Phytochemistry, 1975, Vol. 14, pp. 543–552. Pergamon Press. Printed in England.

ENZYMATIC AND HYDROLYTIC DEGRADATION OF BENZYLARYL ETHERS RELATED TO HARDWOOD LIGNINS

DAVID CIARAMITARO and CORNELIUS STEELINK

Department of Chemistry, University of Arizona, Tucson, AR 85721, U.S.A.

(*Revised Received* 22 *July* 1974)

Key Word Index—lignin; benzylaryl ethers; degradation; peroxidase; free-radical intermediates.

Abstract—A number of 4-hydroxybenzylphenyl ethers and their acetates were synthesized as models for hardwood lignin and used as substrates in acid hydrolysis and enzymatic oxidation reactions. Under hydrolytic conditions, the acetates underwent ether cleavage at a slower rate than the free phenols. Evidence for carbonium ion intermediates is presented. Cleavage of the ether substrates by peroxidase–peroxide oxidation was much faster than by acid hydrolysis for all substrates except the acetates which did not react. Subsequent oxidation of the component parts of the ether substrates was selective: the syringyl moieties were oxidized in preference to the guaiacyl moieties. Electron spin resonance studies of the oxidation reaction showed that removal of the phenolic hydrogen atom was the first step, followed by quinone–methide formation. A mechanism is proposed to account for the oxidative degradation of the lignin models.

INTRODUCTION

Degradation of wood lignin by fungi and bacteria has been a subject of intense interest for plant scientists [1]. In recent years, it has become an important factor in the microbiological treatment and utilization of wood wastes. Most studies have shown that fungi and bacteria degrade lignin by hydrolytic and/or oxidative processes.

To gain insight into the mechanism of microbiological attack, investigators have used monomeric model compounds as substrates in their model reactions. Most of these substrates have been patterned on softwood lignin species: i.e. guaiacyl compounds [2–4]. Fewer studies have appeared on hardwood lignin species, owing in part to the scarcity of good model compounds [5, 6] and in part to the greater industrial interest in softwoods.

In recent years, our own studies have focused on the oxidative reactions of hardwood model compounds: i.e. syringyl-derived monomers [5, 7–10]. These syringyl compounds show substantially different reaction patterns from their guaiacyl analogues. Many useful deductions can be drawn about the reaction of the hardwood lignin polymer from the reactions of monomeric models. However, depolymerization of lignin involves breaking monomer–monomer bonds; to gain insight into this process, one must use dimeric or oligomeric models as substrates. The benzylarylether bond is one of the most important bonds in hardwood lignin. A recent report [11] has suggested that 31% of all monomer–monomer bonds in hardwood lignin are of this type. It is certainly one of the bonds most sensitive to hydrolytic cleavage. Model dimers incorporating this ether bond would be representative of important structural units in the lignin polymer. A study of their reactions should elucidate the behavior of lignin under similar conditions.

To carry out this objective, we synthesized a number of substituted benzylaryl ethers incorporating guaiacyl and syringyl moieties representative of hardwood lignin. These were subjected to mild acid hydrolysis and treatment with hydrogen peroxide and peroxidase under physiological conditions. In some cases, acetates of the parent phenols were used to mimic ester structural types in lignin [12].

RESULTS

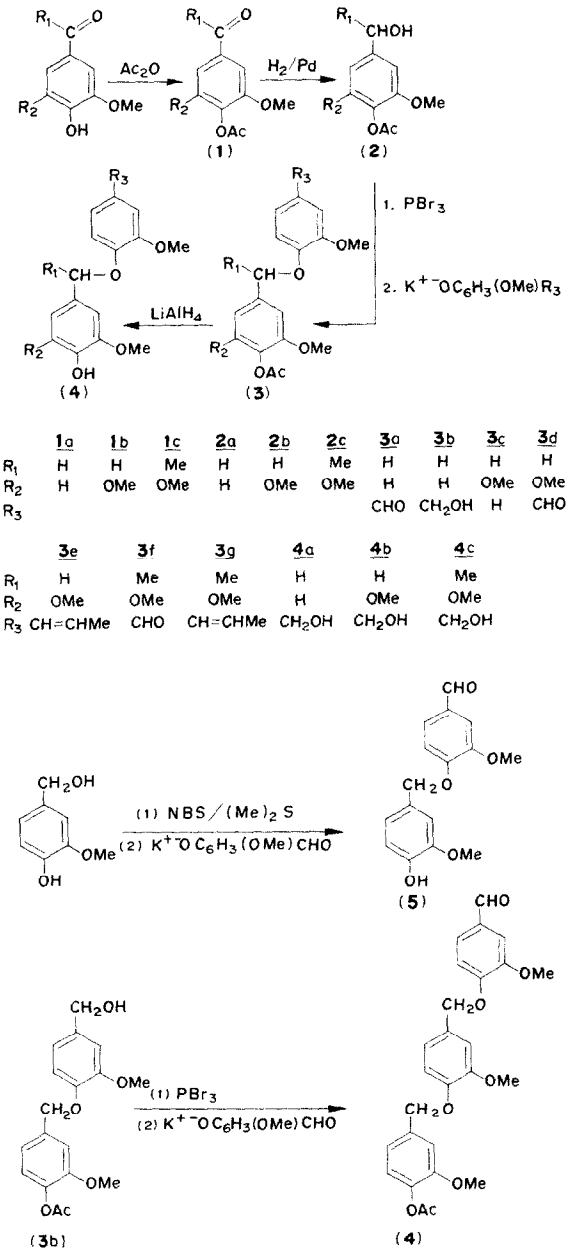
Synthesis of benzylaryl ethers

The model ethers were prepared by a procedure based on those of Mikawa [13] and Adler [14].

The reaction sequence is illustrated in Scheme 1. Vanillin, syringaldehyde or acetosyringone was treated with acetic anhydride in pyridine to afford a protecting group for the phenol; the resulting acetate (**1**) was hydrogenated to yield the 4-*O*-acetyl benzyl alcohol (**2**). This alcohol was brominated with PBr_3 in chloroform and the resulting bromide was coupled with the potassium salt of a phenol. The acetate protecting group was removed by reduction with $LiAlH_4$ to yield the 4-hydroxybenzylphenyl ether (**4**).

An alternate synthesis, utilizing the bromination technique of Corey *et al.* [15] was made in an effort to eliminate the acetate blocking step. Vanillyl and syringyl alcohols were reacted with a dimethylsulfide-*N*-bromosuccinimide brominating complex at low temperature and high dilution; the resulting bromides were coupled with potassium phenolates as before. This method worked fairly well for vanillyl alcohol producing a moderate yield of **5**; the syringyl derivative, however, reacted only to a slight extent.

A route to ether trimers as possible lignin model oligomers, was also investigated. The dimer **3b** was brominated and coupled with the potassium salt of vanillin to afford a good yield of trimer **6**.



Scheme 1. Synthesis of model ethers.

