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Purification and Properties of an *S*-Adenosylmethionine: 2,4-Disubstituted Phenol *O*-Methyltransferase from *Phanerochaete chrysosporium*

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An enzyme catalyzing the *O*-methylation of acetovanillone (3-methoxy-4-hydroxyacetophenone) by *S*-adenosylmethionine was isolated from *Phanerochaete chrysosporium* and purified 270-fold by ultrafiltration, anion-exchange chromatography, and gel filtration. The enzyme exhibited a pH optimum between 7 and 9 and was rapidly denatured at temperatures above 55°C. The K_m values for acetovanillone and *S*-adenosylmethionine were 34 and 99 μM, respectively. *S*-Adenosylhomocysteine acted as a powerful competitive inhibitor of *S*-adenosylmethionine, with a K_i of 41 μM. The enzyme was also susceptible to inhibition by thiol reagents and low concentrations of heavy metal ions. Gel filtration and sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that the enzyme was monomeric and had a molecular weight of approximately 53,000. Substrate specificity studies showed that 3-methoxy- and 3,5-dimethoxy-substituted 4-hydroxy-benzaldehydes, -benzoic acids, and -acetophenones were the preferred substrates for the enzyme. The corresponding 3,4-dihydroxy compounds were methylated relatively slowly, while the 3-hydroxy-4-methoxy compounds were almost inactive as substrates. Substituents in both the 2 and 4 positions relative to the hydroxyl group appeared to be essential for significant enzyme attack of a substrate. Provided that certain steric criteria were satisfied, the nature of the substituent was not critical. Hence, xenobiotic compounds such as 2,4-dichlorophenol and 2,4-dibromophenol were methylated almost as readily as acetovanillone. However, an extended side chain in the 4 position was not compatible with activity as a substrate, and neither homovanillic, caffeic, nor ferulic acid was methylated. The substrate range of the *O*-methyltransferase tends to imply a role in the catabolism or detoxification of lignin degradation products such as vanillic and syringic acids.

Investigations by Harper et al. (14, 16) have demonstrated that chloromethane (CH_3Cl) acts as a methyl donor in the biosynthesis of veratryl alcohol (3,4-dimethoxybenzyl alcohol) in *Phanerochaete chrysosporium* and in the biosynthesis of methyl esters and anisoles in *Phellinus pomaceus*. In both of these white rot fungi, CH_3Cl production is closely coupled to its utilization so that actual release of gaseous CH_3Cl is not detected at any stage of growth in *P. chrysosporium* and is observed only in the idiophase in *P. pomaceus*. There is some evidence that CH_3Cl biosynthesis and utilization occur in a membrane-bound multienzyme complex (20), but all attempts to isolate and purify these enzyme systems have so far been unsuccessful.

In recent studies in this area (7), attention has been focused on determining the extent of participation of CH_3Cl in methylation reactions in white rot fungi and the biochemical rationale for the utilization of this unusual methyl donor in certain metabolic processes in preference to the conventional biological methylating agent *S*-adenosylmethionine (SAM). Although it is now well established that CH_3Cl is derived ultimately from methionine (15, 30), the question of whether SAM itself is the immediate metabolic precursor has yet to be resolved.

Unfortunately, comparatively little information about the nature and function of methylating enzymes in white rot fungi exists in the literature. 4-*O*-methylation of lignin degradation products such as vanillic acid in ligninolytic cultures of *P. chrysosporium* with methionine acting as a source of the methyl group has been reported (6). The methylation system was apparently of relatively low specificity. Eriksson et al. (11) showed methylation of the 4-hydroxyl group of syringic acid by *P. chrysosporium* and several other white rot and soft rot fungi. These workers suggested that methylation could serve as a detoxification route for phenolics in some species. However, it is still far from clear what role, if any, 4-*O*-methylation plays in lignin breakdown.

Intriguingly, the degradation of chlorinated phenols and dioxins by *P. chrysosporium* has been shown to proceed via methoxylated intermediates (25, 26). Thus, 2,4-dichlorophenol, after initial dechlorination by lignin peroxidase and intracellular reduction to 2-chloro-1,4-dihydroquinone, is methylated to form 2-chloro-1,4-dimethoxybenzene before further dechlorination by lignin peroxidase and eventual ring cleavage. The function of methylation in this context appears to be to regenerate a substrate capable of attack by lignin peroxidase.

Methylation is also important in the biosynthesis of secondary metabolites by wood-rotting fungi. Reference to the utilization of CH_3Cl in the formation of veratryl alcohol and methyl benzoate has already been made (14, 16). For the biosynthesis of the natural product methyl *p*-methoxycinnamate by the fungus *Lentinus lepideus*, SAM is used as the methyl donor (28). An *O*-methyltransferase that catalyzes *O*-methylation of esters of hydroxycinnamic acids has been

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isolated from this organism. The enzyme has a limited substrate range, being restricted to methylation of *p*-hydroxy groups. Neither free acids nor methyl esters of hydroxybenzoic acids act as substrates. The presence of a SAM-dependent carboxyl-methylating enzyme catalyzing the formation of methyl esters from free aromatic acids such as cinnamic and *p*-methoxybenzoic acid in cell extracts from this organism has also been reported (21).

