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Original

Arylglycerol Formation in Degradation of β-*O*-4 Lignin Substructures with and without α-*O*-γ Bond by *Phanerochaete chrysosporium*

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Abstract——Hydrogen (deuterium) atoms at C_α and C_β positions of β-*O*-4 lignin substructure model compounds with and without α-*O*-γ bond were found to be retained at C_α and C_β positions of arylglycerol in degradation by *Phanerochaete chrysosporium.* It was accordingly proved that any compound which lost deuterium atoms at C_α and/or C_β positions (e.g. α- and/or β-keto derivatives) is not the intermediate compound to give the arylglycerol.

1. Introduction

Investigations using oligolignols (lignin substructure model compounds) have contributed to the elucidation of the specific reactions of lignin metabolism by white-rot fungi and other microorganisms. For the degradation of β-*O*-4 substructure (arylglycerol-β-aryl ether), which is the most frequent substructure of lignin[1], involvement of arylglycerol as a metabolic intermediate was proposed in 1960's[2~4] and it was confirmed recently[5]. Based on the identification of metabolites by chromatography, Fukuzumi and coworkers reported in 1969 possible involvement of direct hydroxylation at C_β position of β-*O*-4 substructure by an enzyme preparation from a white-rot fungus, *Poria subacida* to produce arylglycerol in the degradation of a β-*O*-4 dimer[4].

We recently found that the C_β hydroxylation (Fig. 4(1)) was not involved in the formation of arylglycerol from β-*O*-4 substructure with α-*O*-γ bond by a white-rot fungus, *Phanerochaete chrysosporium,* based on tracer experiments with α, β-dideuterated substrates and mass spectrometric identification of metabolites[6]. In the previous investigation we used a simplified trimer model containing both β-*O*-4 and α-*O*-γ bonds, arylglycerol-β-vanillin-γ-benzyl diether (I-Bzl), which represents a β-*O*-4 bond adjacent to α-*O*-γ bond in lignin (Fig. 1). By the investigation, C_β-C_γ fragment compound in C_α-C_β cleavage of the β-*O*-4 substructure was characterized for the first time as benzyloxyethanol. A γ-benzyl ether dimer (α-*O*-γ dimer) was isolated from hydrogenolysis products of Yachidamo (*Fraxinus mandshurica*) wood[7], and the γ-benzyl

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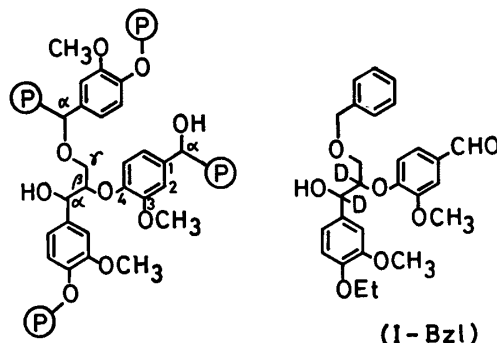

Fig. 1. A portion of lignin polymer which contains β-*O*-4 and α-*O*-γ substructures and its model compound. Left structure represents arylglycerol-β-aryl-γ-benzyl diether substructure (β-*O*-4 and α-*O*-γ substructure). P: continuation of lignin polymer. Right compound (I-Bzl) is an arylglycerol-β-aryl-γ-benzyl diether lignin substructure model trimer.

ether substructure (α-*O*-γ substructure) was suggested to be present in protolignin. However, it has been considered that most of the β-*O*-4 substructure has no etherified hydroxyl function at the γ-position[8]. We, therefore, reinvestigated the degradation of deuterated β-*O*-4 dimer without α-*O*-γ bond by *P. chrysosporium*.

2. Experimental

2.1 *Microorganism and culture conditions*

Phanerochaete chrysosporium Burds. (ME-446) was maintained at 30°C on 2% malt agar slants. Experimental culture (10 ml in 100 ml Erlenmeyer flask) was grown at 39°C without agitation in a nitrogen-limiting (2.6 mM nitrogen, equimolar NH_4NO_3 and L-asparagine), glucose, dilute mineral salts medium buffered with poly (acrylic acid) (pH 4.5, 0.01 M in carboxyl)[6,9].

