CHEMICAL SYNTHESIS AND FUNGAL METABOLISM OF RADIOLABELED LIGNIN MODEL COMPOUNDS

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This thesis is dedicated with much love to my parents.

Acknowledgements

I want to extend my thanks to Dr. Michael H. Gold, for his patience, understanding and guidance throughout my stay at the Oregon Graduate Center.

I also express my gratitude to the faculty, staff and students with whom I have had the pleasure of working.

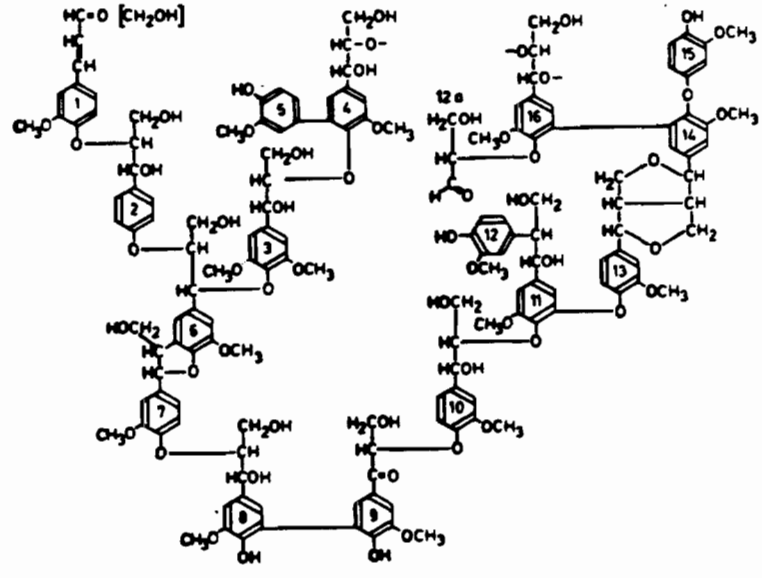
Tables and Figures

ABSTRACT

Culture parameters influencing metabolism of synthetic ^{14}C-labeled lignin model compounds to $^{14}CO_2$ in defined media by the fungi, Polyporus versicolor and Phanerochaete chrysosporium, were examined. Model compound metabolism was oxygen-dependent. Agitation of the cultures, resulting in formation of mycelial pellets, suppressed $^{14}CO_2$ evolution by P. chrysosporium, to a greater extent than by P. versicolor. The concentration of nutrient nitrogen was critical; $^{14}CO_2$ evolution was retarded at 12 mM ammonium tartrate relative to 1.2 mM ammonium tartrate. Cultures evolved more $^{14}CO_2$ when grown on xylose than on either glucose or glycerol. Initial glucose at 0.1% concentration was significantly less supportive of growth and $^{14}CO_2$ evolution than cultures with 0.5-1.0% glucose. Studies with cycloheximide, a protein synthesis inhibitor, demonstrated that the lignin model compound degrading enzyme system was constitutive. 4-Methoxyl-[^{14}C]veratryl alcohol was found to be a catabolic product in the metabolism of 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether and 4-methoxyl-[^{14}C]veratric acid to $^{14}CO_2$.

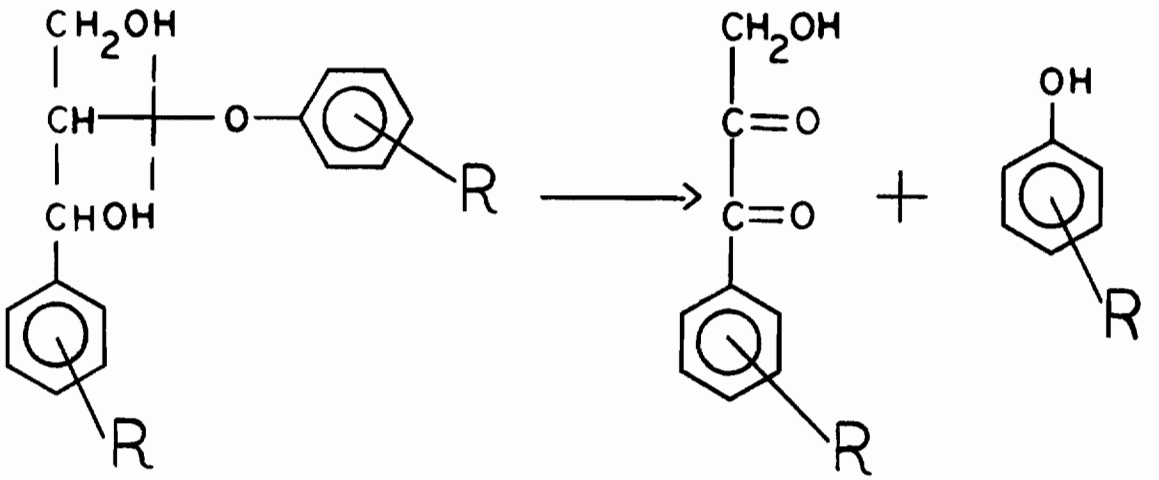
INTRODUCTION

Lignins are complex three-dimensional polymers derived from phenylpropanoid precursors; they comprise approximately one-third of the polymeric material in woody plants. The nature of the structure of lignin, heterogeneous with respect to monomeric units and bond type, was not elucidated until the 1960's (1,2). The following schematic formula shows the major intermonomer linkages in conifer lignin.

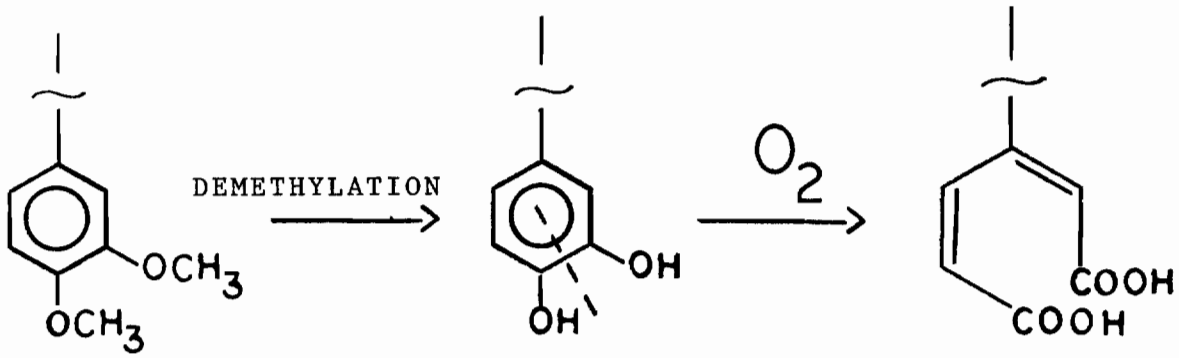


Knowledge of the factors influencing lignin biodegradation is necessary in order to understand wood decay, the formation of humic substances and to explore the potential for utilizing lignin degrading organisms or enzymes in lignocellulose bioconversion processes (3).

Only a limited number of organisms, principally basidiomycete fungi, have been found to cause extensive degradation of lignin in woody plants (3). Although the initial lignin depolymerization steps are not understood, several possible pathways have been advanced (4,5): a) early cleavage either oxidative or hydrolytic, of alkyl-aryl ether bonds, releasing principally monomeric phenolic fragments, viz.,



and b) early cleavage of aromatic groups in the polymer, followed by a series of degradative reactions releasing various one to six carbon fragments, viz.,



With either pathway lignin breakdown would probably yield a large variety of fragments, each in low concentration and therefore difficult to separate and characterize. For this reason simple chemically defined ^{14}C-labeled lignin models [10], [11], [13] and [14] were used as alternative substrates to help delineate the pathway(s) by which fungi metabolize lignin (6,7).

A review of earlier work on both guaiacylglycerol-β-guaiacyl ether and its veratryl analog with microoganisms or their enzymes has not provided strong evidence for any one pathway by which these compounds are metabolized (8-12).

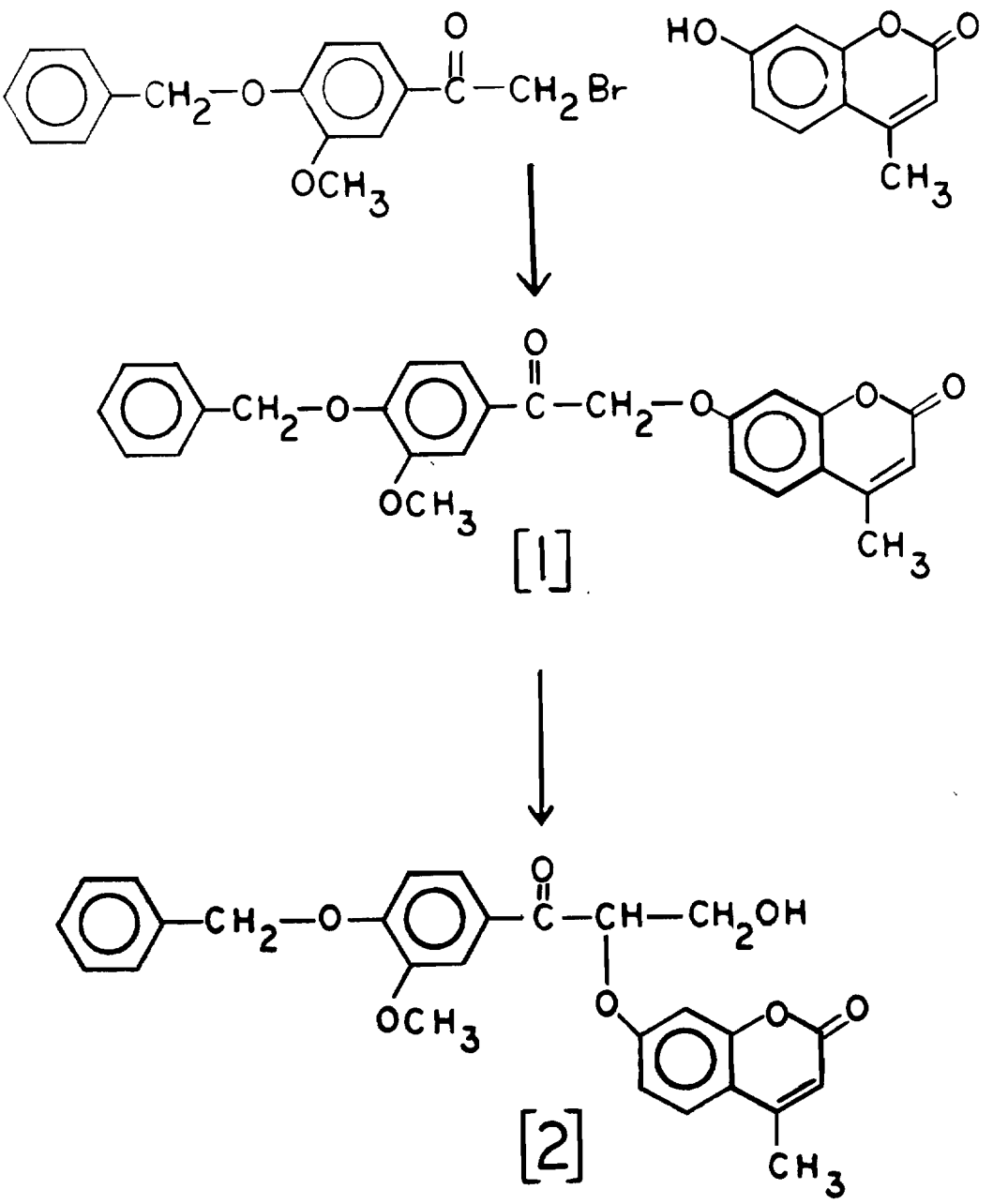
Previous workers have used the isolation and characterization of catabolic products from cultures of these lignin degrading organisms to help delineate the metabolic pathways and the enzymes which are involved. Using this approach, Kirk et al. (13) was able to demonstrate alkylphenol carbon-to-carbon bond cleavage in syringlyglycol-β-guaiacyl ether and α-guaiaoxyacetosyringone. Fukuzumi et al. (10), and Crawford et al. (12) were able to demonstrate aryl-alkyl ether bond cleavage in veratrylglycerol-β-guaiacyl ether by fungi and bacteria respectively.

In the present study several compounds were synthesized for use in the elucidation of the nutritional and environmental factors that influence the biodegradation of lignin models, as well as to elucidate the metabolic steps involved in the degradation of some β-arylether compounds.

The compounds whose syntheses are first described are substrates for the fluorimetric assay of β-etherases (Scheme I). Guaiacylglycerol-β-guaiacyl ether and other related dimeric compounds ^{14}C-labeled at

specific positions in the ring, side chain and 4-methoxyl position were also synthesized (Scheme II). Metabolic studies on these compounds were undertaken using two white-rot fungi, Phanerochaete chrysosporium and Polyporus versicolor.

