

Degradation of Phenolic Compounds and Ring Cleavage of Catechol by *Phanerochaete chrysosporium*

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POL-88, a mutant of the white-rot fungus *Phanerochaete chrysosporium*, was selected for diminished phenol-oxidizing enzyme activity. A wide variety of phenolic compounds were degraded by ligninolytic cultures of this mutant. With several *o*-diphenolic substrates, degradation intermediates were produced that had UV spectra consistent with muconic acids. Extensive spectrophotometric and polarographic assays failed to detect classical ring-cleaving dioxygenases in cell homogenates or in extracts from ligninolytic cultures. Even so, a sensitive carrier-trapping assay showed that intact cultures degraded [U - ^{14}C]catechol to [^{14}C]muconic acid, establishing the presence of a system capable of 1,2-intradiol fission. Significant accumulation of [^{14}C]muconic acid into carrier occurred only when evolution of $^{14}CO_2$ from [^{14}C]catechol was inhibited by treating cultures with excess nutrient nitrogen (e.g., L-glutamic acid) or with cycloheximide. L-Glutamic acid is known from past work to repress the ligninolytic system in *P. chrysosporium* and to mimic the effect of cycloheximide. The results here indicate, therefore, that the enzyme system responsible for degrading ring-cleavage products to CO_2 turns over faster than does the system responsible for ring cleavage.

White-rot wood-destroying basidiomycetes are among the most important degraders of lignin. An important step during lignin degradation is aromatic ring cleavage, which probably occurs in the polymer (8, 11, 18), as well as in low-molecular-weight phenolics (4). To facilitate studies of individual chemical reactions involved in ligninolytic activity, low-molecular-weight phenolic compounds structurally related to lignin are often used as substrates. However, phenol-oxidizing enzymes commonly produced by white-rot fungi hamper detailed studies by catalyzing oxidative coupling and polymerization. *Phanerochaete chrysosporium*, an active lignin-degrading fungus, has been chosen for degradation studies in part because it has low phenol-oxidizing enzyme (PO) activity (17). Nevertheless, with wild-type strains of *P. chrysosporium*, even the low titer of PO activity hampers detailed studies.

The purpose of this investigation was to characterize the ability of a mutant of *P. chrysosporium* with ligninolytic activity and diminished PO activity to degrade phenolic compounds and to cleave aromatic rings. Ligninolytic activity in *P. chrysosporium* was induced by nitrogen starvation (17).

MATERIALS AND METHODS

Chemicals. 3,4-Dimethylcatechol and 3,5-dimethylcatechol were prepared by the method of Loudon and Scott (24). 2-Chloroisovanillic acid had been prepared earlier (9). *cis,cis*-Muconic acid was prepared by the method of Pandell (27) and *cis,trans*-muconic acid was prepared by the method of Elvidge et al. (12); these were verified by proton magnetic resonance spectroscopy. The dimethyl esters of muconic acids were prepared by methylation for 15 min with an ether solution of diazomethane at room temperature. Excess diazomethane and diethylether were removed by gentle heating. [U - ^{14}C]catechol (30 mCi/mmol) was obtained from Research Products International Corp. (Elk Grove Village, Ill.). [U -ring- ^{14}C]acetovanillone was prepared from [U -ring- ^{14}C]guaiacol and acetic acid (23). Other chemicals were reagent grade from commercial sources.

Organism and production and selection of mutants. *P. chrysosporium* Burds. ME-446 (wild type; ATCC 34540) was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Madison, Wis. Strain POL-88 and 10 other mutants were produced from ME-446 by UV light-induced mutagenesis of conidia. Conidia were suspended in sterile distilled water, filtered through glass wool, and diluted to an absorbance at 650 nm of 0.5 ($\approx 2 \times 10^6$ conidia per ml); 10 ml of the conidial suspension in a 9-cm Petri dish was exposed to UV light (Mineralight UVS-11,

