

Efficient Biomass Conversion: Delineating the Best Lignin Monomer-Substitutes

Investigators

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[This project has now completed its second full year]

Abstract

The three year plan is to delineate a set of approaches for successfully altering lignin structure, in a way that allows plant cell wall breakdown to produce biofuels in a more energy-efficient manner, by providing alternative plant-compatible monomers to the lignification process. In fact, the bold plan for much of the work under the GCEP 2.3 “Renewable Energy Biomass” program is *to ‘redesign’ lignin to be chemically labile* (but obviously not biologically labile) so that the processing costs are vastly reduced and the net energy balance of biomass conversion is significantly higher.

The approach in this part of the coordinated group of GCEP projects is to synthesize and test various classes of novel plant-compatible monomer-substitutes for their abilities to incorporate into lignins, and then to determine how such incorporation affects biomass processing in biomimetic cell wall systems. The ability of a chosen monomer to incorporate into lignins (copolymerizing with the traditional monomers) is determined by *in vitro* biomimetic lignification involving the phenolic radical coupling reactions that typify the lignification process. Those that successfully make co-polymers are polymerized into a suspension-cultured cell wall system to further delineate their polymerization efficacy and to provide biomimetic cell wall material for preliminary testing of conversion efficiency following selected pretreatments and in a variety of processes.

The most promising monomer-substitutes are revealed to other GCEP researchers (notably The Boerjan and Chapple groups) so that the process of understanding the pathways that produce the monomers and obtaining the required genes can proceed most expediently. At the same time, these same researchers involved in gene and biosynthetic pathway studies reveal those monomers for which pathways can be deduced in their labs so that our basic chemical lignification studies can highlight the likely success for

compatible lignification of those monomers and determine the possible digestibility implications resulting from such lignin modification (via studies using our model cell wall system).

Accomplishments include the following... We have synthesized a further set of monomer replacements for testing; some of these compounds (particularly the 1-*O*-hydroxycinnamoyl glucoses and the 1,2-di-*O*-hydroxycinnamoyl glucoses) have been long-sought as important biological intermediates and are now available in significant quantities. The classes of monomer-substitutes include: a) Difunctional monomers or monomer conjugates linked via cleavable ester or amide (and/or hydrophilic) functionality; b) Monomers that produce novel cleavable functionality in the polymer; c) Hydrophilic monomers; d) Monomers that minimize lignin-polysaccharide cross-linking; and e) Monomers that produce simpler lignins. Compounds have been sent to another GCEP research group, the Boerjan lab, for identification and authentication of metabolites in Arabidopsis lines. Synthetic lignification studies of the compounds synthesized last year and this year are revealing the compatibility (or otherwise) of monolignol substitutes with the lignification process. In collaboration with Boerjan's group, we are embarking on a metabolomics approach toward using LC-MS methods to characterize novel monomer cross-coupling reactions that are important for their compatibility with lignification. Isolated primary cell walls have been ectopically lignified with several more sets of monomer-substitutes to provide materials for testing the digestibility (saccharifiability) of lignin-modified cell walls, with and without pretreatment. In particular, introducing various catechins and gallates (from plant tannin pathways) into lignification produces quite remarkable improvements in degradability; the improvement is related quite strikingly to the phenolic-OH content of the monomer-substitute; the first in a series of papers on monolignol replacements has been published, and a provisional patent was filed last year and is now being converted into a full patent; new compounds have been added to this series this year, including catechin vanillate and catechin ferulate which also introduce readily cleavable linkages into the lignin polymer. Last year, in collaboration with Boerjan's group, we tested a new multi-misregulation approach to produce the first transgenics that derive their lignins primarily from non-traditional monomers. The approach was aimed at improving digestibility by reducing the extent of cell-wall-polymer cross-linking, but the plants are not healthy, and not worthy of testing for digestibility implications. Nevertheless, the approach was informative in delineating just how far lignification can be perturbed away from normal monolignols and sheds light on how biosynthetic pathways interact. The paper has now appeared as a cover article in *The Plant Journal*. An approach toward providing gram-quantities of secondary wall elements in a culture system is progressing to allow testing in relevant dicot systems. Overall, a picture is emerging of the kinds of monolignol-substitutes that are compatible with lignification and allow for improved and/or less energy-intensive saccharification of pretreated and non-pretreated cell walls. The final year is gearing up to result in a slew of publications describing the approach, including papers on syntheses, characterization of synthetic and cell wall modified lignins, assaying the altered

(improved) digestibility properties of the walls, and evaluation of the most promising approaches toward improving the processing of plant biomass for bioenergy via lignin modification.

Introduction

The objective of this work is to reduce the energy requirements for processing lignocellulosic materials by structurally altering lignin, by modifying its monomer complement, to allow the biomass resources to be more efficiently and sustainably utilized. It aims to identify lignin monomer-substitutes that are fully compatible with the polymerization processes inherent in plant lignification and that, additionally, can produce modified lignin polymers that render plant cell walls less recalcitrant toward processing to biofuels. The use of lignocellulosics for biofuels, and the improvements if feedstocks can be selected/engineered for easier processing, will contribute enormously to minimizing greenhouse gas production in the transportation fuels sector.

The approach is to synthesize and test a strategic range of novel plant compatible monomer-substitutes for their abilities to incorporate into lignins, and then to determine how such incorporation affects biomass processing in biomimetically lignified cell wall systems. The classes of monomer substitutes include: a) Difunctional monomers or monomer conjugates linked via cleavable ester or amide (and/or hydrophilic) functionality; b) Monomers that produce novel cleavable functionality in the polymer; c) Hydrophilic monomers; d) Monomers that minimize lignin-polysaccharide cross-linking; and e) Monomers that produce simpler lignins.

The ability of a chosen monomer to incorporate into lignins (copolymerizing with the traditional monomers) is determined by *in vitro* biomimetic lignification involving the phenolic radical coupling reactions that typify the lignification process. Those that successfully make co-polymers are next polymerized into a suspension-cultured cell wall system to further delineate their polymerization efficacy and to provide biomimetic cell wall material for preliminary testing of conversion efficiency following selected pretreatments and in a variety of processes. These materials also provide the cell-wall-NMR database to allow the success of plant transformations to be determined.

Monomer-substitutes that are most promising are revealed to other GCEP researchers so that the process of understanding the pathways that produce the monomers, obtaining the required genes, and testing plant transformations, can proceed most expediently.

