CONJUGATED OLEFIN HYDROXYLATION BY *PHANEROCHAETE CHRYSOSPORIUM* AND HORSERADISH PEROXIDASE

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The thesis "Conjugated Olefin Hydroxylation by *Phanerochaete chrysosporium* and Horseradish Peroxidase" by Robert M. Kuhn has been examined and approved by the following Examination Committee:

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DEDICATION

To my parents and my aunt Anna for their love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Conjugated Olefin Hydroxylation by *Phanerochaete chrysosporium* and Horseradish Peroxidase

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The hydroxylation and cleavage of conjugated aryl olefins by horseradish peroxidase and *Phanerochaete chrysosporium* were investigated, and optimum incubation conditions for the enzyme reaction were developed. Substrate specificity experiments showed that the enzyme specificity corresponded roughly to that exhibited by the fungus, with the exception that *P. chrysosporium* also readily degraded the mono-substituted *m*- and *p*-methoxycinnamyl alcohols to their corresponding anisyl alcohols. The pathways employed by the two systems were shown to be different. ^{18}O tracer studies showed that the organism probably utilized the hydroxylation product as an intermediate, confirming earlier reports by other workers. (The peroxidase, however, appears to cleave the olefin directly, in addition to catalyzing the hydroxylation reaction. It is not able to cleave the hydroxylated products.) Both peroxidase and laccase purified from *Polyporus versicolor* incorporated labeled oxygen only onto the β-carbon of 4-O-ethylisoeugenol, whereas *P. chrysosporium* incorporated a significant amount at the benzylic carbon. In addition, the ability of the fungus

to perform the hydroxylation reaction in the presence of catalase suggests that the phenol oxidase(s) of *P. chrysosporium* are not the sole catalytic agent(s) in the metabolism of lignin-related aryl olefins.

CHAPTER 1

INTRODUCTION

Wood has long been used as a raw material for construction and for fuel. It is likely that the vast deserts of the Middle East were once heavily forested and denuded by man in prehistoric times (1). Despite the introduction of new materials for many of its uses and the widespread use of fossil fuels, the use of wood has not decreased in recent years (2). In fact, in 1977 7.8 billion board feet of wood were harvested in the state of Oregon alone (2).

Wood is largely composed of cellulose, hemicellulose and lignin, the three most abundant polymers on earth. Together they comprise nearly 100% of softwood (3). Cellulose and its derivatives have found many uses, including textiles, cellophane and paper. The lignin component is partly responsible for the strength and resiliency that makes wood so well suited for construction. Together with bark and many extractives, lignin contributes to the strength and resistance to biological attack that makes wood a most useful material.

Unlike cellulose, the polymeric structure of lignin is not a linear repeating sequence, with regularity in both composition (4) and bond type. Rather, it is a heterogeneous material, formed by randomly aggregating subunits in a free radical process (5,6) (See Figure 1). It is insoluble and has no optical activity or crystallinity (7). The presence of at least eight nonhydrolyzable bond types, including

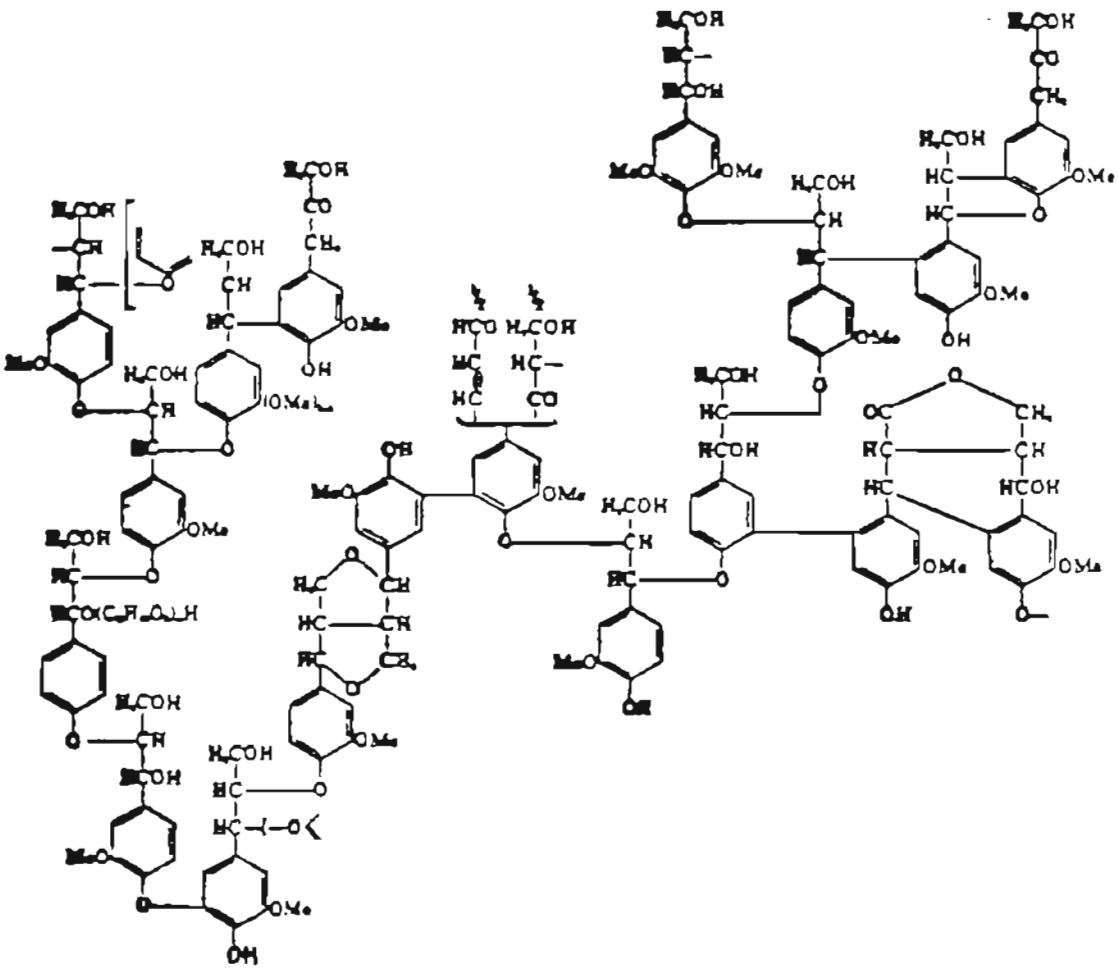


Figure 1. Lignin polymeric substructure (5).

both carbon-carbon and ether bonds among phenyl and alkyl moieties account for some of the difficulty with which it is modified (8).

Aside from its use as a low grade fuel in pulp mills and the extraction of small amounts of usable chemicals such as catechol, DMSO and vanillin, no suitable method exists for fully utilizing the high energy content of the aromatic residues of lignin. The Kraft and acid sulfite processes that have been developed by the paper industry to effect the removal of lignin from wood have resulted in the need for methods to deal with large quantities of waste lignin. In addition, the pollution problems and attendant costs associated with the use of sulfur and chlorine in the pulping and bleaching processes make the study of lignin worthwhile.

As the study of the biodegradation of wood developed, early attention focused on the pathogenicity of microbes attendant to the search for better wood preservatives and the need to understand and enhance the resistance of trees to disease (9). Subsequent studies have followed the path of least chemical resistance. In work pioneered by the U. S. Army Natick Laboratories in Massachusetts (10,11,12), the biodegradability of cellulose by fungi has been thoroughly studied, pathways have been ascertained (13) and enzymes isolated and characterized (14). New mutant strains of organisms have been isolated that exhibit enhanced cellulolytic capacity (15,16) and work on scale up to the industrial level is underway (17-19).

Lignin has been more recalcitrant in yielding its secrets. Its chemical structure was not clearly understood until the 1960's (20) as work on its biodegradation was beginning. Screening of wood-degrading fungi revealed that only the so-called "white rot" Basidiomycetes were efficient lignin degraders capable of degrading ^{14}C lignin completely to $^{14}CO_2$. Of these, the two species used in this study, *Phanaerochaete chrysosporium* and *Polyporus versicolor*, were found to be among the most active degraders of lignin and are the most extensively studied (21,22).

The observation that phenol oxidase activity was found in most of these fungi but not in closely related brown rot fungi, which do not degrade lignin as well, has been taken by some as evidence that phenol oxidases are involved in the biodegradation of lignin (23). Steelink (24) suggested that the increased numbers of stable free radicals observed in white rotted wood indicate that phenol oxidase induced depolymerization is a major biodegradative mechanism.

Mutant strains of fungi that completely lack phenol oxidase have been isolated (25,26). These organisms cannot degrade lignin, but also showed an inability to degrade glucans when phenols are present, indicating a more general metabolic disorder. Complete understanding of the role of phenol oxidases in lignin degradation awaits isolation of mutants lacking only phenol oxidase and isolation of ligninolytic enzymes from fungi.

There are white rot fungi with low phenol oxidase activity, among them *P. chrysosporium* (known variously as *Chrysosporium lignorum*,

Polyporus dichrous, *Chrysosporum pruinosum*, and in the imperfect state, as *Sporotrichum pulverulentum*) (23). Because phenol oxidase activity obscures identification of the products of lignin biodegradation by further oxidation or polymerization, the low phenol oxidase activity of *P. chrysosporium* has made it a good organism for the study of the metabolism of lignins.

Lignin is biosynthesized in plants by polymerization of free radical subunits in a process initiated by dehydrogenation of the phenolic moieties of substituted cinnamyl alcohols (27). Using the techniques of Freudenberg (28) the phenol oxidase horseradish peroxidase has been used by Haider & Trojanowski (29) and others (30) to polymerize ^{14}C-coniferyl alcohols to make specifically labeled ^{14}C-synthetic lignins.

The existence of these "low phenol oxidase" strains among the most active lignin degraders also supports the view that the lignin polymer is degraded in an orderly manner. Cleavage of the bond between carbons α and β to a substituted ring has been demonstrated using model dimers of lignin substructure moieties which lack phenolic hydrogen (31). Metabolism of aryl dimers with release of guaiacol and vanillic acid by β-ether cleavage has also been reported (31,32).

In addition to the study of lignin degradation by white-rot fungi, microbiological interest in these organisms has addressed itself to the problem of color formation in wood and pulps (33). Quinones and conjugated ketones and olefins are the strongest color formers in lignin (34). Cellobiose:quinone oxidoreductase has been isolated from white rot fungus in which, interestingly, reduction of the quinone is linked to oxidation of another compound found in

decaying wood, cellobiose (35). The fungal decarboxylation of vanillic acid to methoxyhydroquinone has been reported (36,37) and the enzyme isolated from *P. chrysosporium* by Yajima et al. (36). The oxidative decarboxylation of vanillic acid is also catalyzed by horseradish peroxidase (38,39).

While reductive degradation of conjugated olefins has been reported (40,41), it appears that the major catabolic pathway in olefin degradation is oxidative, furnishing aldehyde or alcohol products via a dihydroxy intermediate (28).

The similarity of action between peroxidase and the fungal attack on lignin related compounds (23) as well as the oft-repeated contention that phenol oxidases play an important role in the fungal metabolism of lignin has led to the observation that peroxidase could also catalyze conversion of 1-(4'-ethoxy,3'-methoxyphenyl)-propene X to 1-(4'-ethoxy,3'-methoxyphenyl)-1,2-propanediol XI (42).

In contrast to vanillic acid, which has been shown to be a poor model substrate for the study of lignin degradation (36), the conjugated olefin degradative system responds to the same physiological conditions required for lignin degradation. Vanillic acid was shown to be metabolized by *P. chrysosporium* in shaking cultures containing high nitrogen concentrations. Both are conditions which inhibit ligninolytic activity.

Additionally, vanillic acid decarboxylation occurs during the primary growth phase of the fungal life cycle. The degradation of lignin and the hydroxylation of X, however, are regulated as secondary

metabolic events, taking place only after the depletion of either carbon or nitrogen from the culture medium and primary growth has stopped. This occurs typically after six days under the culture conditions employed. These two systems also display similar behavior with respect to oxygen concentration and agitation, and neither lignin nor 4-O-ethylisoeugenol X can serve as a sole source of carbon for the fungus.

Insofar as the hydroxylation reaction may be used by the fungus to degrade nonpolymerized olefinic side chains in the lignin macromolecule and appears to be under the same physiological control as the lignin degrading system of *P. chrysosporium*, it is a useful object of study and can serve to increase our knowledge of lignin biodegradation.

The present study investigates the effect of peroxidase on lignin related olefinic compounds and examines the hydroxylation reaction in detail. It is shown that although *P. chrysosporium* degrades the conjugated olefin system by way of hydroxylation and cleavage of the subsequent *vic*-diol (43), horseradish peroxidase and laccase from *P. versicolor* also produce these products, but by different mechanisms. The implication is that although white rot fungi contain phenol oxidases, these enzymes are not solely responsible for the observed transformations.

CHAPTER 2

MATERIALS AND METHODS

Instrumentation

Gas chromatographic analyses were performed on a Varian Series 1200 programmable chromatograph fitted with a glass column (180 × 0.2 cm ID) packed with 3% OV 101 on Chromosorb Q 100/120 (Applied Science) with a Linear chart recorder. Oven temperature was programmed from 80 or 130°C - 290°C at 15°/min unless stated otherwise. Proton NMR spectra were obtained using a JEOL FX90Q Fourier Transform NMR: chemical shifts are expressed as ppm (δ) downfield from an internal standard of tetramethylsilane. Gas chromatography/mass spectrometry (GC/MS) was performed on a CEC DuPont Model 21-491B spectrometer operated at 70 eV in the GC mode using a Varian Series 2700 gas chromatograph and OV 101 packing as above. Capillary column GC/MS was performed on a Finnegan 4021 GC/MS/DS spectrometer operated at 70 eV with 0.25 mm ID × 25 m column with SE-54 packing (J & W Scientific). Optical density measurements and enzyme kinetic measurements were obtained using a Cary 15 spectrophotometer with 1 ml quartz cells, 1 cm light path. Melting points were determined on a Kofler hot stage microscope and are uncorrected.

Chemicals

All chemicals were reagent grade, from Aldrich (Milwaukee, WI), Sigma (St. Louis. MO) or Mallinckrodt (St. Louis, MO) or as indicated,

used as purchased. All gases were purchased from Airweld, Inc., Portland, Oregon. Samples of 1,2-dihydro-6-methoxynaphthalene XII; 1,2-dihydro-7-methoxynaphthalene XIII; 1,2-dihydroxy-1,2,3,4-tetrahydronaphthalene XIV; 1,2-dihydroxy-6-methoxy-1,2,3,4-tetrahydronaphthalene XV and 1,2-dihydroxy-7-methoxy-1,2,3,4-tetrahydronaphthalene XVI were generously provided by Drs. Frank M. Hauser and Subbarao Prasanna of this department. Others were used as previously synthesized in this laboratory (31,44).

