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Synthesis and Characterization of New 5-Linked Pinoresinol Lignin Models

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Abstract: Pinoresinol structures, featuring a β-β′-linkage between lignin monomer units, are important in softwood lignins and in dicots and monocots, particularly those that are down-regulated in syringyl-specific genes. Although readily detected by NMR spectroscopy, pinoresinol structures largely escaped detection by β-ether-cleaving degradation analyses presumably due to the presence of the linkages at the 5 positions, in 5-5′- or 5-*O*-4′-structures. In this study, which is aimed at helping better understand 5-linked pinoresinol structures by providing the required data for NMR characterization, new lignin model compounds were synthesized through biomimetic peroxidase-mediated oxidative coupling reactions between pre-formed (free-phenolic) coniferyl alcohol 5-5′- or 5-*O*-4′-linked dimers and a coniferyl alcohol monomer. It was found that such dimers containing free-phenolic coniferyl alcohol moieties can cross-couple with the coniferyl alcohol producing pinoresinol-containing trimers (and higher oligomers) in addition to other homo- and cross-coupled products. Eight new lignin model compounds were obtained and characterized by NMR spectroscopy, and one tentatively identified cross-coupled β-*O*-4′-product was formed from a coniferyl alcohol 5-*O*-4′-linked dimer. It was demonstrated that the 5-5′- and 5-*O*-4′-linked pinoresinol structures could be readily differentiated by using heteronuclear multiple-bond correlation (HMBC) NMR spectroscopy. With appropriate modification (etherification or acetylation) to the newly obtained model compounds, it would be possible to identify the 5-5′- or 5-*O*-4′-linked pinoresinol structures in softwood lignins by 2D HMBC NMR spectroscopic methods. Identification of the cross-coupled dibenzodioxocin from a coniferyl alcohol 5-5′-linked moiety suggested that thioacidolysis or derivatization followed by reductive cleavage (DFRC) could be used to detect and identify whether the coniferyl alcohol itself undergoes 5-5′-cross-linking during lignification.

Keywords: lignins · NMR spectroscopy · oxidation · radical reactions · synthetic methods

Introduction

In gymnosperm (softwood) cell walls, lignin dominantly consists of guaiacyl units derived from coniferyl alcohol, with minor levels of *para*-hydroxyphenyl units derived from *para*-coumaryl alcohol and elevated levels of the latter in compression wood zones.[1] It has been documented that lignification starts with dehydrodimerization of monolignols through radical coupling, resulting in three (for coniferyl alcohol) dimeric products that can further couple with the coniferyl alcohol or oligomers to ultimately produce lignin (Figure 1). The majority of linkages between the structural units of lignin are (in descending order) β-*O*-4′ (β-aryl ether), β-5′ (phenylcoumaran), and β-β′ (resinol). Other linkages present at relatively minor levels are 5-5′ (biaryl, almost universally present as dibenzodioxocins), β-1′ (spirodienone), and 5-*O*-4′ (diaryl ether).[2]

Historically, degradative methods such as acidolysis, thioacetolysis, hydrogenolysis, and the more recently widely used thioacidolysis and derivatization followed by reductive cleavage (DFRC) methods play an important role in our current knowledge of lignin structure.[3] The principle behind these methods is based on the efficient cleavage of β-aryl ether units so that any substructures linked through β-ethers (at both the 4-*O*- and β-positions, or with the phenolic end free) are released as low molecular mass compounds suitable for analysis by GC, GC-MS, and NMR spectroscopy. Due to the complexity of lignins, there are some limitations when applying such methods. For instance, it is difficult to identify and quantify degradation products larger than dimers due to the lack of authentic reference compounds and, because dimers or higher oligomers may have many isomeric forms, unfortunate side reactions also complicate the results. Today, NMR spectroscopy has emerged as a particularly powerful tool for the structural elucidation of polymeric lignins mainly due to the availability of advanced instruments with high-field magnets, sensitive cryogenically-cooled probes, and digital processing techniques

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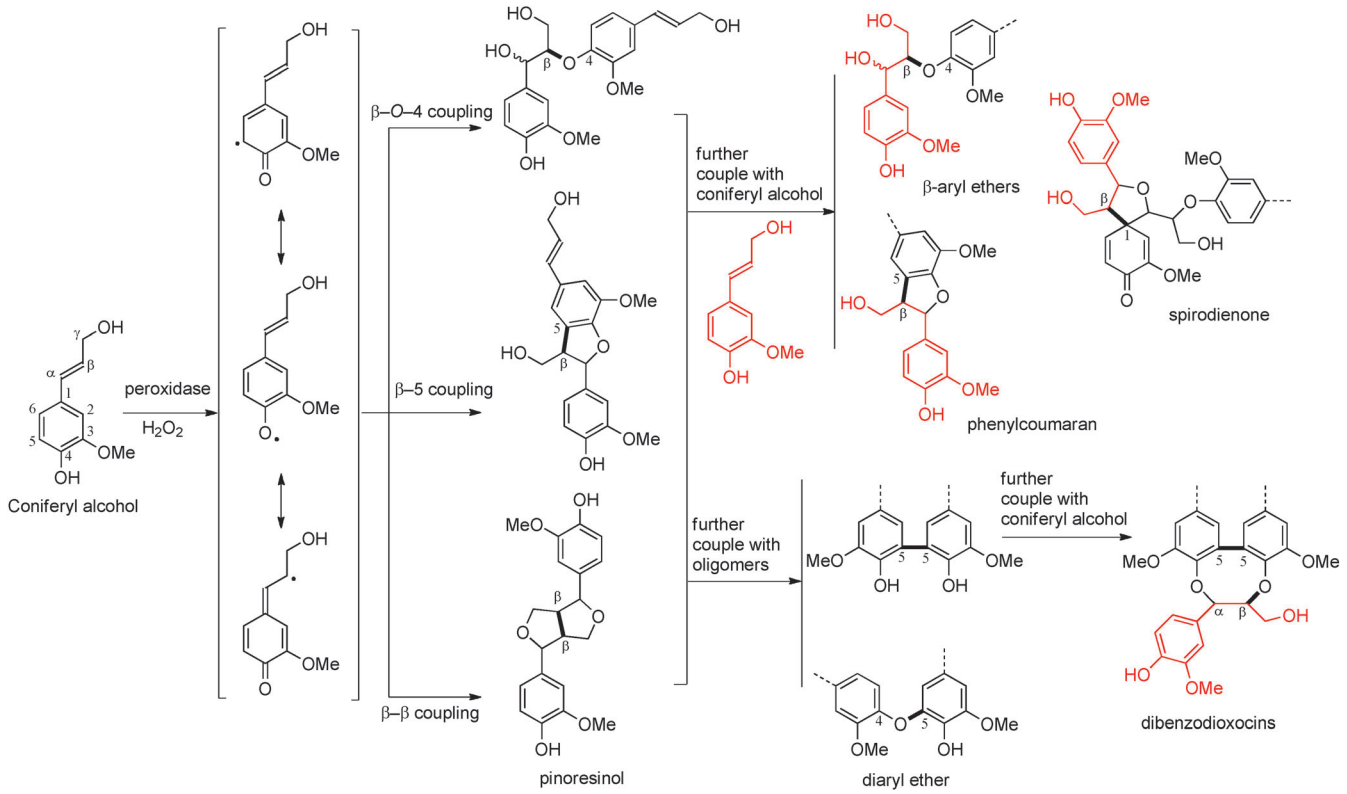

