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Biosynthetic Pathway for Veratryl Alcohol in the Ligninolytic Fungus *Phanerochaete chrysosporium*

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Veratryl alcohol (VA) is a secondary metabolite of white-rot fungi that produce the ligninolytic enzyme lignin peroxidase. VA stabilizes lignin peroxidase, promotes the ability of this enzyme to oxidize a variety of physiological substrates, and is accordingly thought to play a significant role in fungal ligninolysis. Pulse-labeling and isotope-trapping experiments have now clarified the pathway for VA biosynthesis in the white-rot basidiomycete *Phanerochaete chrysosporium*. The pulse-labeling data, obtained with ^{14}C-labeled phenylalanine, cinnamic acid, benzoic acid, and benzaldehyde, showed that radiocarbon labeling followed a reproducible sequence: it peaked first in cinnamate, then in benzoate and benzaldehyde, and finally in VA. Phenylalanine, cinnamate, benzoate, and benzaldehyde were all efficient precursors of VA in vivo. The isotope-trapping experiments showed that exogenous, unlabeled benzoate and benzaldehyde were effective traps of phenylalanine-derived ^{14}C. These results support a pathway in which VA biosynthesis proceeds as follows: phenylalanine → cinnamate → benzoate and/or benzaldehyde → VA.

The fungi that cause white rot of wood employ a variety of extracellular oxidative enzymes to cleave lignin. The best-characterized of these are the lignin peroxidases (LiPs) (5, 10, 18, 23, 25), which cleave polymeric lignin between C_α and C_β of its propyl side chain to give oxidized lignin oligomers (12, 13). Although other ligninolytic mechanisms exist, perhaps allowing certain LiP-negative fungi to degrade lignin (12, 22, 27, 34), LiPs are common to many white-rot basidiomycetes, and the evidence is strong that they play a role in ligninolysis (12). In LiP-producing fungi, lignin degradation coincides with LiP secretion, and in most cases both processes are functions of secondary (idiophasic) metabolism (10, 18).

White-rot fungi that produce LiPs generally secrete a secondary metabolite, veratryl alcohol (VA) (14, 19), which is a substrate for LiP (31). VA protects LiP against H_2O_2-mediated inactivation and has been proposed to act in vivo as a stabilizer for the enzyme (33). This stabilizing effect is probably responsible for the observation that exogenous addition of VA enhances LiP levels in certain white-rot fungi (7). VA also promotes the LiP-catalyzed oxidation of other molecules that are either inaccessible to the enzyme or recalcitrant, by themselves, to enzymatic attack (1, 11, 13, 15, 21, 24). Some researchers have proposed that VA participates in these reactions by acting as a redox shuttle between LiP and substrate (1, 15, 24). Others have presented evidence that only one of the redox states in the LiP catalytic cycle is capable of oxidizing certain substrates and that VA is required in these cases as a cosubstrate that allows the enzyme to complete its turnover (21, 30). VA thus plays diverse roles in white-rot fungal metabolism.

The biosynthetic pathway for VA was the subject of a study some years ago. Shimada et al. (28) performed ^{14}C isotope-trapping experiments with the ligninolytic fungus *Phanerochaete chrysosporium* ATCC 34541 and concluded from their results that the pathway proceeds as follows: phenylalanine → cinnamic acid → 3,4-dimethoxycinnamyl alcohol → veratryl glycerol → VA. New results from ^{14}C pulse-labeling and isotope-trapping experiments now show that only the first step in the originally proposed pathway is correct. Cinnamate is not metabolized to 3,4-dimethoxycinnamyl alcohol as proposed but rather is cleaved to benzoate, benzaldehyde, or a closely related derivative. One of these metabolites then serves as a substrate for hydroxylation and methylation to give VA.

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MATERIALS AND METHODS

Unlabeled reagents. Unlabeled benzoic acid and *trans*-cinnamic acid (Aldrich) were used as received. Benzaldehyde (Aldrich) was glass distilled and stored under N_2 in the dark at −5°C. 3,4-Dimethoxycinnamyl alcohol was prepared by ethylating 3,4-dimethoxycinnamic acid (Aldrich) and reducing the resulting ester with diisobutyl aluminum hydride (DIBAL-H; Aldrich) (26). The resulting crude 3,4-dimethoxycinnamyl alcohol was recrystallized twice from petroleum ether-ethyl acetate.