Scheme I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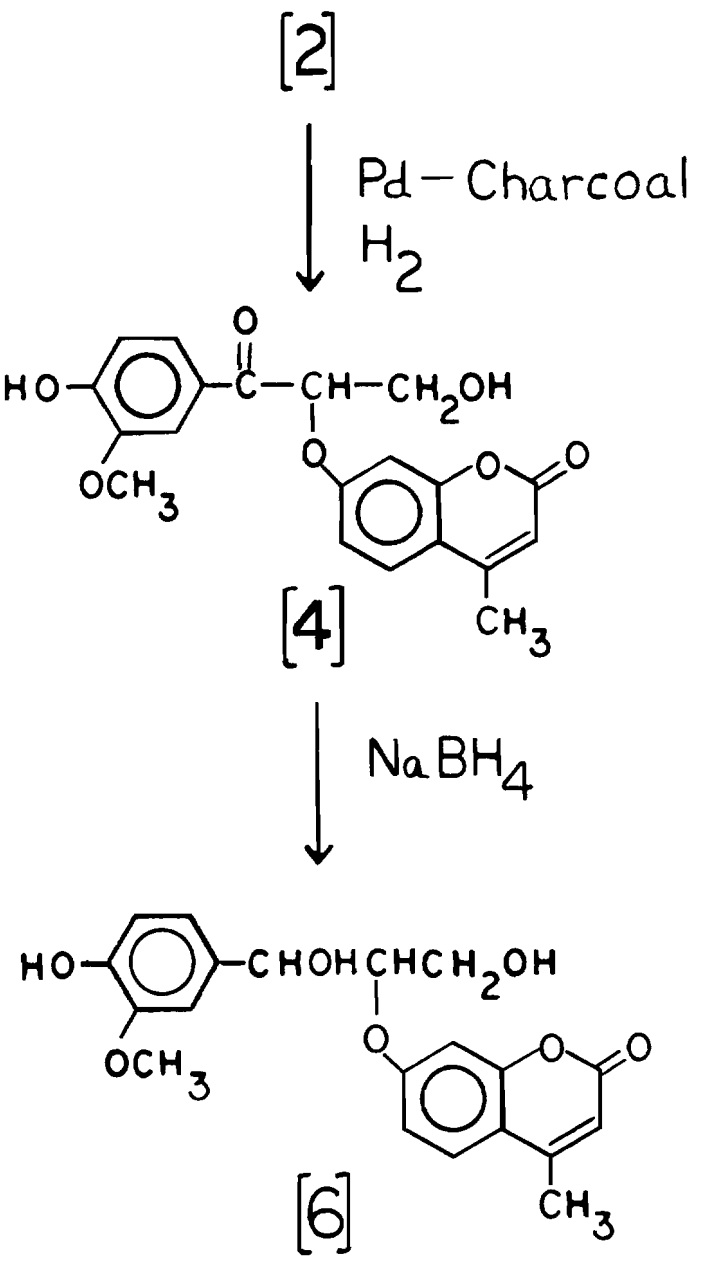
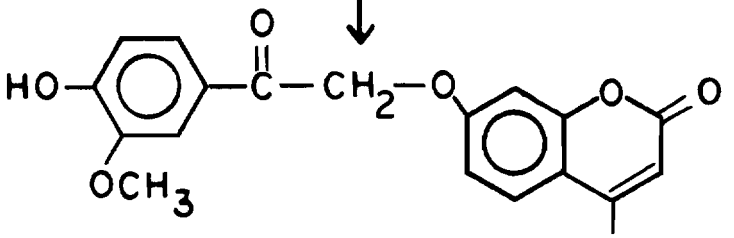
O
O
CH3
CH2
O
C
CH2 Br
OCH3
CH2
O
C
CH2
O
O
O
CH3
OCH3
[1]
CH2
O
C
CH
CH2OH
O
O
O
OCH3
CH3
[2]

Scheme I

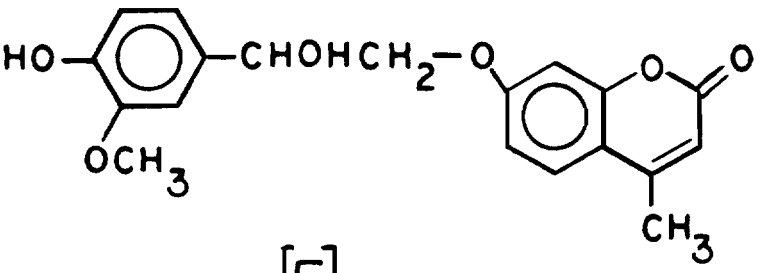

[2]
Pd – Charcoal
H_2
HO
O
C
CH
CH_2OH
O
OCH_3
O
O
O
CH_3
[4]
$NaBH_4$
HO
CHOH
CHCH$_2$OH
O
OCH_3
O
O
O
CH_3
[6]

Scheme I

[1]

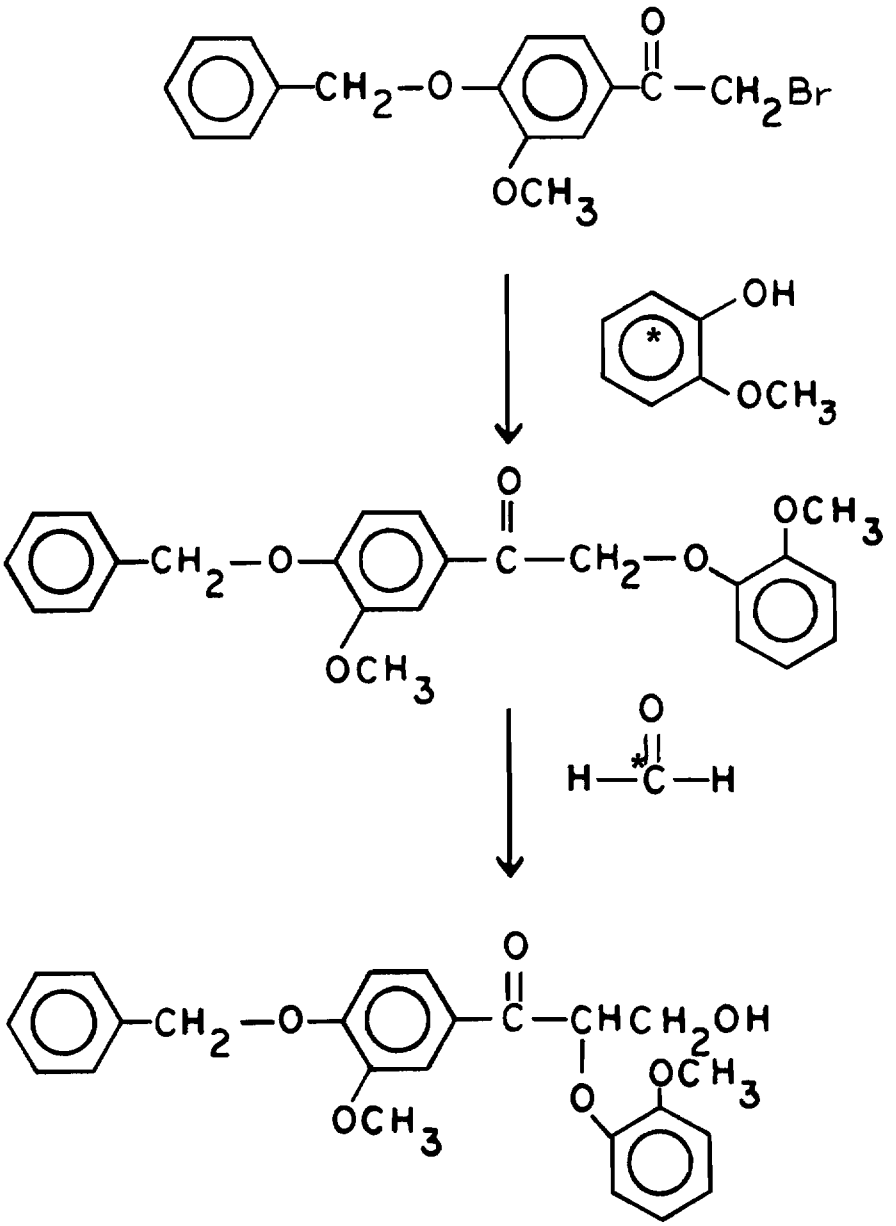


[3]



[5]

Scheme II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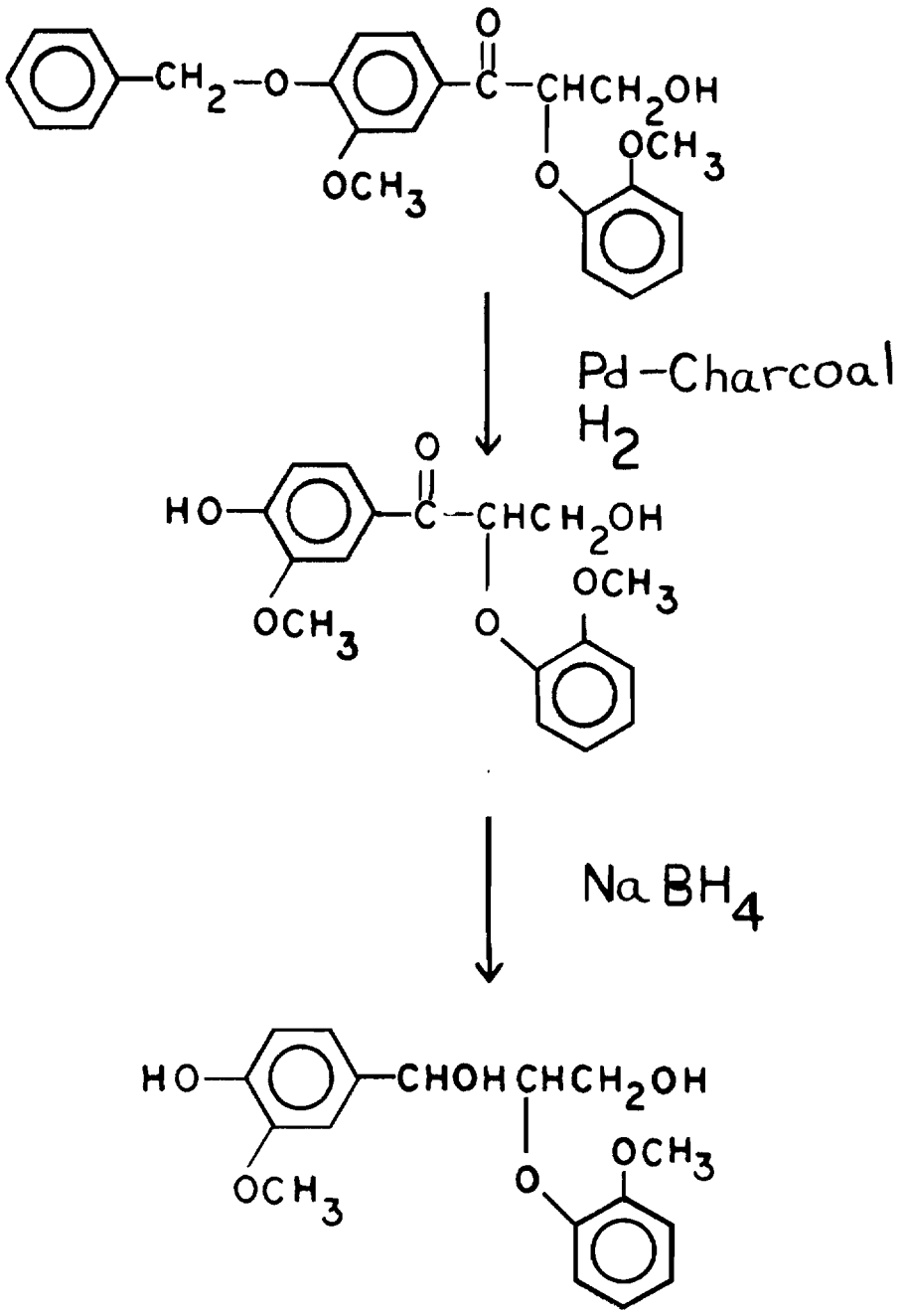
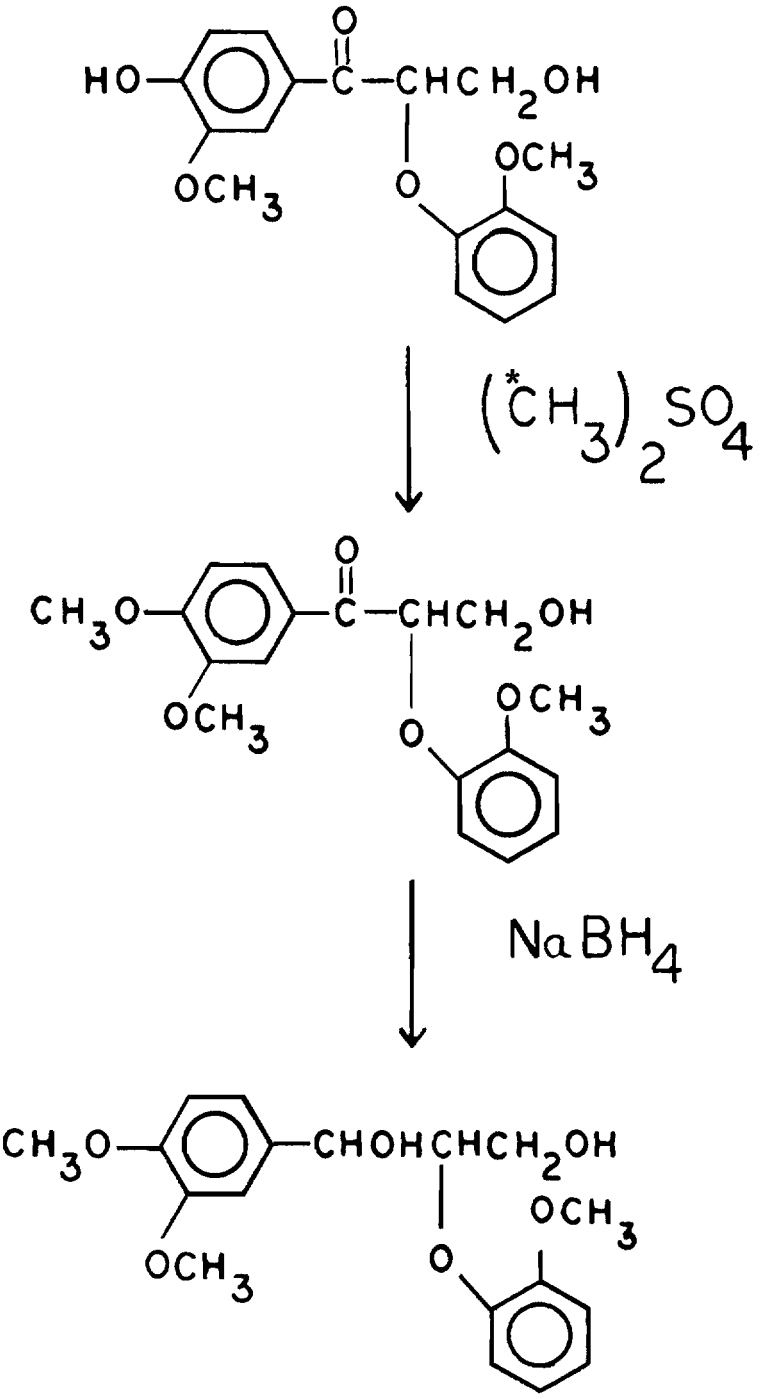
C–CH2Br
OCH3
OH
*
OCH3
O
CH2–O
C–CH2–O
OCH3
OCH3
O
H–*C–H
O
CH2–O
C–CHCH2OH
OCH3
OCH3
O

Scheme II



Scheme II



MATERIALS and METHODS

Instrumentation

^{1}H NMR spectra were determined with a Varian HA-100 spectrometer; chemical shifts are expressed as parts per million (δ) downfield from an internal standard of tetramethylsilane. Mass spectra were obtained on a CEC DuPont Model 21-110B spectrometer operated at 70 eV in the direct inlet mode. Ultraviolet spectra were run on a Cary 15 spectrometer, using 1 cm path length quartz cells. Gas chromatographic analyses were performed on a Varian series 1700 programable chromatograph. High pressure liquid chromatographic analyses were performed on a Waters Associates high pressure liquid chromatograph, M-440 absorbance detector, M-6000 A pump, M-U6K injector. Trapped $^{14}CO_2$ was counted on a Beckman LS-3133P liquid scintillation counter. Melting points were determined on a Kofler hot stage microscope and are uncorrected. Elemental analyses were performed at the Department of Chemistry, University of Oregon, Eugene, Oregon.

