Rapid Paper

Degradation Pathway of Arylglycerol- β -aryl Ethers by *Phanerochaete chrysosporium*

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The degradation pathway for the most important β -O-4 lignin substructure with a white rot fungus, *Phanerochaete chrysosporium*, was investigated, and the following conclusions were obtained. a) The allyl alcohol end group attached to the β -O-4 substructure was degraded to a formyl group via a glycerol group. b) Arylglycerol was formed by the cleavage of the β -O-4 substructure without involvement of the hydroxylation reaction at C_β . c) ^{18}O was not incorporated from $^{18}\text{O}_2$ into arylglycerol nor phenol liberated by the degradation of the β -O-4 substructure, but was incorporated into the benzyl alcohol derivative formed by C_α - C_β cleavage. d) Two alternative degradation pathways of the β -O-4 substructure, a pathway via arylglycerol and direct oxygenative C_α - C_β cleavage, are proposed.

The β -O-4 lignin substructure is the most frequent interphenylpropane linkage¹⁾ which functions as a junction to give three-dimensional lignin polymers, and the cleavage of the linkage results in low molecular weight lignin fragments. Therefore, many biodegradation studies have been carried out using β -O-4 substructure models. Earlier investigations^{2,3)} suggested the formation of arylglycerol in the degradation of β -O-4 substructure models by white rot fungi, and this suggestion was confirmed recently by Enoki *et al.*⁴⁾ by mass spectrometric identification. However, the mechanism of arylglycerol formation from the β -O-4 substructure remains uncertain, and the following pathways have been assumed^{2~6)}: (i) hydrolysis of the β -ether bond to

give arylglycerol; (ii) monooxygenase-catalyzed hydroxylation at C_β , subsequent cleavage of the formed hemiketal, and reduction of aryldihydroxyacetone to give arylglycerol.

The present investigation using the α,β -dideuterated, γ -benzyl ether of the β -O-4 substructure model and $^{18}\text{O}_2$ showed that two alternative pathways which do not involve C_β hydroxylation, the pathway (ii), functioned in degradation of the β -O-4 substructure by *Phanerochaete chrysosporium*, and that the allyl alcohol end group was degraded to a formyl group via a glycerol group. The use of deuterated β -1 lignin substructure models in the study of fungal degradation was reported by Kirk and Nakatsubo.⁷⁾

MATERIALS AND METHODS

Culture conditions. *Phanerochaete chrysosporium* BURDS. (ME-446) was used. The culture medium was the same as reported earlier⁸⁾ except that the medium contained 2.6 molar nitrogen (equimolar NH_4NO_3 and L-asparagine). Cultures for degradation studies (20 ml in a 300 ml Erlenmeyer flask or 10 ml in a 100 or 50 ml Erlenmeyer flask) were grown at 39°C without agitation.

Analysis of metabolites. Addition of substrates and extraction of metabolites were the same as reported earlier.⁹⁾ For the $^{18}\text{O}_2$ experiment, immediately after the addition of substrate, the 50 ml flasks which were fitted with a septum, rubber (Aldrich), were flushed with N_2 gas through a sterilized cotton filter at ca. 500 ml/min for 15 min, then 50 ml of $^{18}\text{O}_2$ (CEA, 98.75%) was introduced with a syringe. The metabolites were analyzed by gas chromatograph-mass spectrometry (GC-MS) directly or after acetylation (Ac_2O -pyridine = 1:1, r.t.) and subsequent TLC separation (silica gel plates: Kieselgel 60 F₂₅₄ or PF₂₅₄, Merck). The metabolites were identified by comparison of mass spectra with those of authentic compounds. The following instruments were used: a Varian XL-200 FT NMR spectrometer (200 MHz, TMS as an internal standard), and a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer (70 eV).

Syntheses of substrates and authentic compounds. Structures of substrates and authentic compounds are shown in Fig. 2. Compounds (I) and (III) were prepared by the methods previously reported.^{10,11)} Compound (II) was prepared by treatment of (I) with *m*-chloroperbenzoic acid, followed with sodium methoxide. MS m/z (%): 424 (M^+ , 1.4), 240 (20.5), 222 (10.3), 210 (10.3), 204 (9.2), 192 (25.6), 179 (100), 167 (81), 166 (82.1), 153 (56.4), 151

