

DEGRADATION MECHANISMS
OF LIGNIN SUBSTRUCTURE MODEL COMPOUNDS
BY *CORIOLUS VERSICOLOR*

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CONTENTS

INTRODUCTION	1
CHAPTER 1	
DEGRADATION OF NONPHENOLIC β -O-4 LIGNIN SUBSTRUCTURE MODEL COMPOUNDS BY THE CULTURE OF <i>CORIOLUS VERSICOLOR</i>	12
1.1 Degradation Pathways for a Nonphenolic β - O-4 Lignin Substructure Model	13
1.2 Aromatic Ring Cleavage of Nonphenolic β -O- 4 Lignin Substructure Models	35
1.3 Formation of <i>p</i> -Benzoquinone Monoketals as Novel Degradation Products	44
CHAPTER 2	
DEGRADATION OF NONPHENOLIC β -O-4 LIGNIN SUBSTRUCTURE MODEL COMPOUNDS BY LIGNIN PEROXIDASE OF <i>CORIOLUS</i> <i>VERSICOLOR</i>	56
CHAPTER 3	
DEGRADATION OF PHENOLIC LIGNIN MODEL COMPOUNDS BY LACCASE OF <i>CORIOLUS VERSICOLOR</i>	70
3.1 Ca-C β Cleavage of a Phenolic β -1 Lignin Substructure Model	71
3.2 Degradation Mechanisms of Phenolic β -1 Lignin Substructure Models	81
3.3 Degradation Mechanisms of Phenolic β -O-4 Lignin Substructure Models	101
3.4 Aromatic Ring Cleavage of 4,6-Di- <i>tert</i> - butyl-2-methoxyphenol	115

3.5 Oxidation of Nonphenolic Benzyl Alcohols in the Presence of Syringaldehyde	123
CONCLUSION	132
REFERENCES	137
ACKNOWLEDGMENTS	145

ABBREVIATIONS

Ac	acetyl
	acetate derivative
anhyd	anhydrous
CI	chemical ionization
concd	concentrated
d	doublet (NMR)
<i>d</i>	dextrorotatory
DDQ	2,3-dichloro-5,6-dicyano-1,4-benzoquinone
DI	direct inlet system (MS)
DMF	dimethylformamide
ϵ	molar absorptivity
EI	electron impact (MS)
	electron ionization (MS)
equiv	equivalent
ESR	electron spin resonance
Et	ethyl ester derivative
eV	electronvolt
FID	flame ionization detector
g	gram
<i>g</i>	gravitation constant
GC	gas chromatography
h	hour
Hz	herz (s^{-1})
i.d.	inside diameter
IR	infrared
kat	katal
<i>l</i>	levorotatory
L	liter
LAH	lithium aluminum hydroxide
m	meter
	milli (10^{-3})
	multiplet (NMR)
μ	micro (10^{-6})
<i>m</i>	meta
M	$mol \cdot L^{-1}$ (molar)
min	minute
mol	mole
MS	mass spectroscopy
	mass spectrum
MWL	milled wood lignin
<i>m/z</i>	mass-to-charge ratio
n	nano (10^{-9})

<i>n</i>	normal (e.g. <i>n</i> -hexane)
<i>v</i>	frequency (wavenumber)
<i>N</i>	normal (concentration)
NMR	nuclear magnetic resonance
<i>o</i>	ortho
<i>p</i>	para
Pd-C	palladium on activated carbon
Ph	phenyl
pH	hydrogen ion concentration
ppm	parts per million
<i>q</i>	quartet (NMR)
<i>R_f</i>	retardation factor
<i>s</i>	singlet (NMR)
soln	solution
<i>t</i>	triplet (NMR)
temp	temperature
<i>tert</i>	tertiary (e.g. <i>tert</i> -butyl)
THF	tetrahydrofuran
TLC	thin-layer chromatography
<i>U</i>	uniformly labeled
<i>v/v</i>	volume-to-volume ratio
<i>w/w</i>	weigh-to-weight ratio

INTRODUCTION

Lignin occurs in cell walls of true vascular plants, ferns, and club mosses but does not in those of mosses, algae, and microorganisms (Kawamura & Higuchi, 1964; Higuchi, 1985a). Lignin is covalently bonded to hemicelluloses, and distributed in the spaces of cellulose microfibrils. Lignin is the most abundant renewable resource next to cellulose, and its content in woody plants ranges from 15-36%. A considerable part of the photosynthesis in plant is contributed to the conversion of atmospheric carbon dioxide to lignin. Lignin constitutes to about 40% of the solar energy store in plant (Janshekar & Fiechter, 1983). Hence lignin metabolism plays an important role in the carbon cycle on earth.

Chemical and spectrometric studies of lignin have been performed by many scientists, and the outline of lignin structure was shown in the late of 1960s (Adler, 1977; Sakakibara, 1983). Lignin is an amorphous, water-insoluble, three-dimensional aromatic polymer formed by the dehydrogenative polymerization of *p*-hydroxycinnamyl alcohols, *p*-coumaryl, coniferyl, and sinapyl alcohols, by peroxidase as shown in Fig. 1. Lignin contains of various stable carbon-to-carbon and ether linkages between monomeric phenylpropane units, such as arylglycerol- β -aryl ether [β -O-4' substructure, (intermonomer linkages 1-2, 2-3, 4-5, 6-7, 7-8, and 13-14 in Fig. 1.)], phenylcoumarane [β -5' substructure, (3-4)], diarylpropane [β -1' substructure, (8-9)], biphenyl [5-5' substructure, (5-6 and 11-12)], resinol [β - β ' substructure, (10-11)] etc. Most of the substructures are not easily hydrolyzable. Thus, lignin is resistant to the degradation by most microorganisms.

Lignin biodegradation has received a great attention during the past 10 years (Crawford & Crawford 1984; Chen & Chang, 1985; Harvey et al., 1985; Higuchi, 1985b; Kirk & Shimada, 1985; Leisola & Fiechter, 1985; Higuchi, 1986; Kirk & Farrell, 1987;

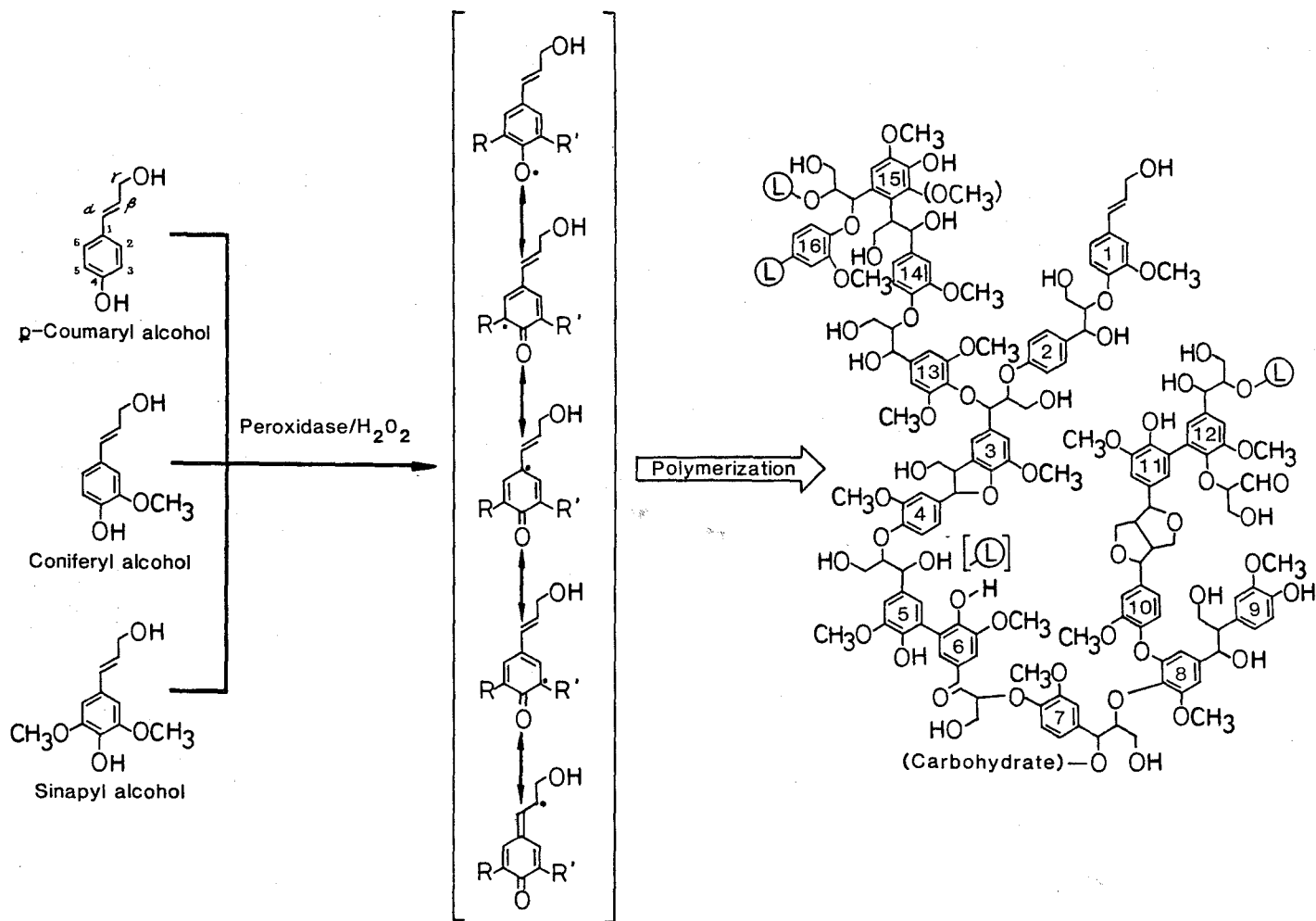


Fig. 1. Dehydrogenative polymerization of *p*-hydroxycinnamyl alcohols and the structure of lignin (Adler, 1977).

INTRODUCTION

Buswell & Odier, 1987; Umezawa, 1988). Elucidation of the lignin biodegradation mechanisms is essential not only for understanding the earth's carbon cycle, but also for establishing new technologies on pulping, bleaching, converting lignins to useful chemicals etc., and for protecting the environment from lignin related pollutions. From the structural features of lignin, biodegradative systems must be extracellular, non-specific, and non-hydrolytic, and are different from those for other natural polymers such as polysaccharides, proteins, and nucleic acids.

Lignin is more or less degraded by fungi, which classified as white-rot (Basidiomycetes and a few Ascomycetes), brown-rot (Basidiomycetes), and soft-rot (Ascomycetes and Fungi Imperfecti) fungi, based on the type of decay. Among these, white-rot fungi are the most powerful lignin-degrader, and have been used frequently for lignin biodegradation research [e.g. *Phanerochaete chrysosporium* (= *Sporotrichum pulverulentum*), *Coriolus* (= *Polyporus*, *Trametes*, *Polystictus*) *versicolor*, *Phlebia radiata*, *Lentinula* (= *Lentinus*) *edodes* (shiitake)]. Some of bacteria (e.g. *Streptomyces*, *Pseudomonas*) degrade lignin partly.

Two complementary approaches have been applied to lignin biodegradation researches: (i) characterization of polymeric degraded lignin and identification of low molecular weight products from fungus-decayed wood (Chen & Chang, 1985), and (ii) degradation of synthesized oligolignols (lignin substructure model compounds) by fungus and identification of the degradation products (Higuchi, 1985b). From the former approach general degradation manners and some biochemical features for lignin degradation as follows have been deduced: (i) C α -C β cleavage of side chains, (ii) cleavage of β -aryl ether bond, and (iii) aromatic ring cleavage (Chen & Chang, 1985). However, the specific reactions on the degradation pathways could not be elucidated sufficiently for the complex structure of lignin. In this point,

INTRODUCTION

the latter approach with the substructure model compounds is useful, and makes possible the detailed studies of specific reactions of lignin biodegradation. Indeed, by the use of model compounds lignin peroxidase (ligninase) was first detected from the culture of *P. chrysosporium* (Tien & Kirk, 1983; Glenn et al., 1983).

In the 1960s, *p*-diphenol oxidase, laccase (*p*-diphenol:oxygen oxidoreductase, EC 1.10.3.2), received a considerable attention as a lignin-degrading enzyme (Harkin, 1967). Because laccase is commonly excreted by lignin-degrading fungi, induced by phenols, and causes Bavendamm's reaction, by which wood-rotting fungi have been roughly classified into lignin decomposing and nondecomposing types (Higuchi, 1971).

Kirk et al. (1968a,b) reported the alkyl-aryl cleavage and α oxidation of phenolic β -O-4 lignin substructure model compounds by the laccase and the culture of *C. versicolor*. However, both laccase and the culture did not catalyze the oxidation of the nonphenolic β -O-4 model compounds, which represent the structure having etherified phenolic units. Later, they found that the culture conditions used in the above investigation did not suitable for the expression of a ligninolytic system. They developed a new biodegradation assay of lignin based on the decomposition of synthetic ^{14}C -labeled lignins to $^{14}\text{CO}_2$ (Kirk et al., 1975) and defined culture parameters for the ligninolytic system of *P. chrysosporium*, that is, nitrogen-limiting, high O_2 partial pressure and stationary cultures (Kirk et al., 1978). It was subsequently reported that *C. versicolor* exhibits ligninolytic activity under these culture conditions (Reid & Seifert, 1981; Leatham & Kirk, 1983; Evans & Palmer, 1983). These ligninolytic culture conditions were adopted for the degradation of lignin substructure model compounds by *P. chrysosporium* (see Higuchi, 1985b; 1986) and brought the discovery of the ligninolytic enzyme,

INTRODUCTION

lignin peroxidase (Tien & Kirk, 1983; Glenn et al., 1983). On the other hand, Kamaya and Higuchi found that a nonphenolic β -1 lignin substructure model compound (Kamaya & Higuchi, 1984a) and 3,4-dimethoxycinnamyl alcohol (Kamaya & Higuchi, 1984b) are degraded by the ligninolytic culture of *C. versicolor*.

The present author, first of all, investigated the degradation of a nonphenolic β -O-4 lignin substructure model compound, 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol (1), by the ligninolytic culture of *C. versicolor* (Section 1.1). Chemical structures of substrates, degradation products, etc. are shown in Fig. 2.

Because β -O-4 substructure is the major interphenylpropane unit in lignin, elucidation of the degradation pathways for this substructure is very important. The substrate (1) was degraded rapidly by the ligninolytic culture of *C. versicolor* and gave many degradation products, (2), (3), (8), (9), (24)-(28), (43)-(45), and (52). The chemical structures of the products suggested the involvement of the following reactions in the degradation: (i) oxidation and reduction of benzylic position, (ii) β -ether cleavage, (iii) C_α - C_β cleavage of side chain, (iv) aromatic ring cleavage, and (v) the formation of *p*-benzoquinone monoketals (8) and (9). The cleavage of β -etherated aromatic rings were proved by use of ^{13}C -labeled compounds (Section 1.2). *p*-Benzoquinone monoketal (8) was found for the first time as a degradation product of β -O-4 lignin model compounds by lignin peroxidase of *P. chrysosporium*, and the formation mechanisms of (8) was discussed based on the incorporation experiment of $^{18}\text{O}_2$ (Section 1.3). The degradation pathways of nonphenolic β -O-4 lignin substructure model compounds by the culture of *C. versicolor* were similar to those proposed for *P. chrysosporium* and its lignin peroxidase (see Umezawa, 1988). The results suggested that *C. versicolor* also produces the lignin peroxidase. Recently, lignin

INTRODUCTION

peroxidase was purified from the culture filtrate of *C. versicolor* (Dodson et al., 1987), but the degradation of β -O-4 substructure by this enzyme has not been reported.

In Chapter 2, the degradation of nonphenolic β -O-4 lignin substructure model compounds by lignin peroxidase of *C. versicolor* is discussed. Most of the degradation reactions, except reduction of benzaldehyde to benzyl alcohol, by the culture of *C. versicolor* (Kawai et al., 1985a,b; 1987) were found to be caused by lignin peroxidase.

In Chapter 3, the degradation of phenolic lignin model compounds by laccase of *C. versicolor* is discussed. As mentioned above, laccase is commonly excreted by lignin-degrading fungi. Ander and Eriksson (1976) reported that a phenol oxidase less-mutant of *S. pulverulentum* (= *P. chrysosporium*) could not degrade lignin, but the addition of purified fungal laccase to this mutant restored the ability for lignin degradation suggesting that laccase is involved in the lignin-degrading system. On the other hand, earlier papers reported that polymerization is major reaction of laccase for lignin preparations (see Kirk & Shimada, 1985). Polymerization of lignin by lignin peroxidase was also reported recently (Haemerli et al., 1986). This is understandable because the initial steps of the degradation reaction catalyzed by both enzymes are one-electron oxidations of phenolic moieties in lignin to the corresponding phenoxy radicals which are polymerizes by coupling.

In relation to the lignin degradation, a few papers on the oxidation of phenolic β -O-4 model compounds by laccase (Kirk et al., 1968a,b; Wariishi et al., 1987) but nothing on β -1 model compounds have been reported. In Section 3.1 and 3.2, C α -C β cleavage, alkyl-aryl cleavage, and C α oxidation of phenolic β -1 lignin substructure model compounds (17)-(19) by laccase are described. Based on the isotopic experiments with ¹⁸O-labeled

INTRODUCTION

water and molecular oxygen, the degradation mechanisms for phenolic β -1 lignin substructure model compounds are proposed. In addition, the degradation of phenolic β -O-4 lignin substructure model compounds (12) and (13) was examined. The model compounds were degraded via the similar reactions proposed for phenolic β -1 model compounds (17)-(19) (Section 3.3). In Section 3.4, the first identification of aromatic ring cleavage product (62) from (60) by laccase is described. On the basis of the tracer experiments with H_2^{18}O and $^{18}\text{O}_2$, the formation mechanisms of (62) are discussed. Finally the possibility of the degradation of non-phenolic lignin model compounds by laccase is examined (Section 3.5). Nonphenolic benzyl alcohols, (47) and (56), were found to be oxidized to corresponding aldehydes, (46) and (55), by laccase in the presence of syringaldehyde (51).

INTRODUCTION

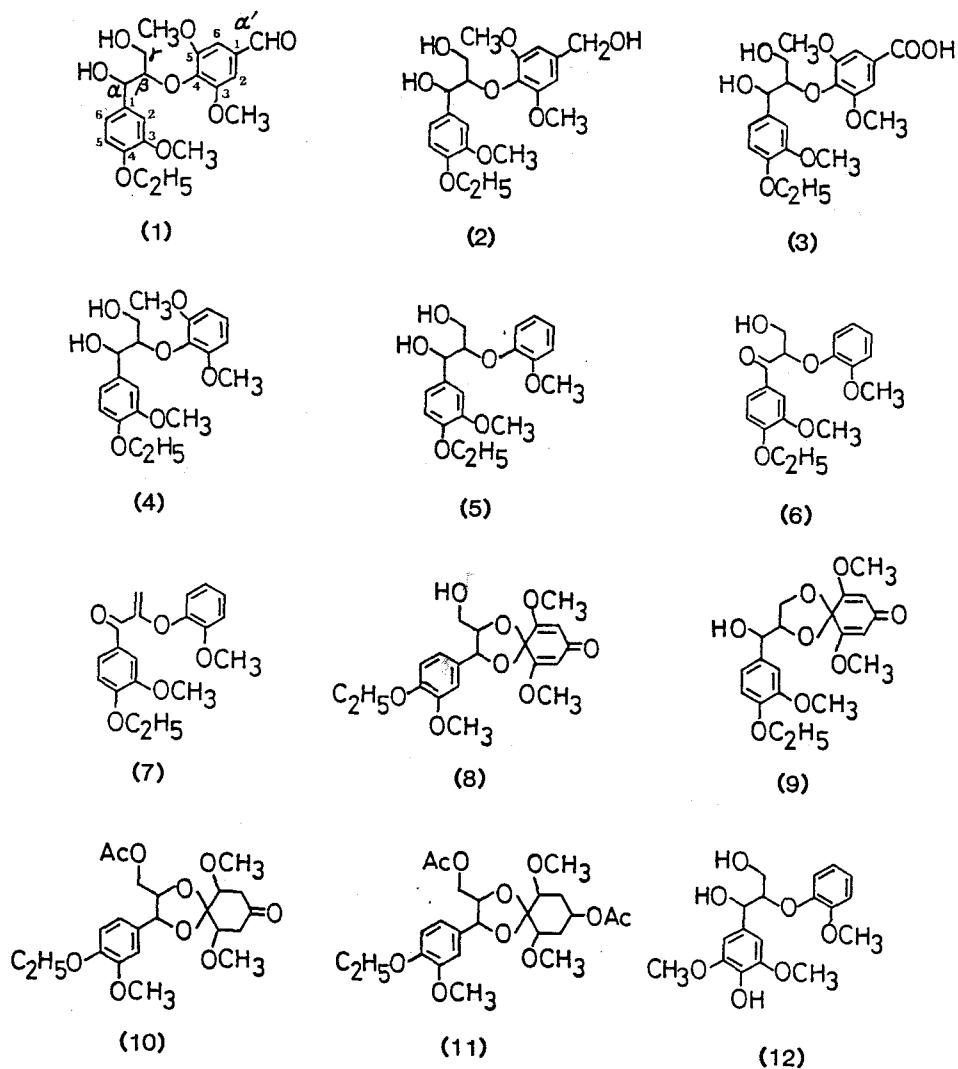
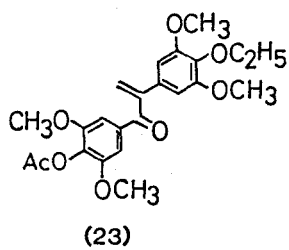
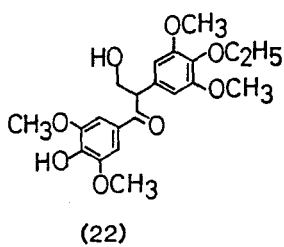
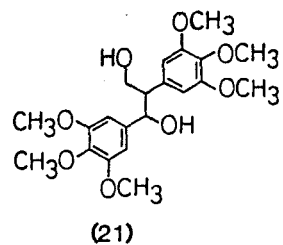
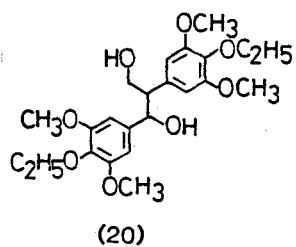
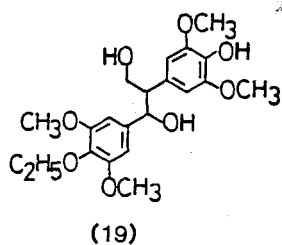
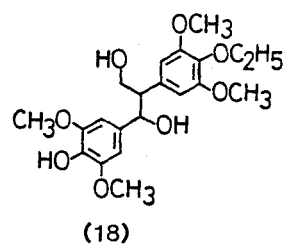
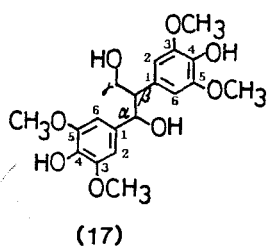
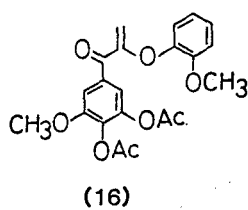
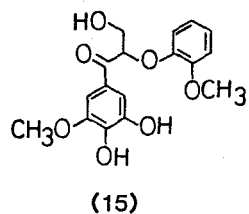
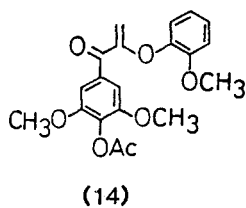
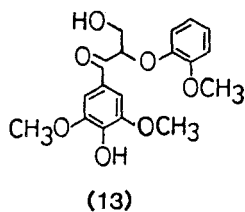


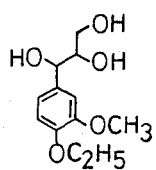
Fig. 2. Structural formulae of the compounds.

The numbering system illustrated in (1) and (17) is commonly used in lignin chemistry.

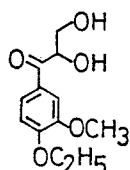
INTRODUCTION



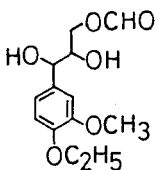
INTRODUCTION



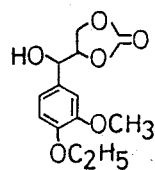
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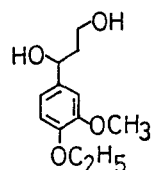
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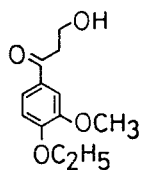
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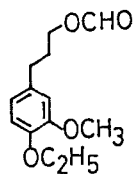
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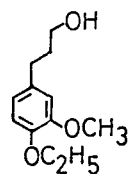
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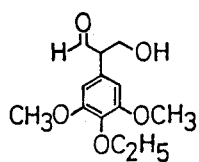
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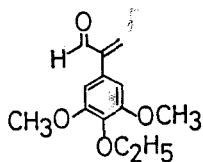
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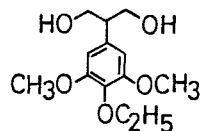
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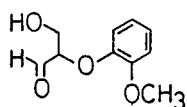
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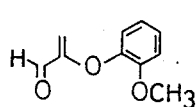
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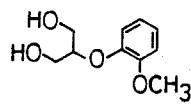
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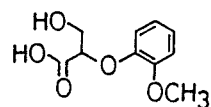
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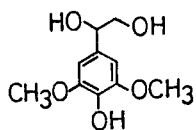
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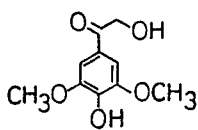
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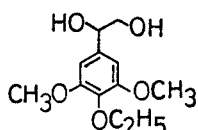
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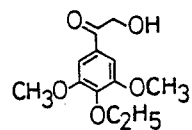
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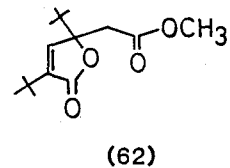
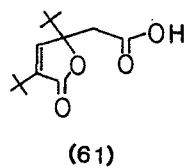
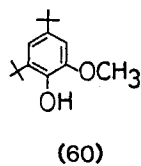
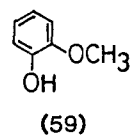
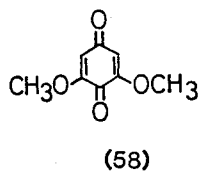
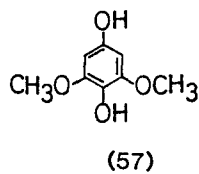
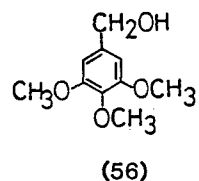
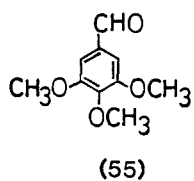
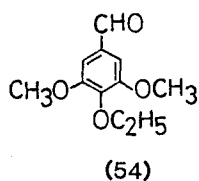
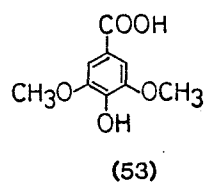
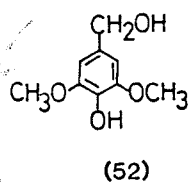
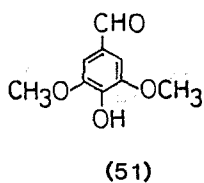
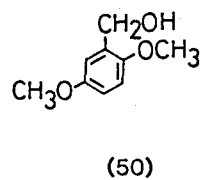
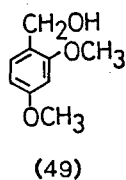
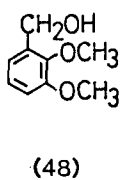
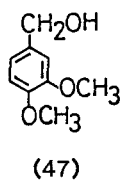
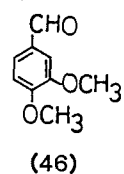
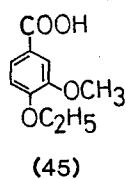
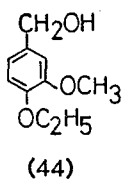
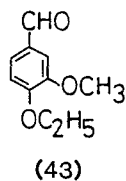


(41)



(42)

INTRODUCTION



CHAPTER 1

DEGRADATION OF NONPHENOLIC β -O-4 LIGNIN SUBSTRUCTURE MODEL COMPOUNDS BY THE CULTURE OF *CORIOLUS VERSICOLOR*

The arylglycerol- β -aryl ether bond (β -O-4 substructure) is the most frequent interphenylpropane linkage in lignin (48% and 60% in spruce and birch lignins, respectively) (Adler, 1977). It is, therefore, very important to elucidate the degradation pathways for the β -O-4 substructure by fungi in view of the chemistry and biochemistry of lignin biodegradation.

Coriolus versicolor is one of the most common lignin degrading fungi. Consequently, it is likely that *C. versicolor* degrades the β -O-4 substructure of lignin. Nevertheless, earlier papers in 1960s (Russell et al., 1961; Kirk et al., 1968a,b) reported that phenolic β -O-4 lignin models were degraded, but nonphenolic β -O-4 models could not be degraded by this fungus.

Later, many researchers (Reid & Seifert, 1981; Leatham & Kirk, 1983; Evans & Palmer, 1983) reported that *C. versicolor* exhibits ligninolytic activity under the culture conditions (nitrogen-limiting, high O₂ partial pressure and stationary cultures) defined for *Phanerochaete chrysosporium* by Kirk et al. (1978). Kamaya and Higuchi (1984a,b) reported that a nonphenolic β -1 lignin substructure and 3,4-dimethoxycinnamyl alcohol were degraded by *C. versicolor* under these culture conditions.

The present chapter describes the catabolism of nonphenolic β -O-4 lignin substructure model compounds by the ligninolytic culture of *C. versicolor*.

Section 1.1

1.1 DEGRADATION PATHWAYS FOR A NONPHENOLIC β -O-4 LIGNIN SUB-STRUCTURE MODEL

1.1.1 INTRODUCTION

The purpose of the present section is to determine the chemical structures of the degradation products of a nonphenolic β -O-4 lignin substructure model, 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol [β -syringaldehyde ether (1)], by the ligninolytic culture of *Coriolus versicolor*, and elucidate the degradation pathways for the nonphenolic β -O-4 lignin substructure.

β -Syringaldehyde ether (1) was catabolized rapidly and various degradation products were identified by spectroscopic analysis. On the basis of the chemical structures of the products, the degradation pathways of the β -O-4 lignin substructure by *C. versicolor* were proposed (Kawai et al., 1985a,b).

1.1.2 MATERIALS AND METHODS

Organism and culture conditions

Coriolus versicolor Ps4a was supplied from the culture collection of Forestry and Forest Products Research Institute, Tsukuba, Japan, and maintained at 30 °C on malt agar slants. The mycelia have clamp connection (Fig. 1.1A), and the fruiting bodies are formed on Shirakanba (*Betula platyphylla*) sawdust-rice bran (5/1, w/w) medium as shown in Fig. 1.1B.

Experimental culture (20 mL in 300-mL Erlenmeyer flasks) was inoculated with a small mycelial mat from a slant and grown without agitation at 29-30 °C (Kamaya & Higuchi, 1984a). The culture medium was prepared as described for *Phanerochaete*

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

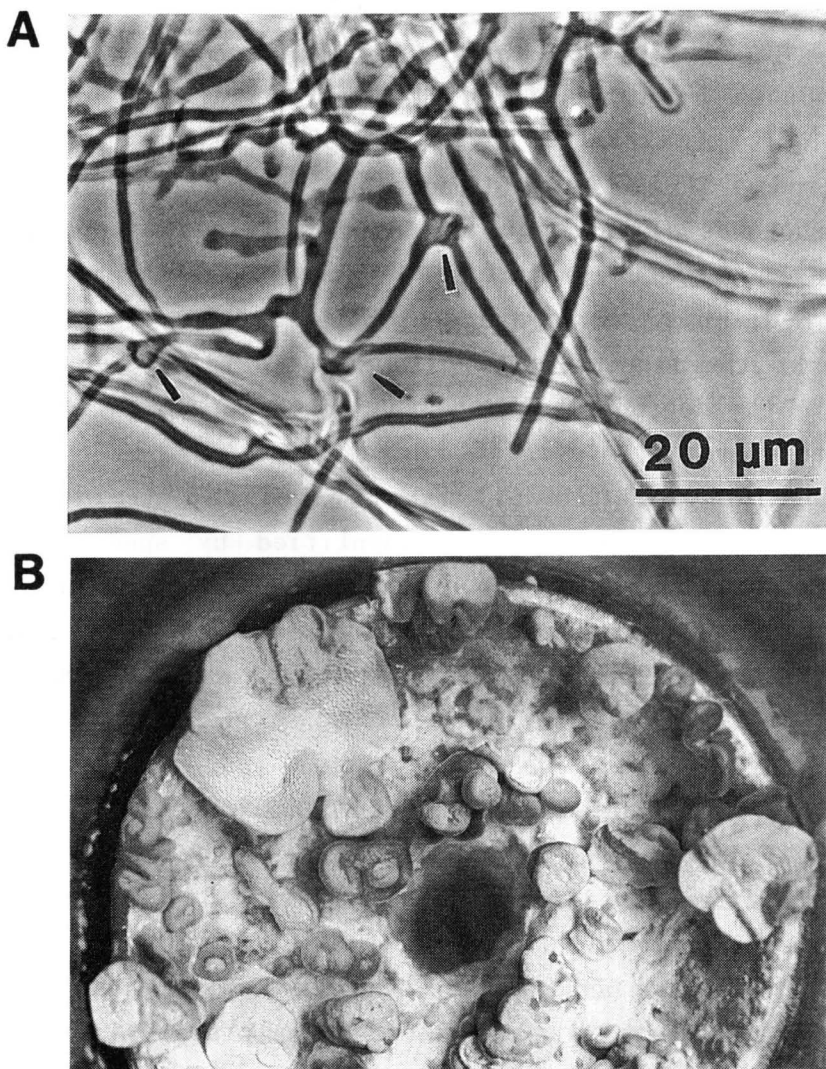


Fig. 1.1 Mycelia (A) and fruiting bodies (B) of *C. versicolor* Ps4a.

(A) The mycelia have clamp connections (arrowhead). Phase contrast, x 250.

(B) The mycelia grown in 3% malt shaking culture were inoculated on Shirakanba (*Betula platyphylla*) sawdust-rice bran (5/1, w/w) medium, and grown for 1 month in the dark at 25 °C, for 1 month under continuous light (300 lux) at 25 °C, for 3 days in the dark at 4 °C, and for 2 months under continuous light at 25 °C, successively.

1.1. DEGRADATION OF A *B-O-4* DILIGNOL BY *C. VERSICOLOR*

chrysosporium (Kirk et al., 1978). Medium contained the following per liter of distilled water: glucose, 10 g; L-asparagine·H₂O, 0.1 g; NH₄NO₃, 50 mg; KH₂PO₄, 0.2 g; MgSO₄·7H₂O, 50 mg; CaCl₂, 10 mg; mineral solution, 1 mL; and vitamin solution, 0.5 mL. Minerals (per liter of distilled water), consisted of nitrilotriacetate, 1.5 g; MgSO₄·7H₂O, 3.0 g; MnSO₄·H₂O, 0.5 g; NaCl, 1.0 g; FeSO₄·7H₂O, 0.1 g; CoSO₄, 0.1 g; CaCl₂, 82 mg; ZnSO₄, 0.1 g; CuSO₄·5H₂O, 10 mg; AlK(SO₄)₂, 10 mg; H₃BO₃, 10 mg; NaMoO₄, 10 mg. Vitamins (per liter of distilled water), biotin, 2 mg; folic acid, 2 mg; thiamine·HCl, 5 mg; riboflavin, 5 mg; pyridoxine·HCl, 10 mg; cyanocobalamine, 0.1 mg; nicotinic acid, 5 mg; DL-calcium pantothenate, 5 mg; *p*-aminobenzoic acid, 5 mg; thiocctic acid, 5 mg. The medium was buffered with polyacrylic acid (0.01 M in carboxyl, pH 4.5) (Nakatsubo et al., 1981).