Background

Over the past decade it has become apparent that the metabolic malleability of lignification, the process of polymerization of phenolic monomers to produce lignin polymers, provides enormous potential for engineering the resistant polymer to be more amenable to processing, as reviewed.¹⁻⁵ Massive compositional changes can be realized by perturbing single genes in the monolignol pathway, particularly the hydroxylases. More strikingly, monomer substitution has been observed in the process

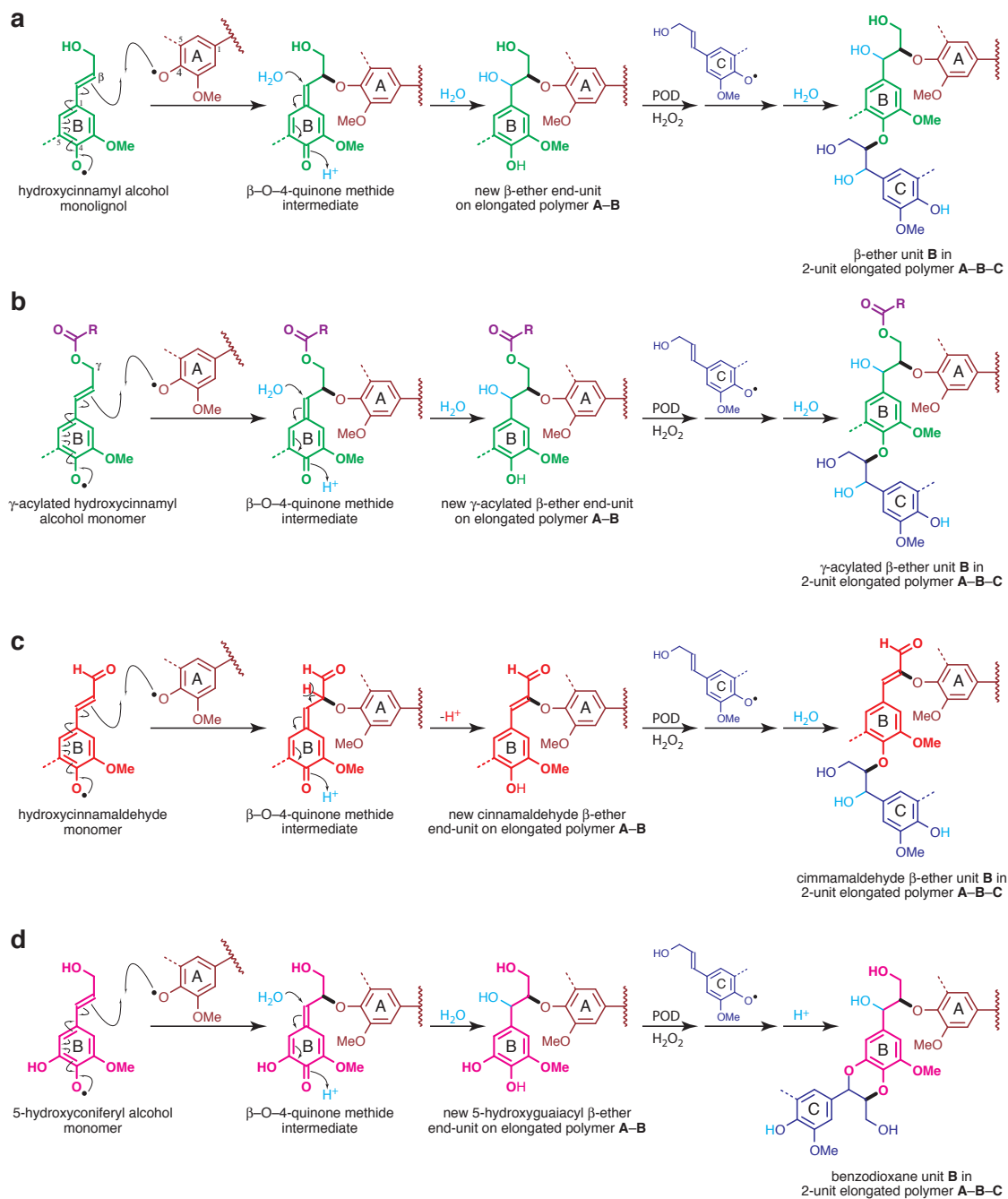


Figure 1: Cross-coupling and post-coupling reactions for various well-suited “monomers” incorporated into lignification.² Illustration is for the major β -O-4-coupling only. a) Normal hydroxycinnamyl alcohol radicals **B** cross-couple with the phenolic end of the growing polymer **A**, mainly by β -O-4-coupling, to produce an intermediate quinone methide which rearomatizes by nucleophilic water addition to produce the elongated lignin chain **A-B**. The subsequent chain elongation via a further monolignol radical **C** etherifies the unit created by the prior monomer **B** addition, producing the

Figure 1: (ctd) C2-unit-elongated polymer unit **A–B–C**. b) Various γ -acylated monolignols (*p*-coumarate, *p*-hydroxybenzoate, and acetate) cross-couple equally well producing analogous products but with the β -ether unit **B** γ -acylated in the lignin polymer unit **A–B–C**. c) Hydroxycinnamaldehydes **B** may also cross-couple with the phenolic end of the growing polymer **A**, again mainly by β –O–4-coupling, to produce an intermediate quinone methide again, but one which rearomatizes by loss of the acidic β -proton, producing an unsaturated cinnamaldehyde- β –O–4-linked **B** end-unit. Incorporation further into the polymer by etherification is analogous to a). The unsaturated aldehyde units **B** give rise to unique thioacidolysis markers. d) 5-Hydroxyconiferyl alcohol monomer **A** also cross-couples with the phenolic end of the growing polymer **A**, mainly by β –O–4-coupling, to produce an intermediate quinone methide as usual which rearomatizes normally by nucleophilic water addition to produce the elongated lignin chain **A–B** bearing a novel 5-hydroxyguaiacyl phenolic end-unit. The subsequent chain elongation via a further monolignol radical **C** coupling β –O–4 to the new phenolic end of **A–B**, but this time the rearomatization of the quinone methide (not shown) is via internal attack of the 5-OH producing novel benzodioxane units **B–C** in the 2-unit-elongated polymer unit **A–B–C**. 5-Hydroxyconiferyl alcohol incorporation produces a lignin with a structure that deviates significantly from the “normal” lignin. The bolded bonds are the ones formed in the radical coupling steps.

of lignification, particularly in cases where a plant’s ability to biosynthesize the usual complement of monolignols is compromised. These substitutions include products of incomplete monolignol biosynthesis such as 5-hydroxyconiferyl alcohol,⁶ ferulic acid,⁷ and coniferaldehyde and sinapaldehyde,⁶ in some cases at quite high levels and without obvious pleiotropic effects. This suggests that lignin composition and structure can be altered, leading to plants with characteristics for improved processing to biofuels.

Replacing the entire monomer component of lignification with a novel monomer is unlikely to be an effective strategy that is ‘acceptable’ to the growing plant. Introducing strategic monomers into the normal monolignol pool is, however, a viable proposition. To date, incorporation of up to 30% novel monomer has produced plants with no pleiotropic effects or obvious growth phenotypes. A range of alternative monomers appears to be consistent with the GCEP RFP criteria of maintaining the plant’s structural and functional integrity, but any approach will require empirical testing. The key here is to home in on the best strategies for plant-compatible monomer substitution that will produce lignins that substantially ease processing of the cell wall.

