EPR Investigation into the Effects of Substrate Structure on Peroxidase-Catalyzed Phenylpropanoid Oxidation

Wendy R. Russell,*,† Mark J. Burkitt,‡ Lorraine Scobbie,† and Andrew Chesson§

Molecular Nutrition Group, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen, AB21 9SB United Kingdom, Gray Cancer Institute, P.O. Box 100, Mount Vernon Hospital, Northwood, HA6 2JR United Kingdom, and School of Biological Sciences, University of Aberdeen, Aberdeen, AB24 5UA United Kingdom

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The plant polymer lignin represents one of the most structurally diverse natural products and results from the oxidative coupling of phenylpropanoid monomers. Peroxidase catalyses the oxidation of phenylpropanoids to their phenoxyl radicals, and the subsequent nonenzymatic coupling controls the pattern and extent of polymerisation. Using EPR spectroscopy, we have demonstrated that for a series of substrates increased methoxylation increases peroxidase-catalyzed oxidation and that this is most easily achieved with the monomeric alcohols. Dimeric compounds, synthesized to represent the initial products of phenylpropanoid coupling, showed a marked decrease in their ability to be oxidized when compared with the monomeric substrates. These findings demonstrate that the structure of the monomer determines the final composition of lignin, which ultimately effects the overall structure. The results indicate that the polymer grows primarily as a result of the reactivity of the monomers and that polymerization to high molecular weight may be restricted to methoxylated species.

Introduction

Oxidative coupling of phenolic compounds results in the formation of a variety of natural products of which lignin represents one of the most structurally diverse. Lignin is the second most abundant natural polymer and not only plays a vital role in plant growth and development but also in the carbon cycle. Lignin restricts the degradation of plant polysaccharides, and although this may be an important aspect in maintaining gastrointestinal health in humans, it also limits the bioavailability of these vital resources for commercial exploitation. The complexity of lignin is predominantly due to the variety of phenylpropanoid radicals and the number of resonance structures that can be incorporated into its three-dimensional conformation. Phenylpropanoid polymerization to form lignin occurs in plant cell walls under control of the peroxidase family of enzymes.[1] A number of products arising via the phenylpropanoid pathway are exported from the cell and are able to act as potential substrates for this process. Hydroxylation of cinnamic acid to give 4-hydroxycinnamic acid (Figure 1, **1a**) is the first step in the pathway leading to the generation of lignin precursors. Methoxylation at the ortho positions gives 4-hydroxy-3-methoxycinnamic acid (Figure 1, **1b**) and 3,5-dimethoxy-4-hydroxycinnamic acid (Figure 1, **1c**). These acids may then be reduced via the aldehydes to the corresponding alcohols (Figure 1, **2a**−**c**). Plants exert a high degree of selection on the phenylpropanoid metabolites contributing to lignin structure.[2] In most vascular plants, the bulk of the lignin molecule is formed from the mono- and dimethoxylated alcohols, with only a small contribution from the nonmethoxylated 4-hydroxycinnamyl alcohol (Figure 1, **2a**). The corresponding acids are found only in trace amounts in the lignin of most plants, the exception being members of the *Poaceae (Gramineae)* and closely related families.[3,4] In these plants, 4-hydroxycinnamic acid and 4-hydroxy-3-methoxycinnamic acid may contribute one-third or more of the total phenolic content of the cell wall. The pattern of deposition of these two acids differs greatly. 4-Hydroxy-3-methoxycinnamic acid appears to be wholly ester-linked to wall polysaccharide, before being exported from the cell and incorporated into the expanding cell wall, where it can function as a cross-linking agent between adjacent polysaccharide chains and between lignin and polysaccharides.[5−8] The nonmethoxylated acid, 4-hydroxycinnamic acid, apparently serves a different function: It is more closely involved in lignin deposition and appears to be primarily ester-linked to other phenylpropanoid units, being linked only rarely to carbohydrate.[9,10]

Lignin polymerization proceeds by an oxidative mechanism involving the coupling of substrate phenoxyl radicals. The site at which coupling occurs is determined by the distribution of the unpaired electron within parent phenylpropanoid radicals.[11] Spin density measurements have shown the distribution of the unpaired electron on the carbon nuclei to be greater than that on the phenolic oxygen of the 4-hydroxycinnamyl alcohol derived phenoxyl radical, leading to a preference for initial C−C coupling.[11] Increasing the level of methoxylation results in a higher degree of unpaired electron density on the phenolic oxygen and accordingly, the occurrence of more C−O linkages. Similar observations have been reported for the corresponding alcohols.[11] Although the extent of methoxylation determines bond formation and therefore the characteristics of lignin, it does not wholly account for the advantage to the plant of the methoxylated alcohols for lignin formation or, in members of the *Poaceae*, the occurrence of 4-hydroxy-3-methoxycinnamic acid as the dominant cross-linking agent.[3,4] One additional factor may be the relative ease with which the various lignin precursors undergo initial oxidation to their phenoxyl radicals by peroxidases.

