Non-enzymatic reduction of quinone methides during oxidative coupling of monolignols: implications for the origin of benzyl structures in lignins†

Anders Holmgren,[a] Gösta Brunow,[b] Gunnar Henriksson,[a] Liming Zhang*[a] and John Ralph[c]

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Lignin is believed to be synthesized by oxidative coupling of 4-hydroxyphenylpropanoids. In native lignin there are some types of reduced structures that cannot be explained solely by oxidative coupling. In the present work we showed *via* biomimetic model experiments that nicotinamide adenine dinucleotide (NADH), in an uncatalyzed process, reduced a β-aryl ether quinone methide to its benzyl derivative. A number of other biologically significant reductants, including the enzyme cellobiose dehydrogenase, failed to produce the reduced structures. Synthetic dehydrogenation polymers of coniferyl alcohol synthesized (under oxidative conditions) in the presence of the reductant NADH produced the same kind of reduced structures as in the model experiment, demonstrating that oxidative and reductive processes can occur in the same environment, and that reduction of the *in situ*-generated quinone methides was sufficiently competitive with water addition. *In situ* reduction of β–β-quinone methides was not achieved in this study. The origin of racemic benzyl structures in lignins therefore remains unknown, but the potential for simple chemical reduction is demonstrated here.

Introduction

The cell walls of mature secondary xylem cells in woody plants are generally strong, stiff, relatively resistant to microbial degradation, and have low water absorbing capacity. These properties are necessary for fulfilling the functions of water conductivity and mechanical support of various types of wood cells, as tracheids, vessels and libriform fibers. Plants have developed a composite type material suitable for these functions consisting mainly of *cellulose*, a β-1,4-homoglucan, *hemicelluloses*, a group of often branched heteropolysaccharides, and *lignin*, a racemic, branched phenylpropanoid polymer. The woody cell walls also contain smaller amounts of proteins, extractives (various types of low molecular mass organic compounds), and inorganic materials.[2,3] The cellulose chains form crystalline bundles, *fibrils*, that function as enforcing fibers, while hemicelluloses and lignin play the role of a flexible connecting plastic.[3,4] The biosynthesis of this composite is complex: cellulose is synthesized directly outside the cell-membrane by complexes of membrane proteins that create a cellulose fibril consisting of about 64 or more chains,[3] while hemicelluloses are synthesized in the Golgi apparatus, excreted to the cell wall through budding of Golgi vesicles, and thereafter precipitated.[5] As for cellulose biosynthesis, hemicellulose polymerization is thermodynamically-driven by a coupled hydrolysis of nucleotide triphosphates.[6,7] Lignin, on the other hand, is believed to be synthesized from its monomers, primarily the three monolignols (Fig. 1a), *in situ* into a gel consisting of mainly cellulose and hemicellulose. According to a widely

