ACTA CHEMICA SCANDINAVICA 27 (1973) 2909–2922

Studies on the Enzymatic Degradation of Lignin

The Action of Peroxidase and Laccase on Monomeric and Dimeric Model Compounds*

JOSEF GIERER and AUGUSTINE E. OPARA

Swedish Forest Products Research Laboratory, Box 5604, S-114 86 Stockholm, Sweden

Monomeric and dimeric phenolic lignin model compounds were treated with one equivalent of hydrogen peroxide in the presence of *peroxidase*. During this treatment products of higher molecular weight were formed by oxidative carbon-to-carbon and carbon-to-oxygen coupling. The oligomeric components of the mixtures obtained were separated by chromatographic methods and characterised by NMR-spectra, mass spectra, and osmometric determination of the molecular weights. Non-phenolic model compounds remained unaffected by the enzyme system.

Oxidation of the phenolic models by *laccase* gave essentially the same pattern of reaction products as was obtained with peroxidase.

The results support the view that both enzymes are able to generate the mesomeric aryloxy radicals by hydrogen abstraction, the subsequent coupling reactions proceeding spontaneously. No indication was obtained that peroxidase or laccase are directly involved in the degradation of the aromatic moieties of lignin.

White rot fungi *(Basidiomycetes)* are well known for their ability to degrade wood lignins.[1–3] The fungi of this type produce varying amounts of extracellular phenol-oxidising enzymes,** of which peroxidase and laccase have been isolated. The good correlation between the production of these enzymes and the ability of the fungi to degrade lignin underlies the concept that these enzymes are directly involved in the degradation process.[4,5] This argument is strengthened by the fact that the taxonomically related species termed "brown-rot fungi" which do not exude any detectable amounts of phenol-oxidising enzymes, leave the lignin essentially undegraded. Other findings indicating a possible role of phenoloxidases in the fungal degradation of lignin

* The terms monomeric and dimeric refer to model compounds containing one and two aromatic nuclei, respectively.

** The terms phenol-oxidising enzymes and phenoloxidases are used interchangeably here and include peroxidase (EC. 1.11.1.7, Donor: H_2O_2 oxidoreductase) and laccase (EC. 1.10.3.1, *o*-Diphenol: oxygen oxidoreductase) which catalyse oxidation of phenols to polymeric dark products.

are the increased number of free radicals in wood after decay by white-rot fungi [6,7] and the oxidative cleavage of simple phenols with phenol-oxidising enzymes.[8]

Numerous studies on the effect of white-rot fungi or their enzymes on lignin and lignin model compounds have been carried out and have led to divergent theories on the microbial degradation of lignin. The main theories so far advanced have been reviewed recently [9–12] and may be summarised as follows:

(1) Side chains in lignin units are oxidised at the α- or β-carbon atoms with formation of structures containing keto groups and liberation of phenolic units. Methyl-aryl ether bonds are also cleaved in the process.

(2) β-Aryl ether linkages are cleaved hydrolytically giving rise to alcoholic and phenolic structures.

(3) The fragmentation of lignin is brought about by cleavage of alkyl aryl carbon-carbon bonds. In this type of reaction side-chains are removed from the aromatic nuclei by oxidative coupling between radical intermediates of the phenoxyl and cyclohexadienonyl types. Intermediary *p*-quinoid structures and aldehydic or acidic fragments are formed.

(4) The enzymes catalyse the cleavage of the aromatic nuclei. After introducing the required hydroxylation pattern (formation of *ortho*- or *para*-diphenol structures) by demethylation or hydroxylation, the phenolic rings are cleaved to give aliphatic degradation products (usually carboxylic acids).

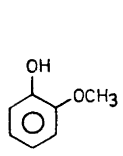
Separately, or in coordination, reactions of these types should bring about extensive degradation of lignin. However, the pathway and mechanism of biodegradation of lignin is obscure and the results from many studies in this field are contradictory and confusing. No precise information is available as to the specific enzymes involved in the microbial degradation nor are the sequential changes occurring during the process known in detail. It is commonly held that the degradation of lignin is oxidative because the composition of degraded lignin resembles that of humic acid,[13,14] both products containing less methoxyl but more phenolic hydroxyl, carbonyl, and carboxyl groups. These changes apparently result from demethylations, hydroxylations, oxidative ring cleavages, and side chain oxidations. The extracellular enzymes, laccase and peroxidase, which are detected in culture media where lignin digestion has been demonstrated, are believed to play an important role in these transformations. Laccase is thought to be more involved in the degradation because of its wide distribution in fungi and other microorganisms effecting wood decay.[15,16] However, the significance of these enzymes in the process is uncertain.

This paper deals with model studies carried out with the aim of elucidating the role of peroxidase and laccase in the degradation of lignin. The low molecular weight components formed were separated by column chromatography after reductive acetylation of the crude mixtures. The structural assignments are based on molecular weight determinations and on NMR and mass spectra. In particular, the mode of coupling in the isolated compounds was deduced from the ratio of acetyl to methoxyl protons in the NMR-spectra. The identity of isolated compounds with previously known compounds was also corroborated by their melting points and mixed melting points.

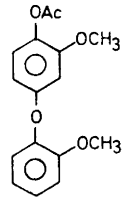
RESULTS

Oxidation of monomeric models

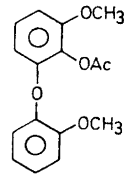
Guaiacol. Peroxidase and laccase catalysed the conversion of guaiacol (I) into mixtures of products which precipitated from the buffered (pH 5) dark-brown solution. Thin-layer chromatography of the resulting mixtures showed the presence of residual guaiacol. Column chromatography after reductive acetylation gave a diaryl ether (II), two diaryl compounds (IV and V) and a trimeric compound (VII). The structures assigned to compounds II and VII are arbitrary, the choice between possible isomers (II – III) and (VII – X) being based solely on the preferential *p,p'*-coupling mode.* Compound VII should arise from the *p,p'*-coupled diaryl V by C – O coupling.



