DEGRADATION OF LIGNIN SUBSTRUCTURE MODEL COMPOUNDS BY *FUSARIUM SOLANI* M-13-1

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INTRODUCTION

Lignin is one of major components of cell walls in vascular plants higher than ferns, particularly in wood tissues of trees[1]. The lignin contents of coniferous wood, dicotyledonous wood, and grass are 25% to 33%, 17% to 25%, and 15% to 20%, respectively. Lignin occurs as a matrix component with hemicelluloses in the spaces of intercellulose microfibrils in cell walls and as a cementing component in intercellular layer to connect cells one another and to harden the cell walls[2]. Thus, the lignified tissues gain mechanical strength and resist microbial attacks. Lignin hinders the permeation of water across the cell walls in the conductive xylem tissues by its hydrophobic nature[3]. Distribution of lignin in individual cell wall is heterogeneous. The lignin concentration is high in the middle lamella and low in the secondary wall. But, the largest portion of lignin is located in the secondary wall because of its high thickness[4].

Lignin is dehydrogenative polymers of p-hydroxycinnamyl alcohols by peroxidase interconnected by various types of linkages such as arylglycerol-β-aryl ether (β-O-4), phenylcoumaran (β-5), biphenyl (5-5'), 1,2-diarylpropane-1,3-diol (β-1), non-cyclic benzyl aryl ether (α-O-4), pinoresinol (β-β'), diphenyl ether (3-O-4')[5]. Coniferous lignin is a dehydrogenative polymer of coniferyl alcohol. Angiosperm lignin is a mixed dehydrogenative polymer of coniferyl alcohol and sinapyl alcohol. Grass lignin is a mixed dehydrogenative polymer of coniferyl, sinapyl, and p-

hydroxycinnamyl alcohols, and some of the γ-position of the polymer are esterified with p-hydroxycinnamic acid[6]. Lignin is linked and associated with hemicelluloses[7]. Lignin structure as well as its distribution in cell wall is heterogeneous. In hard woods the lignin in the secondary wall of the fibers contains predominantly syringyl units, and the vessel lignin consists mostly of guaiacyl units[8].

Therefore, lignin is an irregular and complex aromatic polymer joined by various bonds which are chemically and biochemically stable carbon-to-carbon and ether bonds, and has no hydrolyzable repeating units. Furthermore lignin is a racemic compound. Thus, lignin is different from other biopolymers, such as polysaccharides, proteins, and nucleic acids which are easily hydrolyzed and catabolized stereospecifically.

Lignin is the most abundant and renewable biomass next to cellulose on the earth. The amount of carbon fixed in lignin by photosynthesis is comparable to that in cellulose. Lignin plays an important role in carbon cycle[9,10] on the earth and in humus formation. On the other hand, forestry, agriculture, wood industry, and pulp and paper industry produce large quantities of lignocellulosic waste materials.

Twice oil crisis of the 1970's allowed us to recognize the finiteness of fossil resources. It is predicted that recent increase in world population will result in the aggravation of the food situation. Environmental preservation is required for living of human being. It has been recognized that biological reactions produce desired products selectively in good yields at ordinary temperature and

atmospheric pressure without by-products. The biological reaction which is resource saving, energy saving, and non-environmental pollution is expected for various industrial applications. It has became feasible to increase enzyme production efficiency by improvement of microbial function using genetic engineering techniques such as recombination of DNA, cell fusion, and mutation. Development of bioreactor with immobilized enzyme and microorganism made possible the stable and efficient utilization of enzyme, and the use of contineous and large scale bioprocess.

However, lignin biodegradation has not fully been clarified. Elucidation of lignin biodegradation is very important for pure science such as biochemistry and ecology, and for industrial application of lignins, woody resources, and lignocellulosic waste materials.

Lignin utilization is very restricted in spite of the efforts by many investigators. In kraft pulping, lignin released in waste liquor is concentrated and burned only to recycle the reagent and to save energy. In sulfite pulping, a part of lignosulfonate is used as dispersing agent, such as admixtures for the preparation of cement and concrete, and as raw materials of vanillin. New biochemical methods, to be applied for lignin utilization, are expected.

The lignin degrading microorganism has been used recently in several applied and industrial projects. Pulp industry consumes much resource and energy, and release much waste liquor. To improve such disadvantages, remarkable is biological pulping proposed by Eriksson et al., who isolated and exploited cellulase-less mutant of a white-rot

fungus[11,12]. Biomechanical pulping and biobleaching of kraft pulp are considered to be practical application, even if the microbial delignification is not complete but partial[13]. Fungal treatment of waste liquor is important to their decolorization[14] and to removal of mutagen and carcinogen such as lignin-derived chlorinated phenols[15].

In addition to pulping, biochemical conversion of cellulose and hemicellulose in wood and lignocellulose such as straw and baggase to useful substances is important. Biochemical removal of the lignin barrier is necessary to increase accesibility of cellulase and hemicellulases to cell wall polysaccharides. Following applications could be possible[16]: preparation of food and feedstuff for ruminants as single-cell protein, saccharification and fermentaion giving fine chemicals, and alcohol or methane as energy resources.

Thus, lignin biodegradation and bioconversion research are very important for the subjects on resource, food, and environment.

Lignin is considerablly resistant for both chemical and enzymic attacks, and chemistry of lignin biodegradation has been studied through the following approaches: a) characterization of polymeric degraded lignin separated from decayed wood, b) identification of low molecular weight degradation products extracted from decayed wood, c) degradation of dimeric and trimeric lignin-substructure model compounds.

Early studies on the analysis of white-rotted lignin showed the decrease of structures yielding vanillin on nitrobenzene oxidation[17], less content of carbon, methoxyl,

and hydrogen than in sound lignin, more content of oxygen, carbonyl group, and carboxyl group, and increasing of structures yielding vanillic acid on hydrolysis with dioxane-water[18,19,20]. Hata[20] suggested that lignin was degraded by oxidative shortening of the terminal α - or β -coniferyl alcohol ether moiety to the corresponding vanillic acid ether moiety followed by the cleavage of the ether linkage.

Kirk and Chang[21,22] characterized heavily degraded lignin isolated from spruce wood decayed by white-rot fungi, Coriorus versicolor and Polyporus anceps, by elemental and methoxyl analysis, functional group analysis, spectroscopy (UV, IR, ^{1}H-NMR), and chemical degradation. The degraded milled wood lignins (MWLs) were about one atom less in hydrogen, one atom richer in oxygen, and about 25% deficient in methoxyl as compared to MWL from the sound wood. The degraded MWL was lower in phenolic hydroxyl group and higher in conjugated carbonyl and carboxyl groups than in the sound MWL. The carboxyl group consisted of aromatic (16%) and aliphatic (43%). They presumed that the aliphatic acid was formed by the cleavage of aromatic rings.

Chen and Chang[23] characterized degraded lignins isolated from spruce and birch woods decayed by a white-rot fungus, Phanerochaete chrysosporium, by ^{13}C-NMR spectroscopy. The biodegraded lignin contained substructures of the types α -oxoarylpropanes, 4-O-alkylvanillins, 4-O-alkylvanillic acids, 4-O-alkylvanillyl alcohols, alkoxyacetic acids, aroxyethanols, and aroxyacetic acids, and their syringyl derivatives. From the results and isolated degradation products described below, they considered that those struc-

tures were formed by way of Cα -Cβ cleavage, a combination of Cα oxidation in β -O-4 structure, Cβ -Cγ cleavage, and the reductive cleavage of the ether linkage, demethylation, oxidative cleavage of aromatic rings, and other reactions.

So far, as degradation products of lignin by white-rot fungi, vanillin, vanillic acid, syringaldehyde, syringic acid, 2,6-dimethoxy-p-benzoquinone, methoxyhydroquinone, methoxy-p-benzoquinone, coniferaldehyde, guaiacylpyruvic acid, guaiacylglycerol-β -coniferyl ether, p-hydroxybenzoic acid, ferulic acid, p-hydroxycinnamic acid, dehydrodivanillin, p-hydroxycinnamaldehyde, and guaiacylglycerol were reported[24]. Among which, however, identification of the first eight compounds was secure but that of the other compounds was not adequate[24].

Chen and Chang[22] systematically analyzed low molecular weight degradation products from the above decayed wood, and identified five phenols, especially including acetosyringone, and nineteen aromatic acids by gas chromatography (GC), high-performance liquid chromatography (HPLC), and GC-mass spectrometry (MS) using authentic samples.

To clarify the mechanism of lignin biodegradation, a limitation is present in the use of polymeric lignin which is irregular and complex. It is difficult to follow precisely the conversion of the functional groups and the cleavage of the specific linkages in lignin polymer, during degradation. Since lignin is a complicated and unique polymer and it formes a composite structure in the cell wall, as mentioned above, the biodegradation rate of polymeric lignin or native lignin is slow. The low molecular weight degrada-

tion products are not accumulated, because a variety of the degradation products are produced in a small amount and their degradation rate is faster than that of polymeric lignin[24]. It is indispensable to use various lignin substructure model compounds for the elucidation of lignin degradation mechanism.

Since arylglycerol-β-aryl ether (β-O-4) is the most abundant substructure and is contained 40-60% in lignin, such substructure models have been mainly used for biodegradation studies. Ishikawa et al.[25] reported that veratrylglycerol-β-guaiacyl ether was demethylated at C_4 position by Fomes formentarius and Poria subacida to give guaiacylglycerol-β-guaiacyl ether whose β-O-4 linkage was further cleaved to afford guaiacylglycerol. They considered that the formation of guaiacylglycerol was resulted from the direct hydrolysis of the β-O-4 linkage. Fukuzumi and Shibamoto[26] also found that veratrylglycerol-β-guaiacyl ether was transformed to guaiacylglycerol-β-guaiacyl ether whose β-O-4 linkage was split to yield guaiacol and guaiacylglycerol by an enzyme from Poria subacida. Fukuzumi et al.[27] further found that the enzyme required NADH in both cleavage reactions. They speculated that the formation of two degradation products resulted by hydroxylation of β-carbon of the β-guaiacyl ether by a monooxygenase followed by the hydrolysis of the resulting hemiketal. Ishikawa and Oki[28] also reported the cleavae of guaiacylglycerol-β-guaiacyl ether by C. versicolor and F. formentarius.

However, Higuchi[29] pointed out that such degradation

studies were carried out on a small scale, and the degradation products isolated were analyzed only by UV spectroscopy, papaer chromatography, and thin layer chromatography (TLC), which resulted in inconclusive identification.

Kirk et al.[30] found that guaiacylglycerol-β-guaiacyl ether and veratrylglycerol-β-guaiacyl ether were converted to dehydrodiveratrylglycerol-β-guaiacyl ether and α-guaiacoxy-β-hydroxypropioveratrone, respectively, by C. versicolor and Stereum frustulatum, and no cleavage of their β-ether linkages occurred. They[31] further found that syringylglycol-β-guaiacyl ether was oxidized to α-guaiacoxyacetosyringone, whose alkyl-aryl C-C bond was cleaved to yield guaiacoxyacetaldehyde, guaiacoxyacetic acid, and 2,6-dimethoxy-p-benzoquinone, and that the degradation was catalyzed by a laccase.

In 1978, Kirk et al.[32] established ligninolytic culture conditions of P. chrysosporium. Since then, it has been recognized that the culture condition was suitable for most Bacidiomycetes. By using the ligninolytic culture of P. chrysosporium, Gold et al. studied the degradation of guaiacylglycerol-β-guaiacyl ether[33] and 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether[34,35].

Bacterial cleavage of the β-O-4 model was reported by some investigators. Crawford et al.[36,37] found that initial transformation of veratrylglycerol-β-guaiacyl ether by Pseudomonas acidovorans was demethylation at C_4 position and oxidation at Cα hydroxyl group, and then its β-ether bond was cleaved to give guaiacol and vanillic acid. Fukuzumi and Katayama[38] found that guaiacylglycerol-β-coniferyl

ether was degraded by Pseudomonas sp.[39] to give α-hydroxypropiovanillone and coniferyl alcohol indicating the cleavage of the β-O-4 linkage. They also investigated the degradation of dehydrodiconiferyl alcohol[40], a β-5 substructure model, and of a modified β-1 substructure model[41].

Toms and Wood[42] examined the degradation of α-conidendrin, a lignan, by Pseudomonas multivorans.

In this investigation, model compounds as substrates and authentic samples of catabolic products were synthesized. The design of the model compounds must be exact in conformity with enzymic specificity. Identification of some catabolic products in earlier studies were unreliable. Definite identification by comparison with authentic samples was restricted to the case of simple compounds. Since, generally, the yield of a catabolic product isolated is low, it is neccesary to use the substrate in large scale. Preparation of all of the substrate and catabolic products from dehydrogenative oligomers of coniferyl alcohol are difficult. Therefore, syntheses of lignin substructure models and authentic samples of catabolic products are very important. Nakatsubo[43] developed a general synthetic method of dimeric and trimeric lignin substructure models consists of β-O-4, β-5, β-1, and β-β' linkages. In this study, model compounds as substrates were synthesized by his methods and their modifications. Most of the authentic samples were synthesized from the model compounds and their derivatives. Almost all catabolic products were identified by the chromatographic and spectrometric comparison with the authentic samples.

Iwahara et al.[44] isolated about 50 strains of microorganisms, including bacteria, yeasts, and molds which grow on a medium containing DHP of conifery alcohol as a sole source of carbon, from soils, rotted wood, and sewers using enrichment techniques. The molds among these isolated microorganims degraded the DHP extensively, and almost all of the isolated molds were identified as Fusarium spp., among which Fusarium solani M-13-1 exhibited best growth on a glucose-peptone medium and it degraded DHP and dilignols such as guaiacylglycerol-β-coniferyl ether, dehydrodiconiferyl alcohol, and dl-pinoresinols. Thus, F. solani M-13-1 was used in this study. Ohta et al. reported degradation of dehydrodiconiferyl alcohol by the fungus[45].

After their investigations, Norris[46] found that Fusarium solani AF W1 was degraded ^{14}C-labelled DHP as a sole carbon source to generate $^{14}CO_2$. Sutherland et al.[47] found that eighteen Fusarium spp. degraded lignocellulose containing ^{14}C-labelled lignin. However, degradation mechanism of lignin by their strain has not been investigated.

In chapter 1, degradation of arylglycerol-β-aryl ethers by F. solani M-13-1 was described. Firstly, arylglycerol-β-aryl ethers were synthesized to use as substrates and synthetic authentic samples of catabolic products[48] (section 1.1). And then, initial degradative reaction of side chain of guaiacylglycerol-β-coniferyl ether[49] (section 1.2), cleavage of alkyl-aryl C-C bonds of arylglycerol-β-aryl ethers[50] (section 1.3), and cleavage of 2- (β-) aryl ether bond of glycerol-2-aryl ethers formed by the cleavage of alkyl-aryl C-C bonds[51] (section

1.4) were described.

In chapter 2, catabolism of a phenylcoumaran and a phenylcoumarone by the fungus was described[52,53]. The phenylcoumarone was found to be a catabolic intermediate of the phenylcoumaran.

In chapter 3, degradation of an arylglycerol-α,β-diaryl ether, a trimeric non-cyclic benzyl aryl ether substructure model, was described. Firstly, an adequate model compound used in this biodegradation experiment was synthesized[54,55] (section 3.1). And then, biodegradation of the model compound was investigated[54] (section 3.2).

In chapter 4, degradation and stereoselective reduction of α-carbonyl derivative of an arylglycerol-β-aryl ether by the fungus was described. Firstly, degradation and reduction of the α-carbonyl derivative were investigated[56] (section 4.1). The reduction product, major catabolic intermediate, was found to be optically active. Thus, the enantiomeric purity of the reduction product was examined[57] and then its specific rotation was determined (section 4.2).

CHAPTER 1

DEGRADATION OF ARYLGLYCEROL-β-ARYL ETHERS BY *FUSARIUM SOLANI* M-13-1

1.1 Syntheses of Arylglycerol-β-Aryl Ethers

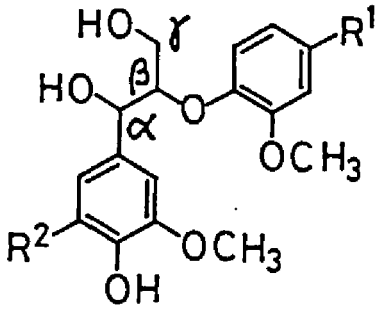
1.1.1 INTRODUCTION

Arylglycerol-β-aryl ether (β-*O*-4) bond is a major intermonomer linkage in lignin[58]. Syntheses of the β-*O*-4 substructure models are, therefore, very important to study chemical structure, reactivity, and biodegradation of lignin. Guaiacylglycerol-β-guaiacyl ether has been synthesized by many investigators and widely used as a lignin model. The compound is, however, not fully adequate as the β-*O*-4 model because it has no side chain at the *para* position of the phenoxy moiety. Freudenberg *et al.* obtained guaiacylglycerol-β-coniferyl ether (*6*) by dehydrogenation of coniferyl alcohol[59] and synthesized it *via* a β-hydroxy ketone derivative[60]. Nimz[61] isolated guaiacylglycerol-β-vanillin ether (*1*) from the hydrolysis products of spruce lignin and *1* was again synthesized *via* a β-hydroxy ketone derivative. But the yields in both cases were low. Very recently, Nakatsubo and Higuchi[62] established the high yield syntheses of guaiacylglycerol-β-coniferaldehyde ether (*5*) and β-coniferyl ether (*6*) from coniferaldehyde and vanillin.

In the study of degradation of guaiacylglycerol-β-coniferyl ether (*6*) by *F. solani* M-13-1 (section 1.2 and 1.3), various β-*O*-4 dimers, from *1* to *5* in Fig. 1-1, as the

catabolic products were obtained. These dimers have to be synthesized to confirm their chemical structures and to investigate their further catabolism.

This section describes a high yield synthesis of guaiacylglycerol-β-vanillin ether (1) which could be converted to other β-O-4 dimers, such as 2–5, by functional group transformation and side chain extention.



	R^1	R^2
	α′	
1	CHO	H
2	CH_2OH	H
3	COOH	H
	α′ β′ γ′	
4	CH=CH–COOH	H
5	CH=CH–CHO	H
6	CH=CH–CH_2OH	H
7	CHO	OCH_3

Fig. 1-1 Arylglycerol-β-aryl ethers

1.1.2 RESULTS AND DISCUSSION

Guaiacylglycerol-β-vanillin ether (1) was synthesized as shown in Fig. 1-2 through six reaction steps from vanillin. Methyl (4-formyl-2-methoxyphenoxy)acetate (8) was prepared by stirring the reaction mixture of vanillin, methyl monochloroacetate, K_2CO_3, and KI in acetone at reflux temperature. Under this condition the reaction proceeded through 3 hr without hydrolysis of the methyl ester group. Treatment of 8 with methyl orthoformate and p-toluenesulfonic acid (p-TsOH) in methanol afforded dimethylacetal derivative 9. Condensation of 9 with O-benzylvanillin by use of lithium diisopropylamide (LDA) in tetrahydrofuran (THF) at -78℃ gave β-hydroxy ester derivative 10 in high yield. The reduction of 10 with $LiAlH_4$ in THF at 50℃ yielded 1,3-diol derivative (11), which was converted to 1 by catalytic reduction with palladium/charcoal (Pd-C) in methanol and by subsequent treatment with 1N HCl in dioxane. The over-all yield of 1 from vanillin was 65%. These acetal derivatives, 10, 11, and 12 were found to be unstable and must be kept in a refrigerator. Cleavage of the acetal group occurred when those compounds were allowed to stand for several days at room temperature or submitted to silica gel column chromatography (Wako gel C-100), although those were able to purify with a silica gel TLC plate (Merck silica gel 60 PF_{254}). Therefore, the respective steps from 10 to 12 were performed as soon as possible without purification. Compound 1 was easily purified by silica gel column chromatography, since the respective reaction steps from 10

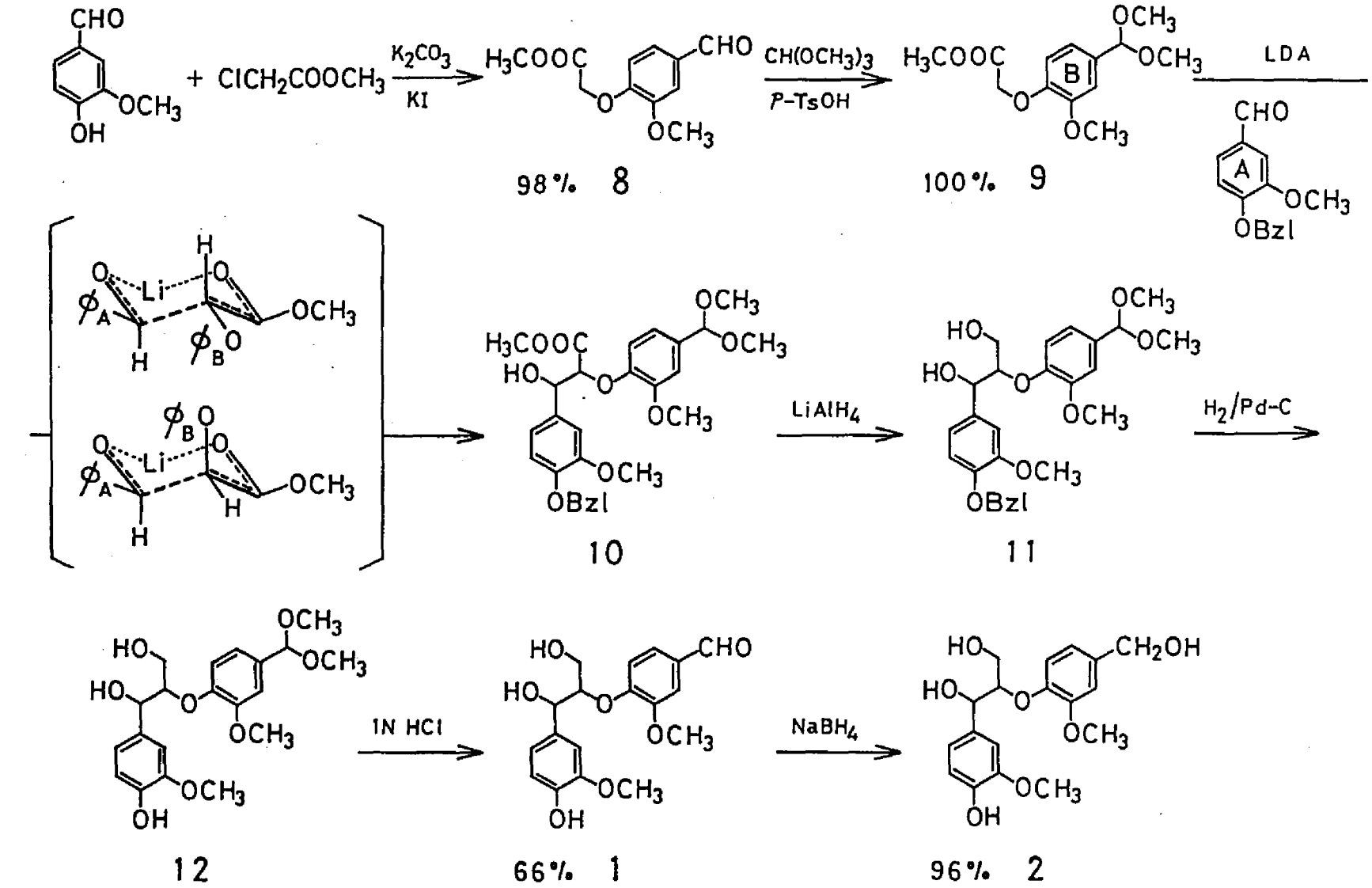


Fig. 1-2 Synthetic route for guaiacylglycerol-β-vanillin ether (1) and β-(vanillyl alcohol) ether (2)

to 1 proceeded almost quantitatively.

Various β-O-4 dimers composed of guaiacyl, syringyl, and p-hydroxyphenyl nuclei could be obtained by the present method. As an example, syringylglycerol-β-vanillin ether (7) was synthesized from vanillin and syringaldehyde by the same procedure as in 1 in 63% over-all yield, which indicated that the present synthetic method is a generally applicable one.

The condensation of lithium enolate of 9 with O-benzylvanillin conceivably proceeded via a six-membered transition state, in which the trans diequatorial orientation of the bulky groups (aryl and aroxy groups) would be more favorable than cis orientation because of the steric repulsion[63]. The trans diequatorial orientation leads to erythro form, and the cis orientation does threo form. Consequently, erythro form would predominate over threo form. The ratio of erythro and threo form of 10 was estimated to be 3:1 by the separation of the respective isomers and also by ^{1}H-NMR spectroscopy of the mixture. Compound 1 was obtained as an inseparable mixture of erythro and threo forms with the same ratio of compound 10.

Guaiacylglycerol-β-(vanillyl alcohol) ether (2) was obtained by reduction of 1 with $NaBH_4$ in methanol at 0℃ and also prepared from 11 by cleavage of the acetal group and by subsequent catalytic reduction with 10% Pd-C. Miksche[64] synthesized syringylglycerol-β-(syringyl alcohol) ether via a β-hydroxy ketone derivative, but the yield was not reported.

Guaiacylglycerol-β-(vanillic acid) ether (3) which was

prepared from 13 by Ag_2O oxidation and by subsequent catalytic reduction did not crystallize because 13 was obtained as a mixture of erythro and threo forms. To obtain 3 as crystals, 13 was converted to its acetonide (isopropylidene ketal) derivative 14 with 2,2-dimethoxypropane and camphorsulfonic acid (CSA) in acetone[65] (Fig. 1-3) and each isomer of 14 was separated by silica gel column chromatography. It was found that 1,3-O-alkylidene structures, as protecting groups of the 1,3-diol of 13, were useful to separate the both isomers chromatographycally. It would be ascribed that the formation of six-membered ring fixed the conformation of the 1,3-diol structure. Oxidation of erythro-14 with $KMnO_4$ in dioxane afforded erythro-15 which gave erythro-16 by cleavage of the cyclic ketal with 1N HCl in dioxane without isomerization at α-position. Catalytic reduction of erythro-16 with Pd-C in methanol yielded erythro-3 as a colorless crystal. Threo-14 also gave threo-3 in almost the same yield as in the case of erythro form. The over-all yield of 3 from 11 was 60%. Compound 3 was also synthesized from 13 through formation of benzylidene protecting group (benzaldehyde dimethylacetal/ p-TsOH/ benzene/ room temperature (r.t.)/ 80%), $KMnO_4$ oxidation ($KMnO_4$/ dioxane/ r.t./ 95 %), and deprotection by catalytic reduction (H_2/ 10% Pd-C/ acetic acid/ 50 ℃/ 80%).

The use of the isopropylidene protecting group does not lead a new chiral center, and hence 14 is believed to be an important intermediate to synthesize oligomeric lignin models, different from the case of other alkylidene group. A trilignol composed of β-O-4 and β-1 substructures was

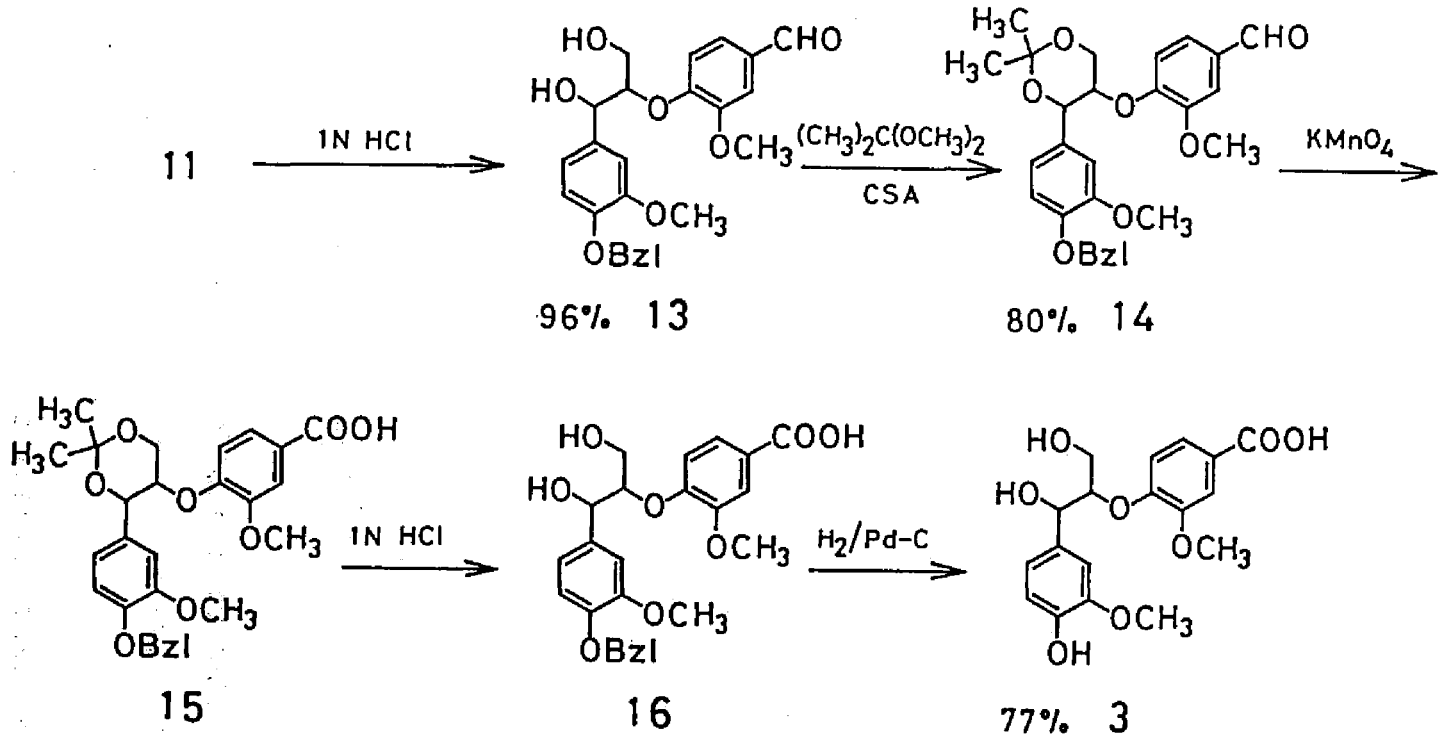


Fig. 1-3 Synthetic route for guaiacylglycerol-β-(vanillic acid) ether (3)

recently synthesized via this isopropylidene derivative 14[65].

Guaiacylglycerol-β-(ferulic acid) ether (4) was prepared by the Knoevenagel reaction of 1 with malonic acid and piperidine in pyridine at 80℃ (Fig. 1-4).

Guaiacylglycerol-β-coniferaldehyde ether (5) was synthesized from 1 as shown in Fig. 1-4. Compound 1 was converted to tri-tetrahydropyranyl (THP) ether derivative 17 with 2,3-dihydro-4H-pyran and p-TsOH in dichloromethane at 0 ℃. The Knoevenagel reaction of 17 under the same condition as above afforded β-(ferulic acid) ether derivative 18 which was converted to 19 by treatment with diazomethane. Reduction of 19 with $LiAlH_4$ in THF at -25 ℃ gave 20, whose allyl alcohol group was oxidized to the corresponding aldehyde group with active MnO_2[66] in carbon tetrachloride. The removal of the tri-THP ethers of 21 with 1N HCl in dioxane at room temperature yielded 5 without isomerization at the α-position. The over-all yield of 5 from 1 was 55%. Side chain extention of 17 by the Wittig reaction (1,3-dioxane-2-ylmethyltriphenylphosphonium bromide,[63,67]/ THF / t-BuOK / t-BuOH) also gave 21. Guaiacylglycerol-β-coniferyl ether (6) was easily obtained by $NaBH_4$ reduction of 5[62]. On the other hand, cleavage of the tri-THP ethers of 20 did not proceed smoothly[62].