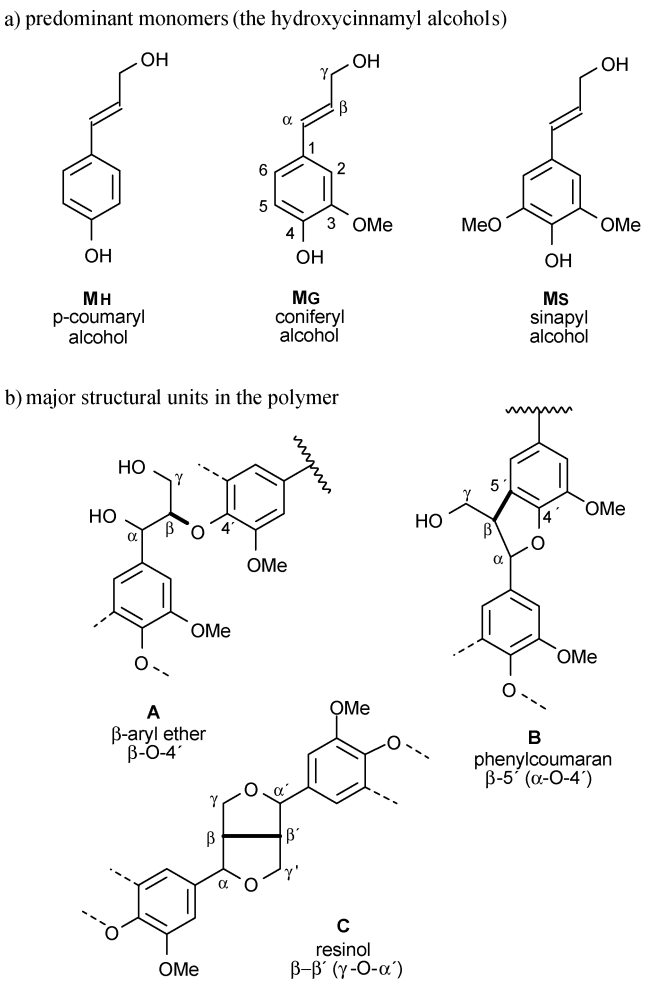
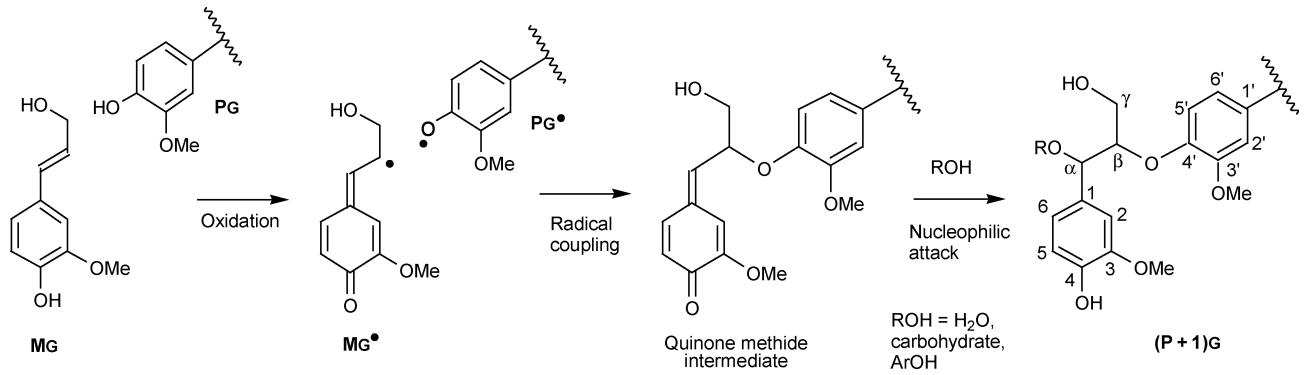


Fig. 1 (a) Monolignols (the lignin monomers) and (b) the major structures in lignins.

[a]*Department of Fiber and Polymer Technology, School of Chemical Sciences, Royal Institute of Technology, KTH, 100 44 Stockholm, Sweden. E-mail: liming@polymer.kth.se; Fax: +46 8 7096166*
[b]*Department of Chemistry, University of Helsinki, Helsinki, Finland*
[c]*US Dairy Forage Research Center, USDA-Agricultural Research Service, Madison, WI 53706, USA*
† A new model in lignin biosynthesis II (for part I, see ref. 16).

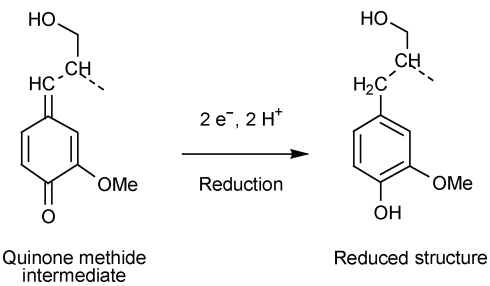


Scheme 1 Mechanism for formation of a covalent bond, the β-O-4′-bond, in lignins. In the last structure, the nomenclature for inter-monomer bonds in lignins is explained; the aliphatic side-chain carbons are denoted α, β and γ with α being the benzylic position, *i.e.* closest to the aromatic ring. The carbons in the aromatic ring are numbered with '1' for the carbon connected to the aliphatic carbon chain. **MG** is coniferyl alcohol, **PG** is a guaiacyl end-group on the lignin polymer and **(P + 1)G** is the coupling product of coniferyl alcohol to the polymer.