The common features of all peroxidases are that their active sites contain very similar prosthetic groups. For nearly all known

* To whom correspondence should be addressed. Telephone: +44 1224 716619. Fax: +44 1224 716619. E-mail: W.Russell@rowett.ac.uk.
† Rowett Research Institute.
‡ Gray Cancer Institute.
§ University of Aberdeen.

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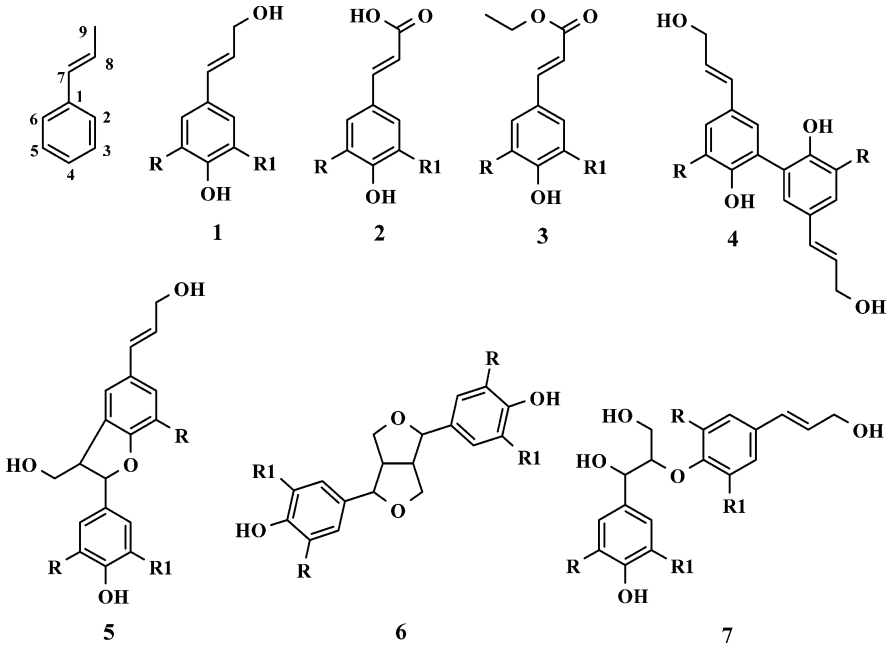

Figure 1. Structures of the substituted cinnamyl alcohols; **1a** 4-hydroxycinnamyl alcohol (R and R1 = H), **1b** 4-hydroxy-3-methoxycinnamyl alcohol (R = H and R1 = OCH_3), **1c** 4-hydroxy-3,5-dimethoxycinnamyl alcohol (R and R1 = OCH_3), substituted cinnamic acids; **2a** 4-hydroxycinnamic acid (R and R1 = H), **2b** 4-hydroxy-3-methoxycinnamic acid (R = H and R1 = OCH_3), **2c** 4-hydroxy-3,5-dimethoxycinnamic acid (R and R1 = OCH_3), substituted ethyl cinnamates; **3a** ethyl 4-hydroxycinnamate (R and R1 = H), **3b** ethyl 4-hydroxy-3-methoxycinnamate (R = H and R1 = OCH_3), **3c** ethyl 4-hydroxy-3,5-dimethoxycinnamate (R and R1 = OCH_3) and models of the predominant linkages found within lignin, **4** 5−5 (R1 = OCH_3), **5a** 8−5, (R and R1 = H), **5b** 8−5 (R = H and R1 = OCH_3), **6a** 8−8 (R and R1 = H), **6b** 8−8 (R = H and R1 = OCH_3), **6c** 8−8 (R and R1 = OCH_3), **7a** 8-O-4 (R and R1 = H), **7b** 8-O-4 (R = H and R1 = OCH_3) and **7c** 8-O-4 (R and R1 = OCH_3).