Both guaiacylglycerol-β-coniferyl ether (6) and β-coniferaldehyde ether (5) were obtained as dehydrogenation products of coniferyl alcohol[58,68], and also isolated from the hydrolysis products of spruce lignin[61,69]. Recently, Nakatsubo and Higuchi[62] found that synthetic compounds 5

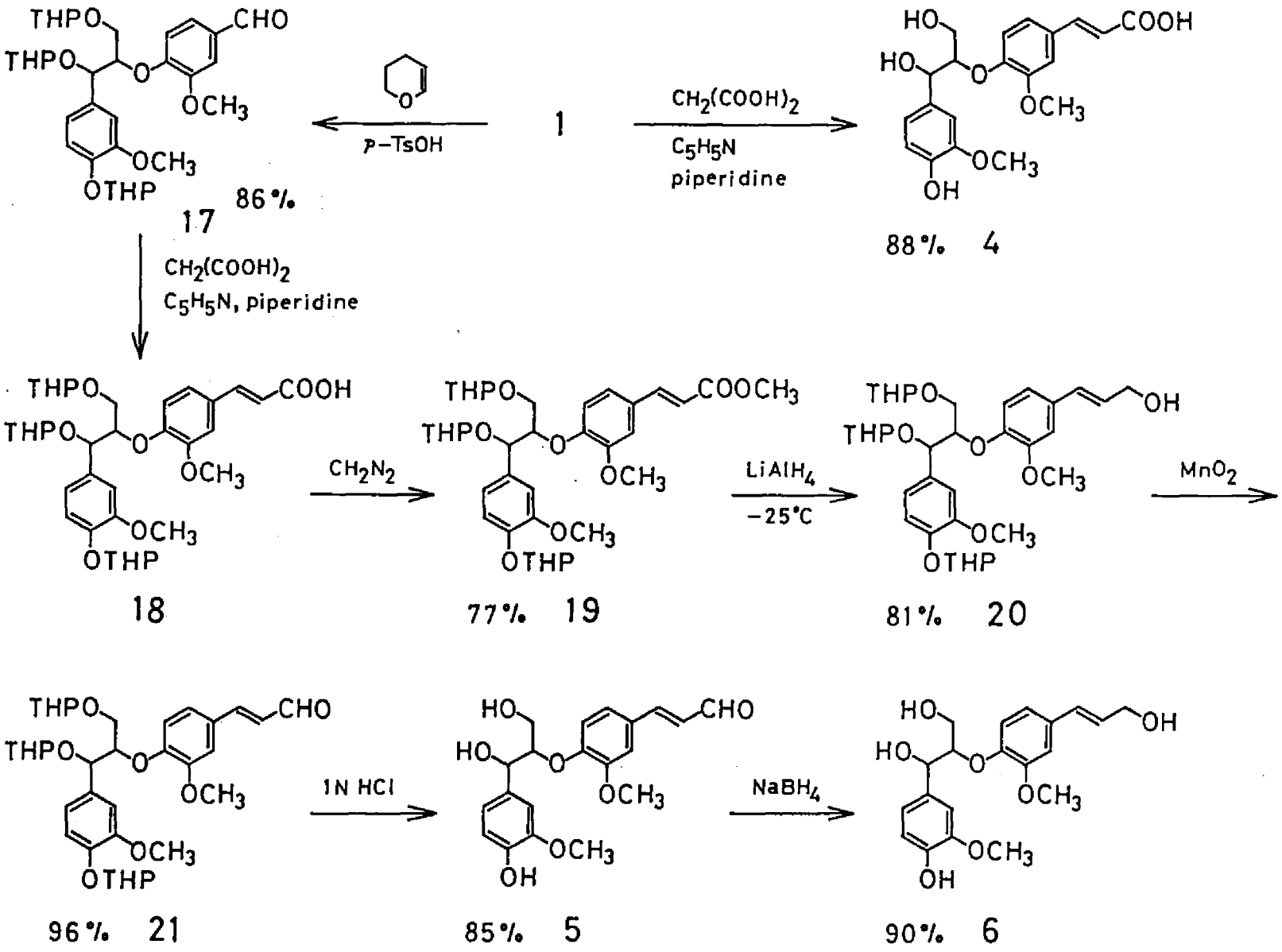


Fig. 1-4 Synthetic route for guaiacylglycerol-β-(ferulic acid) ether (4), β-coniferaldehyde ether (5), and β-coniferyl ether (6)

and 6 from coniferaldehyde and vanillin were identical with those obtained by dehydrogenation of coniferyl alcohol.

Next section shows that all arylglycerol-β-aryl ethers described here are identical with those obtained in catabolism of 6 by F. solani M-13-1.

1.1.3 EXPERIMENTAL

All the melting points were uncorrected. Analytical and preparative TLC were conducted using precoated plates with Merck silica gel 60 F_{254} (0.25 mm thickness) and plates coated with Merck silica gel 60 PF_{254} (2 mm). Ultraviolet (UV) spectra and infrared (IR) spectra were taken by a Hitachi model 200-20 double beam spectrometer and by a Jasco model IR-S, respectively. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a Hitachi R-22 high resolution NMR spectrometer (90 MHz), with tetramethylsilane as an internal standard. Chemical shifts (δ) and coupling constant (J) are expressed in ppm and Hz, respectively. Peak multiplicities are abbreviated singlet s, doublet d, triplet t, quartet q, and multiplet m. Mass spectra (MS) were determined with a Shimadzu LKB 9000 gas chromatograph-mass spectrometer with a direct inlet system at an ionizing voltage of 70 eV; the relative intensity of each peak is designated in parentheses.

Methyl (4-formyl-2-methoxyphenoxy)acetate (8)

To a stirred solution of 100 g (0.657 mol) of vanillin in 1.4 liters of acetone were added 78.8 g (0.723 mol) of

methyl monochloroacetate, 99.9 g (0.723 mol) of K_2CO_3, and 12.0 g (0.0723 mol) of KI. The mixture was refluxed for 3 hr with vigorous stirring and then cooled to room temperature. The inorganic salts were filtered off and washed with EtOAc. The filtrate and the washings were combined and concentrated in vacuo. The residue was dissolved in EtOAc. The solution was washed successively with water and saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. Crystallization of the residue from EtOAc-n-hexane gave 144.9 g (98 %) of colorless needles. Mp. 92-93℃ (EtOAc). Anal. Calcd. for $C_{11}H_{12}O_5$: C, 58.93; H, 5.39, Found: C, 58.86; H, 5.25. UV λ_{max}^{EtOH} nm (log ε): 228 (4.21), 272 (4.06), 308 (3.90). IR ν_{max}^{KBr} cm^{-1}: 1747 (C=O), 1688 (C=O). ^{1}H-NMR ($CDCl_3$): δ 3.81 (3H, s, $-COOCH_3$), 3.93 (3H, s, $Ar-OCH_3$), 4.78 (2H, s, $-CH_2-$), 6.82-7.44 (3H, Ar-H), 9.79 (1H, s, -CHO). MS m/z (%): 224 (100, M^+), 165 (36), 151 (41), 150 (24), 149 (12), 137 (11), 119 (16), 105 (20).

Methyl (4-dimethoxymethyl-2-methoxyphenoxy)acetate (9)

To a stirred solution of 6.72 g (30 mmol) of 8 in a mixture of 32.9 ml (31.8 g, 300 mmol) of methyl orthoformate and 60 ml of MeOH was added 80 mg of p-TsOH at room temperature. After 30 min the reaction solution was neutralized by the addition of $NaHCO_3$, which was then filtered off and washed with EtOAc. The filtrate and the washings were combined and concentrated in vacuo. The residue was dissolved in EtOAc. The solution was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. Crystallization of the residue from EtOAc-n-hexane gave 8.06 g

(100 %) of colorless needles. Mp. 55-56℃ (EtOAc). Anal. Calcd. for $C_{13}H_{18}O_6$: C, 57.77; H, 6.71, Found: C, 57.65; H, 6.78. UV λ_{max}^{EtOH} nm (log ε): 228 (3.91), 277 (3.41). IR ν_{max}^{KBr} cm^{-1}: 1785 (C=O). ^{1}H-NMR ($CDCl_3$): δ 3.32 (6H, s, $-CH(OCH_3)_2$), 3.78 (3H, s, $-COOCH_3$), 3.89 (3H, s, $Ar-OCH_3$), 4.68 (2H, s, $-CH_2-$), 5.31 (1H, s, $-CH(OCH_3)_2$), 6.75-7.04 (3H, Ar-H). MS m/z (%): 270 (11, M^+), 240 (14), 239 (100), 224 (11), 211 (5), 165 (10), 151 (21), 137 (5), 119 (4), 105 (3).

β-Hydroxy ester 10

To a stirred solution of 2.52 ml (1.82 g, 18.0 mmol) of diisopropylamine (freshly distilled from sodium metal) in 20 ml of anhydrous THF (freshly distilled from pottasium metal and benzophenone) was added dropwise 10.09 ml (18.0 mmol) of a solution of 1.65 N n-butyl lithium in n-hexane over a period of 30 min at 0℃ under nitrogen. The stirring was continued for additional 30 min at the same temperature, and then the resulting lithium diisopropylamide solution was cooled to -78℃. To the stirred cold solution was added dropwise a solution of 4.05 g (15.0 mmol) of 9 in 40 ml of anhydrous THF over a period of 30 min at -78℃. The stirring was continued for additional 30 min at the same temperature. To the stirred solution was added dropwise a solution of 3.63 g (15.0 mmol) of O-benzylvanillin in 40 ml of anhydrous THF over a period of 30 min at -78℃. After stirring for additional 90 min below -70℃, the reaction solution was neutralized by the addition of powdered dry ice and partitioned between EtOAc and water. The aqueous layer was

extracted twice with EtOAc. The organic layers were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give 7.9 g of a crude glass which was used for the subsequent $LiAlH_4$ reduction without further purification. An aliquot (45 mg) of the crude product was purified by TLC developed with EtOAc-*n*-hexane (= 1:3) to give 27 mg of *erythro* form (R_f 0.25) and 10 mg of *threo* form (R_f 0.23). UV λ_{max}^{EtOH} nm (log ε): 231 (4.28), 279 (3.81). IR ν_{max}^{KBr} cm^{-1}: 1755 (C=O). ^{1}H-NMR ($CDCl_3$): (*erythro*): δ 3.30 (6H, s, $-CH(OCH_3)_2$), 3.65 (3H, s, $-COOCH_3$), 3.82 (3H, s, Ar-OCH_3), 3.87 (3H, s, Ar-OCH_3), 4.70 (1H, d, $J\alpha\,\beta$ = 5.6, β -CH-), 5.09 (1H, d, $J\alpha\,\beta$ = 5.6, α -CH-), 5.11 (2H, s, $-OCH_2Ph$), 5.27 (1H, s, $-CH(OCH_3)_2$), 6.25-7.50 (11H, Ar-H), (*threo*): δ 3.32 (6H, s, $-CH(OCH_3)_2$), 3.56 (3H, s, $-COOCH_3$), 3.86 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 4.51 (1H, d, $J\alpha\,\beta$ = 6.6, β -CH-), 5.06 (1H, d, $J\alpha\,\beta$ = 6.6, α -CH-), 5.12 (2H, s, $-OCH_2Ph$), 5.27 (1H, s, $-CH(OCH_3)_2$), 6.75-7.50 (11H, Ar-H). MS *m/z* (%): 494 (0.2, M^+), 464 (0.1), 463 (0.2), 448 (0.3), 403 (0.6), 270 (8), 242 (11), 240 (12), 239 (79), 224 (8), 211 (5), 179 (6), 167 (8), 165 (9), 151 (20), 137 (4), 136 (5), 135 (5), 119 (5), 105 (5). 95 (7), 91 (100).

Diol 11

To a stirred suspension of 1.78 g (46.8 mmol) of $LiAlH_4$ in 40 ml of anhydrous THF was added dropwise a solution of 7.9 g (15 mmol, a crude glass) of 10 in 50 ml of anhydrous THF over a period of 30 min at 50℃ under nitrogen. The stirring was continued for additional 15 min at the same

temperature. The reaction mixture was then cooled to 0℃ and the excess $LiAlH_4$ was decomposed by the addition of wet Et_2O followed by the dropwise addition of water. The resulting mixture was partitioned between EtOAc and water. The aqueous layer was extracted twice with EtOAc. All organic layers were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo to give 7.5 g of a crude glass which was used for the subsequent reaction without further purification. Erythro-11 obtained from erythro-10 by the same method described above was purified by TLC (EtOAc-n-hexane = 1:1) for spectroscopy. UV λ_{max}^{EtOH} nm (log ε): 231 (4.25), 279 (3.76). ^{1}H-NMR ($CDCl_3$-D_2O): (erythro): δ 3.33 (6H, s, $-CH(OCH_3)_2$), 3.6-3.8 (2H, m, γ $-CH_2-$), 3.86 (6H, s, $Ar-OCH_3$), 4.08-4.24 (1H, m, β -CH-), 4.92 (1H, d, $J\alpha\beta$ = 5, α -CH-), 5.09 (2H, s, $-OCH_2Ph$), 5.28 (1H, s, $-CH(OCH_3)_2$), 6.78-7.42 (11H, Ar-H). MS m/z (%): 484 (0.8, M^+), 466 (0.1), 454 (0.8), 453 (3), 345 (5), 268 (5), 256 (9), 243 (8), 224 (54), 198 (12), 193 (59), 179 (34), 167 (100), 152 (36), 151 (37), 137 (14), 123 (8), 109 (8), 91 (97).

Compound 12

Compound 11 (7.5 g, a crude glass) was dissolved in 100 ml of MeOH, and 3.6 g of 10 % palladium on carbon was added to the solution. The mixture was stirred for 30 min at room temperature under hydrogen. The catalyst was filtered off and washed with MeOH. The filtrate and the washings were combined and evaporated in vacuo to give 6.2 g of a crude glass which was used for the subsequent reaction without

further purification. Erythro-12 obtained from erythro-11 was purified by TLC (EtOAc-n-hexane = 3:2) for spectroscopy. UV λ_{max}^{EtOH} nm (log ε): 231 (4.22), 280 (3.82). ^{1}H-NMR ($CDCl_3$-D_2O): (erythro): δ 3.32 (6H, s, -CH(OCH_3)$_2$), 3.6-3.8 (2H, m, γ -CH_2-), 3.82 (3H, s, Ar-OCH_3), 3.84 (3H, s, Ar-OCH_3), 4.06-4.22 (1H, m, β -CH-), 4.90 (1H, d, $J\alpha\beta$ = 5, α -CH-), 5.27 (1H, s, -CH(OCH_3)$_2$), 6.76-6.98 (6H, Ar-H). MS m/z (%): 376 (0.1, M^+-H_2O), 363 (10), 362 (35), 346 (10), 314 (15), 299 (5), 214 (6), 209 (6), 198 (9), 193 (58), 179 (26), 167 (100), 152 (36), 151 (44), 137 (29), 123 (9), 119 (10), 109 (8).

Guaiacylglycerol-β-vanillin ether (1)

To a stirred solution of 6.2 g (1.6 mmol, a crude glass) 12 in 80 ml of dioxane was added 1 ml of 1N HCl at room temperature. After 10 min the reaction solution was partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was chromatographed on a silica gel column (Wako gel C-100, 5 X 30 cm) with EtOAc-n-hexane (= 3:2) to give 3.46 g of a colorless glass. The yield of 1 from 9 was 66 %. UV λ_{max}^{EtOH} nm (log ε): 230 (4.27), 279 (4.11), 310 (3.99). IR ν_{max}^{KBr} cm^{-1}: 3500-3400, 2950, 1690 (C=O), 1593, 1524, 1468, 1433, 1280, 1245, 1160, 1140, 1030, 868, 819, 786, 738. ^{1}H-NMR ($CDCl_3$-D_2O): (erythro): δ 3.80-3.95 (2H, m, γ -CH_2-), 3.84 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 4.41 (1H, m, β -CH-), 4.95 (1H, d, $J\alpha\beta$ = 5.5, α -CH-), 6.80-7.38 (6H, Ar-H), 9.71 (1H, s, -CHO), (threo): δ 3.60-3.72 (2H, m, γ -CH_2-),

3.84 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 4.41 (1H, m, β -CH-), 4.95 (1H, d, $J\alpha\beta$ = 5.5, α -CH-), 6.80-7.38 (6H, Ar-H), 9.73 (1H, s, -CHO). MS m/z (%): 348 (0.3, M^+), 330 (1), 312 (1), 300 (30), 271 (3), 211 (10), 178 (63), 166 (11), 162 (12), 153 (35), 152 (93), 151 (100), 137 (48), 123 (23), 119 (16), 109 (22).

Syringylglycerol-β-vanillin ether (7)

UV λ_{max}^{EtOH} nm (log ε): 230 (4.28), 278 (4.08), 310 (4.02). IR ν_{max}^{KBr} cm^{-1}: 3500-3400, 2950, 1690 (C=O), 1590, 1520, 1510, 1465, 1435, 1335, 1280, 1235, 1140, 1120, 1025, 815, 784, 733. ^{1}H-NMR ($CDCl_3$-D_2O): δ 3.50-4.10 (2H, m, γ -CH_2-), 3.76-3.93 (9H, Ar-OCH_3), 4.26-4.46 (1H, m, β -CH-), 4.85-5.00 (1H, d, $J\alpha\beta$ (erythro) = 6, α -CH-), 6.55-7.45 (5H, Ar-H), 9.76 (erythro) and 9.78 (threo) (1H, two s, -CHO). MS m/z (%): 378 (2, M^+), 360 (2), 330 (20), 241 (4), 226 (4), 208 (8), 196 (12), 183 (46), 182 (100), 181 (29), 178 (28), 167 (52), 152 (54), 151 (64), 149 (18), 137 (16), 123 (51), 109 (25).

Guaiacylglycerol-β-(vanillyl alcohol) ether (2)

To a stirred solution of 174 mg (0.5 mmol) of 1 in 8 ml of MeOH was added 19 mg (0.5 mmol) of $NaBH_4$ at 0℃ under nitrogen. After 15 min at the same temperature the reaction mixture was partitioned between EtOAc and brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane = 3:1) to give 168 mg (96 %) of a colorless glass. UV λ_{max}^{EtOH} nm (log ε): 231 (4.10),

280 (3.74). IR ν_{max}^{KBr} cm^{-1}: 3500-3400, 1605, 1520, 1505, 1465, 1425, 1275, 1230, 1155, 1130, 1030, 855, 815. ^{1}H-NMR (acetone-d_6-D_2O): δ 3.7-3.9 (2H, m, γ -CH_2-), 3.82 (6H, s, Ar-OCH_3), 4.15-4.35 (1H, m, β -CH-), 4.48-4.58 (2H, s, α'-CH_2-), 4.82-4.95 (1H, m, α -CH-), 6.70-7.15 (6H, Ar-H). MS m/z (%): 350 (1, M^+), 332 (4), 302 (28), 255 (5), 241 (4), 225 (5), 211 (8), 196 (7), 180 (100), 167 (15), 166 (13), 154 (66), 153 (67), 150 (53), 149 (24), 137 (84), 125 (30), 123 (30), 109 (20), 107 (22).

Guaiacylglycerol-β-(ferulic acid) ether (4)

To a stirred solution of 427 mg (1.23 mmol) of 1 in 10 ml of pyridine were added 1.28 g (12.3 mmol) of malonic acid and 1 drop of piperidine. The reaction solution was heated to 80℃. After 12 hr at the same temperature the reaction solution was evaporated in vacuo and then the residue was dissolved in EtOAc. The solution was washed successively with 1N HCl and saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. An aliquot of the residue was purified by TLC (5% MeOH in CH_2Cl_2) for spectroscopy. Treatment of the remaining residue with diazomethane in MeOH followed by the purification of the resulting methyl ester by TLC (2% MeOH in CH_2Cl_2) gave 436 mg (88 %) of a colorless glass. Since diazomethane reacts with the carboxyl group more rapidly than the phenolic hydroxyl group, the methylation was stopped before the formation of the methyl ether. UV λ_{max}^{MeOH} nm (log ε): 229 (4.14), 285.5 (4.23), 311 (4.16). IR ν_{max}^{KBr} cm^{-1}: 3500-3400, 2950, 1705 (C=O), 1635, 1600, 1520, 1510, 1425, 1270, 1160, 1140, 1028, 980, 850, 825.

^{1}H-NMR (methyl ester; $CDCl_3$-D_2O): δ 3.50-3.90 (2H, m, γ-CH_2-), 3.79 (3H, s, -$COOCH_3$), 3.83 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 3.90-4.30 (1H, m, β-CH-), 4.85-5.00 (1H, m, α-CH-), 6.27 (1H, d, $J\alpha'\beta' = 16$, β'-CH=), 6.70-7.15 (6H, Ar-H), 7.56 (1H, d, $J\alpha'\beta' = 16$, α'-CH=). MS (methyl ester) m/z (%): 404 (0.8, M^+), 386 (6), 368 (7), 356 (48), 327 (7), 295 (18), 234 (70), 208 (100), 193 (7), 177 (74), 167 (9), 151 (26), 149 (31), 147 (16), 145 (38), 137 (53), 133 (27), 117 (27), 105 (20).

Compound 13

To a stirred solution of 7.5 g (15 mmol, a crude glass) of 11 in 80 ml of dioxane was added 1 ml of 1N HCl at room temperature. After 10 min the reaction solution was partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was chromatographed on a silica gel column (Wako gel C-100, 5 X 30 cm) with EtOAc-n-hexane (= 1:1) to give 4.85 g of a colorless glass. The yield of 13 from 9 was 74 %. IR λ_{max}^{KBr} cm^{-1}: 1692 (C=O). ^{1}H-NMR ($CDCl_3$-D_2O): δ 3.53-4.15 (2H, m, γ-CH_2-), 3.85 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 4.25-4.50 (1H, m, β-CH-), 4.90-5.00 (1H, d, $J\alpha\beta = 5$, α-CH-), 5.09 (2H, s, -OCH_2Ph), 6.80-7.44 (6H, Ar-H), 9.77 (erythro) and 9.78 (threo) (1H, two s, -CHO).

Compound 14

This compound was prepared from 13 by the method of Namba et al.[65].

Compound erythro-15

To a stirred solution of 138 mg (0.289 mmol) of erythro 14 in 6 ml of dioxane was added a solution of 68 mg (0.43 mmol) of $KMnO_4$ in 1 ml of water at room temperature. After 90 min 1 ml of MeOH was added to the reaction mixture and the stirring was continued for additional 30 min to decompose excess $KMnO_4$ to MnO_2. The MnO_2 was then filtered off and washed successively with MeOH and hot water. The filtrate and the washings were combined, acidified to pH 2 with concentrated HCl and extracted three times with EtOAc. The organic layers were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue (170 mg) was used for subsequent reaction without further purification. An aliquot of the residue was methylated with diazomethane in MeOH, and the methyl ester derivative was purified by TLC (EtOAc-n-hexane = 1:3) for spectroscopy. ^{1}H-NMR (methyl ester, $CDCl_3$): δ 1.51 (3H, s, C-CH_3), 1.62 (3H, s, C-CH_3), 3.79 (3H, s, -$COOCH_3$), 3.83 (3H, s, Ar-OCH_3), 3.86 (3H, s, Ar-OCH_3), 3.9-4.2 (2H, m, γ -CH_2-), 4.1-4.4 (1H, m, β -CH-), 4.89 (1H, d, $J\alpha\beta$ = 8, α -CH-), 5.08 (2H, s, -OCH_2Ph), 6.43-7.50 (11H, Ar-H). MS (methyl ester) m/z (%): 508 (0.8, M^+), 329 (3), 242 (43), 208 (95), 182 (4), 179 (12), 177 (19), 167 (4), 151 (19), 149 (11), 137 (4), 123 (6), 119 (7), 105 (6), 91 (100).

Compound erythro-16

To a stirred solution of 170 mg of erythro-15 (a crude glass) in 8 ml of dioxane was added 0.5 ml of 1N HCl at room temperature. After 18 hr the reaction solution was parti-

tioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give 120 mg of a colorless glass which was used for subsequent reaction without further purification. An aliquot of the crude glass was methylated with diazomethane in MeOH and then purified by TLC (EtOAc-*n*-hexane = 2:1) for spectroscopy. ^{1}H-NMR (methyl ester, $CDCl_3$): δ 3.83 (6H, s, $-COOCH_3$ and Ar-OCH_3), 3.86 (3H, s, Ar-OCH_3), 3.55-4.15 (2H, m, γ $-CH_2-$), 4.20-4.40 (1H, m, β -CH-), 4.92 (1H, d, $J\alpha\ \beta$ = 5, α -CH-), 5.08 (2H, s, $-OCH_2Ph$), 6.76-7.60 (11H, Ar-H). MS (methyl ester) *m/z* (%): 468 (0.3, M^+), 450 (0.1), 437 (0.3), 420 (1), 329 (5), 256 (7), 242 (13), 208 (20), 182 (31), 167 (5), 151 (60), 137 (7), 123 (12), 108 (7), 91 (100).

Erythro-guaiacylglycerol-β-(vanillic acid) ether (3)

Erythro-16 (120 mg, a crude glass) was dissolved in 4 ml of MeOH and 50 mg of 10% Pd-C was added to the solution. The mixture was stirred for 30 min at room temperature under hydrogen. The catalyst was filtered off and washed with MeOH. The filtrate and the washings were combined and evaporated *in vacuo*. Crystallization of the residue from MeOH-CH_2Cl_2 gave 81 mg of a colorless powder. The yield of 3 from 14 was 77 %. UV λ_{max}^{EtOH}: 235 (sh), 250 (sh), 283. IR ν_{max}^{KBr} cm^{-1}: 3400, 2950, 1700 (C=O), 1600, 1517, 1460, 1430, 1275, 1230, 1183, 1150, 1125, 1032, 950, 770. ^{1}H-NMR (methyl ester, $CDCl_3$-D_2O): δ 3.60-4.00 (2H, m, γ $-CH_2-$), 3.84 (3H, s, $-COOCH_3$), 3.89 (6H, s, two Ar-OCH_3), 4.20-4.40 (1H, m, β -CH-), 4.94 (1H, d, $J\alpha\ \beta$ = 5, α -CH-), 6.80-7.60 (6H, Ar-

H). MS (methyl ester) m/z (%): 360 (1, M^+-H_2O), 347 (0.8, M^+-OCH_3), 342 (0.6, 360-H_2O), 330 (18, 360-CH_2O), 299 (2), 270 (4), 208 (73), 182 (39), 167 (13), 153 (33), 151 (100), 137 (47), 123 (24), 119 (13), 108 (13).

Threo-guaiacylglycerol-β-(vanillic acid) ether (3)

^{1}H-NMR (methyl ester, $CDCl_3-D_2O$): δ 3.55-3.75 (2H, s, γ-CH_2-), 3.84 (3H, s, -$COOCH_3$), 3.89 (3H, s, Ar-OCH_3), 3.91 (3H, s, Ar-OCH_3), 4.1-4.4 (1H, m, β-CH-), 4.93 (1H, d, $J\alpha\ \beta$ = 6, α-CH-), 6.83-7.68 (6H, Ar-H).

Compound 17

To a stirred solution of 173 mg (0.497 mmol) of 1 and 627 mg (7.46 mmol) of 2,3-dihydro-4H-pyran in a mixture of 10 ml of CH_2Cl_2 and 0.5 ml of dioxane was added 4 mg of p-TsOH at 0℃ under nitrogen. After 30 min at the same temperature the reaction solution was neutralized by the addition of triethylamine and partitioned between EtOAc and saturated $NaHCO_3$ solution. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane = 1:2) to give 257 mg (86 %) of a colorless glass.

Compound 18

To a stirred solution of 257 mg (0.428 mmol) of 17 in 15 ml of pyridine were added 445 mg (4.28 mmol) of malonic acid and 1 drop of piperidine. The reaction solution was heated to 80℃. After stirring for 12 hr at the same temperature, the reaction solution was evaporated in vacuo,

and pyridine was removed azeotropically by evaporation with benzene. The residue was used for the subsequent reaction without further purification.

Compound 19

Compound 18 (a crude oil) was dissolved in 5 ml of MeOH. To the stirred solution was added dropwise an ethereal solution of diazomethane at room temperature until the yellow color was not discharged. After 10 min the reaction solution was evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane = 1:3) to give 215 mg of a colorless glass. The yield from 17 was 77 %. IR λ_{max}^{KBr} cm^{-1} 1735 (C=O). ^{1}H-NMR ($CDCl_3$): δ 6.20-6.30 (1H, d, $J\alpha'\beta'$ = 16, β'-CH=), 7.48-7.58 (1H, d, $J\alpha'\beta'$ = 16, α'-CH=).

Compound 20

To a stirred suspension of 63 mg (1.6 mmol) of $LiAlH_4$ in 5 ml of anhydrous THF was added dropwise a solution of 215 mg (0.328 mmol) of 19 in 10 ml of anhydrous THF over a period of 30 min at -25℃ under nitrogen. The stirring was continued for additional 15 min at the same temperature. After the same work up as described in the preparation of 11, the product was purified by TLC (EtOAc-n-hexane = 2:3, X 2) to give 167 mg (81 %) of a colorless glass. ^{1}H-NMR ($CDCl_3$): δ 4.26 (2H, d, $J\beta'\gamma'$ = 6, γ'-CH_2-).

Compound 21

To a stirred solution of 155 mg (0.247 mmol) of 20 in 6 ml of CCl_4 was added 322 mg (3.71 mmol) of active MnO_2[66] at

room temperature. After 24 hr MnO_2 was filtered off and washed with $CHCl_3$. The filtrate and the washings were combined and evaporated *in vacuo*. The residue was purified by TLC (EtOAc-*n*-hexane = 1:1) to afford 148 mg (96 %) of a colorless glass, which gave a red purple color on TLC plate with phloroglucinol-HCl. IR λ_{max}^{KBr} cm^{-1}: 1680 (C=O). ^{1}H-NMR ($CDCl_3$): δ 9.61 (1H, d, $J\beta'\gamma'$ = 7.5, γ'-CHO).

Guaiacylglycerol-β-coniferaldehyde ether (5)

To a stirred solution of 120 mg (0.192 mmol) of 21 in 8 ml of dioxane was added 1 ml of 1N HCl at room temperature. After 18 hr the reaction solution was partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was purified by TLC (EtOAc-*n*-hexane = 2:1, X 2) to give 61 mg (85 %) of a pale yellow glass. UV λ_{max}^{EtOH} nm (log ε): 231 (4.10), 250 (3.88, sh), 290 (4.00), 335 (4.20). IR λ_{max}^{KBr} cm^{-1}: 3500-3400, 2950, 1665 (C=O), 1625, 1600, 1520, 1510, 1465, 1430, 1280, 1225, 1135, 1028, 970, 810. ^{1}H-NMR ($CDCl_3$): δ 3.6-4.1 (2H, m, γ-CH_2-), 3.86, 3.88, and 3.92 (6H, s, Ar-OCH_3), 4.17-4.40 (1H, m, β-CH-), 4.95 (1H, d, $J\alpha\beta_{(erythro)}$ = 5.5, β-CH-), 6.58 (*erythro*) and 6.59 (*threo*) (1H, dd, $J\alpha'\beta'$ = 15.5, $J\alpha'\beta'$ = 7.5, α'-CH=), 6.82-7.25 (6H, Ar-H), 7.35 (*erythro*) and 7.36 (*threo*) (1H, d, $J\alpha'\beta'$ = 15.5, α'-CH=), 9.61 (*erythro*) and 9.62 (*threo*) (1H, d, $J\beta'\gamma'$ = 7.5, γ'-CHO). MS *m/z* (%): 374 (1, M^+), 356 (3), 326 (36), 297 (7), 265 (10), 237 (4), 204 (100), 178 (86), 161 (43), 153 (37), 151 (51), 147 (45), 137 (83), 135 (53), 124 (32), 119 (25).

1.2 Initial Degradative Reactions of Guaiacylglycerol-β - Coniferyl Ether

1.2.1 INTRODUCTION

Lignin is a complex aromatic polymer which is formed by the coupling of the phenoxy radicals of p-hydroxycinnamyl alcohols, and it contains a variety of intermonomer linkages[70]. The pathways by which the complex lignin polymer is biodegraded are not known[71]. Because the structure of the lignin is heterogeneous, it is advantageous to use model compounds containing major lignin substructures to elucidate the degradation pathway of lignin. For this purpose, large scall synthetic methods for preparing such model compounds, for use both as substrates and as authentic samples of suspected intermediary metabolites have been developed. Arylglycerol-β -aryl ether substructures are the most common interphenylpropane linkage in lignin. Syntheses of the substructure model compounds were described in section 1.1.

Ohta et al. reported degradation of dehydrodiconiferyl alcohol, a model for a phenylcoumaran substructure, by Fusarium solani M-13-1[45]. The fungus was isolated from soil by an enrichment technique, using a medium containing a dehydrogenation polymer of coniferyl alcohol as sole carbon source[44].

In this section, initial degradative reactions of guaiacylglycerol-β -coniferyl ether (6), a model for the arylglycerol-β -aryl ether substructure, by F. solani M-13-1 are described.

1.2.2 EXPERIMENTAL

Microorganism

Fusarium solani M-13-1 was used[44].