^{14}C-labeled compounds. [U-^{14}C]phenylalanine (474 mCi mmol^{-1}; Amersham) and [α-^{14}C]benzoic acid (40.0 mCi mmol^{-1}; ICN), both with radiochemical purities of >98%, were used as received. [*ring*-^{14}C]benzaldehyde (7.3 mCi mmol^{-1}; Sigma) was >90% radiochemically pure, the major ^{14}C-labeled impurity consisting of benzoate. Attempts to obtain a more homogeneous preparation by preparative high-performance liquid chromatography (HPLC) did not result in significant purification, probably because benzaldehyde tends to autoxidize.

[*trans*-U-^{14}C]cinnamic acid was prepared from [U-^{14}C]phenylalanine by deamination with phenylalanine ammonia-lyase (PAL; grade 1 [Sigma], from *Rhodotorula glutinis*). Labeled phenylalanine (13.8 μCi) was dissolved in 0.5 ml of Tris HCl (pH 9.0; 0.1 M), whereupon PAL (0.1 U) was added and the solution was magnetically stirred for 5 h. The solution was then acidified to approximately pH 2 with HCl and extracted six times with 0.75 ml of ethyl acetate. The combined organic phases were dried over anhydrous Na_2SO_4 and concentrated under a stream of N_2. The resulting crude [^{14}C]cinnamic acid was purified by preparative thin-layer chromatography on a plate of silica gel 60 (20 by 20 by 0.1 cm; Merck) in a 50:8