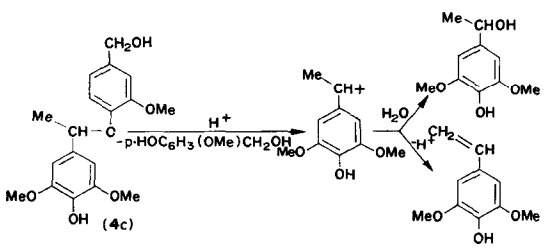
Mild acid hydrolysis

All model ethers were hydrolyzed under standard lignin conditions [16]: 0·2 M HCl in 9:1 dioxane–water at 50° for varying periods of time. The products of hydrolysis were identified by TLC. The results of a number of experiments are listed in Table 1; the model ethers are listed in order of decreasing reactivity towards hydrolysis.

In general, free phenols (**4a–c, 5**) were completely hydrolyzed in 8 hr. Acetate derivatives (**3a–d, 6**), on the other hand, required up to 24 hr for complete cleavage of the ether and ester linkages. The trimer (**6**), was most resistant to hydrolysis. Elimination and hydrolysis were competing reactions in **4c**, an expected consequence of the formation of a benzyl carbonium ion intermediate. Among the hydrolysis products of **4c** was a small amount of 1-(3,5-dimethoxy-4-hydroxyphenyl)-ethylene. Although it has been substantiated that diphenylmethane derivatives can also be formed in the course of these hydrolysis reactions with lignin and lignin models [16, 17], none has been found thus far in the hydrolyses of these benzylaryl ether models. In other respects, these ethers appear to be good models for lignin in hydrolysis reactions. Styrenes and stilbene derivatives [18, 19] and various low-MW phenols including vanillin and vanillyl alcohol derivatives are hydrolysis products of spruce lignin and lignin model phenols.

Table 1. Products and estimated (to within 5%) yields from acid hydrolysis of model compounds. Listed in order of decreasing reactivity with 0·2 M hydrochloric acid in 9:1 dioxane–water

Compound	Time (hr)	Product	% Yield
4a	8	Vanillyl alcohol	95
		low R_f products	Trace
5	8	Vanillyl alcohol	45
		Vanillin	45
		Low R_f products	10
4c	8	1-(3,5-Dimethoxy-4-hydroxyphenyl)-ethylene	15
		α-Methyl syringyl alcohol	20
		Vanillyl alcohol	20
		Low R_f products	25
4b	8	Starting material	20
		Syringyl alcohol	40
		Vanillyl alcohol	40
	24	Syringyl alcohol	45
		Vanillyl alcohol	45
		Low R_f products	Trace
3b	8	Vanillyl alcohol	50
		4-*O*-Acetylvanillyl alcohol	30
		Low R_f products	20
	24	Vanillyl alcohol	70
		Low R_f products	30
3d	8	Starting material	60
		Vanillin	20
		4-*O*-Acetylsyringyl alcohol	10
		Syringyl alcohol	10
	24	Starting material	50
		Vanillin	25
		Syringyl alcohol	20
6	8	Starting material	60
		Vanillin	25
		Vanillyl alcohol	10
		4-*O*-Acetylvanillyl Alcohol	5
	24	Starting material	50
		Vanillin	25
		Compound **5**	10
		Vanillyl alcohol	10
		Low R_f products	5



Oxidation with hydrogen peroxide and horseradish peroxidase

All oxidation reactions were carried out by mixing ether substrate (10^{-3} M) with horseradish peroxidase (10^{-6} M) and two or more equivalents of hydrogen peroxide in MeOH–H_2O (1:4). These reaction mixtures were allowed to stand at room temperature for varying periods of time.

In contrast to the acid hydrolysis reactions (previous section), all of the free phenol ether dimers (**4a–c**) were rapidly cleaved at the ether bond by enzyme and hydrogen peroxide at pH 6. The acetates, on the other hand, remained unaltered by the oxidizing system. Also, in contrast to their hydrolysis reactions, all of the free phenol ethers were completely cleaved during the reaction period by the enzyme system. No dimeric starting material was found in the product mixtures, except in cases where supersaturated solutions of starting materials precipitated out. Since the substrate must have

a free phenolic hydrogen to be attacked by peroxidase–peroxide, it appears that 1-electron dehydrogenation is the first step in the reaction sequence. This is borne out by esr studies (described later) and is consistent with our previous studies on monomers [7].

Table 2 shows the products and estimated yields for the oxidation of model compounds. In all cases, five to six identifiable oxidation products were found. Most of these compounds represent further oxidation of the two phenyl moieties in the benzylaryl ethers. Both side chain oxidation and oxidative cleavage of the side chain occur. Excess hydrogen peroxide only causes further side chain cleavage of syringyl moieties to 2,6-dimethoxybenzoquinone and some cleavage of the guaiacyl moiety to 2-methoxybenzoquinone. The syringyl moiety also suffered some demethylation to yield the *o*-quinone.

In all experiments, the syringyl moiety gave higher concentrations of oxidation products than did the guaiacyl moiety. In order to investigate more fully the relative ease of oxidation of these two moieties, a competing reaction was carried out with equimolar amounts of syringyl and vanillyl alcohols with two equivalents of hydrogen peroxide. The product mixture was analyzed by TLC. It showed syringaldehyde to be the major product, with smaller amounts of 2,6-dimethoxybenzoquinone and vanillin with small, but equal, amounts of starting materials. Thus, syringyl alcohol is preferentially oxidized to the aldehyde and also preferentially cleaved to the quinone.

The length of the side chain also affects the course of the oxidation reaction. When **4c** (ether of α-methylsyringyl alcohol), is treated with two equivalents of hydrogen peroxide and enzyme, the major product is acetosyringone, and the minor products are vanillin, vanillyl alcohol, syringyl alcohol and 1-(3,5-dimethoxy-4-hydroxyphenyl)-ethylene. No benzoquinone is detected. Although the syringyl moiety is oxidized preferentially as in the previous experiments, side chain cleavage (i.e. ejection of acetaldehyde) is more difficult than ejection of formaldehyde, and no quinone is formed. The ethylene derivative could arise from a rearrangement of the intermediate quinone methide or simple acid hydrolysis.

Two alternate mechanisms could explain the appearance of syringyl and vanillyl alcohols from the oxidation of **4b**. Simple hydrolysis, catalyzed by the weakly acidic peroxide solution or radical initiated cleavage are both possible.

To determine whether or not hydrolysis occurs under the reaction conditions, a 20% MeOH–H_2O solution of IVb was allowed to stand with two equivalents of hydrogen peroxide and no enzyme for time periods of 1 day, 1 week and 4 weeks. After 24 hr, very small quantities of hydrolysis products were detected. After one week, some precipitated starting material was found in addition to small amounts of syringyl and vanillyl alcohols and a relatively large amount of 4-hydroxy-3,5-dimethoxybenzylmethyl ether. The 4-week reaction mixture yielded the above products plus small amounts of syringaldehyde and 2,6-dimethoxybenzoquinone. Thus, it appears that the syringyl and vanillyl alcohols formed in the enzymatic oxidations (24 hr) are largely products of oxidation rather than hydrolysis.

