

Oxygenation of 4-Alkoxy Groups in Alkoxybenzoic Acids by *Polyporus dichrous*

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The degradation of several alkyl ethers of vanillic acid, of 3-ethoxy-4-hydroxybenzoic acid, and of syringic acid, by the lignin-decomposing fungus *Polyporus dichrous* included (i) 4-dealkylation (e.g., 3-ethoxy-4-isopropoxybenzoic acid was in part dealkylated to 3-ethoxy-4-hydroxybenzoic acid), (ii) hydroxylation of the 4-alkoxy groups (e.g., 3-ethoxy-4-isopropoxybenzoic acid was oxidized in part to 2-[4-carboxy-2-ethoxyphenoxy]-propane-1-ol), and (iii) reduction of carboxyl groups (older cultures) (e.g., 3-ethoxy-4-isopropoxybenzoic acid was reduced to 3-ethoxy-4-isopropoxybenzaldehyde and 3-ethoxy-4-isopropoxybenzyl alcohol). Some ethers (e.g., tri-*O*-methyl gallic acid and glycerol- β -[4-carboxy-2-ethoxyphenyl]-ether) were not affected. The dealkylations and hydroxylations indicate that the fungus has a relatively nonspecific mechanism for oxygenating various 4-alkoxy groups of alkoxybenzoic acids; no evidence for oxygenation of 3-alkoxy groups was obtained. Hydroxylation products were generally degraded further, probably via dealkylation. The vanillic acid and 3-ethoxy-4-hydroxybenzoic acid formed by dealkylations were readily metabolized. Although the isopropyl ether of syringic acid was hydroxylated to 2-(4-carboxy-2,6-dimethoxyphenoxy)-propane-1-ol, neither this compound nor the parent isopropyl ether was dealkylated; syringic acid itself was only slowly and incompletely metabolized. The relationship of these results to lignin degradation is discussed.

Wood-destroying basidiomycetes or ascomycetes that decompose lignin ("white-rot fungi") cleave alkyl-aryl ether linkages in certain low molecular weight compounds related to structural elements in lignin. Thus vanillic acid (I) is dealkylated to protocatechuic acid (II) by *Polyporus vesicolor* (3, 8), and various 3,4-dimethoxyphenyl compounds are *para*-demethylated by *Fomes fomentarius* to 4-hydroxy-3-methoxyphenyl compounds: e.g., veratric acid (III) is converted to vanillic acid (I) (14). Higher alkyl ethers have not been investigated, with the exception of β -glycerol ethers of guaiacol, which have been selected for study because of the importance of glycerol- β -aryl ether structures in lignin. There are conflicting reports about the metabolism of the glycerol ethers; some authors have presented evidence that this ether linkage in such compounds as guaiacylglycerol- β -(2-methoxyphenyl)-ether (IV) is cleaved by white-rot fungi, whereas other investigators have found this linkage in IV and other compounds apparently resistant to dealkylation (21, 26).

To gain further information on the capacities of white-rot fungi to cleave alkyl-aryl ether

bonds, we have studied the metabolism of several alkyl ethers of vanillic (I), 3-ethoxy-4-hydroxybenzoic (V), and syringic (VI) acids.

MATERIALS AND METHODS

Fungus and culture conditions. *Polyporus dichrous* Fr., isolate no. FP 106899-Sp, was used for these studies because it grows well in submerged culture, and because extracellular phenoloxidase activity, which could interfere with the studies, cannot be detected in the growing cultures (19). The isolate was obtained from the Center for Forest Mycology Research, USDA, Forest Service, Madison, Wis. In nature, this fungus causes a white rot of the wood of various species of broad-leaved trees (23) and has been shown to decompose lignin under laboratory conditions (19).

The organism was maintained on ground aspen wood (20 mesh) that was supplemented with 2.5% peptone (Difco Laboratories, Detroit, Mich.) and adjusted initially to 200% moisture content. The liquid culture medium contained (grams per liter): glucose, 4; yeast extract (Difco), 1; peptone (Difco), 1; MgSO₄·7H₂O, 1, dispensed in 600-ml portions per 2-liter Erlenmeyer flask and autoclaved for 15 min at 121 C. After cooling, it was seeded with a 5% volume of a thick slurry made by blending the above decaying wood with the sterile medium. The dry weight of

inoculum was approximately 100 mg/100 ml of culture. The inoculated medium had a pH of 5.0, which decreased during growth to 4.5. Flasks were incubated on a rotary shaker at or near the optimum temperature for the organism (28 C).

Compounds (75 mg/600 ml) were added as sodium salts in a few milliliters of water (pH 5.5), or as solids, to the culture medium before autoclaving. At this concentration no noticeable toxicity was encountered.

Compounds. 3-Ethoxy-4-hydroxybenzoic acid (V; melting point 163 to 165 C; 164 to 165 C [16]) was prepared by oxidation of the corresponding aldehyde using Ag_2O and base (24).

3,4-Diethoxybenzoic acid (VII; melting point 164 to 167 C) was prepared by ethylation of protocatechuic acid by diethyl sulfate and NaOH (18). A similar procedure allowed the preparation of vanillic acid ethyl ether (VIII; melting point 195 to 196 C) from vanillic acid. 3-Ethoxy-4-methoxybenzoic acid (IX; melting point 164 to 165 C; 165 C [16]) was prepared by methylation of 3-ethoxy-4-hydroxybenzoic acid by using dimethyl sulfate and NaOH. Various other alkyl ethers of vanillic acid (I), 3-ethoxy-4-hydroxybenzoic acid (V), and syringic acid (VI) were prepared by reacting equivalent amounts of the sodium salts of the corresponding aldehydes with the appropriate alkyl bromide and subsequent oxidation of the product aldehyde with KMnO_4 . Preparation of 3-ethoxy-4-isopropoxybenzoic acid (X) is described in the following as an example.