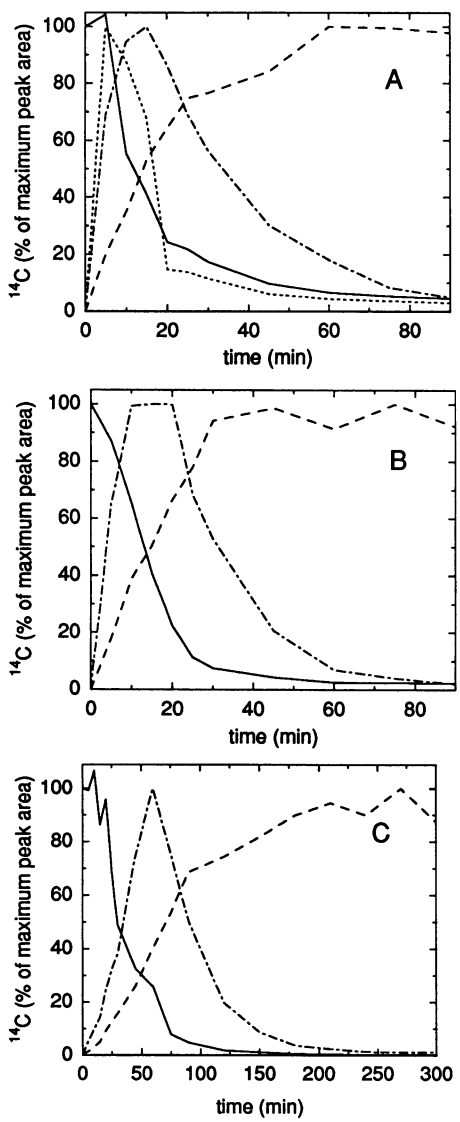

FIG. 1. Time course of ^{14}C incorporation from various compounds into VA precursors and VA by *P. chrysosporium* ATCC 34541. Pulse-labeling kinetics are shown as a percentage of the maximum peak area for each metabolite. (A) ^{14}C incorporation from [^{14}C]phenylalanine. The curves show labeling in phenylalanine (——), cinnamic acid (· · ·), benzoic acid (— · —), and VA (– – –). The maximum peak areas for each metabolite were as follows: phenylalanine, 8.80×10^4 dpm (at time zero); cinnamic acid, 3.34×10^3 dpm; benzoic acid, 1.72×10^4 dpm; and VA, 3.39×10^4 dpm. (B) ^{14}C incorporation from [^{14}C]cinnamic acid. The curves show labeling in cinnamic acid (——), benzoic acid (— · —), and VA (– – –). The maximum peak areas for each metabolite were as follows: cinnamic acid, 7.72×10^4 dpm (at time zero); benzoic acid, 1.79×10^4 dpm; and VA, 5.41×10^4 dpm. (C) ^{14}C incorporation from [^{14}C]benzoic acid. The curves show labeling in benzoic acid (——), benzaldehyde (— · —), and VA (– – –). The maximum peak areas for each metabolite were as follows: benzoic acid, 1.06×10^5 dpm (at time zero); benzaldehyde; 2.42×10^4 dpm; and VA, 5.18×10^4 dpm.

mixture of formic acid-saturated benzene and methanol. The product (approximately 90% radiochemical purity) was then further purified by preparative reversed-phase HPLC on a styrene divinylbenzene column (Hamilton PRP-1; 5 μm; 4.1 by 150 mm). The column was preequilibrated with methanol-H_2O (3:1) containing 0.1% H_3PO_4, and [^{14}C]cinnamic acid was eluted with a 25-min linear gradient (0.8 ml min^{-1}, room temperature) to 100% methanol in the presence of 0.1% H_3PO_4. The pooled peak fractions, which eluted between 14 and 17 min, were diluted to about 30 ml with aqueous 0.1% H_3PO_4 and extracted three times with 40 ml of CH_2Cl_2. The combined organic extracts were dried over anhydrous Na_2SO_4, evaporated to dryness on a rotary vacuum evaporator at room temperature, and redissolved in H_2O to give [^{14}C]cinnamic acid with a radiochemical purity of >99%, as determined by analytical HPLC.

Metabolic experiments. Two isolates of *P. chrysosporium* were used: ATCC 34541 (ME-446) and ATCC 24725 (BKM-F-1767). Cultures (10.0 ml in 125-ml Erlenmeyer flasks) in N-limited medium with basal trace elements (17) were inoculated with conidia and grown without agitation at 39°C under O_2. The resulting mycelial mats were used for ^{14}C-labeling studies.

For pulse-labeling experiments, 6-day-old cultures of ATCC 34541 were used. ^{14}C-labeled phenylalanine, cinnamate, benzoate, or benzaldehyde (2.2×10^{-3}, 1.9×10^{-3}, 3.1×10^{-2}, or 9.2×10^{-2} μmol, respectively) was pipetted onto the mats of eight replicate cultures in 1.0 ml of sterile water. Samples (0.175 ml) were taken from underneath the mats of the eight replicates at the following times after ^{14}C addition: 5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, 270, and 300 min. The samples from each time point were immediately pooled and frozen by immersing the sample vial in dry ice-ethanol. The cultures were maintained under O_2 between samples.

The isotope-trapping experiments employed 6-day-old cultures of ATCC 34541 or 3-day-old cultures of ATCC 24725. Labeled phenylalanine (1.8×10^{-3} μmol for ATCC 34541; 2.2×10^{-3} μmol for ATCC 24725) and suspected intermediary metabolites (2.0 mg, unlabeled), as indicated, were pipetted together in 1.0 ml of sterile water (adjusted to pH 7.0) onto the mats of four replicate cultures. At intervals, samples (0.3 ml) were taken and pooled from the replicates as described above. All cultures were maintained under O_2 between samples.

The course of VA synthesis from labeled benzoate (1.8×10^{-2} μmol) by ATCC 24725 was monitored by the same protocol, using 3-day-old cultures without the addition of an isotope trap.

Metabolite analysis. The previously frozen samples were thawed, and aliquot portions (0.51 ml) were filtered through 0.45-μm-pore-size microcentrifuge filters. The filters were rinsed twice by centrifugation with 0.045 ml of acetonitrile-acetic acid (85:15), and the rinses were combined with the original filtrates. From each combined filtrate, 0.50 ml was removed and injected for HPLC analysis on the PRP-1 styrene divinylbenzene column described above. The mobile phases consisted of water–1.0% acetic acid (A) and acetonitrile–1.0% acetic acid (B), delivered at 1.0 ml min^{-1} and ambient temperature according to the following elution program: 15% B from 0 to 15 min, followed by a linear gradient to 50% B from 15 to 40 min, followed by a linear gradient to 100% B from 40 to 42 min. Fractions (1.0 ml) were collected and assayed for ^{14}C in Polyfluor cocktail (Packard) in an LKB Wallac 1214 liquid scintillation counter. The elution positions of unlabeled isotope traps were monitored with an in-line UV detector at 260 nm.

