

Metabolism of Radiolabeled β -Guaiacyl Ether-Linked Lignin Dimeric Compounds by *Phanerochaete chrysosporium*

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Phanerochaete chrysosporium metabolized the radiolabeled lignin model compounds [γ - ^{14}C]guaiacylglycerol- β -guaiacyl ether and [4-methoxy- ^{14}C]veratrylglycerol- β -guaiacyl ether (VI) to $^{14}\text{CO}_2$ in stationary and in shaking cultures. $^{14}\text{CO}_2$ evolution was greater in stationary culture. $^{14}\text{CO}_2$ evolution from [γ - ^{14}C]guaiacylglycerol- β -guaiacyl ether and [4-methoxy- ^{14}C]veratrylglycerol- β -guaiacyl ether in stationary cultures was two- to threefold greater when 100% O_2 rather than air (21% O_2) was the gas phase above the cultures. $^{14}\text{CO}_2$ evolution from the metabolism of the substrates occurred only as the culture entered the stationary phase of growth. The presence of substrate levels of nitrogen in the medium suppressed $^{14}\text{CO}_2$ evolution from both substrates in stationary cultures. [^{14}C]veratryl alcohol and 4-ethoxy-3-methoxybenzyl alcohol were formed as products of the metabolism of VI and 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether, respectively.

Approximately 30% of most woody plants is comprised of lignin, a polymer consisting of substituted phenylpropanoid monomeric units. After cellulose, lignin is the second most abundant natural polymer, and therefore its catabolism and utilization as a renewable resource are of great interest. Although a variety of white rot fungi are capable of catabolizing lignin (10) the enzymes responsible have not been determined. Since lignin is such a complex molecule (19) manifesting heterogeneity with respect to monomeric units as well as bond type, biochemical studies on its degradation face considerable obstacles. For this reason lignin model compounds (6, 12, 13) have been used extensively to investigate possible lignin transformation reactions. Since a major intermonomeric linkage in lignin is the arylglycerol- β -aryl ether bond (15), a variety of studies have been undertaken to examine the degradability by white rot fungi of dimeric lignin compounds containing this linkage. Fukuzumi (6) reported the existence of an enzyme in *Poria subabscida* which cleaves the β -ether and methoxyl ether bonds in veratrylglycerol- β -guaiacyl ether (VI) to produce guaiacol and guaiacylglycerol. No subsequent studies on that fungal enzyme have appeared. In addition, Kirk et al. (13) have examined the degradation of syringylglycol- β -guaiacyl ether by *Polyporus versicolor* and *Stereum frustulatum*. They reported the cleavage of the aryl phenyl bond by

a fungal phenol oxidase-catalyzed reaction yielding guaiacoxycetaldehyde and 2,6-dimethoxy-*p*-benzoquinone. In the present study we investigated the metabolism of guaiacylglycerol- β -guaiacyl ether (IV) and VI. We report on the culture conditions affecting the rate of their metabolism and on the existence of an enzyme system capable of cleaving the α,β bond on the side chain of the etherated dimers or their metabolites.

MATERIALS AND METHODS

Synthesis of substrates. *O*-benzyl- α -bromoacetovanillone was prepared as described by Leopold (16). *O*-benzyl- α -*O*-(guaiacyl)acetovanillone (I) was prepared from *O*-benzyl- α -bromoacetovanillone essentially as described by Adler et al. (1). The crude product was recrystallized from ethyl acetate-chloroform, mp 101 to 102°C.

For *O*-benzyl- α -*O*-(guaiacyl)- β -[^{14}C](hydroxy)propiovanillone (II) a solution of I (1.42 g, 3.7 mmol), anhydrous potassium carbonate (55.3 mg, 0.4 mmol), and a dry [^{14}C]formaldehyde-ethyl acetate mixture (30 ml) obtained by extraction of aqueous formaldehyde (10 ml, 37%) with ethyl acetate (3 \times 50 ml) and subsequent addition of 10 mCi of [^{14}C]formaldehyde was gently heated under reflux for 5 min and subsequently stirred at 25°C for 5 h. Water (50 ml) was added to the reaction, and the mixture was extracted with chloroform-ethyl acetate (1:1, 3 \times 50 ml). The combined extracts were dried over sodium sulfate and evaporated under reduced pressure. The crude product was recrystallized from ethanol to yield 1.24 g (82%) of II, mp 85 to 86°C. ^1H nuclear magnetic resonance (CDCl_3) of the unlabeled compound showed the following: δ = 6.80 to 6.74 (aromatic, 12H), 5.40 (t, J = 4 Hz, 1H), 5.20 (s, 2H), 4.05 (d, J = 4 Hz, 2H), 3.90

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(s, 3H), 3.80 (s, 3H), 2.28 (s, 1H). Hydrogenolysis of II in 95% ethanol over palladium-charcoal was effected under hydrogen (35 lb/in²) after 2 h. The resulting product α -O-(guaiacyl)- β -[¹⁴C]hydroxyproprioanilone(III) was recrystallized from ethanol-water to yield 1.39 g (95%), mp 88 to 89°C.

