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Functional diversity of cytochrome P450s of the white-rot fungus *Phanerochaete chrysosporium*

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Abstract

The functional diversity of cytochrome P450s (P450s) of the white-rot basidiomycete, *Phanerochaete chrysosporium*, was studied. A series of compounds known to be P450 substrates of other organisms were utilized for metabolic studies of *P. chrysosporium*. Metabolic conversions of benzoic acid, camphor, 1,8-cineol, cinnamic acid, *p*-coumaric acid, coumarin, cumene, 1,12-dodecanediol, 1-dodecanol, 4-ethoxybenzoic acid, and 7-ethoxycoumarin were observed with *P. chrysosporium* for the first time. 1-Dodecanol was hydroxylated at seven different positions to form 1,12-, 1,11-, 1,10-, 1,9-, 1,8-, 1,7-, and 1,6-dodecandiols. The effect of piperonyl butoxide, a P450 inhibitor, on the fungal conversion of 1-dodecanol was also investigated, indicating that hydroxylation reactions of 1-dodecanol were inhibited by piperonyl butoxide in a concentration-dependent manner. With 11 substrates, 23 hydroxylation reactions and 2 deethylation reactions were determined and 6 products were new with the position of hydroxyl group incorporated. In conclusion, fungal P450s were shown to have diverse and unique functions.

Keywords: Basidiomycetes; Biodiversity; Cytochrome P450; 1-Dodecanol; Metabolic conversion

White-rot fungi are capable of degrading a wide variety of recalcitrant aromatic compounds, including polymeric lignin and environmentally persistent pollutants. The extracellular ligninolytic enzymes involved in the metabolism of aromatic compounds have been extensively studied [8,19]. Intracellularly, cytochrome P450 (P450)-mediated oxygenation reactions were shown to play an important role during fungal metabolism of recalcitrant xenobiotic compounds [3,12,15,22,39]. *Phanerochaete chrysosporium* is the most extensively studied white-rot fungus with regard to ligninolysis and xenobiotic metabolism. The gene diversity of fungal P450s was recently suggested via a whole genomic sequence, where as many as 148 P450 genes were found in the *P. chrysosporium* genome [21]. However, little information on their function has been reported.

In the present study, to better understand fungal P450s, a series of well-characterized P450 substrates known for other organisms were utilized for metabolic studies of *P. chrysosporium*. Benzoic acid, camphor, 1,8-cineol, cinnamic acid, *p*-coumaric acid, coumarin, cumene, 1,12-dodecanediol, 1-dodecanol 4-ethoxybenzoic, and 7-ethoxycoumarin were utilized in this study. Against these 11 substrates, 25 P450 derived hydroxylation and deethylation reactions were observed, indicating that *P. chrysosporium* P450s have diverse and unique functions.

Materials and methods

Chemicals. Benzoic acid, cinnamic acid, 1,12-dodecanediol, 1,12-dodecanedioic acid, 1-dodecanol, 4-hydroxybenzoic acid, 7-hydroxycoumarin, vanillic acid, and Triton X-100 were obtained from Wako Pure Chemicals. 1,8-Cineol, coumarin, cumen, 4-ethoxybenzoic acid, 7-ethoxycoumarin, 2-hydroxybenzoic acid, and salicylic acid were

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purchased from Tokyo Chemical Industry, and caffeic acid, camphor, and *p*-coumaric acid were from Sigma–Aldrich. Deionized water was obtained with a Milli Q System (Millipore).

Culture conditions. P. chrysosporium ATCC 34541 was grown from conidial inocula at 37 °C in stationary culture (10 mL of medium in a 100-mL Erlenmeyer flask) under air. The medium (pH 4.5) used in this study was the same as that previously described for ligninolytic culture conditions, with 1% glucose as the carbon source and 1.2 mM ammonium tartrate as the nitrogen source [20].