plant peroxidases the prosthetic group is ferriprotoporphyrin IX. During activation by hydrogen peroxide, peroxidase undergoes a two-electron oxidation to generate an active species known as compound I.[12−15] This intermediate stores two oxidizing equivalents from hydrogen peroxide as an oxyferryl iron center and a radical (either on the porphyrin ring or on a protein residue). The oxidation of an organic substrate, such as a phenolic by compound I involves the transfer of a single electron and proton to the peroxidase radical, yielding the substrate free radical and an oxyferryl haem intermediate known as compound II. The subsequent one-electron reduction of compound II by a second substrate molecule completes the reaction cycle. The exact location of the radical on compound I has been a topic of interest, and detailed kinetic and spectroscopic data are beginning to provide important information with regard to the control of porphyrin-protein radical exchange.[16] The primary intermediate product of the two-electron oxidation of peroxidase has demonstrated that the π-cation radical is located on the porphyrin ring.[17−20] However, in the active site of both prostaglandin H synthase-1 and prostaglandin H synthase-2, a catalytically active tyrosyl radical has been detected[21,22] and similarly an active protein-based radical (Trp191) has been identified in cytochrome *c* peroxidase[23,24] and catalase-peroxidase (Tyr 353).[25] Lignin peroxidase appears to operate by the same mechanism, and again a protein-based radical (Trp 171) has been shown to be an active intermediate.[26−28] It is likely that, since the prosthetic group in peroxidases is inaccessible, the electron must penetrate the protein matrix in order for the enzyme to function physiologically.

The ease with which the various lignin precursors undergo initial oxidation to their phenoxyl radicals by peroxidase is expected to be dependent on their electron/proton-donating abilities. Since the reaction cycle of horseradish peroxidase (HRP) can be monitored directly by EPR spectroscopy,[17,29,30] we used this technique in the present investigation to compare the abilities of a range of phenylpropanoid compounds to act as substrates for the enzyme. Conclusions are presented concerning the relationship between the structure of phenylpropanoid substrates and their ease of oxidation by HRP, which provide, in turn, important information on how substrate structure influences the final structure of the lignin polymer.

Experimental Section

General laboratory reagents and substituted 4-hydroxy cinnamic acids were obtained from Aldrich (U.K.). HRP (Type II) and hydrogen peroxide (27.5% w/v) were from Sigma Chemical Co Ltd (U.K.). All other compounds were synthesized as reported previously.[11] HRP was dialyzed against EDTA (1 mmol dm^{-3}) in potassium phosphate buffer (25 mmol dm^{-3}; pH 7.0) for 24 h and then against water over chelating resin (iminodiacetic acid) for 24 h. All stocks solutions were kept over chelating resin to remove contaminating metal ions. EPR experiments showed that the enzyme treated as above remained contaminated by copper ions. Therefore, where indicated, the concentration of EDTA used in the dialysis was increased to 50 mmol dm^{-3}.

ICP-MS Measurement of Copper. HRP (100 mg) was denatured by microwave digestion in nitric acid (1.8 cm^3; 69−70% w/w) and H_2O_2 (0.2 cm^3; 30% w/w). Copper was measured in the resulting digest using a Plasma Quad (PQ2+) ICP-MS instrument supplied by VG Elemental (U.K.).

EPR Spectroscopy. Reaction mixtures were prepared in 2.5 mm internal diameter EPR tubes (Wilmad, NJ) and contained HRP (5 mmol dm^{-3}), methanol (35% v/v), and glycerol (16% w/w) in potassium phosphate buffer (40 mmol dm^{-3}; pH 7.0). When indicated, hydrogen peroxide (5 mmol dm^{-3}) was included in reactions. Added substrates were predissolved in methanol, to give a final concentration of 3.5 mmol dm^{-3}. Immediately after preparation, reaction mixtures were cooled to 125 K in the cavity of a Bruker E106 spectrometer using a liquid nitrogen unit (Bruker, UK). Spectra (X-band) were then recorded using the following instrument settings: modulation frequency, 100 kHz; center field, 2250 G; sweep width, 3500 G; modulation amplitude 1.0, gauss; time constant, 163 ms; sweep time 327 s; power, 20 mW; and a suitable receiver gain setting. The *g* values were calculated with

