models chosen to mimic pMDI are PI and 4-BPI. **Figure 1.A** compares the chemistry of these models with pMDI adhesive so as to establish rationale behind these choices.

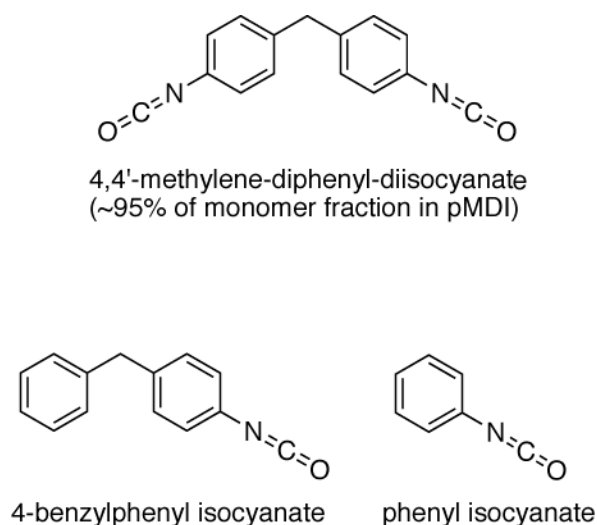


Figure 1.A. The difunctional pMDI adhesive (top) and the two monofunctional model compounds used in this thesis research.

Appendix B. Evidence for cleavage of lignin by a brown rot basidiomycete

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SUMMARY

Biodegradation by brown-rot fungi is quantitatively one of the most important fates of lignocellulose in nature. It has long been thought that these basidiomycetes do not degrade lignin significantly, and that their activities on this abundant aromatic biopolymer are limited to minor oxidative modifications. Here we have applied a new technique for the complete solubilization of lignocellulose to show, by one-bond ^1H - ^{13}C correlation NMR spectroscopy, that brown rot of spruce wood by *Gloeophyllum trabeum* resulted in a marked, nonselective depletion of all intermonomer sidechain linkages in the lignin. The resulting polymer retained most of its original aromatic residues and was probably interconnected by new unprotonated linkages that are invisible in one-bond ^1H - ^{13}C correlation spectra. Additional work is needed to characterize these linkages, but it is already clear that the aromatic polymer remaining after extensive brown rot is no longer recognizable as lignin.

INTRODUCTION

Lignin comprises roughly 15% of all terrestrial biomass and must be continuously broken down to maintain the global carbon cycle, yet is one of the most recalcitrant to biodegradation of all natural polymers. Few organisms possess the extracellular mechanisms to attack its combinatorial, stereoirregular linkages, which are formed in the cell walls of vascular plants via free radical coupling reactions between various 4-hydroxycinnamyl alcohols and the growing

polymer (Boerjan et al., 2003). Efficient ligninolysis is apparently confined to certain filamentous fungi that grow on the cellulose and hemicelluloses of dead plant cell walls (Kirk and Farrell, 1987). It is well-documented that the porosity of intact lignocellulose is too low for cellulases and hemicellulases to penetrate, and is generally accepted that most lignocellulolytic fungi circumvent this problem by degrading some of the lignin that encrusts the polysaccharides (Blanchette et al., 1997).

One important group of lignocellulolytic fungi is generally thought to contradict this model. Brown rot basidiomycetes cause a rapidly destructive decay that is the primary cause of failure in wooden structures (Zabel and Morrell, 1992), but is also an essential source of carbon cycling and humus formation in coniferous forests (Gilbertson and Ryvarden, 1986). Extensively brown-rotted wood is markedly depleted in polysaccharides and consists largely of a partially oxidized aromatic residue derived from the lignin. As compared with sound lignin, brown-rotted lignin has a lower aromatic methoxyl content and has a higher content of phenolic hydroxyls, conjugated carbonyls, and carboxyls. However, it is still polymeric, which has led most researchers to conclude that brown-rot fungi modify lignin without degrading it significantly (Kirk, 1975; Agosin et al., 1989; Eriksson et al., 1990; Jin et al., 1990; Irbe et al., 2001; Filley et al., 2002).

There are two reasons why this view might be incorrect. First, there is considerable evidence that brown rot fungi initiate lignocellulose degradation by producing extracellular hydroxyl radicals ($\bullet\text{OH}$), highly reactive, nonselective oxidants that depolymerize cellulose via hydrogen abstraction but also attack lignin via electrophilic addition to its aromatic rings (Goodell et al., 1997; Hammel et al., 2002; Suzuki et al., 2006). As one consequence of the latter reaction is aryl methyl ether cleavage (Gierer et al., 1992), which would explain why brown-rotted lignin is

depleted in methoxyls, there is no obvious reason why the arylglycerol- β -aryl ether linkages that interconnect the majority of lignin subunits would not be analogously cleaved (Fig. 1; Tatsumi and Terashima, 1985). Second, it appears that brown rot fungi do not depend solely on $\bullet$ OH to degrade wood polysaccharides, but rather augment this system with hydrolytic enzymes (Cohen et al., 2005). If this is the case, it is unclear how the enzymes could operate without some prior ligninolysis to increase wood porosity. To address this problem, we have adopted a new technique for lignocellulose solubilization that permits, for the first time, a closer look at brown-rotted lignin via 2D solution-state NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Reagents and fungal cultures

DMSO (99%), NMI (%), hydroiodic acid (57%), phenol (recrystallized), bromine (99%), sodium thiosulfate (0.1N), acetyl bromide (99%), dioxane (99%), acetic acid, and zinc dust were supplied by Aldrich Chemical (Milwaukee, WI). Acetic anhydride (99%) was supplied by Mallinckrodt Baker. *Gloeophyllum trabeum* (ATCC 11539) was grown as described earlier on wafers of spruce wood (*Picea glauca*, $3 \times 10 \times 30$ mm, each approximately 300 mg dry weight) that had been cut with the large face perpendicular to the grain (Suzuki et al., 2006).

Milling of spruce

After 16 weeks of decay, those wafers that had lost 50-70% of their dry weight were pooled to give an average of 63.7% with a standard deviation of 8.5%, dried overnight at 50°C, and ground in the presence of dry ice using a coffee grinder. The degraded spruce and control were dried at 50 °C, ground in the presence of dry ice using a coffee grinder, then added to a Retsch

PM100 planetary ball-mill equipped with a 50 ml ZrO₂ cup, eight 10 mm and three 30 mm ZrO₂ balls. The degraded spruce (300 mg) was milled at 300 rpm for 80 min. with an interval of 20 min. and pause time of 10 min. between each interval, giving 60 min. total mill time. The control spruce (500 mg) was milled at 600 rpm for 260 min. with 20 min. interval and 10 min. pause, giving 180 min.

Dissolution and acetylation

Dissolution of the milled spruce samples closely followed the procedure introduced by Lu and Ralph (2003). Ball-milled degraded and control spruce (100 mg) was added to 2 ml of DMSO and 1 ml NMI, dissolved with stirring overnight at room temperature, acetylated with 600 μ l acetic anhydride for 1.5 hr., and precipitated quantitatively in 400 ml of water with vigorous stirring for 30 min. The settled precipitate was collected by vacuum filtration through a 0.2 μ m Whatman nylon membrane and washed with 2 $\times$ 200 ml of distilled water, and dried in a 50 $^{\circ}$ C vacuum oven to give a WPG of ~30%.

Cell wall analytical methods

i. Quantification of cell wall components

Klason lignin and acid-soluble lignin analyses were performed on the degraded and control spruce utilizing the ASTM D 1106 standard. Carbohydrate analysis was conducted according to the methods described by Davis. Table 1 shows the analytical results of both brown-rotted and control spruce samples.

ii. Methoxyl content analysis

The quantification of methoxyls in the degraded and control spruce was conducted according

to a modification of the Zeisel-Vieböck-Schwappach method. Briefly, 100 mg of vacuum oven (50 °C) dried wood material (80 mesh) was placed into a reaction flask with 50 mg red phosphorus, 0.5 g phenol, 6 drops of acetic anhydride, and 5 ml of hydroiodic acid. The flask was attached to the apparatus with a washing vessel of red phosphorus. The first absorption tube was filled with 7 ml of 20% sodium acetate (in glacial acetic acid) and 8 drops of bromine. The second tube was filled with 4 ml of 20% sodium acetate (in glacial acetic acid) and 5 drops of bromine. Nitrogen flowed through the apparatus at a rate of 1 bubble per second. The reaction flask was heated to 140 °C and maintained for 2 hr. Absorption tube contents were transferred quantitatively (3 × 20 ml of water) into a flask containing 25 ml of 20% sodium acetate (in water). Formic acid (4%) was added dropwise (5-10 drops) to the flask with gentle shaking until the brown color (bromine) completely discharged. Methyl red (few drops) was added to help indicate the disappearance of bromine. Then, 10 ml of sulfuric acid (10%) and 10 ml of potassium iodide (10%) were added to the flask and let stand for 5 minutes. The iodine was then titrated with 0.1N sodium thiosulfate until the color of the liberated iodine nearly disappears. Just as the color changes to light straw, 1-2 ml of starch solution (1%) is added to change the color of the solution a light blue. The titration is continued until the iodo-starch complex is completely discharged. A blank (pure potassium iodate), two vanillin samples, two brown-rot degraded spruce samples, and two control spruce samples were analyzed as shown in Table 2. Methoxyl content was calculated through the following equation:

$$[\text{OCH}_3] = \frac{(V_s - V_b) \cdot N \cdot 5.1706 \cdot f}{W} \times 100$$

where: V_s = the volume of standard $\text{Na}_2\text{S}_2\text{O}_3$ solution required for titration

of the sample, ml

V_b = the volume of standard $\text{Na}_2\text{S}_2\text{O}_3$ solution required for titration of the

blank (pure potassium iodate), ml

N = the normality of the $\text{Na}_2\text{S}_2\text{O}_3$ solution

5.1706 = the quantity of $-\text{OMe}$ groups, corresponding to 1 ml of 0.1N

$\text{Na}_2\text{S}_2\text{O}_3$, mg

f = the titer factor for 0.1N $\text{Na}_2\text{S}_2\text{O}_3$

W = the weight of the moisture free sample, mg

iii. Derivatization Followed by Reductive Cleavage (the DFRC method)