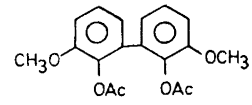
I



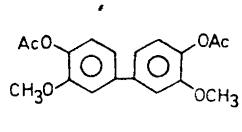
II



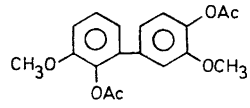
III



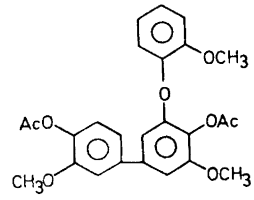
IV



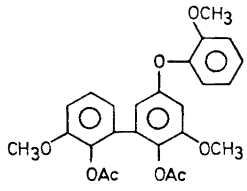
V



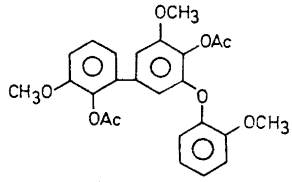
VI



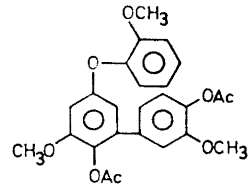
VII



VIII



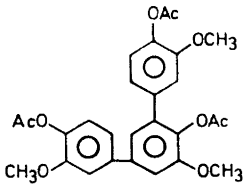
IX



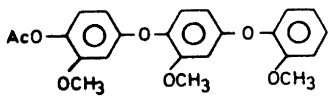
X

* The predominant yield of *p,p'*-coupled isomers is consistent with measurements of the electron density in various positions of phenoxy radicals (Scott, A. T. *Quart. Rev. Chem. Soc.* **19** (1965) 2–5). Compound VI resulting from *o-p'*-coupling was not isolated from the reaction mixture but cannot be ruled out.

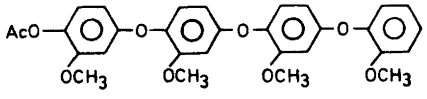
The crude fraction from which the trimeric compound VII had been crystallised and the subsequent fractions could not be separated into their components. The first fraction containing residual compound VII showed at least two further spots on TLC and the mass spectrum contained peaks at m/e 410, 452, and 494. The two subsequent fractions also gave overlapping spots on TLC and the mass spectra contained peaks at *m/e* 532, 574, 616, 658 and 654 696, 738, respectively. The difference in 42 mass units may be due to the loss of ketene from acetylated compounds and/or to the presence in the reaction



XI

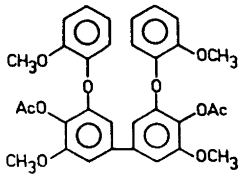


XII

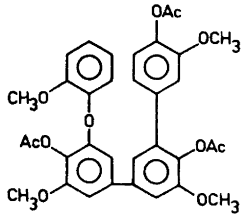


XIII

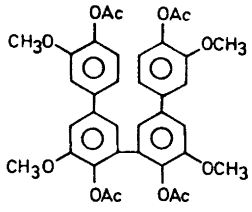
mixture of aryl-aryl and aryl-O-aryl linked oligomers differing by 42 units in molecular weight. For example, compounds VII and XII should give rise to molecular ion peaks at *m/e* 452 and 410, respectively. On the basis of the



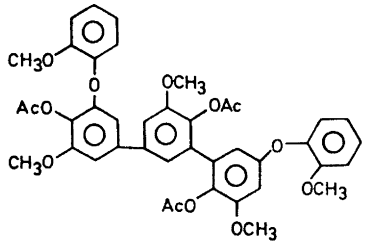
XIV



XV



XVI

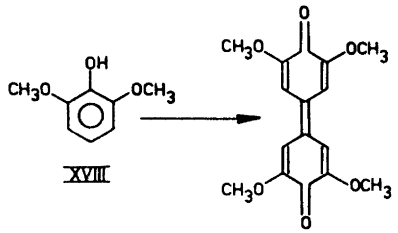


XVII

chromatographic behaviour (TLC) of the fractions eluted from the column it seems therefore most probable that some of these ions originate from non-separated oligomers with different modes of coupling between the aryl residues. Thus, the presence of different trimers (*e.g.* XI, M = 494, VII, M = 452, XII, M = 410), tetramers (*e.g.* XVI, M = 658, XV, M = 616, XIV, M = 574, XIII, M = 532) and of a pentamer (*e.g.* XVII, M = 738) is indicated.