Syntheses

Meso-dl-1-phenyl-1,2-propanediol I: *Trans*-1-phenylpropene (1.22 g, 10.3 mmole), sodium bicarbonate (0.5 g) and 3.8 g (17.6 mmol) *m*-chloroperoxybenzoic acid in 80 ml methylene chloride were stirred at room temperature for 3 h. The reaction was transferred to a separatory funnel, and washed in 2 × 50 ml water. The organic phase was dried over sodium sulfate, filtered and evaporated under reduced pressure at ~70°C. The yellow-white solid obtained was immediately dissolved in 20 ml dioxane, and sodium hydroxide (20 ml 1.0 *N*) was added, turning the solution a turbid brown color. After stirring for 1 h at 25°C, 40 ml water was added and the product extracted with 2 × 60 ml ethyl acetate. The ethyl acetate extracts were combined and washed with 20 ml each of 5% sodium bicarbonate and water, dried over sodium sulfate and the solvent removed at 70°C under reduced pressure yielding a yellow oil. The oil was purified by flash chromatography using a 30 mm glass column containing 30 g 230-400 mesh Silica gel 60

(Merck) and eluted under air pressure (45) with hexane:ethyl acetate 10:1 at approximately 10 ml/min to yield 1.2 g of a clear oil (78%). ^{1}H NMR ($CDCl_3$): δ 7.29 (s, 5H), 3.57 (d, 1H, J = 2 Hz), 3.03 (double quartet, 1H, J = 2, 5 Hz), 1.45 (d, 3H, J = 5 Hz). Mass spectrum of the trimethylsilyl (TMSi) derivative: m/e, relative intensity: 281 (M-15); 207; 180; 179, 98%; 147, 27%; 118; 117, 100%; 91.

Methyl 3-methoxycinnamate II: A solution of 3.0 g (16.8 mmoles) 3-methoxycinnamic acid, 2.02 ml methanol and 250 µl concentrated sulfuric acid in 8.0 ml methylene chloride was heated under reflux for 16 h as previously described (46). The reaction mixture was washed with 2 × 30 ml each of water and saturated sodium bicarbonate. The solvent was removed under reduced pressure at 70°C yielding 2.81 g (86.7%) of a yellow liquid. Thin layer chromatography (TLC) on precoated Silica gel 60 F-254 with fluorescent indicator (Merck) was performed using hexane:ethyl acetate 1:1, R_f = 0.75. ^{1}H NMR ($CDCl_3$): 7.66 (d, 1H, J = 16 Hz), 6.88 - 7.39 (aromatic, 4H), 6.44 (d, 1H, J = 16 Hz), 3.82, 3.79 (2s, 6H). Mass spectrum: m/e, relative intensity: 193; 192 (M^+), 100%; 191; 177; 162; 161, 91%; 134; 133, 21%; 118, 17%; 103; 102.

3-Methoxycinnamyl alcohol III: A solution of 2.71 g (14.1 mmoles) II and lithium aluminum hydride ($LiAlH_4$)(1.07 g, 28.2 mmoles) in 70 ml dry ethyl ether was stirred in a dry ice-acetone bath for 1.5 h (28, 40). Ethyl acetate (40 ml) was added and the mixture was allowed to come to room temperature. Saturated potassium tartrate (100 ml) was

added and the solution was extracted with 3 × 60 ml ethyl acetate, dried over sodium sulfate, evaporated under reduced pressure and purified by flash chromatography using hexane:ethyl acetate 1:1, to yield 1.67 g (72%) of a clear liquid III plus recovery of 200 mg of ester II. ^{1}H NMR ($CDCl_3$): 6.75 - 7.32 (aromatic, 4H), 6.65 (d, 1H, J = 17 Hz), 6.31 (double triplet, 1H, J = 17, 5 Hz), 4.32 (broad t, 2H, J = 5 Hz), 3.81 (s, 3H). ^{1}H NMR ($CDCl_3$) irradiated at 4.32 δ triplet: 6.80 - 7.34 (aromatic, 4H), 6.66 (d, 1H, J = 17 Hz), 6.34 (d, 1H, J = 17 Hz), 3.80 (s, 3H). Mass spectrum of TMSi derivative: m/e, relative intensity; 236 (M^+), 65%; 221, 29%; 205, 17%; 165; 148; 147, 100%; 146; 131; 117; 115; 103, 20%.

Methyl 3,4 dimethoxycinnamate IV: Methylation of 900 mg (4.33 mmoles) 3,4-dimethoxycinnamic acid in the same manner as described for II (46) yielded 370 mg (39%) of a pale yellow solid IV. ^{1}H NMR (d_6-acetone): 7.62 (d, 1H, J = 16 Hz), 6.93 - 7.32 (aromatic, 4H), 6.42 (d, 1H, J = 16 Hz), 3.89, 3.86, 3.63 (3s, 9H). Mass spectrum: m/e, relative intensity; 222 (M^+), 100%; 207, 12%; 191, 26%; 179; 164; 163; 147; 119.

3,4 Dimethoxycinnamyl alcohol V: A solution of 370 mg (1.67 mmole) IV was reduced with $LiAlH_4$ as described in the preparation of III and purified by flash chromatography using ethyl acetate: hexane 1:1 to yield 270.3 mg (84.9%) of a white solid V. ^{1}H NMR ($CDCl_3$): 6.76 - 7.00 (aromatic, 3H), 6.59 (d, 1H, J = 16 Hz), 6.22

(double triplet, 1H, J = 16, 6 Hz), 4.31 (broad t, 2H, J = 6 Hz), 3.89, 3.88 (2s, 6H). Homonuclear decoupling by irradiation at the triplet, δ = 4.24, collapsed the double triplet centered at δ = 6.16 to a doublet, J = 16 Hz. Mass spectrum of the TMSi derivative: m/e, relative intensity; 266 (M^+), 100%; 251; 239, 52%; 235, 68%; 222, 80%; 191, 37%; 177, 48%; 147.

Methyl 4-methoxycinnamate VI: A solution of 2.0 g (12.2 mmoles) of *p*-hydroxycinnamic acid and 3.7 g potassium carbonate (26.8 mmoles) in refluxing redistilled acetone was reacted overnight with 3.4 g (26.8 mmoles) dimethyl sulfate under positive pressure of nitrogen as previously described (47). Methanol (10 ml) was added to the cooled flask, the mixture was filtered, dried under reduced pressure and redissolved in diethyl ether (20 ml). Triethylamine (5.0 ml) was then added, the mixture stirred for one hour at 25°C, washed successively with water, 10% hydrochloric acid and saturated sodium chloride (10 ml each), dried over sodium sulfate and evaporated under reduced pressure to yield 1.99 g (91.6%) of a pale yellow solid VI, mp 85-88°C. Lit. mp 89°C (48). TLC in ethyl acetate:hexane 1:2 showed a single spot, R_f = 0.70. ^{1}H NMR ($CDCl_3$): 7.67 (d, olefinic, 1H, J = 16 Hz), 7.50 (d, aromatic, 2H, J = 8 Hz), 6.91 (d, aromatic, 2H, J = 8 Hz), 6.29 (d, olefinic, 1H, J = 16 Hz), 3.84, 3.79 (2s, 6H). Coupling and peak assignments were confirmed with the aid of homonuclear decoupling experiments. Mass spectrum: m/e, relative intensity; 192 (M^+), 74%; 162; 161, 100%; 147, 134; 133, 29%; 119; 90; 89; 79, 80%.

4-Methoxycinnamyl alcohol VII: The reduction of 1.9 g VI by lithium aluminum hydride in redistilled tetrahydrofuran was accomplished as described above for III, yielding 1.28 g (80%) of a pale yellow hygroscopic solid VII, mp 72-78°C. Lit. mp 79°C (49). ^{1}H NMR ($CDCl_3$): 7.30 (d, 2H, J = 9 Hz), 6.86 (d, 2H, J = 9 Hz), 6.67 (d, 1H, J = 16 Hz), 6.20 (double triplet, 1H, J = 16, 5 Hz), 4.69 (broad s, 1H), 4.28 (d, 2H, J = 5 Hz), 3.79 (s, 3H). Homonuclear decoupling by irradiation at the doublet, δ = 4.28 resulted in resolution of the double triplet at δ = 6.20 into a doublet (J = 16 Hz). Addition of deuterium oxide followed by sonication resulted in disappearance of the singlet at δ = 4.69 after one hour. Mass spectrum of TMSi derivative: m/e, relative intensity; 236 (M^+), 100%; 235, 36%; 221; 209; 205, 30%; 165; 161; 147, 94; 131.

$\alpha\beta$-Phenyl glycol VIII: A solution of 2.0 g (19.2 mmoles) styrene was reacted with *m*-chloroperoxybenzoic acid as described for I, to yield 1.08 g of an oil which was analyzed by GC/MS without further purification. The gas chromatogram showed three compounds in addition to VIII which were not identified. Mass spectrum of the TMSi derivative: m/e, relative intensity; 267 (M-15), 1.0%; 193; 179, 100%; 147, 24%; 133; 131; 105; 104, 4%; 103; 91; 73, 84%.

3-Phenyl-1,2-propanediol IX: A solution of 2.81 g (24 mmoles) allylbenzene was oxidized with *m*-chloroperoxybenzoic acid as described for I, yielding 2.33 g of a clear liquid which was analyzed by GC/MS as the TMSi derivative without further purification. Mass spectrum of

the TMSi derivative: m/e, relative intensity; 281 (M-15); 209; 208; 207, 100%; 194; 193, 71%; 147, 56%; 132; 117, 59%.

1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI: The *threo* form of XI was prepared from 4-O-ethyl isoeugenol X, available in our laboratory (31) by a modification of the procedures of Baran (50). X (200 mg) was dissolved in 5 ml of pyridine and stirred with 250 mg (1.0 mmole) osmium tetroxide for 16 h. Sodium bisulfite (2.0 g), 30 ml water and 20 ml pyridine were added and the mixture stirred for an additional hour. The reaction was then extracted twice with 200 ml of ethyl acetate, washed with 100 ml of saturated NaCl, dried over sodium sulfate and evaporated to dryness under reduced pressure. The product XI was purified by preparative TLC in hexane:ethyl acetate 1:1 (R_f = 0.45), yielding a thick yellow oil which showed a single peak on the gas chromatogram. Mass spectrum of the TMSi derivative: m/e, relative intensity; 370 (M^+), 0.8%; 355, 1.4%; 254; 253, 100%.

Fungi and Inocula

Phanerochaete chrysosporium (ME 446) and *Polyporus versicolor* were obtained from the Center for Forest Mycology Research, USDA Forest Products Laboratory, Madison, Wisconsin. Both were maintained at 28°C on 3% malt extract, 0.25% yeast extract, 2% agar slants as described previously (51). *P. chrysosporium* conidia were washed from mature slants at least one week old with 5 ml sterile distilled water. One hundred µl, or 10^5 conidia were used to inoculate a standard 25 ml

culture. *P. versicolor* inoculum was prepared by scraping off the top 2-3 mm of a mature slant, making a slurry with 5 ml sterile distilled water by blending for 10 seconds in a Waring blender and inoculating with 100 µl of this mycelial suspension.

Cultures containing 25 ml of a previously described medium (52, 53) buffered with 20 mM Na-2,2-dimethylsuccinate (54) were prepared containing ammonium tartrate (1.2 mM) and glucose 0.5%. Erlenmeyer flasks (250 ml) fitted with two-hole rubber stoppers and glass tubing for purging were incubated in stationary culture at 38°C. When the cultures were three days old, they were purged with oxygen, air or argon, as indicated, and sealed (55). On the sixth day, 3.0 mg substrate was added as a 5% or 10% solution in N,N-dimethylformamide (DMF), purged again with oxygen and sealed for two days as described previously (44).

Metabolism Experiments: Extraction and Analysis

Extraction of metabolic products was accomplished by a modification of a previously described procedure (44). Ethyl acetate (7 ml) was added to cultures grown as indicated. They were shaken vigorously and poured through a Büchner funnel without filter paper into a large test tube. When the phases had separated the ethyl acetate was removed by pipette, the water layer and mycelium recombined in the culture flask and the procedure repeated twice more with 5 ml aliquots of ethyl acetate. The combined ethyl acetate fractions were dried over anhydrous sodium sulfate and the solvent removed in a stream of

nitrogen at room temperature. Substrates, and when available, authentic diol products were extracted with substrates from water to ensure complete recovery was possible by this method.

The dried extracts (in 7 ml scintillation vials with teflon cap liners) were derivatized using *bis*(trimethylsilyl)trifluoroacetamide: pyridine 1:1. These trimethylsilyl (TMSi) derivatives were analyzed by gas chromatography using a temperature program of 80 or 130°-300°C or by gas chromatography/mass spectrometry and compared with known standards as described previously (44).

Modification of 1-(4'-ethoxy,3'-methoxyphenyl)propene X with $^{18}O_2$ by *P. chrysosporium*

Minimal salts medium (3.0 ml)(52,53) containing 1.2 m*M* ammonium tartrate and 2% glucose was inoculated with 100 µl of a standard spore suspension in modified 10 ml Erlenmeyer flasks with O-ring pressure fittings. After eight days of growth, 1.0 mg X (10% in DMF) was added, followed by 1.0 ml sterile water. Duplicate flasks were sealed to a manifold provided with a pressure valve (Figure 2). The flasks were then evacuated to 30 mm pressure using a vacuum pump with liquid nitrogen traps and a stream of argon gas was allowed to fill the flasks. The argon was scrubbed of any trace of oxygen by bubbling through a pale blue solution of 0.1 *M* chromic sulfate in 0.002 *M* perchloric acid reduced to chromous ion over a zinc-mercury amalgam (56). This purging was repeated four times, during each of which the apparatus was reduced to 2.0 mm pressure and refilled to 200 mm pressure with argon to ensure complete removal of gases.

