

Efficient Biomass Conversion: Delineating the Best Lignin Monomer-Substitutes

Investigators

John Ralph, Professor, Biochemistry; Xuejun Pan, Professor, Biological Systems Engineering; Sara Patterson, Professor, Horticulture; Dino Ress, Postdoctoral Researcher; (other Postdoctoral Researchers not yet in place); Jenny Bolivar, Graduate Researcher; Christy Davidson, Technician, U. Wisconsin-Madison. Involved coworkers (unfunded): Hoon Kim and Fachuang Lu, Research Scientists, U. Wisconsin-Madison. Collaborators: John Grabber, Research Scientist, and Paul Schatz, Technician, US Dairy Forage Research Center, USDA-ARS.

[This project only began 2 months ago]

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.

The approach 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) will be determined by *in vitro* biomimetic lignification involving the phenolic radical coupling reactions that typify the lignification process. Those that successfully make co-polymers will next be 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 will be revealed to other GCEP researchers so that the process of understanding the pathways that produce the monomers and obtaining the required genes can proceed most expediently.

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 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 biomimetic 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.

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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 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.

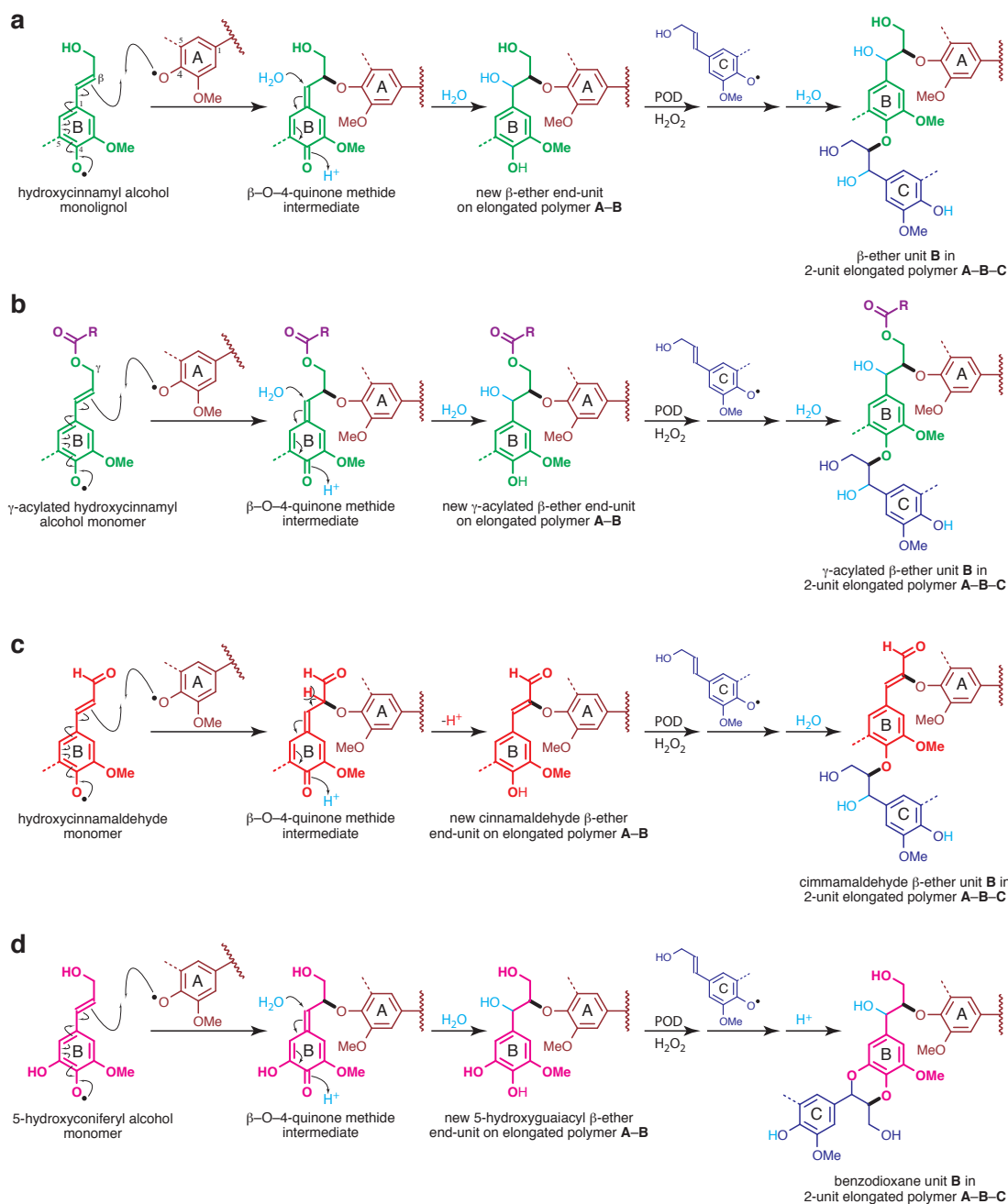
Replacing the entire monomer component of lignification with a novel monomer is unlikely to be an effective strategy that is "acceptable" to the growing plant. Introducing strategic monomers into the normal monolignol pool is, however, a viable proposition. To date, incorporation of up to 30% novel monomer 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.