Scheme 2 is postulated to account for the formation of the aldehyde and the quinone. The failure of the acetate-blocked ether to react and previous reports of the reactions of syringyl alcohols [7] indicate that the phenolic hydrogen is homolytically cleaved in the first step, to yield radical **7**, which can react further. The proposed reaction sequence leading to the 4-methoxy ether and the *p*-quinone (from radical **7b**) does not occur under the reaction conditions employed here. No veratryl or trimethoxybenzyl derivatives were ever found in product mixtures from oxidations of **4a** or **4b**.

A more feasible mechanism (route A) involves the aldehyde with subsequent oxidation and liberation of formaldehyde as a byproduct. Evidence for this pathway was obtained by carrying out the oxidation in and detecting formaldehyde with Nash's [20] reagent. The possibility that solvent methanol might oxidize to formaldehyde was eliminated by a blank determination.

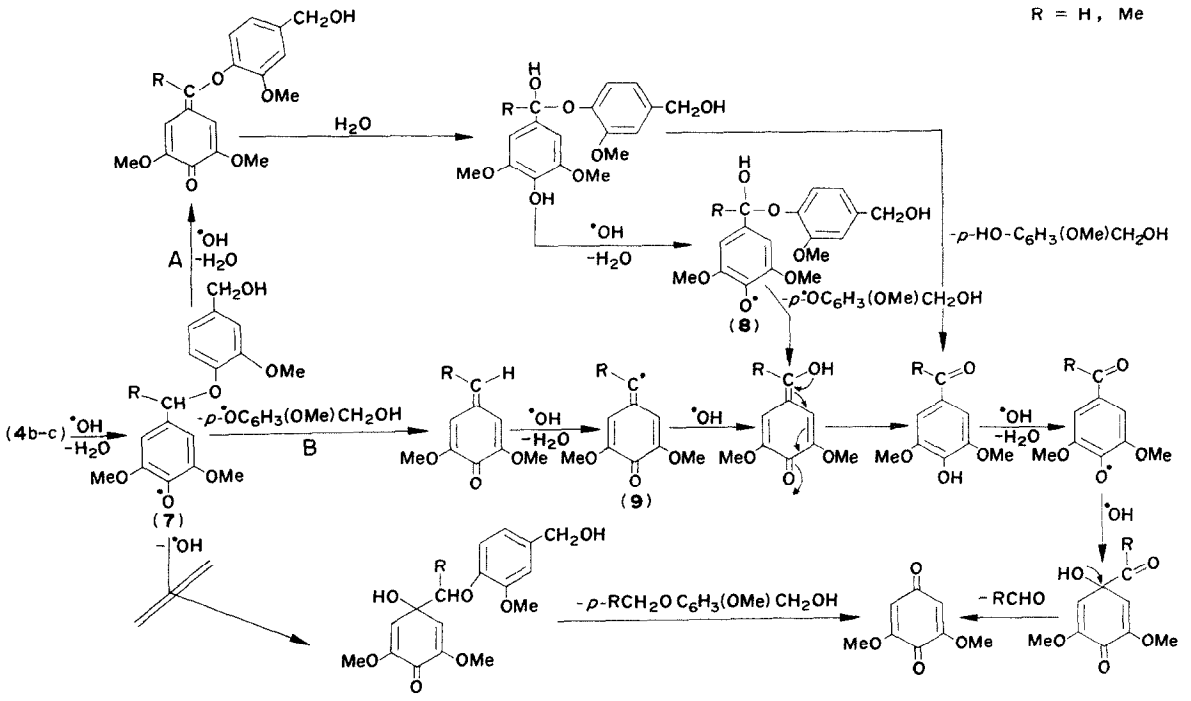
The guaiacyl dimers **4a** and **5** reacted with enzyme and peroxide to yield products analogous to those of the syringyl compounds. Larger amounts of unidentified low-R_f products were detected by TLC, indicating that dimerization of the guaiacyl radicals had probably taken place.

A small amount of low-R_f red chromophore appeared on the chromatograms of all reaction mixtures containing syringyl derivatives. This chromophore was conspicuously absent in all oxi-

Table 2. Products and estimated (to within 5%) yields from enzymatic oxidations of model compounds

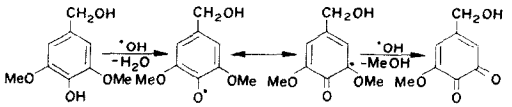
Compound	Method*	Product	%
4a	A	Vanillin	10
		Vanillyl alcohol	10
		Compound R_f 0·37	15
		Compound R_f 0·30	15
		Compound R_f 0·16	15
		Compound R_f 0·09	15
4b	A	Syringaldehyde	42
		2,6-Dimethoxy-*p*-benzoquinone	9
		Syringyl alcohol	12
		Vanillyl alcohol	22
		Vanillin	5 (variable)
		Compound R_f 0·27	3
		Chromophore R_f 0·15	3
		Compound R_f 0·10	3
4b	B	Syringaldehyde	30
		2,6-Dimethoxy-*p*-benzoquinone	20
		2-Methoxy-*p*-benzoquinone	5
		Vanillin	10
		Syringyl alcohol	8
		Vanillyl alcohol	10
		Chromophore R_f 0·12	Trace
		Low R_f products	10
4b	C	3,5-Dimethoxy-4-hydroxybenzyl methyl ether	45
		Starting material	10
		Syringyl alcohol	20
		Vanillyl alcohol	25
4b	D	3,5-Dimethoxy-4-hydroxybenzyl methyl ether	45
		Starting material	10
		Syringaldehyde	5
		2,6-Dimethoxy-*p*-benzoquinone	Trace
		Vanillyl alcohol	25
		Syringyl alcohol	15
4c	A	Acetosyringone	35
		Vanillin	20
		1-(3,5-Dimethoxy-4-hydroxyphenyl)-ethylene	5
		Vanillyl alcohol	10
		α-Methyl syringyl Alcohol	5
		Chromophore R_f 0·17	6
		Low R_f products	10
5	A	Vanillin	25
		2-Methoxy-*p*-benzoquinone	20
		Vanillyl alcohol	10
		Compound R_f 0·39	10
		Compound R_f 0·34	15
		Compound R_f 0·24	15
Equimolar mixture of vanillyl and syringyl alcohols	A	Syringaldehyde	45
		Vanillin	5
		2,6-Dimethoxy-*p*-benzoquinone	15
		Vanillyl alcohol	10
		Syringyl alcohol	10
		Low R_f compounds	10

* A—2 Equivalents peroxide, 0·5 mg peroxidase, 24 hr; B—excess peroxide, 1·0 mg peroxidase, 24 hr; C—2 equivalents peroxide, no peroxidase, 1 week; D—2 equivalents peroxide, no peroxidase, 4 weeks.



Scheme 2. Enzymatic oxidation of model ethers **4b** and **4c**.