The yields of the isolated components were considerably lower than the amounts actually formed, due to losses during the repeated chromatographic separation used in the purification procedure. Since the acetylated compounds were prepared from the reddish-brown reaction mixture under reducing conditions, it is likely that some of the parent phenolic components, particularly the biaryls and their carbon-to-carbon and carbon-to-oxygen coupled products, originated from the corresponding diphenoquinones.[17]

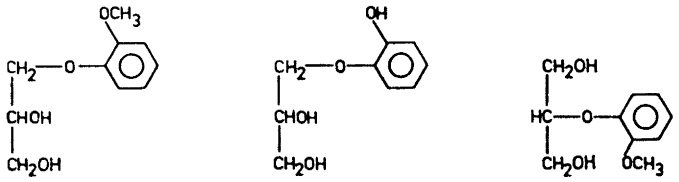
2,6-Dimethoxyphenol (XVIII). Oxidation of this compound by peroxidase-hydrogen peroxide afforded exclusively the *p,p'*-coupled quinoid product,



XVIII XIX

coerulignone (XIX), in almost quantitative yield. The same reaction catalysed by laccase had been reported previously.[18]

Glyceryl-aryl ethers. Treatment of the glyceryl-α- and β-guaiacyl ethers (XX and XXI, respectively) with peroxidase-hydrogen peroxide did not yield any

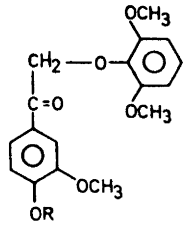


XX XXII XXI

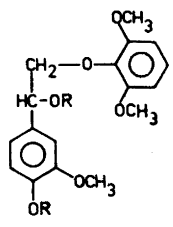
detectable reaction product. The model compounds were recovered unchanged after the enzymatic treatment. The phenolic analogue to XX (XXII), however, gave a reddish-brown mixture of products similar to that observed in the enzymatically catalysed oxidation of guaiacol (see above). The mixture was shown to be free from both glycerol and catechol (TLC) showing that no oxidative or hydrolytic cleavage of the ether bond had taken place.

Oxidation of dimeric models

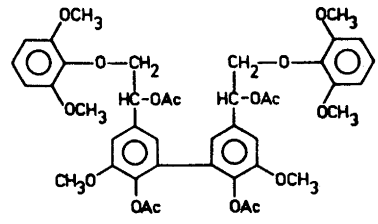
On treatment with peroxidase–hydrogen peroxide or with laccase the dimeric compounds XXV [1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy)-ethanol], XXIX (pinoresinol), and XXXII (dihydro-dehydrodiisoeugenol) underwent carbon-to-carbon and carbon-to-oxygen couplings



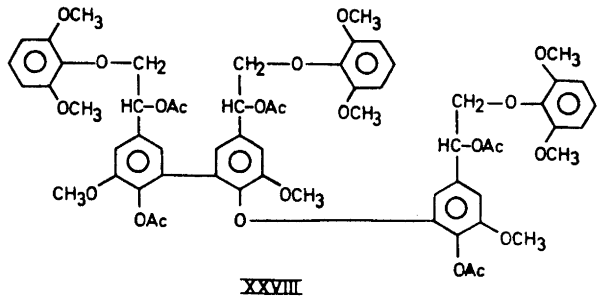
XXIII R = Ac
XXIV R = CH_2–C_6H_5



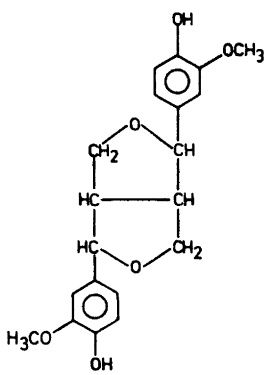
XXV R = H
XXVI R = Ac



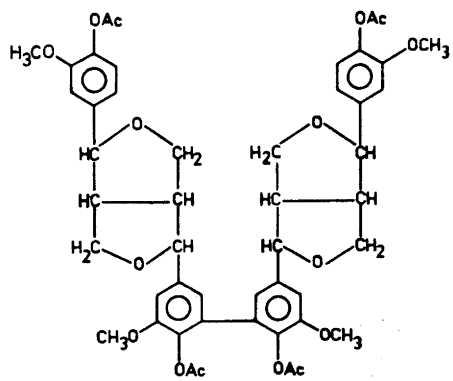
XXVII



XXVIII



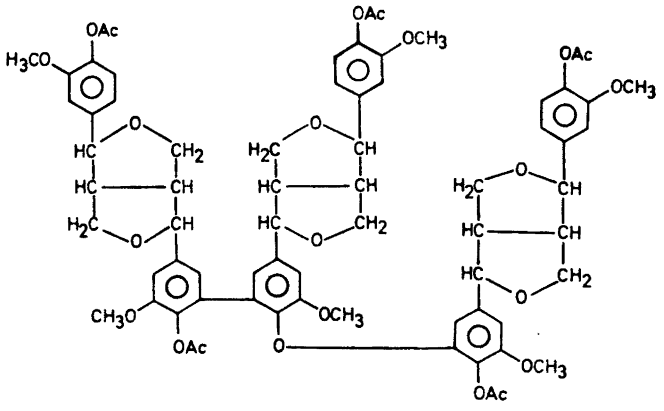
XXIX



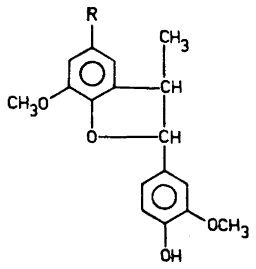
XXX

at the free positions *ortho* to the phenolic hydroxyl groups. The diaryl compounds XXVII, XXX, and XXXIV, respectively, constituted the main reaction products. In minor amounts, the oxygen-to-carbon coupling products between these diaryl compounds and the corresponding starting compounds were formed and isolated as their acetates (XXVIII, XXXI, and XXXVIII, respectively). From dihydro-dehydro-diisoeugenol (XXXII) an additional product of oxygen-to-carbon coupling was formed and characterised as the acetate XXXVI.

Most of these coupling products were unstable under electron impact and did not exhibit the expected molecular ions peaks. In these instances the molecular weights were determined using an osmometric method. Comparison of the thin-layer chromatograms of the acetylated mixtures of oxidation products with those of the reductively acetylated mixtures of the same oxidation products did not reveal any significant differences. This indicates that no oxidation of the *o,o'*-dihydroxy-diaryl compounds to the corresponding dipheno-quinones (*cf.* behaviour of I, see above) had taken place.