Figure 1. Dehydrodimerization of coniferyl alcohol through free-radical coupling produces three dehydrodimers that further couple with coniferyl alcohol or lignin oligomers forming the lignin polymer with various linkages between structural units. Bonds formed by radical coupling are bolded.

that allow increased sensitivity and higher dispersion/resolution in NMR experiments.[4] Meanwhile, many lignin model compounds have been synthesized and characterized, providing essential data for interpreting and validating the NMR spectra of lignins and advancing our understanding of lignin structure; however, more model compounds, particularly of certain types, are still needed.[5]

Dimeric products with β-5′-, β-1′-, and 5-5′-linkages are readily identified and quantified following β-ether-cleaving methods including acidolysis, thioacidolysis, and DFRC methods.[6] However, dimers derived from pinoresinol structures in softwood lignin have escaped detection by acidolysis,[7] or thioacidolysis.[8] If they exist in degradation as products from softwood lignin, they must be below the detection limit (less than 0.1 % in the case of acidolysis). Those results appear to contradict NMR spectroscopic estimates of at least 2 % pinoresinol structures present in softwood lignin.[9] Low levels have been validated by DFRC methods raising questions about why they are not detected in acidolytic methods.[10] Such discrepancies were considered to result from pinoresinol structures having at least one linkage (5-5′ or 5-*O*-4′) at their 5 positions on the aromatic rings,[11] (Figure 2), and were supported by thioacidolysis indications that pinoresinol-derived trimeric products were tentatively found to have 5-5′- and 5-*O*-4′-linkages.[12] A pathway to form such structures was proposed, as summarized on the left hand side of Figure 2, although no definitive evidence has been presented.[11a] Based on the current (conventional) theory of lignin formation, 5-5′- or 5-*O*-4′-linkages between lignin units are formed between two preformed lignin oligomers (or polymeric molecules), not from two monolignol molecules (right hand side of Figure 2).[10,13] It has not been established whether 5-5′- or 5-*O*-4′-linkages can be formed between coniferyl alcohol and a pre-formed lignin oligomer (polymer) as described on the left-hand side of Figure 2; however, 5-5′- or 5-*O*-4′-linked pinoresinol model compounds (**3** and **10**, Figure 3) could be conveniently obtained if compounds **1** and **2**, bearing 5-5′- or 5-*O*-4′-linked coniferyl alcohol moieties, respectively, were able to cross-couple (through β-β′-coupling) with the coniferyl alcohol monomer under peroxidase-mediated oxidation conditions (Figure 3).

In this paper, we therefore report the synthesis of the 5-5′- or 5-*O*-4′-linked coniferyl alcohol models **1** and **2**, and their use in cross-coupling reactions with coniferyl alcohol under peroxidase-catalyzed oxidation conditions, to establish whether the 5-5′- or 5-*O*-4′-linked pinoresinol model compounds can be produced, and to secure model compounds for more detailed NMR analysis of lignin polymers. We show that the expected 5-linked pinoresinol compounds were obtained, along with six other new lignin model compounds. Examination of the HMBC spectra of these model compounds suggested that unambiguous differentiation between 5-5′-linked and 5-*O*-4′-linked pinoresinol structures in

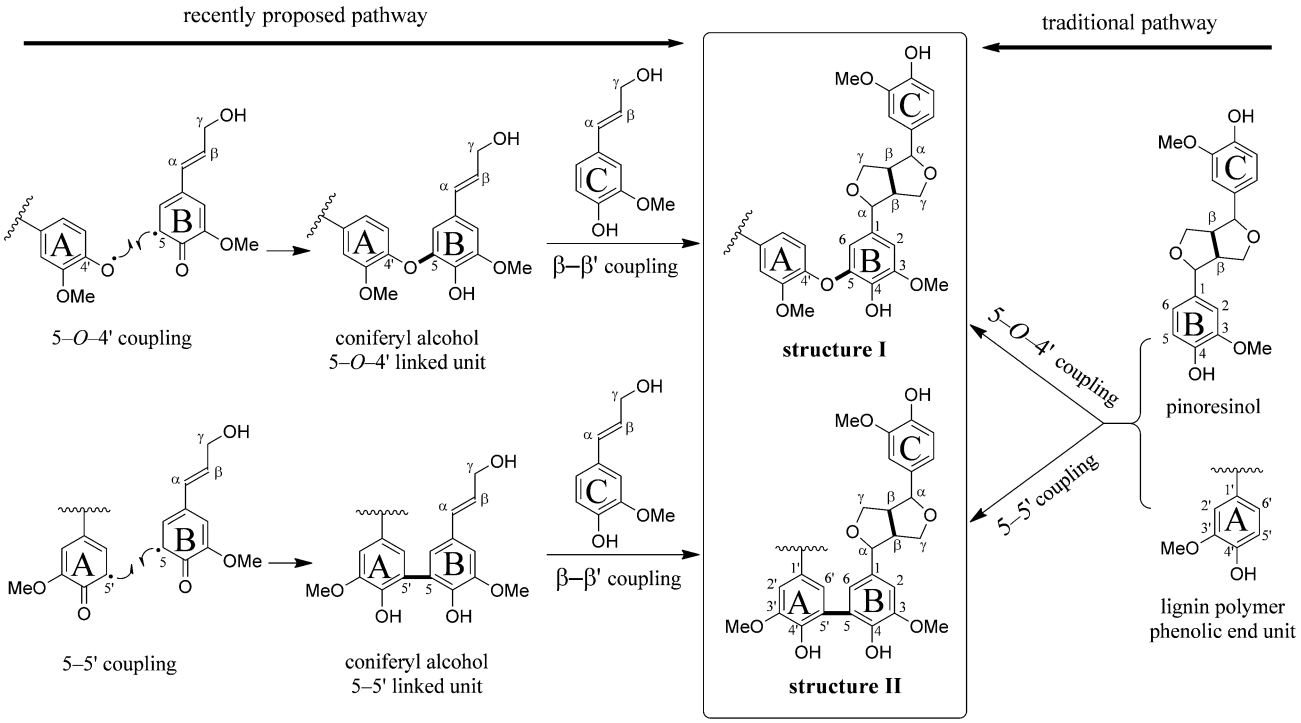

Figure 2. 5-Linked pinoresinol structures (structures **I** and **II**) in softwood lignin and possible biosynthetic pathways leading to such structures. Bonds formed by radical coupling are bolded.