Culture conditions

Inorganic medium contained the following salts in 1000 ml of distilled water: NH_4NO_3, 2 g; K_2HPO_4, 1 g; KCl, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 10 mg; $MnCl_2 \cdot 4H_2O$, 5 mg; $CaCl_2 \cdot 2H_2O$, 20 mg; $CuSO_4 \cdot 5H_2O$, 1 mg. The medium was adjusted to pH 6.8 and glucose, 20 g; yeast extract, 1 g; peptone, 5g; casamino acid, 2 g were added as nutrients. The nutrient medium, 100 ml in a 500 ml Sakaguchi flask, was autoclaved for 15 min at 120℃. Mycelia from the stock culture were inoculated into the nutrient medium and cultured on a reciprocating shaker (145 strokes per minute) for 4 days at 28℃. Mycelia were centrifuged and washed with sterile water. The washed mycelia were suspended in 100 ml of sterile water, 1 ml of which was taken for a dry weight determination. The remaining mycelia were centrifuged, suspended in 100 ml of the inorganic medium, and shaken for about 5 hr before use.

Substrates of biodegradation

Guaiacylglycerol-β-coniferyl ether (6), guaiacylglycerol-β-(ferulic acid) ether (4), and guaiacylglycerol-β-vanillin ether (1) were used as the substrate.

Biodegradation

To 100 ml of the inorganic medium in a 500 ml Sakaguchi flask, previously autoclaved, was added a solution of about 100 mg of the substrate in 1 ml of acetone, followed by about 200 mg of mycelia (dry weight). Two control flasks which contained only mycelia or substrate in the inorganic medium were similarly prepared; all flasks were shaken at 28℃.

Analyses of Catabolic Products

Degradation of substrates and formation of catabolic products were monitored by UV spectroscopy and TLC analysis of the culture filtrates. When catabolic products were detected by TLC, mycelia were removed by centrifugation and washed with distilled water. The supernatant and the washings were combined, acidified to pH 2 with 1N HCl, and extracted five times with an equal volume of EtOAc. The combined EtOAc extracts were concentrated to about 100 ml and back-extracted with three 100 ml portions of saturated $NaHCO_3$ solution. The combined aqueous $NaHCO_3$ layers were washed with 100 ml of EtOAc. The EtOAc extracts and the washings were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated to dryness *in vacuo* (Fraction A). The aqueous $NaHCO_3$ layer was acidified to pH 2 with concentrated HCl solution and extracted with four 150 ml portions of EtOAc. The combined extracts were then washed with saturated brine, dried over Na_2SO_4, and evaporated to dryness *in vacuo* (Fraction B). To a solution of Fraction B in MeOH was added dropwise with stirring a limited amount of ethereal solution of diazomethane until

only the carboxyl group of the products was methylated. The reaction was followed by TLC analysis. The solvent was removed by evaporation under reduced pressure. Both fractions were then subjected to column chromatography and TLC on silica gel, and isolated compounds were identified from their NMR, mass, and IR spectra, supplemented for specific color reactions. Authentic samples served as references for identifications. Molecular weight of 5,5'-dehydrodiguaiacylglycerol-β-(ferulic acid) ether (22) was determined by high-pressure gel permeation chromatography (GPC).

Chromatography and analytical instruments were the same as in section 1.1. GPC was taken by a Shimadzu 830 liquid chromatograph (column, μ-styragel 500 Å, 7 mm ID X 30 cm; solvent, THF; flow rate, 0.74 ml/min). A calibration curve was prepared with standard polystyrenes (molecular weight = 4000 and 10000), liriodendrin octaacetate (1078)[72], and guaiacylglycerol-β-(methyl ferulate) ether (4') (404).

Preparation of compounds

Guaiacylglycerol-β-coniferyl ether (6).

Coniferyl alcohol was synthesized by the reduction of methyl ferulate with $LiAlH_4$ in Et_2O at -30℃[73] (yield 82%). A solution of 30 g (0.167 mmol) of coniferyl alcohol in a minimum amount of acetone and 4 mg of horseradish peroxidase (Sigma, crude, 33 purpurogallin units/mg) were added to 2 liters of distilled water. With vigorous stirring, 1 liter of 0.6% H_2O_2 (0.176 mol) was added dropwise over a period of 1 hr. The mixture was stirred until the spot of

coniferyl alcohol disappeared on silica gel TLC developed with 5% MeOH in CH_2Cl_2. The reaction mixture was then acidified to pH 2 with concentrated HCl and extracted with three 2 liters portions of EtOAc. The combined extracts were washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated to dryness in vacuo. The residue was then chromatographed on a silica gel column (Wako gel C-100, 850 g, 5 X 80 cm) by means of a gradient elution with benzene-acetone, 10:1 to 1:1. Compound 7 was eluted at the ratio of 3:1. Purification of the eluate by TLC with 5% MeOH in CH_2Cl_2 gave 7 as a colorless syrup, which was a mixture of the erythro and threo forms, with a ratio of about 1:1 determined by its ^{1}H-NMR spectrum.

Guaiacylglycerol-β-coniferaldehyde ether (5), guaiacylglycerol-β-(ferulic acid) ether (4), guaiacylglycerol-β-vanillin ether (1), and guaiacylglycerol-β-(vanillic acid) ether (3).

Syntheses of these compounds were described in section 1.1.

1.2.3 RESULTS

Degradation of guaiacylglycerol-β-coniferyl ether (6)

Fig. 1-5 shows the changes in UV absorption spectra of filtrates of a mycelial suspension of F. solani M-13-1 incubated with compound 6. The absorbance at 280 nm decreased gradually with a concomitant slight increase of the absorb-

ance at 340 and 310 nm. The absorbance at 280 nm then rapidly decreased, with a shoulder appearing transiently at 310-320 nm.

From Fraction A obtained from the culture filtrate after 40 hr of incubation, 32 mg of a syrup was isolated by TLC developed with 5% MeOH in $CHCl_3$). The yield was 6.4% from 500 mg of the substrate 6. The compound gave red purple and bright blue colors on TLC plates with phloroglucinol-HCl and 2,6-dichloroquinone-4-chloroimide, respectively, indicating the presence of a cinnamaldehyde group and p-hydroxybenzyl alcohol group. The ^{1}H-NMR spectrum of the compound showed the aldehydic proton as a doublet at δ 9.57 (erythro) and 9.58 (threo) (Fig. 1-6(a)), the α'-methine proton as a doublet at7.50 (threo) and 7.53 (erythro), the β'-methine proton as a double doublet at 6.60 (threo) and 6.63 (erythro), and the α-methine proton at 4.84-4.94. The MS of the compound showed a molecular ion peak at m/z 374, and an ion peak for coniferaldehyde at m/z 178. From the above results and the following data, the compound was identified as guaiacylglycerol-β-coniferaldehyde ether (5). ^{1}H-NMR (acetone-d_6): δ 3.35-3.80 (2H, γ-CH_2-), 3.78-3.92 (6H, Ar-OCH_3), 4.35-4.60 (1H, β-CH-), 4.84-4.94 (1H, α-CH-), 6.60 (0.5H, dd, J = 15.5, J = 7.5, threo-β'-CH=), 6.63 (0.5H, dd, J = 15.5, J = 7.5, erythro-β'-CH=), 6.62-7.40 (6H, Ar-H), 7.50 (0.5H, d, J = 15.5, threo-α'-CH=), 7.53 (0.5H, d, J = 15.5, erythro-α'-CH=), 9.57 (0.5H, d, J = 7.5, erythro-γ'-CHO), 9.58 (0.5H, d, J = 7.5, threo-γ'-CHO). MS m/z : 374 (M^+), 356 (M^+-H_2O), 342, 338, 326 (356-CH_2O), 297, 265, 243, 237, 204 (M^+-H_2O-vanillin), 196, 178

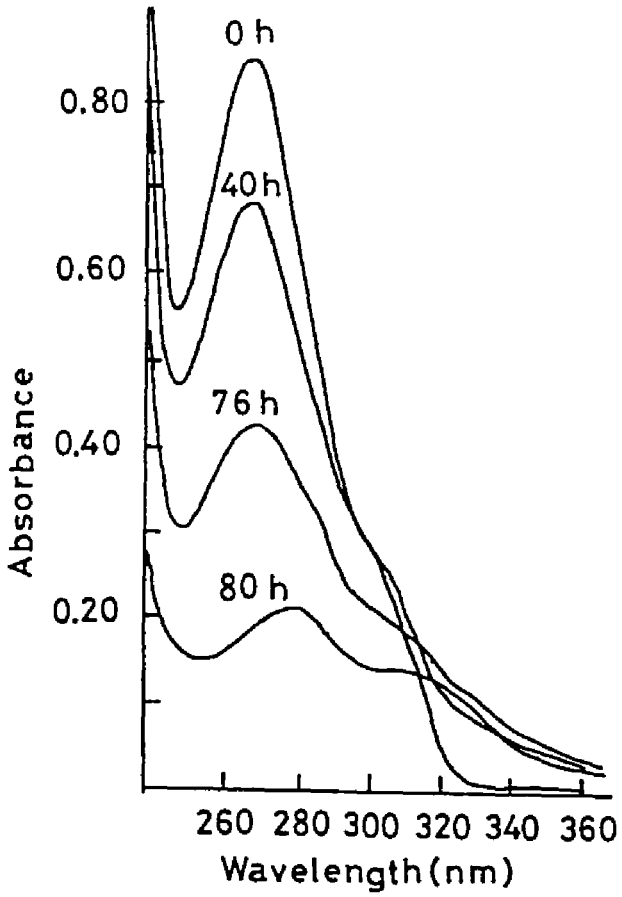


Fig. 1-5 Changes in the UV absorption of culture filtrates containing guaiacylglycerol-β-coniferyl ether (6) during incubation with Fusarium solani M-13-1

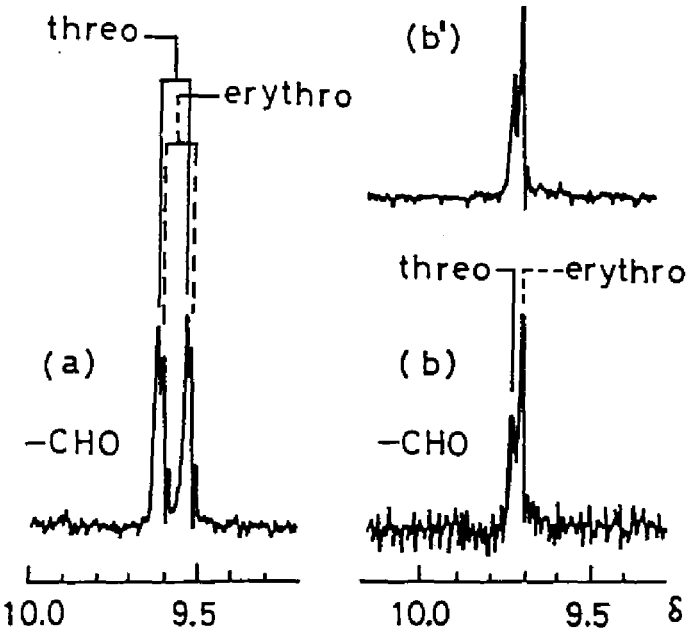


Fig. 1-6 ^{1}H-NMR spectra of the aldehyde group of (a) catabolic guaiacylglycerol-β-coniferaldehyde ether (5), (b) catabolic guaiacylglycerol-β-vanillin ether (1), and (b') synthetic 1

(coniferaldehyde, base peak), 177 (178-H), 151 (vanillin-H), 147, 137, 135, 124, 119, 91, 77, 65.

Fig. 1-6(a) shows the ^{1}H-NMR spectrum of the aldehydic proton of 5. The two doublets are due to the erythro and threo forms. Since the height of each peak is approximately equal, the erythro/threo ratio of the compound was about 1:1 as in the case of the substrate 6.

Fraction B obtained from the culture filtrate after 76 hr of incubation was esterified with diazomethane, from which 90 mg of a syrup was isolated by TLC developed with 5% MeOH in $CHCl_3$. The yield was 4.5% from 2.0 g of the substrate 6. The compound gave a bright blue color on TLC plates with 2,6-dichloroquinone-4-chloroimide, indicating the presence of a p-hydroxybenzyl alcohol group. The ^{1}H-NMR spectrum of the compound indicated the three methyl ester protons as a singlet at δ 3.80, the α'-methine proton as a doublet at 7.58, the β'-methine proton as a doublet at 6.30, and the α-methine proton at 4.47-5.02. The MS of the compound showed the molecular ion peak at m/z 404 and an ion peak for methyl ferulate at m/z 208. The IR spectrum showed a carboxyl stretching vibration band at 1730 cm^{-1}, due to the methyl ester group. From the above results and the following data the compound was identified as the methyl ester of guaiacylglycerol-β-(ferulic acid) ether (4). All data for the compound were completely identical with those of the authentic samples. The compound was found by ^{1}H-NMR to be a mixture of erythro and threo forms. ^{1}H-NMR ($CDCl_3$) : δ 2.80-3.02 (2H, alcoholic-OH), 3.55-3.80 (2H, γ-CH_2-), 3.80 (3H, s, -$COOCH_3$), 3.86 and 3.90 (6H, Ar-OCH_3), 3.80-

4.40 (1H, β-CH-), 4.47-5.02 (1H, α-CH-), 5.65-6.00 (1H, Ar-OH), 6.30 (1H, d, J = 16.0, β'-CH=), 6.70-7.15 (6H, Ar-H), 7.58 (1H, d, J = 16.0, α'-CH=). MS m/z: 404 (M^+), 386 (M^+-H_2O), 372, 368, 356 (386-CH_2O), 295, 234 (M^+-H_2O-vanillin), 208 (methyl ferulate, base peak), 177, 167, 166, 151 (vanillin-H), 147, 137, 133, 117, 105, 91, 89, 77, 65. IR $\lambda_{max}^{CH_2Cl_2}$ cm^{-1}: 3640, 3030, 1730 (C=O), 1640, 1600, 1515, 1180, 1040.

Fraction B obtained from the culture filtrates after 80 hr of incubation with 6 was esterified with diazomethane, from which 14.8 mg of a syrup was obtained by column chromatography on silica gel with 2% MeOH in $CHCl_3$ as eluent, and subsequently by silica gel TLC with EtOAc as solvent. The compound gave a bright blue color on TLC plates with 2,6-dichloroquinone-4-chloroimide, indicating the presence of a p-hydroxybenzyl alcohol group. The ^{1}H-NMR and IR spectra of the compound were similar to those of the methyl ester of 4. The ^{1}H-NMR spectrum of the acetate of the compound indicated the peak of the Ar-$OCOCH_3$ at δ 1.98-2.07, which overlapped that of the aliphatic-$OCOCH_3$ and shifted upfield (20-30 Hz) from that of common Ar-$OCOCH_3$. The shift is characteristic of biphenyl structures[74]. The MS of the compound indicated an ion peak for methyl ferulate at m/z 204. Molecular weight of the compound, determined by high-pressure gel permeation chromatography, was about 800-900 (Fig. 1-7). From these results and the following data the compound was identified as the dimethyl ester of 5,5'-dehydrodiguaiacylglycerol-β-(ferulic acid) ether (22). ^{1}H-NMR (dimethyl ester, $CDCl_3$): δ 3.50-3.80 (4H, γ-CH_2-),

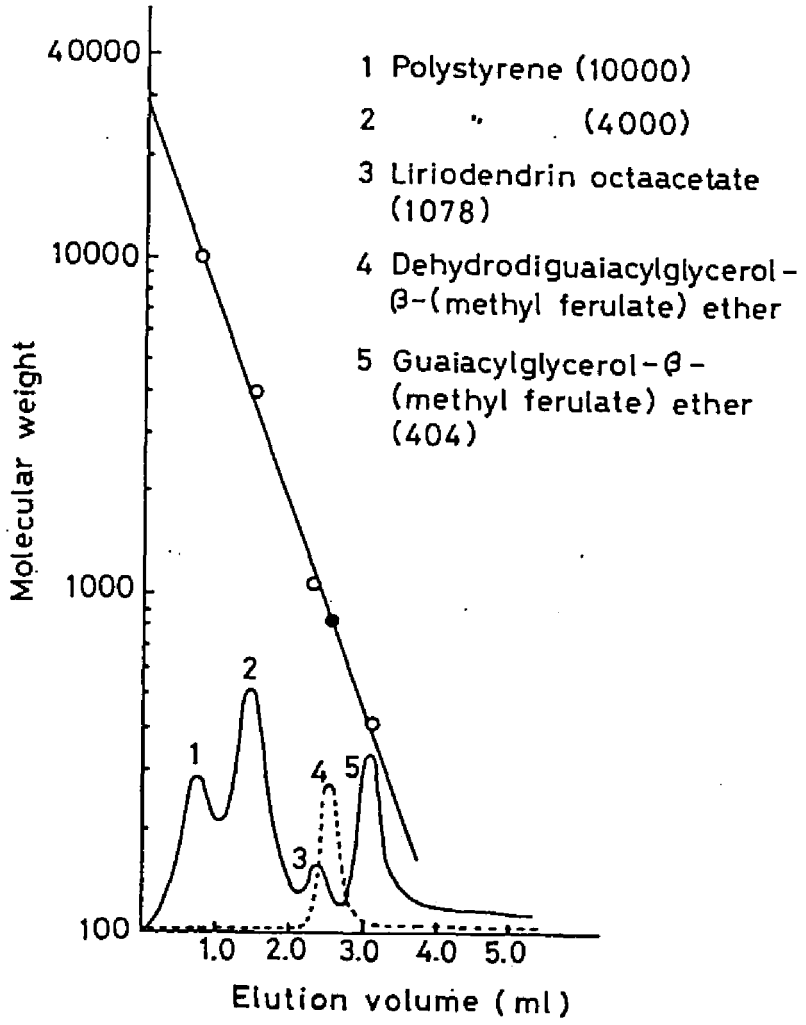


Fig. 1-7 Molecular weight determination of 5,5'-dehydrodiguaiacylglycerol-β-(methyl ferulate) ether (22') by high-performance gel filtration chromatography. Condition: Column, μ-styragel 500 A, 7 mm X 30 cm; eluent, tetrahydrofran; flow rate, 0.74 ml/min, detection, UV at 254 nm.

3.70-3.95 (18H, $-COOCH_3$ and $Ar-OCH_3$), 3.95-4.30 (2H, β -CH-), 4.88-5.07 (2H, α -CH-), 6.31 (2H, d, J = 15.5, β '-CH-), 6.60-7.15 (10H, Ar-H), 7.61 (2H, d, J = 15.5, α '-CH-). IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3640, 3030, 1730 (C=O), 1640, 1600, 1515, 1135, 1040. ^{1}H-NMR (hexaacetate of dimethyl ester, $CDCl_3$): δ 1.98, 2.03, and 2.07 (18H, aliphatic and Ar-OAc), 3.79, 3.82, 3.85, and 3.87 (18H, $-COOCH_3$ and $Ar-OCH_3$), 3.90-4.45 (4H, γ $-CH_2-$), 4.55-4.80 (2H, β -CH-), 5.95-6.15 (2H, α -CH-), 6.29 (2H, d, J = 15.5, β '-CH=), 6.60-7.15 (10H, Ar-H), 7.58 (2H, d, J = 15.5, α '-CH=).

Degradation of guaiacylglycerol-β-(ferulic acid) ether (4)

Since it was found that the degradation of 6 by F. solani M-13-1 gave 4 via 5, the fungus was shake-cultured in a medium containig 4. Fig. 1-8 shows the changes in the UV absorption spectrum of culture filtrates during incubation. The absorbance at 280 and 310 nm decreased continuously until all absorption disappeared.

From Fraction A obtained from the culture filtrate after 106 hr of incubation, 2.8 mg of a syrup was isolated by silica gel TLC developed with 3 % MeOH in CH_2Cl_2. The yield was 0.8 % (2.8 mg) of 357 mg of the substrate 4. The product gave orange and bright blue colors on TLC plates with 2,4-dinitrophenylhydrazine-HCl and 2,6-dichloroquinone-4-chloroimide indicating the presence of an aldehyde group and a p-hydroxybenzyl alcohol group, respectively. The ^{1}H-NMR spectrum of the compound showed the aldehydic proton at δ 9.72 (erythro) and 9.74 (threo) (Fig. 1-6(b)), and α-

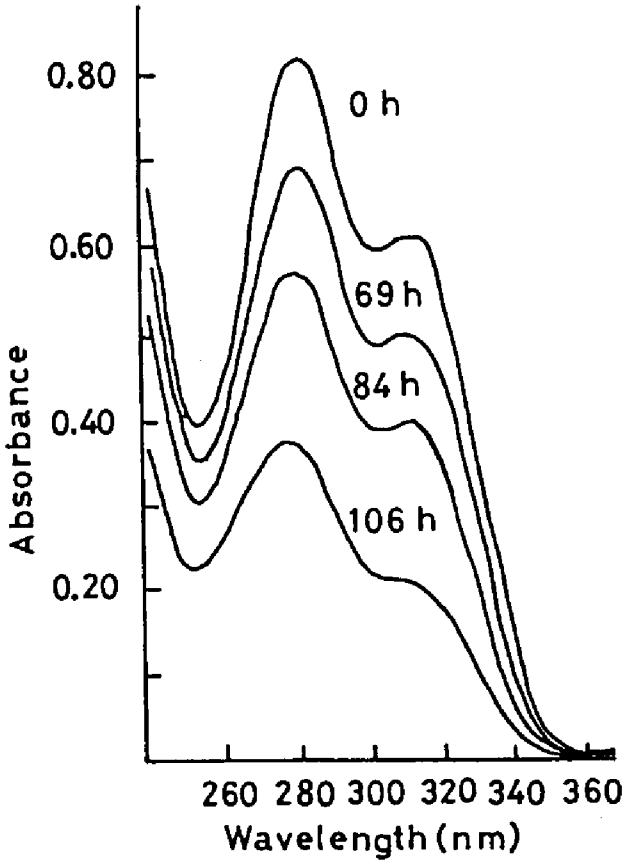


Fig. 1-8 Changes in the UV absorption of culture filtrates containing guaiacylglycerol-β-(ferulic acid) ether (4) during incubation with Fusarium solani M-13-1

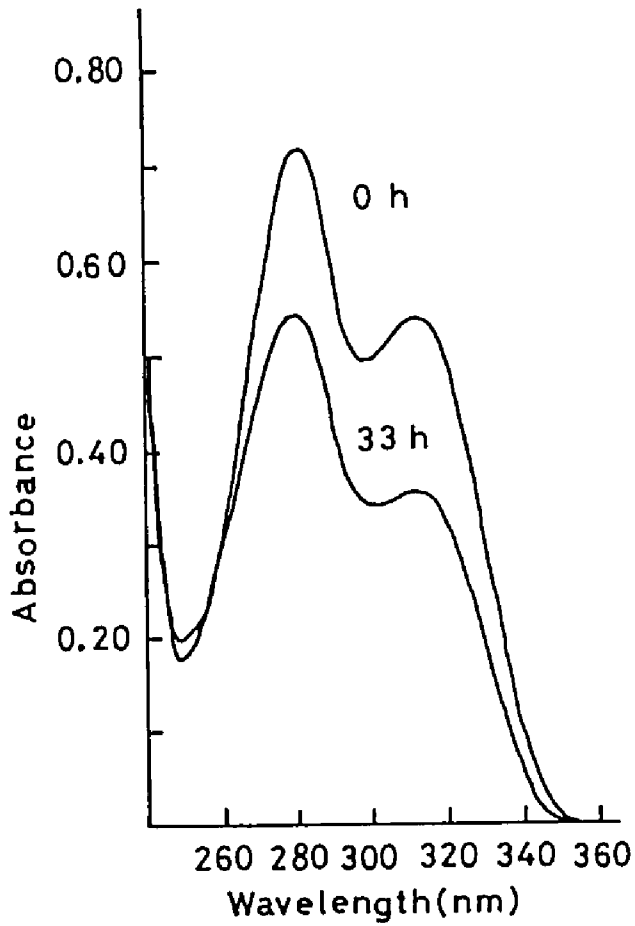


Fig. 1-9 Changes in the UV absorption of culture filtrates containing guaiacylglycerol-β-vanillin ether (1) during incubation with Fusarium solani M-13-1

methine proton at 4.88-5.02. The β'- and γ'-methine protons were absent. The MS of the compound indicated the molecular ion peak at m/z 348 and an ion peak of vanillin at m/z 152. The IR spectrum of the compound showed carbonyl stretching vibration band at 1710 cm^{-1}, due to the aryl aldehyde group. From the above results and the following data, the compound was identified as guaiacylglycerol-β-vanillin ether (1). All data for the catabolic product were completely identical with those of the authentic sample. ^{1}H-NMR ($CDCl_3$): δ 3.55-3.75 (2H, γ-CH_2-), 3.83, 3.88 and 3.92 (6H, Ar-OCH_3), 4.30-4.50 (1H, β-CH-), 4.88-5.02 (1H, α-CH-), 5.60 (1H, Ar-OH), 6.78-7.40 (6H, Ar-H), 9.72 (5/7H, s, erythro-CHO), 9.74 (2/7H, s, threo-CHO). MS m/z: 348 (M^+), 330 (M^+-H_2O), 316, 300 (M^+-CH_2O), 271, 211, 194, 178 (M^+-H_2O-vanillin), 152 (vanillin, base peak), 151, 137, 123, 119, 109, 91. IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}: 3640, 3030, 1710 (Ar-CHO), 1600, 1515, 1235, 1130, 1035.

Fig. 1-6 shows the ^{1}H-NMR spectra of the aldehydic protons of the catabolic and synthetic products.
Since both signals were identical, the erythro/threo ratios in the synthetic and catabolic products was about 2.5/1; the larger singlet in both spectra is due to the erythro form.

Degradation of guaiacylglycerol-β-vanillin ether (1)

Since it was found that the degradation of 4 by F. solani M-13-1 gave 1 as a transformation product, the fungus was shake-cultured in a medium containing 1. Fig. 1-9 shows that the UV absorption at 280 and 310 nm decreased

continuously; all absorption finally disappeared.

Fraction B obtained from the culture filtrate after 33 hr of incubation was esterified with diazomethane, from which 54 mg of a syrup was isolated by silica gel TLC, developed four times with EtOAc-n-hexane (= 3:2) as solvent. The yield was 5.9 % from 912 mg of the substrate 1. The product gave a bright blue color on TLC plates with 2,6-dichloroquinone-4-chloroimide, indicating the presence of a p-hydroxybenzyl alcohol group. The ^{1}H-NMR spectrum of the compound revealed the three methyl ester protons as a singlet at δ 3.84, the β-methine proton at 4.0-4.2 (threo) and 4.30 (erythro), and α-methine proton at 4.89-4.94. The MS of the compound showed the (M^+-H_2O) peak at m/z 360 and an ion peak for methyl vanillate at m/z 182. The IR spectrum of the compound showed carbonyl stretching vibration band at 1728 cm^{-1}, due to the methyl ester group. From the above results and the following data, the compound was identified as the methyl ester of guaiacylglycerol-β-(vanillic acid) ether (3). All data of the compound were completely identical with those of the authentic sample. The compound was found to be a mixture of erythro and threo forms by ^{1}H-NMR spectrum. (Methyl ester 3'): ^{1}H-NMR ($CDCl_3$): δ 3.20-3.70 (2H, alcoholic-OH), 3.55-3.80 (2H, γ-CH_2-), 3.84 (3H, s, -$COOCH_3$), 3.87, 3.89, and 3.91 (6H, Ar-OCH_3), 4.0-4.2 (2/7H, m, threo-β-CH-), 4.30 (5/7H, m, erythro-β-CH-), 4.89-4.94(1H, α-CH-), 6.78-7.65 (6H, Ar-H). MS m/z: 360 (M^+-H_2O), 342, 330 (360-CH_2O), 315, 299, 270, 208 (M^+-H_2O-vanillin), 182 (methyl vanillate), 167, 151 (base peak), 137, 123, 119, 108, 91, 79, 77, 65. IR $\nu_{max}^{CH_2Cl_2}$ cm^{-1}:

3640, 3030, 1728 (C=O), 1603, 1515, 1220, 1185, 1140, 1040.

1.2.4 DISCUSSION

Based on the chemical structures of catabolic products obtained from filtrates of cultures containing compound 6, 4, and 1, the proposed scheme is shown in Fig. 1-10 as the catabolic pathway of 6 by F. solani M-13-1. The γ'-cinnamyl alcohol group of 6 is initially oxidized to a γ'-aldehyde group, then to a γ'-carboxyl group, yielding 5 and 4. Compound 4 is converted to 3 by the release of a C_2 fragment (β' and γ'-C); compound 23 and acetate are possible intermediates in this reaction. The α'-aldehyde group of 1 is oxidized to a α'-carboxyl group, yielding 3.

In the pathway from 6 to 3, it is evident that the fungal attack occurs preferentially in the terminal side chain (α', β', and γ'-C), resulting in oxidative shortening of the side chain. Neither oxidation of the α-secondary alcohol to ketone nor cleavage of the arylglycerol-β-aryl ether linkage was observed.

The pathway from 6 to 4 is entirely consistent with that from dehydrodiconiferyl alcohol to 5-formyl-2-guaiacyl-3-hydroxy-7-methoxycoumaran reported previously[45]; dehydrodiconiferyl alcohol is initially oxidized, by F. solani M-13-1, to the corresponding γ'-aldehydic compound, which is then oxidized to the γ'-carboxylic compound. Subsequent release of a C_2 fragment from the carboxylic acid leads to the 5-formyl-2-guaiacyl-3-hydroxy-7-methoxycoumaran. Toms and Wood[74] found that trans-ferulic acid was converted to

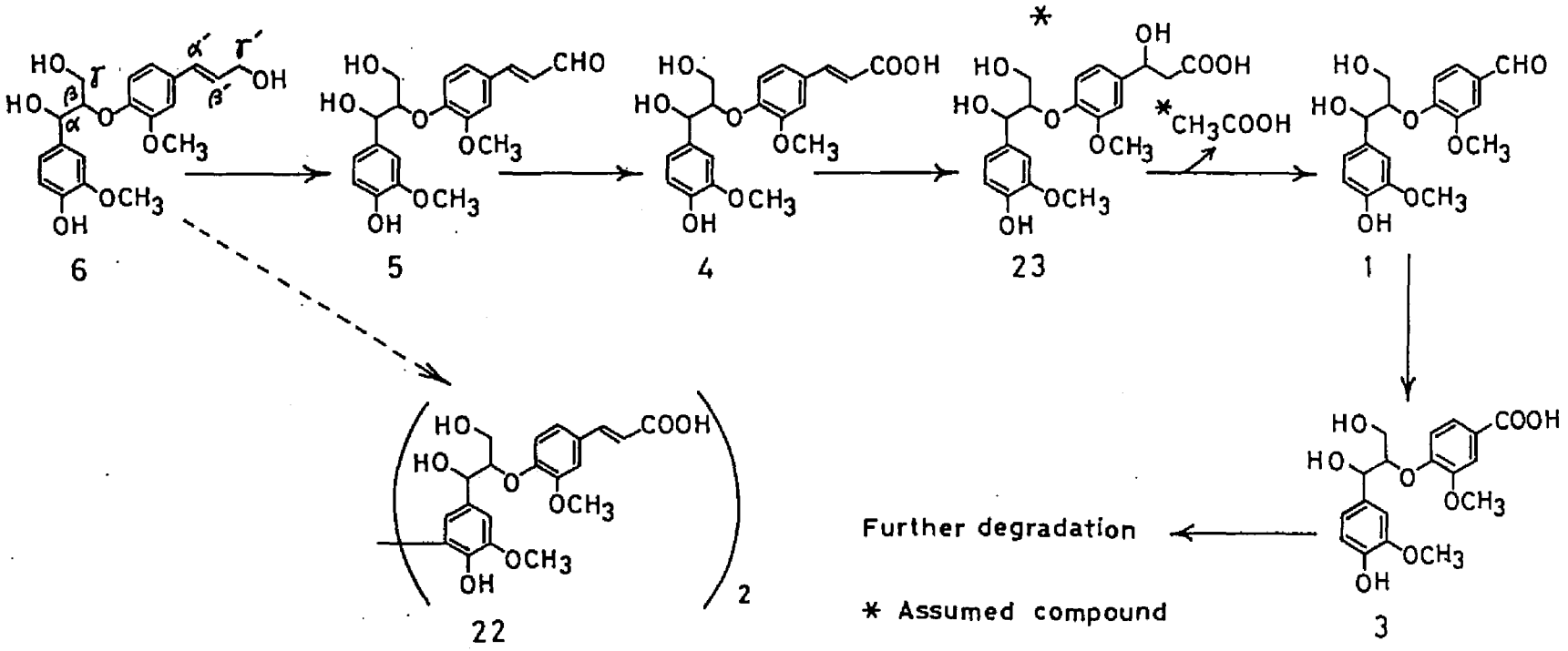


Fig. 1-10 Proposed pathway for the catabolism of guaiacylglycerol-β-coniferyl ether (6) by Fusarium solani M-13-1

vanillin, with release of acetate, by Pseudomonas acidovorans; cell extracts oxidized vanillin to vanillic acid in the presence of NAD^+. They proposed that 3-hydroxy-3-(4-hydroxy-3-methoxy)phenylpropionic acid is an intermediate in the conversion of trans-ferulic acid to vanillin. In the proposed pathway (Fig. 1-10), compound 23 is inferred by analogy with the 3-hydroxy-3-phenylpropionic acid of Toms and Wood[75].