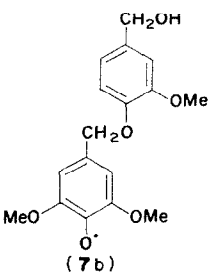
dations of guaiacyl model ethers. We suspected that this compound might be an orthoquinone [10] but comparison with an authentic sample of 5- hydroxyvanillin *o*-quinone by TLC did not show identity. However, identity with 5-hydroxyvanillyl alcohol *o*-quinone was determined by R_fs and a MS of the TLC eluate. Thus, a side reaction of demethylation occurs in these oxidations:



As in the acid hydrolysis experiments, the model ethers compare well with lignin under the conditions of enzymatic oxidation. Vanillin and syringaldehyde [21] and 2,6-dimethoxybenzoquinone [22] all have been isolated from the products of fungal attack of hardwoods. Various syringyl and guaiacyl ketone and alcohol derivatives have also been detected from hardwood products [22].

ESR studies

In order to gain insight into the nature of the radical species produced during the enzymatic oxidation of ether model compounds, a number of these reactions were monitored by ESR spectroscopy. Model ether substrate (10^{-2} M) was mixed with hydrogen peroxide (3×10^{-2} M) and horseradish peroxidase (10^{-6} M) and examined immediately after mixing. As expected, all model compounds underwent immediate dehydrogenation to yield radical species. For example, **4b** yielded radical **7b**, identified as a triplet of septuplets. Except for slightly different coupling constants, this ESR spectrum was identical to that found for syringyl alcohol [8] (see Table 3).



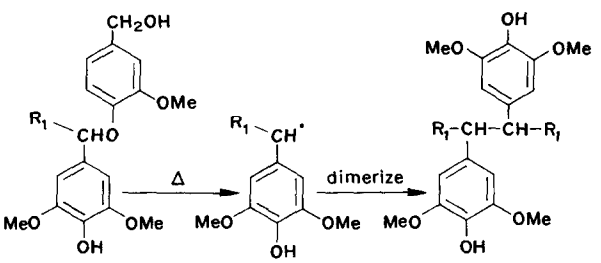
After 14 min, the radical disappeared and a new spectrum (a doublet of quintets) began to form. This spectrum corresponds to a syringyl nucleus

Table 3. Hyperfine coupling constants (in G) of radical intermediates and products of ether and alcohol oxidations

Radical	Solvent*	Coupling constants $A^{H}_{\alpha,\beta}$ OMe	A^{H}_{other}
7	A	1·36 (8)	9·50 (2)
8 or **9**	A	1·48 (8)	4·88 (1)
Syringaldehyde	A	1·55 (8)	
9c	B	0·69	5·60 (1)
Acetosyringone	B	1·61 (8)	
Syringyl alcohol[8]	A	1·35 (8)	9·32 (2)
α-Methyl syringyl alcohol	A	0·70	4·34 (1)

* A—22·5% MeOH–H_2O. B—12·5% Cellosolve–H_2O.

with one benzyl proton. After another 15 min, the spectrum changes to a strong seven-line pattern, identical to the radical derived from syringaldehyde. In the oxidation of compound **4c** (which contains an α-methyl group in place of a hydrogen atom), only two ESR spectra were observed. The first spectrum was a doublet of septuplets, resembling that of α-methylsyringyl alcohol. After its disappearance in 35 min, a second spectrum appeared which was characteristic of acetosyringone [5]. The lack of an intermediate spectrum (as was the case with **4b**) is consistent with the lack of a benzyl hydrogen atom.



(4b) MW = 320
(4c) MW = 334

(10b) MW = 334
(10c) MW = 362

These results are entirely consistent with the mechanism proposed in Scheme 2 route A, although alternative routes such as B cannot be excluded. In the case of the oxidation of **4b**, the sequence of ESR spectra differ from those reported for the oxidation of syringyl alcohol [8]. In the alcohol spectra, there were no time intervals between the appearance of different radical species. In the corresponding ether spectra, complete disappearance of radicals occurred before emergence of new radicals, indicating that intervening non-radical processes were taking place.

Route A could account for this phenomenon. Initial dehydrogenation of the phenol of compound **4b** or **4c** produces a phenoxy radical (**7b** or **7c**), which subsequently loses a benzylic hydrogen to form a quinone–methide. This hydrates rapidly to form a second phenol. In the case of **4b**, this hydrated intermediate can dehydrogenate as before to yield **8**, giving the intermediate radical seen on the ESR. In the case of **4c**, the intermediate simply loses the guaiacyl phenol and tautomerizes to acetosyringone, which undergoes the final oxidation step.

Route B is a possible alternate in Scheme 1, since there is no compelling evidence to exclude it. Loss of phenoxy radical from the radical **7** yields a quinone methide which could be responsible for the intermediate radical spectrum. However, since quinone methides of this type are extremely susceptible to hydrolysis, and would most likely hydrolyze before oxidation, route B would probably be a minor pathway in the reaction. When guaiacyl derivatives were oxidized under identical conditions, no ESR signals were observed. Apparently, dimerization to biphenyl derivatives is rapid and scavenges all radical species.

DISCUSSION

To account for the degradation of hardwood lignin by fungi, many investigators have proposed the existence of hydrolytic enzymes. Presumably, these enzymes catalyze the depolymerization of lignin by cleaving ether and ester bonds. Our studies show that non-enzymatic acid hydrolysis can effect these cleavage reactions, although the conditions used in these experiments are much more severe than those at physiological pH values.

The most significant result of this work emerges from the enzymatic oxidation experiments. At physiological pH, hydrogen peroxide and peroxidase are capable of cleaving both benzylaryl ether bonds are arylalkyl ether bonds with remarkable ease. An absolute requirement for this reaction, however, is the availability of a free phenolic group. For hardwood lignins, this oxidation reaction alone could effect the loss of more than 30% of the bonds which cross-link the monomers in the polymer. Oxidative side chain cleavage could account for further depolymerization.

EXPERIMENTAL

General. Horseradish peroxidase enzyme Type VI (MW 44100) was purchased from Sigma and a stock soln of $1{\cdot}8 \times 10^{-6}$ M enzyme in glass-dis. H_2O was used in the enzymatic oxidations. NMR and ^{13}C-NMR spectra were taken using tetramethylsilane. DMSO, and D_2O as internal standards. Chemical shift values are given in δ (ppm) units in the text. ESR spectra were recorded on a Varian E-3 spectrometer. Solutions were placed in flat quartz cells and the spectra were monitored at 95·9 gHz with a frequency modulation of 100 kHz. M.ps are uncorrected. Elemental analyses were performed by Huffman Laboratories of Wheatridge, Colo., and Scandanavian Microanalytical Laboratories, Herlev, Denmark.