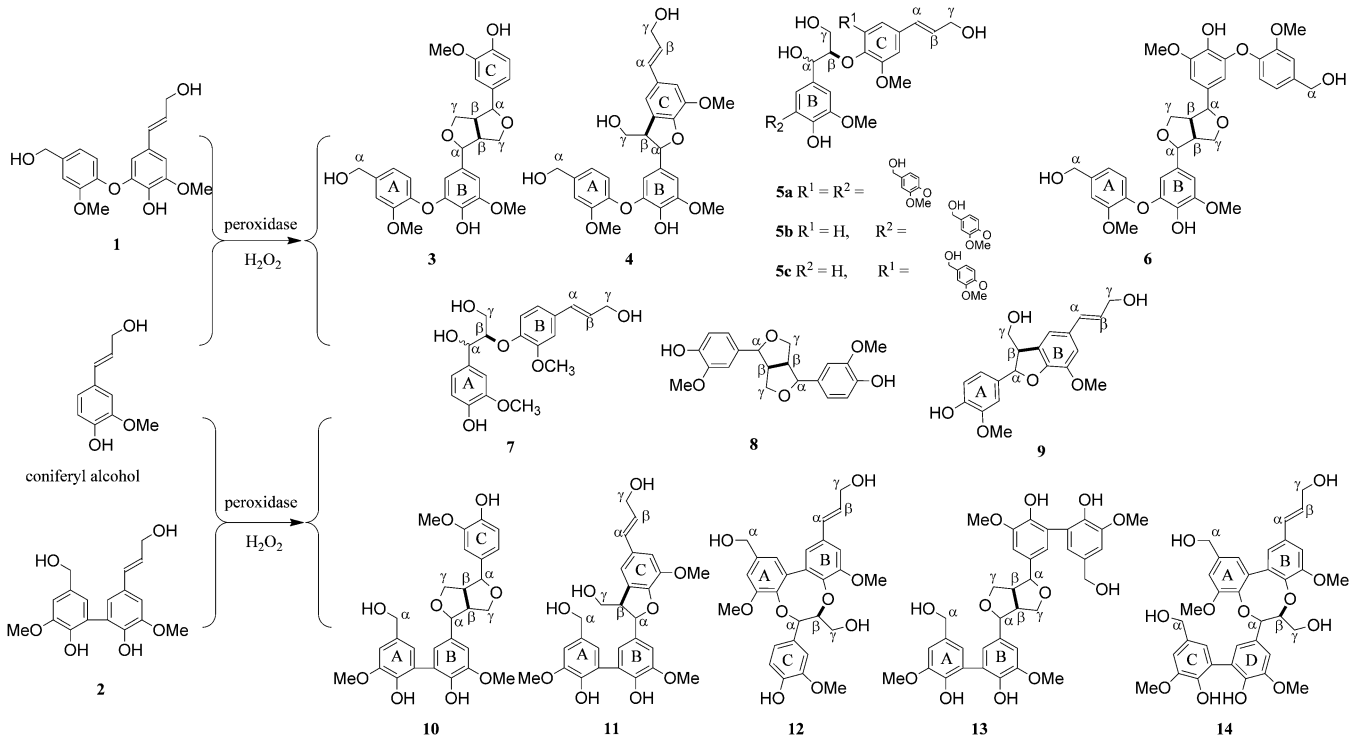

Figure 3. Coupling products produced from peroxidase-catalyzed H_2O_2 oxidative free-radical coupling between coniferyl alcohol and the 5-linked coniferyl alcohol model compounds **1** or **2**. Bonds formed by radical coupling are bolded.

lignin by NMR spectroscopy is possible, whereas detection of 5-5′-linked coniferyl alcohol in lignin may need more sensitive analytical methods such thioacidolysis or DFRC methods coupled with GC- or LC-MS.

Results and Discussion

Mechanisms leading to pinoresinol structures: Based on the current hypothesis of lignin formation, lignification of plant cell walls starts with dimerization of monolignols (Figure 1) or, in special cases such as in grasses, perhaps from ferulates on arabinoxylans acting as nucleation sites.[14] From coniferyl alcohol, three dimers, namely β-5′-, β-β′-, and β-*O*-4′-coupled dehydrodimers, are produced through free-radical coupling reactions. Although theoretically possible, no 5-5′- or 5-*O*-4′-coupled dimers are formed in vitro from the radical coupling of coniferyl alcohol.[10,15] After phenolic oxidation, each of the formed dimers can further cross-couple with a new incoming coniferyl alcohol monolignol radical producing trimers through 4-*O*-β′- or 5-β′-coupling. The trimers can then also cross-couple with a monolignol in a similar way to form larger oligomers, building up the polymer. Although 5-5′- and 5-*O*-4′-coupling between a growing oligomer or polymer molecule and a monolignol (again, both as radicals) is yet again conceptually possible, no concrete evidence has been found; in fact, all evidence is that coniferyl alcohol always couples at its β-position (except in dehydrodimerization reactions in which at least one of the coniferyl alcohol radicals couples at its β-position).[10,13] Thus, as in the traditional hypothesis, all the evidence suggests that the 5-5′- or 5-*O*-4′-linkages are formed from coupling between two dimers or higher oligomers (right hand side of Figure 2), not from reactions involving monolignol coupling. As for the fate of a pinoresinol dimer formed through dimerization of coniferyl alcohol during early lignification in softwoods, there are two possible positions, namely the 4-*O*- and 5-positions, on each aromatic ring available for coupling to other units or to a coniferyl alcohol, to produce 4-*O*- (4-*O*-β′- or 4-*O*-5′-) and 5- (5-β′-, 5-5′, or 5-*O*-4′-) linked pinoresinol structures. However, out of the 30 possible pinoresinol-containing structures, only the ones with 4-*O*-β′-ether linkages on both aromatic rings, or units with a 4-*O*-β′-ether linkage on one ring with the other remaining terminal (free-phenolic), can be released and detected as β-β dimers by β-ether-cleaving methods including acidolysis, thioacidolysis, or the DFRC method (Figure 4). Therefore, it is distinctly unsurprising why NMR spectroscopy, which detects all pinoresinol structures in the lignin, gives much higher estimations than β-ether-cleaving methods that measure only those structures connected by β-ethers at both 4-*O*-positions (or at one with the other remaining free-phenolic moiety). In a recent report, the failure to detect any pinoresinol structures by β-ether-cleaving methods was attributed to a proposed alternative lignification pathway leading to pinoresinol structures with 5-linkages (left hand side of Figure 2).[11a] So far no evidence has been found to prove or disprove this alternative pathway although, again, all indications are that coniferyl alcohol does not couple at its 5-position with guaiacyl oligomers. The potential for the putative intermediate 5-linked coniferyl alcohols to couple with a coniferyl alcohol forming the expected 5-linked pinoresinol structures, however, could be easily tested in vitro if the corresponding models **1** and **2** were available. These are model compounds

