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# Structural Transformation of Isolated Poplar and Switchgrass Lignins during Dilute Acid Treatment

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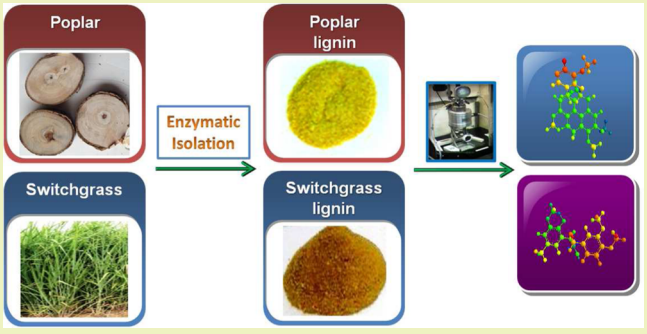
Supporting Information

**ABSTRACT:** A key step in conversion of cellulosic biomass into sustainable fuels and chemicals is thermochemical pretreatment to reduce plant cell wall recalcitrance. Obtaining an improved understanding of the fundamental chemistry of lignin, the most recalcitrant component of biomass, during pretreatment is critical to the continued development of renewable biofuel production. To examine the intrinsic chemistry of lignin during dilute acid pretreatment (DAP), lignin was isolated from poplar and switchgrass using a cellulolytic enzyme system and then treated under DAP conditions. Our results highlight that lignin is subjected to depolymerization reactions within the first 2 min of dilute acid pretreatment and these changes are accompanied by increased generation of aliphatic and phenolic hydroxyl groups of lignin. This is followed by a competing set of depolymerization and repolymerization reactions that lead to a decrease in the content of guaiacyl lignin units and an increase in condensed lignin units as the reaction residence time is extended beyond 5 min. A detailed comparison of changes in functional groups and molecular weights of cellulolytic enzyme lignins demonstrated different structural parameters, related to the recalcitrant properties of lignin, are altered during DAP conditions.




**KEYWORDS:** *Poplar, Switchgrass, Dilute acid pretreatment, Cellulolytic enzyme lignin, Reaction mechanism*

## INTRODUCTION

Lignocellulosic biomass is one of the most abundant, sustainable resources for biofuels, biochemicals, and biobased products that not only meet the growing global demand for green energy and materials but also decrease net greenhouse gas emissionss.[1] To fully realize this potential, an in-depth understanding of lignocellulosic recalcitrance, which refers to plant cell walls' inherent resistance to deconstruction from microbes and enzymes, needs to be advanced. Lignin is considered as one of the most recalcitrant components in the plant cell wall[2] because of its structure, content, distribution, and associations with plant polysaccharides within the cell wall.[3] Lignin is derived from hydroxycinnamyl monomers with various degrees of methoxylation forming a racemic, cross-linked, and highly heterogeneous aromatic macromolecule.[4] Lignin and hemicellulose are embedded between and around cellulose microfibrils, providing rigidity and structural support to plant cell walls, which evolved over millions of years to resist biological and chemical attack. The intrinsic structural complexity and correlated recalcitrance has become the major barrier in biomass deconstruction and conversion-to-biofuel biorefinery processes.[2c]

To date, biochemical conversion of lignocellulosic biomass to bioethanol primarily involves three essential steps: pretreatment to reduce the inherent plant cell wall recalcitrance, enzymatic hydrolysis to deconstruct polysaccharides into fermentable sugars, and fermentation to convert those sugars into ethanol. The goal of pretreatment is to modify physiochemical features of the plant cell wall so that the resulting biomass is more amenable to enzymatic deconstruction. Dilute acid pretreatment (DAP) has been recognized as a cost-effective pretreatment technology that can enhance biomass sugar yield.[5] The important role of lignin morphological and structural change after DAP in enzymatic hydrolysis has been the focus of several studies but is still under considerable debate. DAP pretreatment research studies have revealed that lignin tends to coalesce into aggregates