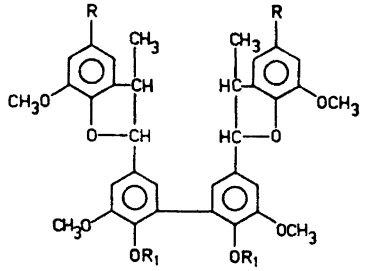


XXXI



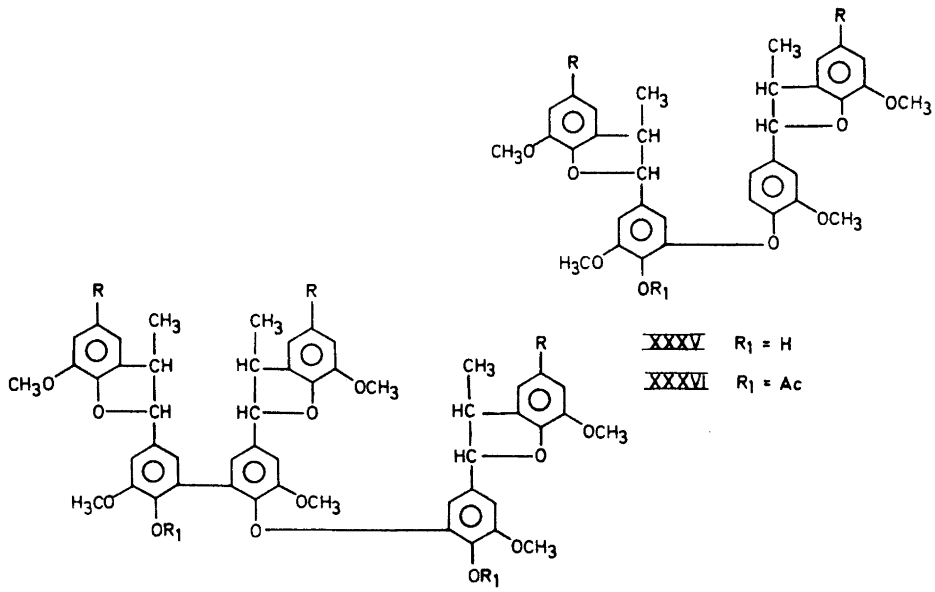
XXXII

R= propyl



XXXIII R_1 = H

XXXIV R_1 = Ac



XXXV R_1 = H
XXXVI R_1 = Ac

XXXVII R_1 = H
XXXVIII R_1 = Ac

R = propyl

DISCUSSION AND CONCLUSION

Although guaiacol is commonly used as substrate in the assay of phenoloxidases,[19] no thorough investigation of its oxidation products has been undertaken so far. The bright red oxidation mixture was termed "tetraguaiacone" [20] and considered as a polymer constructed exclusively by carbon-to-carbon bonds.[17,21] The isolation of carbon-to-oxygen linked dimers and oligomers in the present investigation suggests that this type of interconnecting linkage is also an intrinsic structural feature of the high molecular weight tetraguaiacone.

The stability of the glycerylguaiacyl ethers XX and XXI and the sensibility of compound XXII towards peroxidase-hydrogen peroxide and laccase shows that the catalytic effect of these enzymes is oxidative rather than hydrolytic in nature. It appears to be restricted to phenolic structures.

The monomeric and dimeric phenolic models investigated here afforded only carbon-to-carbon and carbon-to-oxygen coupled products. Thus, it may be concluded that the two enzyme systems function primarily – if not exclusively – to catalyse oxidative coupling with the formation of aryl-aryl- and aryl-O-aryl linkages. No evidence for any extensive cleavage of ether linkages was obtained. The isolated coupling products contained all aryl alkyl ether bonds originally present. In particular, the methyl aryl ether and β-aryl ether bonds were shown to remain *essentially* unaffected.

Therefore, any degradative effect of these enzymes should be connected with the oxidative coupling reactions which they catalyse. Recently, de-

hydrogenative coupling reactions of this kind brought about by laccase (*p*-diphenol oxidase)[22] or peroxidase[23,24] have been observed using model compounds of the *p*-hydroxy-α-carbonyl[22,24] and *p*-hydroxy-α-carbinol[22,23] types. In these reactions the α-carbonyl- and α-carbinol side chains are displaced as carboxylic acids and aldehydes, respectively, by coupling of phenoxyl radicals to *p*-cyclohexadienonyl radicals. However, this elimination of side chains has been observed with phenolic units of the syringyl type only. Guaiacyl type units containing a free *ortho*- and an substituted *para*-position, undergo preferentially, if not exclusively, *o,o'*-coupling. The formation of such *o,o'*-dihydroxydiaryl structures has been described previously.[21,23–25,27] In the present work it is shown that, in addition to these carbon-to-carbon coupled compounds, carbon-to-oxygen coupled products are formed. (*cf.* the behaviour of compounds XXV, XXIX, and XXXII). The coupled products are remarkably stable towards further oxidation.

The identical pattern of coupling products obtained with peroxidase-hydrogen peroxide and with laccase, as well as the lack of substrate specificity of these two enzymes suggests that only the first step in the reactions, the abstraction of hydrogen from the phenolic hydroxyl group, is enzymatically catalysed. The subsequent couplings appear to proceed spontaneously. This behaviour of the two enzymes is well known from the extensive studies on the biosynthesis of lignin[26,27] in which coniferylalcohol and other *p*-hydroxycinnamyl alcohols have been used as substrates.