accepted model originally presented by Freudenberg and Neish,[8] this takes place by an uncatalyzed radical coupling of resonance-stabilized monolignol radicals and phenolic radicals on the lignin polymer, followed by nucleophilic additions to the quinone methide intermediates, as explained in Scheme 1. Aromatization of β-ether quinone methides, usually *via* water addition, yields β-aryl ethers **A** (Fig. 1b). Covalent bonds (ethers and esters) between lignin and polysaccharide can form if quinone intermediates are trapped by polysaccharide hydroxy groups.[9–11] Phenolic or aliphatic hydroxy groups on the lignin itself can also perform the nucleophilic attack creating intra-lignin ethers in phenylcoumaran (β-5′-coupled) units **B** and in resinol (β–β′coupled) units **C**.[8] The phenolic radicals are probably generated either directly by an enzyme such as peroxidase,[12–14] or possibly indirectly *via* a redox shuttle of some kind.[15,16]

Although the Freudenberg model explains the large majority of lignin structures, a number of structures that cannot be explained by this chemistry alone have long been noted in lignins, including the reduced lignin structures[1,17] described in Fig. 2. Reduced β–β′-structures are especially interesting, since they are routinely observed in softwood lignins by NMR,[1,18] and by degradative methods.[19] There is some evidence that the common type of β–β′-structure, *i.e.* pinoresinol units **C** (Fig. 1 and Scheme 1 for a nomenclature explanation of lignin bonds), is frequently 5-linked in the structure of lignin, whereas the reduced β–β′-structures normally seem to be simply etherified (*i.e.* by β-O-4-bonds).[1]

A possible explanation for the presence of reduced structures in native lignins is that the quinone methide intermediate formed during lignin biopolymerization is, in some way, two-electron reduced (Scheme 2), instead of being subject to a nucleophilic attack by a hydroxy group. Although enzyme-driven reductions could possibly explain reduced structures in lignin, secoisolariciresinol products released from lignins (by methods shown to not scramble the β–β′-stereochemistry) have been shown to be racemic.[18] ‡ There is a number of potential reducing agents synthesized by living organisms that could potentially reduce quinone methide structures, generated during lignification, in uncatalyzed reactions. In this work we study and discuss the formation of the reduced lignin structures based on results of model experiments and synthetic dehydrogenation polymers.



Scheme 2 Reduced structures can be created by two-electron reduction of quinone methide intermediates.

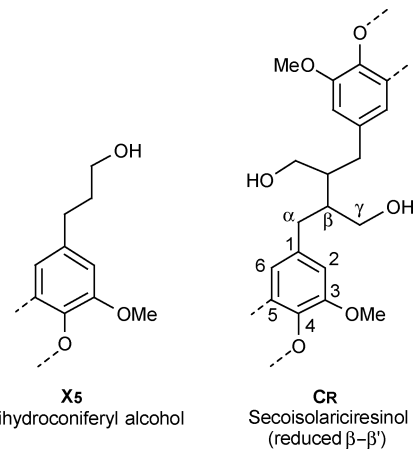


Fig. 2 Reduced lignin sub-structures that cannot be directly explained by radical coupling reactions and nucleophilic attack, according to Scheme 1.

Results and discussion

We tested four low molecular mass reducing agents produced by living cells for their abilities to reduce a model compound for the β-ether quinone methide intermediate in lignin biopolymerization: nicotinamide adenine dinucleotide (NADH), reduced glutathione,

‡ Note that in this reference, as detailed recently,[16] the DFRC product of secoisolariciresinol units was originally incorrectly attributed to pinoresinol units.

both being common reducing agents produced in virtually all living cells,[20] ascorbic acid, known as a redox buffer agent in plant cell walls,[21] and α-ketoglutaric acid, intermediate in the citric acid cycle. Furthermore, the extracellular enzyme cellobiose dehydrogenase (CDH), that carries both flavin and heme prosthetic groups,[22] known to have a broad specificity for reducing various compounds including quinones, was included in the screen as an example of a reducing enzyme. Sodium borohydride was used as a positive control reducing agent.