Observations to date have allowed us to detail some ideal properties of monolignol substitutes.² When such compounds are introduced into lignins, even at significant levels, the plants show no obvious growth/development phenotype. Monomers that have accessible conjugation into the sidechain allowing for so-called “endwise” β –O–4-coupling seem to fare the best. Examples are: 5-hydroxyconiferyl alcohol, the hydroxycinnamaldehydes, hydroxycinnamate esters, and acylated hydroxycinnamyl

alcohols, Figure 1. Due to incompatibilities in radical coupling reactions, *p*-hydroxyphenyl moieties fare less well than guaiacyl or syringyl moieties, at least when incorporating into guaiacyl-syringyl lignins, but other phenolics have not been well studied.

Without regard to plant biochemistry, it is easy to come up with a set of weird and wonderful monomers from simple chemical principles, from chemical catalogs, or by design. At this initial stage, however, the *only* monomers in contention are those that plants can biosynthesize; i.e., for which *in planta* biosynthetic pathways (and hence enzymes and genes) exist. All of the potential lignin monomers we intend to test have been isolated from various plant materials. The derivation of some is not entirely obvious but, if plants are truly making them, then enzymes and genes for the required biochemical pathways must be in place. The classes of monomers are considered the most fruitful to explore are as described above.

Approach

The project has now completed its second full year. The steps required to select and then test promising monomer substitutes are briefly described here. The actual progress is summarized in the next section (Progress).

1. *Delineate monomer compatibility.* Determining the compatibility of the chosen monomers with lignification via *in vitro* model coupling reactions is essential to test as any monomer that does not couple integrally into lignins is unlikely to be valuable. And, for as much as we know about radical coupling, coupling and cross-coupling propensities must be tested empirically! We have used such methods to define how ferulates couple into lignins, for example.⁸ The models and model polymers also provide the NMR database required to identify the resulting products and pathways in the more complex cell wall models and in transformed plants, and oligomers can be used for GC- or LC-MS (metabolomics) screening.

2. *Biomimetically lignify the selected monomers into cell walls.* Selected monomers, at varying levels relative to the normal monolignols, need to be incorporated into cell walls. Strategically ¹³C-labeled monomers are used as appropriate.

3. *Delineate the resultant cell wall lignin structure.* Structural characterization of the walls reveals whether the monomers integrate into wall lignins as planned. Ectopic lignification of isolated cell walls also provides materials for conversion testing. Structure is examined by degradative methods and, most importantly, via our whole-cell-wall dissolution and NMR procedures (where strategic labeling can help reveal the bonding patterns).⁹⁻¹²

4. *Test biomass processing impacts.* Monomers are all selected for their potential to improve biomass processing efficiency. The walls from step 2 are tested under a variety of biomass conversion methods to delineate how much improvement might be expected *in planta* from utilization of the monomer substitutes at various levels.

Progress

Progress in the main areas is outlined below (with some reference to progress in the prior year).

1. Synthesis of lignin monomer-substitutes

Building upon the series of compounds synthesized and, in part, tested last year, we now have successful syntheses of a number of further important groups of plant-compatible monomer-substitutes that have the potential, when incorporated into lignification, to improve lignin degradability or access to cell wall polysaccharides.

As the compound set is getting rather unwieldy to track and report on, we have simplified the figures in a way that allows the progress on each to be monitored as we attempt to finish up this study in a massive push this year. Figure 2 (on the next two pages) highlights the compounds we are studying.

Compounds reported on in last year's report include the syntheses of large amounts of normal lignin monomers (coniferyl **1G** and sinapyl **1S** alcohols), as well as the first round of potential monomer-substitutes: dihydroconiferyl alcohol (not shown), **3, 5-10, 15, 21, 26-27** (Figure 2). These include examples from the above classes of: a) Difunctional monomers or monomer conjugates linked via cleavable ester and/or hydrophilic functionality and c) Hydrophilic monomers, along with some miscellaneous monomers of interest because they have been found at quite high levels in lignins (e.g., dihydroconiferyl alcohol). Caffeoyl quinic acid **10C** had been implicated as an intermediate in the 3-hydroxylation step catalyzed by cinnamate 3-hydroxylase (C3H);¹³ one of the GCEP targets in Boerjan's group is in the export of quinates to the wall. The analog, feruloyl quinic acid **10G**, was selected as a useful target having both the polar quinic acid moiety and a lignification-compatible ferulate unit. Caffeoyl quinic acid **10C** (= chlorogenic acid) is readily available, but the ferulate analog is not; its non-trivial synthesis was provided in last year's report as Figure S1 (although it was not available in sufficient quantities for lignification into our model cell wall system or for digestibility-testing – this continues to be a problem despite significant effort this year from one postdoc), along with the method for synthesizing the various feruloyl polyols **7-9, 15**. Compounds **9** and **15** are particularly attractive, having two ferulate moieties; their incorporation into lignins would potentially produce polymer chains with cleavable ester links in the backbone. Catechins, available from tannin pathways are also intriguing. So-called Round 1 compounds included catechins **21**, and **26-27** (Figure 2b).

So-called Round 2 compounds also largely synthesized last year (as outlined in last year's Figures S3-S4) included: diferuloyl tartarate **13G**, a more polar *bis*-feruloyl component and the γ -glucosides of all three monolignols **34H/G/S**¹⁴ – these polar monolignol derivatives have been shown to produce higher-MW synthetic lignins using *in vitro* methods.¹⁵