A 3-ml amount of 10 N NaOH and 5.7 g of 2-bromopropane were added to a solution of 5 g of 3-ethoxy-4-hydroxybenzaldehyde in 60 ml of 95% ethanol. The progress of the reaction was monitored by thin-layer chromatography (Merck silica gel HF_{254} ; benzene:methanol:acetic acid, 45:4:2, as developing solvent). After 48 h, 60 ml of water was added, and the mixture was adjusted to pH 12. This was extracted with ether; the ether was washed with water, dried with anhydrous Na_2SO_4 , and removed by vacuum distillation; the colorless oil, 3-ethoxy-4-isopropoxybenzaldehyde, was dried under vacuum over P_2O_5 at 50 C (yield 4 g = 64%).

A 3-g amount of the resulting oil in 10 ml of *t*-butyl alcohol was added to a vigorously stirred solution of 1% Na_2CO_3 at 80 C. Five percent KMnO_4 was added in 1-ml portions until the purple color no longer rapidly disappeared (about 30 ml). Residual KMnO_4 was then reduced with ethanol, and the mixture was filtered and cooled. Acidification of the resulting clear, colorless solution with 2 M sulfuric acid gave a solid precipitate which was recovered on a filter, washed several times with water, and dried (yield 2.9 g = 90%) (melting point 112 C).

By a similar procedure the following compounds were prepared: vanillic acid isopropyl ether (XI; melting point 150 to 151 C; 146 to 148 C [23]), syringic acid isopropyl ether (XII; melting point 142 to 143 C), 3-ethoxy-4-*n*-propoxybenzoic acid (XIII; melting point 152 C), 3-ethoxy-4-*sec*-butoxybenzoic acid (XIV; melting point 88 to 89 C), and 3-ethoxy-4-benzyloxybenzoic acid (XV, melting point 194 to 195 C).

3-Ethoxy-4-isopropoxybenzyl alcohol (XVI), a pale yellow liquid, was prepared by NaBH_4 reduction of

the corresponding aldehyde.

Nuclear magnetic resonance spectra of the above compounds were consistent with the expected structures.

Glycerol- β -(4-carboxy-2-ethoxyphenyl)-ether (XVII): preparation of diethylmalonate-2-(2-ethoxy-4-formylphenyl)-ether. A 7.2-g amount of diethyl-bromomalonate, 3.0 ml of 10 N NaOH, and 5.2 g of 3-ethoxy-4-hydroxybenzaldehyde were refluxed for 1.5 h in 60 ml of 95% ethanol. When worked up as the 3-ethoxy-4-isopropoxybenzaldehyde above, an oil consisting of a mixture of products was obtained. The mixture (6.5 g) contained approximately 5.5 g of the desired malonate ether, which was purified by column chromatography. The column (2.5 by 36 cm) was packed with a slurry of dry silica gel (SilicAR, cc-4, 100 to 200 mesh; Mallinckrodt), in benzene. The product mixture was applied in 1.5- to 2-g amounts, and elution was with ethyl acetate in benzene: first with 200 ml of 2.5%, then with 400 ml of 5%, and finally with 200 ml of 10%. The desired product was eluted between 400 and 750 ml of effluent volume.

Preparation of glycerol- β -(2-ethoxy-4-hydroxymethylphenyl)-ether. A 5.0-g amount of the above purified malonate:ether was reduced with LiAlH_4 (5, 10) in tetrahydrofuran. The product mixture (about 80% glycerol- β -(2-ethoxy-4-hydroxymethylphenyl)-ether) was freed of impurities by column chromatography on silica gel. The column was prepared as above, half of the product mixture was applied, and elution was with methanol in benzene: first with 400 ml of 5%, then with 300 ml of 10%, and finally with 300 ml of 15%. The desired product was in the effluent volume of 700 to 900 ml.

Preparation of glycerol- β -(4-formyl-2-ethoxyphenyl)-ether. A 1.25-g amount of the purified LiAlH_4 reduction product was dissolved in 40 ml of purified dioxane in a 125-ml Erlenmeyer flask, and 2.35 g of solid 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) was stirred in. This solution was held in the dark at 25 C for 18 h, and the crystalline dichlorodicyanohydroquinone was filtered off (1.6 g) (see ref. 2 for similar oxidations with DDQ). A 50-ml amount of 5% sodium bisulfite solution was added, the pH was adjusted to 9 to 10, and the mixture was extracted successively with chloroform, chloroform:acetone (1:1 by volume), and chloroform. The combined extracts were washed with water and dried over Na_2SO_4 , and solvents were removed by vacuum distillation to give a clear, colorless oil (single product by gas chromatography).