(56.4), 139 (56.4) and 137 (38.5). Compound (VI) was synthesized from benzyl alcohol *via* the following three steps: 1) 2,2-diethoxyethyl bromide, sodium hydride, DMF, r.t.; 2) 1 N HCl, dioxane, 50°C; 3) NaBD₄, D₂O, dioxane, r.t. ¹H-NMR (CDCl₃) δ (ppm): 3.57 (2H, d, J = 4.8, Ar-O-CH₂-), 3.72 (1H, m, -CHD-O-), 4.55 (2H, s, Ar-CH₂-), 7.33 (5H, m, Ar-H). MS m/z (%): 153 (M⁺, 15), 107 (25), 92 (25), 91 (100). Compound (VII) was prepared from vanillin *via* ethylation and reduction with NaBD₄. ¹H-NMR (CDCl₃) δ (ppm): 1.46 (3H, t, J = 7.0, -O-C-CH₃), 3.87 (3H, s, -O-CH₃), 4.09 (2H, q, J = 7.0, -O-CH₂-), 4.59 (1H, broad s, Ar-CHD-), 6.85 (2H, s, Ar-H), 6.92 (1H, s, Ar-H). MS m/z (%): 183 (M⁺, 100), 155 (48), 138 (44). Compound (VII') was prepared in the same manner as above except for using NaBH₄ as reducing reagent. Compound (VIII') was prepared from dimethyl acetal of benzyl-5-iodovanillin synthesized by the method of Nakatsubo and Higuchi¹²⁾ *via* the following steps: 1) *n*-BuLi, Et₂O, -75°C; 2) CH₃OD, Et₂O, -35°C; 3) 10% Pd-C, H₂, CH₃OH; 4) 1N HCl, acetone; 5) NaBH₄, CH₃OH. ¹H-NMR (CDCl₃) (acetate) δ (ppm): 2.11 (3H, s, alcoholic Ac), 2.31 (3H, s, phenolic Ac), 3.84 (3H, s, -OCH₃), 5.07 (2H, s, Ar-CH₂-), 6.94~6.97 (2H, m, Ar-H). MS (acetate) m/z (%): 239 (M⁺, 4.2), 197 (100), 155 (61), 138 (93), 137 (38). Compound (VIII) was kindly provided by Mr. Tanahashi (Wood Res. Inst., Kyoto Univ.) Syntheses of compounds (IV), (IV'), (V) and (V') will be reported elsewhere. Compound (IV); MS m/z (%): 468 (M⁺, 0.3), 450 (1.3), 449 (3.3), 207 (28), 181 (70), 179 (40), 152 (63), 151 (83), 149 (37), 91 (100). Compound (IV'); ¹H-NMR (CDCl₃) (acetate, mixture of *erythro* and *threo*) δ (ppm): 1.45 (3H $\times$ 2, t, J = 7.0, -O-C-CH₃); 1.98 and 1.98 (3H $\times$ 2, s, -OAc); 3.5~3.7 (2H $\times$ 2, dd, -C _{γ} H₂-); 3.82, 3.85, 3.85 and 3.90 (3H $\times$ 4, s, -OCH₃); 4.07 (2H $\times$ 2, q, J = 7.0, -O-CH₂-C), 4.46 and 4.50 (2H $\times$ 2, s, Ar-CH₂-); 4.74 and 4.91 (1H $\times$ 2, m, -C _{β} H-); 6.00 and 6.09 (1H $\times$ 2, d, -C _{α} H-); 6.8~7.5 (10H $\times$ 2, m, Ar-H); 9.83 and 9.84 (1H $\times$ 2, s, -CHO); MS m/z (%): 467 (M⁺, 0.2), 449 (3.0), 206 (19), 180 (45), 179 (28), 153 (43), 152 (60), 151 (31), 149 (25), 91 (100). Compound (V); ¹H-NMR (CDCl₃) (acetate) δ (ppm): 1.46 (3H, t, J = 7.0, -O-C-CH₃), 2.05 (3H, s, -OAc), 2.09 (3H, s, -OAc), 3.28 (1H, dd, $J_{\gamma\gamma}$ = 11, $J_{\gamma\beta}$ = 5, -C _{γ} H-), 3.48 (1H, dd, $J_{\gamma\gamma}$ = 11, $J_{\gamma\beta}$ = 4, -C _{γ} H-), 3.83 (3H, s, -OCH₃), 4.09 (2H, q, J = 7.0, -O-CH₂-C), 4.37 (1H, d, J = 12, Ar-CH-), 4.47 (1H, d, J = 12, Ar-CH-), 5.35 (1H, m, -C _{β} H-), 5.99 (1H, d, $J_{\alpha\beta}$ = 8, -C _{α} H-),

6.7~7.3 (8H, m, Ar-H); MS (acetate) m/z (%): 416 (M⁺, 12), 356 (5.9), 314 (8.8), 223 (26), 181 (100), 180 (5.9), 91 (65). Compound (V'); MS (acetate) m/z (%): 419 (2.2), 418 (M⁺, 8.3), 417 (0), 416 (0), 357 (1.8), 315 (5.6), 224 (19), 182 (100), 181 (8.3), 91 (50). Degrees of deuteration of substrates and authentic compounds are shown in Table I.

