Polymerization of Monolignols by Redox Shuttle–Mediated Enzymatic Oxidation: A New Model in Lignin Biosynthesis I

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Lignin is one of the most abundant biopolymers, and it has a complex racemic structure. It may be formed by a radical polymerization initiated by redox enzymes, but much remains unknown about the process, such as how molecules as large as enzymes can generate the compact structure of the lignified plant cell wall. We have synthesized lignin oligomers according to a new concept, in which peroxidase is never in direct contact with the lignin monomers coniferaldehyde and coniferyl alcohol. Instead, manganese oxalate worked as a diffusible redox shuttle, first being oxidized from Mn(II) to Mn(III) by a peroxidase and then being reduced to Mn(II) by a simultaneous oxidation of the lignin monomers to radicals that formed covalent linkages of the lignin type. Furthermore, a high molecular mass polymer was generated by oxidation of coniferyl alcohol by Mn(III) acetate in a dioxane and water mixture. This polymer was very similar to natural spruce wood lignin, according to its NMR spectrum. The possible involvement of a redox shuttle/peroxidase system in lignin biosynthesis is discussed.

INTRODUCTION

Lignin is a necessary constituent in the cell walls of all vascular plants. It is one of the most abundant biopolymers, and a considerable part of the carbon fixed by photosynthesis is consumed by lignin biosynthesis. The biological functions of the polymer are as follows: (1) They give stiffness and strength to the secondary wall of vascular plants. The lignified cell wall can be seen as a composite, with cellulose microfibrils as reinforcing fibers and lignin as a plastic matrix giving stiffness to the material. (2) They make the cell wall hydrophobic. This allows the development of tissues for efficient water transport in vascular plants. (3) They are an obstacle to microbial attack. The presence of lignin makes woody tissues so compact that molecules as large as proteins cannot penetrate the tissue (Blanchette et al., 1997). Thus, wood is much more resistant to microbial attack than are nonlignified cellulosic materials, such as cotton, and many wood-degrading organisms, such as brown rot fungi, do not degrade lignin completely; rather, they leave a modified residual lignin that is one of the main constituents of humus.

Lignin is polymerized in the wood from the monolignols sinapyl, coniferol, and p-coumaryl alcohol (Figure 1) (Freudenberg, 1968). Hardwood lignin (in dicotyledons) originates mainly from sinapyl alcohol and coniferol alcohol, whereas softwood lignin (in coniferous trees and gingko) is made mainly from coniferyl alcohol. However, compression wood in coniferous trees also contains significant amounts of lignin that originates from the monolignol p-coumaryl alcohol (Sjöström, 1993).

As a biomacromolecule, lignin is unusual in having a complex network–type structure that at a first glance appears chaotic (Figure 1). In fact, it is optically inactive (Sjöström, 1993). “Normal” biomolecules, such as cellulose and proteins, generally are synthesized in the active site of an enzyme (or enzyme complex) in nucleotide triphosphate–driven condensations, and the polymers formed have very defined structures (Mathews and van Holde, 1990). It is unlikely that lignin is synthesized in this way. First, lignin polymerization from monolignols is not a condensation. Second, many different types of bonds connect the monolignol residues in a somewhat random pattern. Third, lignin is racemic, which does not accord with enzyme-controlled biosynthesis.

Freudenberg (1959) demonstrated in a classic work that a polymer with the same types of chemical bonds as those in lignin could be obtained through the oxidation of coniferyl alcohol to resonance-stabilized radicals by a peroxidase. He suggested that the coupling of two unpaired electrons formed the covalent bonds. The polymerization could continue if a phenolic group on the lignin polymer became oxidized to a radical, either by a peroxidase or by a peroxidase-generated monolignol radical, and the phenolic radical on the polymer underwent coupling to a second monolignol radical. This theory accounted satisfactorily for the types of
covalent linkages that occur in lignin and for its racemic structure (Freudenberg, 1968).