Hata[20] concluded that coniferyl alcohol groups of the lignin polymer were mainly converted to vanillic acid groups by Poria subacida, and both he and Kirk et al.[22] proposed the presence of β-vanillic acid ether structure as a characteristic of lignin degraded by white-rot fungi. Compound 3 produced by F. solani M-13-1 corresponds to these proposed β-vanillic acid ether structures.

Under the present culture conditions, vanillic acid, which is a prominent product of lignin degradation by white-rot fungi, was not detected by TLC in the culture filtrate of 6, 4, and 1, and was also not obtained in the degradation of dehydrodiconiferyl alcohol by F. solani M-13-1[45]. It seems that vanillic acid is catabolized too rapidly to be detected or that it may not be an intermediate.

Fukuzumi and Katayama[38] reported cleavage of the β-ether linkage of 6 by Pseudomonas sp.[39] to yield β-hydroxypropiovanillone and coniferyl alcohol. The catabolic pathway of 6 by F. solani M-13-1 is, however, completely different from that of Pseudomonas.

With respect to stereospecificity in the catabolism, it was shown here that F. solani M-13-1 attacks erythro and

threo forms without distinction, at least in the terminal side chain shortening, because the erythro and threo ratios in the catabolic products was approximately equal to that of the substrate.

Compound 22 was presumably produced by the oxidative free radical coupling of 6 or 4. However, the fungus gave no color reaction in the Bavendamm test, suggesting that it possesses only very weak phenoloxidase activity.

1.3 Cleavage of Alkyl-Aryl C-C Bond of Arylglycerol-β-Aryl Ethers

1.3.1 INTRODUCTION

Previous section showed that an initial degradation reaction of guaiacylglycerol-β-coniferyl ether (6) by F. solani M-13-1 is an oxidative shortening of the allyl alcohol moiety in the side chain of 6 to form guaiacylglycerol-β-(vanillic acid) ether (3). The present section describes the degradation of 3 and syringylglycerol-β-vanillin ether (7) by the fungus. The latter compound was used to help determine the origin of the aromatic rings in the degradation products and to avoid condensation reactions at the 5 position by the action of phenol-oxidizing enzymes.

1.3.2 EXPERIMENTAL

Preparation of fungal mycelia

Mineral salts base and nutrient medium (both pH 6.0) were prepared as described in section 1.2. Mycelia from a stock culture were inoculated into the nutrient medium (100 ml in a 500 ml of Sakaguchi flask) and cultured on a reciprocated shaker (145 strokes per minute) for 2 days at 30℃. Mycelia were separated from the medium by filtration, washed with the mineral salts base, and used immediately for the degradation experiments.

Biodegradation

A solution of 50 mg of the substrate in 1 ml of sterile water and 350 mg of mycelia (dry weight) was added to 100 ml of sterile mineral salts base in a 500 ml Sakaguchi flask. When a water insoluble substrate was used, 50 mg of the substrate was dissolved in a minimum amount of N,N-dimethylformamide (DMF) and the DMF solution was poured into 1 ml of water. The resulting solution or suspension was added to the inorganic base and used for degradation experiments. Two control flasks which contained only mycelia or substrate in the inorganic base were similarly prepared. All flasks were shaken at 30℃.

Analyses of catabolic products

Mycelia were filtered off and washed with distilled water. The filtrate and the washings were combined and extracted as follows. Filtrates of guaiacylglycerol-β-(vanillic acid) ether (3) and syringylglycerol-β-vanillin ether (7) cultures were extracted three times with CH_2Cl_2. The aqueous layer was then acidified to pH 2 with concentrated HCl and extracted three times with EtOAc. The filtrates of veratrylglycerol-β-vanillin ether (26) and glycerol-2-(vanillic acid) ether (29) cultures were acidified to pH 2 with concentrated HCl and extracted three times with EtOAc.

All extracts were washed with saturated brine, dried over anhydrous Na_2SO_4, and the solvent was removed under reduced pressure. Carboxylic acids in the EtOAc extracts were methylated with a limited amount of diazomethane in MeOH so that only the carboxyl groups were methylated; the methyla-

tion was followed by TLC. The residue was then processed by preparative TLC on silica gel, and isolated compounds were identified by their NMR and mass spectra. Authentic samples were used as references for identification.

Chromatography and analytical instruments were the same as in section 1.1.

Preparation of compounds

Guaiacylglycerol-β-(vanillic acid) ether (3), guaiacylglycerol-β-(vanillyl alcohol) ether (2), and syringylglycerol-β-vanillin ether (7)

Syntheses of these compounds were described in section 1.1.

Syringylglycerol-β-(vanillic acid) ether (24) and syringyl glycerol-β-(vanillyl alcohol) ether (25).

These compounds were synthesized from vanillin and syringaldehyde by the same procedures as for compounds 3 and 2, respectively. Compound 24' (methyl ester derivative of 24) (erythro form), ^{1}H-NMR ($CDCl_3$): δ 3.45-5.00 (2H, m, γ-CH_2-), 3.78 (6H, s, -$COOCH_3$ and Ar-OCH_3), 3.83 (3H, s, Ar-OCH_3), 3.86 (3H, s, Ar-OCH_3), 4.20-4.45 (1H, m, β-CH-), 4.91 (1H, d, J = 5.0, α-CH-), 6.55-7.58 (5H, Ar-H); MS m/z (%): 408 (0.4, M^+), 390 (2), 372 (2), 360 (18), 300 (5), 208 (29), 183 (15), 182 (68), 181 (18), 167 (26), 151 (100), 123 (26), 108 (11).

Compound 25, ^{1}H-NMR (acetone-d_6): δ 3.55-4.15 (2H, m, γ-CH_2-), 3.73-3.84 (9H, Ar-OCH_3), 4.10-4.35 (1H, m, β-CH-),

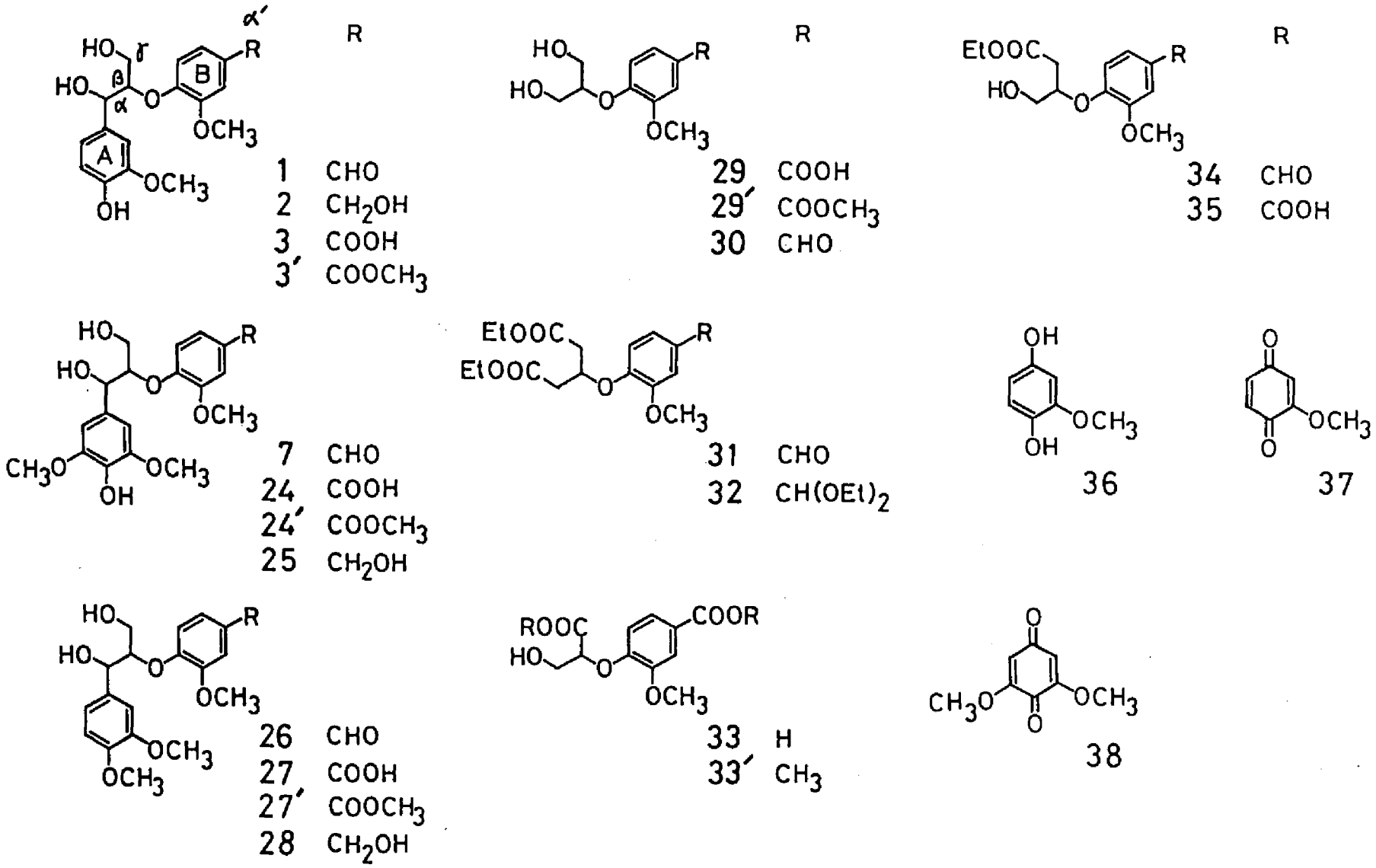


Fig. 1-11 Structures of compounds synthesized and possible catabolic products. (section 1.3)

4.44-4.60 (2H, Ar-CH_2OH), 4.78-4.93 (1H, α -CH-), 6.70-7.04 (5H, Ar-H); MS m/z (%): 380 (1, M^+), 362 (6), 332 (8), 226 (9), 210 (19), 208 (13), 183 (24), 182 (35), 181 (48), 180 (100), 167 (42), 154 (44), 151 (23), 137 (40), 123 (38).

Veratrylglycerol-β-vanillin ether (26), veratrylglycerol-β-(vanillic acid) ether (27), and veratrylglycerol-β-(vanillyl alcohol) ether (28).

These compounds were synthesized from vanillin and veratraldehyde through similar routes as used for compound 1, 3, and 2, respectively. Compound 26, ^{1}H-NMR ($CDCl_3$): δ 3.56-3.90 (2H, m, γ -CH_2-), 3.79-3.87 (9H, Ar-OCH_3), 4.22-4.50 (1H, m, β -CH-), 4.87-5.02 (1H, α -CH-), 6.57-7.38 (6H, Ar-H), 9.77 and 9.78 (1H, two s, erythro and threo -CHO); MS m/z (%): 362 (2, M^+), 354 (0.7), 324 (8), 210 (5), 192 (16), 178 (29), 167 (49), 166 (96), 165 (44), 152 (59), 151 (100), 139 (36), 123 (17), 109 (21).

Compound 27' (methyl ester derivative of 27) (erythro form), ^{1}H-NMR ($CDCl_3$): δ 3.58-3.78 (2H, m, γ -CH_2-), 3.82-3.90 (12H, -$COOCH_3$ and Ar-OCH_3), 4.24-4.42 (1H, m, β -CH-), 4.90-5.04 (1H, m, α -CH-), 6.80-7.00 and 7.49-7.63 (6H, Ar-H); MS m/z (%): 392 (0.9, M^+), 384 (2), 361 (1), 356 (2), 344 (18), 208 (71), 192 (9), 182 (35), 167 (38), 166 (37), 165 (19), 151 (100), 139 (23), 123 (15), 108 (11).

Compound 28, ^{1}H-NMR ($CDCl_3$): δ 3.50-4.00 (2H, m, γ -CH_2-), 3.77-3.87 (9H, Ar-OCH_3), 4.00-4.22 (1H, m, β -CH-), 4.56 (2H, s, Ar-CH_2OH), 4.84-4.98 (1H, α -CH-), 6.69-7.00 (6H, Ar-H); MS m/z (%): 364 (0.7, M^+), 346 (0.7), 316 (8), 210 (4), 192 (14), 181 (20), 180 (100), 167 (29), 166 (28),

165 (35), 154 (23), 151 (49), 139 (27), 137 (22), 123 (14), 107 (16).

Glycerol-2-(vanillic acid) ether (29)

This compound was prepared by a modification of the method reported by Kirk and Lorenz[76]. Diethyl malonate-2-vanillin ether (31) was prepared in 85 % yield by stirring a reaction mixture of vanillin, diethyl bromomalonate, and K_2CO_3 in acetone for 24 hr at room temperature. Treatment of 30 with ethyl orthoformate and p-TsOH in EtOH at room temperature gave diethyl acetatal of 31 (32) in 95 % yield.

To a stirred solution of 7.66 g (19.9 mmol) of 32 in 70 ml of MeOH was added 3.02 g (79.7 mmol) of $NaBH_4$ at 0℃ under nitrogen. After 90 min at the same temperature the reaction mixture was partitioned between EtOAc and brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was dissolved in 60 ml of dioxane. To the stirred dioxane solution was added 1 ml of 1N HCl at room temperature. After 10 min the reaction solution was worked up in a similar way as above to give 4.05 g (90 % yield) of glycerol-2-vanillin ether (30) as colorless powder.

To a stirred solution of 4.25 g (25 mmol) of $AgNO_3$ in 5 ml of water was added 5 ml of 10N NaOH solution (50 mmol). To the resulting mixture containing Ag_2O were added 20 ml of EtOH and a solution of 1.13 g (5 mmol) of 30 in 10 ml of EtOH at room temperature. After 2 hr, the Ag_2O was filtered off and washed successively with EtOH and hot water. The filtrate and the washings were combined, acidified to pH 2

with concentrated HCl and extracted three times with EtOAc. The organic layers were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give 1.18 g (98 % yield) of 29 as colorless powder, which was recrystallized from MeOH. For NMR and MS, 29 was converted to its methyl ester 29' with diazomethane. ^{1}H-NMR (methyl ester 29', $CDCl_3$): δ 3.86 (4H, d, *J* = 4.5, $-CH_2-$), 3.86 (3H, s, $-COOCH_3$), 3.88 (3H, s, Ar-OCH_3), 4.33 (1H, quintet, *J* = 4.5, -CH-), 7.00 (1H, d, *J* = 8.0, Ar-C_5-H), 7.48 (1H, d, *J* = 2.0, Ar-C_2-H), 7.56 (1H, dd, *J* = 8.0, *J* = 2.0, Ar-C_6-H). MS (methyl ester 29') *m/z* (%): 256 (18, M^+), 225 (5), 183 (12), 182 (97), 167 (10), 152 (12), 151 (100), 123 (14), 108 (7).

Compound 29' was acetylated with Ac_2O and pyridine, giving diacetate of 29' (29"). ^{1}H-NMR ($CDCl_3$): δ 2.06 (6H, s, -OAc), 3.89 (6H, s, $-COOCH_3$ and Ar-OCH_3), 4.35 (4H, d, *J* = 5.0, $-CH_2-$), 4.71 (1H, quintet, *J* = 5.0, -CH-), 7.02 (1H, d, *J* = 8.0, Ar-C_5-H), 7.53 (1H, d, *J* = 2.0, Ar-C_2-H), 7.60 (1H, dd, *J* = 8.0, *J* = 2.0, Ar-C_6-H). MS *m/z* (%): 340 (2, M^+), 309 (1), 215 (1), 193 (1), 182 (13), 167 (2), 160 (4), 159 (78), 151 (16), 99 (12), 57 (7), 43 (100).

Glyceric acid-2-(vanillic acid) ether (33)

To a stirred solution of 351 mg (0.913 mmol) of 31 in 10 ml of MeOH was added 138 mg (3.65 mmol) of $NaBH_4$ at 0℃ under nitrogen. After 6 min the reaction solution was partitioned between EtOAc and brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was dissolved in 8 ml

of dioxane. To the stirred dioxane solution was added 0.1 ml of 1N HCl at room temperature. After 10 min the reaction solution was worked up in similar way as above. The resulting residue was purified by TLC (EtOAc-n-hexane = 1:1) to give 139 mg (57 % yield) of ethyl glycerate-2-vanillin ether (34) as colorless crystals.

A solution of 262 mg (1.66 mmol) of $KMnO_4$ in 2.5 ml of water was added to a solution of 197 mg (1.11 mmol) of 34 in 12 ml of dioxane with stirring at room temperature. After 60 min, 1 ml of MeOH was added to the reaction mixture and the stirring was continued for additional 20 min. The resulting MnO_2 was then filtered off and washed successively with MeOH and hot water. The filtrate and the washings were combined, acidified to pH 2 with 1N HCl, and extrated three times with EtOAc. The organic layers were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give 230 mg (73 % yield) of ethyl glycerate-2-(vanillic acid) ether (35) as colorless crystals.

Compound 35 (220 mg, 0.775 mmol) was dissolved in 11.6 ml (2.32 mmol) of 0.2 N NaOH solution and the solution was stirred for 2 hr at room temperature. The solution was then acidified to pH 1 with concentrated HCl and extracted three times with EtOAc. The organic layers were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo* to give 1.18 g (98 % yield) of 33 as colorless crystals. For NMR and MS, 33 was converted to its methyl ester 33' with diazomethane. ^{1}H-NMR (33', $CDCl_3$): δ 3.78 (3H, s, >CHCOOC$\underline{H}_3$), 3.88 (3H, s, Ar-COOC$\underline{H}_3$), 3.91 (3H, s, Ar-OC$\underline{H}_3$), 4.06-4.12 (2H, d, $\underline{J}$ = 4.5, -C$\underline{H}_2$-), 4.79 (1H, t,

J = 4.5, >CH-), 6.87 (1H, d, J = 8.0, Ar-C_5-H), 7.54 (1H, d, J = 2.0, Ar-C_2-H), 7.59 (1H, dd, J = 8.0, J = 2.0, Ar-C_6-H). MS (33') m/z (%): 284 (50, M^+), 266 (2), 253 (10), 225 (7), 207 (4), 195 (14), 182 (91), 181 (16), 167 (11), 152 (12), 151 (100), 123 (13), 119 (12).

Methoxyhydroquinone (36), methoxy-p-benzoquinone (37), and 2,6-dimethoxy-p-benzoquinone (38)

Methoxyhydroquinone (36) was commercially available (Tokyo Kasei Co.). Methoxy-p-benzoquinone (37) was prepared by oxidation of 36 with Ag_2O in Et_2O at room temperature. 2,6-dimethoxy-p-benzoquinone (38) was obtained by oxidation of syringic acid with DDQ in MeOH[77]: MS m/z (%): 168 (50, M^+), 140 (13), 138 (25), 112 (10), 97 (16), 80 (43), 69 (100).

1.3.3 RESULTS

Degradation of guaiacylglycerol-β-(vanillic acid) ether (3)

When F. solani M-13-1 was incubated in the medium containing 3, the UV absorption of the culture filtrate at 282 nm decreased continuously as shown in Fig. 1-12(a). After 20 hr incubation a compound was detected in the filtrate by TLC. After 28 hr incubation the compound was the main product present along with a trace of 3.

An ethyl acetate extract of a 28 hr culture filtrate was methylated with diazomethane. The methyl ester of the compound (2.4 mg, 4.8% from 50 mg of 3) was isolated from the treated extract by TLC (EtOAc-n-hexane = 3:2, X 3). The

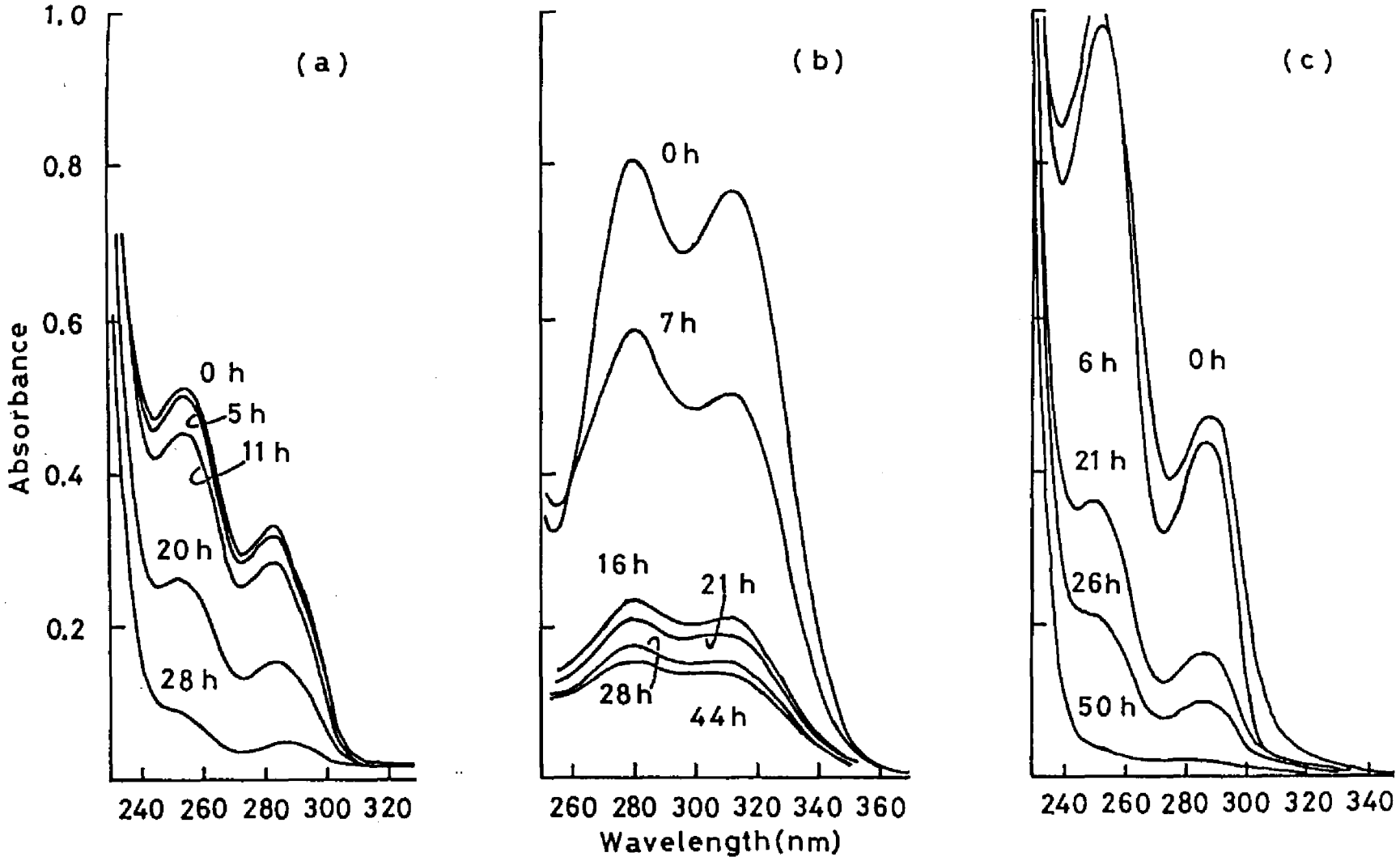


Fig. 1-12 Changes in the UV absorption of culture filtrate containing (a) guaiacylglycerol-β-(vanillic acid) ether (3), (b) syringylglycerol-β-vanillin ether (7), and (c) glycerol-2-(vanillic acid) ether (29) during incubation with Fusarium solani M-13-1

isolated compound was identified as the methyl ester (29') of glycerol-2-(vanillic acid) ether (29) by ^{1}H-NMR and MS. The spectra and R_f value on TLC with those of 29' and its diacetate (29") were identical with those of authentic samples. Methoxyhydroquinone (36), methoxy-p-benzoquinone (37) and/or guaiacol were not detected chromatographically at any stages.

Degradation of syringylglycerol-β-vanillin ether (7)

Figure 1-12(b) shows that UV absorption at 280 and 312 nm of the culture filtrate using 7 as substrate decreased gradually during incubation. Methylene chloride extracts of the culture filtrates after 16, 21, 28, and 44 hr incubations were combined and subjected to TLC (EtOAc-n-hexane = 1:2) which separated three fractions, F_1, F_2, and F_3. Purification of F_1 by TLC (EtOAc-n-hexane = 1:3, X 3) afforded yellow crystals which were identified as 2,6-dimethoxy-p-benzoquinone (38) by MS. Purification of F_3 by TLC (EtOAc-n-hexane = 2:1 X 3, and 3% MeOH in CH_2Cl_2 X 3) gave a syrup which was identified as syringylglycerol-β-(vanillyl alcohol) ether (25).

Ethyl acetate extracts of the culture filtrates after 16, 21, 28, and 44 hr incubations were combined, methylated with diazomethane, and separated to five fractions (F_4-F_8) by TLC (3% MeOH in CH_2Cl_2, X 3). Purification of F_8 by TLC (EtOAc-n-hexane = 1:1, X 4) gave two colorless syrups, which were identified by MS as glycerol-2-(methyl vanillate) ether (29') an syringylglycerol-β-(methyl vanillate) ether (24').

The MS and R_f values on TLC of the isolated compounds were identical with those of authentic samples. The yields of the isolated compounds were about 0.2-1.5 mg (from 200 mg of substrate 7).

Degradation of veratrylglycerol-β-vanillin ether (26)

Two new compounds and residual substrate (26) were present in culture filtrates after 20 hr incubation. The two compounds became dominant with the disappearance of 26 after 48 hr incubation. The ethyl acetate extract of the 48 hr culture filtrate was methylated with diazomethane and purified by TLC (EtOAc-n-hexane = 2:1, X 3) to give the two catabolic compounds which were identified as veratryl-glycerol-β-(methyl vanillate) ether (27') (27.8 mg, 56% from 50 mg of 26) and veratrylglycerol-β-(vanillyl alcohol) ether (28) (8.4 mg, 17%) by comparison of their ^{1}H-NMR and MS with those of authentic samples.

Compound 27 was found to be stable in the culture medium, and not catabolized by F. solani M-13-1.

Degradation of glycerol-2-(vanillic acid) ether (29)

Since it was found that the degradation of 3 and 7 by mycelial suspensions of F. solani M-13-1 yielded glycerol-2-(vanillic acid) ether (29) as a transformation product, the fungus was shake-cultured in a medium containing 29. Fig. 1-12(c) shows the continuous decrease with time of the UV absorption of the culture filtrate at 288 nm. Compound 29

was completely degraded, but the patterns of the chromatogram of the culture filtrate were the same as that of control solution except for the occurrence of substrate 29. Vanillic acid and guaiacol, a catabolic product of vanillic acid by the fungus[78], were not detected.

1.3.4 DISCUSSION

The degradation pathway indicated by these results was depicted in Fig. 1-13. The aldehyde group of three kinds of arylglycerol-β-vanillin ether (1, 7, and 26) were oxidized and/or reduced to the corresponding α'-carboxyl and/or α'-alcohol groups. Both reactions took place at the same time during incubation with the β-vanillin ethers as substrate. The oxidation and reduction between the α'-aldehydic (1, 7, and 26) and α'-alcoholic (2, 25, and 28) compounds are apparently reversible, and only the α'-carboxylic compounds (3, 24, and 27) seem to be degraded further. Reduction of the α'-carboxyl groups in arylglyceol-β-(vanillc acid) ethers (3, 24, and 27) did not occur under the present experimental condition. A similar reaction by the fungus was reported for the allyl aldehyde moiety of 5-(2-formyl-vinyl)-2-(4-hydroxy-3-methoxyphenyl)-3-hydroxymethyl-7-methoxycoumaran[45].

Syringylglycerol-β-vanillin ether (7) was degraded to give glycerol-2-(vanillic acid) ether (29) and 2,6-dimethoxy-p-benzoquinone (38), which were derived from guaiacyl and syringyl nuclei, respectively, indicating the cleavage of alkyl-aryl C-C bond. Guaiacylglycerol-β-(vanillic acid)

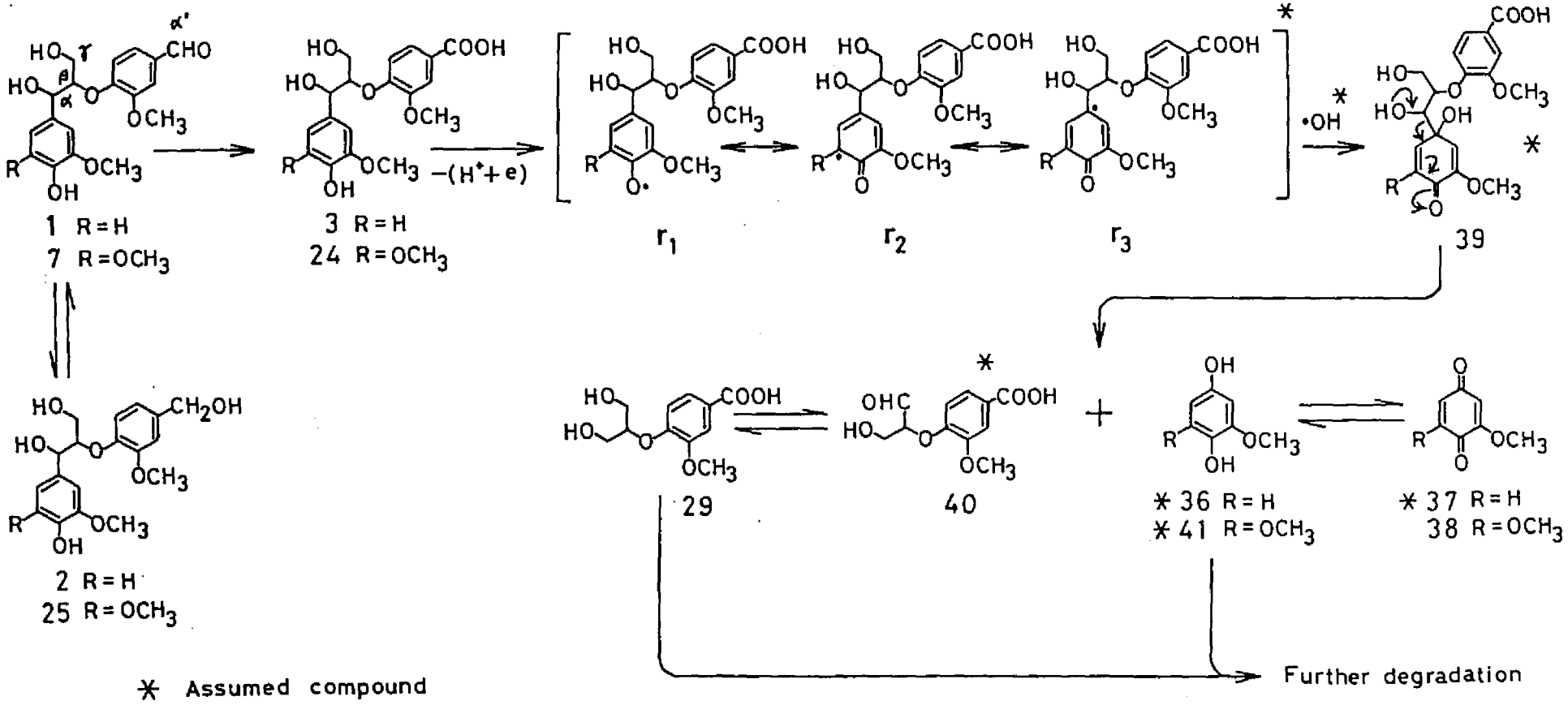


Fig. 1-13 Proposed pathway for the catabolism of guaiacylglycerol-β-vanillin ether (1) and syringylglycerol-β-vanillin ether (7) by Fusarium solani M-13-1

ether (3) was also decomposed to 29, which indicates the cleavage of the C-C linkage. Since veratrylglycerol-β-(vanillic acid) ether (27) was not degraded under the same condition, the cleavage of the alkyl-aryl C-C bond was assumed to be due to the activity of phenol oxidizing enzymes. Both laccase and a phenol oxidase which is remarkably more active for syringaresinol than for guaiacol and other laccase substrates have been isolated from F. solani M-13-1 by Iwahara and Kaoka[79]. The free radicals formed by phenol oxidase-catalyzed oxidation of 3 and 24 could consist of three resonance structures (r_1, r_2, and r_3). Coupling of r_3 with hydroxyl radical (· OH) would give an intermediate compound, 1-hydroxycyclohexadienone derivative 39, which is converted to glyceraldehyde-2-(vanillic acid) ether (40) and methoxyhydroquinone (36) as shown in Fig. 1-13. According to Kirk's[80] recent proposal, disproportionation of the bimolecular phenoxy radicals would give the parent molecule 3 and a cationic chemical species, and then addition of water to the cationic chemical species afforded the same intermediate compound 39, which is converted to 40 and 36 as above.