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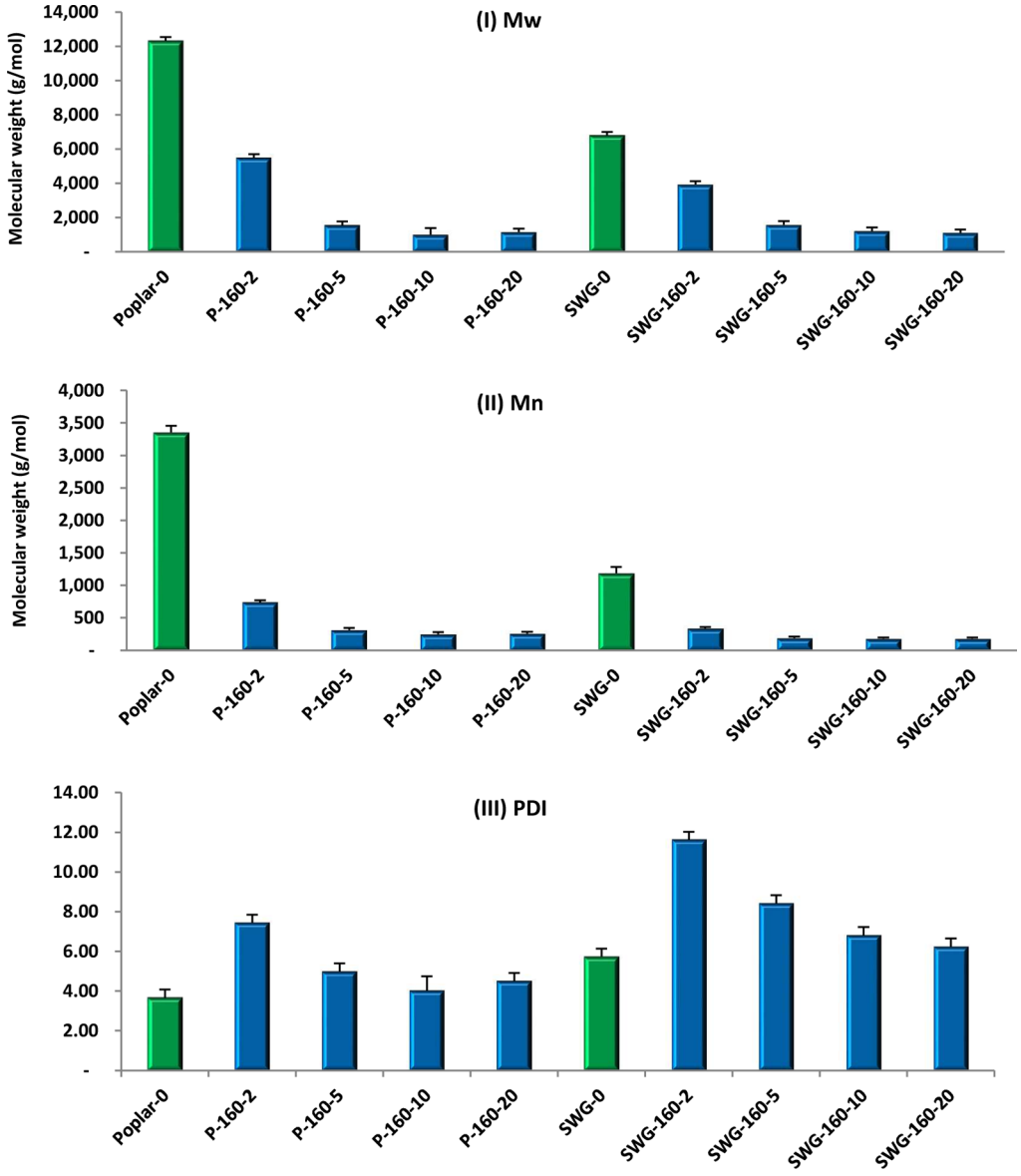


**Figure 1.** Molecular weight change of cellulolytic enzyme lignin (determined as acetate) after DAP at various residence time. (I) Weight-average molar mass ($M_w$), (II) number-average molar mass ($M_n$), and (III) polydispersity index (PDI).

accompanying their relocalization as "droplets" on the surface of biomass cell walls as the reaction temperatures are above the glass transition point of lignin. It is proposed to open up the cell wall matrix structure and thereby improve cellulose accessibility for enzymes.[6] Recent studies have also revealed that the efficiency of downstream enzyme hydrolysis is impeded due to the blockage of the cellulose surface layer by lignin droplets, prevention of enzymes accessibility to inner layers,[7] and the formation of cellulase–lignin interactions to deactivate the enzymes.[8] As a result, no clear picture has been given to date about lignin chemistry and how these structural changes limit efficient sugar release.

More recent work, which investigated the fate of cellulolytic enzyme lignin under autohydrolysis pretreatment conditions, reported a drastic decrease in the molecular weight,[9] which is different from research investigating the changes in structural characteristics of lignin isolated from pretreated biomass.[10] The difference in the fate of lignin during pretreatments is attributed to the partial protection of lignin by polysaccharides in the biomass matrix.[9] This implies that lignin structural parameters are relevant to plant cell wall recalcitrance and how those parameters individually and synergistically affect enzymatic saccharification are vital for improving current bioconversion process.

To provide fundamental information on lignin chemistry under dilute acid pretreatment at high severity with the least influence of plant cell wall polysaccharides, cellulolytic enzyme lignin isolated from poplar and switchgrass were pretreated with 0.1 M $H_2SO_4$ at 160 °C for 0–20 min residence time and structurally analyzed by two-dimensional (2D) $^{13}C$-$^{1}H$ heteronuclear single quantum coherence (HSQC) nuclear magnetic resonance (NMR) spectroscopy, phosphitylation followed by $^{31}P$ NMR and gel permeation chromatography (GPC) analysis.

