Title	<Original>Aromatic Ring Cleavage of Various β-O-4 Lignin Model Dimers by Phanerochaete chrysosporium
Author(s)	UMEZAWA, Toshiaki; KAWAI, Shingo; YOKOTA, Shinso; HIGUCHI, Takayoshi
Citation	Wood research : bulletin of the Wood Research Institute Kyoto University (1986), 73: 8-17
Issue Date	1986-12-28
URL	http://hdl.handle.net/2433/53302
Right	
Type	Departmental Bulletin Paper
Textversion	publisher

Original

Aromatic Ring Cleavage of Various β-*O*-4 Lignin Model Dimers by *Phanerochaete chrysosporium**

Toshiaki UMEZAWA**, Shingo KAWAI**,
Shinso YOKOTA** and Takayoshi HIGUCHI**

(Received September 1, 1986)

Abstract——In the degradation of β-*O*-4 lignin model dimers with different aromatic substituents on β-etherated aromatic ring by intact cells of *Phanerochaete chrysosporium*, esters of arylglycerol, cyclic carbonates, formate and methyl oxalate, were formed as aromatic ring cleavage products of the models. Substituents of aromatic nuclei considerably influenced the formation of aromatic ring cleavage products and O-C_4 cleavage product, arylglycerol.

1. Introduction

Elucidation of specific degradative reactions involved in lignin degradations by white-rot fungi are being performed mainly by using lignin substructure models. Otherwise it is very difficult because lignin is a complex polymer, consisting of phenylpropane units connected via many C-C and C-O-C linkages.

The β-*O*-4 lignin substructure which has the basic structure Ph_A-C_αHOH-C_βH(OPh_B)-$C_\gamma H_2OH$, where Ph_A and Ph_B represent substituted aromatic rings, is the most frequent intermonomer linkage in lignin[1]. Hence, β-*O*-4 lignin model compounds are the most important ones for elucidating the mechanism of lignin biodegradation. Many degradative reactions of the β-*O*-4 models by intact cells of white-rot fungi especially *Phanerochaete chrysosporium* and *Coriolus versicolor* have been reported: C_α-C_β cleavage in propyl side chain[2~5], arylglycerol formation[3,5,6] which was finally proved to arise via O-C_4 cleavage and not via C_β-O cleavage[3,7~9], aromatic ring cleavage[10,11], alkyl-phenyl cleavage[2] and oxidation of C_α benzyl alcohol to C_α carbonyl group[12]. The C_α-C_β cleavage[13~19], the formation of arylglycerol[14,16~19] and the oxidation of C_α benzyl alcohol[18,19] were found to be catalyzed by an extracellular lignin-degrading enzyme (lignin peroxidase) from *Phanerochaete chrysosporium*. Moreover, we recently demonstrated for the first time aromatic ring

* A part of this study was presented at the 36th Annual Meeting of the Japan Wood Research Society (Shizuoka), April, 1986.

** Research Section of Lignin Chemistry.

cleavage of β-*O*-4 lignin substructure models by the lignin peroxidase[19,20]. In the investigation[19,20] four esters of arylglycerol were identified as products of aromatic ring cleavage of β-*O*-4 lignin models by the enzyme. The present paper shows that all the products of aromatic ring cleavage are also formed in the degradation of the β-*O*-4 lignin models by intact cells of *P. chrysosporium*. In addition, the effects of the aromatic ring substituents on the degradation of β-*O*-4 lignin substructure model dimers by the intact cells of *P. chrysosporium* are discussed in focusing on aromatic ring cleavage and arylglycerol formation.

2. Materials and Methods

2.1 Culture Conditions

Cultures (20 ml/300 ml Erlenmeyer flask) of *Phanerochaete chrysosporium* Burds. (ME-446) were grown without agitation in a nitrogen-limiting medium at 39°C[3,21].

2.2 Degradation of lignin substructure model dimers

4-Ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (I) (2 mg), 4-ethoxy-3-methoxyphenylglycerol-β-(2,6-dimethoxyphenyl) ether (II) (2 mg), 4-ethoxy-3-methoxyphenylglycerol-β-([U-ring-^{13}C]-2,6-dimethoxyphenyl) ether (II-^{13}C) (ca. 1 mg), 4-ethoxy-3-methoxyphenylglycerol-β-phenyl ether (III) (2 mg) and 4-ethoxy-3-methoxyphenylglycerol-β-vanillin ether (IV) (2 mg) were added respectively to 5 or 6-day-old cultures (20 ml/culture) as solutions of *N,N*-dimethylformamide (0.1 ml/culture). The flasks were flushed with O_2 and then incubated for additional 87–94 hr at 39°C without agitation[22]. Degradation products were extracted with ethyl acetate (20 ml × 3 per 20 ml culture) from whole cultures. The extract was acetylated (Ac_2O/pyridine = 1 : 1, v/v, room temp., 24 hr). The acetylated products were dissolved in 100 μl of acetone, and 1 μl of the acetone solution was injected into GC-MS.