Chemistry of DFRC release and quantification of acetylated monolignols by reductive cleavage of β -aryl ethers was conducted as described by Lu and Ralph utilizing the “cell walls” protocol. Prior to GC analysis, solid-phase extraction was performed. A cyclohexane and ethyl acetate mixture (5:1 v/v) was dripped on a silica-gel LC syringe, and 1 ml of CH_2Cl_2 was added to the degraded sample and dripped on the silica-gel. Then, the cyclohexane/ethyl acetate mixture (6×2 ml) was pushed through the silica gel and the combined filtrates collected in a round-bottom flask. After rotary evaporation, the monomer sample was dissolved in 2 ml of CH_2Cl_2 and a 2 μl sample injected directly into a Shimadzu GCMS model QP2010: column, 0.20 mm $\times$ 30 mm SPB-5 (Supelco); He carrier gas, 1 ml/min; 20:1 split ratio; injector 220 $^\circ\text{C}$, FID detector 300 $^\circ\text{C}$, temperature program as previously described. The amounts of individual

coniferyl diacetate (G) monomers were determined using a response factor derived from pure monomer standard with tetracosane as an internal standard. See Table 3 for relative retention times and the GC response factor relative to the tetracosane internal standard.

Solution-state NMR spectroscopy

One-bond ^1H - ^{13}C correlation (HSQC) NMR spectra were acquired at 300 K on a Bruker BioSpin (Rheinstetten, Germany) DMX-750 instrument equipped with a cryogenically cooled 5-mm TXI $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ gradient probe with inverse geometry (proton closest to the sample). Samples (approximately 60 mg each) of ball-milled, acetylated wood were added to 5-mm diameter NMR tubes and dissolved in 500 μl of $\text{DMSO-}d_6$. The central DMSO solvent peak was used as an internal reference ($\delta_{\text{C}} = 39.5$ ppm, $\delta_{\text{H}} = 2.49$ ppm). HSQC experiments had the following parameters: sweep width, 8.6-2.4 ppm in F2 (^1H) using 1864 data points (acquisition time, 200 ms) and 160-40 ppm in F1 (^{13}C) using 512 increments (F1 “acquisition time,” 11.3 ms). The number of scans was 32 with a 1-s interscan delay, the d24 delay was set to 1.72 ms ($\sim 0.25/J$, where J is an average 145 Hz ^{13}C - ^1H coupling constant), and the total acquisition time was 5 hr 34 min. Processing used typical matched Gaussian apodization in F₂ and a squared cosine-bell in F₁. Integration calculations of the 2D contours in HSQC spectra were accomplished with Bruker TopSpin v. 2.0 software (Ralph et al., 2006).

RESULTS AND DISCUSSION

Milling, Dissolution, and Derivatization

The milling process employed for the control spruce was found to be the most effective and efficient procedure, which maintains a low temperature (< 50 °C), eliminates paramagnetic iron,

and mills the cell walls to a particle size ($< 10 \mu\text{m}$) necessary for dissolution. However, the brown-rot spruce was considerably decayed to a state where the structure integrity of the cell wall was highly degraded, allowing the use of a much milder ball-milling regime. Taking this into consideration, the brown-rot spruce was milled at half the normal rpm, thus minimizing excessive pulverization of the cell wall structure. Some differences were noted in the dissolution and derivatization steps: 1. The brown-rot spruce dissolved at a slower rate (usually requiring overnight) than the control (usually 3 hr. was sufficient); 2. The color of the brown-rot solution was completely black as opposed to an amber color of the control prior to derivatization, most likely caused by the increased concentration of lignin-type structures; 3. The filtration of the brown-rot spruce after derivatization and precipitation was considerable slower than that of the control, suggesting a finer particle size in the brown-rot sample due to the increased quantity of short polymer chains from lignocellulolytic cleavage; 4. Unlike the acetylated control spruce cell wall sample, the acetylated brown-rot sample did not fully dissolve in chloroform, only DMSO.

Brown-rot spruce analyses

We grew the brown rot basidiomycete *Gloeophyllum trabeum* on wafers of spruce wood for 16 weeks (Fig. 2) and then selected a subset of the biodegraded wood samples that had lost $64\% \pm 9\%$ ($n = 9$) of their dry weight and were 36% deficient in methoxyl groups as shown by the wet chemical analysis generally used (Table 1, see Experimental Procedures). In these properties, our *G. trabeum*-degraded spruce samples closely resembled those analyzed earlier by Kirk, which showed an average dry weight loss of 70% and were 35% deficient in methoxyls (Kirk, 1975). Our samples had lost 85% of their glucan and over 90% of their other polysaccharides, but only 16% of the residual material (Klason lignin and acid-soluble lignin) that is generally interpreted

as modified but intact lignin (Table 2). These results agree well with those generally obtained after extensive decay of gymnosperm woods by *G. trabeum* (Eriksson et al., 1990; Worrall et al., 1997).

i. Klason lignin and sugar analyses

Table 1 shows the quantitative percentages of each cell wall component for both the brown-rot and control spruce. Cellulose, which is known to be the primary target of the fungus, has been metabolized extensively (45% → 19% of the cell wall), as have the hemicelluloses. Expectedly, the lignin content has been highly concentrated (28% → 65% of the cell wall).

ii. Methoxyl analysis

Researchers have shown that *Gloeophyllum trabeum* has the capability of demethoxylating the aromatic ring of guaiacyl lignin. To better understand what has been modified with the spruce cell wall after brown-rot decay, we quantified the lignin methoxyls utilizing the Zeisel-Vieböck-Schwappach method. The results shown in Table 2 are frequencies of methoxyls based on total lignin and based on the weight of dry wood. Since equal weights of wood samples were analyzed for both brown-rot and control spruce, the increase in methoxyl content for the brown-rot sample was expected. Thus, taking into account the increased concentration of lignin in the brown-rot sample, the frequency of methoxyls based on total lignin is a more accurate interpretation, showing that approximately 1/3 of the lignin methoxyls have been removed by the fungus in the course of decay of spruce in this study. This methoxyl content corresponds fairly closely to data from previous researchers studying *G. trabeum* degradation of softwoods after 60-70% wt. loss (Kirk, 1975).

iii. Derivatization Followed by Reductive Cleavage analysis

To determine how *G. trabeum* alters the lignin in wood, we utilized a wet chemical procedure that is generally called the DFRC method. DFRC analysis cleaves the predominating arylglycerol- β -aryl ether linkages of lignin in high yield to give 4-acetoxycinnamyl acetates that are then quantified by gas chromatography (Lu and Ralph, 1997a, b). The results showed that *G. trabeum* reduced the assayable arylglycerol- β -aryl ether content of the residual aromatic polymer to 18% of its original value on a Klason lignin plus acid-soluble lignin basis, and decreased it to 28% of its original value on a methoxyl basis (Table 1). It does not necessarily follow that the lignin was degraded, because nondegradative free radical coupling reactions between phenolic lignin subunits, which are likely in the highly oxidizing environment of brown rot, would also reduce the 4-acetoxycinnamyl acetate yield upon subsequent DFRC analysis. However, the DFRC results make it clear that the aromatic polymer remaining after extensive brown rot by *G. trabeum* bears little resemblance to native lignin.

iv. Cell wall dissolution and acetylation

To identify some of the structural changes that had occurred in the brown-rotted lignin, we next applied a recently developed solubilization method that has been used successfully on native lignocellulose samples. In this procedure, the samples are ball-milled, completely dissolved in an NMI/DMSO mixture, acetylated, precipitated in H₂O, and finally redissolved in a suitable solvent (Lu and Ralph, 2003). We found that this approach was also applicable to extensively brown-rotted wood, which dissolved completely in DMSO after acetylation to give a nearly black solution (Fig. 2). Unlike earlier approaches that involved extracting a small proportion of the total lignin from biodegraded lignocellulose with organic solvents (Chua et al.,