## MATERIALS AND METHODS

**Enzymatic Isolation of Lignin.** Poplar used in this study was provided by Oak Ridge National Laboratory, Oak Ridge, TN 37831,




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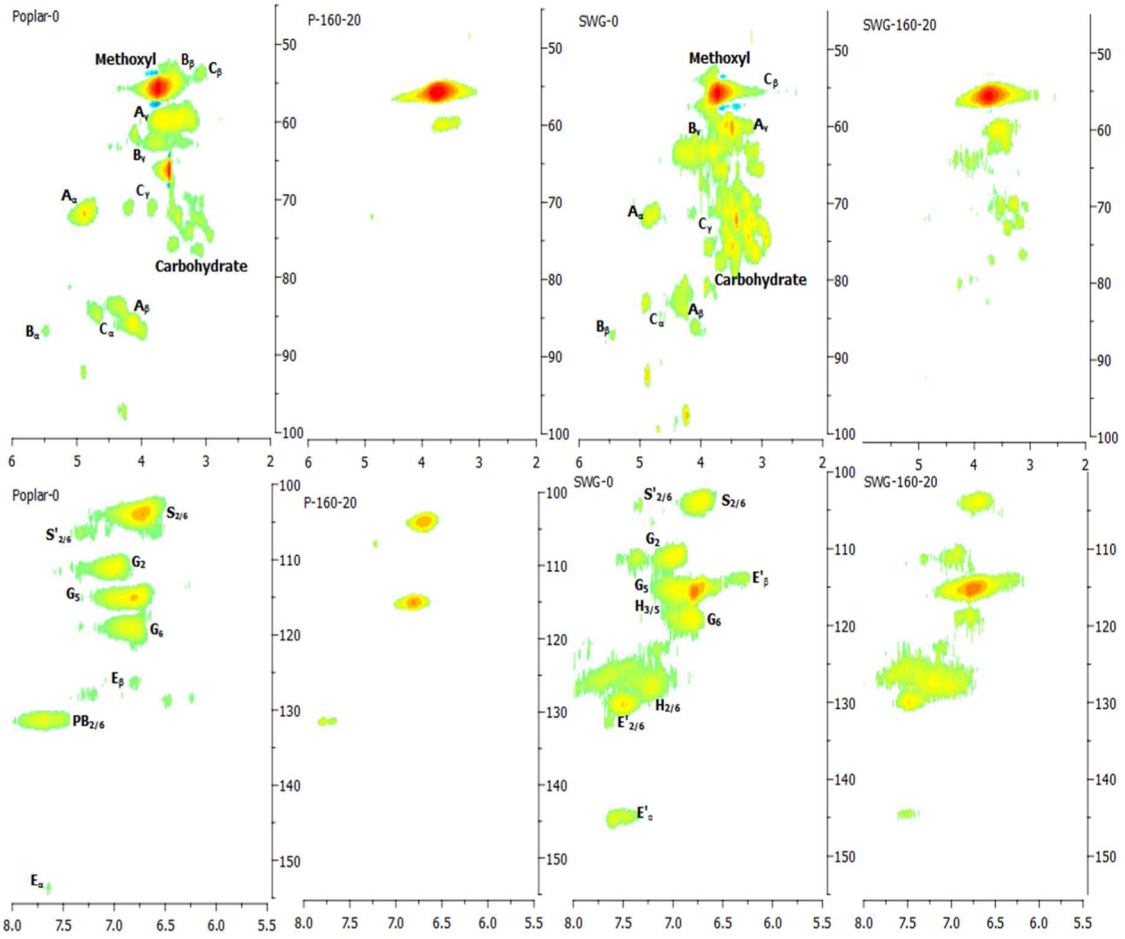


**Figure 2.** Selected 2D-HSQC spectra of poplar and switchgrass cellulolytic enzyme lignins before and after DAP. A, $\beta$-O-4 ether; B, phenylcoumaran; C, resinol; G, guaiacyl; S, syringyl; S′, syringyl with C$\alpha$ oxidized to keto group; H, *p*-hydroxyphenyl; PB, *p*-hydroxybenzoate; E, cinnamaldehyde; E′, *p*-coumarate unit (structure shown in Scheme 1).