For γ -¹⁴C-labeled compound IV, the reduction of III as previously described (12) gave a noncrystalline solid. The specific activity of IV was 1.0×10^5 cpm/mg. The mass spectrum exhibited *m/e* 320 (*M*⁺).

For α -O-(guaiacyl)- β -(hydroxy)proprio-4-[methoxy-¹⁴C]veratrone (V), a solution of III (unlabeled, 1.87 g, 5.9 mmol), potassium hydroxide (0.33 g, 5.9 mmol), deacidified dimethylsulfate (0.75 g, 5.9 mmol), and 250 μ Ci of [¹⁴C]dimethylsulfate were heated under reflux for 3 h. KOH in dry methanol was added to maintain the orange color. The product was recrystallized from methanol to yield 1.59 g (80%) of V, mp 117 to 118°C. Mass spectrum of the tetramethylsilyl (TMSi) derivative of the unlabeled compound exhibited *m/e* 404 (*M*⁺).

For 4-methoxy-¹⁴C-labeled compound VI, V was reduced with sodium borohydride in 95% ethanol as described previously (12). The specific activity of VI was 5.0×10^5 cpm/mg.

For 4-ethoxy-3-methoxyphenylglycerol- β -guaiacyl ether (VII), diazoethane was generated from *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine as previously described for diazomethane (10). An ethereal solution of diazoethane was added to a solution of IV (unlabeled) in dimethylformamide. By using a large excess of diazoethane, the reaction was completed in 2 h as measured by thin-layer chromatography. Excess diazoethane was destroyed by acetic acid. Solvents were removed under reduced pressure with frequent addition of water to form an azeotrope. 4-Ethoxy-3-methoxybenzyl alcohol (VIII) was produced from vanillyl alcohol in the same manner.

GC and GC-mass spectrometry. Gas chromatography (GC) was carried out with a Varian model 1700 instrument fitted with a glass column (180- by 0.2-cm inside diameter) packed with 3% OV-101 on Chromosorb Q 100/120 (Applied Science). The oven temperature was programmed from 130 to 270°C at 10°/min unless indicated otherwise. Mass spectrometry was carried out with a DuPont model 21-491B equipped with the same instrument and column for GC. The spectra were obtained at 70 eV. Several mass spectra were obtained with a CEC DuPont model 21-110B operated at 70 eV.

Thin-layer chromatography was done on precoated Silica Gel G plates. The radioactive zones on the thin-layer plates were detected by autoradiography with Kodak NS-5T No-Screen film. High-pressure liquid chromatography (HPLC) was performed on a reverse-phase μ -Bondapak C₁₈ column fitted to run in the isocratic mode on a Waters Associates chromatographic system. Solvents for thin-layer chromatography were (i) benzene-dioxane-formic acid (9:25:4) and (ii) benzene-acetone-methanol (4:1:1), and those for HPLC were methanol-water (iii) 7:3 and (iv) 1:1.

Growth of mycelia. A culture of *P. chrysosporium* ME446 was maintained on slants as previously described (7). The organism was grown at 38°C from a

conidial inoculation in stationary culture in 250-ml Erlenmeyer flasks containing 20 ml of medium. The medium was as previously described (14) except that 20 mM dimethylsuccinate replaced the *O*-phthalate buffer, and unless stated otherwise 1.2 mM NH₄ tartrate was used as the N source. The labeled compounds (5×10^4 cpm) in *N,N*-dimethylformamide were added to cultures as indicated. Cultures were incubated at 38°C unless indicated otherwise. In experiments with agitated cultures, the 250-ml flasks containing 100 ml of media were placed on a New Brunswick G-10 shaker operating at a speed of 150 rpm and describing a 2-inch (ca. 5.08-cm) circle.