The features of the reactions described in this work are consistent with the view that the function of the extracellular enzymes peroxidase and laccase consists in detoxifying low molecular weight phenolic compounds which may be released from lignin during its fungal decomposition, and in maintaining the metabolic balance around the fungi.[28] No experimental evidence for a *direct* involvement of these enzymes in the microbial degradation of lignin was obtained (*cf.* also Ref. 28a). However, this result does not exclude the possibility that the above oxidative coupling reactions may constitute the first step of a reaction sequence, in which peroxidase and laccase in concert with other (unknown) enzymes participate in the microbial lignin breakdown.

EXPERIMENTAL

All melting points are corrected. Evaporations were carried out under reduced pressure.

Chromatography. Both analytical and preparative separations by thin-layer chromatography (TLC) were carried out using silica gel HF_{254} (E. Merck A G., Darmstadt) as adsorbant. The solvent systems used varied with the model compound under examination and are indicated below. Vanillin-sulphuric acid [vanillin (3.0 g), conc. sulphuric acid (0.5 ml)] in ethanol (100 ml) was used as spray reagent and gave characteristic color spots after 10 min heating (120°).

Silicic acid used for column chromatography was supplied by Mallinckrodt, USA (marketed as SilicAR CC-1 100 – 200 mesh).

Spectroscopy. The NMR spectra were run on a Perkin-Elmer R-12 spectrometer with deuteriochloroform ($CDCl_3$) as solvent and tetramethylsilane (TMS) as internal standard. The mass spectra were determined on a Perkin-Elmer 270 instrument at 20 – 35 eV using the direct inlet system with the temperature of the probe heater between 50 and 120°.

Acetylation. Acetylations and reductive acetylations were carried out according to standard procedures.[29]

Model compounds. 1-Guaiacoxy-2,3-dihydroxy-propane (XX) was commercially available. 2-Guaiacoxy-1,3-dihydroxy-propane (XXI),[30] 1-*o*-hydroxyphenoxy-2,3-dihydroxy-propane (XXII),[31] ω-bromo-4-acetoxy-3-methoxy-acetophenone,[32] ω-bromo-4-benzyloxy-3-methoxy-acetophenone,[32] pinoresinol (XXIX),[33] and dihydro-dehydro-diisoeugenol (XXXII)[34] have been prepared as previously described. Compound XXV was prepared as follows:

4-Acetoxy-3-methoxy-ω-(2,6-dimethoxyphenoxy)-acetophenone (XXIII). 2,6-Dimethoxyphenol (10.8 g, 0.7 mol) and ω-bromo-4-acetoxy-3-methoxyacetophenone (13.0 g, 0.045 mol) were reacted as described for the preparation of a related compound.[35] Recrystallisation of the crude product from ethanol gave compound XXIII as colourless prisms (7.4 g, 45 %), m.p. 95–96°. ν_{max} (KBr) 1760 cm^{-1} (ester C=O), 1700 cm^{-1} (ketone C=O). [Found: C 63.64; H 5.62; *m/e* 360. $C_{19}H_{20}O_7$ (M 360) requires C 63.70; H 5.55]. δ 7.85–7.60 (m, 3 H); 7.38–6.56 (m, 3 H); 5.20 (s, 2 H); 3.91 (s, 3 H); 3.82 (s, 6 H); 2.30 (s, 3 H).

1-(4-Hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy)-ethanol (XXV). Compound XXIII (3 g) in THF (50 ml) was reduced with lithium aluminium hydride (1.2 g) in THF (80 ml) using a standard procedure.[36] The residue (2.7 g) obtained after working-up was recrystallised from benzene to give XXV as colourless prisms, m.p. 82–84°. ν_{max} (KBr) 3500–3200 cm^{-1} (OH). (Found: C 63.92; H 6.35. *m/e* 320. $C_{17}H_{20}O_6$ (M 320) requires: C 63.77; H 6.25). δ 7.30–6.80 (m, 3 H); 6.67–6.50 (m, 3 H); 5.85 (b, 2 H); 3.85 (s, 9 H).

The compound gave an intense blue colour with 2,6-dibromoquinone-4-chloroimide and alkali indicating the presence of a *p*-hydroxybenzyl alcohol grouping.[37]

The diacetate (XXVI) was obtained as needles, m.p. 122–125°. ν_{max} (KBr) 1750, 1725 cm^{-1} (ester C=O). δ 7.39–6.98 (m, 3 H); 6.75–6.50 (m, 3 H; 6.37 (t, 1 H; J=6 cps). 4.20 (d, 2 H; J=6 cps); 3.75 (s, 9 H); 2.25 (s, 3 H); 2.02 (s, 3 H.) *m/e* 404. Compound XXV was also prepared by hydrogenolysis (Pd/C) of the benzyl ether XXIV which was obtained by reacting ω-bromo-4-benzyloxy-3-methoxy-acetophenone[32] with 2,6-dimethoxyphenol as described above.

4-Benzyloxy-3-methoxy-ω-(2,6-dimethoxyphenoxy)-acetophenone (XXIV) was obtained as colourless crystals by reacting ω-bromo-4-benzyloxy-3-methoxy-acetophenone[32] with 2.6-dimethoxyphenol, m.p. 94–95°. δ 7.70–6.50 (m, 11 H); 5.19, 5.10 (s, 2 H, 2 H); 3.90, 3.89 (s, 3 H, 6 H). *m/e* 408.