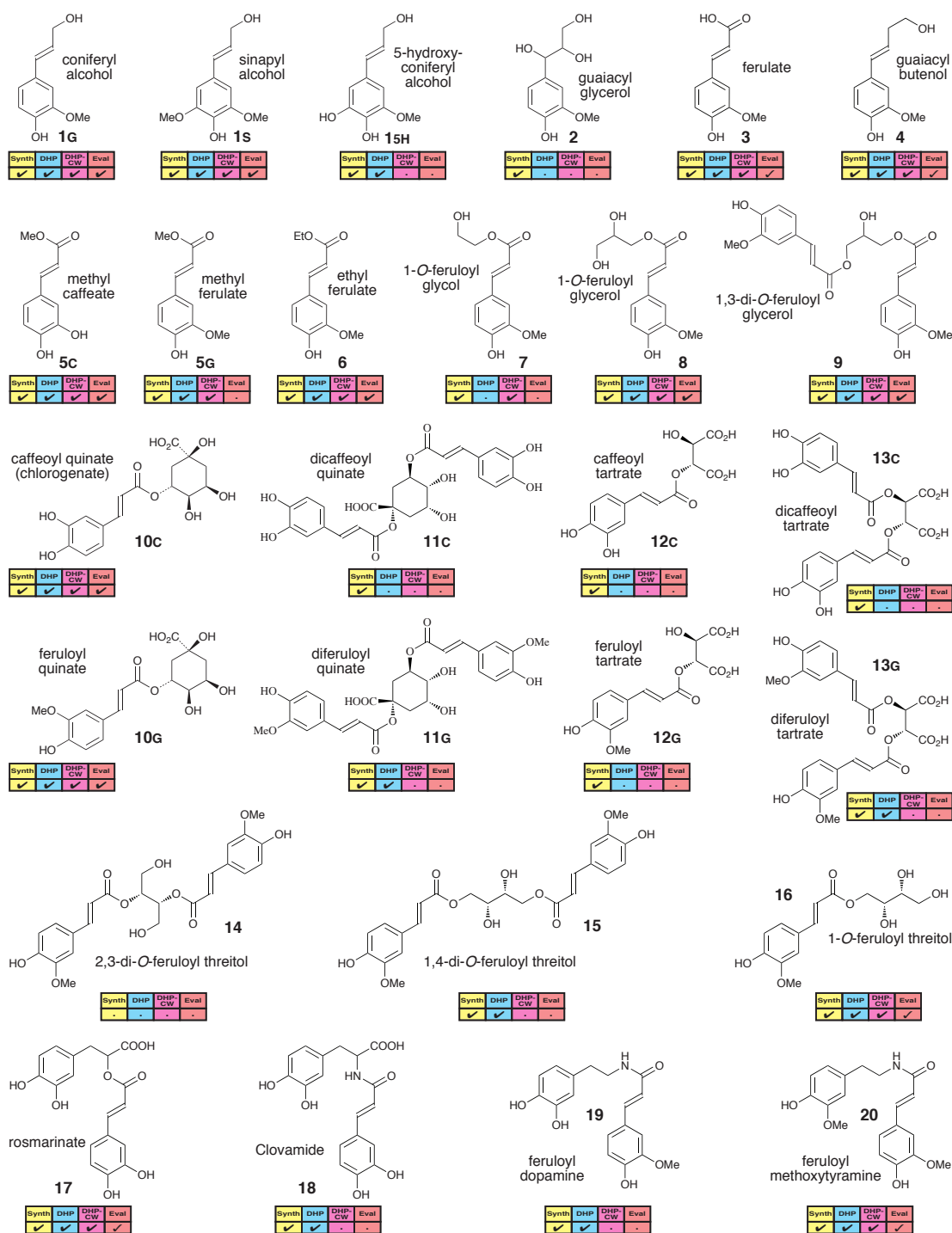


Figure 2. Compounds, synthesized or acquired, for lignification studies. Names are on all of the compounds. The color coded section under each compound is to indicate whether: **Synth** – it has been obtained (synthesized or acquired); **DHP** – it has been subjected to *in vitro* lignification, coupled with NMR to determine... (continued on next page)

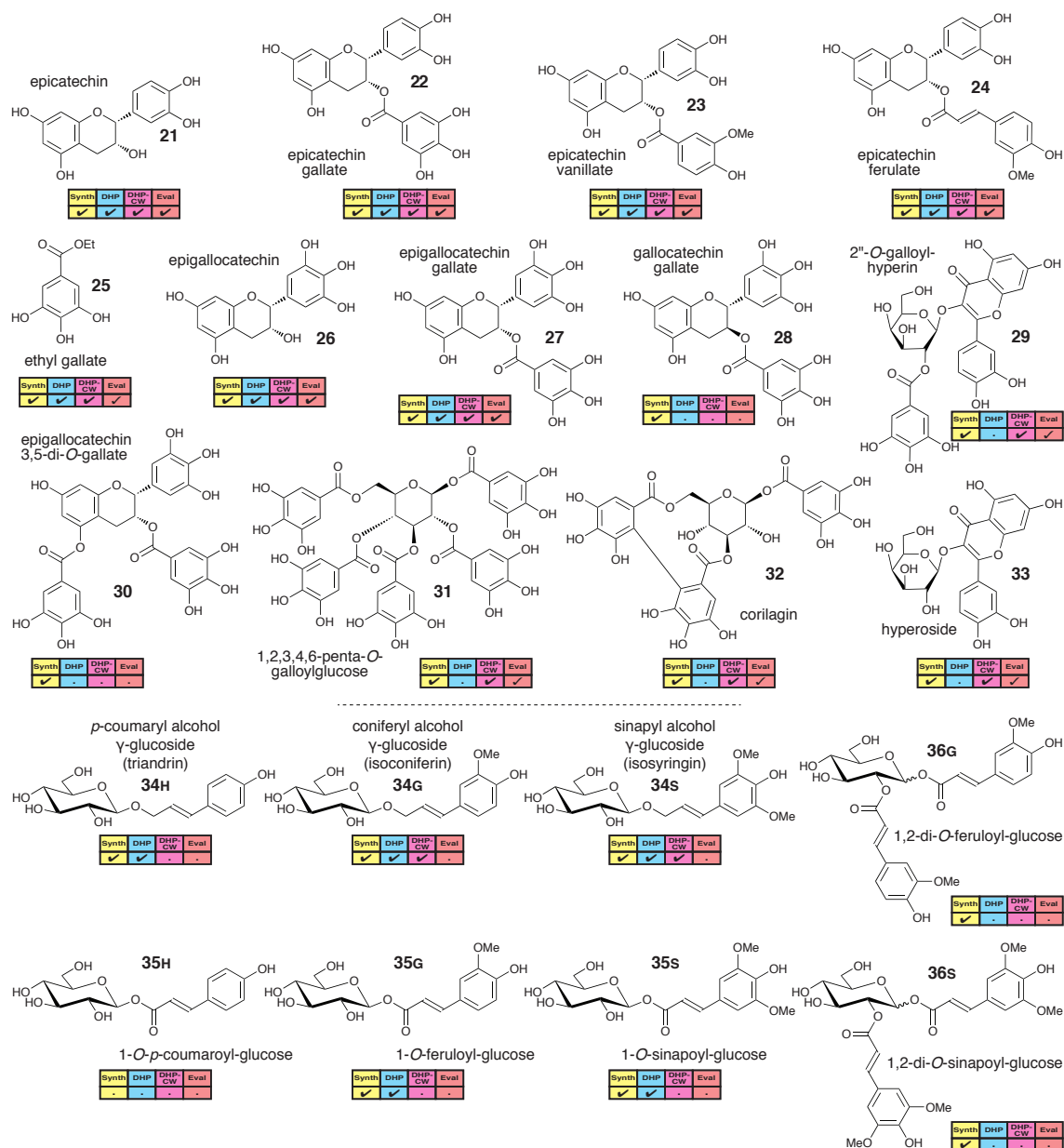


Figure 2 (ctd). ...whether it is compatible with lignification (and cross-couples into the polymer); **DHP-DW** – it has been incorporated (along with the traditional monolignols, coniferyl and sinapyl alcohols **1**) into lignins within the suspension-cultured corn cell wall system, as well a subjected to NMR to again characterize the *in muro* cross-coupling; and **Eval** – the cell walls have undergone (or are undergoing) analysis, pretreatment, and enzymatic saccharification to determine the efficacy of the monomer substitution. A lighter tick indicates partial progress.