It is likely that 29 was formed by the reduction of 40, and that methoxyhydroquinone (36 and 41) were further catabolized *via* ring fission and/or oxidized to methoxy-*p*-benzoquinone (37 and 38). The fact that 36 or 37 could not be obtained from 3 as a catabolic product is probably due to oxidative condensation of the hydroquinone (36) or compound 3 at 5 position by phenol oxidizing enzymes or to their rapid catabolism.

It was previously found that 5,5'-biphenyl tetrameric

compound (22) is formed from guaiacylglycerol-β-coniferyl ether (6) by the action of phenol oxidizing enzymes of F. solani M-13-1 (section 1.2). Kirk et al.[30] found that the alkyl-aryl C-C bond in syringylglycol-β-guaiacyl ether was cleaved by a culture filtrate of C. versicolor with formation of guaiacoxyacetaldehyde and 38, and that a laccase purified from the culture filtrate effected the reaction. Pew and Connors[81] reported that glyceraldehyde-2-guaiacyl ether was obtained by dehydrogenation of guaiacylglycerol-β-guaiacyl ether by a horseradish peroxidase. Those reports seem to indicate that in F. solani M-13-1 a similar reaction occurred. It should be concluded that phenol oxidizing enzymes are responsible for biodegradation of phenolic moieties in lignin. Ander and Eriksson[82] obtained evidencefor an obligatory role of phenol oxidase in lignin biodegradation.

Non-oxidative cleavage of alkyl-aryl C-C bond has been proposed for degradation of α-conidendrin by Pseudomonas multivorans[42] and of phloretin by mold[83]. If non-oxidative cleavage of 3 or 24 would occur, 29 and either guaiacol or 2,6-dimethoxyphenol would be produced. However, the possibility of such cleavage was excluded by the formation of 38 and no detection of guaiacol or 2,6-dimethoxyphenol.

On the other hand, cleavage of the β-aryl ether linkage in arylglycerol-β-aryl ether was reported in white-rot fungi[84,27] and in bacteria[38]. Recently, cleavage of the Cα-Cβ linkage in veratrylglycerol-β-aryl ether was also reported in white-rot fungi[34] and in bacteria[85].

1.4 Cleavage of Alkyl-Aryl Ether Bond of Glycerol-2-Aryl Ethers

1.4.1 INTRODUCTION

Previous section showed that F. solani M-13-1 degraded phenolic arylglycerol-β-(vanillic acid) ethers (3 and 24), yielding glycerol-2-(vanillic acid) ether (29) and methoxy-p-benzoquinones (37 and 38). Compound 29 was further degraded by the fungus, but none of catabolic products were detected. Predicted intermediates such as glyceric acd-2-(vanillic acid) ether (33) and ethylene glycol mono-(vanillic acid) ether were not catabolized by the fungus[50]. Kuwahara et al. reported the catabolism of vanillic acid by F. solani M-13-1[78]. It was assumed that in the catabolism of 29 vanillic acid was catabolized too fast to be detected under the culture conditions.

In the present investigation, glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether (42) which has an ethoxyl group instead of a methoxyl group was used as a substrate for the fungus. It is assumed that 3-ethoxy-4-hydroxybenzoic acid (43) which is predicted as a degradation product of 2-(β-)aryl ether linkage is catabolized slower than vanillic acid and accumulated enough to be detected. Consequently, 43 as a major catabolic product and other two products were isolated and identified, indicating the cleavage of the 2-(β-) aryl ether linkage.

1.4.2 EXPERIMENTAL

Microorganism

Fusarium solani M-13-1[44] was used. Composition of the basal inorganic salts medium and nutrient medium (both pH 6.0) were the same as described in section 1.2. Fungal mycelia were prepared as described in section 1.3.

Biodegradation

Glycerol-2-(4-hydroxy-3-ethoxybenzoic acid) ether (42) was used as a substrate. The substrate was dissolved in 0.5 ml of *N,N*-dimethylformamide (DMF). The DMF solution and 350 mg (dry weight) of the mycelial suspension in 5 ml of the basal inorganic medium were added to 95 ml of the basal inorganic medium in a 500 ml of Erlenmeyer flask. A control flask without mycelia in the medium was prepared. All flasks were shaken on the rotary shaker at 28℃.

Analysis of catabolic products

Degradation of the substrate and formation of catabolic products were monitored by UV spectroscopy, TLC, and high-performance liquid chromatography (HPLC) of the culture filtrate. Mycelia were removed from the two culture flasks by filtration after 2 and 8 days incubation and washed with distilled water. The filtrate and the washings were combined, acidified to pH 2 with 1N HCl, and extracted three times with EtOAc. The extracts were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The resulting residue after 2 days incubation was

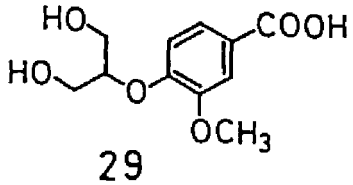
methylated with an excess of diazomethane in MeOH and purified by preparative TLC. The residue after 8 days incubation was purified by preparative TLC without methylation.

Preparation of compounds

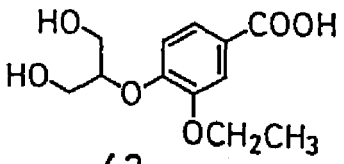
Glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether (42)

This compound was synthesized *via* following seven steps which are the same method of the synthesis of 29 (section 4.1). 1) 3-Ethoxy-4-hydroxybenzaldehyde/ ethyl bromomalonate/ K_2CO_3/ dry DMF/ 74%; 2) $CH(OEt)_3$/ EtOH/ *p*-TsOH/ r.t.; 3) $NaBH_4$ (4.0 equivalent)/ MeOH-THF/ 0℃; 4) Ac_2O/ pyridine/ r.t.; 5) 1N HCl/ dioxane-H_2O; 70% (2)-5)); 6) $KMnO_4$/ dioxane/ r.t.; 7) 1N NaOH/ r.t./97% (6)-7)). ^{1}H-NMR (CD_3OD): δ 1.43 (3H, t, *J* = 7.0, $-OCH_2CH_3$), 3.79 (4H, d, *J* = 5.1, $-CH(CH_2OH)_2$), 4.13 (2H, q, *J* =7.0, $-OCH_2CH_3$), 4.41 (1H, quintet, *J* = 5.1, O-CH<), 7.15 (1H, d, *J* = 9.0, Ar-C_5-H), 7.55-7.60 (1H, Ar-C_2-H), 7.65 (1H, dd, *J* = 9.0, *J* = 2.0, Ar-C_6-H). ^{13}C-NMR (CD_3OD): δ 15.06 ($-OCH_2CH_3$), 62.03 (2C, $-OCH(CH_2OH)_2$), 65.92 ($-OCH_2CH_3$), 82.55 ($-O-CH-(CH_2OH)_2$), 116.01 (Ar-C_2), 117.07 (Ar-C_5), 124.73 (Ar-C_6), 125.24 (Ar-C_1), 150.33 (Ar-C_3), 153.46 (Ar-C_4), 169.40 (Ar-COOH). MS *m/z* (%): 256 (24.9, M^+), 183 (6.9), 182 (62.1), 165 (6.9), 155 (8.3), 154 (100), 153 (7.5), 137 (29.1), 109 (5.8). IR ν_{max}^{KBr} cm^{-1}: 3320, 2875, 2650 (sh), 1675 (C=O), 1585, 1510, 1388, 1338, 1265, 1213, 1142, 1110, 1032, 970, 870, 765, 750, 660, 515. UV λ_{max}^{MeCN} nm: 259, 292.

Methyl ester derivative, glycerol-2-(methyl 3-ethoxy-4-hydroxybenzoate) ether, was prepared by the treatment of 42



29



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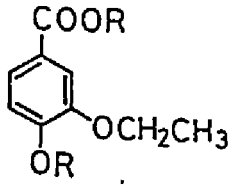

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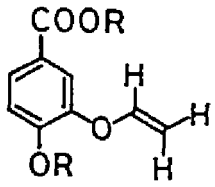
43′ R=CH_3



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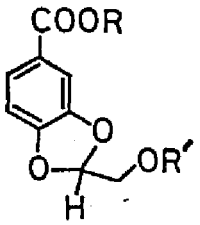
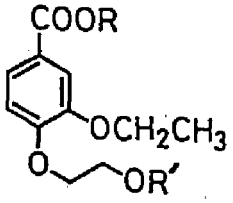


45 R=R′=H
45′ R=CH_3 R′=Ac



46 R=R′=H
46′ R=CH_3 R′=Ac

Fig. 1-14 Structures of the substrates used and the compounds isolated from the culture filtrate. (section 1.4)

with diazomethane. ^{1}H-NMR ($CDCl_3$): δ 1.48 (3H, t, J = 7.0, $-OCH_2CH_3$), 2.10 (broad, -OH), 3.85 (4H, d, J = 4.5, $-CH(CH_2OH)_2$), 3.90 (3H, s, $-COOCH_3$), 4.16 (2H, q, J = 7.0, $-OCH_2CH_3$), 4.27 (1H, quintet, J = 4.7, O-CH<), 7.13 (1H, d, J = 8.6, Ar-C_5-H), 7.55-7.60 (1H, Ar-C_2-H), 7.64 (1H, dd, J = 8.7, J = 2.0, Ar-C_6-H).

3-Ethoxy-4-hydroxybenzoic acid (43)

This compound was synthesized by the similar method of the preparation of vanillic acid with Ag_2O [86]. ^{1}H-NMR (CD_3OD): δ 1.43 (3H, t, J = 7.0, $-OCH_2CH_3$), 4.13 (2H, q, J =7.0, $-OCH_2CH_3$), 6.84 (1H, d, J = 8.8, Ar-C_5-H), 7.54 (1H, d, J = 1.8, Ar-C_2-H), 7.55 (1H, dd, J = 8.7, J = 2.0, Ar-C_6-H). ^{13}C-NMR (CD_3OD): δ 15.00 ($-OCH_2CH_3$), 65.61 ($-OCH_2CH_3$), 115.02 (Ar-C_2), 115.77 (Ar-C_5), 122.89 (Ar-C_1), 125.08 (Ar-C_6), 147.54 (Ar-C_3), 152.66 (Ar-C_4), 169.83 (Ar-COOH). MS m/z(%): 182 (50.3, M^+), 165 (2.5), 155 (8.3), 154 (99.1), 38 (8.9), 137 (100), 125 (3.0), 109 (14.7), 97 (4.6). IR ν_{max}^{KBr} cm^{-1}: 3360, 2870, 2600, 1675 (C=O), 1590, 1510, 1430, 1380, 1275, 1225, 1210 (sh), 1110, 1038, 950, 877, 801, 762, 624, 574, 505. UV λ_{max}^{MeCN} nm: 259, 290.

Methyl 3-ethoxy-4-methoxybenzoate (43')

This compound was prepared by the treatment of 43 with excess amount of diazomethane in MeOH at 0℃. ^{1}H-NMR ($CDCl_3$): δ 1.48 (3H, t, J =7.0, $-OCH_2CH_3$), 3.88 (3H, s, $-COOCH_3$), 3.92 (3H, s, Ar-OCH_3), 4.16 (2H, q, J = 7.0, $-OCH_2CH_3$), 6.88 (1H, d, J = 8.5, Ar-C_5-H), 7.54(1H, d, J = 2.0, Ar-C_2-H), 7.66 (1H, dd, J = 8.4, J= 2.0, Ar-C_6-H). ^{13}C-NMR ($CDCl_3$): δ

14.71 (-$OCH_2\underline{C}H_3$), 51.85(-$COO\underline{C}H_3$), 55.95 (Ar-OCH_3), 64.43 (-$O\underline{C}H_2CH_3$), 110.43 (Ar-C_2), 113.33 (Ar-C_5), 122.55 (Ar-C_1), 123.35 (Ar-C_6), 147.81 (Ar-C_3), 153.15 (Ar-C_4), 166.69 (-$\underline{C}OOCH_3$). MS m/z (%): 210 (43.8, M^+), 182 (41.4), 179 (13.0), 167 (7.0), 152 (9.8), 151 (100), 139 (3.1), 136 (2.6), 123 (10.8), 108 (4.6). IR ν_{max}^{KBr} cm^{-1}: 2920, 2875, 1705 (C=O), 1587, 1512, 1430, 1390, 1342, 1296, 1272, 1217, 1194, 1135, 1105, 1046, 1018, 991, 869, 838, 763.

Methyl 4-ethoxy-3-methoxybenzoate

This compound was synthesized from vanillin via following three steps: 1) vanillin/ C_2H_5I/ K_2CO_3/ dry DMF/ r.t./ 80%; 2) $KMnO_4$/ dioxane/ r.t.; 3) CH_2N_2/ MeOH/ 0℃. ^{1}H-NMR ($CDCl_3$): δ 1.49 (3H, t, J = 7.0, -$OCH_2\underline{C}H_3$), 3.89 (3H, s, -$COOCH_3$), 3.92 (3H, s, Ar-OCH_3), 4.16 (2H, q, J = 7.0, -$O\underline{C}H_2CH_3$), 6.87 (1H, d, J = 8.3, Ar-C_5-H), 7.44 (1H, d, J = 2.0, Ar-C_2-H), 7.65 (1H, dd, J = 8.3, J = 2.0, Ar-C_6-H). MS m/z (%): 210 (40.4, M^+), 182 (40.7), 179 (9.1), 167 (6.8), 152 (9.9), 151 (100), 139 (2.7), 136 (2.5), 123 (12.9), 108 (4.5). IR ν_{max}^{KBr} cm^{-1}: 2930, 2890, 1705 (C=O), 1585, 1505, 1482, 1435, 1392, 1340, 1290, 1258, 1216, 1183, 1132, 1030, 981, 900, 875, 822, 777, 763, 640.

Derivatization of the catabolic products

Hydrogenation of catabolic product B'

Catabolic product B' (0.7 mg) was dissolved in 1 ml of MeOH, and 0.7 mg of 10 % Pd-C was added to the solution. The mixture was stirred under hydrogen at room temperature

for 1 min. The catalyst was filtered off and washed with MeOH. The filtrate and the washings were combined and evaporated in vacuo. The residue was purified by TLC (CH_2Cl_2-n-hexane = 1:1) to give as colorless crystals.

Acetylation and subsequent methylation of product C and D

Product C was dissolved in 5 drops of pyridine, and 5 drops of Ac_2O and 1 ml of EtOAc were added to the solution at room temperature. After stirring for over night, the solvent was removed by azeotropic distillation with EtOAc. The residual colorless powder was dissolved in MeOH, and 2 ml of an ethereal solution of diazomethane was added to the solution at 0℃. After 10 min the reaction solution was evaporated in vacuo. The residue was purified by TLC (CH_2Cl_2) to give C' as pale yellow powder.

Product D was also acetylated and methylated by the same method to give D' as pure colorless powder.

Chromatography and spectrometry

Analytical TLC was conducted by using precoated plates with Merck silica gel 60 F_{254} (0.25 mm thickness). Preparative TLC was conducted by using precoated plates with Merck silica gel 60 F_{254} (0.5 and 2.0 mm thickness) and plates coated with Merck silica gel 60 PF_{254} (2 mm). Column chromatography was performed on the FMI high performance low to medium pressure chromatograph equipped with a column of Merck silica gel 60 (230-400 ASTM mesh). HPLC was performed by using a Jasco BIP-I HPLC pump system with a Jasco UVIDEC--100-IV UV spectrophotometer as a detector. Peak area was

calculated by using a Shimadzu Chromatopac C-R3A. The column used was a Chemco Pak Finesil C_{18}-5 (4.6 mm ID X 15 cm) with a precolumn (4.6 mm ID X 5.0 cm).

^{1}H and ^{13}C-NMR spectra were recorded on a Hitachi R-90H FT-NMR spectrometer (90 MHz) with tetramethylsilane as an internal standard. Mass spectra (MS) were taken by a JEOL JMS DX-300 mass spectrometer with a direct inlet system at an ionizing voltage of 70 eV; relative intensity of each peak was designated in parenthese. Infrared (IR) and UV spectra were taken by a Jasco A-302 infrared spectrophotometer and a Hitachi model 200-20 double beam spectrometer, respectively.

1.4.3 RESULTS

Glycerol-2-(vanillic acid) ether (29) was completely degraded, but vanillic acid and guaiacol were not detected from the culture filtrate. Minor peaks in the HPLC of the culture filtrate showed the same patterns as those of the control solution.

Glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether (42) was also degraded completely, but slower than 29. Fig. 1-15 shows degradation of 42 monitored by UV absorption at 251 nm and 287 nm of the culture filtrate during incubation.

Two cultures were extracted after 2 days incubation, because TLC analysis of the culture filtrate (15% MeOH in CH_2Cl_2) showed two new spots A and B. Both spots gave blue color for spraying a mixture of $FeCl_3$-$K_3Fe(CN)_6$, indicating the presence of a phenolic hydroxyl group. The extract was

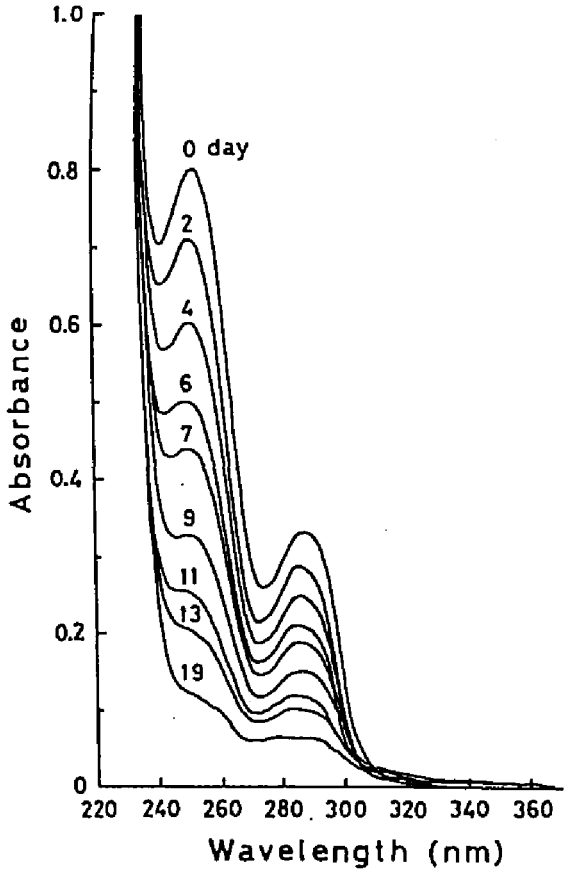


Fig. 1-15 Changes in the UV absorption of culture filtrates containing glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether (42) during incubation of Fusarium solani M-13-1

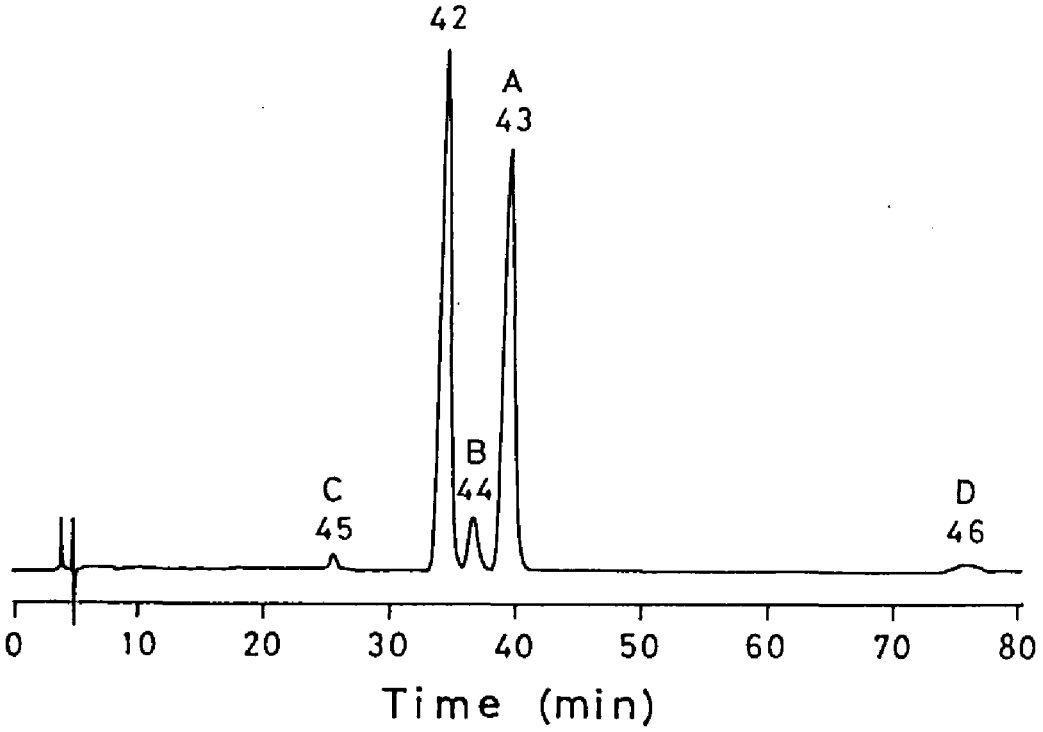


Fig. 1-16 High-performance liquid chromatogram of the culture filtrate containing glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether (42) after 7 days incubation. A, 3-ethoxy-4-hydroxybenzoic acid (43); B, 4-hydroxy-3-vinyloxybenzoic acid (44); C, 3,4-(hydroxymethyl)methylenedioxybenzoic acid (45); D, 3-ethoxy-4-(2-hydroxyethoxy)benzoic acid acid (46)

treated with diazomethane for 2 hr to methylate both carboxyl and phenolic hydroxyl groups. The methylated extract was separated by preparative TLC (CH_2Cl_2-n-hexane = 1:1, X 3) to give three main bands, A' (9.7 mg), B' (1.4 mg), and methyl ester of 42 recovered (77.0 mg).

After 7 days incubation, the two catabolic products increased related to the substrate (Fig. 1-16). Two cultures were extracted after 8 days incubation. The extract was separated by TLC (15% MeOH in CH_2Cl_2) without methylation to give A (20.3 mg), B (5.3 mg), C (1.2 mg), D (1.3 mg), and recovered 42.

Fig. 1-17(b) shows ^{1}H-NMR of A' indicating the presence of two $-OCH_3$ ($Ar-OCH_3$ and $-COOCH_3$), $-OCH_2CH_3$, and aromatic ring protons, and the absence of glycerol moiety in the substrate. Table 1-1 shows ^{13}C-NMR data of A' indicating the presence of one $Ar-OCH_3$, one $-COOCH_3$, the $-OCH_2CH_3$, and the aromatic ring carbons. The presence of the ester was confirmed by its IR spectrum (1705 cm^{-1}). Its MS showed a molecular ion peak at m/z (%): 210 (71) and major fragment ion peaks at m/z 182 (51, $M^+-C_2H_4$), 179 (17, M^+-OCH_3), and 151 (100, $M^+-C_2H_4-OCH_3$ or $M^+-COOCH_3$). These spectra and chromatographic behavior were completely identical with those of synthetic 43'. Therefore, A' was identified as methyl 3-ethoxy-4-methoxybenzoate (43').

Two methyl groups of A' were derived from diazomethane methylation. A was identified as 3-ethoxy-4-hydroxybenzoic acid (43), which was confirmed by the following data. ^{1}H-NMR (CD_3OD): δ 1.43 (3H, t, J = 7.0, $-OCH_2CH_3$), 4.13 (2H, q, J = 7.0, $-OCH_2CH_3$), 6.84 (1H, d, J = 8.8, $Ar-C_5-H$), 7.54 (1H,

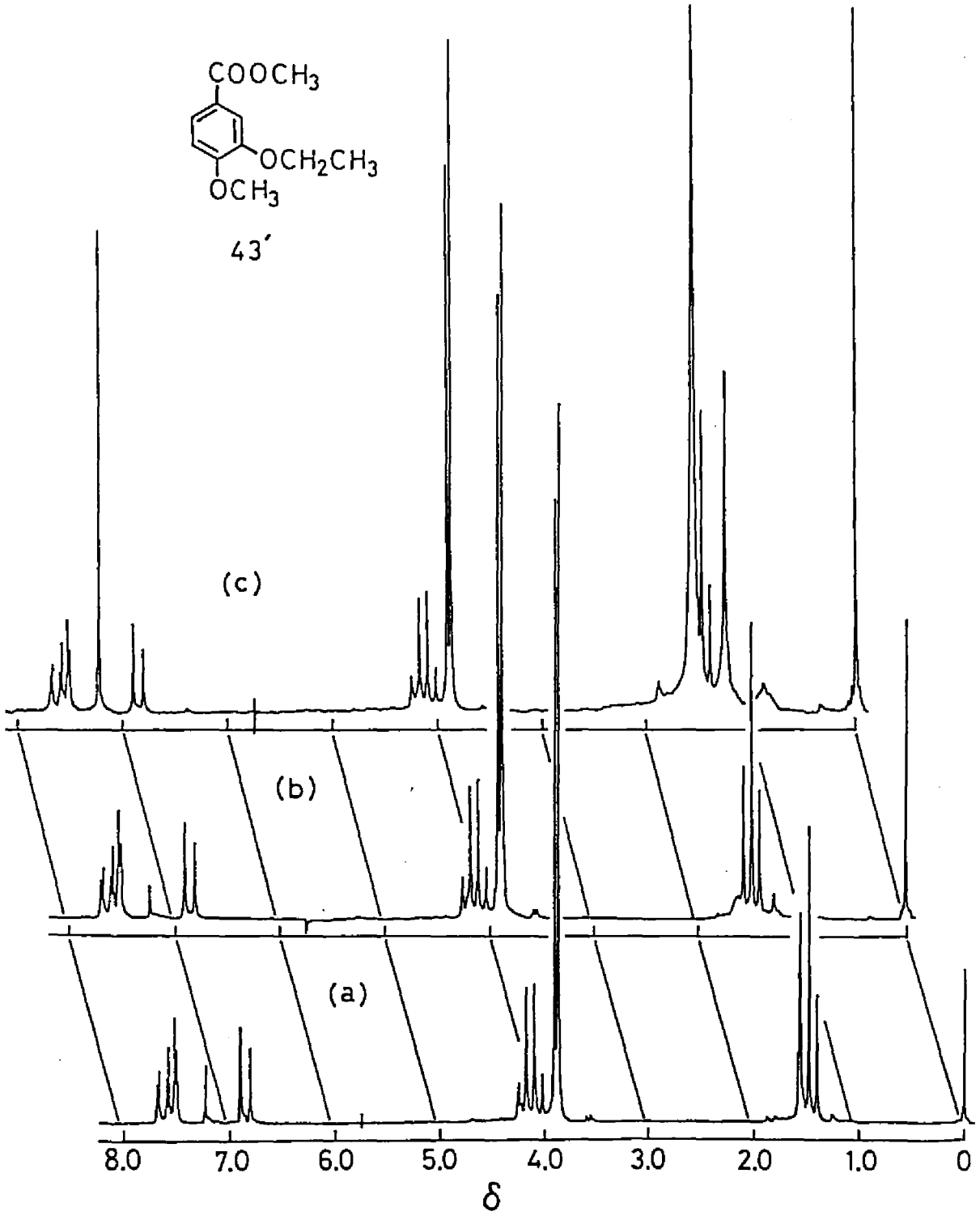


Fig. 1-17 ^{1}H-NMR spectra of methyl 3-ethoxy-4-methoxybenzoate (43')
(a) Synthetic compound. (b) Catabolic product A'.
(c) Derivative formed by the catalytic reduction of catabolic product B'.

Table 1-1 ^{13}C-NMR data of catabolic A (43), A' (43'), and B (44)

	A (43)	A' (43')	B (44)
(solvent)	(CD_3OD)	($CDCl_3$)	($CDCl_3$)
-COOH	169.84	------	170.42
-COO$\underline{C}H_3$	------	51.83	------
-$\underline{C}OOCH_3$	------	166.69	------
-$OCH_2\underline{C}H_3$	15.02	14.70	------
-$O\underline{C}H_2CH_3$	65.66	64.39	------
-OCH=$\underline{C}H_2$	------	------	97.02
-O$\underline{C}$H=CH_2	------	------	143.10
Ar-OCH_3	------	55.95	------
Ar-C_1	122.98	122.52	121.59
Ar-C_2	115.10	110.40	115.47
Ar-C_3	147.59	147.78	147.10
Ar-C_4	152.71	153.12	151.03
Ar-C_5	115.80	113.30	117.74
Ar-C_6	125.10	123.33	127.25

A, 3-Ethoxy-4-hydroxybenzoic acid (43)

A', Methyl 3-ethoxy-4-methoxybenzoate (43')

B, 4-Hydroxy-3-vinyloxybenzoic acid (44)

d, J =1.9, Ar-C_2-H), 7.55(1H, dd, J = 8.8, J = 2.0, Ar-C_6-H). ^{13}C-NMR: (Table 1-1). MS m/z (%): 182 (51.1, M^+), 165 (2.9, M^+-OH), 155 (8.5), 154 (98.8, M^+-C_2H_4), 138 (8.9), 137 (100, M^+-C_2H_5O or M^+-OH-C_2H_4), 125 (3.1), 109 (14.4), 97 (4.7). IR ν_{max}^{KBr} cm^{-1}: 1675 (C=O). These spectra and chromatographic behavior were completely identical with those of synthetic 43.

^{1}H-NMR spectrum of methyl 4-ethoxy-3-methoxybenzoate was very similar to that of 43', although some of the former chemical shift was different from the latter one. The possibility that A is 4-ethoxy-3-hydroxybenzoic acid was neglected by the careful comparison of their chemical shifts and IR spectra.

^{1}H-NMR spectrum of B' was assigned as follows, ($CDCl_3$): δ 3.89 (3H, s, -$COOCH_3$), 3.93 (3H, s, Ar-OCH_3), 4.46 (1H, dd, J_{cis} = 6.2, J_{gem} = 2.0, $^{O}_{H}>C=C<^{H}_{H}$), 4.75 (1H, dd, J_{trans} = 13.7, J_{gem} = 1.9, $^{O}_{H}>C=C<^{H}_{H}$), 6.62 (1H, dd, J_{trans} = 13.7, J_{cis} = 6.2, $^{O}_{H}>C=C<^{H}_{H}$), 6.95 (1H, d, J = 8.6, Ar-C_5-H), 7.67 (1H, d, J = 2.0, Ar-C_2-H), 7.81 (1H, dd, J = 8.5, J = 2.0, Ar-C_6-H): The data indicated the presence of a vinyl ether group and the absence of the glycerol moiety and the ethoxyl group. Its MS showed the molecular ion peak at m/z (%): 208 (100, M^+) and major fragment ion peaks at 193 (3.7, M^+-CH_3), 179 (18.8), 178 (11.5), 177 (99.4, M^+-OCH_3), 167(7.4), 165 (3.7), 151 (20.3), 149 (31.8, 177-CO). Therefore, compound B' was identified as methyl 4-methoxy-3-vinyloxybenzoate (44'). Two methyl groups were introduced by the treatment with diazomethane. The structure was confirmed by the fact that hydrogenation of B' with 10% Pd-C in MeOH

under hydrogen for 1 min gave quantitatively a product, whose ^{1}H-NMR (Fig. 1-17 (c)), MS, and TLC were identical with those of methyl 3-ethoxy-4-methoxybenzoate (43').

