Aromatic ring cleavage of β-*O*-4 lignin model dimers without prior demeth(ox)ylation by lignin peroxidase

Toshiaki Umezawa and Takayoshi Higuchi

Research Section of Lignin Chemistry, Wood Research Institute, Kyoto University, Uji, Kyoto 611, Japan

Received 11 July 1986

Methyl oxalate of arylglycerol was formed as an aromatic ring cleavage product in degradation of arylglycerol-β-aryl ether (β-*O*-4) type lignin substructure model dimers by extracellular lignin peroxidase of *Phanerochaete chrysosporium*. The enzymatic cleavage of arylglycerol-β-(*o*-[$^2H_3$]methoxyphenyl) ether indicated that the methyl group of the methyl ester was derived from the methoxy group of the β-*O*-4 model dimer. It is thus concluded that demeth(ox)ylation was not essential for the enzymatic aromatic ring cleavage of the methoxylated aromatic substrates, β-*O*-4 lignin substructure models.

Aromatic ring cleavage *Lignin peroxidase* *β-O-4 lignin substructure* *Methyl oxalate* *Demethylation* *Demethoxylation*

1. INTRODUCTION

In the accompanying paper [1], we demonstrated for the first time aromatic ring cleavage of β-*O*-4 lignin substructure models by extracellular lignin peroxidase from *Phanerochaete chrysosporium*: Three esters of 4-ethoxy-3-methoxyphenylglycerol, cyclic carbonate, formate, and monomethyl oxalate were formed from β-*O*-4 lignin substructure model dimers by the enzyme/H_2O_2 system.

This paper confirmed that the three esters formed by the enzyme reaction were aromatic ring cleavage products based on tracer experiments with arylglycerol-β-[U-*ring*-^{13}C]aryl ethers. Furthermore, the tracer experiment with arylglycerol-β-(*o*-[$^2H_3$]methoxyphenyl) ether provided the evidence that demethylation (or demethoxylation) was not essential for the enzymatic ring cleavage of the methoxylated aromatic substrates, β-*O*-4 lignin substructure models.

2. MATERIALS AND METHODS

2.1. *Preparation of substrates and authentic compounds*

4-Ethoxy-3-methoxyphenylglycerol-β-(*o*-[$^2H_3$]-methoxyphenyl) ether (I-D′) was prepared as in [2] from *o*-[$^2H_3$]methoxyphenol prepared previously [3]. MS (acetate:(I-D′-Ac)) *m/z* (%): 435(M^+, 13.2), 223(7.8), 212(16.3), 207(23.9), 206(22.4), 181(100). 1-(4-Ethoxy-3-methoxyphenyl)-2-(2,6-dimethoxyphenoxy)-1-methoxy-3-hydroxypropane (II-Me) was prepared by methylation (BF_3-Et_2O in CH_3OH, reflux) of 4-ethoxy-3-methoxyphenylglycerol-β-(2,6-dimethoxyphenyl) ether (II) prepared in [4]. ^{1}H-NMR (acetate:(II-Me-Ac)) ($CDCl_3$) δ(ppm): 1.46(3H×2,t,J=6.9,-O-C-CH_3×2); 1.92, 2.04,2.04 and 2.06(3H×4,s,-OAc×4); 3.23 and 3.24(3H×2,s,Cα-O-CH_3×2); 3.87 and 3.87(3H×2,s,Ph-O-CH_3×2); 3.89(1H,dd,γ-H); 4.09(2H×2, q,J=7.0,-O-CH_2-C×2); about 4.3(4H,m,γ-H×2 and α-H×2); 4.39(1H,dd,γ-H), 5.26(1H×2,m,β-H×2); 6.85(3H×2,m,aromatic). MS *m/z* (%): 340(M^+, 1.5), 196(12.0), 195(100), 167(42.5),152 (8.3),151(7.0). The following compounds were prepared previously: 4-Ethoxy-3- methoxyphenyl-