Synthesis. Procedures previously [23, 24] reported were used in the synthesis of the 4-*O*-acetyl derivatives of vanillin (**1a**), syringaldehyde (**1b**), and acetosyringone (**1c**) a new compound, yield 100%; m.p. 154–157°; NMR ($CDCl_3$) δ 2·34 (*s*,3), 2·58 (*s*,3), 3·82 (*s*,6), 7·19 (*s*,2); MS *m/e* (rel. int.) 238 (15), 197 (43), 196 (100), 182 (43), 181 (100), 153 (21), 66 (21), 43 (82). The 4-*O*-acetylbenzyl alcohols were made according to known procedures [13]. Compound **2b** gave a yield of 96%; m.p. 93·5–95·5°; NMR ($CDCl_3$): δ 2·21 (*s*,3), 3·68 (*s*,6), 4·42 (*s*,2), 6·54 (*s*,2); MS *m/e* (rel. int.): 226 (14), 185 (18), 184 (100), 167 (20), 123 (16), 109 (14), 43 (23). Compound **2c** gave a 99% yield; m.p. 76–77·5° NMR ($CDCl_3$): δ 2·01 (*d*,3), 2·12 (*s*,3), 3·76 (*s*,6), 5·44 (*q*,1), 6·88 (*s*,2); MS *m/e* (rel. int.): 240 (20), 198 (85), 183 (100), 155 (80), 137 (30), 123 (80), 109 (40).

3-Methoxy-5-hydroxymethyl-o-benzoquinone. To a soln of 5 g 5-hydroxyvanillin in 25 ml isopropanol was added 3 g $NaBH_4$. The soln was refluxed for 2 hr and allowed to stand overnight. It was then poured into satd H_3BO_3 and extracted 2× with $CHCl_3$. The extracts were evaporated; and the oily product was dissolved in MeOH and poured into a soln of 4 g $NaIO_4$ in 200 ml H_2O. The mixture was shaken until it was judged that the red color of the *o*-quinone had reached a maximum in intensity, and then extracted with $CHCl_3$. The extract was dried (K_2CO_3) evaporated and chromatographed on silica gel with 50% ether–pentane. The resultant red compound was identified by its MS. MS *m/e* (rel int.): 168 (4), 149 (16), 139 (5), 129 (24), 87 (25), 85 (93), 83 (100), 71 (30), 57 (50); λ_{max} (95% EtOH) 950 nm (ϵ 25·0).

3-Methoxy-4-O-acetylbenzyl-2′-methoxy-4′-formylphenyl ether (**3a**). A 20 ml $CHCl_3$ soln of 10 g (0·05 mol) **2a** was prepared and reacted with a 10-ml $CHCl_3$ soln of 14 g (0·05 mol) PBr_3. After addition was complete, the reaction mixture was boiled gently for 20 min. The soln was cooled, washed quickly with H_2O, dried ($MgSO_4$) and added dropwise to a refluxing suspension of 7·8 g vanillin (0·05 mol) and 7 g anhyd. K_2CO_3 in 150 ml Me_2CO. The reaction was refluxed in N_2 for 1 hr after addition was complete, then cooled, poured into H_2O and extracted with $CHCl_3$. The extracts were combined, washed, dried (Na_2SO_4) and evaporated to leave a brown oil which could be eluted on a silica gel column with 15–25% Et_2O–pentane or recrystallized from C_6H_6–pentane. Yield 10·2 g (61%); m.p. 121–123°; NMR ($CDCl_3$) δ 2·22 (*s*,3), 3·68 (*s*,3), 3·83 (*s*,3), 5·07 (*s*,2), 7·00 (*m*,6), 9·97 (*s*,1); MS *m/e* (rel. in.): 330 (1), 180 (71), 153 (33), 152 (21), 139 (56), 138 (100), 123 (26), 108 (18); IR: 2750 (CHO), 1745 (C=O), 1250 (C–O–C), 1112 (OMe).

The following new benzylaryl ethers were prepared from appropriate compounds by the method outlined for **3a** above: *3-Methoxy-4-O-acetylbenzyl-2′-methoxy-4′-hydroxymethylphenyl ether* (**3b**). Yield, 44%; m.p. 132–137°; NMR (DMSO-*d*,6): δ 2·23 (*s*,3), 3·78 (*s*,6), 4·41 (*d*,2), 5·03 (*s*,2), 5·10 (*m*,1), 7·02 (*m*,6); MS *m/e* (rel. int.): 332 (5), 196 (24), 154 (81), 138 (22), 137 (100), 122 (12); IR: 3610 (OH), 1760 (C=O), 1265 (C–O–C), 1155 (OMe). *3,5-Dimethoxy-4-O-acetylbenzyl-2′-methoxyphenyl ether* (**3c**). Yield, 13·6%; m.p. 96–99°; NMR ($CDCl_3$): δ 2·30 (*s*,3), 3·79 (*s*,6), 3·89 (*s*,3), 5·08 (*s*,2), 6·70 (*s*,2), 6·90 (*s*,4); MS *m/e* (rel. int.): 332 (15), 209 (84), 168 (58), 167 (100), 151 (25), 136 (15), 123 (20), 95 (11). *3,5-Dimethoxy-4-O-acetylbenzyl-2′-methoxy-4′-formylphenyl Ether* (**3d**). Yield, 63%; m.p. 151·5–154°; NMR ($CDCl_3$) δ 2·30 (*s*,3), 3·80 (*s*,6), 3·93 (*s*,3), 5·17 (*s*,2), 6·71 (*s*,2), 7·10 (*m*,3), 10·08 (*s*,1); MS *m/e* (rel. int.): 360 (4), 209 (82), 167 (100), 151 (40), 147 (49), 136 (22), 123 (30), 106 (18), 95 (25); IR: 2850 (CHO), 1780 (C=O), 1190 (OMe), 1140 (C–O–C). *3,5-Dimethoxy-4-O-acetylbenzyl-2′-methoxy-4′-(1-propenyl)-phenyl ether* (**3e**). Yield, 15%, m.p. 109–111°; NMR ($CDCl_3$) δ 1·75 (*d*,3), 2·17 (*s*,3), 3·63 (*s*,6), 3·72 (*s*,3), 4·88 (*s*,2), 6·38 (*m*,7); MS *m/e* (rel. int.): 372 (31), 206 (91), 167 (100), 165 (77), 164 (100), 163 (42), 149 (20), 123 (17), 107 (21); IR 1725 (C=O), 1570 (C=C), 1245 (C–O–C), 1135 (OCH_3). *Anal.* Found: C, 67·16; H, 6·51. $C_{21}H_{24}O_6$ requires: C, 67·73; H, 6·50%. *1-(3,5-Dimethoxy-4-O-acetylphenyl)-ethyl-2′-methoxy-4′-formylphenyl ether* (**3f**). Yield, 68%; m.p. 118–119·5°. NMR ($CDCl_3$): δ 1·65 (*d*,3), 2·22 (*s*,3), 3·70 (*s*,6), 3·84 (*s*,3), 5·30 (*q*,1), 7·05 (*m*,5), 9·97 (*s*,1); MS *m/e* (rel. int.): 374 (1), 223 (93), 197 (12), 182 (96), 181 (100), 162 (21), 151 (40), 137 (12), 121 (23); IR: 2885 (CHO), 1760 (C=O), 1260 (C–O–C), 1140 (OCH_3). *Anal.* Found: C, 64·01; H, 6·14. $C_{20}H_{22}O_7$ requires: C, 64·16; H, 5·92). *1-(3-5-Dimethoxy-4-O-acetylphenyl)-ethyl-2′-methoxy-4′-(1-propenyl)-phenyl ether* (**3g**). Yield, 10%; m.p. 114·5–117°; NMR ($CDCl_3$): δ 1·64 (*d*,3), 1·81 (*d*,3), 2·25 (*s*,3), 3·72 (*s*,6), 3·82 (*s*,3), 5·20 (*q*,1), 6·45 (*m*,7); MS *m/e* (rel. int.) 386 (6), 223 (15), 206 (16), 181 (100), 165 (52), 163 (100), 149 (14), 121 (16), 91 (28); IR: 1750 (C–O), 1600 (C=C), 1210 (C–O–C), 1135 (OMe). (*Anal.* Found: C, 68·51; H, 6·70. $C_{22}H_{26}O_6$ requires: C, 68·38; H, 6·78%). *3-Methoxy-4-O-acetylbenzyl-2′-methoxyphenyl-4′-benzyl-2″-methoxy-4″-formylphenyl- Ether* (**6**). In a procedure similar to that of the syntheses of the dimers, 1·7 g of **3b** was dissolved in 10 ml $CHCl_3$ and reacted with 1·4 g PBr_3. An acetone soln of 0·8 g vanillin was refluxed in N_2 with 3 g K_2CO_3 and the washed and dried $CHCl_3$ soln was added to the acetone slurry as before. The reaction mixture was refluxed for 2 hr after addition was complete and allowed to stir in N_2 at room temp. overnight. Workup followed the same procedure as that of the dimers. The product was recrystallized with difficulty from $CHCl_3$–pentane; an amorphous white powder was obtained. Yield 1·5 g (63%); m.p. 145–147°; NMR (DMSO-d_6): δ 2·25 (*s*,3), 3·78 (*s*,6), 3·81 (*s*,3), 5·02 (*s*,2), 5·09 (*s*,2), 7·19 (*m*,9), 10·12 (*s*,1); MS *m/e* (rel. int.): 466 (1), 315 (39), 179 (62), 151 (17), 137 (100), 122 (13), 107 (19); IR: 2790 (CHO), 1735 (C=O), 1255 (C–O–C), 1135 (OMe). (*Anal.* Found: C, 64·98; H, 5·85. $C_{26}H_{26}O_8$ requires: C, 66·95; H, 5·57%). *3-Methoxy-4-hydroxybenzyl-2′-methoxy-4′-hydroxymethylphenyl ether* (**4a**). A stirred suspension of 1 g (0·003 mol) **3a** was made in 50 ml Et_2O. To