Enzymes. The peroxidase (horseradish) type II used in this study was purchased from Sigma Chemical Co., St Louis, Mo. and contained approximately 135 purpurogallin units/mg. Laccase, isolated from *Neurospora crassa* was kindly supplied by Dr. K. E. Eriksson of this institute. The activity of this enzyme was measured using D,L-dopa as substrate,[38] 1 mg/ml, at pH 6 and 25°, gave 3500 KU (Klett units).

Solution. Acetate buffer solution (pH 5, 10 mM) was used. Acetone used to increase the solubility of some of the model compounds was purified by drying the technical grade solvent with P_2O_5 and distilling the decanted liquid over potassium permanganate.

Enzyme-catalysed reactions. The appropriate model compound (0.5–1.0 g) was dissolved in acetate buffer (pH 5.0), in some cases in a mixture of buffer and acetone, and a small amount (*ca.* 5 mg) of laccase or peroxidase and one equivalent of hydrogen peroxide was added. The solution was stirred (2 h) and then worked up by extraction with ethyl acetate. The major oligomeric products were characterised as the acetates. Evidence that the oxidation products described here resulted from enzymatic dehydrogenation rests upon controls carried out with a heat-denaturated solution of laccase and with a solution of active peroxidase omitting the addition of hydrogen peroxide.

Oxidation of guaiacol. (a) with peroxidase /H_2O_2. Hydrogen peroxide (30.0 ml, 1.0 %, 0.0088 mol) was added during 30 min to a stirred solution of guaiacol (2.1 g, 0.0169 mol) in acetate buffer (200 ml) containing peroxidase (4 mg). Stirring was continued for a further 30 min after which the brown mixture was extracted with ethyl acetate (3 × 100 ml). The combined ethyl acetate extracts were dried (Na_2SO_4) and evaporated to give a dark oily residue (1.7 g). Reductive acetylation of the residue gave a colourless solid (1.9 g) which showed several spots on TLC (benzene-ethyl acetate 15:1, developed four times with intermittent drying). The main oligomeric components were separated by chromatography on a silicic acid column (2 × 100 cm). Elution with benzene-ethyl acetate (15:1) gave in the following order:

(1) *4-Acetoxy-2',3-dimethoxydiphenyl ether* (II). The oil gave a yellow spot with vanillin-H_2SO_4. It was eluted simultaneously with guaiacol acetate (bright orange spot with vanillin-

H_2SO_4) and was separated from the latter by preparative thin-layer chromatography using benzene-chloroform (10:1) as eluent. The plate was developed three times with intermittent air drying. The oil gave only one spot (orange) on TLC (benzene-chloroform 10:1). Structure II was assigned to the compound which probably is indistinguishable from isomer III. ν_{max} 1765 cm^{-1} (ester C=O) [Found: C 64.61; H 5.55. *m/e* 288 $(M)^+$. $C_{16}H_{16}O_5$ (M 288) requires: C 64.43; H 5.38] δ 7.20 – 6.29 (m, 7 H); 3.80, 3.73 (s, 3 H, 3 H); 2.25 (s, 3 H). *m/e* 288 (40), 246/247 (100/70), 199 (20), 183 (12),171 (11), 149 (16), 136 (56), 92/91 (30/27), 79/77 (20/38), 51 (10), 43 (50).

(2) *2,2'-Diacetoxy-3,3'-dimethoxydiphenyl* (IV). Colourless prisms, m.p. 132 – 133° (lit: 133 – 134°),[17] gave a blue spot with vanillin-H_2SO_4. ν_{max} (KBr) 1760 cm^{-1} (ester C=O). δ 7.30 – 6.60 (m, 6 H); 3.84 (s, 6 H) 2.29 (s, 6 H). *m/e* 330 (2), 288 (65), 246/247), (100/56), 231 (15), 213/214 (22/15), 203 (20), 191 (8), 185/186 (8/5), 171 (4), 43 (7).

(3) *4,4'-Diacetoxy-3,3-dimethoxydiphenyl* (V). Colourless needles, m.p. 198 – 200° (lit: 195 – 198°),[17] gave a pale grey spot with vanillin-H_2SO_4. ν_{max} (KBr) 1765 cm^{-1} (ester C=O) δ 7.30 (s, 2 H); 7.19 (s, 4 H); 3.87 (s, 6 H); 2.20 (s, 6 H). *m/e* 330, 288 (15), 246 (100), 231 (10), 203 (9), 185 (3), 171 (2), 160 (2), 131 (4), 115 (4), 57 (3), 43 (20). The diaryls IV and V were eluted simultaneously and purified by fractional crystallisation from ethanol.

(4) *4,4'-Diacetoxy-3,3'-dimethoxy-5-guaiacoxydiphenyl* (VII) was obtained as fine needles, m.p. 172 – 173°, after several crystallisations of a fraction following the diaryls IV and V. It gave a pink spot with vanillin-H_2SO_4. ν_{max} (KBr) 1760, 1754 cm^{-1} (ester C=O). [Found: C 66.67; H 5.46. *m/e* 452 (M^+). $C_{25}H_{24}O_8$ (M 452) requires: C 66.40; H 5.31]. δ 7.05 – 6.95 (m, 9 H); 3.83, 3.80, 3.75 (s, 3 × 3 H); 2.25 (s, 3 H); 2.11 (s, 3 H). *m/e* 452 (78), 410 (83), 370 (83), 369/368 (55/95), 329/328 (40/85), 288 (26), 209 (60), 196 (44), 193 (60), 152/151 (82/100), 137/136 (82/83), 123 (57), 91 (25), 79/78 (32/32), 77 (48), 51 (40).