Metabolic reactions. After a 3-day incubation, the substrates (500 mM acetone solution) were added to the cultures to a final concentration of 1.0 mM. Triton X-100 (0.01%, w/v) was added when 1-dodecanol was utilized as the substrate. After additional incubation, metabolic reactions were stopped by adding 10 mL acetone. Metabolic products were analyzed by HPLC after homogenization and filtration (0.45 μm), and by GCMS after homogenization, extraction with chloroform at pH 3, drying over $MgSO_4$, evaporation, and derivatization using *N*,*O*-bis(trimethylsilyl)trifluoroacetamide/pyridine (2:1, v/v) for trimethylsilylation, or acetic anhydride/pyridine (1:1, v/v) for acetylation [24]. An extraction standard (1-decanol) was added to each flask to a final concentration of 25 μM when the metabolic reactions were stopped. To qualify the products, the areas of individual product peaks were estimated after integration of each peak and normalization to the area of the 1-decanol peak. As a control, uninoculated samples and samples without additional substrates were utilized. All assays were performed at least three times.

Enzyme reaction. Lignin peroxidase (LiP) isozyme H8 and manganese peroxidase (MnP) isozyme H4 were purified from the extracellular medium of *P. chrysosporium* as previously reported [37,38]. Both enzymes were electrophoretically homogeneous with RZ (A_{407}/A_{280}) values of 4.8 and 4.9, respectively.

For LiP reactions, the reaction mixtures (1 mL) consisted of the enzyme (20 μg), substrate (0.5 mM), veratryl alcohol (0 or 0.1 mM), and H_2O_2 (0.1 mM) in 20 mM sodium succinate, pH 3.0. MnP reaction mixtures (1 mL) contained the enzyme (10 μg), substrate (0.5 mM), $MnSO_4$ (0.5 mM), and H_2O_2 (0.1 mM) in 50 mM sodium malonate, pH 4.5. Reaction products were analyzed by HPLC or GCMS as described above.

Instrumentation. GCMS was performed at 70 eV on a JEOL AMII-15 A equipped with a 30-m fused silica column (DB-5, J & W scientific). The oven temperature was programmed from 80 to 320 °C at 8 °C/min, 80 to 320 at 6 °C/min or 40 to 320 at 8 °C/min with an injection temperature of 280 °C. Products were identified by comparing their retention time on GC and HPLC and mass fragmentation pattern with authentic standards. Some products were identified as trimethylsilyl (TMS) and *O*-diacetyl derivatives. Products for which authentic standards are not available were determined by comparing their mass data with reported spectra or with the National Institute of Standard Technology (NIST) mass spectral library. Dodecanediols were identified by a detailed analysis of their mass fragmentation patterns.

Results and discussion

Fungal metabolism of compounds known to be substrates for other P450s

The 18 metabolites from 10 substrates, benzoic acid, cinnamic acid, *p*-coumaric acid, coumarin, cumene, camphor, 1,8-cineol, 1,12-dodecanediol, 4-ethoxybenzoic, and 7-ethoxycoumarin, used in this study, are listed in Table 1. Sequential annotation data accessible via the web (http://www.jgi.doe.gov/programs/whiterot.htm) were utilized for the *P. chrysosporium* P450 genes; thus, no accession numbers are given in the present work.

Cinnamic acid was hydroxylated at aryl 4-position to form *p*-coumaric acid (4-hydroxycinnamic acid); the latter was further hydroxylated at 3-position to form 3,4-dihydroxycinnamic acid (caffeic acid). Cinnamic acid is reported to be hydroxylated to form *p*-coumaric acid by plant P450 CYP73 and *p*-coumaric acid is hydroxylated by other plant P450 CYP98 [29,33]. Thus, *P. chrysosporium* possesses functions similar to those of CYP73 and CYP98; however, phylogenetic analysis of *P. chrysosporium* P450 genes revealed that no P450 genes in the *P. chrysosporium* genome fell into either CYP73 or CYP98 families.

Cumene is hydroxylated as reported for bacterial P450 CYP102. However, the incorporation position for the hydroxyl group is unknown for this reaction [11]. CYP102 is also known as P450 BM3, a fusion protein of P450 and P450 reductase. Seven sequences of the *P. chrysosporium* P450s were found to be in the same phylogenetic cluster as CYP102 (unpublished data[1]).

1,12-dodecanediol was oxygenated as reported for yeast P450 CYP52 [32] and seven sequences from the *P. chrysosporium* genome were categorized into the CYP52 family [21].

4-Ethoxybenzoic acid and 7-ethoxycoumarin were deethylated as reported for bacterial P450s (P450-RR2, CYP105A1, and CYP105B1), mammalian P450s (CYP2B subfamily), plant P450 (CYP76B1), and fungal P450 (CYP510A1) [1,2,14,16,18]. No sequences of the *P. chrysosporium* P450 genes showed a sequential similarity above 35% to CYP2, CYP76, or CYP105. On the other hand, at lease nine sequences of the *P. chrysosporium* P450 genes showed a sequential identity above 35% to CYP510A1 where sequential overlaps more than 470 amino acid residues were observed.