this suspension was added 3·3 g (0·008 mol) finely crushed $LiAlH_4$. The mixture was stirred and refluxed for 2 hr. Excess hydride was destroyed by the addition of wet Et_2O to the reaction mixture, which was then poured into satd aq. H_3BO_3. The Et_2O layer was drawn off and the aqueous phase extracted with $CHCl_3$. All organic phases were combined, dried (Na_2SO_4) and evaporated. The oil which resulted crystallized from $CHCl_3$–Et_2O. Yield 0·1 g (11%); m.p. 173–176°; NMR (DMSO-d_6) δ 3·63 (*s*,6), 4·26 (*d*,2), 4·57 (*m*,1), 4·70 (*s*,2), 6·52 (*m*,6), 8·21 (*s*,1); MS *m/e* (rel. int.): 290 (4), 154 (71), 138 (77), 137 (100), 123 (68), 107 (11), 95 (25); IR (Nujol): 3480 (OH), 1280 (C–O–C), 1140 (OMe).

The same procedure was used with the appropriate acetate derivatives to prepare the following compounds: *3,5-Dimethoxy-4-hydroxybenzyl-2'-methoxy-4'-hydroxymethylphenyl ether* (**4b**). Yield, 68%; m.p. 100–105°; NMR (DMSO-d_6): δ 3·72 (*s*,9), 4·43 (*d*,2), 4·92 (*s*,2), 5·10 (*d*,1), 6·84 (*m*,5), 8·30 (*s*,1); MS *m/e* (rel. int.): 334 (30), 320 (11), 167 (100), 154 (4), 137 (4), 122 (5), 106 (3); ^{13}C NMR (DMSO-d_6): δ 55·2, 56·6, 62·2, 70·0, 105·4, 110·0, 113·3, 117·9, 126·4, 134·6, 135·0, 145·6, 147·0, 148·4; IR (Nujol): 3650 (OH), 1225 (OMe), 1120 (C–O–C). (*Anal.* Found: C, 63·07; H, 6·27 $C_{17}H_{19}O_6$ requires: C, 63·74; H, 6·29%). *1-(3,5-Dimethoxy-4-hydroxyphenyl)-ethyl-2'-methoxy-4'-hydroxymethyl-phenyl ether* (**4c**). Yield, 35·8%; m.p. 109 111°; NMR (DMSO-d_6): δ 1·58 (*d*,3), 3·69 (*s*,6), 3·78 (*s*,3), 4·37 (*d*,2), 4·95 (*d*,1), 5·29 (*q*,1), 6·70 (*m*,5), 8·14 (*s*,1); MS *m/e* (rel. int.): 362(9), 334 (4), 181 (100), 162 (70), 154 (99), 137 (94), 125 (87), 107 (69), 93 (94), 77 (80), 65 (92); IR (KBr): 3350 (OH), 1250 (C–O–C), 1140 (OMe). (*Anal.* Found: C, 64·98; H, 5·85. $C_{18}H_{22}O_6$ requires: C, 64·66; H, 6·33%).

It is to be noted that the MS of both **4b** and **4c** have parent peaks greater than the MWs of the compounds: **4b** is 14 m. u. smaller than the *m/e* 334 peak obtained and **4c** is 28 units smaller than the *m/e* 362 parent peak. This anomaly caused enough concern that the ^{13}C NMR spectrum of **4b** was run in an effort to see if there was indeed any extra methylene group that had eluded analysis. The spectrum obtained shows clearly that there is not.