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

glycerol-β-[U-*ring*-^{13}C]guaiacyl ether (I-^{13}C) (^{13}C: ~90 atom%, statistically uniform labeling) [5]; 4-ethoxy-3-methoxyphenylglycerol-β-(2,6-[U-*ring*-^{13}C]dimethoxyphenyl) ether (II-^{13}C) (^{13}C: ~90 atom%, statistically uniform labeling) [4]; diethyl ether of (II), (II-Et) [1]; 1-(4-ethoxy-3-methoxyphenyl)-1,3-diethoxy-2-hydroxypropane (IV-Et) [1]; acetate of β,γ-cyclic carbonate (V-Ac) [5]; acetate of α,β-cyclic carbonate (V'-Ac) [1]; acetate of γ-formate (VI-Ac) [6]; and 1-(4-ethoxy-3-methoxyphenyl)-1,3-diethoxypropyl methyl oxalate (VII-Et) [1]. The symbol 'Ac' in the number of compounds denotes that the compounds with 'Ac' are the acetate of the corresponding compounds, e.g. (V-Ac) is the acetate of (V).

Compound (IV-Me-Ac), diacetate of 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxy-1-methoxypropane (IV-Me), was prepared from 4-ethoxy-3-methoxyacetophenone prepared previously [2] via the following steps. (1) $CuBr_2$ in ethyl acetate, reflux; (2) CH_3COONa in DMF, room temperature; (3) 28% CH_3ONa in CH_3OH solution in CH_3OH/CH_2Cl_2, 2:8 (v/v), 0°C; (4) ethyl vinyl ether/dl-10-camphorsulfonic acid in CH_2Cl_2, 0°C; (5) paraformaldehyde/K_2CO_3 in DMSO, room temperature; (6) ethyl vinyl ether/dl-10-camphorsulfonic acid in CH_2Cl_2, 0°C; (7) $NaBH_4$ in CH_3OH, 0°C; (8) CH_3I/NaH in DMF, room temperature; (9) 1 N HCl in acetone, room temperature; (10) acetic anhydride/pyridine in ethyl acetate, room temperature. ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.46(3H×2,t,J=6.9,-O-C-CH$_3$×2); 1.92, 2.04,2.04 and 2.06(3H×4,s,-OAc×4); 3.23 and 3.24(3H×2,s,Cα-O-CH$_3$×2); 3.87 and 3.87(3H×2,s,Ph-O-CH$_3$×2); 3.89(1H,dd,γ-H); 4.09(2H×2, q,J=7.0,-O-CH$_2$-C×2); about 4.3(4H,m,γ-H×2 and α-H×2); 4.39(1H,dd,γ-H), 5.26(1H×2,m,β-H×2); 6.85(3H×2,m,aromatic). MS *m/z* (%): 340(M$^+$, 1.5), 196(12.0), 195(100), 167(42.5),152 (8.3),151(7.0). The following compounds were prepared previously: 4-Ethoxy-3- methoxyphenylglycerol-β-[U-*ring*-^{13}C]guaiacyl ether (I-^{13}C) (^{13}C: ~90 atom%, statistically uniform labeling) [5]; 4-ethoxy-3-methoxyphenylglycerol-β-(2,6-[U-*ring*-^{13}C]dimethoxyphenyl) ether (II-^{13}C) (^{13}C: ~90 atom%, statistically uniform labeling) [4]; diethyl ether of (II), (II-Et) [1]; 1-(4-ethoxy-3-methoxyphenyl)-1,3-diethoxy-2-hydroxypropane (IV-Et) [1]; acetate of β,γ-cyclic carbonate (V-Ac) [5]; acetate of α,β-cyclic carbonate (V'-Ac) [1]; acetate of γ-formate (VI-Ac) [6]; and 1-(4-ethoxy-3-methoxyphenyl)-1,3-diethoxypropyl methyl oxalate (VII-Et) [1]. The symbol 'Ac' in the number of compounds denotes that the compounds with 'Ac' are the acetate of the corresponding compounds, e.g. (V-Ac) is the acetate of (V).

2.2. *Enzymes*

Enzyme preparation and enzyme assay were reported in the accompanying paper [1].