Coumarin, which is hydroxylated at 7-position by mammalian CYP2A and at 3-position by CYP1A, CYP2E1, and CYP3A4 [4,13,31,36,40], was hydroxylated at four different positions to form 5-, 6-, 7-, and 8-hydroxycoumarin by *P. chrysosporium*. No sequence of *P. chrysosporium* P450 genes showed a sequential similarity above 35% to either CYP1, 2, or CYP3 families.

Benzoic acid, which is hydroxylated only at 4-position by the fungal P450 (CYP53) from *Aspergillus niger* [35], was hydroxylated at three different positions to form 2- and 4-hydroxybenzoic acid, and 3-methoxy-4-hydroxybenzoic acid by *P. chrysosporium*. A methylation reaction seems to occur after the incorporation of a hydroxyl group at 3-position. One sequence of *P. chrysosporium* P450 gene fell into the CYP53 family.

[1] Unpublished results by N. Hiratsuka, H. Ichinose, O. Gotoh, H. Wariishi.

Table 1
Mass spectra and chromatographic data of the substrates and metabolites

Substrate	Metabolites	Retention time (min)		Mass spectrum *m/z* (relative intensity)
		GC	HPLC	
Benzoic acid	Salicylic acid (TMS[b])	13.81[a]	19.00	267 (M-15, 13), 209 (3.2), 193 (3.7), 149 (4.8), 135 (10.2), 117 (7.1), 103 (5.6), 91 (7.8), 73 (100), 65 (4.1), 45 (13.0)
	p-Hydroxybenzoic acid (TMS[b])	15.53[a]	9.43	282 (M^+, 12.3), 267 (59.1), 223 (66.1), 207 (3.1), 193(53.3), 179 (3.7), 149 (4.0), 135 (5.0), 126 (12.4), 103 (3.7), 91 (7.8), 73 (100), 59 (4.2), 45 (19.1)
	Vanillic acid (TMS[b])	17.63[a]	—	312 (M^+, 28.2), 297 (52.6), 282 (7.0), 267 (38.0), 253 (24.9), 223 (39.5), 207 (2.4), 193 (13.1), 179 (3.4), 117 (47.7), 89 (8.5), 73 (100), 59 (12.5), 45 (17.3), 28 (12.3)
Camphor	4-Hydroxycamphor	9.37[d]	—	168 (M^+, 9.0), 125 (25.7), 107 (7.2), 97 (15.2), 83 (100), 69 (35.7), 67 (13.5), 55 (46.7), 41 (58.2), 29 (28.4)
	5-Hydroxycamphor	13.28[d]	—	168 (M^+, 3.5), 153 (38.4), 135 (16.6), 125 (7.9), 111 (40.6), 108 (100), 95 (26.3), 93 (70.2), 91 (17.2), 83 (19.5), 81 (26.7), 79 (22.4), 69 (42.6), 67 (35.9), 57 (32.4), 55 (61.9), 43 (76.9), 41 (85.0), 39 (43.1), 29 (34.1), 27 (29.9)
