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Biosynthesis of the Secondary Metabolite Veratryl Alcohol in Relation to Lignin Degradation in *Phanerochaete chrysosporium*

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Abstract. The lignin-degrading basidiomycete *Phanerochaete chrysosporium* synthesizes veratryl alcohol (3,4-dimethoxybenzyl alcohol) via phenylalanine, 3,4-dimethoxycinnamyl alcohol and veratrylglycerol. Study of the conversion of 3,4-dimethoxycinnamyl alcohol to veratrylglycerol and veratryl alcohol showed is to be (a) catalyzed by a secondary metabolic system, (b) markedly suppressed by culture agitation, and (c) strongly inhibited by L-glutamate. The amount of veratryl alcohol synthesized de novo was positively correlated with the O_2 concentration after primary growth. Other work has shown that the cinnamyl alcohol terminal residue in a lignin substructure model compound is degraded via arylglycerol and benzyl alcohol structures in ligninolytic cultures of *P. chrysosporium*, and that the ligninolytic system exhibits traits (a)–(c) above. Ligninolytic activity is also strongly and positively correlated with O_2 concentration. The results here suggest, therefore, that the actual biosynthetic secondary metabolic product is 3,4-dimethoxycinnamyl alcohol, but that this is degraded by the ligninolytic system to veratryl alcohol via veratrylglycerol. Veratryl alcohol is only slowly metabolized by the fungus, and accumulates.

Key words: White-rot fungi – Secondary metabolism – Wood decay – Mycelial pellets – Fungus physiology – L-Glutamic acid repression – Phenylalanine

A recent investigation has shown that the lignin-degrading basidiomycete *Phanerochaete chrysosporium* metabolizes the lignin substructure model compound methyl dehydrodiconiferyl alcohol (I) via the initial transformations A and B in Fig. 1 (Nakatsubo et al. 1981). To study reactions A and B in more detail, we have used the simple compound 3,4-dimethoxycinnamyl alcohol (II). When added to ligninolytic cultures, II was rapidly converted to veratrylglycerol [1-(3,4-dimethoxyphenyl)-glycerol, III] and veratryl alcohol (3,4-dimethoxybenzyl alcohol, IV) (see Fig. 2). Because veratryl alcohol is also known to be synthesized de novo by *P. chrysosporium* (Lundquist and Kirk 1978), the question arose whether the de novo product might also be synthesized via 3,4-dimethoxycinnamyl alcohol and veratrylglycerol. Results here show that it is, and that the biosynthetic pathway and the lignin-degrading pathway apparently overlap.

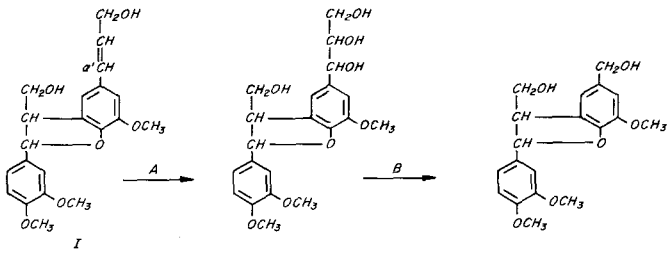

Fig. 1. Initial steps (**A** and **B**) in the degradation of methyl dehydrodiconferyl alcohol (I) by ligninolytic cultures of *Phanerochaete chrysosporium*. The C_α-aldehyde is interconverted with the C_α-alcohol in the product after step B (Nakatsubo et al. 1981)

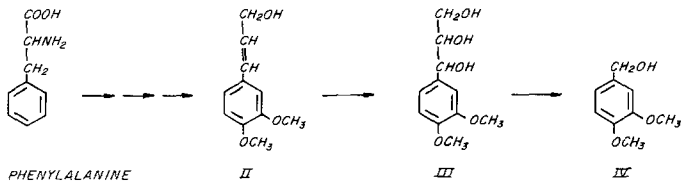

Fig. 2. Conversion of phenylalanine to veratryl alcohol (IV), via 3,4-dimethoxycinnamyl alcohol (II) and veratrylglycerol (III) in 6-day-old cultures of *Phanerochaete chrysosporium*