Compound B was identified as 4-hydroxy-3-vinyloxybenzoate (44), which was confirmed by the following data. The presence of the vinyloxy carbons were indicated by ^{13}C-NMR (δ 97.02 and 143.10) as shown in Table 1-1. ^{1}H-NMR ($CDCl_3$) : δ 4.61 (1H, dd, J_{cis} = 6.0, J_{gem} = 2.2, $\overset{O}{H}>C=C<\overset{H}{H}$), 4.89 (1H, dd, J_{trans} = 13.5, J_{gem} = 2.2, $\overset{O}{H}>C=C<\overset{H}{H}$)), 5.92 (1H, broad, Ar-OH), 6.66 (1H, dd, J_{trans} = 13.5, J_{cis} = 6.0, $\overset{O}{H}>C=C<\overset{H}{H}$), 7.03 (1H, d, J = 8.4, Ar-C_5-H), 7.71 (1H, d, J = 1.8, Ar-C_2-H), 7.81 (1H, dd, J = 8.4, J = 2.0, Ar-C_6-H). MS m/z (%): 180 (38.2, M^+), 166 (10.0), 165 (100), 154 (5.7), 149 (6.5), 137 (28.4), 119 (4.7), 109 (8.3). IR ν_{max}^{KBr} cm^{-1}: 3400, 2900, 1670 (C=O), 1643 (vinyl C=C), 1595 (aromatic C=C), 1515 (aromatic C=C), 1440, 1282, 1195, 763. UV λ_{max}^{MeCN} nm : 256, 287.

Compound C gave the following ^{1}H-NMR and MS : ^{1}H-NMR ($CDCl_3$): δ 3.96 (2H, d, J = 3.2, -CH_2OH), 6.29 (1H, t, J = 3.2, $\overset{O}{O}>CH-$), 6.85 (1H, d, J = 8.2, Ar-C_5-H), 7.49 (1H, d, J = 1.7, Ar-C_2-H), 7.71 (1H, dd, J = 8.1, J = 1.7, Ar-C_6-H). MS m/z (%): 196 (19.1, M^+), 179 (1.4), 166 (9.9), 165 (100), 119 (4.1). Compound C was acetylated with acetic anhydride and pyridine followed by the methylation with diazomethane to give a derivative C' which showed following ^{1}H-NMR and MS. ^{1}H-NMR ($CDCl_3$): δ 2.07 (3H, s, alcoholic-OAc), 3.88 (3H, s, -$COOCH_3$), 4.39 (2H, d, J = 3.6, -CH_2OAc), 6.37 (1H, t, J = 3.7, $\overset{O}{O}>CH-$), 6.83 (1H, d, J = 8.2, Ar-C_5-H), 7.46 (1H, d, J = 1.7, Ar-C_2-H), 7.66 (1H, dd, J = 8.1, J = 1.7

Ar-C_6-H). MS m/z (%): 252 (10.9, M^+), 221 (6.1), 210 (11.3), 193 (6.4), 192 (22.9), 180 (10.9), 179 (100), 136 (6.4), 120 (6.6), 43 (19.9). Therefore, C was identified as 3,4-(hydroxymethyl)methylenedioxybenzoic acid (45).

Compound D was also acetylated and then methylated to give D'. From the following ^{1}H-NMR and MS of D and D', D was identified as 3-ethoxy-4-(2-hydroxyethoxy)benzoic acid (46). ^{1}H-NMR ($CDCl_3$): δ 1.47 (3H, t, J = 7.0, $-OCH_2CH_3$), 3.90-4.04 (2H, m) and 4.12-4.28 (2H, m) ($Ar-OCH_2CH_2OH$), 4.16 (2H, q, J = 7.0, $-OCH_2CH_3$), 6.95 (1H, d, J = 8.4, Ar-C_5-H), 7.60 (1H, d, J = 2.0, Ar-C_2-H), 7.71 (1H, dd, J = 8.4, J = 2.0, Ar-C_6-H). MS m/z (%): 226 (M^+, 27.1), 182 (26.4), 165 (3.9), 154 (100), 137 (37.6), 45 (23.0). D': ^{1}H-NMR ($CDCl_3$): δ 1.45 (3H, t, J = 6.9, $-OCH_2CH_3$), 2.09 (3H, s, alcoholic-OAc), 3.88 (3H, s, $-COOCH_3$), 4.13 (2H, q, J = 7.0, $-OCH_2CH_3$), 4.20-4.33 (2H, m) and 4.39-4.53 (2H, m) ($ArOCH_2CH_2$-OAc), 6.90 (1H, d, J = 8.1, Ar-C_5-H), 7.56 (1H, d, J = 1.9, Ar-C_2-H), 7.64 (1H, dd, J = 8.2, J = 2.0, Ar-C_6-H). MS m/z (%): 282 (1.4, M^+), 251 (0.6), 239 (0.2), 179 (1.4), 87 (100), 43 (38.2). However, a small amount (1.5%) of D (46) was detected in the control solution.

Methyl ester of the recovered substrate after 2 days incubation was confirmed by its ^{1}H-NMR.

Fig. 1-18 shows time course of degradation of the substrate 42 and formation of the catabolic products. The substrate decreased with the gradual formation of 43, 44, and 45, among which 43 was a major product and accumulated most after 8 days incubation and then decreased. The substrate disappeared completely after 10 days incubation.

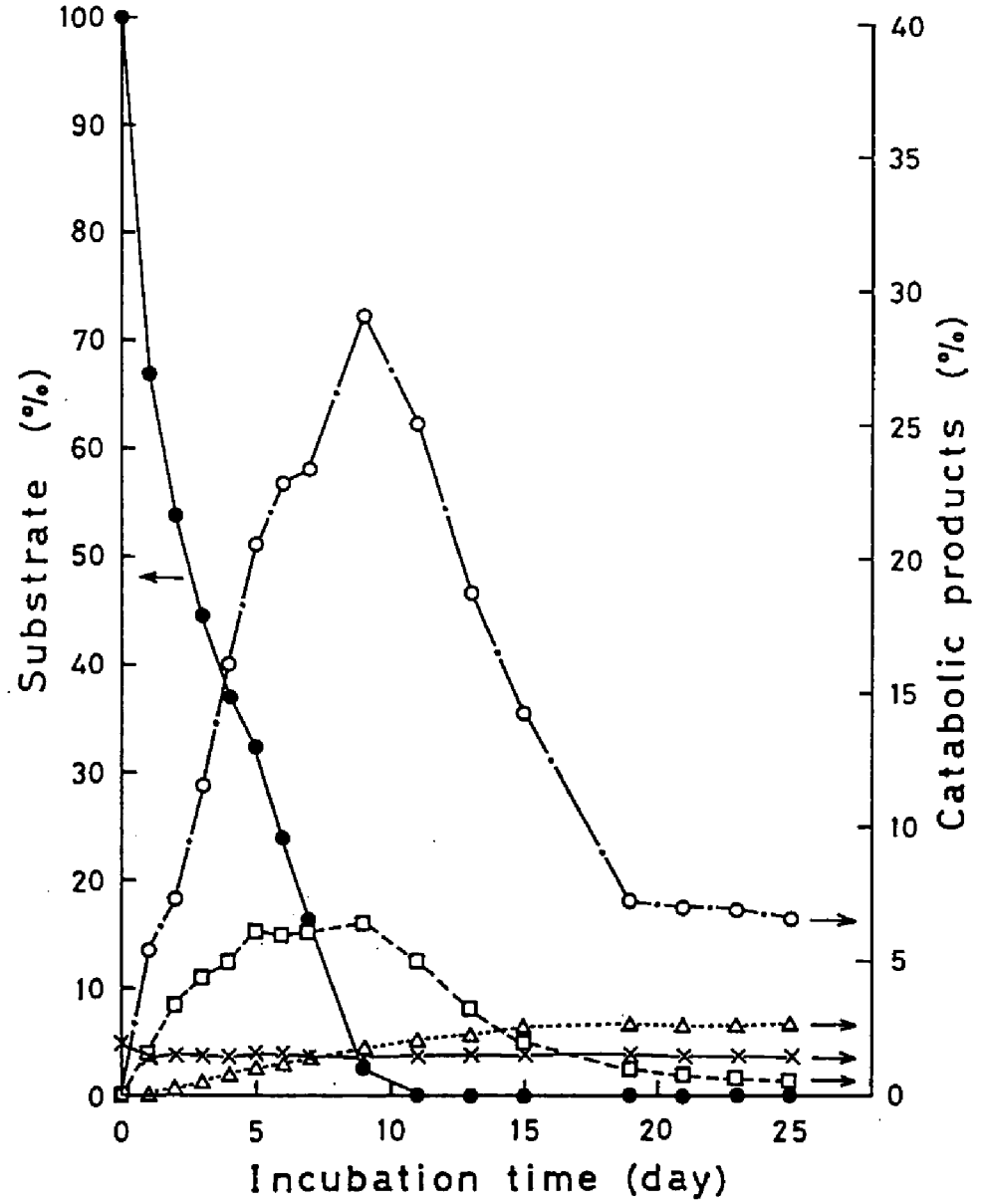


Fig. 1-18 Time course of the degradation of the substrate and the formation of catabolic products in the culture filtrate.
●, Substrate, glycerol-2-(3-ethoxy-4-hydroxybenzoic acid (42); ○, A, 3-ethoxy-4-hydroxybenzoic acid (43); □, B, 4-hydroxy-3-vinyloxybenzoic acid (44); △, C, 3,4-(hydroxymethyl)methylenedioxybenzoic acid (45); ×, D, 3-ethoxy-4-(2-hydroxyethoxy)benzoic acid (46).

Product 44 also accumulated most after 8 days incubation and then decreased. Product 45 was formed gradually followed by the constant accumulation. Compound 46 may be an impurity which was present in the substate. Compound 46 was constantly present in only a small amount, about 1.5 %, in all stages, indicating that 46 was stable and not catabolized as in the case of 4-(2-hydroxyethoxy)-3-methoxybenzoic acid (ethylene glycol mono-(vanillic acid) ether)[50]. o-Ethoxyphenol was not detected from the culture filtrate by HPLC.

1.4.4 DISCUSSION

Glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether (42) was completely degraded, although the degradation rate was remarkably slower than that of glycerol-2-(vanillic acid) ether (29). 3-Ethoxy-4-hydroxybenzoic acid (43) and 4-hydroxy-3-vinyloxybenzoic acid (44) were formed from 42 (Fig. 1-19). The fact indicated that the aryl ether at C_2 ($C\beta$) position of 42 was cleaved by this fungus. Detection of a degradation product derived from the C_3 side chain of 42 was not examined. It is assumed that glycerol-2-(vanillic acid) ether (29) was degraded *via* vanillic acid which was not isolated because of its rapid catabolism. Compounds 43 and 44 were further degraded. 3,4-(Hydroxymethyl)methylenedioxybenzoic acid (45) isolated was a cyclic acetal of protocatechuic acid and glycol aldehyde. Mechanism of the formation of 45 is now under study.

Fig. 1-20 shows an assumed degradation pathway of arylglycerol-β-aryl ether moiety of terminal molecular

chain of lignin. In section 1.2 and 1.3, cleavage of alkyl-aryl, $C\alpha$ -C_1, bond was described. A repetition of the alkyl-aryl cleavage and subsequent β -ether cleavage may most contribute to the depolymerization of lignin by F. solani M-13-1.

Kamaya et al.[87] identified many degradation products of guaiacylglycerol-β -syringaresinol ether by F. solani M-13-1. They identified many products from its culture filtrate and discussed their formation; glycerol-2-syringaresinol ether and glyceric acid-2-syringaresinol ether were suggested to be formed by the alkyl-aryl cleavage of the guaiacylglycerol moiety, and syringaresinol by the cleavage of 2-aryl ether bond of glyceraldehyde-2-syringaresinol ether. Because incubation of glycerol- and glyceric acid-2-syringaresinol ether did not give the products expected by their 2-aryl ether cleavage. However, a direct precursor of syringaresinol was unknown, since they did not carry out incubation of the glyceraldehyde-2-syringaresinol ether. No detection of the products by the cleavage of the 2-aryl ether in glycerol-2-syringaresinol ether may be due to the rapid oxidation of their syringaresinol moiety, since the alkyl-aryl ether bond of glycerol-2-aryl ether was demonstrated in this investigation.

In contrast to the case of F. solani M-13-1, Polyporous dichrous, a white rot fungus, was reported not to metabolize 29[76], although the investigation was before the establishment of ligninolytic culture condition by Kirk et al.[32].

Enoki et al.[34] and Goldsby et al.[33] obtained glycerol-2-guaiacyl ether as a degradation product of

veratrylglycerol-β-guaiacyl ether and α-deoxy-guaiacyl-glycerol-β-guaiacyl ether by Phanerochaete chrysosporium under the ligninolytic culture condition. However, they did not report the catabolism of glycerol-2-guaiacyl ether. They[33] examined catabolism of glycol-2-guaiacyl ether (guaiacoxyethanol) and detected guaiacol as a degradation product, indicating the cleavage of the ether bond.

Recently, Morohoshi and Haraguchi[88] reported that laccase III-c from Coriorus versicolor cleaved the 2-aryl ether of glyceraldehyde-2-syringyl ether, which is formed by the alkyl-aryl cleavage of syringylglycerol-β-syringyl ether, giving 2,6-dimethoxyphenol.

Direct cleavage of the β-ether linkage in aryl-glycerol-β-aryl ethers was not found in the case of F. solani M-13-1.

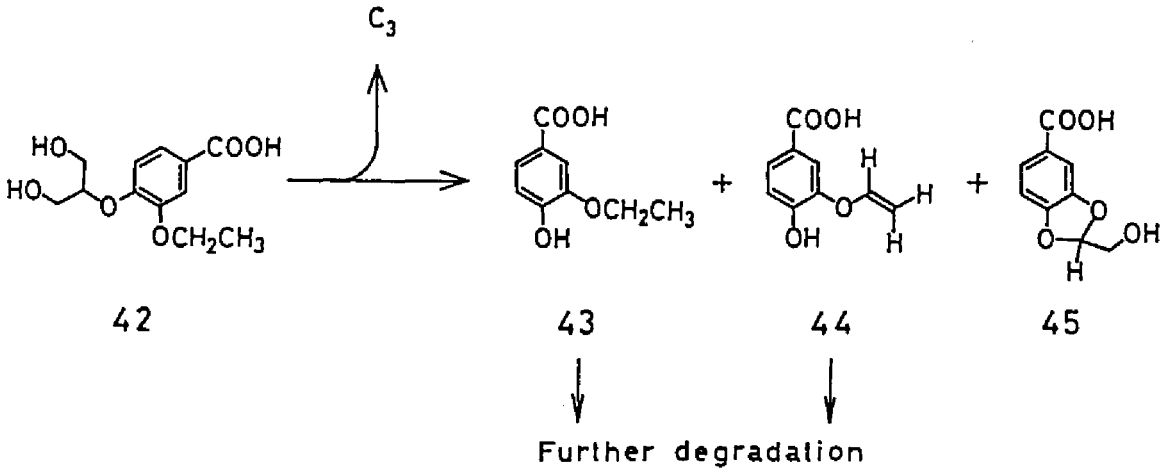


Fig. 1-19 Degradation of glycerol-2-(3-ethoxy-4-hydroxy-benzoic acid) ether (42) by Fusarium solani M-13-1

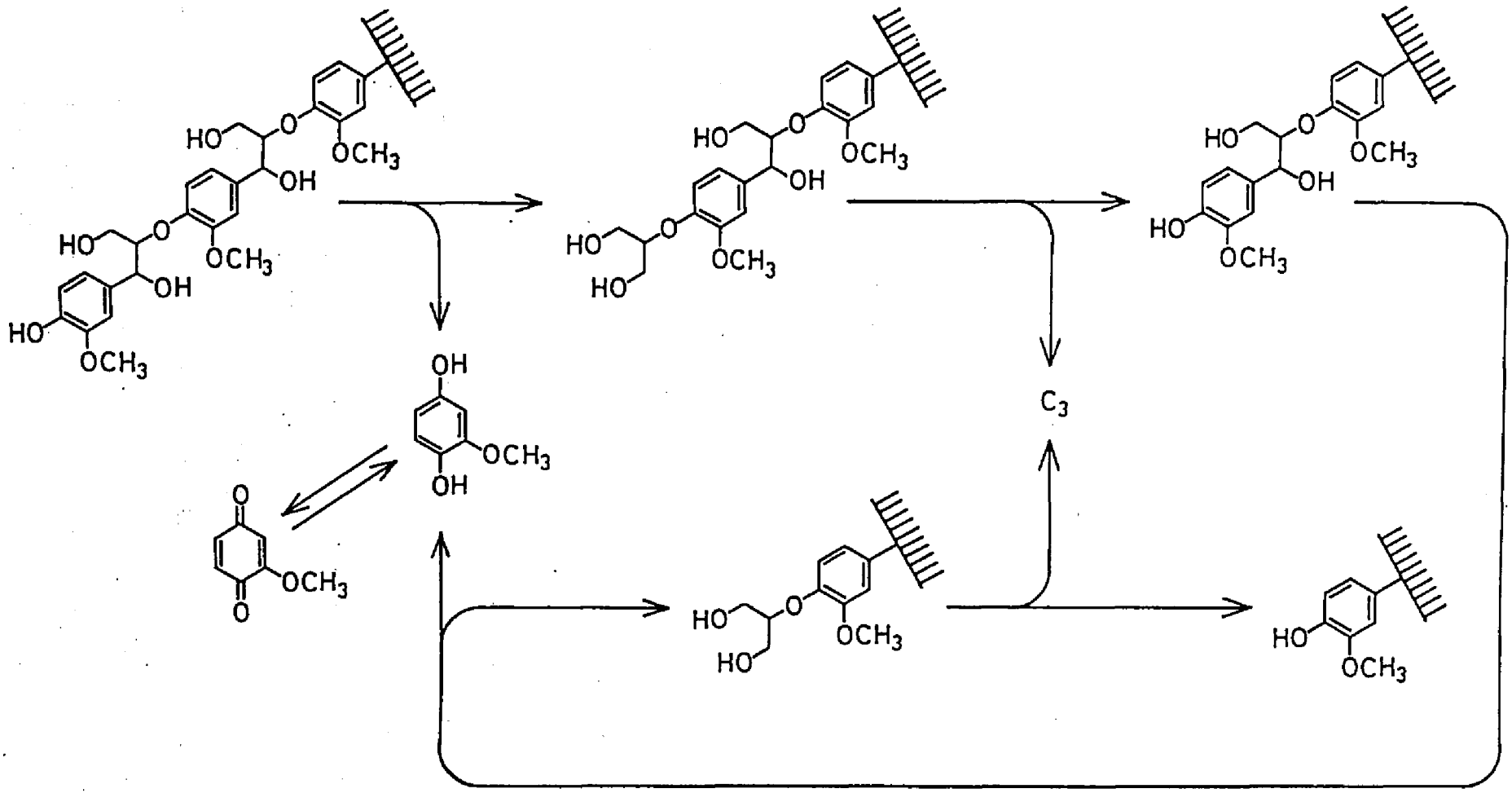


Fig. 1-20 Assumed degradation pathway of a terminal guaiacylglycerol-β-aryl ether moiety of lignin by Fusarium solani M-13-1

CHAPTER 2

DEGRADATION AND TRANSFORMATION OF A PHENYLCOUMARAN AND A PHENYLCOUMARONE BY FUSARIUM SOLANI M-13-1

2.1 INTRODUCTION

A phenylcoumaran is one of the main substructure and consists 9-12% of spruce lignin and 6% of birch lignin[5]. Previously Ohta et al.[45] showed that an initial degradation reaction of dehydrodiconiferyl alcohol, a phenylcoumaran substructure model, by F. solani M-13-1 is an oxidative shortening of its allyl alcohol moiety followed by the cleavage of the coumaran ring to form 5-substituted vanillyl alcohols. The degradation of dehydrodiconiferyl alcohol by Phanerochaete chrysosporium[89,90] and by Pseudomonas sp.[40,39] also was reported. However, the degradation mechanism of the coumaran ring was not clear.

This chapter describes degradation of 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxy-coumaran (47), which is an analog of an intermediate, 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3-methoxyphenyl)-7-methoxycoumaran, in the degradation of dehydrodiconiferyl alcohol[45] by F. solani M-13-1. It was found that initial reactions of 47 were oxidation or reduction of the aldehyde group and dehydrogenation of the phenolic hydroxyl group, and the dehydrogenation products were partly cleaved between $C\alpha$-C_{aryl} and $C\alpha$-$C\beta$ bonds. Side chain reactions of the dehydrogenation products, phenylcoumarones, by the fungus were also described.

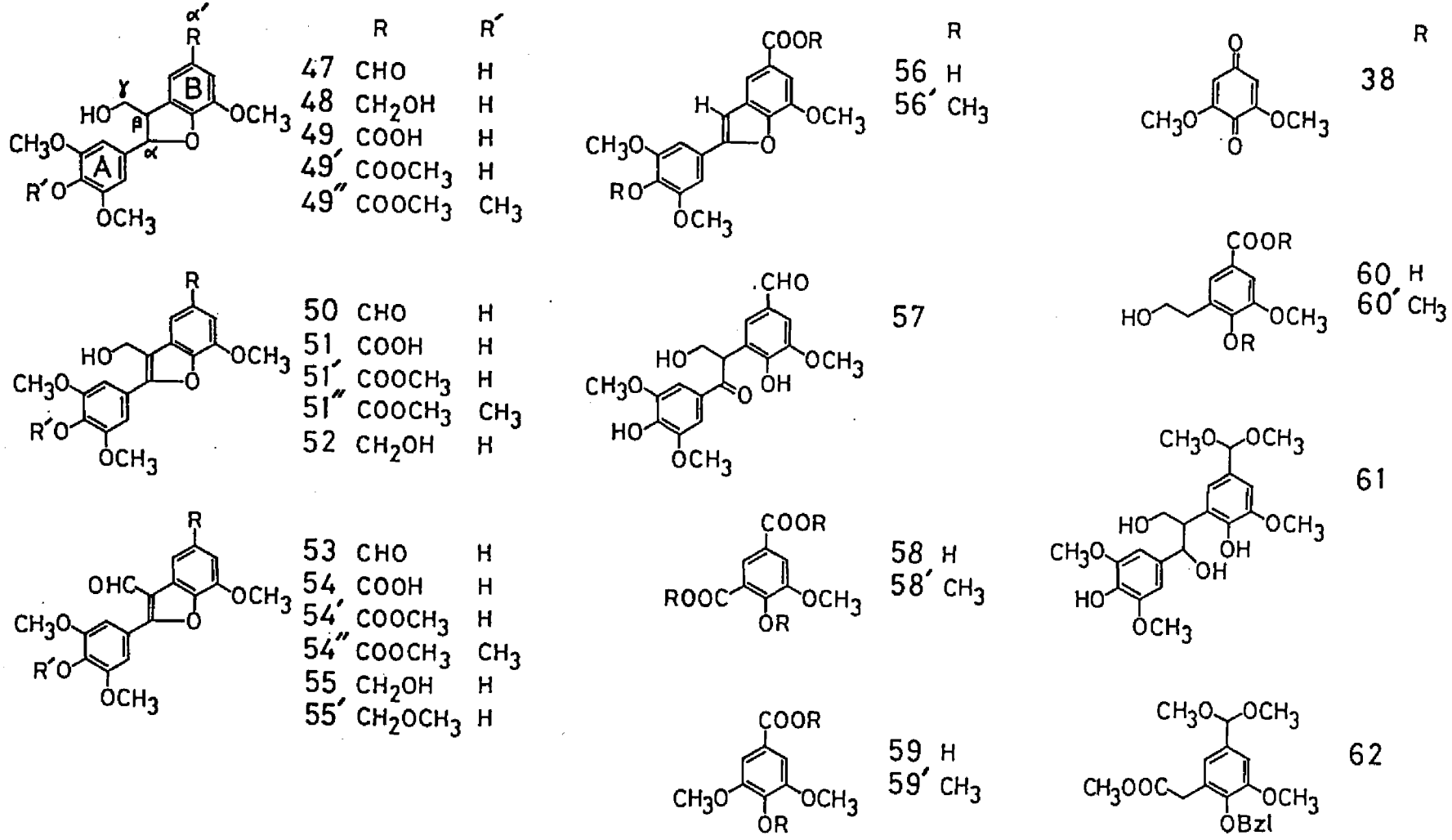


Fig. 2-1 Structures of compounds synthesized and identified as catabolic products (chapter 2)

2.2 EXPERIMENTAL

Preparation of fungal mycelia

Composition of the basal mineral salts medium and nutrient medium (both pH 6.0) were the same as described in section 1.2. Fungal mycelia were prepared as described in section 1.3.

Cultural conditions of catabolic experiments

5-Formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (47) (200 mg), 3,5-dihydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (48) (50 mg), 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (50) (50 mg), and 3,5-diformyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (53) (40 mg) were used as substrates. Compound 47 and 48 were dissolved in the minimum amount of DMF (less than 0.5 ml per 50 mg). Compound 47 and 48 were dissolved in 1 ml of DMF (per 50 or 40 mg). The DMF solution and 350 mg (dry weight) of the mycelial suspension in 10 ml of the basal medium were added to 90 ml of the basal medium on a 500 ml Sakaguchi flask. Two control flasks which contained only mycelia or substrate in the medium were prepared similarly. All flasks were shaken on a reciprocated shaker (145 strokes per minute) at 30℃.

Analysis of catabolic products

Monitoring of the degradation was described in section 1.3. Mycelia were filtered off and washed with distilled

water. The filtrate and the washings were combined and extracted twice with an equal volume of CH_2Cl_2. The aqueous layer was then acidified to pH 2 with concentrated HCl and extracted three times with an equal volume of EtOAc. Both extracts were washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The EtOAc extracts were methylated with diazomethane in MeOH. Both CH_2Cl_2 extracts and methylated EtOAc extracts were submitted to preparative TLC and the compounds isolated were identified by ^{1}H-NMR and MS. Synthetic authentic samples were used as references for identification.

Chromatography and spectrometry

Analytical and preparative TLC and column chromatography were described in section 1.4. ^{1}H-NMR spectra were recorded on Varian XL-200 (200 MHz) and Hitachi R-90H (90 MHz) FT-NMR spectrometers, with $CDCl_3$ as a solvent and tetramethylsilane as internal standard. Chemical shifts (δ) and coupling constants (J) are described in ppm and Hz, respectively. Peak multiplicities are abbreviated singlet s, doublet d, triplet t, quartet q, and multiplet m. Mass spectra (MS) and UV spectra were taken by the same instruments as described in section 1.1.

Syntheses of compounds

5-Formyl-3-hydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (47).

This compound was synthesized from 5-iodovanillin and

syringaldehyde by a similar method of Nakatsubo and Higuchi[91]. ^{1}H-NMR: δ 3.55-4.20 (3H, β-CH- and γ-CH_2-), 3.87 (6H, s, Ar-A-OCH_3), 3.96 (3H, s, Ar-B-OCH_3), 5.53 (1H, s, Ar-OH), 5.68 (1H, d, J = 7.0, α-CH-), 6.63 (2H, s, Ar-A-H), 7.37-7.43 (2H, Ar-B-H), 9.84 (1H, s, -CHO). MS m/z (%): 360 (58, M^+), 343 (23), 342 (100), 330 (65), 328 (24), 327 (33), 311 (22), 310 (19), 282 (10), 281 (11), 267 (14), 239 (12), 167 (24).

3,5-Dihydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (48)

To a stirred solution of 180 mg (0.5 mmol) of 47 in 20 ml of MeOH was added 19.5 mg (0.5 mmol) of $NaBH_4$ at 0℃ under nitrogen. After 15 min the reaction mixture was partitioned between EtOAc and water. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated *in vacuo*. The residue was purified by TLC (EtOAc-*n*-hexane = 2:1) giving 167 mg (97%) of 48. ^{1}H-NMR: δ 3.60-3.74 (1H, m, β-CH-), 3.87 (6H, s, Ar-A-OCH_3), 3.92 (3H, s, Ar-B-OCH_3), 3.94-4.20 (2H, m, γ-CH_2-), 4.66 (2H, s, α'-CH_2-), 5.52 (1H, broad s, Ar-OH), 5.57 (1H, d, J = 7.5, α-CH-), 6.66 (2H, s, Ar-A-H), 6.87-6.88 (2H, Ar-B-H). MS m/z (%): 362 (73, M^+), 344 (100), 332 (35), 329 (33), 313 (49), 167 (32).

5-Carboxy-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (49) and its methylated derivatives 49' and 49"

To a stirred solution of 10 mg (0.028 mmol) of 47 in 3

ml of CH_2Cl_2 and 0.5 ml of DMF were added 35 mg (0.42 mmol) of 2,3-dihydro-4H-pyran and 0.5 mg of p-TsOH successively at 0℃ under nitrogen. After stirring at 0-5℃ for 2 hr the reaction solution was neutralized by the addition of triethylamine, and then partitioned between Et_2O and a saturated $NaHCO_3$ solution. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane = 1:2) giving 11.7 mg(80%) of ditetrahydropyranyl ether of 47.

To a stirred solution of 11.7 mg (0.022 mmol) of the tetrahydropyranyl ether in 1.5 ml of dioxane was added a solution of 7.0 mg (0.044 mmol) of $KMnO_4$ im 0.5 ml of water at room temperature. After 60 min 1 ml of MeOH was added to the reaction mixture, and the stirring was continued for an additional 20 min. The precipitate of MnO_2 was then filtered off and washed successively with MeOH and hot water. The filtrate and the washings were combined, acidified to pH 2 with 1N HCl, and extracted with EtOAc. The extract was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo.

The residue was dissolved in 1.5 ml of dioxane. To the stirred solution was added 0.2 ml of 1N HCl at room temperature. After 2 hr the reaction solution was partitioned between Et_2O and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC ($MeOH-CH_2Cl_2$ = 5:95, X 6) giving 3 mg of 49.

Methyl ester of 49 (49') was prepared by treatment of 49 with a limited amount of diazomethane in MeOH for 3 min.

Methyl ether of 49' (49") was obtained by treatment of 49 with excess diazomethane for 6 hr. Both compounds were purified by TLC (EtOAc-n-hexane = 1:1). Compound 49' : ^{1}H-NMR: δ 3.62-3.78 (1H, m, β -CH-), 3.86 (6H, s, Ar-A-OCH_3), 3.92 (3H, s, -$COOCH_3$), 3.96 (3H, s, Ar-B-OCH_3), 3.92-4.06 (2H, m, γ -CH_2-), 5.53 (1H, broad s, Ar-OH), 5.67 (1H, d, J = 7.5, α -CH-), 6.64 (2H, s, Ar-A-H), 7.58-7.62 (2H, Ar-B-H). MS m/z (%): 390 (65, M^+), 372 (100), 360 (91), 341 (26), 167 (34). Compound 49": ^{1}H-NMR: δ 3.64-3.74 (1H, m, β -CH-), 3.83 (3H, s, Ar-A-OCH_3), 3.84 (6H, s, Ar-A-OCH_3), 3.91 (3H, s, -$COOCH_3$), 3.95 (3H, s, Ar-B-OCH_3), 3.9-4.04 (2H, m, γ -CH_2-), 5.69 (1H, d, J = 7.5, α -CH-), 6.63 (2H, s, Ar-A-H), 7.59-7.61 (2H, Ar-B-H).

5-Formyl-3-hydroxy methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (50) and 3,5-diformyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (53).

These compounds were prepared by oxidation of 47 with 1 and 2 equivalents of 2,3-dichloro-5,6-dicyano-p-benzoquinone (DDQ), respectively. Compound 50 : ^{1}H-NMR: δ 3.99 (6H, s, Ar-A-OCH_3), 4.09 (3H, s, Ar-B-OCH_3), 4.99 (2H, s, γ -CH_2-), 5.71 (1H, s, Ar-OH), 7.17 (2H, s, Ar-A-H), 7.40 (1H, d, J = 1.4, Ar-B_6-H), 7.84 (1H, s, J = 1.4, Ar-B_4-H), 10.03 (1H, s, -CHO). MS m/z (%): 358 (100, M^+), 343 (11), 341 (9), 325 (8), 309 (18), 299 (10), 297 (9).

Compound 53: ^{1}H-NMR: δ 4.01 (6H, s, Ar-A-OCH_3), 4.10 (3H, s, Ar-B-OCH_3), 5.92 (1H, s, Ar-OH), 7.14(2H, s, Ar-A-H), 7.49 (1H, d, J = 1.3, Ar-B_6-H), 8.36 (1H, d, J = 1.3, Ar-B_4-H), 10.08 (1H, s, Ar-CHO), 10.38 (1H, s, γ -CHO). MS

m/z (%): 356 (100, M^+), 328 (27), 313 (15), 285 (27).

3-Hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxy-5-(methoxycarbonyl)coumarone (51'), 3-hydroxymethyl-7-methoxy-5-methoxycarbonyl-2-(3,4,5-trimethoxyphenyl)-coumarone (51"), 3-formyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxy-5-(methoxycarbonyl)coumarone (54'), and 3-formyl-7-methoxy-5-methoxycarbonyl-2-(3,4,5-trimethoxyphenyl)-coumarone (54").