	Hydroxydamphor	12.43[d]	—	168 (M^+, 6.4), 153 (39.6), 125 (18.8), 111 (53.5), 109 (14.2), 97 (22.4), 95 (9.4), 83 (21.9), 81 (18.0), 70 (100), 55 (73.4), 41 (50.0), 39 (32.5), 27 (12.3)
1,8-Cineol	2-Hydroxycineol	14.38[c]	—	170 (M^+, 3.3), 126 (31.3), 111 (40.2) 108 (66.8), 97 (11.9) 93 (56.7), 83 (42.2), 79 (16.9) 71 (100), 69 (68.0)
	3-Hydroxycineol	14.98[c]	—	170 (M^+, 8.9), 155 (12.0), 141 (2.3), 137 (12.9), 126 (6.6), 119 (3.2), 111 (30.0), 108 (50.2), 97 (10.2), 93 (100), 87 (84.8), 84 (55.8), 79 (28.8), 67 (20.4), 65 (13.1)
Cinnamic acid	*p*-Coumaric acid (TMS[b])	20.71[a]	—	308 (M^+, 41.3), 293 (77.0), 249 (15.2), 219 (63.5), 179 (1.5), 147 (1.7), 73 (100)
p-Coumaric acid	Caffeic acid (TMS[b])	22.52[a]	12.30	396 (M^+, 72.9), 379 (19.4), 363 (4.6), 307 (15.5), 249 (5.7), 219 (100), 191 (16.1), 173 (5.2), 159 (13.2), 141 (4.6), 125 (6.9), 111 (8.6), 45 (35.5)
Coumarin	8-Hydroxycoumarin (TMS[b])	17.65[a]	—	234 (M^+, 6.8), 219 (100), 191 (38.9), 175 (3.0), 160 (8.1), 133 (2.9), 117 (9.3), 102 (10.4), 88 (24.1), 75 (38.8), 63 (13.9)
	5-Hydroxycoumarin (TMS[b])	18.85[a]	—	234 (M^+, 26.8), 219 (7.4), 191 (19.2), 161 (5.1), 133 (4.2), 117 (2.7), 105 (4.9), 95 (4.3), 89 (7.4), 73 (100), 63 (19.9)
	6-Hydroxycoumarin (TMS[b])	19.25[a]	—	234 (M^+, 49.4), 219 (100), 191 (7.9), 161 (4.0), 135 (7.3), 117 (7.1), 105 (5.1), 95 (14,8), 89 (14.8), 73 (69.9), 63 (22.2)
	7-Hydroxycoumarin (TMS[b])	19.40[a]	—	234 (M^+, 56.6), 219 (82.4), 191 (9.5), 163 (66.4), 133 (14,3), 89 (31.9), 73 (100), 63 (44.9)
Cumene	Hydroxycumene	14.70[c]	—	136 (M^+, 12.5), 121 (60.4), 103 (12.5), 91 (11.3), 77 (14.5), 73 (42.1), 60 (100)
1,12-Dodecanediol	1,12-Dodecanedioic acid (TMS[b])	22.38[a]	—	374 (M^+, 0.07), 359 (39.0), 343 (4.4), 315 (3.0), 243 (6.9), 217 (7.9), 147 (10.1), 129 (20.6), 117 (16.6), 73 (100)
p-Ethoxybenzoic acid	*p*-Hydroxybenzaldehyde (TMS[b])	11.40[d]	—	194 (M^+, 57.2), 179 (100), 161 (8.0), 151 (46.1), 135 (7.4), 105 (5.5), 95 (12.3), 91 (18.7), 73 (42.3), 45 (39.8)
7-Ethoxycoumarin	7-Hydroxycoumarin (TMS[b])	19.35[a]	13.62	234 (M^+, 75.2), 219 (100), 163 (32.5), 151 (6.1), 96 (11.9), 73 (40.7), 57 (25.8), 45 (20.8)

