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p-Benzoquinone monoketals, novel degradation products of *β-O*-4 lignin model compounds by *Coriolus versicolor* and lignin peroxidase of *Phanerochaete chrysosporium*

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2-(4-Ethoxy-3-methoxyphenyl)-3-hydroxymethyl-6,10-dimethoxy-1,4-dioxaspiro[4,5]deca-6,9-diene-8-one (**III**) and its isomer **IV** were identified as catabolites of 4-ethoxy-3-methoxyphenylglycerol-*β*-syringaldehyde ether (**I**) by the culture of *Coriolus versicolor*. Compound **III** was also produced from 4-ethoxy-3-methoxyphenylglycerol-*β*-syringic acid ether (**II**) by lignin peroxidase of *Phanerochaete chrysosporium*. An isotopic experiment showed that molecular oxygen was incorporated into the quinone oxygen of **III** in the degradation of **II** by lignin peroxidase.

p-Benzoquinone monoketal; *β-O*-4 lignin substructure; Aryl cation radical; Lignin peroxidase; (White-rot fungus)

1. INTRODUCTION

The arylglycerol-*β*-aryl ether bond (*β-O*-4 substructure) is the most abundant intermonomer linkage in lignin [1]. We previously found that ligninolytic cultures of *Coriolus versicolor* degraded non-phenolic *β-O*-4 lignin model dimers via C*α*-C*β* cleavage, C*α*-oxidation, aromatic ring cleavage, etc. [2,3]. Recent investigations demonstrated that lignin peroxidase, an extracellular heme protein from *Phanerochaete chrysosporium*, catalyzed not only C*α*-C*β* cleavage but also aromatic ring cleavage of veratryl alcohol [4] and lignin model dimers [5,6].

In the present paper, we report that *p*-benzoquinone monoketal (**III**), a novel product, was formed in the degradation of *β-O*-4 lignin model dimers by both the culture of *C. versicolor* and lignin peroxidase of *P. chrysosporium*, and discuss the formation mechanism of **III**.

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2. MATERIALS AND METHODS

2.1. *Syntheses of substrates*

4-Ethoxy-3-methoxyphenylglycerol-*β*-syringaldehyde ether (**I**) was prepared as described [2].

4-Ethoxy-3-methoxyphenylglycerol-*β*-syringic acid ether (**II**) was prepared from the diacetate of **I** via the following two steps: (i) Jones reagent [7] in acetone at 0°C; and (ii) sodium methylate (28% in MeOH) in MeOH/CH_2Cl_2 (= 1/4) at 0°C. MS *m/z* (%): 422($M^{\cdot+}$, 3), 224(100), 198(52), 195(40), 183(23), 181(42), 180(56), 152(21), 151(35) and 137(23).

2.2. *Culture conditions of* C. versicolor

C. versicolor was maintained at 30°C on 2% malt agar slants. Experimental cultures (20 ml in 300-ml Erlenmeyer flasks) were inoculated with a small mycelial mat from the slant and grown

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without agitation at 30°C in nitrogen-limited medium [2].

2.3. *Degradation of I by* C. versicolor

Substrate **I** was added to 7-day-old cultures and incubated for 76 h. The cultures were then extracted with ethyl acetate as in [2]. The extracts were submitted to TLC (Kieselgel 60, F_{254}, Merck) directly or after acetylation (Ac_2O/pyridine = 1/1, room temperature, 24 h). **III** from the non-acetylated extracts, and **III**-Ac, **IVa**-Ac and **IVb**-Ac from the acetylated extracts were separated by TLC, respectively (developing solvents: **III**, MeOH/CH_2Cl_2 = 1/99 × 3 and EtOAc/*n*-hexane = 1/2 × 4; **III**-Ac, MeOH/CH_2Cl_2 = 1/99 × 2 and EtOAc/*n*-hexane = 1/3 × 3; **IVa**-Ac, MeOH/CH_2Cl_2 = 2/98 × 4 and EtOAc/*n*-hexane = 1/2 × 4; and **IVb**-Ac, MeOH/CH_2Cl_2 = 1/99 × 2 and EtOAc/*n*-hexane = 1/2 × 3).