1982), experiments with fully dissolved samples allow solution-state spectroscopic analyses to be done without concerns that the sample may have been selectively fractionated.

v. Solution-state NMR spectroscopy

We analyzed the biodegraded wood and an undegraded control sample by ^1H - ^{13}C HSQC NMR spectroscopy (Fig. 3). The advantage of an HSQC spectrum is that it correlates the chemical shift of every hydrogen atom in a molecule with the chemical shift of the carbon it is attached to, thus providing excellent peak resolution in two dimensions and increased confidence in signal assignments. We have set the contour intensities of the aromatic signals in the two spectra to approximately equal volumes so that signals from other cell wall components can be compared visually. In the control spruce spectrum (1a), most cell wall components are fairly well dispersed, depicting the guaiacyl lignin aromatic region (G), the dominant lignin side-chain units (β -aryl ether (A), phenylcoumaran (B), and pinoresinol (C), lignin methoxyls, the anomeric region (orange) of the polysaccharides cellulose and hemicelluloses, and aliphatic region (black) of polysaccharides. The cellulose correlations (not labeled) are the dominant peaks in the spectrum: H-1/C-1 (4.67, 102.3), H-2/C-2 (4.53, 74.3), H-3/C-3 (5.07, 75.1), H-4/C-4 (3.66, 79.1), H-5/C-5 (3.82, 74.5), and H-6/C-6 (3.99, 4.22, 65.2). Looking at the brown-rot spruce spectrum (1b), again most cell wall components are well dispersed. However, distinct changes are evident in this spectrum, most predominant of which is the dramatic decrease in the lignin side-chain units (e.g., β -aryl ethers, phenylcoumaran, and pinoresinol).

As expected, the results show that polysaccharide signals were depleted relative to aromatic ones in the brown-rotted sample. However, the salient feature in the brown-rotted spectrum is the marked, nonselective loss of lignin sidechain signals relative to aromatic and methoxyl signals.

For example, a quantitative comparison of the NMR signals for C_α in arylglycerol- β -aryl ether structures (structure A in Fig. 3) shows that, on a methoxyl basis, this predominant structure of lignin was depleted to 29% of its original value after brown rot (Table 3). This value agrees remarkably well with the value of 28% obtained from our wet chemical analyses (Table 1).

Table 4 shows contour integrations of both the control and brown-rot whole cell wall HSQC spectra and compare the relative frequencies of each major lignin side-chain at their alpha position. The integrations are all calculated relative to their respective methoxyl integral. We noticed some interesting trends within samples. The ratios of A:OMe and ABC:OMe have decreased. This suggests that, even though the methoxyl content has decreased 1/3, the β -aryl ether, phenylcoumaran, and pinoresinol were cleaved at a much higher frequency. What is quite interesting is that the percentages of A, B, and C in both control and brown-rot cell wall samples are 70%, 21%, and 9%, respectively. This shows that even though the spruce lignin side-chains were degraded quite heavily by *G. trabeum*, there was no preference for cleavage of one linkage over another. Two possible explanations may exist: 1. The fungus has penetrated all areas of the cell wall, allowing the hydroxyl radicals from Fenton chemistry to degrade the components in a non-selective manner, and 2. The fungus penetrates the cell wall to a certain degree, leaving some of the cell wall, and its lignin, intact and undegraded. Another interesting note from spectra is the strong presence of cellulose in the degraded spruce, thus giving more support to explanation 2 above. Most hemicelluloses have been removed by the fungus, as noted by the absence of much aliphatic and anomeric polysaccharides.

The aromatic region of the brown-rot spectra does show some changes in its structure, as noted by the variation in contour chemical shifts. A technique known as spectrum subtraction

may was used to get a better picture what chemical shifts changed between the control and brown-rot spectra. This subtraction spectrum is shown in Figure 2. This spectrum shows that *G. trabeum* has modified the aromatic region of the lignin in a fairly extensive manner, creating three new large contours. The exact nature of these new chemical shifts are not understood. It is quite possible that there are new condensed structures present in the degraded spruce, thus altering the shielding on C-2/H-2, C-5/H-5, and C-6/H-6. However, our efforts to assign these new contours from several lignin model compounds have not yet come to fruition.

The decreased amplitude of lignin sidechain NMR signals after brown rot cannot have resulted simply from oxidation of the sidechains without cleavage, because most of the resulting signals would have predictable chemical shifts, yet cannot be found in the spectrum. For example, the HSQC H_β - C_β cross-peak near 5.8, 80 ppm expected after benzylic oxidation of arylglycerol- β -aryl ether structures to give ketones at C_α is undetectable (Ralph et al., 2004). The result also cannot be due to crosslinking of the aromatic rings via new linkages, because these reactions would have a negligible effect on the sidechain chemical shifts (Ralph et al., 2004). Therefore, it appears that lignin sidechains must be degraded during brown rot. Moreover, they are removed from the lignin even more rapidly than the methoxyls are.

New, oxidatively cleaved sidechain structures that might take the place of those depleted during brown rot are not evident in our spectrum. For example, the HSQC H_β - C_β cross-peak near 5.4, 72 ppm expected for acetylated phenylglycerols appears to be absent (Ralph et al., 2004), even though phenylglycerols are likely products from the attack of $\bullet OH$ at the aromatic 4-position of arylglycerol- β -aryl ether structures (Fig. 1). The only new structures clearly apparent in the HSQC spectrum after brown rot are in the aromatic region, where shifts to higher ppm along the ^1H coordinate have occurred (Fig. 3b). These signals may reflect additional acetoxyl

substituents on the aromatic rings (Ralph et al., 2004), which would be consistent with the earlier observation that brown-rotted lignin has an increased phenolic content (Kirk, 1975).

The disappearance of lignin sidechains during decay by *G. trabeum* must be reconciled with the facts that brown-rotted lignin retains most of its aromatic residues and is still polymeric (Kirk, 1975; Agosin et al., 1989; Jin et al., 1990). We surmise that the lignin was first depolymerized to permit penetration by polysaccharide hydrolases, but later reconnected via new linkages formed via radical coupling of the phenolic units that become enriched during attack on the aromatic rings by $\bullet\text{OH}$. As these coupling reactions occur between the 5-positions of two lignin rings or between the 5-position of one ring and the phenoxy oxygen of another ring, the resulting products would include biphenyls and diphenyl ethers. Our NMR experiment could not have detected these products, because their linkages contain no hydrogen atoms and consequently are invisible in HSQC spectra.

In addition, the lack of discernible new sidechain structures in our HSQC spectrum of decayed wood probably reflects the fact that $\bullet\text{OH}$ exhibits very little regioselectivity in reactions with aromatic compounds (Walling, 1975; Gierer et al., 1992). As a result, the fungus probably oxidized many of the lignin substructures at multiple sites to give a large number of products so dispersed across the spectrum that they fail to stand out above the baseline noise. During this study, we considered the possibility that an examination of less extensively decayed wood specimens would circumvent this problem, but the results showed that new sidechain structures were also undetectable after 2-3 weeks and 7-10% weight loss (data not shown). One solution to this problem in future work might be to supplement the wood beforehand with a synthetic lignin enriched at specific positions with ^{13}C , which would amplify potentially interesting signals in the NMR spectra (Shary et al., 2007).

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We thank M. W. Davis and F. J. Matt for the chemical analyses in Table 2, and C. J. Houtman for helpful discussions. This work was supported by U.S. Dept. of Energy grant DE-AI02-07ER64480 to K.E.H. and J.R., and made use of a 750 MHz instrument at the National Magnetic Resonance Facility at the University of Wisconsin-Madison.

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Table 1. Methoxyl and uncondensed arylglycerol- β -aryl ether content in sound and decayed spruce wood

Sample	Methoxyls	Arylglycerol- β -aryl ethers by DFRC analysis	
	g/g lignin ^a	g/g lignin ^a	g/g methoxyls
Sound wood	0.168	0.111	0.661
Decayed wood	0.107	0.020	0.187

^aKlason lignin plus acid-soluble lignin

Table 2. Chemical composition of sound and decayed spruce wood samples

Sample	Klason lignin	ASL ^a	Glucose	Mannose	Xylose	Galactose	Arabinose	Rhamnose
Sound wood	mg							
per g sound wood	273	7	451	108	60	13	11	1
Decayed wood	mg							
per g sound wood	215	20	69	8	6	3	1	nd ^b
per g decayed wood	591	54	191	22	16	8	2	nd ^b

^aAcid-soluble lignin^bnd: not detected

Table 3. Relative integrals of contours in HSQC spectra of acetylated, solubilized spruce wood

Lignin Structure ^a	Sound wood	Decayed wood
	Integral (rel)	Integral (rel)
OMe	1.00	1.00
A α	0.079 (69)	0.023 (70)
B α	0.024 (21)	0.007 (21)
C α	0.011 (10)	0.003 (9)

^aSee Fig. 3 for abbreviations used for structures. Numbers in parentheses show what percent of the sum of the integrals for structures A α , B α , and C α is accounted for by the integral for each individual structure.