Reduction of the β-ether quinone methide model was achieved using NADH or $NaBH_4$, but with none of the other reducing agents tested, including the enzyme CDH. The treated model compound was analyzed by GC–MS. The gas chromatogram of the sodium borohydride-treated sample displayed a single peak. An equivalent peak was found in the chromatogram of the NADH-treated sample (peak labeled with **2**, Fig. 3). In each case the peak exhibited a mass spectrum corresponding to the silylated reduced model compound **2** (Scheme 3). To confirm the identity of the product, three NMR experiments (^1H, ^{13}C-DEPT-135 and 2-D HSQC) were run on the sodium borohydride-treated sample. DEPT-135 spectra confirmed that the α-carbon was a methylene group by its inversion, like the γ-carbon, relative to the methoxy, β- and aromatic C–Hs.

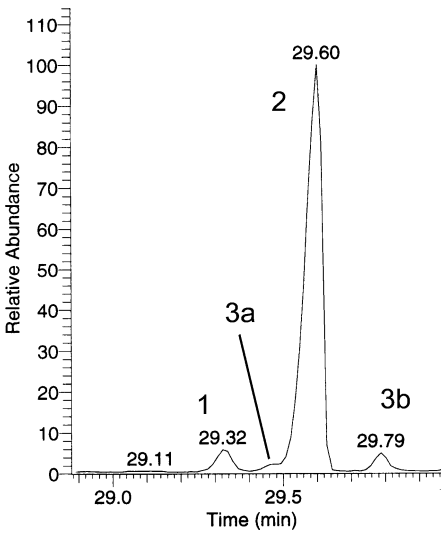
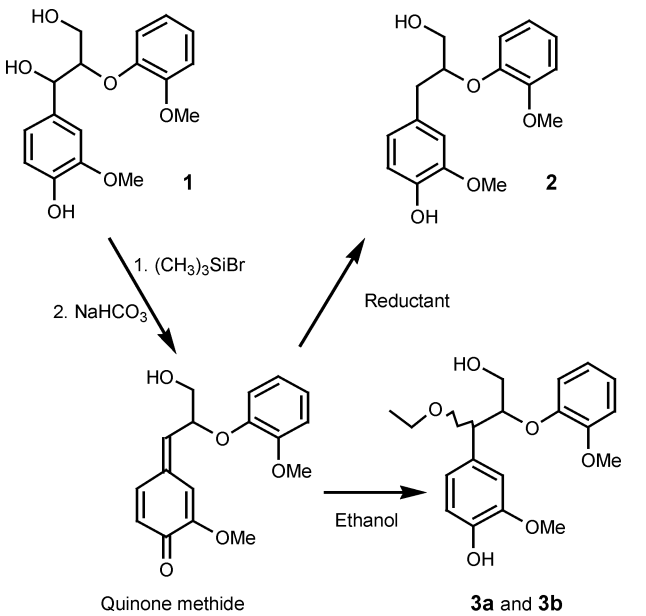


Fig. 3 GC chromatogram of the silylated products of the quinone methide reduction with NADH. Peaks are labeled with their structures from Scheme 3; **1** is guaiacylglycerol-β-guaiacyl ether, **3a** and **3b** are the two isomers of the ethanol addition product, and **2** is the reduced quinone methide.

Synthetic dehydrogenation polymers (DHPs) were prepared in the presence NADH to test the reducing capabilities of NADH in an oxidizing environment such as that involved in lignin polymerization. The interest here was to determine if oxidation (radical generation from the phenol) and reduction could proceed in the same reaction medium. Even though conventional dehydrogenation polymers differ in unit proportions from isolated lignins, as reviewed,[19] they reproduce essentially all of the known inter-unit linkages, and derive from the same chemical steps to achieve polymerization: oxidation of monolignols/oligomer, radical–radical coupling forming a quinone methide intermediate which is finally