2.2 *Metabolism of substrate*

2.2 mg of [α, β-D_2]4-ethoxy-3-methoxyphenylglycerol-β-vanillin ether (I) was added to 6-day-old culture as *N, N*-dimethylformamide (DMF) solution (0.01 ml)[10]. The flask was flushed with sterilized 100% O_2 immediately after addition of the substrate and stational incubation was continued at 39°C for additional 120hr. The culture was extracted with ethyl acetate[10] and the extract was acetylated (acetic anhydride/pyridine=1/1, by volume, r.t., 24hr). The acetylated product was separated by preparative TLC (solvent: ethyl acetate/*n*-hexane=1/1, by volume, developed twice) to give a fraction, Rf value of which was approximately equal to that of triacetate of 4-ethoxy-3-methoxyphenylglycerol (II) (G-fraction). The G-fraction was analyzed by GC-MS [1% OV-1 on chromosorb W (AW-DMCS) (Shinwakakou Co., Ltd., Japan), glass column: 2 m×0.3 cm (i.d.), carrier gas: He (31 ml/min)]

and the retention degree of deuterium in 4-ethoxy-3-methoxyphenylglycerol (II) was examined.

2.3 *Syntheses of substrate and authentic compounds*

[α, β-D_2]4-ethoxy-3-methoxyphenylglycerol-β-vanillin ether (I) was prepared as follows from 4-ethoxy-3-methoxyphenyl 2′-methoxy-4′-formylphenoxymethyl ketone, which was prepared by a modification of the method reported earlier[11]: 1) methyl orthoformate/*dl*-10-camphorsulfonic acid/CH_2Cl_2/CH_3OH/r.t. (90.9%), 2) NaH/D_2O (CEA, 99.85%)/dioxane/r.t., 3) paraformaldehyde/K_2CO_3/DMSO/r.t., 4) $NaBD_4$ (CEA, 97%)/D_2O (CEA, 99.85%)/DMSO/r.t. (The protecting group for formyl group, dimethyl acetal, was deprotected during working up process and/or purification using TLC). MS (diacetate, I-Ac) m/z(%): 462 (M^++2, 8.30), 461 (M^++1, 3.65), 460 (M^+, 0.174), 401 (1.3), 252 (6.4), 251 (3.8), 250 (2.4), 238 (7.9), 224 (17.0), 209 (8.8), 208 (11.1), 207 (14.9), 196 (8.4), 182 (100). ^{1}H-NMR (diacetate, I-Ac) ($CDCl_3$) δ (ppm): 1.45 (t, O-C-CH_3); 2.00, 2.01, 2.02 and 2.04 (s, AcO); 3.86, 3.87, 3.88 and 3.91 (s, O-CH_3); 4.07 (q, O-CH_2-C); 4.0–4.4 (m, γ-CH_2), 4.8–5.0 (m, trace of β-CH); about 6 (α-H was not detected); 6.8–7.5 (aromatic protons); 9.84 and 9.86 (s, CHO). 4-Ethoxy-3-methoxyphenylglycerol-β-vanillin ether (I′) and triacetate of 4-ethoxy-3-methoxyphenylglycerol (II-Ac) were prepared previously[12]. Triacetate of [α, β-D_2]4-ethoxy-3-methoxyphenylglycerol (II-D-Ac) was prepared from 4-ethoxy-3-methoxyphenyl hydroxymethyl ketone prepared previously[13] *via* the following steps: 1) ethyl vinyl ether/*dl*-10-camphorsulfonic acid/CH_2Cl_2/0°C, 2) NaH/D_2O (CEA, 99.85%)/dioxane/0°C, 3) paraformaldehyde/K_2CO_3/DMSO/r.t., 4) acetic anhydride/pyridine/ethyl acetate/r.t., 5) $NaBD_4$ (CEA, 97%)/D_2O (CEA, 99.85%)/dioxane/0°C, 6) 1 N HCl/acetone/r.t. followed by separation of *erythro* and *threo* forms with TLC (solvent: ethyl acetate/*n*-hexane=2/1, by volume, developed twice), 7) acetic anhydride/pyridine/ethyl acetate/r.t. MS (*erythro*) m/*z* (%): 370 (M^++2, 7.5), 369 (M^++1, 0.51), 368 (M^+, 0), 309 (6.3), 267 (2.9), 252 (2.6), 249 (1.5), 224 (6.5), 207 (21.6), 182 (100); (*threo*) m/z (%): 370(M^++2, 6.7), 369 (M^++1, 0.38), 368 (M^+, 0), 309 (3.4), 267 (2.4), 252 (1.7), 249 (1.2), 224 (6.9), 207 (14.7), 182 (100). ^{1}H-NMR ($CDCl_3$) (*erythro*) δ (ppm): 1.46 (3H, t, J=7.00, O-C-CH_3); 1.98, 2.04 and 2.11 (3H×3, s, AcO); 3.88 (3H, s, OCH_3); 4.08 (2H, q, J=7.00, O-CH_2-C); 4.22 (2H, s, γ-CH_2); 6.8-6.9 (3H, m, aromatic protons); (*threo*) δ (ppm): 1.46 (3H, t, J=7.03, O-C-CH_3); 2.05, 2.07 and 2.07 (3H×3, s, AcO); 3.78 (1H, d, J=12.1, γ-CH); 3.88 (3H, s, OCH_3); 4.09 (2H, q, J=7.02, O-CH_2-C); 4.23 (1H, d, J=12.2, γ-CH); 6.8-7.0 (3H, m, aromatic protons). Protons at α- and β-positions were not detected in the ^{1}H-NMR spectra.