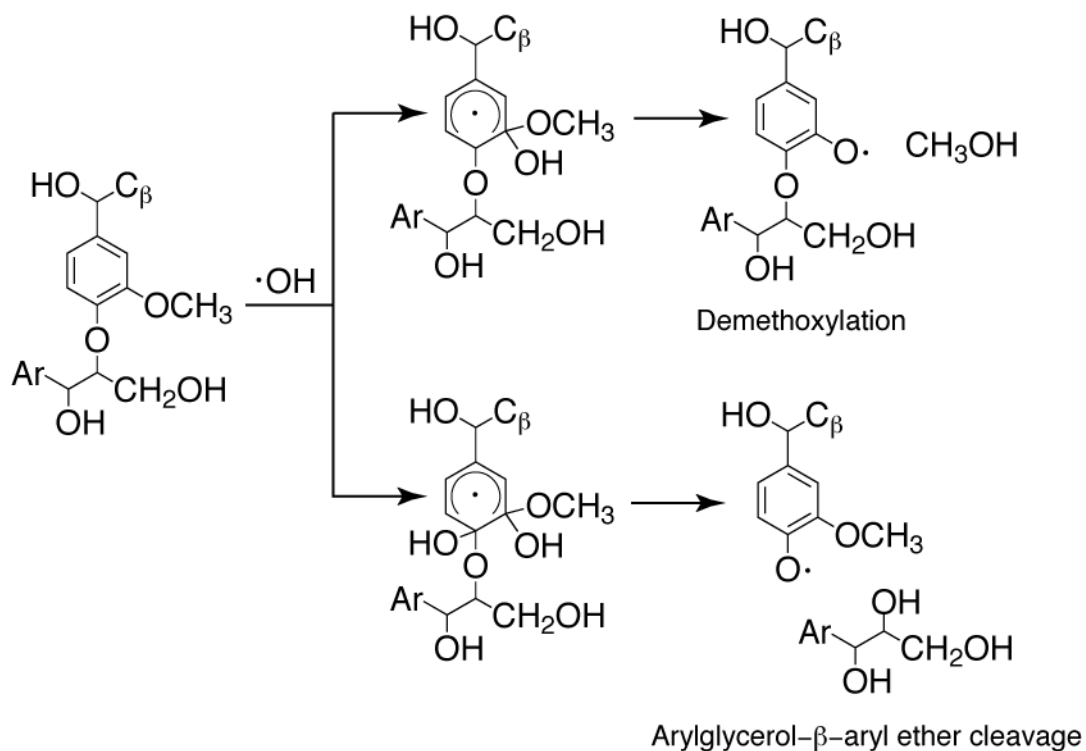


Fig. 1. Two expected reactions of $\cdot\text{OH}$ with the predominant arylglycerol- β -aryl ether structure of lignin (structure A in Fig. 3). The upper pathway shows attack at the aromatic 3-position to eliminate methanol, and the lower pathway shows attack at the 4-position to eliminate a phenylglycerol. Ar: aryl.

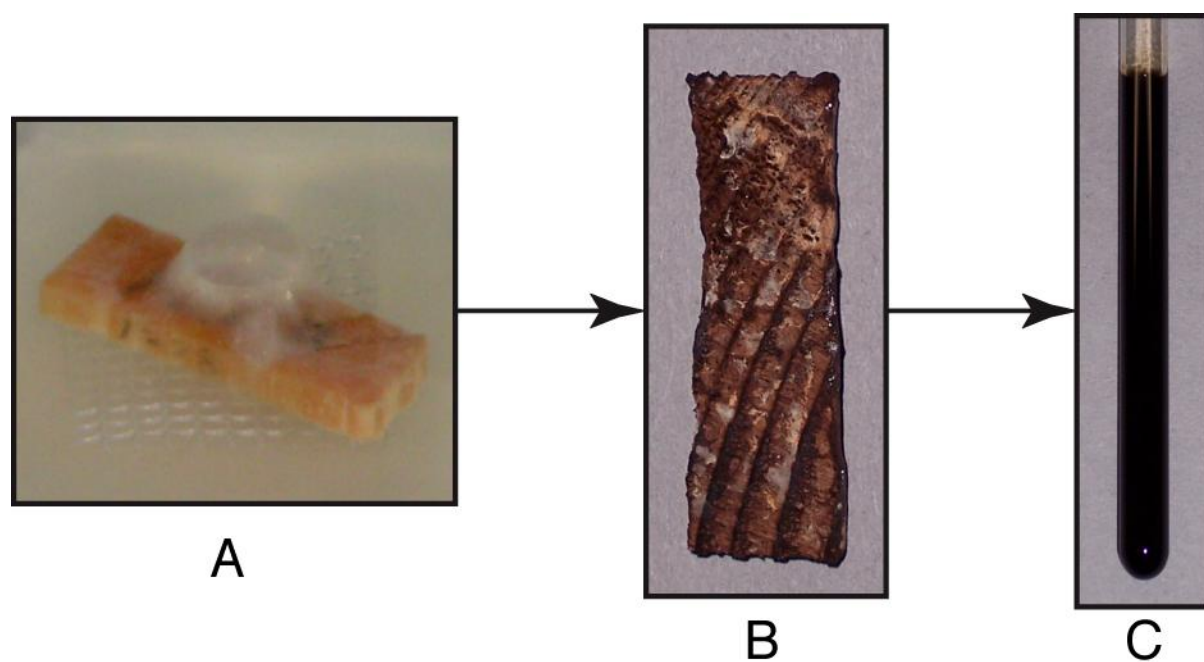


Fig. 2. Summary of the steps prior to NMR analysis, showing (A) *G. trabeum* growing on a spruce wafer one week after inoculation, (B) appearance of a typical spruce wafer 16 weeks after inoculation, and (C) the decayed wood sample after milling, acetylation, and complete dissolution in $\text{DMSO-}d_6$.

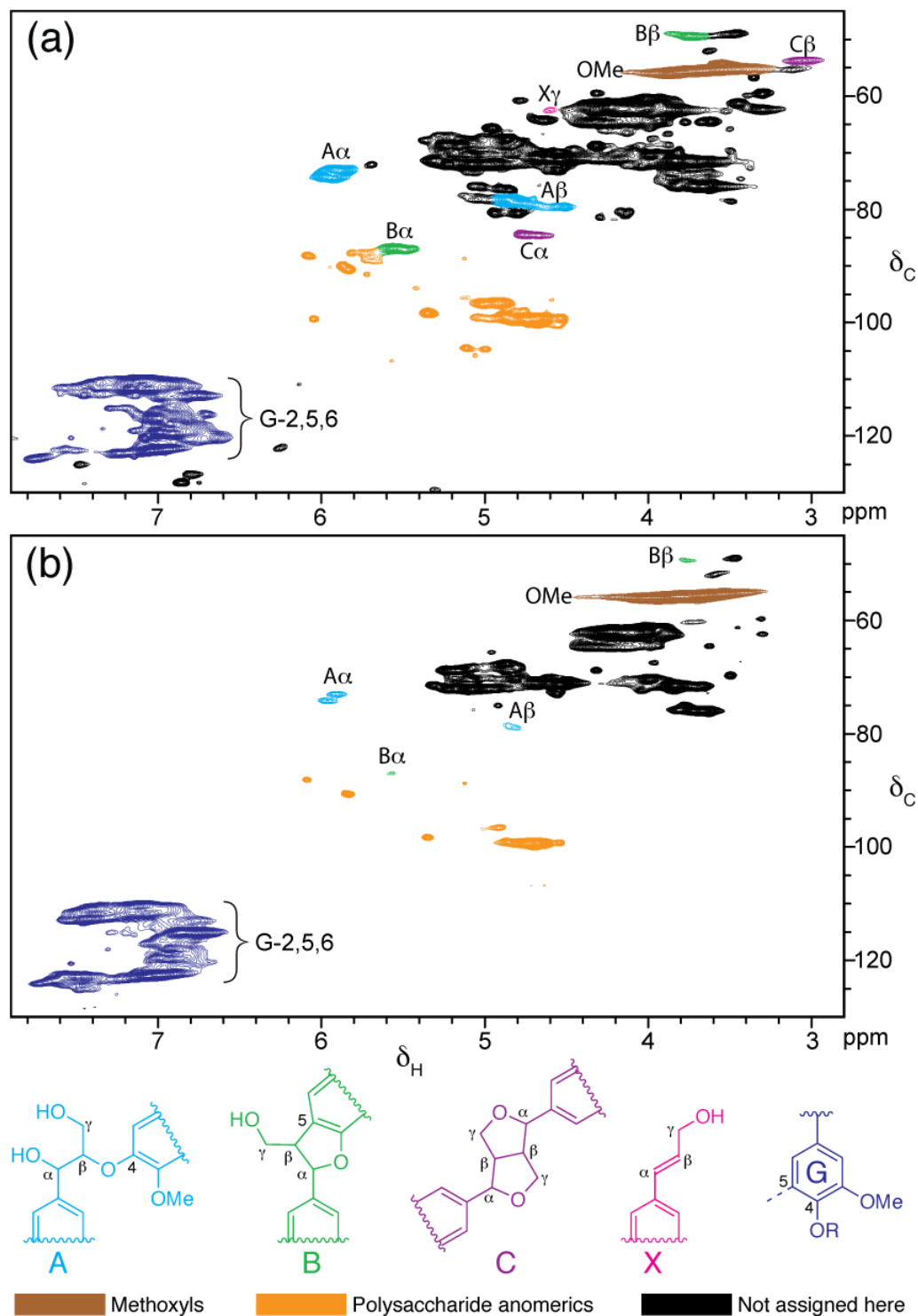


Fig. 3. HSQC spectra of acetylated sound spruce wood (a) and acetylated, brown-rotted spruce wood (b) in DMSO- d_6 . Most of the unassigned structures are attributable to non-anomeric carbon-hydrogen bonds in polysaccharides. OMe: methoxyl.