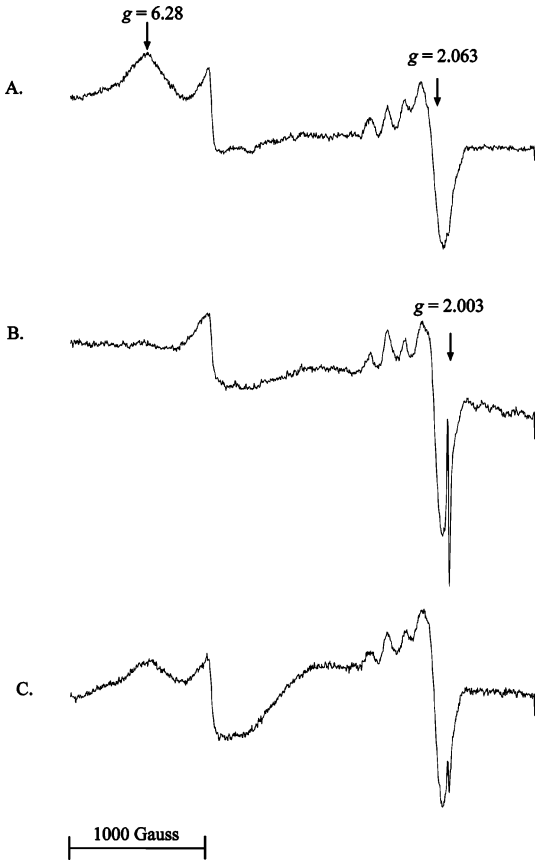

Figure 2. EPR spectra (125 K) of HRP: (A) native HRP (5 mmol dm^{-3}); (B) HRP (5 mmol dm^{-3}) plus H_2O_2 (5 mmol dm^{-3}); and (C) HRP (5 mmol dm^{-3}), H_2O_2 (5 mmol dm^{-3}) plus **1b** 4-hydroxy-3-methoxycinnamyl alcohol (3.5 mol dm^{-3}). Reaction mixtures were prepared in potassium phosphate buffer (40 mmol dm^{-3}; pH 7.0) containing methanol (35% v/v) and glycerol (16% w/w).

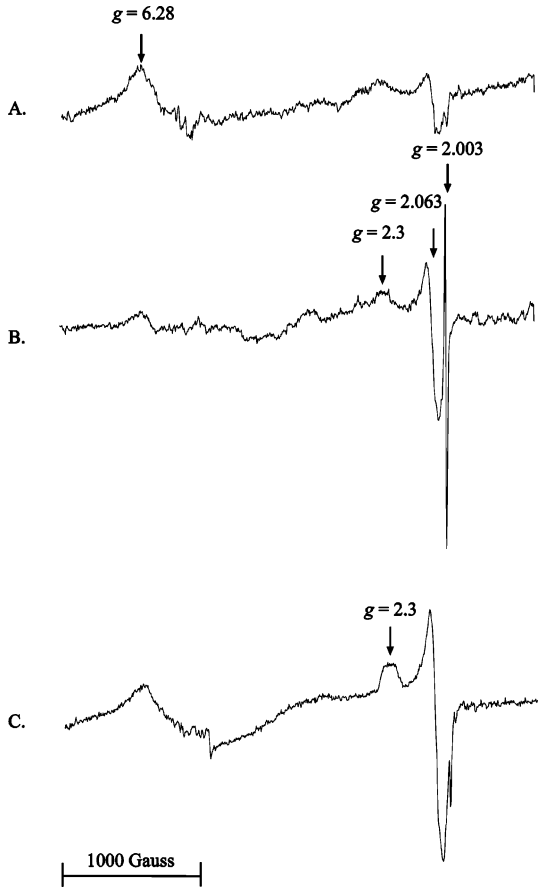

Figure 3. EPR spectra (125 K) of HRP following extensive dialysis against EDTA: (A) native HRP (5 mmol dm^{-3}); (B) HRP (5 mmol dm^{-3}) plus H_2O_2 (5 mmol dm^{-3}); and (C) HRP (5 mmol dm^{-3}), H_2O_2 (5 mmol dm^{-3}) plus **1b** 4-hydroxy-3-methoxycinnamyl alcohol (3.5 mol dm^{-3}). Reaction mixtures were prepared in potassium phosphate buffer (40 mmol dm^{-3}; pH 7.0) containing methanol (35% v/v) and glycerol (16% w/w).

reference to 1,1-diphenyl-2-picrylhydrazyl (6.25 mmol dm^{-3} in ethanol), contained in a capillary tube and placed within the sample tube (g = 2.0036). To correct spectra for cavity signals, background spectra were recorded (40 mmol dm^{-3}, pH 7.0 potassium phosphate buffer containing 35% v/v methanol and 16% w/w glycerol) and subtracted from spectra using Bruker E106 software.

Computational Chemistry. Calculation of the substrate highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) were made using AM1 theory on geometry optimized structures within the MOPAC program (Accelrys, U.K.) running on an SGI Indigo 2 workstation.