In pulse-labeling experiments with [^{14}C]phenylalanine as the radiocarbon source, a portion of each sample was treated with PAL before HPLC analysis to convert residual [^{14}C]phenylalanine to [^{14}C]cinnamate. The difference in [^{14}C]cinnamate

between PAL-treated and untreated samples then afforded a measure of the phenylalanine remaining at each time in the pulse-labeling experiment. This approach was taken because phenylalanine itself eluted with the void volume of the HPLC column.

^{14}C peak areas for the individual metabolites were calculated and corrected, when necessary, for the loss of radiolabeled β and γ carbons from [U-^{14}C]phenylalanine and [U-^{14}C]cinnamate. Time courses of radiolabeling are reported as point-to-point graphs, i.e. without any curve fitting, and scatter due to variability between samples is apparent in the plots. Early in this investigation, we considered the possibility that agitated pellet cultures of *P. chrysosporium* might give less scatter than stationary cultures in pulse-labeling experiments, but we rejected this approach for three reasons: (i) the original work by Shimada et al. (28) was done with stationary cultures of ATCC 34541, and our goal was to reproduce their conditions as closely as possible; (ii) little is known about the secondary metabolism of ATCC 34541 in agitated culture; and (iii) although agitated cultures of ATCC 24725 exhibit lignin-olytic metabolism (16), these systems are dissimilar from the culture method used by Shimada et al. because they require the addition of surfactants or other amendments.

RESULTS

Pulse-labeling studies. Cultures of *P. chrysosporium* ATCC 34541 rapidly converted phenylalanine to VA. The results of typical experiments are shown in Fig. 1A and Fig. 2. In the Fig. 1 experiment, when 0.20 μM [U-^{14}C]phenylalanine was supplied to the fungus, incorporation of ^{14}C into extracellular VA peaked at 60 min, by which time 50% of the benzylic ^{14}C initially added could be found in this metabolite. In addition, three transiently labeled extracellular compounds were observed in the HPLC analyses. At ≤5 min after the [^{14}C]phenylalanine pulse, a labeled metabolite running identically with cinnamic acid peaked, at which time it contained 4% of the ^{14}C initially added, and then fell off rapidly. Then, at 15 min, two metabolites eluting with benzoic acid and benzaldehyde peaked and declined. The maximal yields of initially added benzylic ^{14}C coeluting with benzoate and benzaldehyde were 25 and 3%, respectively. The kinetics of ^{14}C labeling in these two benzylic metabolites were too similar to indicate which compound was the precursor of the other. 3,4-Dimethoxycinnamyl alcohol, which was previously proposed to be an intermediate in VA biosynthesis (28), was not observed in any of the HPLC analyses.

ATCC 34541 also converted cinnamic acid to VA, and with kinetics similar to those observed when phenylalanine was used as the precursor (Fig. 1B). When 0.17 μM [U-^{14}C]cinnamic acid was supplied to the fungus, incorporation of ^{14}C into VA peaked at 45 min, by which time 90% of the benzylic ^{14}C initially added could be found in this metabolite. The greater incorporation of ^{14}C into VA from cinnamate, compared with phenylalanine, can probably be attributed to the fact that the amino acid has metabolic destinations other than VA, e.g., in protein synthesis. Protein turnover in idiophasic *P. chrysosporium* occurs rapidly, with a half-time of about 10 h (8). Benzoate and benzaldehyde were observed as transient metabolites in this experiment, both peaking at 10 to 20 min and then declining. The maximal yields of initially added benzylic ^{14}C found in benzoate and benzaldehyde were 30 and 7%, respectively, and again the labeling kinetics for these two metabolites were too similar to infer a precursor-product relationship. Labeled 3,4-dimethoxycinnamyl alcohol was not observed in any of the analyses.