Appendix C. 2D solution-state NMR of non-derivatized aspen and kenaf cell walls.

Two-dimensional NMR experiments, using gradient-HSQC one-bond ^{13}C – ^1H correlation spectroscopy, on non-derivatized cell wall material from a representative angiosperm *Populus tremuloides* (quaking aspen) and a herbaceous plant *Hibiscus cannabinus* (kenaf) have been acquired and analysed. Natural acetates are found on syringyl units of kenaf lignin and acylate a substantial fraction of the sidechains of lignin units¹⁻³ and hemicelluloses like glucuronoxylan contain acetyl side groups off their main chain.

Methods can be found in Chapter 2, with slight deviations described here. Ball-milled wood was prepared from *Populus tremuloides* sapwood, and *Hibiscus cannabinus* bast fiber (1 year old Tainung2 kenaf, kindly supplied by Jim Han). After the mixer mill grinding, *Hibiscus cannabinus* was the only species soxhlet extracted sequentially with water, 80% ethanol, and acetone to isolate the cell wall (essentially free of chlorophyll and other extractives).

The next paragraphs analyse the HSQC spectra, which are shown following the text. Angiosperm lignins, because of their derivation from both coniferyl and sinapyl alcohols, are known to have a higher β -aryl ether (A) content, a lower phenylcoumaran (B) content, and have an increased proportion of resinol (C) units compared to the gymnosperms which are derived (almost) solely from coniferyl alcohol. It is also quite noticeable that the kenaf has the lowest lignin content of the three, and consequently contains a proportionately larger quantity of polysaccharides, as seen by the intense anomeric correlations. The β -aryl ether units are well dispersed. For example, if a coniferyl or sinapyl alcohol monomer couples at its β -position with a guaiacyl unit, the resulting β -aryl ether linkage ($\text{A}\beta_{\text{G}}$) has its $\text{A}\beta$ correlation at 4.37/84.0 ppm.⁴ This correlation clearly separates from the correlations where a monolignol couples with a

syringyl unit, where the β -position ($A\beta_S$) appears at 4.19/86.3 ppm and 4.02/87.7 ppm.⁴

Resinol correlations ($C\alpha$, 4.64/85.3 ppm; $C\beta$, 3.05/53.9 ppm; $C\gamma_1$, 4.20/71.5 ppm)⁴ are shown to be more significant in the aspen and kenaf than in pine. Spirodienone (β -1) structures were detected in the aspen spectra; however, because of the low quantity of such linkages in lignin (about 1.8% in aspen lignin),⁵ the spectrum showed these only at low contour intensity.

Spirodienone correlations $S\alpha$, $S\beta$, and $S\beta'$ (not shown) were found at 5.14/81.4 ppm, 2.81/60.3 ppm, and 4.18/79.8 ppm.^{5,6}

Some interesting differences between the aspen and the kenaf spectra can be seen. In the aspen spectra, it is evident that the syringyl:guaiacyl ratio is much lower than with the kenaf; the pine displays exclusively guaiacyl units. With this dissolution method, there is the capability of estimating the syringyl:guaiacyl (S:G) ratio in the aromatic region of the lignin. Notice the syringyl (S) and guaiacyl (G) correlations of the aromatic C-H groups in four basic conglomerates: S-2/6, G-2, G-5/6, and G-5. Previous literature suggested that the S:G ratio in kenaf and aspen lignin to be between 4.3-7.8 and 1.6-2.2, respectively.⁷⁻⁹ Through volume integration of the S (S-2/6) and G (G-2) contours of the kenaf and aspen cell wall HSQC spectra, we determined the ratios to be 5.6 and 2.3, respectively, which falls into the expected ranges. The syringyl correlations shown are from β -aryl ether (A) and resinol (C) lignin units, while the guaiacyl correlations are from β -aryl ether (A) phenylcoumaran (B) and resinol (C) lignin units. The correlation at 7.07/120.5 ppm is from the residually protonated H-5/C-5 of N-methylimidazole solvent which, even though greatly suppressed, is still visible in the aromatic region.

An interesting feature of kenaf lignin is its extensive acetylation of syringyl units with all the acetate on the γ -position of the β -aryl ether side-chain. Thus, it appears that approximately two-

thirds of the β -aryl ether units are acetylated.^{1,10} The $A\gamma$ -acetate contours, undoubtedly present in the kenaf spectrum, are unfortunately found in a region of the spectrum (4.15-4.45, 63.2 ppm) highly overlapping with polysaccharide contours. We cannot, using these spectra, assign any clear correlation to these acetylated $A\gamma$'s; however, the extended contour in this region suggests its existence. Logically, if the γ -OH is acetylated, an $A\beta$ correlation around 4.62/84.6 ppm should be present (compound 3071 in the NMR database⁴). In the syringyl $A\beta$ region for aspen and kenaf, there are two contours, 4.19/86.3 ppm and 4.02/87.7 ppm, as discussed previously. Aspen shows some guaiacyl $A\beta$ correlation (4.37/84.0 ppm); however in the kenaf spectrum a strong correlation exists in a similar region where a guaiacyl $A\beta$ typically resides (4.41/83.4 ppm). As noted in Figure 2.6f, kenaf contains few guaiacyl units. Hence, this strong correlation is consistent with the $A\beta$ on γ -acetylated β -aryl ether units, e.g., likely evidence of the $A\gamma$ acetate. Thus, the kenaf $A\beta$ at 4.41/83.4 ppm has been tentatively labeled $A\beta_{\gamma Ac}$.

We integrated the natural acetate-CH₃ contour regions in all spectra and compared them to those of the methoxyl correlations. The values obtained show that for pine, aspen, and kenaf, the ratio of methoxyl to acetate was 1:0.1, 1:0.3, and 1:0.6, respectively. Knowing that the syringyl (β -aryl ether type) units of kenaf are ~60% acetylated,¹⁰ and that syringyl units predominate the lignin portion, a 1:0.6 ratio of methoxyl:acetate is a reasonable estimate and an indication that a large portion of the acetates are on lignins. Furthermore, a distinctive difference between the kenaf acetate contour compared to those in aspen and pine is an extended contour region at 1.75/20.3 ppm. Aspen syringyl units are only ~4% acetylated;^{10,11} acetylated xylans are the largest contributors to the acetate content. The major component of angiosperm hemicelluloses is

an *O*-acetyl-4-*O*-methylglucurono- β -D-xylan (~16% of aspen and ~13% of kenaf cell wall),¹² where about 7 out of 10 xylose residues contain an *O*-acetyl group at C-2 or C-3.¹³ The 2-acetylated xylan (2-*O*-Ac- β -D-Xylp) and the 3-acetylated xylan (3-*O*-Ac- β -D-Xylp) H-2/C-2 and H-3/C-3 correlations (shown in Figure 4b and 4c) are depicted at 4.56/73.8 ppm and 4.85/75.2 ppm, respectively, in aspen and kenaf.^{14,15} The relative ratio of 3-*O*-Ac- β -D-Xylp : 2-*O*-Ac- β -D-Xylp was estimated from contour integration to be 1:1.12 in aspen and 1:1.29 in kenaf. Previous literature on an isolated 4-*O*-methylglucuronoxylan in aspen shows that the 2-*O*-acetylated xylose units are approximately one-third less abundant than the 3-*O*-acetylated xylose units.¹⁴ However, *O*-acetyl migration during isolation methodologies makes this estimation difficult to ascertain.¹⁶ For the angiosperms, glucomannans are a secondary hemicellulose component, comprising ~2% of aspen and kenaf hemicelluloses.¹² The C-2 and C-3 positions of the mannan chain are partially substituted with *O*-acetyl groups at a frequency of about 1 out of 3-4 hexose units.¹³

In the anomeric region the kenaf spectra show anomeric correlations of a terminal β -D-galactopyranosyl (β -D-Galp^T), (1 $\rightarrow$ 6)-linked to β -D-Manp, at 4.32/105.7 ppm,^{17,18} which is absent in aspen. Acetylated xylan structures show their respective anomeric correlations at 4.53/99.8 ppm (2-*O*-Ac- β -D-Xylp) and 4.44/102.0 ppm (3-*O*-Ac- β -D-Xylp).^{14,15} The 4-*O*-MeGlcA in the aspen and kenaf, (1 $\rightarrow$ 2)-linked in glucuronoxylan depict their anomeric correlations at 5.20/97.7 ppm and 5.25/97.7 ppm.¹⁴ This variation in δ_H may be caused by the presence of the carboxylic acid; chemical shift changes in the DMSO-*d*₆/NMI-*d*₆ solvents would be expected. For aspen, the reducing α and β ends of D-Xylp residues are shown at 4.98/94.2 ppm and 4.33/97.8 ppm.¹⁴ In the kenaf spectrum, only the reducing β end of D-Xylp is observed.