Degradation of substrate

Substrate (1) (4.4 mg/20-mL culture) was added to 7-day-old cultures as a DMF solution (100 μ L/20-mL culture) without sterilization. The cultures were flushed with sterile 100% O₂ immediately after addition of the substrate and incubated under the same conditions.

After incubation for the desired period of time, whole culture was acidified with 1 N HCl to pH 2 and extracted with ethyl acetate (20 mL, three times). The combined organic layer was washed with saturated NaCl soln, dried over anhyd Na₂SO₄, and evaporated under reduced pressure. Then residual DMF was removed under high vacuum.

Degradation of the substrate in the culture was monitored by examining the extracts by TLC (Kieselgel 60, F254, Merck).

The extracts after 6-50 h of incubation and 72-80 h of incubation were combined, respectively, and combined extracts were acetylated with acetic anhydride and pyridine (1/1, v/v) in

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY C. VERSICOLOR

ethyl acetate for 24 h at room temp. The acetylated extracts were partitioned between ethyl acetate and saturated NaHCO_3 soln. The ethyl acetate layer was washed with saturated NaCl soln, dried over anhyd Na_2SO_4 and evaporated under reduced pressure (neutral fraction).

The NaHCO_3 layer was acidified with 1 N HCl to pH 2 and extracted three times with ethyl acetate. The combined ethyl acetate layer was washed with saturated NaCl soln, dried over anhyd Na_2SO_4 , and evaporated under reduced pressure (acidic fraction).

These products were analyzed directly or after TLC separation by ^1H NMR, DI-MS and GC-MS [column, 1% OV-1 on Chromosorb W (AW-DMCS) (Shinwa Kakou), glass column, 2 m x 3 mm (i.d.)].

Catabolites were identified by comparison of the spectra with those of authentic compounds.

The yields of catabolites were determined by gravimetric analysis after TLC separation.

Isolation and identification of veratryl alcohol (47)

The cultures (28 cultures, total 560 mL) were inoculated with a small mycelial mat from the slant and incubated without agitation at 30 °C. The 7-day-old cultures were flushed with sterile 100% O_2 and incubated under the same conditions for three days. The whole cultures were combined, acidified to pH 2 with 1 N HCl , and extracted with 1 L of ethyl acetate. The organic layer was washed with saturated NaCl soln, dried over Na_2SO_4 , and evaporated under reduced pressure. Veratryl alcohol (47) was separated by TLC (solvent: CH_2Cl_2) and identified by ^1H NMR.

Syntheses of the substrate and authentic compounds

1-(4-Ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxy-phenoxy)-1,3-propanediol [β -syringaldehyde ether (1)] was synthesized

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY C. VERSICOLOR

from acetovanillone (4'-hydroxy-3'-methoxyacetophenone, Tokyo Chemical Industry) by modification of the method of Adler and Eriksoo (1955): (i) CuBr₂ in refluxing ethyl acetate (66.8%); (ii) ethyl vinyl ether/*dl*-10-camphorsulfonic acid in CH₂Cl₂ at 0 °C; (iii) syringaldehyde (4-hydroxy-3,5-dimethoxybenzaldehyde, Tokyo Chemical Industry)/K₂CO₃ in acetone at room temp (ii-iii: 60.8%); (iv) paraformaldehyde/K₂CO₃ in (CH₃)₂SO at room temp; (v) 1 N HCl in acetone at room temp (iv, v: 58.8%); (vi) ethylene glycol/*dl*-camphorsulfonic acid in refluxing temp; (vii) NaBH₄ in methanol at 0 °C; (viii) 1 N HCl in acetone at room temp (vi-viii: 56.0%); (ix) iodoethane/K₂CO₃ in DMF at room temp → 50 °C (61.9%).

¹H NMR (CDCl₃) (acetate) δ 1.45 (t, $J=7.0$ Hz, 3H, -O-C-CH₃), 1.87 (s, 3H, -OAc), 2.02 (s, 3H, -OAc), 3.86 (s, 3H, -OCH₃), 3.88 (s, 6H, -OCH₃), 4.07 (q, $J=7.0$ Hz, 2H, -O-CH₂-), about 3.90 (1H, C3-H), 4.34 (dd, $J=4.0, 12$ Hz, 1H, C3-H), 4.69-4.84 (m, 1H, C2-H), 6.11 (d, $J=7.8$ Hz, 1H, C1-H), 6.76-6.97 (m, 3H, Ph(guaiacyl)-H), 7.12 (s, 2H, Ph(syringyl)-H), 9.89 (s, 1H, -CHO).

MS (acetate) m/z 490 (M⁺).

The diacetate of 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol [formate (26-Ac)] was synthesized from isoeugenol (2-methoxy-4-propenylphenol, Nakarai Chemicals) via the following steps: (i) iodoethane/K₂CO₃ in DMF at room temp; (ii) DDQ in water-saturated benzene at room temp (Nakamura & Higuchi, 1976); (iii) NaBH₄ in methanol at 0 °C; (iv) ethyl vinyl ether/*dl*-10-camphorsulfonic acid in CH₂Cl₂ at 0 °C; (v) *m*-chloroperbenzoic acid in CH₂Cl₂ at 0 °C; (vi) sodium methylate (28% in methanol)/CH₂Cl₂ (1/4, v/v) at 0 °C; (vii) benzyl chloride/NaH in DMF at room temp; (ix) 1 N HCl in acetone at room temp; (x) methane-sulfonyl chloride/triethylamine in THF at 0 °C → room temp; (xi) 10% Pd-C under H₂ in methanol at room temp; (xii) acetic anhy-

1.1. DEGRADATION OF A *B-O-4* DILIGNOL BY *C. VERSICOLOR*

dride/pyridine in ethanol at room temp; (xiii) sodium formate/trioctylmethylammonium chloride in DMF at 60 → 100 °C.

¹H NMR (CDCl₃) (acetate) δ 1.50 (t, *J*=7.0 Hz, 3H, -O-C-CH₃), 2.07 (s, 3H, -OAc), 2.08 (s, 3H, -OAc), 3.88 (s, 3H, -OCH₃), 4.09 (q, *J*=7.0 Hz, 2H, -O-CH₂-), about 3.85 (1H, C3-H), 4.34 (dd, *J*=4.0, 12 Hz, 1H, C3-H), 5.36-5.50 (m, 1H, C2-H), 5.91 (d, *J*=7.2 Hz, 1H, C1-H), 6.76-6.98 (m, 3H, Ph-H), 8.03 (s, 1H, -CHO).

1-(4-Ethoxy-3-methoxyphenyl)-1,3-propanediol [*β*-deoxy diol (28)] was synthesized from 3-(4-hydroxy-3-methoxyphenyl)-1-propanol kindly provided by Dr. F. Nakatsubo, Faculty of Agriculture, Kyoto University, via the following steps: (i) DDQ in water-saturated benzene at room temp; (ii) NaBH₄ in methanol at 0 °C; (iii) diazoethane in methanol at room temp.

¹H NMR (CDCl₃) (acetate) δ 1.45 (t, *J*=7.0 Hz, 3H, -O-C-CH₃), 2.04 (s, 3H, -OAc), 2.06 (s, 3H, -OAc), 2.30-2.36 (m, 2H, C2-H₂), 3.88 (s, 3H, -OCH₃), 4.05 (q, *J*=7.0 Hz, 2H, -O-CH₂-), 3.98-4.21 (m, 2H, C3-H), 5.79 (dd, *J*=7.2, 8.8 Hz, 1H, C1-H), 6.78-6.92 (m, 3H, Ph-H).

Ethyl 4-ethoxy-3-methoxybenzoate (45-Et) was prepared from vanillic acid (4-hydroxy-3-methoxybenzoic acid, Nakarai Chemicals) by ethylation with diazoethane.

2,3-Dimethoxybenzyl alcohol (48) was prepared from 2-hydroxy-3-methoxybenzaldehyde (*o*-vanillin, Nakarai Chemicals) via the following two steps: (i) iodomethane/K₂CO₃ in DMF at room temp; (ii) NaBH₄ in methanol at 0 °C.

¹H NMR (CDCl₃) δ 3.87 (s, 3H, -OCH₃), 3.89 (s, 3H, -OCH₃), 4.70 (s, 2H, Ph-CH₂-), 6.82-7.12 (m, 3H, Ph-H).

2,4-Dimethoxybenzyl alcohol (49) was prepared from 2,4-dihydroxybenzaldehyde (*β*-resorcylaldehyde, Nakarai Chemicals) via the following two steps: (i) iodomethane/K₂CO₃ in DMF at room temp; (ii) NaBH₄ in methanol at 0 °C.

1.1. DEGRADATION OF A *B-O-4* DILIGNOL BY *C. VERSICOLOR*

¹H NMR (CDCl₃) δ 3.80 (s, 3H, -OCH₃), 3.83 (s, 3H, -OCH₃), 4.60 (s, 2H, Ph-CH₂-), 6.40-6.46 (m, 2H, Ph-H_{3,5}), 7.16 (d, *J*=9.0 Hz, 1H, Ph-H₆).

2,5-Dimethoxybenzyl alcohol (**50**) was prepared from 2,5-dimethoxybenzaldehyde (Nakarai Chemicals) by reduction with NaBH₄ in methanol at 0 °C.

¹H NMR (CDCl₃) δ 3.77 (s, 3H, -OCH₃), 3.81 (s, 3H, -OCH₃), 4.65 (s, 2H, Ph-CH₂-), 6.76-6.90 (m, 3H, Ph-H).

3,4-Dimethoxybenzyl alcohol (veratryl alcohol) (**47**) was commercially available (Tokyo Chemical Industry).

¹H NMR (CDCl₃) δ 3.87 (s, 3H, -OCH₃), 3.88 (s, 3H, -OCH₃), 4.61 (s, 2H, Ph-CH₂-), 6.78-6.94 (m, 3H, Ph-H).

Other compounds, 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol [glycerol (**24**)] (Umezawa & Higuchi, 1984), 1-(4-ethoxy-3-methoxyphenyl)-2,3-dihydroxypropanone [propanone (**25**)] (Kamaya & Higuchi, 1984b), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol-2,3-cyclic carbonate [cyclic carbonate (**27**)] (Umezawa & Higuchi, 1985c), 4-ethoxy-3-methoxybenzaldehyde [benzaldehyde (**43**)] (Umezawa et al., 1983b), 4-ethoxy-3-methoxybenzyl alcohol [benzyl alcohol (**44**)] (Umezawa et al., 1983b), and syringyl alcohol [4-hydroxy-3,5-dimethoxybenzyl alcohol (**51**)] (Kamaya & Higuchi, 1984c), were prepared as described previously and acetylated before use as authentic compounds.

Mass spectral data of (**24**-Ac)-(**28**-Ac), (**43**), (**44**-Ac), (**45**-Ac) and (**51**-Ac) are listed in Table 1.1.

Instruments

NMR spectra were obtained with a Varian XL-200 FT-NMR spectrometer (200 MHz) using (CH₃)₄Si as an internal standard. Chemical shifts and coupling constants are given in δ value (ppm) and Hz, respectively. Mass spectra were taken with a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer (EI, 70 eV).

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY C. VERSICOLOR

TABLE 1.1

Relative Intensity of the Important Fragment Ions of the Authentic Compounds and Degradation Products.

Authentic Compounds	
Compound	Mass Spectral Data m/z (%)
Glycerol (24-Ac)	368 (M^+ , 7), 308 (6), 266 (5), 223 (8), 207 (13), 206 (33), 182 (25), 181 (100), 178 (13).
Propanone (25-Ac)	324 (M^+ , 9), 266 (6), 180 (22), 179 (100), 152 (10), 151 (81).
Formate (26-Ac)	354 (M^+ , 7), 294 (4), 223 (6), 206 (23), 182 (12), 181 (100).
Cyclic carbonate (27-Ac)	310 (M^+ , 12), 250 (6), 223 (4), 206 (5), 182 (15), 181 (100), 154 (12), 150 (12).
β -Deoxy diol (28-Ac)	310 (M^+ , 32), 250 (61), 207 (48), 191 (49), 181 (100), 179 (67), 177 (28), 163 (54), 151 (37), 147 (30).
Benzaldehyde (43)	180 (M^+ , 70), 152 (80), 151 (100), 137 (10), 113 (16), 109 (24).
Benzyl alcohol (44-Ac)	224 (M^+ , 45), 182 (14), 165 (23), 154 (30), 153 (12), 151 (12), 137 (100), 136 (20), 125 (14), 122 (16).
Benzoic acid (45-Et)	224 (M^+ , 64), 196 (33), 181 (12), 179 (28), 168 (47), 153 (18), 152 (26), 151 (100), 123 (20).
Syringyl alcohol (51-Ac)	268 (M^+ , 3), 227 (14), 226 (100), 184 (62), 183 (15), 167 (84), 123 (26).

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY C. VERSICOLOR

TABLE 1.1 (cont'd)

Degradation Products	
Compound	Mass Spectral Data m/z (%)
Glycerol (24-Ac)	368 (M^+ , 5), 308 (5), 266 (4), 223 (9), 207 (11), 206 (28), 182 (15), 181 (100).
Propanone (25-Ac)	324 (M^+ , 9), 266 (4), 180 (24), 179 (100), 152 (10), 151 (79).
Formate (26-Ac)	354 (M^+ , 7), 294 (5), 223 (5), 206 (26), 182 (11), 181 (100).
Cyclic carbonate (27-Ac)	310 (M^+ , 13), 250 (8), 223 (5), 206 (8), 182 (12), 181 (100), 154 (12), 150 (17).
β -Deoxy diol (28-Ac)	310 (M^+ , 31), 250 (50), 207 (33), 191 (43), 181 (100), 179 (60), 177 (22), 163 (42), 151 (38), 147 (23).
Benzaldehyde (43)	180 (M^+ , 66), 152 (82), 151 (100), 137 (13), 113 (17), 109 (34).
Benzyl alcohol (44-Ac)	224 (M^+ , 56), 182 (20), 165 (34), 154 (40), 153 (18), 151 (15), 137 (100), 136 (28), 125 (22), 122 (22).
Benzoic acid (45-Et)	224 (M^+ , 72), 196 (34), 181 (12), 179 (23), 168 (48), 153 (17), 152 (22), 151 (100), 123 (17).
Syringyl alcohol (51-Ac)	268 (M^+ , 3), 227 (14), 226 (100), 184 (58), 183 (16), 167 (72), 123 (30).

1.1.3 RESULTS

Substrate (1) (4.4 mg/culture) was added to the eight cultures of *Coriolus versicolor* and incubated. Extraction was carried out after 6, 12, 24, 50, 72, 76, and 80 h of incubation, respectively.

Degradation of β -syringaldehyde ether (1) in the culture was monitored by examining the extracts by TLC. Substrate (1) disappeared almost completely for 72 h after addition to the culture. The extracts after 6-50 h of incubation and 72-80 h of incubation were combined, respectively, and the combined extracts were acetylated. The acetylated extracts were separated to the neutral and acidic fractions, respectively, and analyzed.

Analysis of catabolites (6-50 h of incubation)

The acetylated extracts were submitted to TLC as shown in Fig. 1.2, and five products could be separated and identified by ^1H NMR and DI-MS.

The triacetate of 1-(4-ethoxy-3-methoxyphenyl)-2-(4-hydroxymethyl-2,6-dimethoxyphenoxy)-1,3-propanediol [β -syringyl alcohol ether (2-Ac)] (13.9%, mol product formed/mol initial substrate $\times$ 100) was isolated from the neutral fraction and identified by ^1H NMR and DI-MS.

^1H NMR (CDCl_3) δ 1.45 (t, $J=7.0$ Hz, 3H, $-\text{O}-\text{C}-\text{CH}_3$), 1.93 (s, 3H, $-\text{OAc}$), 2.02 (s, 3H, $-\text{OAc}$), 2.11 (s, 3H, $-\text{OAc}$), 3.80 (s, 6H, $-\text{OCH}_3$), 3.85 (s, 3H, $-\text{OCH}_3$), 4.08 (q, $J=7.0$ Hz, 2H, $-\text{O}-\text{CH}_2-$), about 3.85 (1H, C3-H), 4.31 (dd, $J=4.0, 12$ Hz, 1H, C3-H), 4.50-4.62 (m, 1H, C2-H), 5.02 (s, 2H, $\text{Ph}-\text{CH}_2-$), 6.11 (d, $J=7.3$ Hz, 1H, C1-H), 6.56 (s, 2H, $\text{Ph}(\text{syringyl})-\text{H}$), 6.76-6.98 (m, 3H, $\text{Ph}(\text{guaiacyl})-\text{H}$).

MS m/z 534 (M^+).

Additionally, the acetate derivatives of propanone (25-Ac)(<

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

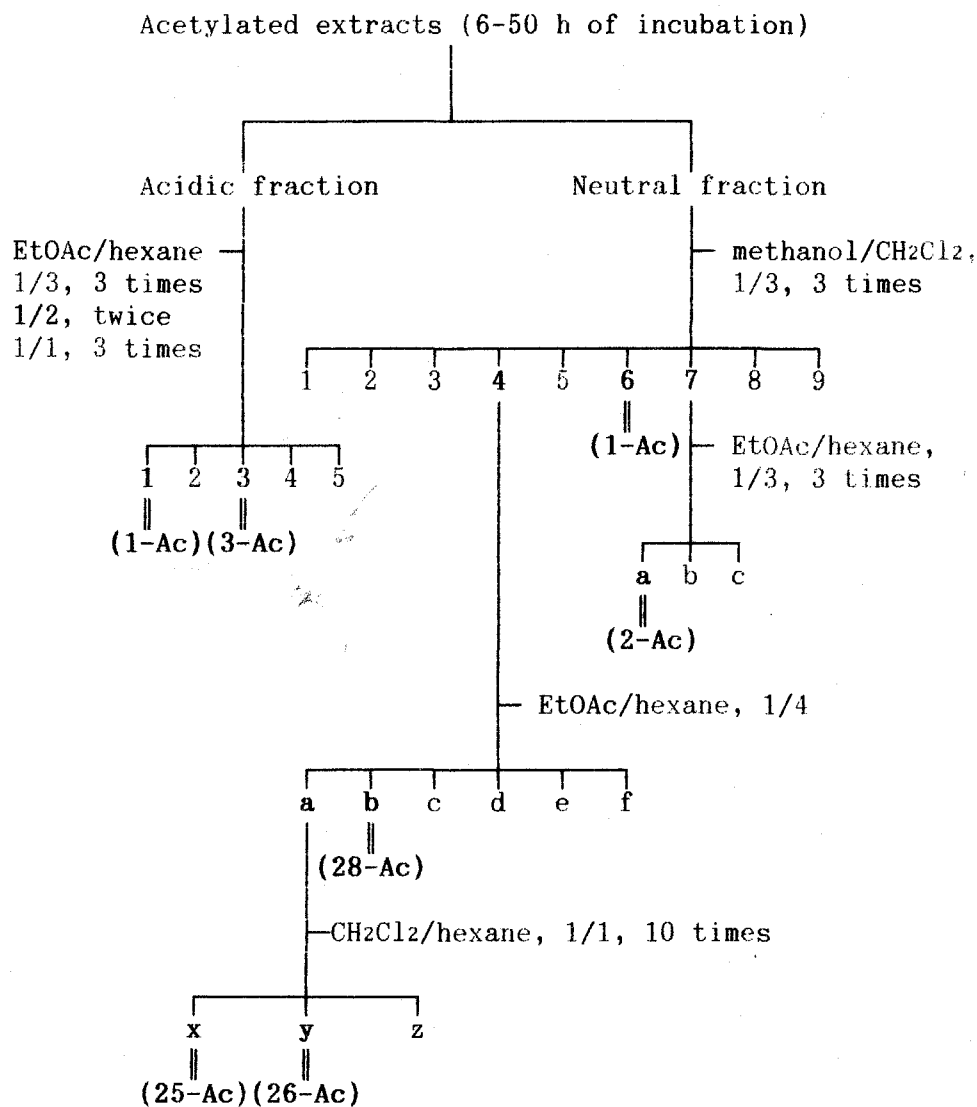


Fig. 1.2 Separation methods of the acetylated degradation products of substrate (1) by *C. versicolor*.

EtOAc, ethyl acetate; hexane, *n*-hexane.

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY C. VERSICOLOR

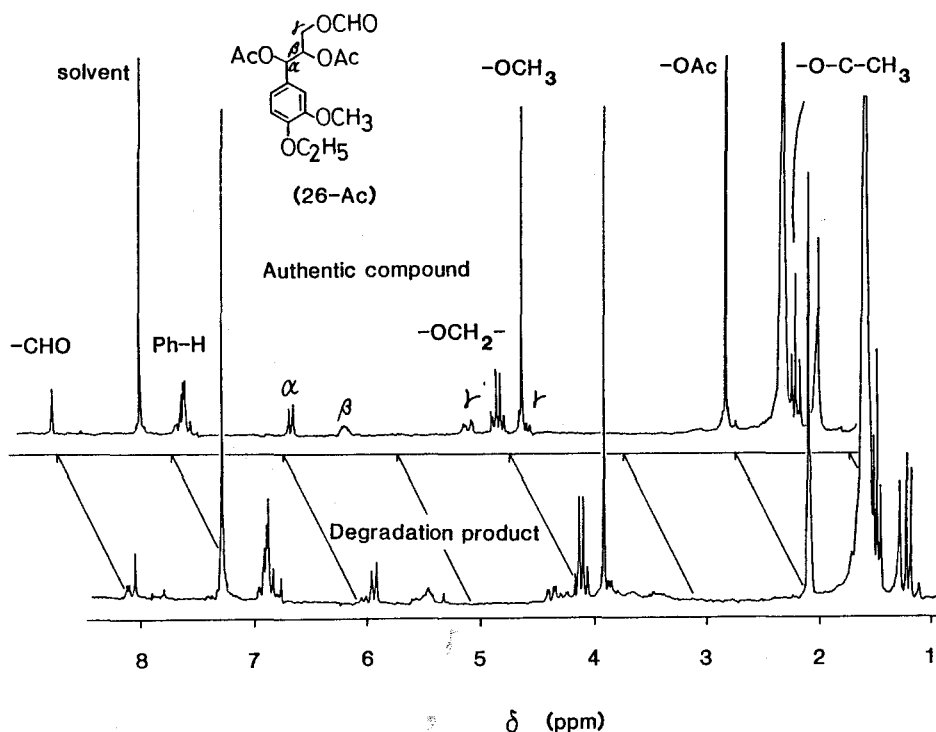


Fig. 1.3 ^1H NMR spectra of the diacetate of 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol (26-Ac).

1 mg), formate (26-Ac) (< 1 mg), and β -deoxy diol (28-Ac) (< 1 mg) were separated by TLC and identified by ^1H NMR and DI-MS. The ^1H NMR spectra of formate (26-Ac) and β -deoxy diol (28-Ac) are shown in Fig. 1.3 and 1.4, respectively. The spectra of these products were identical with those of the authentic compounds.

The diacetate of 2-(4-carboxy-2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol [β -syringic acid ether (3-Ac)] (4.0%) was also isolated from the acidic fraction by TLC and identified by ^1H NMR and DI-MS.

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

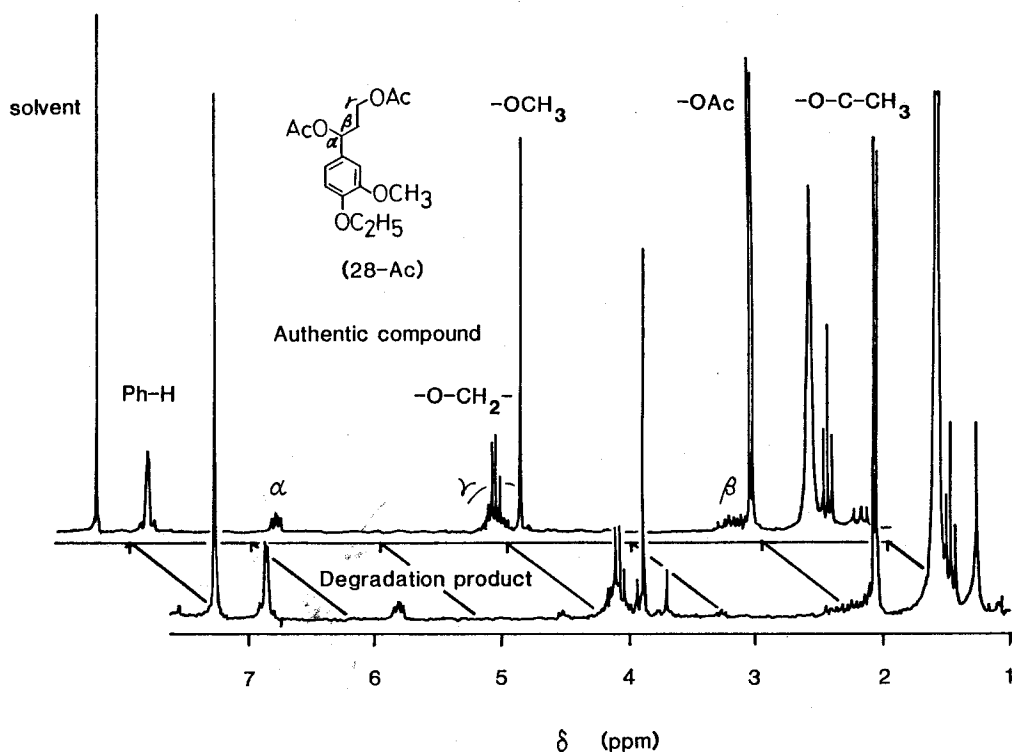


Fig. 1.4 ^1H NMR spectra of the diacetate of 1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (28-Ac).

^1H NMR (CDCl_3) δ 1.44 (t, $J=7.0$ Hz, 3H, $-\text{O}-\text{C}-\text{CH}_3$), 1.88 (s, 3H, $-\text{OAc}$), 2.02 (s, 3H, $-\text{OCH}_3$), 3.80 (s, 6H, $-\text{OCH}_3$), 3.85 (s, 3H, $-\text{OCH}_3$), 4.07 (q, $J=7.0$ Hz, 2H, $-\text{O}-\text{CH}_2-$), about 3.9 (1H, C3-H), 4.34 (dd, $J=4.0, 12$ Hz, 1H, C3-H), 4.64-4.80 (m, 1H, C2-H), 6.09 (d, $J=7.2$ Hz, 1H, C1-H), 6.70-7.00 (m, 3H, Ph(guaiacyl)-H), 7.32 (s, 2H, Ph(syringyl)-H).

MS m/z 506 (M^+).

Fifty percent of substrate (1) remained in the extracts after 6-50 h of incubation.

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

Analysis of catabolites (72-80 h of incubation)

The diacetate of formate (26-Ac), benzaldehyde (43), and the acetate of benzyl alcohol (44-Ac) were identified in the neutral fraction by GC-MS (column temp, 170-210 °C, 5 °C/min). As the triacetate of glycerol (24-Ac) and the acetate of cyclic carbonate (27-Ac) could not be separated from other compounds by GC, (24-Ac) and (27-Ac) were separated by TLC [solvents, (24-Ac), ethyl acetate/*n*-hexane, 1/6, six times; (27-Ac), ethyl acetate/*n*-hexane, 2/5] and identified by GC-MS (column temp, 210 °C).

The diacetate of β -deoxy diol (28-Ac) and the acetate of syringyl alcohol (52-Ac) were also identified by GC-MS after separation by TLC (column temp, 176 °C; solvent, ethyl acetate/*n*-hexane, 3/14, twice).

The mass spectra and retention times were identical with those of the authentic compounds (Table 1.1).

The acidic fraction was ethylated by diazoethane and separated by TLC (solvent, ethyl acetate/*n*-hexane, 1/8). Benzoic acid ethyl ester (45-Et) was identified by GC-MS (column temp, 174 °C). The mass spectrum and retention time were identical with those of the authentic compound (Table 1.1).

For gravimetical analysis, β -syringaldehyde ether (1) (64 mg) was incubated in 14 cultures. After 76-h incubation, the cultures were extracted with ethyl acetate and acetylated, and the extracts were separated into the neutral and acidic fractions. The residual water layer was lyophilized, and the residue was extracted with ethyl acetate and acetylated.

The diacetate of substrate (1-Ac) (12.2%, mol product formed/mol initial substrate x 100), the triacetate of β -syringyl alcohol ether (2-Ac) (5.3%), the diacetate of β -deoxy diol (28-Ac) (<1 mg), benzaldehyde (43) (15.2%), and the acetate of benzyl

1.1. DEGRADATION OF A *B-O-4* DILIGNOL BY *C. VERSICOLOR*

alcohol (44-Ac) (5.7%) were isolated from the neutral fraction and identified by ^1H NMR. The acetate derivatives of glycerol (24-Ac), propanone (25-Ac), and formate (26-Ac) (total 5.7 mg) could not be separated completely from each other by TLC. The mixture contained approximately equimolar amounts of (24-Ac)-(26-Ac), on the basis of the integral values of ^1H NMR. The triacetate of glycerol (24-Ac) (<1 mg) was also isolated from the water layer and identified by GC-MS (column temp, 214 °C). The yield of the acetate of cyclic carbonate (27-Ac) (0.5%) was calculated on the basis of the integral value of ^1H NMR measured by use of benzyl vanillin as an internal standard.

The acidic fraction was ethylated with diazoethane. The diacetate of ethyl ester of β -syringic acid ether (3-Ac-Et) (<1 mg) and the ethyl ester of benzoic acid (45-Et) (<1 mg) were separated and identified by DI-MS (3-Ac-Et) or GC-MS (45-Et) (column temp, 174 °C).

Mass spectral data of identified catabolites are listed in Table 1.1.

Two controls which consisted of only myceria or substrate (1) in the culture medium were incubated, and then extracted under the same conditions as described above. The catabolites (2), (3), (24)-(28), (43)-(45), and (52) were not detected in these extracts.

The extracts in the control that consisted of only myceria, however, contained veratryl alcohol (47) detected by GC-MS. Then, to confirm the structure by ^1H NMR, 28 cultures (560 mL medium) were incubated and extracted with ethyl acetate. Veratryl alcohol (47) was isolated by TLC and submitted to ^1H NMR. Figure 1.5 shows the ^1H NMR spectra of the metabolite and authentic dimethoxybenzyl alcohols (47)-(50). Possibility of 3,5-dimethoxybenzyl alcohol is ruled out, because the protons of the two methoxyl

1.1. DEGRADATION OF A *B-O-4* DILIGNOL BY *C. VERSICOLOR*

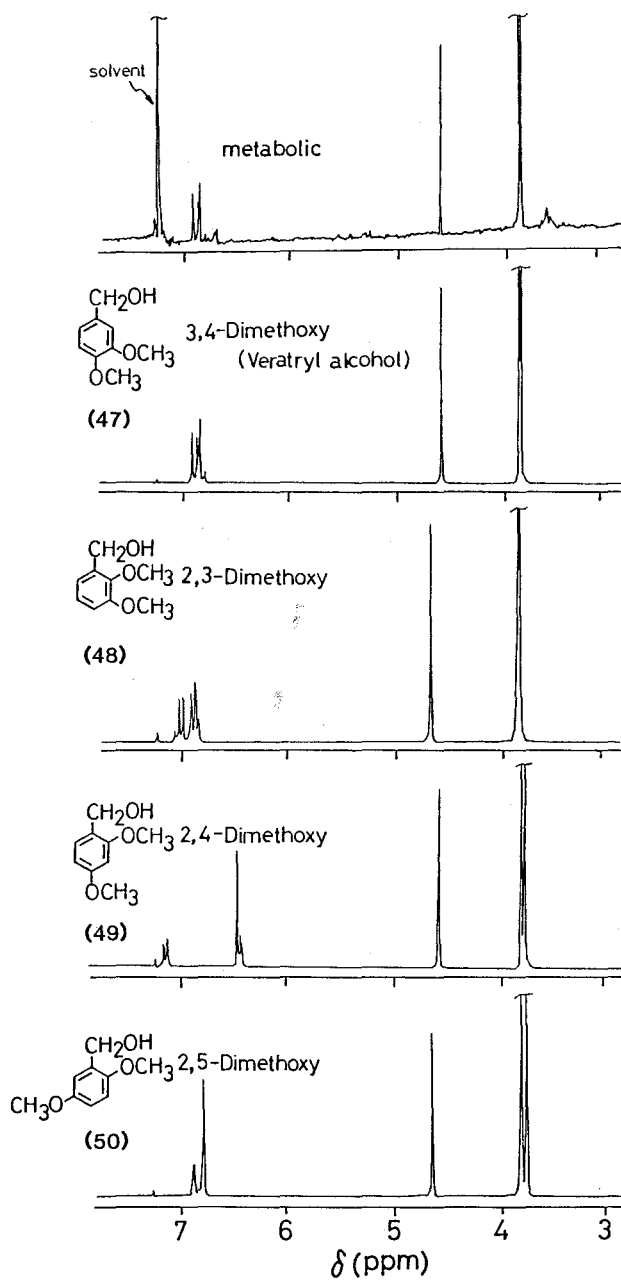


Fig. 1.5 ¹H NMR spectra of metabolic veratryl alcohol and authentic compounds (47)-(50).

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

groups of the metabolite have different chemical shifts in ^1H NMR spectrum, while the chemical shifts of methoxyl groups of 3,5-dimethoxybenzyl alcohol are identical. From the ^1H NMR spectra shown in Fig. 1.5, it is evident that the metabolite is veratryl alcohol (47) and not other isomers (48)-(50).

1.1.4 DISCUSSION

Degradation pathways of 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol (1)

On the basis of the chemical structures of the degradation products (2), (3), (24)-(28), (43)-(45), and (52), the degradation pathways in Fig. 1.6-1.9 for substrate (1) in the ligninolytic culture of *Coriolus versicolor* were proposed.

β -Syringaldehyde ether (1) was partly reduced to the corresponding β -syringyl alcohol ether (2) and partly oxidized to the corresponding β -syringic acid ether (3) (Fig. 1.6). The conversion of benzaldehyde to the corresponding benzyl alcohol and benzoic acid have generally been found in white-rot fungi (Farmer et al., 1959; Zenk & Gross, 1965; Ander et al., 1980; Nakatsubo et al., 1981). β -Syringic acid ether (3) was further oxidized to form *p*-benzoquinone monoketals (8) and (9). The formation pathways of (8) and (9) will be discussed in Section 1.3.

Substrate (1) was cleaved at the β -ether to give glycerol (24) and propanone (25) (Fig. 1.7). Kamaya and Higuchi (1984b) reported the interconversion between 1-(3,4-dimethoxyphenyl)-1,2,3-propanetriol and its α -carbonyl derivative.