2.4. *Preparations of* P. chrysosporium *lignin peroxidase*

Lignin peroxidase which was prepared by the modified method of Tien and Kirk [5,8] from the culture filtrate of *P. chrysosporium* Burds. ME-446 was provided by Nagase Biochemicals. Enzyme activity was assayed by spectrometric quantification of veratraldehyde (ϵ_{310} = 9.3 $\mu mol^{-1} \cdot cm^2$) formed by oxidation of veratryl alcohol [8].

2.5. *Degradation of II by* P. chrysosporium *lignin peroxidase*

Enzyme reactions were carried out in a total volume of 3.3 ml, containing 0.45 mM H_2O_2, 0.3 mM substrate **II**, 20 μl lignin peroxidase (0.1–0.15 unit) and polyacrylic acid buffer (0.01 M in carboxyl, pH 4.5). Reactions were started by the addition of lignin peroxidase, and the reaction mixture was incubated at 37°C under air for 90 min.

Products in the reaction mixture were extracted with three portions of 10 ml of ethyl acetate and acetylated. **III**-Ac was separated by TLC (developing solvent: EtOAc/*n*-hexane = 1/3, × 2). The identity of the product was determined by MS.

2.6. *Incorporation of ^{18}O from $^{18}O_2$*

Reaction vessels which contained substrate **II**, H_2O_2 and buffer were evacuated, flushed with argon, reevacuated, and finally injected with $^{18}O_2$ (^{18}O:99 atom%, Amersham). Reactions were started by the addition of lignin peroxidase and the reaction mixture was incubated at 37°C for 45 min.

2.7. *Instruments*

^{1}H-NMR spectra were obtained with a Varian XL-200 FT-NMR spectrometer (200 MHz). Mass spectra and high-resolution mass spectra were taken with a Shimadzu GC-MS QP-1000 gas chromatograph-mass spectrometer (EI-MS, 70 eV) and a Jeol JMS-DX 300 gas chromatograph-mass spectrometer (EI-MS, 70 eV). IR spectra were measured with a Jasco IR-810.

3. RESULTS

3.1. *Degradation of I by* C. versicolor

III was separated as a catabolite of **I** degraded by *C. versicolor*. The structure of **III**, which is cyclized between Cα and Cβ oxygens, was determined based on the following experiments. (i) When **III** was acetylated, the chemical shifts of Cγ protons in the ^{1}H-NMR spectrum shifted downfield owing to the acetyl group introduced (table 1). (ii) Since **III** is a spiro compound whose Cα and Cβ carbon atoms are asymmetric, the chemical shifts of the methoxyl groups and protons derived from the *p*-benzoquinone moiety in the ^{1}H-NMR spectrum differ from each other as shown in table 1. **III** ^{1}H-NMR (table 1), MS *m/z* (%): 393(7), 392($M^{\cdot+}$, 30), 331(8), 212(24), 169(100) and 154(23). **III**-Ac ^{1}H-NMR (table 1), MS *m/z* (%): 435(7), 434($M^{\cdot+}$, 30), 331(15), 206(60), 169(37), and 154(100). High-resolution MS for $C_{22}H_{26}O_9$ 434.15767 (calcd), 434.15781 (found). IR: $\nu_{max}^{CCl_4}$ 1665 cm^{-1}. The structures of **IVa**-Ac and **IVb**-Ac, which are cyclized between Cβ and Cγ oxygens, were also confirmed by ^{1}H-NMR and MS. The chemical shifts of Cα protons of **IVa**-Ac and **IVb**-Ac in the ^{1}H-NMR spectra were considerably shifted downfield compared with that of the Cα proton of **III**-Ac. These results indicated that acetoxyl groups were attached to the Cα-positions of **IVa**-Ac and **IVb**-Ac. **IVa** and **IVb** were found to be diastereomers of each other, but the assignment to *erythro* and *threo* was not made. **IVa**-Ac ^{1}H-NMR (table 1), MS *m/z* (%): 435(5),

Table 1

^{1}H-NMR spectral data of compounds **III**, **III**-Ac, **IVa**-Ac and **IVb**-Ac