Ultra-Violet Products, Inc., San Gabriel, Calif.) at a distance of 20 cm for 5 min, with stirring once per minute; this gave 99% mortality (1). Surviving conidia were plated on a medium that restricted colony growth (15). It contained (per liter of distilled water): 40 g of sorbose, 2.5 g of citric acid, 0.1 g of desoxy-cholic acid, 5 g of KH_2PO_4 , 2 g of NH_4Cl , 5 mg of thiamine-hydrochloride, 1 ml of a trace metals solution (29), and 15 g of agar (Difco Laboratories, Detroit, Mich.); the final pH was adjusted to 4.0 with KOH. Approximately 25 colonies were introduced per plate. After incubation at 37°C for at least 10 days, plates were flooded with 1 mM *o*-anisidine in sterile water so that a liquid film surrounded the colonies. After an additional 1 to 2 days of incubation, phenol-oxidizing colonies became pigmented due to oxidative polymerization of the *o*-anisidine. Colorless colonies were selected as potential PO-negative mutants.

Culture conditions. All strains were maintained at room temperature on glucose-malt extract-peptone-yeast extract agar slants, which promoted rapid, heavy conidiation. This agar contained (per liter of distilled water): 10 g of glucose, 10 g of malt extract, 2 g of peptone, 2 g of yeast extract, 1 g of L-asparagine-hydrochloride, 2 g of KH_2PO_4 , 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg of thiamine-hydrochloride, and 20 g of agar. The low-nitrogen liquid medium used was that of Kirk et al. (22), except that the only vitamin was thiamine-hydrochloride (0.1 mg/liter), and polyacrylic acid (0.01 M in COOH groups, adjusted to pH 4.2 with KOH) was used as buffer (25). All components were prepared as 10-fold-concentrated solutions and were sterilized by filtration, except for the buffer, which was autoclaved. Medium (10 ml) plus $\sim 10^6$ conidia were added to 125-ml Erlenmeyer flasks. Cultures were incubated at $39 \pm 1^\circ\text{C}$ without agitation and were flushed every third day with O_2 . Cultures became nitrogen starved and developed ligninolytic activity (i.e., the capability of degrading [^{14}C]lignin to $^{14}\text{CO}_2$ after 3 to 4 days [17]).

Biodegradation assays. Radiorespirometric assay procedures for monitoring the degradation of ^{14}C -labeled compounds to $^{14}\text{CO}_2$ were described previously (22). Assays for the degradation of unlabeled aromatic compounds were carried out by the addition of 1 mg of compound in 10 μl of *N,N*-dimethylformamide to a 7-day-old ligninolytic culture. After 3 days of incubation at 39°C under O_2 , 1 ml of culture filtrate was removed and diluted with 5 ml of 95% ethanol. After 4 h, the UV-visible absorption spectrum was recorded. The percent degradation for each compound was measured by the reduction in absorbance at the wavelength of maximal absorbance (260 to 300 nm) relative to the absorbance of the compound incubated with an identical heat-killed culture. Heat killing was accomplished by autoclaving cultures at 116°C for 5 min. Assay variation was always 10% or less (of the values shown) between duplicates and 20% or less on repetition of experiments.

Assay of catechol ring-cleaving activity. Unless otherwise stated, 1 μg (10^5 dpm) of [^{14}C]catechol in 100 μl of distilled water and 1,000 μg of unlabeled *cis,cis*-muconic acid in 10 μl of *N,N*-dimethylformamide were added to a 7-day-old ligninolytic culture (final concentrations, 0.9 μM and 0.7 mM, respectively). After 6 h at 39°C under O_2 , reactions were terminated by acidifying cultures to pH 1.8 (with 100 μl of concentrated HCl per culture) and adding 10 ml of 1:1 (vol/vol)