Formate (26) and cyclic carbonate (27) were formed as aromatic ring cleavage products of substrate (1) and further degraded to give glycerol (24) (Fig. 1.7). Isotopic experiments indicated that formyl carbon of formate (26) and carbonate carbon of cyclic carbonate (25) were derived from β -etherated aromatic

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

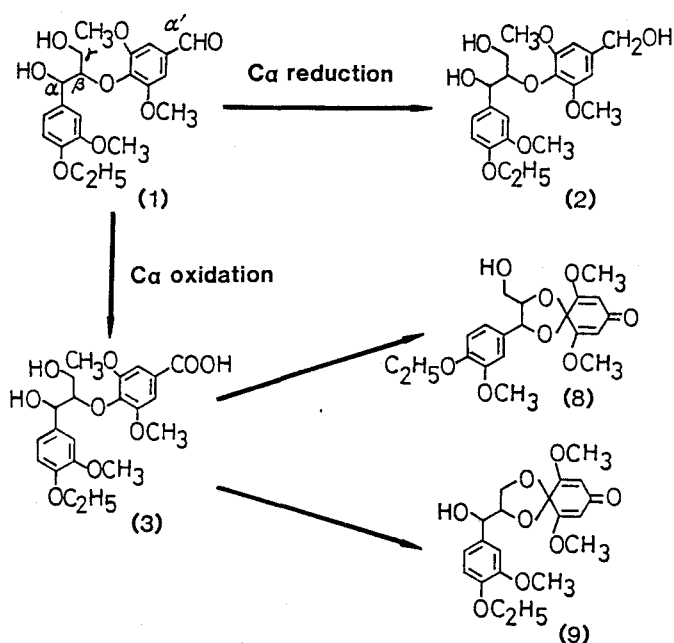


Fig. 1.6 Reduction and oxidation of the benzylic aldehyde of β -syringaldehyde ether (1) by *C. versicolor*. β -Syringic acid ether (3) was further oxidized to form (8) and (9).

ring. These results will be discussed in Section 1.2.

Propyl side chain of glycerol (24) was cleaved between C_{α} and C_{β} to yield benzaldehyde (43), benzyl alcohol (44), and benzoic acid (45) (Fig. 1.8). Kamaya and Higuchi (1984b) reported the C_{α} - C_{β} cleavage of a similar compound of glycerol (24), 1-(3,4-dimethoxyphenyl)-1,2,3-propanetriol, to give C_6 - C_1 monomers by the culture of *C. versicolor*.

The mechanism of formation of syringaldehyde (52) was not elucidated in this experiment, but the following three assumptions can be made as to its formation: (i) Syringyl alcohol (52) could be formed as a counterpart compound for glycerol (24) or propanone (25) through the β -ether cleavage of β -O-4 dimers (1),

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

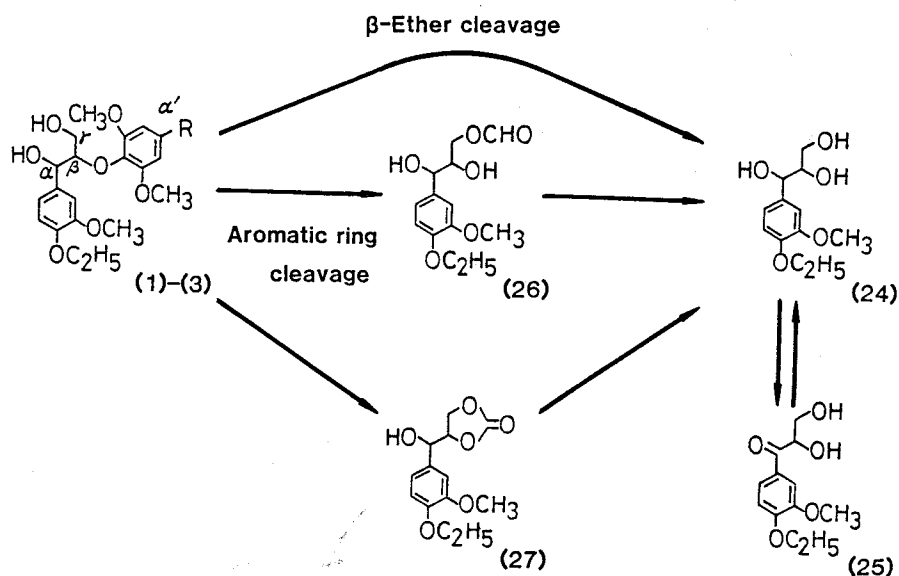


Fig. 1.7 β -Ether cleavage and aromatic ring cleavage of β -O-4 dilignols (1)-(3) by *C. versicolor*.

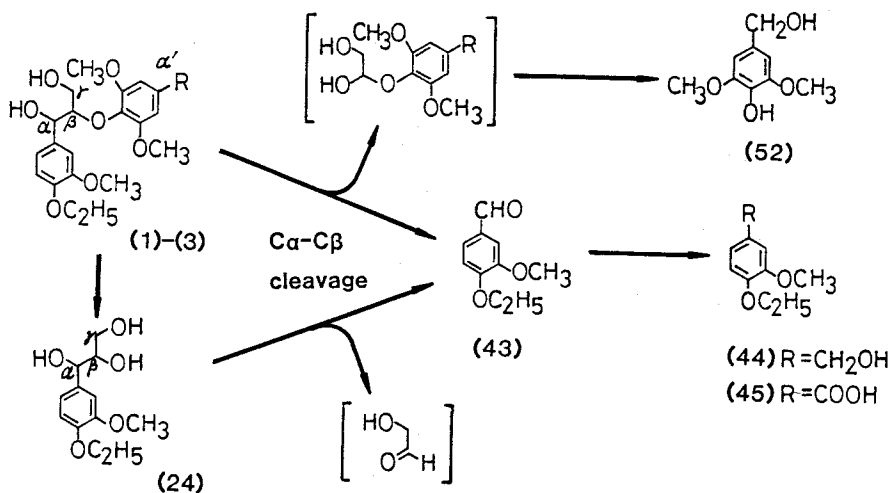


Fig. 1.8 α -C- β cleavage of β -O-4 dilignols (1)-(3) and arylglycerol (24) by *C. versicolor*.

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

(2), and (3). In earlier papers (Ishikawa et al., 1963; Fukuzumi et al., 1969; Enoki et al., 1981), this pathway was proposed for white-rot fungi. However, Umezawa and Higuchi (1985b) found that glycerol (24) and guaiacol (59), degradation products of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (5) by ligninolytic culture of *Phanerochaete chrysosporium*, were formed via different pathways, respectively. Therefore, this pathway is unlikely in *C. versicolor*. (ii) Direct C α -C β cleavage of the β -O-4 dimers (1), (2) and (3) occurred to give benzaldehyde (43) and a C δ -C γ hemiketal intermediate which was spontaneously degraded to give syringyl derivatives [e.g. (52)] and C γ -fragment (Fig. 1.8). Direct C α -C β cleavage of β -1 and β -O-4 lignin substructure models has been proved with the lignin peroxidase of *P. chrysosporium* (Tien & Kirk, 1983; Glenn et al., 1983; Tien & Kirk, 1984; Gold et al., 1984; Habe et al., 1985b). Kamaya and Higuchi (1984a) reported the C α -C β cleavage of a β -1 lignin substructure model by the culture of *C. versicolor*. Recently, lignin peroxidase of *C. versicolor* was isolated by Dodson et al. (1987), and found to catalyze C α -C β cleavage of β -1

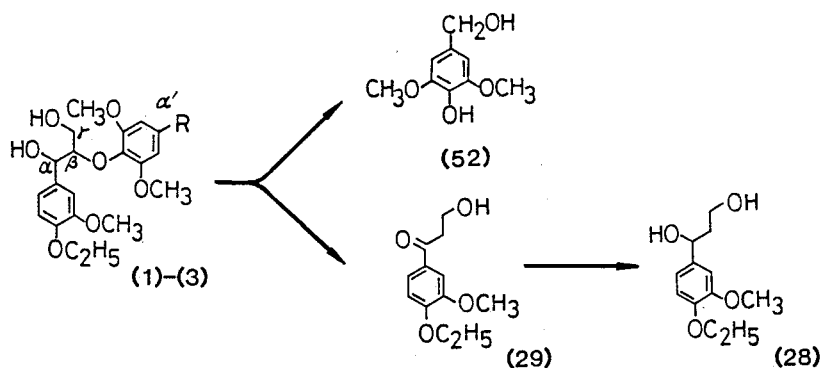


Fig. 1.9 The possible formation pathway of β -deoxy diol (28) from β -O-4 dilignols (1)-(3) by *C. versicolor*.

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

(Dodson et al. 1987) and β -O-4 (Kawai et al., in press, see Chapter 2) lignin substructure models. These results suggested that syringyl alcohol (52) could be formed via these pathways. (iii) Alternatively, syringyl alcohol (52) could be formed as a counterpart compound of β -deoxy diol (28), which was found for the first time as a catabolite of β -O-4 lignin substructure models by white-rot fungi (Fig. 1.9). Intermediate compound (29) was recently found as the degradation product of a β -O-4 model by lignin peroxidase of *C. versicolor*. The formation mechanisms will be discussed in Chapter 2.

De novo synthesis of veratryl alcohol

Veratryl alcohol (47) was identified as a secondary metabolite of *C. versicolor*. Russell et al. (1961) found veratraldehyde (46) in a culture of *C. versicolor*. De novo synthesis of veratryl alcohol (47) by *P. chrysosporium* was reported previously (Lundquist & Kirk, 1978). Afterward many papers were published in relation to physiological and biochemical role of veratryl alcohol (47) in lignin degradation by *P. chrysosporium*. Shimada et al. (1981) reported the biosynthesis of veratryl alcohol (47) in relation to lignin degradation of *P. chrysosporium*. The addition of veratryl alcohol (47) to the culture of *P. chrysosporium* increased the ligninolytic activity and the production of lignin peroxidase in the culture (Leisola et al., 1984; Faison & Kirk, 1985).

The present investigation conclusively suggested that a nonphenolic β -O-4 lignin substructure was mainly degraded through a degradation pathway similar to that proposed for *P. chrysosporium* and its lignin peroxidase (see Umezawa, 1988). Later, lignin peroxidase was isolated from the culture of *C. versicolor* (Dodson et al., 1987) and found to mediate C α -oxidation, β -ether

1.1. DEGRADATION OF A β -O-4 DILIGNOL BY *C. VERSICOLOR*

cleavage, C α -C β cleavage and aromatic ring cleavage of β -O-4 lignin substructures (Kawai et al., in press). The oxidation of β -O-4 lignin substructure model compounds by lignin peroxidase of *C. versicolor* will be discussed in Chapter 2.

Section 1.2

1.2 AROMATIC RING CLEAVAGE OF NONPHENOLIC β -O-4 LIGNIN SUBSTRUCTURE MODELS

1.2.1 INTRODUCTION

As described in Section 1.1., the degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol [β -syringaldehyde ether (1)] was catalyzed by the ligninolytic culture of *Coriolus versicolor* to give many degradation products (Kawai et al., 1985a,b).

In the present section, the formation pathways of the degradation products, 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol [formate (26)] and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol-2,3-cyclic carbonate [cyclic carbonate (27)], are described. To elucidate whether the formyl carbon of formate (26) and the carbonate carbon of cyclic carbonate (27) are derived from β -etherated aromatic ring or not, isotopic experiments with ^{13}C -labeled β -O-4 dilignols (4- ^{13}C) and (5- ^{13}C) were performed. The results indicated that formate (26) and cyclic carbonate (27) were formed as aromatic ring cleavage products of β -O-4 lignin substructure model compounds (Kawai et al., 1985b).

1.2.2 MATERIALS AND METHODS

Organism and culture conditions

Coriolus versicolor Ps4a was maintained at 30 °C on 2% malt agar slant. Experimental culture (20 mL in 300-mL Erlenmeyer flasks) was inoculated with a small mycelial mat from the slant and grown without agitation at 30 °C in nitrogen-limited medium as described in Section 1.1.

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

Degradation of substrates

Substrates (1.2-2.0 mg/20-mL culture) were added to 7-day-old cultures as a DMF soln (100 μ L/20-mL culture) without sterilization. The culture was flushed with sterile 100% oxygen immediately after addition of substrates and incubated under the same condition.

After incubation for the desired period of time, whole culture was acidified with 1 N HCl to pH 2 and extracted with ethyl acetate (20 mL, three times). The combined organic layer was washed with saturated NaCl soln, dried over anhyd Na_2SO_4 , and evaporated under reduced pressure. Then, residual DMF was removed under high vacuum. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v) at room temp for 24 h.

The acetylated products were analyzed by GC-MS [column: 1% OV-1 on Chromosorb W AW-DMCS (Shinwa Kakou), glass column, 2 m x 3 mm (i.d.)] directly or after separation by TLC (Kieselgel F254, Merck).

Catabolites were identified by comparison of these spectra with those of authentic compounds.

Syntheses of substrates and authentic compounds

2,6-[U-Ring- ^{13}C]dimethoxyphenol was synthesized from 2-(2-[U-ring- ^{13}C]methoxyphenoxy)-5-nitrobenzophenone, which was prepared from [U-ring- ^{13}C]aniline (CEA; minimum isotopic purity, 90%) (Kratzl & Vierhapper, 1971; Umezawa & Higuchi, 1985c), by a modified method of Loudon and Scott (1953): (i) hydroxylation with concd H_2SO_4 /acetic acid/30% H_2O_2 at room temp (67.5%); (ii) methylation with diazomethane in methanol at room temp (quantitative); (iii) elimination of the 5-nitrobenzophenone with refluxing piperidine (84.8%).

MS (acetate) m/z (%) 202 (4.0), 201 (2.8), 200 (0.65), 161 (3.1), 160 (100), 159 (69), 158 (20), 157 (3.9), 145 (29),

1.2. AROMATIC RING CLEAVAGE BY C. VERSICOLOR

144 (20), 143 (7.0), 130 (3.9), 129 (2.4), 116 (11), 115 (11), 114 (7.8), 113 (6.1), 112 (3.5).

2-(2,6-Dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol [β -syringyl ether (4)] and 2-(2,6-[U-ring- ^{13}C]dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol [^{13}C -labeled β -syringyl ether (4- ^{13}C)] were prepared from acetovanillone (Tokyo Chemical Industry) by a modification method of Adler et al. (1952) via the following steps: (i) iodoethane/ K_2CO_3 in DMF at room temp; (ii) CuBr_2 in refluxing ethyl acetate; (iii) 2,6-dimethoxyphenol (Nakarai Chemicals) or 2,6-[U-ring- ^{13}C]dimethoxyphenol/ K_2CO_3 in DMF at room temp; (iv) paraformaldehyde/ K_2CO_3 in $(\text{CH}_3)_2\text{SO}$ at room temp; (v) NaBH_4 in methanol at 0°C .

(4): ^1H NMR (CDCl_3) (acetate) δ 1.45 (t, $J=7.0$ Hz, 3H, $-\text{O}-\text{C}-\text{CH}_3$), 1.93 (s, 3H, $-\text{OAc}$), 2.02 (s, 3H, $-\text{OAc}$), 3.79 (s, 3H, $-\text{OCH}_3$), 3.85 (s, 3H, $-\text{OCH}_3$), 4.08 (q, $J=7.0$ Hz, 2H, $-\text{O}-\text{CH}_2-$), about 3.85 (1H, C3-H), 4.34 (dd, $J=4.0, 12$ Hz, 1H, C3-H), 4.50-4.66 (m, 1H, C2-H), 6.12 (d, $J=7.6$ Hz, 1H, C1-H), 6.56 (d, $J=8.3$ Hz, 2H, Ph(syringyl)-H3,5), 6.90 (d, $J=8.8$ Hz, 1H, Ph(guaiacyl)-H5), 6.89-6.90 (m, 2H, Ph(guaiacyl)-H2,6), 6.98 (d, $J=8.3$ Hz, 1H, Ph(syringyl)-H4).

MS (acetate) m/z (%) 463 (2.0), 462 (M^+ , 7.9), 309 (10), 266 (7.9), 250 (14), 249 (13), 239 (13), 208 (8.3), 207 (67), 206 (74), 181 (46), 154 (100), 153 (25).

(4- ^{13}C): MS (acetate) m/z (%) 469 (1.7), 468 (8.2), 467 (6.0), 466 (1.5), 309 (12), 266 (7.9), 250 (14), 249 (13), 245 (13), 244 (7.2), 208 (12), 207 (88), 206 (100), 181 (61), 160 (63), 159 (57), 158 (23).

3-(4-Ethoxy-3-methoxyphenyl)-1-propanol (31) was prepared from 4-ethoxy-3-methoxycinnamyl alcohol by hydrogenation with 10% Pd-C under H_2 in methanol at room temp.

3-(4-Ethoxy-3-methoxyphenyl)-1-formyloxypropane (30) was prepared from (31) by esterification with acetic formic anhydride

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

(Reber et al., 1954) in pyridine at 0 °C → room temp.

¹H NMR (CDCl₃) δ 1.45 (t, *J*=7.0 Hz, 3H, -O-C-CH₃), 1.90-2.05 (2H, C2-H₂), 2.60-2.70 (2H, C3-H₂), 3.86 (s, 3H, -OCH₃), 4.08 (q, *J*=7.0 Hz, 2H, -O-CH₂-), 4.10-4.25 (2H, C1-H₂), 6.70-6.90 (3H, Ph-H), 8.08 (s, 1H, -CHO).

MS *m/z* (%) 239 (7.8), 238 (M⁺, 50), 210 (7.8), 165 (26), 164 (18), 163 (13), 138 (13), 137 (100).

Other compounds, 1-(4-ethoxy-3-methoxyphenyl)-2-(2-[U-ring-¹³C]methoxyphenoxy)-1,3-propanediol [¹³C-labeled β-guaiacyl ether (2-¹³C)] and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol-2,3-cyclic carbonate [cyclic carbonate (27)] (Umezawa & Higuchi, 1985c), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-proapnetriol [glycerol (24)] (Umezawa & Higuchi, 1984), the diacetate of 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol [formate (26-Ac)] (Kawai et al., 1985a), were prepared previously.

Instruments

NMR spectra were obtained with a Varian XL-200 FT NMR spectrometer (200 MHz) with (CH₃)₄Si as an internal standard. Chemical shifts and coupling constants are given in δ value (ppm) and Hz, respectively. Mass spectra were taken with a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer (EI, 70 eV).

1.2.3 RESULTS

Catabolism of 2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (4) and ¹³C-labeled β-syringyl ether (4-¹³C)

β-Syringyl ether (4) (2.0 mg/20-mL culture) was added to the cultures. The cultures were extracted after 72-h incubation, and then the extract was acetylated. The acetylated products were

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

directly submitted to GC-MS (column temp, 211 °C), and triacetate of glycerol (24-Ac) and the diacetate of formate (26-Ac) were identified. The acetate of cyclic carbonate (27-Ac) was also identified by GC-MS (column temp, 211 °C) after separation by TLC (solvent, ethyl acetate/*n*-hexane, 2/5). The mass spectra and the retention times were identical with those of the authentic compounds. These products, (24-Ac), (26-Ac) and (27-Ac), could not be detected in the extract of the uninoculated control.

In an isotopic experiment with ^{13}C -labeled β -syringyl ether (4- ^{13}C) (1.1 mg/20-mL culture) under the same conditions, the triacetate of glycerol (24-Ac), the diacetate of formate (26-Ac) and the acetate of cyclic carbonate (27-Ac) were identified from the extracts after 72 h of incubation. The mass spectrum of the diacetate of formate (26-Ac) is shown in Fig. 1.10. The mass spectra of formate (26-Ac) and cyclic carbonate (27-Ac) derived from (4- ^{13}C) gave higher molecular ion peaks by one mass unit than those of the unlabeled authentic compounds. These results clearly indicated that the formyl carbon of (26) and carbonate carbon of (27) contained ^{13}C derived from the β -etherated aromatic carbons of β -syringyl ether (4- ^{13}C).

Catabolism of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-[U-ring- ^{13}C]-methoxyphenoxy)-1,3-propanediol (5- ^{13}C)

The cultures incubated with ^{13}C -labeled β -guaiacyl ether (5- ^{13}C) (2.0 mg/20-mL culture) were extracted after 60 h of incubation. The triacetate of glycerol (24-Ac) was identified by GC-MS (column temp, 209 °C). The mass spectrum and the retention time were identical with those of the authentic compound. The acetate of cyclic carbonate (27-Ac) was separated by TLC (solvent, ethyl acetate/*n*-hexane, 2/5) and identified by GC-MS (column temp, 211 °C). From the mass spectrum, the carbonate carbon of (27-Ac) formed contained ^{13}C derived from the β -etherated aromatic

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

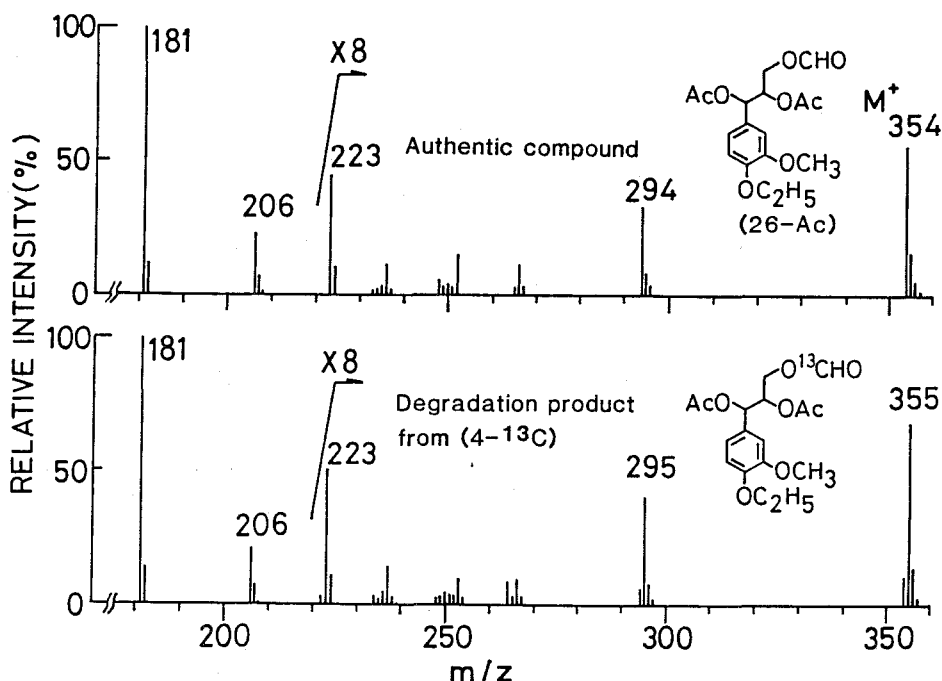


Fig. 1.10 Mass spectra of the diacetate of 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol (26-Ac).

Upper, authentic compound; lower, catabolite from 2-(2,6-[U-ring- ^{13}C]dimethoxyphenoxy)-2-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (4- ^{13}C) by *C. versicolor*.

carbons of β -guaiacyl ether (5- ^{13}C).

The acetate of formate (26), however, could not be detected in the degradation products of guaiacyl ether (5- ^{13}C).

Catabolism of 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol-2,3-cyclic carbonate (27)

The acetylated extracts of cyclic carbonate (27) (1.2 mg/20-mL culture) after 24 h of incubation were analyzed by GC-MS (column temp, 210 °C), and the triacetate of glycerol (24-Ac) was identified by comparison of the mass spectrum and retention time with those of the authentic compound. However, glycerol (24-Ac)

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

was also detected in the acetylated extracts after 24 h of incubation of the uninoculated control culture with cyclic carbonate (27).

Catabolism of 3-(4-ethoxy-3-methoxyphenyl)-1-formyloxypropane (30)

To confirm the possible cleavage of formate (26) to glycerol (24) by the fungus ($-OCHO \rightarrow -OH$), compound (30) having formyl ester was synthesized and used as a substrate. Compound (30) (1.2 mg/20-mL culture) added to the culture disappeared almost completely after 24 h of incubation, and the acetate of compound (31-Ac) was identified by GC-MS (column temp, 188 °C) as a main degradation product in the acetylated extracts. The mass spectra and retention time were identical with those of the authentic compound.

1.2.4 DISCUSSION

As described in section 1.1, formate (26) and cyclic carbonate (27) were identified as the degradation products of β -syringaldehyde ether (1) by *Coriolus versicolor* (Kawai et al., 1985a,b). In the present investigation, cyclic carbonate (27) was found as a catabolite from both β -syringyl ether (4) and β -guaiacyl ether (5) in the culture of *C. versicolor*. Formation of formate (27) from β -syringyl ether (4) was confirmed by GC-MS, but interestingly formate (26) could not be detected in the degradation products of guaiacyl ether (5) by *C. versicolor*. The similar results were obtained in the case of *Phanerochaete chrysosporium* (Umezawa et al., 1986). The results suggests that the number of the alkoxyl groups substituted aromatic nuclei influences the degradation of β -O-4 substructure by *C. versicolor* and *P. chrysosporium*.

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

To elucidate whether the formyl group of formate (27) was derived from β -etherated aromatic ring as a degradation fragment or other compounds [e.g. formic acid produced by oxalic decarboxylase commonly distributed in white-rot fungi (Shimazono, 1955; Shimazono & Hayaishi, 1957)], ^{13}C -labeled β -syringyl ether (4- ^{13}C) was synthesized and used as substrate. The isotopic experiment indicated that formate (26) was formed by the culture of *C. versicolor* as an aromatic ring cleavage product of β -syringaldehyde ether (1) and β -syringyl ether (4), which contain two methoxyl groups in β -phenoxyl groups.

That compound (30) was degraded to give compound (31) suggested that the formyl group of formate (26) is cleaved to give glycerol (24).

The present investigation showed that the cyclic carbonate (27) was also formed as an aromatic ring cleavage product from β -syringaldehyde ether (1), β -syringyl ether (4) and β -guaiacyl ether (5), and cleaved to give glycerol (24) by the culture of *C. versicolor*.

Figure 1.11 shows the formation pathways of glycerol (24) via aromatic ring cleavage products (26) and (27), from aryl-glycerol- β -aryl ethers (1), (4) and (5). Umezawa and Higuchi (1985c) reported that the cyclic carbonate (27) was formed as an aromatic ring cleavage product of β -guaiacyl ether (5) and that cyclic carbonate (27) was further degraded to give glycerol (24) by the culture of *P. chrysosporium*. The degradation pathway via formate (26) by *C. versicolor* was found for the first time in the present investigation, and the involvement of this pathway in *P. chrysosporium* was proved later (Umezawa et al., 1986).

Recent reports (Leisola et al., 1985; Umezawa et al., 1986a; Umezawa & Higuchi, 1986; Miki et al., 1987) demonstrated that aromatic ring cleavage of lignin model compounds were catalyzed by lignin peroxidase of *P. chrysosporium*, and degradation mecha-

1.2. AROMATIC RING CLEAVAGE BY *C. VERSICOLOR*

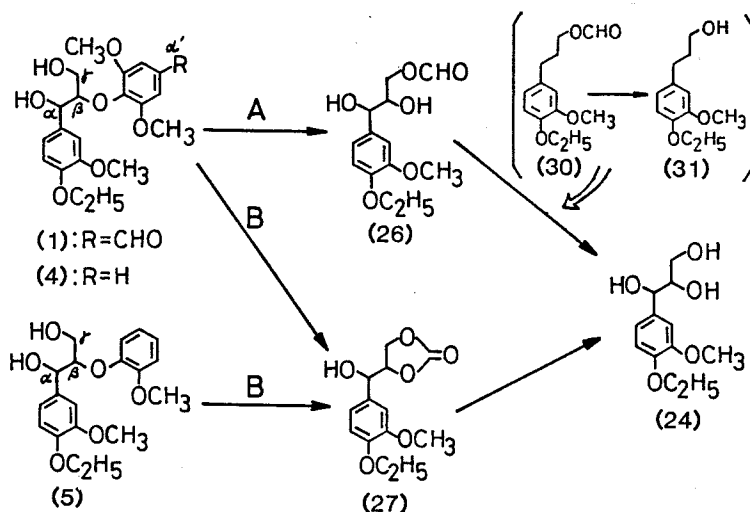


Fig. 1.11 Formation pathways of glycerol (24) via aromatic ring cleavage products, formate (26) and cyclic carbonate (27), from β -O-4 dilignols (1), (4) and (5) by the culture of *C. versicolor*.
Because the pathway (30) $\rightarrow$ (31) was established, the pathway (26) $\rightarrow$ (24) was suggested.

nisms were investigated in detail by use of ^{18}O -labeled molecular oxygen and water (Umezawa & Higuchi, 1987; Shimada et al., 1987; Miki et al., 1988).

Very recently, lignin peroxidase of *C. versicolor* was found to mediate the aromatic ring cleavage. The result will be discussed in Chapter 2 (Kawai et al., in press).

Section 1.3

1.3 FORMATION OF *p*-BENZOQUINONE MONOKETALS AS NOVEL DEGRADATION PRODUCTS

1.3.1 INTRODUCTION

Preceding sections (Section 1.1 and 1.2) described that the ligninolytic culture of *Coriolus versicolor* degraded nonphenolic β -O-4 lignin substructure model compounds via Ca-C β cleavage, aromatic ring cleavage etc. (Kawai et al., 1985a,b). Recent investigations demonstrated that lignin peroxidase, an extracellular heme protein from *Phanerochaete chrysosporium* (Tien & Kirk, 1983; Glenn et al, 1983; Tien & Kirk, 1984; Kuwahara et al., 1984, Gold et al., 1984), catalyzed not only Ca-C β cleavage but also aromatic ring cleavage of veratryl alcohol (Leisola et al., 1985) and lignin substructure model dimers (Umezawa et al., 1986a; Umezawa & Higuchi 1986).

The present section describes that *p*-benzoquinone monoketal, 2-(4-ethoxy-3-methoxyphenyl)-3-hydroxymethyl-6,10-dimethoxy-1,4-dioxaspiro[4,5]deca-6,9-diene-8-one [monoketal (8)], was formed in the degradation of β -O-4 lignin substructure model dimers by both the culture of *C. versicolor* and lignin peroxidase of *P. chrysosporium*, and the formation mechanism of monoketal (8) is discussed on the basis of the isotopic experiment (Kawai et al., 1987a).

1.3.2 MATERIALS AND METHODS

Syntheses of substrates

1-(4-Ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol [β -syngaldehyde ether (1)] was prepared as

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

described in Section 1.1 (Kawai et al., 1985).

2-(4-Carboxy-2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxy-phenyl)-1,3-propanediol [β -syringic acid ether (3)] was prepared from the diacetate of β -syringaldehyde ether (1-Ac) via the following two steps: (i) Jones' reagent (Fieser & Fieser, 1967a) in acetone at 0 °C; (ii) sodium methylate (28% in methanol) in methanol/CH₂Cl₂, 1/4, at 0 °C.

MS m/z (%) 422 (M⁺, 3), 224 (100), 198 (52), 195 (40), 183 (23), 181 (42), 152 (21), 151 (35), 137 (23).

Organism and culture conditions

Coriolus versicolor Ps4a 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 without agitation at 30 °C in nitrogen-limited medium as described in Section 1.1.

Degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol (1) by C. versicolor

β -Syringaldehyde ether (1) was added to 7-day-old cultures and incubated for 76 h. The cultures were then extracted with ethyl acetate as described in Section 1.1. The extracts were submitted to TLC directly or after acetylation with acetic anhydride and pyridine (1/1, v/v) at room temp for 24 h.

Monoketal (8) was separated from the nonacetylated extracts by TLC (solvents, methanol/CH₂Cl₂, 1/99, three times, and ethyl acetate/hexane, 1/2, four times). The acetate of monoketals (8-Ac), (9a-Ac), and (9b-Ac) were separated from acetylated extracts by TLC [solvents, (8-Ac), methanol/CH₂Cl₂, 1/99, three times, and ethyl acetate/*n*-hexane, 1/3, four times; (9a-Ac), methanol/C₂Cl₂, 2/98, four times, and ethyl acetate/*n*-hexane, 1/2, four times; (9b-Ac), methanol/CH₂Cl₂, 1/99, twice, and ethyl acetate/*n*-

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

hexane, 1/2, three times].

Preparation of Phanerochaete chrysosporium lignin peroxidase

Lignin peroxidase which was prepared by the modified method of Tien and Kirk (1984) from the culture filtrate of *P. chrysosporium* Burds. ME-446 was provided by Nagase Biochemicals Co., Ltd. (Umezawa et al., 1986). Enzyme activity was assayed by spectrometric quantification of veratraldehyde (46) ($\epsilon_{310} = 9300 \text{ M}^{-1} \cdot \text{cm}^{-1}$) formed by oxidation of veratryl alcohol (47) (Tien & Kirk, 1984).

Degradation of 2-(4-carboxy-2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (3) 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 β -syringic acid ether (3), 20 μL of lignin peroxidase (1.7-2.5 nkat) and polyacrylic acid buffer (10 mM in carboxyl, pH 4.5). Reactions were started by addition of lignin peroxidase, and the reaction mixture was incubated at 37 °C under air for 90 min.

The reaction mixture was extracted with ethyl acetate (10 mL, three times), and the combined organic layer was washed with saturated NaCl soln, dried over anhyd Na_2SO_4 and evaporated under reduced pressure. The extracts were acetylated with acetic anhydride and pyridine (1/1, v/v) for 24 h.

Monoketal (8-Ac) was separated by TLC (solvent, ethyl acetate/*n*-hexane, 1/3, twice). The identity of the product was determined by DI-MS.

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

Reaction flask which contained β -syringic acid ether (3), H_2O_2 , and buffer was evacuated and then filled with argon gas and

1.3. FORMATION OF *p*-BENZOQUINONE MONOKETALS

this procedure was repeated five times. Finally, $^{18}\text{O}_2$ (^{18}O : 99%, Amersham) was introduced into the evacuated flask. Reaction was started by the addition of lignin peroxidase and the reaction mixture was incubated at 37 °C for 45 min.

Instruments

^1H NMR spectra were obtained with a Varian XL-200 FT-NMR spectrometer (200 MHz) with $(\text{CH}_3)_4\text{Si}$ as an internal standard. Chemical shifts and coupling constants are given in δ value (ppm) and Hz, respectively. Mass spectra were taken with a Shimadzu GCMS QP-1000 gas chromatograph-mass spectrometer (EI, 70 eV) and a Jeol JMS-DX 300 gas chromatograph-mass spectrometer (EI, 70 eV). IR spectra were measured with a Jasco IR-810.

1.3.3 RESULTS

Degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol (1) by *Coriolus versicolor*

Monoketal (8) was separated as a catabolite of β -syringaldehyde ether (1) degraded by *C. versicolor*. The structure of monoketal (8), which has cyclized C_α and C_β oxygens, was determined on the basis of the following experiments. (i) When monoketal (8) was acetylated, the chemical shifts of C_r protons in the ^1H NMR spectrum shifted downfield owing to the acetyl group introduced (Table 1.2). (ii) Since monoketal (8) is a spiro compound whose C_α and C_β carbon atoms are asymmetric, the chemical shifts of the methoxyl groups and protons derived from the *p*-benzoquinone moiety in the ^1H NMR spectrum differ from each other as shown in Table 1.2.

(8): ^1H NMR (Table 1.2).