Chemicals

All chemicals were reagent grade, and were used as purchased except β-methylumbelliferone; recrystallized once from dioxane:water (1:1), and anhydrous potassium carbonate; oven dried at 120°C for 24 hrs. All solvents; acetone, benzene, chloroform, ethyl acetate and methanol were further purified according to Vogel (14). All gases were purchased from Airweld Inc., Portland, Oregon.

Syntheses

Synthesis of guaiacylglycol and glycerol-β-O-(β-methylumbelliferyl) ethers (Scheme I).

O-benzyl-α-O-(β-methylumbelliferyl)acetovanillone [1]

A solution of O-benzyl-α-bromoacetovanillone 2.0 g (6.0 mmol.) prepared as described by Leopold (15), β-methylumbelliferone 1.1 g (6.1 mmol.) and anhydrous potassium carbonate 1.5 g (11.0 mmol.) in dry acetone (100 mL) was heated under reflux for three hrs. Water (200 mL) was added to the reaction and the mixture was extracted with chloroform (3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The resulting crude product was recrystallized from ethyl acetate-chloroform, to yield 2.1 g (80%) of [1] : m.p. 139-140°C; ^{1}H NMR (acetone-d_6) δ 6.90-7.80 (aromatic, 11 H), 6.10 (s, 2 H), 5.58 (s, 2 H), 5.24 (s, 2 H), 3.89 (s, 3 H), 2.41 (s, 3 H).

Anal. Calcd. for $C_{26}H_{22}O_6$: C, 72.58; H, 5.11. Found: C, 72.20; H, 5.05.

O-benzyl-α-O-(β-methylumbelliferyl)-β-(hydroxy)propriovanillone [2]

A solution of [1] 5.9 g (3.7 mmol.), anhydrous potassium carbonate 55.3 mg (0.4 mmol.) and a dry formaldehyde-ethyl acetate mixture (30 mL) obtained by extraction of aqueous formaldehyde (10 mL, 37%) with ethyl acetate (3 x 50 mL) in dry ethyl acetate (15 mL) was gently heated under reflux for five min. and subsequently stirred at 25°C for five hrs. Water (50 mL) was added to the reaction and the mixture was extracted with chloroform:ethyl acetate (1:1; 3 x 50 mL). The combined extracts were dried

over sodium sulfate, filtered and then evaporated under reduced pressure. The resulting product was recrystallized from benzene-chloroform-ethyl acetate to yield 1.4 g (82%) of [2] : m.p. 164-165°C; ^{1}H NMR (acetone-d_6) δ 6.90-8.00 (aromatic, 11 H), 6.79 (t, 1 H), 6.10 (s, 1 H), 5.14 (s, 2 H), 4.15 (d, 2 H), 3.81 (s, 3 H), 2.32 (s, 3 H).

Anal. Calcd. for $C_{27}H_{24}O_7$ H_2O: C, 67.81; H, 5.44. Found: C, 68.38; H, 5.30.

α-O-(β-methylumbelliferyl)acetovanillone [3] and α-O-(β-methylumbelliferyl)-β-(hydroxy)propriovanillone [4]

A solution of either [1] 2.0 g (4.6 mmol.) or [2] 2.0 g (4.5 mmol.) and Pd—Charcoal (100 mg, 10%) in 95% ethanol (25 mL) was shaken under hydrogen (35 psi) for two hrs., and then filtered through celite. Water (100 mL) was added to the filtrate and the mixture was extracted with ethyl acetate (3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The resulting crude products were recrystallized from benzene-ethyl acetate to yield either 1.6 g (95%) of [3] or 1.4 g (95%) of [4] .

Compound [3] : m.p. 174-175°C; ^{1}H NMR ($CDCl_3$-DMSO-d_6) δ 6.70-7.50 (aromatic, 6 H), 6.04 (s, 1 H), 5.30 (s, 2 H), 3.84 (s, 3 H), 2.33 (s, 3 H); mass spectrum m/e 340 (M^+).

Compound [4] : m.p. 189-190°C; ^{1}H NMR ($CDCl_3$-DMSO-d_6) δ 6.64-7.40 (aromatic, 6 H), 6.00 (s, 1 H), 5.64 (t, J = 4 Hz., 1 H), 5.04 (br. m., 1 H), 3.81 (s, 3 H), 3.34 (d, J = 4 Hz., 2 H), 2.30 (s, 3 H); mass spectrum m/e 370 (M^+).

Guaiacylglycol-β-O-(β-methylumbelliferyl) ether [5] and Guaiacylglycerol-β-O-(β-methylumbelliferyl) ether [6].

A solution of either [3] 2.0 g (5.9 mmol.) or [4] 2.0 g (5.4 mmol.) and sodium borohydride 1.1 g (28 mmol.) in 95% ethanol (50 mL) was stirred at 4°C for ten hrs. Water (75 mL) was added to the reaction and the mixture was extracted with chloroform:ethyl acetate (1:1; 3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The crude products were recrystallized from carbon tetrachloride-ethyl acetate to yield either 1.6 g (85%) of [5] or 1.9 g (85%) of [6].

Compound [5]: m.p. 157-158°C; ^{1}H NMR ($CDCl_3$-DMSO-d_6) δ 6.74-7.44 (aromatic, 6 H), 6.00 (s, 1 H), 4.93 (t, J = 6 Hz., 1 H), 4.02 (d, J = 6 Hz., 2 H), 3.80 (s, 3 H), 2.30 (s, 3 H); mass spectrum m/e 342 (M^+).

Anal. Calcd. for $C_{19}H_{18}O_6$: C, 66.70; H, 5.26. Found: C, 66.83; H, 5.03.

Compound [6]: decomp. 74-76°C; ^{1}H NMR ($CDCl_3$-DMSO-d_6) δ 6.64-7.40 (aromatic, 6 H), 6.00 (s, 1 H), 4.80 (d, J = 6 Hz., 1 H), 4.40 (m, J = 6 Hz., 2 H), 3.80 (d, J = 6 Hz., 2 H), 3.74 (s, 3 H), 2.28 (s, 3 H); mass spectrum m/e 372 (M^+).

Anal. Calcd. for $C_{20}H_{20}O_7 \cdot 2\ H_2O$: C, 58.86; H, 5.88. Found: C, 58.98; H, 5.58.

Syntheses of ^{14}C-labeled Lignin Model Compounds (Scheme II).

O- benzyl-α-O-(guaiacyl)acetovanillone [7]

A solution of O-benzyl-α-bromoacetovanillone 2.0 g (6.0 mmol.) prepared as described by Leopold (15), guaiacol 0.76 g (6.1 mmol.) and anhydrous potassium carbonate 1.5 g (11.0 mmol.) in dry acetone (100 mL) was heated under reflux for three hrs. Water (200 mL) was added to the reaction and the mixture was extracted with chloroform (3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The resulting crude product was recrystallized from ethyl acetate-chloroform to yield 1.8 g (80%) of [7]: m.p. 101-102° C, Lit. m.p. 101-102°C (8a).

O-benzyl-α-O-(guaiacyl)-β-[^{14}C]-(hydroxy)propriovanillone [8]

A solution of dry ethyl acetate (15 mL), [7] 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 x 50 mL) with 10 mCi [^{14}C] formaldehyde (New England Nuclear; NEC-039) was gently heated under reflux for five min. and subsequently stirred at 25°C for five hrs. Water (50 mL) was added to the reaction and the mixture was extracted with chloroform: ethyl acetate (1:1; 3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The resulting crude product was recrystallized from ethanol to yield 1.24 g (82%) of [8]; the following data was taken on unlabeled compound, m.p. 85-86°C, Lit. m.p. 85-86°C (8a); ^{1}H NMR ($CDCl_3$) δ 6.80-7.74 (aromatic,

12 H), 5.40 (t, J = 4 Hz., 1 H), 5.20 (s, 2 H), 4.05 (d, J = 4 Hz., 2 H), 3.90 (s, 3 H), 3.80 (s, 3 H), 2.28 (br. s, 1 H).

α-O-(guaiacyl)-β-[^{14}C]-(hydroxy) propriovanillone [9]

A solution of [8] 1.87 g (4.6 mmol.) and Pd-Charcoal (100 mg; 10%) in 95% ethanol (25 mL) was shaken under hydrogen (35 psi) for two hrs., then filtered through celite. Water (100 mL) was added to the filtrate and the mixture was extracted with ethyl acetate (3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The resulting crude product was recrystallized from ethanol-water to yield 1.39 g (95%) of [9]: m.p. 88-89°C, Lit. m.p. 88-89°C (8a).

[γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10]

A solution of [9] 1.87 g (5.9 mmol.) and sodium borohydride 1.1 g (28 mmol.) in 95% ethanol (50 mL) was stirred at 4°C for ten hrs. Water (75 mL) was added to the reaction and the mixture was extracted with chloroform :ethyl acetate (1:1; 3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The yield of [10] was 1.70 g (90%). Mass spectrum m/e 320 (M^+). Specific activity was 7.3 x 10^4 cpm/mg.

guaiacylglycerol-β-[^{14}C] guaiacyl ether [11]

The synthetic procedure for [11] is essentially the same as that described above for [10], with 25 μC [^{14}C] guaiacol (8b) being used in the first reaction. The specific activity of [11] was 14 x 10^4 cpm/mg.

α-O-(guaiacyl)-β-(hydroxy)proprio-4-methoxyl-[^{14}C] veratrone [12]

A solution of [9] 1.87 g (5.9 mmol.), potassium hydroxide 0.33 g (5.9 mmol.), de-acidified dimethylsulfate 0.74 g (5.9 mmol.) and 250 μC [^{14}C] dimethylsulfate were heated under reflux for three hrs. Potassium hydroxide (1M) in dry methanol was added to the reaction to maintain the orange color. After three hrs. water (250 mL) was added to the reaction and the mixture was extracted with ether (3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and evaporated under reduced pressure. The product was recrystallized from methanol to yield 1.59 g (80%) of [12]: m.p. 117-118°C; The following data were taken on unlabeled material; ^{1}H NMR ($CDCl_3$) δ 6.82-7.84 (aromatic, 7 H), 5.40 (t, J = 4 Hz., 1 H), 4.08 (d, J = 4 Hz., 2 H), 3.95 (s, 3 H), 3.92 (s, 3 H), 3.87 (s, 3 H), 3.28 (br s, 1 H); mass spectrum of TMS derivative m/e 404 (M^+).

4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13]

A solution of [12] 1.59 g (4.8 mmol.) and sodium borohydride 1.1 g (28 mmol.) in 95% ethanol (50 mL) was stirred at 4°C for ten hrs. Water (75 mL) was added to the reaction and the mixture was extracted with chloroform:ethyl acetate (1:1; 3 x 50 mL). The combined extracts were dried over sodium sulfate, filtered and then evaporated under reduced pressure. The yield of [13] was 1.44 g (90%). Specific activity was 113.1 x 10^4 cpm/mg.

Veratrylglycerol-β-[^{14}C] guaiacyl ether [14]

The synthetic procedure for [14] is essentially the same as that given for [11] with the addition of the methylation reaction from [12],

The second glass tube extended one cm into the flask, and outside was connected via rubber tubing to a three cm, 18-gauge, Luer-type needle. Clamps on the rubber tubing allowed the flask to be opened or closed. Flasks were flushed with 100% O_2, immediately following inoculation and periodically thereafter (see below), at 100-200 mL per min for 15 min. This procedure was found to remove all evolved $^{14}CO_2$, which was then trapped by placing the exit needle in 5 mL of ethanolamine-containing scintillation fluid (17). Studies demonstrated that $^{14}CO_2$ trapping was 98% efficient.