Scheme 3 Model compound guaiacylglycerol-β-guaiacyl ether **1** is converted to a quinone methide by a two-step reaction and then exposed to a reductant in the presence of ethanol to give the benzyl compound **2** along with ethanol addition product **3** (as a mixture of isomers).

attacked by some intra- or extra-molecular nucleophile. In this work, HSQC spectra of the DHPs showed the presence of β-aryl ether **A**, phenylcoumaran **B**, resinol **C**, and α,β-diaryl ether **A2** structures (Fig. 4) which are common in conventional DHPs.[10,23] Furthermore, reduced β-ether structures **AR** (Fig. 4) of the same type as compound **2** (Scheme 3) were observed from DHPs made in the presence of NADH. The diagnostic coupling network is elegantly revealed by HSQC-TOCSY experiments (Fig. 5). Disappointingly, secoisolariciresinol units **CR** (Figs. 2 and 4) were not observed, nor were dihydroconiferyl alcohol (**X5**, Fig. 2) moieties found.

Biological significance

The results in this work demonstrate that it might be possible to obtain lignin-reduced structures in uncatalyzed protein-independent reactions using a biologically significant reductant such as NADH. Whether it is this compound that *in vivo* is responsible for the reduction, a similar compound, or an enzyme system, is not known. The inability of CDH to reduce the quinone methide here does not imply that there are no other enzymes that can catalyze the reaction. The present results show that direct involvement of enzymes may not be *necessary* to clarify the occurrence of these lignin structures. The detection of reduced β-aryl ether units **AR**, at substantial levels (*ca.* 48% of the β-ethers) in the synthesized lignins, indicates that NADH was able to trap and reduce the β-aryl ether quinone methide in an oxidative radical–radical polymerization system. NADH was not, however, able to produce reduced β–β′-structures in detectable amounts, so conditions and/or reductants for such a process remain elusive. Presumably the reduction of the α-carbon of a β-aryl ether quinone methide competes with a nucleophilic attack by an external nucleophile (water or a phenol), whereas

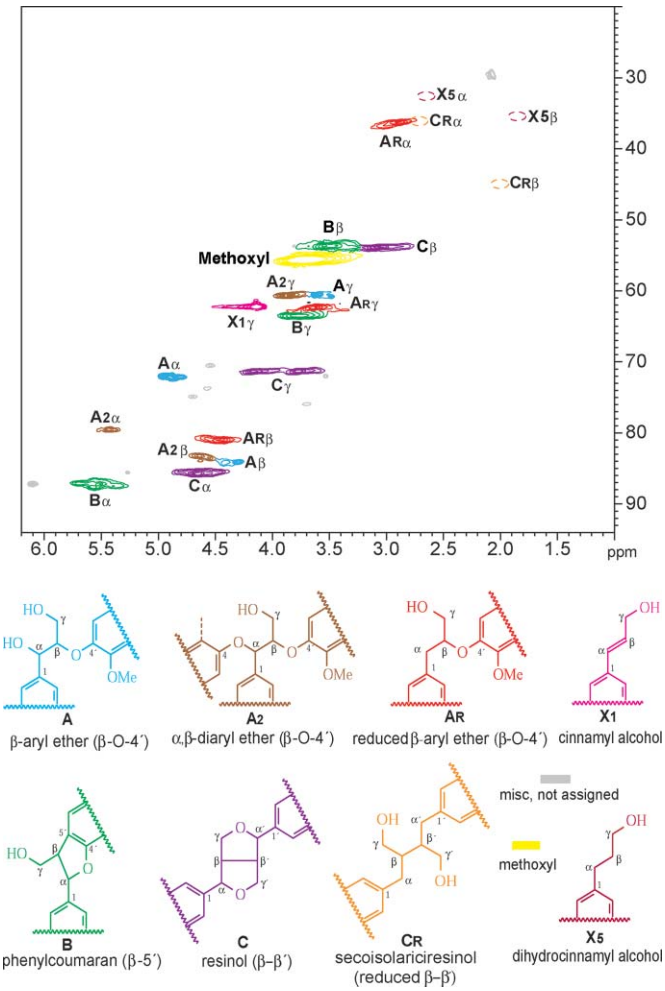


Fig. 4 HSQC spectra of a DHP synthesized in the presence of NADH. **A** corresponds to β-aryl ether, **A2** to α,β-diaryl ether, **AR** to reduced β-aryl ether, **B** to phenylcoumaran, **C** to resinol, **X1** to coniferyl alcohol end-groups. **CR** and **X5** correspond to secoisolariciresinol (reduced β–β′) units and dihydroconiferyl alcohol end-groups, with their expected correlations according to data from Zhang *et al*.[1]