2.3 Preparation of compounds

Chemical structures of substrates, products of their degradation by *P. chrysosporium*, and some related compounds are shown in Fig. 1.

Syntheses of the following substrates and authentic compounds were reported previously: (I)[10], (II)[11], (II-^{13}C)[11], (III)[19], (IV)[7], acetate of 4-ethoxy-3-methoxyphenylglycerol (V-Ac)[7], acetate of 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropane-1-one (V-CO-Ac)[19], acetate of 4-ethoxy-3-methoxyphenylglycerol-β,γ-cyclic carbonate (VI-Ac)[10], acetate of 4-ethoxy-3-methoxyphenylglycerol-α,β-cyclic carbonate (VI′-Ac)[19], acetate of 4-ethoxy-3-methoxyphenylglycerol-γ-formate (VII-Ac)[5], acetate of 4-ethoxy-3-methoxybenzyl alcohol (VIII-Ac)[3]. As for acetate of 1-(4-ethoxy-3-methoxyphenyl)dihydroxypropyl methyl oxalate (IX-Ac), the degradation

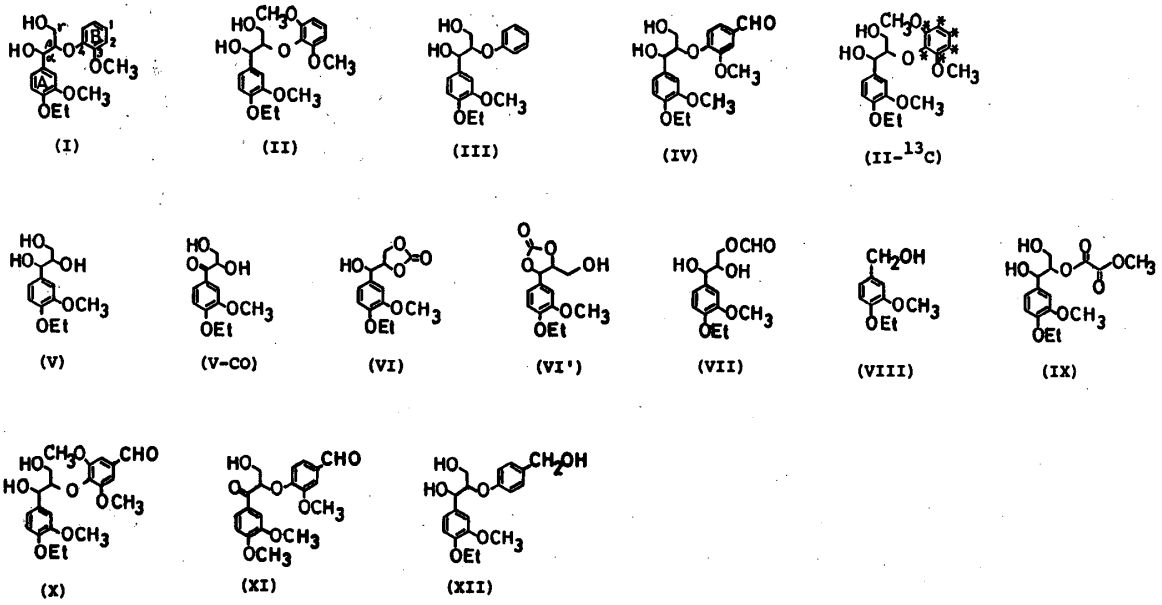

Fig. 1. Chemical structures of substrates, products of their degradation by *Phanerochaete chrysosporium*, and related compounds. (I)–(II-^{13}C): Substrates, (V)–(IX): degradation products, (X)–(XII): other compounds. Acetate of each compound is expressed with the symbol "Ac" (e.g. (V-Ac) is the acetate of (V)).
Et: CH_2CH_3, *: ^{13}C.

product of (I) or (II) by lignin peroxidase of *P. chrysosporium*[19] was used as an authentic sample.