However, synthetic lignin, or dehydrogenated polymer (DHP), prepared by the Freudenberg method displays a different ratio of covalent linkages and contains some bonds that do not occur in native lignin or that at least are very unusual, such as the combination of \(\alpha\)-O-4 and \(\beta\)-O-4 on the same monolignol residue (see Figure 1 for an explanation of the nomenclature). The most striking difference is that the \(\beta\)-O-4 linkage is much more common in lignin than in DHP, whereas carbon-carbon links such as 5-5 bonds are much more common in DHP than in native lignin (Lewis et al., 1998). However, a very slow addition of the monolignol to the polymerization mixture can give DHP, or at least dimers, with a very large proportion of \(\beta\)-O-4 linkages (Syrjanen and Brunow, 2000).

From a theoretical perspective, the high content of condensed structures in DHP is not unexpected, and the Freudenberg model of lignification has been challenged (Davin et al., 1997) by an alternative model in which the biological control over the lignification process is emphasized. It was suggested that the couplings of the peroxidase-generated monolignol and phenol radicals are performed by a separate enzyme, the dirigent protein (DiP); that is, the lignin polymerization is a two-stage process. Such a mechanism is known in the synthesis of lignans, a group of dimers and oligomers of monolignols (Davin et al., 1997). Biological control by DiP provides an explanation for the structural difference between DHP and native lignin (Lewis and Davin, 2000), and in support of the theory, DiP genes are expressed in cells during lignification. Immune microscopy also has revealed DiP inside cell walls under lignification (Kwon et al., 1999).

However, Ralph et al. (1999) emphasized that lignin is racemic and that a DiP-directed lignin ought to be optically active, as lignans actually are. “Abnormal” monolignols can be included in a functional lignin structure, which suggests a plasticity of lignin biosynthesis that does not accord with the idea of enzymatic control (Meyermans et al., 2000). Steric aspects also contradict the idea of DiP-controlled polymerization, because lignified cell walls are so compact that proteins are too large to diffuse within them (Blanchette et al., 1997). If DiPs controlled the formation of all covalent bonds in lignin, the lignin would be expected to contain large amounts of this enzyme trapped by the polymerization; to our knowledge, that has never been demonstrated. Certainly, there is room for new models for the polymerization of monolignols to lignin.

Westermark (1982) suggested that the primary oxidant for both phenolic end groups on the lignin and monolignols is a...
complex between Ca$^{2+}$ and the superoxide anion. The superoxide anion was supposed to be created by a redox enzyme and thus is involved in a kind of redox shuttle. DHP with lignin-type covalent bonds also was synthesized (using xanthine oxidase). However, to our knowledge, no superoxide-generating enzyme for lignifying tissues has been described. In this article, we present a modified Westermark model with Mn(III)/Mn(II) as a redox shuttle and demonstrate that such a system can generate lignin-type covalent bonds from monolignols.

**RESULTS**

The aim of the present study was to investigate the possibility that manganese peroxidase (MnP) could initiate the polymerization of monolignols into lignin-type structures without any direct contact between the enzyme and the monolignol. The function of Mn(II)/Mn(III) oxalate is as a redox shuttle carrying the oxidation potential (Figure 2).

We tested the idea by an experiment in which Mn(III) was...
generated by MnP and the enzyme was removed by ultrafiltration before the reactive species was mixed with the monolignol solution (see Methods). The resulting mixture was subjected to thioacidolysis and analyzed by size exclusion chromatography and gas chromatography combined with mass spectrometry. To test the plasticity of this system, both coniferyl alcohol and coniferaldehyde were used as monomers.

A second study was performed with a larger amount of Mn(III) (chemically generated) in a slightly acidic dioxane:water mixture, which is a good solvent for lignin oligomers. This experiment yielded an amount of DHP sufficient for subsequent analysis by NMR spectroscopy.

**Thioacidolysis Degradation Products of the DHPs**

Thioacidolysis is a lignin degradation method proceeding mainly by the cleavage of β-O-4 aryl ether structures. All noncondensed phenylpropane units participating in β-O-4 structures give rise to phenylpropane units with thioethyl groups on the side chain carbons. If two phenylpropane units are connected to each other via some of the common linkages in lignin (i.e., β-1, β-5, β-6, β-β, 5-5, or 4-O-5 with one or more β-O-4 linkages to the rest of the lignin polymer), the corresponding dimeric structure will be formed upon thioacidolysis. By analogy, trimers, tetramers, and higher oligomers form if the phenylpropane units contain the proper arrangement of acid-stable linkages.