chloroform:acetone per flask. Assay flasks were extracted 1 to 4 days after termination. Under these conditions, the *cis,cis*-muconic acid isomerized to the more stable *cis,trans*-isomer (cf. reference 12). Duplicate cultures were pooled and extracted three times with 30 ml of 1:1 (vol/vol) chloroform:acetone (total, 90 ml) and then once with 30 ml of chloroform, and evaporated to dryness. The residues recovered by evaporation were redissolved in 25 ml of anhydrous methanol, the solvent again was removed, and the residues were redissolved in methanol. Efficiencies of ^{14}C extraction ($\sim 80\%$) were determined by removing aliquots of the methanol solutions and counting in a dioxane-naphthalene scintillation fluid (19). The remaining methanol solution was concentrated by evaporation to 0.5 ml, transferred to a small vial, and methylated with diazomethane. After methylation, samples were evaporated to 0.1 to 0.2 ml. A 5- μl sample of the concentrate was applied as a band onto a precoated silica gel-60 thin-layer chromatography plate (2 by 10 cm; 0.2 mm thick; aluminum support) containing a fluorescent indicator (E. Merck, no. 5539; Darmstadt, West Germany). The plate was developed to 9 cm in 16:1 (vol/vol) methylene chloride:ethylacetate. *R_f* values for *cis,trans*-muconic acid dimethyl ester, catechol, guaiacol, and veratrol (1,2-dimethoxybenzene) were 0.43, 0.16, 0.50, and 0.53, respectively. Muconic acids did not migrate under these conditions. Guaiacol and veratrol were included as standards because they are formed by methylation of residual catechol. Formation of muconic acid was quantitated as the percentage of radioactivity that comigrated on thin-layer chromatography plates with the dimethyl ester of *cis,trans*-muconic acid. Its identity was established by co-chromatographing the labeled product with *cis,trans*-muconic acid in several solvent systems (both as the free acid and the dimethyl ester) and by observing consistent co-crystallization of the ^{14}C metabolite with *cis,trans*-muconic acid; four successive recrystallizations did not change the ratio of radioactivity to absorbance at 260 nm.

RESULTS AND DISCUSSION

Characteristics of strain POL-88. Eleven mutant strains of *P. chrysosporium* were selected for their inability to polymerize *o*-anisidine oxidatively on agar plates. The frequency of this phenotype was about 1 in 10^5 among survivors, as also reported by Ander and Eriksson (1). Spectrophotometric assays, using catechol, guaiacol, L-dopa, *p*-phenylenediamine, *o*-tolidine, and syringaldehyde as substrates, revealed no PO activity in strain POL-88. However, 7-day-old cultures did cause limited dimerization of 4-*t*-butylguaiacol during a 24-h incubation at 39°C under O_2 . This highly sensitive but qualitative test indicated that some PO activity was present (17). Nevertheless, the activity was weak; phenolic compounds listed in Table 1 were not polymerized (colored) by 7-day-old cultures, even with 2- to 3-day incubations under O_2 . For instance, when 500 μg of [^{14}C]acetovanillone was added to cultures of ME-446 (wild-type strain), 30% had polymerized within 5

h, coating the cell walls with a dioxane-extractable radioactive pigment. In contrast, only 20% of that amount of pigment was formed by strain POL-88 after 48 h of incubation.

The degradative abilities of POL-88 were superior to those of many other mutants and similar to those of wild-type strain ME-446, as determined by evolution of $^{14}\text{CO}_2$ from [*U*-ring- ^{14}C]acetovanillone, [*U*- ^{14}C]catechol, and [*U*-ring- ^{14}C]synthetic lignin. With this strain, the lower titer of PO activity did not result in lower ligninolytic activity, as seen with the mutants characterized by Ånder and Ericksson (1) and by Gold et al. (16). It is unclear whether PO activity has an essential role in ligninolytic activity.

