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A New In Vitro Model of Lignin Biosynthesis

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There are still many open questions concerning the structure as well as the peculiar biosynthesis of lignin.[1–3] The most accredited theories go back to the early 1960s, when Freudenberg and co-workers proposed dehydrogenation reactions as the dominating framework of a polymerization process which involves *p*-hydroxycinnamyl alcohols as the primary precursors and building blocks of all lignins.[4, 5] Numerous structural investigations of isolated and synthetic lignins and of model compounds then outlined a composite picture of a very complex, three-dimensional polymeric structure devoid of ordered repeating units, such as in the case of other biological macromolecules.[1, 5–11] According to these studies lignin production is triggered by the enzymatic formation of resonance-stabilized phenoxy radicals, while the polymerization is generally known to run as a non-enzymatic process which advances in a random fashion through radical and ionic steps. We now report that under in vitro homogeneous conditions—which, in principle, could be related to the natural microenvironment[12, 13]—a synthetic lignin is produced. The beginning of the polymerization process appears to be an alternating sequence of radical and ionic steps which generate ordered structures of oligolignols.

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The polymerization of the monomeric precursor by random coupling reactions cannot be studied in vivo; many theories on lignin structure and biosynthesis rely upon in vitro experiments on the polymerization of coniferyl alcohol (**1**, Figure 1),[5, 7, 14] which is the most common natural lignin

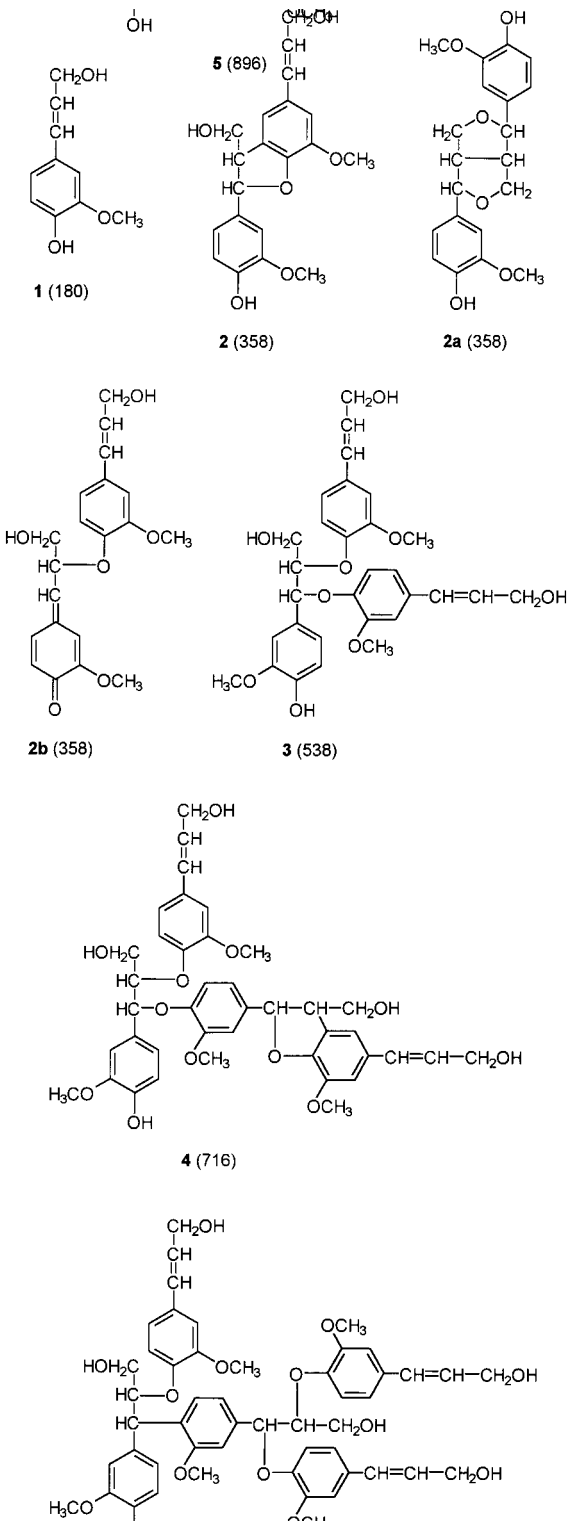

Figure 1. Schematic representation of oligolignols (molecular weights are given in parentheses).