If progress is to be made in defining the role of CH_3Cl as a methyl donor in wood-rotting fungi and more generally in assessing the degree to which *O*-methylation is an integral part of lignin degradation, a much clearer understanding of the nature and function of SAM-dependent enzymes in methyl transfer in wood-rotting fungi is necessary. This article describes the purification and characterization of one such enzyme from *P. chrysosporium* capable of methylating substituted phenols.

MATERIALS AND METHODS

Organism and fungal culture. *P. chrysosporium* Burds INA-12 (CNCM I-398) is a strain utilized by Harper et al. (14). The fungus was grown without agitation at 37°C in 2-liter conical flasks in 200 ml of a medium which contained the following (in grams per liter): KH_2PO_4, 0.2; $MgSO_4 \cdot 7H_2O$, 0.05; NaCl, 0.59; $CaCl_2 \cdot 2H_2O$, 0.013; thiamine, 0.0025; yeast extract (Difco), 0.1; glycerol, 10; L-asparagine monohydrate, 1.0; NH_4NO_3, 0.5; and 2,2-dimethylsuccinic acid, 1.46. It also contained 1 ml of trace element solution per liter (2). The medium was adjusted to pH 5.0 with 2 M KOH, filter sterilized, and inoculated with approximately 5×10^5 conidia per ml prepared as described by Eriksson and Johnsrud (12).

The flasks were flushed with 100% O_2 for 2 min before being stoppered with sterile polytetrafluoroethylene-coated rubber stoppers. Cultures were harvested by filtration after 70 h of growth. Superficial moisture was removed by pressing mycelia gently between pieces of filter paper before freezing them at −15°C until required. A yield of ~0.4 g (wet weight) of mycelia per liter of culture medium was obtained.

Preparation of cell extracts. Extracts were prepared by suspending frozen mycelia (100 g) in 100 ml of 0.1 M potassium phosphate buffer (pH 6.5) containing 1 mM dithiothreitol and macerating the cells with a tissue grinder attached to a Black & Decker power drill at maximum speed for 10 min. Cooling in an ice bath ensured that the temperature did not rise above 4°C. The resulting homogenate was centrifuged at 50,000 × *g* for 30 min at 4°C, and the clear supernatant (150 ml) was decanted and retained for purification.

Enzyme and protein assays. *O*-Methyltransferase activity was measured by assaying production of 3,4-dimethoxyacetophenone (DMAP) when the preparation was incubated with 3-methoxy-4-hydroxyacetophenone (acetovanillone) in the presence of SAM. The standard assay was performed at 37°C in duplicate 40-ml screw-cap septum vials sealed with polytetrafluoroethylene-coated silicone discs. The vials contained, in a total volume of 2 ml, Tris buffer (pH 8.0; 50 mM), acetovanillone (1 mM), SAM (1 mM), and 0.05 ml of the enzyme preparation to be assayed. After incubation for 2 h, the reaction was terminated by quickly freezing the mixture at −15°C, and the mixture was stored at this temperature until extraction and quantification of DMAP. The assay of the enzyme after preincubation with inhibitor was performed by the standard procedure except that incubation was for only 1 h. Protein was determined by the method of Lowry et al. (18) with bovine serum albumin as the standard.

Extraction and determination of DMAP. After addition of 4-methoxyacetophenone (0.2 ml of a 50-μg/ml solution in acetone) as an internal standard, the assay mixture (2 ml) was extracted three times with chloroform (10 ml per extraction), and the bulked extract was washed with saturated NaCl (5 ml). The extract was dried over anhydrous $MgSO_4$ and evaporated to approximately 0.5 ml under a stream of nitrogen. Finally, the volume of the solution was increased to 1 ml with chloroform.

The DMAP concentration was determined by gas chromatography-mass spectrometry (GC-MS) on a Hewlett-Packard 5890 gas chromatograph linked to an HP5970 mass-selective detector controlled by an HP300 series computer. The gas chromatograph was fitted with an Ultra 1 fused silica wall-coated open tubular capillary column (12 m by 0.2 mm) with 100% dimethylpolysiloxane as the bonded phase (thickness, 0.33 μm). Helium was used as the carrier gas at a flow rate of 1.5 ml min^{-1}. After splitless injection of the sample (1 μl), the oven temperature was maintained at 40°C for 1 min and then programmed to increase at 5°C min^{-1} up to 60°C and then at 15°C min^{-1} up to 300°C. DMAP was quantified by comparing the ion current at *m/e* 180 (the molecular ion of DMAP) at the retention time of the compound with that given by a standard of the authentic compound. The ion current at *m/e* 150 (the molecular ion of the internal standard) was also monitored to allow correction for incomplete recovery of DMAP.