2.4 Instrumentation

Mass spectra (EI-MS, ionizing voltage: 70 eV) were taken with a Shimadzu GCMS-QP 1000 gas chromatograph-mass spectrometer [glass column, 1 m×2.6 mm; 1.5% OV-17 on Chromosorb W AW DMCS, 80–100 mesh (Shinwa Kakou Co., Ltd., Japan); column temp.: 170–240°C (5°C/min); carrier gas, He: 40 ml/min].

3. Results

Fig. 2 shows the total ion chromatograms of the acetylated products. The following products were identified by comparison of the mass spectra with those of authentic compounds (Table 1).

Arylglycerol (V), propiophenone (V-CO), cyclic carbonates (VI) and (VI'), formate (VII) and C_α-C_β cleavage product (VIII) were formed from (II) and identified (as acetates) (Table 1-b, Fig. 2-A). (II) was completely degraded during the degradation. In the degradation of (III), (VIII) was detected (Table 1-c). As for the substrates (I) and (IV), the formation of compounds (V), (V-CO), (VI), (VI') and (VIII) was observed (Table 1-a, d; Fig. 2-B, C). Although the cor-

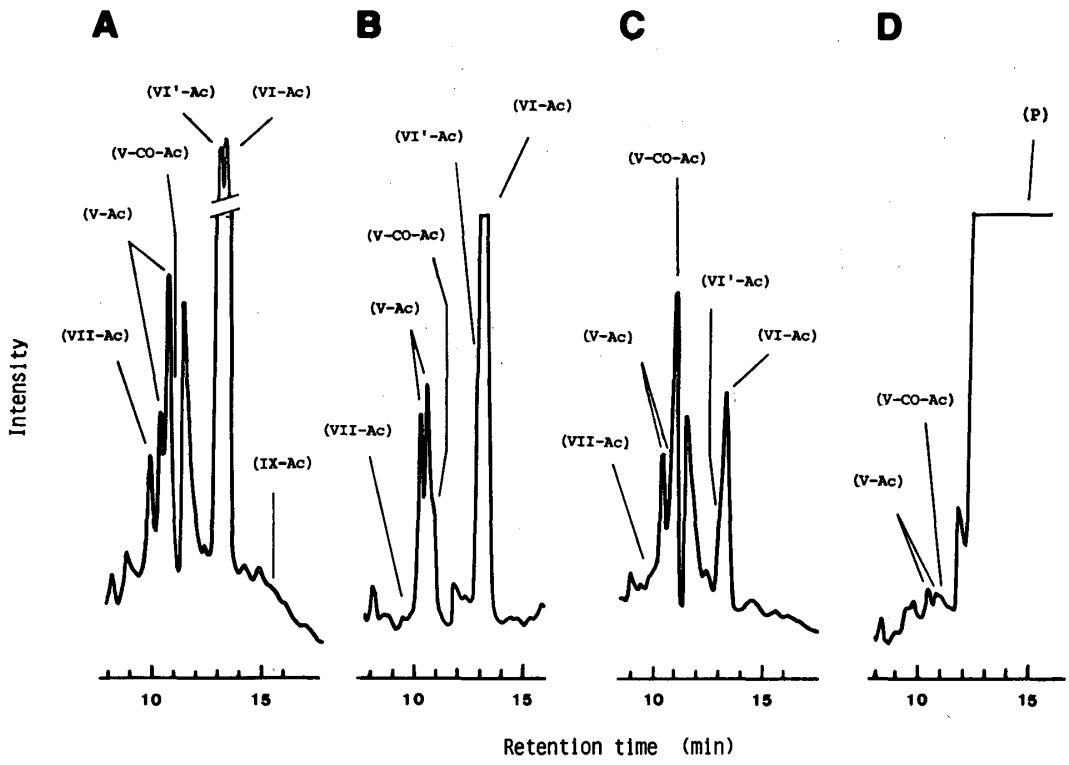

Fig. 2. Total ion chromatograms of the acetylated products of the degradation of β-*O*-4 lignin models, (I), (II), (III) and (IV), by intact cells of *Phanerochaete chrysosporium*. A, B, C and D: Products of the degradation of (II), (I), (IV) and (III). (P) in D: 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxy-2-phenoxypropane-1-one (data not shown). The total ion chromatograms were obtained under the same conditions. Thus, the same amount of each substrate was added to the culture, and incubated, and then the products were extracted and acetylated as described in the text. Each acetylated sample was dissolved in the same amount of acetone (100 μl) and 1 μl of the solution was injected into gas chromatograph-mass spectrometer, which was operated under the same condition including gain throughout the analysis of the four samples. Operation conditions of the GC-MS are described in the text. Retention time of (VIII-Ac) was 2.4 min. As for (V-Ac), the peak eluted earlier was *erythro* isomer, the other was *threo* isomer.