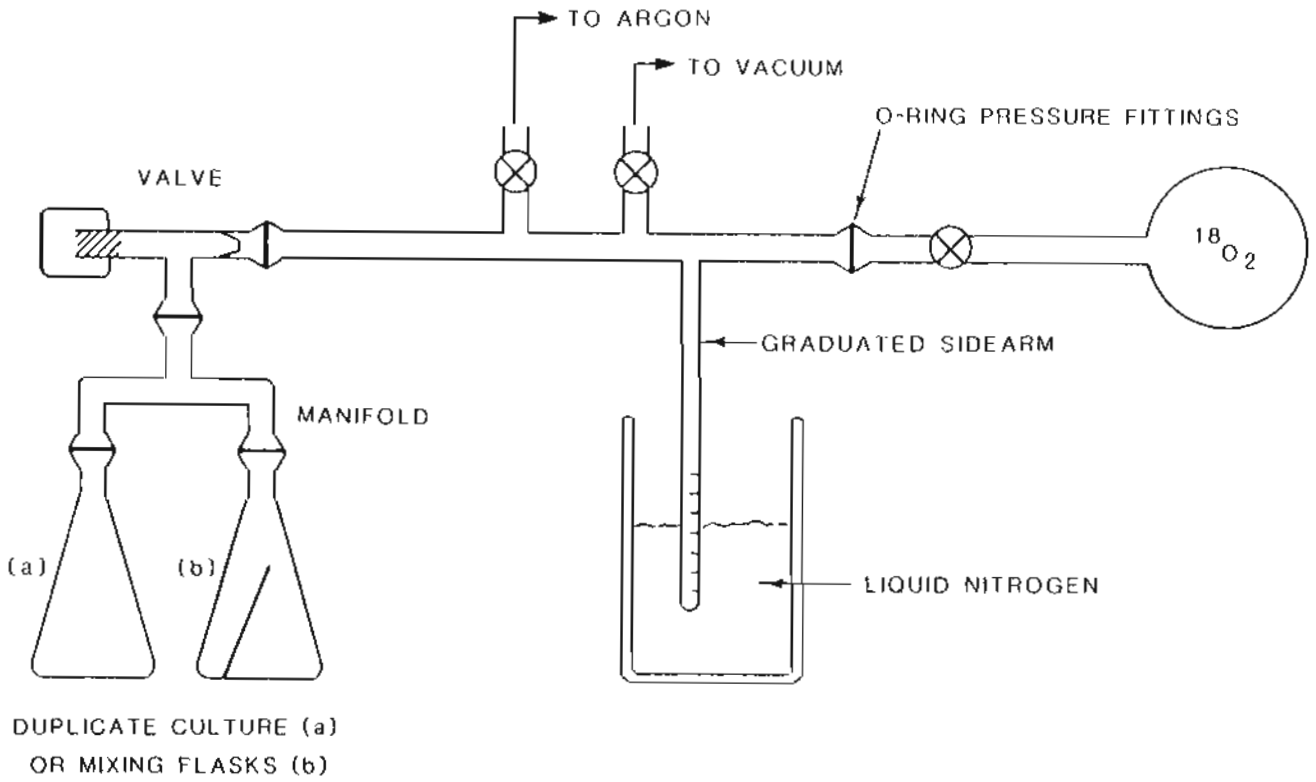


Figure 2. Apparatus for reaction of phenol oxidases and *P. chrysosporium* in $^{18}O_2$ atmosphere. The system is alternately evacuated and purged with argon to remove atmospheric oxygen. $^{18}O_2$ is then condensed into the graduated sidearm and equilibrated with the reaction vessels. Valve permits recondensation of unused $^{18}O_2$ in storage bulb and removal of reaction vessel for incubation at controlled temperature.

Following removal of atmospheric oxygen, the cultures were reduced to 30 mm pressure and sealed from the system, which was then reduced to a pressure of 2.0 mm Hg. $^{18}O_2$ gas (99.8 atom % isotopic purity, Prochem, Summit, New Jersey) was then condensed to a liquid by plunging a sidearm graduated in atmospheres into liquid nitrogen. When enough liquid had collected to bring the cultures to 1.5 atmospheres partial pressure in oxygen, the gas bottle was sealed, the oxygen allowed to evaporate, then equilibrated with the cultures. The cultures were sealed and incubated at 38°C. After 24 hours, a small portion of mycelium was taken in a glass pipette tip, washed three times with water and placed on an agar slant containing malt extract, yeast extract as before to determine if exposure to vacuum killed the fungus. Cultures were extracted as usual with 2 × 5 ml ethyl acetate, dried under nitrogen gas at room temperature and analyzed by gas chromatography/mass spectrometry. Results are compared with duplicate cultures treated in an identical manner but incubated under $^{16}O_2$ condensed from a bulb purged with bottled gas.

Enzyme Assays

Laccase purified from *P. versicolor* was assayed using a modification of the method of Herzog and Fahimi (57). A solution of Knox gelatin 2 mg/ml is prepared at 70°C, to 5 ml of which is added 5 ml 0.2 *N* citric acid/Na_2HPO_4 buffer pH 5.4. To this solution is

added 750 µl of 3,3'-diaminobenzidine (DAB, Sigma) prepared by adding 40 ml 2% DMF in methanol to 100 mg DAB in a serum vial.

To assay laccase, a final volume of 1.0 ml of DAB assay solution with 10 or 50 µl enzyme solution was used. The Cary 15 spectrophotometer is zeroed against DAB solution without enzyme, the reaction is followed as an increase in absorbance at 465 nm.

To assay horseradish peroxidase, 10 or 50 µl of enzyme solution in a final volume of 1.00 ml of DAB stock solution was used. The reaction is started by the addition of 40 µl 100 mM H_2O_2 to the test cuvette; the spectrophotometer is zeroed against the enzyme solution containing no peroxide. Activity is reported as increase in absorbance at 465 nm.

Horseradish peroxidase and laccase were also assayed by a modification of the procedure of Polis & Shmukler (58). Two ml of 5% pyrogallol in water is mixed with 1.0 ml of 147 mM hydrogen peroxide and 2.0 ml of 100 mM phosphate buffer, pH 6.0 and brought to 10 ml with distilled water. 950 µl of this solution is added to a 1 ml, 1 cm cuvette and the reaction started with 50 µl of protein solution. The same assay solution is used for laccase, omitting the peroxide. The reaction is followed spectrophotometrically at 420 nm.

Protein determination was by the method of Lowry et al. (59).

Preparation of laccase from *Polyporus versicolor*

Using a modification of the procedure of Fåhraeus & Reinhammar (60), a 200 ml culture of *P. versicolor* was inoculated with a mycelial slurry as described above, and grown for five days in air at 28°C on a New Brunswick rotary shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) describing a 2.0 cm circle at a speed of 125 rpm. The culture medium was as described above for stationary cultures using 2.0% glucose and 12 mM ammonium tartrate.

This five day culture was used as inoculum for a 1 liter culture containing Vogel's medium (61) with 2% glucose and 500 mg/liter casein hydrolysate. The inoculum culture was poured into a sterilized Waring blender and blended four times for 5 seconds. This slurry was transferred aseptically into the medium, in a 2.8 liter Erlenmeyer flask (final volume 1200 ml) and incubated as above on the rotary shaker.

When the culture was three and six days old, laccase production was induced by additions of 30 mg 2,5-xylidine (as 480 µl of a 0.5 M solution in ethanol: water 1:1) (60). A red color developed overnight after the first addition and persisted thereafter. Enzyme was harvested after 8-10 days of growth.

The culture medium was suction filtered through Miracloth (Calbiochem) in a Büchner funnel and chilled to 4°C. All subsequent steps were performed at this temperature. Three 1.0 ml aliquots were removed and frozen for assay (fraction 1) and total protein was precipitated by saturation with 700 g/l ultra pure ammonium sulfate

(Schwarz/Mann), maintaining pH 6.0 with additions of solid *tris*(hydroxymethyl) aminomethane buffer. The medium was centrifuged at 16,000 × g for 15 min in a Sorvall RC2B refrigerated centrifuge and the supernatant carefully decanted. The pellet was taken up in a minimal amount of 100 m*M* phosphate buffer, pH 6.0, dialysed overnight against 50 m*M* phosphate buffer, pH 6.0, and frozen at -15°C. The frozen protein was removed from the dialysis bags, ground with sand at 4°C, and filtered through Miracloth (fraction 2).

After removal of two 1.0 ml aliquots, the preparation was frozen in a dry ice-acetone bath and lyophilized using a Virtis Unitrap II model 10-MR-ST lyophilizer (fraction 2A).

The dry protein obtained in this manner was dissolved in a saturated sucrose solution until it reached saturation (~ 350 mg/ml) filtered through glass wool and applied to a 2.0 × 35 cm column of Sephadex G-100. The column was eluted with phosphate buffer (20 m*M*, pH 6.0) at 4°C and 1.5 ml fractions collected on an automatic fraction collector. Laccase (*p*-diphenol:O_2 oxidoreductase, EC 1.10.3.2) activity was located using the 3,3'-diaminobenzidine assay (57). These fractions were lyophilized again and frozen (fraction 3).

Lignin Model Substrate Modification by Enzymes

The reaction mixture (2.0 ml) contained 100 m*M* phosphate buffer, pH 6.0, and 100 µl of 1.0 mg/ml horseradish peroxidase, type II. Twenty µl of a 10% solution of substrate in DMF is added and the reaction started by addition of 20 µl 100 m*M* hydrogen peroxide, or as indicated.

Products were extracted with 3 × 2 ml ethyl acetate, and dried over sodium sulfate. The solvent was removed under a stream of nitrogen, followed by gas chromatographic analysis of the TMSi derivatives. Laccase modification of the lignin substrates was analyzed in the same manner, without addition of peroxide.

Potassium Superoxide Reactivity

A solution of potassium superoxide (Apache, Seward, Illinois) was prepared by the method of Valentine & Curtis (62). 18-Crown-6 ether (0.3 *M*) was dissolved in freshly distilled DMSO, followed by addition of 10.7 mg/ml (0.15 *M*) potassium superoxide. This yellow solution shows a positive superoxide test with nitroblue tetrazolium, and is stable for several days at room temperature.

Two hundred fifty µl of this solution is mixed with 100 µl 10% (DMF) solution of substrate (0.75:1 mole ratio) and incubated for 5 min. The reaction is stopped by addition of water (1.0 ml) and shaken for 10 minutes, followed by addition of two drops 2 *N* HCl, 100 mg of solid sodium chloride and extracted into 3 × 2 ml ethyl acetate. The combined extracts are washed with saturated sodium bicarbonate (2 ml), dried over sodium sulfate and evaporated to dryness in a stream of nitrogen at room temperature. Products were analyzed as their TMSi derivatives by gas chromatography as described above.

Modification of 4-O-ethylisoeugenol X by Laccase Under $^{18}O_2$ Atmosphere

One hundred twenty units (390 µl) of laccase fraction 2A (a saturated solution of fraction 2) were placed in one well of a two-well mixing flask. To the other, 1.0 mg of 4-O-ethylisoeugenol X (10% in DMF) was added to 600 µl of phosphate buffer, 100 m*M*, pH 6.0. The flask was sealed to the purging apparatus described above (Figure 2) and alternately degassed to 2.0 mm Hg vacuum and refilled to atmospheric pressure with scrubbed argon. This was repeated three times, after which enough $^{18}O_2$ gas was condensed into the sidearm to bring the final oxygen partial pressure to 1.3 atm. The liquid oxygen was allowed to evaporate, then vented into the reaction vessel. The two wells were mixed and the flask removed and incubated at 35°C with shaking for 5 hours. Products were analyzed as their TMSi derivatives by GC/MS as previously described (44).

Modification of 4-O-ethylisoeugenol X by Horseradish Peroxidase Using ^{18}O-Hydrogen Peroxide Generated *in situ* from $^{18}O_2$

In one well of a two-well reaction vessel was placed 100 µg horseradish peroxidase, 40 µl of a 100 m*M* glucose solution and phosphate buffer, 100 m*M*, pH 6.0 to 800 µl. In the other well was placed 0.1 units of glucose oxidase (63) and 1.0 mg substrate X in the phosphate buffer (800 µl). Atmospheric oxygen was removed as described above, the vessel pressurized to 1.3 atm partial pressure of

$^{18}O_2$ and the reactants were mixed and incubated as before. Products were analyzed by GC/MS of their TMSi derivatives as previously described (44).

CHAPTER 3

RESULTS

Activity of Horseradish Peroxidase on Model Compounds

It has long been thought that phenol oxidases play an important part in the biodegradation of lignin (6,24). These enzymes are often found in the medium of lignin degrading fungi (9) and are easily purified from the broth of the white rot fungus *P. versicolor* (60). Kirk et al. (23) have observed the liberation of side-chain fragments from lignin model dimers and hydroxylation of a coniferyl alcohol derivative has been reported under conditions in which lignin degradation has been shown to take place (64).

The effect of horseradish peroxidase on lignin related olefin model compounds was investigated in the present study. Assay conditions were standardized using 1-(4'-ethoxy-3'-methoxyphenyl)propene X (4-O-ethylisoeugenol), measuring the production of 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI. No reaction of X was observed using boiled peroxidase. Similarly, omitting either H_2O_2 or enzyme resulted in complete recovery of substrate.

