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OLEFIN SATURATION AND ACID REDUCTION OF 3,4-DIMETHOXYCINNAMIC ACID AND DERIVATIVES BY *PHANEROCHAETE CHRYSOSPORIUM*

AKIO ENOKI, YASUO YAJIMA and MICHAEL H. GOLD

Department of Chemistry and Biochemical Sciences, Oregon Graduate Center, Beaverton, OR 97006, U.S.A.

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Key Word Index—*Phanerochaete chrysosporium*; basidiomycete; dimethoxycinnamic acid; ferulic acid; veratryl alcohol; acid reduction; olefin saturation; metabolism.

Abstract—The white rot fungus *Phanerochaete chrysosporium* metabolized 3,4-dimethoxycinnamic acid in shaking and nitrogen sufficient cultures. Metabolites identified included 3-(3,4-dimethoxyphenyl)propionic acid, dimethoxycinnamyl alcohol and 3-(3,4-dimethoxyphenyl)-1-propanol. Significantly smaller amounts of veratryl and vanillyl alcohol were also present. The abundance of the propionic acid and the propanol as metabolic products indicate that olefin saturation and acid reduction are important reactions catalysed under these conditions. Metabolites identified from the metabolism of 3-(3,4-dimethoxyphenyl)-propionic acid included the above 1-propanol as well as veratryl and vanillyl alcohol; the identification of these benzyl alcohol derivatives as metabolites suggests that α,β-bond cleavage of this substrate was preceded by alkane hydroxylation at the α-position.

INTRODUCTION

Recently the metabolism and utilization of lignin and its industrially produced derivatives as renewable resources has aroused considerable interest. Although a variety of white rot fungi are capable of catabolizing lignin [1], the responsible enzymes have not been determined. Since lignin is such a complex polymer, metabolic studies concerned with the biochemical details of its degradation face considerable obstacles. For this reason the metabolism of various well-defined lignin model compounds has been studied under a variety of culture conditions. In this report we describe studies on the metabolism of the 3,4-dimethoxycinnamic acid (**1**) and its derivatives by the white rot fungus *Phanerochaete chrysosporium* in shaking culture under conditions where neither carbon nor nitrogen are limiting. We report on the reduction of the carboxylic acid group of **1** yielding 3,4-dimethoxycinnamyl alcohol, on an olefin saturation reaction yielding 3-(3,4-dimethoxyphenyl)propionic acid and finally 3-(3,4-dimethoxyphenyl)-1-propanol, as well as on the cleavage of the α,β-bond yielding veratryl alcohol. The reduction of cinnamic acid derivatives to cinnamyl alcohols has been well established in plants [2] and fungi [3, 4]. Olefin saturation and α,β-cleavage of ferulic acid have been reported in the fungus *Trametes* [4].

RESULTS

Metabolism of 3,4-dimethoxycinnamic acid

Products of the fungal metabolism of **1** are shown in Table 1. The major products formed were 3,4-dimethoxycinnamyl alcohol **5**, 3-(3,4-dimethoxyphenyl)-propionic acid and 3-(3,4-dimethoxyphenyl)-1-propanol. During this 6-day period, all of the substrate was converted to products.

Table 1. Metabolism of 3,4-dimethoxycinnamic acid

	Mol product/initial mol of substrate (%)	
	4 days	6 days
Substrate	28	trace
3-(3,4-Dimethoxyphenyl)propionic acid **2**	5.6	13.7
3,4-Dimethoxycinnamyl alcohol **5**	11.3	21.9
3-(3,4-Dimethoxyphenyl)-1-propanol **6**	32.1	42.2
Veratryl alcohol	0.1	2.48
Vanillyl alcohol	0.3	0.8

The organism was grown at 28° from a conidial inoculation in shaking culture (50 ml). After 2 days **1** was added to a final concentration of 0.05% and the cells were incubated for an additional period as indicated above. The products were isolated and identified as described in the Experimental.

Metabolism of 3-(3,4-dimethoxyphenyl)propionic acid

Products of the fungal metabolism of **2** are shown in Table 2. The major product formed was 3-(3,4-dimethoxyphenyl)-1-propanol **6**. Significantly smaller amounts of veratryl and vanillyl alcohol were also formed. During this 4-day period 87% of the substrate was converted to products.