The main thioacidolysis monomer products (i.e., phenylpropane units with three thioethyl groups in the side chain) can be identified as their silylated derivatives using gas chromatography, and this gives the content of noncondensed β-O-4 structures present in the lignin sample (Rolando et al., 1992). By incorporating a second step consisting of desulfuration over Raney nickel (a metal catalyst), thioethyl groups are removed, which makes it possible to identify oligomers using gas chromatography–mass spectrometry after acetylation and/or silylation of the products (Lapierre et al., 1991).

The yield of the enzyme-generated DHPs was ~5%, based on a gravimetric analysis of the DHP obtained relative to the amount of monolignol used. In the DHP prepared from coniferyl alcohol, several monomeric and dimeric products from these reactions were obtained (Figures 3 and 4). Compound 1 (Figure 3), corresponding to the main thioacidolysis monomer, was found at a concentration of 309 μmol/g lignin in the coniferyl alcohol DHP. Moreover, compounds 2 and 3 (Figure 3) correspond to the thioacidolysis products of coniferyl alcohol (Pan and Lachenal, 1993). Several dimeric products of the desulfuration step were identified (Figure 4). The compounds are mainly of the 5-5 and 4-O-5 biphenyl types. The dimers containing unsaturated side chains originate from a coniferyl alcohol phenylpropane unit, and those containing a saturated side chain originally were connected as β-O-4 structures. A β-5 dimer also was found in small amounts. Similar dimer analyses and typical mass fragments of thioacidolysis monomers and dimers have been described previously (Lapierre et al., 1991; Rolando et al., 1992).

**Size Exclusion Chromatography**

In Figure 5, chromatograms of the thioacidolysis products of normal spruce wood lignin and of enzyme-generated coniferyl alcohol DHP are compared. The DHP contains signif-
A New Model in Lignin Biosynthesis

Significant amounts of thioacidolysis monomers eluting at ~50 min and also a peak of dimers and oligomers that eluted somewhat earlier (Suckling et al., 1994). In the chromatograms, the arrow indicates the elution time of thioacidolyzed pinoresinol, which is a dimer used for calibration. The coniferyl alcohol DHP contains relatively more oligomer compounds than the spruce wood lignin. The coniferaldehyde DHP (Figure 6) shows even more oligomers than were found in the coniferyl alcohol DHP. Clearly, both of the monolignols have polymerized, and the DHP, upon thioacidolysis, gives monomers from noncondensed β-O-4 structures and dimer oligomers from acid-stable intermonomeric linkages, such as the 5-5 biphenyl bond.

NMR Spectroscopy

Quantitative 13C-NMR spectra of spruce milled wood lignin (MWL) and the DHP made by Mn(III) acetate in a dioxane:water solution are presented in Figure 7. The assignments and integrals listed in Table 1 show that the two lignins are very similar. Signal 1 is aromatic C2, which has an integration of ~1 in both samples. The abundance of the β-O-4 structure is very similar in these two samples, as indicated by signals 3 and 4. Signal 2 corresponds to C4 from β-5 structures, which also are fairly equal. Signal 6 contains the methoxy peaks and the Cβ from the β-β structures.

The content of the β-β structure is slightly higher in the DHP than in the spruce lignin. Signal 7 also corresponds to β-5 structures, but for the spruce MWL, this signal also contains β-1 structures, which explains the higher amount from signal 7 than from signal 2. The β-1 structure is not present in the DHP. The sample amount available for the DHP was not sufficiently large to allow an optimal signal-to-noise ratio, as shown in Figure 7 by comparison with the MWL spectrum.

DISCUSSION

It appears that MnP can initiate the polymerization of monolignols into lignin-type structures without any direct contact between the enzyme and the monolignol. Mn(II)/Mn(III) oxalate works as a redox shuttle, carrying the oxidation potential (Figure 2). The MnP used in this experiment is a fungal enzyme involved in lignin biodegradation (Gold et al., 2000). Whether such activity occurs in plants is an open question.