Materials and Methods

Experimental Cultures. Phanerochaete chrysosporium Burds. (ME-446; ATCC 35540) was obtained from the Center for Forest Mycology Research, Forest Products Laboratory, Forest Service, U.S. Department of Agriculture, Madison WI, and was maintained at 30° C on 2% malt agar slants. The basal medium contained mineral salts and vitamins at the concentrations previously reported (Kirk et al. 1978) plus L-asparagine (0.62 mM) and NH_4NO_3 (0.62 mM) as nitrogen sources, and D-glucose (56 mM) as carbon source. Cultures were buffered at pH 4.5 with poly-(acrylic acid) (Aldrich Chem. Co. No. 18, 128-5, MW 250,000), 0.01 M in carboxyl, or with 0.01 M sodium 2,2-dimethylsuccinate (Fenn and Kirk 1979). Culture inoculation was with a conidial suspension (Kirk et al. 1978). Eight or 10 ml cultures in 100 ml Erlenmeyer flasks, or 20 ml cultures in 300 ml Erlenmeyer flasks, were incubated under 100% O_2 without agitation at 37–39°C, unless otherwise stated. Five or six day old cultures were used for the experiments; these possessed maximum lignin-degrading activity (Fenn and Kirk 1981).

Chemicals. 3,4-Dimethoxycinnamyl alcohol (II) was prepared by reducing the methyl ester of 3,4-dimethoxycinnamic acid with $LiAlH_4$ in dry tetrahydrofuran at −35°C (m.p. 74°C, M^+, 194 *m/e*).

Threo-veratrylglycerol (III). II (0.6 mmol) in 3 ml of dioxane was added dropwise with stirring to 3 ml of pyridine containing 0.6 mmol of OsO_4, and the mixture held overnight in the dark. After completion of the reaction, as determined by thin layer chromatography (tlc, < 12 h), the

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Non-standard abbreviation: tlc – thin layer chromatography

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mixture was evaporated to a syrup, dissolved in 5 ml of ethanol, and treated with 6 mmol of Na_2SO_3 in 5 ml of water to reductively cleave the osmium complex. The byproduct osmic acid was removed by filtration, and the solution was evaporated to a syrup. Purification by preparative silica gel tlc (5% methanol in CH_2Cl_2) yielded crystalline III, which was recrystallized from methanol: CH_2Cl_2. Figure 3 includes the ^{1}H-nmr spectrum of the acetylated product (M^+, 354 *m/e*; $\varepsilon_{280\,nm}$ 2.8×10^3 in ethanol).

For comparison, an *erythro/threo* mixture of III was prepared by oxidation of II with m-chloroperbenzoic acid followed by treatment with methanolic sodium methoxide (triacetate: M^+, 354 *m/e*). Assignments of ^{1}H-nmr signals for the *threo* and *erythro* forms were made by comparison (cf. Higuchi et al. 1974; Lundquist 1979).

Other unlabeled chemicals were purchased reagent grade (Nakarai Chemicals Co., Tokyo).

3,4-Dimethoxycinnamyl alcohol-4-[O^{14}CH$_3$] (62 μCi/mmol) was prepared from coniferyl alcohol (Nakamura and Higuchi 1976) by methylation in ether with [^{14}C] diazomethane prepared from N [^{14}C]-methyl-N-nitroso-p-toluenesulfonamide (Daiichi Pure Chemicals Co., Ltd., Tokyo).

L-Phenylalanine-[U-^{14}C] (382 mCi/mmol) and [^{14}CH$_3$] L-methionine (5.8 mCi/mmol) were purchased from Daiichi Pure Chemicals Co., Ltd. (Tokyo).

Extraction of Compounds from Cultures. Cultures were inactivated by addition of an equal volume of ethanol, and the mycelial mats removed by glass wool filtration and rinsed well with ethanol. Combined filtrates and washings were evaporated to about 2 ml, to which 1 ml each of saturated NaCl and $NaHCO_3$ were added. The basic solutions were exhaustively extracted with ethyl acetate. The $NaHCO_3$ was omitted for extraction of ferulic and isoferulic acids. Extracts were dried over Na_2SO_4 and evaporated to dryness.

Identification and Quantitation of Compounds. Metabolic products were identified by comparison with authentic samples by ^{1}H-nmr and UV spectroscopy, and by mass spectrometry. Thereafter, they were routinely identified by silica gel tlc comparisons with authentic samples (Kieselgel 60 PF_{254}, Merck), with 5–8% methanol in CH_2Cl_2 or ethylacetate: n-hexane (1:1 or 1:2 by vol) as developing solvent. Alternatively, compounds were routinely identified by high performance liquid chromatography, HPLC) with a μ-Bondapak C-18 column (8 mm i.d. × 10 cm long; solvent, 50% methanol; flow rate, 1 ml/min; detector, UV absorbance at 254 nm).

Quantitative determinations of unlabeled products were also done with HPLC, using the above conditions and standard calibration curves for each product.