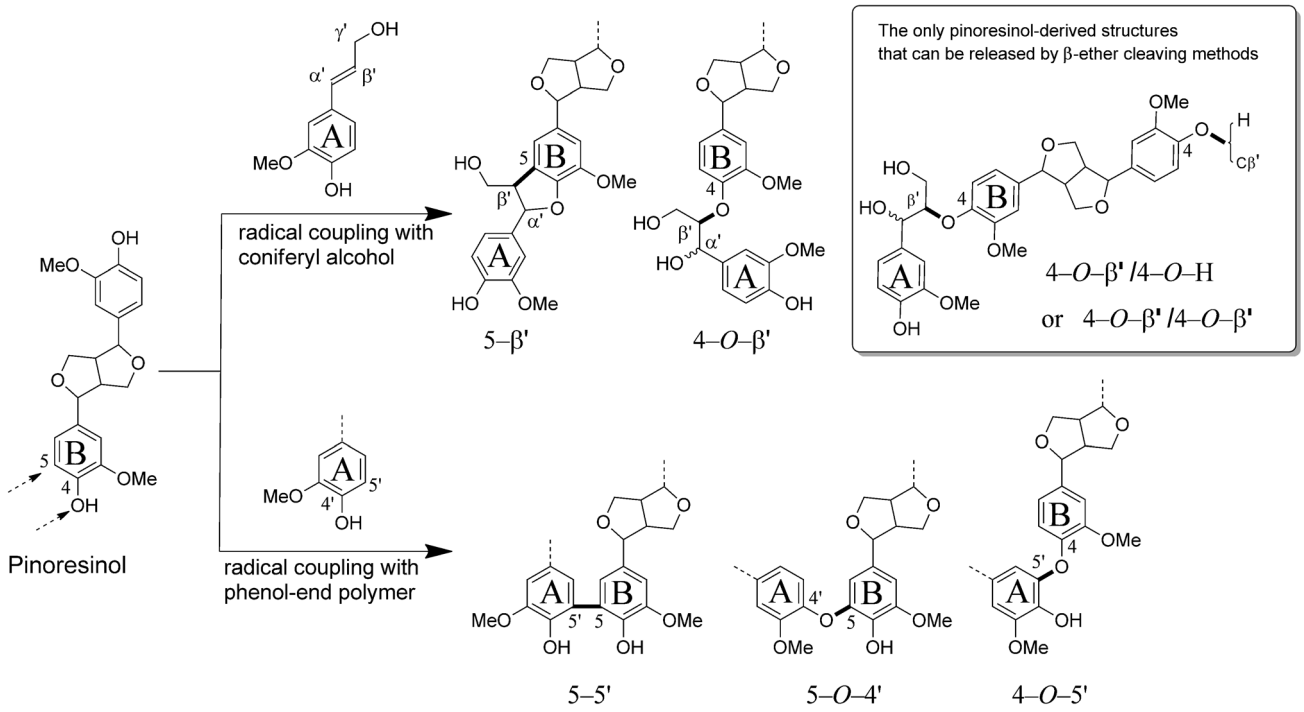

Figure 4. Possible structures derived from cross-coupling reactions between pinoresinol and coniferyl alcohol or a phenolic end-unit in a lignin polymer (only coupling structures derived from the pinoresinol "B ring" are shown), as well as (inset) pinoresinol linked by a 4-*O*-β′-ether at one phenolic end or linked by 4-*O*-β′-ethers at both phenolic ends, which are the pinoresinol-derived structures that can be released by β-ether-cleaving methods.

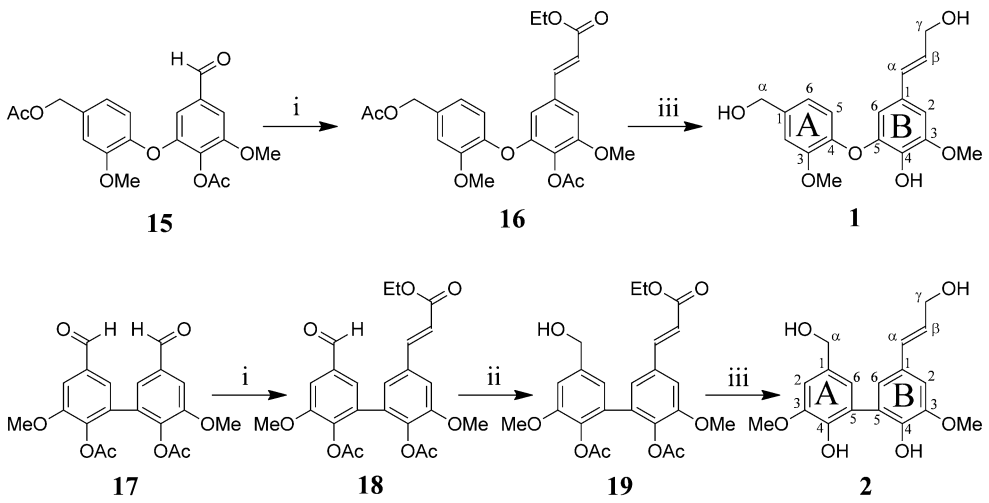

Figure 5. Synthetic routes to make 5-linked coniferyl alcohol model compounds **1** and **2**. i) NaH, triethyl phosphonoacetate, THF; ii) *tert*-butylamine-borane complex, CH_2Cl_2; iii) diisobutylaluminium hydride, hexanes.

in which a simple apocynol moiety (having no conjugated α,β-unsaturation) is used to model the guaiacyl unit in the lignin polymer. Although verification of such a possibility does not prove the alternative pathway, free-radical coupling reactions between 5-linked coniferyl alcohol models and coniferyl alcohol could produce various valuable lignin model compounds with 5-linkages. Preparation and NMR characterization of such 5-linked lignin models will clearly help to advance our understanding of lignin structures. In this work, model compounds **1** and **2** were synthesized (Figure 5) and an in vitro biomimetic free-radical coupling reaction of coniferyl alcohol with **1** or **2** was performed to test whether the expected compounds **3** and **10** could be produced and, most importantly, to obtain various 5-linked lignin model compounds and the spectral data to authenticate such structures, if produced, in the polymer.