Cultures were incubated at either 28°C or 38°C, the optimal growth temperature for P. versicolor and P. chrysosporium respectively (18). All cultures were incubated without shaking unless otherwise indicated. For agitated cultures, flasks were shaken on a New Brunswick shaker (New Brunswick Scientific Co., Inc.) operating at a speed of 125 rpm and describing a 2.5 cm circle. Two types of $^{14}CO_2$ evolution assays were conducted: A) Flasks containing ^{14}C-labeled compounds were inoculated, incubated, and purged with O_2 every three days for approximately twenty days. B) Flasks containing only basal media were inoculated and incubated for six days after which ^{14}C-labeled compounds were introduced, and subsequently flasks were purged approximately every eight hrs. for two days. Data is presented as the sum total amount of $^{14}CO_2$ evolved in cpm/flask as a function of incubation time.

Extraction of Cultures and the Isolation of Metabolic Products

Metabolic products of ^{14}C-labeled model compounds were extracted from acidified cultures with chloroform:acetone (1:1; 3 x 50 mL). Extracts were dried over sodium sulfate, filtered and then evaporated under reduced

pressure. The residue was dissolved in one-two mL methanol:acetone (1:1) for thin-layer or high pressure liquid chromatography.

Thin-layer chromatography (TLC) was done on precoated silica gel plates (E. Merck Co., 60-F 254). Developing solvents used were as follows: A) benzene-dioxane-formic acid, (a, 56:16:28; b, 30:90:10; c, 90:25:4); B) benzene-acetone-methanol, (4:1:1) (19). High pressure liquid chromatography (HPLC) was done on a reverse phase μ-Bondapak C_{18} column (Waters Associates). Solvent systems used were as follows: C) methanol-water, (a, 6:4; b, 3:7); D) acetonitrile-water (6:4).

Thin-layer plates were examined for radioactivity using autoradiography. Following TLC development, X-ray film (Kodak; NS-5T) was placed in contact with the plate for one to three days. Development of the exposed film revealed which areas of the plate possessed radiolabeled compounds. These spots were removed from the plate by scraping off the silica gel from the desired region of the plate and washing the radiolabeled material from the gel with methanol:acetone (1:1). Further purification, if possible, was achieved using HPLC. Directly following their recovery from the HPLC, ultraviolet spectra (200-400 nm) of the radiolabeled compounds were taken and compared with the spectra of standard compounds. Gas chromatographic analyses were performed on a Varian chromatograph, with an OV-101 column using a temperature program of 100-300°C, 10°C/min.

RESULTS

Syntheses and Properties of Lignin Model Compounds

The synthesis of guaiacylglycol and glycerol-β-O-(β-methylumbelliferyl) ether [5] and [6] presented no special problems. Yields of 77 and 65% were obtained for [5], [6] respectively. Elemental analysis of the final products confirmed their expected C and H composition. Compound [6] existed as the dihydrate, and decomposed at 74-76°C. Compound [5] melted at 157-158°C. Both compounds fluoresce only slightly when excited under a mineral lamp, (λ= 256 and 356 nm), as compared with β-methylumbelliferone. Preliminary evidence by Gold (20) indicated that these compounds can be used as useful substrates for assaying bacterial catabolism of arylglycerol and glycol-β-aryl ethers.

[γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] , 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13] , guaiacylglycerol-β-[^{14}C] guaiacyl ether [11] and veratrylglycerol-β-[^{14}C] guaiacyl ether [14] were prepared in overall yields of 56, 45, 56 and 45% from [^{14}C] formaldehyde, [^{14}C] dimethylsulfate and [^{14}C] guaiacol respectively. The specific activities of compounds [10] , [13] , [11] and [14] were 7.3 x 10^4, 113.1 x 10^4, 14 x 10^4 and 8.3 x 10^4 cpm/mg respectively.

Effects of Oxygen and Nitrogen on the Decomposition of [γ-^{14}C] guaiacylglycerol-β-guaiaxyl ether [10] and 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$.

Because lignin degradation has been shown to be an oxidative

process (Hackett et al., 1977; Kirk and Chang, 1975; Gold et al., 1978) model compound metabolism as a function of O_2 or N_2 in the flask was examined. The decomposition of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10] and 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$, following their addition to six-day-old cultures maintained initially under 100% O_2, but maintained after their addition under either 100% O_2 or 100% N_2, is shown in Table 1. Assay method B was used. The evolution of $^{14}CO_2$ was measured every eight hrs., and was observed only in those cultures maintined entirely under O_2, with no significant $^{14}CO_2$ detected from the N_2 cultures.

Effect of Culture Agitation on Decomposition of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10] to $^{14}CO_2$.

^{14}C-labeled lignins have been shown to be degraded to varying degrees depending upon culture agitation (Kirk et al., 16); model compound metabolism as a function of culture agitation was therefore examined. The degradation of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10] and 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$ in cultures maintained from the time of inoculation either on a rotary shaker (150 rpm; 2.5 cm amplitude), or as non-agitated (stationary) cultures is shown in Table 2. Assay method A was used. $^{14}CO_2$ evolution was measured every three days over a twenty day period. Agitated cultures containing P. chrysosporium showed suppressed $^{14}CO_2$ evolution, compared to non-agitated cultures. In parallel experiments with P. versicolor, no such suppression of $^{14}CO_2$ was observed in agitated cultures. Cultures that were agitated all formed two- to three-mm mycelial pellets, while non-agitated cultures formed mycelial mats.

Table 1

Effect of O_2 vs N_2 Atmosphere on Production of $^{14}CO_2$ from ^{14}C-labeled Lignin Model Compounds by *P. chrysosporium*[a]

Model Compound		Hours after Addition of Labeled Compound			
		12	24	36	48
4-methoxy-[^{14}C]veratrylglycerol-β-guaiacyl ether					
	100% O_2	730	1110	1430	1610
	100% N_2	52	70	100	122
[γ-^{14}C]guaiacylglycerol-β-guaiacyl ether					
	100% O_2	100	240	510	850
	100% N_2	45	52	90	112

a) Stationary cultures containing 1.2 mM ammonium tartrate. The values in the table represent the sum total amount of $^{14}CO_2$ cpm trapped. Assay method B was used.

Table 2

Effect of Culture Agitation on Decomposition of ^{14}C-labeled Lignin Model Compounds to $^{14}CO_2$.[a]

Fungus	Model Compound	Culture Age (days) 6		12		18	
		Sta.	Agit.	Sta.	Agit.	Sta.	Agit.
P. versicolor							
	[γ-^{14}C] guaiacylglycerol-β-guaiacyl ether	1362	757	1490	990	2040	153C
	4-methoxy-[^{14}C] veratrylglycerol-β-guaiacyl ether	62	66	171	153	740	41C
P. chrysosporium							
	[γ-^{14}C] guaiacylglycerol-β-guaiacyl ether	4266	115	7033	280	9400	48C
	4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether	2062	175	3100	569	4125	110C

a) Medium contained 1.2 mM ammonium tartrate. The values in the table represent the sum total amount of $^{14}CO_2$ cpm trapped. Assay method A was used.

Decomposition of ^{14}C-labeled Lignin Model Compounds to $^{14}CO_2$

The degradation of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10], 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13], guaiacylglycerol-β-[^{14}C]guaiacyl ether [11], and veratrylglycerol-β-[^{14}C]guaiacyl ether [14] to $^{14}CO_2$ by P. chrysosporium is depicted in Figure 1. Assay method A was used. A three day lag period preceded any detectable $^{14}CO_2$, after which experiments with each compound showed essentially the same initial rate of $^{14}CO_2$ evolution, even though the two ring-labeled compounds were inoculated with 1/2 the normal amounts; 2.5 x 10^4 cpm/flask. The degradation of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10] and 4-methoxyl-[^{14}C]veratryl-glycerol-β- guaiacyl ether [13] to $^{14}CO_2$ by P. versicolor is depicted in Figure 2. $^{14}CO_2$ evolution was measured daily. A one day lag period preceded any detectable $^{14}CO_2$, after which all cultures released considerable amounts of $^{14}CO_2$ from all of the ^{14}C-labeled compounds. The specific activities of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10], 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13], guaiacylglycerol-β-[^{14}C]guaiacyl ether [11] and veratrylglycerol-β-[^{14}C]guaiacyl ether [14] were 7.3 x 10^4, 113.1 x 10^4, 14.0 x 10^4 and 8.3 x 10^4 cpm/mg respectively.

Decomposition of ^{14}C-labeled Lignin Model Compounds to $^{14}CO_2$ in Cultures Containing Initially 12 or 1.2 mM Ammonium Tartrate Concentrations

The relationships between the initial ammonium tartrate concentration in the cultures and the initial rates of decomposition of [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10], 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13], guaiacylglycerol-β-[^{14}C]guaiacyl ether [11],

Figure 1

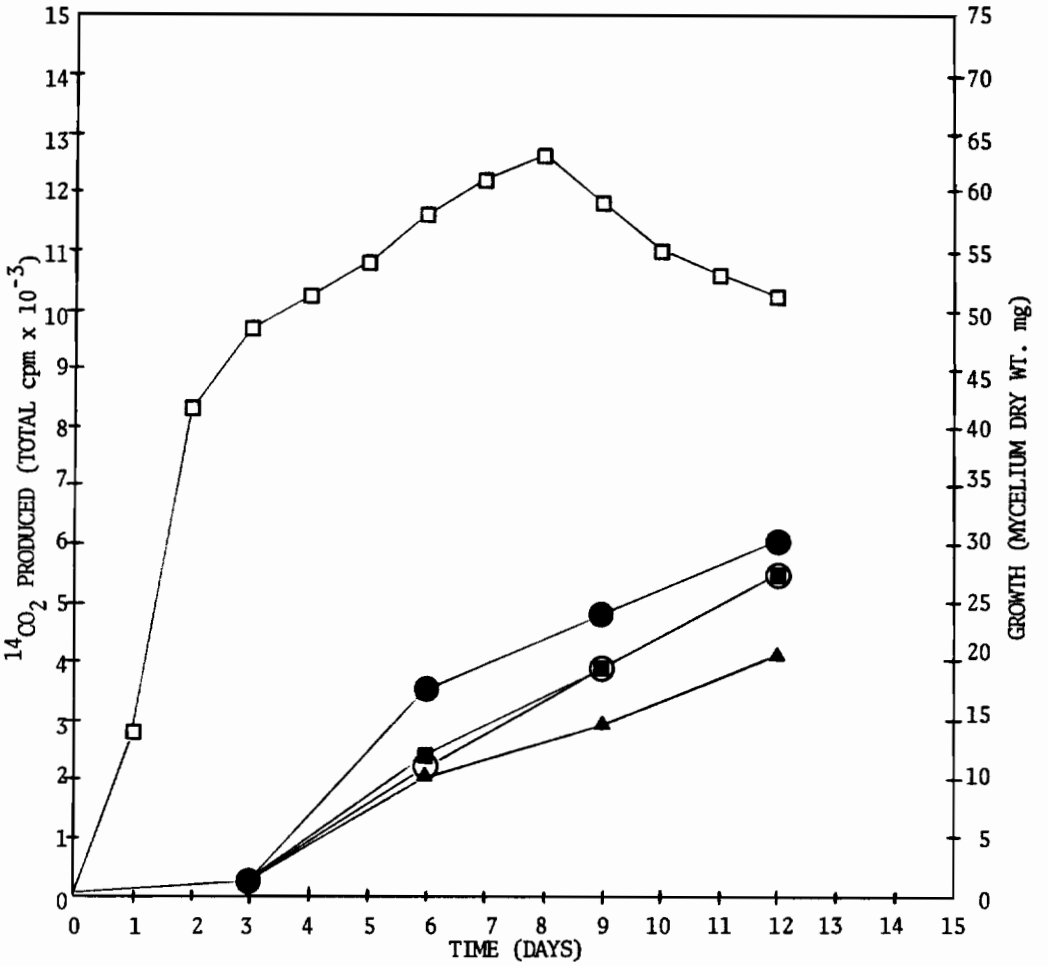


Figure 1. Degradation of ^{14}C-labeled lignin model compounds to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 1.2 mM ammonium tartrate. Assay method A was used. ● , [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] ; ▲ , 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13] ; ■ , guaiacylglycerol-β-[^{14}C] guaiacyl ether [11] ; ○ , veratrylglycerol-β-[^{14}C] guaiacyl ether [14] . Growth, mycelium dry wt., □ .