United States. Alamo switchgrass was provided as a courtesy by University of Georgia, Athens, GA 30602, United States.[11] Cellulolytic enzyme lignins of poplar and switchgrass were isolated by modifying the procedures described in the literature.[12] After Soxhlet extraction with dichloromethane, poplar and switchgrass with toluene (about 4 mL/g dry biomass) were put into milling jars and ground in a rotary ball mill at room temperature for 1 week at 96 rpm with mixed porcelain balls ranging from 1.270−3.175 cm in diameter at the ball/biomass weight ratio of 20:1. Those extractives-free ball-milled materials were then subjected to two consecutive rounds of enzymatic hydrolysis. Biomass (15 g/L) was suspended in 20 mM, pH 5.0 sodium acetate buffer solution containing 2 mg/mL CELLULYSIN cellulase (Calbiochem, *Trichoderma viride*, activity > 10 000.0 U/g) for 48 h at 50 °C shaken at a frequency of 150 rpm. Following hydrolysis, the residue was collected by centrifugation, washed with distilled water, centrifuged, and freeze-dried. The freeze-dried residue was then subjected to two rounds of extraction with dioxane/water (96:4, v/v, 10 mL/g biomass) under a nitrogen atmosphere for 24 h. After extraction, the supernatant was collected, combined, concentrated, precipitated in water, and freeze-dried. The yields of poplar and switchgrass lignins after enzymatic isolation are around 10 and 15%, respectively.

**Dilute Acid Pretreatment of Cellulolytic Enzyme Lignins.** Poplar and switchgrass cellulolytic enzyme lignins were transferred to a 4560 mini-Parr 300 mL pressure reactor at 5.0% dry solids in 0.1 M $H_2SO_4$ solution and then sealed. The impeller speed was set to about 100 rpm, and the vessel was heated to the temperature 160 °C over 25−30 min (at 6 °C/min). The reactor was held at the pretreatment temperature ±2 °C (160 °C; 0.65−0.69 MPa) for the specified residence time ±30 s. The reactor was then quenched in an ice bath (5 min).[13] The pretreated slurry was filtered to remove the solid material and washed with an excess of deionized (DI) water. Paramagnetic impurities were removed by washing the solids with a 2% aqueous solution of ethylenediaminetetraacetic acid (EDTA) and DI water. Following DAP, the aqueous phase was extracted with ethyl acetate, dried with anhydrous $MgSO_4$, concentrated with a rotary evaporator, and combined with solid lignin residues, which were then dried in the fume hood overnight and the structural changes were examined by $^{13}C$-$^{1}H$ HSQC, $^{31}P$ NMR, and GPC analysis.[14]

**Molecular Weight Analysis of Lignins before and after DAP.** The lignin samples (dried under vacuum at 40 °C overnight) were acetylated with acetic anhydride/pyridine (1/1, v/v) at RT for 24 h in a sealed flask under an inert atmosphere. The concentration of the lignin in this solution was approximately 20 mg/mL. After 24 h, the solution was diluted with ∼20 mL of ethanol and stirred for an additional 30 min, after which the solvents were removed with a rotary evaporator followed by drying in a vacuum oven at 40 °C. The number-average molecular weight ($M_n$) and weight-average molecular weight ($M_w$) were determined by GPC after acetylation of lignin. Prior to GPC analysis, the acetylated lignin sample was dissolved in tetrahydrofuran (1.0 mg/mL), filtered through a 0.45 $\mu$m filter, and placed in a 2 mL autosampler vial. The molecular weight distributions of those samples were then analyzed on an Agilent GPC SEC 1200 system equipped with four Waters Styragel columns (HR1, HR2, HR4, HR6), Agilent refractive index (RI) detector, and Agilent ultraviolet detector (Waters, Inc., Milford, MA, United States) (270 nm) using tetrahydrofuran (THF) as the mobile phase (1.0 mL/min) with injection volumes of 20.0 $\mu$L. A calibration curve was constructed