Preparation of glycerol- β -(4-carboxy-2-ethoxyphenyl)-ether (XVII). A 1-g amount of the DDQ oxidation product was dissolved in 100 ml of 95% ethanol containing 1% KOH. A 3.9-g amount of freshly prepared, ethanol-washed Ag_2O was added with stirring, and then air was bubbled through vigorously with continued stirring (7). After 30 min, the solution was filtered and neutralized, and ethanol was removed by vacuum evaporation to about 30 ml. A 25-ml amount of water was added, the solution was acidified, and then extracted successively with chloroform, chloroform:acetone (1:1 by volume), and chloro-

form. The combined extracts were dried with Na_2SO_4 (yield 700 mg = 66%). Recrystallization from chloroform:ethanol gave fine white needles of a melting point of 157 C. Calculated for XVII, $\text{C}_{12}\text{H}_{16}\text{O}_6$: C, 56.25; H, 6.25. Found: C, 56.19; H, 6.16. Neutralization equivalent 263 (molecular weight 256). Nuclear magnetic resonance (NMR) (d_6 acetone), δ 1.35 (t, $J = 7\text{Hz}$, 3, CH_2CH_2); δ 3.77 (d, $J = 5\text{Hz}$, 4, CHCH_2OH); 64.20 (quartet, $J = 7\text{Hz}$, 2, $\text{CH}_2\text{CH}_2\text{O}$); δ 4.5 (quintet, $J = 5\text{Hz}$, 1, CH_2CHCH_2); 67.22 (d, $J = 8\text{Hz}$, 1, aromatic portion ortho to glyceryl ether); m centered at 67.67 (2, aromatic protons ortho to carboxyl); 64-7 (3, two $-\text{CH}_2\text{OH}$ and one $-\text{COOH}$, removed by D_2O).

The chlorinated compounds 5-chloroveratric acid (XVIII), 5-chlorovanillic acid (XIX), 2-chloroveratric acid (XX), and 2-chlorovanillic acid (XXI) were prepared as described in earlier research (6).

Other compounds were obtained from commercial sources. They were recrystallized when the thin-layer and gas chromatographic data indicated they were not chemically pure.

Spectroscopy. Spectra were taken on the following instruments: ultraviolet irradiation (UV), Beckman DK-2; infrared (IR), Beckman IR-12; NMR, Varian T-60. UV spectra were taken using ethanol solutions, IR spectra were taken with KBr pellets, and NMR (proton magnetic resonance) spectra were taken with CDCl_3 or d_6 -acetone solutions.

Detection, isolation, and identification. Detection of conversion products. After various periods of incubation, 25-ml samples of the cultures were acidified and extracted thoroughly with chloroform:acetone (1:1 vol/vol) followed by chloroform. Mycelial pellets did not interfere with the extraction. The washed extract was freed of solvents by vacuum evaporation. The product was dissolved in a few milliliters of dioxane and, 1 to 2 mg of docosane or tetracosane in chloroform was added as internal standard for gas chromatography. This solution was transferred to a 25-ml flask, and solvents were removed by vacuum distillation. The residue was dissolved in 100- μ liters of dimethylformamide and 100 μ liters of bis-(trimethylsilyl)-trifluoroacetamide containing 1% trimethylchlorosilane added to form the trimethylsilyl (TMS) derivatives, which were analyzed by gas chromatography. Extracts of control cultures (no compounds added) contained no interfering compounds.

Gas chromatography. Gas chromatography was performed with a Hewlett-Packard 5750 instrument with a flame ionization detector. The columns were 2.06 mm inside diameter ss, packed with Chromosorb G, AW-DMCS, 80/100 mesh: (A) 200 cm, SE-30, 5% by weight of solid support; (B) 200 cm OV-17, 5%; and (C) 150 cm XE-60, 0.5%. Temperatures were: columns, various constant temperatures; injection, 270 C; and detector, 250 C. Carrier gas was N_2 , 25 ml/min.

Quantitative estimates of compounds were made by comparing peak areas with that of the hydrocarbon internal standard; an initial reading was made before compounds began disappearing from cultures.

Isolation of products. Whole cultures were extracted in the same way as samples. Products were

then purified by preparative thin-layer chromatography (100- μ m thick layers of silica gel HF₂₅₄).

Identification of products. Vanillic acid (I), formed on dealkylation of its methyl and ethyl ethers (III) and (VIII), was identified by comparison with authentic vanillic acid by gas chromatography of the TMS derivatives, using columns A, B, and C. 3-Ethoxy-4-hydroxybenzoic acid (V) produced from its methyl (IX), ethyl (VII), and *n*-propyl (XIII) ethers was identified similarly. Also, this product was isolated from cultures containing 3-ethoxy-4-isopropoxybenzoic acid (X) and was identified by comparison of its UV, IR, and NMR spectra with those of the authentic material. The chlorinated vanillic acids XXI and XXIII formed from the chlorinated veratric acids XX and XXII were also identified, after isolation, by UV, IR, and NMR comparisons with the authentic materials.

The hydroxylation product isolated from cultures initially containing 3-ethoxy-4-isopropoxybenzoic acid (X) was recrystallized from chloroform (melting point 138 to 139 C) and identified from its chromatographic behavior, and spectral and analytical data as 2-(4-carboxy-2-ethoxyphenoxy)propane-1-ol (XXII). Elemental analysis: C, 60.09; H, 6.62; calculated for $\text{C}_{12}\text{H}_{16}\text{O}_5$: C, 60.00; H, 6.66. Neutralization equivalent = 237 (molecular weight = 240). UV maxima same as starting material (242, 292 nm in ethanol solution; 242, 291 nm after addition of NaOH). IR very similar to starting material, but with a hydroxyl stretching band at 3,360 cm^{-1} . NMR (d_6 -acetone), δ 1.32 (d, $J = 6\text{Hz}$, 3 CH_2CH); δ 1.40 (t, $J = 7\text{Hz}$, 3, CH_2CH_2); δ 3.68 (d, $J = 5\text{Hz}$, 2, HOCH_2CH); δ 4.13 (quartet, $J = 7\text{Hz}$, 2, OCH_2CH_2); δ 4.55 (center of m, 1, $\text{CH}_2\text{CHCH}_2\text{OH}$); δ 4-6 (broad adsorption, ~ 2 , CH_2OH , COOH , removed by D_2O); 67.10 (d, $J = 8\text{Hz}$, 1, aromatic proton *ortho* to propoxyl); δ 7.65 (center of m, 2, aromatic protons ortho to carboxyl).