MS m/z (%) 393 (7), 392 (M^+ , 30), 331 (8), 212 (24), 169

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

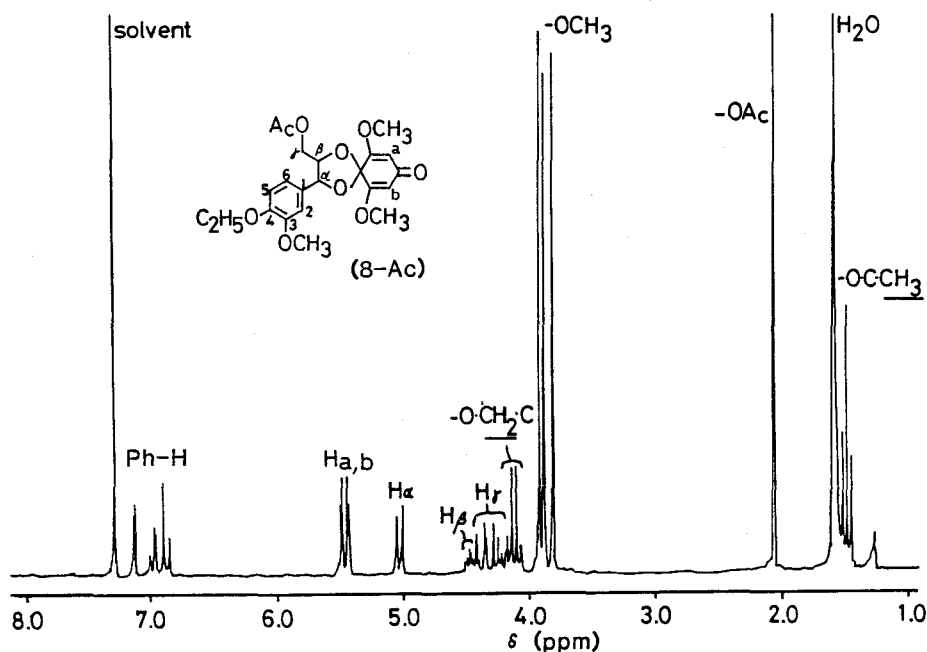


Fig. 1.12 ^1H NMR spectrum of 2-(4-ethoxy-3-methoxyphenyl)-3-hydroxymethyl-6,10-dimethoxy-1,4-dioxaspiro[4,5]deca-6,9-dien-8-one (8-Ac).

(100), 154 (23).

(8-Ac): ^1H NMR (Table 1.2, Fig 1.12).

MS m/z (%) 435 (7), 434 (M^+ , 30), 331 (15), 206 (60), 169 (37), 154 (100).

High-resolution MS for $\text{C}_{22}\text{H}_{26}\text{O}_9$ 434.15767 (calculated), 434.15781 (found).

IR ν_{max} (CCl_4) 1665 cm^{-1} .

The structures of the acetate of monoketals (9a-Ac) and (9b-Ac), which cyclized between C_β and C_γ oxygens, were also confirmed by ^1H NMR and DI-MS. The chemical shifts of C_α protons of (9a-Ac) and (9b-Ac) in the ^1H NMR spectra were considerably shifted downfield compared with that of the C_α proton of (8-Ac).

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

TABLE 1.2

¹H NMR Spectral Data of Monoketals (8), (8-Ac), (9a-Ac) and (9b-Ac)

	Chemical shifts	
	(8)	(8-Ac)
Ethoxyl	1.51 (t, $J=6.9$, 3H) 4.10 (q, $J=7.0$, 2H)	1.47 (t, $J=7.0$, 3H) 4.10 (q, $J=7.0$, 2H)
Acetyl	—	2.05 (s)
Methoxyl	3.82 (s) 3.87 (s) 3.90 (s)	3.80 (s) 3.87 (s) 3.90 (s)
C α -H	5.30 (d, $J=9.1$)	5.01 (d, $J=8.9$)
C β -H	4.30-4.40 (m)	4.40-4.50 (m)
C γ -H	3.65 (dd, $J=12.6$, 2.7) 3.98 (dd, $J=12.5$, 3.5)	4.22 (dd, $J=11.9$, 6.8) 4.35 (dd, $J=11.9$, 2.9)
>C=CH-CO-	5.45 (d, $J=1.7$) 5.48 (d, $J=1.7$)	5.42 (d, $J=1.7$) 5.47 (d, $J=1.7$)
Aromatic-H	6.86 (d, $J=8.2$, H5) 6.99 (dd, $J=8.3$, 2.0, H6) 7.10 (d, $J=1.9$, H2)	6.85 (d, $J=8.3$, H5) 6.96 (dd, $J=8.3$, 1.9, H6) 7.10 (d, $J=1.9$, H2)

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

TABLE 1.2 (cont'd)

	Chemical shifts	
	(9a-Ac)	(9b-Ac)
Ethoxyl	1.46 (t, $J=7.0$, 3H) 4.09 (q, $J=7.0$, 2H)	1.45 (t, $J=7.0$, 3H) 4.10 (q, $J=7.0$, 2H)
Acetyl	2.08 (s)	2.12 (s)
Methoxyl	3.78 (s) 3.87 (s) 3.89 (s)	3.71 (s, 6H) 3.87 (s)
Ca-H	5.81 (d, $J=8.9$)	5.94 (d, $J=6.2$)
Cβ-H	4.75-4.95 (m)	4.65-4.85 (m)
Cγ-H	about 3.85 about 4.05	about 4.20 4.37 (dd, $J=7.3$, 6.4)
>C=CH-CO-	5.37 (d, $J=1.7$) 5.41 (d, $J=1.7$)	5.33 (d, $J=1.5$) 5.38 (d, $J=1.7$)
Aromatic-H	6.80-7.00 (m, 3H)	6.80-7.00 (m, 3H)

Chemical shifts and coupling constants (J) are given in δ value (ppm) and Hz, respectively. CDCl_3 and $(\text{CH}_3)_4\text{Si}$ were used as solvent and internal standard, respectively. For structural formulae see Fig. 1.13.

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

These results indicated that acetoxyl groups were attached to the Ca positions of (9a-Ac) and (9b-Ac). Monoketals (9a) and (9b) were found to be diastereomers of each other, but the assignment to *erythro* and *threo* was not made.

(9a-Ac): ^1H NMR (Table 1.2).

MS m/z (%) 435 (5), 434 (M^+ , 18), 212 (14), 181 (100), 169 (29), 125 (15).

(9b-Ac): ^1H NMR (Table 1.2).

MS m/z (%) 435 (7), 434 (M^+ , 31), 223 (36), 212 (16), 181 (100), 169 (32), 154 (19), 125 (16).

The structure of monoketal (8) was further confirmed by mass spectrometric analysis of the reduced compounds by Pd-C/ H_2 and NaBH_4 as shown in Fig. 1.13.

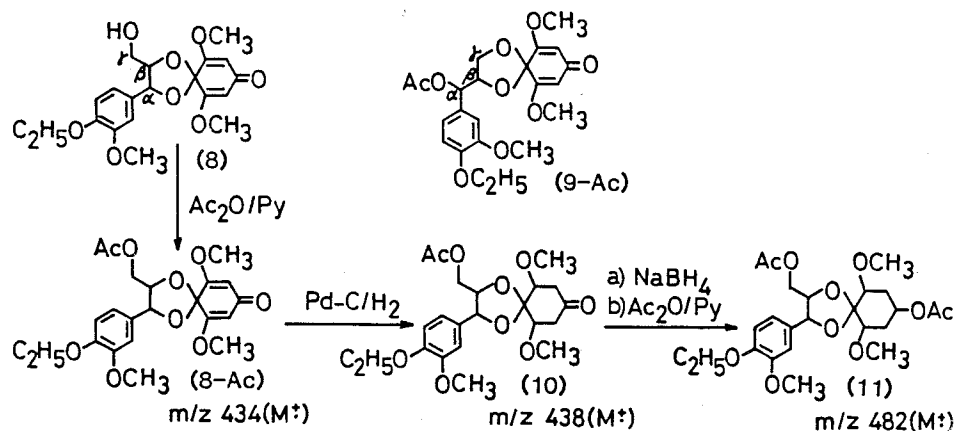


Fig. 1.13 Chemical structures of monoketals (8), (8-Ac), (9-Ac), and the reduced compounds of (8-Ac).

(i) When the acetate of monoketal (8-Ac) was hydrogenated with 10% Pd-C under H_2 in methanol at room temp for 20 min, two major products cyclohexanones (10a) and (10b) were isolated by

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

TLC (solvent, ethyl acetate/*n*-hexane, 1/3, twice), but both the compounds gave the same molecular ion peak at m/z 438. The result indicated that cyclohexanones (10a) and (10b) were stereoisomers each other.

(ii) Cyclohexanones (10a) and (10b) were further reduced with NaBH₄ in methanol at 0°C for 10 min, and the reduced products were acetylated. By this treatment, the molecular ions of the products (11a) and (11b) increased to give m/z 482, 44 mass units higher than original cyclohexanones (10a) and (10b), respectively.

Degradation of 2-(4-carboxy-2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (3) by lignin peroxidase

Monoketal (8) was also found to be formed by lignin peroxidase degradation of β-syringic acid ether (3), which is a catabolite of β-syringaldehyde ether (1) by the culture of *C. versicolor* as described in Section 1.1 (Kawai et al., 1985a). The acetate of monoketal (8-Ac) was identified by MS analysis.

(8-Ac): MS m/z (%) 435 (11), 434 (M⁺, 38), 331 (13), 206 (58), 169 (38), 154 (100).

In addition, the isotopic experiment with ¹⁸O₂ showed that 80% of the quinone oxygen of the acetate of monoketal (8-Ac) was derived from ¹⁸O₂ (Fig. 1.14).

1.3.4 DISCUSSION

The present investigation showed that monoketal (8) is formed as a degradation product of β-syringaldehyde ether (1) by the culture of *Coriolus versicolor*. In previous section (Kawai et al., 1985a,b), many degradation products of nonphenolic β-O-4 lignin substructure model dimers (1), (4) and (5) via Cα-Cβ cleavage and aromatic ring cleavage by *C. versicolor* were

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

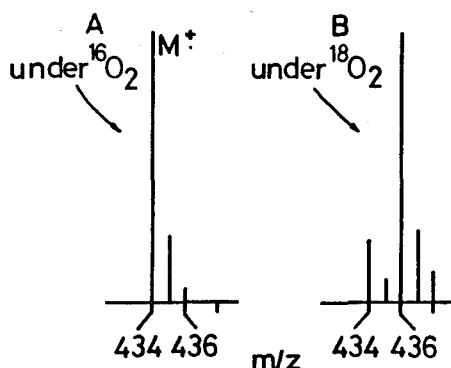


Fig. 1.14 Molecular ion region of the acetate of monoketal (8-Ac).

(A) Degradation product from β -syringic acid ether (3) by lignin peroxidase under air ($^{16}\text{O}_2$); (B) degradation product from (3) under $^{18}\text{O}_2$.

identified. Similar degradation products of β -O-4 lignin substructure models by lignin peroxidase of *Phanerochaete chrysosporium* were found (Tien & Kirk, 1983; Glenn et al., 1983; Tien & Kirk, 1984; Gold et al., 1984; Habe, 1985b; Miki et al., 1986a; Umezawa et al., 1986; Umezawa & Higuchi, 1986).

Kirk and co-workers (Kersten et al., 1985; Hammel et al., 1985) demonstrated by use of ESR that lignin peroxidase catalyzed one-electron oxidation of aromatic nuclei to form aryl cation radicals. Figure 1.15 shows the proposed degradation mechanisms of monoketal (8) from β -syringic acid ether (3) via cation radical of β -etherated aromatic ring (B-ring). It is conceivable that the B-ring of (3) is oxidized by lignin peroxidase to give a cation radical, which is subsequently attacked by the C α hydroxyl group of (3). The resulting radical reacts with molecular oxygen and the peroxide intermediate formed could be decarboxylated to give monoketal (8). The incorporation of molecular oxygen ($^{18}\text{O}_2$)

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

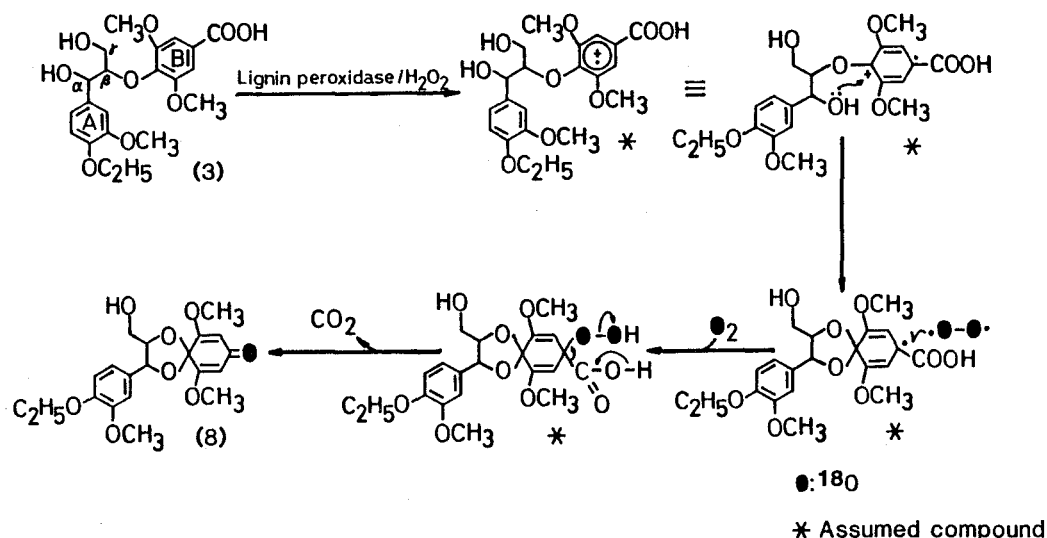


Fig. 1.15 Possible mechanism for the formation of monoketal (8) from β -syringic acid ether (3) by lignin peroxidase.

Incorporation of molecular oxygen to p -benzoquinone moiety of monoketal (8) was proved by the isotopic experiment using $^{18}O_2$.

into monoketal (8) (Fig. 1.13) supported the mechanism.

The formation mechanism of monoketal (9) which is cyclized between C_β and C_r oxygens is also explicable in a similar manner to that shown in Fig. 1.15.

Umezawa and Higuchi (1985c) reported that the migration of the β -aryl group from C_β to C_r oxygen during degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (5) to give guaiacoxyethanol in the culture of *P. chrysosporium*. There, it was suggested that migration occurs via attack of the hydroxyl group on the β -aryl cation radical produced by lignin peroxidase. Recently, similar rearrangements were reported by use of lignin peroxidase of *P. chrysosporium* (Miki et al., 1986b).

1.3. FORMATION OF p-BENZOQUINONE MONOKETALS

Kirk et al. (1986) detected an ESR signal corresponding to the β -aryl cation radical of a C_α carbonyl-containing β -O-4 lignin model formed by lignin peroxidase and suggested that aryl cation radicals were attacked by the hydroxyl group to form a cyclohexadienone ketal similar to monoketals (8) and (9).

In conclusion, these results suggest that monoketals (8) and (9) and other degradation products identified in Section 1.1 (Kawai et al., 1985a,b) from β -O-4 lignin substructure dilignols could be formed via the attack of hydroxyl groups on the cation radical, C_α - C_β cleavage and aromatic ring cleavage catalyzed by the lignin peroxidase in the culture of *C. versicolor*.

CHAPTER 2

DEGRADATION OF NONPHENOLIC β -O-4 LIGNIN SUBSTRUCTURE MODEL COMPOUNDS BY LIGNIN PEROXIDASE OF *CORIOLUS VERSICOLOR*

2.1 INTRODUCTION

Chapter 1 (Kawai et al., 1985a,b, 1987a) described that the nonphenolic β -O-4 lignin substructure model compounds were degraded by the ligninolytic culture of *C. versicolor*, and that the degradation could be catalyzed by a similar enzyme to that isolated from *P. chrysosporium* (lignin peroxidase) (Tien & Kirk, 1983; Glenn et al. 1983). Dodson et al. (1987) recently isolated lignin peroxidase from the culture filtrate of *C. versicolor*. Jönsson et al. (1989) reported very recently that the amino-terminal regions of lignin peroxidases from *C. versicolor* were quite similar to those of an isozyme from *P. chrysosporium*, and that β -O-4 lignin models were degraded by lignin peroxidase of *C. versicolor*. However, they did not study on the structure of degradation products and the degradation mechanisms of β -O-4 lignin model compounds.

The purpose of this investigation is to elucidate the degradation mechanisms of β -O-4 lignin substructure models, β -syringyl ether (4) and β -guaiacyl ether (5), by lignin peroxidase of *C. versicolor*, through identification of the degradation products (Kawai et al., in press).

2.2 MATERIALS AND METHODS

Enzyme preparation

Coriolus versicolor Ps4a was maintained at 25 °C on 3% malt agar slants. The cultures (100 mL in 1-L Erlenmeyer flasks x 10)

2. DEGRADATION OF *B-O-4* DILIGNOLS BY LIGNIN PEROXIDASE

were inoculated with small mycelial mats from the slants and grown without agitation at 25 °C. The culture medium was the same reported by Jönsson et al. (1987) except that 0.1 % Tween 80 [polyoxyethylene (20) sorbitan monoarate, Wako Pure Chemical Industrials] was added. Medium contained the following compounds per liter of distilled water: glucose, 2.20 g; ammonium tartrate, 0.94 g; KH₂PO₄, 1.00 g; NaH₂PO₄·H₂O, 0.26 g; MgSO₄·7H₂O, 0.50 g; Thiamine·HCl, 0.1 mg; CaCl₂, 5.1 mg; FeSO₄·7H₂O, 5.0 mg; MnSO₄·4H₂O, 0.48 mg; ZnSO₄·7H₂O, 0.64 mg and veratryl alcohol, 0.5 mg.

After 13 days, ammonium sulfate was added to the culture filtrate (950 mL) to 80 % concentration, and the soln was allowed to stand for 18 h at 4 °C. The precipitate was collected on celite (No. 545, Johns-Manville), resuspended in 0.2 M sodium tartrate buffer, pH 4.5, and dialyzed against the same buffer for 20 h at 4 °C. The dialyzed tube containing crude enzyme proteins was dehydrated with polyethylenglycol 6000 at 4 °C to 7 mL. The concentrated protein was used as a crude enzyme soln. The preparation contained both lignin peroxidase and laccase activities. Lignin peroxidase and laccase activities were assayed spectrophotometrically by using veratryl alcohol (46) (Tien & Kirk, 1984, see Section 1.3) and syringaldazine (Aldrich Chemical) (Leonowicz & Grzywnowicz, 1981, see Section 3.1), respectively.

Enzyme reactions

Substrate (1 µmol) was incubated in a total volume of 2.8 mL with the enzyme (lignin peroxidase activity, ca. 1 nkat) and 0.5 mM H₂O₂ in 0.2 M sodium tartrate buffer (pH 4.5) at 30 °C for 2 h under air. Reaction was terminated by extraction with ethyl acetate (5 mL, three times). The ethyl acetate layer was dried over Na₂SO₄ and evaporated under a reduced pressure. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v) in

2. DEGRADATION OF *B-O-4* DILIGNOLS BY LIGNIN PEROXIDASE

ethyl acetate for 15 h and analyzed by GC-MS [column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 11 m x 0.53 mm (i.d.), 120-220 °C, 5 °C/min, carrier gas; He (15 mL/min)].

Degradation products were identified by comparison of the mass spectra and the retention times with those of the authentic compounds.

In a control experiment, H₂O₂ was replaced by tartrate buffer.

Semi-quantitative experiments were performed as follows. The diacetate of 3-(4-hydroxy-3-methoxyphenyl)-1-propanol (3 µg in 10-µL dioxane soln) as an internal standard was added before extraction, and the extracts were analyzed as described previously. The quantities of degradation products were determined by comparing peak area of the internal standard in total ion chromatogram with those of degradation products.

Syntheses of substrates and authentic compounds

Following compounds were prepared by the methods reported previously: 2-(2,6-Dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol [β -syringyl ether (4)] (Kawai et al. 1985b), 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol [β -guaiacyl ether (5)] (Kawai et al., 1985b), 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol [glycerol (24)] (Kamaya & Higuchi, 1984) and 4-ethoxy-3-methoxybenzaldehyde [benzaldehyde (43)] (Umezawa et al., 1983).

1-(3-Ethoxy-4-methoxyphenyl)-2-(2-methoxyphenoxy)-3-hydroxypropanone [α -carbonyl dimer (6)] was obtained as intermediate compound of (5) before reduction with NaBH₄.

The acetates of 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol [formate (26)] (Kawai et al. 1985a) and 1-(4-ethoxy-3-methoxyphenyl)-1,2,3-propanetriol-2,3-cyclic carbonate

2. DEGRADATION OF *B-O-4* DILIGNOLS BY LIGNIN PEROXIDASE

[cyclic carbonate (27)] (Umezawa & Higuchi, 1985) were previously prepared.

1-(4-Ethoxy-3-methoxyphenyl)-3-hydroxypropanone [β -deoxy compound (29)] was synthesized from coniferyl alcohol prepared by the method of Kamaya et al. (1980) via three reaction steps; (i) ethylation of the phenolic hydroxyl group with iodoethane and K_2CO_3 in DMF at room temp, (ii) hydrogenation of double bond with 5% Pd-C under H_2 gas at room temp and (iii) oxidation of benzylic methylene with DDQ in benzene at room temp.

1H -NMR ($CDCl_3$) (acetate) δ 1.15 (t, $J=7.0$ Hz, 3H, -O-C- CH_3), 2.04 (s, 3H, -OAc), 3.27 (t, $J=6.4$ Hz, 2H, C2- H_2), 3.93 (s, 3H, -O CH_3), 4.18 (q, $J=7.0$ Hz, 2H, -O- CH_2 -C), 4.51 (t, $J=6.4$ Hz, 2H, C3- H_2), 6.89 (d, $J=8.3$ Hz, 1H, Ph- H_5), 7.55 (d, $J=1.9$ Hz, 1H, Ph- H_2), 7.56 (dd, $J=8.3, 2.0$ Hz, 1H, Ph- H_6).

Mass spectral data of the authentic compounds are listed in Table 2.1.

Instruments

NMR spectra were recorded by a Bruker AM-500 FT-NMR spectrometer (500 MHz). Chemical shifts and coupling constants are given in δ values (ppm) and Hz, respectively. Mass spectra were taken by a Hitachi M-80B gas chromatograph-mass spectrometer (EI, 70 eV).

2.3 RESULTS

Degradation of 2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (4)

The acetates of the degradation products were directly submitted to GC-MS. Figure 2.1 shows the gas chromatogram of products and mass chromatograms of base ion peaks of identified

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

TABLE 2.1

Relative Intensity of the Important Fragment Ion of the Authentic and Degradation Products.

Authentic compounds	
Compounds	Mass spectral data m/z (%)
α -Carbonyl dimer (6-Ac) ^a	388 (M^+ , 5), 328 (9), 265 (15), 180 (11), 179 (100), 151 (41), 150 (20), 123 (10)
(7)	328 (M^+ , 29), 180 (13), 179 (100), 177 (26), 151 (37), 150 (13)
Benzaldehyde (43)	180 (M^+ , 68), 152 (59), 151 (100)
Glycerol (24a-Ac) ^b	368 (M^+ , 10), 308 (10), 206 (33), 181 (100)
Glycerol (24b-Ac) ^b	368 (M^+ , 10), 308 (5), 206 (23), 181 (100)
β -Deoxy (28-Ac)	266 (M^+ , 25), 206 (40), 179 (64), 151 (100)
Formate (26-Ac)	354 (M^+ , 12), 206 (17), 181 (100)
Cyclic carbonate (27-Ac)	310 (M^+ , 12), 181 (100)
Degradation products of β -syringyl ether (4)	
Compounds	Mass spectral data m/z (%)
Glycerol (24a-Ac) ^b	368 (M^+ , 7), 308 (7), 206 (30), 181 (100)
Glycerol (24b-Ac) ^b	368 (M^+ , 10), 308 (4), 206 (23), 181 (100)
β -Deoxy (29-Ac)	266 (M^+ , 18), 206 (42), 179 (65), 151 (100)
Formate (26-Ac)	354 (M^+ , 9), 206 (20), 181 (100)
Cyclic carbonate (27-Ac)	310 (M^+ , 13), 181 (100)

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

TABLE 2.1 (cont'd)

Degradation products of β -guaiacyl ether (5)	
Compounds	Mass spectral data m/z (%)
(7)	328 (M^+ , 61), 179 (100), 177 (30), 151 (46)
Benzaldehyde (43)	180 (M^+ , 58), 152 (51), 151 (100)
Glycerol (24b-Ac) ^b	368 (M^+ , 19), 308 (8), 206 (23), 181 (100)

^a Direct ionization. (6-Ac) thermally degraded to give α,β -unsaturated compound (7).

^b *erythro* and *threo* forms

products. The acetate derivatives of glycerol (24-Ac), formate (26-Ac) and cyclic carbonate (27-Ac) were identified. Furthermore, the occurrence of β -deoxy compound (29-Ac) was suggested. Then, the acetylated extract was separated by TLC (Kieselgel 60 F254, Merck; solvent, ethyl acetate/*n*-hexane, 1/2, twice). The fraction whose *R_f* value corresponded to that of the acetate of synthetic β -deoxy compound (29-Ac) was analyzed by GC-MS, and β -deoxy compound (29-Ac) was identified (Fig. 2.2). The data of mass spectra and quantities of identified degradation products are listed in Table 2.1 and 2.2, respectively.

Trace amounts of glycerol (24-Ac), formate (26-Ac), cyclic carbonate (27-Ac) and β -deoxy compound (29-Ac) were detected in the acetylated extract of the control experiment by GC-MS analysis. But the amounts were less than 5-15% of those in complete one.

Veratryl alcohol (47) added to the culture as enzyme inducer and not removed completely from the enzyme solution was found as the acetate (the peak at about 3 min in Fig. 2.1).

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

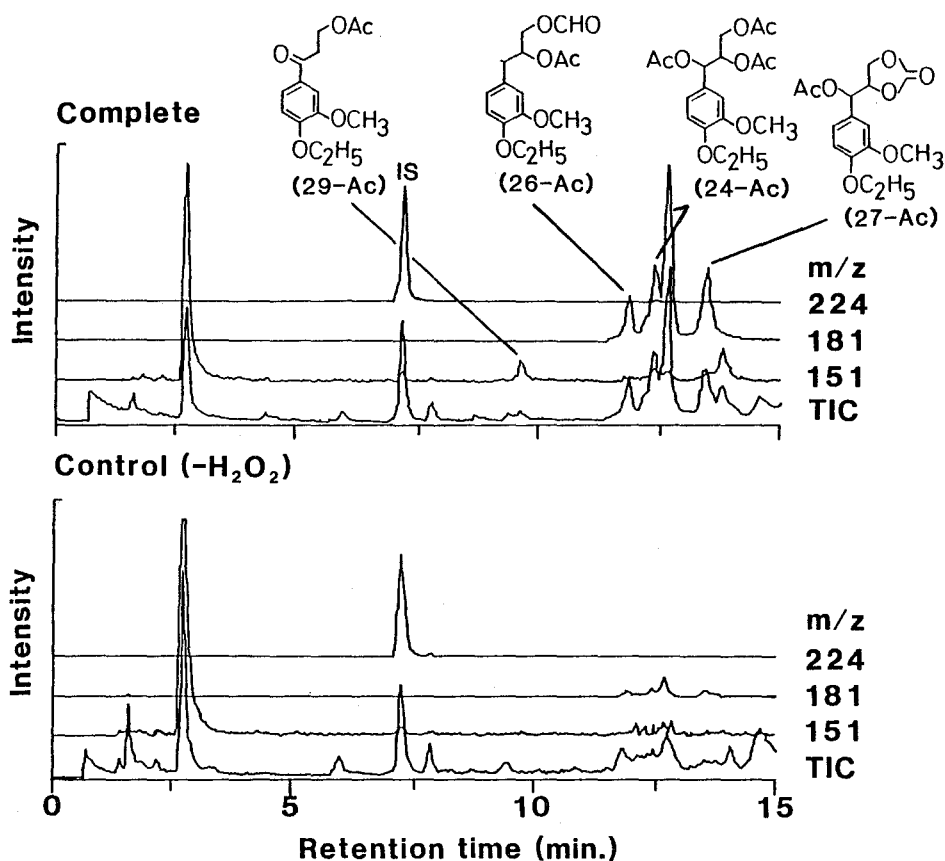


Fig. 2.1 Gas chromatogram (total ion chromatogram in GC-MS analysis) and mass chromatograms (base ion peaks) of acetylated degradation products formed from 2-(2,6-dimethoxyphenoxy)-1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol (4) by lignin peroxidase of *C. versicolor*.

Upper, complete system (enzyme/substrate/H₂O₂); lower, control system (without addition of H₂O₂). Before extraction of the reaction mixture, the diacetate of 1-(4-hydroxy-3-methoxyphenyl)-1-propanol (I.S., 3 μ g) was added as an internal standard.

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

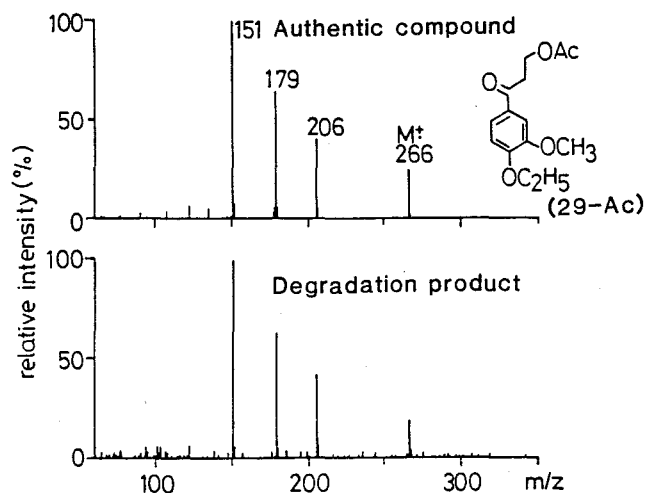


Fig. 2.2 Mass spectra of the acetate of 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone (29-Ac). Upper, authentic compound; lower, degradation product formed from β -syringyl ether (4).

TABLE 2.2

Yields of the Degradation Products of β -Syringyl Ether (4) and β -Guaiacyl Ether (5)

Substrates	Products (n mol)					
	(7)	(24-Ac)	(26-Ac)	(27-Ac)	(29-Ac)	(43)
β -Syringyl ether (4)	—	18.8	3.1	7.6	1.1	—
β -Guaiacyl ether (5)	1.6	—	6.4	—	—	8.7

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

Degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (5)

The acetates of the degradation products were directly submitted to GC-MS. Figure 2.3. shows the gas chromatogram of the products, and mass chromatograms of base ion peaks of identified products. Benzaldehyde (43) and the triacetate of glycerol (24-Ac) were identified. The diacetate of α -carbonyl dimer (6-Ac) was thermally decomposed to release acetic acid in the process of GC-MS and to form α,β -unsaturated carbonyl compound (7). The data of mass spectra and quantities of identified degradation products are listed in Table 2.1 and 2.2, respectively.

While, control experiment indicated that no products were formed from β -guaiacyl ether (5).

2.4 DISCUSSION

Lignin peroxidase of *Coriolus versicolor* was isolated by Dodson et al. (1987) and found to catalyze the oxidation of veratryl alcohol (47) to veratraldehyde (46) and C α -C β cleavage of non-phenolic β -1 lignin substructure model. The enzyme property was similar to that of lignin peroxidase of *Phanerochaete chrysosporium*. However, the oxidation of nonphenolic β -O-4 lignin substructure, which is most frequent linkage in lignin, by lignin peroxidase of *C. versicolor* has been poorly understood. Hence, the degradation of β -syringyl ether (4) and β -guaiacyl ether (5) by this enzyme was attempted.

β -Syringyl ether (4) and β -guaiacyl ether (5) were degraded to give many products, such as (6), (24), (26), (27), (29) and (43). Possible degradation pathways of (4) and (5) were proposed on the basis of the identified products as shown in Fig. 2.4. The degradation reactions did not occurs when H₂O₂ was not added to

2. DEGRADATION OF *B*-O-4 DILIGNOLS BY LIGNIN PEROXIDASE

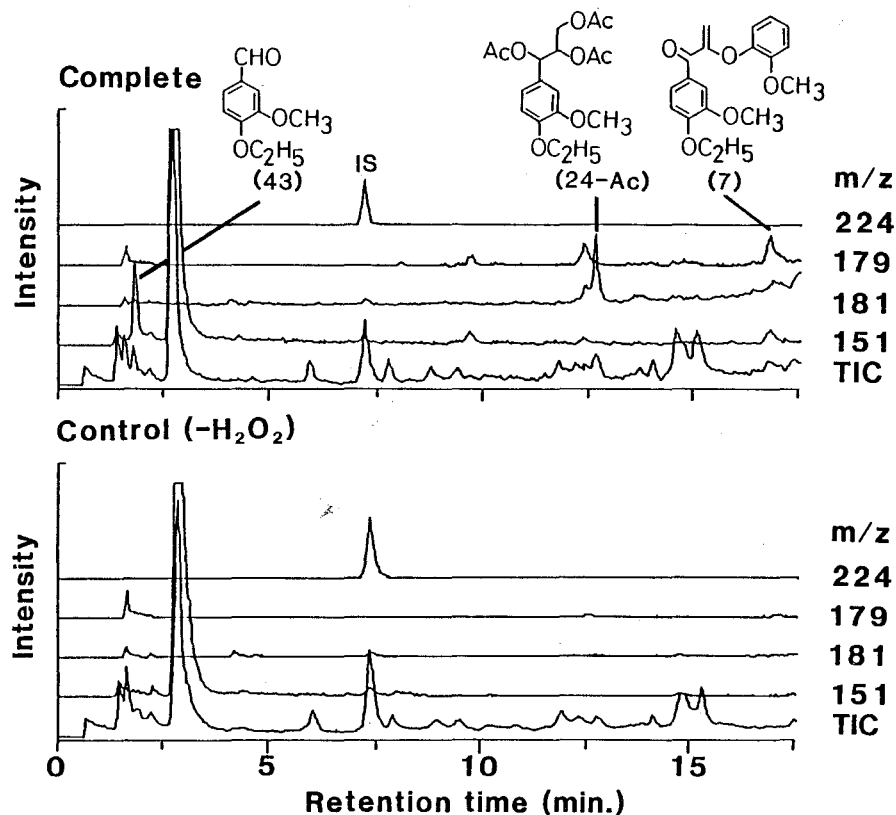


Fig. 2.3 Gas chromatogram (total ion chromatogram in GC-MS analysis) and mass chromatograms (base ion peaks) of acetylated degradation products formed from 1-(4-ethoxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (5) by lignin peroxidase of *C. versicolor*. Upper, complete system (enzyme/substrate/ H_2O_2); lower, control system (without addition of H_2O_2). Before extraction of the reaction mixture, the diacetate of 1-(4-hydroxy-3-methoxyphenyl)-1-propanol (I.S., 3 μ g) was added as an internal standard.