Extraction and determination of methylated products formed from substrates other than DMAP. Extraction and determination of methoxylated benzaldehydes and acetophenones after incubation with enzyme was conducted as described above for DMAP, with the appropriate molecular ion being monitored by GC-MS in each case.

After adjustment to pH 2, extraction and determination of the methylated products from phenol, halophenols, 2,3-dimethoxyphenol, guaiacol, 3-methoxyphenol, hydroquinone, chlorohydroquinone, and catechol were conducted as described above for DMAP by monitoring the appropriate molecular ion, except that 4-chloroanisole was used as the internal standard. Veratryl alcohol was assayed in a similar manner, except that 3,4,5-trimethoxybenzyl alcohol was used as the internal standard.

Following extraction after adjustment to pH 2, determination of all methoxylated benzoic and cinnamic acids was conducted by derivatization with *N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA) using *p*-anisic acid as the internal standard. The chloroform extract (100 μl) was evaporated to dryness under nitrogen, and the residue was reconstituted in MSTFA (100 μl). This solution (1 μl) was then injected splitless onto the gas chromatograph, which was fitted with a CP-SIL-19 fused silica wall-coated open tubular capillary column (25 m by 0.25 mm) with a bonded phase of 7% cyanopropyl, 7% phenyl, 85% methyl, and 1% vinylpolysiloxane (thickness, 0.19 μm). Helium was used as the carrier gas at a flow rate of 1.5 ml min^{-1}. The oven temperature was held initially at 100°C for 1 min and then programmed to increase at 10°C min^{-1} up to 300°C. Quantification was by monitoring the ion current at the *m/e* of the appropriate molecular ion in each case.

Determination of K_m. The rate of *O*-methylation was measured at acetovanillone concentrations between 0.03 and 1 mM while the concentration of SAM was held at a number of fixed values between 0.2 and 1 mM and the usual double reciprocal plot for two substrate reactions was obtained (9).

K_m values were determined by plotting intercepts of the primary plots against the reciprocal of substrate concentration for each substrate.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Pharmacia Phastsystem gel electrophoresis unit. Commercially prepared gels consisting of 12.5% polyacrylamide (2% cross-linked) containing 0.112 M acetate–0.112 M Tris (pH 6.5) were used. Samples were prepared for application by the addition of 2.5% SDS and 5% mercaptoethanol followed by heating at 100°C for 5 min. An equal volume of bromophenol blue (0.01%) was then added. Electrophoresis was carried out with Phastgel SDS buffer strips containing 0.2 M *N*-[tris (hydroxymethyl)methyl]glycine, 0.2 M Tris, and 0.55% SDS at pH 8.1. The gels were stained with a 0.1% solution of Coomassie blue R, washed and destained with methanol-acetic acid-water (30/10/60), and finally preserved in glycerol-acetic acid-water (5/10/85). For molecular weight determination, the following proteins were used in calibration (molecular weight of subunit in parentheses): rabbit muscle myosin (205,000), *Escherichia coli* β-galactosidase (116,000), rabbit muscle phosphorylase *b* (97,000), bovine serum albumin (66,000), ovalbumin (45,000), rabbit glyceraldehyde 3-phosphate dehydrogenase (36,000), bovine erythrocyte carbonic anhydrase (29,000), bovine pancrease trypsinogen (24,000), soybean trypsin inhibitor (20,100), and bovine milk α-lactalbumin (14,200).

Protein purification. Column chromatography was performed on a Pharmacia fast protein liquid chromatography (FPLC) system. Each column was connected to a UV-M monitor linked to an LCC-500 liquid chromatography controller which regulated the flow of buffers from two P-500 pumps. Fractions were collected with a FRAC-100 fraction collector.

Determination of molecular weight. Two methods of molecular weight measurement were used. The molecular weight of the enzyme was determined by gel filtration of the purified enzyme in 100 mM phosphate buffer (pH 6.5) containing 1 mM dithiothreitol on a Pharmacia Sepharose 12 column previously calibrated with the following reference proteins (molecular weight in parentheses): β-amylase (200,000), aldolase (145,000), β-phosphorylase (95,000), bovine serum albumin (67,000), peroxidase (40,000), and carbonic anhydrase (29,000). The molecular weight of the enzyme was also estimated by comparing the mobility of the purified enzyme with that of standard reference proteins under SDS-PAGE.

Chemicals. Acetovanillone, DMAP, and all substituted benzyl alcohols, benzaldehydes, benzoic acids, and cinnamic acids were acquired from Aldrich Chemical Co., Gillingham, Dorset, United Kingdom. MSTFA reagent was obtained from Pierce Chemical Co., Rockford, Ill. *S*-Adenosylmethionine *p*-toluenesulfonate salt and *S*-adenosylhomocysteine (SAH) were obtained from Sigma Chemical Co., Poole, Dorset, United Kingdom. Proteins used for calibration of Sephadex columns and as standards in SDS-PAGE were also obtained from Sigma.