Figure 1 (next page): 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 2-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.



Results

As the project has literally just started, the steps required to select and then test promising monomer substitutes are briefly described here. The actual progress in the first two months is summarized at the end of this section.

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 will also provide the NMR database required to identify the resulting products and pathways in the more complex cell wall models and in transformed plants.

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 will be used as appropriate.

3. *Delineate the resultant cell wall lignin structure.* Structural characterization of the walls will reveal whether the monomers integrate into wall lignins as planned, and provide materials for conversion testing. Structure will be examined by degradative methods and, most importantly, via our whole-cell-wall dissolution and NMR procedures (where the strategic labeling will 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 will be 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.

Actual Progress to Date (in the <2-months since this grant has been in place)

Syntheses of the large amounts of normal lignin monomers (coniferyl **1** and sinapyl **2** alcohols), as well as several potential monomer-substitutes **3-18** (Figure 2), have been completed, in part from (non-GCEP-funded) work done prior to, and in preparation for, this project. 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., dihydro-coniferyl alcohol **3**). Quinate **9** has recently 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. A major undertaking has been the synthesis of the interesting tannin monomer derivatives **17-18** in which a phenolic acid (vanillic or ferulic acid) has been esterified onto the tannin monomer to provide multifunctional compounds that are anticipated to lignify; synthesis of these latter compounds is progressing well now using improved methods for selective protection of epicatechin.

Only very preliminary *in vitro* lignification experiments have been completed. Several of these monomers are already being incorporated into the biomimetic cell wall system to produce lignified cell wall materials for structural analysis and for digestibility testing. Data from these, to be reported on next time, will be used to ascertain whether

improved cell wall degradability appears to be promising with such classes of compounds, and will help guide the choice of the next most promising monomer-substitutes for testing.