2.3. *Enzyme reactions*

Unless otherwise noted, enzyme reactions, acetylation and GC-MS analysis of products were run in the same way as in the accompanying paper [1].

^{18}O experiments with a reaction mixture containing $H_2{}^{18}O$ (total volume 1.1 ml) were performed as follows. 1065 μl of 100 mM sodium tartrate buffer (pH 3.0) in a 20 ml Fernbach flask was lyophilized and redissolved into 545 μl of $H_2{}^{18}O$ (CEA, ^{18}O: 97.53%) and then 520 μl distilled water was added. To the ^{18}O-enriched buffer solution, 25 mM H_2O_2 (20 μl), 0.3 μmol substrate, (II-Me) or (II-Et), in 10 μl CH_3OH and 5 μl lignin peroxidase (0.3 unit) were added, and the reaction mixture was incubated for 5 min at 37°C under air. Reactions were terminated by extraction with ethyl acetate (10 ml). The ethyl acetate layer was dried over Na_2SO_4 and evaporated. As for compound (II-Me), half of the ethyl acetate extract was re-incubated at 37°C for 5 min in the $H_2{}^{16}O$ medium without addition of the enzyme (total volume 550 μl, 535 μl of 100 mM sodium tartrate (pH 3.0), 10 μl of 25 mM H_2O_2, 5 μl CH_3OH). Products were analyzed by GC-MS (mass chromatography) immediately (less than 3 min) or after acetylation (acetic anhydride/pyridine, 10 h, room temperature).

2.4. *Instruments*

^{1}H-NMR spectra were taken with a Varian XL-200 FT-NMR spectrometer (TMS as an internal standard). GC-MS was done on a Shimadzu GCMS QP-1000 (EI-MS, 70 eV, column: 1.5% OV-17 on Chromosorb W AW DMCS (Shinwa Kakou, Japan), glass column 1 m × 0.26 cm (i.d.), column temperature: 170–240°C, 5°C/min).

3. RESULTS

Compounds (I-^{13}C) and (II-^{13}C) were degraded by the lignin peroxidase/H_2O_2 system. GC-MS analysis (mass chromatography) of acetates of the products (fig.1) showed that β,γ-cyclic carbonate (V-Ac), α,β-cyclic carbonate (V'-Ac), formate (VI-Ac) and methyl oxalate (VII-Ac) were formed as aromatic ring cleavage products by the enzyme system: Molecular ion peaks of the cyclic carbonate and the formate from (I-^{13}C) and/or (II-^{13}C) were *m/z* 311 and 355, respectively, one mass unit higher than that from unlabeled (II) and those of unlabeled authentic samples, while the molecular ion peak of the methyl oxalate from both (I-^{13}C) and (II-^{13}C) was *m/z* 414, 2 mass units higher than that from unlabeled (II) (fig.1).

Compound (I-D'), which has a deuterated methoxyl group on the B-ring, was used as a substrate and degraded by the lignin peroxidase system. GC-MS analysis (mass chromatography) showed that the molecular ion peak was *m/z* 415, 3 mass units higher than that from (II) having non-deuterated methoxyl groups on the β-aryl group (fig.1). Accordingly, the methyl group of the methyl ester of the oxalate was demonstrated to be derived from the methoxyl group on the B-ring of (I-D').

In further investigations, etherated substrates (II-Et) and (II-Me) were used in ^{18}O-incorporation experiments, since some products from these substrates can be submitted directly to GC-MS analysis without acetylation. In a preliminary experiment, compound (II-Me), the α-methyl ether of (II) which represents β-*O*-4 substructures etherated at the α-position, was incubated with the enzyme system under $H_2{}^{16}O$. The arylglycerol (IV-Me-Ac) [MS *m/z* (%): 340(M^+, 2.3), 196(14.3), 195(100), 167(40.4), 152(8.6), 151(8.4)], cyclic carbonate (V-Me) [MS *m/z* (%): 282(M^+, 5.6), 196(12.1), 195(100), 167(61.0), 152(14.7), 151(9.4)], formate (VI-Me-Ac) [MS *m/z* (%): 326(M^+, 2.8), 196(11.7), 195(100), 167(41.3), 152(8.4), 151(6.3)] and methyl oxalate (VII-Me-Ac) [MS *m/z* (%): 384(M^+, 3.7), 196(12.5), 195(100), 167(33.2), 152(7.7), 151(6.3)] were iden-