Maximum activity of peroxidase has been reported to range from pH 4.3 using 3,3'-diaminobenzidine (57) to 6.0 using pyrogallol (58, 65) and 7.0 using *o*-dianisidine (66). In the present study, the effect of pH on the reaction was studied using a variety of buffers over the pH range 3.5 to 9.0 (Figure 3). No attempt was made to

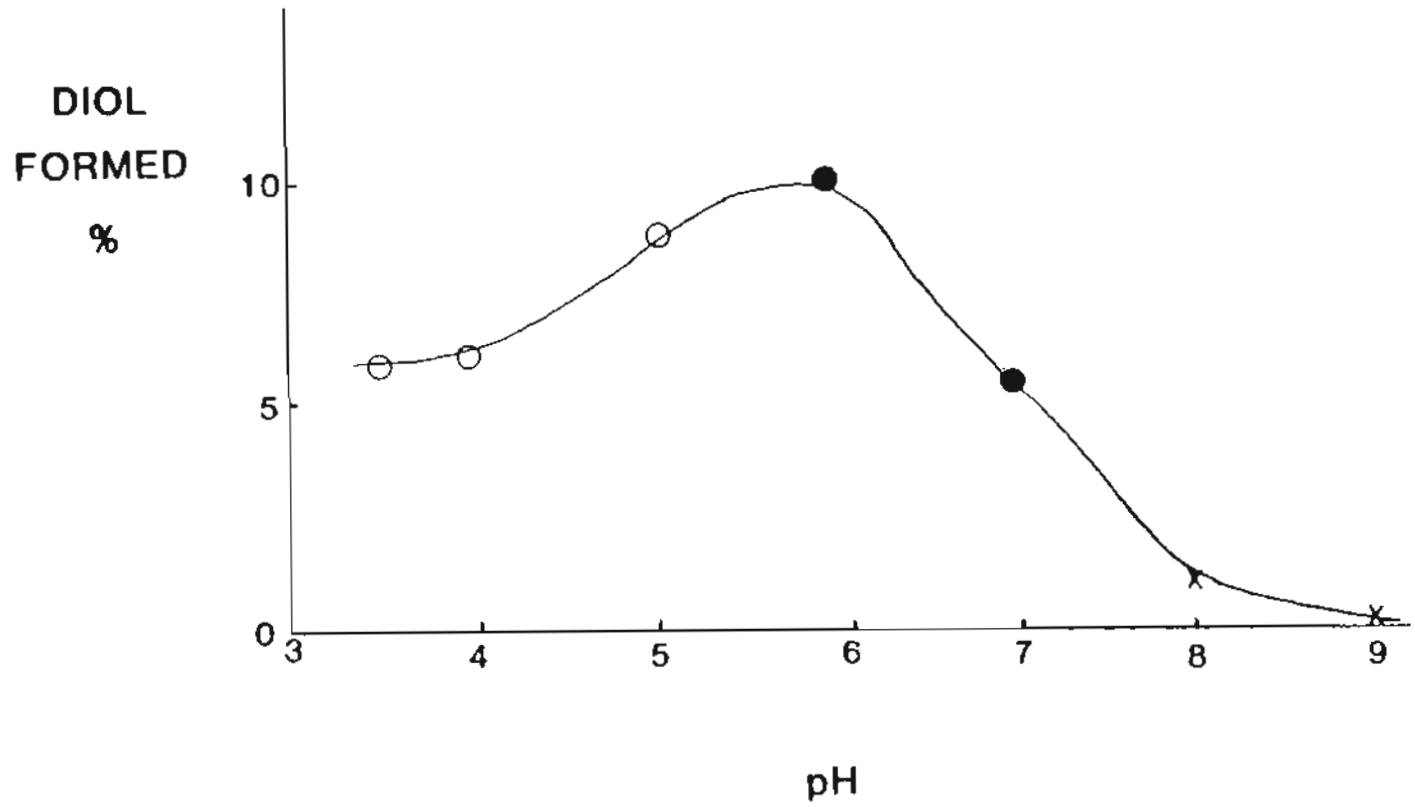


Figure 3. Effect of pH on hydroxylation of 4-O-ethylisoeugenol X by peroxidase. Formation of 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI is shown. The reaction mixture (2.0 ml) contained 100 µg horseradish peroxidase and 2.5 mg substrate in 100 m*M* buffer. Buffers used were acetate (○), phosphate (●) and *tris*(hydroxymethyl)aminomethane (×). The reaction was stopped after 3 h at 35°C by addition of ethyl acetate and extraction as described in the text.

determine what, if any, effect was due to the variation in buffer. Modification of X was at a maximum over a range from pH 5.0 to 6.0, and fell off at higher pH values. At pH 8.0 and above, a number of products in addition to the diol XI were formed. Oxidations of lignin and of conjugated phenyl olefins by alkaline hydrogen peroxide have been reported (34).

Herzog and Fahimi (57) have reported that high concentrations of hydrogen peroxide adversely affect the activity of peroxidase. This effect was also evident with the hydroxylation of X and 1-(4'-ethoxy-3'-methoxyphenyl)propene-3-ol (4-O-ethylconiferol XVII) (Figure 4). A concentration of approximately 1.0 m% H_2O_2 produced the greatest amount of hydroxylation product from both compounds. It is possible that the enzyme itself is oxidized by high peroxide concentrations.

Figure 5 shows the course of the reaction with time. When the reaction mixture contained 1 m% hydrogen peroxide, hydroxylation of 1.0 mg of substrate proceeded to 25% completion in 3 h. This represents a 60% consumption of available H_2O_2; although, as indicated in Figure 4, additional peroxide failed to result in the accumulation of more product, even in the presence of a large amount (4.0 mg) of substrate. Thus, it appears that the enzyme modifies this compound at a constant rate and becomes inactive in about 3 h. The enzyme was always present in excess (specific activity can be found in Table 9).

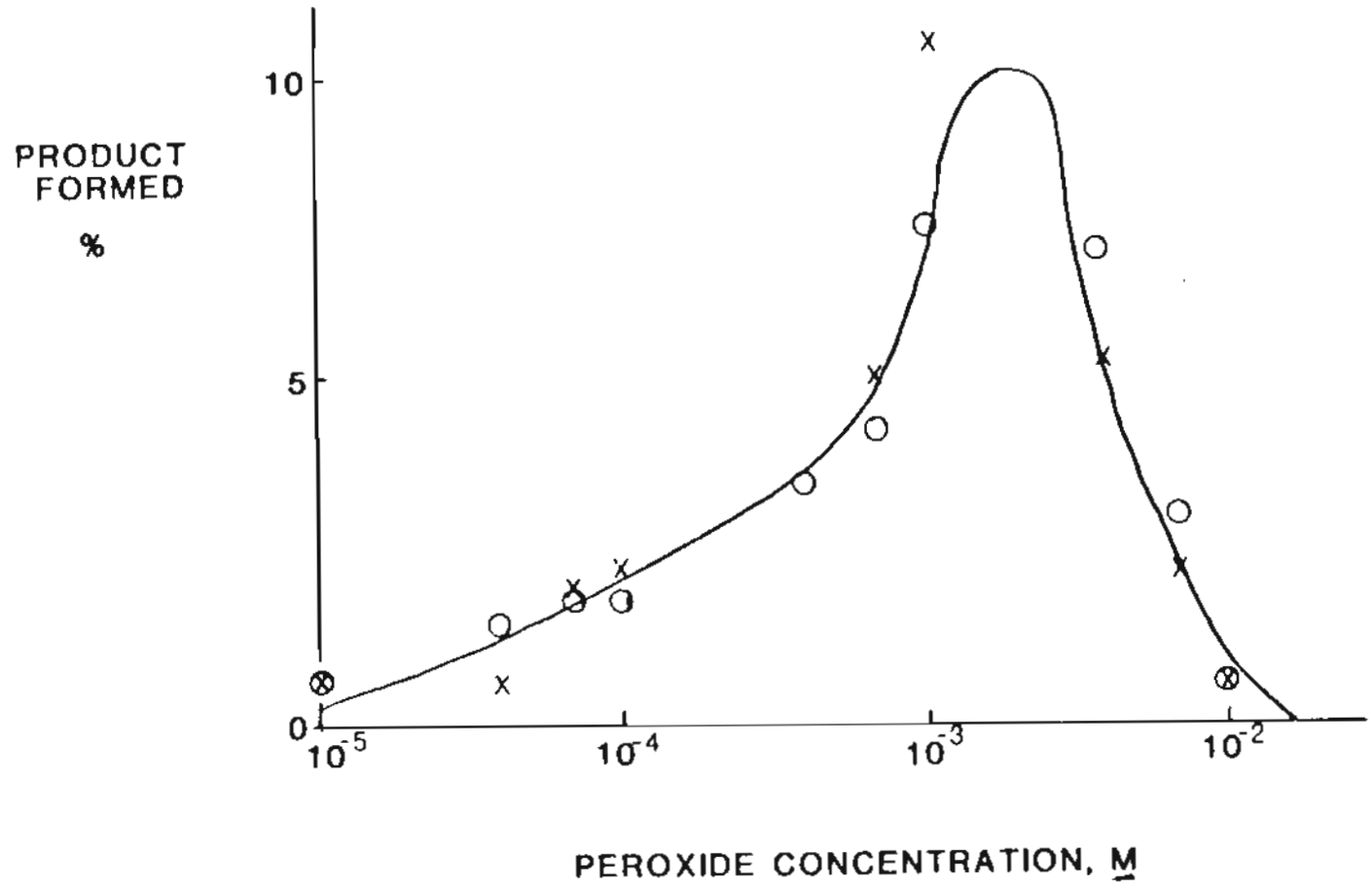


Figure 4. Effect of peroxide concentration on hydroxylation of 4-O-ethylisoeugenol X and 4-ethoxy-3-methoxycinnamyl alcohol XVII by peroxidase. Formation of 1-(4'-ethoxy-3'-methoxyphenyl-1,2-propanediol XI (×) from X and 1-(4'-ethoxy-3'-methoxyphenyl) glycerol XX (○) from XVII is shown. Reaction mixture (2 ml) contained 100 µg horseradish peroxidase and 1.0 mg substrate in phosphate buffer, 100 m*M*, pH 6.0, and was incubated for 3 h at 35°C.

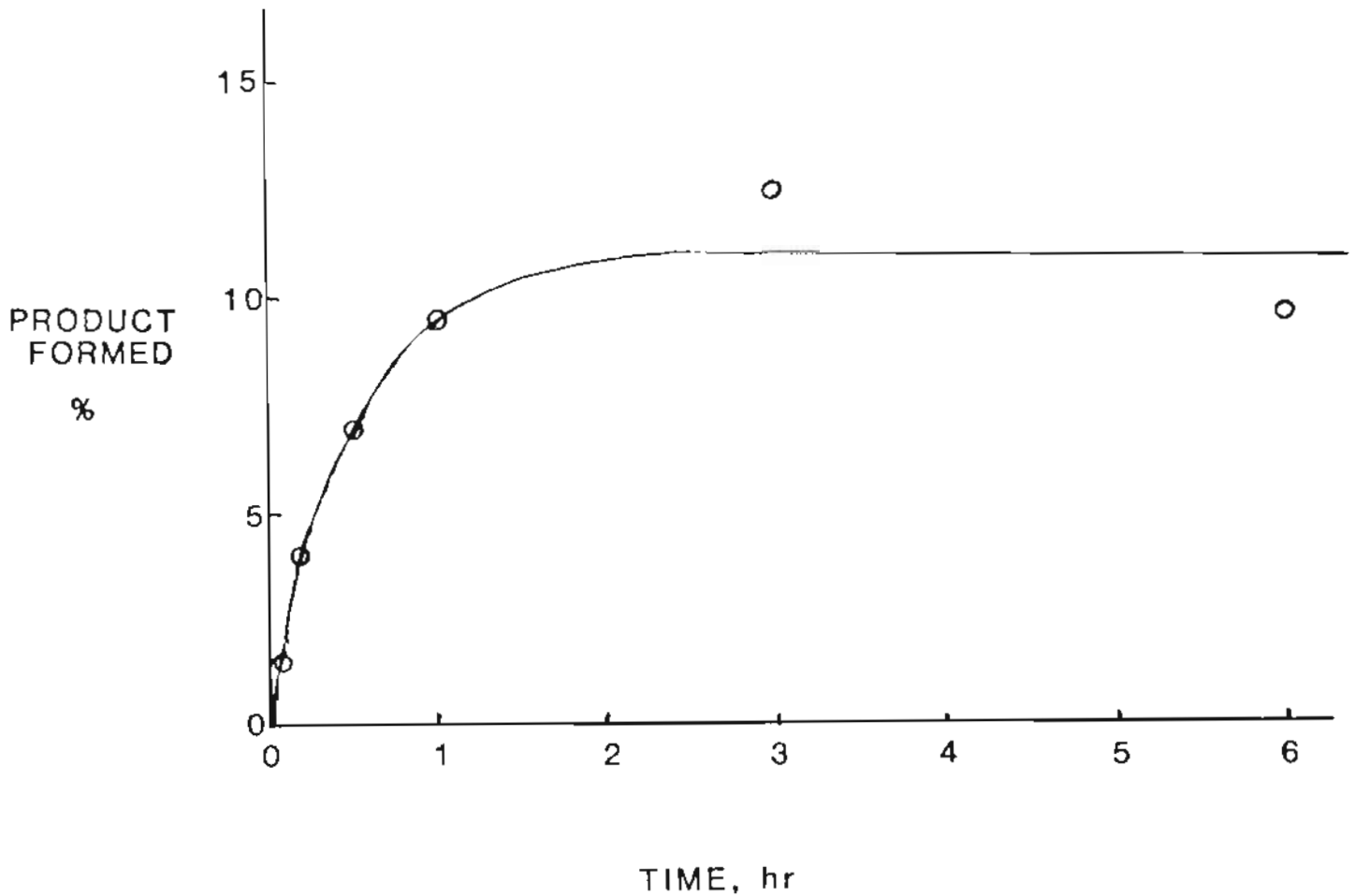


Figure 5. Time course of hydroxylation of 4-O-ethylisoeugenol X by horseradish peroxidase. Formation of 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI is shown. The reaction conditions are the same as described in the legend to Figure 4, except separate flasks were stopped at the indicated times by addition of 5 ml ethyl acetate.

To determine the substrate specificity of peroxidase on non-phenolic compounds, incubations were carried out under the conditions indicated in Table 1. The results indicate that while peroxidase can hydroxylate many olefins, the activity is limited to olefins conjugated to substituted benzene rings. Nonsubstituted rings such as styrene XXI, *trans*-β-methylstyrene XXII, allylbenzene XXIII and 1,2-dihydronaphthalene XXIV were not modified. The singly substituted systems *m*- and *p*-methoxycinnamyl alcohols III and VII were also not suitable substrates; however, the mono-methoxynaphthalenes XII and XIII, were hydroxylated by horseradish peroxidase.

Conjugation is not strictly required. The homoconjugated system 3-(4'-ethoxy-3'-methoxyphenyl)propene XXV was hydroxylated, although this occurred to a lesser extent than with its conjugated isomer X.

Peroxidase was unable to react with extended conjugated olefins. The stilbene derivatives XXVI and XXVII as well as 4-O-ethylferulic acid XXVIII were recovered unmodified (Tables 1, 2). Though this could be the result of an absence of protons on a carbon alpha to the double bond, eliminating the possibility of stabilization by resonance, it seems more likely that the inductive effect of electronegative groups reduces the electron density at the olefinic site, therefore lowering its reactivity. Additionally, 4-ethoxy-3-methoxystyrene XIX has no alpha protons, yet it does serve as a suitable substrate for the hydroxylation reaction.