The new compounds synthesized (or otherwise obtained) this year include: the class of caffeoyl and feruloyl amides **18-20** (which, as with the esters, introduce a cleavable bond into the backbone of the polymer) – feruloyl dopamine **19** and feruloyl methoxytyramine **20** were synthesized as outlined in Supplementary Figure S1; guaiacylbutenol **4** (which potentially reduces lignin polysaccharide cross-linking by internally trapping its own quinone methide intermediates); various of the other gallate and tannin-related compounds in the series **21-33**; the polar 1-*O*-hydroxycinnamoyl glucoses **35H/G/S**; and 1,2-di-*O*-hydroxycinnamoyl-glucoses **36G/S** that are again additionally capable of introducing readily cleavable units into the backbone of the lignin polymer. Some of these compounds are particularly valuable beyond this study, as discussed in more detail below.

Guaiacylbutenol 4. This compound does appear to be produced naturally but the guaiacylbutenoid C6-C4 (instead of the usual guaiacylpropanoid C6-C3) unit was initially rather a biochemical mystery. The biosynthesis of the production of the C6-C4 skeleton is, however, succumbing to investigation, particularly in rhubarb and ginger.¹⁶ Boerjan's group are tracking down the genes required for this pathway. The synthesis proved to be more difficult than anticipated, but was accomplished as outlined in Supplementary Figure S2.

1-O-β-Feruloyl-glucose 35G and 1-O-β-sinapoyl-glucose 35S. In addition to being monomer-replacement candidates that we wanted to examine, these compounds are common substrates for serine carboxypeptidase-like acyltransferases and serve as acyl donors in the biosynthesis of numerous secondary metabolites.¹⁷ An example is the feruloyl arabinosyl transferase that acylates arabinoxylans in the plant cell wall with ferulate,¹⁸ providing the grasses with powerful methods for cross-linking wall polysaccharides (by ferulate dehydrodimerization) and, as we have postulated,¹⁹ forming the very nucleation sites for lignification – ferulates have been shown to cross-couple with monolignols and are integrally incorporated into grass lignins.^{20,21} We consequently developed, and have now submitted for publication,²² the first chemical (and multigram-scale) synthesis of 1-*O*-β-feruloyl and 1-*O*-β-sinapoyl glucopyranoses **35G** and **35S**, as outlined in Supplementary Figure S3.

1,2-di-O-Feruloyl-glucose 36G and 1,2-di-O-sinapoyl-glucose 36S. Chapple's group (with contributions from ours) had identified 1,2-disinapoylglucose **36S** and its associated pathway from 1-sinapoylglucose **35S** in *Arabidopsis*.¹⁷ It was authenticated by NMR, but had not been synthesized. We now have a synthesis for this compound and its ferulate analog (which, to our knowledge, has not yet been identified *in planta*), Figure S4.

2. Monomer compatibility with lignification

It is important that monomer substitutes are compatible with the radical coupling reactions of lignification. As we do not envision fully replacing normal monolignols, this means that monomers need to cross-couple with monolignols and with, primarily,

guaiacyl and syringyl phenolic end-units in the growing polymer. We have already sufficiently documented the compatibility of ferulates with lignification,^{20,21} so reaffirming this for each of the ferulate-based monomers was not seen as crucial. [Incorporation into cell wall lignins, for feruloylquinic acid **8** and 1,4-di-*O*-feruloyl threitol **16**, were provided in last year's report (Figure S7)]. That said, we shall be producing synthetic lignins (based solely on coiferyl alcohol, or both coniferyl and sinapyl alcohols), typically using 25% or less of the monomer-substitute, to provide the necessary database for recognizing all of these components in lignins.

More important has been to explore how well other phenolics, those for which we know less about their radical coupling behavior, incorporate into lignin polymers. For example, it was unknown whether the caffeates (such as in **5C** and **10C**, Figure 2, and rosmarinic acid **17**), rather commonly produced plant metabolites, will efficiently couple into lignins. Consequently, last year we reported on synthetic lignins in which methyl caffeate **5C** and rosmarinic acid **17** were used as low-level monomer-substitutes. As was seen from the NMR evidence (last year's report, Figures S5 and S6), caffeates incorporate integrally into guaiacyl lignins, even making the anticipated benzodioxane structures anticipated when the 3-OH of a caffeoyl-derived moiety internally traps the intermediate quinone methide produced after a monolignol adds, at its β -position, to the catechol unit, at its 4-*O*-position; analogous benzodioxanes have been well authenticated products of lignification with 5-hydroxyconiferyl alcohol in COMT-deficient angiosperms.⁶ Those findings were important – although we were originally not convinced that caffeates and other *o*-diphenols were compatible with lignification, they appear to be so (although the full range of coupling and cross-coupling propensities remains to be determined). This means that the large number of plant metabolites in this class are open for consideration as possible lignin monomer substitutes.

Preparation of synthetic lignins incorporating the potential monomer-substitutes from Figure 2 has escalated this year, as revealed in the color-coded status boxes. An impression of the detail afforded by the NMR experiments is presented in the three sets of examples in Figure 3 and Supplementary Figures S5-6. The exact nature of the structures in the polymer in the case of guaiacylbutenol **4** incorporation, Figure 3, is still under elucidation, but it is eminently clear that the compound has incorporated and it appears that it has indeed internally trapped its quinone methide intermediate, as hoped – this implies that replacement with this monomer should reduce lignin-polysaccharide cross-linking and consequently improve the wall saccharifiability.

New approach to determining cross-coupling success. In collaborations with our group, Boerjan's group has developed significant new LC-MS-based methodologies for metabolomics, allowing detailed profiling of oligolignols.²³⁻²⁶ Although our NMR-based methods allow us to readily detect novel monomer incorporation, the details of the important cross-coupling reactions (between the novel monomer and normal monolignols) remain somewhat elusive. MS methods can help us determine that, for example, 'a trimer comprises one novel monomer X and two coniferyl alcohol units,' thus

establishing that cross-coupling has occurred. In the coming year, starting right now, we are preparing lower-molecular-mass synthetic lignins (DHPs) incorporating many or all of the monomers of Figure 2, for such analysis by the Boerjan group under GCEP. This collaborative project will significantly enhance our knowledge of the compatibility of each of the monolignol-replacement compounds with the process of lignification *in vitro* and, by extension, *in vivo*.