Table 1

^{18}O incorporation into the products in the degradation of (II-Me) and (II-Et) by the lignin peroxidase/H_2O_2 system under $H_2{}^{18}O$

Substrates		% incorporation of ^{18}O[a]			
		Formate (VI-Me)	Cyclic carbonate (V-Me)	Oxalate[b] (VII-Me)	(VII-Et)
(II-Me)	not acetylated[c]	NA	96	NA	–
	acetylated[c]	86	98	100	–
(II-Me) re-incubated under $H_2{}^{16}O$[d]	not acetylated[c]	NA	98	NA	–
	acetylated[c]	51	98	94	–
(II-Et)	not acetylated[c]	–	–	–	90
	acetylated[c]	–	–	–	90

[a] % incorporation of ^{18}O = 100 × (^{18}O content in the ester carbonyl oxygen of the products)/(^{18}O content in $H_2{}^{18}O$ of the medium) (49 atom%)

[b] One atom of ^{18}O was incorporated into the carboxyl oxygen of the products, since $M^+ + 4$ of both the products was negligible

[c] Ethyl acetate extracts of the products were submitted to the gas chromatograph-mass spectrometer directly ('not acetylated') or after acetylation ('acetylated')

[d] Half of the products in the degradation of (II-Me) by the lignin peroxidase/H_2O_2 system under $H_2{}^{18}O$ was re-incubated under $H_2{}^{16}O$