RESULTS

Metabolism of compound (I)

The ethyl acetate extract after 18 hours incubation with (I) was shown to contain three main compounds; the substrate, (II) and (III) by comparing mass spectra with those of the authentic samples.

Metabolism of compounds (IV) and (IV')

50 mg of (IV) was added to 10 cultures (5 mg/20 ml of culture) which were incubated for 43 hr (4 cultures) and 117 hr (6 cultures). The extracts (43 hr: 23.1 mg, 117 hr: 30.7 mg) were combined. GC-MS analysis (1.5% SE-30, 1 m, 170°C) of the products showed the occurrence of compounds (VI) and (VII). Retention degrees of deuterium in (VI) and (VII) were estimated to be about 90 and 80%, respectively, based on the mass spectra. After acetylation and removal of acetates of (IV) and veratryl alcohol, the extract was found to contain acetates of (V') and (VIII) by GC-MS (1% OV-1, 2 m, 260 or 190°C). The mass spectrum of the metabolite (V'), m/z (%): 419 (0.87), 418 (M⁺, 3.04), 417 (1.74), 416 (0), 357 (1.5), 315 (7.6), 224 (22), 182 (100), 181 (8.6), 91 (91), showed that the metabolite was a mixture, double-deuterated at C _{α} and C _{β} (M⁺, 418) and mono-deuterated at C _{α} (M⁺, 417), and that the ratio of the former/the latter was 6:4. In other words, the degree of deuteration at C _{β} in the metabolite (V') was 60% which

TABLE I. DEGREES OF DEUTERATION (DD) OF SUBSTRATES AND AUTHENTIC COMPOUNDS

Compound	IV		IV'	V'		VI	VII	VIII'
	C _{α}	C _{β}		C _{α}	C _{β}			
DD ^a (%)	100	59	>91	100	100	100	100	100

^a Determined by integration of ¹H-NMR except for IV' (by MS).

was equal to that of the substrate (IV) (see Table I). Thus the deuterium at C_β was found to be retained on the formation of (V') from (IV). (V'), (VII) and 4-ethoxy-3-methoxy-benzaldehyde were not detected in the extract from a non-inoculated control culture. The possible *de novo* synthesis of (VIII) by the fungus which is known to synthesize the 4-*O*-methylated analog of (VIII), veratryl alcohol,¹³ was ruled out, because a deuterated compound (VIII') which is not biosynthesized was found to be formed from (IV') by the fungus.

Metabolism of compound (V')

When the ethyl acetate extract of the culture incubated with 1.3 mg of (V') for 73.5 hr was analyzed by GC-MS (1% OV-1, 2m, 151°C), (VI) and (VII) were identified. Retention degrees of deuterium were 97% for (VI) and 70% for (VII) based on MS, respectively, whereas the retention degree of deuterium of (VII) in the extract from the culture incubated with

(V') for 23.5 hours was 87%. (VI) and (VII) were not detected in the extract from a non-inoculated control.

Incorporation of ^{18}O from $^{18}\text{O}_2$

After incubation with 5 mg of (IV') under an atmosphere of $^{18}\text{O}_2$ for 113 hours, the culture extract (4.4 mg) was analyzed as mentioned above. MS showed that (V) and (VIII') did not contain ^{18}O . On the other hand, 25% of (VII') contained 1 atom of ^{18}O and *de novo* synthesized veratryl alcohol contained 1 atom or 2 atoms of ^{18}O , respectively. MS also indicated that ^{18}O was incorporated into the benzyl position of (VII') (Fig. 1-b). As for veratryl alcohol, ^{18}O was found to be incorporated into methoxyl groups but not into the benzyl position (Fig. 1-a).

DISCUSSION

Based on the metabolites identified and the isotopic experiment, we propose Fig. 2 as the

FIG. 1-a,b. Molecular Ion (M^+) and Principal Mass Spectrometry Fragment Ion Peaks of Acetates of Veratryl Alcohol (1-a) and (VII') (1-b).