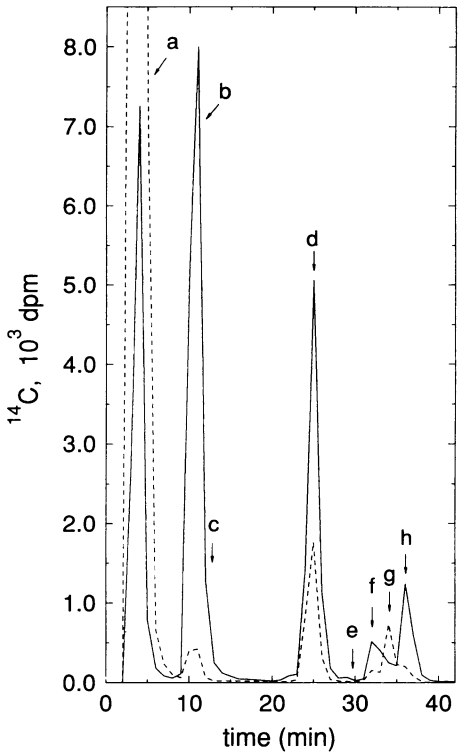

FIG. 2. HPLC analysis of the extracellular fluid from cultures of *P. chrysosporium* ATCC 34541 supplied with [U-^{14}C]phenylalanine. The radiograms shown are of samples taken 10 min (——) and 1 h (– – –) after addition of [^{14}C]phenylalanine. The designations a to h correspond to the elution positions of authentic standards of phenylalanine (a), VA (b), benzyl alcohol (c), benzoic acid (d), 3,4-dimethoxycinnamyl alcohol (e), veratraldehyde (f), cinnamic acid (g), and benzaldehyde (h). In this experiment, maximal VA labeling occurred 3 h after the [^{14}C]phenylalanine pulse.

The fungus also converted benzoate to VA (Fig. 1C). The low specific activity of the benzoate available to us made it necessary to administer a much more dilute ^{14}C pulse than in the foregoing experiments, which resulted in a slower time course of radiolabeling. When 2.8 μM [α-^{14}C]benzoic acid was supplied to the fungus, incorporation of ^{14}C into VA peaked at 200 to 300 min, by which time 49% of the radiolabel initially added could be found in this metabolite. This relatively low maximum, compared with the result using [^{14}C]cinnamate as the source, is probably due to the diffuse nature of the [^{14}C]benzoate pulse. Benzaldehyde was observed as a transient metabolite in this experiment, peaking at 60 min and then declining. The maximal yield of initially added benzylic ^{14}C found in benzaldehyde was 23%. The HPLC peak corresponding to [^{14}C]cinnamate did not appear when [^{14}C]benzoate was used as a precursor.

Finally, ATCC 34541 converted benzaldehyde to VA (data not shown). When 8.4 μM [*ring*-^{14}C]benzaldehyde was supplied to the fungus, the incorporation of ^{14}C into VA reached 31% of the radiolabel initially added after 300 min. The HPLC peak corresponding to [^{14}C]cinnamate did not appear when [^{14}C]benzaldehyde was used as a precursor. Trace levels of [^{14}C]benzoate were found in the HPLC analyses of early samples, but it was not possible to determine whether these resulted from fungal metabolism or autoxidation of benzaldehyde.

Variability was observed between experiments in the absolute rate of VA synthesis. For example, in some repetitions of the above work, ^{14}C incorporation into VA from phenylalanine peaked at 3 h rather than 1 h (Fig. 2; Fig. 3B). However, the same pulse-labeling sequence was always observed regardless of the absolute rate: radiocarbon labeling peaked first in cinnamate, then in benzoate or benzaldehyde, and finally in VA.