Metabolism of 3-(4-ethoxy-3-methoxyphenyl)propionic acid

Because of the ambiguity generated with respect to some apparently ring-demethylated products formed in the metabolism of **2**, we synthesized and examined the metabolism of **13**, a compound with different substituents at the 3- and 4-positions. Products of the fungal

Table 2. Metabolism of 3-(3,4-Dimethoxyphenyl)propionic acid

	Mol of product/initial mol of substrate (%)	
	2 days	4 days
Substrate	20.3	13.1
3-(3,4-Dimethoxyphenyl)-1-propanol **6**	66.0	62.7
Veratryl alcohol	0.25	0.80
Vanillyl alcohol	0.44	1.69

Experimental details as described under Table 1.

metabolism of **13** are shown in Table 3. The major product formed was 3-(4-ethoxy-3-methoxyphenyl)-1-propanol **14**. The following products were also formed but in significantly smaller amounts: 3-(4-ethoxy-3-hydroxyphenyl)propionic acid **15**, 3-(4-ethoxy-3-hydroxyphenyl)-1-propanol **16**, 3-(4-hydroxy-3-methoxyphenyl)-1-propanol **9** and 3-(4-hydroxy-3-methoxyphenyl)propionic acid **7**. The formation of these metabolic products indicates that the fungus is capable of ring de-etherification at either the 3- or 4-position.

Metabolism of ferulic acid

Products of the fungal metabolism of ferulic acid are shown in Table 4. The major products formed were coniferyl alcohol, 3-(4-hydroxy-3-methoxyphenyl)-propionic-acid and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol. Additional products formed in significantly smaller amounts were vanillyl alcohol, vanillic acid and methoxy-*p*-hydroquinone.

DISCUSSION

The results in this study indicate that in high nitrogen conditions in shaking cultures *P. chrysosporium* metabolizes various cinnamic acid derivatives, including 3,4-dimethoxycinnamic acid and ferulic acid. Under these conditions the two pathways which predominate are the reduction of the terminal carboxylic acid to an alcohol and saturation of the double bond. Reduction of the terminal carboxylic group and saturation of the double bond of 3,4-dimethoxycinnamic acid were also found to take place

Table 3. Metabolism of 3-(4-ethoxy-3-methoxyphenyl)propionic acid

	Mol of product/mol of initial substrate (%)	
	2 days	4 days
Substrate	18.1	12.0
3-(4-Ethoxy-3-methoxyphenyl)-1-propanol **14**	68.3	64.0
3-(4-Ethoxy-3-hydroxyphenyl)-propionic acid **15**	1.0	0.9
3-(4-Ethoxy-3-hydroxyphenyl)-1-propanol **16**	0.9	2.3
3-(4-Hydroxy-3-methoxyphenyl)-propionic acid **7**	0.9	0.8
3-(4-Hydroxy-3-methoxyphenyl)-1-propanol **9**	0.9	0.7
4-Ethoxy-3-methoxybenzyl alcohol	0.2	1.5
4-Ethoxy-3-hydroxybenzyl alcohol	0.8	5.6
Vanillyl alcohol	0.2	0.4

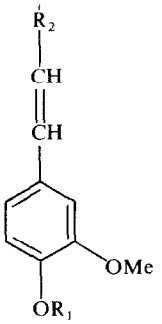
Experimental details as described under Table 1.

under limiting nitrogen conditions in stationary culture where lignin degradation [5] and lignin dimeric model compound metabolism are facilitated [6]. The reduction of some cinnamic acids to alcohols has been reported in

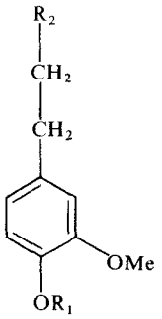
Table 4. Metabolism of ferulic acid

	Mol product/initial mol of substrate (%)	
	2 days	4 days
Substrate	trace	—
3-(4-Hydroxy-3-methoxyphenyl) propionic acid **7**	72.0	60.1
3-(4-Hydroxy-3-methoxyphenyl)-1-propanol **9**	17.2	30.1
Coniferyl alcohol	8.1	1.1
Vanillyl alcohol	0.2	0.3
Vanillic acid	2.3	1.4
Methoxy-*p*-hydroquinone	0.3	0.6

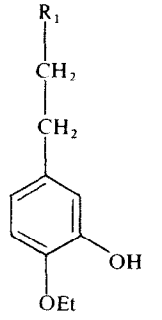
Experimental details as described under Table 1.