The crude fraction from which compound VII was separated showed mass ion peaks at *m/e* 410, 452, and 494. Indication that the fraction consisted of a mixture of different compounds was obtained from TLC, which showed overlapping coloured spots with the pink colour of compound VII, (*m/e* 452) predominating. Subsequent fractions showed ion peaks at higher mass numbers (*m/e* 532, 574, 616 and 658. Final elution with 25 – 50 % ethyl acetate produced fractions which exhibited mass ion peaks at *m/e* 654, 696, and 738. Each fraction showed at least two overlapping spots giving different colours with the spray reagent, indicating mixtures. Methoxyl analysis of the combined fractions gave a value of 17.05 %. (Calculated OCH_3 content for acetylated guaiacol 18.67 %) indicating that no extensive demethylation had taken place during the enzymatic treatment.

(*b*) *with laccase.* Laccase (2 mg) was added to a solution of guaiacol (0.6 g) in acetate buffer (100 ml) and the mixture was stirred for 30 min. Another batch containing laccase (3.2 mg) and guaiacol (2.8 g) in acetate buffer (250 ml) was stirred overnight. The preparations were worked up separately and the residues were reductively acetylated. The two resulting mixtures showed the same spots on TLC (*RF*-values and colours) as did a corresponding reaction mixture obtained by peroxidase/H_2O_2 oxidation.

Oxidation of 2,6-dimethoxyphenol (XVIII) with peroxidase/H_2O_2. 2,6-Dimethoxyphenol (1.0 g, 0.0065 mol) was dissolved in acetate buffer (200 ml) and peroxidase (5 mg) was added. To the stirred solution hydrogen peroxide (12 ml, 1 %, 0.0035 mol) was added during 30 min and the mixture was stirred for another 2 h. Purple crystals (0.6 g) precipitated [coerulignone (XIX, m.p. 293° (lit: 294°) [18] 0.6 g]. Reductive acetylation gave the diacetate, m.p. 231 – 233° (lit: 239°).[18] δ 6.73 (s, 4 H); 3.85 (s, 12 H); 2.83 (s, 6 H).

Oxidation of guaiacylglyceryl ethers with peroxidase/H_2O_2. 1-Guaiacoxy-2,3-dihydroxypropane (XX) and 2-guaiacoxy-1,3-dihydroxy-propane (XXI) (0.22 g of each) were separately dissolved in acetate buffer solution (100 ml) and peroxidase (5 mg) was added. To the stirred solutions a 1 % hydrogen peroxide solution (4 ml) was added during 30 min. The mixtures were stirred for 2 h and then worked up as described above. Compounds XX and XXI were recovered quantitatively. The identity of the unchanged compounds was proven by determination of melting points with admixture of authentic samples and also by TLC (chloroform-methanol-acetic acid 100:10:3).

A solution of 1-(*o*-hydroxyphenoxy)-2,3-dihydroxy-propane (XXII) (13 mg) in acetate buffer (20 ml), containing peroxidase (3 mg) was similarly treated with a solution of 1 % hydrogen peroxide (3 ml). The mixture turned reddish-brown. Extraction with ethyl acetate yielded a light oil. The TLC (chloroform, methanol, acetic acid (100:10:3) showed

residual starting material and coloured polymeric material but no glycerol or catechol.

Oxidation of 1-(4-hydroxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy)-ethanol (XXV). *(a) with peroxidase/H_2O_2.* Compound XXV (4.0 g, 0.0125 mol) was dissolved in acetone (120 ml) and acetate buffer solution (500 ml), followed by peroxidase (10 mg), was added. Hydrogen peroxide (36 ml, 1 %, 0.0105 mol) was then added dropwise during 30 min to the stirred solution. The stirring was continued for 2 h and the mixture was then extracted with ethyl acetate (3 × 100 ml). The oily residue (3.8 g) obtained after evaporation of the solvent, consisted of two major overlapping components (TLC, $CHCl_3$:EtOH, 100:1, developed three times). Better resolution of the mixture was obtained after acetylation, the TLC (benzene-ethyl acetate, 3:1, developed three times) showing three components. The acetylated mixture (1.8 g) was separated on a silicic acid column (2 × 75 cm) to yield in the order given:

(1) o,o-*Diaryl tetraacetate* XXVII (0.69 g) (37 %) solid resin, ν_{max} (KBr) 1760, 1740 cm^{-1} (ester C=O). [Found: C 62.51; H 5.91, *m/e* 806. $C_{42}H_{46}O_{16}$ (M 806) requires: C 62.53; H 5.73]. δ 7.35–6.43 (m, 10 H); 6.05 (t, 2 H, J = 6 cps); 4.25 (d, 4 H, J = 5 cps); 3.89, 3.76 (s, 6 H, 12 H); 2.05, 2.03 (s, 6 H, 6 H). Mol. wt. 881 (osmometry).

(2) *Compound XXVIII*, solid resin (0.15 g, 8.3 %). ν_{max} 1760, 1740 cm^{-1} (ester C=O). [Found: 62.66; H 5.87. $C_{61}H_{66}O_{23}$ (M 1166) requires: C 62.77; H 5.66]. δ 7.35–7.45 (m, 15 H); 6.20–5.90 (b, 3 H); 4.31–4.08 (b, 6 H); 3.85–3.70 (b, 27 H); 2.1, 2.0 (15 H). Mol. wt. 1095 (osmometry).

The third component was obtained in a small yield which did not allow identification.