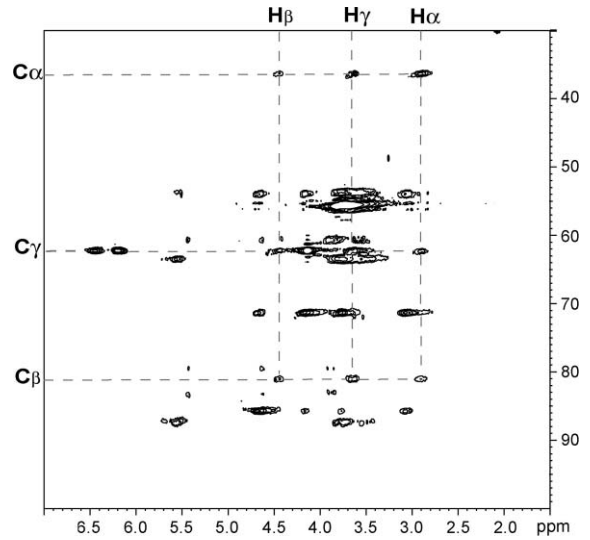


Fig. 5 HSQC-TOCSY of a DHP synthesized in the presence of NADH. The dashed lines represent the proton–carbon correlations on the propanoid chain of the reduced β-aryl ether structure **AR** (*cf.* **2** in Scheme 3).

the β–β′- or β-5′-quinone methides have intramolecular hydroxy groups out-competing the attack on the α-carbon. Since we observed resinol and phenylcoumaran structures in the DHPs, NADH was apparently not effective in competing against the fast intramolecular reactions in the polymerization process under these conditions.

So far, reduced structures have only been discovered in end-groups **X5** and in β–β′ **CR** units in natural lignins.[1,17,24,25] Unfortunately, in this work we have not detected any reduced β–β′-structures **CR**, but succeeded in producing reduced β-O-4′-structures **AR**. Whether this is due to limitations in the detection methods or, more probably, to a difference in the structure and the polymerization environment between lignin and DHPs cannot be definitively confirmed. DHP systems are known to be sensitive to polymerization conditions.[26] If the latter explanation is the case, then there must be some kind of mechanism directing the reducing effect towards β–β′-units in lignin polymerization. Enzymatic catalysis remains possible, but other options such as low molecular weight catalysts, or unusual conditions during the reduction, remain to be eliminated. One possibility is that the conditions during part of the natural biosynthesis of lignin in some way give more long-lived β–β′-quinone methides than in our *in vitro* experiments.

A reduction of a quinone methide with NADH costs the plant three ATP equivalents of energy.[27] It is therefore logical to question the reason for reduced structures in lignins unless they are simply to be attributed to the results of coincidental side-reactions. In an earlier work[1] it was shown that the reduced β–β′-units seemed to have been created as dimers, *i.e.* according to bulk polymerization. Thus, it might be possible that under some period in the cell wall synthesis, high biochemical activity includes the presence of reducing agents in concert with fast lignin polymerization leading to bulk polymerization and reduction of quinone methides, whereas under other periods, reducing agents are absent and the lignification rate is slower, giving an end-wise polymerization. A lignin polymer with reduced structures would have different properties than 'normal' lignins, such as higher hydrophobicity and a lower propensity for hydrogen bonding or formation of covalent bonds to polysaccharides through α-ethers and α-esters. Additionally, a higher flexibility of the β–β′-bonds would be expected due to the absence of the tetrahydrofuran ring structures. Therefore, reduction might be the way for the plant to manipulate the properties of some of its lignin.