	Chemical shifts			
	III	**III**-Ac	**IVa**-Ac	**IVb**-Ac
Ethoxyl	1.51 (3H,t,J=6.9) 4.10 (2H,q,J=7.0)	1.47 (3H,t,J=7.0) 4.10 (2H,q,J=7.0)	1.46 (3H,t,J=7.0) 4.09 (2H,q,J=7.0)	1.45 (3H,t,J=7.0) 4.10 (2H,q,J=7.0)
Acetyl		2.05 (3H,s)	2.08 (3H,s)	2.12 (3H,s)
Methoxyl	3.82 (3H,s) 3.87 (3H,s) 3.90 (3H,s)	3.80 (3H,s) 3.87 (3H,s) 3.90 (3H,s)	3.78 (3H,s) 3.87 (3H,s) 3.89 (3H,s)	3.71 (6H,s) 3.87 (3H,s)
Cα-H	5.30 (1H,d,J=9.1)	5.01 (1H,d,J=8.9)	5.81 (1H,d,J=8.9)	5.94 (1H,d,J=6.2)
Cβ-H	4.30–4.40 (1H,m)	4.40–4.50 (1H,m)	4.75–4.95 (1H,m)	4.65–4.85 (1H,m)
Cγ-H	3.65 (1H,dd,J=12.6,2.7) 3.98 (1H,dd,J=12.5,3.5)	4.22 (1H,dd,J=11.9,6.8) 4.35 (1H,dd,J=11.9,2.9)	about 3.85 (1H) about 4.05 (1H)	about 4.20 (1H) 4.37 (1H,dd,J=7.3, 6.4)
>C=CH-CO-	5.45 (1H,d,J=1.7) 5.48 (1H,d,J=1.7)	5.42 (1H,d,J=1.7) 5.47 (1H,d,J=1.7)	5.37 (1H,d,J=1.7) 5.41 (1H,d,J=1.7)	5.33 (1H,d,J=1.5) 5.38 (1H,d,J=1.7)
Aromatic	6.86 (1H,d,J=8.2,H5) 6.99 (1H,dd,J=8.3,2.0, H6) 7.10 (1H,d,J=1.9,H2)	6.85 (1H,d,J=8.3,H5) 6.96 (1H,dd,J=8.3,1.9, H6) 7.10 (1H,d,J=1.9,H2)	6.80–7.00 (3H,m)	6.80–7.00 (3H,m)

Chemical shifts and coupling constants (J) are given in δ value (ppm) and Hz, respectively. Peak multiplications are abbreviated singlet, s; doublet, d; triplet, t; quartet, q; and multiplet, m. $CDCl_3$ and tetramethylsilane were used as solvent and internal standard, respectively

434($M^{\cdot+}$, 18), 212(14), 181(100), 169(29) and 125(15). **IVb**-Ac ^{1}H NMR (table 1), MS m/z (%): 435(7), 434($M^{\cdot+}$, 31), 223(36), 212(16), 181(100), 169(32), 154(19) and 125(16).

The structure of **III** was further confirmed by mass spectrometric analysis of the reduced compounds by Pd-C/H_2 and $NaBH_4$ as shown in fig.1. (i) When **III**-Ac was hydrogenated with 10% Pd-C in MeOH at room temperature for 20 min, two major products **Va** and **Vb** were isolated by TLC (developing solvent: EtOAc/n-hexane = 1/3, × 2), but both the compounds gave the same molecular ion peak at m/z 438. We therefore concluded that **Va** and **Vb** were stereoisomers of each other. (ii) **Va** and **Vb** were further reduced with $NaBH_4$ in MeOH at 0°C for 10 min and the reduced products were acetylated. By this treatment, the molecular ions of the two products **VIa** and **VIb** increased to give m/z 482, 44 mass units higher than original compounds **Va** and **Vb**, respectively.

3.2. *Degradation of II by lignin peroxidase*

III was also found to be formed by lignin peroxidase degradation of **II**, which was a catabolite of **I** in the culture of *C. versicolor* [2]. **III**-Ac was identified by MS analysis.