[a] RT on the GC temperature program: 80 °C (0–5 min), 80–320 °C (5–35 min).
[b] TMS, trimethylsilylated.
[c] RT on the GC temperature program: 40 °C (0–5 min), 40–280 °C (5–35 min).
[d] RT on the GC temperature program: 80 °C (0–5 min), 80–320 °C (5–45 min).

Camphor is hydroxylated at 5- and 6-position by bacterial P450cam (CYP101) and P450camr [9,17]. On the other hand, *P. chrysosporium* hydroxylated camphor at three different positions to form 4- and 5-hydroxycamphor, and to hydroxycamphor with an unknown hydroxyl group position [6,34]. 1,8-Cineol, which is hydroxylated at 2- and 3-position by bacterial CYP176A1 (P450cin) and mammalian CYP3A [10,27,28], is hydroxylated at these two positions by *P. chrysosporium*. No *P. chrysosporium* P450 genes exhibited a sequential similarity above 35% to P450 camr, CYP101, CYP176, and CYP3 families.

Fungal metabolism of 1-dodecanol

1-Dodecanol was converted to seven hydroxylated products by *P. chrysosporium*. A total peak area of seven metabolites was found to be equivalent to 10% of added 1-dodecanol. As shown in Fig. 1, the mass spectra of the TMS derivatives of the seven metabolites showed

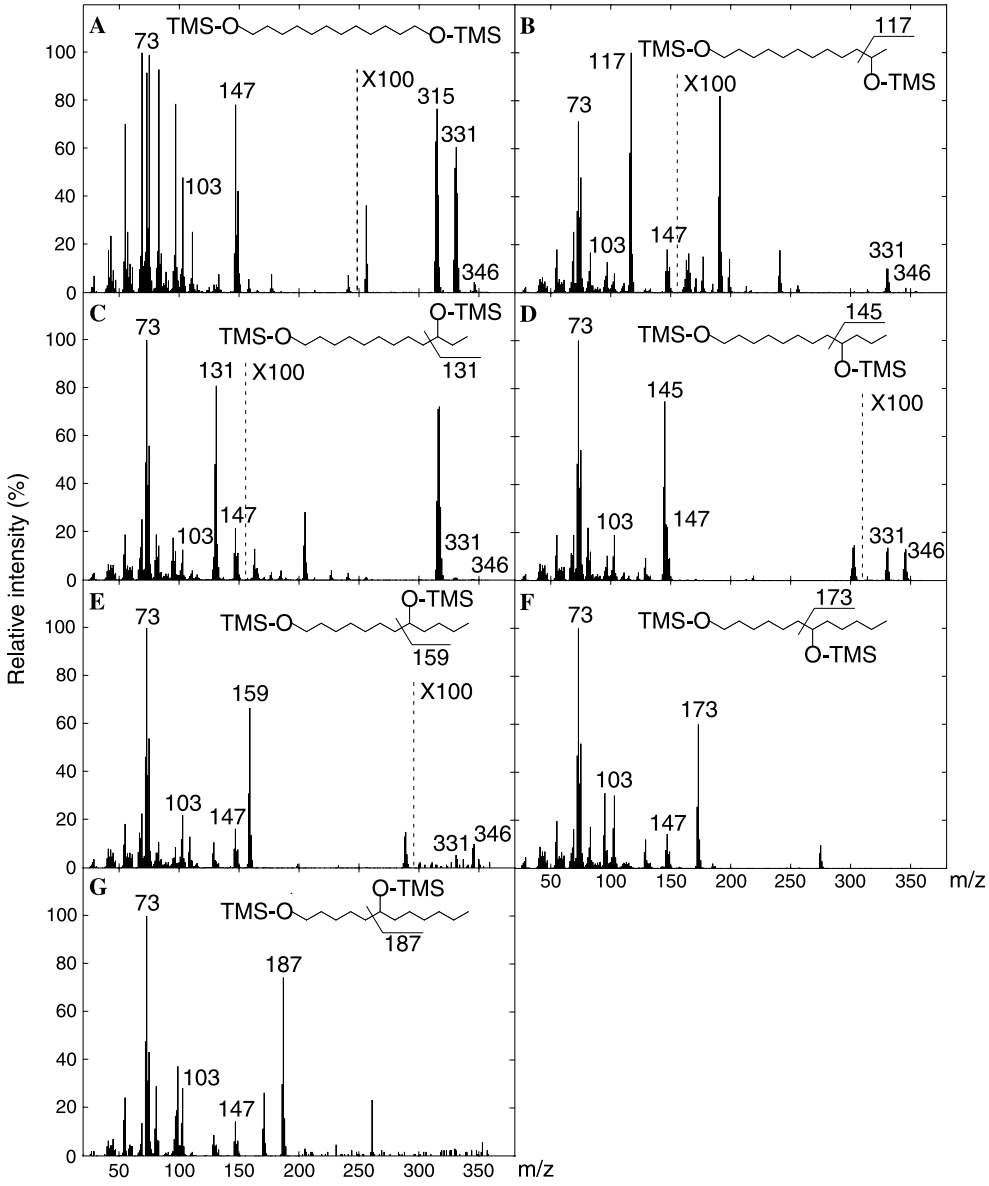

Fig. 1. Mass spectral analysis of 1-dodecanol metabolites produced by the fungus. The mass spectra were obtained from the GC peak appearing at retention times (in min) of 22.63 (1,12-dodecanediol; A), 21.36 (1, 11-dodecanediol; B), 21.15 (1,10-dodecanediol; C), 20.72 (1, 9-dodecanediol; D), 20.43 (1,8-dodecanediol; E), 20.28 (1, 7-dodecanediol; F), and 20.20 (1,6-dodecanediol; G).