The analogous hydroxylation products (XXIII, XXIV) from the isopropyl ethers of vanillic and syringic acids (XI and XII) were also isolated in crystalline form (melting point XXIII = 144 to 146 C; XXIV = 175 to 176 C) and identified from their spectral data. The latter, XXIV, was examined for optical activity with a Perkin-Elmer Polarimeter 141, and found to be active ($[\alpha]_{365\text{nm}}^{26\text{C}} = -14.7$ [$c = 2.5$, EtOH]). NMR XXIII (CDCl_3), δ 1.34 (d, $J = 6\text{Hz}$, 3, CH_2CH); 153.59 (d, $J = 5\text{Hz}$, 2, HOCH_2CH); δ 3.98 (s, 3, OCH_2); δ 4.60 (sextet, $J = 6\text{Hz}$, 1, CH_2CHCH_2); 65.47 (s, 2, $\text{CH}_2\text{OH} + \text{COOH}$, removed by D_2O); δ 7.05 (d, $J = 8\text{Hz}$, 1, aromatic proton ortho to propoxyl); δ 7.77 (center of m, 2, aromatic protons ortho to carboxyl). XXIV (CDCl_3), δ 1.41 (d, $J = 6\text{Hz}$, 3, CH_2CH); δ 3.63 (d, $J = 5\text{Hz}$, 2, HOCH_2CH); δ 3.95 (s, 6, OCH_2); δ 4.31 (center of m, 1, CH_2CHCH_2); δ 7.42 (s, aromatic protons); δ 7.43 (s, 2, $\text{CH}_2\text{OH} + \text{COOH}$, removed by D_2O).

The two hydroxylation products of XIII — 1-(4-carboxy-2-ethoxyphenoxy)propane-3-ol (XXV) and 1-(4-carboxy-2-ethoxyphenoxy)propane-2-ol (XXVI) — were separated as follows. The mixture of their methyl esters, prepared by brief treatment with diazomethane in methanol:ether, was applied as a streak to preparative thin-layer chromatography

plates (silica gel HF₂₅₄) and developed three times with 5% isopropanol in benzene, with evaporation of solvent between runs. This gave a broad band that was arbitrarily divided into upper and lower portions, and the products were eluted with acetone. Repeating this procedure twice with the recovered compounds yielded the two products separate from each other. NMR methyl ester of XXV (CDCl₃), δ 1.43 (t, J = 7Hz, 3, CH₂CH₃); δ 2.08 (quintet, J = 6Hz, 2, CH₂CH₂CH₃); δ 2.67 (s, 1, CH₂OH, removed by D₂O); multiple peaks at δ 3.8 to 4.4 (9, CH₂CH₂O + OCH₂CH₂CH₂OH + COOCH₃, latter at δ 3.88); δ 6.87 (d, 1, J = 8Hz, aromatic proton *ortho* to propoxyl); 67.67 (center of m, 2, aromatic protons *ortho* to carboxyl). NMR methyl ester of XXVI (CDCl₃), δ 1.32 (d, 3, J = 6Hz, CH₃CH); δ 1.44 (t, 3, J = 7Hz, OCH₂CH₃); δ 2.73 (s, 1, CH₂CHOHCH₃, removed by D₂O); multiple peaks at δ 3.8 to 4.4 (8, CH₂CH₂O + OCH₂CHOHCH₃ + COOCH₃, latter at 3.88); δ 6.90 (d, 1, J = 8Hz, aromatic proton *ortho* to propoxyl); δ 7.64 (center of m, 2, aromatic protons *ortho* to carboxyl).

The methyl esters were saponified in methanolic KOH, and the free acids were recrystallized from chloroform/hexane (melting point XXV = 164 to 7 C, XXVI = 142 C). Elemental analyses: XXV C, 60.27; H, 6.70; XXVI C, 60.09; H, 6.77; calculated for C₁₂H₁₆O₅: C, 60.00; H, 6.66.

RESULTS

Dealkylations and other conversions of alkyl ethers: ethers of vanillic acid (I). Vanillic acid methyl ether (veratric acid, III) and vanillic acid ethyl ether (VIII) were rapidly 4-dealkylated by *P. dichrous* to vanillic acid (I) (Fig. 1). The vanillic acid was present only in small quantities in the cultures (maximum -2% of the 111 added initially), because it was

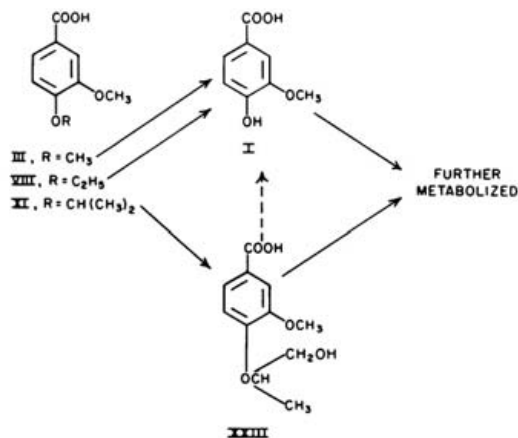


FIG. 1. Dealkylation and hydroxylation of alkyl ethers of vanillic acid (I) by *P. dichrous*. Compound XXIII is further metabolized, presumably via dealkylation to I (dashed arrow).

rapidly metabolized (20). The ethyl ether was dealkylated less rapidly than the methyl ether, so that the amount of vanillic acid present in the cultures at any time was even lower (maximum < 1% of the VIII added initially).