responding peaks were not observed in total ion chromatograms as shown in Fig. 2, background subtraction of mass spectra indicated the formation of trace amounts of the following products (Table 1): Formate (VII) from (I), methyl oxalate (IX) from (II), arylglycerol (V) and propiophenone (V-CO) from (III). Moreover mass chromatographic analysis of the acetylated degradation products of (IV) suggested the formation of (VII). The substrates (I), (III) and (IV) were not completely degraded during the incubation. Uninoculated control culture gave none of the products.

The carbonyl carbon atoms of (VI), (VI'), (VII) and (IX) were found to be derived from the 2,6-dimethoxyphenyl nucleus of (II) based on tracer experiment

Table 1. Relative Intensity of the Important Fragment Ions of the Metabolic Products

1-a: Metabolic products derived from (I)

Metabolic product	Mass spectral data, m/z, (Relative intensity, %)
(V-Ac)	368 (M^+, 6.4), 308 (7.9), 223 (4.7), 206 (32.7), 181 (100), 153 (9.9), 151 (11.1)
(V-CO-Ac)	324 (M^+, 6.2), 179 (100), 151 (57.6), 123 (12.4)
(VI-Ac)	310 (M^+, 10.3), 223 (4.0), 206 (3.5), 181 (100), 153 (14.8), 151 (9.7)
(VI'-Ac)	310 (M^+, 84.2), 206 (46.5), 179 (22.7), 178 (40.5), 177 (20.0), 165 (13.0), 151 (100)
(VII-Ac)	354 (M^+, 9.0), 294 (4.7), 223 (5.9), 206 (23.1), 181 (100), 153 (12.4), 151 (11.6)
(VIII-Ac)	224 (M^+, 62.4), 196 (5.2), 182 (16.7), 165 (25.1), 154 (29.6), 153 (11.8), 151 (12.1), 139 (8.4), 138 (9.7), 137 (100)

1-b: Metabolic products derived from (II)

Metabolic product	Mass spectral data, m/z, (Relative intensity, %)
(V-Ac)	368 (M^+, 8.2), 308 (5.0), 223 (5.5), 206 (19.4), 181 (100), 153 (9.5), 151 (8.0)
(V-CO-Ac)	324 (M^+, 5.9), 179 (100), 151 (51.7), 123 (10.2)
(VI-Ac)	310 (M^+, 10.4), 223 (4.1), 206 (2.6), 181 (100), 153 (15.2), 151 (12.7)
(VI'-Ac)	310 (M^+, 78.6), 206 (54.4), 179 (17.3), 178 (41.2), 177 (20.7), 165 (8.5), 151 (100)
(VII-Ac)	354 (M^+, 8.2), 294 (4.0), 223 (4.6), 206 (18.6), 181 (100), 153 (11.9), 151 (10.2)
(VIII-Ac)	224 (M^+, 50.7), 196 (5.4), 182 (15.2), 165 (23.5), 154 (27.8), 153 (12.1), 151 (13.5), 139 (9.9), 138 (10.5), 137 (100)
(IX-Ac)	412 (M^+, 8.6), 352 (2.6), 223 (3.9), 207 (14.7), 206 (17.9), 181 (100)

1-c: Metabolic products derived from (III)

Metabolic product	Mass spectral data, m/z, (Relative intensity, %)
(V-Ac)	368 (M^+, 7.5), 308 (3.0), 223 (4.1), 206 (16.5), 181 (100), 153 (7.3), 151 (7.6)
(V-CO-Ac)	324 (M^+, 5.8), 179 (100), 151 (52.6), 123 (9.8)
(VIII-Ac)	224 (M^+, 54.9), 196 (4.8), 182 (16.4), 165 (24.3), 154 (28.3), 153 (12.4), 151 (13.9), 139 (8.2), 138 (9.4), 137 (100)

1-d: Metabolic products derived from (IV)