An explanation of this curious phenomenon has been offered by Dr. Ward Scott[25] and will be the subject of another communication. The temperatures involved in volatilizing the samples of **4b** and **4c** may have homolytically cleaved these molecules. The syringyl and α-methyl syringyl radicals may have combined to form 1,2-diphenylethane structures **10b** + **10c** with molecular weights higher than those of the original ethers. Since these structures are more stable to heat than the ethers, a larger proportion of them arrive whole at the ion collector, giving large M + 14 and M + 28 peaks. A sample of **10b**, synthesized in our laboratory, gave essentially the same mass spectrum as **4b**. In all other properties, **10b** differed from **4b**. *3-Methoxy-4-hydroxybenzyl-2'-methoxy-4'-formylphenylether* (**5**). A 20 ml soln 2·68 g *N*-bromosuccinimide was stirred in N_2 at 0°. To this soln was added a soln of 1 g Me_2S in 10 ml CH_2Cl_2. After addition was complete, it was observed that the soln had turned opaque yellow. Stirring was continued, the temp. was lowered to −25°, and a soln of 1·54 g vanillyl alcohol in 300 ml. CH_2Cl_2 was added quickly to the reaction mixture. The −25° temp. was maintained for 4 hr and the stirring continued. After about 15 min, the opaque yellow of the reaction mixture changed to a clear, faint yellow soln with white particles floating through it. At the end of 4 hr, the soln was washed twice with cold brine and dried ($MgSO_4$). An acetone soln of 1·52 g vanillin was refluxed in N_2 with 1·38 g K_2CO_3 until the characteristic milky-yellow slurry formed. The CH_2Cl_2 soln prepared above was quickly added with vigorous stirring. When addition was complete, the heat was shut off and the reaction allowed to stir in N_2 at room temp. overnight. The reaction mixture was then washed 2× with H_2O, dried (Na_2SO_4) and evaporated to yield a brown oil which crystallized on standing. The product was recrystallized from Et_2O–pentane. Yield 1 g (35%); m.p. 154–155·5°; NMR ($CDCl_3$): δ 3·81 (*s*,3), 3·88 (*s*,3), 5·04 (*s*,2), 7·08 (*m*,6), 10·09 (*s*,1); MS *m/e* (rel. int.): 288 (1), 152 (56), 151 (58), 137 (100), 123 (20), 109 (21); IR: 3450 (OH), 2770 (CHO), 1260 (C–O–C), 1140 (OMe).

Acid hydrolysis reactions: hydrolysis of **4a**. A 2 ml solution in 9:1 dioxane–H_2O which was 0·2 M in HCl was made of 50 mg of **4a**. The mixture was and left at 50° for 8 hr. The mixture was then cooled and poured into dil. aq. $NaHCO_3$. This soln was extracted 2× with 15-ml portions of $CHCl_3$ and the extracts were combined, dried (Na_2SO_4) and evaporated. The product mixture was chromatographed against standards on layer of silica gel. Found: vanillyl alcohol (R_f 0·27).

Hydrolysis of **4b**. A 4-ml soln of 100 mg **4b** was made in 9:1 dioxane–H_2O which was 0·2 M in HCl. This solution was divided into two portions and warmed to 50°; one portion was left for 8 hr and the other for 24 hr. Workup for both reactions was the same as in the above procedure. Found: after 8 hr, **4b** (R_f 0·24), syringyl alcohol (R_f 0·25), vanillyl alcohol (R_f 0·27); after 24 hr, syringyl alcohol (R_f 0·25), vanillyl alcohol (R_f 0·27).

Hydrolysis of **4c**. A 2-ml soln in the acidic dioxane–H_2O soln described above was made of 10 mg of **4c**. This soln was heated at 50° for 8 hr, worked up and chromatographed as before. The silica gel plate was developed in iodine. Found: 1-(3,5-dimethoxy-4-hydroxyphenyl)-ethylene (R_f 0·64), α-Methyl syringyl alcohol (R_f 0·20), vanillyl alcohol (R_f 0·27).

Hydrolysis of **5**. A 2-ml soln of 40 mg **5** was made in the dioxane–H_2O acid solvent described above. This soln was reacted at 50° for 8 hr. Found: vanillin (R_f 0·57), vanillyl alcohol (R_f 0·25).

Hydrolysis of **3b**. A soln of 100 mg **3b** in 4 ml of dioxane–H_2O acid solvent was made and divided into two portions. The portions were kept at 50° for 8 and 24 hr, respectively. Found: after 8 hr, 4-*O*-acetylvanillyl alcohol (R_f 0·40), vanillyl alcohol (R_f 0·27); after 24 hr, vanillyl alcohol (R_f 0·27).

Hydrolysis of **3d**. A soln of 40 mg of **3d** was made in 4 ml of the acid solvent; this was divided into two parts, which were reacted at 50° for 8 and 24 hr. Found: after 8 hr, **3d** (R_f 0·72), vanillin (R_f 0·57), 4-*O*-acetylsyringyl alcohol (R_f 0·44), syringyl alcohol (R_f 0·25); after 24 hr, **3d** (R_f 0·72), vanillin (R_f 0·57), syringyl alcohol (R_f 0·25).

Hydrolysis of **6**. A soln of 50 mg **6** in 4 ml of the acid solvent was made, divided up into halves, reacted at 50° for 8 and 24 hr and worked up as before. Found: after 8 hr, **6** (R_f 0·72), vanillin (R_f 0·57), 4-*O*-acetylvanillyl alcohol (R_f 0·40), vanillyl alcohol (R_f 0·27); after 24 hr, **6** (R_f 0·72), vanillin (R_f 0·57), compound **5** (R_f 0·62), vanillyl alcohol (R_f 0·27).

Enzymatic oxidations: *oxidation of vanillyl and syringyl alcohols*. A 10-ml MeOH soln of 92 mg vanillyl alcohol and 110 mg syringyl alcohol was prepared. This soln was raised in vol. to 100 ml by the addition of dis. H_2O and 0·7 ml (two equivalents) of 3% H_2O_2 and 24 ml peroxidase soln (1 mg of enzyme) were added to the MeOH–H_2O solution. The mixture was swirled to mix the components (at this time it turned yellow), allowed to stand at room temp. overnight and worked up by extraction of the aqueous solutions with $CHCl_3$. The product mixture was chromatographed against standards as before. Found: 2,6-dimethoxy-*p*-benzoquinone (R_f 0·63), vanillin (R_f 0·57), syringaldehyde (R_f 0·49), vanillyl alcohol (R_f 0·27), syringyl alcohol (R_f 0·25).

Oxidation of **4a**. A solution was made of 50 mg **4a** in 20 ml MeOH. The soln was diluted to 100 ml by the addition of dist. H_2O and to this was added 0·31 ml (two equivalents) H_2O_2 and

12·5 ml enzyme stock solution. The soln was swirled to mix the contents and allowed to stand at room temp. overnight. Found: vanillin (R_f 0·57), vanillyl alcohol (R_f 0·27).