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precursor, followed by isolation and structural determination of the produced polymer. The reaction is in general conveniently accomplished[15] by slow mixing, over a period of several hours, of a highly diluted solution of the precursor[16] with a buffered solution of H_2O_2 in the presence of horseradish peroxidase,[17] which serves to initiate the polymerization process. The presence of a small quantity of a polar, water-soluble material like guaiacylglycerol, which is able to copolymerize with coniferyl alcohol, is also recommended in order to keep the growing polymer in solution as long as possible. This synthetic lignin, commonly referred to as dehydrogenative polymerizate (DHP),[5] generally very rapidly separates from the reaction medium, thus subtracting itself from a possible higher degree of polymerization. We have analyzed this material[18] by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS)[19] and deduced that the radical mechanism is preferred over the competing ionic mechanism for the polymerization process. Following our continuing interest in lignin biosynthesis, we have found that horseradish peroxidase is still highly active in a phosphate buffer solution of cetyltrimethylammonium sulfate ($(CTA)_2SO_4$) at a concentration higher than the critical micelle concentration (c.m.c. $= 2.7 \times 10^{-4}$ M). When tested at a surfactant concentration of 2.7×10^{-2} M with *o*-phenylenediamine as the model substrate in a buffered solution of H_2O_2, a decrease in activity of only twofold with respect to the experiment performed in the absence of surfactant is observed. We also checked that, under these experimental conditions, micelles actually formed. Thus, we were able to polymerize coniferyl alcohol, keeping the final polymer in solution for an indefinite period.

Fundamental results regarding the structures of the oligolignols formed in our experiments and the polymerization mechanism involved came from electrospray ionization mass spectrometry (ESI-MS)[20] combined with HPLC; the reaction mixture was analyzed when the polymerization process was interrupted at the beginning, that is, after five minutes. We used a mass spectrometer with an ESI interface as a detector after HPLC separation of the mixture (Figure 2), employing a postcolumn doping of the eluate with ammonium acetate. The instrument[21] gave the masses of the $[M+NH_4]^+$ ionic species of each oligomer (**1**–**6** (see Figure 1 for the structures of **1**–**5**).[22] By direct infusion of the whole mixture into the mass spectrometer ion source, even the heptamer (**7**) and the octamer (**8**) are revealed, as it is apparent from the mass spectrum (Figure 3). Also it is clearly evident that all the oligomers have definite masses, since other products with masses close to the indicated ones are practically absent (the satellite peaks at higher masses are due to the sodiated and potassiated oligomers).

What is new and totally unexpected from our experiment is the high regularity of the oligomeric units, which differ from one other by mass units of, alternatingly, about 178.1 and 180.1. As the molecular weight of coniferyl alcohol is

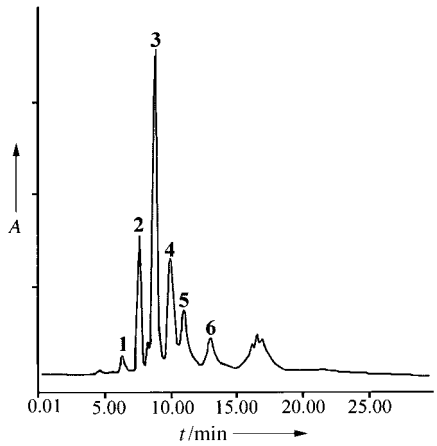

Figure 2. HPLC profile of the oligolignol mixture. Column: RP-18 (12.5 cm, 4.6 mm inner diameter); eluent A: H_2O; eluent B: CH_3CN; gradient B: 0→40% in 15 min; flow rate: 0.8 mL min^{-1}; UV detection at 268 nm. Compound **2a** coelutes with **2**.

180.1, these mass increments imply that the two reaction mechanisms, the radical and the ionic ones, are not operating randomly, but in parallel in a coordinate process. Coupling of two radical moieties would give a mass increment of 178.1; conversely, the ionic mechanism, formally consisting of the addition of one monomer unit to a quinone methide intermediate, leads to an increment of 180.1.

On the grounds of the ESI-MS data it is possible to propose the biosynthetic pathway which is reasonably followed to produce the oligolignols we have detected under our biomimetic conditions. Both the dimers **2** and **2a** ($M_r = 358$ each) are formed by one radical step (formally, the monomer (with 180) plus 178). In all the other cases, the key step is the production of the dimeric quinone methide intermediate (**2b**, also formed by one radical step). This compound is then able to add coniferyl alcohol (**1**) to form the trimer **3** (i.e., guaiacylglycerol-β,γ-bis-coniferyl ether, $M_r = 538$); alternatively, it can add dehydrodiconiferyl alcohol (**2**), or pinoresinol (**2a**), to form the tetramer **4** (i.e., guaiacylglycerol-β-coniferyl-γ-dehydro-diconiferyl ether, $M_r = 716$). In the former case, one radical step and a ionic step are involved (formally the dimer (with 358) plus 180); in the latter case, two radical steps (to form the intermediate dimers **2b** and **2**) and an ionic step are necessary (formally the trimer (with 538) plus 178). The pentamer **5** ($M_r = 896$) is formed through two ionic and two radical steps, by the addition of the trimer **3** to the quinone methide **2b** (formally the tetramer (with 716) plus 180).