Because of limited access to the enzyme, we could not synthesize sufficient amounts of DHP for NMR characterization; therefore, we performed DHP synthesis with chemically generated Mn(III) in an acidic water:dioxane mixture. The NMR spectrum of this DHP was very similar to that of isolated softwood lignin. Although the conditions of the polymerization differed from natural conditions to a greater extent than in the enzymatic experiments, this result further supports the conclusion that lignin can be polymerized by a redox shuttle mechanism. Furthermore, a DHP similar to lignin can be made without the involvement of DIP.

Figure 5. Size Exclusion Chromatogram for the Thioacidolyzed Coniferyl Alcohol DHP and Normal Spruce Wood Lignin.

Thioacidolyzed pinoresinol, a dimer, was used for calibration. Its elution time is indicated by the arrow. This figure emphasizes the similarities between the natural lignin and the redox shuttle-made DHP. See Methods for details. The chromatograms are presented using a relative scale; that is, each chromatogram is normalized with respect to the highest peak in that chromatogram.

Figure 6. Size Exclusion Chromatogram for the Thioacidolyzed Coniferaldehyde DHP and Normal Spruce Wood Lignin.

Coniferaldehyde is an unusual monolignol. That lignin type bonds can be generated from this monomer by the redox shuttle mechanism illustrates the plasticity of the system, a feature known from experiments in vivo. The chromatograms are plotted on a relative scale as in Figure 5, and the elution time of thioacidolyzed pinoresinol is indicated by the arrow.
As demonstrated in the enzymatic experiment [the ultrafiltration of the Mn(III) solution took several minutes], Mn(III) is relatively stable, with a lifetime of several minutes or more, and can function as a diffusing redox shuttle as well as or better than the superoxide anion–calcium complex suggested by Westermark (1982). There are advantages to introducing a diffusing redox shuttle over the classic Freudenberg model. A small redox shuttle can easily penetrate compact structures to which proteins such as peroxidases and DiP do not have access and will oxidize all phenolic groups, both on monolignols and on the lignin polymer, much more easily than if this were done directly by an enzyme (for steric reasons).

The radicals on a monolignol and on the polymer are both primary oxidation products (i.e., oxidized by the same oxidant), whereas if the radical on the lignin polymer is generated by the oxidation of a monolignol radical, the polymer radical is a secondary oxidation product (i.e., oxidized by the primary oxidation product) and therefore is expected to occur at a lower concentration than the monolignol radical (the primary product). Therefore, the redox shuttle mechanism should lead to a greater frequency of polymer-monolignol coupling (the opposite of monolignol-monolignol coupling); that is, coupling one by one to a growing lignin chain is favored over dimerization.

The radicals on the polymer in the form of phenolic coniferyl alcohol end groups can couple to a monolignol through the phenolic oxygen, the 5-carbon, or the 1-carbon (Figure 8). The latter possibility is less likely (but not impossible) for steric reasons; thus, it seems probable that the couplings between the phenolic oxygen and the 5-carbon will predominate. If the monolignol radical couples preferably to the β-carbon, the predominant bond will be β-0-4, as in native lignin. In the classic Freudenberg model with the dimerization of monolignol radicals as the predominant reaction, on the other hand, different carbon–carbon bonds would be expected to constitute a considerable part of the bonds between monolignol residues, as is seen in synthetic DHP.
In support of the assumptions that a low molecular mass redox shuttle is involved in lignin biopolymerization, Landucci and co-workers made DHPs in an organic solvent using chemically generated Mn(III) as oxidant, and these DHPs displayed a pattern of covalent bonds similar to those in native lignin (Landucci, 1995; Landucci et al., 1995). It should be noted that polymeric lignin dissolves in organic solvents such as dioxane and acetone more easily than in an ethanol:water mixture. This could affect both the yield and the molecular mass of the polymers synthesized.