Degradation of aromatic compounds by strain POL-88. Thirty-six aromatic compounds were tested for degradability by ligninolytic cultures (Table 1). *P. chrysosporium* appears to be among the most versatile and nonspecific degraders of aromatic molecules yet examined. Based on disappearance of UV absorbance, using 1 mg of compound per culture, 16 compounds were degraded by more than 50% and 28 compounds were degraded by at least 20% in 3 days. The degradation assay used here was based on the difference in soluble phenolic compound concentration between live and heat-killed controls. Similar results were obtained when control cultures were killed with 1 mM sodium azide instead of heat. This effectively rules out artifacts caused by phenolic compounds binding to the mycelium. This degradation assay probably underestimated the extent of degradation, because UV-absorbing degradation products are not distinguishable from starting materials, and especially because some of the probable intermediates—e.g., muconic acids—have higher extinction coefficients than do the starting materials. Furthermore, at the high concentrations tested, toxicity of an aromatic compound might cause an underestimation of degradability as compared with degradation rates at lower concentrations—e.g., subsequent study showed that 4-methylcatechol is toxic.

The most extensively degraded substrates were the *p*-hydroxybenzoic acids (e.g., vanillic acid) and compounds that were probably converted to them, such as vanillin (4-hydroxy-3-methoxybenzaldehyde). Vanillic acid and related compounds are oxidatively decarboxylated by *P. chrysosporium* (2, 3, 5, 6, 30) and subsequently may be demethylated to form hydroxyquinol (1, 2, 4-trihydroxybenzene). The aromatic ring of the latter is rapidly cleaved and metabolized further by *P. chrysosporium* (4). Ånder et al. (2) and Buswell et al. (5), however, presented evidence that ring cleavage occurs without demethylation, the ring-cleavage sub-

TABLE 1. Degradation of aromatic compounds by ligninolytic cultures of *P. chrysosporium* POL-88

Compound	% Degradation ^a
Monophenols, <i>m</i>- and <i>p</i>-diphenols	
Vanillic acid	100
Ferulic acid	100
2,4-Dihydroxybenzoic acid	98
4-Hydroxy-3-methoxybenzaldehyde	96
Isovanillic acid	83
Syringic acid	79
Curcumin	74
4-Hydroxy-3-methoxyphenylacetic acid	61
7-Hydroxy-4-methylcoumarin	55
2,6-Dihydroxybenzoic acid	41
2'-Hydroxy-3'-methoxyacetophenone	39
4'-Hydroxy-3'-methoxyacetophenone	38
6,7-Dimethoxycoumarin	25
7-Hydroxycoumarin	20
Gentisic acid	19
Guaiaicol	11
4-Hydroxy-3-methoxymandelic acid	10
2-Chloroisovanillic acid	4
<i>o</i>-Di- and triphenols (catechols)	
Protocatechuic acid	94
3',4'-Dihydroxyacetophenone	74
Gallic acid	74
2',3'-Dihydroxy-4'-methoxyacetophenone	67
6,7-Dihydroxy-4-methylcoumarin	65
3,5-Dimethylcatechol	54
2',3',4'-Trihydroxyacetophenone	51
2,3-Dihydroxybenzoic acid	41
3,4-Dihydroxyphenylacetic acid	37
4-Isopropylcatechol	36
7,8-Dihydroxy-4-methylcoumarin	35
2,3,4-Trihydroxybenzoic acid	30
Pyrogallol	29
Catechol	22
3-Methylcatechol	19
3,4-Dimethylcatechol	11
3,5-Disulfonylcatechol	11
4-Methylcatechol	8

^a Average percent degradation (loss of UV absorbance) of 1 mg of aromatic compound per culture during a 3-day incubation at 39°C under O₂ relative to duplicate heat-killed controls. Duplicate cultures varied less than ±10% of the values shown.

strate probably being a hydroxylated methoxyhydroquinone. Because vanillic and related acids are prominent intermediates of lignin degradation (7), enzymes involved in vanillic acid degradation are clearly of importance in lignin degradation.

Strain POL-88 did not significantly degrade 2-chloroisovanillic acid, which is in accord with earlier studies with the white-rot fungus *Polyporus dichrous* (21).