2. DEGRADATION OF *B*-O-4 DILIGNOLS BY LIGNIN PEROXIDASE

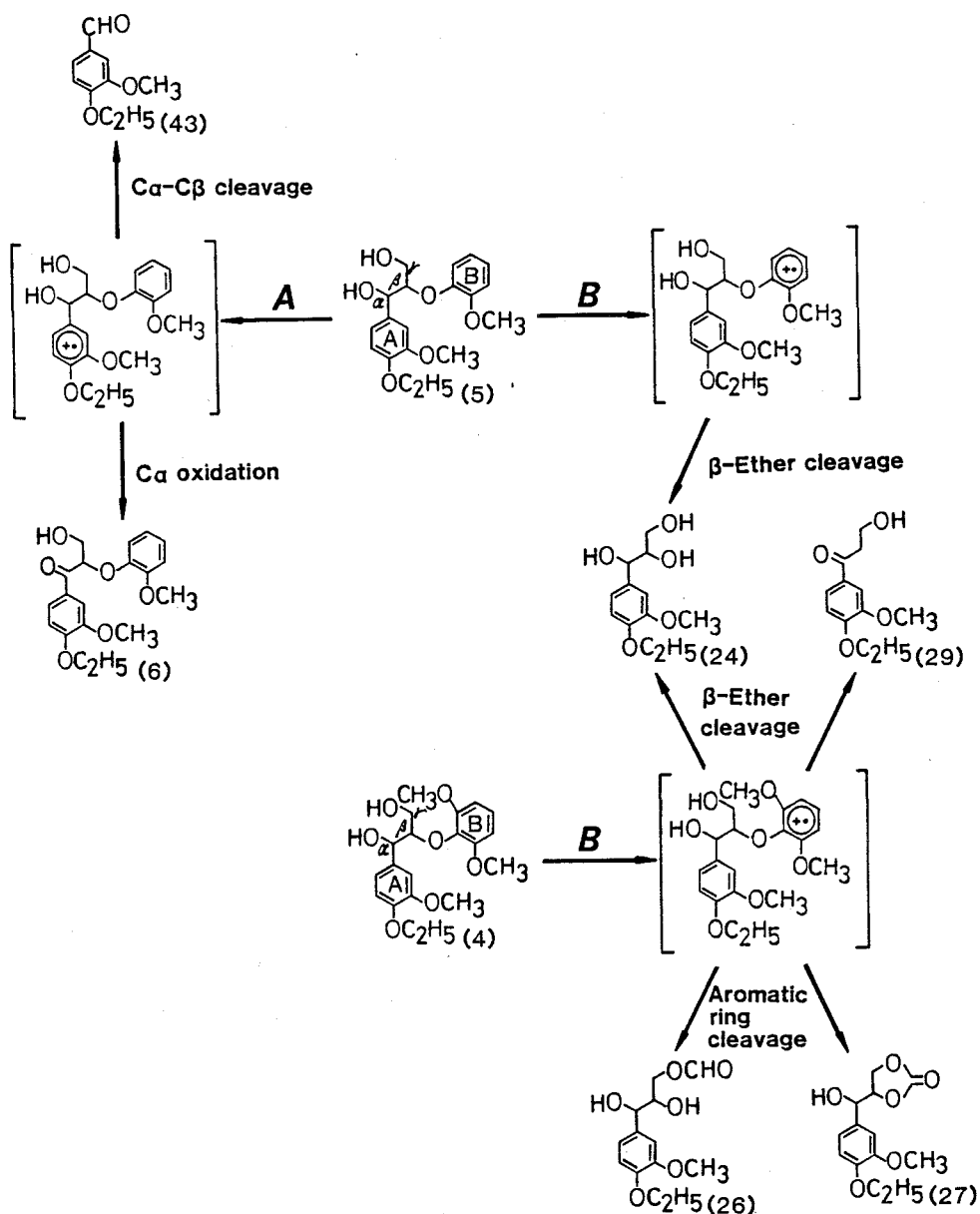


Fig. 2.4 Proposed degradation pathways of β -syringyl ether (4) and β -guaiacyl ether (5) by lignin peroxidase of *C. versicolor*.

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

the reaction mixtures. Therefore, all of the degradation reactions could be catalyzed by lignin peroxidase.

It was demonstrated by use of ESR that lignin peroxidase of *P. chrysosporium* catalyzed one-electron oxidation of alkoxyated aromatic nuclei to form the aryl cation radicals (Kersten et al., 1985; Hammel et al., 1985; Kirk et al., 1986). Formed cation radicals reacted with nucleophiles (e.g. H₂O or hydroxyl group of the side chain) and radicals (e.g. O₂) to give various degradation products (Hammel et al., 1985; Schoemaker, 1985; Habe et al., 1985a; Kirk et al., 1986; Renganathan et al., 1986; Miki et al., 1986a,b; Umezawa and Higuchi, 1986; Kawai et al., 1987a; Umezawa & Higuchi, 1987b; Shimada et al., 1987; Miki et al., 1988; Umezawa, 1988). The present experiment demonstrated that four types of reactions: (i) C α -C β cleavage, (ii) C α -oxidation, (iii) β -ether cleavage and (iv) aromatic ring cleavage, were mediated by lignin peroxidase of *C. versicolor*. These results were almost same as found in the degradation of β -O-4 lignin substructures by intact culture of *C. versicolor* as described in Chapter 1 (Kawai et al. 1985a,b, 1987a).

It was also evident that higher alkoxyated aromatic rings are preferentially attacked by lignin peroxidase of *C. versicolor* as found in the degradation of these substrates by the culture (Yokota et al., 1988) and lignin peroxidase (Umezawa & Higuchi, 1986a,b) of *P. chrysosporium*.

β -Deoxy compound (29) was identified for the first time as degradation product of these substrates by lignin peroxidase. Two possible formation mechanisms of (29) were shown in Fig. 2.5. One-electron oxidation of β -etherated aromatic ring is first catalyzed by lignin peroxidase to form the aryl cation radical. At the pathway A, benzylic hydrogen is released and the bond between C β and the etheric oxygen atom is cleaved to form an enol intermediate. At the pathway B, C α hydroxyl group attacks to the

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

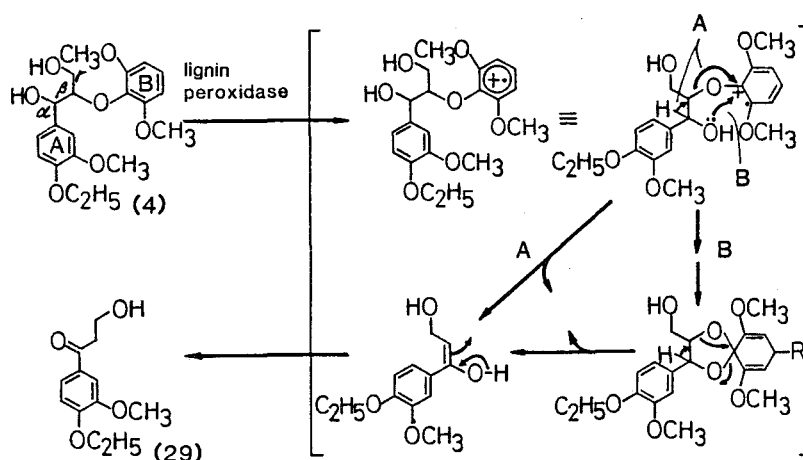


Fig. 2.5 Possible mechanisms for the formation of β -deoxy compound (29) from β -syringyl ether (4) by lignin peroxidase of *C. versicolor*.

cation. Then a cyclohexadienone intermediate is formed via several steps and converted to the enol compound. The attack by hydroxyl groups of side chain to β -aryl cations was important reaction for lignin peroxidase oxidation (Umezawa and Higuchi, 1985c; Kirk et al., 1986; Miki et al., 1986a,b; Kawai et al., 1987a; Umezawa and Higuchi, 1987; Miki et al., 1988). The enol compound is spontaneously converted to give β -deoxy compound (29). Chen and Chang (1985) proposed the reductive cleavage of β -O-4 bond by white-rot fungi. The carbon atom at C β position of this compound (29) is reduced, but C α is oxidized. Earlier investigation in Section 1.1 showed that a similar β -deoxy derivative, 1-(4-ethoxy-3-methoxyphenyl)-1,3-propanediol [β -deoxy diol (28)], was formed as a degradation product of β -O-4 model, β -syringaldehyde ether (1), by the culture of *C. versicolor* (Kawai et al., 1985a). This product could be formed by reduction of β -deoxy compound (29) by the culture. Since the present enzyme

2. DEGRADATION OF β -O-4 DILIGNOLS BY LIGNIN PEROXIDASE

preparation was crude, involvement of other enzymes for the formation of β -deoxy compound (29) might be possible. β -Deoxy derivatives were identified as degradation products of β -O-4 models by *Pseudomonas* species (Vicuña, 1988) and Mn-dependent peroxidase of *P. chrysosporium* in the presence of Mn^{2+} , H_2O_2 and a thiol (Gold et al., 1989; Wariishi et al., 1989b). Mn-dependent peroxidase was recently found in the culture of *C. versicolor* (Johansson & Nyman, 1987).

The present investigation indicated that many oxidative reactions were catalyzed by lignin peroxidase of *C. versicolor*. In conclusion, lignin peroxidase is one of the most important lignin-degrading enzyme of *C. versicolor*.

CHAPTER 3

DEGRADATION OF PHENOLIC LIGNIN MODEL COMPOUNDS BY LACCASE OF *CORIOLUS VERSICOLOR*

Laccase is commonly distributed in white-rot fungi and is known to cause Bavendamm's reaction. *Coriolus versicolor* excretes a phenol oxidizing enzyme, laccase (Fåhræus & Reinhammer, 1967).

It has hitherto been shown that laccase catalyzes only subtraction of one-electron from phenolic hydroxyl groups to give the phenoxy radicals which generally undergo polymerization via radical coupling accompanied by partial depolymerization of propyl side chains via alkyl-aryl cleavage. In fact, earlier reports (Crawford, 1981; Higuchi, 1985; Kirk & Shimada, 1985; Evans, 1985; Lundquist & Kristersson, 1985; Morohoshi & Haraguchi, 1987; Morohoshi et al., 1987) showed that lignin preparations treated with laccase are partly depolymerized but mostly polymerized. In spite of considerable efforts by many researchers, the inherent role of laccase in lignin biodegradation has not been elucidated sufficiently.

The main purpose of this chapter is to elucidate the specific reactions of lignin by laccase of *C. versicolor* through the chemical structures of the degradation products of various lignin model compounds.

Section 3.1

3.1 Ca-C₈ CLEAVAGE OF PHENOLIC β -1 LIGNIN SUBSTRUCTURE MODEL

3.1.1 INTRODUCTION

1,2-Diarylpropane-1,3-diol structure (β -1 substructure) is one of the major interphenylpropane linkage in lignin, and the proportion of β -1 substructure in lignin is estimated 7-15% (Adler, 1977; Sakakibara, 1983). Nonphenolic β -1 lignin substructure model compounds played an essential role in discovery of lignin peroxidase from the culture of *Phanerochaete chrysosporium* (Tien & Kirk, 1983; Glenn et al., 1983; Tien & Kirk, 1984; Kuwahara et al., 1984; Gold et al., 1984) and made possible to explain the mechanism of the oxidative Ca-C₈ cleavage of nonphenolic lignin substructure models via cation radicals by lignin peroxidase and its mimetic system (Hammel et al., 1985; Shoemaker et al., 1985; Habe et al., 1985a). Thus, it is conceivable that elucidation of the reaction mechanism of laccase for β -1 lignin substructures give information on the role of the laccase in lignin biodegradation.

The present section describes the degradation of β -1 lignin substructure model compounds by laccase of *Coriolus versicolor*. Phenolic substrate, 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol [disyringylpropane (17)], was degraded via Ca-C₈ cleavage of the propyl side chain, but nonphenolic substrate, 1,2-bis(4-ethoxy-3,5-dimethoxyphenyl)-1,3-propanediol (20), could not be oxidized (Kawai et al., 1987b).

3.1. *Ca-CB CLEAVAGE OF A B-1 DILIGNOL BY LACCASE*

3.1.2 MATERIALS AND METHODS

Laccase preparation

The electrophoretically homogeneous laccase preparation of *Coriolus versicolor* IFO-30340 was kindly provided by Oji Paper Co., Ltd. (Sugiura et al., 1987). It was stocked at -20 °C as a 50% glycerol soln. Laccase activity was determined spectrophotometrically by measuring the absorption at 525 nm using syringaldazine ($\epsilon = 6500 \text{ M}^{-1} \cdot \text{cm}^{-1}$, Aldrich Chemical) (Leonowicz & Grzywnowicz, 1981). The reaction mixture contained 10 μL of laccase, 400 μL of 0.5 mM syringaldazine in ethanol, and 3 mL of 0.2 M acetate buffer (pH 5.3); as a control the reaction mixture containing 10 μL of buffer instead of enzyme was used.

Enzyme reaction of lignin substructure models

The flask containing 5 mL of 0.2 M acetate buffer (pH 4.0) was previously flushed with 100% oxygen gas for 1 min. The reaction was started by addition of substrate (2.5 μmol in 50 μL of DMF soln) and 1 μL of enzyme soln (400-800 nkat) into the buffer, and the reaction mixture was shaken at 30 °C for 15 min.

In a control experiment, the enzyme was replaced by a boiled enzyme (100 °C, 5 min), the inactivation of which was spectrophotometrically confirmed.

The reaction mixture was extracted with five portions of ethyl acetate (total, 25 mL). The combined organic layer was washed with saturated NaCl Soln, dried over anhyd Na_2SO_4 , and evaporated under a reduced pressure. The residual DMF was removed under a high vacuum. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate for 15 h at room temp.

The acetylated products were analyzed directly by GC-MS [column, 1.5% OV-17 on Chromosorb W AW-DMCS (Shinwa Kakou), glass

3.1. *Ca-CB CLEAVAGE OF A β -1 DILIGNOL BY LACCASE*

column 1 m x 2.6 mm (i.d.), column temp, 180-220 °C, 5 °C/min; and Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m x 0.53 mm (i.d.), column temp, 130-220 °C, 5 °C/min].

Identification of degradation products were performed by comparison of the mass spectra and the retention times with those of the authentic compounds.

Syntheses of substrates and authentic compounds

erythro-1,2-Bis(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol [disyringylpropane (17)] was prepared by the method of Namba (1981; see Nakatsubo, 1988).

¹H NMR (CDCl₃) (acetate) δ 2.00 (s, 3H, alcoholic-OAc), 2.01 (s, 3H, alcoholic-OAc), 2.31 (s, 3H, Ph-OAc), 2.32 (s, 3H, Ph-Ac), 3.25-3.45 (m, 1H, C2-H), 3.73 (s, 6H, -OCH₃), 3.75 (s, 6H, -OCH₃), 4.20 (dd, $J=7.1$, 12 Hz, 1H, C3-H), 4.42 (dd, $J=7.1$, 11 Hz, 1H, C3-H), 6.06 (d, $J=6.2$, 1H, C1-H), 6.33 (s, 2H, Ph-H), 6.35 (s, 2H, Ph-H).

erythro-1,2-Bis(4-ethoxy-3,5-dimethoxyphenyl)-1,3-propanediol (20) was prepared from disyringylpropane (17) by ethylation with iodoethane and K₂CO₃ in DMF at room temp.

¹H NMR (CDCl₃) (acetate) δ 1.34 (t, $J=7.1$ Hz, -O-C-CH₃), 1.35 (t, $J=7.1$ Hz, -O-C-CH₃), 1.97 (s, 3H, -OAc), 1.98 (s, 3H, -OAc), 3.26-3.38 (m, 1H, C2-H), 3.77 (s, 6H, -OCH₃), 3.78 (s, 6H, -OCH₃), 4.01 (q, $J=7.1$ Hz, 2H, -O-CH₂-), 4.03 (q, $J=7.1$ Hz, 2H, -O-CH₂-), 4.14 (dd, $J=6.7$, 11 Hz, 1H, C3-H), 4.33 (dd, $J=6.7$, 11 Hz, 1H, C3-H), 6.03 (d, $J=7.0$ Hz, 1H, C1-H), 6.35 (s, 2H, Ph-H), 6.38 (s, 2H, Ph-H).

Diacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-hydroxyethanone [syringylethanone (40-Ac)] was prepared from α -bromo-4'-acetoxy-3',5'-dimethoxyacetophenone (Kamaya & Higuchi, 1984a), by treatment with sodium acetate in DMF at room temp.

¹H NMR (CDCl₃) δ 2.24 (s, 3H, alcoholic-OAc), 2.36 (s, 3H,

3.1. *Ca-CB CLEAVAGE OF A β -1 DILIGNOL BY LACCASE*

Ph-OAc), 3.88 (s, 6H, -OCH₃), 5.31 (s, 2H, C2-H₂), 7.16 (s, 2H, Ph-H).

MS Table 3.1.

Triacetate of 1-(4-hydroxy-3,5-dimethoxyphenyl)-1,2-ethanediol [syringylglycol (39-Ac)] was prepared from the acetate of syringylethanone (40-Ac) by reduction with NaBH₄ in methanol at 0 °C and acetylation with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate at room temp.

¹H NMR (CDCl₃) δ 2.07 (s, 3H, alcoholic-OAc), 2.13 (s, 3H, alcoholic-Ac), 3.83 (s, 6H, -OCH₃), 4.26 (dd, $J=7.6$, 12 Hz, 1H, C2-H), 4.34 (dd, $J=4.5$, 12 Hz, 1H, C2-H), 5.98 (dd, $J=4.5$, 7.6 Hz, 1H, C1-H), 6.60 (s, 2H, Ph-H).

MS Table 3.1.

1-(4-Ethoxy-3,5-dimethoxyphenyl)-1,2-ethanediol [phenylglycol (41)] was prepared from the acetate of syringylethanone (40-Ac) via following three steps: (i) sodium methylate (28% in methanol) in methanol/CH₂Cl₂ (1/4) at 0 °C, (ii) bromoethane/K₂CO₃ in DMF at room temp, (iii) NaBH₄ in methanol at 0 °C.

¹H NMR (CDCl₃) (acetate) δ 1.35 (t, $J=7.0$ Hz, -O-C-CH₃), 2.07 (s, 3H, -OAc), 2.13 (s, 3H, -OAc), 3.85 (s, 6H, -OCH₃), 4.03 (q, $J=6.9$ Hz, 2H, -O-CH₂-), 4.28 (d, $J=7.0$ Hz, 1H, C2-H), 4.29 (d, $J=5.1$ Hz, 1H, C2-H), 5.94 (dd, $J=6.8$, 5.1 Hz, 1H, C1-H), 6.56 (s, 2H, Ph-H).

MS Table 3.1.

The acetate of 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-hydroxyethanone [phenylethanone (42-Ac)] was prepared from acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone, Aldrich Chemical) via the following three steps: (i) iodoethane/K₂CO₃ in DMF at room temp, (ii) CuBr₂ in refluxing ethyl acetate, (iii) sodium acetate in DMF at room temp.

¹H NMR (CDCl₃) δ 1.37 (t, $J=7.1$ Hz, 3H, -O-C-CH₃), 2.24 (s, 3H, -OAc), 3.90 (s, 6H, -OCH₃), 4.14 (q, $J=7.1$ Hz, 2H, -O-

3.1. Ca-CB CLEAVAGE OF A β -1 DILIGNOL BY LACCASE

CH₂-), 5.31 (s, 2H, C₂-H₂), 7.15 (s, 2H, Ph-H).

MS Table 3.1.

4-Ethoxy-3,5-dimethoxybenzaldehyde [benzaldehyde (54)] was prepared from syringaldehyde (51) (Tokyo Chemical Industry) by ethylation with bromoethane and K₂CO₃ in DMF at room temp.

(51-Ac): MS Table 3.1.

(54): MS Table 3.1.

2,6-Dimethoxy-*p*-benzoquinone [benzoquinone (58)] was prepared from pyrogallol (1,2,3-trihydroxybenzene, Nakarai Chemicals) by the method of Baker (1941).

¹H NMR (CDCl₃) δ 3.82 (s, 6H, -OCH₃), 5.85 (s, 2H, >C=CH-CO-).

MS Table 3.1.

2,6-Dimethoxy-*p*-hydroquinone [hydroquinone (57)] was prepared from benzoquinone (58) by reduction with NaBH₄ in methanol at 0 °C.

¹H NMR (CDCl₃) (acetate) δ 2.29 (s, 3H, -OAc), 2.33 (s, 3H, -OAc), 3.79 (s, 6H, -OCH₃), 6.38 (s, 2H, Ph-H).

MS Table 3.1.

Instruments

Enzyme activity was measured using a Hitachi Model 200-20 double-beam spectrophotometer. NMR spectra were recorded by a Varian XL-200 FT-NMR spectrometer (200 MHz) with (CH₃)₄Si 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 GCMS-QP 1000 gas chromatograph-mass spectrometer (EI, 70 eV).

3.1. Ca-CB CLEAVAGE OF A β -1 DILIGNOL BY LACCASE

TABLE 3.1

Relative Intensity of the Important Fragment Ions of the Authentic Compounds^a

Compound	Mass spectral data m/z (%)
Syringyl-glycol (39-Ac)	341 (0.2), 340 (M^+ , 1.2), 298 (17), 239 (8), 238 (46), 197 (14), 196 (100), 184 (7), 183 (65), 181 (9), 180 (7), 167 (13), 155 (9), 123 (9)
Syringyl-ethanone (40-Ac)	296 (M^+ , 0.3), 254 (28), 182 (17), 181 (100), 153 (11)
Phenyl-glycol (41-Ac)	327 (7), 326 (M^+ , 42), 267 (13), 266 (62), 237 (21), 225 (14), 224 (82), 212 (12), 211 (100), 197 (18), 196 (69), 195 (45), 183 (51), 182 (17), 181 (18), 180 (11), 167 (25), 155 (20), 123 (26)
Phenyl-ethanone (42-Ac)	283 (3), 282 (M^+ , 20), 210 (12), 209 (96), 182 (10), 181 (100), 153 (14), 123 (6)
Syringaldehyde (51-Ac)	225 (0.2), 224 (M^+ , 1.6), 183 (10), 182 (100), 181 (40), 167 (8)
Benzaldehyde (54)	211 (6.7), 210 (M^+ , 55), 183 (10), 182 (100), 181 (83), 167 (17), 153 (6), 139 (9), 135 (6), 125 (9), 111 (8), 110 (9)
Hydroquinone (57-Ac)	255 (0.2), 254 (M^+ , 1.2), 212 (19), 171 (9), 170 (100), 155 (21)
Benzoquinone (58)	169 (8.6), 168 (M^+ , 100), 153 (7), 140 (24), 138 (37), 137 (10), 125 (32), 112 (24), 110 (10)

^a Column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone).

3.1. Ca-Cb CLEAVAGE OF A β -1 DILIGNOL BY LACCASE

3.1.3 RESULTS

Degradation of 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (17)

The acetate of degradation products were submitted directly to GC-MS. The triacetate of syringylglycol (39-Ac), diacetate of syringylethanone (40-Ac), the acetate of syringaldehyde (51-Ac), and benzoquinone (58) were identified as shown in Fig. 3.1. The mass spectra and retention times of these products were identical with those of the authentic compounds.

The extracts of control experiment gave negligible amounts of syringylglycol (39-Ac) and syringaldehyde (51-Ac). However, GC analysis using diacetate of 3-(4-hydroxy-3-methoxyphenyl)-1-propanol as an internal standard showed that the amounts of syringylglycol (39-Ac) and syringaldehyde (51-Ac) produces nonenzymatically were less than 1% of those produced enzymatically.

Degradation of 1,2-bis(4-ethoxy-3,5-dimethoxyphenyl)-1,3-propanediol (20)

The acetates of incubated products were analyzed by GC-MS. However, benzaldehyde (54) and phenylglycol (41-Ac), which would be expected when Ca-Cb cleavage of nonphenolic substrate (20) occurred, were not detected in the extracts. The results confirmed that laccase can not oxidize the nonphenolic β -1 substructure model compounds.

Degradation of syringaldehyde (51)

The acetate of degradation products were submitted to GC-MS, and benzoquinone (58) and hydroquinone (57) were identified as degradation products. Other products were observed by GC-MS and TLC, but the structures were not determined.

3.1. Ca-CB CLEAVAGE OF A β -1 DILIGNOL BY LACCASE

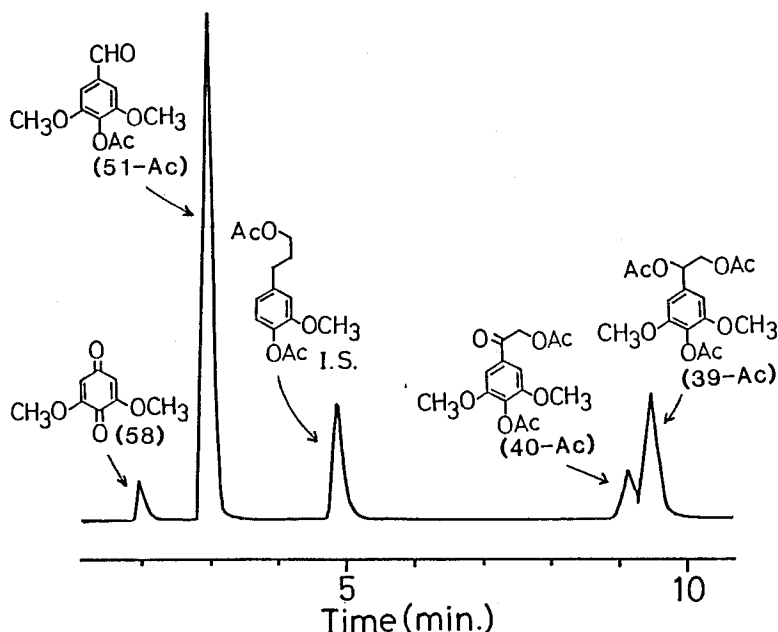


Fig. 3.1 Gas chromatogram (total ion chromatogram in GC-MS analysis) of the degradation products formed from disyringylpropane (17) by laccase of *C. versicolor*.

Before extraction of the reaction mixture, 100 μ g (0.38 μ mol) of the diacetate of 3-(4-hydroxy-3-methoxyphenyl)-1-propanol was added as an internal standard (I.S.).

Column, 1.5% OV-17, 1 m x 2.6 mm (i.d.), column temp, 180-220 $^{\circ}$ C, 5 $^{\circ}$ C/min.

3.1.4 DISCUSSION

It is known that the initial step in the catalytic mechanism of laccase involves one-electron donation to specific Cu^{2+} site with the formation of free radical products of organic substances (Reinhammer & Malmström, 1981). Actually, Nakamura (1960) demonstrated by use of ESR the oxidative formation of *p*-benzosemiquinone from *p*-hydroquinone by laccase.

In the present investigation, the phenolic β -1 lignin sub-

3.1. C α -C β CLEAVAGE OF A β -1 DILIGNOL BY LACCASE

structure model compound (17) was found to be degraded by laccase of *C. versicolor*. However, nonphenolic β -1 substrate (20) could not be degraded as expected.

Disyringylpropane (17) was degraded to give syringylglycol (39), syringylethanone (40), syringaldehyde (51) and benzoquinone (58). The structures of the degradation products indicated that disyringylpropane (17) was cleaved between C α and C β via phenoxy radicals of (17) by the degradation mechanisms shown in Fig. 3.2. The phenoxy radicals, which could be formed from both phenolic hydroxyl groups (A or B-ring), give rise to the C α -C β cleavage to form syringylglycol (39) and syringaldehyde (51).

It is presumed that syringylglycol (39) and syringaldehyde (51) were oxidized further to give syringylethanone (40) and benzoquinone (58), respectively.

Benzoquinone (58) identified in this experiment may not be ascribed to the alkyl-aryl cleavage of disyringylpropane (17)

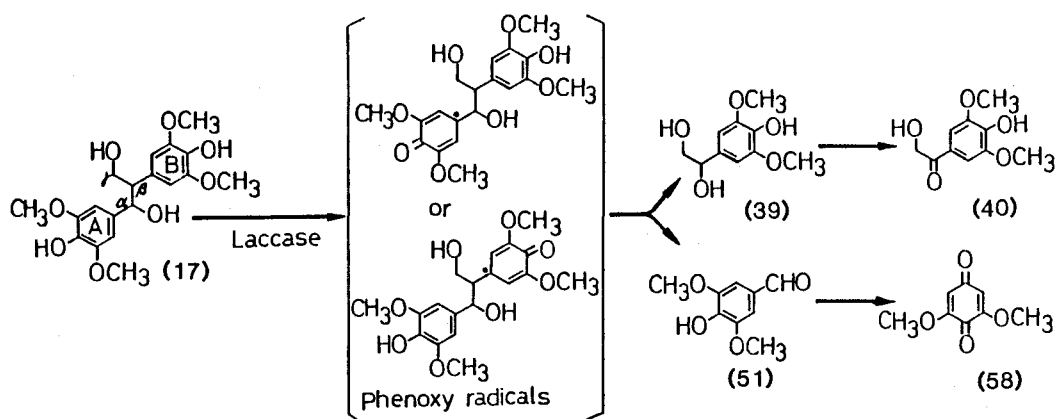


Fig. 3.2 Proposed degradation pathways of disyringylpropane (17) by laccase *C. versicolor*.

3.1. *Ca-CB* CLEAVAGE OF A β -1 DILIGNOL BY LACCASE

because, (i) the expected counterpart compound in the alkyl-aryl cleavage, 2-(4-hydroxy-3,5-dimethoxyphenyl)-3-hydroxypropanal, was not detected by GC-MS, (ii) when the degradation of (17) was performed with laccase of low activity (< 10 nkat), syringylglycol (39) and syringaldehyde (51) were detected but benzoquinone (58) could not be detected by GC-MS, and (iii) the benzoquinone (58) was formed as the degradation products of syringaldehyde (51) by laccase of *C. versicolor*.

C_{α} oxidation and cleavage of the alkyl-aryl bond were found as degradation pathways of phenolic lignin substructure models by laccase (see Higuchi, 1985b; Kirk & Shimada, 1985). However, the present investigation showed for the first time that the laccase catalyzed not only alkyl-aryl cleavage but also C_{α} - C_{β} cleavage.

Detailed degradation mechanisms for phenolic β -1 lignin substructure model compounds will be discussed in the next section.

Section 3.2

3.2 DEGRADATION MECHANISMS OF PHENOLIC β -1 LIGNIN SUBSTRUCTURE MODELS

3.2.1 INTRODUCTION

To elucidate the degradation mechanisms by laccase of *Coriolus versicolor*, previous section reported the degradation of a phenolic β -1 lignin substructure model compound, 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol [disyringylpropane (17)] by laccase, and it was found for the first time that the cleavage between C α and C β of the propyl side chain of disyringylpropane (17) was caused by laccase (Kawai et al., 1987b). These results showed that laccase catalyzed not only alkyl-aryl cleavage via disproportionation of phenoxy radicals and C α oxidation, as previously found (Kirk et al., 1968b), but also C α -C β cleavage.

This section describes further investigation on the degradation of new phenolic β -1 lignin substructure model compounds that have one phenolic hydroxyl group, 2-(4-ethoxy-3,5-dimethoxyphenyl)-1-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (18) and 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (19), by laccase of *C. versicolor*. Substrate (18) was degraded via C α -C β cleavage, alkyl-aryl cleavage and C α oxidation to give products (22), (32), (42), (51), (57) and (58). On the other hand, substrate (19) was degraded via C α -C β cleavage to give the products (39), (40) and (54).

On the basis of isotopic experiments with $^{18}\text{O}_2$ and H_2^{18}O , the detailed reaction mechanisms for the degradation of phenolic β -1 lignin substructure model compounds by laccase are discussed (Kawai et al., 1988a).

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

3.2.2 MATERIALS AND METHODS

Laccase preparation

A purified homogeneous laccase preparation of *Coriolus versicolor* IFO-30340 was kindly provided by Oji Paper Co., Ltd. (Sugiura et al., 1987). It was stored at -20 °C as a 50% glycerol solution. The enzyme activity was determined spectrophotometrically by measuring the absorption at 525 nm using syringaldazine (Aldrich Chemical) as described in Section 3.1 (Leonowicz & Grzywnowicz, 1981).

Enzymic reaction of β -1 lignin substructure models

The flask containing 5 mL of 0.2 M acetate buffer (pH 4.0) was previously flushed with 100% oxygen gas for 1 min. The reaction was started by addition of substrate (2.5 μ mol in 50 μ L of DMF soln) and 1 μ L of enzyme soln (400-800 nkat) into the buffer, and the reaction mixture was shaken at 30 °C for 15 min.

In a control experiment, the enzyme was replaced by a boiled enzyme (100 °C, 5 min), the inactivation of which was spectrophotometrically confirmed.

The reaction mixture was extracted with five portions of ethyl acetate (total, 25 mL). The combined organic layer was washed with saturated NaCl soln, dried over anhyd Na₂SO₄, and evaporated under reduced pressure. Then residual DMF was removed under high vacuum. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate for 15 h at room temp.

The acetylated products were analyzed by GC-MS directly or after TLC separation [column, 1.5% OV-17 on Chromosorb W AW-DMCS (Shinwa Kakou): glass column 1 m x 2.6 mm (i.d.), column temp, 180-220 °C, 5 °C/min; and Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m x 0.53 mm (i.d.), column temp,

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

130-220 °C, 5 °C/min].

Identification of degradation products was performed by comparison of the spectra and retention times with those of the authentic compounds or by ^1H NMR and mass spectra analysis after separation by TLC (Kieselgel 60 F254, Merck).

Isotopic experiments

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

The flask containing 1 mL of 0.2 M acetate buffer and 1 μL of enzyme soln (100-200 nkat) were evacuated and then filled with argon gas, and this procedure was repeated five times. Finally, $^{18}\text{O}_2$ gas (^{18}O : 99%, Amersham, or 98.58%, CEA) was introduced into the evacuated flask. The reaction was then started by the addition of 0.25 μmol of substrates (2 μL of DMF soln), and the reaction mixture was shaken at 30 °C for the desired time. The reaction was terminated by extraction with ethyl acetate (10 mL). The ethyl acetate layer was dried over anhyd Na_2SO_4 and evaporated.

The degradation products were trifluoroacetylated with trifluoroacetic anhydride for 10 min at room temp, or acetylated with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate for 10 h at room temp. Then acylated products were immediately analyzed by GC-MS.

Incorporation of ^{18}O from H_2^{18}O

The flask containing 100 μL of H_2^{18}O (^{18}O : 98%, CEA), 100 μL of 0.2 M acetate buffer (pH 4.0), and 1 μL of the enzyme soln (30 nkat) was flushed with $^{16}\text{O}_2$ gas for 1 min. The reaction was started by addition of 0.1 μmol of substrates (2 μL of DMF soln), and the reaction mixture was incubated at 30 °C for 15 min. The reaction mixture was then extracted with ethyl acetate,

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

and the ethyl acetate layer was dried over anhyd Na_2SO_4 and evaporated. Half of the extracts was reincubated for 15 min at 30 °C in 100 μL of acetate buffer under air. The extracts were acetylated with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate for 10 h at room temp and submitted to GC-MS immediately.

Syntheses of substrates and authentic compounds

Substrates, the mixtures of *erythro* and *threo* of 2-(4-ethoxy-3,5-dimethoxyphenyl)-1-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (**18**) and 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (**19**), were synthesized by the method of Namba (1981; see Nakatsubo, 1988).

(**18**): ^1H NMR (CDCl_3) δ (*erythro*), 1.35 (t, $J=7.1$ Hz, 3H, -O-C-CH₃), 1.98 (s, 6H, alcoholic-OAc), 2.31 (s, 3H, Ph-OAc), 3.24-3.44 (m, 1H, C2-H), 3.73 (s, 6H, -OCH₃), 3.78 (s, 6H, -OCH₃), 4.02 (q, $J=7.1$ Hz, 2H, -O-CH₂-), 4.16 (dd, $J=11$, 6.5 Hz, 1H, C3-H), about 4.38 (1H, C3-H), 6.06 (d, $J=6.7$ Hz, 1H, C1-H), 6.33 (s, 2H, Ph-H), 6.39 (s, 2H, Ph-H); (*threo*), 1.32 (t, $J=7.0$ Hz, 3H, -O-C-CH₃), 2.05 (s, 3H, alcoholic-OAc), 2.14 (s, 3H, alcoholic-OAc), 2.29 (s, 3H, Ph-OAc), 3.24-3.44 (m, 1H, C2-H), 3.67 (s, 6H, -OCH₃), 3.72 (s, 6H, -OCH₃), 3.98 (q, $J=7.1$ Hz, 2H, -O-CH₂-), about 4.38 (1H, C3-H), 4.52 (dd, $J=11$, 7.1 Hz, 1H, C3-H), 5.90 (d, $J=8.2$ Hz, 1H, C1-H), 6.17 (s, 2H, Ph-H), 6.26 (s, 2H, Ph-H).