Solubility is not strictly related to reactivity. 4-O-ethylisoeugenol X, which is used extensively throughout this study, is not soluble in water and immediately forms a milky precipitate, floating

Table 1. Horseradish Peroxidase Modification of 4'-Ethoxy-3'-Methoxyphenyl Olefins[a]

Compound	Product	Amount µg	Amount %
4-O-ethylisoeugenol X	1-(4'-ethoxy-3'-methoxyphenyl)1,2-propanediol XI	265	10.6
	4-O-ethylvanillin XVIII	150	6.0
4-O-ethylconiferyl alcohol XVII	1-(4'-ethoxy-3'-methoxyphenyl)glycerol XX	190	7.6
	4-O-ethylvanillin XVIII	58	2.3
4-ethoxy-3-methoxystyrene XIX	1-(4'-ethoxy-3'-methoxyphenyl)glycol XXXI	150	6.0
	4-O-ethylvanillin XVIII	90	3.6
3-(4'-ethoxy-3'-methoxyphenyl)propene XXV	3-(4'-ethoxy-3'-methoxyphenyl)1,2-propanediol XXXVII	108	4.3
4-O-ethylferulic acid XXVIII	None	0	0

[a]Reactions contained 2.5 mg substrate in 2.0 ml 100 mM phosphate buffer, pH 6.0, and 100 µg horseradish peroxidase. Reactions were started by addition of 1.0 mM H_2O_2 and incubated with shaking at 35°C for 3 h.

Table 2. Effect of Horseradish Peroxidase on Other Olefins[a]

Compound	Product
Cyclohexene	None
Stilbene XXVI	None
4,4'-Dimethoxystilbene XXVII	None

[a]Reaction conditions were the same as in Table 1.

on the reaction mixture after its addition as a solution in DMF. In contrast, 4-O-ethylferulic acid XXVIII and the cinnamyl alcohol derivatives III and VII are soluble, but unreactive.

The effect of the orientation of the olefin was investigated using the dihydronaphthalene derivatives XII, XIII and XXIV. With the olefin fixed in the *cis* configuration, hydroxylation still occurred (Table 3).

Recovery of 4-O-ethylvanillin XVIII in addition to hydroxylation products from the peroxidase reaction mixture of X, XVII and 4-ethoxy-3-methoxystyrene XIX led to investigation of the mechanism of the peroxidase reaction (Table 3). Initially, it was thought that the aldehyde XVIII was the result of oxidative cleavage of the respective hydroxylated products XI, XX, XXXI. This pathway has been postulated for production of veratryl alcohol XXX from 3,4-dimethoxycinnamyl alcohol V in *P. chrysosporium* (43,67). In addition, this fungus has been shown to directly cleave the C-C bond of a *vicinal* diol with recovery of the resulting benzyl alcohol derivative (31).

To test this hypothesis, the hydroxylated compounds XI, XX and XXXI were incubated with peroxidase under standard assay conditions. The data presented in Table 4 show that in each case, the hypothesized intermediate is not modified by peroxidase. This result suggests that the aldehyde XVIII is formed by a direct cleavage of the olefin rather than by C_{α}-C_{β} cleavage of the diol in the peroxidase reaction. This is in sharp contrast to the fungal reaction, in which the hydroxylated compound may be an intermediate in the cleavage pathway.

Table 3. Effect of Substitution Position on Modification of Aryl Olefins by Horseradish Peroxidase[a]

Compound	Product	Amount µg	Amount %
Trans-β-methylstyrene XXII	None	0	0
4-O-ethylisoeugenol	1-(4'-ethoxy-3'-methoxy-phenyl)1,3-propanediol XI	265	10.6
	4-O-ethylvanillin XVIII	150	6.0
3-methoxycinnamyl alcohol III	None	0	0
4-methoxycinnamyl alcohol VII	None	0	0
3,4-dimethoxycinnamyl alcohol V	Veratryl aldehyde XXXVIII	320	9.3
4-ethoxy-3-methoxycinnamyl alcohol XVII	1-(4'-ethoxy-3'-methoxy-phenyl)glycerol XX	190	7.6
	4-O-ethylvanillin XVIII	58	2.3
1,3-dihydronaphthalene XXIV	None	0	0
1,2-dihydro-7-methoxy-naphthalene XIII	1,2-dihydroxy-6-methoxy-1,2,3,4-tetrahydro-naphthalene XV	550	22.0
1,2-dihydro-6-methoxy-naphthalene XII	1,2-dihydroxy-7-methoxy-1,2,3,4-tetrahydro-naphthalene XIV	175	7.0
Allylbenzene XXIII	None	0	0
3-(4'-ethoxy-3'-methoxy-phenyl)propene XXV	3-(4'-ethoxy-3'-methoxy-phenyl)1,2-propanediol XXXVII	108	4.3
Styrene XXI	None	0	0
4-ethoxy-3-methoxy-styrene XIX	1-(4'-ethoxy-3'-methoxy-4-O-phenyl)glycol XXXI	150	6.0

[a]Reaction conditions same as Table 1.

Table 4. Effect of Horseradish Peroxidase on Non-Olefinic Compounds[a]

Compound	Product
1-(4'-ethoxy-3'-methoxyphenyl)glycerol XX	None
1-(4'-ethoxy-3'-methoxyphenyl)propane-1,2-diol XI	None
1-(4'-ethoxy-3'-methoxyphenyl)propane	None
1-(4'-acetyl-3'-methoxyphenyl)-1,2-oxo-propane	*b*
1-(4'-ethoxy-3'-methoxyphenyl)glycol XXXI	None

[a]Reaction conditions same as Table 1.

[b]Hydrolyzes in water.

Reaction of Potassium Superoxide with Model Compounds

Generation of the superoxide anion ($O_2^{-\cdot}$) in peroxidase reactions has been demonstrated by Nilsson et al. (68) by detection of its electron spin resonance signal, and others (69,70). To determine if this species could effect hydroxylation of the conjugated olefins X and XVII, they were reacted with potassium superoxide (KO_2) in DMSO and crown ether. Under these admittedly unphysiological conditions. KO_2 reacted with the substrate in an aprotic environment, then is provided with water and acid in the workup.

While not proving that superoxide is the reactive species in the peroxidase reaction, the isolation of XI and XX (Table 5) from the reaction of the corresponding olefins does show that superoxide can perform the stated transformations. The dismutation products, oxygen and hydrogen peroxide, cannot modify these compounds. The highly basic nature of KO_2 in crown ether, a ligand specific for sodium and potassium ions, caused dehydration of 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI and 1-(4'-ethoxy-3'-methoxyphenyl)glycerol XX. No C_α-C_β cleavage product was observed.

The chemistry of active oxygen is very complex, with many transient, interconvertable forms present in solution. Michelson (71) reports that peroxidase will consume $O_2^{-\cdot}$. The dismutation of the superoxide ion can form singlet oxygen (72), another reactive form of oxygen which has been implicated, though not yet convincingly, in lignin biodegradation (73). Therefore, singlet oxygen cannot be ruled

Table 5. Reaction of Potassium Superoxide with Lignin Model Compounds[a]

Compound	Product[b]	Amount mg	Amount %
4-O-ethylisoeugenol X	1-(4'-ethoxy-3'-methoxy-phenyl)-1,2-propanediol XI	0.5	5
4-O-ethylconiferyl alcohol XVII	1-(4'-ethoxy-3'-methoxy-phenyl)glycerol XX	trace	
1-(4'-ethoxy-3'-methoxy-phenyl)-1,2-propanediol XI	1-(4'-ethoxy-3'-methoxy-phenyl)propene-1-ol[c]	4.0	40
1-(4'-ethoxy-3'-methoxy-phenyl)glycerol XX	1-(4'-ethoxy-3'-methoxy-phenyl)propene-1,3-diol[c]	2.0	20
4-O-ethylvanillin XVIII	4-O-ethylvanillic acid	trace	

[a]Reaction of 10.0 mg substrate with 0.75 equivalents KO_2, followed by H_2O, H_2SO_4 and NaCl; extracted into ethyl acetate as described in text.

[b]Only products pertinent to the present investigation are presented. Other higher oxidation products were also obtained.

[c]Dehydration product.

out as the reactive species in either the KO_2 or the peroxidase reactions. It is beyond the scope of this study to define the exact species of active oxygen responsible for the observed transformation.

Fungal Metabolism of Model Compounds

It has been widely reported that the ability of *P. chrysosporium* to degrade ^{14}C-lignins and ^{14}C-lignin dimers to $^{14}CO_2$ is at a maximum in cultures in which nutrient nitrogen was limiting (44,52,55,74). This was not tested in the present study.

Veratryl alcohol XXX has been shown to be a secondary metabolic product in *P. chrysosporium* (67,75,76). This organism is capable of degrading veratryl alcohol but at a slower rate than its production. The accumulation of veratryl alcohol in the culture medium is concurrent with the onset of ligninolytic activity (43,75). Hence, the presence of veratryl alcohol in the culture extracts was taken as a sign that the fungus had entered the physiological stage in which it was capable of degrading lignin model compounds.

In addition, a qualitative observation is used to judge culture maturity. It has been noted that cultures of wild type *P. chrysosporium* take on a distinctive yellow color after about six days of growth, roughly coincident with the onset of ligninolytic activity (77). A phenol oxidase negative mutant (104-2) isolated in this laboratory is unable to degrade DHP and fails to develop the yellow color in several weeks of growth in liquid medium (26,77). These two indicators, color and presence of veratryl alcohol, were used to judge the competence of *P. chrysosporium* to degrade lignin model substrates.

Modification of model compounds by *P. chrysosporium* is shown in Table 6. The substrate specificity of fungal degradation of these compounds shows some similarities to that of horseradish peroxidase. Lignin model compounds with unsubstituted phenyl rings were not modified by *P. chrysosporium*. 4-O-ethylferulic acid XXVIII and the stilbene derivatives XXVI and XXVII were recovered unmodified.

The naphthalene derivatives XII, XIII and XXIV, in which the olefin is fixed in the *cis* configuration, were all modified by *P. chrysosporium*, without regard to the position of the substituent. The amount of product recovered was considerably less than was observed for the *trans* olefins. In contrast, horseradish peroxidase modified *cis* and *trans* olefins in approximately equivalent amounts (Table 2).

The most striking difference between this organism and peroxidase lies in the ability of *P. chrysosporium* to readily degrade *m*- and *p*-methoxycinnamyl alcohols III and VII. It is probable that the hydroxylated compounds *m*- and *p*-methoxyphenylglycerols XXXII and XXXIII are formed as intermediates in the fungal formation of the anisyl alcohols XXXIV and XXXV. A closely related compound, 1-(4'-ethoxy-3'-methoxyphenyl)glycerol XX has been shown to be rapidly degraded by *P. chrysosporium* under these conditions (44).

Peroxidase was unable to hydroxylate these mono-substituted cinnamyl alcohols, lending support to the suspicion that peroxidase is not the primary agent for this reaction *in vivo*.

Table 6. Modification of Aryl Olefins by *P. chrysosporium*[a]

Substrate	Product	Amount mg	Amount %
4-O-ethylisoeugenol X	1-(4'-ethoxy-3'-methoxy-phenyl)-1,2-propanediol XI	1.76	58.8
	4-O-ethylvanillyl alcohol XXIX	0.77	25.6
4-O-ethylconiferyl alcohol XVII	1-(4'-ethoxy-3'-methoxy-phenyl)glycerol XX	0.66	22.0
	4-O-ethylvanillyl alcohol XXIX	1.26	42.0
4-O-ethylferulic acid XXVIII	None	0	0
4-methoxycinnamyl alcohol VII	*p*-anisyl alcohol XXXIV	2.70	90.2
3-methoxycinnamyl alcohol III	*m*-anisyl alcohol XXXV	2.69	89.5
3,4-dimethoxycinnamyl alcohol V	veratryl alcohol XXX[b]	2.94	98.0
trans-β-methylstyrene XXII	1-phenyl-1,2-propanediol	trace	
allylbenzene XXI	None	0	0
1,2-dihydronaphthalene XXIV	1,2-dihydroxy-1,2,3,4 tetrahydronaphthalene XIV	0.27	9.0
1,2-dihydro-6-methoxy-naphthalene XII	1,2-dihydroxy-7-methoxy-1,2, 3,4 tetrahydronaphthalene XIV XIV	0.72	24.0
cyclohexene	None	0	0
stilbene XXVI	None	0	0
3,3'-dimethoxystilbene XXVII	None	0	0

[a]Stationary 25 ml cultures contained minimal salts medium with 1.2 mM ammonium tartrate and 2.0% glucose. Incubation of six day old cultures with 3.0 mg substrate was under 100% oxygen for 48 h at 35°C.

[b]The amount of veratryl alcohol XXX shown was in excess of the amount produced as a secondary metabolite in control cultures without added substrate.

The effect of oxygen concentration on fungal degradation of X is shown in Table 7. The ligninolytic system of *P. chrysosporium* has been shown to be more effective under increased oxygen tension (52,55,78,79). This effect was also observed here, resulting in a greater degree of C_α-C_β cleavage under 1.5 atm partial pressure O_2 than under 1.0 atm oxygen.

Effect of Catalase on *P. chrysosporium*

To help determine if the peroxidase system of *P. chrysosporium* is responsible for hydroxylation of ethylated isoeugenol X, the peroxide scavenger catalase (EC. 1.11.1.6) was added to fungal cultures. Catalase, as seen in Table 8, inhibited the ability of the fungus to react with the two peroxidase substrates *o*-anisidine and guaiacol. Both of these compounds are oxidized to a brown color in the presence of hydrogen peroxide and horseradish peroxidase.

Bovine serum albumin (BSA) was added to each flask to make the final protein concentration 22 mg/ml to protect the catalase from the presence of any protease activity and as a control to insure that any observed effect was not the result of addition of nonspecific protein. Thymol (200 ng) was added to one culture to control for the amount present as a stabilizer in commercial catalase (0.1%). Adsorption of the colored product to the mycelial mass prevented quantitation of the effect in the guaiacol and *o*-anisidine cultures. It is evident that catalase, and not thymol or protein, inhibited oxidation of these two compounds. The hydroxylation of 4-O-ethylisoeugenol X was not inhibited by addition of catalase, however.

Table 7. Effect of Oxygen Concentration on Metabolism of 4-O-Ethylisoeugenol X by *P. chrysosporium*[a]

Product	Partial Pressure of Oxygen			
	1.0 atm		1.5 atm	
	Amount		Amount	
	mg	%	mg	%
1-(4'-ethoxy,3'-methoxy-phenyl)1,2-propanediol XI	0.58	58	0.07	7
4-O-ethylvanillyl alcohol XXIX	0.25	25	0.71	71

[a]Three ml cultures, six days old, containing minimal salts medium, 1.2 mM ammonium tartrate, 2.0% glucose were pressurized by evaporating a measured amount of liquid oxygen into the cultures. Cultures were incubated with 1.0 mg substrate at 35°C for 48 h.