RESULTS

Purification of 4-*O*-methyltransferase. All stages of enzyme purification were performed at approximately 4°C. The crude cell extract prepared as described in Materials and Methods was concentrated to approximately 10 ml with an Amicon Centriprep 30,000 ultrafiltration unit. Concentrated enzyme preparation (2.5 ml) was applied to a Pharmacia FPLC Mono Q 10/10 anion-exchange column equilibrated with 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) containing 1 mM dithiothreitol. The column was eluted with this buffer (16 ml) and then with a linear gradient of 0 to 0.5 M NaCl in 20 mM MES buffer (pH 6.5) containing 1 mM dithiothreitol, and fractions (1 ml each) were collected. Enzyme activity emerged as a single discrete peak confined to the fractions eluted at NaCl concentrations between 0.31 and 0.34 M. Those fractions with the highest specific activity were pooled and applied to a Pharmacia FPLC Sepharose 12 gel filtration column equilibrated with 10 mM phosphate buffer containing 1 mM dithiothreitol. The enzyme eluted from the column at an elution volume of 1.90 relative to the void volume of the column. SDS-PAGE of the purified enzyme yielded a single band, confirming the homogeneity of the preparation. Purification by this procedure was 266-fold, and the overall yield was 8%. The results of a typical enzyme purification procedure are summarized in Table 1.

TABLE 1. Purification of phenolic 4-*O*-methyltransferase from *P. chrysosporium*

Step no.	Stage of purification	Vol (ml)	Total enzyme activity (mU)[a]	Yield (%)	Protein (mg ml^{-1})	Sp act (mU mg of protein^{-1})
1	Cell extract	160	480	100	8.0	0.38
2	Concentrate after ultrafiltration	9.8	349	73	45.9	0.78
3	Anion-exchange chromatography	3.0	126	26	3.3	12.7
4	Gel filtration	0.9	36	8	0.4	101

[a] One milliunit of enzyme activity is defined as the amount of enzyme required to catalyze the formation of 1 nmol of DMAP min^{-1} at 37°C under the conditions of the standard assay in 50 mM Tris buffer (pH 8.0) with 1 mM SAM and 1 mM acetovanillone.

Stability of 4-*O*-methyltransferase. Preparations of the enzyme from stage 4 of the purification procedure (Table 1) had a half-life of approximately 30 h in 100 mM potassium phosphate buffer containing 1 mM dithiothreitol at 2°C but lost 75% of their activity after being frozen. The addition of 10% glycerol to the preparations allowed the retention of 95% of their activity after freezing. Preparations from stage 3 had a half-life of about 10 days under similar conditions and could be frozen at −15°C for several weeks without significant loss of activity. In view of the low overall yield of the purification procedure and the lability of the pure enzyme, preparations from stage 3 were generally used for enzyme characterization.

Influence of enzyme concentration. The initial velocity of the enzyme reaction in the standard assay was directly proportional to the enzyme concentration at protein concentrations from 20 to 150 μg ml^{-1}. The rate of DMAP formation was linear with respect to time over at least 4 h at 37°C provided that utilization of SAM was not allowed to exceed 4%.

Influence of pH and temperature. The effect of pH on enzyme activity was measured under standard conditions in 50 mM 2,2-dimethylsuccinate, Tris, and sodium carbonate buffers spanning a pH range from 4 to 10. The enzyme displayed a relatively broad pH optimum of between 7 and 9, with the maximum velocity being recorded at pH 8.0 (Fig. 1).

The velocity of *O*-methylation was determined at temperatures ranging from 15 to 55°C under standard assay conditions. The optimum temperature for the reaction under the

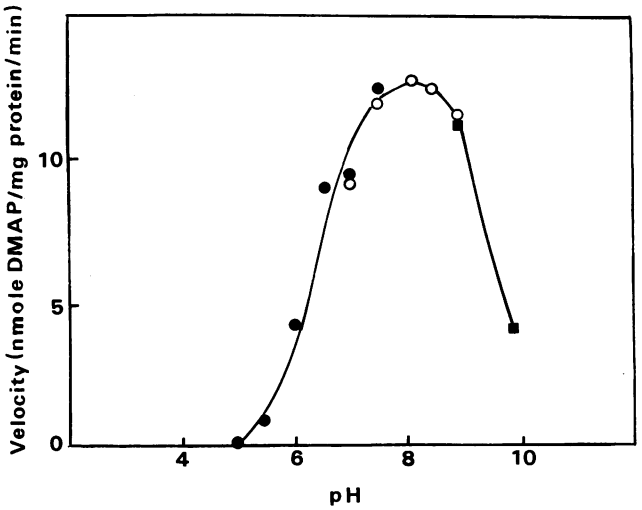

FIG. 1. Effect of pH on O-methyltransferase activity. The enzyme was incubated at the appropriate pH under standard assay conditions at 37°C in 50 mM 2,2-dimethylsuccinate buffer (●), 50 mM Tris buffer (○), or 50 mM sodium carbonate buffer (■). SAM and acetovanillone concentrations were both 1 mM.

conditions used was 45°C. Rapid denaturation of the enzyme occurred above 55°C. The activation energy of the enzyme reaction between 15 and 45°C, determined by an Arrhenius plot, was 28.4 kJ mol^{-1}.