Products from the peroxidase-catalyzed coupling of 1 or 2 with coniferyl alcohol: It is well known that lignification is a chemical process involving combinatorial coupling of phenolic radicals generated by enzyme-catalyzed oxidation. These coupling reactions include coupling between two monolignol radicals (monolignol dehydrodimerization, lignin initiation), coupling between a growing oligomer or polymer radical and a monolignol radical (end-wise polymerization, lignin polymer growth), and coupling between two growing oligomer or polymer radicals (lignin polymer branching).[10,13a] The peroxidase/H_2O_2 system has often been used to simulate lignification and to synthesize lignin model compounds.[16] One of the main goals here was to synthesize compounds **3** and **10** from compounds **1** and **2** by using a peroxidase-catalyzed H_2O_2 oxidative coupling reaction. (Figure 3) When compound **1** and coniferyl alcohol were oxidized in the peroxidase/H_2O_2 system, various coupling products were produced as evidenced by the NMR spectrum (Figure 6 A), in which almost all of the correlation peaks can be assigned. A total of six coupling products were isolated by chromatography and identified by NMR spectroscopy. Compounds **7**–**9** are homo-coupled dimerization products from coniferyl alcohol and compound **6** was the only homo-coupled product from compound **1**. The expected β-β′-cross-coupled product **3** along with the β-5′-cross-coupled product **4** was also produced. Although isolation of β-*O*-4′-cross-coupled products such as **5** was not successful, the heteronuclear single quantum coherence (HSQC) spectrum of the acetylated whole product mixture (Figure 6) suggested the existence of β-*O*-4′-coupling products other than **7** in the coupling reaction products. The correlations at $\delta_H/\delta_C = 6.04/74.8$ ppm and 6.14/75.08 ppm were characteristic of C_α–H_α correlations of an acetylated β-*O*-4′ lignin model compound, whereas the corresponding C_β–H_β correlations were found at $\delta_H/\delta_C = 4.89/81.42$ and 4.98/81.42 ppm. When we looked at correlations at lower contour levels in these regions (circled regions, Figure 6), more correlations were observed. The COSY (see the Supporting Information, Figure S1) and HSQC-TOCSY (Supporting Information, Figure S2) data revealed that such C_α–H_α correlations and C_β–H_β correlations were co-correlated, belonging to one molecule, and their γ-CH correlations were also evident. Moreover, HMBC data (Supporting Information, Figure S3) showed that the C_α signals at $\delta_C \approx 75.0$ ppm were correlated with protons at $\delta_H = 6.45$–6.55 ppm, characteristic of C_2 protons in 5-*O*-4′-linked guaiacyl units, that is, the B ring of compounds **5a** and **b**. Two more pairs of correlations at $\delta_H/\delta_C = 6.97/75.0$ and 7.15/75.0 ppm and at $\delta_H/\delta_C = 7.02/75.80$ and 7.18/75.80 ppm were also observed in the HMBC spectrum suggesting that there are another guaiacyl-type β-*O*-4′-dimeric products besides compound **7**, that is, compound **5c**. Therefore, besides the correlations at $\delta_H/\delta_C = 6.05/74.40$ and 6.09/75.26 ppm (C_α–H_α) and at $\delta_H/\delta_C = 4.85/80.14$ and 4.80/80.55 ppm (C_β–H_β), which were assigned to compound $\mathbf{7}_{\mathbf{Ac}}$, correlations at $\delta_H/\delta_C = 5.90$–6.15/74.0–76.10 ppm and at $\delta_H/\delta_C = 4.70$–5.00/79.5–82.0 ppm were tentatively assigned to C_α–H_α and C_β–H_β correlations of 5-*O*-4-

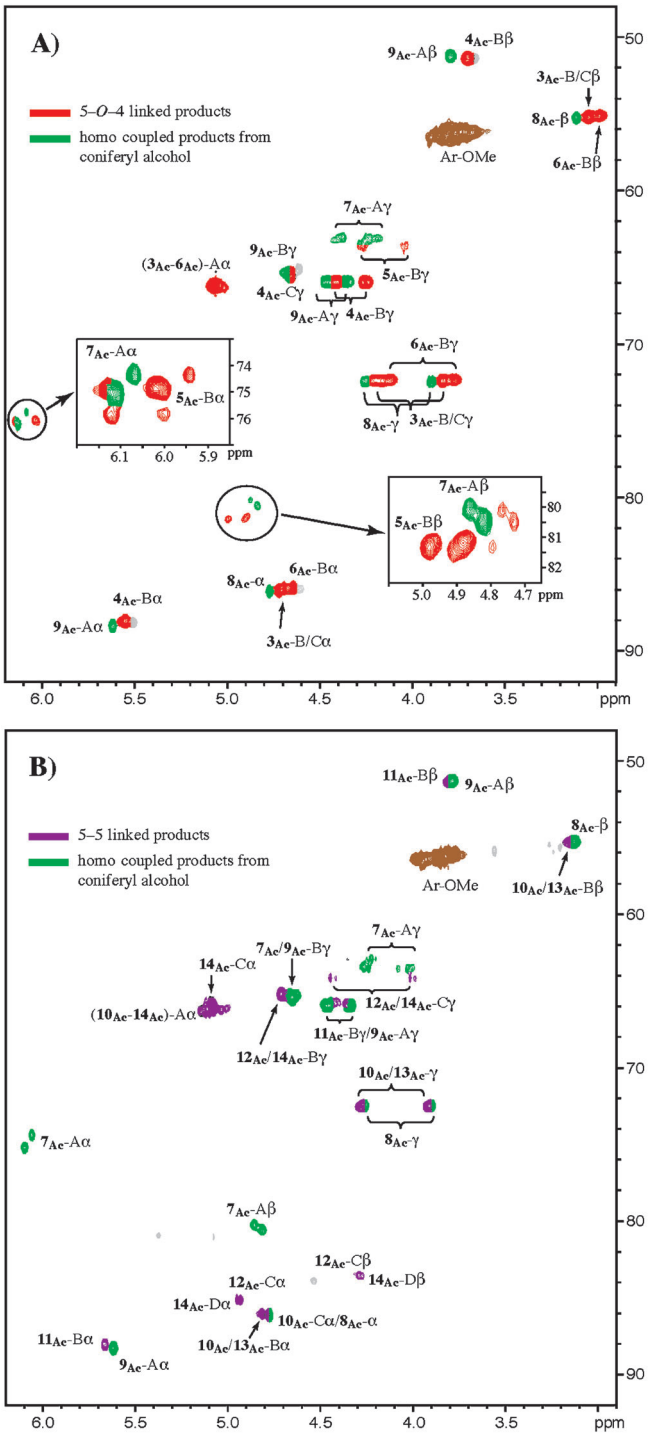

Figure 6. HSQC spectra of acetylated oxidation product mixtures from peroxidase-catalyzed H_2O_2 oxidative free-radical coupling reactions; A) between coniferyl alcohol and 5-*O*-4′-linked coniferyl alcohol **1**; B) between coniferyl alcohol and 5-5′-linked coniferyl alcohol **2**.

linked β-*O*-4′-coupled products, compounds **5** (Figure 3). These six compounds accounted for most of the coupling products from compound **1** and coniferyl alcohol, as clearly demonstrated by HSQC NMR spectroscopic analysis of the whole (acetylated) product (Figure 6). To confirm the formation of homo-coupled compound **6**, peroxidase-catalyzed coupling of compound **1** was also carried out and compound **6** was isolated and identified by NMR spectroscopy. Thus, comparing the HSQC NMR spectrum of compound $\mathbf{6}_{\mathbf{Ac}}$ with that of the acetylated products allowed ready identification of compound $\mathbf{6}_{\mathbf{Ac}}$ in the cross-coupling product mixture (Figure 6 A).