Metabolic product	Mass spectral data, m/z, (Relative intensity, %)
(V-Ac)	368 (M^+, 6.0), 308 (7.1), 223 (5.8), 206 (29.8), 181 (100), 153 (11.1), 151 (20.0)
(V-CO-Ac)	324 (M^+, 5.3), 179 (100), 151 (58.3), 123 (11.6)
(VI-Ac)	310 (M^+, 12.0), 223 (3.8), 206 (4.3), 181 (100), 153 (15.8), 151 (12.0)
(VI'-Ac)	310 (M^+, 60.8), 206 (39.5), 179 (23.3), 178 (32.9), 177 (17.5), 165 (14.0), 151 (100)
(VIII-Ac)	224 (M^+, 60.9), 196 (6.2), 182 (19.3), 165 (25.6), 154 (31.9), 153 (13.6), 151 (16.1), 139 (9.4), 138 (10.3), 137 (100)

1-e: Authentic compounds

Compound	Mass spectral data, m/z, (Relative intensity, %)
(V-Ac)	368 (M^+, 5.9), 308 (7.6), 223 (4.1), 206 (29.8), 181 (100), 153 (10.0), 151 (10.6)
(V-CO-Ac)[a)]	324 (M^+, 5.9), 179 (100), 151 (54.8), 123 (10.5)
(VI-Ac)	310 (M^+, 11.1), 223 (3.8), 206 (2.0), 181 (100), 153 (14.5), 151 (8.5)

(VI′-Ac)[b]	310 (M^+, 72.5), 206 (52.4), 179 (16.9), 178 (41.2), 177 (19.2), 165 (7.9), 151 (100)
(VII-Ac)	354 (M^+, 7.6), 294 (3.7), 223 (4.1), 206 (18.6), 181 (100), 153 (9.7), 151 (6.0)
(VIII-Ac)	224 (M^+, 55.0), 196 (6.0), 182 (18.2), 165 (24.7), 154 (31.7), 153 (13.1), 151 (10.8), 139 (8.4), 138 (9.8), 137 (100)
(IX-Ac)	412 (M^+, 12.1), 352 (3.4), 223 (5.0), 207 (14.6), 206 (18.8), 181 (100)

a) In the previous paper[19], we reported the mass spectrum of synthesized (V-CO-Ac): m/z (%), 324 (M^+, 6.9), 264 (15.5), 222 (7.6), 191 (33.4), 179 (100), 151 (70.0), 123 (16.3). In the present analysis, however, the same compound did not give fragment ions, m/z 264, 222, 191, although conditions of GC-MS analysis were the same in both the present and the previous investigations.

b) In the previous paper[19], we reported the mass spectrum of synthesized (VI′-Ac): m/z (%) 310 (M^+, 61.4), 206 (41.6), 181 (12.0), 179 (14.6), 178 (37.1), 177 (18.9), 165 (13.8), 151 (100). The present analysis of the same compound showed that the fragment ion m/z 181 was overestimated in the previous paper because of a small contamination of the isomer (VI-Ac). As shown in Fig. 2, the separation in GC of (VI-Ac) and (VI′-Ac), the conditions of which were the same as in the previous paper, is not satisfactory.