Effect of substrate concentration. The K_m values of the enzyme were 34 μM for acetovanillone and 121 μM for SAM [99 μM if allowance is made for 18% unnatural S-(+)-isomer present in commercial SAM]. The reciprocal plot for acetovanillone departed from linearity at concentrations above 100 μM, indicating some inhibition at higher concentrations of this substrate. However, even at an acetovanillone concentration of 1 mM (in the presence of 1 mM SAM), inhibition was slight, with the enzyme exhibiting 89% of the activity predicted on the basis of simple Michaelis-Menten kinetics.

Molecular weight. Upon gel filtration, the enzyme activity emerged as a single discrete peak at a relative elution volume corresponding to a molecular weight of 52,000. SDS-PAGE of the purified enzyme indicated a molecular weight of 54,000, signifying that the active enzyme was monomeric.

Effect of inhibitors and metal ions. A characteristic feature of transmethylation reactions involving SAM is that the reaction is strongly inhibited by low concentrations of the demethylated product SAH (22). The effect of SAH concentrations between 0 and 0.2 mM was determined at SAM concentrations of 0.25, 0.5, 0.75 and 1.0 mM. The results were analyzed graphically by a Dixon plot (8). SAH acted as a powerful competitive inhibitor of the enzyme reaction; the K_i for SAH (41 μM) was considerably less than the K_m for SAM.

The effect of thiol-blocking agents, metal ions, and chelating agents on enzyme activity was examined after dialysis of the enzyme against 100 mM phosphate buffer (pH 6.5). The dialyzed enzyme was preincubated in the presence of possible inhibitors for 10 min before addition of substrate. The influences of the various compounds on activity are shown in Table 2. The enzyme was strongly inhibited by certain thiol-blocking agents such as phenylmercuriacetate and was sensitive to low concentrations of heavy metal ions,

TABLE 2. Effect of possible inhibitors and metal ions on O-methyltransferase activity

Inhibitor or metal ion added[a]	Final concn (mM)	Relative activity[b]
N-Ethylmaleimide	0.05	72
Iodoacetamide	0.5	104
Phenylmercuriacetate	0.01	0
Mg^{2+}	1.0	98
Ca^{2+}	1.0	99
Hg^{2+}	0.1	0
Ag^{+}	0.1	0
Ag^{+} + 1 mM EDTA	0.1	0
Cu^{2+}	0.1	0
Cu^{2+} + 1 mM EDTA	0.1	74
Zn^{2+}	0.1	45
Fe^{2+}	1.0	103
Mn^{2+}	1.0	98
EDTA	1.0	115

[a] The dialyzed enzyme was preincubated with inhibitor for 10 min before addition of substrate.
[b] The activity of the enzyme without addition was assigned a value of 100.

though inhibition by certain heavy metal ions appeared partially reversible by EDTA. These observations suggest the presence of a thiol group at the active site. It has been reported that some plant and animal catechol O-methyltransferases require a bivalent ion such as Mg^{2+} for maximal activity (3, 10, 23). However, there is no evidence that Mg^{2+} has an appreciable effect on the enzyme from *P. chrysosporium*, which appears more similar in this respect to the O-methyltransferases involved in lignin biosynthesis (22). The lack of a metal ion requirement was confirmed by the failure of chelating agents such as EDTA to affect activity significantly.

Substrate specificity. The relative rates of O-methylation of a variety of phenolic substrates by the O-methyltransferase are shown in Table 3. It is clear that 4-hydroxy-3-methoxybenzaldehyde, 4-hydroxy-3-methoxybenzoic acid, and 4-hydroxy-3-methoxyacetophenone were excellent substrates. The corresponding 3,5-dimethoxy-4-hydroxy compounds were also readily methylated. Aldehydes and acids of this type are frequent products of fungal lignin degradation, with both 3-methoxy-4-hydroxy- and 3,5-dimethoxy-4-hydroxy-substituted structures being present in hardwood lignins but predominantly the former occurring in softwood lignins (1, 5, 6, 17). In contrast, the corresponding 3-hydroxy-4-methoxy compounds were very poor substrates. Thus, the rate of isovanillin methylation was less than 1% of that of vanillin. Attack on 3,4-dihydroxy compounds was also considerably slower than attack on 4-hydroxy-3-methoxy compounds and occurred predominantly at the 4 position. Isovanillin, vanillin, and veratraldehyde are all potential products of the action of the O-methyltransferase on 3,4-dihydroxybenzaldehyde. Under the conditions of the assay, only isovanillin and veratraldehyde were detected as products from this substrate. As isovanillin is methylated at only 0.7% of the rate of vanillin, the main precursor of the dimethylated compound, veratraldehyde, must be vanillin. Hence, on the basis of the relative proportions of the products arising from 3,4-dihydroxybenzaldehyde shown in Table 3, the rate of attack on the 4 position in this substrate was approximately sixfold greater than the rate of attack on the 3 position.