The isopropyl ether (XI) of vanillic acid disappeared from cultures in ~6 days—i.e., less rapidly than III or VIII, which took 3 to 4 days—and no vanillic acid was detected in extracts of the cultures. A single product was present in the cultures in a maximum amount exceeding 50% of the XI originally in the medium. This compound was isolated and identified as 2-(4-carboxy-2-methoxyphenoxy)propane-1-ol (XXIII; i.e., the product was formed by hydroxylation of the isopropyl group of XI) (Fig. 1). It disappeared from culture on continued incubation, but more slowly than XI, and without formation of detectable amounts of intermediates.

If vanillic acid (I) was formed from its isopropyl ether (XI) or from the hydroxylation product of XI (XXIII), or both, it disappeared too rapidly to be detected. To slow this rapid loss of the phenolic product of dealkylation in subsequent studies, we used ethers of the ethyl analog of vanillic acid: 3-ethoxy-4-hydroxybenzoic acid (V). Although V was metabolized by the fungus, it disappeared more slowly than I (4 to 5 days versus 3 days for 75 mg to disappear from 600-ml cultures).

Ethers of 3-ethoxy-4-hydroxybenzoic acid (V). Results are summarized in Table 1. Both 3-ethoxy-4-methoxybenzoic acid (IX) and 3,4-diethoxybenzoic acid (VII) were rapidly 4-dealkylated, and a substantial amount of V could be detected; the estimated maximum amount in culture was 60% of the weight of the IX and VII originally in the medium. With the ethyl ether (VII), a small amount (maximum about 3%) of a nonphenolic product was detected but was not identified. Its chromatographic behavior indicated that it was a product analogous to that formed from the isopropyl ether (X) described below.

Two products were found in cultures to which 3-ethoxy-4-isopropoxybenzoic acid (X) had been added (Table 1; Fig. 2). One of these was 3-ethoxy-4-hydroxybenzoic acid (V), formed by dealkylation and present in a maximum amount of about 10% of the X originally added. The second compound (maximum of about 45% of the X) was identified as 2-(4-carboxy-2-ethoxyphenoxy)propane-1-ol (XXII), produced by hydroxylation of the isopropyl group, just as XXIII was produced from XI. The hydroxylation product (XXII), isolated in crystalline form, was fed back to the organism and was

TABLE 1. Degradation of 3-ethoxy-4-hydroxybenzoic acid (V) and its ethers by *P. dichrous*

Substrate ^a	Days to disappear from culture ^b	V	Other products detected		
		Estimated ^c maximum level in culture (% of ether added initially)	No. of other products	Estimated ^c maximum level in culture, all products combined ^d (% of ether added initially)	Days for disappearance of all products ^e
V (3-ethoxy-4-hydroxybenzoic acid)	4-5				4-5
IX (methyl ether of V)	5	60 (3-4 days)	0		6
VII (ethyl ether of V)	7	60 (5-6 days)	1	3 (5-6 days)	8
X (isopropyl ether of V)	5-6	10 (5-6 days)	1 ^f	45 (5-6 days)	8
XIII (<i>n</i> -propyl ether of V)	8-9	1 (7 days)	2 ^f	>80 (7 days)	11
XIV (<i>sec</i> -butyl ether of V)	6-7	— ^g	3	>80 (7 days)	>18
XV (benzyl ether of V)	7-8	— ^g	0	—	8
XVII (β -glycerol ether of V)	— ^h	— ^g	0	—	—

^a 75 mg per 600 ml of medium.

^b Number of days for original substrate ether per se to disappear completely from time of inoculation; conditions were standardized.

^c Estimated by gas chromatography with use of internal standard. Not corrected for differences between starting materials and products in response factor of flame ionization detector.

^d Except V; probably mostly hydroxylation products (see text).

^e Number of days for all products, including V, to disappear from culture.

^f Products of hydroxylation of propyl groups (XXIII, XXII, XXIV, XXV and XXVI).

^g Not detected.

^h Not metabolized.

found to disappear without V or other products being detected; XXII disappeared more slowly than X. Since no 3-ethoxy-4-hydroxybenzoic acid (V) was detected from XXII it is apparent that the V observed during metabolism of the isopropyl ether (X) was formed directly from X.

The *n*-propyl ether of V (XIII) also was both dealkylated and hydroxylated. A small amount of 3-ethoxy-4-hydroxybenzoic acid (V) was detected, showing dealkylation (Table 1). Most of the *n*-propyl ether (XIII) was converted into a mixture of two products which appeared simultaneously and built up in approximately equal amounts; the maximum level of these two products together was at least 80% of the originally added XIII (Table 1). They were identified as two hydroxylation products, namely 1-(4-carboxy-2-ethoxyphenoxy)-propane-3-ol (XXV) and 1-(4-carboxy-2-ethoxyphenoxy)-propane-2-ol (XXVI) (Fig. 2). When fed back to the fungus they disappeared slowly and without detected intermediates.

Results with 4-*sec*-butoxy-3-ethoxybenzoic acid (XIV) were analogous to those with the *n*-propyl ether (XIII), except that no dealkylation product (V) was detected, and three (instead of two) products were formed, presumably also by hydroxylation (since the isolated, crystalline mixture was similar to the other hydrox-

ylation products in its IR and UV spectra and thin-layer and gas chromatographic behavior). These three compounds were only partially metabolized, with formation of two additional products, which were themselves slowly and incompletely metabolized.