When compound **2** was treated with the coniferyl alcohol under similar peroxidase-catalyzed H_2O_2 oxidation conditions, the main cross-coupled products isolated were compounds **10–12**, which were formed through β-β′-, β-5′- and β-*O*-4′-cross-coupling reactions, respectively (Figure 3). The other isolated products were homo-coupling products **7–9** from coniferyl alcohol, and products **13** and **14** from compound **2**. According to the NMR data of these isolated products, almost all of the correlations in the HSQC NMR spectrum of the whole product mixture (acetylated; (Figure 6 B) were assigned.

As compound **1** and compound **2** each have a linkage at their aromatic ring 5-positions, their propensity to couple with coniferyl alcohol was expected to be analogous to that of sinapyl alcohol. The identification of the cross-coupled products from the coupling reactions between the coniferyl alcohol and compound **1** or **2** suggested that compounds **1** and **2** indeed behave similarly to sinapyl alcohol, forming β-β′-, β-5′-, and β-*O*-4′-cross-coupled products. Having the *ortho*-bisphenol structure, compound **2** was able to couple with coniferyl alcohol or with another compound **2** producing dibenzodioxocin compound **12** or **14**, after internal trapping of the post-coupling quinone methide intermediate by the other phenol, forming these characteristic 8-membered rings.[17] These dibenzodioxocin structures are usually found in softwood lignins,[4b,17–18] and it was contended that essentially all 5-5′-linked units are incorporated into such structures because the *ortho*-bisphenol (derived from 5-5′ coupling) is very active and tends to couple with a monolignol forming a dibenzodioxocin efficiently under in vitro oxidative coupling conditions. As discussed above, the β-*O*-4′-cross-coupled products between coniferyl alcohol and compound **1** were not isolated; however, analysis of 2D NMR (HSQC, HMBC and HSQC-TOCSY) spectra (see the Supporting Information) suggested the existence of these compounds and this coupling pathway. The main β-*O*-4′-cross-coupled product was tentatively identified as compound **5**. The homo-coupled products **6**, **13**, and **14** were not isolated directly from the cross-coupling reaction of coniferyl alcohol with compounds **1** or **2**. To verify their formation, they were synthesized from coupling reactions under similar conditions in separate experiments in which only compound **1** or **2** was used as substrate. It is evident based on the results from this study (Figure 3, compound **6**) that 5-linked-coniferyl alcohols **1** and **2** readily cross-couple at the β-positions of the coniferyl alcohol moiety with coniferyl alcohol (at its β-, 5- or 4-*O*-positions) forming pinoresinol (compounds **3** and **10**), phenylcoumaran (compounds **4** and **11**), and β-*O*-4′-ether (compounds **5** and **12**) units in the products. However, these results do not necessarily prove the pathway recently

proposed (left hand side of Figure 2) for the biosynthesis of 5-linked pinoresinol structures in softwood lignin because whether the 5-linked coniferyl alcohols can be formed by coupling between a growing polymer and a coniferyl alcohol monomer is still not clear; in fact, because such coupling pathways have never been observed in vitro, it remains unlikely.

Implications:

Possibility of distinguishing 5-linked pinoresinol structures in softwood lignins: Pinoresinol structures are readily recognized by HSQC NMR spectroscopy because of the well-resolved and diagnostic C_β–H_β correlations in the tetrahydrofuran rings.[19] However, structural information about the aromatic rings of pinoresinol structures is obscured in an HSQC spectrum of lignin, and the available pinoresinol-related lignin model compounds are still very limited. Although 5-linked pinoresinol structures **I** and **II** (Figure 2) have been thought to be primary pinoresinol structures in spruce lignin (based on thioacidolysis results), they have not been confirmed by NMR spectroscopy because of lack of reliable model compounds. In this work, compounds **3** and **10**, which are reasonable models for 5-linked pinoresinol structures **I** and **II**, have been synthesized and characterized. We therefore looked for the possibility of distinguishing them by comparing the NMR data of compounds **3** and **10**. For model compounds **3** and **10**, the C–H correlations of the side-chains in their HSQC spectra are separated sufficiently to differentiate them from each other (Figure 6). But an HSQC spectrum of a lignin sample normally does not have such good resolution due to the complexity and polymeric nature of lignin. HMBC is a long-range C–H correlation experiment, ideally correlating all protons with carbons within a three-bond distance.[4b] It has been used to identify structures in complex natural polymers such as polysaccharides and lignins.[20] When looking at molecular structures of compounds **3** and **10**, we found that both the carbon and proton at the α positions are key starting points to correlate all carbons or protons within the three-bond distance. In the literature, HMBC correlations starting from H_α to three aromatic carbons (C_1, C_2, and C_6) have often been used to distinguish guaiacyl from syringyl units in various structures (β-*O*-4′, β-5′, and β-β′).[21] However, the correlations starting from C_α to the two aromatic protons (H2 and H6) has scarcely been used for structural characterization.[4b] In this study, we attempted to use correlations starting from α positions (H_α and C_α) in the HMBC spectra (Figure 7) to distinguish compounds **3** and **10** aimed at providing a basis for identifying 5-linked pinoresinol structures **I** and **II** in softwood lignins. It is clear that compound **10** stands out when looking at correlations (in the left panel of Figure 7) from α proton of the B ring having a 5-5′-linkage, whereas the H_α–C_2 correlations in compound **3** are also well differentiated. When we look through correlations (in the right panel of Figure 7) starting at C_α it is easy to find compound **3** with its unique correlations from C_α–B_6 at $\delta_C/\delta_H = 86.2/6.48$ ppm. If hydroxyl groups in compounds **3** and **10** are appropriately derivatized,