NA, not assayed

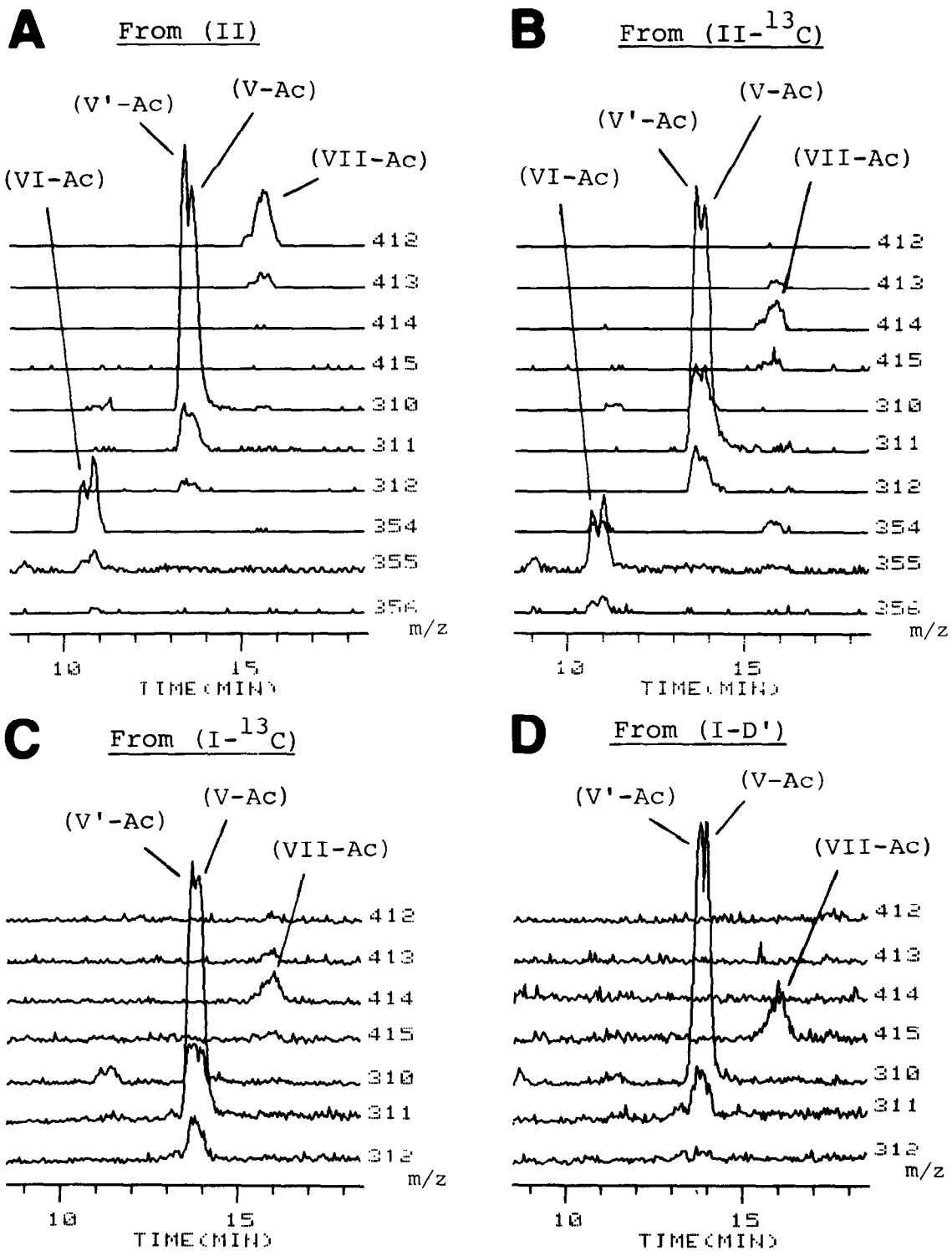

Fig.1. Mass chromatogram of molecular ion peaks of degradation products from (II), (II-^{13}C), (I-^{13}C) and (I-D') by the lignin peroxidase/H_2O_2 system. (A) Degradation products from unlabeled 4-ethoxy-3-methoxyphenylglycerol-β-

(2,6-dimethoxyphenyl) ether (II). Molecular ion peaks of (VII-Ac), (V-Ac), (V'-Ac) and (VI-Ac) are *m/z* 412, 310, 310 and 354, respectively. (B,C) Degradation products from 4-ethoxy-3-methoxyphenylglycerol-*β*-(2,6-[U-*ring*-^{13}C]dimethoxyphenyl) ether (II-^{13}C) and -*β*-[U-*ring*-^{13}C]guaiacyl ether (I-^{13}C), respectively. Molecular ion peaks of (VII-Ac), (V-Ac), (V'-Ac) and (VI-Ac) are *m/z* 414, 311, 311 and 355, respectively. (D) Degradation products from 4-ethoxy-3-methoxyphenylglycerol-*β*-(*o*-[^{2}H$_3$]methoxyphenyl) ether (I-D'). Molecular ion peak of (VII-Ac) is *m/z* 415.

tified as products by GC-MS analysis after acetylation. The spectrum and retention time of (IV-Me-Ac) were identical to those of synthesized authentic compound. Formation of methyl oxalate (VII-Et) in the degradation of (II-Et) by the enzyme system was reported in the accompanying paper [1]. Furthermore, arylglycerol (IV-Et-Ac) [MS *m/z* (%): 340(M^+, 2.0), 209(100), 181(16.0), 153(8.1), 151(4.3), 125(8.8), 93(11.0)] was identified as another product by comparison of the mass spectrum and the retention time with those of the authentic compound. In the degradation of compounds (II-Me) and (II-Et) under $H_2{}^{18}O$ (^{18}O: 49 atom%), ^{18}O atoms were incorporated into aromatic ring cleavage products. Table 1 summarizes the ^{18}O contents of the products. To evaluate oxygen exchange between the oxygen atoms of carboxyl groups of products and H_2O, half of the reaction products from (II-Me) was reincubated under $H_2{}^{16}O$. Residual ^{18}O contents of products are also shown in table 1.