Substitutions in both the 2 and 4 positions relative to the hydroxyl group appear to be required for substantial methylation of a substrate to occur. Compounds such as *p*-hy-

TABLE 3. Substrate specificity of *O*-methyltransferase

Substrate[a]	Rate[b]	Substrate	Rate[b]
4-Hydroxy-3-methoxyacetophenone (acetovanillone)	100	2-Hydroxy-3-methoxybenzaldehyde	<0.2
4-Hydroxy-3-methoxybenzaldehyde (vanillin)	88	Phenol	<0.2
4-Hydroxy-3-methoxybenzoic acid (vanillic acid)	98	2-Methoxyphenol (guaiacol)	7
4-Hydroxy-3-methoxybenzyl alcohol (vanillyl alcohol)	13	Catechol	7[e]
4-Hydroxy-3,5-dimethoxyacetophenone (acetosyringone)	102	Hydroquinone	<0.2
4-Hydroxy-3,5-dimethoxybenzaldehyde (syringaldehyde)	88	2-Chlorohydroquinone	<0.2
4-Hydroxy-3,5-dimethoxybenzoic acid (syringic acid)	89	2,3-Dimethoxyphenol	1
3-Hydroxy-4-methoxybenzaldehyde (isovanillin)	0.6	3-Methoxyphenol	<0.2
3-Hydroxy-4-methoxybenzoic acid (isovanillic acid)	1	2,4-Difluorophenol	<0.2
3-Hydroxy-4-methoxybenzyl alcohol (isovanillyl alcohol)	<0.2	2,4-Dichlorophenol	91
3,4-Dihydroxybenzaldehyde	22[c]	2,4-Dibromophenol	95
3,4-Dihydroxybenzoic acid	8.5[d]	2,6-Dichlorophenol	9
4-Hydroxybenzaldehyde	2	2-Bromophenol	9
4-Hydroxybenzoic acid	8	4-Bromophenol	3
4-Hydroxybenzyl alcohol	<0.2	4-Hydroxy-3-methoxyphenylacetic acid (homovanillic acid)	<0.2
2-Hydroxybenzaldehyde	<0.2		

[a] Ferulic, isoferulic, caffeic, and sinapic acids did not act as substrates.
[b] Rate of methylation on a molar basis relative to that with acetovanillone as the substrate in 100 mM Tris buffer (pH 8.0) with 1 mM SAM and 1 mM phenolic substrate at 37°C.
[c] Product composed of isovanillin, vanillin, and veratraldehyde in the proportion 19:<0.2:3.
[d] Product composed of isovanillic, vanillic, and veratric acids in the proportion 8:<0.2:0.5.
[e] Guaiacol was the only product detected.

droxybenzaldehyde and guaiacol acted as relatively poor enzyme substrates, but phenol itself was not attacked. Diphenols in general displayed little activity as substrates. Although some transformation of catechol occurred, no methylation of either hydroquinone or chlorohydroquinone was detected.

The nature of the substituents in the 2 and 4 positions does not appear critical provided certain steric criteria are satisfied. Accordingly, 2,4-disubstitution of phenol with certain halogens is consistent with activity as a substrate. Thus, 2,4-dichlorophenol and 2,4-dibromophenol were almost as readily methylated as acetovanillone. This behavior is largely dependent on the steric rather than the electronic effects of the substituents, since no significant methylation was observed when the substrate was disubstituted in the 2 and 4 positions by fluorine. The van der Waals radius of fluorine is only 1.35 Å (1Å = 0.1 nm), much closer to that of H (1.20 Å) than to those of Cl and Br (1.80 and 1.95 Å, respectively) (27). Neither 2,6-dihalosubstituted phenols nor phenols monosubstituted in the 2 or 4 position with halogen were readily utilized as substrates, demonstrating yet again the necessity for substitution in both the 2 and 4 positions relative to the hydroxyl group if significant activity as a substrate for the *O*-methyltransferase is to be achieved. Surprisingly, despite the finding that vanillic acid was an excellent substrate, no methylation of homovanillic acid could be detected; this result implies the existence of a strict limit on the maximum as well as the minimum size of the substituent *para* to the hydroxyl group. This may also explain why hydroxycinnamic acids such as ferulic, isoferulic, and caffeic acids were not methylated by the enzyme. Also unexpected in view of the ready enzymatic transformation of both vanillin and vanillic acid was the comparative resistance of vanillyl alcohol to enzymatic attack, with the rate of methylation of the compound being only 13% of that of the vanillic acid.