Table 8. Effect of Catalase on Phenol Oxidase Activity of *P. chrysosporium*[a]

Treatment[b]	Substrate			
	Guaiacol[c]	*o*-Anisidine[c]	4-O-Ethylisoeugenol X	
			Products, % of Substrate	
			1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI	4-O-ethyl-vanillyl alcohol XXIX
Control	+	+	25	51
Bovine serum albumin	+	+	14	50
Thymol	+	Not Done	Not Done	Not Done
Catalase 60,000 U	-	-	32	35
Catalase 120,000 U	-	-	30	37

[a] Culture conditions were the same as described in the legend to Table 6.

[b] Control cultures contained substrate only: 1.25 mg *o*-anisidine, 2.50 mg guaiacol or 4.0 mg ethylisoeugenol. BSA cultures contained substrate plus 22 mg bovine serum albumin. Catalase cultures contained the indicated amount of catalase plus enough BSA to make 22 mg total protein. Thymol control contained substrate, BSA and 200 ng thymol per culture.

[c] + or - indicate presence or absence, respectively, of colored oxidation product.

Addition of hydrogen peroxide to samples of medium showed by immediate evolution of bubbles that the catalase was still active at the end of the experiment. Bits of mycelium inoculated onto malt-yeast extract agar were still viable, indicating that the fungus was not killed by incubation with catalase. Careful filtration of a separate control culture showed that no ability to colorize *o*-anisidine or guaiacol remained in the medium and the activity was not removed from the mycelium by washing three times with buffer. This suggests that the peroxidative activity is associated with the cell surface.

The ability of *P. chrysosporium* to modify 4-O-ethylisoeugenol X in the presence of catalase suggests that extracellular hydrogen peroxide is not essential for this reaction. This does not rule out the possibility that internal peroxidase(s) may fulfill this function. The inability to oxidize the peroxidase indicators when incubated with catalase implies, however, that internal peroxidase(s) make a small contribution, if any, to the activity observed. These findings support the view that X is either modified by some agent other than peroxidase or is transported inside the cell and is modified there.

Purification of Laccase from *P. versicolor*

Laccase from *P. versicolor* was purified by a modification of the method of Fåhraeus and Reinhammar (60). Table 9 shows the results of the purification and a comparison of two assay methods. 3,3'-Diamino-benzidine is shown to be a more sensitive indicator of phenol oxidase

Table 9. Purification of Laccase from *P. versicolor*

Fraction	Assay[a]					
	Pyrogallol		3,3'-Diaminobenzidine			
	ΔA_{420}/min•ml	Units/ml[b]	ΔA_{465}/min•ml	Units/ml[c]	µg/ml[d]	Units/mg
Laccase[e]						
1	0.064	2.0	0.304	96	390	0.25
2	0.340	11.6	2.204	698	-	-
			0.694[f]	220[f]		
2A	-	-	19.62	6200	4960	1.25
3	2.20	75.6	8.440	2670	255	10.47
Peroxidase	0.172	6.0	2.880	912	0.1	9120

[a] Assay conditions are described in Materials and Methods.

[b] One pyrogallol unit is the amount of enzyme required to catalyze the conversion of pyrogallol to 1 µmole purpurogallin per minute at pH 6.0, 25°C.

[c] One diaminobenzidine unit is the amount of enzyme required to consume 1 µmole hydrogen peroxide per minute at pH 5.4, 25°C.

[d] Determined by the method of Lowry et al. (59).

[e] Laccase fractions are defined as follows: (1) *P. versicolor* culture filtrate following induction by 2,5-xylidine; (2) Preparation following ammonium tartrate precipitation, grinding with sand, filtration, resuspension in phosphate buffer, 20 m*M*, pH 6.0; (2A) Fraction 2 following lyophilization and resuspension in distilled water to saturation: (3) Eluant of Sephadex G-100 column having maximum activity, concentrated 7.5 times by lyophilization and resuspension.

[f] This determination contained 4 m*M* H_2O_2.

activity, particularly for peroxidase. Also shown is the inhibitory effect of hydrogen peroxide on laccase activity (Fraction 2).

Separation and Identification of Isomers of 1-(4'-Ethoxy-3'-Methoxyphenyl)-1,2-Propanediol XI

In order to determine the identities of the two isomers of XI, the *threo* form was synthesized by osmium tetroxide oxidation of X (50). This was compared (80) to the *erythro-threo* mixture available in our laboratory (31) and produced in the reactions described in this work. Figure 6a shows a gas chromatogram of enzymatically (laccase) produced XI. Figure 6b shows a mixture of this sample with the authentic *threo* isomer. It can be seen that the *threo* isomer elutes after the *erythro* form.

As further confirmation, mass spectra were taken of successive dilutions of the $^{18}O_2$-laccase produced diol mixture. Samples of the authentic *threo* form of XI were added and mass spectra taken of the resulting isotope dilution. Table 10 shows that as the ^{18}O-labeled diol was diluted by the ^{16}O *threo* sample, the amount of ^{18}O-label decreased in the isomer that eluted last.

Incorporation of ^{18}O into Products of 4-O-Ethylisoeugenol X Modified by Phenol Oxidases and *P. chrysosporium*

The use of $^{18}O_2$ to determine the mode of action of hydroxylation and cleavage of X was successful in part. The inefficiencies of removing atmospheric oxygen by the method described resulted in incorporation of $^{16}O_2$ into XI, XVIII and XXIX in an undetermined amount.

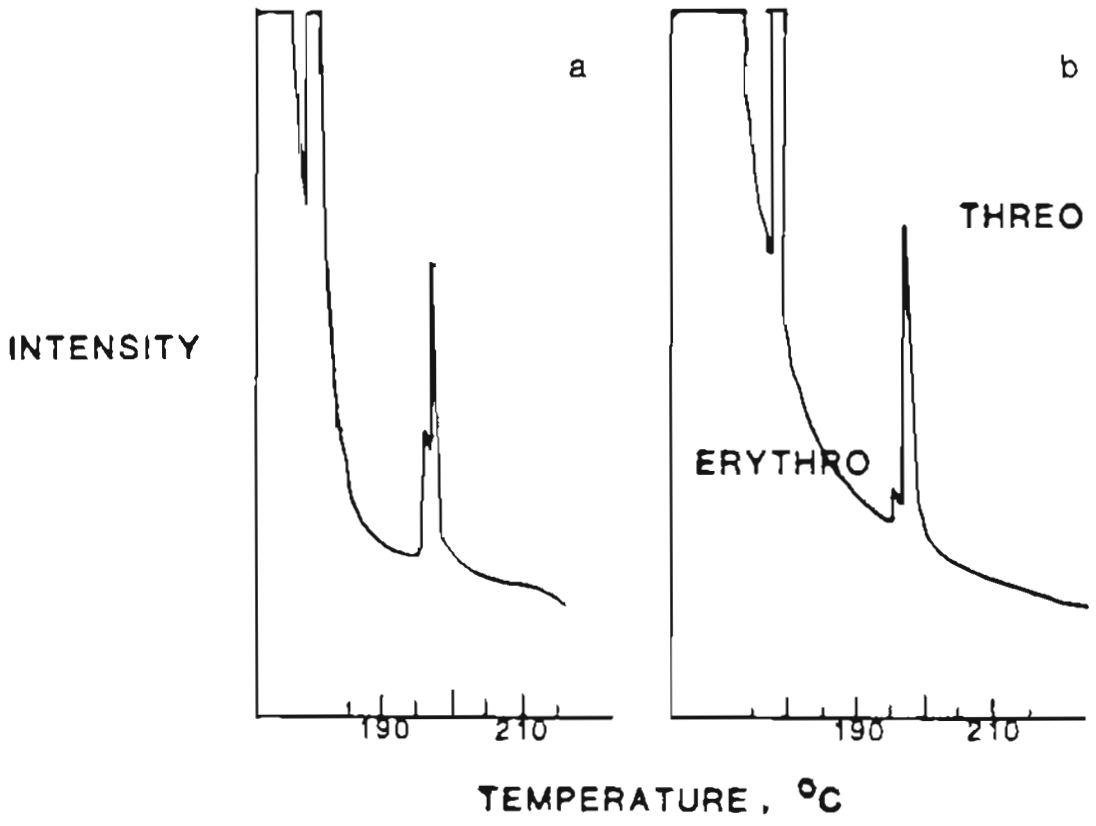


Figure 6. Separation and identification of isomers of 1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI.

a. *Erythro-threo* mixture of XI produced by laccase reaction with X under 1.3 atm partial pressure $^{18}O_2$.

b. Sample (a) mixed with authentic *threo* isomer produced by OsO_4 oxidation of X. Gas chromatograms were run on Varian 2700 instrument on a temperature program of 4°/min from 185-210°C.

Table 10. Effect on Mass Spectrum of Mixture of 2-^{18}O-1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI Isomers by Authentic ^{16}O-*threo* Compound

Peak[a] Ion	Relative Intensity		
	Source[b]		
	Laccase	Laccase + OsO_4 1	Laccase + OsO_4 2
1			
370	80	79	Not Done
372	100	100	
2			
370	93.9	185	352
372	100	100	100

[a]Peak 1 is the isomer with the shortest retention time on the gas chromatograph. Peak 2 eluted later.

[b]Laccase sample was produced under 1.3 atm partial pressure $^{18}O_2$ as described in Materials and Methods. Laccase + OsO_4 1 is a 7.5:1 v/v dilution of the laccase sample by authentic ^{16}O-*threo* isomer of undetermined concentration produced by osmium tetroxide oxidation. Laccase + OsO_4 2 is a 45:1 dilution of the same. Chromatograms of laccase and laccase + OsO_4 1 are shown in Figure 6a and 6b, respectively.

However, an examination of the data that were obtained (Table 11) makes several observations possible. These are summarized in Figure 7.

1. Phenol oxidases and *P. chrysosporium* all incorporate a single atom of ^{18}O in formation of XI from X. Although peroxidase was exposed to both $^{18}O_2$ atmosphere and $H_2{}^{18}O_2$ generated *in situ* by glucose oxidase, as indicated above, peroxidase is unable to modify X in oxygen alone. The reacting species in the peroxidase reaction is therefore $H_2{}^{18}O_2$ (63). The presence of the 374 ion in the spectrum of XI from the $^{18}O_2$ reactions is the result of the natural abundance of the atoms in the 372 ion. The 374:372 ratio in these products is not significantly higher than the 372:370 ratio observed in the ^{16}O standard.

2. The β-carbon receives the ^{18}O preferentially in oxidation by phenol oxidases. The formation of the base peak in the spectrum of XI is shown in Scheme 1. Cleavage of the C_α-C_β bond in the mass spectrometer results in loss of one of the oxygen atoms that were incorporated in the formation of XI from X. In the resultant ion, therefore, the 255:253 ratio represents an approximation of the amount of ^{18}O added to the α-carbon. Bearing in mind the natural abundance of 255 ion seen in the ^{16}O standard (silicon contains 3.4% of the A+2 isotope, ^{30}Si), and comparing to the 372:370 ratio, it appears that most of the ^{18}O incorporated by the phenol oxidases adds to the β-carbon. It is reasonable to postulate from this evidence that if an epoxide intermediate is formed, it is hydrolyzed by addition of the oxygen from water to a benzylic carbonium ion intermediate (Scheme 2).