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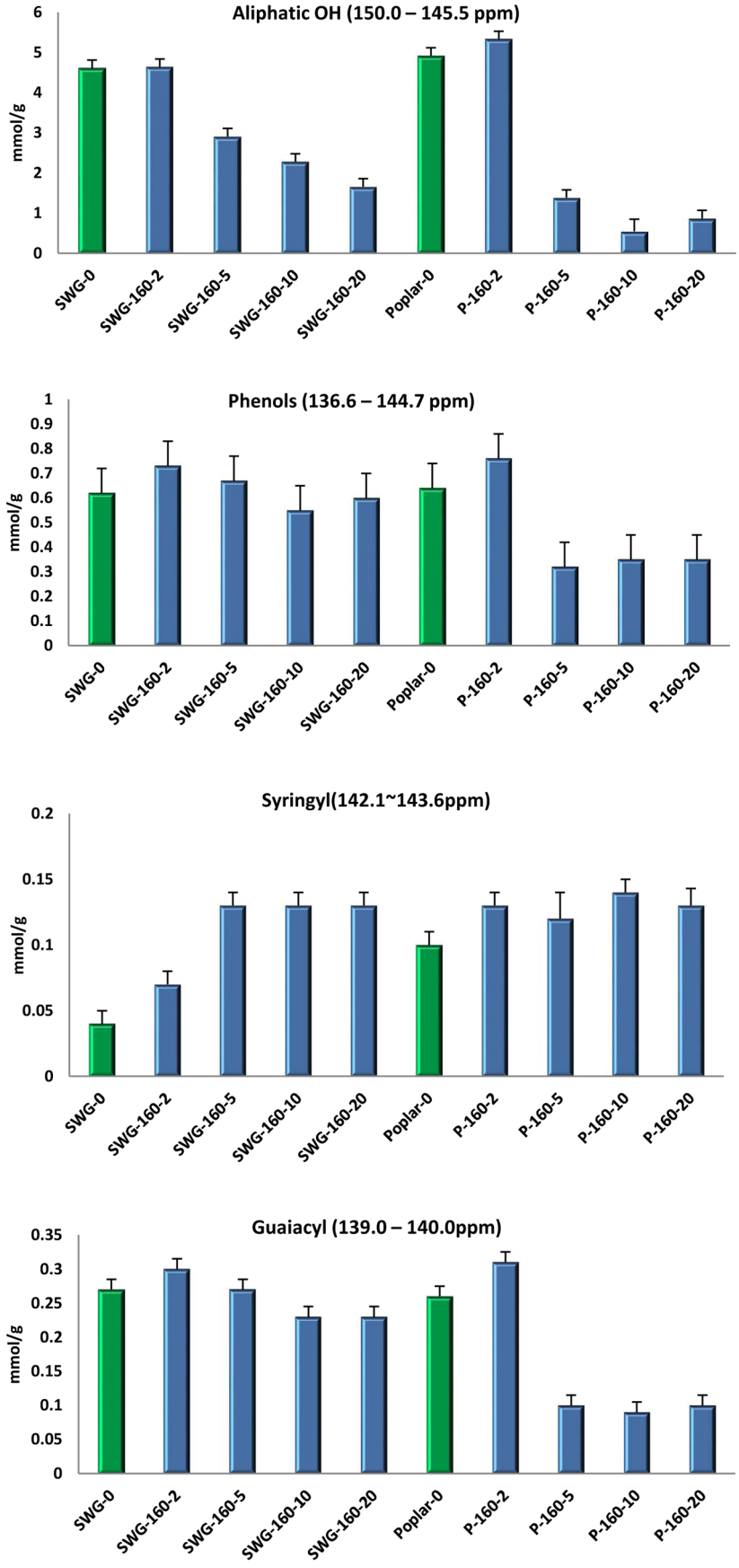


**Figure 3.** continued





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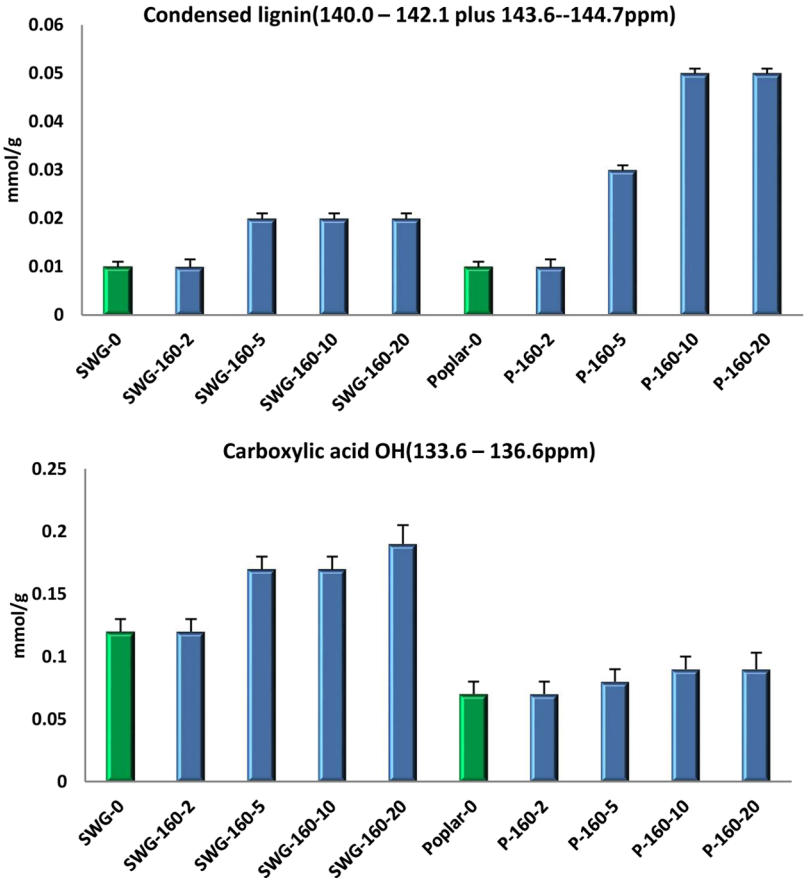


**Figure 3.** Hydroxyl group contents in poplar and switchgrass cellulolytic enzyme lignins before and after DAP calculated from quantitative $^{31}$P NMR spectra.

based on eight narrow polystyrene standards ranging in molecular weight from $1.5 \times 10^3$ to $3.6 \times 10^6$ g/mol. Data collection and processing were performed using Polymer Standards Service WinGPC Unity software (version 7.2.1, Polymer Standards Service USA, Inc., Warwick, RI, United States). Error analysis was conducted by performing three individual isolations and GPC peak integrations.