Results

The EPR spectrum of HRP at 125 K in phosphate buffer (pH 7.0) exhibited a broad signal at $g = 6.28$ (Figure 2A), which is characteristic of the $g_\perp$ component of a high-spin ferric haem complex (d,[5] $S = 5/2$). Such complexes are also characterized by a $g_{||}$ component with a value of $g \sim 2.0$.[30] In agreement with previous studies,[29] the $g_\perp$ component of the EPR spectrum appears to be due to a normal axial haem. However, rhombic distortion in spectra obtained at 1.4 K has also been reported, and it was suggested that the rhombic nature of the signal could be obscured by partial saturation.[30] Alternatively, it has also been suggested that, at the higher temperatures employed here and in other studies,[29] spin relaxation might occur fast enough to broaden and obscure resolution of the two components of the $g_\perp$ signal.[31] Furthermore, the $g \sim 2.0$ region of the spectrum containing the $g_{||}$ component of the high-spin ferric haem complex (Figure 2A) is obscured by additional signals, which are believed to be from a Cu^{2+} species. Cu^{2+} signals have been observed previously in the EPR spectra of Japanese radish peroxidase, HRP, and other peroxidases.[29] The spectrum shown in Figure 2A also displays a signal at $g = 4.26$, which is believed to reflect the presence of contaminating, nonhaem iron.[31]

The addition of one equivalent of hydrogen peroxide to the enzyme before freezing resulted in the loss of the $g_\perp = 6.28$ component of the high-spin ferric haem signal, which was accompanied by the appearance of a sharp signal at $g = 2.003$ (Figure 2B). Closer examination of the spectra shown in Figure 2, panels A and B, suggests that other changes have occurred as a result of hydrogen peroxide addition, but again these changes were largely obscured by the Cu^{2+} signal. To address this matter, experiments were repeated using HRP that had been dialyzed against EDTA at a higher concentration to remove bound copper ions. As shown in Figure 3A, the EPR spectrum of the treated enzyme still showed the characteristic $g_\perp = 6.28$ component of the high-spin ferric haem signal. The signal at $g = 4.26$ was absent, confirming that it was indeed from contaminating, nonhaem ferric iron. Having also eliminated the interfering Cu^{2+} signals, it was now possible to discern at least two additional signals: one at $g = 2.063$ and a relatively broad signal at $g \sim 2.3$ (Figure 3A). Again, H_2O_2 addition resulted in the loss of the high-spin ferric haem signal ($g_\perp = 6.28$), which was accompanied by the appearance of the sharp signal at $g = 2.003$. The addition of H_2O_2 to the enzyme also caused a

prominent increase in the signal at $g = 2.063$ (Figure 3B), which may indicate that upon peroxide binding the high-spin ferric iron undergoes conversion to a low-spin ferric complex as suggested by Yamaguchi and co-workers.[32] The signal at $g = 2.3$ is believed to be the g_z component of the same species, but the g_x component of this signal could not be resolved. Similar studies that also obtained very poor resolution of this component suggested that the species is heterogeneous or suffers random deformations upon freezing.[31]

In a recent review comparing the reaction of haem proteins and enzymes with hydroperoxides, it was shown that the EPR spectra of the primary oxyferryl porphyrin π-cation radical is extremely variable.[33] It has a wide and unusual line shape caused by ferromagnetic or antiferromagnetic exchange interactions between the ferryl iron and the porphyrin radical. The line shape differs between proteins and is likely to differ in the same protein depending on preparation procedures and this is further complicated by the potential porphyrin-protein radical exchange. To identify the radical involved in the principal purpose of our investigation, which is to compare the abilities of a series of phenylpropanoids to act as substrates for HRP, we next examined the spectra obtained from reaction mixtures containing HRP, H_2O_2, and the potential substrate, 4-hydroxy-3-methoxycinnamyl alcohol. As shown in Figure 2C, the addition of 0.7 equiv of 4-hydroxy-3-methoxycinnamyl alcohol to HRP in the presence of 1.0 equiv of H_2O_2, followed by freezing, resulted in the detection of an EPR spectrum that was very similar to that of the resting enzyme (Figure 2A): the $g_\perp = 6.28$ component of the high-spin ferric haem signal was present, and only a very weak signal at $g = 2.003$.