FIG. 2. Proposed Degradation Pathway of Veratrylglycerol- β -conferyl Ether by *Phanerochaete chrysosporium*, Involving Two Alternative Pathways for Cleavage of the β -O-4 Substructure.

metabolic pathway for veratrylglycerol- β -conferyl ether (I) in a ligninolytic culture of *Phanerochaete chrysosporium*. (I) was first oxidized at its allyl alcohol end group to give a glycerol (II) which was degraded to a vanillin ether (III) with the loss of a C_2 unit. Thus, the allyl alcohol and group attached to the β -O-4 substructure was degraded in a similar manner to that attached to the coumaran substructure.⁸⁾ Consequently, allyl alcohol end groups in lignin which comprise about 6% as end groups of lignin¹⁾ could be degraded *via* glycerol end groups.

The following two pathways have hitherto been suggested for β -ether cleavage and arylglycerol formation from the β -O-4 substructure²⁻⁶⁾ but without reliable evidence: (i) monooxygenase-catalyzed hydroxylation at C_β , subsequent degradation of the formed hemiketal, and reduction of the β -keto intermediate to give arylglycerol and phenol; (ii) hydrolysis of the β -O-4 linkage to give arylglycerol and phenol. The present results showed that pathway (i) is not valid for degradation of the β -O-4 substructure by *P. chrysosporium*, because deuterium at C_β in (V') was retained. Arylglycerol derivative (V') was further degraded to (VI) and (VII). Vicinal diol cleavage is well known for the fungus^{4,7,8,14-16)}. At first glance, the MS of (VI) and (VII) suggested that C_β -D was almost completely retained and that some loss of C_α -D occurred. However, longer incubation decreased the retention degree of deuterium of

(VII). Hence, loss of deuterium of (VII) may be caused by interconversion between (VII) and the corresponding benzoic acid. It was concluded that C_α -D and C_β -D were almost completely retained in the diol cleavage. Similar retention of 3H in vicinal diol cleavage by the fungus was reported only qualitatively by Shimada and Gold.¹⁶⁾

It was further established that in the degradation of (IV'), ^{18}O was not incorporated into (V) or (VIII'), but incorporated in the benzyl position of (VII'), and the methoxyl groups of veratryl alcohol synthesized *de novo*. Two possible mechanisms were considered for the incorporation of ^{18}O into (VII'). One is incorporation of ^{18}O during oxidation of (VII') to the corresponding benzoic acid which may be reduced to give ^{18}O -(VII'). This mechanism was eliminated by the discovery that vanillyl alcohol (VIII') did not contain ^{18}O . It was thus concluded that ^{18}O was incorporated into (VII') during oxygenative C_α - C_β cleavage. Since veratryl alcohol was found to be synthesized *via* $C_\alpha OH$ - $C_\beta OH$ cleavage of veratrylglycerol by *P. chrysosporium*,¹⁴⁾ and since ^{18}O was found not to be incorporated into the benzyl position of veratryl alcohol, if all of (VII') is formed from (V), ^{18}O is not expected to be incorporated into the benzyl position of (VII'). Hence, incorporation of ^{18}O into the benzyl position of (VII') suggests the participation of another pathway, in which (V) is not involved, to yield (VII'). In conclusion, we propose the following two alternative pathways for the

degradation of the β -O-4 substructure based on the degradation products identified and isotopic experiments: (a) pathways via (V) (arylglycerol), (a-1) hydrolysis to give (V) and (VIII') or (a-2) initial attack of the vanillin portion in (IV') to result in (V); (b) direct oxygenative C $_{\alpha}$ -C $_{\beta}$ cleavage of the β -O-4 substructure to give (VI), (VII) and (VIII), which is completely different from the mechanism reported by Enoki *et al.*¹⁷⁾: Initial C $_{\gamma}$ -oxidation and subsequent non-oxygenative retro-aldol condensation type cleavage of the C $_{\alpha}$ -C $_{\beta}$ linkage. In the pathway (b), $^{18}\text{O}_2$ was incorporated into the C $_{\alpha}$ position, while it was presumed that $^{18}\text{O}_2$ was not incorporated into the C $_{\alpha}$ position on C $_{\alpha}$ -C $_{\beta}$ cleavage of a β -1 type lignin model dimer by *P. chrysosporium*.¹⁵⁾

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