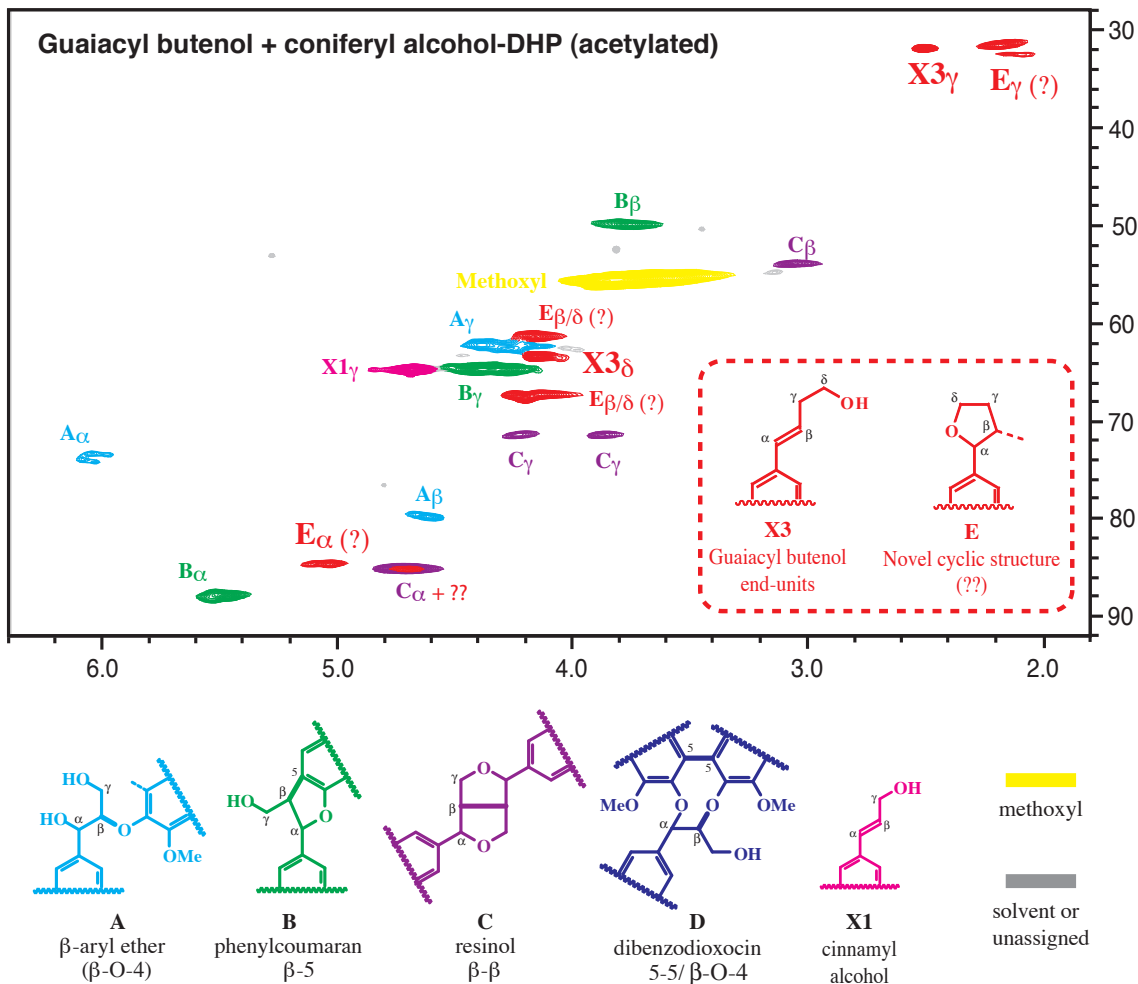


Figure 3. 2D HSQC NMR of the sidechain region of a synthetic lignin derived from coniferyl alcohol **1G** and guaiacyl butenol **4** (Figure 2). New signals derived from incorporation of the guaiacyl butenol are in red; normal lignin structures **A-D** and **X1** are also colored in our normal manner. The exact nature of the new cyclic structures **E** is still under investigation, but it is eminently clear that the compound has incorporated and it appears that it has indeed internally trapped its quinone methide intermediate, as hoped.

3. Cell Wall Lignification and Digestibility Testing

We have, via John Grabber's collaboration, an excellent grass (corn) cell wall system for testing cell wall lignification and providing materials for digestibility testing (as well as for whole-cell-wall NMR characterization).²⁷ This allows us to determine the likely efficacy of a monolignol-substitute strategy before undertaking a search for genes (involved in the substitute's biosynthesis) and plant engineering. As noted below, in Section 4, we are also seeking a dicot secondary wall system to improve the validity of testing beyond the grass system. Thus, we artificially lignified cell walls from maize cell suspensions with various combinations of normal monolignols (coniferyl and sinapyl alcohols **1**) plus a variety of phenolic monolignol substitutes (**2-36**, Figure 2). Cell walls were then incubated *in vitro* with anaerobic rumen microflora to assess the potential impact of lignin modifications on the enzymatic degradability and fermentability of fibrous crops used for ruminant livestock or biofuel production.

As described in last year's report, compounds from 'Round 1' [dihydroconiferyl alcohol (not shown), **3**, **5-10**, **15**, **21**, **26-27** (Figures 2)] have all been incorporated into cell wall lignins. The enzymatic saccharification of intact and chemically pretreated cell walls lignified by these and other monolignol substitutes has been elucidated and continues to be characterized to identify promising genetic engineering targets for improving plant fiber utilization (see below). As alluded to above, in our search for monomer-substitutes that improve digestibility, one path explores reducing the hydrophobicity of lignin to permit greater penetration and hydrolysis of fiber by polysaccharidases. Lignin hydrophobicity could be modulated by the incorporation of phenolics with extensive sidechain or aromatic ring hydroxylation (e.g., guaiacyl glycerol **2** or epigallocatechin gallate **27**) or substitution with hydrophilic groups (e.g., feruloylquinic acid **10G** or 1-*O*-feruloyl glycerol **8**). Another approach is to incorporate phenolics with *ortho*-diol functionality (e.g., methyl caffeate **5C**, caffeoylquinic acid **10C**, epicatechin **21**, epigallocatechin **26**, and epigallocatechin gallate **27**). The presence of such *o*-diphenols provides an intramolecular pathway to trap lignin quinone methide intermediates which form cross-links between lignin and structural polysaccharides;² such cross-links appear to limit the enzymatic hydrolysis of cell walls.²⁸ Another route, first illustrated by our work with coniferyl ferulate,^{29,30} would be to incorporate readily cleaved *bis*-phenolic conjugates (e.g., epigallocatechin gallate **27**, 1,3-di-*O*-feruloyl glycerol **9**, or 1,4-di-*O*-feruloyl threitol **15**) to facilitate lignin depolymerization during pretreatment of biomass for saccharification.

A study on catechin derivatives is now published;³¹ In the absence of anatomical constraints to digestion, lignification with normal monolignols hindered both the rate and extent of cell wall hydrolysis by rumen microflora. Inclusion of methyl caffeate **5C**, caffeoylquinic acid **10C**, or feruloylquinic acid **10G** with monolignols considerably depressed lignin formation and strikingly improved the degradability of cell walls. In contrast, dihydroconiferyl alcohol, guaiacylglycerol **2**, epicatechin **21**, epigallocatechin **26**, and epigallocatechin gallate **27** readily formed copolymer-lignins

with normal monolignols; cell wall degradability was moderately enhanced by greater hydroxylation or 1,2,3-triol functionality (in **26-27**). Mono- or diferuloyl esters with various aliphatic or polyol groups readily copolymerized with monolignols, but in some cases they accelerated inactivation of wall-bound peroxidase and reduced lignification; cell wall degradability was influenced by lignin content and the degree of ester group hydroxylation.