Assay of ¹⁴CO₂ evolution. Flasks were fitted with ports which allowed periodic purging with 100% oxygen or air as indicated and permitted trapping of evolved ¹⁴CO₂ in a basic scintillation fluid as described previously (11). The efficiency of ¹⁴CO₂ trapping after purging for 15 min was greater than 98%. Radioactivity was measured in a Beckman LS-3133P spectrometer. Counting efficiency > 70% was monitored with an automatic external standard.

Isolation and purification of metabolic products. After incubation for 7 days, 2 ml of 2 N H₂SO₄ was added to the flasks, and the cultures were filtered. The mycelial mat was frozen on Dry Ice, ground in a mortar, and extracted twice with ethyl acetate (20 ml). The culture filtrate was also extracted twice with ethyl acetate (30 ml). The combined organic fractions were dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure and a stream of nitrogen. Aliquots of the samples were redissolved in methanol and chromatographed on HPLC with solvent system iii or iv. Portions of the collected product peaks were either counted or rechromatographed after silanation on GC or GC-mass spectrometry for identification and measurement of quantity.

Trimethylsilanation of products and standards was carried out by adding bis-(*N,O*-trimethylsilyl)trifluoroacetamide-pyridine (1:1) to the dry residue and heating at 80°C for 5 min.

[¹⁴C]formaldehyde (40 mCi/mmol) and [methyl-¹⁴C]dimethylsulfate (10 mCi/mmol) were acquired from New England Nuclear Corp. All other chemicals were reagent grade.

RESULTS

Effect of culture parameters on metabolism. The evolution of ¹⁴CO₂ from γ -¹⁴C-labeled IV and from 4-methoxy-¹⁴C-labeled VI is shown in Fig. 1A and B. With these high-specific-activity substrates, more than 40% of the carbon is converted to ¹⁴CO₂ in stationary cultures grown at 38°C in approximately 20 days. In shaking culture the volume of media and cell mass was sixfold greater. Thus, on a weight of cell basis, ¹⁴CO₂ evolution in shaking culture from compound IV is approximately 12.5% and that from compound VI is approximately 7.5% of that in stationary culture. The organism grows as 3- to 4-mm spheres in shaking culture. Although oxygen transfer may have been slightly lower in

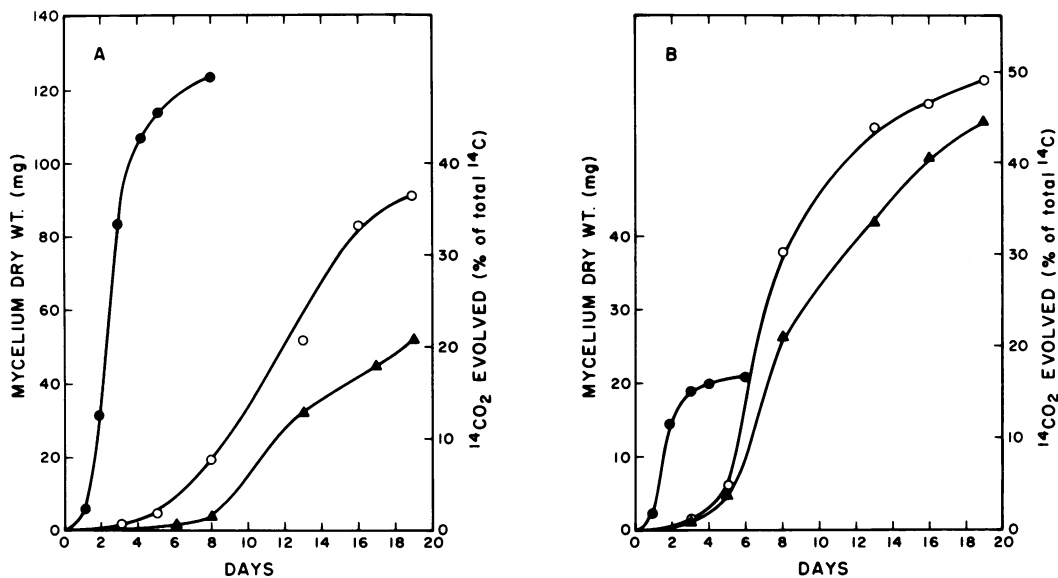


FIG. 1. Evolution of $^{14}\text{CO}_2$ from cultures containing radiolabeled lignin dimeric substrates. (A) Shaking and (B) stationary cultures were incubated with γ - ^{14}C -labeled IV (○) and 4-methoxy- ^{14}C -labeled VI (▲). Shaking and stationary flasks contained 100 and 20 ml of medium, respectively. Flasks were purged periodically with 100% O_2 , and evolved $^{14}\text{CO}_2$ was counted as described in the text. Mycelium dry weight (●) was measured with identical cultures.