Figure 2

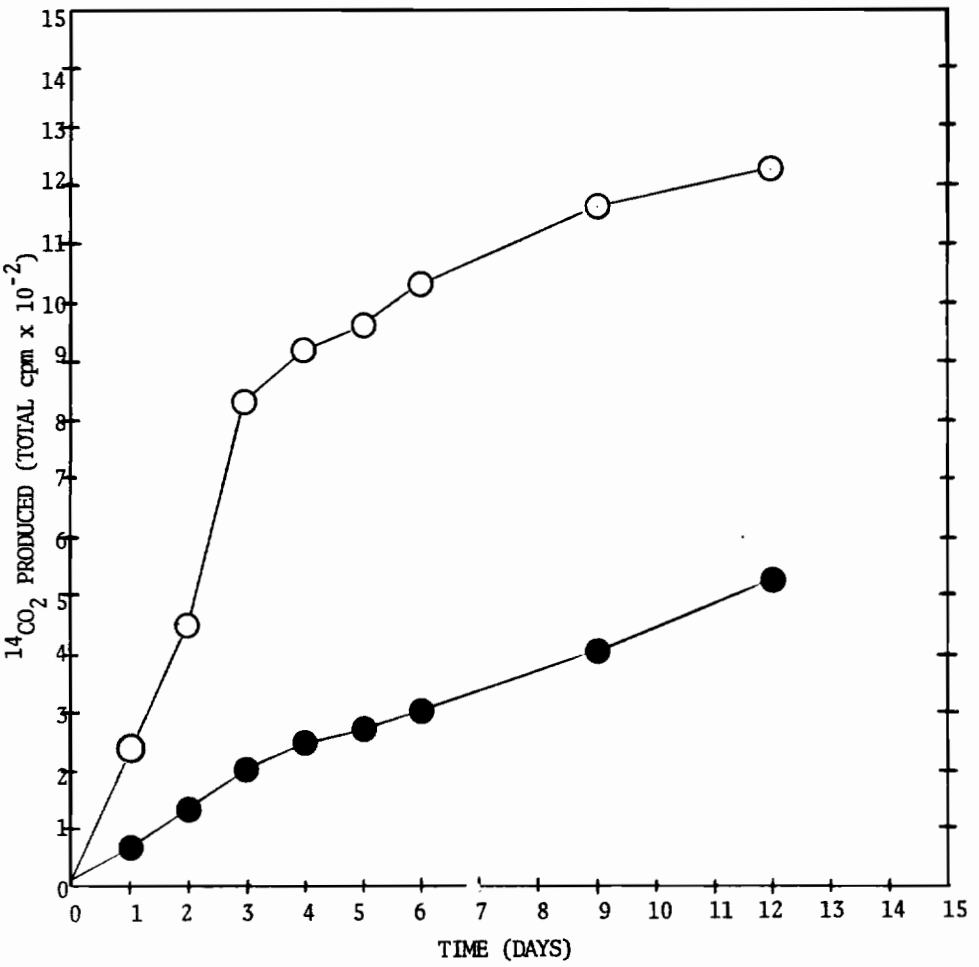


Figure 2. The degradation of ^{14}C-labeled lignin model compounds to $^{14}CO_2$ by P. versicolor. Cultures contained initially 1.2 mM ammonium tartrate. Assay method A was used. ● , [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] ; ○ , 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13] .

veratrylglycerol-β-[^{14}C]guaiacyl ether [14] to $^{14}CO_2$ by P. chrysosporium are shown in Figure 3, A-D respectively. All cultures were incubated for six days prior to the addition of ^{14}C-labeled materials. Assay method B was used. Those cultures which initially contained 1.2 mM ammonium tartrate and either [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10], 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13], guaiacylglycerol-β-[^{14}C]guaiacyl ether [11], veratrylglycerol-β-[^{14}C] guaiacyl ether [14] were found to evolve $^{14}CO_2$ with initial rates of 18, 4.4, 2.2 or 2.0 times greater than did cultures containing the same ^{14}C-labeled materials and 12 mM ammonium tartrate.

Constitutive Nature of Ligninolytic Activity

Experiments were conducted to examine the activity of cultures treated with the protein synthesis inhibitor cycloheximide. [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] was added to six-day-old cultures of P. chrysosporium. Previous addition of cycloheximide on day 4 prevented subsequent evolution of $^{14}CO_2$. When added on the sixth day, the inhibitor appeared to have no effect on $^{14}CO_2$ evolution during the first 24 hrs., but later activity was affected. This indicates that the model compound degradation activity appears between the fourth and sixth day, and in the absence of the substrate compound (see Figure 4).

Effect of Various Growth Substrates on the Decomposition of [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] to $^{14}CO_2$.

Media containing 1.0% of either glucose, xylose, or glycerol were inoculated with [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] and

Figure 3_A

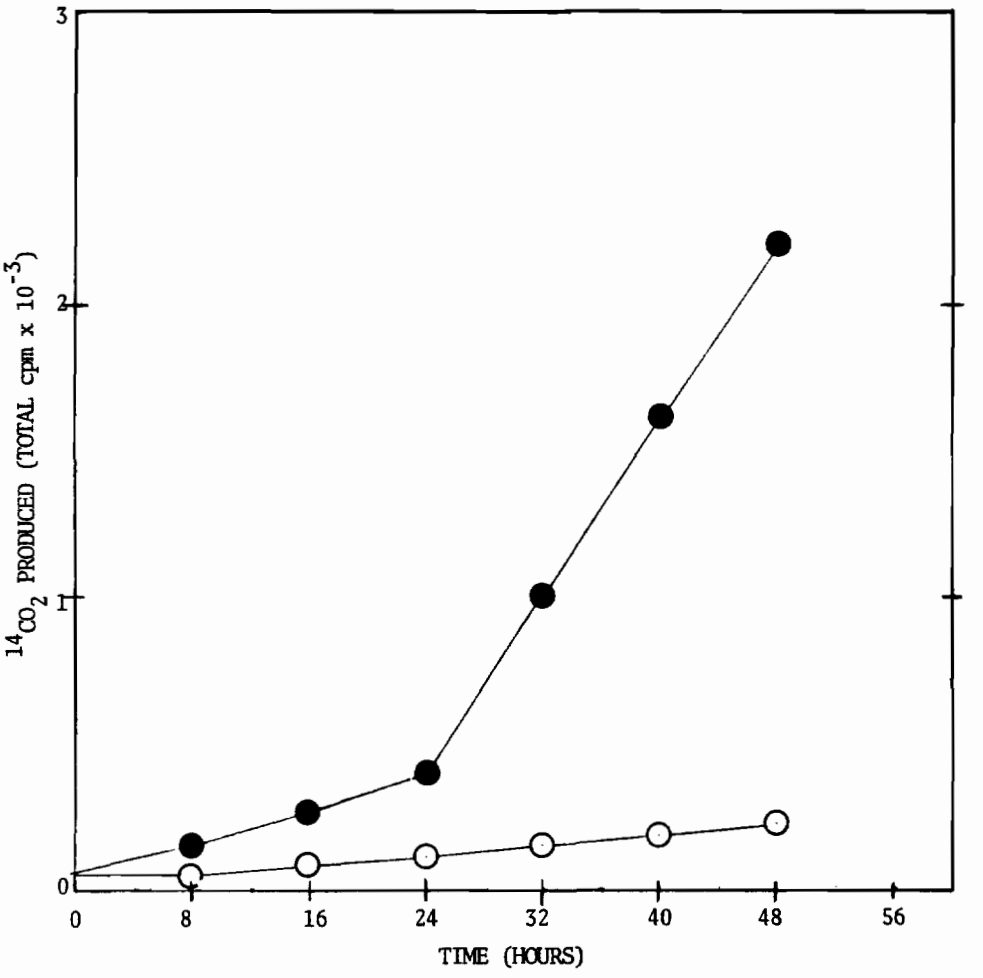


Figure 3_A. Decomposition of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10], to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 1.2 or 12 mM ammonium tartrate. Assay method B was used. ● , 1.2 mM ammonium tartrate; ○ , 12 mM ammonium tartrate.

Figure 3_B

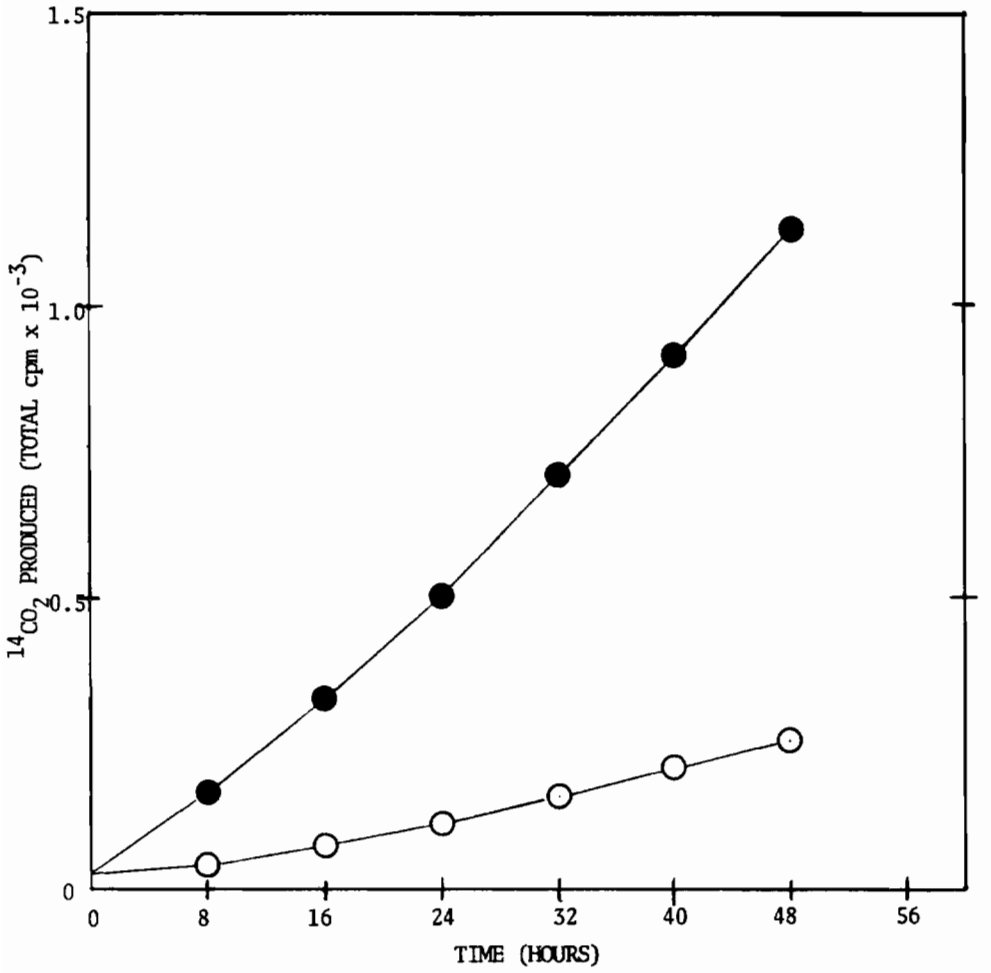


Figure 3_B. Decomposition of 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 1.2 or 12 mM ammonium tartrate. Assay method B was used. ● , 1.2 mM ammonium tartrate; ○ , 12 mM ammonium tartrate.

Figure 3_C

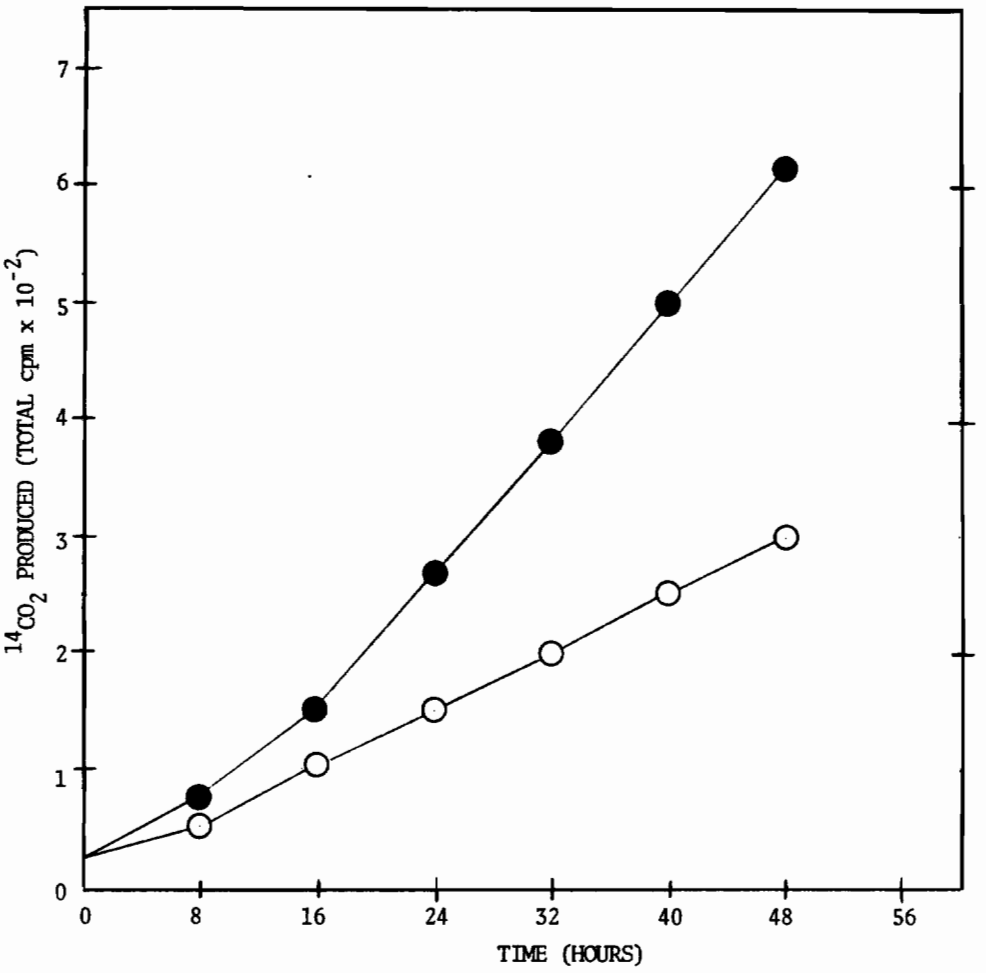


Figure 3_C. Decomposition of guaiacylglycerol-β-[^{14}C]guaiacyl ether [11], to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 1.2 or 12 mM ammonium tartrate. Assay method B was used. ●, 1.2 mM ammonium tartrate; ○, 12 mM ammonium tartrate.