DISCUSSION

The *O*-methyltransferase from *P. chrysosporium* differs from most enzymes of this type isolated from plant sources in that it is strongly *para* specific. The majority of *O*-methyltransferases involved in lignin biosynthesis exhibit an absolute specificity for the *meta* hydroxyl group of 3,4-dihydricphenols (22). For such enzymes, phenylpropanoid substrates such as caffeic, 5-hydroxyferulic, and 3,4,5-trihydroxycinnamic acids are in general the best substrates, although occasionally activity on simpler phenolic compounds such as 3,4-dihydroxybenzaldehyde and catechol is observed. Although some *para*-specific *O*-methyltransferase from plants have been characterized, they have mainly possessed a very limited substrate range and have been involved in the biosynthesis of more complex natural products such as the alkaloids norbelladine in *Nerine bowdenii* (19) and mescaline in *Lophophora williamsii* (4) or isoflavones in *Cicer arietinum* (29). The only fungal phenolic *O*-methyltransferase hitherto investigated has been that from *Lentinus lepideus*, which, although it exhibited *para* specificity, was restricted in substrate range to the methyl esters of phenylpropanoid compounds such as *p*-coumaric, caffeic, and ferulic acids (28).

In contrast, the *O*-methyltransferase from *P. chrysosporium* attacked 3-methoxy- and 3,5-dimethoxy-substituted 4-hydroxybenzaldehydes, 4-hydroxybenzoic acids, and 4-hydroxyacetophenones with almost equal facility. The presence of this enzyme not only explains the 4-*O*-methylation of vanillic acid and acetovanillone observed in ligninolytic cultures of *P. chrysosporium* by Chen et al. (6) but also the methylation of syringic acid observed in cultures of the fungus by Eriksson et al. (11). The failure of the enzyme to utilize homovanillic acid as a substrate despite the reported methylation of the compound by *P. chrysosporium* (6) suggests that other *O*-methyltransferases must be present in the organism. Similarly, although the methylation of 2,4-dichlorophenol to 2,4-dichloromethoxybenzene observed by Valli and Gold (25) in cultures of *P. chrysosporium* can be attributed to the *O*-methyltransferase described in this article, the enzyme is obviously not responsible for the methylation of 2-chlorohydroquinone to form 2-chloro-1,4-dimethoxybenzene, which was also identified as a metabolite of 2,4-dichlorophenol. No methylation whatsoever of 2-chlorohydroquinone by the enzyme was detected in this study.

Furthermore, the lack of activity of ferulic, isoferulic, and caffeic acids as substrates of the O-methyltransferase indicates that the enzyme is not involved in the biosynthesis of veratryl alcohol via the route postulated by Shimada et al. (24), which involved methylation of ferulic acid. This finding does not rule out the possibility that coenzyme A derivatives rather than the free acids are substrates of the enzyme in the biosynthetic pathway. Nevertheless, it seems doubtful that such coenzyme A intermediates are attacked by the O-methyltransferase in view of the steric constraints on the size of the substituent located *para* to the hydroxyl in substrates of the enzyme.

In light of the considerations described above, it is clear that other phenolic O-methyltransferases must exist in *P. chrysosporium*. The high incorporation of C^2H_3Cl into the methoxyl groups of veratryl alcohol demonstrated by Harper et al. (14), together with the stimulatory effects of CH_3Cl on veratryl alcohol biosynthesis (13), indicates that CH_3Cl participates in at least some of these methylation reactions. The sensitivity of SAM-dependent methylation to inhibition by SAH should allow the latter compound to be used as a biochemical probe to identify the extent of CH_3Cl-dependent methylation. The precise metabolic function of the SAM-dependent O-methyltransferase described in this report cannot yet be defined, but the substrate specificity of the enzyme implies a role in catabolism or detoxification of lignin degradation products.