Isotope-trapping studies. When [^{14}C]phenylalanine was supplied to cultures of ATCC 34541, the appearance of labeled benzoate and benzaldehyde as intermediary metabolites was transient. However, when unlabeled benzoate was included in such experiments as an isotope trap, a different result was obtained: the unlabeled benzoate was progressively reduced to benzaldehyde by the fungus, and ^{14}C was trapped in both metabolites. The increase in radiolabeled benzoate plus benzaldehyde was not transient, but rather continuous (Fig. 3). In early samples, most of the trapped ^{14}C was present in benzoate, whereas by the end of the experiment the exogenous benzoate trap had been reduced to benzaldehyde, and most of the trapped ^{14}C was present in this compound.

The effect of exogenous benzoate in the fungal cultures was not limited to isotope trapping; it also clearly slowed the entire pathway, presumably via some uncharacterized regulatory phenomenon. This effect is evident from the observation that ^{14}C accumulation in benzoate plus benzaldehyde at 1 h in cultures with isotope trap was only about 25% of the labeling at this time in cultures with no trap. However, by 3 h, the ability of benzoate and benzaldehyde to trap phenylalanine-derived ^{14}C was readily apparent. ^{14}C labeling of benzoate and benzaldehyde in the controls without trap had declined by this time, whereas radiolabel continued to accumulate in the two metabolites when trap was included (Fig. 3A).

^{14}C accumulation into VA was lower in the presence of exogenous benzoate than it was in the absence of trap. In early samples, this effect is due at least in part to the inhibitory effect on the overall pathway by benzoate or benzaldehyde. However, in the 3- and 4-h samples, the decrease in VA labeling can probably be attributed as well to the dilution of phenylalanine-derived ^{14}C in the benzoate or benzaldehyde pools by the isotope traps (Fig. 3B).

^{14}C was also trapped when unlabeled benzaldehyde was included in [^{14}C]phenylalanine labeling experiments. The results were similar to those shown above for benzoate, except that the benzaldehyde trap was not detectably oxidized to benzoate by fungal cultures, and ^{14}C was therefore trapped only in benzaldehyde (data not shown).

An isotope-trapping experiment was also attempted with unlabeled cinnamate, with the result that [^{14}C]VA synthesis was completely inhibited and radiolabel failed to appear in any intermediary metabolite. A likely explanation for this finding is that cinnamate inhibits PAL, the enzyme responsible for cinnamate formation from phenylalanine. PAL from *P. chrysosporium* has not been characterized, but inhibition by cinnamate is typical of PALs from all sources (2).

Finally, an isotope-trapping experiment was attempted with 3,4-dimethoxycinnamyl alcohol, the compound proposed earlier by Shimada et al. (28) to be a pathway intermediate. This compound failed to trap any radiolabel and did not inhibit the formation of VA.

The isotope-trapping results with benzoate and benzaldehyde were confirmed with another strain of *P. chrysosporium*, ATCC 24725. This experiment was conducted on a longer schedule than investigations with the other strain, and on this time scale the reduction of benzaldehyde to benzyl alcohol became significant in fungal cultures. Otherwise, the results

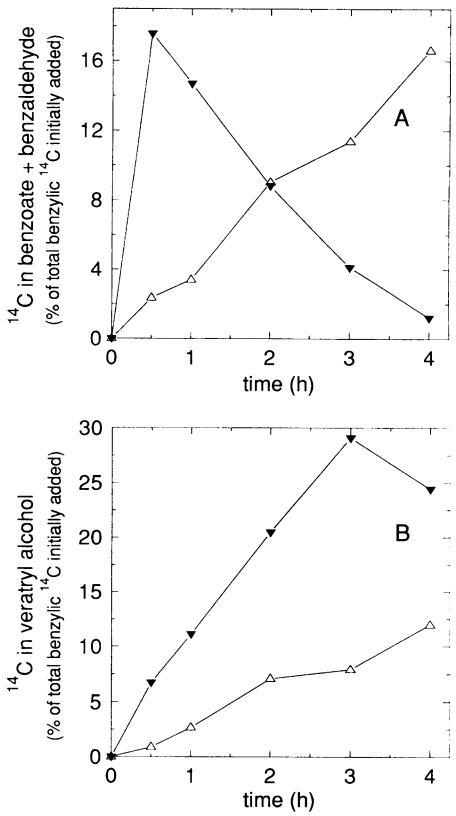

FIG. 3. Kinetics of radiolabel incorporation into [^{14}C]phenylalanine-derived metabolites in an isotope-trapping experiment with *P. chrysosporium* ATCC 34541. (A) ^{14}C in benzoic acid and benzaldehyde in the presence (△) and absence (▼) of unlabeled benzoate added as an isotope trap. (B) ^{14}C in VA in the presence (△) and absence (▼) of the benzoate isotope trap.