Overall, monolignol substitutes improved the inherent degradability of non-pretreated cell walls by restricting lignification or possibly by reducing lignin hydrophobicity or cross-linking to structural polysaccharides. For example, when epicatechin derivatives were included in the lignification, gas production via rumen microbes (a good measure of cell wall fermentability) was markedly enhanced with increasing phenolic hydroxyl content of the monomer-substitute (in a virtually linear fashion).³² Results are shown in Table 1. All catechin derivatives were added with normal monolignols to cell walls to potentially form a quantity of lignin equal to the high lignin control. Epicatechin (EC **21**), epicatechin gallate (ECG **22**) and epigallocatechin gallate (EGCG **27**) readily polymerized with monolignols to form lignin in concentrations equal to the high lignin control. Lignin concentrations were somewhat depressed when epigallocatechin **26** and epicatechin vanillate **23** were used to lignify cell walls. Lignified cell walls were incubated *in vitro* with rumen microflora, which produce a potent array of cell wall degrading enzymes. Gas production during incubation of cell walls (positively and highly related to cell wall fermentation and utilization by rumen microflora) was considerably enhanced by most catechin derivatives, especially epicatechin gallate **22**, epigallocatechin gallate **27**, or epigallocatechin **26**. Improved fermentability with these catechin

Table 1. Klason lignin and *in vitro* ruminal fermentability of maize cell walls artificially lignified with a binary mixture of coniferyl alcohol (CA **1G**) and sinapyl alcohol (SA **1S**) or trinary mixtures of CA and SA with epicatechin (EC **21**), epigallocatechin (EGC **26**), epicatechin gallate (ECG **22**), epigallocatechin gallate, (EGCG **27**), or epicatechin vanillate (ECV **23**).

Monolignols	Lignin mg/g	Gas production (mL/g)			NP ^a (mg/g)	GRL ^b (mL/mg);	NPAL ^c (mg/mg)
		6 h	12h	48 h			
Nonlignified control	25.5c ^d	235a	318a	356a	20e	--	--
CA:SA low lignin control	148.8b	72c	177cd	257c	174b	0.80ab	1.26ab
CA:SA high lignin control	174.7a	52cd	129e	218d	239a	0.92a	1.48a
CA:SA:EC (21)	181.6a	38d	88f	224d	213a	0.84ab	1.24bc
CA:SA:EGC (26)	147.2b	100b	232b	283b	100d	0.60c	0.66e
CA:SA:ECG (22)	178.9a	65cd	166d	259c	146bc	0.63c	0.83de
CA:SA:EGCG (27)	173.4a	73c	186c	264c	120cd	0.62c	0.68e
CA:SA:ECV (23)	153.1b	68c	182cd	272bc	116cd	0.66c	0.76de

^a Nonfermented polysaccharides (NP)

^b Gas reduction per unit lignin (GRL) calculated as [Nonlignified – Lignified gas production at 48 h]/Klason lignin

^c Nonfermented polysaccharide accumulation per unit lignin (NPAL) calculated as [Lignified – Nonlignified NP at 48 h]/Klason lignin

^d Means within columns with unlike letters differ ($P < 0.05$).

derivatives was further supported by lower levels of nonfermented polysaccharides (NP) remaining after incubation with rumen microflora and by lower gas reduction per unit lignin (GRL) and lower nonfermented polysaccharide accumulation per unit lignin (NPAL) in cell walls. Enhanced fermentability of cell walls was positively related to the degree of catechin hydroxylation (see Figure 4).

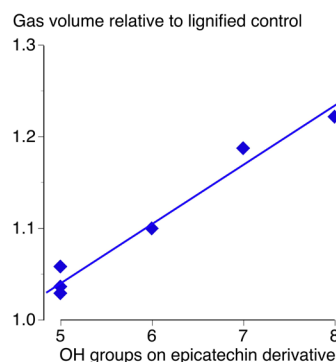


Figure 4: Gas production (relative to a normally-lignified control) for lignins augmented with various epicatechins having 5-8 phenolic hydroxyls. Gas production was markedly enhanced with increasing phenolic hydroxyl content of the monomer-substitute (in a virtually linear fashion).¹⁹

Cell walls described in Table 1 and cell wall residues collected after a weak acid or weak alkaline pretreatment were also subjected to enzymatic hydrolysis with a crude fungal cellulase and xylanase mixture, Table 2. The release of glucose, the main sugar of interest for fermentation into biofuels, was monitored during enzymatic hydrolysis. As noted for Table 1, the inclusion of most catechin derivatives enhances glucose production, especially at the early stages of hydrolysis (6 h) and this was more pronounced after weak acid or base pretreatment of cell walls. Table 2 highlights the most important findings. The supplementary report S7 provides more details.

The results with catechins and gallates were sufficiently encouraging that a provisional patent was filed on “Incorporation of flavan-3-ols and gallic acid derivatives

Table 2. Klason lignin of cell walls and enzymatic release of glucose from cell walls and from cell wall residues collected after pretreatment with 0.05% solutions of H₂SO₄ or NaOH at 100 °C for 1 h. Cell walls were artificially lignified with a binary mixture of coniferyl alcohol (CA **1G**) and sinapyl alcohol (SA **1S**) or ternary mixtures of CA and SA with epicatechin (EC **21**), epigallocatechin (EGC **26**), epicatechin gallate (ECG **22**), epigallocatechin gallate, (EGCG **27**), or epicatechin vanillate (ECV **23**).

Monolignols	Klason lignin mg/g	Glucose release (mg/g)					
		Cell walls		H ₂ SO ₄ residues		NaOH residues	
		6 h	48 h	6 h	48 h	6 h	48 h
Nonlignified control	25.5c ^a	590a	830a	885a	972a	910a	995a
CA:SA low lignin control	148.8b	392b	620bc	517d	807d	646bc	895bc
CA:SA high lignin control	174.7a	310c	530de	495d	808d	581c	857c
CA:SA:EC (21)	181.6a	324c	505e	544cd	801d	698bc	920bc
CA:SA:EGC (26)	147.2b	419b	660b	680b	936ab	787ab	964ab
CA:SA:ECG (22)	178.9a	396b	590cd	656b	898bc	763ab	946ab
CA:SA:EGCG (27)	173.4a	358bc	550de	629bc	842cd	750b	919bc
CA:SA:ECV (23)	153.1b	361bc	548de	708b	908b	675bc	869c

^a Means within columns with unlike letters differ ($P < 0.05$).

into lignin to improve biomass utilization” – this has just been filed as a full patent application.

Some monolignol substitutes, chiefly readily cleaved bi-phenolic conjugates like epigallocatechin gallate **27** or diferuloyl polyol esters (such as **9** and **15**), are expected to greatly boost the enzymatic degradability of cell walls following chemical pretreatment. In ongoing work, we are characterizing the enzymatic saccharification of intact and chemically pretreated cell walls lignified by these and other monolignol substitutes to identify promising genetic engineering targets for improving plant fiber utilization. Although producing cell walls that can be saccharified without requiring chemical pretreatments remains a goal, it is likely that pretreatment will be required to allow the wall polysaccharides to be fully utilized. Thus, monomer-substitutes that produce lignins that are essentially ‘designed’ for simplified pretreatment, are particularly attractive targets.