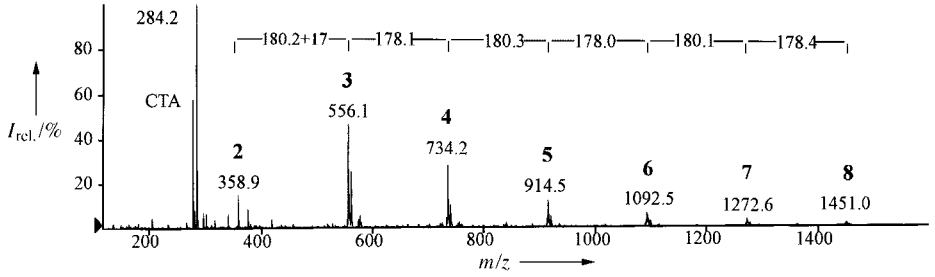

Figure 3. ESI mass spectrum of the oligolignol mixture (direct infusion): NH_4^+ adducts (H^+ adduct for **2**); CTA = cetyltrimethylammonium ion. The mass differences between the peaks are reported.

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Our study is the first reported demonstration that model lignins can be efficiently produced in vitro, in an aqueous environment, under homogeneous conditions; the presence of the micelles is able to keep in solution all the lipophilic reagents and reaction products, with practically no detriment to the catalytic efficacy of the enzyme. Moreover, since the activity of an enzyme under micelle conditions could be considered more similar to the cell system than in buffer alone,[12, 13] we propose that in our case the polymerization mechanism which drives oligolignol formation is closer to the natural process than are the other, well-established in vitro biosynthetic experiments. In conclusion, our results show that in lignin biosynthesis the ionic and the radical mechanisms operate effectively in parallel to the same extent, and they formally appear as strictly alternate steps. These results are also in favor of a more recent concept of a structural scheme for natural lignins which assumes an ordered structure with repeating units.[23, 24]

Experimental Section

In a typical experiment 20 mg of coniferyl alcohol are dissolved in 3.6 mL of sodium phosphate buffer (0.01M; pH 6.5) containing $(CTA)_2SO_4$ at a concentration of 2.7×10^{-2}M; to this solution are added sequentially 130 μL of 3% H_2O_2 and 20 μL of buffer containing seven purpurogallin units of horseradish peroxidase (type II, 200 U mg^{-1}, Sigma). The clear solution, where the substrate is at a concentration of 5.3 g L^{-1}, is stirred over a period of 5 min. After this time, which is sufficient for the substrate to be consumed (HPLC analysis), the reaction is stopped by the addition of a few drops of 5% $Na_2S_2O_7$, and the mixture thoroughly extracted with ethyl acetate. The organic layer is then washed with brine and dried, and the solvent evaporated under vacuo to give approximately 23 mg of product.

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Highly Substituted Spiro[4.4]nonatrienes from a β-Amino-Substituted α,β-Unsaturated Fischer Carbene Complex and Three Molecules of an Arylalkyne**

Heiko Schirmer, Michael Duetsch, Frank Stein, Thomas Labahn, Burkhard Knieriem, and Armin de Meijere*

Dedicated to Professor Klaus Kühlein on the occasion of his 60th birthday

In recent years α,β-unsaturated Fischer carbene complexes have established themselves as valuable functional building blocks in organic chemistry.[1] In particular, β-amino-substituted alkenylcarbene complexes exhibit a highly diverse reactivity depending upon the nature of the substituents and reaction conditions.[2] These complexes are readily available from alkynylcarbene complexes by 1,4-addition of amines to 1-metalla-1-en-3-ynes[3] and undergo inter alia formal [3+2],[4] [2+2+1],[5] and [4+2] cycloadditions[4c, 6] with alkynes. In contrast, (3-dimethylaminoalkenylidene)chromium complexes with sterically demanding substituents on the alkenyl terminus, and therefore normally with a *Z* configuration, react preferentially by sequential insertion of two alkyne units and carbon monoxide and only then undergo cyclization, that is, they give cyclopenta[*b*]pyrans by a formal [3+4+1] cycloaddition, frequently in high yields.[7] Although it also has a sterically demanding substituent and a *Z* configuration, the

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