4-Benzoyloxy-3-ethoxybenzoic acid (XV) was relatively rapidly metabolized, but no products were detected.

The β -glycerol ether of 3-ethoxy-4-hydroxybenzoic acid (i.e., glycerol- β -(4-carboxy-2-ethoxyphenyl)-ether, XVII, was not metabolized, even on prolonged incubation.

Ethers of syringic acid (VI). The methyl ether of syringic acid, i.e., tri-*O*-methylgallic acid (XXVII), was not metabolized. The isopropyl ether (XII) was hydroxylated to form 2-(4-carboxy-3,5-dimethoxyphenoxy)-propane-1-ol (XXIV), which accumulated quantitatively, and was not further metabolized even on prolonged incubation. Syringic acid (VI) was metabolized only slowly and incompletely.

Ethers of chlorinated vanillic acid. Products arising by dealkylation of the 3-alkoxy group of I, V, or VI, or any of their ethers were not observed. Only the dealkylations of the alkoxy group in the 4-position were seen. An attempt was made to block this 4-dealkylation, and thereby allow the accumulation of a postu-

lated 3-dealkylated product by using 5-chloroveratric acid (XVIII). This approach was taken because it was recently shown that a chloro-substituent ortho to a methoxy group prevents demethylation by *Nocardia corallina*, so that XVIII is converted to 5-chloroisovanillic acid (6). As with veratric acid (III), *P. dichrous*, however, rapidly demethylated XIX in the 4-position, and the resulting 5-chlorovanillic acid (XIX) was metabolized further. 2-Chloroveratric acid (XX) was also rapidly 4-dealkylated to 2-chlorovanillic acid (XXI), which, in contrast to XIX, was resistant to further degradation, and accumulated quantitatively.

Reduction of aromatic acids. In connection with attempts to prepare large amounts of the hydroxylation product (XXII) of 3-ethoxy-4-isopropoxybenzoic acid (X), the latter compound was added to cultures that had hydroxylated or dealkylated the X originally present. Rather than being hydroxylated, the newly added X was quantitatively reduced to 3-ethoxy-4-isopropoxybenzyl alcohol (XVI), with intermediate formation of the aldehyde (identifications were by gas chromatographic comparisons with the synthetic materials using TMS derivatives and columns A and C).

Subsequent experiments indicated that such reductions were associated with cultures older than about 8 days. A similar reduction occurred with vanillic acid isopropyl ether (XI), but other compounds were not investigated. The alcohol, XVI, produced by reduction of X was only very slowly metabolized (> 18 days), even by fresh cultures.

DISCUSSION

Dealkylations and other conversions. 3-Ethoxy-4-hydroxybenzoic acid (V) was not detected as a product during degradation of its *sec*-butyl or benzyl ethers (XIV and XV), and only a small amount of V was seen in extracts of cultures metabolizing the *n*-propyl ether (XIII). Similarly, degradation of the hydroxylation product (XXII) of the isopropyl ether (X) and the various conversion products of XIII and XIV did not give rise to detectable quantities of V. Nevertheless, these compounds presumably were metabolized ultimately via a dealkylation with formation of V, just as the methyl, ethyl, and propyl ethers were; the dealkylations of the higher ethers evidently proceeded more slowly than metabolism of the released V, precluding detection of the latter.

The alkyl products of the dealkylations were

not identified but would be expected to be carbonyl compounds. Dealkylation of methyl and higher alkyl ethers of *p*-hydroxybenzoic acid by *N. corallina* and by a *Pseudomonas* has been reported recently (4). Dealkylation in these bacterial systems is catalyzed by mono-oxygenases and requires NADPH₂ and O₂. The alkyl products of the dealkylations are carbonyl compounds, e.g., acetaldehyde from the ethyl ether.

The enzymatic dealkylation of alkyl ethers, such as those with the bacteria above involves, in effect, an oxygenation of the alkyl carbon atom of the ether linkage; the resulting unstable intermediate decomposes to release the phenol and a carbonyl compound (1, 4, 25). Assuming that the same mechanism is operative here, oxygenation of the center carbon of the isopropyl group in the isopropyl ethers would lead to dealkylation.

On the other hand, it was hydroxylation of one of the non-ether carbon atoms of the isopropyl group in X, XI, and XII that produced the products XXII, XXIII, and XXIV. Similarly, hydroxylation of one of the two non-ether carbon atoms of the *n*-propyl group in XIII gave the observed mixture of XXV and XXVI, and the single product from the ethyl ether VII and the three products from the *sec*-butyl ether XIV also were probably hydroxylation products. Single hydroxylations of the non-ether carbon atoms in the 4-alkoxy groups of these various starting compounds would give one product from VII, X, XI, and XII; two from XIII; and three from XIV; these were the numbers of products observed in each case. These results indicate that *P. dichrous* has a nonspecific mechanism for the oxygenation of the 4-alkoxy groups in this series of compounds. There was

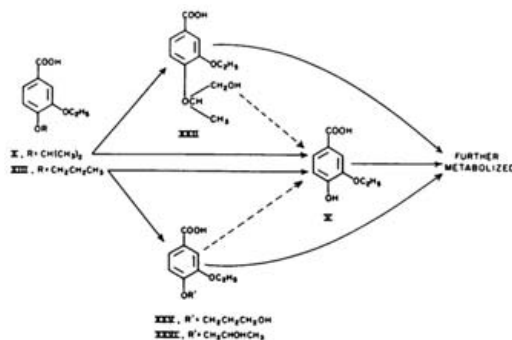


FIG. 2. Dealkylation and hydroxylation of propyl ethers of 3-ethoxy-4-hydroxybenzoic acid (V). Hydroxylation products presumably are metabolized via dealkylation to V (dashed arrows).

no evidence that the 3-ethoxyl group in any of the compounds was affected by the oxygenating system. Hydroxylation of the non-ether carbon atom of higher alkyl ethers, such as observed here, was apparently not observed in the bacterial systems referred to above (4).