Extracts containing radiolabeled products were co-chromatographed with authentic compounds using silica gel tlc. Compounds were located under UV light, eluted with dioxane, and their radioactivity determined by liquid scintillation spectrometry.

The following instruments were used: R-22 Hitachi High Resolution NMR spectrometer (90 MHz); Shimadzu-LKB 9000 gas chromatograph-mass spectrometer (70 eV); Hitachi 124 double beam UV-Visible spectrometer; Waters 440 high performance liquid chromatograph, and Beckman LS-100 liquid scintillation spectrometer.

Results

Biosynthesis of Veratryl Alcohol

Six-day-old cultures of *Phanerochaete chrysosporium* rapidly converted 3,4-dimethoxycinnamyl alcohol (II) to vertrylglycerol (III) and veratryl alcohol (IV) (Fig. 2). Six hours after addition of 2.7×10^4 dpm (5 μmol) of 3,4-dimethoxycinnamyl alcohol-[4-O^{14}CH$_3$] per 10 ml culture, approximately 19% and 15% of the ^{14}C was found by tlc in compounds III and IV, respectively. Prolonged incubation resulted in disappearance of III and accumulation of IV. Approximately 20 mg of III were isolated by tlc 16 h after addition of a total of 200 mg of II to 6 day old 20 ml cultures (10 mg/culture). The product was acetylated and the purified triacetate was found by ^{1}H-nmr to be a mixture of *threo* and *erythro* isomers in a ratio of approximately 2:1 (Fig. 3) (cf. Higuchi et al. 1974; Lundquist 1979; Nakatsubo et al. 1981).

In the absence of exogenously added 3,4-dimethoxycinnamyl alcohol (II), small amounts of veratrylglycerol (III) and substantial amounts of veratryl alcohol (IV) were observed. Compound III was identified by comparison of its triacetate with that of authentic III by mass spectrometry. III was detected in 4-day-old cultures, whereas IV was found only after 5 days' incubation (Table 1).

When [U-^{14}C] L-phenylalanine was added to 6-day-old cultures, veratryl alcohol was rapidly labeled. Five hours after addition of 2.6 nmol of the labeled phenylalanine (2.2×10^6 dpm), only veratryl alcohol was detected as a labeled product by tlc. Shorter incubation periods disclosed the transient appearance of labeled veratrylglycerol (Fig. 4). These results were confirmed by the use of unlabeled carriers to facilitate trapping, which also allowed 3,4-dimethoxycinnamyl alcohol (II) to be established as an intermediate (Table 2). Trapping experiments indicated that one or both of the methyl groups of II came from methionine, and that ferulic acid but not isoferulic acid was also labeled by [^{14}CH$_3$] methionine (Table 2).

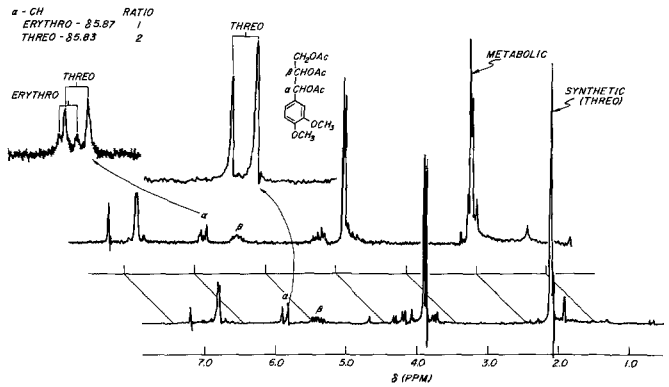

Fig. 3. ^{1}H-nmr spectra of the triacetates of authentic *threo*-veratrylglycerol and of the product from conversion of 3,4-dimethoxycinnamyl alcohol by *Phanerochaete chrysosporium*. Spectra were taken in $CDCl_3$ with tetramethylsilane as internal standard

Table 1. Production of veratrylglycerol and veratryl alcohol as natural secondary metabolites in cultures of *Phanerochaete chrysosporium*[a]

Culture age (day)	Amount of metabolite (nmol/10 ml culture)	
	Veratrylglycerol (III)	Veratryl alcohol (IV)
1–3	0	0
4	71	0
5	71	1786
6	179	3887

[a] Stationary cultures (10 ml) were grown under 100% O_2 at 37–39° C. Duplicate cultures were harvested each day and inactivated by the addition of 10 ml of ethanol. Products were determined after extraction with ethylacetate as described in the text

Culture Factors Influencing Biosynthesis of Veratryl Alcohol

Veratrylglycerol (III) and veratryl alcohol (IV) were not found in cultures before days 4 and 5, respectively (Table 1). Experiments showed that the enzyme system for synthesis of these compounds from 3,4-dimethoxycinnamyl alcohol (II) was not induced by II; addition of cycloheximide to 6-day-old cultures prior to addition of II did not prohibit formation of III and IV.