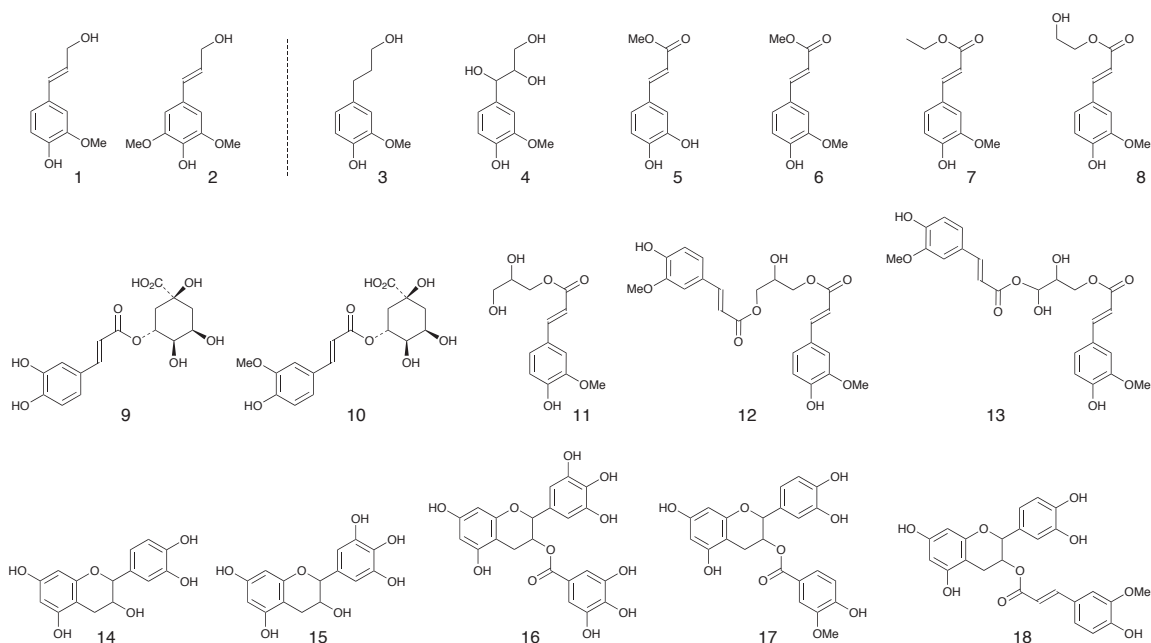


Figure 3: Compounds synthesized (or obtained) for first-round lignification studies: coniferyl alcohol **1**, sinapyl alcohol **2**, dihydroconiferyl alcohol **3**, guaiaacylglycerol **4**, methyl caffeate **5**, methyl ferulate **6**, ethyl ferulate **7**, feruloyl ethanol **8**, caffeoyl quinic acid **9**, feruloyl quinic acid **10**, 1-*O*-feruloyl glycerol **11**, 1,3-di-*O*-feruloyl glycerol **12**, 2,3-di-*O*-feruloyl threitol **13**, epicatechin **14**, epigallocatechin **15**, epigallocatechin gallate **16**, epicatechin vanillate **17**, epicatechin ferulate **18**.

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.

We have been exploring one novel monomer system under prior auspices.¹⁴ 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. 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

[Again, this research project is only just beginning. However, before the award was finalized but the intention was known, the following presentations, referencing the GCEP approach, were given.]

1. Ralph, J.; Grabber, J. H.; Lu, F.; Kim, H.; Marita, J. M.; Hatfield, R. D. Designing lignins for improved biomass processing, Global Climate and Energy Change (GCEP) Research Symposium, Frances C. Arrillaga Alumni Center, Stanford University, Stanford, CA, October 1-3, 2008.
2. Ralph, J.; Hatfield, R. D.; Grabber, J. H.; Lu, F.; Kim, H.; Marita, J. M. Designing lignins for improved biomass processing, FuncFiber 2008 International Symposium on the biology and biotechnology of wood, Umeå, Sweden, March 10-12, 2008, 2008; Umeå, Sweden, 2008; p 14.
3. Ralph, J., A basis for overcoming the 'lignin problem' in biomass conversion. 2008 Contemporary Biochemistry, Biofuels Seminar Series, Ebling Symposium Center, Microbial Sciences Building, U. Wisconsin-Madison, 2008.
4. Ralph, J., Modification of cell wall structure to improve biomass processing. Scion Seminar Series, Scion, Rotorua, New Zealand, 2009.

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Contacts

John Ralph: jralph@wisc.edu

Xuejun Pan: xpan@wisc.edu

Sara Patterson: spatters@wisc.edu