This suggests that 4-hydrox-3-methoxycinnamyl alcohol is easily oxidized by HRP and that the signal at $g = 2.003$ represents the radical center being reduced. Presence of the high-spin ferric haem signal also shows that iron(IV) is also being reduced to iron(III). It is likely that this signal at $g = 2.003$ represents a protein-based radical, but its exact location is uncertain. The same results were obtained when the reaction was performed using the enzyme that had been treated with high-concentration EDTA. From these observations, it appears that the reaction cycle of HRP in the presence of 4-hydroxy-3-methoxycinnamyl alcohol can be clearly monitored by EPR spectroscopy. Also, the contaminating copper ions do not effect this reaction, which is in agreement with previous findings.[29] The above observations support our proposal that EPR spectroscopy provides a useful means by which to determine the ability of HRP to oxidize a range of phenylpropanoid substrates. Therefore, we synthesized a series of substituted cinnamyl alcohols (Figure 1, **1a**−**c**), cinnamic acids (Figure 1, **2a**−**c**), cinnamate esters (Figure 1, **3a**−**c**), as well as a range of dehydrodimers (Figure 1, **4**−**7**), representing the products of initial coupling of the lignin precursors. The EPR spectra observed upon the inclusion of the monomeric substrates (Figure 1, **1**−**3**) with HRP and H_2O_2 are presented in Figure 4. Increasing the degree of methoxylation within each substrate group (i.e., alcohol, acid, or ester) resulted in increased attenuation of the $g = 2.003$ signal. This suggests that increasing the degree of methoxylation of the substrate increases the ease of oxidation.

The fact that all of the spectra in Figure 4 display the characteristic $g_\perp = 6.28$ component of the high-spin ferric haem signal, as well as varying contributions from the radical at $g = 2.003$, suggests that the spectra consist of signals from both the ferric peroxidase and compound I (compound II is EPR-silent). Therefore, although the nonmethoxylated substrates each

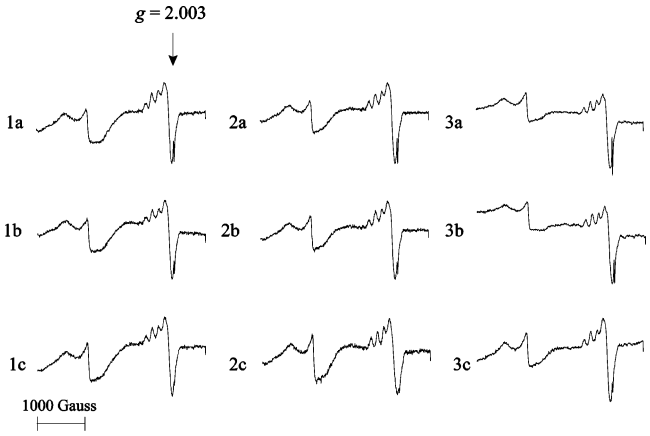

Figure 4. EPR spectra (125 K) of HRP (5 mmol dm^{-3}) in the presence of H_2O_2 (5 mmol dm^{-3}) and the various monomeric substrates (3.5 mmol dm^{-3}): **1a** 4-hydroxycinnamyl alcohol (R and R1 = H); **1b** 4-hydroxy-3-methoxycinnamyl alcohol (R = H and R1 = OCH_3); **1c** 4-hydroxy-3,5-dimethoxycinnamyl alcohol (R and R1 = OCH_3); **2a** 4-hydroxycinnamic acid (R and R1 = H); **2b** 4-hydroxy-3-methoxycinnamic acid (R = H and R1 = OCH_3); **2c** 4-hydroxy-3,5-dimethoxycinnamic acid (R and R1 = OCH_3); **3a** ethyl 4-hydroxycinnamate (R and R1 = H); **3b** ethyl 4-hydroxy-3-methoxycinnamate (R = H and R1 = OCH_3) and **3c** ethyl 4-hydroxy-3,5-dimethoxycinnamate (R and R1 = OCH_3). Reaction mixtures were prepared in potassium phosphate buffer (40 mmol dm^{-3}; pH 7.0) containing methanol (35% v/v) and glycerol (16% w/w).