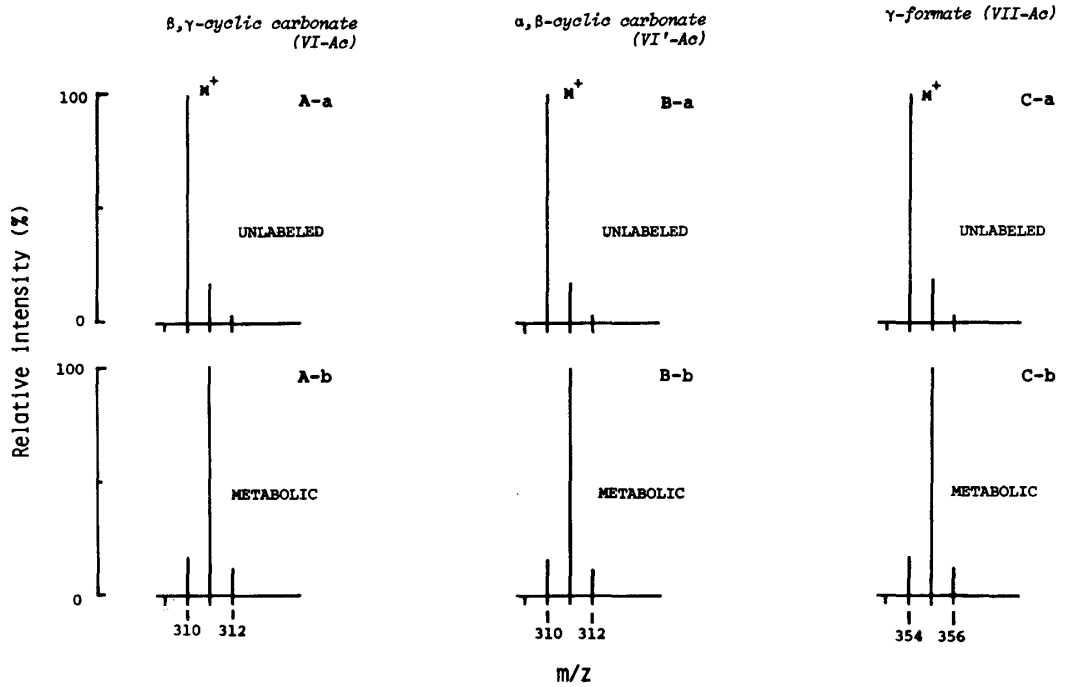

Fig. 3. Molecular ion regions of the mass spectra of aromatic ring cleavage products (acetates) in the degradation of (II-^{13}C) by *Phanerochaete chrysosporium* and their authentic samples. A-a, B-a and C-a are molecular ion regions of the mass spectra of unlabeled, synthesized authentic samples [A-a: β,γ-cyclic carbonate (VI-Ac), B-a: α,β-cyclic carbonate (VI′-Ac), C-a: γ-formate (VII-Ac)]. A-b, B-b and C-b are those of metabolic products [(VI-Ac), (VI′-Ac) and (VII-Ac), respectively] in the degradation of (II-^{13}C), B-ring of which is uniformly labeled with ^{13}C (^{13}C: 90 atom %), by intact cells of *Phanerochaete chrysosporium*.

with ^{13}C. Fig. 3 shows the mass spectra of (VI-Ac), (VI′-Ac) and (VII-Ac) from (II-^{13}C). The spectra showed that the molecular ion peaks of the products were

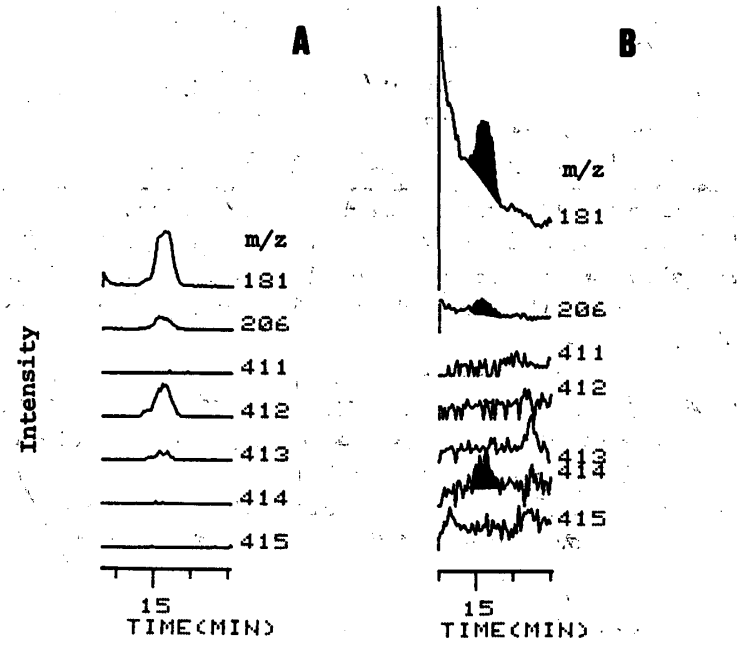

Fig. 4. Mass chromatograms of molecular ion regions and important fragment ions of acetate of methyl oxalate (IX-Ac).
A: Unlabeled authentic sample (molecular weight: 412), mass spectral data of which are shown in Table 1-e, B: product of degradation of (II-^{13}C) by *Phanerochaete chrysosporium*. The chromatograms of m/z 411–415 are magnified five times. Operation conditions of the GC-MS are described in the text.

higher by one mass unit than those of unlabeled authentic compounds. Fig. 4 shows the mass chromatogram of the molecular ion region and important fragment ions of (IX-Ac) produced from (II-^{13}C). The chromatogram showed that the molecular ion peak of the product (m/z 414) was higher by two mass units than that of unlabeled authentic sample (m/z 412).