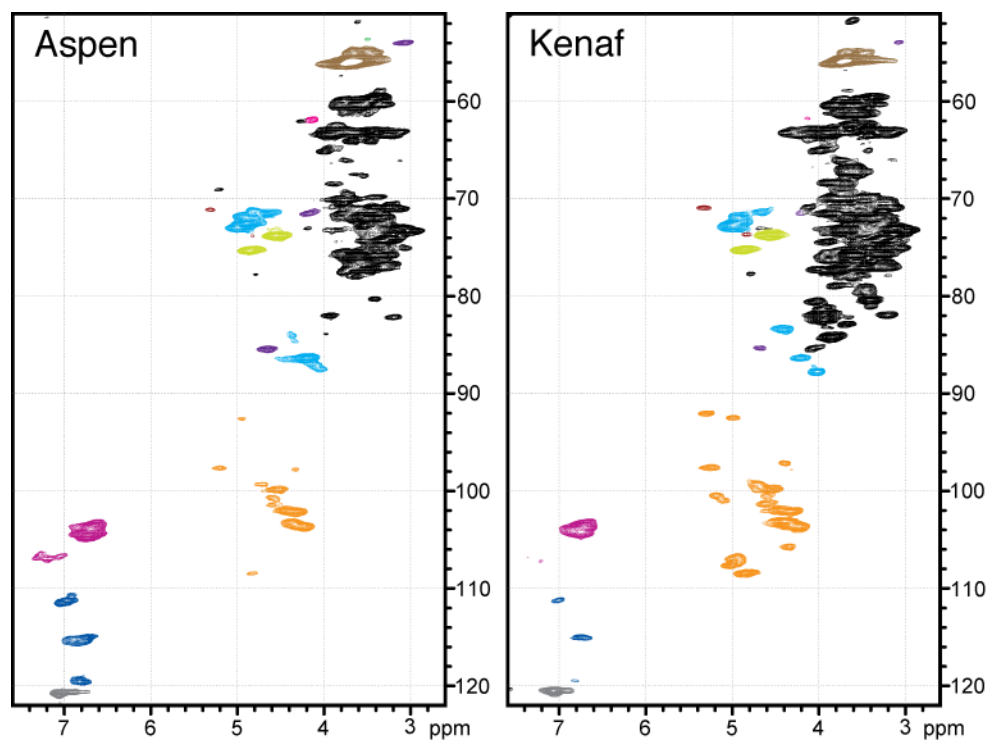
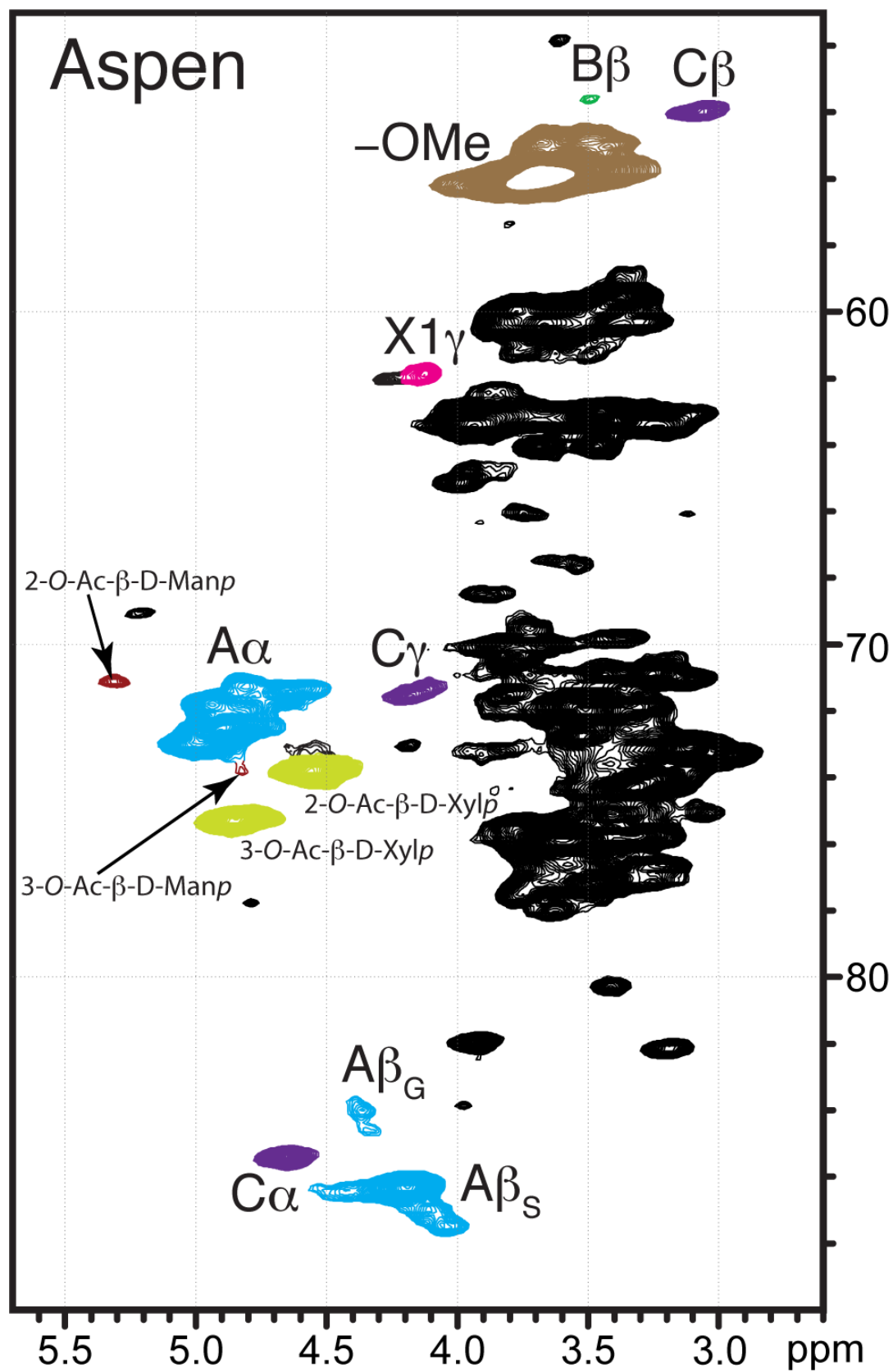
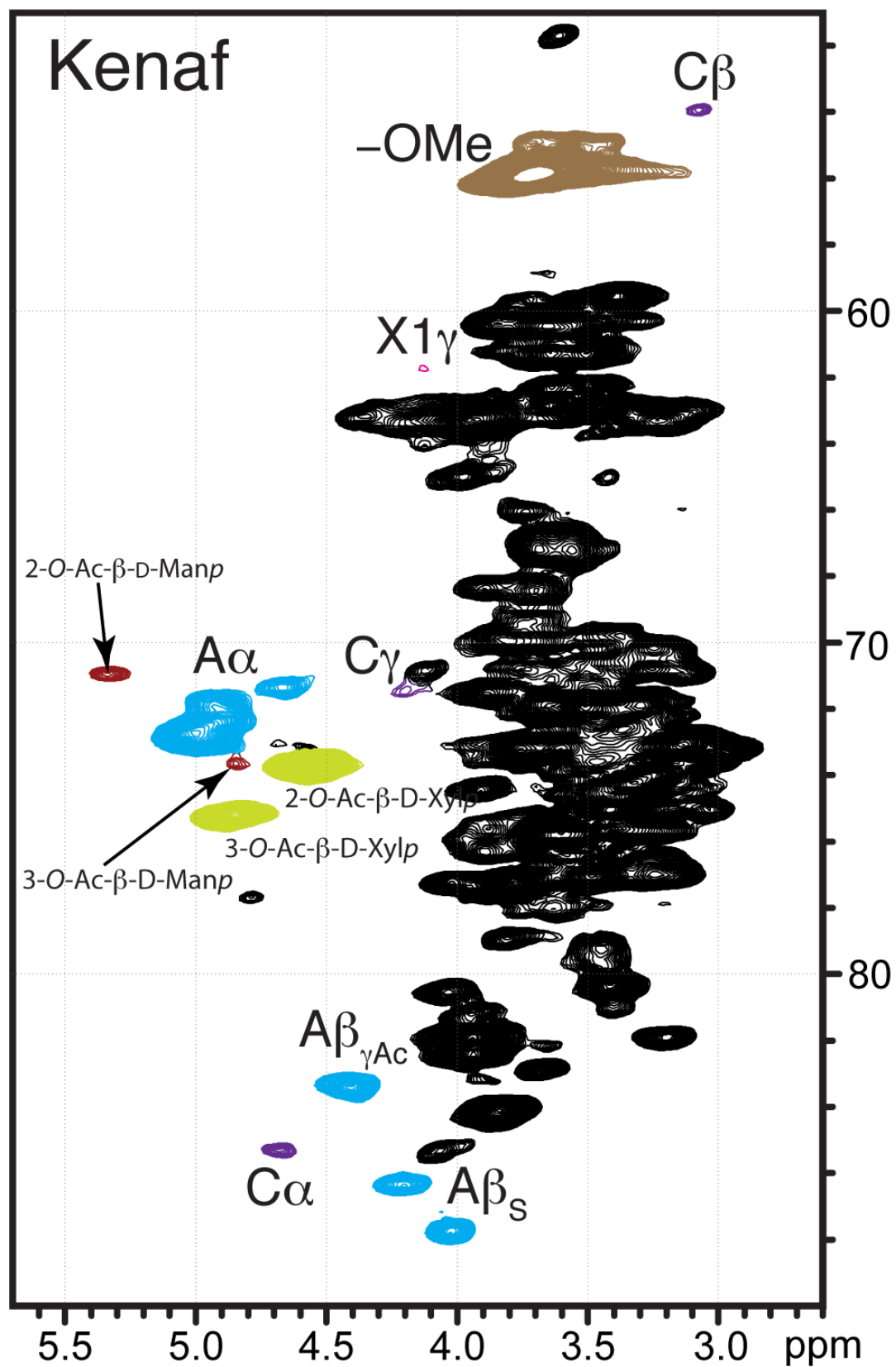
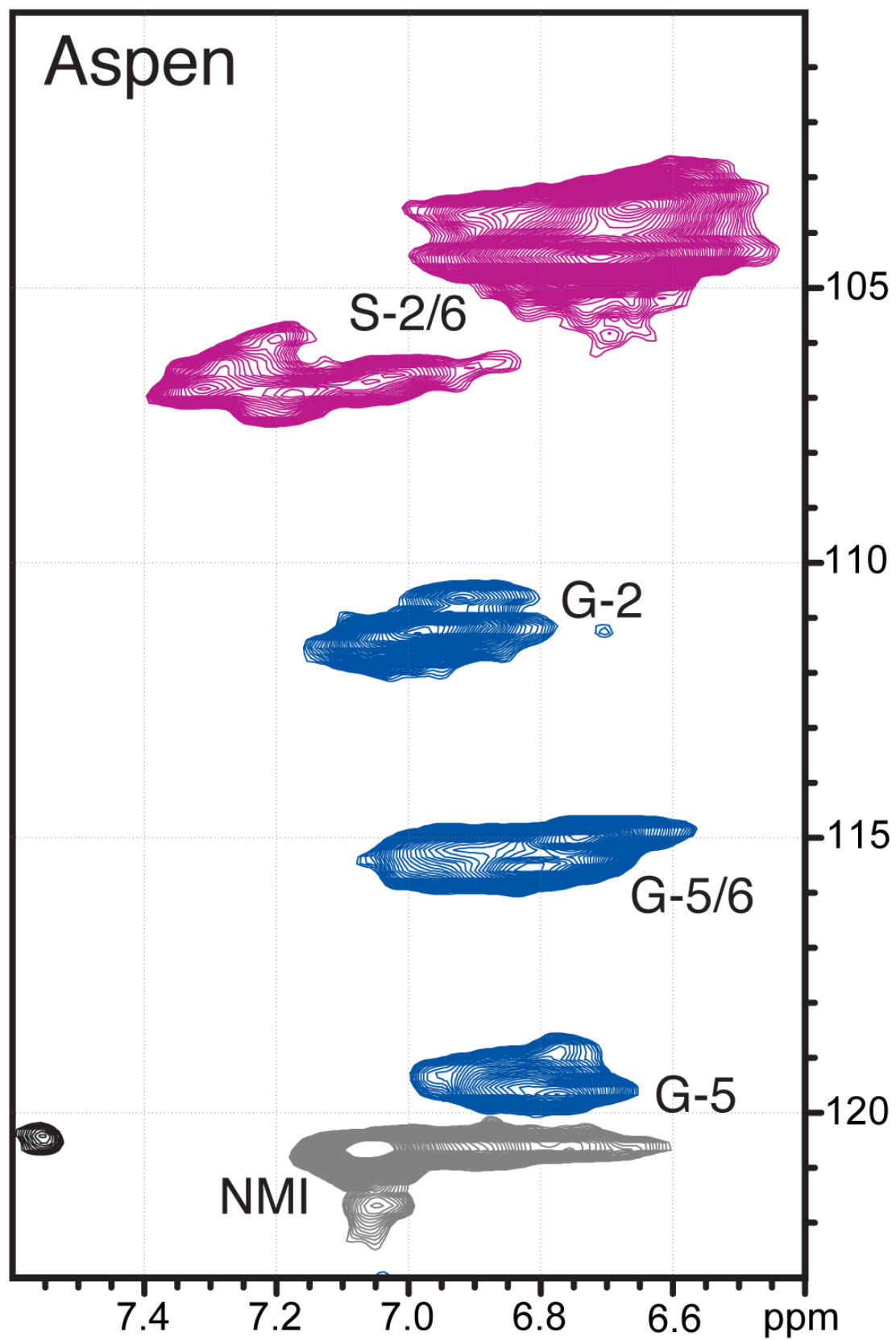