2.4 *Spectrometry and chromatography*

^{1}H-NMR spectra were taken with a Varian XL-200 FT-NMR spectrometer (200

MHz) with TMS as an internal standard. Chemical shifts and coupling constants are given in δ-values (ppm) and Hz, respectively. Mass spectra were taken with a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer (electron ionization mass spectrometry, ionizing voltage: 70eV). M^+ in mass spectral data of the deuterated compounds represents molecular ion of unlabeled analogs. Preparative TLC was done on silica gel plates (Kieselgel 60 F_{254}, Merck).

3. Results

GC-MS analysis showed that the G-fraction contained triacetate of 4-ethoxy-3-methoxyphenylglycerol. The metabolite gave a mass spectrum and a retention time identical to (II-D-Ac). It is well known that deuterated compounds have a little smaller retention times than non-deuterated analogs in gas chromatograpy. In order to avoid the isotope effect on the calculation of the content of deuterium atoms, ten mass spectra were taken successively at certain points of the peak in GC. Fig. 2

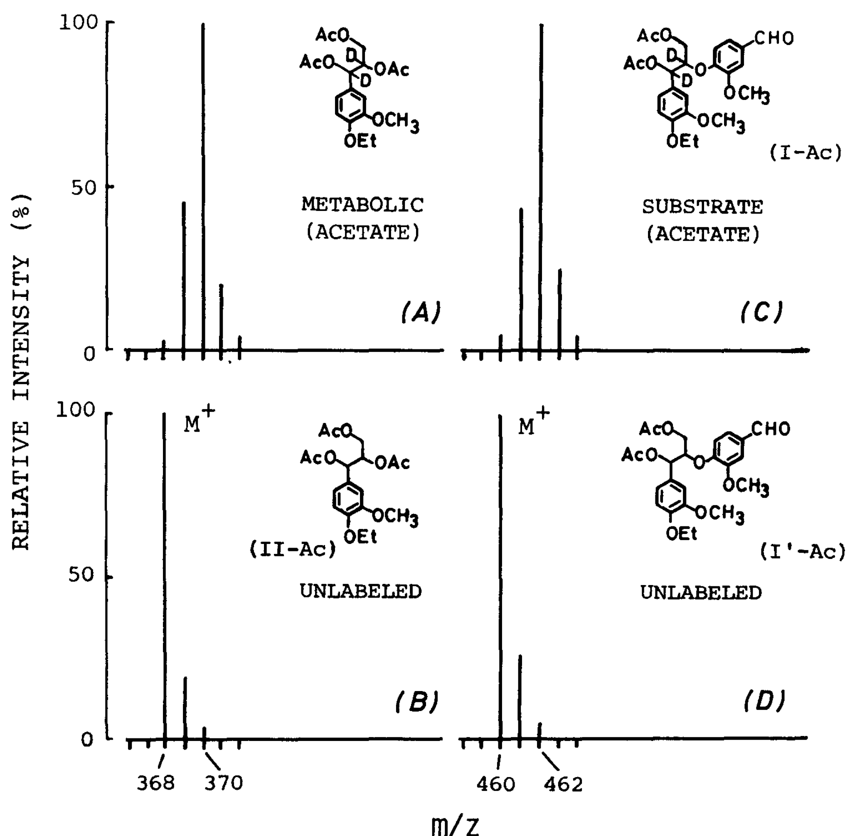

Fig. 2. Molecular ion region of the mass spectra of the metabolic arylglycerol and substrate. (A): The metabolic product, 4-ethoxy-3-methoxyphenylglycerol (acetate). (B): Unlabeled acetate of 4-ethoxy-3-methoxyphenylglycerol (II-Ac). (C): Acetate of substrate (I-Ac). (D): Acetate of unlabeled analog of the substrate (I′-Ac).