(Table 1) were similar to those obtained with ATCC 34541. Both [^{14}C]phenylalanine and [^{14}C]benzoate were efficiently converted to VA by the fungus. The addition of unlabeled benzoate to cultures given [^{14}C]phenylalanine suppressed VA synthesis significantly and caused radiolabel to accumulate in the original trap and its reduction products, benzaldehyde and benzyl alcohol. The addition of unlabeled benzaldehyde to cultures given [^{14}C]phenylalanine also suppressed VA synthesis significantly and caused radiolabel to accumulate in benzaldehyde and benzyl alcohol. The unlabeled benzaldehyde trap was not detectably oxidized in vivo to benzoate, and no label was trapped in benzoate.

DISCUSSION

These experiments establish the intermediacy of benzoate and/or benzaldehyde in the metabolic pathway that leads from phenylalanine to VA. First, transient metabolites with HPLC mobilities identical to those of benzoate and benzaldehyde were formed from both of the proposed upstream metabolites, phenylalanine and cinnamate. Second, benzoate and benzaldehyde functioned as ^{14}C traps when labeled phenylalanine was supplied to the fungus. The isotope trapping experiments with exogenous benzoate also showed that the HPLC method we used to identify benzoate was valid, because they resulted in a shift of phenylalanine-derived ^{14}C into radiolabeled HPLC peaks that ran identically with two benzoate reduction prod-

TABLE 1. ^{14}C labeling of metabolites derived from phenylalanine and benzoate in cultures of *P. chrysosporium* (ATCC 24725), with and without isotope traps

Compound(s) added to cultures	Time in culture (h)	^{14}C in fungal metabolite (% of benzylic ^{14}C initially added)				
		Benzoate	Benzaldehyde	Benzyl alcohol	Total in benzyl metabolites	VA
[^{14}C]phenylalanine	6	0.2	0.1	0.2	0.5	15.7
	24	0.3	0.1	0.4	0.8	20.1
[^{14}C]phenylalanine + unlabeled benzoate	6	4.5	4.5	0.7	9.7	10.6
	24	3.2	6.9	5.2	15.3	7.3
[^{14}C]phenylalanine + unlabeled benzaldehyde	6	0.3	5.0	6.2	11.5	4.2
	24	0.3	0.4	13.2	13.9	2.0
[^{14}C]benzoate	6	0.2	0.1	0.2	0.5	32.2
	24	0.1	0.1	0.4	0.6	18.4

ucts: benzaldehyde and benzyl alcohol. It is extremely unlikely that some other, misidentified metabolite running at the position of benzoate would exhibit this behavior. Finally, the proposed pathway is supported by our observation that benzoate and benzaldehyde themselves served as efficient precursors of VA.

Although the pulse-labeling results fail to establish a precursor-product relationship between benzoate and benzaldehyde, the results obtained with unlabeled isotope traps show that benzoate was rapidly reduced in fungal cultures, whereas benzaldehyde was not detectably oxidized to benzoate. It is therefore a plausible inference that benzoate precedes benzaldehyde in the biosynthetic sequence leading to VA. However, the resolution of our experimental method is limited by the fact that it only detects extracellular metabolites. We still lack information about the pool sizes and intracellular compartmentation of the pathway intermediates and cannot rule out the possibility that benzaldehyde might precede benzoate or that one of the two metabolites might be in equilibrium with the main pathway without lying directly on it.

The evidence is strong that cinnamate is a pathway intermediate. A transient metabolite derived from phenylalanine, and exhibiting an HPLC mobility identical to that of cinnamate, was detected in very early samples from the [^{14}C]phenylalanine pulse-labeling experiment. [^{14}C]cinnamate was a very efficient precursor for VA biosynthesis, and in pulse-labeling experiments it yielded the same transient metabolites as phenylalanine did: benzoate and benzaldehyde. We and others have observed that *P. chrysosporium* produces PAL, the enzyme required for the conversion of phenylalanine to cinnamate (6, 29). The only caveats required are that the identification of cinnamic acid in pulse-labeling experiments depended on HPLC mobility alone and that an isotope-trapping experiment with unlabeled cinnamate was not possible.