4. Development of dicot (Poplar, Arabidopsis) nodule culture system

Although we have a cell wall system that allows us to produce ectopically lignified cell walls with lignins of our choice, the cell walls from the current maize system are in some ways too easy to delignify, even when lignified with traditional monolignols. Having a dicot system, and particularly a secondary cell wall system, would be advantageous. Although there are a number of culture systems that produce tracheary elements (secondary wall structures), the amounts required for lignification studies and the follow-up digestibility studies here are beyond the realms of many of these.

The Patterson lab has primarily focused on optimizing a method of plant cell culture that will yield large amounts of purified tracheary elements to be used in biochemical experiments. We continue to focus on establishing nodule cultures from poplar and Arabidopsis in order to differentiate and isolate high amounts of TEs. These TEs will be enriched and used in *in vitro* lignification and digestibility studies. Supplementary Document S8 summarizes the results of our latest attempts.

5. Miscellaneous

A number of other research endeavors accompany this work. We have continued collaborating with the Belgian GCEP group (Boerjan) on modeling synthetic lignification, and on identifying and authenticating plant phenolics that might be utilized as monolignol-substitutes. We also continue to identify novel components in the lignins in natural, mutant, and transgenic plants (with Boerjan’s and Chapple’s GCEP groups, as well as others), to provide further insight into the malleability of the lignification process and to provide new leads into potential modifications.

Future Plans

Most of the compounds required have now been synthesized at sufficient scale to allow the cell wall materials to be prepared and tested (although a few compounds that are emerging as interesting will still be produced). We are now furiously making and characterizing the synthetic *in vitro* lignins to evaluate the cross-coupling compatibility of the new monomers. In a new project with Boerjan's lab, we will make additional lower-MWt lignins for characterization by LC-MS methods, again to firmly establish whether or not (and how) the new monomers are cross-coupling with the traditional units. We are also hoping to explore cleavage reactions on those lignins that contain readily cleavable groups, via developing GPC-MS methods, but that will almost certainly require an extension of time. The preparation of cell wall material lignified with all of these materials is also progressing well in batches – we intend to finish up all of the available compounds over the next few months. Full analysis and digestibility testing of those materials will fully occupy the remaining grant period.

The project has required a lot of up-front studies that are just now coming to fruition. Although some papers have been published, including a cover article in *The Plant Journal*, the real flurry of papers (and possible further patent disclosures) will come from now on. In addition to papers on the novel syntheses of many of the compounds required here, the impacts of lignin modification via the following classes of compounds will be reported on in (likely) separate papers.

1. Mono- and dicaffeoylated molecules.
2. Monolignol glucosides and hydroxycinnamoyl glucosides (could be split).
3. Mono- and diferuloylated molecules (could be split into two papers).
4. Bifunctional amides.

Patenting potential for some of the other components is not yet clear but further patents will be pursued if digestibility results are encouraging.

We are hoping to be granted a no-cost extension to allow all of this work to be completed – it is a massive project producing some good results and with a LOT to finish up to fully capitalize on this research. [We also got off to a late start due to issues with our University, and have had delays in recruiting the required postdocs. We have been fortunate to have the contributions from crucial collaborators not funded directly from this grant, particularly from Yuki Tobimatsu who is a Postdoctoral Fellow supported by Japan Society for the Promotion of Science (JSPS)]. It is anticipated that, by the end of this project, we will have a very good idea of what types of targets are the best to pursue for producing plant cell walls that are ideally tailored toward industrial utilization. We hope to have the opportunity to continue working with our valuable GCEP collaborators to bring such ideas fully to fruition, in plants modified to vastly improve processing efficiency and the consequent net energy balance of deploying biofuels.

Conclusions

The project described here, when coupled with collaborating studies from other labs, will help delineate just how far lignification can be perturbed in various directions, and will develop new leads toward altered lignins with improved processing or utilization potential — structurally altering lignin by altering its monomer complement will allow the biomass polysaccharides to be more efficiently and sustainably utilized. Ideally, we will develop modified lignins that are chemically labile (but biologically sound). There is currently no way of knowing which of these lignin-alteration strategies might be tolerated by plants, but classes of targets that confer significant potential for improved biomass processing will be identified. Although it is impossible to detail the exact anticipated energy savings at this point, reducing the recalcitrance of plant cell walls to industrial processing, for biofuels and beyond, will have an enormous impact on biofuels production and, consequently, on mitigating net GHG emissions. As noted previously, we have been exploring one novel monomer system under prior auspices.²⁵ The preliminary results already attest to the potential of the GCEP approach for success. The prospective energy savings are indicated by the remarkable processing improvements on cell walls. For example, with 25% of the monomer-substitute coniferyl ferulate incorporated into lignins, alkaline pulping at 100 °C resulted in the same degree of delignification (and produced 16% higher fiber yield) as from the normally-lignified material at 160 °C. Introducing 60% of the monomer-substitute allowed the pulping to be carried out to the same level at 30 °C and produced 67% higher fiber yield. Such gains portend enormous potential for sustainable local (and even small-scale) processing without massive facility costs; a conventional pulp mill digester facility currently costs ~\$1 billion, for example. Similar energy savings, and consequent reductions in greenhouse gas emissions, are anticipated from reducing the energy requirements for processing biomass into liquid fuels. Similar gains are anticipated for some of the novel monomer-replacements being examined here. Along with the near carbon neutrality of utilizing plant biomass (instead of fossil fuel sources), these lignin-modified plant materials have the potential to significantly ameliorate greenhouse gas emission in the transportation fuel industry globally.

Publications

The following papers and presentations cite this GCEP grant. One provisional patent was filed last year; it is just now been converted into a full patent application.

Refereed Publications

1. D. K. Ress, J. H. Grabber and J. Ralph. Lignin monomer replacement strategies: Syntheses of (-)-epicatechin ferulate, (-)-epicatechin vanillate, and 1,2-diferuloyl-(L)-tartaric Acid. 2011, in preparation.
2. S. Elumalai, J. H. Grabber, J. Ralph and X. Pan. Incorporation of epicatechin derivatives into cell walls, and assessment of responses to pretreatment methods and subsequent enzymatic hydrolysis. 2011, in preparation.
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1. S. Elumalai, J. H. Grabber, J. Ralph and X. Pan. Evaluating the response of artificially lignified maize cell wall to pretreatments and enzymatic hydrolysis. 16th International Symposium on Wood, Fiber, and Pulp Chemistry, Tanjing, China, 2011.
2. Y. Zhu, Y. Tobimatsu, J. H. Grabber and J. Ralph. Potential monolignol replacements: Synthesis and lignification of β -hydroxycinnamoyl glucosides and monolignol γ -glucosides. Global Climate and Energy Project (GCEP) Annual Symposium, Stanford, CA, 2010.
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1. J. H. Grabber, J. Ralph. Incorporation of flavan-3-ols and gallic acid derivatives into lignin to improve biomass utilization. USA Provisional Patent, #61/325,695, April 19, 2010.
2. The above has just been converted to a full Patent Application – we do not yet have the filing number.

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