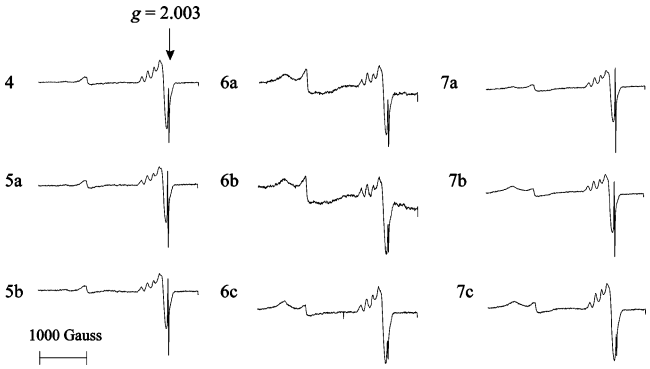

Figure 5. EPR spectra (125 K) of HRP (5 mmol dm^{-3}) in the presence of H_2O_2 (5 mmol dm^{-3}) and the various dimeric substrates (3.5 mmol dm^{-3}): **4** 5−5 (R = OCH_3); **5a** 8−5 (R and R1 = H); **5b** 8−5 (R = H and R1 = OCH_3); **6a** 8−8 (R and R1 = H); **6b** 8−8 (R = H and R1 = OCH_3); **6c** 8−8 (R and R1 = OCH_3); **7a** 8-O-4 (R and R1 = H); **7b** 8-O-4 (R = H and R1 = OCH_3) and **7c** 8-O-4 (R and R1 = OCH_3). Reaction mixtures were prepared in potassium phosphate buffer (40 mmol dm^{-3}; pH 7.0) containing methanol (35% v/v) and glycerol (16% w/w).

display the strongest signal from compound I, some oxidation has occurred because the ferric peroxidase is also present. This signal is not present when an equivalent amount of H_2O_2 is added to the enzyme in the absence of a substrate (Figure 2B). The results do, however, show that there is an almost complete conversion of compound I to the ferric peroxidase in the presence of the dimethoxylated substrates, indicating that progressive methoxylation increases reactivity toward compound I (Figure 4). Similar experiments revealed that, compared with the monomers, the dimeric phenylpropanoids (Figure 1, **4**−**8**) are poor substrates for compound I (Figure 5). In particular, substrates **4**, **5a**, and **5b** were found to be essentially unreactive, as revealed by the presence of a prominent signal at $g = 2.003$ from the radical and the absence of the signal at $g = 6.28$ from the $g_\perp$ component of the high-spin ferric haem peroxidase. Increasing the degree of methoxylation of substrate **6** did, however, result in increased suppression of the radical signal

Table 1. Energy (eV) of the Frontier Orbitals for the Monomeric Substrates

	a		b		c	
substrate	HOMO	LUMO	HOMO	LUMO	HOMO	LUMO
cinnamyl alcohols (**1a**−**c**)[a]	−8.76362	−0.12819	−8.45336	−0.11646	−8.38945	−0.07973
cinnamic acids (**2a**−**c**)[a]	−9.1199	−0.78821	−8.84913	−0.82485	−8.76742	−0.83769
ethyl cinnamates (**3a**−**c**)[a]	−9.16465	−0.77723	−8.95258	−0.82792	−8.80779	−0.83035

[a] **1a** 4-hydroxycinnamyl alcohol, **1b** 4-hydroxy-3-methoxycinnamyl alcohol, **1c** 4-hydroxy-3,5-dimethoxycinnamyl alcohol, **2a** 4-hydroxycinnamic acid, **2b** 4-hydroxy-3-methoxycinnamic acid, **2c** 4-hydroxy-3,5-dimethoxycinnamic acid, **3a** ethyl 4-hydroxycinnamate, **3b** ethyl 4-hydroxy-3-methoxycinnamate and **3c** ethyl 4-hydroxy-3,5-dimethoxycinnamate. HOMO (highest occupied molecular orbital) and LUMO (lowest unoccupied molecular orbital).

at $g = 2.003$, and a similar trend was observed for substrate **7**, in which the progressive regeneration of the high-spin ferric haem signal ($g_{\perp} = 6.28$) was observed (Figure 5).

Since the electron provided for the reduction of compound I is expected to reside in the substrate HOMO, the energy of this frontier orbital was calculated for each of the monomeric substrates. Increasing the degree of methoxylation was found to increase the energy of the HOMO for a given substrate group (Table 1), which reflects the observed increase in reactivity of the more methoxylated substrates toward HRP-compound I (Figure 4). It was also found that the alcohols have higher energy HOMO than their corresponding acids and esters, which was also reflected, though to a lesser degree, in the EPR measurements (Figure 4).

Discusssion

In a previous investigation, we have demonstrated that a knowledge of the delocalization of the unpaired electron onto the carbon nuclei of phenoxyl radicals, generated from a series of substituted 4-hydroxycinnamyl alcohols and cinnamic acids, can be used to predict the sites at which coupling occurs during polymerization.[11] However, because the phenoxyl radicals observed by EPR in this study were generated from the phenylpropanoids using Ce^{4+} within a fast-flow system, information was provided only on the reactivity and fate of the phenoxyl radicals. No information was provided on the relative ease with which the various substrates undergo oxidation to the observed phenoxyl radicals by the physiological oxidizing system, peroxidase. Such findings would not wholly account for the advantage to the plant of the methoxylated alcohols for lignin formation or, in members of the *Poaceae*, the occurrence of 4-hydroxy-3-methoxycinnamic acid as the dominant cross-linking agent.[3,4] Consequently, the present investigation was undertaken to address this issue, employing EPR spectroscopy to examine the ability of various substrates to reduce the oxidized form of HRP.