The UV spectra of many of the phenolic compounds examined were not altered during degradation; such changes are commonly ob-

served with *Fusarium solani* (26). Instead, the absorbance simply diminished. Some of the catechols, however, underwent substantial spectral changes. Transient peaks at 258 to 268 nm were observed with catechol, 3,4-dimethylcatechol, 3,5-dimethylcatechol, and 2',3'-dihydroxy-4'-methoxy-acetophenone. Appearance of these peaks was consistent with formation of muconic acid intermediates. When tested by the UV spectrum disappearance method, *cis,cis*-muconic acid and *cis,trans*-muconic acid were degraded by ligninolytic cultures to 54 and 86%, respectively, in 3 days.

Assay of aromatic ring-cleavage activity. Polarographic and spectrophotometric assay techniques (9,10), using catechol, protocatechuic acid, gentisic acid, homogentisic acid, methoxyhydroquinone and other *o*- and *p*-dihydroxybenzenoid compounds as substrates, failed to reveal any ring-cleavage dioxygenases in many different enzyme preparations from ligninolytic cultures. Because these cultures could respire ring carbons, failure to detect activity indicated either that it was lost during preparation of crude extracts or that the assays were not sufficiently sensitive. Therefore, a sensitive isotope-trapping method was developed involving intact cultures and [U - 14 C]catechol. With this assay, ring-cleavage activity was detected.

We initially determined the response of cultures to different concentrations of catechol. Experiments in which increasing concentrations were added to ligninolytic cultures showed complex kinetics for the evolution of $^{14}\text{CO}_2$ from [U - 14 C]catechol. Two distinct respiration responses were observed, both of which followed saturation kinetics. The first response showed half-saturation (K_m) at 13 μM catechol and had a V_{max} of 9.4 nM/min catechol evolved as CO_2 per culture. The second response was half-saturated at 218 μM catechol and had a V_{max} of 22.2 nM/min evolved as CO_2 per culture. Because of the presence of the second, low-affinity response, evolution of CO_2 was higher than if the first response (higher-affinity system) alone had been saturated. In subsequent studies, a low concentration of substrate was used (0.9 μM) to ensure that only the high-affinity system was significantly involved.

Incubation of [U - 14 C]catechol in ligninolytic cultures in the presence of excess unlabeled *cis,cis*-muconic acid resulted in the trapping of only a small amount of labeled muconic acid—i.e., there was no strong pooling effect (Table 2). Heat-killed cultures and the medium alone gave similar yields. Most of the [U - 14 C]catechol degraded by live cultures was trapped as $^{14}\text{CO}_2$. The remaining ^{14}C was predominantly in starting material (determined by thin-layer chromatography). Further experiments revealed that

TABLE 2. Ring cleavage and respiration of [U - 14 C]catechol by ligninolytic cultures of *P. chrysosporium* POL-88 during a 6-h assay in the presence of *cis,cis*-muconic acid carrier

Culture	Total radioactivity as:	
	<i>cis,trans</i> -Muconic acid (%)	CO_2 (%)
Medium only	2	0
Heat-killed control	2	0
Culture ^a	2	16
Culture + L-glutamic acid ^b (12 h earlier)	17	7
Culture + cycloheximide (25 mM, 12 h earlier)	27	1
Culture ^a under N_2 instead of O_2	0	0
Culture + sodium azide (1 mM, 5 min earlier)	0	0

^a Control culture in the defined medium, under O_2 .

^b Three times more nitrogen than originally present.

$^{14}\text{CO}_2$ formation from [U - 14 C]catechol could not be suppressed by the addition of common metabolically important organic acids (e.g., succinic, malic, oxalic, or glycolic acids) with which cleavage fragments might pool. However, selective suppression of $^{14}\text{CO}_2$ formation was obtained by pretreating the cultures with cycloheximide, a potent protein synthesis inhibitor. Although unlabeled *cis,cis*-muconic acid did not in itself suppress $^{14}\text{CO}_2$ evolution from [U - 14 C]catechol, significant accumulation of labeled product occurred only when unlabeled *cis,cis*-muconic acid was present in cycloheximide-treated cultures. Under these conditions, labeled muconic acid was trapped in good yield as the major product of degradation. L-Glutamic acid had an effect similar to that of cycloheximide (Table 2).