(**19**): ^1H NMR (CDCl_3) δ (*erythro*), 1.34 (t, $J=7.0$ Hz, -O-C-CH₃), 1.99 (s, 6H, alcoholic-OAc), 2.32 (s, 3H, Ph-OAc), 3.29-3.44 (m, 1H, C2-H), 3.75 (s, 6H, -OCH₃), 3.77 (s, 6H, -OCH₃), 4.00 (q, $J=7.1$ Hz, 2H, -O-CH₂-), 4.16 (dd, $J=11$, 6.7 Hz, 1H, C3-H), about 4.35 (1H, C3-H), 6.03 (d, $J=6.8$ Hz, 1H, C1-H), 6.35 (s, 2H, Ph-H), 6.36 (s, 2H, Ph-H); (*threo*), 1.31 (t, $J=7.0$ Hz, -O-C-CH₃), 2.05 (s, 3H, alcoholic-OAc), 2.14 (s, 3H, alcoholic-OAc), 2.30 (s, 3H, Ph-OAc), 3.29-3.44 (m,

3.2. DEGRADATION MECHANISMS OF *B*-1 DILIGNOLS BY LACCASE

1H, C2-H), 3.67 (s, 6H, -OCH₃), 3.71 (s, 6H, -OCH₃), 3.97 (q, $J=7.1$ Hz, 2H, -O-CH₂-), about 4.37 (1H, C3-H), 4.52 (dd, $J=11$, 7.2 Hz, 1H, C3-H), 5.89 (d, $J=8.1$ Hz, 1H, C1-H), 6.18 (s, 2H, Ph-H), 6.22 (s, 2H, Ph-H).

1-(4-Ethoxy-3,5-dimethoxyphenyl)-2-hydroxyethanone [phenylethanone (42)] was prepared from acetosyringone (Aldrich Chemical) via the following four steps: (i) iodoethane/K₂CO₃ in DMF at room temp, (ii) CuBr₂ in refluxing ethyl acetate, (iii) sodium formate in DMF at room temp, (iv) saturated sodium bicarbonate soln in methanol/CH₂Cl₂ (1/4, v/v) at room temp.

¹H NMR: (42-Ac), see Section 3.1.

MS: Table 3.2 [the acetyl derivative (42-Ac) and the trifluoroacetyl derivative (42-TFA)].

The other compounds, 1-(4-hydroxy-3,5-dimethoxyphenyl)-1,2-ethanediol [syringylglycol (39)], 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-hydroxyethanone [syringylethanone (40)], 4-ethoxy-3,5-dimethoxybenzaldehyde [benzaldehyde (54)], 2,6-dimethoxy-*p*-hydroquinone [hydroquinone (57)] and 2,6-dimethoxy-*p*-benzoquinone [benzoquinone (58)], were prepared as described in Section 3.1. Syringaldehyde (51) was commercially available (Tokyo Chemical Industry). The mass spectral data of these compounds are listed in Table 3.2.

Instruments

Enzyme activity was measured using a Hitachi Model 200-20 double-beam spectrophotometer. NMR spectra was recorded by a Varian XL-200 FT-NMR spectrometer (200 MHz) with (CH₃)₄Si as an internal standard. Chemical shifts and coupling constants are given in δ values (ppm) and Hz, respectively. Mass spectra were taken by a Shimadzu GCMS-QP 1000 gas chromatograph-mass spectrometer [EI (70 eV) and CI mode].

3.2. DEGRADATION MECHANISMS OF *B*-1 DILIGNOLS BY LACCASE

TABLE 3.2

Relative Intensity of the Important Fragment Ions of the Authentic Compounds^a

Compound	Mass spectral data m/z (%)
Syringyl-glycol (39-Ac)	341 (0.2), 340 (M^+ , 1.2), 298 (17), 239 (8), 238 (46), 197 (14), 196 (100), 184 (7), 183 (65), 181 (9), 180 (7), 167 (13), 155 (9), 123 (9)
Syringyl-ethanone (40-Ac)	296 (M^+ , 0.3), 254 (28), 182 (17), 181 (100), 153 (11)
Syringyl-ethanone (40-Ac-CI) ^b	298 (15), 297 (MH^+ , 100), 256 (11), 255 (82), 254 (22), 240 (12), 239 (93), 198 (12), 197 (91), 196 (21), 181 (20)
Phenyl-ethanone (42-Ac)	283 (3), 282(M^+ , 20), 210 (12), 209 (96), 182 (10), 181 (100), 153 (14), 123 (6)
Phenyl-ethanone (42-TFA) ^c	337 (3), 336 (M^+ , 21), 209 (24), 182 (10), 181 (100), 153 (9)
Syringaldehyde (51-Ac)	225 (0.2), 224 (M^+ , 1.6), 183 (10), 182 (100), 181 (40), 167 (8)
Benzaldehyde (54)	211 (6.7), 210 (M^+ , 55), 183 (10), 182 (100), 181 (83), 167 (17), 153 (6), 139 (9), 135 (6), 125 (9), 111 (8), 110 (9)
Hydroquinone (57-Ac)	255 (0.2), 254 (M^+ , 1.2), 212 (19), 171 (9), 170 (100), 155 (21)
Benzoquinone (58)	169 (8.6), 168 (M^+ , 100), 153 (7), 140 (24), 138 (37), 137 (10), 125 (32), 112 (24), 110 (10)

^a Column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone).

^b Chemical ionization mode; reagent gas, 2-methylpropane.

^c Trifluoroacetyl derivative.

3.2.3 RESULTS

Degradation of 2-(4-ethoxy-3,5-dimethoxyphenyl)-1-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (18)

The acetates of degradation products were directly submitted to GC-MS. Figure 3.3 shows the gas chromatogram of the degradation products. Benzoquinone (58), the diacetate of hydroquinone (57-Ac) and the acetate of phenylethanone (42-Ac) were identified. The acetate of syringaldehyde (51-Ac) was also identified by GC-MS after separation by TLC (solvent, CH₂Cl₂). The mass spectra and retention times of these products were identical with those of the authentic compounds. The diacetate of 2-(4-ethoxy-3,5-dimethoxyphenyl)-1-(4-hydroxy-3,5-dimethoxyphenyl)-3-hydroxypropanone [diphenylpropanone (22-Ac)] was confirmed in the reaction mixture by GC-MS. Then the acetylated extracts were separated by TLC (solvent, ethyl acetate/*n*-hexane, 1/2, twice), and the diacetate of diphenylpropanone (22-Ac) was identified by ¹H NMR and DI-MS.

¹H NMR (acetone-*d*₆) δ 1.22 (t, *J*=7.0 Hz, -O-C-CH₃), 1.93 (s, 3H, alcoholic-OAc), 2.24 (s, 3H, Ph-OAc), 3.79 (s, 6H, -OCH₃), 3.85 (s, 6H, -OCH₃), about 3.90 (2H, -O-CH₂-), 4.27 (dd, *J*=11, 5.5 Hz, 1H, C3-H), 4.73 (dd, *J*=11, 8.5 Hz, 1H, C3-H), 5.10 (dd, *J*=8.8, 5.6 Hz, 1H, C2-H), 6.75 (s, 2H, Ph-H), 7.42 (s, 2H, Ph-H).

MS *m/z* 490 (M⁺).

Furthermore, the occurrence of the 2-(4-ethoxy-3,5-dimethoxyphenyl)-3-hydroxypropanal [phenylpropanal (32)] in the reaction mixture was suggested by GC-MS. However, the mass spectra indicated that the acetate derivatives of phenylpropanal (32-Ac) and diphenylpropanone (22-Ac), which have β -acetoxy ketone, release acetic acid thermally in the process of GC-MS to give α,β -unsaturated carbonyl compounds, (33) and (23), respec-

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

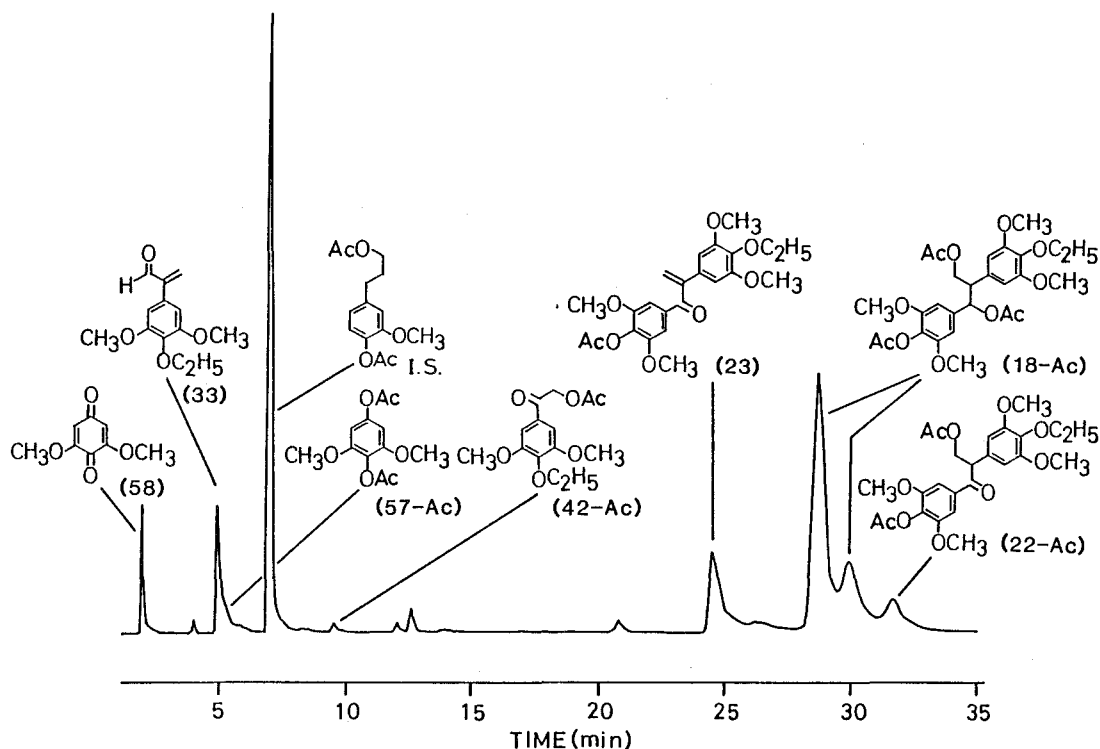


Fig. 3.3 Gas chromatogram (total ion chromatogram in GC-MS analysis) of the degradation products formed from 2-(4-ethoxy-3,5-dimethoxyphenyl)-1-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (18) by laccase of *C. versicolor*.

Before extraction of the reaction mixture, 100 μ g (0.38 μ mol) of the diacetate of 3-(4-hydroxy-3-methoxyphenyl)-1-propanol (I.S.) was added as an internal standard. The acetate of syringaldehyde (51-Ac) was also detected by GC-MS after separation by TLC.

Column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m x 0.53 mm (i.d.); column temp, 130-220 $^{\circ}$ C, 5 $^{\circ}$ C/min.

3.2. DEGRADATION MECHANISMS OF *B*-1 DILIGNOLS BY LACCASE

tively (Fig. 3.3).

Then phenylpropanal (**32**) was separated from the degradation products without acetylation by TLC (solvent, methanol/CH₂Cl₂, 1/99, twice), and the occurrence and the structure were confirmed by ¹H NMR and DI-MS.

¹H NMR (CDCl₃) δ 1.36 (t, *J*=7.1 Hz, 3H, -O-C-CH₃), 3.76 (dd, *J*=6, 8 Hz, 2H, C3-H), 3.84 (s, 6H, -OCH₃), about 3.9 (m, 1H, C2-H), 4.00 (q, *J*=7.1 Hz, 2H, -O-CH₂-), 4.19 (dd, *J*=11, 8 Hz, 1H, C3-H), 6.38 (s, 2H, Ph-H), 9.775 (d, *J*=0.8 Hz, 1H, C1-HO).

MS *m/z* (%) 255 (5), 254 (M⁺, 28), 236 (15), 225 (42), 224 (38), 195 (39), 180 (23), 179 (29), 167 (100), 165 (25), 151 (21), 137 (39), 109 (25), 107 (20).

Phenylpropanal (**32**) separated was reduced by NaBH₄ and then acetylated to give the diacetate of 2-(4-ethoxy-3,5-dimethoxy-phenyl)-1,3-propanediol (**34-Ac**) which was purified by TLC (solvent, ethyl acetate/*n*-hexane, 1/3) and identified by ¹H NMR and GC-MS.

¹H NMR (CDCl₃) δ 1.35 (t, *J*=7.1 Hz, 3H, -O-C-CH₃), 2.05 (s, 3H, -OAc), 3.24 (quintet, *J*=6.7 Hz, 1H, C2-H), 3.83 (s, 6H, -OCH₃), 4.03 (q, *J*=7.1 Hz, 2H, -O-CH₂-), 4.30 (d, *J*=6.8 Hz, 2H, C1 or C3-H), 4.31 (d, *J*=6.6 Hz, 2H, C1 or C3-H), 6.43 (s, 2H, Ph-H).

MS (1.5% OV-17), *m/z* (%) 341 (5), 340 (M⁺, 25), 281 (17), 280 (100), 253 (10), 252 (67), 251 (87), 210 (24), 209 (13), 182 (10), 181 (11), 180 (17), 179 (23), 177 (17), 167 (14), 165 (10), 149 (11), 137 (12), 121 (11).

These degradation products were not detected in the reaction mixtures of control experiment by GC-MS.

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

Incorporation of ^{18}O from $^{18}\text{O}_2$ in the degradation of substrate (18)

The reaction was terminated after 1 h by extraction of the reaction mixture with ethyl acetate. An aliquot of the extract was trifluoroacetylated and analyzed by GC-MS. The mass spectrum showed that 69% of the ^{18}O was incorporated into the C1 position of phenylethanone (42) (Fig. 3.4). Alternatively the extracts were acetylated and the acetate of the extracts were analyzed by GC-MS. The results showed that 54% of the ^{18}O was incorporated into phenylethanone (42).

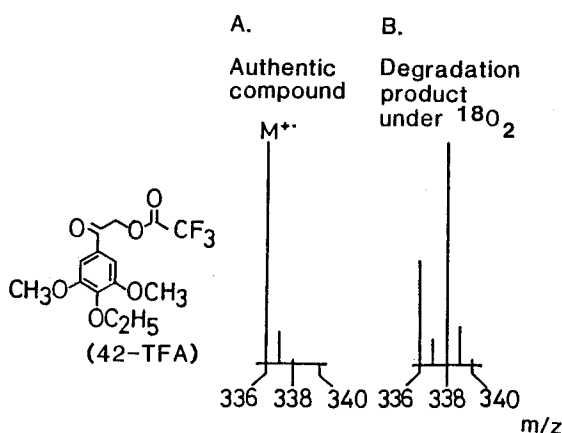


Fig. 3.4 Molecular ion region of trifluoroacetate of 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-hydroxyethanone (42-TFA).

(A) authentic compound; (B) degradation product from substrate (18) under $^{18}\text{O}_2$ (^{18}O : 99%).

Incorporation of ^{18}O from H_2^{18}O in the degradation of substrate (18)

The acetates of the degradation products and reincubated products were submitted to GC-MS. Table 3.3 shows the data of mass spectra of degradation products. Incorporation of ^{18}O from

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

H_2^{18}O (^{18}O : 49%) to hydroquinone (57-Ac) and benzoquinone (58) was confirmed by their mass spectra. However, a part of the oxygen of hydroquinone (57) was found to be exchanged with the oxygen of water during incubation. It was found consequently that two atoms of ^{18}O were incorporated into a part of hydroquinone (57-Ac). One atom of ^{18}O was exchanged almost completely with ^{16}O of H_2^{16}O during the reincubation, while another atom of ^{18}O was not exchanged with water. These results suggested that only one molecule of water is incorporated relative to hydroquinone (57) formation during degradation of diphenylpropane (18) by laccase.

TABLE 3.3

The Relative Intensity of Molecular Ion Regions of the Degradation Products Formed from Substrate (18)^a

	Authentic compound	Formed in H_2^{18}O ^b	Reincubated in H_2^{16}O ^c
Hydroquinone (57-Ac)			
254 (M^+)	100	76.4	100
256	2.5	100	84.4
258	0.4	32.3	5.1
Benzoquinone (58)			
168 (M^+)	100	100	100
170	1.2	78.3	68.8
% incorporation of ^{18}O ^d		90	83

^a Column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone).

^b The reaction mixture containing 100 μL of H_2^{18}O (^{18}O : 99%), 100 μL of 0.4 M acetate buffer (pH 4.0), 1 μL of enzyme, and 0.1 μmol of substrates was incubated at 30 °C for 15 min.

^c The half of the extracts formed in H_2^{18}O was reincubated for 15 min at 30 °C in 100 μL of 0.2 M acetate buffer.

^d Percentage incorporation of ^{18}O = $100 \times (^{18}\text{O} \text{ content of the products}) / [^{18}\text{O} \text{ content in } \text{H}_2^{18}\text{O} \text{ of the medium (49\%)]$.

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

Degradation of 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (19)

The acetate of degradation products were directly submitted to GC-MS. Figure 3.5 shows the gas chromatogram of the degradation products. Benzaldehyde (54), the diacetate of syringyl-ethanone (40-Ac) and the triacetate of syringylglycol (39-Ac) were identified. The mass spectra and retention times of these

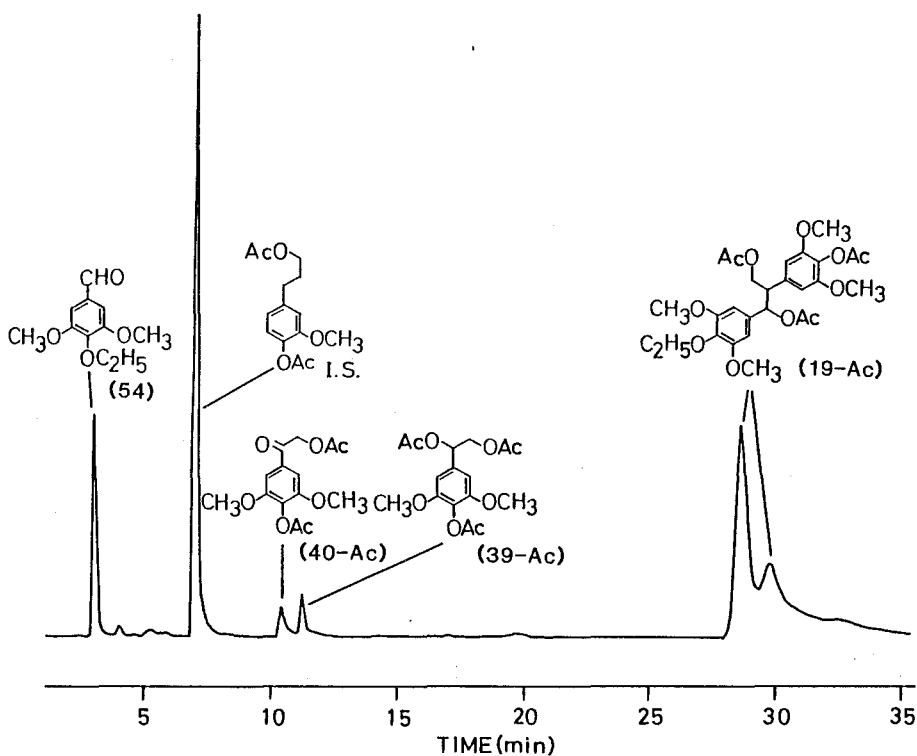


Fig. 3.5 Gas chromatogram (total ion chromatogram in GC-MS analysis) of the degradation products formed from 1-(4-ethoxy-3,5-dimethoxyphenyl)-2-(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (19) by laccase of *C. versicolor*.

Experimental procedures were the same as in Fig. 3.3.

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

products were identical with those of the authentic compounds.

Small amounts of benzaldehyde (54) and syringylglycol (39-Ac) were detected by GC-MS in the extracts of the control experiment. However, the amount of syringylglycol (39-Ac) and benzaldehyde (54) formed in the control experiment were negligible (less than 5% of those formed by enzymatic degradation) by GC-MS analysis using the diacetate of 3-(4-ethoxy-3,5-dimethoxyphenyl)-1-propanol as an internal standard.

Incorporation of ^{18}O from $^{18}\text{O}_2$ in the degradation of substrate (19)

The reaction was terminated by extraction of the reaction mixture with ethyl acetate. The extracts were acetylated and they were submitted to GC-MS. However, the mass spectrum showed no incorporation of ^{18}O into the degradation products.

Incorporation of ^{18}O from H_2^{18}O in the degradation of substrate (19)

The acetates of the degradation products and reincubated products were submitted to GC-MS. As shown in Table 3.4, ^{18}O from H_2^{18}O (^{18}O : 49%) was incorporated to syringylglycol (39-Ac) and syringylethanone (40-Ac). ^{18}O of syringylglycol (39) but not syringylethanone (40) was exchangeable with ^{16}O of water or acetic acid during reincubation or the acetylation.

3.2.4 DISCUSSION

As demonstrated in Section 3.1, the Ca-C β cleavage of a phenolic β -1 lignin substructure model, disyringylpropane (17), was mediated by laccase of *Coriolus versicolor*. On the other hand, a nonphenolic β -1 substructure model (20), was not oxidized

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

TABLE 3.4

The Relative Intensity of Molecular Ion Regions of the Degradation Products Formed from Substrate (19)^a

	Authentic compound	Formed in H ₂ ¹⁸ O ^b	Reincubated in H ₂ ¹⁶ O ^c
syringylglycol (39-Ac)			
340 (M ⁺)	100	100	100
342	2.5	46.4	38.2
% incorporation of ¹⁸ O ^d		65	56
syringyl ethanone (40-Ac-CI) ^e			
297 (MH ⁺)	100	100	100
299	2.5	65.6	76.6
% incorporation of ¹⁸ O ^d		81	89

^a Column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone).

^b The reaction mixture containing 100 μ L of H₂¹⁸O (¹⁸O: 99%), 100 μ L of 0.4 M acetate buffer (pH 4.0), 1 μ L of enzyme, and 0.1 μ mol of substrates was incubated at 30 °C for 15 min.

^c The half of the extracts formed in H₂¹⁸O was reincubated for 15 min at 30 °C in 100 μ L of 0.2 M acetate buffer.

^d Percentage incorporation of ¹⁸O = 100 x (¹⁸O content of the products) / [¹⁸O content in H₂¹⁸O of the medium (49%)].

^e Chemical ionization mode; reagent gas, 2-methylpropane.

by laccase (Kawai et al., 1987b). These observations showed that the phenolic hydroxyl group was necessary for the oxidation of β -1 substrates by laccase.

Furthermore, the initial step in catalytic mechanism of laccase involves one-electron donation to a specific Cu²⁺ site with the formation of free radical products of organic substances (Reinhammer & Malmström, 1981). Actually, Nakamura (1960) reported by using ESR the oxidative formation of *p*-benzosemiqui-

3.2. DEGRADATION MECHANISMS OF *B*-1 DILIGNOLS BY LACCASE

none from *p*-hydroquinone by laccase.

These observations indicated that the degradation of disyringylpropane (17) was caused by laccase via phenoxy radicals. However, the degradation mechanisms were not elucidated clearly, because disyringylpropane (17) has two phenolic hydroxyl groups attacked by laccase, and from which phenolic hydroxyl group some of the degradation products were derived could not be specified.

In the present investigation, phenolic substrates (18) and (19), which have one phenolic hydroxyl group (A or B-ring), were synthesized and used for elucidation of the degradation mechanisms.

Substrate (18) was degraded by laccase and diphenylpropane (22), phenylpropanal (32), phenylethanone (42), hydroquinone

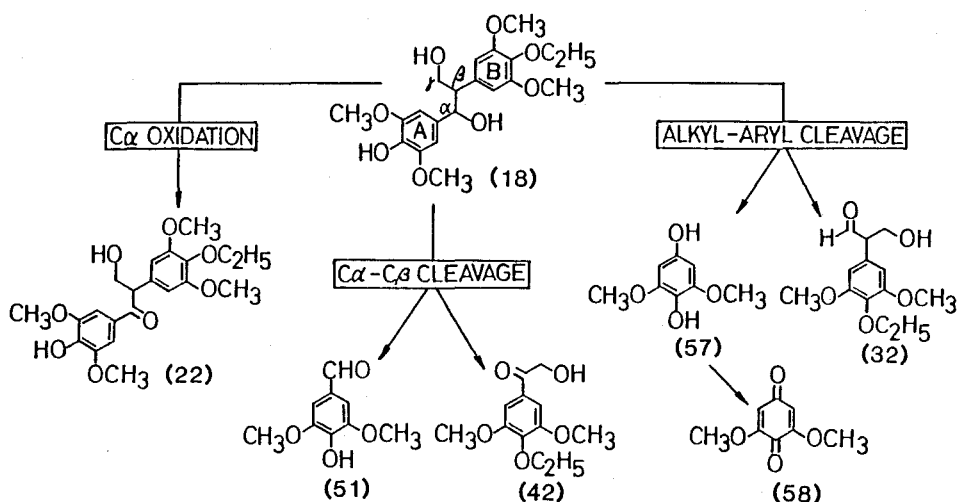


Fig. 3.6 Proposed degradation pathways of substrate (18) by laccase of *C. versicolor*.

Chemical reaction scheme showing the conversion of compound (19) to compound (40) via intermediate (39).

Compound (19) is a dimer of 3,4,5-trimethoxybenzoic acid, with a central C-C bond labeled α and β . It undergoes $C_{\alpha}-C_{\beta}$ CLEAVAGE to form 3,4,5-trimethoxybenzaldehyde (54) and 3,4,5-trimethoxybenzyl alcohol (39).

Compound (39) then undergoes C_{α} OXIDATION to form 3,4,5-trimethoxybenzoic acid (40).

(57), and benzoquinone (58) were identified as degradation products. Substrate (19), on the other hand, gave syringylglycol (39), syringylethanone (40), and benzaldehyde (54) as degradation products by laccase. On the basis of the structures of these products and isotopic experiments, possible degradation pathways of substrates (18) and (19) are shown in Figs. 3.6 and 3.7, respectively. These reactions may proceed via phenoxy radicals of (18) and (19) formed by their oxidation by laccase. Three types of degradation, (i) C_a-C_b cleavage, (ii) C_a oxidation, and (iii) alkyl-aryl cleavage, which are suspected to be the primary degradation reactions by laccase, are discussed below.

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

Ca-C β cleavage of phenolic β -1 substrates (18) and (19)

Phenylethanone (42) and syringaldehyde (51), and syringylglycol (39) and benzaldehyde (54) could be formed by $Ca-C\beta$ cleavage of the phenoxy radicals of substrates (18) and (19), respectively, as shown in Fig. 3.8.

The phenoxy radicals formed by the enzymatic oxidation of (18) and (19) are cleaved between Ca and $C\beta$ carbons to form radicals and quinone intermediates. In the case of substrate (18) (Fig. 3.8, pathway A), the radicals reacts with molecular oxygen to yield phenylethanone (42), and quinone methide is converted to

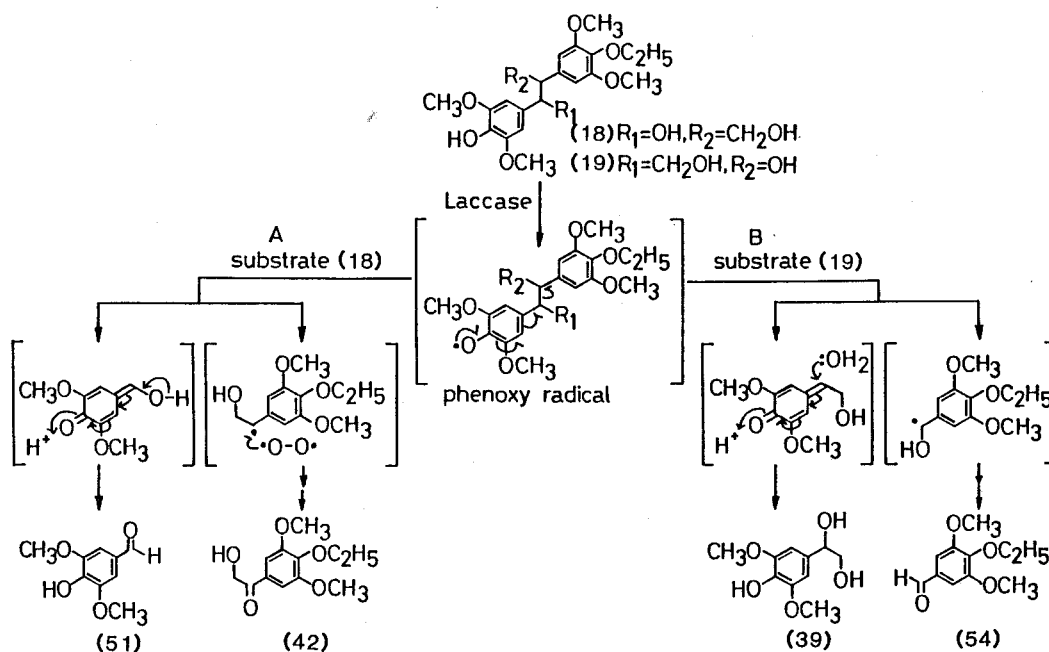


Fig. 3.8 Possible mechanisms for $Ca-C\beta$ cleavage of substrates (18) (pathway A) and (19) (pathway B).

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

syringaldehyde (51). Incorporation of $^{18}\text{O}_2$ into phenylethanone (42) is in good agreement with this pathway. On the other hand, in the case of substrate (19) (Fig. 3.8 , pathway B), the radical is converted to benzaldehyde (54) and quinone methide which reacts with water to yield syringylglycol (39). However, isotopic experiments with H_2^{18}O showed that the exchange of the C_α hydroxyl group of syringylglycol (39) with ^{16}O of water or acetic acid proceeds slowly. Therefore, the above degradation mechanism was not completely verified, but it could be concluded that this pathway was one of the major pathways for the formation of syringylglycol (39) from substrate (19).

C_α oxidation and alkyl-aryl cleavage of phenolic β -1 substrate (18)

The mechanisms of C_α oxidation and alkyl-aryl cleavage are illustrated in Fig. 3.9. Disproportionation of the phenoxy radicals of substrate (18) occurs to form the cation intermediate. The release of the C_α proton from the cation leads to the formation of diphenylpropanone (22) (C_α oxidation, Fig. 3.9 , pathway A). And the attack by water to the cation intermediate leads to the formation of phenylpropanal (32) and hydroquinone (57) by alkyl-aryl cleavage (Fig. 3.9 , pathway B). Incorporation of H_2^{18}O into hydroquinone (57) is in good agreement with this pathway. The mechanism of conversion of hydroquinone (57) to benzoquinone (58) by laccase proceeded as previously described (Nakamura, 1960). It is suggested that syringylglycol (39) formed from substrate (18) via C_α - C_β cleavage is also oxidized to syringylethanone (40) via a pathway similar to pathway A in Fig. 3.9.

C_α oxidation and alkyl-aryl cleavage are well known reactions via phenoxy radicals caused by laccase (see Higuchi, 1985b; Kirk & Shimada, 1985). In an earlier paper Kirk et al. (1968b)

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

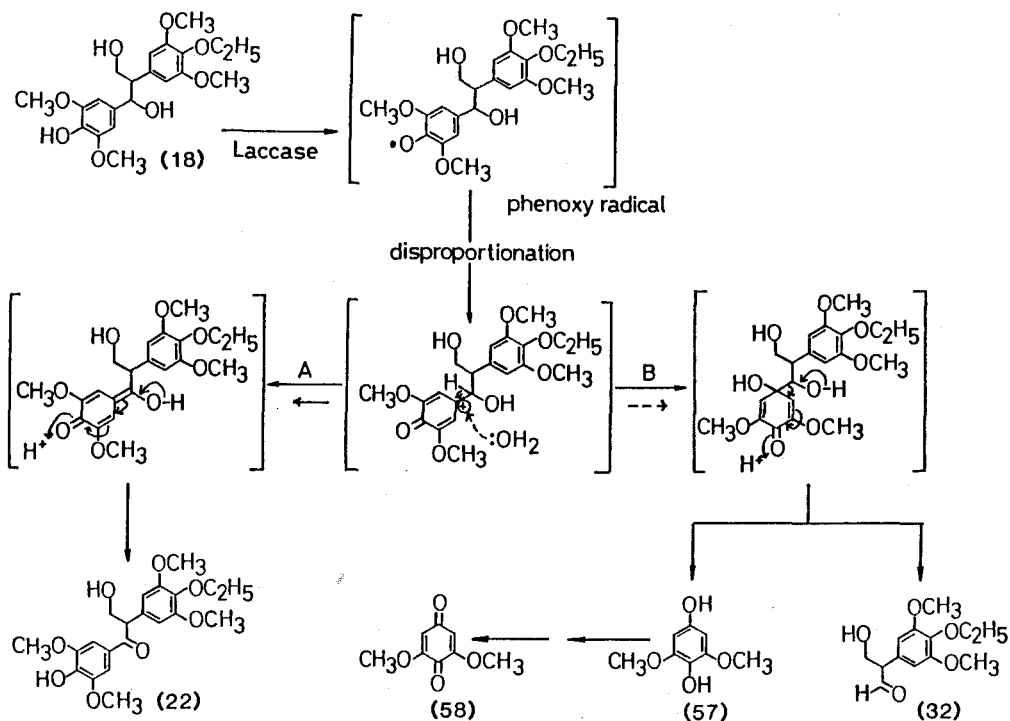


Fig. 3.9 Possible mechanisms for Ca oxidation (pathway A) and alkyl-aryl cleavage (pathway B) of substrate (18).

reported that the phenolic β -O-4 lignin models were oxidized with laccase of *C. versicolor* at the Ca position and cleaved between alkyl and aryl group. Higuchi and co-workers (see Higuchi, 1985b) found that many phenolic lignin substructure model compounds, β -O-4, β -5, β - β , and β -1 substructures, were degraded by *Fusarium solani* M-13-1, and suggested that the Ca oxidation and alkyl-aryl cleavage proceeded by laccase activity of the fungus (Iwahara, 1983).

In the previous section, direct Ca-C β cleavage of the di-

3.2. DEGRADATION MECHANISMS OF β -1 DILIGNOLS BY LACCASE

syringylpropane (17) by laccase was proposed (Kawai et al., 1987b). The present investigations strongly supported that the Ca-C β cleavage is directly caused via phenoxy radical intermediates of substrates as in Ca-C β cleavage of nonphenolic β -1 lignin substructure dimers via aryl cation radicals mediated by lignin peroxidase of *Phanerochaete chrysosporium* and its mimetic system (Hammel et al., 1985; Shoemaker et al., 1985; Habe et al., 1985a; Renganathan et al., 1986).

Kamaya and Higuchi (1984c) reported that the Ca-C β cleavage of disyringylpropane (17) was catalyzed by the ligninolytic culture of *P. chrysosporium* and horseradish peroxidase. Namba et al. (1983) also found that syringylglycol (39) and syringaldehyde (51) were degradation products of the same substrate (17) as well as the alkyl-aryl cleavage products. These reactions could be explained by degradation mechanisms similar to those shown in Figs. 3.8 and 3.9.