1 R_1 = Me, R_2 = COOH
3 R_1 = H, R_2 = COOH
5 R_1 = Me, R_2 = CH_2OH
8 R_1 = H, R_2 = CH_2OH
11 R_1 = Et, R_2 = COOH
12 R_1 = Et, R_2 = CH_2OH



2 R_1 = Me, R_2 = COOH
6 R_1 = Me, R_2 = CH_2OH
7 R_1 = H, R_2 = COOH
9 R_1 = H, R_2 = CH_2OH
13 R_1 = Et, R_2 = COOH
14 R_1 = Et, R_2 = CH_2OH



15 R_1 = COOH
16 R_2 = CH_2OH

plants [2] and in several fungal systems [3, 4, 7]. In addition, the reduction of anisic and veratric acid to their corresponding alcohols has been reported in *Polyporus versicolor* [8, 9].

Evidence for the saturation of double bonds in cinnamic acid derivatives is not well documented. To our knowledge only one reference with no supporting data for the saturation of the double bond in ferulic acid has been previously reported in fungi [4]. Thus, the results in this study demonstrate conclusively that the fungal-catalysed saturation of double bonds in cinnamic acid derivatives can be a major reaction in their metabolism. Our results using the saturated cinnamic acid derivatives 3-(3,4-dimethoxyphenyl)propionic acid **2** and 3-(4-ethoxy-3-methoxyphenyl)propionic acid **13** indicate that the presence of an olefin in the substrate is not a prerequisite for reduction of the acid to an alcohol.

The results using 3-(4-ethoxy-3-methoxyphenyl)-propionic acid **13** also clearly indicate that aryl ether cleavage at the 3- or 4-position of these substituted cinnamic acid derivatives is possible. The low yield of the products of aryl ether cleavage indicate that the conditions used in this study are probably not optimal. To our knowledge this is also the first well-documented report of the 3-demethylation of a lignin model substrate where both the 3- and 4-positions are initially blocked. The relevance of 3-demethylation to the possible 3-demethylation of guaiacyl lignin will be examined. The de-etherification of alkoxybenzoic acids at the 4-position by white rot fungi to yield vanillic acid has been previously described [10].

The identification of veratryl alcohol as a minor metabolic product of **1** and **2** and the identification of 4-ethoxy-3-methoxybenzyl alcohol as a product of the metabolism of 3-(4-ethoxy-3-methoxyphenyl)propionic acid **15** indicates that substituted phenylpropionic acids can be metabolized by fungi to α,β-bond cleavage products as described previously [4]. The presence of benzyl alcohol derivatives as products suggests the possibility that the substituted phenylpropionic acids are initially hydroxylated at the α-position. α,β-Cleavage of the postulated α-hydroxy intermediate might proceed through an aldolase-type mechanism after initial reduction of the acid to an aldehyde [11, 12]. The resulting substituted benzaldehyde α,β-cleavage products should be reduced quickly by white rot fungi as has been described earlier [9].

The metabolism by white rot fungi of veratryl alcohol and 4-ethoxy-3-methoxybenzyl alcohol is likely to occur via oxidation of the benzyl alcohol to an acid [13, 14] with simultaneous de-etherification at the 4-position to yield vanillic acid [10]. Vanillate hydroxylase, an enzyme which oxidatively decarboxylates vanillic acid to methoxyhydroquinone, has recently been isolated from white rot fungi [15, 16]. Where ferulic acid was the substrate, olefin saturation and subsequent α-hydroxylation would yield an intermediate which might undergo α,β-cleavage by the mechanism described above to yield vanillin or undergo possible alkyl–phenyl cleavage which has previously been shown to be catalysed by white rot fungi [17, 18] to yield methoxy-*p*-hydroquinone.