For this reason, we prepared a synthetic lignin from coniferyl alcohol in a slightly acidic dioxane:water mixture using chemically generated Mn(III). To our knowledge, this kind of solvent has not been used before, and the lignin obtained is very similar to an isolated lignin such as MWL. The DHP obtained from the experiment using coniferaldehyde indicates that the redox shuttle concept, as expected, is rather unspecific, which is in accord with the observed plasticity of lignin biosynthesis.

The results presented in this work suggest that a redox shuttle is involved in lignin biosynthesis, and this finding supports the hypothesis that a low molecular mass redox shuttle is involved in lignin biopolymerization.

### Table 1. Assignment and Integrals for Selected Peaks of the $^{13}$C-NMR Spectra for the Spruce MWL and the Coniferyl Alcohol Mn(III) Acetate DHP Given in Figure 7

<table>
<thead>
<tr>
<th>Signal Number</th>
<th>Assignment</th>
<th>Integral$^{\text{a}}$ Mn(III) Acetate DHP</th>
<th>Integral$^{\text{b}}$ Spruce MWL</th>
<th>Chemical Shift (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Aromatic C&lt;sub&gt;2&lt;/sub&gt; in G units$^{\text{c}}$</td>
<td>0.970</td>
<td>0.976</td>
<td>108.0–115.4</td>
</tr>
<tr>
<td>2</td>
<td>Aliphatic C&lt;sub&gt;4&lt;/sub&gt;, in β-5 structures</td>
<td>0.090</td>
<td>0.105</td>
<td>87.0–89.0</td>
</tr>
<tr>
<td>3&lt;sup&gt;\text{d}&lt;/sup&gt;</td>
<td>Mainly aliphatic C&lt;sub&gt;4&lt;/sub&gt; in β-O-4 structures</td>
<td>0.403</td>
<td>0.404</td>
<td>78.5–82.7</td>
</tr>
<tr>
<td>4&lt;sup&gt;\text{d}&lt;/sup&gt;</td>
<td>Mainly aliphatic C&lt;sub&gt;4&lt;/sub&gt; in β-O-4 structures</td>
<td>0.395</td>
<td>0.374</td>
<td>73.6–77.6</td>
</tr>
<tr>
<td>5&lt;sup&gt;\text{e}&lt;/sup&gt;</td>
<td>Aliphatic C&lt;sub&gt;8&lt;/sub&gt; in –CH&lt;sub&gt;2&lt;/sub&gt;OH structures</td>
<td>0.680</td>
<td>0.725</td>
<td>60.0–68.0</td>
</tr>
<tr>
<td>6&lt;sup&gt;\text{f}&lt;/sup&gt;</td>
<td>C in –OCH&lt;sub&gt;3&lt;/sub&gt;</td>
<td>1.093</td>
<td>1.060</td>
<td>53.5–59.5</td>
</tr>
<tr>
<td>7&lt;sup&gt;\text{g}&lt;/sup&gt;</td>
<td>Mainly aliphatic C&lt;sub&gt;8&lt;/sub&gt; in β-5 structures</td>
<td>0.075</td>
<td>0.136</td>
<td>49.0–53.0</td>
</tr>
</tbody>
</table>

$^{\text{a}}$Integrals were normalized by setting signal 6 to a value of 1.09, assuming 1.00 from the methoxy carbon and 0.09 from the β-β structure, which could be estimated according to the small signal at 73 ppm.

$^{\text{b}}$Normalization of integrals was performed according to Robert (1992).

$^{\text{c}}$G, guaiacyl.

$^{\text{d}}$Also contains small amounts of unassigned structures for MWL.

$^{\text{e}}$Not containing C<sub>4</sub> in pinoresinol structures or other C<sub>4</sub>-O-C<sub>4</sub> or α structures.

$^{\text{f}}$Also contains small amounts of β-β structures.

$^{\text{g}}$Also contains β-1 structures for MWL.

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**Figure 8.** Resonance Forms of Radicals on Monolignol and Lignin Polymer.