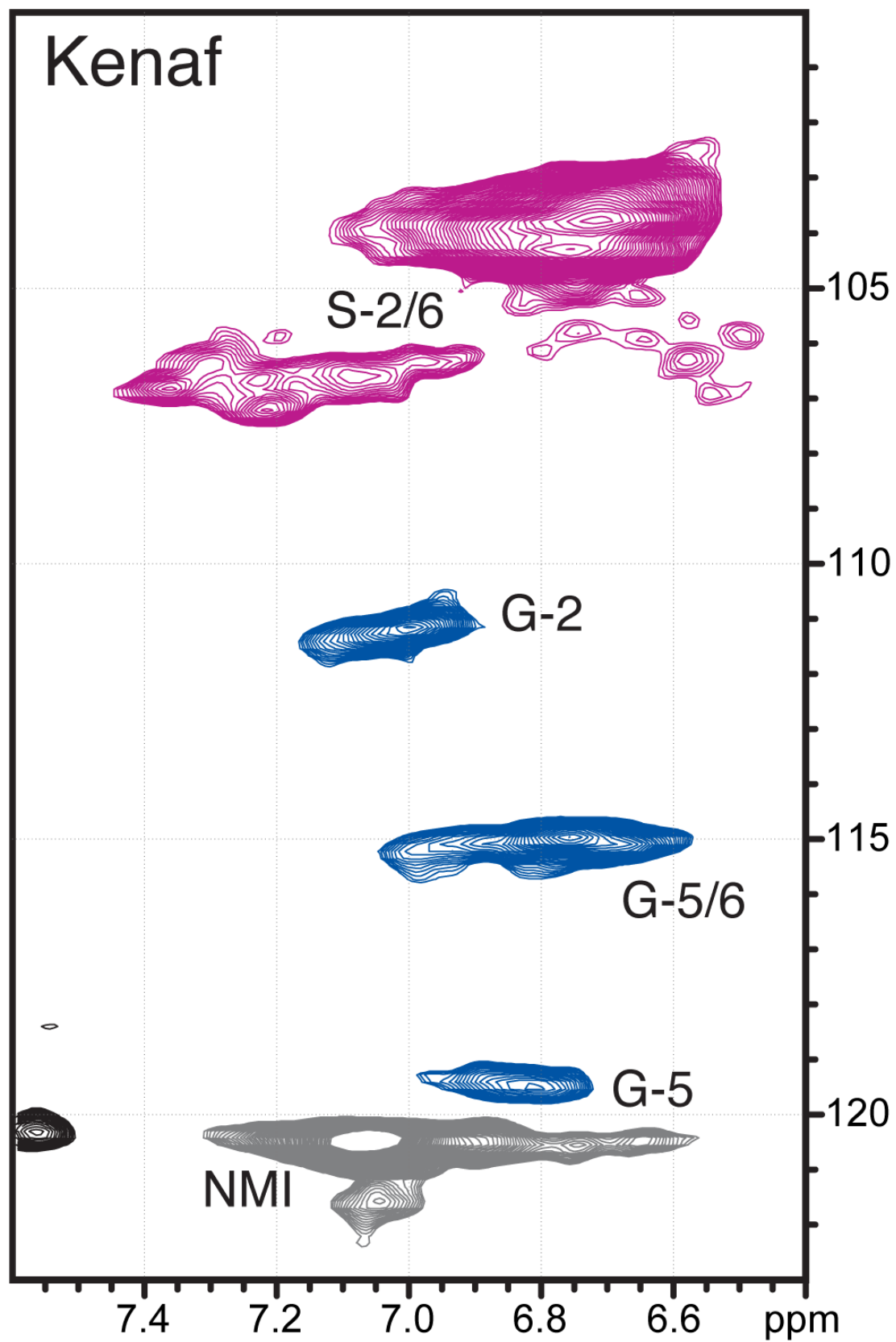
Figure C.1. HSQC spectra of aspen and kenaf cell walls.