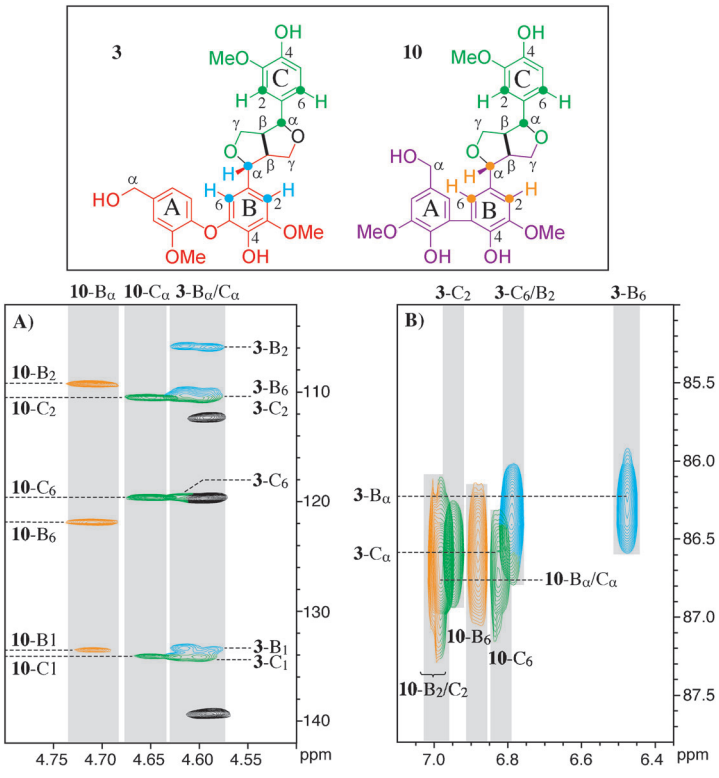

Figure 7. Partial HMBC spectra of compounds **3** and **10** (spectra from the individual compounds were overlaid showing the diagnostically different correlations). A) Correlations from protons α and carbons 2, 6, and 1 in the B units of compounds **10** (orange) and **3** (blue), and in the C units of compounds **10** and **3** (green). B) Correlations from carbons α and protons 2 and 6 in B units of compounds **10** (orange) and **3** (blue), and in the C units of compounds **10** and **3** (green).

they can be valid lignin models for NMR characterization of 5-linked pinoresinol structures in softwood lignin. For example, as acetylated lignin samples are usually used for NMR characterization, alkylation or etherification of phenolic hydroxyl and acetylation the primary hydroxyl in compounds **3** and **10** will produce models suitable for modeling 5-linked pinoresinol structures in acetylated softwood lignin; simple acetylation of all hydroxyls of compounds **3** and **10** gives rise to $\mathbf{3}_{\mathbf{Ac}}$ and $\mathbf{10}_{\mathbf{Ac}}$, which are good models for 5-linked pinoresinol structures in the case where phenolic hydroxyls are free, that is, for terminal phenolic units.

The potential to identify (detect) 5-linked coniferyl alcohol units: As discussed above, the recently proposed pathway for formation of 5-linked pinoresinol structures requires 5-linked coniferyl alcohols as precursors. In addition to our in vitro study here demonstrating that 5-5′- or 5-*O*-4′-linked coniferyl alcohols can cross-couple with the coniferyl alcohol forming 5-linked pinoresinol structures **I** and **II** (Figure 2), it is also required to prove the existence of such precursors during lignification in cell walls. From the compounds resulting from this work (Figure 3), it can be seen that only compounds **5c** and **12** (or **5a** and **14**) are unique and interesting because the primary structures of the 5-

linked coniferyl alcohols are retained during coupling reactions, that is, the cinnamyl moiety remains intact. So finding such structures in lignin by using NMR spectroscopy or other methods certainly could prove the existence of 5-linked coniferyl alcohols. The abundance of such structures could be extremely low so that NMR spectroscopy may not be the ideal option for such a task even though the required model compounds **12** or **14** have been synthesized and characterized here. More sensitive analytical tools than NMR spectroscopy will likely be required to detect any possible 5-linked coniferyl alcohol units. Our experience in lignin structural research suggests that thioacidolysis or DFRC method coupled with GC- or LC-MS analysis have the potential to detect such 5-linked pinoresinol structures in lignin because 1) both MS methods have high resolution and sensitivity to detect trace compounds in a complex mixture; 2) thioacidolysis and DFRC methods are high-yielding degradation methods for lignin producing very diagnostic products; 3) both methods produce diagnostic products from cinnamyl alcohol end-units,[22] which is exactly what is needed in this case. However, the most important piece required for solving the puzzle using a strategy involving degradation methods and GC- or LC-MS is the identification of the diagnostic products derived from the structures of interest. Currently we are testing some strategies to synthesize those diagnostic products that would be expected to be produced by thioacidolysis or DFRC methods. As might be appreciated, this cannot be a direct extension of this work as the A-ring moiety in **12** or the A and C ring moieties in **14** are simple models for a G-unit to provide the required NMR data; in lignin, these would need to be full β-ether units to release trimers with the diagnostic coniferyl alcohol signatures.

Conclusion

It has been demonstrated in vitro that 5-5′- or 5-*O*-4′-linked coniferyl alcohol units can cross-couple with coniferyl alcohol monomer forming the 5-5′- or 5-*O*-4′-linked pinoresinol products (in addition to other homo- and cross-coupled products). Eight new lignin model compounds have been synthesized and isolated from peroxidase-catalyzed biomimetic oxidative coupling reactions between two 5-linked coniferyl alcohol dimeric model compounds and coniferyl alcohol. It has been found that 5-linked coniferyl alcohols cross-couple with a coniferyl alcohol forming β-β′-, β-5′-, and β-*O*-4′-cross-coupled products analogously with the way that a sinapyl alcohol monomer cross-couples with a coniferyl alcohol monomer. With synthesized model compounds, it has been shown that 5-5′-linked and 5-*O*-4′-linked pinoresinols can be differentiated from each other in HMBC NMR spectra by examining correlations starting from α positions (both carbons and protons) of side-chains, which suggests the possibility of identifying 5-5′-linked and 5-*O*-4′-linked pinoresinol structures in softwood lignin once appropriate models are obtained through alkylation of the obtained model compounds in this work.

Experimental Section

Materials: All chemicals and solvents used in this study were purchased from Aldrich (Milwaukee, WI, USA) and used as supplied. Flash chromatography was performed with Biotage snap silica cartridges on an Isolera One (Biotage, Charlottesville, VA). All synthesized compounds were characterized by NMR spectroscopy and/or GC-MS methods. NMR spectra were acquired on a Bruker Biospin (Billerica, MA, USA) AVANCE 500 (500 MHz) spectrometer fitted with a cryogenically-cooled 5 mm TCI gradient probe with inverse geometry (proton coils closest to the sample) and spectral processing used Bruker's Topspin 3.1 (Mac) software. Standard Bruker implementations of one- and two-dimensional (gradient-selected COSY, HSQC and HMBC) NMR experiments were used for routine structural assignments of newly synthesized compounds. The conditions used for all samples were 5–10 mg in 0.5 mL NMR solvent ([D_6]acetone, [D_6]acetone) with the central solvent peaks ($\delta_H/\delta_C = 2.04/29.80$ ppm) used as internal reference. NMR assignment and high resolution mass data for all compounds synthesized in this work can be found in the Supporting Information.