Resonance forms of a monolignol (coniferyl alcohol) radical and a phenolic radical on a lignin polymer. Covalent bonds are formed when two radicals meet. This figure shows that the possibilities for bonding are different for monomer and polymer radicals.
Manganese peroxidase (MnP) was obtained from Tienzyme (University Park, PA). Coniferaldehyde was obtained from Aldrich. Coniferyl alcohol was a kind gift from the Swedish Pulp and Paper Research Institute (Stockholm, Sweden). Mn(III) acetate dihydrate was purchased from Aldrich. All other chemicals were of analytical grade. Ultrafiltration units (for centrifugation) were from Filtron (Northborough, MA).

Polymerization of Coniferaldehyde and Coniferyl Alcohol Using Redox Shuttle–Mediated Enzymatic Oxidation

MnP (0.15 unit) was mixed in an ultrafiltration unit with a 10,000-D cutoff with Mn(ll)SO₄ and oxalic acid adjusted to pH 5.0 with NaOH to final concentrations of 1.5 mM Mn(ll) and 30 mM oxalic acid and a final volume of 1.6 mL. The manganese oxalate was taken from a colorless stock solution. [Fresh manganese oxalate solutions often are pink/violet as a result of contamination by Mn(III), but the color disappears quickly in solution as a result of disproportionation.] After 5 min of incubation at room temperature, a pink/violet color corresponding to the formation of Mn(III) oxalate by the “oxidase”-type reaction catalyzed by MnP (Gold et al., 2000) had developed.

The ultrafiltration units were placed in a centrifuge and spun for 12 min at 4000 rpm until most of the liquid had passed through the filter. The color remained in the now enzyme-free solution, and ~1 mL was added at room temperature to separate solutions of coniferyl alcohol and coniferaldehyde in water:ethanol (6:1, v/v). The reaction proceeded in a round-bottomed flask with magnetic stirring. A new portion of manganese sulfate and oxalic acid was added to the MnP in the ultrafiltration unit, incubated, and filtered, and the protein-free Mn(III) was added to the coniferyl alcohol and coniferaldehyde solutions. This procedure was repeated until four additions of Mn(III) oxalate had been made.

The monolignol solutions (coniferyl alcohol or coniferaldehyde) were incubated at room temperature for an additional 1 h, by which time the experiment had taken ~2.5 h. To ensure that no protein had leaked though the used filter, a solution of MnP was centrifuged until most of the liquid had passed through the unit. No protein was detectable in the filtrate by UV spectroscopy, nor did the filtrate have any detectable ability to oxidize Mn(III) oxalate to Mn(III) oxalate.

The coniferyl alcohol and coniferaldehyde solutions were rotary evaporated. The solvent-free material was rinsed three times with ~30 mL of water to remove salts and water-soluble products. The drops of water remaining after the last rinse were removed by rotary evaporation. One milliliter of acetone and then 1 mL of dioxane were added to dissolve the product mixture in the flask. The solutions were transferred to 5-mL glass vials, and the solvent was evaporated under a stream of nitrogen gas. The solvent-free glass vial was placed in a desiccator to dry the products.

Polymerization of Coniferyl Alcohol Using Mn(III) Acetate

A second dehydrogenated polymer (DHP) was obtained by dissolving coniferyl alcohol (200 mg) in 30 mL of dioxane:water (1:1, v/v). This solution was added dropwise to a solvent consisting of 40 mL of dioxane:water (1:1, v/v) containing 2 mL of acetic acid and saturated with Mn(III) acetate dihydrate (~3 g was used) under stirring. After the coniferyl alcohol solution had been added, the solution was filtered and the clear filtrate was rotary evaporated to remove the dioxane.

As the dioxane evaporated, DHP precipitated in the water. The water was removed by freeze-drying, and the solid DHP obtained was washed with 20 mL of dichloromethane in a beaker for 1 h, using a magnetic stirrer, to remove low molecular mass lignin compounds. The dichloromethane was removed by filtration on a filter funnel, and the washed DHP was acetylated (Gellerstedt, 1992). The acetylated DHP was dissolved in acetone-d₆ and characterized by NMR. The yield of the DHP was 50%.