Figure 3_D

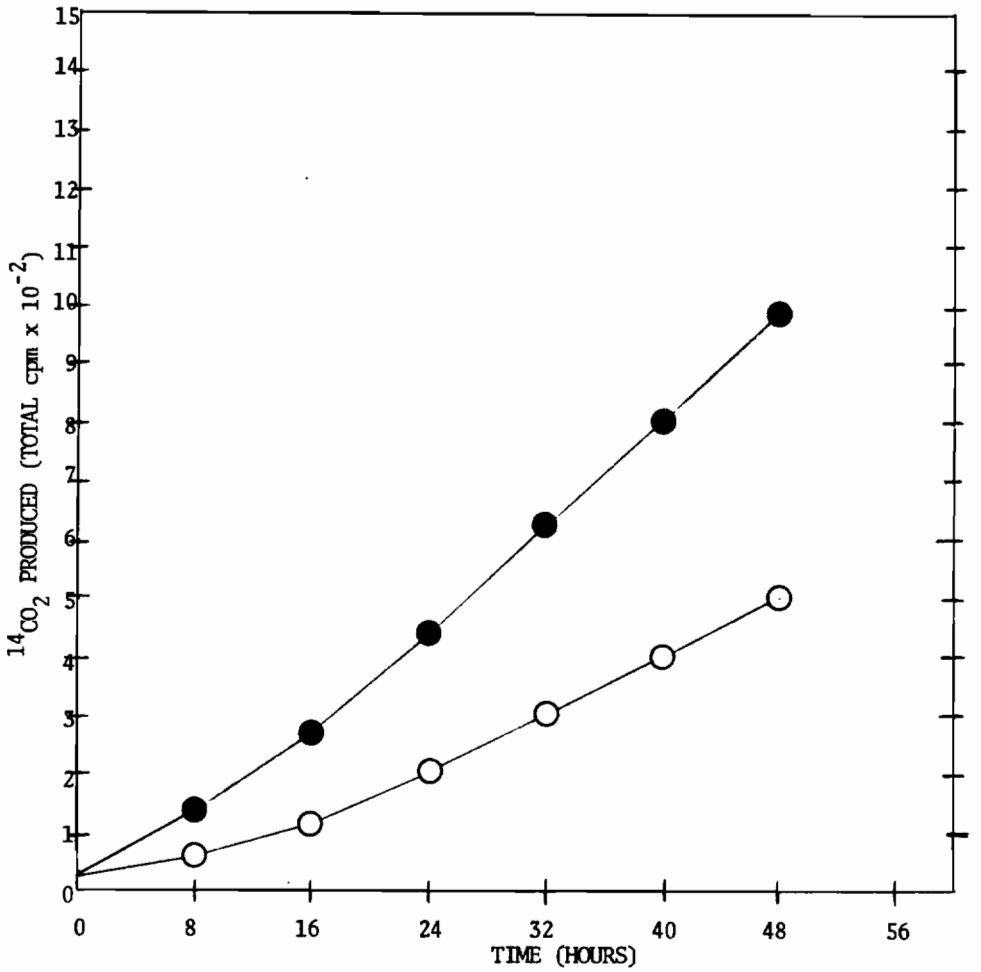


Figure 3_D. Decomposition of veratrylglycerol-β-[^{14}C]guaiacyl ether [14], to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 1.2 or 12 mM ammonium tartrate. Assay method B was used. ● , 1.2 mM ammonium tartrate; ○ , 12 mM ammonium tartrate.

Figure 4

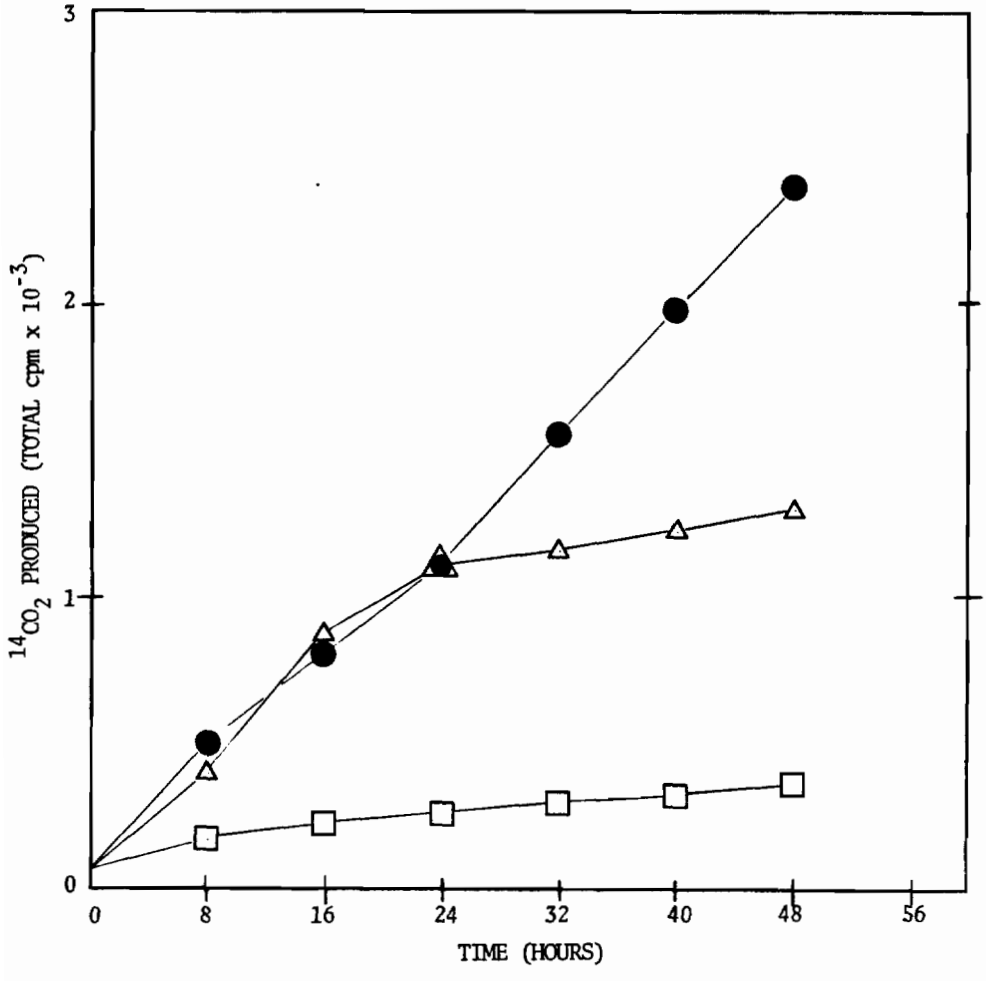


Figure 4. Effect of cycloheximide on the decomposition of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10], to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 1.2 mM ammonium tartarte. Assay method B was used. ● , no cycloheximide; □ , cycloheximide addition at four days; △ , cycloheximide addition at seven days.

P. chrysosporium, and $^{14}CO_2$ evolution was measured every three days (assay method A). Cultures containing xylose evolved more $^{14}CO_2$ than did either glucose or glycerol cultures (see Figure 5).

Effect of Glucose Concentration on the Decomposition of [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] to $^{14}CO_2$

Cultures of P. chrysosporium containing 0.01, 0.1, 0.5 or 1.0% initial glucose levels, were inoculated with [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10] to determine what effect initial culture glucose levels had on the decomposition of the ^{14}C-labeled lignin model compound to $^{14}CO_2$. In cultures containing less than 0.1% initial glucose significantly lower yields of evolved $^{14}CO_2$ were seen (see Table 3). Assay method A was used.

Inhibitory Effect of Unlabeled Veratric Acid or Unlabeled Veratryl Alcohol on the Decomposition of 4-methoxy-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$

The degradation of 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$ in cultures of P. chrysosporium was followed before and after addition of unlabeled veratric acid or unlabeled veratryl alcohol (see Figure 6). Cultures containing 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] and P. chrysosporium were incubated for nine days prior to the addition of veratric acid (0.1 or 1.0%) or veratryl alcohol (1.0%). Assay method A was used. Following the second addition, an inhibition of $^{14}CO_2$ evolution was observed. The effects of the addition, however, were not observed until after an additional six days had passed. Parallel experiments with unlabeled glycolic acid (0.1%) or glycerol (1.0%) showed no inhibition.

Figure 5

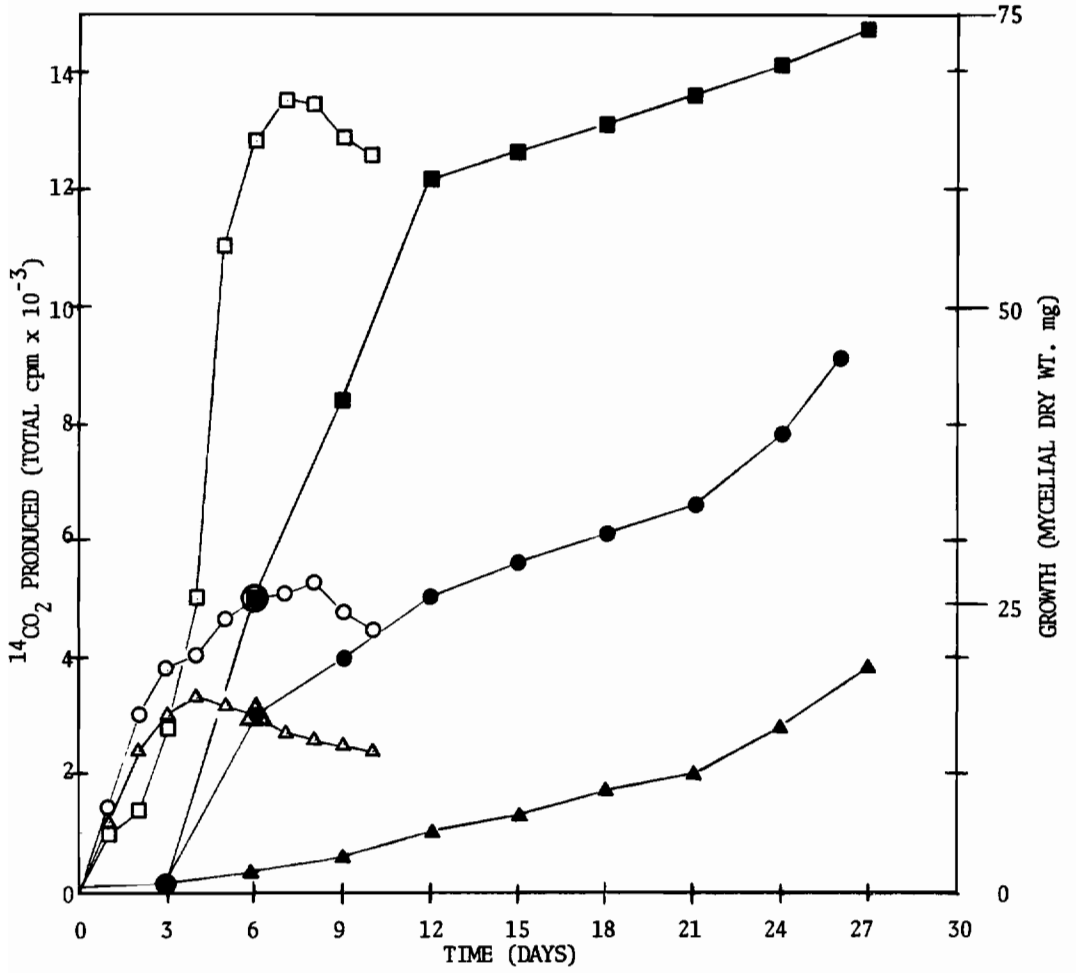


Figure 5. Decomposition of [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] to $^{14}CO_2$ by P. chrysosporium. Each culture contained initially 1.2 mM ammonium tartrate, and 1% of each carbon source. Assay method A was used. ●, glucose; ■, xylose; ▲, glycerol. Growth, mycelium dry wt., ○, glucose; □, xylose; △, glycerol.

Table 3

Effect of Glucose Concentration on the Decomposition of [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether to $^{14}CO_2$.[a]

Fungus	Initial % Glucose in Cultures	Culture Age (days) 6	12	18
P. chrysosporium	1.0	1600	6350	9400
	0.5	1300	6350	9150
	0.1	900	2600	3200
	0.01	94	322	583
P. versicolor	1.0	1786	1965	2206
	0.1	159	384	632
	0.01	240	352	536

a) Medium contained 1.2 mM ammonium tartrate. The values in the table represent the sum total amount of $^{14}CO_2$ cpm trapped. Assay method A was used.

Figure 6

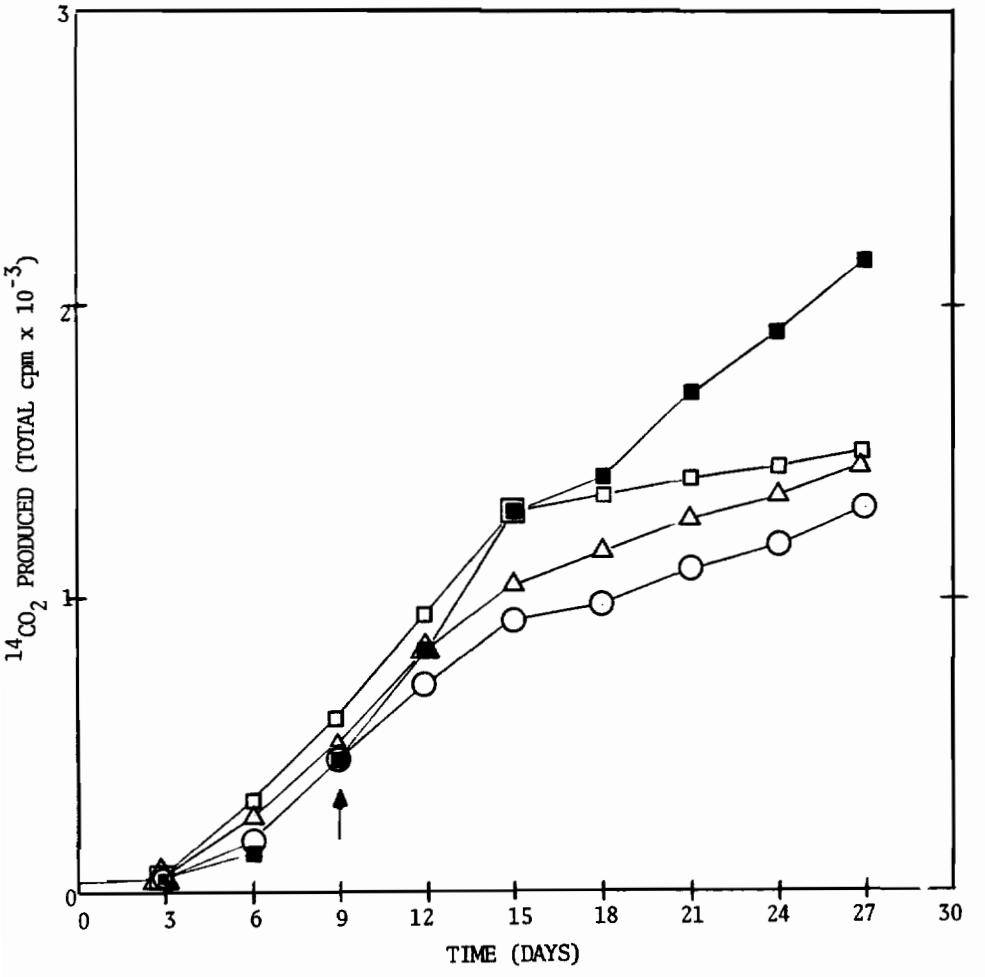


Figure 6. Inhibitory effect of unlabeled veratric acid and unlabeled veratryl alcohol on the decomposition of 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] to $^{14}CO_2$ by P. chrysosporium. At nine days, veratric acid (final concentration 0.1% or 1%) or veratryl alcohol (final concentration 1%) was added to the appropriate experimental flasks, while 50 µL DMF was added to the control. All cultures contained initially 1.2 mM ammonium tartrate. Assay method A was used. ■ , control; ○ , 1% veratric acid; △ , 0.1% veratric acid; □ , 1% veratryl alcohol.