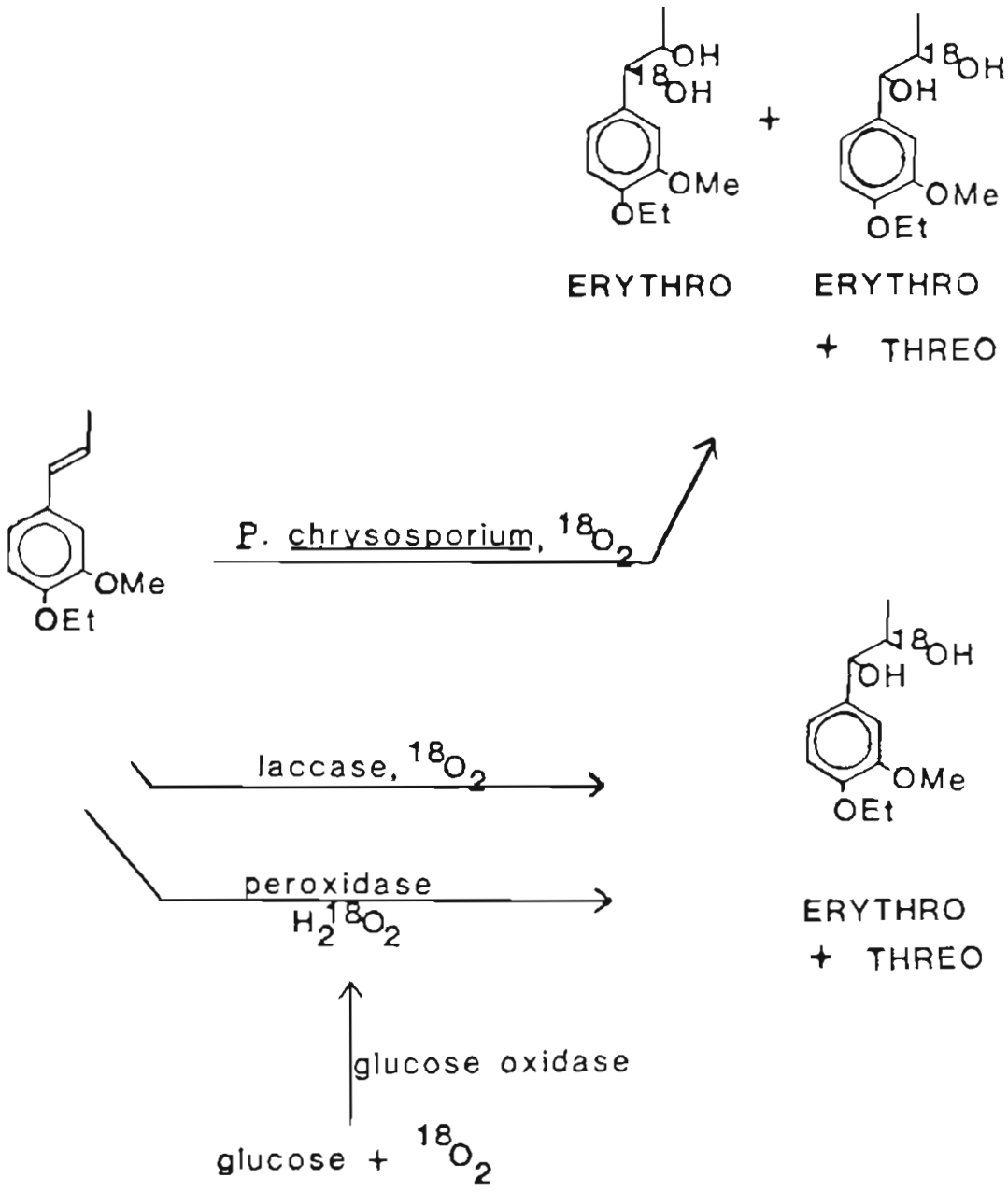
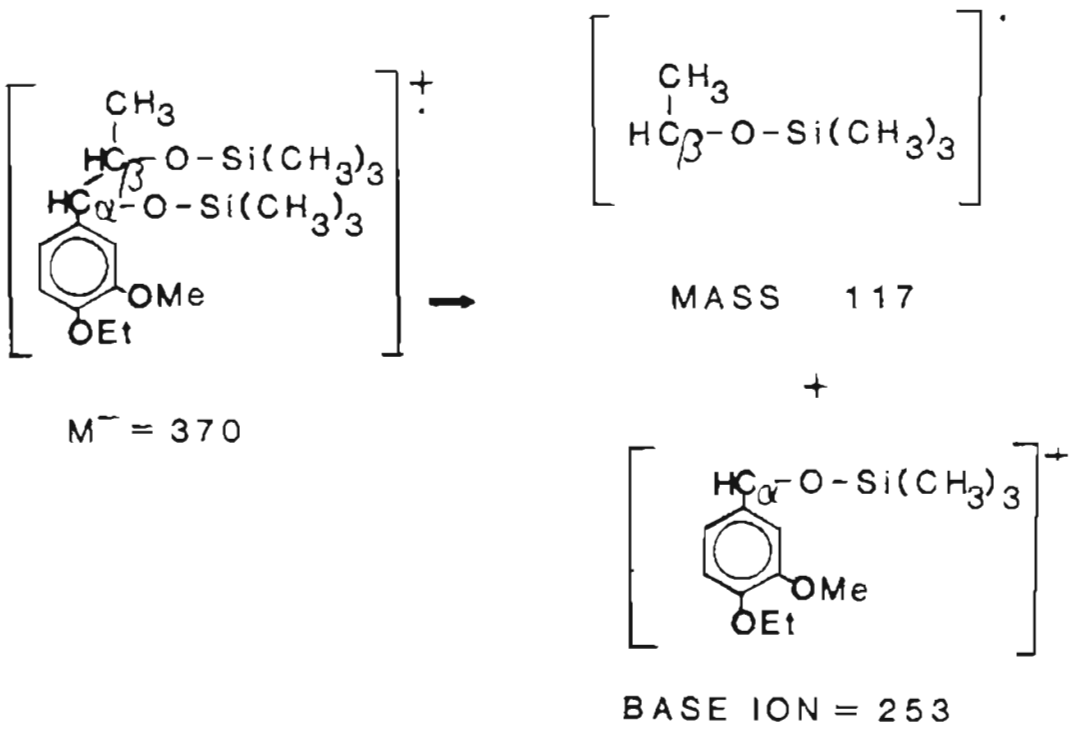
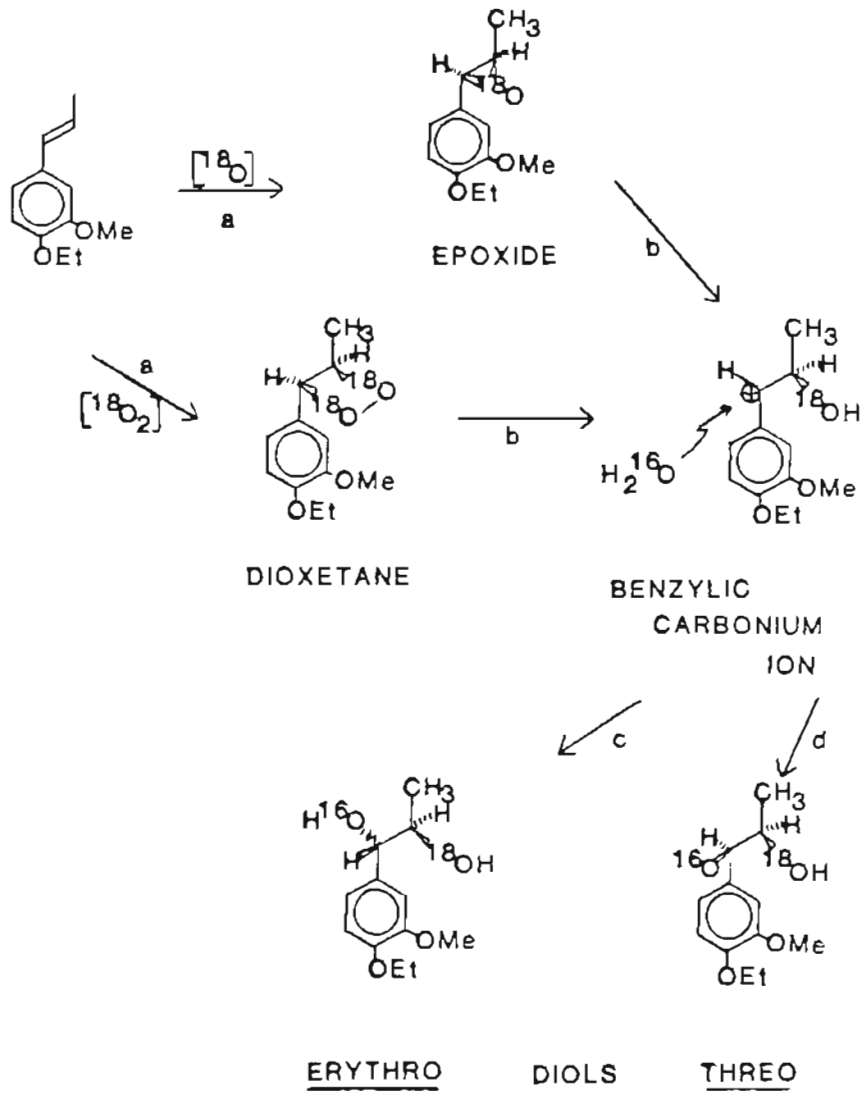


Figure 7. Summary of mass labeling data for diol products of 4-O-ethylisoeugenol oxidation.



Scheme 1. Formation of base peak ion of 1-(4'-ethoxy-3'-methoxyphenyl) -1,2-propanediol XI in the mass spectrometer.

Cleavage of the C_α-C_β bond of the TMSi derivative of XI yields the base peak 253 ion in the spectrum. The opposite distribution of free radical and positive charge results in formation of an ion of m/e 117, which is also observed in the spectrum, but to a much lesser extent.



Scheme 2. Proposed pathway for hydroxylation of 4-O-ethylisoeugenol X by phenol oxidases.

a. Formation of epoxide or dioxetane by laccase and $^{18}O_2$ or peroxidase and $H_2{}^{18}O_2$.

b. Formation of carbonium ion at benzylic carbon.

c. Formation of *erythro* isomer of 2-^{18}O-1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI by addition of $H_2{}^{16}O$.

d. Formation of *threo* isomer.

3. *P. chrysosporium* may form the *erythro* form of the diol XI by a different mechanism than do the phenol oxidases. The data presented in Table 11 suggest that *P. chrysosporium* appears to have incorporated more ^{18}O into the α-carbon of the diol XI than did the phenol oxidases.

To examine the possibility that the two forms of the diol were ^{18}O-hydroxylated in different positions, the *erythro* and *threo* isomers were separated and analyzed by capillary column GC/MS. Table 12 shows that a significantly larger amount of ^{16}O was incorporated by the fungus into the α-carbon of the *erythro* isomer by *P. chrysosporium* than by the phenol oxidases. The apparently higher value for the *threo* isomer may not be significant.

The implication of this finding is that if an epoxide or, perhaps, a dioxetane intermediate (81) is formed by *P. chrysosporium* in the formation of the diol XI, it may be cleaved by a different mechanism than that employed by the phenol oxidases. A mechanism that is consistent with the data involves utilization of a fungal epoxidase to effect diol formation by *trans* addition of water to the β-carbon of the epoxide intermediate (82) (Scheme 3). In this way, the *erythro* isomer could be formed with ^{18}O incorporated into the α-carbon.

The dioxetane structure has been postulated elsewhere as an intermediate to explain the incorporation of two atoms from molecular oxygen onto adjacent carbons of a benzene ring (83). The dioxetane has been postulated only for bacterial systems, however, and has never been directly observed.

Table 11. Incorporation of Mass-Labeled Oxygen into Products of 4-O-Ethylisoeugenol X[a]

Product Ion	Relative Intensity			
	$^{16}O_2$	$^{18}O_2$		
	Standard	Peroxidase	Laccase	*P. chrysosporium*
1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI				
370	100[b]	48.9	93.9	35.9
372	19.1	100[b]	100[b]	100[b]
374	0	11.4	16.7	14.5
253	100	100	100	100
255	4.9	8.3	7.7	15.2
4-O-ethylvanillin XVIII				
180	100	100	100	-
182	1.4	4.9	7.2	-
4-O-ethylvanillyl alcohol XXIX				
254	100	-	-	100
256	8.3	-	-	35.6

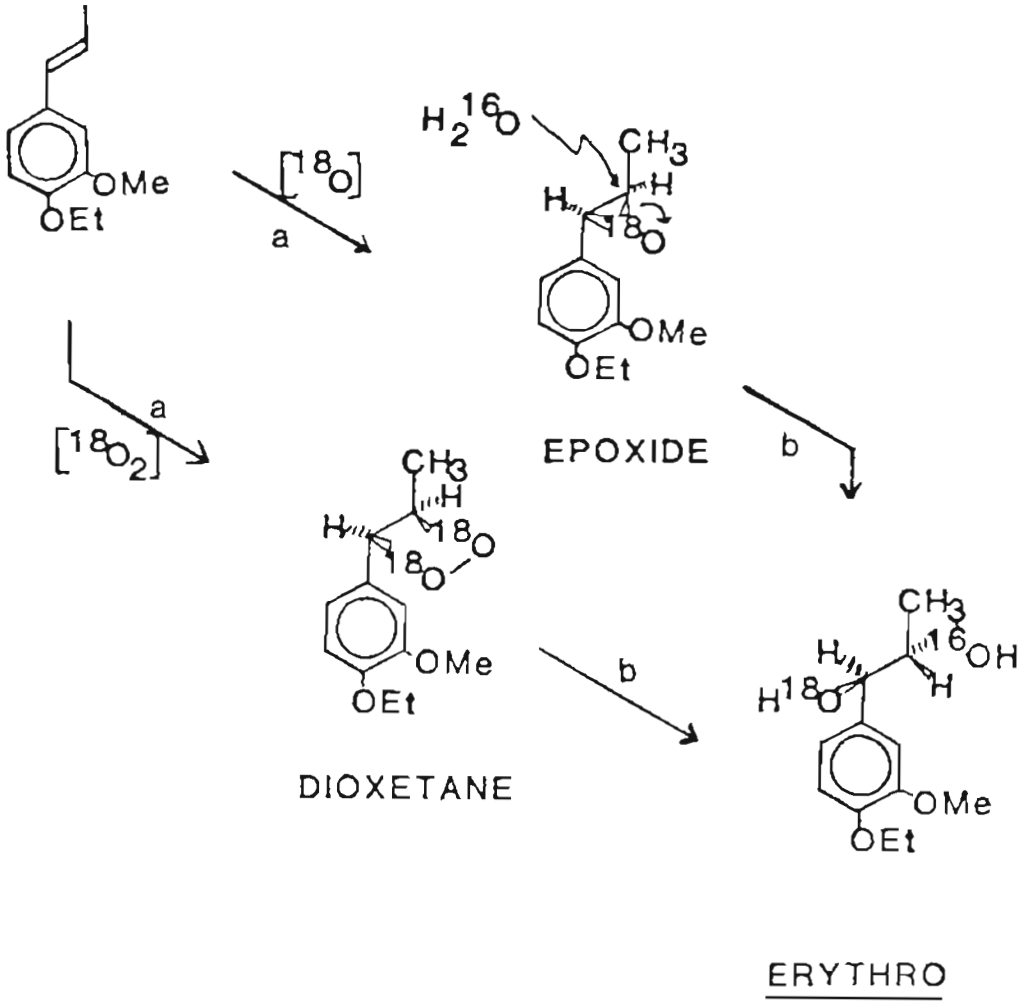
[a]Reaction conditions are described in Materials and Methods.

[b]The actual relative abundance of the 370 ion is 0.8% of the 253 ion in the ^{16}O-standard. In each case, the molecular ion for this compound is normalized to 100% for clarity.

Table 12. Composition of *Erythro* and *Threo* Isomers of 1-(4'-Ethoxy-3'-methoxyphenyl)-1,2-propanediol XI Formed in $^{18}O_2$ by Phenol Oxidases and *P. chrysosporium*[a]

Source	Relative Intensity			
	Erythro		*Threo*	
Ion:	253	255	253	255
Laccase	100	8.9	100	4.8
Peroxidase	100	9.0	100	6.2
P. chrysosporium	100	38.3	100	13.9

[a]Reaction conditions are described in Materials and Methods. Complete separation of isomers was achieved with a Finnigan capillary column GC/MS with INCOS data system.



Scheme 3. Proposed mechanism for formation of *erythro*-1-^{18}O-1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI by *P. chrysosporium*.

a. Formation of ^{18}O-epoxide or $^{18}O_2$-dioxetane by *P. chrysosporium*.

b. Concerted *trans* addition of $H_2{}^{16}O$ to C-2 results in formation of *erythro* isomer.

The possible presence of an epoxide does not rule out an additional pathway employing a carbonium ion intermediate. Indeed, *threo*-β-^{18}O diol formation by *P. chrysosporium* implies this pathway. The isolation of two isomers implies a nonenzymatically controlled step or alternate pathway, as does the instability of the proposed epoxide intermediate. Attempts to synthesize this epoxide in this laboratory have been unsuccessful (84).

4. No significant amount of ^{18}O is incorporated into 4-O-ethylvanillin XVIII by the phenol oxidases. Comparison of the 182:180 and 153:151 ratios, which represent the molecular ion and loss of an aldehyde or ethyl group, respectively, show that the data in Table 11 do not support any conclusions about ^{18}O incorporation into XVIII. The apparently higher value of the 182:180 ratio observed for laccase and peroxidase is within the limits of natural abundance and experimental error. Whether this compound is formed by cleavage of the diol XI or by direct cleavage of the olefin X, as suggested by the inability of peroxidase to cleave XI (Table 4), remains unclear, since the majority of the ^{18}O is found on the β-carbon in XI and would not appear in the aldehyde XVIII.