GC-MS analysis showed that (VIII) was a major product in the degradation of all the substrates, especially (I), (III) and (IV). Preliminary quantification by means of stable isotope dilution procedure showed that the yields of 4-ethoxy-3-methoxybenzyl alcohol (VIII) and arylglycerol (V) (sum of *erythro* and *threo* isomers) were 167 μg and 18 μg, respectively, after 90 hr-incubation of (I) (2.0 mg) with the culture (20 ml) of *P. chrysosporium*, where the incubation conditions were the same as these in the present study. Quantitative studies on time course of degradation of β-*O*-4 dimers by *P. chrysosporium* are now in progress.

4. Discussion

Our previous study of the degradation of arylglycerol-β-guaiacyl ether (I) by intact cells of *P. chrysosporium* showed the formation of aromatic ring cleavage prod-

uct, β,γ-cyclic carbonate (VI)[10]. We further reported that (VI) was also formed from (I), arylglycerol-β-(2,6-dimethoxyphenyl) ether (II) and arylglycerol-β-syringaldehyde ether (X) in the culture of *C. versicolor,* and that γ-formate (VII) was identified as another aromatic ring cleavage product in the degradation of (II) and (X), which have two methoxyl substituents on the B-ring, by the fungus[11]. In addition, our recent study on the degradation of β-*O*-4 models by lignin peroxidase from *P. chrysosporium* showed that the enzyme catalyzed aromatic ring cleavage of β-*O*-4 lignin model dimers (I) and (II)[19,20], to yield new aromatic ring cleavage products, α,β-cyclic carbonate (VI′) and methyl oxalate (IX) which were not detected in the previous investigations with ligninolytic cultures of both the fungi[10,11], as well as (VI) and (VII). The present investigation confirmed that α,β-cyclic carbonate (VI′), formate (VII) and methyl oxalate (IX) as well as β,γ-cyclic carbonate (VI) were also formed in the degradation of the β-*O*-4 lignin model dimers by intact cells of *P. chrysosporium.* These results strongly suggested that aromatic ring cleavage (formation of (VI), (VI′), (VII) and (IX)) in the culture of *P. chrysosporium* was catalyzed by lignin peroxidase.

In the present study, we examined the degradation of β-*O*-4 lignin models with different aromatic substituents on the B-nucleus by intact cells of *P. chrysosporium.* Although detailed quantification of the products were not made, total ion chromatograms (Fig. 2) showed that the number of methoxyl groups considerably influenced the reactivity of the β-*O*-4 models. In the degradation of (II) which has two methoxyl substituents on the B-nucleus by the intact cells, arylglycerol (V), propiophenone (V-CO), β,γ-cyclic carbonate (VI), α,β-cyclic carbonate (VI′), formate (VII), α,β-cleavage product (VIII) and trace amounts of methyl oxalate (IX) were detected. As for (I), which has one methoxyl substituent on the B-nucleus, formation of (V), (V-CO), (VI), (VI′), (VIII) and trace amounts of (VII) were detected. As for the degradation of (IV) which has one methoxyl and one formyl substituents on the B-nucleus, (V), (V-CO), (VI), (VI′) and (VIII) were formed as products, and formation of trace amounts of formate (VII) was also suggested by mass chromatography. In the degradation of (III), which has no methoxyl substituent on the B-nucleus, by the fungus, (VIII) and trace amounts of (V) and (V-CO) were produced. However, formation of aromatic ring cleavage products could not be detected. The relationship between the products and the substrates is in accord with our previous results with lignin peroxidase from *P. chrysosporium*[19,20] and intact cells of *C. versicolor*[11]. Renganathan *et al.*[23] also reported that (V) was not detected in the degradation of another β-*O*-4 model (XII) which has no methoxyl group on the B-nucleus as (III) by lignin peroxidase from *P. chrysosporium.* Since evidence has been provided that lignin peroxidase functions by single-electron oxida-

tion of the aromatic nuclei to give the unstable cation radicals[18,24], it seems to be reasonable that the A-nucleus of (III) having two electron donating alcoxyl groups was preferentially oxidized by lignin peroxidase than the B-nucleus having one alcoxyl group resulting in the formation of (VIII) via 4-ethoxy-3-methoxybenzaldehyde.