In addition, the isotopic experiment with $^{18}O_2$

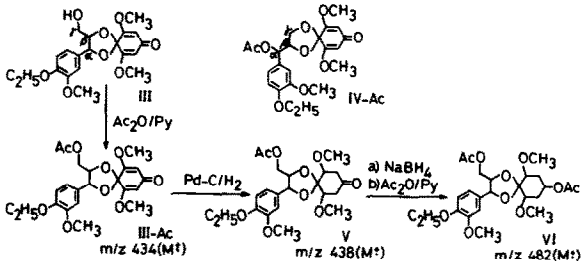

Fig.1. Chemical structures of p-benzoquinone monoketals **III**, **III**-Ac, **IV**-Ac, and the reduced compounds of **III**-Ac. Ac, -CO-CH_3; Py, pyridine; Pd-C, palladium carbon.

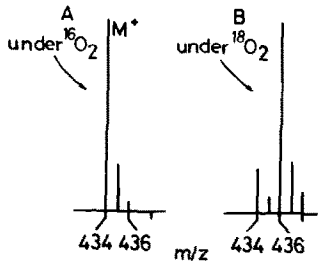

Fig.2. Molecular ion region of the acetate of *p*-benzoquinone monoketal **III**-Ac. (A) Degradation product from **II** under air ($^{16}O_2$); (B) degradation product from **II** under $^{18}O_2$.

showed that 80% of the quinone oxygen of **III** was derived from $^{18}O_2$ (fig.2B).

4. DISCUSSION

Kirk and co-workers [12,13] demonstrated by use of ESR that lignin peroxidase catalyzed single-electron oxidation of aromatic nuclei to form cation radicals. We propose the formation mechanism of **III** from **II** via the cation radical of *β*-etherated syringyric acid (B-ring) shown in fig.3. It is conceivable that the B-ring of **II** is oxidized by lignin peroxidase to give a cation radical, which is subsequently attacked by the C*α* hydroxyl group of **II**. The resulting radical reacts with molecular oxygen and the peroxide intermediate formed could be decarboxylated to give **III**. The incorporation of molecular oxygen into **III** supported the mechanism.

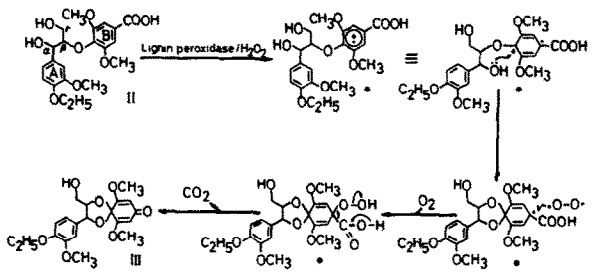

Fig.3. Possible mechanism for the formation of *p*-benzoquinone monoketal **III** from **II** by lignin peroxidase. Incorporation of molecular oxygen to the *p*-benzoquinone moiety of **III** was proved by the isotopic experiment using $^{18}O_2$. * Assumed compound.

The present investigations showed that **III** is formed as a degradation product of **I** by the culture of *C. versicolor*. We previously identified many degradation products of non-phenolic *β-O*-4 lignin model dimers via C*α*-C*β* cleavage and aromatic ring cleavage by *C. versicolor* [2,3]. Similar degradation products of *β-O*-4 lignin model dimers by lignin peroxidase of *P. chrysosporium* have been found [5,6,8–11]. These results suggest that **III** and the above degradation products could be formed via the attack of a hydroxy group on the cation radical, C*α*-C*β* cleavage and aromatic ring cleavage catalyzed by the lignin peroxidase in the culture of *C. versicolor*.

The formation mechanism of **IV** which is cyclized between C*β* and C*γ* oxygens is also explicable in a similar manner to that shown in fig.3.

Umezawa and Higuchi [14] reported the migration of the *β*-aryl group from C*β* to C*γ* oxygen during degradation of 4-ethoxy-3-methoxyphenylglycerol-*β*-guaiacyl ether to give guaiacoxyethanol in the culture of *P. chrysosporium*. There, it was suggested that migration occurs via the attack of the C*γ* hydroxyl group on the *β*-aryl cation radical produced by lignin peroxidase. Recently, Kirk et al. [15] detected an ESR signal corresponding to the *β*-aryl cation radical of a C*α* carbonyl-containing *β-O*-4 model compound formed by lignin peroxidase and suggested that aryl cation radicals were attacked by C*γ*-hydroxyl groups to form cyclohexadienone ketals similar to **III**.

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