These compounds were used for identification of the catabolic products 51 and 54. Compound 51' and 54' were prepared by oxidation of 49' with 1 and 2 equivalents of DDQ, respectively. Compounds 51" and 54" were obtained by treatment of 51' and 54' with diazomethane in MeOH at 0℃ for 4 hr, respectively.

Compound 51': ^{1}H-NMR: δ 3.96 (3H, s, $-COOCH_3$), 3.99 (6H, s, Ar-A-OCH_3), 4.09 (3H, s, Ar-B-OCH_3), 4.97 (2H, s, γ $-CH_2-$), 5.74 (1H, broad s, Ar-OH), 7.19 (2H, s, Ar-A-H), 7.55 (1H, d, J = 1.4, Ar-B_4-H), 8.05 (1H, d, J = 1.4, Ar-B_4-H). MS m/z (%): 388 (100, M^+), 372 (18), 357 (8), 355 (17).

Compound 51": ^{1}H-NMR: δ 3.93 (3H, s, $-COOCH_3$), 3.96 (9H, s, Ar-A-OCH_3), 4.09 (3H, s, Ar-B-OCH_3), 4.99 (2H, s, γ $-CH_2-$), 5.74 (1H, broad s, Ar-OH), 7.17 (2H, s, Ar-A-H), 7.56 (1H, d, J = 1.4, Ar-B_4-H), 8.07 (1H, d, J = 1.5, Ar-B_4-H).

Compound 54': ^{1}H-NMR: δ 3.97 (3H, s, $-COOCH_3$), 4.01 (6H, s, Ar-A-OCH_3), 4.09 (3H, s, Ar-B-OCH_3), 5.90 (1H, s, Ar-OH), 7.14 (2H, s, Ar-A-H), 7.64 (1H, d, J = 1.4, Ar-B_6-H), 8.55 (1H, d, J = 1.4, Ar-B_4-H), 10.36 (1H, s, γ -CHO).

MS m/z (%): 386 (100, M^+), 371 (5), 358 (16), 355 (15), 343 (8), 315(18), 289 (12).

Compound 54": ^{1}H-NMR: δ 3.94 (3H, s, $-COOCH_3$), 3.95 (9H, s, Ar-A-OCH_3), 4.08 (3H, s, Ar-B-OCH_3), 7.08 (2H, s, Ar-A-H), 7.61 (1H, d, J = 1.5, Ar-B_6-H), 8.52 (1H, d, J = 1.5, Ar-B_4-H), 10.34 (1H, s, γ -CHO).

3,5-Dihydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxy-coumarone (52).

This compound was obtained by treatment of 50 with $NaBH_4$ in MeOH. ^{1}H-NMR: δ 3.98 (6H, s, Ar-A-OCH_3), 4.04 (3H, s, Ar-B-OCH_3), 4.77 (2H, s, α'-CH_2-), 4.92 (2H, s, γ -CH_2-) 5.70 (1H, broad s, Ar-OH), 6.84-6.87 (2H, Ar-B-H), 7.16 (2H, s, Ar-A-H). MS m/z (%): 360 (100, M^+), 358 (47), 344 (50), 343 (20), 342 (24), 327 (16), 312 (15), 299 (13). Triacetate derivative 52' was prepared by treatment of 52 with Ac_2O and pyridine. ^{1}H-NMR: δ 2.11 (3H, s, alcoholic-OAc), 2.13 (3H, s, alcoholic-OAc), 2.36 (3H, s, Ar-OAc), 3.91 (6H, s, Ar-A-OCH_3), 4.04 (3H, s, Ar-B-OCH_3), 5.20 (2H, s, α'-CH_2-), 5.41 (2H, s, γ -CH_2-), 6.86 (1H, d, J = 1.2, Ar-B_6-H), 7.13 (2H, s, Ar-A-H), 7.32 (1H, d, J = 1.2, Ar-B_4-H).

3-Formyl-5-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (55).

This compound was prepared by oxidation of 52 with 1 equivalent of DDQ in dioxane (yield 60%). ^{1}H-NMR: δ 4.00 (6H, s, Ar-A-OCH_3), 4.05 (3H, s, Ar-B-OCH_3), 4.80 (2H, s, α'-CH_2-), 5.85 (1H, s, Ar-OH), 6.98 (1H, d, J = 1.5, Ar-B_6-H), 7.12 (2H, s, Ar-A-H), 7.78 (1H, d, J = 1.5, Ar-B_4-H),

10.33 (1H, s, γ -CHO).

2-(5-Formyl-2-hydroxy-3-methoxyphenyl)-3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-propanone (57).

This compound was prepared by DDQ oxidation of 1-(4-hydroxy-3,5-dimethoxyphenyl)-2-(2-hydroxy-3-methoxy-5-(dimethoxymethyl)phenyl)-1,3-propanediol (61), an intermediate in the synthesis of 47[91], and by subsequent treatment with 1N HCl. ^{1}H-NMR: δ 3.8-4.4 (2H, m, γ -CH_2-), 3.88 (6H, s, Ar-A-OCH_3), 3.97 (3H, s, Ar-B-OCH_3), 5.15-5.33 (1H, m, β -CH-), 5.91 (1H, broad s, Ar-A-OH), 6.63 (1H, broad s, Ar-B-OH), 7.30-7.37 (4H, Ar-H), 9.73 (1H, s, -CHO). MS m/z (%): 376 (0.1, M^+), 358 (3.3), 356 (1.7), 346 (6.3), 328 (33), 313 (3.1), 285 (6.0), 181 (100), 167 (4.8), 153 (6.9).

5-(2-Hydroxyethyl)vanillic acid (60) and its dimethylated derivative 60'.

Compound 60 was synthesized from methyl (2-benzyloxy-5-dimethoxymethyl-3-methoxyphenyl)acetate (62), an intermediate in the synthesis of 47[91], via the following four steps[53]: (a) $LiAlH_4$/ THF/ 50℃; (b) 1N HCl/ dioxane/ r.t.; (c) Ag_2O ($AgNO_3$-NaOH)/ EtOH/ r.t.; (d) H_2/ 10% Pd-C/ MeOH/ r.t.. For ^{1}H-NMR and MS analyses, 60 was converted to 60' with diazomethane. ^{1}H-NMR: δ 2.94 (2H, t, J = 6.5, $ArCH_2$-CH_2OH), 3.87 (2H, t, J = 6.5, $ArCH_2CH_2OH$), 3.92 (6H, s, $-COOCH_3$ and Ar-OCH_3), 3.94 (3H, s, Ar-OCH_3), 7.52(1H, d, J = 1.0, Ar-H), 7.57 (1H, d, J= 1.0, Ar-H). MS m/z (%): 240 (100, M^+), 210 (40), 209 (99), 195 (66), 194 (77), 181 (25), 163 (28).

5-Carboxyvanillic acid (58) and its trimethylated derivative (58').

Compound 58 was synthesized from 2-benzyloxy-5-dimethoxymethyl-3-methoxybenzaldehyde, an intermediate in the synthesis of 47[91], via the following three steps[53]: (a) 1N HCl/ dioxane/ r.t.; (b) $KMnO_4$/ dioxane/ r.t.; (c) H_2/ 10% Pd-C/ MeOH/ r.t.. For ^{1}H-NMR and MS analyses, 58 was converted to 58' with diazomethane. ^{1}H-NMR: δ 3.92, 3.93, and 3.96 (6H, 3H, and 3H, respectively, three s, four $-OCH_3$), 7.70 (1H, d, J = 2.0, Ar-C_2-H), 8.02 (1H, d, J = 2.0, Ar-C_6-H). MS m/z (%): 254 (66, M^+), 223 (100), 221 (93), 207 (20), 195 (12), 193 (18), 181 (13), 180(28), 177 (10), 165 (21).

Methyl 3,4,5-trimethoxybenzoate (59').

This compound was prepared by treatment of syringic acid (59) with diazomethane. ^{1}H-NMR: δ 3.90 (12H, s, four $-OCH_3$), 7.30 (2H, s, Ar-H). MS m/z (%): 226 (100, M^+), 211 (55), 195 (29), 183 (12), 155 (25), 151 (12), 125 (12), 124 (10), 66 (16), 59 (19).

2,6-Dimethoxy-p-benzoquinone (38).

This compound was obtained by the oxidation of syringic acid (59) with DDQ in MeOH[77]. ^{1}H-NMR: δ 3.81 (6H, s, $-OCH_3$), 5.86 (2H, s, ring-H). MS m/z (%): 168 (50, M^+), 140 (13), 138 (25), 125 (18), 112 (10), 97 (16), 80 (43), 69 (100).

2.3 RESULTS

Degradation of 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (47).

UV absorption of the culture filtrate containing 47 (λ_{max} = 305 nm) changed gradually and several absorption peaks appeared at 263, 282, and 295 nm after 32-38 hr (Fig. 2-2). The absorbance at 320-370 nm gradually increased. TLC analysis showed a decrease of 47, followed by the formation of 48 and 49, and the subsequent production of phenylcoumarones which exhibited fluorescence under a long-wave UV light (365 nm) on a TLC plate. Compound 48, 50, 52, 53, 55', 57, and 38 were isolated and identified from CH_2Cl_2 extract. Compound 49, 51, 54, 56, 58, 59, and 60 were identified from EtOAc extract.

The CH_2Cl_2 extract from two cultures after 12 hr incubation was separated into eleven fractions (Fr-N1 - N11) by TLC (EtOAc-n-hexane = 1:1, v/v). Fr-N4 was a colorless syrup (1 mg) which gave one spot on TLC, and it was found to be one of the two main products in the initial stage of incubation. The compound was identified as 3,5-dihydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (48) by comparison with the synthetic compound (^{1}H-NMR, MS, and TLC). It was found that the aldehyde group of 47 was reduced to the corresponding primary hydroxyl group. Purification of Fr-N5 by TLC (MeOH-CH_2Cl_2 = 4:96, X 3) afforded four fractions (Fr-N5-1 - N5-4). Further purification of Fr-N5-3 by TLC (MeOH-CH_2Cl_2 = 3:97, X 2) gave three

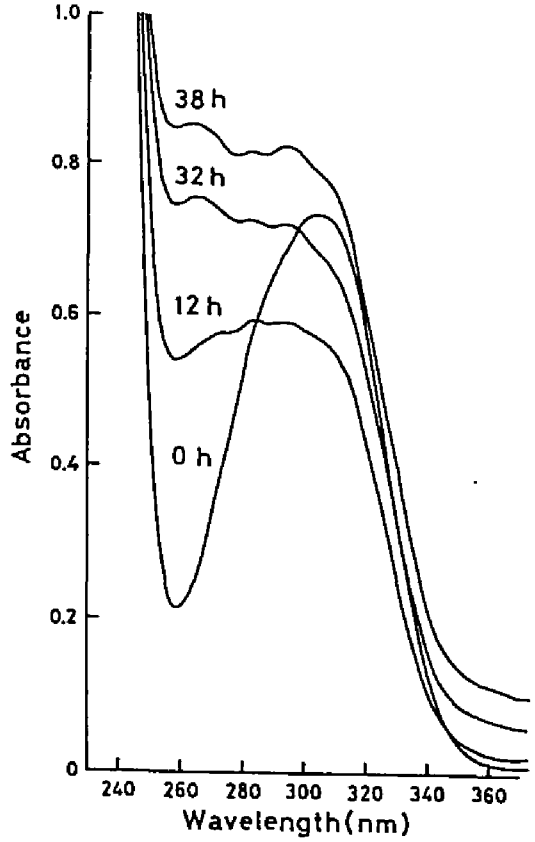


Fig. 2-2 Changes in the UV absorption of culture filtrates containing 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxy)-7-methoxycoumaran (47) during incubation of Fusarium solani M-13-1

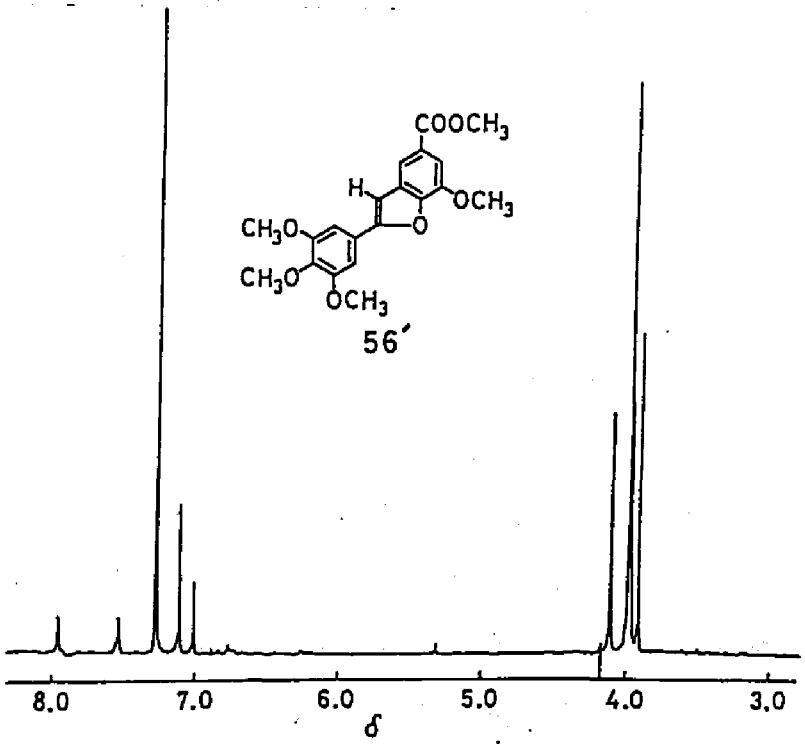


Fig. 2-3 ^{1}H-NMR spectrum of catabolic 7-methoxy-5-methoxycarbonyl-2-(3,4,5-trimethoxyphenyl)coumarone (56')

bands among which the lowest band was characterized by MS and TLC. The MS and R_f value of the compound (< 1 mg) were identical with those of the authentic 2-(5-formyl-2-hydroxy-3-methoxyphenyl)-3-hydroxy-1-(4-hydroxy-3,5-dimethoxyphenyl) 1-propanone (57). The compound indicated that the coumaran ring of 47 was opened oxidatively but α'-aldehyde remained. TLC of Fr-N10 (< 1 mg) showed a single spot which exhibited a bluish fluorescence under a long-wave UV light. The compound was identified as 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (50) by comparison with the synthetic compound (^{1}H-NMR, MS, and TLC). It indicated that dehydrogenation of 47 between Cα-Cβ occurred. Fr-N11 was further subjected to TLC (CH_2Cl_2, X 2), on which a yellow substance (< 1 mg) appeared. Its MS and R_f value on TLC were identical with those of authentic 2,6-dimethoxy-p-benzoquinone (38). It was ascribed to be formed from the syringyl nuclei in 47 by oxidative cleavage of the Cα-C_{aryl} bond.

The CH_2Cl_2 extract from two cultures after 38 hr incubation was separated into five fractions (Fr-N'1 - N'5) by TLC (EtOAc-n-hexane = 1:2, X 6). Fr-N'4(< 1 mg) gave one spot on TLC which exhibited a bluish fluorescence under a long-wave UV light. The compound was identified as 3-formyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxy-5-(methoxymethyl)coumarone (55'), an α'-methyl ether of 55. ^{1}H-NMR: δ 3.44 (3H, s, α'-CH_2OCH_3), 4.02 (6H, s, Ar-A-OCH_3), 4.08 (3H, s, Ar-B-OCH_3), 4.59 (2H, s, α'-CH_2OCH_3), 5.90 (1H, s, Ar-OH), 6.99 (1H, d, J = 1.5, Ar-B_6-H), 7.14 (2H, s, Ar-A-H), 7.80 (1H, d, J = 1.5, Ar-B_4-H), 10.35 (1H, s, -CHO).

MS m/z (%): 372 (100, M^+), 356 (12), 342 (36), 341 (36), 313 (17). Fr-N'5 was in a trace amount and gave a bluish fluorescence on TLC under a long-wave UV light. The R_f value on TLC was identical with that of authentic 3,5-diformyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (53).

Since thin-layer chromatograms of the methylated EtOAc extracts after 12 hr and 38 hr incubations were similar qualitatively, they were combined and separated into six fractions (Fr-A1 - A6) by TLC (EtOAc-n-hexane = 1:1). Fr-A1 (5.6 mg) gave one spot on TLC, and the compound was one of the two main products in the initial stage of the incubation. The compound was identified as methyl ester of 5-carboxy-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (49), (49'), by comparison with the authentic sample (^{1}H-NMR, MS and TLC). The aldehyde group of 47 was oxidized to the corresponding carboxyl group. The dimethylated derivative 49" (2.7 mg) of 49 was also isolated from Fr-A5.

Fr-A3 gave four bands (Fr-A3-1 - A3-4) on TLC (MeOH-CH_2Cl_2 = 3:97), among which Fr-A3-1 (1.4 mg) was a main product and exhibited a weak fluorescence under a long-wave UV light. The compound was identified as methyl ester of 5-carboxy-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (51), (51'). It is considered to be formed from 47 by the oxidation of α'-aldehyde and the dehydrogenation at Cα-Cβ. The dimethylated derivative 51" of 51 also was isolated from Fr-A5-2 and identified.

Separation of Fr-A5 by TLC (CH_2Cl_2) gave many bands (Fr-A5-1 - A5-11). Further purification of Fr-A5-4 gave a

small amount of colorless syrup (< 1 mg) whose MS revealed a molecular ion peak at m/z 240 and M^+-31 peak, indicating the structure of a monomeric methyl ester derivative. Its ^{1}H-NMR spectrum had two triplet at δ 2.94 and 3.87 (both 2H, J = 6.5) and two doublets at δ 7.52 and 7.57 (both 1H, J = 1.5), indicating the presence of a RCH_2CH_2OR' moiety and a pair of meta coupled protons, respectively. Therefore, the compound was identified as methyl 5-(2-hydroxyethyl)-veratrate (60'), dimethylated derivative of 5-(2-hydroxyethyl)vanillic acid (60). The structure was confirmed by comparison with the synthetic compound(^{1}H-NMR, MS, and TLC).

Purification of Fr-A6 by TLC (CH_2Cl_2, X 3) gave five bands (Fr-A6-1 - A6-5), among which Fr-A6-2 was dominant and Fr-A6-4 was second in abundance. Fr-A6-2 (3.4 mg) was pure and exhibited a bluish fluorescence under a long-wave UV light on TLC. The ^{1}H-NMR spectrum of the compound showed a singlet at δ 10.38 (1H) which shifted downfield compared with that of a usual aldehyde because of the ring current effect for allyl aldehyde adjacent to two aromatic rings. It had five $-OCH_3$ signals (15H) at δ 3.90-4.12 indicating the presence of one $-COOCH_3$ and four $-OCH_3$. The MS of the compound showed a strong molecular ion peak at m/z 400. The compound was therefore identified as 3-formyl-7-methoxy-5-methoxycarbonyl-2-(3,4,5-trimethoxyphenyl)coumarone (54"), dimethylated derivative of 54. The structure was confirmed by comparison with the synthetic compound (^{1}H-NMR, MS, and TLC). The methyl ester derivative of 54 (54') also was isolated from Fr-A5-7. These results showed that oxidation of α'-aldehyde, dehydrogenation between Cα-Cβ, and

oxidation of γ-alcohol in 47 occurred. Compound 54 was a main product among the phenylcoumarones.

TLC analysis indicated that Fr-A6-4 (1 mg) was pure. Its ^{1}H-NMR spectrum (Fig. 2-3) showed only a singlet at δ 7.01 (1H) except for the signals of $-OCH_3$ and Ar-H. Integral of the $-OCH_3$ signal, negative color reaction with $FeCl_3$-$K_3Fe(CN)_6$, and the presence of the M^+-31 ion at m/z 341 in the MS indicated that the compound contained five $-OCH_3$ and was a dimethylated derivative. The $-OCH_3$ signals were assigned as follows: δ 3.92 (3H, s, $-COOCH_3$), 3.97 (3H, s, Ar-A_4-OCH_3), 3.98 (6H, s, Ar-$A_{3,5}$-OCH_3), 4.11 (3H, s, Ar-B_3-OCH_3). The singlet at δ 7.12 (2H) was due to the 2- and 6- positions in the A-ring. The doublets at δ 7.54 and 7.96 (each J = 1.5) were ascribed to meta-coupled and derived from the ortho proton to carbonyl substituent, Ar-B_5-$COOCH_3$. The lower field position of the latter compared with the former may be due to desielding derived from the double bond in the phenylcoumarone structure. Consequently, they were assigned to Ar-B_6-H and Ar-B_4-H, respectively. Desielding due to an allyl carbonyl group such as 54 was not observed indicating the absence of the γ-carbonyl group. The MS showed a strong molecular ion peak at m/z (%): 372 (100) and the following fragment ion peaks at 357 (77), 341 (7.3), 329 (18), 299 (15), 297 (13), 269 (8.2), 243 (14). Therefore, the compound had no γ-carbon, and the singlet at δ 7.01 was assigned to β-CH= olefin proton. The compound was identified as 7-methoxy-5-methoxycarbonyl-2-(3,4,5-trimethoxyphenyl)coumarone (56'), which was formed from 47 byoxidation of the α'-aldehyde and by oxidative elimina-

tion of a γ-hydroxymethyl group.

Fr-A6-5 (< 1 mg) gave one spot on TLC, and its MS and R_f value on TLC, were identical with those of methyl 3,4,5-trimethoxybenzoate (59'), dimethylated deriderivative of syringic acid (59). This fact indicated that the cleavage of the Cα-Cβ bond in the coumaran ring occurred.

In further experiments with 270 mg of 47 (five cultures), two and three cultures were extracted after 72 and 192 hr incubations, respectively. To detect 5-carboxyvanillic acid (58), EtOAc extracts after incubation for 192 hr were methylated with diazomethane at 0℃ for 12 hr and co-chromatographed by TLC (MeOH-CH_2Cl_2 = 2:98, X 2) with trimethylated derivative (58') of 58. The band corresponding to the synthetic standard 58' was further purified by TLC (EtOAc-n-hexane = 1:2) to give 0.7 mg of pure 58. Its ^{1}H-NMR and MS were identical with those of the synthetic compound.

Degradation of 3,5-dihydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (48)

Because many catabolic products appeared after 50 hr incubation and most of the products were present for 333 hr incubation, shaking of cultures was stopped after 333 hr and extracted. The CH_2Cl_2 extracts were separated into ten fractions (Fr-N"1 - N"10). TLC analysis showed Fr-N"5 (1.4 mg) was pure. Its ^{1}H-NMR and MS were assigned as follows: ^{1}H-NMR: δ 4.00 (6H, s, Ar-A-OCH_3), 4.10 (3H, s, Ar-B-OCH_3), 5.65 (1H, broad s, Ar-OH), 6.95 (1H, s, β-CH=), 7.12 (2H,

s, Ar-A-H), 7.55 (1H, d, J = 1.3, Ar-B_6-H), 8.00 (1H, d, J = 1.3, Ar-B_4-H). MS m/z (%): 344 (100, M^+). Therefore, the compound was identified as 5-carboxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (56). Its dimethylated derivative was identical with compound 56' obtained from EtOAc extracts in the degradation of 47.

^{1}H-NMR spectrum showed that Fr-N"3 was a mixture of the substrate 48 and the phenylcoumarone 52. The structure was confirmed by ^{1}H-NMR spectrum after acetylation (Ac_2O-pyridine) and subsequent separation with TLC, and by comparison with synthetic compound 52'.

The EtOAc extract was separated into six fractions (Fr-A'1 - A'6) from which Fr-A'2 (0.8 mg), Fr-A'3 (2.8 mg), and Fr-A'5 (0.4 mg) were identified as 49', 51', and 54', respectively, by comparison with synthetic compounds. α'-Alcohol in 48 was oxidized to the corresponding carboxylic acid.

Catabolism of 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (50).

When DMF solution of 50 was added to the basal medium with shaking, the substrate precipitated partly. It is assumed that only soluble part of 50 was catabolized by the fungus. The culture was extracted after 115 hr incubation. From the EtOAc extract, 54" (3 mg) was isolated by TLC (EtOAc-n-hexane = 1:1) and identified. Its ^{1}H-NMR, MS and TLC were identical with those of synthetic 54". The CH_2Cl_2 extract was separated by TLC (EtOAc-n-hexane = 1:1, X 2). A minor band which exhibited blue fluorescence by a long-

wave UV light overlapped the major band of 50 and they could not be separated. The former band was tentatively identified as 55 by TLC analysis with synthetic 55.

Catabolism of 3,5-diformyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumarone (54).

Precipitation occurred on addition of a DMF solution of 54 to the basal medium. TLC analysis indicated that two compounds were formed after 70 hr incubation, and their amounts increased from 70 hr to 215 hr incubation. After 215 hr the culture was extracted. Both compounds were exhibited blue fluorescence under a long wave UV light. One of them (5.3 mg) was isolated from the CH_2Cl_2 extract by TLC (EtOAc-n-hexane = 2:1) and identified as 55 by ^{1}H-NMR and TLC in comparison with the synthetic 55. Another compound was detected from the EtOAc extract and identified as 53.

2.4 DISCUSSION

Based on the catabolic products identified, the degradation pathway for 5-formyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-3-hydroxymethyl-7-methoxycoumaran (47) in Fig. 2-4 is proposed. The aldehyde group of 47 initially was oxidized or reduced to the corresponding carboxyl or primary alcohol group giving 48 and 49, respectively. Both reactions took place in the culture at the same time. On the other hand, oxidation of the α'-primary hydroxyl group of 48 to the corresponding carboxyl group was demonstrated by the

formation of 49, 51, 54, and 56 from 48. Therefore, the oxidation and the reduction between the α'-aldehyde and the α'-alcohol were reversible, and the α'-position finally was oxidized to the carboxyl group, which was not reduced by this fungus. Similar reactions were reported for aryl-glycerol-β-vanillin ethers (section 1.2 and 1.3) and veratrum aldehyde[92]. P. chrysosporium oxidized or reduced the aldehyde of a non-phenolic phenylcoumaran substructure model[89].

Formation of 50, 51, 52, 53, 54, 55, 56, and 57 from 47 by F. solani M-13-1 could be attributed to the fungal phenol oxidizing activities. Dehydrogenation of 47, 48, and/or 49 by the action of a phenol oxidizing enzyme gives the phenoxy radicals. Since the radicals have a proton at α-position of the side chain, their disproportionation to a quinonemethide 63 and the parent phenol (47, 48, or 49) could occur readily[93].

For the quinonemethide 63, the following three reactions are conceivable: (I) Dehydrogenation of the Cβ-H of 63 to lead to the 3-hydroxymethyl-2-phenylcoumarone derivatives (50, 51, and 52), (II) Elimination of formaldehyde from the γ-position of 63 to afford the phenylcoumarone 56, (III) Nucleophilic addition of H_2O to Cα of 63 to give a 2-hydroxy-2-phenylcoumaran derivative 57' (hemiketal type), which was found to be tautomerized to 57 (keto-ol type). Reaction (II) was demonstrated in this investigation for the first time. Reactions (I) and (III) were found[90] in the degradation of 47 by P. chrysosporium and horseradish peroxidase.

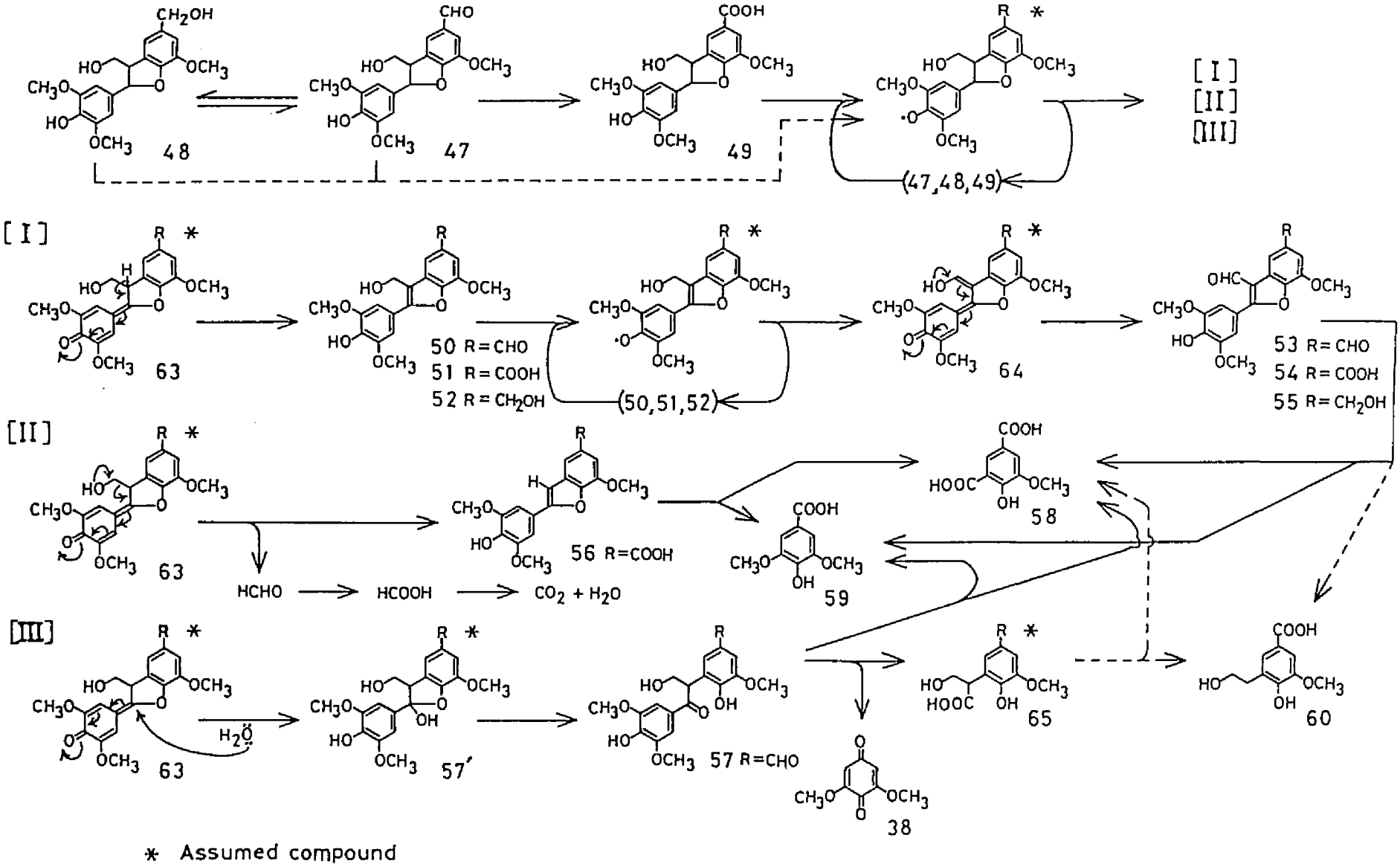


Fig. 2-4 Proposed pathways for the degradation of 5-formyl-3-hydroxymethyl-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran (47) by Fusarium solani M-13-1

The formaldehyde formed in Reaction (II) could be catabolized to CO_2 via formate by formaldehyde dehydrogenase and formate dehydrogenase[78].

Further action of the phenol oxidizing enzyme to 50, 51, and 52 might result in the formation of 3-formyl-2-phenylcoumarone derivatives (53, 54, and 55). Because phenoxy radicals of 50, 51, and 52 have a proton at the γ-position which is conjugated to their unsaturated system, disproportionation of their phenoxy radicals to quinonemethide 64 and the parent phenols may occur. Rearomatization of quinonemethide 64 leads to 53, 54, and 55. This reaction also was reported in the degradation of 47 by P. chrysosporium[90].

3-Carboxy-2-phenylcoumarone derivatives were not detected, in contrast to the degradation of a non-phenolic 3-formyl-2-phenylcoumarone derivative by the white-rot fungus[89]. The same result was obtained when 50 and 53 were used as substrates. Although F. solani M-13-1 is known to oxidize an α,β-unsaturated aldehyde in the terminal side chain of guaiacylglycerol-β-coniferaldehyde ether (5) (section 1.2) and a γ'-aldehyde derivative of dehydrodiconiferyl alcohol[45,94], the oxidation of the γ-aldehyde of the phenylcoumarones (53, 54, and 55) to the corresponding γ-carboxylic acids might not occur under this culture condition.

Cleavage of the $C\alpha$-C_{aryl} bond in 57 by the phenol oxidizing enzyme may result in the formation of 2,6-dimethoxy-p-benzoquinone (38) from the syringyl nuclei (A-ring) in 47. The quinone 38 could not be the oxidation

product of syringic acid (59). Although a white-rot fungus, Sporotrichum pulverulentum, was found to oxidize vanillic acid, an analog of 59, to methoxy-p-benzoquinone[95], F. solani M-13-1 catabolized vanillic acid via guaiacol and/or protochatechuic acid[78].