**HSQC and $^{31}$P NMR Analysis of Lignins before and after DAP.** All experiments were carried out in a Bruker 400 MHz NMR spectrometer. For the HSQC experiment, samples were prepared as follows: 50 mg of lignin sample was added to 0.5 mL of deuterated dimethyl sulfoxide, DMSO-$d_6$, and stirred at 45 °C for 4 h, employing a standard Bruker pulse sequence with 13 ppm spectra width in F2 ($^1$H) dimension with 1024 data points (95.9 ms acquisition time), 210 ppm spectra width in F1 ($^{13}$C) dimension with 256 data points (6.1 ms acquisition time), a 90° pulse, 0.11 s acquisition time, 1.5 s pulse delay, $^1J_{C-H}$ of 145 Hz and 48 scans. NMR data were processed using the TopSpin 2.1 software (Bruker BioSpin) and MestreNova (Mestre Laboratories) software packages. Phosphitylation and $^{31}$P NMR have been exploited to quantitatively determine hydroxyl functional groups in isolated lignin. Quantitative $^{31}$P NMR were acquired after in situ derivatization of the lignin sample using about 15.0 mg of lignin sample with 2-chloro-4,4,5,5-tetramethyl-1,3,2-dioxaphospholane (TMDP) in a solution of (1.6:1, v/v) pyridine/$CDCl_3$, chromium acetylacetonate (relaxation agent), and endo-*N*-hydroxy-5-norbornene-2,3-dicarboximide (NHND, internal standard).[2a,10b] The quantitative $^{31}$P NMR spectra were acquired at a frequency of 161.93 MHz over 32 K data points with acquisition time of 1.29 s, using an inverse gated decoupling pulse sequence with a 25 s pulse delay and 128 scans. Average data of lignin functional groups were presented based on three repeated tests on each lignin sample.

## RESULTS AND DISCUSSION

**Molecular Weight Analysis of Lignin.** To determine the effect of DAP conditions on the molecular weight of lignin, untreated and pretreated lignin samples were acetylated and subsequently analyzed by GPC and these results are summarized in Figure 1 (sample code Poplar-0, indicates native lignin isolated from poplar; SWG-0, native lignin isolated from switchgrass; P-160-20, poplar lignin subjected to DAP at 160 °C for 20 min; SWG-160-20, switchgrass lignin subjected to DAP at 160 °C for 20 min). There was a drastic decrease in molecular weights of pretreated lignins within a short 5 min residence time (i.e., $M_w$ of poplar lignin from 12 000 to 1560 g/mol; $M_w$ of switchgrass lignin from 6800 to 1590 g/mol) followed by a leveling-off with increasing residence time. GPC analysis also revealed that the most significant increase in PDI occurred after a 2 min DAP that is then accompanied by a decrease with longer residence time, which could be attributed to the behavior of $M_n$ and $M_w$. PDI was obtained through dividing $M_w$ by $M_n$ and any changes on those two parameters can result in the alteration of PDI. Compared with previous studies reporting that $M_w$ of lignin isolated from pretreated switchgrass and poplar was reduced by 16.8% and 3.2% respectively,[10,15] it could be concluded that the protection of the plant cell wall matrix offset this significant decrease in the molecular weight of lignin. It suggests in the absence of intact cellulose and hemicelluloses at elevated hydrothermal conditions, lignin is subjected to a significant depolymerization in a very short residence time, which is consistent with previous study on lignin fate under autohydrolysis pretreatment.[9]