the shaking flasks, the growth rate of the cells in both cultures (Fig. 1) was similar, indicating that oxygen starvation was not a factor. With either compound, added at the time of inoculation, significant $^{14}\text{CO}_2$ evolution did not occur until approximately 72 h after inoculation of the cultures and continued at a significant rate for an additional 16 days. In contrast, the primary growth phase ended after approximately 72 h. Although there was a slight increase in cell weight up to approximately day 10 (data not shown), probably due to polysaccharide accumulation, the cultures entered the stationary phase of growth before 72 h indicating that $^{14}\text{CO}_2$ evolution from these lignin dimers is not coincident with the primary growth phase. These results are similar to those obtained with the same organism when ^{14}C -lignins were used as substrates (14). In the present experiments the medium contained only 1.2 mM NH_4 tartrate, and therefore growth probably ceased because nitrogen was a limiting nutrient. The effect of nutrient nitrogen concentration on the complete metabolism of IV and VI can be seen in Table 1. In this experiment cells were incubated in stationary culture in a medium containing either 1.2 or 12 mM NH_4 tartrate for 6 days, after which the ^{14}C -substrates were added and $^{14}\text{CO}_2$ was monitored periodically. Cells previously grown in high-N media are approximately only 10% as

TABLE 1. Effect of nitrogen in the medium on the evolution of $^{14}\text{CO}_2$ from radiolabeled lignin dimeric compounds^a

| Addition to medium | $^{14}\text{CO}_2$ (% of total ^{14}C) at: | |
|--------------------------------|--|------|
| | 48 h | 96 h |
| IV | | |
| 1.2 mM NH_4 tartrate | 4.5 | 11.4 |
| 12.0 mM NH_4 tartrate | 0.4 | 1.6 |
| VI | | |
| 1.2 mM NH_4 tartrate | 6.2 | 16.4 |
| 12.0 mM NH_4 tartrate | 0.5 | 2.6 |

^a Stationary cultures containing either 1.2 or 12 mM NH_4 tartrate were inoculated with *P. chrysosporium* and incubated at 38°C for 6 days. At that time either IV (50×10^4 cpm, 1×10^5 cpm/mg) or VI (50×10^4 cpm, 5.0×10^5 cpm/mg) was added, the cultures were purged periodically, and the $^{14}\text{CO}_2$ was counted as described in the text.

effective as cells grown in low-N media at metabolizing either ^{14}C -substrate to $^{14}\text{CO}_2$. The depressive effect of high nitrogen on the complete metabolism of these lignin-related dimers is similar to that described previously for the metabolism of ^{14}C -lignins to $^{14}\text{CO}_2$ (14).

Because lignin degradation has been shown to be strongly dependent on oxygen concentration (14), we examined the effects of oxygen in the cultures on the metabolism of IV and VI. In this

experiment, cells were grown for 6 days in low-N media in stationary culture and purged either with 100% O₂ or with air (21%) O₂. After 6 days the ¹⁴C-labeled compounds were added, and evolved ¹⁴CO₂ was measured periodically by purging with either air or 100% O₂. The data presented in Fig. 2 indicate that the rate of ¹⁴CO₂ evolution was two- to threefold greater in those cultures purged with O₂. The dry weight of the cells at the end of the linear phase of growth on day 3 was 25 and 27 mg for the air and 100% O₂ cultures, respectively. On day 6 the dry weight of the cells was 29 and 30 mg for the air and 100% O₂ cultures, respectively. This indicates that the difference in the ¹⁴CO₂ evolution is not due to a difference in the mass of the cells.