The hydroxylations observed here are related to those effected by the fungus *Sporotrichum sulfurescens* in dialkylbenzenes. As one example of many conversions shown for the *Sporotrichum*, 1,4-diisopropylbenzene was hydroxylated to 1,4-di-(1-hydroxy)isopropylbenzene (15).

It is interesting that syringic acid isopropyl ether (XII) was hydroxylated to a product (XXIV) which was resistant to further metabolism. Because the organism obtained no energy or carbon from XII, the hydroxylation provides an example of the phenomenon of co-metabolism (12).

The product (XXIV) of hydroxylation of syringic acid isopropyl ether (XII) was optically active, showing that the hydroxylation was stereospecific. By analogy, it is to be expected that the other hydroxylations leading to products with asymmetric carbon atoms (i.e., XXII, XXIII, and XXVI) also were stereospecific, although the optical activity of these products was not determined.

The further degradation of the vanillic acid released by the dealkylations of its ethers has been investigated, and the results are described in a separate investigation (20). Vanillic acid is not metabolized via protocatechuic acid as might be expected (3, 8), but is oxidatively decarboxylated, with formation of methoxyhydroquinone, which is rapidly degraded.

The reductions of the carboxyl groups in the isopropyl ethers of vanillic acid and 3-ethoxy-4-hydroxybenzoic acid (compounds XI and X) by older cultures are analogous to reductions observed for other white-rot fungi. Zenk and Gross (27) studied the reduction of veratric acid (III) to the aldehyde and alcohol by *P. versicolor*. They obtained a cell-free extract that reduced III to veratraldehyde in the presence of adenosine 5'-triphosphate, NADPH₂, and Mg²⁺. Veratraldehyde was reduced to veratryl alcohol by an alcohol dehydrogenase system in the presence of NADH₂ or NADPH₂.

Significance to lignin degradation. In the degradation of the lignin polymer by white-rot fungi, propane side chains of terminal phenylpropane units are oxidized with loss of the two terminal carbons, leaving aromatic acid structures (11). As a consequence, vanillic acid is a product of hydrolysis of white-rotted lignin, indicating that the vanillic acid moieties are

linked through an ether bond to the rest of the polymer in the β -positions in arylglycerol elements (11; T. K. Kirk and H-m. Chang, unpublished data) (Fig. 3). These ether bonds must be cleaved as the fungi degrade the polymer further. The enzyme system effecting this ether cleavage may be specific for aryl ethers such as those of vanillic acid which possess a carboxyl function para to the ether bond, and this may help explain the failure in some past work with white-rot fungi to demonstrate ether cleavage with glycerol ethers that do not have this substitution (21, 26). In the present work the model compounds had this carboxyl substituent, and most of the alkyl ethers indeed were cleaved. However, the compound most closely related to the presumed structural elements in white-rotted lignin, the β -glycerol ether, XVII, was not affected. This may indicate that the structure XVII was not appropriate, which is in accord with the fact that the hydroxylation product XXII, which differs from XVII only by one hydroxyl group, was metabolized. Also in accord with this explanation is recent evidence that the ether bond in an arylglycerol- β -aryl ether compound is not cleaved by a soil pseudomonad until the benzyl alcohol group is oxidized (Crawford et al., unpublished data). Similarly, it has been shown that the amount of such " α -carbonyl" groups in lignin itself increases substantially during degradation by white-rot fungi (T. K. Kirk and H-m. Chang, unpublished data). These results indicate that glycerol- β -aryl ethers are not cleaved per se in biological systems.

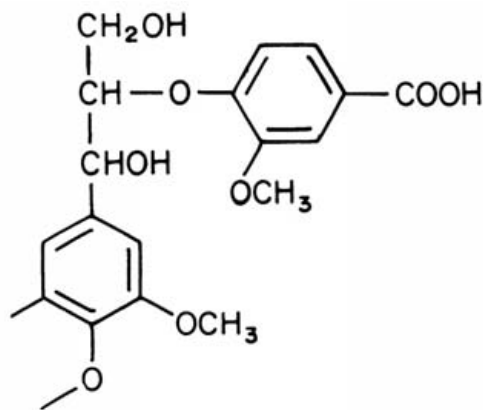


FIG. 3. Vanillic acid moiety linked through an ether bond in the β -position of an arylglycerol element of lignin. Such vanillic acid ethers are formed on fungal oxidation of side chains in terminal units (11, T. K. Kirk and H-m. Chang, unpublished data).

The resistance of syringic acid (VI) and its ethers to degradation suggests that the full complement of enzymes normally present during lignin degradation was not operative. In nature, *P. dichrous* decomposes hardwood lignin, which is comprised in large part of syringyl-type structural units, so the syringyl moiety clearly is not intrinsically resistant.

Further studies are under way, in this laboratory, of the ether-cleaving capacities of white-rot fungi, which employ culture conditions under which the lignin polymer itself is metabolized.