shows that the relative abundance of molecular ion region of the mass spectra of the metabolic arylglycerol (the average value of the ten spectra) and that of the substrate (I) (the average value of eight spectra measured by direct inlet system). The mass spectra showed that 65.3% of the metabolic arylglycerol and 66.13% of the substrate (I) contained two deuterium atoms excess to natural abundance at the α- and β-positions. The values were corrected for $M^{+}+1$ and $M^{+}+2$ of unlabeled and mono-deuterated analogs. Accordingly, 98.7% of hydrogen (deuterium) atoms at α- and β-positions of the arylglycerol were proved to be derived from those of the substrate (I).

4. Discussion

In 1960's, two reaction mechanisms were proposed for the white-rot fungal degradation of β-*O*-4 bond to produce the arylglycerol. One is direct hydroxylation at the β-position of the β-*O*-4 substructure (Fig. 4(1))[4]. The other is hydrolysis of the β-*O*-4 bond[3]. However, our previous study on incorporation of $H_2{}^{18}O$ evidenced that the hydrolysis was not involved in the degradation by *Phanerochaete chrysosporium*[12]. No involvement of the former, direct β-hydroxylation, was also proved by the present and previous[6] investigations.

The arylglycerol-β-aryl-γ-benzyl diether (I-Bzl) which was used in the previous study[6] represents a α-*O*-γ and β-*O*-4 substructure. The compound is a simplified trimer model for a junction substructure connecting two chains of lignin polymer (Fig.1). The previous study evidenced that hydrogen (deuterium) atoms at C_α and C_β of the

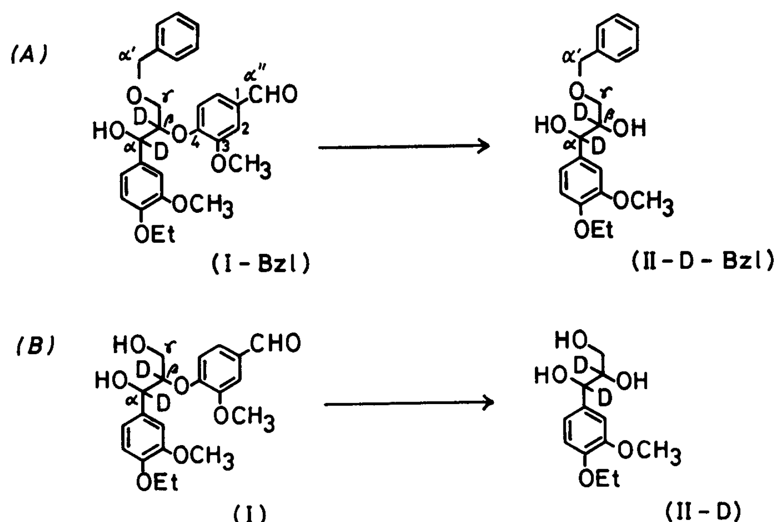

Fig. 3. Arylglycerol formation in the degradation of β-*O*-4 dimers (I) and (I-Bzl) by *Phanerochaete chrysosporium*. Degradation of (I-Bzl) was reported previously[5]. Hydrogen (deuterium) atoms at α- and β-positions of (I) and (I-Bzl) were retained at α- and β-positions of the arylglycerols, (II-D) and (II-D-Bzl).