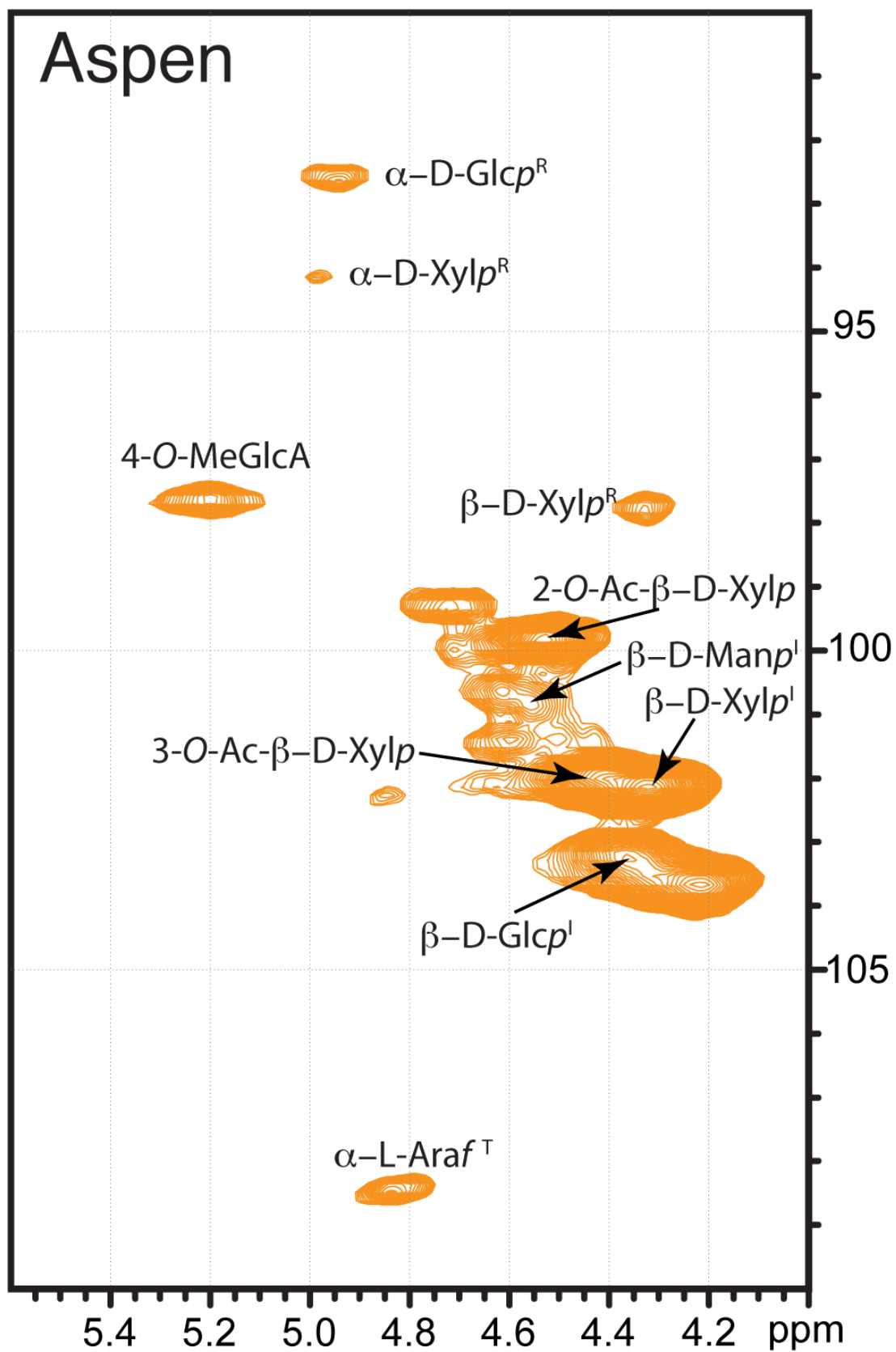
The following figures are HSQC spectra acquired identically to that of loblolly pine in Chapter 2 covering the aliphatic region, lignin aromatic region, and polysaccharide anomeric region, respectively.

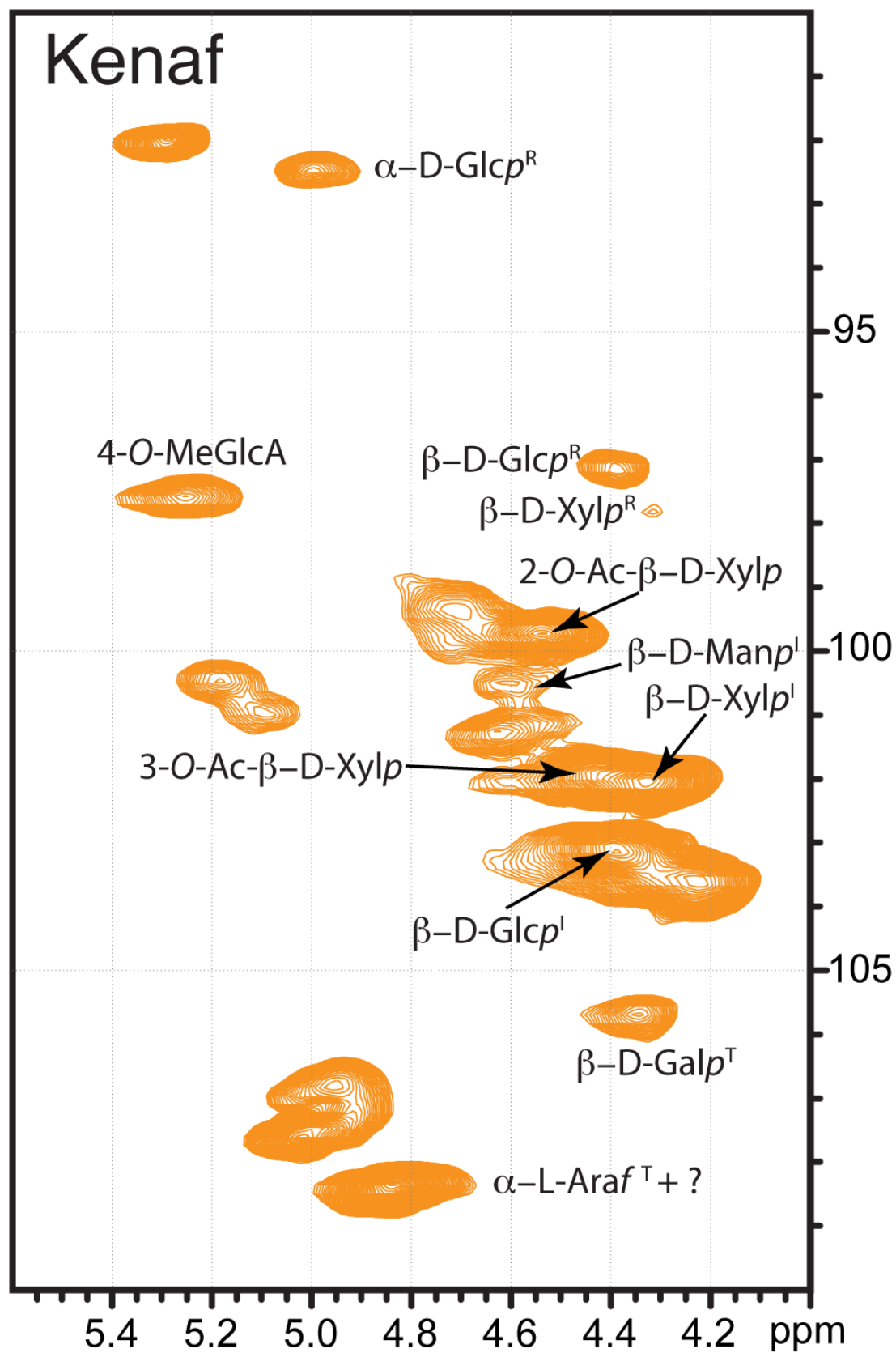












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