Failure to trap labeled muconic acid in the presence of a large excess of unlabeled carrier, in the absence of cycloheximide or glutamic acid, suggests that the ring-cleavage activity was compartmentalized and that sufficient unlabeled muconic acid (or the other acids tested) could not enter the compartment for a significant pooling effect. In the presence of cycloheximide and the absence of the unlabeled carrier, labeled muconic acid also did not accumulate. This result shows that the product, when released from the cleavage site, could pool with the carrier (if present), and that muconic acid could be further altered even by cycloheximide-treated cells. The blocked release of CO_2 from *cis,cis*-muconic acid in the presence of cycloheximide indicates that an enzyme(s) involved in catechol degradation beyond the point of ring cleavage turns over more rapidly than the system responsible for cleavage. Protein turnover is

known to be rapid in nitrogen-starved ligninolytic cultures (5 to 7%/h) (14).

An alternative interpretation for the cycloheximide effect is that the antibiotic eliminated the compartmentalization, permitting pooling of labeled and unlabeled muconic acids. The fact that glutamic acid had an effect similar to that of cycloheximide argues against this interpretation, however, because it is unlikely that the glutamic acid would affect compartmentalization. It is probable that glutamic acid, like cycloheximide, stopped synthesis of certain proteins. This and certain other amino acids are powerful repressors of the ligninolytic system in nitrogen-starved cultures of *P. chrysosporium* (13). By shifting cultures to primary metabolism, they apparently act as general repressors of secondary metabolism, including lignin degradation (14, 28). The inhibitory effect of excess glutamic acid on ligninolytic activity is physiologically indistinguishable from that of cycloheximide (13).

Characteristics of the ring-cleaving system.

Use of the standard 6-h assay with whole ligninolytic cultures of strain POL-88, incubated with cycloheximide and carrier *cis,cis*-muconic acid, allowed some of the characteristics of the catechol-cleaving system to be examined. The ring-cleavage activity was fully constituted in 1-day-old cultures. This was 1 day before the time cultures evolved CO₂ from catechol, and 2 to 3 days before the appearance of ligninolytic activity ([*U*-ring-¹⁴C]synthetic lignin → ¹⁴CO₂). Cultures that had been heat-killed, incubated under

N₂, or treated with the electron transport inhibitors sodium azide (1 mM; a strong ligand of Cu²⁺ and Fe³⁺) or carbonyl cyanide-*m*-chlorophenol hydrazone (CCCP, 0.48 μM) did not exhibit ring-cleaving activity and did not evolve ¹⁴CO₂ from either [*U*-¹⁴C]catechol or [6-¹⁴C]glucose. Ring-cleavage activity was stimulated by O₂. Maximal activity occurred in cultures assayed under 100% O₂; cultures under 20 and 60% O₂ gave 27 and 52%, respectively, of the activity under 100% O₂. No activity was detected in cell pastes or in cell-free extracts.

The effects of O₂, N₂, azide, and CCCP are consistent with the presence of an oxygen- and transition metal (e.g., Cu²⁺ or Fe³⁺)-dependent system capable of intradiol cleavage of catechol with formation of *cis,cis*-muconic acid. The results also suggest that intact cultures and electron transport are essential for ring cleavage. A requirement for active energy metabolism is also suggested because compounds that inhibited glucose respiration also inhibited catechol cleavage to a similar or greater extent (Table 3). White-rot fungi require a growth substrate such as cellulose or glucose to degrade lignin (20), apparently because lignin degradation is a net consumer of energy. Early expression of activity does not rule out participation of this activity in the production of CO₂ from either catechol or lignin. The expression of another enzyme apparently limits the rate of CO₂ production from catechol and perhaps lignin.