EXPERIMENTAL

Synthesis of substrates and intermediates. 3,4-Dimethoxycinnamic acid **1**, 3-(3,4-dimethoxyphenyl)propionic acid **2** and ferulic acid were obtained from Aldrich. Methyl 3,4-dimethoxycinnamate (**4**) was prepared from **1** by a previously described procedure [19]. 3,4-Dimethoxycinnamyl alcohol **5** was prepared by reduction of **4** at −30° for 1 hr in tetrahydrofuran containing a 2-fold excess of $LiAlH_4$.

3-(3,4 Dimethoxyphenyl)-1-propanol **6**: A solution of **5** (2.0 g) and Pd–charcoal (100 mg, 10%) in MeOH (25 ml) was shaken in H_2 (35 psi) for 2 hr and filtered through celite to yield **6**. 3-(4-Hydroxy-3-methoxyphenyl)propionic acid **7**, methyl ferulate, coniferyl alcohol **8** and 3-(4-hydroxy-3-methoxyphenyl)-1-propanol **9** were produced from ferulic acid by procedures described above.

Methyl 4-ethoxy-3-methoxycinnamate **10**: Methylferulate was ethylated with $Et_2SO_4/K_2CO_3/Me_2CO$ for 5 hr, as described previously [20] for Me_2SO_4, to produce **10**.

4-Ethoxy-3-methoxycinnamic acid **11**: **10** was hydrolysed in 1.0 N NaOH for 30 min at 80° to yield **11**. 4-Ethoxy-3-methoxycinnamoyl alcohol **12**, 3-(4-ethoxy-3-methoxyphenyl)-propionic acid **13** and 3-(4-ethoxy-3-methoxyphenyl)-1-propanol **14** were produced from **10** and **11** by procedures described above.

A mixture of methyl-4-ethoxy-3-hydroxycinnamate and methyl 3-ethoxy-4-hydroxycinnamate was produced by ethylating in refluxing Me_2CO methyl 3,4-dihydroxycinnamate with 0.5 equiv. Et_2SO_4 as described above. 3-(4-Ethoxy-3-hydroxyphenyl)propionic acid **15** and 3-(4-ethoxy-3-hydroxyphenyl)-1-propanol **16** were prepared from this mixture by procedures described above.

Gas chromatography. GC was carried out with a Varian Model 1700 instrument fitted with a glass column (180 × 0.2 cm i.d.) packed with 3% OV-101 on chromosorb Q 100.120 (Applied Science). The oven temp. was programmed from 150 to 270° at 15°/min unless indicated otherwise. MS was carried out with a Dupont Model 21-491 B equipped with the same instrument and column for GC. The spectra were obtained at 70 eV.

Growth of mycelia. A culture of *Phanerochaete chrysosporium ME 446* was maintained on slants as previously described [21]. The organism was grown at 28° on a rotary shaker operating at a speed of 175 rpm, in 125 ml Erlenmeyer flasks containing 50 ml of a medium previously described [5] containing 1% glucose except that the phthalate buffer was eliminated, 12 mM ammonium tartrate was used as the nitrogen source, and 0.2% yeast extract was added. Flasks were inoculated with 5×10^7 conidia and incubated for 48 hr after which the substrate was added at a concn of 0.05% as indicated.

Extraction and isolation of metabolic products. At the indicated intervals after the addition of the phenolic substrates, the cultures were suction-filtered. The mycelial mat was frozen on dry ice, ground in a mortar and extracted with EtOAc (3 × 20 ml). The EtOAc fraction was then used to extract the culture filtrate. To the total organic fraction (60 ml) was added 1 ml 20% H_2SO_4, 100 mg sodium dithionite and the mixture was washed twice with 30 ml H_2O, dried and evapd to dryness under N_2. Trimethylsilanation of products and standards was carried out by adding bis-(*N*,*O*-trimethylsilyl)trifluoracetamide–pyridine (1:1) to the dry residue and heating at 80° for 5 min. Metabolic products were identified after comparison of the MS of their TMSi derivative with those of chemically synthesized or commercially acquired standards.

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