It is known that lignin peroxidase of *P. chrysosporium* catalyzes the oxidation of not only nonphenolic substrates and also phenolic substrates (Tien & Kirk, 1984; Pasczynski et al., 1986). Recently, Yokota et al., (1988, submitted) found that substrate (18) was degraded by the culture and lignin peroxidase of *P. chrysosporium* to give the products similar to those formed by laccase in this study. Very recently, Wariishi et al. (1989) reported that Mn-dependent peroxidase of *P. chrysosporium* also catalyzed the similar reactions of a phenolic β -1 model compound. These results indicated that the oxidation of phenolic substructure dimers by lignin peroxidase and Mn-dependent peroxidase may proceed via phenoxy radicals as in the laccase reactions shown in Figs. 3.8 and 3.9.

Section 3.3

3.3 DEGRADATION MECHANISMS OF PHENOLIC β -O-4 LIGNIN SUBSTRUCTURE MODELS

3.3.1 INTRODUCTION

The previous sections 3.1 and 3.2 described that laccase catalyzed not only alkyl-aryl cleavage and Ca oxidation but also Ca-C₈ cleavage of the phenolic β -1 lignin substructure model compounds (17)-(19) (Kawai et al., 1987b, 1988a).

This section describes the degradation of a phenolic β -O-4 lignin substructure model compound by laccase of *Coriolus versicolor*. In earlier paper, Kirk et al. (1968b) demonstrated that alkyl-aryl cleavage and Ca oxidation of a phenolic β -O-4 model, 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol, were catalyzed by laccase of *C. versicolor*. Recently, Wariishi et al. (1987) reported that a similar substrate, 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (12), was mainly cleaved between Ca and C₈ to form syringaldehyde (51) and 2-(2-methoxyphenoxy)ethanol, which further degraded to give guaiacol (59), by laccase, in contrast to earlier results (Kirk et al., 1968b).

To elucidate this question, the degradation of substrate (12) by laccase of *C. versicolor* was performed. The substrate (12) was found to be degraded via alkyl-aryl cleavage and Ca oxidation. However, both syringaldehyde (51) and 2-(2-methoxyphenoxy)ethanol were not detected. A new pathway for the formation of guaiacol (59) is discussed (Kawai et al., 1989a).

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

3.3.2 MATERIALS AND METHODS

Laccase preparation

A purified homogeneous laccase preparation of *Coriolus versicolor* IFO-30340 was kindly provided by Oji Paper Co., Ltd. (Sugiura et al., 1987). A crude laccase preparation from *C. versicolor* Ps4a was prepared by the method of Fåhræus and Reinhammer (1967). 2,5-Xylidine was added to the 4-day-old culture as an inducer of laccase. After eight days, cultures (3 L) were filtered and concentrated to about 100 mL using a Millipore ultrafiltration system (10 kDa pore size). The concentrated soln was saturated with ammonium sulfate and cooled to 4 °C overnight. The precipitate was separated by centrifugation (10,000 g, 20 min, 4 °C), redissolved in 70 mL of 0.1 M phosphate buffer (pH 6.0) and used as a crude enzyme soln. This soln showed an activity of 370 nkat/mL with syringaldazine (Aldrich Chemical) as substrate as described in Section 3.1, but did not oxidize veratryl alcohol (47) to veratraldehyde (46) in the presence of H₂O₂.

Enzyme reactions

To the flask containing 40-400 nkat of enzyme soln [purified enzyme (1 μ L) or crude enzyme (100-500 μ L)] and 5 mL of 0.2 M acetate buffer (pH 4.0), substrate (1-2.5 μ mol in 20 μ L of acetone soln) was added, and the reaction mixture was incubated at 30 °C for 10 min-1 h.

The flask was extracted with ethyl acetate (5 mL, three times). The organic layer was dried over anhyd Na₂SO₄ and evaporated under reduced pressure. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v), and analyzed by GC-MS [column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m x 0.53 mm (i.d.), column temperature, 100-220 °C, 5 °C/min]. The acetylated extract was further

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

ethylated with diazoethane in diethyl ether at room temp, and analyzed by GC-MS.

The degradation products were identified by comparison of the spectra with those of the authentic compounds.

In a control experiment, enzyme was replaced by acetate buffer.

The degradation of the mixture of α -carbonyl dimer (13), syringaldehyde (51) and syringic acid (53) by laccase was also performed by the same procedure. The reaction mixture was extracted after 0, 10 and 30 min of incubation, and the diacetate of 3-(4-hydroxy-3-methoxyphenyl)-1-propanol as an internal standard was added before extraction. The extract was trimethylsilylated with *N,O*-bis(trimethylsilyl)acetamide/trimethylchlorosilane/pyridine (3/1/3, v/v/v) for 2 h, and analyzed by GC [column, 1.5% OV-17 on Chromosorb W AW-DMCS, glass column, 1.5 m x 2.6 mm (i.d.), column temp, 90-220 °C, 6 °C/min].

Syntheses of substrates and authentic compounds

1-(4-Hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (12) was kindly provided from Dr. T. Umezawa, Wood research Institute, Kyoto University.

1-(4-Hydroxy-3,5-dimethoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propanone [α -carbonyl dimer (13)] was prepared from syringaldehyde (Tokyo Chemical Industry) via following steps: (i) benzyl chloride/K₂CO₃/KI in DMF at room temp, (ii) methyl magnesium bromide (2.01 mol/L in THF) (Tokyo Chemical Industry) in anhyd THF (distilled from potassium and benzophenone) at room temp, (iii) Jones's reagent (Fieser & Fieser, 1967a) in acetone at room temp, (iv) ethylene glycol/*dl*-camphor sulfonic acid in refluxing benzene, (v) 5% Pd-C under H₂ gas in methanol/ethyl acetate/benzene (1/2/2) at room temp, (vi) acetic anhydride/pyridine in ethyl acetate at room temp, (vi) 1N HCl in THF at

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

room temp, (vii) CuBr₂ in refluxing ethyl acetate [1-(4-acetoxy-3,5-dimethoxyphenyl)-2-bromoethanone], (viii) guaiacol (Kanto Chemicals)/K₂CO₃/KI in DMF at room temp, (ix) paraformaldehyde/K₂CO₃ in (CH₃)₂SO at room temp, (x) sodium methoxide in methanol/CH₂Cl₂ (1/4) at 0 °C.

¹H NMR (CDCl₃) (acetate) δ 2.07 (s, 3H, alcoholic-OAc), 2.35 (s, 3H, Ph-OAc), 3.76 (s, 3H, -OCH₃), 3.86 (s, 6H, -OCH₃), 4.45 (dd, $J=13$, 7.5 Hz, 1H, C3-H), 4.73 (dd, $J=13$, 3.5 Hz, 1H, C3-H), 5.64 (dd, $J=7.4$, 3.5 Hz, 1H, C2-H), 6.75-7.05 (m, 4H, Ph(guaiacyl)-H), 7.49 (s, 2H, Ph(syringyl)-H).

MS Table 3.5.

1-(3,4-Dihydroxy-5-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propanone (15) was synthesized from pyrogallol (Nakarai Chemicals) via following steps: (i) ethanol/concd H₂SO₄ with Soxhlet extractor charged with molecular sieves 3A or 4A at refluxing temp (Fieser & Fieser, 1967b), (ii) 5% borax (sodium tetraborate decahydrate)/(CH₃)₂SO₄/sodium hydroxide in water at room temp (Scheline, 1966) (ethyl 3,4-dihydroxy-5-methoxybenzoate), (iii) benzyl chloride (2.2 equiv mol)/K₂CO₃/KI in DMF at room temp, (iv) LAH in anhyd THF (distilled from potassium and benzophenone), (v) activated MnO₂ in CH₂Cl₂ at room temp (3,4-dibenzyloxy-5-methoxybenzaldehyde), (vi) methyl magnesium bromide (2.01 mol/L in THF) (Tokyo Chemical Industry) in anhyd THF (distilled from potassium and benzophenone) at room temp, (vi) Jones' reagent (Fieser & Fieser, 1967a) in acetone at room temp, (vii) ethylene glycol/*dl*-camphor sulfonic acid in refluxing benzene, (viii) 5% Pd-C under H₂ gas in methanol at room temp, (ix) acetic anhydride/pyridine (1/1, v/v) in ethyl acetate at room temp, (x) 1N HCl in acetone at room temp, (xi) CuBr₂ in refluxing ethyl acetate, (xii) guaiacol (Kanto Chemicals)/K₂CO₃/KI in DMF at room temp, (xiii) paraformaldehyde/K₂CO₃ in DMF at room temp, (xiv) sodium methoxide in methanol/CH₂Cl₂ (1/4) at 0

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

°C.

^1H NMR (CDCl_3) (acetate) δ 1.97 (s, 3H, alcoholic-OAc), 2.23 (s, 3H, Ph-OAc), 2.25 (s, 3H, Ph-OAc), 3.67 (s, 3H, -OCH₃), 3.82 (s, 3H, -OCH₃), 4.42 (dd, $J=12$, 6.6 Hz, 1H, C3-H), 4.58 (dd, $J=12$, 3.9 Hz, 1H, C3-H), 5.47 (dd, $J=6.3$, 4.0 Hz, 1H, C2-H), 6.75-6.82 (m, 2H, Ph-(guaiacyl)-H_{4,5}), 6.86-6.95 (m, 2H, Ph-(guaiacyl)-H_{3,6}), 7.59 (1H, Ph-(syringyl)-H), 7.62 (1H, Ph-(syringyl)-H).

MS (acetate) m/z (%) 461 (1.0), 460 (M^+ , 2.6), 400 (35), 358 (48), 337 (46), 316 (35), 209 (45), 167 (100), 165 (23), 164 (38), 150 (21), 149 (21), 124 (20).

[3-OC²H₃]Syringaldehyde (51-D) was synthesized from 3,4-dibenzoyloxy-5-methoxyphenylbenzaldehyde [intermediate compound for synthesis of (15) in step (v)] via following step: (i) 5% Pd-C under H₂ gas in methanol and ethyl acetate at room temp, (ii) benzyl chloride (1 equiv mol)/K₂CO₃/KI in DMF at room temp (benzyl chloride preferentially reacts with 4-phenolic hydroxyl group for the electron-attracting effect of the *p*-positioned aldehyde), (iii) C²H₃I (Merck, 99.5%)/K₂CO₃ in DMF at room temp, (iv) 5% Pd-C under H₂ gas in methanol at room temp.

MS (acetate) m/z (%) 228 (0.8), 227 (5.3), 224 (0.2), 186 (11), 185 (100), 184 (26), 182 (3), 170 (3), 167 (4), 142 (3).

[3-OC²H₃]Syringic acid (53-D) was synthesized from ethyl 3,4-dihydroxy-5-methoxybenzoate [intermediate compound for synthesis of (15) in step (ii)] via following step: (i) benzyl chloride (1 equiv mol)/K₂CO₃/KI in DMF at room temp, (ii) C²H₃I (Merck, 99.5%)/K₂CO₃ in DMF at room temp, (iii) sodium methoxide in methanol at room temp, (iv) 5% Pd-C under H₂ gas in ethanol at room temp.

MS (acetate ethyl ester) m/z (%) 272 (0.6), 271 (M^+ , 4.2), 230 (12), 229 (100), 201 (11), 184 (27).

3.3. DEGRADATION OF *β*-O-4 DILIGNOLS BY LACCASE

The diacetate of 2-(2-methoxyphenoxy)-1,3-propanediol [guaiacoxypropanediol (36-Ac)] and the acetate of 3-hydroxy-2-(2-methoxyphenyl)propionic acid ethyl ester [guaiacoxypropionate (37-Ac-Et)] were prepared from guaiacol (Kanto Chemicals) and diethyl bromomalonate (Nakarai Chemicals) by the similar method of Katayama et al. (1981): (i) guaiacol/diethyl bromomalonate/K₂CO₃/KI in DMF at room temp, (ii) NaBH₄ (4 equiv mol) in methanol at 0 °C (5 min), (iii) acetic anhydride/pyridine (1/1, v/v) in ethyl acetate at room temp. The products were separated by TLC (solvents, methanol/CH₂Cl₂, 1/99 and CH₂Cl₂).

(37-Ac): ¹H NMR (CDCl₃) δ 2.05 (s, 6H, -OAc), 3.82 (s, 3H, -OCH₃), 4.322 (d, *J*=5.1 Hz, 2H, C1 or C3-H), 4.324 (d, *J*=5.3 Hz, 2H, C1 or C3-H), 4.54 (dt, *J*=5.1, 5.2 Hz, C2-H), 6.84-6.90 (m, 2H, Ph-H_{4,5}), 6.98-7.28 (m, 2H, Ph-H_{3,6}).

MS: Table 3.5.

(38-Ac-Et): ¹H NMR (CDCl₃) δ 1.26 (3H, -O-C-CH₃), 2.07 (s, 3H, -OAc), 3.83 (s, 3H, -OCH₃), 4.23 (2H, -O-CH₂-), 4.52-4.54 (2H, C3-H), 4.84 (1H, C2-H), 6.83-6.90 (2H, Ph-H_{4,5}), 6.97-7.03 (2H, Ph-H_{3,6}).

MS: Table 3.5.

The following compounds were prepared previously in Section 3.1 : 2,6-dimethoxy-*p*-hydroquinone [hydroquinone (57)] (Kawai et al., 1988b) and 2,6-dimethoxy-*p*-benzoquinone [benzoquinone (58)] (Kawai et al., 1987b). The following compounds were commercially available: syringaldehyde (50) and syringic acid (53) (Tokyo Chemical Industry), and guaiacol (59) (Kanto Chemicals). Mass spectra of these compounds are listed in Table 3.5.

Instruments

¹H NMR spectra were recorded by a Varian XL-200 (200 MHz) and a Bruker AM-500 (500 MHz) FT-NMR spectrometers. Chemical shifts and coupling constants are given in δ value (ppm) and Hz,

3.3. DEGRADATION OF *β*-O-4 DILIGNOLS BY LACCASE

TABLE 3.5

Relative Intensity of the Important Fragment Ions of the Authentic Compounds^a.

Compound	Mass spectral data <i>m/z</i> (%)
α-Carbonyl dimer (13-Ac) (14)	432 (M ⁺ , 0.5), 372 (18), 331 (9), 330 (42), 182 (11) 181 (100), 179 (21). 373 (5), 372 (20), 331 (10), 330 (42), 182 (11), 181 (100), 179 (20).
Guaiacoxy- propanol (36-Ac)	283 (1.6), 282 (M ⁺ , 9.2), 160 (7), 159 (100), 124 (33), 109 (12).
Guaiacoxy- propionate (37-Ac-Et)	283 (1.2), 282 (M ⁺ , 6.7), 209 (5), 160 (8), 159 (100), 131 (6), 124 (10), 109 (5).
Syringaldehyde (51-Ac)	225 (0.9), 224 (M ⁺ , 5.5), 183 (10), 182 (100), 181 (24), 167 (6), 139 (4).
Syringic acid (53-Ac-Et)	269 (0.8), 268 (M ⁺ , 3.9), 227 (13), 226 (100), 198 (11), 181 (31).
Hydroquinone (57-Ac)	255 (0.8), 254 (M ⁺ , 6.5), 212 (29), 171 (10), 170 (100), 169 (6), 155 (12).
Benzoquinone (58)	170 (14), 169 (15), 168 (M ⁺ , 100), 153 (6), 140 (15), 138 (38), 125 (21), 112 (13), 110 (9).
Guaiacol (59)	167 (2.4), 166 (M ⁺ , 11), 125 (9), 124 (100), 109 (46).

^a Instrument, Hitachi M80-B; column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone).

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

respectively. Mass spectra were taken by a Shimadzu GCMS-QP 1000 and a Hitachi M-80B gas chromatograph-mass spectrometers (EI, 70 eV). Gas chromatogram was recorded by a Shimadzu 8A gas chromatograph (FID).

3.3.3 RESULTS

Degradation of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (12)

The acetates of the degradation products were directly submitted to GC-MS. Figure 3.10 shows the gas chromatogram of the products. The acetate of guaiacol (59-Ac), benzoquinone (58), the diacetate of hydroquinone (57-Ac) and α -carbonyl dimer (13-Ac) were identified. The mass spectra and retention times of these products were identical with those of the authentic compounds. The occurrence of the acetate of 3-hydroxy-2-(2-methoxyphenoxy)propanal [guaiacoxyprominal (35-Ac)] was suggested by GC-MS analysis, and then guaiacoxyprominal (35) was identified as follows. The degradation product was reduced with NaBH₄ in methanol at 0 °C, and it was acetylated with acetic anhydride and pyridine in ethyl acetate at room temp. This products was analyzed by GC-MS. The gas chromatogram of the treated product indicated that the peak corresponding the acetate of guaiacoxyprominal (35-Ac) and (36) disappeared. While, the peak of the diacetate of guaiacoxyprominediol (37-Ac) appeared.

To detect the acidic compounds, the acetylated product was ethylated by diazoethane and analyzed by GC-MS. Thus, the acetate of ethyl guaiacoxypromionate (38-Ac-Et) was identified. Compounds (13), (35), (38), (57), (58) and (59) were not detected in the reaction mixture of the control by GC-MS analysis by use of the diacetate of 3-(4-ethoxy-3-methoxyphenyl)-1-propanol as an internal standard.

3.3. DEGRADATION OF *B*-O-4 DILIGNOLS BY LACCASE

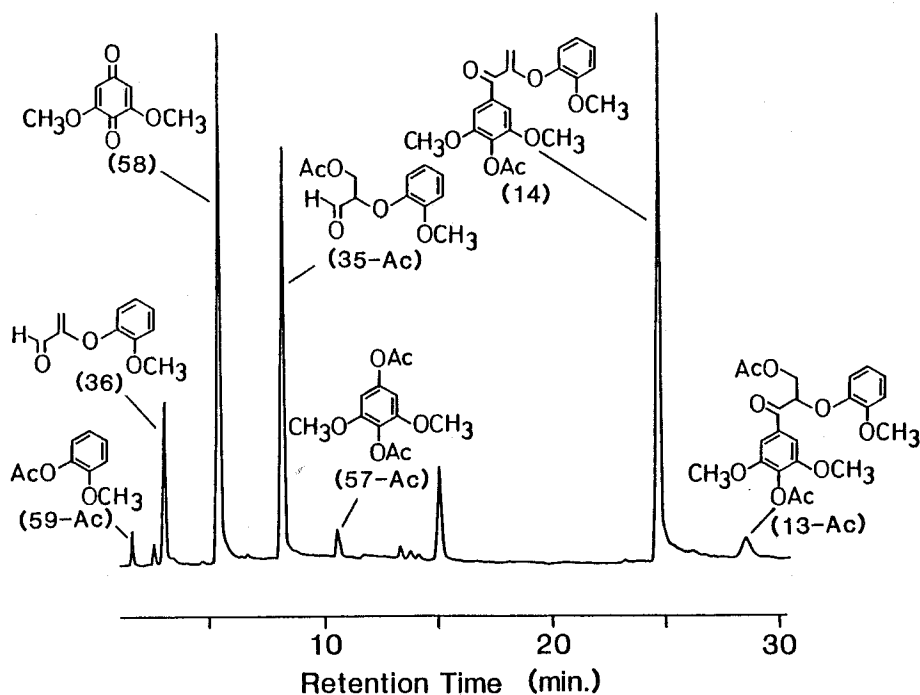


Fig. 3.10 Gas chromatogram (total ion chromatogram in GC-MS analysis) of acetylated degradation products formed from 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (12) by laccase of *C. versicolor*.

Substrate (12) was completely degraded. The acetates of α -carbonyl dimer (13-Ac) and phenylpropanal (35-Ac) thermally released acetic acid in the process of GC-MS analysis to give α,β -unsaturated carbonyl compounds (14) and (36), respectively. Column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m x 0.53 mm (i.d.); column temp, 100-220 °C, 5 °C/min.

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

In further experiment, the occurrence of syringaldehyde (51) and syringic acid (53) was carefully examined by use of the stable isotope labeled compounds (51-D) and (53-D). However, syringaldehyde (51) was not detected. While a trace amount of syringic acid (53) was detected in the degradation product ($< 1 \mu\text{g}$), although almost equal amount of which was found in the reaction mixture of control experiment.

Degradation of 1-(4-hydroxy-3,5-dimethoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propanone (13)

The acetate of the degradation product of α -carbonyl dimer (13) was directly submitted to GC-MS. The acetate of guaiacol (59-Ac), benzoquinone (58), the diacetate of hydroquinone (57-Ac) were identified. The acetylated product was ethylated by diazoethane and analyzed by GC-MS. The acetate of ethyl guaiacoxypionate (38-Ac-Et) was identified. The mass spectra and retention times were identical with those of the authentic compounds. Compounds (38), (57), (58) and (59) were not detected in the reaction mixture of control experiment by GC-MS. The amount of syringic acid (53) formed enzymatically was almost equal to that formed non enzymatically ($< 1 \mu\text{g}$).

Then, the degradation of the mixtures of three substrates, α -carbonyl dimer (13), syringaldehyde (51) and syringic acid (53), by laccase was examined to investigate the degradation rate of the respective substrates (Fig. 3.11). The results showed that syringic acid (53) was preferentially degraded by laccase, and no syringic acid (53) was detected after 30 min reaction, although 60-80% of α -carbonyl dimer (13) and syringaldehyde (51) remained intact.

3.3. DEGRADATION OF *B*-O-4 DILIGNOLS BY LACCASE

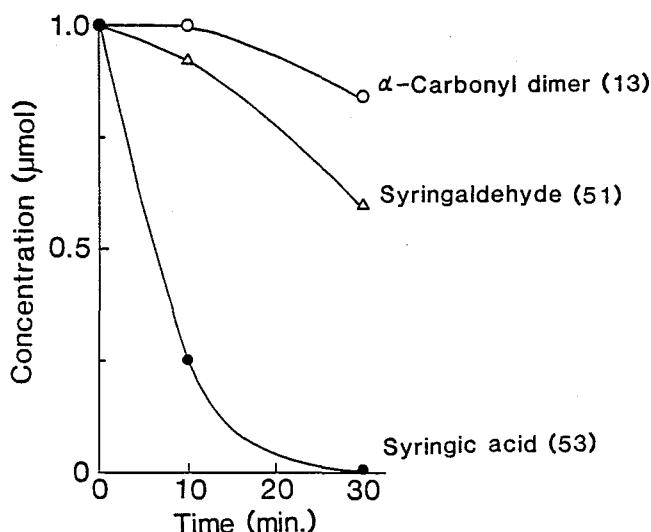


Fig. 3.11 Time course of degradations of the mixture of α -carbonyl dimer(13), syringaldehyde (51) and syringic acid (53) by laccase of *C. versicolor*. Column, 1.5% OV-17 on Chromosorb W (AW-DMCS), 1.5m x 2.6 mm (i.d.); column temp, 90-220 °C, 6 °C/min.

3.3.4 DISCUSSION

On the basis of the structures of the degradation products, the possible degradation pathways of substrates (12) and (13) by laccase of *Coriolus versicolor* were proposed as shown in Fig. 3.12. The reaction may proceed via phenoxy radicals of substrates (12) and (13) formed by laccase as described in Section 3.2.

Guaiacoxypropanal (35) and hydroquinone (57) could be formed by alkyl-aryl cleavage of substrate (12). Ca hydroxyl group of substrate (12) was oxidized to form α -carbonyl dimer (13) (Ca oxidation). α -Carbonyl dimer (13) was further cleaved between

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

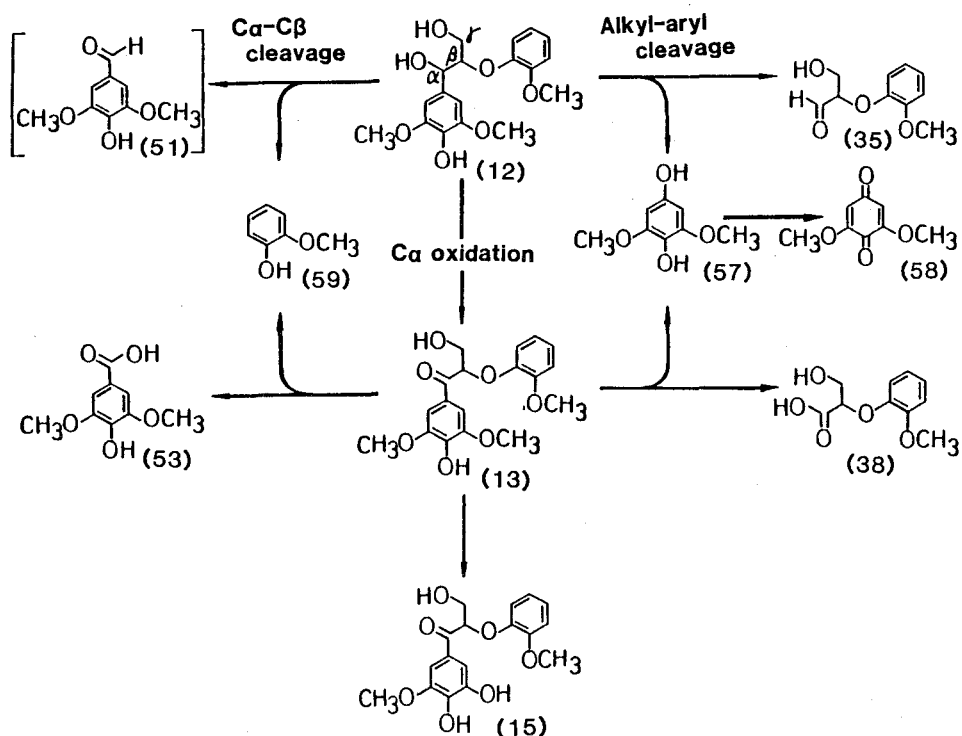


Fig. 3.12 Proposed degradation pathways of substrates (12) and (13) by laccase of *C. versicolor*.

alkyl and aryl groups to give guaiacoxypionate (38) and hydroquinone (57). Detailed oxidation mechanisms were similar to those of phenolic β -1 lignin substructure model compounds (17)-(19) as shown in Fig. 3.9 (Section 3.2) (Kawai et al., 1988a). In earlier paper, Kirk et al. (1968b) reported that Ca oxidation and alkyl-aryl cleavage of the similar substrate, 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-methoxyphenoxy)ethanol, were mediated by laccase of *C. versicolor*. The conversion of hydroquinone (57) to benzoquinone (58) was reported previously (Section 3.2).

In the degradation of nonphenolic β -O-4 lignin substructure model compounds by lignin peroxidase, a phenol from β -aryl moiety

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

and a benzaldehyde were formed by $C\alpha$ - $C\beta$ cleavage of the substrate (Higuchi, 1986; Kirk & Farrell, 1987; Buswell & Odier, 1987; Umezawa, 1988). In Section 3.1 and 3.2, $C\alpha$ - $C\beta$ cleavage of phenolic β -1 lignin substructure models (17)-(19) mediated by laccase, and the degradation mechanisms for $C\alpha$ - $C\beta$ cleavage were proposed (Fig. 3.8., Kawai et al., 1988a). It can therefore be presumed that the guaiacol (59) is formed via direct $C\alpha$ - $C\beta$ cleavage of substrate (12).

Two alternative pathways for the formation of phenols from β -aryl ether moieties of phenolic β -O-4 model compounds were proposed previously. (i) Phenol formation via phenoxypropanal formed by alkyl-aryl cleavage (Morohoshi & Haraguchi, 1987) and (ii) via phenoxyethanol formed with benzaldehyde by $C\alpha$ - $C\beta$ cleavage (Wariishi et al., 1987). However, syringaldehyde (51), which was identified as a major degradation product of substrate (12) by Wariishi et al., (1987), could not be detected.

As a considerable amount of guaiacol (59) was found in the degradation products of α -carbonyl dimer (13), syringic acid (53) as an expected product of $C\alpha$ - $C\beta$ cleavage of α -carbonyl dimer (13) was searched and identified. But the amount of syringic acid (53) was very little and almost equal to that formed nonenzymatically.

Both syringaldehyde (51) and syringic acid (53) are known to be good substrates for laccase (Ishihara & Nishida, 1983; Kawai et al, 1987b, 1988a). Then, the degradation of the mixture of α -carbonyl dimer (13), syringaldehyde (51) and syringic acid (53) by laccase were examined to investigate the degradation rate of the respective substrate (Fig. 3.12). Syringic acid (53) was preferentially degraded by laccase, and the degradation of the former two substrates was comparatively slow. Consequently, it was concluded that guaiacol (59) could be formed from α -carbonyl dimer (13) with syringic acid (53) via $C\alpha$ - $C\beta$ cleavage as shown in Fig. 3.13, but syringic acid (53) was degraded faster.

3.3. DEGRADATION OF β -O-4 DILIGNOLS BY LACCASE

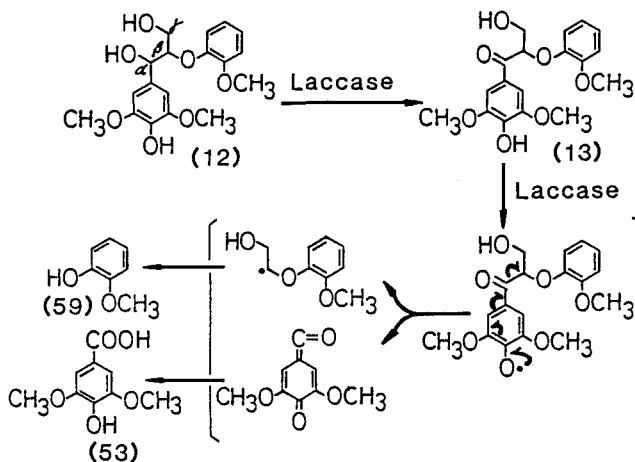


Fig. 3.13. Possible mechanism for the formation of syringic acid (53) and guaiacol (59) from α -carbonyl dimer (13) by laccase of *C. versicolor*.

Very recently, the occurrence of 1-(3,4-dihydroxy-5-methoxyphenyl)-3-hydroxy-2-(2-methoxyphenoxy)propanone (15), the triacetate of which thermally releases acetic acid to give α,β -unsaturated carbonyl compound (16), was suggested by GC-MS analysis. Then the authentic compound (15) was synthesized. The mass spectrum of the degradation product was identical with those of synthesized compound (15) (data not shown). Further investigations are now in progress whether guaiacol (59) is formed by laccase or not as the degradation product from (15).

Section 3.4

3.4 AROMATIC RING CLEAVAGE OF 4,6-DI-*TERT*-BUTYL-2-METHOXYPHENOL

3.4.1 INTRODUCTION

As described in Section 3.1 -3.3 , laccase of *Coriolus versicolor* catalyzed not only alkyl-aryl cleavage but also C α -C β cleavage of the side chain of β -1 and β -O-4 lignin substructure model compounds (Kawai et al., 1987b, 1988a, 1989a). In this section, the possibility of aromatic ring cleavage of the phenolic lignin model compound, 4,6-di-*tert*-butyl-2-methoxyphenol [dibutylguaiacol (60)], by laccase was examined. Dibutylguaiacol (60) was found to be degraded by laccase to form an aromatic ring cleavage product, 2,4-di-*tert*-butyl-4-methoxycarbonylmethyl-2-buten-4-olide [muconolactone (62)]. The following tracer experiments with H₂¹⁸O and ¹⁸O₂ demonstrated the incorporation of ¹⁸O from ¹⁸O₂ into the muconolactone (62) (Kawai et al., 1988b).

3.4.2 MATERIALS AND METHODS

Enzyme preparation

Crude laccase preparation of *Coriolus versicolor* Ps4a was prepared by the method of Fåhræus and Reinhammer (1967) as described in Section 3.3.

Enzyme reaction

Enzyme soln (3 mL, 1100 nkat) and substrate (60) (2 μ mol in 5- μ L acetone) were placed in a flask and the reaction mixture was incubated for 2 h at 30 °C under air. In a control experiment, enzyme was replaced by 0.1 M phosphate buffer (pH 6.0).

The reaction mixture was then extracted with ethyl acetate

3.4. AROMATIC RING CLEAVAGE BY LACCASE

(10 mL). The organic layer was washed with saturated NaCl soln, dried over anhyd Na₂SO₄ and evaporated under reduced pressure. The extract was separated by TLC (Kieselgel F254, Merck; solvent, ethyl acetate/*n*-hexane, 1/10). The fraction whose *R_f* value corresponds to that of muconolactone (62) was analyzed by GC-MS [column, Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone) 12 m x 0.53 mm (i.d.); column temp, 120-140 °C, 5 °C/min].

The amount of muconolactone (62) formed was calculated quantitatively by the stable isotope dilution method. Deuterated muconolactone (62-D) as an internal standard (2 µg in 20-µL dioxane) was added to a flask before extraction, and the fraction containing muconolactone (62) and (62-D) was separated by TLC and analyzed by GC-MS (mass chromatography).

Isotopic experiments

Incorporation of ¹⁸O from ¹⁸O₂

A flask containing 2.9 mL of 0.1 M phosphate buffer (pH 6.0) and 100 µL of enzyme soln (40 nkat) was evacuated and then filled with argon gas, and this procedure was repeated five times. Finally, ¹⁸O₂ (¹⁸O: 98.58%, CEA) was introduced into the evacuated flask. The reaction was then started by the addition of 2 µmol of dibutylguaiacol (60), and the reaction mixture was shaken for 3 h at 30 °C. The reaction was terminated by extraction with ethyl acetate (10 mL). The ethyl acetate layer was dried over anhyd Na₂SO₄ and evaporated. The fraction containing muconolactone (62) was immediately separated by TLC and analyzed by GC-MS.

3.4. AROMATIC RING CLEAVAGE BY LACCASE

Incorporation of ^{18}O from H_2^{18}O

To a flask containing lyophilized enzyme (300 μL , 110 nkat), 150 μL of H_2^{18}O (^{18}O : 97%, CEA), 150 μL of distilled water and 2 μmol of dibutylguaiacol (**60**) were added, and the reaction mixture was shaken for 3 h at 30 $^{\circ}\text{C}$. The reaction mixture was then extracted with ethyl acetate (10 mL), and the ethyl acetate layer was dried over anhyd Na_2SO_4 and evaporated. The fraction containing muconolactone (**62**) was immediately separated by TLC and analyzed by GC-MS.

Syntheses of substrate and authentic compounds

4,6-Di-*tert*-butyl-2-hydroxyphenol [dibutylguaiacol (**60**)] was prepared from guaiacol (Nakarai Chemicals) by the method of Ley and Müller (1956). However, the product contained the isomer, 3,5-di-*tert*-butyl-2-methoxyphenol, of which the chemical property are identical with those of dibutylguaiacol (**60**) (*R_f* value on TLC, retention time in GC, and ^1H NMR and mass spectra). When the product containing both isomers was treated with acetic anhydride and pyridine (1/1, v/v) at room temp for 24 h, one isomer (3,5-di-*tert*-butyl-2-methoxyphenol) was acetylated, but the other was not. The nonacetylated form was selected as the target compound (**60**), since the phenolic hydroxyl group of (**60**) is not susceptible to acetylation with acetic anhydride owing to steric hindrance by the neighboring bulky *tert*-butyl group.

2,4-Di-*tert*-butyl-4-methoxycarbonylmethyl-2-butene-4-olide [muconolactone (**62**)] was synthesized from 4-*tert*-butylcatechol (Nakarai Chemicals) via the following step: (i) introduction of the *tert*-butyl group with 2-methyl-2-propanol and concd H_2SO_4 in acetic acid at room temp (3,5-di-*tert*-butylcatechol) (Matsuura et al., 1972), (ii) aromatic ring cleavage of 3,5-di-*tert*-butylcatechol to muconolactone (**61**) with ferric acetylacetonate (Nakarai Chemicals) and ca. 8% peracetic acid (Findley et al.,

3.4. AROMATIC RING CLEAVAGE BY LACCASE

1945) in acetic acid at room temp [modified method of Pandell (1983)]. The product (61) was recrystallized from petroleum ether (15.7%), and (iii) methylated with diazomethane in diethyl ether at room temp.