Milled Wood Lignin

Spruce milled wood lignin (MWL) was prepared in the standard way (Björkman, 1956). The yield of the MWL was ~20%, calculated as grams of MWL per grams of Klason lignin. The MWL was placed in a beaker and washed with dichloromethane (20 mL) followed by ethyl acetate (20 mL) to remove low molecular mass lignin compounds. The solvent was removed by filtration on a filter funnel, and the residue was acetylated (Gellerstedt, 1992). The acetylated MWL (130 mg) was dissolved in acetone-d₆ (0.6 mL) before NMR analysis.

Thioacidolysis

Thioacidolysis experiments were performed as described by Rolando et al. (1992). In the calculation of the amount of the major monomeric degradation product, a conversion factor based on the yield of monomers (76%) from the thioacidolysis of a β-O-4 model compound (guaiacyl-glycerol β-guaiacyl ether) was used.

Raney Nickel Desulfuration

Raney nickel desulfuration was performed as reported by Lapierre et al. (1991).

Acetylation

Acetylation was performed on the samples degraded by thioacidolysis followed by Raney nickel desulfuration by dissolving the sample in pyridine:acetic anhydride (1:1, v/v) and allowing the reaction to proceed overnight as described by Gellerstedt (1992). Subsequently, the sample was dissolved in dichloromethane before gas chromatography–flame ionization detection (GC-FID) and gas chromatography–mass spectrometry (GC-MS) analyses.
GC-FID

The silylated monomeric products from thioacidolysis were quantified using GC-FID. The GC analysis was performed with a Hewlett-Packard 6890 instrument. For the quantification of monomeric products, the column used was Restek Corporation (Bellefont, PA) Rtx 5 (60 m, 0.25-µm i.d., 0.25-µm film thickness), and the temperature program used was from 180°C to 270°C at a rate of 4°C per min, stay at 270°C for 15 min, and from 270°C to 300°C at a rate of 40°C per min, stay at 300°C for 5 min. The injector was maintained at 250°C, and the detector was maintained at 280°C. Helium was used as carrier gas with an initial inlet pressure of 38.6 kPa using the constant flow mode. Split injection was used.

GC-MS

For the identification of monomeric and dimeric products, a DB 5MS column (J&W Scientific, Folsom, CA) was used (30 m, 0.25-µm film thickness). The detector, the interface, and the ion source were all maintained at 250°C. Helium was used as carrier gas. Split injection was used. For monomers, the temperature program was from 130°C to 260°C at a rate of 5°C per min, at 260°C for 10 min, and the initial helium inlet pressure was 61 kPa using the constant flow mode. For dimers, the temperature program was from 120°C to 200°C at a rate of 15°C per min, from 200°C to 260°C at a rate of 5°C per min, stay at 260°C for 15 min, from 260°C to 300°C at a rate of 20°C per min, stay at 300°C for 5 min, and the initial helium inlet pressure was 59 kPa using the constant flow mode. The mass spectrometer was a Finnigan Trace GC-MS device, 2000 series, operated at 70 eV for all GC-MS analyses.

Size-Exclusion Chromatography

Size exclusion chromatography was used for samples degraded by thioacidolysis and dissolved in dioxane:water (9:1, v/v). The system used consisted of three Ultrastrylar gel (Waters, Milford, MA) columns (100, 500, and 1000 Å, respectively) connected in series. The mobile phase was dioxane:water (9:1, v/v), and the flow rate was 0.6 mL/min using a Waters 515 HPLC pump. Detection was performed with a Waters 2487 UV light detector at 280 nm. Thioacidolyzed pinesosinol, a dimer containing a C3–C3 intermonomeric linkage, was used for calibration.

NMR

NMR experiments were run on an Avance 400-MHz instrument (Bruker, Billerica, MA) with a 5-mm broad-band probe. Quantitative 13C experiments were performed with the inverse gated proton decoupling sequence. A pulse angle of 70° and a delay time of 12 s were applied during data acquisition. A spectral window of 240 ppm was used with 32,000 data points, which gave an acquisition time of 0.678 s. A total of 6800 scans were collected for each sample (experimental time of 1 day).

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