trimer (I-Bzl) were retained at the C_α and C_β of the γ-benzyl etherified arylglycerol (II-D-Bzl) in the cleavage of the β-O-4 bond by *P. chrysosporium*, and that the β-O-4 bond was cleaved without cleavage of the α-O-γ bond (Fig. 3A)[6]. In the present investigation it was proved that the β-O-4 dimer (I) with non-etherified hydroxyl function of the γ-position which occurs more frequently in lignin gave the same result, retention of hydrogen (deuterium) atoms, in the cleavage of the β-O-4 bond (Fig. 3B). Thus, it was established that any compound which lost deuterium atoms at C_α and/or C_β of the β-O-4 type substrates with and without α-O-γ bond was not the intermediate compound to give the arylglycerol. Accordingly, as shown in Fig. 4, the following four intermediates were not involved in the arylglycerol formation accompanied by the β-O-4 bond cleavage: (1) β-keto intermediate which could be produced by direct hydroxylation at β-position of the substrate proposed earlier[4], (2) α-keto intermediate, (3) an olefin with C_α-C_β double bond by possible dehydration of the substrate and (4) α-hydroxycinnamyl alcohol derivative by possible elimination of the phenoxyl group attached to the β-position.

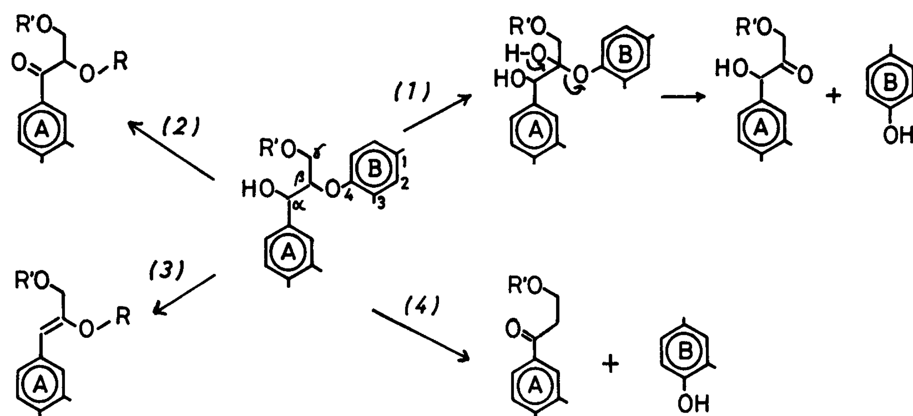

Fig. 4. Reaction mechanisms which do not involve the arylglycerol formation. In all the reactions, (1)–(4), hydrogen (deuterium) atoms at α- or β-position of the β-O-4 substructure are lost. R represents B-ring, hydrogen or an alkyl group derived from B-ring. R′ represents hydrogen or α-O-γ bond.

C_α-oxidation is one of prominent reactions in the degradation of lignin by white-rot fungi[14~16]. α-Keto formation in the degradation of a β-O-4 dimer by *P. chrysosporium* was reported[17]. But the previous[6] and present studies proved that the α-keto derivatives were not involved in the arylglycerol formation in the degradation of the β-O-4 substructures with and without adjacent α-O-γ bond, and that such α-keto derivatives may be degraded *via* other pathway(s) which does not involve the arylglycerol.

Earlier investigations[2,4~6,13] postulated without any evidence that the phenol

etherified at β-position of the β-O-4 dimer is the counterpart compound for the arylglycerol. But we, recently, proved that the β-O-4 bond was cleaved between ethereal oxygen and the 4-position carbon of the etherified phenoxyl group (B-ring in Fig. 4) and that the phenol was not the counterpart compound for the arylglycerol[18]. Consequently, no involvement of the reactions (1) and (4) was further proved, because in the reactions (1) and (4) the counterpart compound for the arylglycerol is expected to be the phenol.

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