4. DISCUSSION

In the accompanying paper [1], we reported that cyclic carbonates (V) and (V'), formate (VI) and

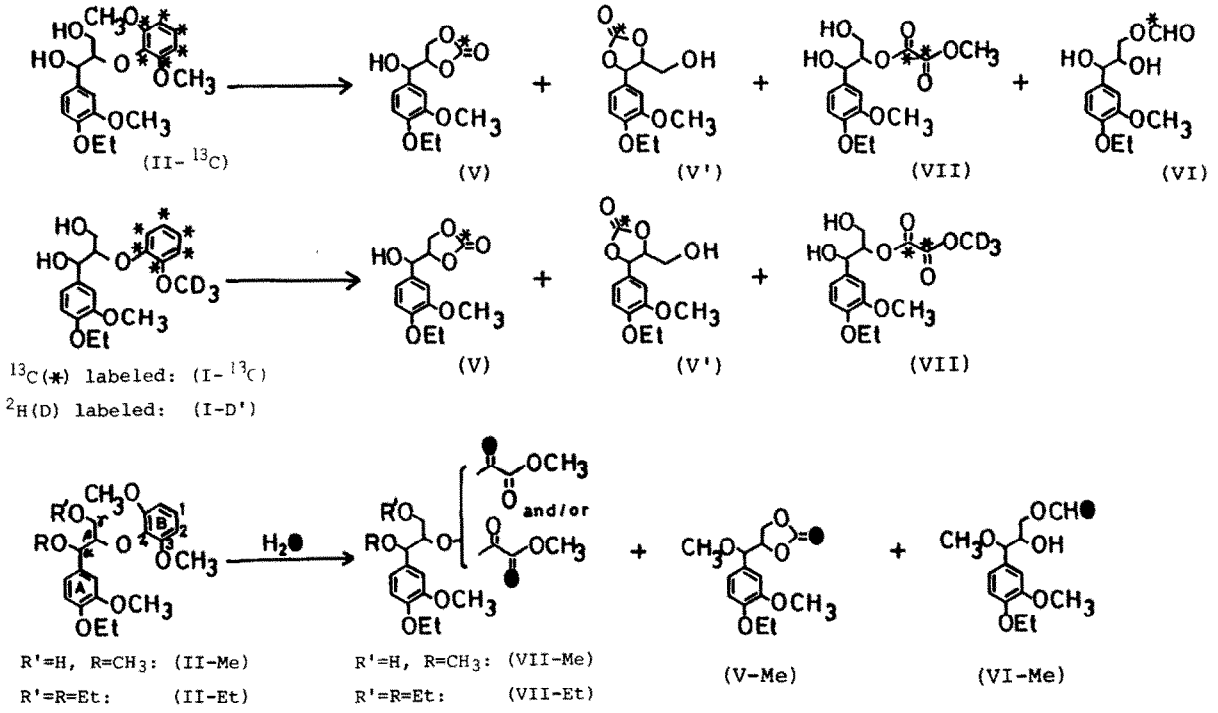

Fig.2. Aromatic ring cleavage products and ^{18}O incorporation into the products in the degradation of *β*-*O*-4 lignin model dimers by the lignin peroxidase/H_2O_2 system. As for methyl oxalates (VII) and (VII-Me), and formate (VI-Me), the positions of the oxalyl group and the formyl group were assigned tentatively at the *β*-position and *γ*-position, respectively, based on identification of (VII-Et) and (VI) from (II-Et) and (II-^{13}C), respectively. (*) ^{13}C, (D) ^{2}H, (●) ^{18}O; Et, CH_2CH_3.

methyl oxalates (VII) and (VII-Et) were formed from 4-ethoxy-3-methoxyphenylglycerol-β-aryl ethers by lignin peroxidase/H_2O_2 system. Here, we have confirmed that the enzymatic reaction products, cyclic carbonates (V) and (V′), formate (VI), and methyl oxalate (VII) were aromatic ring cleavage products based on the tracer experiments with arylglycerol-β-[U-*ring*-^{13}C]aryl ethers as substrates (fig.2).