^1H NMR (CDCl_3) δ 0.98 (s, 9H, $-\text{C}(\text{CH}_3)_3$), 1.24 (s, 9H, $-\text{C}(\text{CH}_3)_3$), 2.80 (d, $J=14$ Hz, 1H, $>\text{C}-\text{CH}_2-\text{COO}-$), 2.96 (d, $J=14$ Hz, 1H, $>\text{C}-\text{CH}_2-\text{COO}-$), 3.59 (s, 3H, $-\text{OCH}_3$), 6.97 (s, 1H, $>\text{C}=\text{CH}-$).

^{13}C NMR (CDCl_3) δ 25.2 (q), 28.0 (q), 31.5, 37.6, 37.8 (t), 51.8 (q), 88.4, 143.5, 146.0 (d), 169.7, 171.2.

EI-MS m/z (%) 268 (M^+ , missing), 213 (12), 212 ($\text{M}^+ - \text{C}_4\text{H}_8$, 100), 198 (11), 197 (95), 153 (64), 137 (15), 109 (13).

CI-MS (2-methylpropane) m/z (%) 270 (16), 269 (NH^+ , 100).

IR (KBr) $\nu_{\text{C=O}}$ 1746, 1733 cm^{-1} , $\nu_{\text{C=C}}$ 1639 cm^{-1} .

Deuterated muconolactone (62-D) was prepared from (61) by treatment with $\text{C}^2\text{H}_5\text{OH}$ (99.5%, CEA) in the presence of trifluoroacetic anhydride at 35 $^\circ\text{C}$.

CI-MS m/z (%) 272 (MH^+ , 100), 269 (4.5).

Instruments

NMR spectra were recorded on a Varian XL-200 FT-NMR spectrometer (200 MHz). Chemical shifts and coupling constants are given in δ value (ppm) and Hz, respectively. Mass spectra were measured on a Shimadzu GCMS QP-1000 gas chromatograph-mass spectrometer [EI-MS (70 eV) and CI-MS (reagent gas, 2-methylpropane)]. IR spectrum was registered using a Hitachi 260-30 infrared spectrometer. Enzyme activity was determined with a Hitachi 200-20 double-beam spectrophotometer.

3.4. AROMATIC RING CLEAVAGE BY LACCASE

3.4.3 RESULTS

It was confirmed by GC-MS analysis that muconolactone (**62**) was formed as an aromatic ring cleavage product by laccase from dibutylguaiacol (**60**), which was not degraded completely for 2-h incubation as shown in Fig. 3.14. The mass spectra (EI and CI) and retention time of the degradation product (**62**) were identical with those of the authentic compound. Furthermore, quantification by stable isotope dilution method showed that 20 nmol (5.4 μ g) of muconolactone was formed as degradation product from 2 μ mol (472.7 μ g) of dibutylguaiacol (**60**), while the amount of muconolactone (**62**) produced nonenzymatically was 1.5 nmol (0.4 μ g).

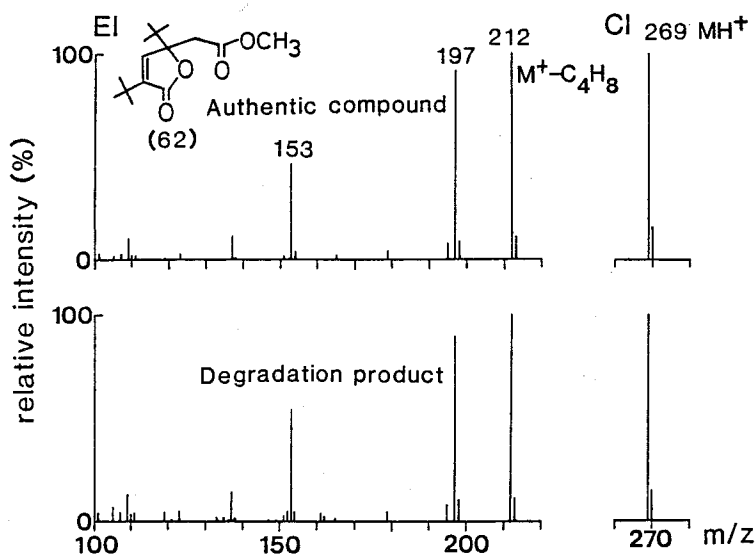


Fig. 3.14 Mass spectra of 2,4-di-*tert*-butyl-4-methoxycarbonylmethyl-2-buten-4-olide (**62**).
Upper panel; authentic compound; lower panel, degradation product formed from dibutylguaiacol (**60**) by laccase.

3.4. AROMATIC RING CLEAVAGE BY LACCASE

Other products were observed by TLC, but their structures were not determined.

Incorporation of ^{18}O into muconolactone (**62**) from $^{18}\text{O}_2$ or H_2^{18}O was investigated by use of GC-MS. Figure 3.15 shows mass chromatograms of the MH^+ region of muconolactone (**62**). Analysis showed that H_2^{18}O was not incorporated to muconolactone (**62**), while one (22%) or two (11%) atoms of ^{18}O were incorporated into muconolactone (**62**) from molecular oxygen (Fig. 3.15).

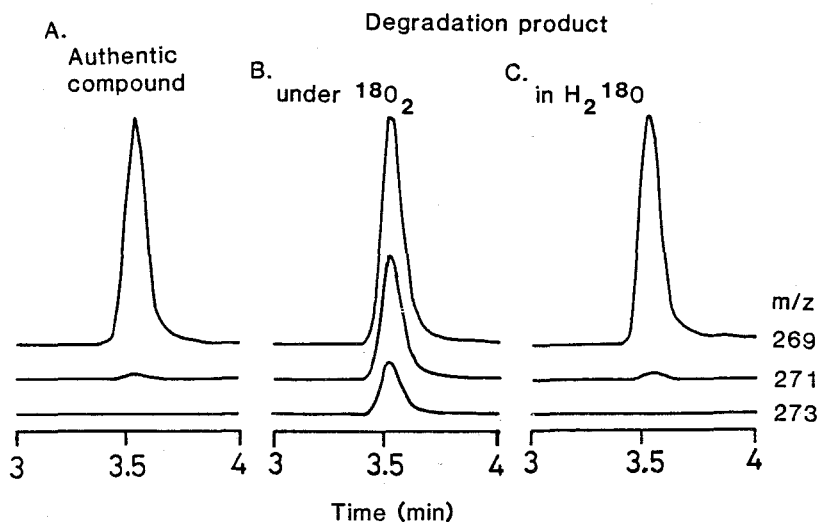


Fig. 3.15 Mass chromatograms of the MH^+ region of muconolactone (**62**).
(A) Authentic compound, (B) product from substrate (**60**) under $^{18}\text{O}_2$ (^{18}O : 98.58%), (C) product from substrate (**60**) in H_2^{18}O (^{18}O : 48.5%).

3.4. AROMATIC RING CLEAVAGE BY LACCASE

3.4.4 DISCUSSION

Our investigation showed that monomeric lignin degradation phenols, vanillyl alcohol, syringyl alcohol, etc., were mostly converted to polymerized or quinone-type compounds by laccase (data not shown). It appeared rather difficult to identify the structures of the aromatic ring cleavage products without having synthetic authentic compounds available, even if a small amount of such products could be obtained from these substrates. Therefore, 4,6-di-*tert*-butyl-2-methoxyphenol (60) was synthesized as substrate for laccase. The ortho and para positions for the phenolic hydroxyl group of this compound (60) were blocked with bulky *tert*-butyl groups to prevent coupling and side chain reactions of phenoxy radical(s). And it was succeeded for the first time the identification of a aromatic ring cleavage product, muconolactone (62), by laccase compared with the authentic compound.

Figure 3.16 depicts two possible mechanisms of formation of muconolactone (62). Dibutylguaiacol (60) is oxidized by laccase to form the phenoxy radical, which is subsequently attacked by molecular oxygen. The resulting hydroperoxide reacts with nucleophilic oxygen species. Since isotopic experiments showed that H₂O was not incorporated into muconolactone (62), formation of (62) via pathway B was ruled out. However, mass spectrometric analysis showed that two ¹⁸O atoms from ¹⁸O₂ were incorporated into a part of muconolactone (62) (Fig. 3.15). These results are in accordance with the contention that the hydroperoxide group of an intermediary O₂ adduct reacts with the adjacent carbonyl group to form a cyclic peroxide, which is converted to a muconate derivative. The muconate compound then undergoes cyclization to yield muconolactone (62).

Muconolactone (62) was isolated previously as a product in

3.4. AROMATIC RING CLEAVAGE BY LACCASE

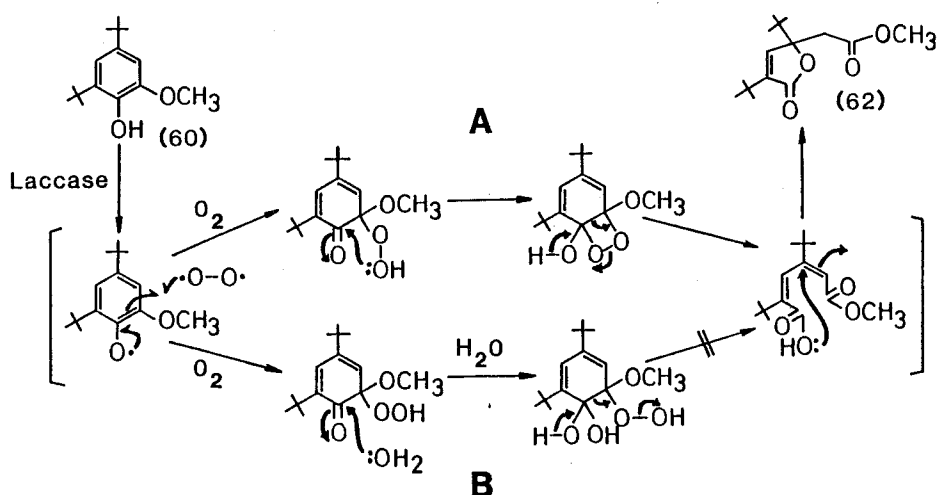


Fig. 3.16 Possible mechanisms for the formation of muconolactone (62) from 2,4-di-*tert*-butyl-2-methoxyphenol (60).

photosensitized (Matsuura et al., 1972) and alkaline-oxygen (Eckert et al., 1973; Gierer & Imsgard, 1977) oxidations of dibutylguaiacol (60). Very recently, muconolactone (62) was identified as a degradation product of dibutylguaiacol (60) by laccase from *Clitopilus* species (data not shown).

It is known that aromatic ring cleavage of phenolic compounds by microorganisms is generally catalyzed by dioxygenase. However, the present investigation showed that in addition to side chain cleavage of phenolic β -1 and β -O-4 lignin substructure model compounds as described previous Sections 3.1 -3.3 (Kawai et al. 1987b, 1988a, 1989a), aromatic ring cleavage of phenolic lignin model compounds is catalyzed by laccase of *C. versicolor*.

Section 3.5

3.5 OXIDATION OF METHOXYLATED BENZYL ALCOHOLS BY LACCASE OF *CORIOLUS VERSICOLOR* IN THE PRESENCE OF SYRINGALDEHYDE

3.5.1 INTRODUCTION

Laccase mediates one-electron oxidation of phenolic substrates to form many degradation products via various pathways (Kawai et al., 1987b; 1988a,b; 1989a), but the enzyme can not oxidize nonphenolic substrate. However, an earlier paper reported that veratrylglycerol- β -guaiacyl ether was converted to its α -carbonyl derivative by laccase in the presence of spruce MWL (Kirk et al., 1968a). They concluded that free radicals participated in the oxidation of veratrylglycerol- β -guaiacyl ether. Recently, the formation of guaiacol (59) from the mixture of guaiacoxymethanol and syringaldehyde (51) by laccase was reported (Wariishi et al., 1987).

In the present section, the oxidation of nonphenolic monomers, veratryl alcohol (47) and 3,4,5-trimethoxybenzyl alcohol (56), by laccase of *C. versicolor* in the presence of syringaldehyde is described, and the mechanisms for the oxidation of these substrates are discussed. Furthermore, the oxidation of nonphenolic β -1 lignin substructure model compounds by laccase in the presence of syringaldehyde or bamboo MWL was investigated. However, β -1 substrates could not be oxidized in our experimental conditions (Kawai et al., 1989b).

3.5.2 MATERIALS AND METHODS

Laccase Preparation

A purified homogeneous laccase preparation of *C. versicolor*

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

IFO-30340 was kindly provided from Oji Paper Co., Ltd.(Sugiura et al., 1987). It was stored at -20 °C as a 50% glycerol solution. The enzyme activity was determined spectrophotometrically by measuring the absorption at 525 nm using syringaldazine (Aldrich Chemical) as substrate at 30 °C as described in Section 3.1 (Leonowicz and Grzywnowicz, 1981).

Enzymic oxidation of methoxylated benzyl alcohols in the presence of syringaldehyde

Substrate, veratryl alcohol (47) or 3,4,5-trimethoxybenzyl alcohol (56) (0.5 μ mol in 5 μ L DMF soln), and syringaldehyde (2 μ mol in 5- μ L DMF soln) were incubated in a total volume of 1 mL with the enzyme (15-25 nkat) in 0.2 M acetate buffer (pH 4.0) at 30 °C for 30 min under air. In a control experiment, no syringaldehyde was added to the flask.

The reaction mixture was extracted with 10 mL ethyl acetate. The organic layer was washed with saturated NaCl soln, dried over anhyd Na₂SO₄ and evaporated under reduced pressure. The extract was acetylated with acetic anhydride and pyridine (1/1, v/v) in ethyl acetate for 10 h and analyzed by GC-MS [instrument; Shimadzu GCMS-QP 1000 gas chromatograph-mass spectrometer (EI, 70 eV), column; Shimadzu capillary column Hicap CBP1-W12-100 (methyl silicone), 12 m x 0.53 mm (i.d.), temperature program; Initial temp at 130 °C was hold for 2 min, then elevated to 150 °C at 5 °C/min]. Degradation products were identified by comparison of the mass spectra and retention times with those of the authentic compounds.

The amounts of benzaldehydes (46) and (55) formed were calculated quantitatively by stable isotope dilution method. Deuterated internal standard, (46-D) or (55-D), (2 μ g) was added to the flask before extraction, and analyzed by GC-MS.

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

Enzymic oxidation of nonphenolic β -1 lignin substructure model compounds in the presence of syringaldehyde or bamboo MWL

Reaction conditions of β -1 lignin model compounds (20) and (21) by laccase are listed in Table 3.6. Reaction mixture was extracted with ethyl acetate (10 mL, twice), and the organic layer was washed with saturated NaCl soln, dried over anhyd Na_2SO_4 , and evaporated under reduced pressure. The extract was acetylated and analyzed by GC-MS.

TABLE 3.6

Reaction Conditions for the Degradation of Nonphenolic β -1 Substructure (20) and (21) by Laccase in the Presence of Syringaldehyde or Bamboo MWL

Nonphenolic compound	Phenolic compound	Medium		Time
(21)	Syringaldehyde	Acetate buffer	pH 4.0	0.5 h
(20)	Syringaldehyde	Acetate buffer	pH 5.3	0.5 h
(20)	Syringaldehyde	Acetate buffer	pH 4.0	0.5 h
(20)	Syringaldehyde	Tartrate buffer	pH 3.0	0.5 h
(21)	MWL	Acetate buffer	pH 4.0	2.0 h
(21)	MWL	Dioxane/water	9/1	2.0 h
(21)	MWL	Dioxane/acetate buffer, pH 5.3	3/1	2.0 h

Syntheses of substrates and authentic compounds

[4-OC²H₃]Veratraldehyde (46-D) was prepared from vanillin by methylation with C²H₃I (²H: 99.5%, Merck) in DMF at room temp. MS m/z (%) 170 (19), 169 (M⁺, 100), 168 (52), 167 (1.7), 166 (0.4), 98 (15), 95 (12), 79 (11).

3,4,5-Trimethoxybenzaldehyde (55) was prepared from

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

syringaldehyde by methylation with iodomethane and K_2CO_3 in DMF at room temp. C^2H_3I (2H : 99.5%, Merck) was used for the preparation of [4- OC^2H_3]3,4,5-trimethoxybenzaldehyde (55-D).

(55): MS m/z (%) 199 (0), 198 (2.3), 197 (12), 196 (M^+ , 100), 195 (5.5), 181 (41), 125 (19), 110 (14).

(55-D): MS m/z (%) 200 (13), 199 (M^+ , 100), 198 (8.7), 196 (3.0), 181 (34), 125 (13).

3,4,5-Trimethoxybenzyl alcohol (56) was obtained by reduction of (55) with $NaBH_4$ in methanol at 0 °C.

MS m/z (%) 241 (15), 240 (M^+ , 100), 198 (43), 197 (12), 183 (17), 181 (67), 169 (19).

Veratryl alcohol (47), veratraldehyde (46) and syringaldehyde (51) were commercially available (Tokyo Chemical Industry).

(47): MS (acetate) m/z (%) 211 (10), 210 (M^+ , 81), 168 (36), 153 (12), 152 (10), 151 (100), 150 (10), 137 (16), 135 (12), 107 (16).

(46): MS m/z (%) 169 (0.6), 167 (15), 166 (M^+ , 100), 165 (52), 151 (13), 95 (28), 77 (18).

1,2-Bis(3,4,5-trimethoxyphenyl)-1,3-propanediol (21) was prepared from 1,2-bis(4-hydroxy-3,5-dimethoxyphenyl)-1,3-propanediol (17) (Kawai et al., 1987b) by methylation with diazomethane. 1,2-Bis(4-ethoxy-3,5-dimethoxyphenyl)-1,3-propanediol (20), 2,6-dimethoxy-*p*-benzoquinone [benzoquinone (58)] (Kawai et al., 1987b) and 2,6-dimethoxy-*p*-hydroquinone [hydroquinone (57)] (Kawai et al., 1988a) were prepared previously as described in Section 3.1. Bamboo MWL was kindly provided by Dr. M. Tanahashi, Wood Research Institute, Kyoto University.

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

3.5.3 RESULTS

Degradation of veratryl alcohol (47) and 3,4,5-trimethoxybenzyl alcohol (56)

As degradation products of the mixture of veratryl alcohol (47) and syringaldehyde (51) by laccase, veratraldehyde (46), hydroquinone (57), and benzoquinone (58) were identified by GC-MS analysis. When no syringaldehyde (51) was added to the flask, little veratraldehyde (46) was detected. Then, the quantification of degradation product (46) was conducted. Figure 3.17 shows the mass chromatograms of the M^+ regions of degradation product (46) (m/z 166) and internal standard (46-D) (m/z 169). The amount of product (46) formed in the presence of syringaldehyde (51) was 7.6×10^{-3} μmol (1.3 μg , average of experiments 1 and 2). While

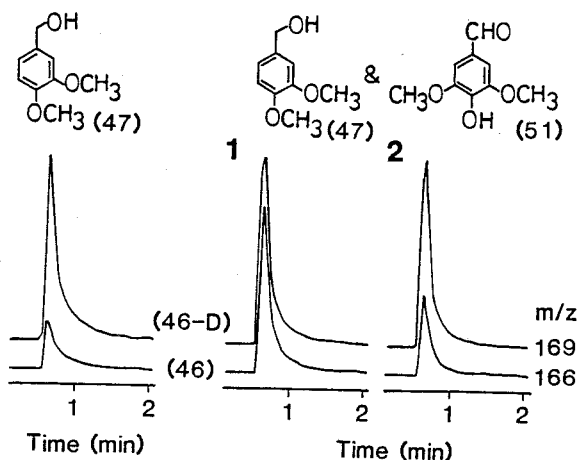


Fig. 3.17 Mass chromatograms of the M^+ regions of the degradation product (46) (m/z 166) and internal standard (46-D) (m/z 169).

Before extraction of the reaction mixture, internal standard (2 μg) was added.

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

the amount of (46) formed in the absence of syringaldehyde (51) was 3.1×10^{-3} μmol (0.5 μg).

As degradation products of the mixture of 3,4,5-trimethoxybenzyl alcohol (56) and syringaldehyde (51) by laccase, 3,4,5-trimethoxybenzaldehyde (55), hydroquinone (57) and benzoquinone (58) were identified by GC-MS analysis. In the control experiment, however, 3,4,5-trimethoxybenzaldehyde (55) was formed very little. Figure 3.18 shows the mass chromatograms of M^+ regions of degradation product (55) (m/z 196) and internal standard (55-D) (m/z 199). The amount of product (55) formed in the presence of syringaldehyde (51) was 7.4×10^{-3} μmol (1.5 μg , average of experiments 1 and 2). While the amount of (55) formed in the absence of syringaldehyde (51) was 9.7×10^{-4} μmol (0.2 μg).

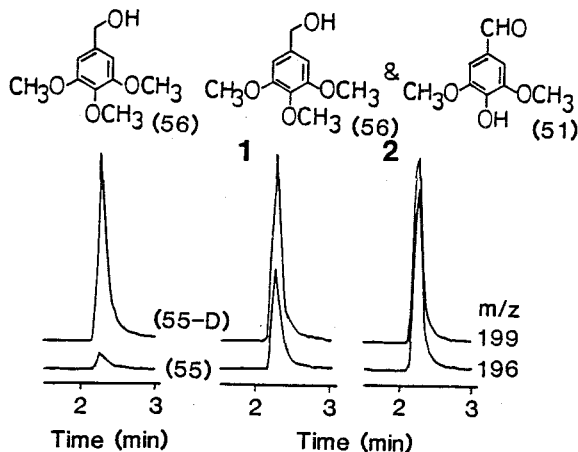


Fig. 3.18 Mass chromatograms of the M^+ regions of the degradation product (55) (m/z 196) and internal standard (55-D) (m/z 199).

Before extraction of the reaction mixture, internal standard (2 μg) was added.

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

Degradation of nonphenolic β -1 lignin substructure model compounds

The acetylated degradation products of the mixture of nonphenolic β -1 model compound (21) and syringaldehyde (51) by laccase were submitted to GC-MS. 3,4,5-Trimethoxybenzaldehyde (55) was little formed as a Ca-C β cleavage product of (21), but almost equal amount of (55) was found in the extracts of (21) by laccase in the absence of syringaldehyde (51). The experimental conditions were the same as in Table 3.6.

3.5.4 DISCUSSION

Coriolus versicolor excretes laccase (Fåhræus & Reinhammer, 1967) and lignin peroxidase (Dodson et al., 1987). Lignin peroxidase catalyzes the oxidation of both nonphenolic and phenolic lignin substructure models (Dodson et al., 1987; Kawai et al., in press, See Chapter 2) as in the case of *Phanerochaete chrysosporium* (Tien & Kirk, 1984). On the other hand, laccase is unable to oxidize nonphenolic lignin dilignols (Kawai et al., 1987b; Wariishi et al., 1987). Lignin macromolecules are composed of phenolic (10-20%) and nonphenolic (80-90%) moieties (Adler, 1977; Higuchi, 1985a). The content of phenolic hydroxyl groups could be increased during side chain cleavage by *P. chrysosporium* (Tien & Kirk, 1984) and laccase (Morohoshi & Haraguchi, 1987; Kawai et al., 1989a). Hence, if laccase or radical intermediates formed by laccase could catalyze the oxidation of nonphenolic moieties of lignin, the degradation rate of lignin macromolecules could be increased.

The present investigation showed that veratryl alcohol (47) or nonphenolic trimethoxybenzyl alcohol (56) was oxidized to the corresponding benzaldehyde (46) or (55) by laccase of *C. versi-*

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

color in the presence of syringaldehyde. These results indicate that the addition of syringaldehyde (51) induces the oxidation of the nonphenolic monomers (47) and (56). Hence, the oxidation mechanisms of nonphenolic methoxylated benzyl alcohols (47) and (56) by laccase in the presence of syringaldehyde (51) were proposed as illustrated in Fig. 3.19. Laccase mediates one-electron oxidation from syringaldehyde (51) to give the phenoxy radical. The phenoxy radical is mainly converted to hydroquinone (57) and benzoquinone (58). However, it seems that some of the radical mediate one-electron oxidation of nonphenolic monomers (47) or (56) to give the corresponding aryl cation radical. The aryl cation radical is converted to benzaldehyde (46) or (55) via several steps.

The oxidation of nonphenolic β -1 lignin substructure model compounds (20) and (21) by laccase in the presence of the phenolic substances, syringaldehyde (51) or bamboo MWL was further attempted. Kirk et al. (1968) reported that incubation of veratrylglycerol- β -guaiacyl ether (25 mg), spruce MWL (50 mg), and laccase in acetate buffer (pH5.0) for 46 h resulted in the formation of a small amount (< 1mg) of its α -carbonyl derivative. However, in the present experiment C α -C β cleavage products, the formation mechanism of which is very similar to that of C α oxidation products, of β -1 model compounds could not be detected.

Kirk et al. (1968) also reported the conversion of veratrylglycerol- β -guaiacyl ether to its α -carbonyl derivative by 2,4,6-triphenylphenoxy dimer, which dissociated to two phenoxy radical monomers, in benzene. These result suggests that the possibility of the oxidation of nonphenolic dilignols by phenoxy radicals formed by laccase is still remained.

3.5. OXIDATION OF NONPHENOLIC SUBSTANCES BY LACCASE

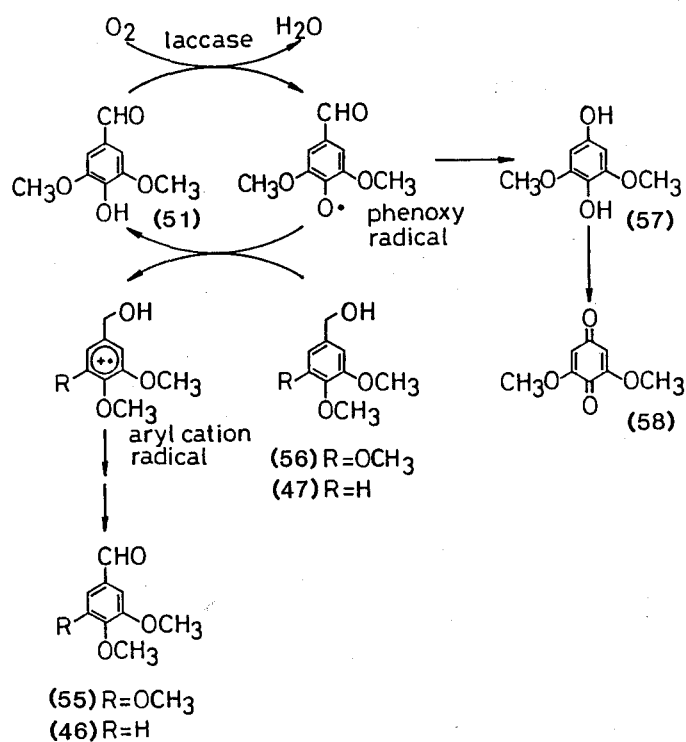


Fig. 3.19 The oxidation mechanisms of nonphenolic monomers, veratryl alcohol (47) and 3,4,5-trimethoxybenzyl alcohol (56), by laccase of *C. versicolor* in the presence of syringaldehyde.

CONCLUSION

The present study was designed to elucidate the degradation mechanisms of lignin by a white-rot fungus, *Coriolus versicolor*, through the degradation of lignin model compounds by the culture and extracellular enzymes of this fungus.

In Chapter 1, the degradation of nonphenolic β -O-4 lignin substructure model compounds by the ligninolytic culture of *C. versicolor* was described. Since β -O-4 substructure is the most frequent intermonomer linkage in lignin and many of this linkage are etherified, elucidation of the degradation mechanisms for this substructure is essential. Earlier paper (Russell et al., 1961; Kirk et al., 1968a) reported that nonphenolic β -O-4 model compounds could not be degraded by *C. versicolor*. This was because the culture parameters which were later determined by Kirk et al. (1978) were not appropriate for ligninolytic system of this fungus. Then, the author attempted the degradation of 1-(4-ethoxy-3-methoxyphenyl)-2-(4-formyl-2,6-dimethoxyphenoxy)-1,3-propanediol by *C. versicolor* under ligninolytic culture conditions (Section 1.1, Kawai et al., 1985a,b). The substrate was rapidly degraded to give many degradation products. The chemical structures of the degradation products indicated that five types of reactions were caused by the ligninolytic culture of *C. versicolor*: (i) oxidation and reduction at the benzylic position of the substrate, (ii) β -ether cleavage to give arylglycerol, (iii) C α -C β cleavage of propyl side chain of the substrate and arylglycerol to give benzaldehyde, (iv) cleavage of β -etherated aromatic ring of the substrate to give formate and cyclic carbonate of arylglycerol (Section 1.2), and (v) the formation of *p*-benzoquinone monoketals (Section 1.3).

The aromatic ring cleavage product, 1-(4-ethoxy-3-methoxyphenyl)-3-formyloxy-1,2-propanediol, was first identified (Kawai et al., 1985a), and an isotopic experiment with the ¹³C-labeled substrate indicated that its formyl carbon was derived from the

CONCLUSION

β -etherated aromatic ring of the β -O-4 model compound (Kawai et al., 1985b). The carbonate carbon of the cyclic carbonate of arylglycerol was found to be derived from β -aryl group of β -O-4 model compounds. These two compounds were further degraded by the culture of *C. versicolor* to give the arylglycerol.

In Section 1.3, the first identification of *p*-benzoquinone monoketals, 2-(4-ethoxy-3-methoxyphenyl)-3-hydroxymethyl-6,10-dimethoxy-1,4-dioxaspiro[4,5]deca-6,9-diene-8-one and its isomer was described. Former compound was also produced from a β -O-4 model compound by lignin peroxidase of *Phanerochaete chrysosporium* (Kawai et al., 1987a). Other reactions (i)-(iv), except reduction, have been confirmed to be involved in the degradation pathways of lignin substructure model compounds by lignin peroxidase of *P. chrysosporium* (see Umezawa, 1988). These results suggested that most of the reactions for nonphenolic β -O-4 model compounds were catalyzed by the lignin peroxidase of *C. versicolor*, which was later isolated from the culture filtrate of *C. versicolor* by Dodson et al. (1987). To confirm the above suggestion, the degradation of nonphenolic β -O-4 lignin substructure model compounds by lignin peroxidase of *C. versicolor* was attempted (Kawai et al., in press), and the following four types of the reactions were confirmed as expected: (i) C α oxidation, (ii) β -ether cleavage, (iii) C α -C β cleavage, and (iv) aromatic ring cleavage. In addition, 1-(4-ethoxy-3-methoxyphenyl)-3-hydroxypropanone was first identified as the degradation product of β -O-4 model compound by lignin peroxidase and the formation pathway via an aryl cation radical intermediate was proposed (Chapter 2).

The results in Chapter 1 and 2 indicated that lignin peroxidase is one of the most important lignin-degrading enzyme of *C. versicolor*.

CONCLUSION

In Chapter 3, the degradation mechanisms for lignin model compounds by laccase of *C. versicolor* were described. Laccase is commonly distributed in white-rot fungi and causes Bavendamm's reaction. In spite of considerable efforts by many researchers, the intrinsic role of laccase in lignin biodegradation has not been elucidated clearly (see Kirk & Shimada, 1985). Kirk et al. (1968b) reported the degradation of a phenolic β -O-4 model compound by laccase via alkyl-aryl cleavage and C α oxidation. The degradation of phenolic β -1 lignin substructure model compounds by laccase was first examined by the present author (Section 3.1 and 3.2, Kawai et al., 1987b; 1988a). The results indicated that laccase catalyzes not only alkyl-aryl cleavage and C α oxidation but also C α -C β cleavage of propyl side chain of β -1 model compounds. Isotopic experiments with ^{18}O -labeled H_2O and O_2 indicated that the quinone methide and radical intermediates formed by direct C α -C β cleavage of propyl side chain by laccase react with water and molecular oxygen, respectively, and converted to the identified products. Possible mechanisms for alkyl-aryl cleavage and C α oxidation were proposed.

In Section 3.3, the degradation of phenolic β -O-4 model compounds by laccase was discussed (Kawai et al., 1989a). The substrate was degraded by alkyl-aryl cleavage and C α oxidation as reported previously (Kirk et al., 1968b). The results further suggested that the α -carbonyl derivative further cleaved between C α and C β carbons to give a new phenol.

The possibility of aromatic ring cleavage by laccase was described in Section 3.4 (Kawai et al., 1988b). When 4,6-di-*tert*-butyl-2-methoxyphenol was used as the substrate for laccase, a muconolactone, 2,4-di-*tert*-butyl-4-methoxycarbonylmethyl-2-buten-4-olide, was first identified as aromatic ring cleavage product. Tracer experiments with ^{18}O labeled H_2O and O_2 indicated that two atoms of molecular oxygen were concerned with the formation mech-

CONCLUSION

anism of the muconolactone.

Laccase is known to oxidize phenolic substrates via various reactions, but not nonphenolic substrates. Here, the oxidation of nonphenolic benzyl alcohols by laccase in the presence of syringaldehyde was investigated (Kawai et al., 1989b). The results suggested that the phenoxy radical of syringaldehyde formed by laccase catalyzed the oxidation of nonphenolic benzyl alcohols to give benzaldehydes. However, nonphenolic β -1 model compounds could not be degraded in the present conditions.

The oxidative reactions by lignin peroxidase and laccase of *C. versicolor* are summarized in the Table. The principle of the reaction by both enzymes is one-electron oxidation of substrates. Autooxidative reactions subsequently proceed via aryl cation radicals and phenoxy radicals. Lignin peroxidase can oxidize both

TABLE

The Oxidative Reactions by Lignin Peroxidase and Laccase of *C. versicolor*

	Lignin peroxidase	Laccase
Enzymic reaction	One-electron oxidation of phenolic and non-phenolic compounds	One-electron oxidation of phenolic compound
Nonenzymic reactions	Ca-C β cleavage β -Ether cleavage Aromatic ring cleavage Ca oxidation	Ca-C β cleavage Alkyl-aryl cleavage Aromatic ring cleavage Ca oxidation

CONCLUSION

phenolic and nonphenolic compounds, but laccase can not oxidize nonphenolic compounds. The oxidation potential of lignin peroxidase is higher than that of laccase.

Lignin peroxidase was recently isolated from *Phlebia radiata* (Niku-Paavola et al., 1988) and its activity was detected in the culture of *Coriolus hirstus* (Yoshihara et al., 1988) and *Chrysosporium pruinsum* (Waldner et al., 1988). However, no lignin peroxidase activity could be detected in the ligninolytic culture of some white-rot fungi, *Lentinula edodes* (Leatham, 1986), *Pleurotus ostreatus*, *Bjerkandera adusta*, *Trametes cingulata* and *Fomes lignosus* (Waldner et al., 1988).

On the other hand, laccase is produced by most of white-rot fungi. Lignin contains 10-20% of phenolic hydroxyl groups which are attacked by both lignin peroxidase and laccase. The phenolic hydroxyl groups could be increased during side chain cleavage catalyzed by lignin peroxidase and laccase. It is therefore concluded that laccase is also an important lignin-degrading enzyme. Recently, Mn-dependent peroxidase was isolated from *P. chrysosporium* (Kuwahara et al., 1984), *L. edodes* (Forrester et al., 1988) and *C. versicolor* (Johansson & Nyman, 1989) and found to catalyze similar reactions via phenoxy radicals as by laccase (Wariishi et al., 1989).

It is concluded that the degradation of lignin in wood by *C. versicolor* is initiated by both lignin peroxidase and laccase.

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