Isolation of metabolites. In the isolation of [4-methoxy-¹⁴C]veratryl alcohol (IX) from cultures incubated with VI, VI (2.0 × 10⁵ cpm; specific activity, 5 × 10⁵ cpm/mg) was added to fungal cultures at the time of inoculation, and cultures were allowed to incubate for 7 days with periodic purging with 100% O₂. After extraction, HPLC analysis (C₁₈ column, methanol-water, 1:1) revealed a major component which was not found at the same concentration in control cultures. The new peak was radioactive and had approximately 1% of the radioactivity found in the remaining substrate peak. The new product

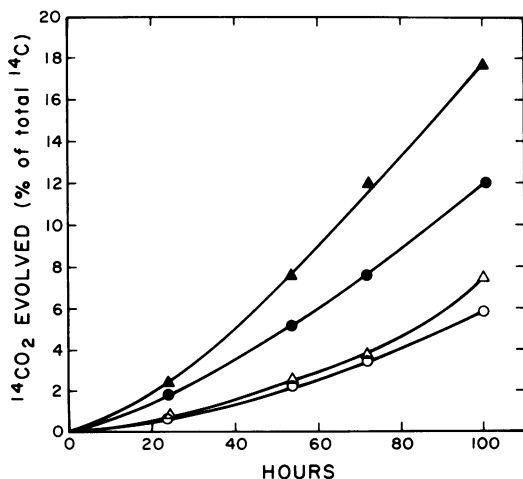
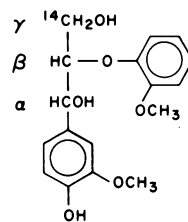


Fig. 2. Effect of O₂ on the evolution of ¹⁴CO₂ from cultures containing radiolabeled lignin dimeric compounds. Stationary cultures were inoculated with *P. chrysosporium* and incubated under air for 6 days. From that time, compound IV (5 × 10⁴ cpm) was added to half of the flasks, and the cultures were purged immediately and then periodically with air (21% O₂) (○) and 100% O₂ (●). Compound VI (5 × 10⁴ cpm) was added to the remaining flasks, and these cultures were also purged immediately and then periodically with air (△) and 100% O₂ (▲).



IV

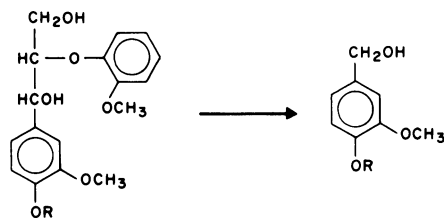
VI R = ¹⁴CH₃VII R = CH₂CH₃IX R = ¹⁴CH₃VIII R = CH₂CH₃

Fig. 3. Structures of substrates used and products identified in this study.

co-chromatographed on HPLC and GC with veratryl alcohol. As shown in Fig. 3, these results indicate that IX is a product of the metabolism of VI. However, the de novo synthesis of veratryl alcohol from glucose by *P. chrysosporium* has been reported elsewhere (14). It was conceivable, therefore, that VI could have been metabolized to low-molecular-weight fragments with subsequent synthesis of [¹⁴C]veratryl alcohol and randomization of the label. This possibility appeared unlikely, however, because the veratryl alcohol peak in control cultures was considerably smaller than in cultures inoculated with VI. To unambiguously demonstrate the metabolism of these dimers to C₆-C₁ products, we synthesized and studied the fungal metabolism of VII.

In the isolation of VIII from cultures incubated with VII, VII was added to fungal cultures at a concentration of 0.05% at the time of inoculation, and cultures were allowed to incubate for 7 days with periodic purging with 100% O₂. After the incubation period, both the mycelium and culture filtrate were extracted as described above and analyzed by HPLC and GC. A major peak which co-chromatographed with VIII was apparent. The mass spectrum of the TMSi derivative of this component (Fig. 4) was as follows: *m/e*, relative intensity = 256, 5%; 255, 20%; 254, 100% (M⁺); 253, 16%; 239, 10%; 225, 15%; 224, 5%; 223, 13%; 211, 19%; 210, 3.0%; 209, 20%; 195, 3%; 179, 8.0%; 167, 2.0%; 166, 16%; 165, 83%; 138, 5%; 137, 55%; 136, 5%; 135, 5%; 122, 7.0% and was essentially identical to that of the TMSi deriv-

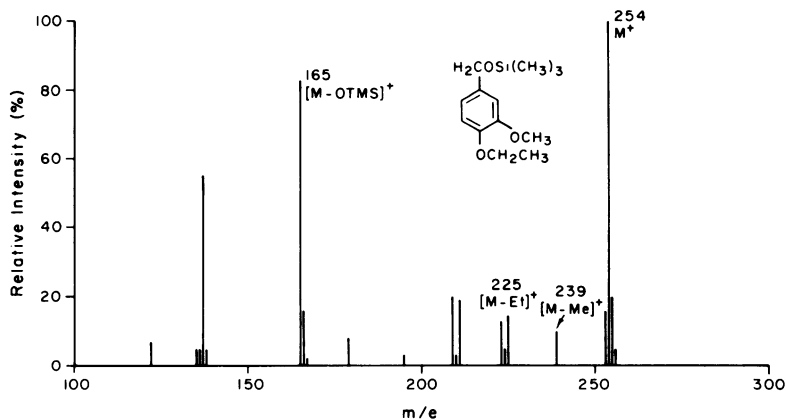


FIG. 4. Mass spectrum of the TMSi derivative of VIII.