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LITERATURE CITED

1. Axelrod, J. 1956. The enzymic cleavage of aromatic ethers. *Biochem. J.* **63**:534-639.
2. Becker, H. C., and E. Adler. 1961. Zur Oxydation mit Chinonen. *Acta Chem. Scand.* **15**:218-219.
3. Cain, R. B., R. F. Bilton, and J. A. Darrah. 1968. The metabolism of aromatic acids by micro-organisms. Metabolic pathways in the fungi. *Biochem. J.* **108**:797-828.
4. Cartwright, N. J., K. S. Holdom, and D. A. Broadbent. 1971. Bacterial attack on phenolic ethers: dealkylation of higher ethers and further observations on *O*-demethylase. *Microbios* **3**:113-130.
5. Chaikin, S. W. 1948. β -Glyceryl phenyl ether and 1,3-dichloro-2-phenoxypropane. *J. Amer. Chem. Soc.* **70**:3522.
6. Crawford, R. L., E. McCoy, J. M. Harkin, T. K. Kirk, and J. Obst. 1973. Degradation of methoxylated benzoic acids by a *Nocardia* from a lignin-rich environment: significance to lignin degradation and effects of chloro-substituents. *Appl. Microbiol.* **26**:176-184.
7. Feather, M. S., and J. F. Harris. 1966. Relationship between some uronic acids and their decarboxylation products. *J. Organ. Chem.* **31**:4018-4021.
8. Flaig, W., and K. Haider. 1961. Die Verwertung phenolischer Verbindungen durch Weissfäulepilze. *Arch. Mikrobiol.* **40**:212-223.
9. Fukuzumi, T., and T. Shibamoto. 1965. Enzymatic degradation of lignin. IV. Splitting veratryl-glycerol- β -guaiacyl ether by enzyme of *Poria subacida*. *J. Jap. Wood Res. Soc.* **11**:248-252.
10. Gierer, J., and J. Kunze. 1961. Zur Spaltung von β -Hydroxy-alkyl-aryläthern durch Alkali. *Acta Chem. Scand.* **15**:803-807.
11. Hata, K. 1966. Investigations on lignins and lignification. XXXIII. Studies on lignins isolated from spruce wood decayed by *Poria subacida* B11. *Holzforchung* **20**:142-147.
12. Horvath, R. S. 1972. Microbial co-metabolism and the degradation of organic compounds in nature. *Bacteriol. Rev.* **36**:146-155.
13. Ishikawa, H., and H. Oki. 1966. The oxidative degradation of lignin. IV. The enzymic hydrolysis of ether linkages in lignin. *J. Jap. Wood Res. Soc.* **12**:101-107.
14. Ishikawa, H., W. J. Schubert, and F. F. Nord. 1963. Investigations on lignin and lignification. XXVIII. The degradation by *Polyporus versicolor* and *Fomes fomentarius* of aromatic compounds structurally related to softwood lignin. *Arch. Biochem. Biophys.* **100**:140-149.
15. Johnson, R. A., C. M. Hall, W. C. Krueger, and H. C. Murray. 1973. Microbial oxygenation of dialkylbenzenes (1). *Bioorg. Chem.* **2**:99-110.
16. King, H. 1939. Synthesis of diphenyl ethers containing methoxy- and ethoxy-groups. *J. Chem. Soc.* p. 1165-1168.
17. Kirk, T. K. 1971. Effects of microorganisms on lignin. *Annu. Rev. Phytopathol.* **9**:185-210.
18. Kirk, T. K., and E. Adler. 1970. Methoxyl-deficient structural elements in lignin of sweetgum decayed by a brown-rot fungus. *Acta Chem. Scand.* **24**:3379-3390.
19. Kirk, T. K., and A. Kelman. 1965. Lignin degradation as related to the phenoloxidases of selected wood-decaying basidiomycetes. *Phytopathology* **55**:739-745.
20. Kirk, T. K., and L. F. Lorenz. 1973. Methoxyhydroquinone, an intermediate of vanillate catabolism by *Polyporus dichrous*. *Appl. Microbiol.* **26**:173-175.
21. Kirk, T. K., J. M. Harkin and E. B. Cowling. 1968. Degradation of the lignin model compound syringyl-glycol- β -guaiacyl ether by *Polyporus versicolor* and *Stereum frustulatum*. *Biochim. Biophys. Acta* **165**:145-163.
22. Mndzhoyan, A. L., V. G. Afrikyan, G. A. Khorenyan, R. A. Alexsanyan, and N. O. Stepanyan. 1965. Derivatives of *p*-alkoxybenzoic acids. XXIII. Synthesis of amino esters of 3-methoxy-4-alkoxy-benzoic acids (in Russian). *Izv. Akad. Nauk. Arm.* 14749b, SSR, *Khim. Nauk.* **18**:193-199.
23. Nobles, M. K. 1948. Studies in forest pathology. VI. Identification of cultures of wood-rotting fungi. *Can. J. Res. C.* **26**:281-431.
24. Pearl, J. A. 1946. Reaction of vanillin and its derived compounds. I. The reaction of vanillin with silver oxide. *J. Amer. Chem. Soc.* **68**:429-432.
25. Ribbons, D. W. 1970. Stoichiometry of *O*-demethylase activity in *Pseudomonas aeruginosa*. *FEBS Lett.* **8**:101-104.
26. Russell, J. D., M. E. K. Henderson, and V. C. Farmer. 1961. Metabolism of lignin model compounds by *Polystictus versicolor*. *Biochim. Biophys. Acta* **52**:565-570.
27. Zenk, M. H., and G. G. Gross. 1965. Reduktion von Veratrumsäure zu Veratryldehyd und Veratrylalkohol durch zellfreie Extrakte von *Polystictus versicolor* L. *Z. Pflanzenphysiol.* **53**:356-362.