Similarly, preincubation with II did not significantly change the time of appearance of the enzyme system converting II to III and IV.

The effect of culture agitation on biosynthesis of IV was examined. Cultures grown on a rotary shaker (2.5 cm radius, 130 rpm) only slowly metabolized II, and no IV was detected. Growth was in pellet form. In sharp contrast, stationary cultures (mat form) rapidly converted II to IV.

Table 2. Incorporation of ^{14}C from labeled phenylalanine or methionine into products in 6-day-old cultures of *Phanerochaete chrysosporium*[a]

Substrate	Product	^{14}C Incorporation %
Phenylalanine	3,4-Dimethoxy-cinnamyl alcohol (II)	31.9
Phenylalanine	Veratrylglycerol (III)	2.2
Phenylalanine	Veratryl alcohol (IV)	31.2
Methionine	3,4-Dimethoxy-cinnamyl alcohol (II)	13.2
Methionine	Ferulic acid	8.9
Methionine	Isoferulic acid	0

[a] Each 10 ml culture received 2.6 nmol of L-phenylalanine-[U-^{14}C] (2.2×10^6 dpm) or 86 nmol of L-methionine-[$^{14}CH_3$] (1.1×10^6 dpm), and 1–2 mg/culture of unlabeled carrier (product). After 1 h (phenylalanine) or 15 min (methionine) at 37–39° under 100% O_2, cultures were extracted and products isolated and their radioactivity determined

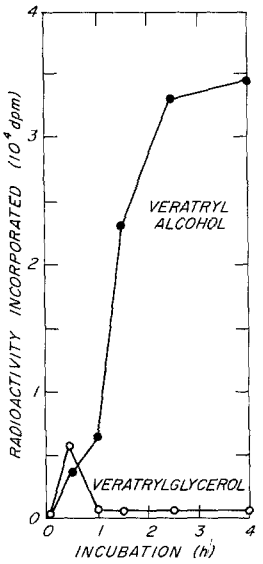

Fig. 4. Incorporation of ^{14}C from [U-^{14}C] L-phenylalanine into veratryl alcohol via veratrylglycerol by *Phanerochaete chrysosporium*. The ^{14}C-phenylalanine was added to 6-day-old 8 ml cultures as 0.5 ml aqueous solution (1.1×10^6 dpm, 1.3 nmol), and the cultures were incubated under 100% O_2 and 37°C. Products were isolated by thin layer chromatography of the ethyl acetate extracts

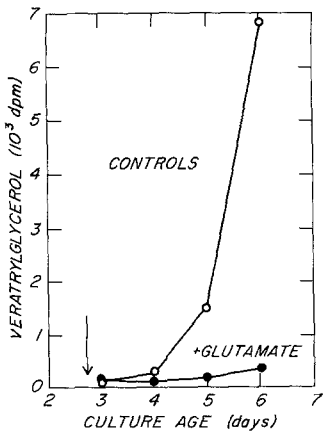

Fig. 5. Effect of L-glutamate on conversion of 3,4-dimethoxycinnamyl alcohol (II) to veratrylglycerol (III). Experiment was conducted as described in Table 3. Arrow indicates glutamate or water (control) addition. After addition of L-glutamate or water, the radioactive 3,4-dimethoxycinnamyl alcohol was added on each day and cultures incubated for 5 h at 37–39°C under 100% O_2. The incubation was stopped by addition of 10 ml of ethanol, and radioactive veratryl alcohol was isolated and determined as described in the text

Table 3. Effect of L-glutamate on formation of metabolites in cultures of *Phanerochaete chrysosporium*[a]

Substrate	Product	Inhibition in % (with respect to control)
[U-^{14}C] phenylalanine	3,4-Dimethoxycinnamyl alcohol (II)	97
[U-^{14}C] phenylalanine	Veratrylglycerol (III)	100
[U-^{14}C] phenylalanine	Veratryl alcohol (IV)	95
3,4-Dimethoxycinnamyl alcohol-4-[O$^{14}CH_3$]	Veratrylglycerol (III)	95
[$^{14}CH_3$] methionine	3,4-Dimethoxycinnamyl alcohol (II)	88
[$^{14}CH_3$] methionine	Ferulic acid	95