(b) with laccase. Laccase (2.5 mg) was added to a solution of XXV (0.11 g) in acetone (10 ml) and acetate buffer (200 ml). The mixture was stirred for 2 h and worked up as described above. The mixture of acetylated products exhibited a TLC pattern identical to that of the mixture obtained by peroxidase/H_2O_2 oxidation.

Oxidation of pinoresinol (XXIX). (a) with peroxidase/H_2O_2. Pinoresinol (XXIX) (1.02 g, 0.0028 mol) was dissolved in acetone (50 ml) and acetate buffer solution (300 ml). To the stirred solution peroxidase (10 mg) and then during 20 min 1 % hydrogen peroxide solution (11 ml, 0.003 mol) was added. The mixture was stirred for 2 h and then worked up as described above. Acetylated and reductively acetylated samples of the resulting mixture showed an identical TLC pattern. The main components of the mixture were separated on a silicic acid column (2 × 70 cm) using benzene-ethyl acetate (3:2) as eluent to give:

(1) *Dehydro-dipinoresinol tetraacetate (XXX)*. Colourless needles (0.31 g), m.p. 202° (lit: 195°).[39] ν_{max} (KBr) 1760 cm^{-1} (ester C=O). (Found: C 64.93; H 5.57. Calc. for $C_{48}H_{52}O_{16}$ (M 884): C 65.01; H 5.84). δ 7.2–6.45 (m, 10 H); 4.70. [b, 4 H(α)]; 4.48–3.9 [m, 8 H(γ)]; 3.85 (s, 12 H); 3.08 [b, 4 H(β)]; 2.14, 2.02 (s, 6 H, 6 H).

(2) *Trimer (XXXI)* solid resin, 0.25 g. ν_{max} (KBr) 1760 cm^{-1} (ester C=O). (Found: C 66.6; H 6.30. $C_{70}H_{72}O_{23}$ (1280) requires: C 65.62; H 5.62). Mol. wt. 1303 (osmometry).

(b) with laccase. To a solution of compound XXIX (0.12 g) in acetone (20 ml) and acetate buffer solution (175 ml) laccase (1.5 mg) was added and the mixture stirred for 2 h. The mixture was worked up as described above. The acetylated mixture was indistinguishable from that obtained by peroxidase/H_2O_2 oxidation of XXIX (TLC).

Oxidation of dihydro-dehydro-diisoeugenol (XXXII). (a) with peroxidase/H_2O_2. Hydrogen peroxide (6 ml, 1 %, 0.0017 mol) was added during 15 min to a stirred solution of XXXII (1.02 g, 0.00305 mol) in acetone (100 ml) and acetate buffer (130 ml) containing peroxidase (10 mg). The mixture was kept with stirring for 2 h and then worked up in the usual way. TLC of the residue (chloroform, developed five times) showed four components which were separated both as phenols and as acetates by chromatography on a silicic acid column (2 × 50 cm). The following compounds were obtained in pure form:

(1) *Dihydro-dehydro-diisoeugenol (XXXII)*, m.p. 87–89°. The infrared spectrum was indistinguishable from that of the starting material. Mixed m.p. with authentic XXXII 87–89°.

(2) o,o-*Dihydroxy-diaryl XXXIII*.[40] Solid resin (0.3 g); ν_{max} 3550–3400 cm^{-1} (OH). (Found: C 73.52; H 6.97. *m/e* 654 (M^+). Calc. for $C_{40}H_{46}O_8$ (M 654): C 73.39; H 7.03). δ 7.0 (s, 4 H); 6.55 (s, 4 H); (b, 2 OH); 5.10 (d, 2 H, J = 7 cps), 3.82 (s, 12 H); 3.60–3.30 (m, 2 H); 2.50 (t, 4 H, J = 6 cps); 1.80–1.30 (m, 10 H); 0.95 (t, 6 H, J = 6 cps). *Diacetate XXXIV*, solid resin, ν_{max} (KBr) 1760 cm^{-1} (ester C=O) *m/e* 738(M^+).

(3) *o-Hydroxydiaryl ether XXXV*. Solid resin (0.1 g); ν_{max} (KBr) 3600–3300 cm^{-1} (OH). (Found: C 73.46; H 6.98. *m/e* 654 (M^+). $C_{40}H_{46}O_8$ (654) requires: C 73.39; H 7.03).

δ 7.35 – 6.55 (m, 10 H); 5.90 (s, 1 H); 5.05 (t, 2 H, $J = 6$ cps); 3.85 (s, 12 H); 3.10 – 3.30 (m, 2 H); 2.70 – 2.45 (t, 4 H, $J = 6$ cps); 1.90 – 1.30 (m, 10 H); 1.0 (t, 6 H, $J = 6$ cps). *Acetate XXXVI*, solid resin, ν_{max} 1760 cm^{-1} (ester C=O) *m/e* 696(M^+).

(4) *Compound XXXVII* was contaminated by the diaryl XXXIII. Purification was only accomplished by preparative TLC of the acetylated mixture using benzene-ethyl acetate (15:1). *Diacetate XXXVIII*, solid resin (0.1 g); ν_{max} (KBr) 1760 cm^{-1} (ester C=O). *m/e* 1064 (M^+). (Calc. for $C_{64}H_{72}O_{14}$: 1064).

(b) with laccase. Compound XXXII (84 mg) and laccase (3 mg) were dissolved in acetone (60 ml) and acetate buffer (150 ml). The mixture was stirred for 2 h and then worked up as described above. The mixture of acetylated products was indistinguishable from the corresponding mixture of acetylated products obtained by peroxidase/H_2O_2 oxidation (TLC).

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Received May 3, 1973.