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REFERENCES

1. **Adler, E.** 1977. Lignin chemistry—past, present and future. Wood Sci. Technol. **11:**169–218.
2. **Ander, P., and K.-E. Eriksson.** 1976. The importance of phenol oxidase activity in lignin degradation by the white rot fungus *Sporotrichum pulverulentum*. Arch. Microbiol. **109:**1–8.
3. **Axelrod, J., and R. Tomchick.** 1958. Enzymic O-methylation of epinephrine and other catechols. J. Biol. Chem. **233:**702–705.
4. **Basmadjian, G. P., S. F. Hussain, and A. G. Paul.** 1978. Biosynthetic relationships between phenylethylamine and tetrahydroisoquinaline alkaloids in peyote. Lloydia **41:**375–380.
5. **Chen, C. L., and H. M. Chang.** 1985. Chemistry of lignin biodegradation, p. 535–556. *In* T. Higuchi (ed.) Biosynthesis and biodegradation of wood components. Academic Press, Inc., Orlando, Fla.
6. **Chen, C. L., H. M. Chang, and T. K. Kirk.** 1982. Aromatic acids produced during degradation of lignin in spruce wood by *Phanerochaete chrysosporium*. Holzforschung **36:**3–9.
7. **Coulter, C., and D. B. Harper.** 1991. Chloromethane and S-adenosylmethionine as methyl donors in *Phanerochaete chrysosporium*, p. B1. Abstr. 1st Congr. U.K. Biotechnology. Leeds, United Kingdom, 1991.
8. **Dixon, M.** 1953. Determination of enzyme inhibitor constants. Biochem. J. **55:**170–171.
9. **Dixon, M., and E. C. Webb.** 1979. The enzymes, 3rd ed., p. 82–87. Longman Group, London.
10. **Ebel, J., K. Hahlbrock, and H. Grisebach.** 1972. Purification and properties of an *o*-dihydricphenol *meta*-O-methyltransferase from cell suspension cultures of parsley and its relation to flavonoid biosynthesis. Biochim. Biophys. Acta **269:**313–326.
11. **Eriksson, K.-E., J. K. Gupta, A. Nishida, and M. Rao.** 1984. Syringic acid metabolism by some white rot, soft rot and brown rot fungi. J. Gen. Microbiol. **130:**2457–2464.
12. **Eriksson, K.-E., and S. C. Johnsrud.** 1983. Mutants of the white rot fungus *Sporotrichum pulverulentum* with increased cellulase and β-D-glucosidase production. Enzyme Microb. Technol. **5:**425–429.
13. **Harper, D. B., J. A. Buswell, and J. T. Kennedy.** 1991. Effect of chloromethane on veratryl alcohol and lignin peroxidase production by the fungus *Phanerochaete chrysosporium*. J. Gen. Microbiol. **137:**2867–2872.
14. **Harper, D. B., J. A. Buswell, J. T. Kennedy, and J. T. G. Hamilton.** 1990. Chloromethane, methyl donor in veratryl alcohol biosynthesis in *Phanerochaete chrysosporium* and other lignin-degrading fungi. Appl. Environ. Microbiol. **56:**3450–3457.
15. **Harper, D. B., and J. T. G. Hamilton.** 1988. Biosynthesis of chloromethane in *Phellinus pomaceus*. J. Gen. Microbiol. **134:** 2831–2839.
16. **Harper, D. B., J. T. G. Hamilton, J. T. Kennedy, and K. J. McNally.** 1989. Chloromethane, a novel methyl donor for biosynthesis of esters and anisoles in *Phellinus pomaceus*. Appl. Environ. Microbiol. **55:**1981–1989.
17. **Henderson, M. E. K.** 1963. Fungal metabolism of certain aromatic compounds related to lignin. Pure Appl. Chem. **7:**589–602.
18. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.
19. **Mann, J. D., H. M. Foles, and S. H. Mudd.** 1963. Alkaloids and plant metabolism VI O-methylation *in vitro* of norbelladine, a precursor of Amaryllidaceae alkaloids. J. Biol. Chem. **238:**3820–3823.
20. **McNally, K. J., J. T. G. Hamilton, and D. B. Harper.** 1990. The methylation of benzoic and n-butyric acids by chloromethane in *Phellinus pomaceus*. J. Gen. Microbiol. **136:**1509–1515.
21. **Ohta, A., M. Shimada, T. Higuchi, and M. Takahashi.** 1991. A new type of O-methyltransferase involved in the biosynthesis of secondary metabolites of a brown rot fungus *Lentinus lepideus*. Mokuzai Gakkaishi **37:**275–280.
22. **Poulton, J. E.** 1981. Transmethylation and demethylation reactions in the metabolism of secondary plant products, p. 667–723. *In* P. K. Stumpf and E. E. Conn (ed.), The biochemistry of plants, vol. 7. Academic Press, London.
23. **Poulton, J. E., K. Hahlbrock, and H. Grisebach.** 1977. O-Methylation of flavonoid substrates by a partially purified enzyme from soyabean cell suspension cultures. Arch. Biochem. Biophys. **180:**543–549.
24. **Shimada, M., F. Nakatsuba, T. K. Kirk, and T. Higuchi.** 1981. Biosynthesis of the secondary metabolite veratryl alcohol in relation to lignin degradation in *Phanerochaete chrysosporium*. Arch. Microbiol. **129:**321–324.
25. **Valli, K., and M. H. Gold.** 1991. Degradation of 2,4-dichlorophenol by the lignin-degrading fungus *Phanerochaete chrysosporium*. J. Bacteriol. **173:**345–352.
26. **Valli, K., H. Wariishi, and M. H. Gold.** 1992. Degradation of 2,7-dichlorodibenzo-*p*-dioxin by the lignin-degrading basidiomycete *Phanerochaete chrysosporium*. J. Bacteriol. **174:**2131–2137.
27. **Walsh, C.** 1982. Fluorinated substrate analogs: routes of metabolism and selective toxicity. Adv. Enzymol. Relat. Areas Mol. Biol. **55:**197–289.
28. **Wat, C. K., and G. H. N. Towers.** 1975. Phenolic O-methyltransferase from *Lentinus lepideus* (Basidiomycete). Phytochemistry **14:**663–666.
29. **Wengenmayer, H., J. Ebel, and H. Grisebach.** 1974. Purification and properties of a S-adenosylmethionine isoflavone 4-O-methyltransferase from cell suspension cultures of *Cicer arietinum* L. Eur. J. Biochem. **50:**135–143.
30. **White, R. H.** 1982. Biosynthesis of methyl chloride in the fungus *Phellinus pomaceus*. Arch. Microbiol. **132:**100–102.