characteristic fragmentation patterns according to dodecanediol with one hydroxyl group incorporated into different positions from ω-position to (ω-6)-position. The spectra also contained ion fragments common to TMS derivatives of diols at *m/z* 147 $[(CH_3)_2Si{=}O{-}Si(CH_3)_3]^+$, 103 $[(CH_3)_3SiO{=}CH_2]^+$, and 73 $[(CH_3)_3Si]^+$. *O*-Diacetyl derivatives of the dodecanediols also exhibited characteristic ion fragments in their expected structure from (ω-1)- to (ω-6)-hydroxylated 1-dodecanols, at *m/z* 87 $[CH_3CHOCOCH_3]^+$, 101 $[C_2H_5CHOCOCH_3]^+$, 115 $[C_3H_7CHOCOCH_3]^+$, 129 $[C_4H_9CHOCOCH_3]^+$, 143 $[C_5H_{11}CHOCOCH_3]^+$, and 157 $[C_6H_{13}CHOCOCH_3]^+$, respectively. From these data, fungal metabolites from 1-dodecanol were tentatively identified as 1,12-, 1,11-, 1,10-, 1,9-, 1,8-, 1,7-, and 1,6-dodecanediol. P450-derived hydroxylation reactions of 1-dodecanol were reported at ω-position with yeast P450 CYP52A3 [32], at ω and (ω-1)-positions with frog and mongolian gerbil microsomes [23,26], and at (ω-1) to (ω-3)-positions with bacterial microsomes [25]. Furthermore, hydroxylation reactions of fatty acid catalyzed by P450s of rainbow trout were reported to occur at ω to (ω-6)-positions [5,41]. However, hydroxylation at (ω-4) to (ω-6)-positions of 1-dodecanol was reported for the first time in the present study.

1,10- and 1,9-dodecanediol were predominate metabolites with a yield of 35% and 40% of the total peak area of dodecanediols, respectively. 1,12-, 1,11-, and 1,8-dodecanediol were yielded at 8%, 11%, and 6% of the total peak area of dodecanediols, respectively, and only

trace amounts of 1,7- and 1,6-dodecanediol were found (Fig. 2).

No involvement of extracellular enzymes in hydroxylation reactions

In the present study, metabolic studies were performed under ligninolytic conditions where extracellular LiP and MnP are secreted [8,19]. Thus, the effect of LiP and MnP on the substrates used in the study was examined. No hydroxylated products were found from benzoic acid, cinnamic acid, coumarin, cumene, 1,12-dodecanediol, and 1-dodecanol by either LiP or MnP. The extracellular culture fluid also had no effect on the substrates. It was assumed that the fungus might uptake the compounds into its cells and that the hydroxylation reactions might occur intracellularly.