Benzaldehydes are known to be initial products in C_α-C_β cleavage of β-*O*-4 and β-1 lignin substructure models and lignin by lignin peroxidase (e.g. 4-ethoxy-3-methoxybenzaldehyde for (I), (II), (III) and (IV))[13~19]. Accordingly, (IV) represents lignin degradation intermediates more accurately than (I), (II) or (III). Recently Kirk *et al.*[18] showed that a β-*O*-4 model compound (XI) which is a benzaldehyde analogous to (IV) was not a substrate for lignin peroxidase suggesting that the electron withdrawing formyl and carbonyl groups render both the A- and B-nuclei resistant to oxidation by lignin peroxidase. Present results, however, showed that the formyl substituent on the B-nucleus of (IV) was not important to the formation of (V), (V-CO), (VI) and (VI′) at least qualitatively by intact cells of *P. chrysosporium*. One of the possible formation mechanisms of (V), (V-CO), (VI) and (VI′) from (IV) in the culture of *P. chrysosporium* is as follows. The formyl group on C_1 position of B-ring of (IV) was first converted to less electron withdrawing functional groups. The B-nuclei of the intermediates thus formed were attacked by lignin peroxidase yielding the products, (V), (V-CO), (VI) and (VI′).

Acknowledgment

This research was partly supported by a Grant-in-Aid for Scientific Research (Nos. 59760124, 60760130, 60440015, 61760142) from the Ministry of Education of Japan.

References

1) A. Sakakibara: Wood Sci. Technol., **14**, 89 (1980).
2) A. Enoki, G.P. Goldsby and M.H. Gold: Arch. Microbiol., **125**, 227 (1980).
3) T. Umezawa, F. Nakatsubo and T. Higuchi: Agric. Biol. Chem., **47**, 2677 (1983).
4) T. Umezawa and T. Higuchi: FEBS Lett., **192**, 147 (1985).
5) S. Kawai, T. Umezawa and T. Higuchi: Agric. Biol. Chem., **49**, 2325 (1985).
6) A. Enoki, G.P. Goldsby and M.H. Gold: Arch. Microbiol., **129**, 141 (1981).
7) T. Umezawa and T. Higuchi: Agric. Biol. Chem., **48**, 1917 (1984).
8) T. Umezawa and T. Higuchi: Wood Res., No. 71, 25 (1985).
9) T. Umezawa and T. Higuchi: FEMS Microbiol. Lett., **26**, 123 (1985).
10) T. Umezawa and T. Higuchi: FEBS Lett., **182**, 257 (1985).
11) S. Kawai, T. Umezawa and T. Higuchi: Appl. Environ. Microbiol., **50**, 1505 (1985).
12) P. Fenn and T.K. Kirk: J. Wood Chem. Technol., **4**, 131 (1984).
13) M. Tien and T.K. Kirk: Science, **221**, 661 (1983).
14) J.K. Glenn, M.A. Morgan, M.B. Mayfield, M. Kuwahara and M.H. Gold: Biochem. Biophys. Res. Commun., **114**, 1077 (1983).
15) M. Tien and T.K. Kirk: Proc. Natl. Acad. Sci. USA, **81**, 2280 (1984).

16) M.H. GOLD, M. KUWAHARA, A.A. CHIU and J.K. GLENN: Arch. Biochem. Biophys., **234**, 353 (1984).
17) T. HABE, M. SHIMADA, T. UMEZAWA and T. HIGUCHI: Agric. Biol. Chem., **49**, 3505 (1985).
18) T.K. KIRK, M. TIEN, P.J. KERSTEN, M.D. MOZUCH and B. KALYANARAMAN: Biochem. J., **236**, 279 (1986).
19) T. UMEZAWA, M. SHIMADA, T. HIGUCHI and K. KUSAI: FEBS Lett., **205**, 287 (1986).
20) T. UMEZAWA and T. HIGUCHI: FEBS Lett., **205**, 293 (1986).
21) F. NAKATSUBO, T.K. KIRK, M. SHIMADA and T. HIGUCHI: Arch. Microbiol., **128**, 416 (1981).
22) T. UMEZAWA, F. NAKATSUBO and T. HIGUCHI: Arch. Microbiol., **131**, 124 (1982).
23) V. RENGANATHAN, K. MIKI and M.H. GOLD: Abstracts Lignin Biodegradation Workshop (Vancouver, Canada), 15 (1985).
24) P.J. KERSTEN, M. TIEN, B. KALYANARAMAN and T.K. KIRK: J. Biol. Chem., **260**, 2609 (1985).