Because *P. chrysosporium* can degrade a wide variety of aromatic compounds, it was of inter-

TABLE 3. Effects of phenolic compounds on production of CO₂ from glucose and catechol and on ring cleavage of catechol by ligninolytic cultures of *P. chrysosporium* POL-88

Addition ^a	¹⁴ CO ₂ production ^b from:		Ring cleavage of [<i>U</i> - ¹⁴ C]catechol ^d (relative rate)
	[6- ¹⁴ C]glucose ^c (relative rate)	[<i>U</i> - ¹⁴ C]catechol ^d (relative rate)	
No addition (control)	100	100	100
Catechol	34	2	5
4-Methylcatechol	26	5	13
3,5-Dimethylcatechol	69	5	4
3',4'-Dihydroxyacetophenone	55	13	3
Protocatechuic acid	86	38	63
Gallic acid	61	22	71
Vanillic acid	66	24	11
Syringic acid	93	34	43
Gentisic acid	85	31	30

^a Phenolic compounds were at a final concentration of 1 mg per culture.

^b Rates are expressed as ¹⁴CO₂ production relative to the no-addition control during a 6-h incubation under O₂ at 39°C.

^c [6-¹⁴C]glucose (1 mg per culture; approximately 200,000 dpm). Control cultures respired approximately 1 mg of glucose during the 6-h assay.

^d [*U*-¹⁴C]catechol (1 mg per culture; 0.9 mM; approximately 100,000 dpm). Ring cleavage assays were in the presence of 25 mM cycloheximide added 12 h before, and in the presence of 1 mg (0.7 mM) of *cis,cis*-muconic acid per culture. Rates are expressed as production of [¹⁴C]muconic acid relative to the control during a 6-h incubation under O₂ at 39°C.

est to determine the specificity of the ring-cleavage system described here. Buswell and Eriksson (4) reported a ring-cleavage system in *P. chrysosporium* capable of cleaving hydroxyquinol. However, no activity was detected for cleavage of catechol, protocatechuic acid, or gentisic acid. In the present study, substrate specificity could not be assessed directly because the various labeled catechols and corresponding muconic acids were unavailable. An indirect approach was used. Phenolic compounds were examined for ability to inhibit (or compete with) formation of labeled muconic acid or $^{14}\text{CO}_2$ or both from [$U\text{-}^{14}\text{C}$]catechol. All phenols tested inhibited evolution of CO_2 from catechol (Table 3). The compounds also inhibited respiration of glucose, but the inhibition was less than one-half that observed for the evolution of CO_2 from catechol. Thus, the inhibition of evolution of CO_2 from catechol by phenolic compounds was selective—i.e., not due simply to an overall toxic effect. Cleavage of the [$U\text{-}^{14}\text{C}$]catechol to muconic acid was inhibited most by catechol itself, and also by 3,5-dimethylcatechol, 3',4'-dihydroxyacetophenone, 4-methylcatechol, and vanillic acid. Surprisingly, other benzoic acids such as protocatechuic and gallic acids, which are also catechols, were much less suppressive. All compounds studied except protocatechuic and gallic acids inhibited both ring cleavage of catechol and evolution of $^{14}\text{CO}_2$ from [$U\text{-}^{14}\text{C}$]catechol to a similar extent. This is consistent with ring cleavage being a rate-limiting step in degradation. Pooling of carbon fragments after a different mechanism of cleavage might explain the inhibition pattern observed for protocatechuic and gallic acids.

Although not conclusive, the selective inhibitory effect of several dihydroxyaromatic compounds on catechol ring cleavage is consistent with the presence of a relatively nonspecific intradiol ring-cleaving system in ligninolytic cultures of *P. chrysosporium*. It is not yet known whether this system is a classical 1,2-catechol dioxygenase, which could not be detected. Further studies into the localization, specificity, and the mechanism of action of this ring-cleavage system, and its possible dependence on electron transport, are of interest as they relate to the mechanism(s) of aromatic compound degradation and lignin degradation by *P. chrysosporium*.

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