Synthesis of 5-linked coniferyl alcohol models 1 and 2: Compound **15** was synthesized from vanillyl alcohol by silver (I) oxide oxidation in dry acetone according to a published method with modifications (Figure 5).[23] Thus, vanillyl alcohol (6.0 g, 38.9 mmol) was dissolved in dry acetone (200 mL) to which silver (I) oxide (13.5 g, 58.3 mmol) was added. The mixture was stirred at room temperature for 40 min when TLC ($CHCl_3$-EtOAc, 1:1, v/v) showed only small amount of starting material remaining. The reaction mixture was filtered through polyamide membrane (Whatman, 0.2 μm) and the resulting greenish filtrate was evaporated to give a brown syrup. The product was acetylated directly with pyridine/acetic anhydride (20 mL, 1:1, v/v) at room temperature overnight. Following co-evaporation with ethanol (5 times), the crude acetylated products were purified by flash chromatography (100 g silica gel column) using hexanes/EtOAc (2:1 v/v) to obtain pure compound **15** (2.0 g, 5.15 mmol, 26.5% yield). Compound **16** was made from the acetylated aldehyde **15** by using the Horner–Wadsworth–Emmons reaction. Diisobutylaluminium hydride (DIBAL-H) reduction of compound **16** produced the required compound **1** as an oil. Acetylated 5-5-divanillin **17** was synthesized from vanillin according to a published method.[23] Starting with compound **17** (Figure 5), a Horner–Wadsworth–Emmons reaction with triethyl phosphonacetate (0.5 equiv) accomplished the monoolefination to compound **18** that was not separated from the product mixture. A reduction using borane-*tert*-butylamine complex was used to reduce the aldehyde and to facilitate isolation of the target compound **19**. Thus, compound **19** was obtained in 78% yield over the two steps, following separation by flash chromatography (hexanes/EtOAc, 1:1, v/v). Finally, reduction of **19** by using DIBAL-H produced compound **2** in 90% yield.

Syntheses of 5-linked pinoresinol model compounds by using peroxidase-catalyzed free radical reactions: *5-O-4-linked coniferyl alcohol* **1** *cross-couples with coniferyl alcohol*: Compound **1** (300 mg, 0.90 mmol) and coniferyl alcohol (162 mg, 0.90 mmol) were dissolved in acetone (50 mL) to which phosphate buffer (150 mL, pH 5.0) was added. Then H_2O_2/urea complex (93.06 mg, 0.90 mmol) dissolved in buffer (5 mL) was added into the acetone-buffer system and followed by addition of horseradish peroxidase (EC 3.2.1.4, 2 mg in 2 mL buffer). The solution became bright yellow and then cloudy once the peroxidase was added in. The mixture was kept stirring at room temperature and monitored by TLC (CH_2Cl_2/MeOH, 20:1, v/v). The starting material disappeared in 60 min as shown by TLC. The reaction mixture was then acidified with HCl (1 N, 4 mL), extracted with ethyl acetate (3×100 mL). The combined organic phase was washed with saturated brine and dried over anhydrous $MgSO_4$, evaporated under reduced pressure at 40 °C. One part of the products (150 mg) were loaded onto 1 mm normal phase silica gel plates (about 50 mg/plate) and developed multiple times with methanol/dichloromethane (1:20) as the eluting solvent. Each isolated product was characterized by NMR. The above reaction was repeated and the products were acetylated with acetic anhydride-pyridine (1:1, v/v). One part of the acetylated products was analyzed directly, as a mixture, by NMR spectroscopy and

the remaining fraction was fractionated by TLC (cyclohexane/ethyl acetate, 2: 1, v/v, multiple development). Compound **3** (11 mg, 7.3% yield) and compound **4** (25 mg, 15% yield) were obtained as pale yellow oil. Compound $\mathbf{3}_{Ac}$ (acetylated **3**) or compound $\mathbf{4}_{Ac}$ (acetylated **4**) was obtained by acetylation of **3** or **4** with acetic anhydride/pyridine (1 mL, 1:1. v/v).

Although formed from the cross-coupling reaction, compound **6** was difficult to isolate. Instead it was readily obtained in 38% yield from a homo-coupling reaction with compound **1** alone under similar peroxidase catalyzed oxidation conditions. Compound $\mathbf{6}_{Ac}$ (acetylated **6**) was obtained by acetylation of **6** with acetic anhydride-pyridine (1 mL, 1:1. v/v).

5-5-linked coniferyl alcohol **2** *cross-couples with coniferyl alcohol*: Under similar conditions to those described above, the 5-5′-linked coniferyl alcohol model **2** (320 mg, 0.96 mmol) was also subjected to cross-couple with coniferyl alcohol. Following the normal workup the reaction mixture, a 96 mg aliquot was subjected to TLC fractionation (CH_2Cl_2/MeOH, 20:1, v/v) resulting in seven fractions. The coupling products **10**, **12–14**, plus the homo-coupled products **7–9** from coniferyl alcohol were obtained. The remaining material was acetylated with acetic anhydride-pyridine (1:1, v/v, 5 mL). The acetylated products were analyzed directly by NMR, then applied to TLC (cyclohexane/ethyl acetate, 2:1, v/v) to separate the compounds $\mathbf{10}_{Ac}$–$\mathbf{12}_{Ac}$.

Compound **10** was identified in fraction 4 (15 mg) accompanied by the β-5-coupled coniferyl alcohol dimer **9**. Further TLC separation of this fraction resulted in about 4 mg (5% yield) of pure compound **10**. Compound $\mathbf{10}_{Ac}$ (acetylated **10**) was obtained by acetylation of **10** with acetic anhydride-pyridine (1 mL, 1:1. v/v) and isolated from acetylated products. Compound **11** was in a fraction accompanied by other products. No attempt was made to further purify it. Instead compound $\mathbf{11}_{Ac}$ (acetylated **11**) was obtained in 6% yield by TLC of the acetylated products. Pure compound **12** (2 mg, 2% yield) was isolated as a clear oil from fraction 5. Compound $\mathbf{12}_{Ac}$ (acetylated **12**) was obtained in 3% yield by using TLC of the acetylated products. Compounds **13** and **14** were minor products of the cross-coupling reaction between **2** and coniferyl alcohol; no attempt made to isolate them from such a mixture. Instead, compounds **13** and **14** were synthesized from a homo-coupling reaction of compound **2** alone under similar conditions. Following normal workup it was possible to isolate compound **14** (15% yield) after two consecutive TLC purifications (CH_2Cl_2/MeOH, 20:1, v/v). However, it was difficult to obtain pure compound **13** from the homo-coupling reaction products. Thus some the products were acetylated and subjected to TLC (cyclohexane/ethyl acetate, 1:1, v/v) separation to isolate compounds $\mathbf{13}_{Ac}$ (acetylated **13**) and $\mathbf{14}_{Ac}$ (acetylated **14**). Compound $\mathbf{13}_{Ac}$ (20% yield) and compound $\mathbf{14}_{Ac}$ (22% yield) were obtained as clear oil. Compound **13** was obtained from $\mathbf{13}_{Ac}$ by deacetylation in 1.0 N NaOMe in MeOH.

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