ative of authentic VIII. The amount of VIII was approximately 0.2% of the amount of starting material. The isolation of IX and VIII as metabolic products of VI and VIII indicates that this fungus is capable of cleaving the α,β bond in these lignin model compounds or their metabolites. This cleavage apparently can take place without prior deethylation at the 4 position, demethylation at the 3 position, or ring cleavage.

DISCUSSION

The evolution of $^{14}\text{CO}_2$ from these dimeric substrates occurs only after the culture has entered the stationary phase of growth. This suggests that at least one of the enzymes involved in the complete metabolism of these dimers is produced or activated during the secondary phase of growth.

Post-growth-phase metabolism can be triggered by the limitation of various nutrients and other stressful conditions contributing to an adverse effect on growth. In the present experiment the low levels of nitrogen were probably the primary limiting nutrient causing a cessation of growth. Nitrogen limitation has been demonstrated to be a key regulator in the synthesis of nigeran by *Aspergillus aculeatus* (8) as well as in the synthesis of gibberellins by *Gibberella fujikuroi* (2). The exact nature of the regulation of the metabolism of these lignin dimers by nitrogen is not known. The regulation of lignin metabolism by nitrogen has been previously documented (9, 14).

The results presented in this paper also demonstrate that in stationary culture, cells grown under 100% O_2 metabolize these lignin dimers at a rate severalfold faster than cells grown under air (21% O_2). This indicates that at least some step or steps in the complete degradation of these dimers to CO_2 is oxidative. Growth of the

organism is no better under 100% O_2 than under air. These results are similar to those previously reported for lignin metabolism. It is important to note, however, that unlike results reported for lignin metabolism (14) where agitation from the time of inoculation completely blocks lignin degradation, the results reported here demonstrate that agitated cultures of *P. chrysosporium* can degrade these lignin dimers at a significant although greatly reduced rate compared with unagitated cultures.

It is noteworthy that several of the culture parameters which affect lignin degradation, such as nitrogen concentration in the media, the concentration of O_2 in the atmosphere, and agitation, have similar effects on the degradation of these lignin model compounds. Although this does not demonstrate that the degradation pathways for the polymer and the models are identical, it suggests that the regulation of these pathways has many similarities. These results also suggest that the effect of these parameters on lignin degradation could occur during the degradation of fragments as well as during the initial depolymerization of the polymer.

In this study we report the isolation of 4-methoxy- ^{14}C -labeled IX from VI and VIII from VII. Although studies on the cleavage of IV (18) and syringylglycol- β -guaiacyl ether (13) by white rot fungi have been reported previously, several earlier studies with a variety of white rot fungi (12, 18) indicated that VI was not readily metabolized to low-molecular-weight fragments. The only previous report of the fungal cleavage of a dimer etherified in the 4 position is that of Fukuzumi et al. (6). This early report indicates the existence of etherase(s) in *P. subacida* which demethylate and cleave VI to yield guaiacyl glycerol and guaiacol. Those results do not rule out IV as an intermediate in the cleavage of the

β -ether bond in VI. No additional documentation of this enzyme has appeared. The existence of β -etherase-type enzymes in bacterial systems has been previously described (3, 5).

The present study suggests the existence in *P. chrysosporium* of a metabolic pathway generating cleavage fragments directly from etherified dimers. This is the first unambiguous demonstration that neither the presence of a free phenolic function on the ring nor ring cleavage is a prerequisite for the cleavage of the α,β bond. This type of side-chain cleavage in etherified dimers may be a good model for internal cleavages in the lignin polymer.

The cleavage of the alkylphenyl bond in syringylglycol- β -guaiacyl ether by white rot fungi and by the phenol oxidase laccase was previously described (13). Since phenol oxidases require a free phenol, however, they cannot be implicated in the initial cleavage of VI and VII to veratryl alcohol and 4-ethoxy-3-methoxybenzyl alcohol. Although the pathways for the cleavage of these etherified dimers remain unclear, an initial cleavage of the α,β bond by an enzyme other than a phenol oxidase is suggested by the present results.

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