The ability to reduce the HRP-compound I was found to increase with increasing extent of methoxylation, essentially regardless of the functional group on C9. The cinnamyl alcohols, the principle monomers forming lignin, were as a group more readily oxidized than the corresponding cinnamic acids, which in turn were more easily oxidized than the ethyl cinnamates. However, the relatively poor ability of the nonmethoxylated cinnamyl alcohol to be oxidized may account for the very low level of active incorporation of this monomer into lignin. This is despite its necessary presence in the plant as a precursor in the synthesis of the methoxylated derivatives.

Considerable attention has been paid to the role of the hydroxycinnamic acids in the cell walls of graminaceaous plants as cross-linking molecules between polysaccharide chains and between polysaccharides and lignin.[6−8] Although all three acids contain the bifunctionality necessary for this role, bridging appears to be restricted to the monomethoxylated acid.[34] The only exceptions are the truxillic/truxinic acid cross-links, which may be between either non- or monomethoxylated acids or a mixture of both.[35,36] However, evidence suggests that formation of this form of cross-link does not proceed by an enzyme-mediated radical mechanism but is initiated by light.[37] Where an enzyme-mediated radical mechanism of coupling is implicated, cross-linking appears to involve only the 4-hydroxy-3-methoxycinnamic acid.[7,38] Of particular significance is the role of 4-hydroxy-3-methoxycinnamic acid as the principal and, in some cases, only, compound participating in a covalent interaction between carbohydrate and lignin in grasses. In these plants, the monomethoxylated acid is ester-linked to cell wall polysaccharide, even in undifferentiated meristematic cells.[39] Some monomethoxylated units cross-link in the primary wall and as the cells further differentiate and their walls undergo secondary thickening, many of the remaining ester-linked units appear to act as nucleation sites for lignification.[39]

As predicted by our previous results on unpaired electron distribution,[11] approximately 90% of the products of the synthetic coupling of 4-hydroxy-3-methoxycinnamic acid esterified to the C5 of methyl-L-arabinofuranoside have been demonstrated to contain a C−C linkage.[39] Substituted cinnamyl alcohols couple to the cinnamic acids exclusively through their C8 positions.[39] Coupling to esterified 4-hydroxy-3-methoxycinnamate would, therefore, be expected to occur predominantly by a 8−5 linkage. The preference for the monomethoxylated analogue as a cross-link to polysaccharide may be to impart some control, made possible by the conformational resistance and strength of this C−C linkage. These studies show that despite the preference for C−C bond formation between the nonmethoxylated monolignols, the nonmethoxylated ester will be a poor substitute for the monomethoxylated ester due to its low reactivity toward HRP-compound I. In contrast, the dimethoxylated ester, although equally able to initiate lignification, would do so exclusively by a less rigid 8-O-4 bond, due to the high electron density residing on the phenoxyl oxygen.[11]

The amount of esterified 4-hydroxycinnamic acid present in the cell wall has been observed to parallel the synthesis of lignin in graminaceous plants.[40] Polymerization at this stage appears largely to involve 8-O-4 coupling of dimethoxylated cinnamyl alcohols. The marked decrease found in the ability of the ethyl 4-hydroxycinnamate to reduce the oxidized form of HRP, coupled with the previously observed delocalization of the unpaired electron away from the phenolic oxygen,[11] suggests its potential role as a terminal unit if introduced at this stage. Evidence for the esterification of 4-hydroxycinnamic acid to the primary alcohol of 3,5-dimethoxy-4-hydroxycinnamyl alcohol has been provided.[10] This reinforces the possibility that the nonmethoxylated cinnamic acid may be incorporated into lignin in order to bestow spatial control.

The ease with which the monomeric substrates were found to reduce HRP-compound I showed good correlation with the energy of the highest occupied molecular orbital, from which

the electron is lost. The lower reactivity observed for the dimeric species indicates that end-wise polymerization results by addition of a reactive monomer to the growing polymer chain. Again, it was observed that increasing methoxylation resulted in an increase in reactivity, suggesting that polymerization to high molecular weight is restricted to di- and, possibly, monomethoxylated compounds. These results, taken with the unpaired electron distribution findings reported earlier,[11] allow to some extent the patterns of polymerization of given substrates by horseradish peroxidase to be predicted.[41] These findings also contribute to the understanding of the role played by precursor structure in the determination of the structure of the polymer lignin and its association with cell wall carbohydrate.

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