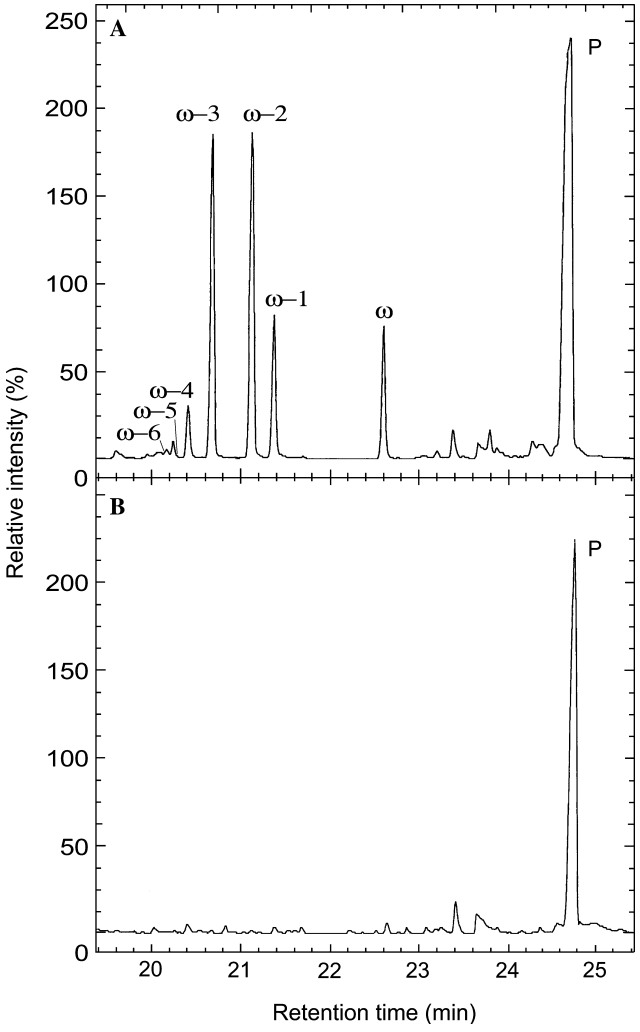

Fig. 2. Total ion chromatograms of fungal products extracted from *P. chrysosporium* incubated for 4 days after the addition of 1-dodecanol without (A) or with (B) 1 mM piperonyl butoxide. The symbols (ω to ω-6) indicate the hydroxylation position of 1-dodecanol. P indicates palmitic acid produced by the fungus. Relative intensity was calculated against the internal standard.

Effect of a cytochrome P450 inhibitor on 1-dodecanol hydroxylations

The effect of piperonyl butoxide (PB), a cytochrome P450 inhibitor [7,30], on the fungal hydroxylation of 1-dodecanol was investigated, indicating that PB inhibited the formation of hydroxylated products in a concentration-dependent manner (Figs. 2 and 3). In contrast, fungal formation of palmitic acid and some other compounds was unaffected by the addition of PB. These data combined with the data showing no participation of LiP and MnP in the hydroxylation reactions suggest the involvement of P450-type catalyst(s) in the hydroxylation reactions of 1-dodecanol.

It is unclear how many and which P450 genes of *P. chrysosporium* are involved in the hydroxylation of 1-dodecanol. Although the concentration-dependent inhibitory patterns of PB on ω to ω-4 hydroxylations were similar (Fig. 2), it is difficult to conclude that only one P450 is responsible for these hydroxylation reactions. There are seven sequences similar to CYP52 in the *P. chrysosporium* genome, which are known to catalyze the hydroxylation of 1-dodecanol [21,32]. Furthermore, a multiple hydroxylation reaction at ω to ω-6-positions of lauric acid was not catalyzed by a single P450, but at least two P450s (CYP2K1 and CYP2M1) and probably more were involved in rainbow trout [5,41]. Thus, further experimental work will be required to determine the *P. chrysosporium* P450(s) involved in 1-dodecanol hydroxylation reactions.

Since at least 148 P450 genes exist in the *P. chrysosporium* genome [21], the functional diversity of P450 catalysis is expected. The present study showed a series of new P450-mediated metabolic reactions. The

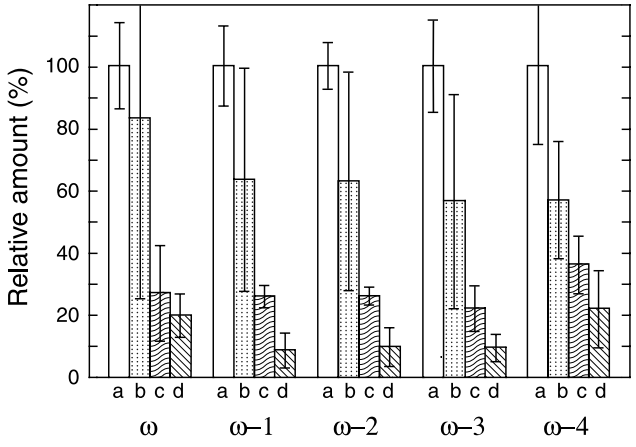

Fig. 3. Inhibitory effect of piperonyl butoxide on fungal hydroxylations of 1-dodecanol. *P. chrysosporium* was preincubated for 3 days and then 1-dodecanol was added (1 mM). Metabolites were analyzed using GCMS after an additional 4-day incubation. Piperonyl butoxide in acetone was added to the culture at the same time as 1-dodecanol was added. A 100-μL acetone solution was added to make final concentrations of a, 0 mM; b, 0.01 mM; c, 0.1 mM; and d, 1 mM.

physiological roles of these metabolic reactions are still unclear, but *P. chrysosporium* P450s were at least shown to have diverse and unique functions. The diversity of P450 molecular species might be a clue as to the functional characteristics of white-rot basidiomycetes in the degradation of lignin and xenobiotics. Extensive heterologous expressions of *P. chrysosporium* P450 genes are now underway.

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