Conclusion

The reduction of a β-aryl ether quinone methide model compound was achieved by non-enzymatic catalysis using the biologically significant reducing agent, NADH. Moreover, synthetic lignins (DHPs) synthesized in the presence of NADH produced analogous reduced structures, indicating that reduction of quinone methides can be achieved by biological reductants present in the oxidative environment of lignin polymerization. We have so

far failed to demonstrate enzyme-free reduction of β–β′-quinone methides. The origin of racemic benzyl structures occurring in lignins therefore remains unknown, but we have been able to demonstrate the potential for simple chemical reduction as a part of the Freudenberg oxidative model for lignin biosynthesis.

Experimental

Materials

Guaiacylglycerol-β-guaiacyl ether was purchased from TCI Europe NV, Zwijndrecht, Belgium, L-glutathione (reduced form), α-ketoglutaric acid, β-nicotinamide adenine dinucleotide reduced disodium salt (NADH), *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), D-(+)-cellobiose, horseradish peroxidase (HRP) type VI and coniferaldehyde were purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden. Bromotrimethylsilane (TMSBr) and L-(+)-ascorbic acid were from E. Merck AB, Sweden. Cellobiose dehydrogenase (CDH) was a kind gift from Gunnar Johansson at the Department of Biochemistry, University of Uppsala, Sweden, and was purified from *Phanerochaete chrysosporium* according to Henriksson *et al*.[28] All other chemicals were of analytical grade. Ultrafiltration units (for centrifugation) Nanosep 10 K were from Pall Corporation, New York.

Quinone methide reduction

Guaiacylglycerol-β-guaiacyl ether [1-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-propane-1,3-diol] **1** (Scheme 3) was used to prepare the quinone methide *via* the benzylic bromide generated *via* TMSBr.[29,30] Dichloromethane was evaporated at high vacuum on ice and the quinone methide dissolved in 99.5% ethanol and kept on ice. The reducing agent was dissolved in potassium phthalate buffer (50 mM, pH 5) and the quinone methide was slowly added to the buffer. The final reaction mixture contained 10% ethanol and the ratio of quinone methide to reducing agent was 1 : 10. The reaction was stirred overnight at room temperature under nitrogen. The mixture was extracted twice with dichloromethane, and dried with Na_2SO_4. The solvent was evaporated and the crude product was silylated with BSTFA for 1 h, as described elsewhere.[31] When testing the enzyme CDH, 0.13 ml of the enzyme solution (10.9 μM) and 6 mg D-cellobiose (co-factor) were dissolved in potassium phthalate buffer prior to quinone methide addition. After the reaction, the enzyme was separated from the mixture by centrifugation in ultrafiltration units and the filtrate extracted and dried as previously described.

A reference sample of compound **2** was prepared by reducing the quinone methide model with $NaBH_4$.[32,33] The quinone methide was prepared as described previously, dissolved in dichloromethane and stirred with aqueous $NaBH_4$ until the pale yellow color disappeared. The organic phase was separated, dried with Na_2SO_4 and evaporated. For GC–MS, the crude product was silylated as described previously.

Synthesis of dehydrogenation polymers (DHPs) under reductive conditions

Coniferyl alcohol was obtained from the reduction of coniferaldehyde with $NaBH_4$.[34] Coniferyl alcohol (200 mg) were dissolved in acetone (10 ml) and mixed together with potassium phosphate buffer (90 ml, 25 mM, pH 6.0) containing NADH (310 mg) and HRP (580 units). The pH was chosen according to the activity pH range of the peroxidase, and following similar DHP synthesis systems in the literature.[26] A hydrogen peroxide solution (29 mM, 100 ml) was added at a rate of 6 ml h^{-1} into the coniferyl alcohol/HRP/NADH solution. The reaction was stirred for a total of 22 h at room temperature. Then, the suspended polymerisate was centrifuged at 5700 rpm for 10 min and the pellet washed twice, resuspended in ultrapure water and freeze-dried and washed with dichloromethane for one hour to extract low molecular weight DHP compounds. The product obtained was a light-brown powder that was subsequently prepared for NMR analysis.