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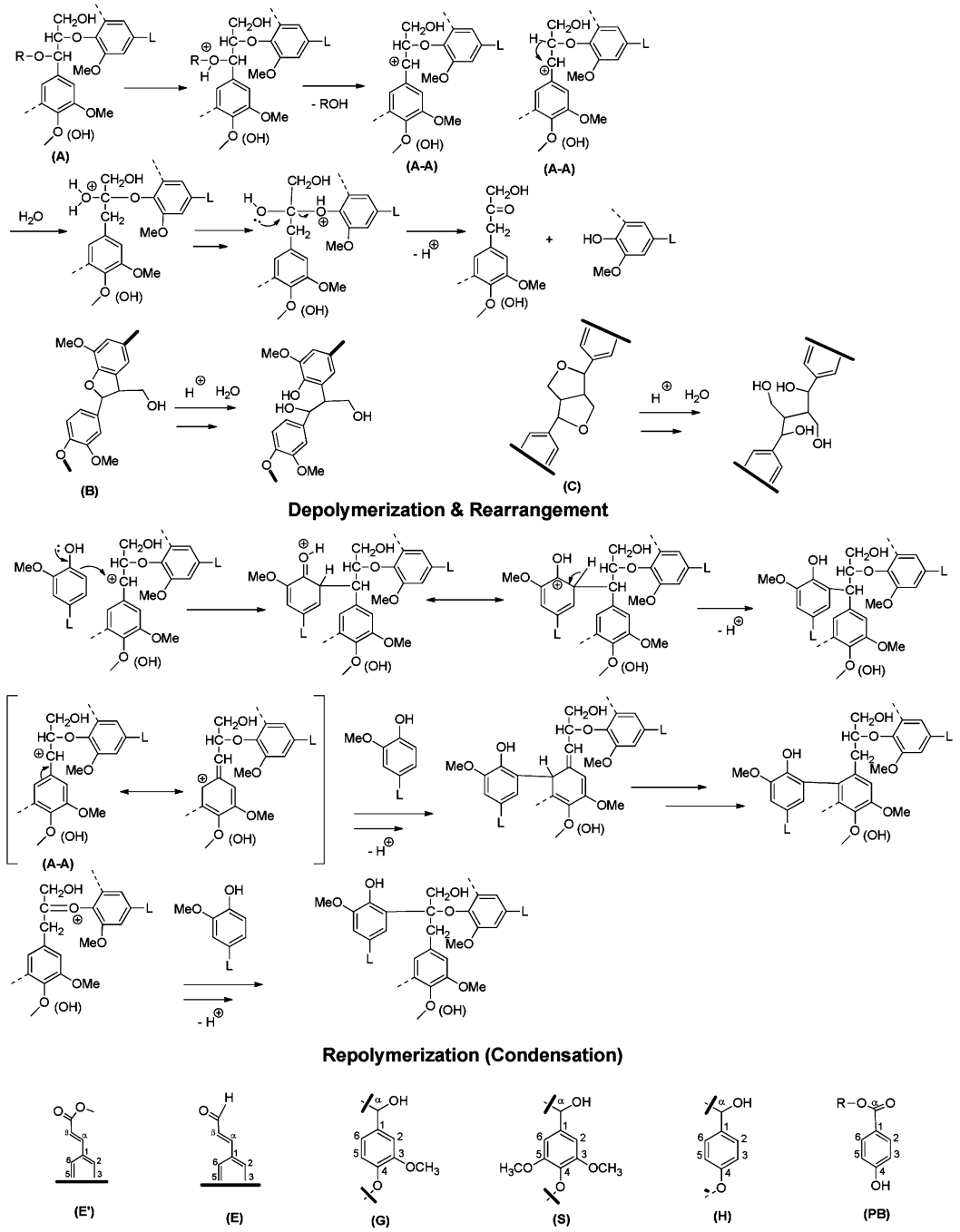
**Scheme 1. Proposed Mechanistic Pathway for Cellulolytic Enzyme Lignin Depolymerization and Repolymerization during DAP**[a]




[a]A, β-O-4 ether; B, phenylcoumaran; C, resinol; G, guaiacyl; S, syringyl; H, *p*-hydroxyphenyl; PB, *p*-hydroxybenzoate; E, cinnamaldehyde; E′, *p*-coumarate unit.

**NMR Analysis.** 2D-HSQC NMR semiquantitative analysis of untreated and pretreated lignin samples provided the evidence of structural changes in lignin side chain and aromatic regions during DAP. The cross peaks were assigned by comparing with literature data and summarized in Figure 2.[10a,16] The major interunits observed in untreated raw poplar and switchgrass lignins were the β-aryl ether linkages (A) with accompanying β-5/α-O-4 phenylcoumaran (B) and resinol (C) units. The presence of lignin syringyl (S), guaiacyl (G), *p*-hydroxybenzoate (PB), and cinnamaldehyde (E) units was confirmed in poplar lignin by the separate contour in the aromatic range, whereas syringyl, guaiacyl, *p*-hydroxyphenyl (H), and *p*-coumarate (E′) units were observed in switchgrass lignin.

The assessment of cross-peak intensity qualitatively suggested a decrease in intensity of β-aryl ether linkages as well as phenylcoumaran and resinol units for both poplar and switchgrass lignins as DAP residence time extended from 2 to 20 min, which are typically correlated with the hydrolytic degradation and/or depolymerization consistent with the observed molecular weight changes. Compared to switchgrass lignin, a more noticeable decrease in poplar lignin aromatic

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signal intensities of PB, and G units was observed under the same DAP conditions. In addition, the HSQC analysis revealed the signal intensity of E unit in untreated poplar lignin dramatically diminished after DAP for 2 min. Moreover, there were still traces in E′ unit in switchgrass lignin after DAP for 20 min. Interestingly, HSQC NMR spectra indicated the presence of the polysaccharides ($\delta_C/\delta_H$ 95.0−105.0/4.2−4.5) associated with lignin−carbohydrate linkages in untreated poplar and switchgrass cellulolytic enzyme lignins. The degradation of lignin−carbohydrate linkages suggested those linkages in switchgrass were more resistant to DAP degradation than the comparable poplar lignin carbohydrate complexes. It could also suggest subsets of hemicelluloses might be the key recalcitrance-causing factors in switchgrass.[3a]