[a] Glutamate (20 nmol in 0.5 ml H_2O) or 0.5 ml H_2O only (controls) was added to 10 ml 6-day-old cultures. After 10 h under 100% O_2 and 37–39°C, labeled amino acid substrates were added (as in Table 2), or 10 nmol (5.5×10^4 dpm) of the cinnamyl alcohol (II). At the time of addition of labeled compounds, 1–2 mg/flask of cold carrier (product) was added. After 1 h (phenylalanine), 15 min (methionine) or 5 h (3,4-dimethoxycinnamyl alcohol), products were isolated and their radioactivity determined

The concentration of O_2 strongly influenced the amount of IV. Cultures (10 ml) grown for 3.75 days under 100% O_2, and then flushed daily with O_2/N_2 mixtures for 3 days, contained the following amounts of IV per culture: 20% O_2/80% N_2, 450 nmol, 40% O_2/60% N_2, 1260 nmol, and 100% O_2, 2170 nmol.

L-Glutamic acid almost completely suppressed conversion of II to III when added to cultures prior to appearance of the converting enzyme system (Fig. 5). Further study with glutamate showed that it strongly suppressed the capacity of cultures to synthesize IV and other intermediate products (Table 3).

Discussion

Biosynthetic Pathway for Veratryl Alcohol

Previous results have established that veratryl alcohol is synthesized de novo from glucose in cultures of *Phanerochaete chrysosporium* (Lundquist and Kirk 1978). The current work establishes that phenylalanine, 3,4-dimethoxycinnamyl alcohol (II) and veratrylglycerol (III) are intermediates. It appears that synthesis proceeds via cinnamic acid, p-hydroxycinnamic acid, caffeic acid, ferulic acid, 3,4-dimethoxycinnamic acid and the corresponding aldehyde. The demonstration that ferulic but not isoferulic acid is labeled by [$^{14}CH_3$] methionine is consistent with this pathway. Shimazono (1959) and others (see Subba Rao et al. 1971) have demonstrated similar transformations of phenylalanine to various cinnamic acid derivatives in other basidiomycetes.

Veratryl Alcohol Synthesis and Lignin Biodegradation

As pointed out in the introduction, this examination of the intermediates of veratryl alcohol biosynthesis was stimulated by studies of the degradation of the lignin substructure model compound I. Results here establish that the initial degradative reactions seen in the model compound (A and B, Fig. 1) are paralleled by the final reactions in veratryl alcohol biosynthesis.

Previous work has shown that both veratryl alcohol synthesis and lignin biodegradation are secondary metabolic events in *P. chrysosporium* and that the onset of secondary metabolism is triggered by nitrogen starvation. Addition of nutrient nitrogen to secondary metabolic cultures suppresses lignin degradation and veratryl alcohol synthesis (Fenn and Kirk 1981; Keyser et al. 1978). The most effective of several examined compounds was L-glutamate. Results here establish that glutamate strongly suppresses intermediate steps in the conversions of phenylalanine and of 3,4-dimethoxycinnamyl alcohol. It is presumed that the suppressive effect of the nitrogenous compounds reflects a shift in metabolism from secondary to primary. Very rapid protein turnover during secondary metabolism in *P. chrysosporium* (Fenn and Kirk 1981) apparently results in rapid loss of certain secondary metabolic functions in response to added nutrient nitrogen.

The inhibition of veratryl alcohol synthesis by culture agitation resulting in pellet formation was unexpected. Lignin degradation is also severely suppressed under these conditions, an effect which has been attributed to low hyphal surface area in contact with O_2, as compared to mycelial mats in stationary culture. The present results suggest that agitation with pellet formation might in fact cause deeper physiological effects in *P. chrysosporium*, perhaps affecting secondary metabolism in general.

Also somewhat surprising was the marked increase in yield of veratryl alcohol in cultures under 100% O_2 as compared to air. This effect is also seen with the lignin-degrading system, in which case it is easily understood because lignin degradation is a heavily oxidative process. Recent studies have demonstrated that O_2 not only affects the activity of the ligninolytic system, but also strongly influences the titer of the system during its formation as cultures enter secondary metabolism (Bar-Lev and Kirk 1981). Conceivably, the effect of O_2 on veratryl alcohol synthesis could also be a dual one, affecting both synthesis of the enzyme system and action of oxidative enzymes involved in the final steps of the biosynthetic pathway.

Our results demonstrate that veratryl alcohol biosynthesis and lignin model compound degradation share cinnamyl alcohol-transforming reactions. It is possible that two separate degradative systems are involved. If this is so, the fact that the glycerol intermediates in both cases are mixtures of *threo* and *erythro* isomers is highly coincidental. It seems more probable that only a single system is involved in the cinnamyl alcohol-transforming reactions of both the synthetic and degradative pathways.

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