Isolation of 4-methoxyl-[^{14}C]veratryl alcohol from Cultures of P. chrysosporium Inoculated with 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13]

4-Methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] was added to six-day-old cultures of P. chrysosporium. After three days the cultures were filtered, and the extracellular fluid was extracted with organic solvents. TLC of the extract followed by autoradiography showed the presence of ^{14}C-labeled material other then the 4-methoxyl-[^{14}C] veratryl compound, [13]. Further purification by high pressure liquid chromatography (HPLC) revealed a single ^{14}C-labeled compound. The ultraviolet spectrum of this compound was identical to the spectrum of standard veratryl alcohol, with λmax. at 228,275 and 280 (sh.) nm (see Figure 7). Absorption maxima for the 4-methoxyl-[^{14}C]veratryl compound [13] were λmax = 228 and 275 nm. Upon gas chromatographic analysis (100-300 C; 10 C/min. program, on OV-101 column) the isolated ^{14}C-labeled compound had the same retention time as standard veratryl alcohol. Upon HPLC analysis (Bondapak C_{18} column, methanol:water (6:4; 3:7)), the ^{14}C-labeled compound had the same retention time as standard veratryl alcohol. The retention times for 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] were different from those observed for veratryl alcohol. This evidence strongly indicates that ^{14}C-labeled veratryl alcohol is a product of 4-methoxy-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] metabolism.

Figure 7

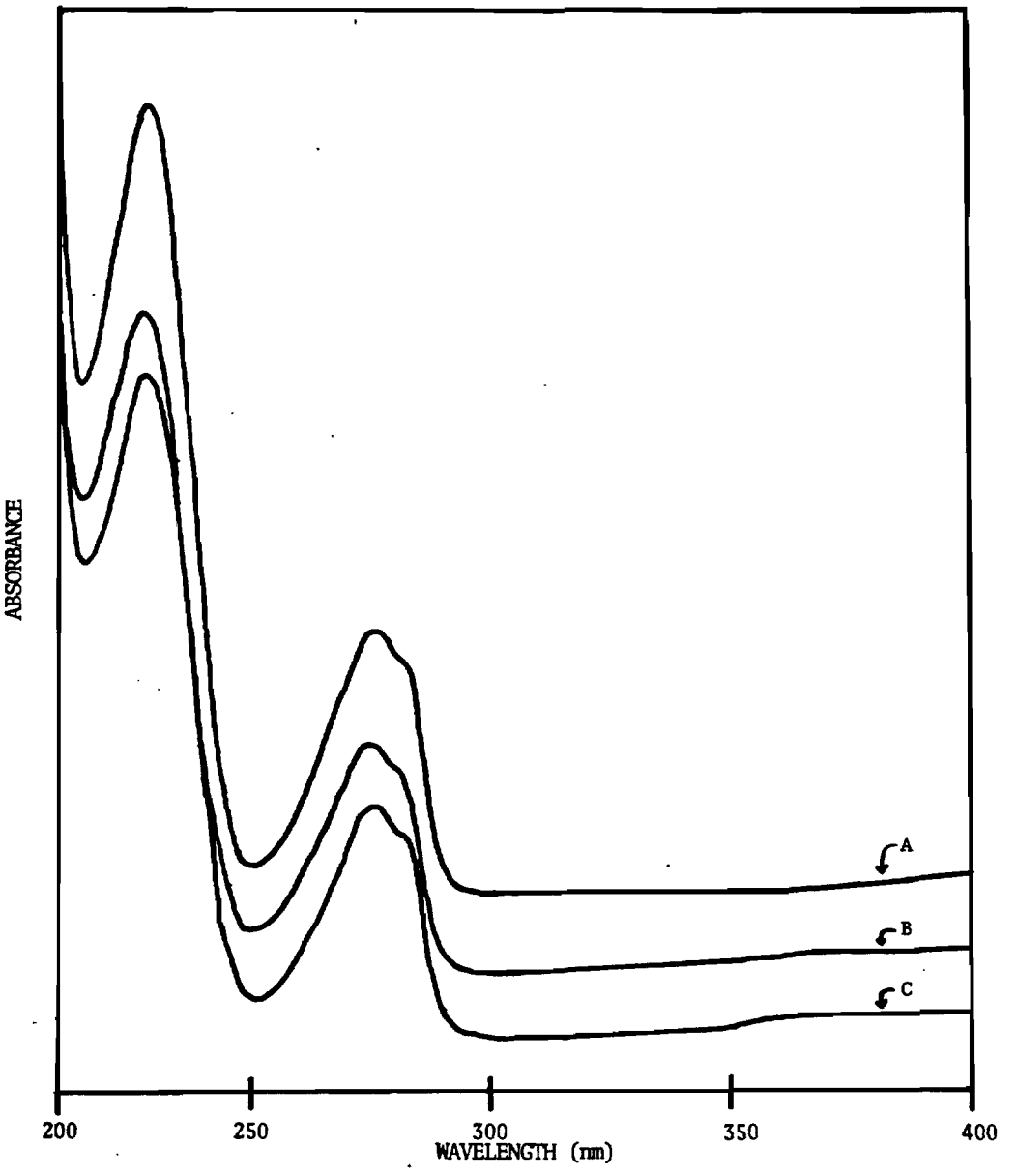


Figure 7. Ultraviolet absorption spectra of: A an authentic sample of veratryl alcohol; B ^{14}C-labeled material extracted and purified from cultures of P. chrysosporium incubated with 4-methoxy-[^{14}C]veratrylglycerol-β-guaiacyl ether [13]; C ^{14}C-labeled material extracted and purified from cultures of P. chrysosporium incubated with 4-methoxyl-[^{14}C]veratric acid.

Decomposition of 4-methoxyl-[^{14}C] veratric acid to $^{14}CO_2$ by P. chrysosporium, and the Isolation of 4-methoxyl-[^{14}C] veratryl alcohol from the Culture Fluid

4-methoxy-[^{14}C] veratric acid was added to six-day-old cultures of P. chrysosporium, and $^{14}CO_2$ evolution was measured by assay method B (figure 8). After an additional three days, the cultures were filtered and the extracellular fluid was extracted with organic solvents. TLC of the extract, followed by autoradiography, showed the presence of ^{14}C-labeled material other than 4-methoxy-[^{14}C] veratric acid. Further purification by HPLC revealed a single ^{14}C-labeled compound. The ultraviolet spectrum of this compound showed λmax at 228, 275 and 280 (sh.) nm. This spectrum was identical to that of standard veratryl alcohol (Figure 7), as were gas chromatographic and HPLC properties.

Figure 8

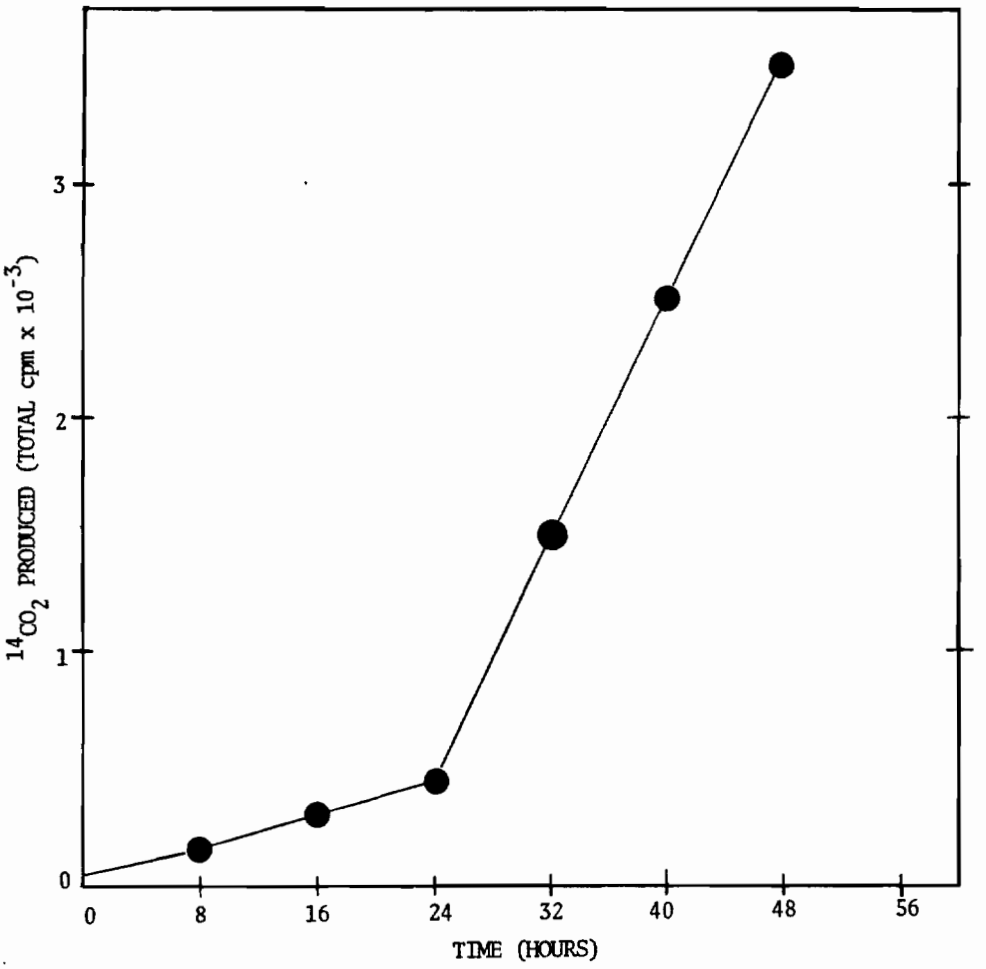


Figure 8. Decomposition of 4-methoxyl-[^{14}C]veratric acid to $^{14}CO_2$ by P. chrysosporium. Cultures contained initially 12 mM ammonium tartrate. Assay method B was used.

DISCUSSION

Lignin biodegradation is not well understood. The sequence of the bond cleavage reactions and the enzymes involved have yet to be elucidated.

The synthesis of ^{14}C-labeled lignin model compounds as well as umbelliferone related substrates for use in the study of these degradative processes was described above. Previous studies of the biodegradation of lignin using both natural and laboratory synthesized ^{14}C-labeled lignins have been reported (17,24). The studies described herein used lignin model compounds in order to simplify the metabolic products in these complex sequences.

The proposed role of β-etherases in some known lignin degrading microorganisms (10, 12) suggested a need for a sensitive assay for β-etherases. With this in mind the synthesis of guaiacylglycerol-β-O-(β-methylumbelliferyl) ether [6] and guaiacylglycol-β-O-(β-methylumbelliferyl) ether [5] was undertaken. Now, using these two compounds, investigations towards a) finding β-etherase activity in previously unscreened organisms, b) the selection of genetic mutants which have nonfunctional β-etherases, and c) the characterization of β-etherases in cell free systems have begun. These two compounds may thus help shed light on the involvement of β-etherases in lignin biodegradation.

Besides this possible role of β-etherases in lignin biodegradation, other factors involved in this degradative process need study. For this purpose ^{14}C-labeled model compounds were synthesized and used as described above.

The requirement for O_2 and the effect of culture agitation on lignin compound metabolism were the first parameters investigated. $^{14}CO_2$ evolution was found to occur in cultures maintained under one atm. O_2, but not one atm. N_2 (Table 1). This suggests that O_2 may play a role in the degradation process. In previous studies, using lignin in aerobic cultures of white-rot fungi, degradation of the lignin was shown to be largely oxidative (25). Whatever the specific requirement for oxygen, agitation, which is generally used to increase the rate of gas exchange between the atmosphere and culture media, was not always beneficial. For example, agitation severely inhibited ^{14}C-labeled lignin model compound decomposition to $^{14}CO_2$ by P. chrysosporium in aerobic cultures, as opposed to stationary incubation (16). A possible explanation for this is that in the continuously shaken cultures most of the lignin model compound bound to the hyphae (26) has become entrapped in the fungal pellets as they form. The O_2 concentration within the pellets might have been so low (27) that lignin model compound decomposition was prevented on all but the outer surface. This explanation does not, however, account for the fact that no such inhibition is seen in identically run experiments using P. versicolor. A plausible explanation for this may be that cultures of P. versicolor contain a higher phenol oxidase content than do cultures of P. chrysosporium (8, 13) (Table 2).