To investigate further the fundamental chemistry of lignin during the DAP during the pretreatment processes, a quantitative $^{31}$P NMR technique was applied to monitor the changes of aliphatic, phenolic hydroxylic, and carboxylic functional groups in poplar and switchgrass lignins after DAP at different residence times, and the results are shown in Figure 3. The relative increase of aliphatic and phenolic OH groups after 2 min DAP at 160 °C can be attributed to the cleavage of aryl ether linkages and a concomitant increase in free phenolics that could allow for lower molecular weight oligomers of lignin to be solubilized. This reaction pathway is supported by the observation of the increased PDI. Moreover, there is no noticeable change in the condensed lignin content for the 2 min pretreated samples, which suggests that the depolymerization has to precede repolymerization reactions and dominate the reaction pathways at the initial stage of DAP.

When residence time was extended beyond 2 min, a decrease in aliphatic OH, total phenols, and G lignins along with an increase in S and condensed lignins and carboxylic acids were observed. The reduction in lignin side chain aliphatic hydroxyl contents resulted not only from the hydrolytic degradation of carbohydrate residue linked to lignin but also from transformation of lignin $\alpha$-OH, $\gamma$-OH groups into ketone, aldehyde, and/or alkene structures like stilbene substructures.[10b]

The increase in phenolic S units content in both switchgrass and poplar lignin indicated that the syringyl unit in lignin underwent a greater extent of ether linkage cleavage during the pretreatment. This increase of S units could be attributed to the lacking of association and protection of lignin by cellulose and hemicelluloses in biomass matrix.[10] Furthermore, the larger values of carboxylic acid groups in pretreated switchgrass lignins were mainly attributed to the hydrolysis of *p*-coumarate units, and partially from the cleavage of the ester bonds from lignin−carbohydrate linkages, which is proposed to improve enzymatic hydrolysis due to the decrease in lignin hydrophobicity and enzyme binding by electrostatic repulsion between enzymes and lignins.[17]

In addition, the possible mechanistic pathways associated with these structural changes during DAP are proposed in Scheme 1 based on data obtained from GPC, NMR, and related literature.[10,18] Both switchgrass and poplar lignins are subjected to a depolymerization reaction (scission of $\beta$-O-4 ether in Scheme 1) at the earlier stage of DAP followed by a competition between depolymerization and repolymerization reactions at a later pretreatment stage. The departure of a leaving group by an $S_N1$ process from a benzylic position (C$\alpha$ of lignin side chain) leads to the formation of resonance-stabilized benzylic carbocation intermediate that could be involved into the key rate-determining step for a reaction and thereby directs the following lignin depolymerization and repolymerization under hydrothermal conditions. With increasing residence time, the resonance stabilized carbocation facilitates several potential condensed structures to be formed, such as $\beta$-5 and $\alpha$-5 linkages (Scheme 1).

Given the results above and previous studies regarding higher severities favoring more condensation reactions,[19] in which higher temperature and longer residence time overcome the higher energy barrier and ultimately produce the equilibrium mixture of condensed lignins, the competition of lignin depolymerization and repolymerization could turn into the competition of kinetic and thermodynamic influence on lignin structural changes during DAP.

## CONCLUSION

Lignin was isolated from poplar and switchgrass using a cellulolytic enzyme system and then treated under DAP conditions in order to determine the intrinsic chemistry of lignin during dilute acid pretreatment. Our results highlight that lignin is subjected to depolymerization within the first 2 min of dilute acid pretreatment time at 160 °C, and these changes are accompanied by increasing values for the aliphatic and phenolic hydroxyl groups of lignin. This is followed by a competing set of depolymerization and repolymerization reactions that lead to a decrease in the content of guaiacyl lignin units and an increase in condensed lignin units as the reaction residence time is extended beyond 5 min at 160 °C. A detailed comparison of changes in functional groups and molecular weights of cellulolytic enzyme lignins demonstrated different structural parameters related to the recalcitrant properties of lignin are altered during the pretreatment conditions. A better and deeper understanding of the fundamental chemical structure of lignin in this study is critical to the continued research on renewable biofuel production.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssuschemeng.5b00426.

Detailed description of $^{31}$P NMR quantification method and definition of molecular weights along with their original data (PDF).

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### Author Contributions

The paper was written through contributions of all authors. All authors have given approval to the final version of the paper.

### Notes

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## ■ ABBREVIATIONS

DAP, dilute acid pretreatment
HSQC, heteronuclear single quantum coherence
NMR, nuclear magnetic resonance
GPC, gel permeation chromatography

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