Oxidation of **4b**. A 10-ml soln of 200 mg **4b** was made in MeOH, and this soln was diluted to 100 ml by the addition of dist. H_2O. The soln was divided into four portions. The first portion was treated with 0·2 ml 3% H_2O_2 and 12·5 ml enzyme stock solution. The second portion received 5 ml peroxide and 25 ml enzyme solution. The third and fourth portions were treated with 0·2 ml peroxide, but no enzyme solution. The first and second portions were reacted for 24 hr; the third portion was allowed to react for 1 week and the fourth reacted for 4 weeks. Samples for mass-spectral analysis were column chromatographed with Et_2O–pentane. Occasionally, enough of a high-R_f product could be obtained for a m.p. or b.p. or a NMR spectrum. Found: first portion, 2,6-dimethoxy-*p*-benzoquinone (R_f 0·63), m.p. 232° (dec), MS *m/e* (rel. int.) 168 (100), 69 (95), syringaldehyde (R_f 0·49) m.p. 114–116°, MS *m/e* (rel. int.): 182 (100), 181 (81), vanillin (R_f 0·57), MS *m/e* (rel. int.): 152 (100), 151 (100), 123 (43), vanillyl alcohol (R_f 0·27), MS *m/e* (rel. int.) 154 (100), 137 (55), syringyl alcohol (R_f 0·25); second portion, 2,6-dimethoxy-*p*-benzoquinone (R_f 0·63), 2-methoxy-*p*-benzoquinone (R_f 0·76), vanillin (R_f 0·57), syringaldehyde (R_f 0·49), vanillyl alcohol (R_f 0·27), syringyl alcohol (R_f 0·25); third portion, 3,5-dimethoxy-4-hydroxybenzylmethyl ether (R_f 0·47), b.p. 189°, NMR ($CDCl_3$) δ 3·37 (*s*,3), 3·74 (*s*,6), 4·35 (*s*,2), 6·57 (*s*,2), MS *m/e* (rel. int.) 198 (19), 168 (88), 167 (57), 137 (100), 122 (23), 71 (27), **4b** (R_f 0·24), syringyl alcohol (R_f 0·25), MS *m/e* (rel. int.) 184 (100), 167 (38), vanillyl alcohol (R_f 0·27),MS *m/e* (rel. int.): 154 (100), 137 (50); fourth portion, 3,5-dimethoxy-4-hydroxybenzylmethyl ether (R_f 0·47), **4b** (R_f 0·24), 2,6-dimethoxy-*p*-benzoquinone (R_f 0·63), syringaldehyde (R_f 0·49), vanillyl alcohol (R_f 0·27), syringyl alcohol (R_f 0·25). MS for the products of the fourth portion were the same as above.

For ESR studies, a 10^{-2} M soln of **4b** was made in 20% MeOH–H_2O and a 3×10^{-2} M soln of hydrogen peroxide was made. Into a test tube were placed 0·5 ml **4b** soln, 0·7 ml hydrogen peroxide soln, 0·35 ml MeOH and 0·35 ml H_2O. At the start of the reaction, 200 μl peroxidase soln was added, the mixture shaken and the soln poured into a flat quartz cell and placed in the cavity of the ESR spectrometer.

Oxidation of **4c**. A 10 ml-soln of 35 mg **4c** was made in MeOH and this was dil. to 100 ml as before by the addition of dist. H_2O. To this soln was added 0·19 ml (two equivalents) of 3% H_2O_2 and 12·5 ml enzyme stock soln. The mixture was swirled and allowed to stand at room temp. overnight. MS samples were obtained by column chromatography as previously described. Found: 1-(3,5-dimethoxy-4-hydroxybenzyl)-ethylene (R_f 0·64), MS *m/e* (rel. int.) 180 (100), 165 (47), 137 (40), 109 (30), 81 (44), acetosyringone (R_f 0·45), MS *m/e* (rel. int.): 196 (87), 181 (100), vanillin (R_f 0·57), MS *m/e* (rel. int.): 152 (94), 151 (100), vanillyl alcohol (R_f 0·27), MS *m/e* (rel. int.): 154 (100), 137 (55), α-methyl syringyl alcohol (R_f 0·20), MS *m/e* (rel. int.): 198 (2), 181 (100).

For ESR studies, a 10^{-2} M soln of **4c** was made in 50% cellosolve–water. The reaction mixture consisted of 0·5 ml **4c** soln, 0·7 ml of 3×10^{-2} M H_2O_2, 0·7 ml H_2O and 200 μl enzyme stock soln. These components were mixed in a test tube and poured into a quartz cell as before.

Oxidation of **5**. A soln 50 mg of **5** was made in 20 ml MeOH. This soln was diluted to 100 ml as before and reacted with 0·31 ml hydrogen peroxide and 12·5 ml of enzyme stock solution. Found: 2-methoxy-*p*-benzoquinone (R_f 0·76), vanillin (R_f 0·57), vanillyl alcohol (R_f 0·27).

Acknowledgements—We wish to acknowledge the generous support of the National Science Foundation, grant No. GP-16492, for this work. We are indebted to Dr. Ward Scott for his helpful comments on the MS.

REFERENCES

1. Sarkanen, K. V. and Ludwig, C. H. (1972) *Lignin: Occurrence, Formation, Structure and Reactions*, Chap. 18, Wiley, New York.
2. Lundquist, K. (1962) *Acta Chem. Scand.* **16**, 2303.
3. Adler, E. and Lundgren, B. O. (1952) *Svensk Papperstidn* **55**, 563.
4. Kratzl, K. and Miksche, G. E. (1963) *Monatsh* **94**, 434.
5. Steelink, C. (1972) in *Recent Advances in Phytochemistry* (Runeckles, V. C. and Watkin, J. E., eds.), Vol. 4, Appleton Century-Crofts, New York.
6. Sakibara, V. A., Takeyama, H. and Morohashi, N. (1966) *Holzforschung* **20**, 45.
7. Young, M. and Steelink, C. (1973) *Phytochemistry* **12**, 2851.
8. Caldwell, E. and Steelink, C. (1969) *Biochim. Biophys. Acta* **184**, 420.
9. Steelink, C. and Fitzpatrick, J. D. (1972) *J. Org. Chem.* **37**, 762.
10. Clare, S. I. and Steelink, C. (1973) *TAPPI* **56**, 119.
11. Nimz, H. (1973) *TAPPI* **56**, 124.
12. Sarkanen, K. V. and Ludwig, C. H., *Op. cit.* p. 2.
13. Mikawa, H. (1954) *Bull. Chem. Soc. Japan* **27**, 50.
14. Adler, E. and Erickson, E. (1955) *Acta. Chem. Scand.* **9**, 341.
15. Corey, E. J., Kim, C. U. and Takeda, M. (1972) *Tetrahedron Lett.* **42**, 4339.
16. Lundquist, K. (1970) *Acta. Chem. Scand.* **24**, 889.
17. Johansson, B. and Miksche, G. (1965) *Acta. Chem. Scand.* **26**, 289.
18. Lundquist, K. and Miksche, G. (1965) *Tetrahedron Lett.* **35**, 2131.
19. Lundgren, R. (1961) *Paper and Timber* **11**, 670.
20. Nash, T. (1953) *Biochem. J.* **55**, 416.
21. Kirk, T. K. and Lundquist, K. (1970) *Svensk Papperstidn* **73**, 294.
22. Ishihara, T. and Miyazaki, M. (1972) *Mokuzai Gakkaishi* **18**, 415.
23. Pisovschi, I. J. (1910) *Chem. Ber.* **43**, 2137.
24. Freudenberg, K. and Hubner, H. H. (1952) *Chem. Ber.* **85**, 1185.
25. Scott, W. C., (1973) United Kingdom Atomic Energy Authority, Aldermaston, Reading, U.K. private communication.