In sum, the pathway in best agreement with the data proceeds as follows: phenylalanine → cinnamate → benzoate and/or benzaldehyde → VA. Even considering the uncertainties discussed above, it is evident that *P. chrysosporium* cleaves the phenylalanine propyl side chain before it hydroxylates and methylates the aromatic ring. This sequence is the opposite to that proposed by Shimada et al. (28).

Some steps in the originally proposed pathway (28) do have a readily confirmed experimental basis. If 3,4-dimethoxycinnamyl alcohol is supplied to idiophasic *P. chrysosporium*, it is expected to undergo hydroxylation by LiP, yielding veratryl glycerol, which in turn will undergo LiP-catalyzed cleavage to give veratraldehyde (9). Veratraldehyde will then be reduced in culture to give VA (20). However, this pathway could have biosynthetic significance only if there existed a prior step in which the fungus formed 3,4-dimethoxycinnamyl alcohol from phenylalanine, a step for which our pulse-labeling and isotope-trapping data provide no evidence.

Harper and coworkers have recently shown that *P. chrysosporium* possesses two mechanisms for the methylation of hydroxylated aromatic compounds, one utilizing *S*-adenosylmethionine as the donor and the other employing methyl chloride. The methoxyl carbons of VA in *P. chrysosporium* are obtained through the methyl chloride-dependent pathway (3, 4, 14). Our data indicate that the first substrate for this methylation reaction is a benzylic (C_7) metabolite bearing an aromatic hydroxyl group.

Our results show that *P. chrysosporium* cleaves cinnamate to give benzoate, benzaldehyde, or a closely related compound. The mechanisms for this reaction remain to be elucidated, but

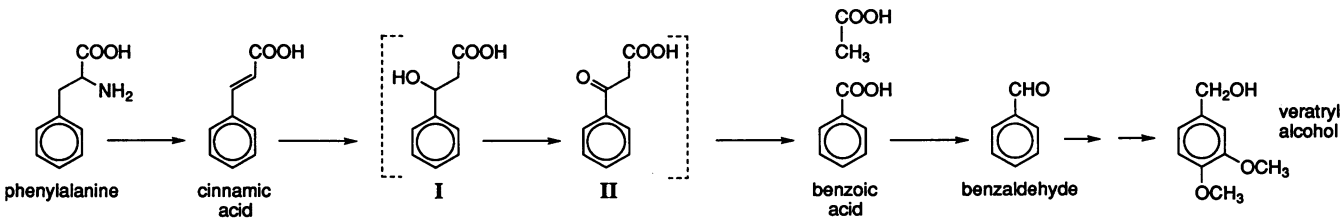

FIG. 4. Likely biosynthetic pathway for VA in *P. chrysosporium*. The intermediates I and II, shown in brackets, are hypothetical. For simplicity, the Claisen cleavage of II is shown to occur with a free carboxyl group present at C_γ of the substrate, but in actuality the reaction would require prior esterification at C_γ (e.g., with coenzyme A) to avoid destabilization of the intermediate enolate. A pathway shorter than the one shown is possible: an ester of I could undergo Claisen cleavage to give acetate and benzaldehyde.

an obvious possibility stands out: cinnamate might be hydrated across the C_α-C_β double bond to give 3-hydroxy-3-phenylpropionate (Fig. 4, intermediate I), which could undergo an enzymatic reversed Claisen condensation to give benzaldehyde and acetate. Alternatively, 3-hydroxy-3-phenylpropionate might be oxidized at C_α to give 3-oxo-3-phenylpropionate (Fig. 4, intermediate II), which could undergo enzymatic Claisen cleavage to yield benzoate and acetate. Sequences of this type are common, e.g., in the β-oxidation of fatty acids, and represent a general biochemical strategy for the cleavage of alkenes (32).

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