5. *P. chrysosporium* incorporates ^{18}O into the cleavage product 4-O-ethylvanillyl alcohol XXIX in approximately the same proportion as found in the *erythro* form of the diol XI. This evidence supports the view that the alcohol XXIX is formed in fungal culture by cleavage of the diol XI to the aldehyde XVIII, followed by reduction (31,40).

CHAPTER 4

DISCUSSION

The widespread occurrence of phenol oxidases in lignin degrading fungi have made them the object of much study (6,24,68). Their ability to modify lignin (23) as well as oxidize phenolic products of lignin biodegradation (40) have led to speculation that they are of central importance in lignin degradation by fungi (25). In the present study, that hypothesis was tested by comparison of reactions known to be carried out by both peroxidase and the white rot fungus *P. chrysosporium* (42).

The substrate specificity of the horseradish peroxidase is not greatly different from that of the fungus on the model compounds listed, with the exception of the methoxycinnamyl alcohols III and VII. These were cleaved to the corresponding anisyl alcohols XXXIV and XXXV by the fungus, but remained unmodified by peroxidase. In all cases, the successful substrates had electropositive ring substituents resembling those found in lignin. These substituents serve to increase the electron density of the double bond to which they are conjugated. This may aid the approach of the oxidizing species. Unsubstituted phenyl olefins were unmodified by either peroxidase or the fungus.

Interestingly, loss of reactivity accompanied introduction of an electronegative group, e.g., the acid moiety of 4-O-ethylferulic acid, despite the disubstituted pattern of the benzene ring. It

should also be noted that this compound has no hydrogen atoms on the carbon atom adjacent to the olefin. Cinnamaldehyde derivatives, which contain both a hydrogen atom adjacent to the olefin and a carbonyl moiety conjugated to it are metabolized by *P. chrysosporium* (43). Incubating this compound with peroxidase should help differentiate between these two explanations.

While the substrate specificity appears to be somewhat similar with the important differences noted above for peroxidase and fungal degradation of these compounds, the pathways show striking dissimilarities: (a) Hydroxylation of 4-O-ethylisoeugenol X by the fungus is not affected by addition of catalase to the medium. The peroxidase indicators *o*-anisidine and guaiacol remained unchanged in the presence of catalase. This implies that the fungal peroxidase may not be responsible for hydroxylation of X. (b) The dihydroxylated compound XI was unmodified by peroxidase. Other *vicinal* polyalcohols are similarly unaffected. Veratryl glycerol XXXVI, which has been shown to be an intermediate in veratryl alcohol biosynthesis in *P. chrysosporium* (43,67) and 1-(4'-ethoxy-3'-methoxyphenyl)glycerol which was also cleaved by the fungus at the C_α-C_β bond, are not substrates for peroxidase. This finding implies that peroxidase and *P. chrysosporium* produce the C_6-C_1 compounds XVIII and XXIX, respectively, by different mechanisms. (c) Isotope incorporation studies using ^{18}O show that molecular oxygen contributes a single oxygen atom at the β-carbon when the oxidation of X is catalyzed by laccase. Similarly, peroxidase incorporates ^{18}O from ^{18}O-hydrogen peroxide at the β-carbon.

P. chrysosporium, however, appears, in these preliminary results, to fix a significant amount of oxygen at the α-carbon of the *erythro* isomer.

The above findings suggest that the biodegradative pathway utilized by *P. chrysosporium* to degrade X and perhaps other aryl olefins does not exclusively utilize phenol oxidase(s).

Further work with this system can yield significant information about lignin biodegradation. Clearly, peroxidases are implicated in lignin degradation; they are important for phenol detoxification as well as their role in modification of the lignin polymer (23). Careful elucidation of biodegradative pathways is important, and identification through use of selective inhibitors of the species of active oxygen responsible for the oxidative transformations of lignin degradation can shed more light on the exact nature of lignin metabolism. The suggestion (6) that lignin biodegradation may take place in a generalized oxidative manner without specific enzymatic mediators needs to be investigated.

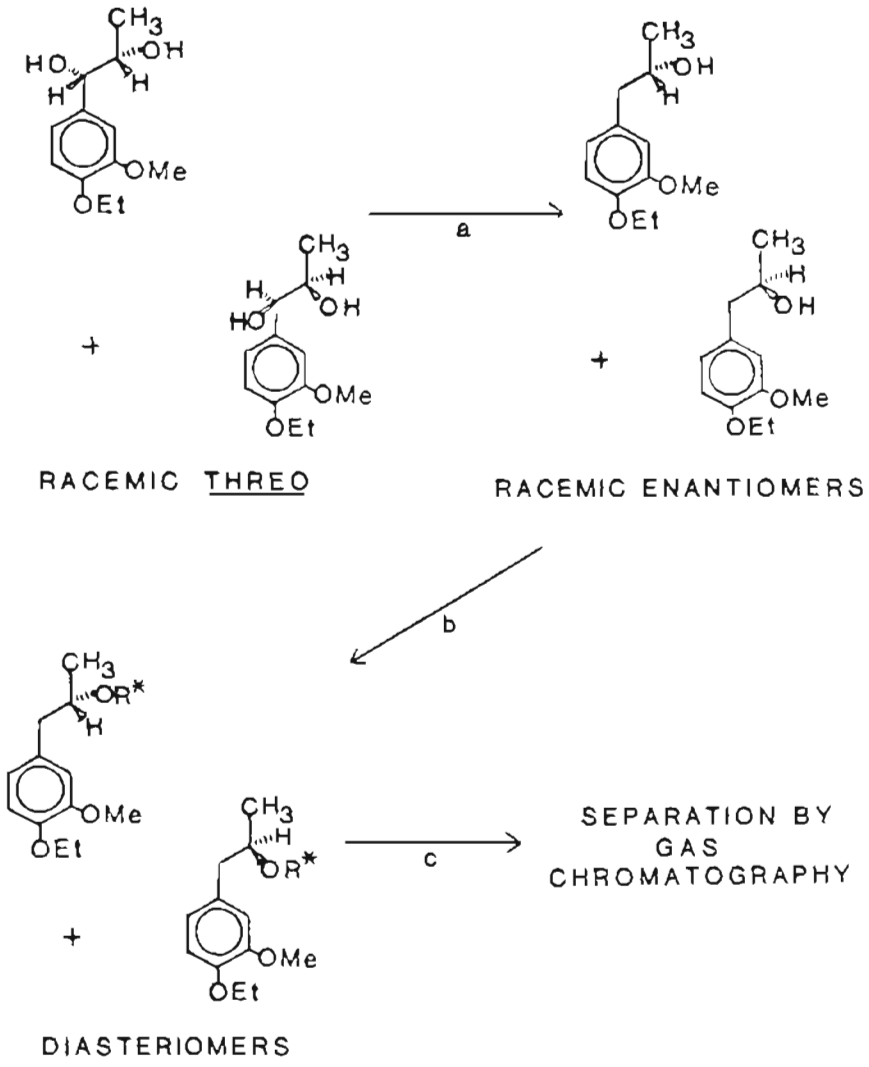
Further work on the problems addressed in this work will also aid in clarifying the role of phenol oxidases in lignin biodegradation. Careful exclusion of $^{16}O_2$ from reaction mixtures will enable quantitative treatment of data obtained. Sensitive O_2 indicators could be employed to ensure that completely anoxic conditions exist before introduction of $^{18}O_2$. The presence of ^{16}O in products will then have quantitative value. Additionally, trapping of O_2 from the head space following a reaction can serve as a check on relative amounts of ^{18}O and ^{16}O actually present, through Raman spectroscopy.

If fungal formation of the diol XI is truly enzymatic, rather than the result of attack by enzymatically formed active oxygen, step (a) in Schemes 2 and 3 should produce optically active epoxides and diols rather than racemic mixtures.

A protocol to determine the optical activity of XI (85) could involve initial separation of the optical isomers of the *threo* product of OsO_4 oxidation. This is accomplished by hydrogenolysis in acetic acid over palladium/carbon catalyst (86) (Scheme 4a). The resultant racemic mixture is reacted with chiral α-methylbenzylamino-isocyanate (85) (Scheme 4b) and the diasteriomers are resolved by gas chromatography. The mixture of racemic *threo* and racemic *erythro* diol produced by *m*-dichloroperoxybenzoic acid also gives these two diasteriomers because the chiral center at the α-carbon is destroyed by hydrogenolysis.

If epoxide formation is stereospecific, however (Schemes 2a, 3a), a chiral center will be formed at the β-carbon, even if the diol is formed through a carbonium ion intermediate. That is, the compounds depicted in Schemes 2 and 3 or their mirror images, but not both, would be formed. Both *erythro* and *threo* isomers would be optically active. Separation by the above procedure would yield only one of the diasteriomers shown in Figure 4b, establishing direct enzymatic participation.

Clearly, the next major step in lignin biodegradation research will be isolation and characterization of the biocatalysts responsible for these transformations. Only when they are found and lignin



Scheme 4. Proposed separation of optical isomers of *threo*-1-(4'-ethoxy-3'-methoxyphenyl)-1,2-propanediol XI.

a. Elimination of chiral center at C_{α}.

b. Reaction with chiral reagent.

c. Separation of diasteriomers by gas chromatography.

biodegradation accomplished *in vitro* will we be able to say with confidence that we know how the fungus does it.

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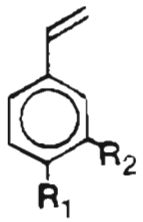
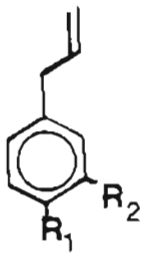
APPENDIX



XIX: R_1 = OEt
R_2 = OMe



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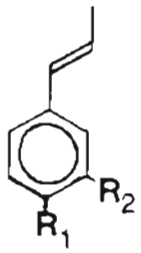
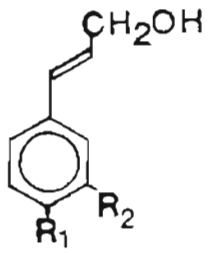


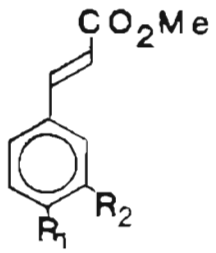
X: R_1 = OEt
R_2 = OMe

XXI: R_1 = R_2 H

XXV: R_1 = OEt
R_2 = OMe

XXII: R_1 = R_2 = H





III: R_1 = H
R_2 = OMe

II: R_1 = H
R_2 = OMe

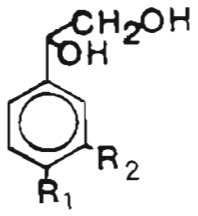
V: R_1 = R_2 = OMe

IV: R_1 = R_2 = OMe

VII: R_1 = OMe
R_2 = H

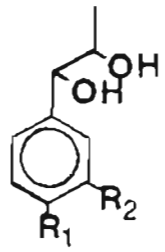
VI: R_1 = OMe
R_2 = H

XVII: R_1 = OEt
R_2 = OMe



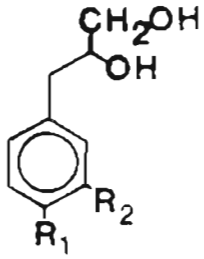
VIII: $R_1 = R_2 = H$

XXXI: $R_1 = OEt$
$R_2 = OMe$



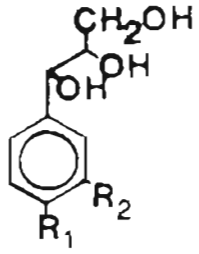
I: $R_1 = R_2 = H$

XI: $R_1 = OEt$
$R_2 = OMe$



IX: $R_1 = R_2 = H$

XXXVII: $R_1 = OEt$
$R_2 = OMe$

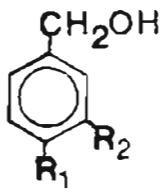


XX: $R_1 = OEt$
$R_2 = OMe$

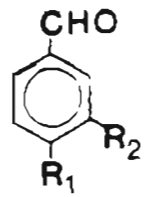
XXXII: $R_1 = H$
$R_2 = OMe$

XXXIII: $R_1 = OMe$
$R_2 = H$

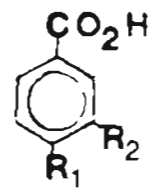
XXXVI: $R_1 = R_2 = OMe$



XXIX: R_1= OEt
R_2= OMe



XVIII: R_1= OEt
R_2= OMe



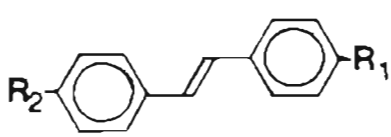
XXVIII: R_1 = OEt
R_2 = OMe

XXX: R_1= R_2= OMe

XXXVIII: R_1= R_2= OMe

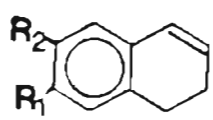
XXXIV: R_1= OMe
R_2= H

XXXV: R_1= H
R_2= OMe



XXVI: R_1 = R_2= H

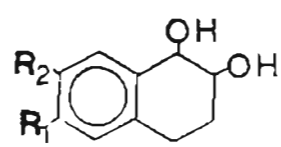
XXVII: R_1 = R_2 = OMe



XII: R_1 = OMe
R_2 = H

XIII: R_1 = H
R_2 = OMe

XXIV: R_1 = R_2 = H



XIV: R_1 = R_2 = H

XV: R_1 = OMe
R_2 = H

XVI: R_1 = H
R_2 = OMe

BIOGRAPHICAL NOTE

The author was born on June 9, 1952 in Cincinnati, Ohio, where he attended elementary school and graduated from Elder High School in 1970. In September 1970 he entered the University of Wisconsin at Madison.

In June of 1972 the author left the University and began a period of nonacademic endeavor which included work as a carpenter, cook, farmer, pipeline inspector, and surgical scrub assistant, primarily in Wisconsin and Kentucky. In March of 1978 he entered the University of Cincinnati and received the Bachelor of Science degree in June of 1979.

Following a summer of work as a microbiology field technician with the Arctic Lakes Process Study at Toolik Lake, Alaska, the author began study at the Oregon Graduate Center in September of 1979, where he completed the requirements for the Master of Science degree in December, 1981, with the degree being awarded formally in July of 1982.

The author is presently pursuing a Ph.D. degree at the University of California, Santa Barbara, in the Department of Biological Sciences.