As for methyl oxalate, we established by the tracer experiment with arylglycerol-β-(*o*-[$^2H_3$]-methoxyphenyl) ether as a substrate that the methyl group of the methyl ester was derived from the methoxyl group on the B-ring (fig.2). The result clearly indicates that demeth(ox)ylation is not essential for aromatic ring cleavage by lignin peroxidase, while earlier work [7] suggested that demethylation of lignin related methoxylated monomeric aromatics to give *o*-diphenols is a prerequisite for ring cleavage by dioxygenases. It is noteworthy and interesting that lignin peroxidase catalyzes both the cleavages of side chains and aromatic rings of lignin substructure models [1] and that the aromatic ring cleavage by the fungus is different from that of simple monomeric phenols by bacteria which need conventional dioxygenases, although the possible involvement of other enzymes including dioxygenases in lignin degradation by the fungus is still open.

The carbonyl oxygen of cyclic carbonate (V-Me) and formate (VI-Me), and one of the carbonyl oxygen of methyl oxalates (VII-Me) and (VII-Et) were derived from $H_2{}^{(18)}O$ (fig.2). Since the ^{18}O contents of oxalates (VII-Me) and (VII-Et) and cyclic carbonate (V-Me) were not reduced and that of formate (VI-Me) had not disappeared completely on re-incubation of the ^{18}O-incorporated products with $H_2{}^{16}O$ medium, the ^{18}O atoms were probably incorporated in the formation of the products.

Furthermore, in the degradation of (II-Et) by the enzyme system another new product was detected, which was eluted from the GC column after the substrate (II-Et) (not shown). The product could not be acetylated by acetylation suggesting that the product has no acetylatable hydroxyl group. The molecular ion of the product was *m/z* 466 (base peak *m/z* 209) which is 32 mass

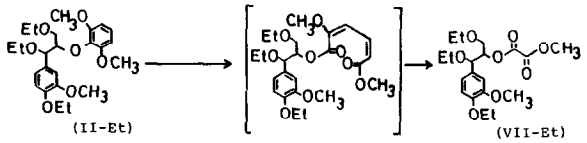

Fig.3. A possible initial product in the aromatic ring cleavage of (II-Et) by the lignin peroxidase/H_2O_2 system.

units higher than that of (II-Et) (*m/z* 434) indicating the addition of two oxygen atoms to the substrate. The $H_2{}^{18}O$ incorporation experiment showed that one of the oxygen atoms of the product was derived from $H_2{}^{(18)}O$. These results suggested that one of the possible structures of the product is methyl arylglyceryl muconate, probably an initial aromatic ring cleavage product, which could be converted to monomethyl oxalate of arylglycerol (VII-Et) (fig.3). Further investigations on the product are in progress.

ACKNOWLEDGEMENTS

The authors thank Nagase Biochemicals, Ltd (Fukuchiyama, Kyoto, Japan) for the generous gift of lignin peroxidase. This research was partly supported by a Grant-in-Aid for Scientific Research (nos 60760130, 60440015, 61760142) from the Ministry of Education of Japan.

REFERENCES

[1] Umezawa, T., Shimada, M., Higuchi, T. and Kusai, K. (1986) FEBS Lett. 205, 287–292.
[2] Umezawa, T. and Higuchi, T. (1985) FEBS Lett. 192, 147–150.
[3] Umezawa, T. and Higuchi, T. (1985) FEMS Microbiol. Lett. 26, 123–126.
[4] Kawai, S., Umezawa, T. and Higuchi, T. (1985) Appl. Environ. Microbiol. 50, 1505–1508.
[5] Umezawa, T. and Higuchi, T. (1985) FEBS Lett. 182, 257–259.
[6] Kawai, S., Umezawa, T. and Higuchi, T. (1985) Agric. Biol. Chem. 49, 2325–2330.
[7] Cain, R.B. (1980) in: Lignin Biodegradation: Microbiology, Chemistry, and Potential Applications (Kirk, T.K. et al. eds) vol.1, pp.21–60, CRC Press, Boca Raton, FL.