The second physiological parameter to be discussed is nutrient nitrogen concentration. Recent studies by Kirk et al., (28) in a system similar to that described above, reported the following sequence of events after inoculation: 0 to 24 hrs.: germination, linear growth, and depletion of nutrient nitrogen; 24 to 48 hrs.: cessation of linear growth and derepression of ammonium permease activity (demonstrating nitrogen starvation); 72 to 96 hrs.: appearance of ligninolytic activity (synthetic ^{14}C-labeled lignin $\longrightarrow$ $^{14}CO_2$). In similar experiments reported here with P. chrysosporium (assay method A) the appearance of detectable levels of $^{14}CO_2$ were observed only after the first 72 hrs. following inoculation (Figure 1), thus suggesting a similar sequence of events. In identical experiments using cultures of P. versicolor, $^{14}CO_2$ evolution began 48 hrs. after inoculation (Figure 2). As stated above, the ligninolytic enzyme system is activated following germination, linear growth and depletion of nutrient nitrogen. Thus, the earlier onset of $^{14}CO_2$ evolution seen in cultures of P. versicolor may be due to a faster growth rate, and a correspondingly earlier nitrogen depletion. This result may also be related to phenol oxidase concentration, as mentioned above.

One might expect, through consideration of the previous discussion, that experiments run using assay method B should show no delay in $^{14}CO_2$ evolution. This expectation was realized only for cultures containing initially 1.2 mM ammonium tartrate. Cultures containing initially 12 mM ammonium tartrate required additional time to achieve starvation conditions, and ligninolytic enzyme system activation (Figure 3).

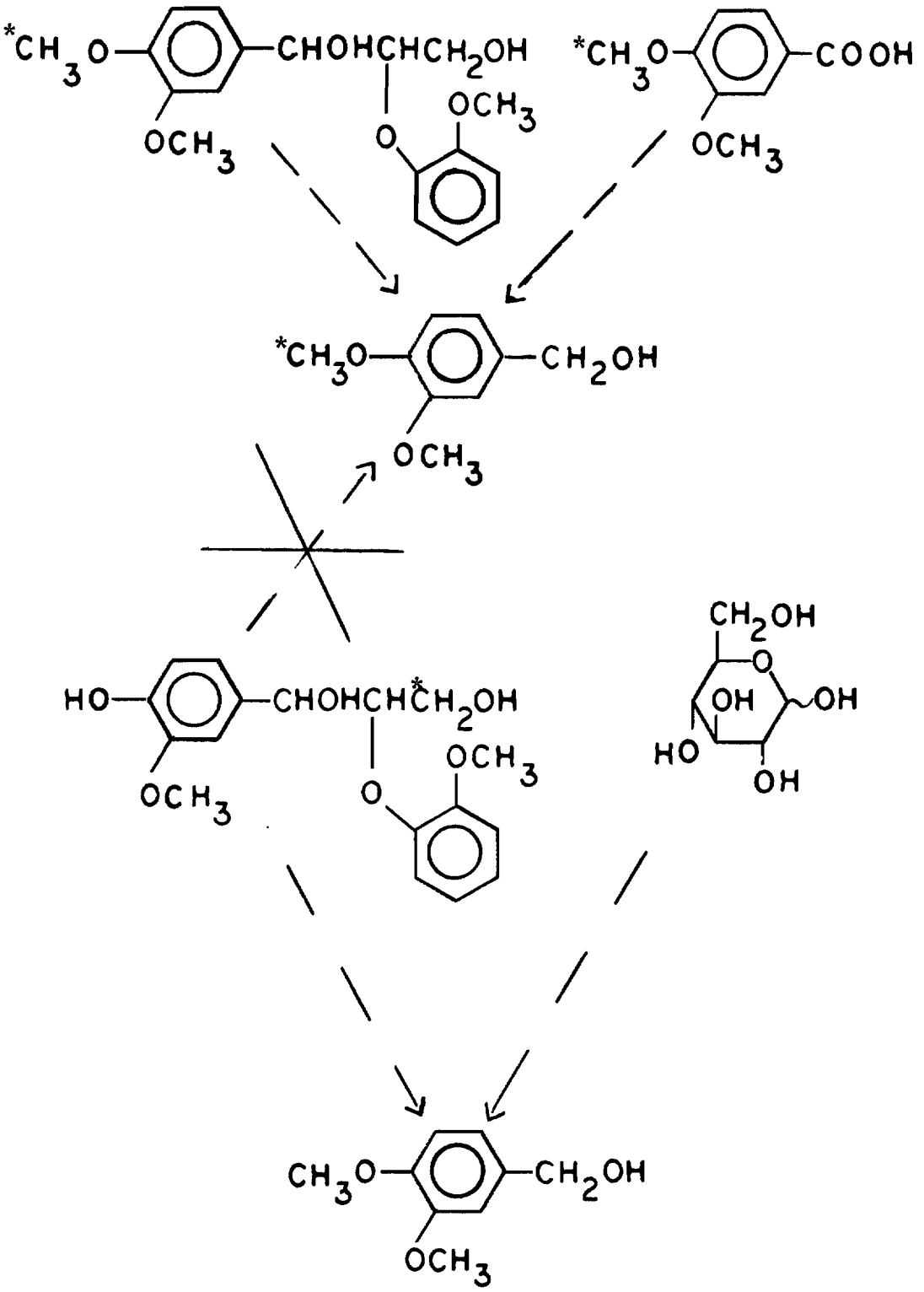
These results and those reported by Kirk et al., (28) offer no explanation for the adverse effect of high nitrogen concentration (12 mM) on the lignin model compound's degradation to $^{14}CO_2$. However three contributing factors can be postulated: a) high nitrogen (12 mM) promotes rapid depletion of the growth substrates known to be necessary for lignin metabolism (29), b) nitrogen metabolism competes with lignin model compound metabolism thorugh requirements for the same cofactor(s), c) nitrogen regulates the synthesis of one or more components of the lignin model compound degrading system (30).

The third physiological parameter to be discussed is the effect of different carbon sources on the degradation of the ^{14}C-labeled lignin model compounds to $^{14}CO_2$. In the experiments performed here xylose, a common wood sugar, and glycerol, an easily metabolized three-carbon fragment, were each compared to glucose in their ability to act as the sole carbon source. All three were able to serve as energy sources (Figure 5), and all three were shown to support [γ-^{14}C]guaiacylglycerol-β-guaiacyl ether [10] decomposition to $^{14}CO_2$ in cultures of P. chrysosporium.

The final aspect of these studies to be discussed pertains to the characterization of the metabolic products isolated from the extracellular culture fluid. The formation of 4-methoxyl-[^{14}C]veratryl alcohol was observed in cultures of P. chrysosporium which contained either 4-methoxyl-[^{14}C]veratrylglycerol-β-guaiacyl ether [13] or 4-methoxyl-[^{14}C] veratric acid. In previous studies by Lundquist and Kirk, de novo synthesis of veratryl alcohol from glucose was observed in cultures of

P. chrysosporium (31). However, when cultures of P. chrysosporium were inoculated with [γ-^{14}C] guaiacylglycerol-β-guaiacyl ether [10] , extracellular extracts showed unlabeled veratryl alcohol but no 4-methoxyl-[^{14}C] veratryl alcohol was isolated (32) (Scheme III). A plausible explanation of these results is as follows: enzymatic oxidative cleavage of the 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether's [13] α-β carbon-carbon bond occurs, releasing 4-methoxyl-[^{14}C] veratric acid (13). No accumulation of the latter or its demethylation products, vanillic acid or 4-methoxyl-[^{14}C] isovanillic acid, were observed. However, 4-methoxyl-[^{14}C] veratric acid is immediately enzymatically reduced to yield 4-methoxyl-[^{14}C] veratryl alcohol (33). No vanillyl alcohol or 4-methoxyl-[^{14}C] isovanillyl alcohol resulting from possible demethylation of the substrate was observed. Further support for this scheme was obtained when both veratric acid and veratryl alcohol were shown to cause a positive dilution effect on the $^{14}CO_2$ evolution, when added to $^{14}CO_2$-evolving cultures of P. chrysosporium previously incubated with 4-methoxyl-[^{14}C] veratrylglycerol-β-guaiacyl ether [13] (Figure 6).

Scheme III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CHOHCHCH2OH
OCH3
OCH3
O
OCH3
*CH3O
COOH
OCH3
*CH3O
CH2OH
OCH3
HO
CHOHCHCH2OH
OCH3
OCH3
O
CH2OH
O
OH
OH
HO
OH
CH3O
CH2OH
OCH3

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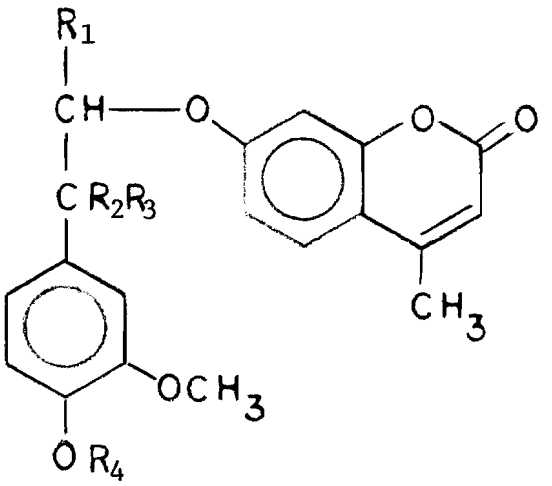
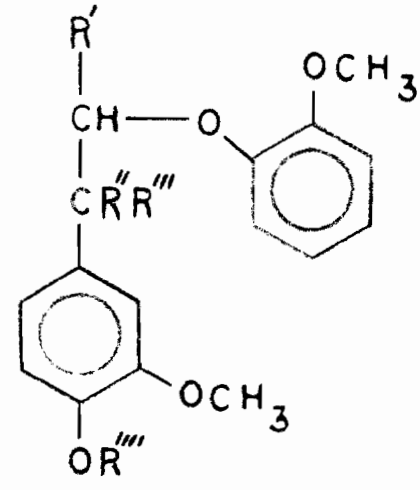
APPENDIX A[a]



1. R_1= H, R_2 R_3= O, R_4= CH_2OH
2. R_1= CH_2OH, R_2 R_3= O, R_4= $CH_2C_6H_5$
3. R_1= H, R_2 R_3= O, R_4= H
4. R_1= CH_2OH, R_2 R_3= O, R_4= H
5. R_1= H, R_2= H, R_3= OH, R_4= H
6. R_1= CH_2OH, R_2= H, R_3= OH, R_4= H

a) Structures of the umbelliferone related compounds synthesized in this study.

APPENDIX B[a]



7. $R' = H$, $R'' R''' = O$, $R'''' = CH_2C_6H_5$
8. $R' = {}^{14}CH_2OH$, $R'' R''' = O$, $R'''' = CH_2C_6H_5$
9. $R' = {}^{14}CH_2OH$, $R'' R''' = O$, $R'''' = H$
10. $R' = {}^{14}CH_2OH$, $R'' = H$, $R''' = OH$, $R'''' = H$
11. $R' = CH_2OH$, $R'' = H$, $R''' = OH$, $R'''' = H$ β-[^{14}C]guaiacyl
12. $R' = CH_2OH$, $R'' R''' = O$, $R'''' = {}^{14}CH_3$
13. $R' = CH_2OH$, $R'' = H$, $R''' = OH$, $R'''' = {}^{14}CH_3$
14. $R' = CH_2OH$, $R'' = H$, $R''' = OH$, $R'''' = CH_3$ β-[^{14}C]guaiacyl

a) Structures of the labeled and unlabeled compounds synthesized in this study.

APPENDIX C [a]

1-(3'-methoxy-4'-phenylmethoxy-phenyl)-2-(4''-methyl-2''H-1''-benzopyran-2-one-7''-oxy)ethanone [1]

1-(3'-methoxy-4'-phenylmethoxy-phenyl)-2-(4''-methyl-2''H-1''-benzopyran-2''-one-7''-oxy)-3-hydroxypropanone [2]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(4''-methyl-2''H-1''-benzopyran-2''-one-7''-oxy)ethanone [3]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(4''-methyl-2''H-1''-benzopyran-2''-one-7''-oxy)-3-hydroxypropanone [4]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(4''-methyl-2''H-1''-benzopyran-2''-one-7''-oxy)ethanediol [5]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(4''-methyl-2''H-1''-benzopyran-2''-one-7''-oxy)propanetriol [6]

1-(3'-methoxy-4'-phenylmethoxy-phenyl)-2-(1''-methoxy-phenoxy)ethanone [7]

1-(3'-methoxy-4'-phenylmethoxy-phenyl)-2-(1''-methoxy-phenoxy)-3-[^{14}C]-hydroxypropanone [8]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(1''-methoxy-phenoxy)-3-[^{14}C]-hydroxy-propanone [9]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(1''-methoxy-phenoxy)-3-[^{14}C] propane-triol [10]

1-(4'-hydroxy-3'-methoxy-phenyl)-2-(1''-methoxy-[^{14}C] phenoxy)propanetriol [11]

1-(3',4'-dimethoxy-phenyl)-2-(1''-methoxy-phenoxy)propanetriol [12]

1-(3'-methoxy-4'-[^{14}C] methoxy-phenyl)-2-(1''-methoxy-phenoxy)propanetriol [13]

1-(3',4'-dimethoxy-phenyl)-2-(1''-methoxy-[^{14}C] phenoxy)propanetriol [14]

a) Chemical Abstract names of the compounds synthesized in this study.