Gas chromatography–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR)

The GC/MS instrument was from Thermo Finnigan Trace GC–MS, 2000 series. The column used was RTX 5MS (30 m, 0.32 mm, 0.25 μm). The silylated products of the quinone methide reduction were analyzed with a carrier gas flow of 0.5 ml min^{-1}, an initial temperature of 120 °C, a ramp rate of 5.0 °C min^{-1} until 200 °C, and then a rate of 4 °C min^{-1} until 280 °C and held there for 30 min. The MS ionization method used was electron impact operating at 70 eV. For the NMR analyses from the quinone methide model experiments, the unmodified reference sample was dissolved in $CDCl_3$. From the DHP experiment the unmodified product was dissolved in acetone-d_6–D_2O (3 : 1). All NMR experiments were run on a Bruker Avance 400 MHz instrument. 2-D NMR experiments were performed with a proton–carbon-selective inverse probe equipped with a Z-gradient coil. Standard Bruker pulse programs were used in all experiments. Pulse lengths of the NMR probes were carefully calibrated and optimum tuning and matching as well as shimming of the NMR probe was performed with each run. The 2-D HSQC spectrum (pulse program 'hsqcetgpsi') was acquired with a spectral window of 12.8 ppm in F2 and 150 ppm in F1 with 512 increments of 2 K points each, giving an acquisition time of 0.2 s in F2. Other parameters for data acquisition included: a delay time of 2 s, an average assumed coupling constant of 150 Hz, 32 scans per increment. The 2-D HSQC-TOCSY spectrum (pulse program 'hsqcetgpml') was acquired with a delay time of 1.3 s, a mixing time of 60 ms and 128 scans per increment. Other acquisition parameters were similar to those of the HSQC experiment. The spectral centre was set at 5.3 ppm in the ^1H dimension and at 91 ppm in the ^{13}C dimension. The acquired 2-D NMR data sets were processed with 2 K × 1 K data points for the HSQC and HSQC-TOCSY spectra, using the $\pi/2$-shifted sine-bell window functions in both dimensions.

Compound 2, 4-[3-hydroxy-2-(2-methoxyphenoxy)-propyl]-2-methoxyphenol (Scheme 3). ^1H NMR (400 MHz; $CDCl_3$): δ 2.87–3.09 (α-CH_2), 2.97 (γ-OH), 3.60–3.68 (γ-CH_2), 3.85–3.89 (OCH_3), 4.20–4.26 (β-CH), 5.52 (phenolic H), 6.75–7.03 (aromatic H). ^{13}C NMR (in $CDCl_3$): δ 37.52 (α-CH_2), 55.86 and 55.96 (2 OCH_3), 63.61 (γ-CH_2), 85.32 (β-CH), 112.14–123.51 (aromatic-C). MS (*m*/*z*) silylated: 448 (M^+, 1%), 324 (2%), 293 (2%), 209 (100%), 179 (12%), 166 (12%), 103 (5%), 73 (72%).

Compounds 3 (a and b), *syn*- and *anti*-4-[1-ethoxy-3-hydroxy-2-(2-methoxyphenoxy)-propyl]-2-methoxyphenol (Scheme 3). MS (*m*/*z*) silylated: 492 (M^+, <1%), 323 (1%), 293 (1%), 253 (100%), 225 (6%), 209 (5%), 181 (7%), 166 (22%), 103 (6%), 73 (62%).

Reduced β-O-4′-structure, AR, from DHP experiment (2-D NMR spectra, Fig. 4). ^{1}H NMR (acetone-d_6–D_2O 3 : 1; 400 MHz): δ 2.91 (α-CH_2), 3.62 (γ-CH_2), 3.75 (OCH_3), 4.45 (β-CH), 6.70–6.99 (aromatic H). ^{13}C NMR (in acetone-d_6–D_2O 3 : 1): δ 36.22 (α-CH_2), 55.51 (OCH_3), 62.22 (γ-CH_2), 80.91 (β-CH), 108.50–121.76 (aromatic-C).

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