The counterpart compound in the Cα -C_{aryl} fission of 57 is assumed to be 2-aryl-3-hydroxypropanoic acid derivative (65) which may convert to 5-carboxyvanillic acid (58) via several steps. Although an α -ketone derivative of guaiacylglycerol-β -aryl ether was found to be reduced to guaiacylglycerol-β -aryl ether followed by the cleavage of the Cα -C_{aryl} bond (section 4.1), occurrence of the α -reduction of 57 was not observed, here.

Formation of 5-carboxyvanillic acid (58) and syringic acid (59) by Cα -Cβ fission of the phenylpropanone 57 and the phenylcoumarones (50-56) could be possible. Umezawa et al. found that 57 was degraded by P. chrysosporium to give 38 and 59 in addition to 58, indicating that the cleavage of the Cα -Cβ and Cα -C_{aryl} bonds in 57 occurred[90]. A non-phenolic 2-(3-methoxy-4-ethoxyphenyl)coumarone derivative was found to be cleaved between Cα -Cβ by the white-rot fungus to give 3-methoxy-4-ethoxybenzoic acid[89].

Ohta et al.[45] obtained 5-acetylvanillyl alcohol as a degradation product of dehydrodiconiferyl alcohol by F. solani M-13-1, and they predicted the presence of a 3-methoxy-2-phenylcoumarone derivative as an intermediate. However, none of them were obtained in this study, although syringic acid (59) was isolated.

It is thus concluded that a phenol-oxidizing enzyme

might play an important role in the degradation of 47 by F. solani M-13-1, and this fact is in good agreement with the degradation study with other oligolignols by F. solani M-13-1. Iwahara[94] found that a laccase purified from Fusarium sp. degraded pinoresinol and syringaresinol oxidatively, and that an enzyme, which is different from laccase and peroxidase catalyzed the hydroxylation of the benzyl position of the two resinols.

However, it is still not clear about the mechanism and initial degradation products of the phenylcoumarones although 5-carboxyvanillic acid (58) and syringic acid (59) were isolated in this study. There is no direct evidence which indicates the cleavage of the double bond in the phenylcoumarones. When phenylcoumarone 53 was used as a substrate, only oxidation and/or reduction products of the α'-aldehyde of the substrate were observed, and the phenylcoumarone structure seemed to be stable under the culture conditions.

The mechanism of the formation of 5-(2-hydroxyethyl)-vanillic acid (60) is not clear.

It seemed that the degradation pathway for 47 was very similar to that by P. chrysosporium[90] but different from that by P. putida[40] which is suggested to cleave the coumaran ring reductively.

CHAPTER 3

DEGRADATION OF A NON-CYCLIC BENZYL ARYL ETHER BY *FUSARIUM SOLANI* M-13-1

3.1 Syntheses of Arylglycerol-α,β-Diaryl Ethers

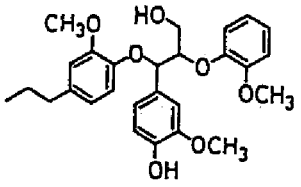
3.1.1 INTRODUCTION

Non-cyclic benzyl aryl ether (α-O-4) is contained (6-8 %) in lignin[5] and is important as a branched structure therein. The branched structure may influence chemical, biological, and physical properties of lignin macromolecules

Guaiacylglycerol-α-guaiacylpropane-β-guaiacyl diether (67) was synthesized by Johanson and Mikshe[96] as a trimeric α-O-4 lignin substructure model and used for studies on the structure and reactivity of lignin. The model is not adequate for lignin biodegradation research because the side chain of the β-aromatic ether is saturateded (Fig. 3-1), and the model is insoluble in culture media due to its low polarity.

In this section, synthesis of guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68), a new trimeric α-O-4 substructure model, was described. As a preliminary synthesis of 68, synthesis of guaiacylglycerol-α-(vanillyl alcohol)-β-guaiacyl diether (69) was examined. Aldehyde and hydroxymethyl groups were introduced at *para* position (α' and α") of β- and α-ether bonds, respectively, to improve the above model with its disadvantages (Fig. 3-1). They are reasonable functional groups as parts of an inter-

mediate structure in the degradation of the C_3-side chain by F. solani M-13-1 (section 1.2).



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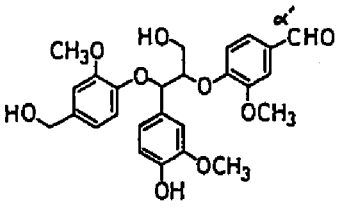

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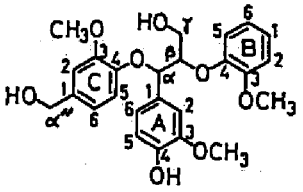

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Fig. 3-1 Structures of guaiacylglycerol-α,β-diaryl ethers, lignin substructure models containing a non-cyclic benzyl aryl ether

3.1.2 RESULTS AND DISCUSSION

Guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68) was synthesized via nucleophilic addition of 1-ethoxyethyl vanillyl ether (75) to a quinonemethide 71 from guaiacylglycerol-β-vanillin ether (1) as shown in Fig. 3-2. The quinonemethide 71 was prepared by bromination of 1 with bromotrimethylsilane in chloroform at room temperature followed by shaking the chloroform solution of a benzyl bromide 70 with a saturated $NaHCO_3$ solution. Bromotrimethyl silane recently was used for the preparation of a benzyl bromide (78) from guaiacylglycerol-β-guaiacyl ether (77) by Ralf and Young[97].

Preliminarily, the addition of three phenols, vanillin (81), benzyl vanillyl ether (84), and 1-ethoxyethyl vanillyl ether (75), to the quinonemethide 79 was examined. Firstly, the reaction of vanillin (81) with 79 was examined, because treatment of guaiacylglycerol-α-vanillin-β-guaiacyl diether with $NaBH_4$ was expected to give 68. The ^{1}H-NMR of the main product 83A showed three signals for methoxyl protons and a doublet for an α-methine proton. Its ^{13}C-NMR showed the presence of α, β, and γ-carbon peaks, and fifteen aromatic carbon signals with three of double intensity. These fact suggested the formation of a trimeric compound. However, no aldehydic proton and carbon were observed, while a singlet at δ 5.67 in the ^{1}H-NMR and a peak at δ 101.42 in the ^{13}C-NMR were present. These signals were assigned to an acetal proton and carbon, respectively. If the acetal moiety is derived from the

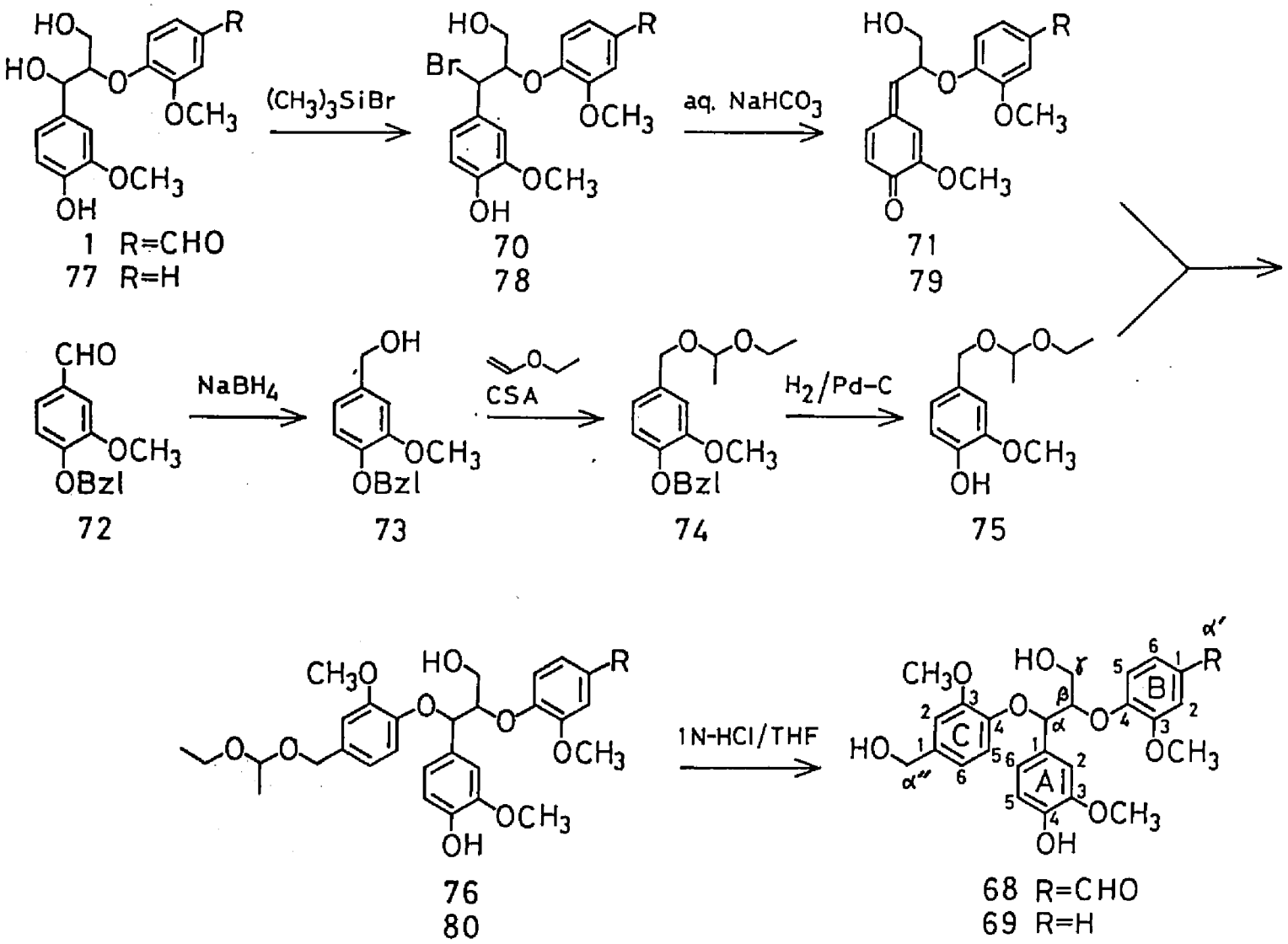


Fig. 3-2 Synthetic routes for guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68) and guaiacylglycerol-α-(vanillyl alcohol)-β-guaiacyl diether (69)

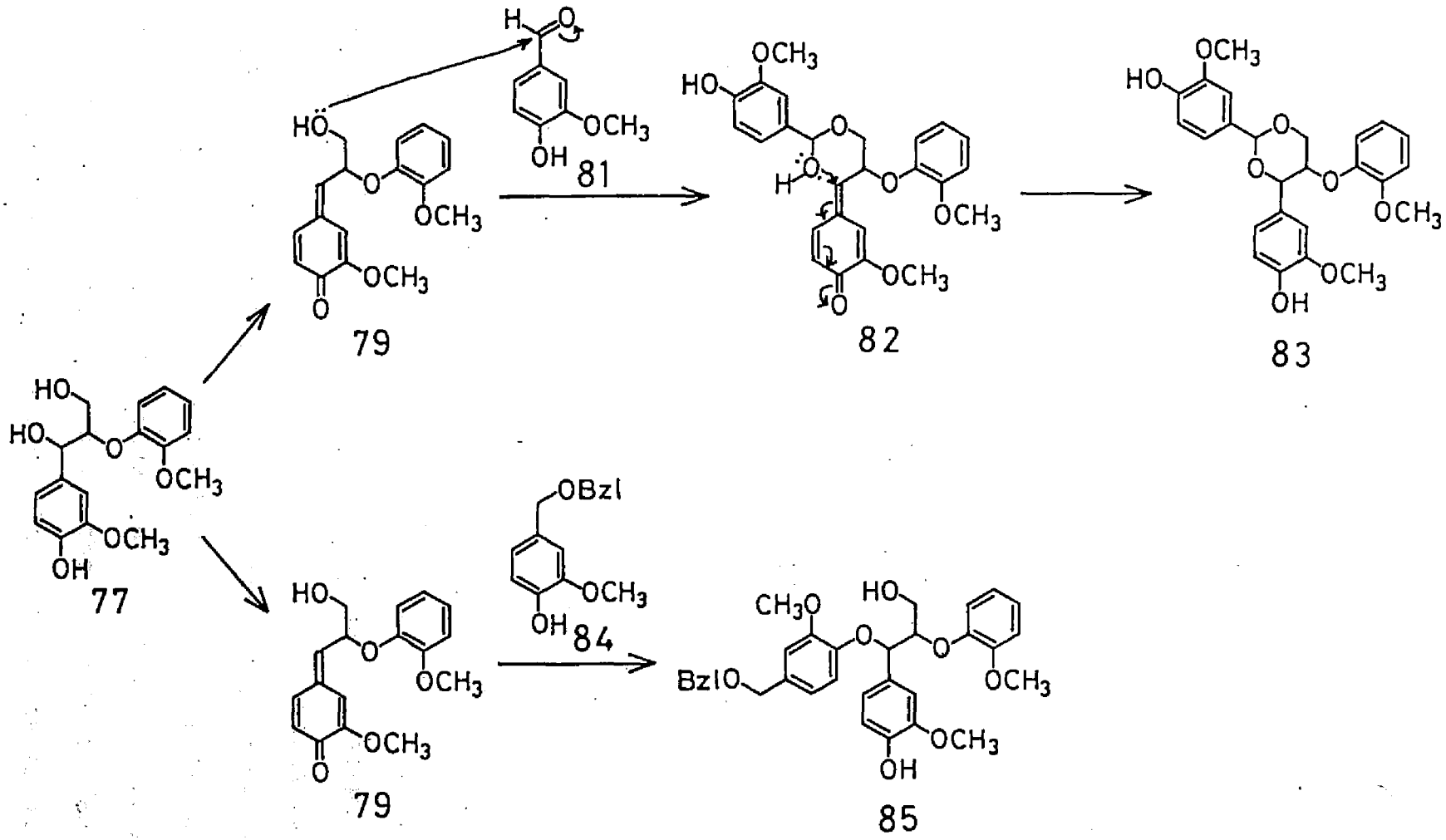


Fig. 3-3 Reactions of vanillin (81) and benzyl vanillyl ether (84) with the quinonemethide 82 from guaiacylglycerol-β-guaiacyl ether (77)

aldehyde of vanillin, the assignment of those peaks and a positive color test of 83 with 2,4-dinitrophenylhydrazine in HCl are reasonable. The ^{1}H-NMR of the acetate of 83A showed two singlets at δ 2.29 and 2.30 which were assigned to two phenolic acetyl protons. The absence of alcoholic acetyl protons indicated a chemical change of the γ-hydroxyl group. It was found that the phenolic hydroxyl group of vanillin was not involved in the formation of the α-O-4 linkage. The MS of the acetate showed the molecular ion peak at m/z 538. Therefore, the product was identified as 2,4-bis(4-hydroxy-3-methoxyphenyl)-5-(2-methoxyphenyl)-5-(2-methoxyphenoxy)-1,3-dioxane (83) whose formation could be shown in Fig. 3-3. The addition of the γ-hydroxyl group to the aldehyde of 81 gave hemiacetal 82 whose hydroxyl group of the hemiacetal moiety attacked the α-carbon intramolecularly to yield the cyclic acetal 83. Relative configulation of the α- and β-protons of 83A was determined to be trans by its coupling constant. The minor product 83B may be cis form.

Secondly, the addition of benzyl vanillyl ether (84) to 79 followed by the deprotection was examined. Since a p-hydroxybenzyl non-cyclic aryl ether bond is susceptible to hydrolysis[98,99], a benzyl protecting group which is cleaved by catalytic reduction in neutral media at room temperature was used. Compound 84 was synthesized from vanillin via four steps: vanillin was converted to its tetrahydropyranyl (THP) ether derivative which was then treated by $NaBH_4$ to give 4-O-THP ether of vanillyl alcohol; its benzylation followed by the cleavage of the THP ether by acidic hydroly-

sis gave 84.

The reaction of 84 with 79 gave a desired trimeric adduct 85 (Fig. 3-3). The ^{1}H-NMR of the main adduct showed three singlets for methoxyl protons, a doublet for an α-methine proton and two singlets for α" and benzyl methylene protons. All of other signals were also assigned. However, deprotection of the benzyl group by the catalytic reduction with 10% Pd-C in methanol did not give the desired trimer 69

Finally, the reaction of 1-ethoxyethyl vanillyl ether (75) with 79 was examined (Fig. 3-2). A 1-ethoxyethyl ether linkage is readily cleaved in a weakly acidic solution. Compound 75 was synthesized from O-benzylvanillin (72) as follows: The reduction of 72 with $NaBH_4$ gave 4-O-benzyl-vanillyl alcohol (73) whose hydroxyl group was protected by 1-ethoxyethyl etherification with ethyl vinyl ether and camphor-10-sulfonic acid (CSA) to give 4-O-benzylvanillyl 1-ethoxyethyl ether (74); its benzyl group was removed by catalytic reduction with 10% Pd-C yielding 75 whose ^{1}H-NMR showed non-equivalence of oxymethylene protons at benzyl and ethoxyl groups because of the presence of an asymmetric carbon adjacent to the oxymethylenes.

The main reaction product 80A was identified as a desired adduct 80 by ^{1}H-NMR and ^{13}C-NMR. The ^{1}H-NMR showed three singlets for methoxyl protons, peaks for 1-ethoxyethyl protons, a doublet for an α-methine proton and two doublets (AB type) for α"-methylene protons whose chemical shifts are not equivalent because of the presence of the asymmetric carbon in the 1-ethoxyethyl group. All peaks in the ^{13}C-NMR were assigned as shown in Table 3-1.

Table 3-1. ^{13}C-NMR data of erythro-80 and erythro-69 (solvent: $CDCl_3$).

	Erythro-80	Erythro-69
$-OCH_2\underline{C}H_3$	15.31	-----
$-OCH(\underline{C}H_3)O-$	19.86	-----
$Ar-O\underline{C}H_3$	55.75	55.75
	55.87	55.84
	55.87	55.90
$-O\underline{C}H_2CH_3$	60.57	-----
γ $-\underline{C}H_2-$	62.20	62.06
α' $-\underline{C}H_2-$	66.85	64.96
α $-\underline{C}H-$	81.80	81.62
β $-\underline{C}H-$	85.54	85.40
$-O\underline{C}H(CH_3)O-$	98.94	-----
$Ar-\underline{A}r-A-C_2$	109.61	109.69
$Ar-\underline{C}-C_2$	111.50	110.83
$Ar-\underline{B}-C_2$	112.11	112.14
$Ar-\underline{C}-C_5$	114.11	114.18
$Ar-\underline{A}-C_5$	115.91	115.98
$Ar-\underline{C}-C_6$	120.05	119.18
$Ar-\underline{A}-C_6$	119.44	119.38
$Ar-\underline{B}-C_5$	120.36	120.37
$Ar-\underline{B}-C_6$	121.12	121.15
$Ar-\underline{B}-C_1$	123.12	123.15
$Ar-\underline{A}-C_1$	130.57	130.47
$Ar-\underline{C}-C_1$	132.11	134.73
$Ar-\underline{A}-C_4$	145.35	145.38
$Ar-\underline{A}-C_3$	146.46	146.40
$Ar-\underline{C}-C_3$	146.46	146.53
$Ar-\underline{B}-C_3$	147.33	147.27
$Ar-\underline{C}-C_4$	149.71	149.81
$Ar-\underline{B}-C_4$	150.82	150.77

Hydrolysis of the 1-ethoxyethyl protecting group by pyridium p-toluenesulfonate and by 1N HCl in THF gave the desired trimer 69. Its ^{1}H-NMR showed three singlets for methoxyl protons, a slightly broad singlet for α"methylene protons and a doublet for an α-methine proton. All peaks in the ^{13}C-NMR were also assigned as shown in Table 3-1. The ^{1}H-NMR of the acetate of 69 showed two singlets for α"- and γ-alcoholic acetyl protons and a singlet for phenolic acetyl protons.

From the result of the preliminary examination, 1-ethoxyethyl vanillyl ether (75) was used for the addition to the quinonemethide 71 from guaiacylglycerol-β-vanillin ether (1). The addition produced a main adduct (76E) and a minor one (76T) joined by a non-cyclic benzyl aryl ether linkage. After separation of both adducts by TLC, their structures were determined by ^{1}H- and ^{13}C-NMR (Table 3-2).

Cleavage of the 1-ethoxyethyl protecting group of the adducts 76E and 76T with 1N HCl in THF gave guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether, 68E and 68T, respectively. Their ^{1}H-NMR and ^{13}C-NMR (Table 3-2) showed three methoxyl protons, α"-benzylic methylene protons and carbon, and eighteen aromatic carbons. The presence of three hydroxyl groups was confirmed by ^{1}H-NMR of the acetate.

The main products, 76E and 68E, and the minor one, 76T and 68T, were tentatively identified as erythro and threo form, respectively, on the basis of the reactivity of the quinonemethide and of the chemical shifts of the γ-CH_2-protons. Nakatsubo et al.[100] reported that the addition

Table 3-2 ^{13}C-NMR data of erythro-76, threo-76, erythro-68, and threo-68 (solvent: $CDCl_3$).

	Erythro-76	Threo-76	Erythro-68	Threo-68
$-OCH_2\underline{C}H_3$	15.32	15.31	-----	-----
$-OCH(\underline{C}H_3)O-$	19.88	19.85	-----	-----
$Ar-O\underline{C}H_3$	55.83	55.81	55.83	55.83
	55.89[a)]	55.90[a)]	55.87[a)]	55.90[a)]
$-O\underline{C}H_2CH_3$	60.61	60.57	-----	-----
γ $-\underline{C}H_2-$	62.64	61.68	62.58	61.67
α "$-\underline{C}H_2-$	66.79	66.82	65.03	65.08
α $-\underline{C}H-$	82.25	81.59	82.15	81.61
β $-\underline{C}H-$	83.89	85.26	83.88	85.34
$-O\underline{C}H(CH_3)O-$	99.02	98.97	-----	-----
$Ar-\underline{A}-C_2$	109.47	109.46	109.43	109.43
$Ar-\underline{B}-C_2$	109.99	110.01	109.96	110.10
$Ar-\underline{C}-C_2$	111.39	111.76	110.68	111.06
$Ar-\underline{C}-C_5$	114.18	114.31	114.17	114.31
$Ar-\underline{B}-C_5$	116.03	116.17	116.04	116.23
$Ar-\underline{A}-C_5$	116.14	117.81	116.17	117.87
$Ar-\underline{C}-C_6$	120.07	120.01	119.20	119.15
$Ar-\underline{A}-C_6$	120.24	120.36	120.22	120.39
$Ar-\underline{B}-C_6$	126.03	126.09	126.03	126.09
$Ar-\underline{A}-C_1$	129.99	128.68	129.92	128.64
$Ar-\underline{B}-C_1$	131.06	131.29	131.06	131.33
$Ar-\underline{C}-C_1$	132.54	132.16	134.99	134.67
$Ar-\underline{A}-C_4$	145.53	145.74	145.51	145.76
$Ar-\underline{A}-C_3$	146.17	146.43	146.18	146.49
$Ar-\underline{C}-C_3$	146.49	146.72	146.47	146.72
$Ar-\underline{C}-C_4$	149.64	149.89	149.78	150.03
$Ar-\underline{B}-C_3$	150.67	150.92	150.65	150.94
$Ar-\underline{B}-C_4$	152.75	153.88	152.69	153.87
α '$-\underline{C}HO$	190.47	190.64	190.52	190.62

a) Two $-OCH_3$

reaction of nucleophiles, such as organic acids, to the quinonemethide (79) from guaiacylglycerol-β-guaiacyl ether (77) gave preferentially the erythro form. Chemical shifts of the γ-CH_2- of 76E (δ 4.03) and 68E (4.03) are at a lower magnetic field than those of 76T (3.60-3.75) and 68T (3.71) in consistency with other α-O-4 substructure models synthesized previously[96].

When 50 mg of 68, as 0.5 ml of DMF solution, was added to 100 ml of the basal medium with shaking, no precipitate was observed, and a clear solution was obtained. It was confirmed that 68 was soluble in this culture medium.

3.1.3 EXPERIMENTAL

Chromatography and spectrometry

Column chromatography and TLC were done by the same manner as described in chapter 2. ^{1}H- and ^{13}C-NMR, mass, and UV spectra were taken by the same instruments as described in section 1.4.

Syntheses of compounds

Guaiacylglycerol-β-vanillin ether (1)

This compound was synthesized by the method described in section 1.1. ^{13}C-NMR ($CDCl_3$): δ 55.83 and 55.89 (E+T, Ar-OCH_3), 61.21 (E+T, γ-CH_2-), 73.22 (E) and 73.60 (T) (α-CH-), 84.61 (E) and 86.58 (T) (β-CH-), 109.21 (E) and 109.44 (T) (Ar-A-C_2), 110.18 (E+T, Ar-B-C_2), 114.29 (E) and 114.41 (T) (Ar-B-C_5), 116.33 (E) and 116.87 (T) (Ar-A-C_5),

119.25 (E) and 119.76 (T) (Ar-A-C_6), 126.09 (E+T, Ar-B-C_6), 131.01 (E) and 131.15 (T) (Ar-B-C_1), 131.32 (T) and 131.87 (E) (Ar-A-C_1), 145.18 (E) and 145.50 (T) (Ar-A-C_4), 146.55 (E) and 146.69 (T) (Ar-A-C_3), 150.57 (T) and 150.65 (E) (Ar-B-C_3), 152.69 (E) and 153.32 (T) (Ar-B-C_4), 190.74 (E+T, -CHO).

Guaiacylglycerol-β-guaiacyl ether (77)

This compound was synthesized by the similar method of Adler and Eriksoo[102]. The erythro and threo ratio was 9:10. ^{1}H-NMR ($CDCl_3$): δ 3.40-3.90 (2H, m, γ -CH_2-), 3.80-3.92(6H, two Ar-OCH_3), 3.90-4.30 (1H, m, β -CH-), 4.80-5.03 (1H, two d, α -CH-), 5.70-5.96 (1H, broad s, Ar-OH), 6.70-7.30 (7H, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 55.86 and 55.92 (E+T, Ar-OCH_3), 60.77 (E) and 61.03 (T) (γ -CH_2-), 72.81 (E) and 73.88 (T) (α -CH-), 86.85 (E) and 88.95 (T) (β -CH-), 108.86 (E) and 109.52 (T) (Ar-A-C_2), 112.16 (E+T, Ar-B-C_2), 114.21 (E) and 114.29 (T) (Ar-A-C_5), 119.02 (E) and 120.08 (T) (Ar-A-C_6), 120.39 (E) and 120.57 (T) (Ar-B-C_5), 121.46 (E) and 121.53 (T) (Ar-B-C_6), 123.80 (E) and 123.90 (T) (Ar-B-C_1), 131.49 (T) and 131.91 (E) (Ar-A-C_1), 144.99 (E) and 145.45 (T) (Ar-A-C_4), 146.52 (E) and 146.58 (T) (Ar-A-C_3), 146.85 (E) and 147.54 (T) (Ar-B-C_3), 151.02 (T) and 151.26 (E) (Ar-B-C_4).

1-Ethoxyethyl vanillyl ether (75)

O-Benzylvanillin (72) was prepared from vanillin and benzyl chloride in the presence of K_2CO_3 and KI in dry DMF at room temperature. O-Benzylvanillyl alcohol (73) was obtained by the reduction of 72 with $NaBH_4$ in MeOH at 0℃.

To a stirred solution of 244.3 mg (1.0 mmol) of 73 in 1 ml of CH_2Cl_2 (dried over basic alumina) were added 0.48 ml (5.0 mmol) of ethyl vinyl ether and 7.5 mg of CSA successively at 0℃ under nitrogen. After stirring for 39 min at the same temperature, the reaction solution was partitioned between CH_2Cl_2 and a saturated $NaHCO_3$ solution. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane =1:4) to give 253 mg (80% yield) of O-benzylvanillyl 1-ethoxyethyl ether (74). ^{13}C-NMR ($CDCl_3$): δ 15.32 ($-OCH_2\underline{C}H_3$), 19.89 ($^{O}_{O}>CH\underline{C}H_3$), 55.89 ($Ar-OCH_3$), 60.54 ($-O\underline{C}H_2CH_3$), 66.91 ($Ar\underline{C}H_2O-$), 71.09 ($-O\underline{C}H_2Ph$), 98.91 ($^{O}_{O}>\underline{C}H-CH_3$), 111.76 ($Ar-C_2$), 114.09 ($Ar-C_5$), 120.02 ($Ar-C_6$), 127.08 (2C, $Bzl-C_2'$), 127.55 ($Bzl-C_4'$), 128.27 (2C, $Bzl-C_3'$), 131.61 ($Ar-C_1$), 137.10 ($Bzl-C_1'$), 147.54 ($Ar-C_3$), 149.64 ($Ar-C_4$).

Compound 74 (47.5 mg, 0.15 mmol) was dissolved in 3 ml of MeOH, and 47.5 mg of 10% Pd-C was added to the solution. The mixture was stirred under hydrogen at room temperature for 20 min. The catalyst was filtered off and washed with MeOH. The filtrate and the washings were combined and evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane = 1:5) to give 31 mg (90% yield) of 75 as colorless oil. ^{1}H-NMR ($CDCl_3$): δ 1.22 (3H, t, J = 7.1, $-OCH_2\underline{C}H_3$), 1.35 (3H, d, J = 5.4, $^{O}_{O}>CH\underline{C}H_3$), 3.35-3.80 (2H, m, $-O\underline{C}H_2CH_3$), 3.89 (3H, s, $Ar-OCH_3$), 4.44 (1H, d, J = 11.4, $Ar-C<^{H}_{H}$), 4.56 (1H, d, J = 11.5, $Ar-C<^{H}_{H}$), 4.79 (1H, q, J = 5.3, $^{O}_{O}>C\underline{H}CH_3$), 5.60 (1H, s, Ar-OH), 6.80-6.90 (3H, m, Ar-H). ^{13}C-NMR ($CDCl_3$): δ 15.34 ($-OCH_2\underline{C}H_3$), 19.93 ($^{O}_{O}>CH\underline{C}H_3$), 55.84 ($Ar-OCH_3$), 60.60 ($-O\underline{C}H_2CH_3$), 67.15 ($Ar\underline{C}H_2O-$), 98.89 ($^{O}_{O}>\underline{C}HCH_3$),

110.57 (Ar-C_2), 114.11 (Ar-C_5), 120.91 (Ar-C_6), 130.22 (Ar-C_1), 145.07 (Ar-C_4), 146.43 (Ar-C_3). MS m/z (%): 226 (14, M^+), 180 (14), 151 (12), 137 (100), 73 (41), 57 (31), 45 (83).

3-(4-(1-Ethoxyethoxymethyl)-2-methoxyphenoxy)-2-(4-formyl-2-methoxyphenoxy)-3-(4-hydroxy-3-methoxyphenyl)-1-propanol (76), (reaction of 75 with 71).

To a stirred solution 209 mg (0.60 mmol) of 1 (dried over P_2O_5 over night) in 25 ml of anhydrous $CHCl_3$ was added 0.158 ml (184 mg, 1.20 mmol) of bromotrimethylsilane (Aldrich) under nitrogen at room temperature. After 22 min the reaction solution was shaken twice with 25 ml of a saturated $NaHCO_3$ solution. The resulting yellow solution of quinonemethide 71 was passed through a column of anhydrous Na_2SO_4 and added dropwise to the next reaction solution.

To a stirred solution of 272 mg (1.20 mmol) of 75 in 10 ml of anhydrous $CHCl_3$ was added dropwise the above solution of 71 over a period of 18 min under nitrogen at room temperature. The stirring was continued for additional 8 hr 17 min. The reaction solution was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The resulting residue was chromatographed on a silica gel column (2 cm ID X 30 cm, EtOAc-n-hexane = 3:1) to give three fractions containing 76. Their further purification by TLC (1.5 % MeOH in CH_2Cl_2) gave 26.5 mg of pure erythro-76 and 8.5 mg of crude threo-76 which was further purified by TLC (3% MeOH in CH_2Cl_2) to give 5.9 mg of pure threo-76. Total yield was 9.7 %.

Erythro-76: ^{1}H-NMR ($CDCl_3$): δ 1.20 (3H, t, J = 7.1, $-OCH_2\underline{CH_3}$), 1.34 (3H, d, J = 5.3, $\overset{O}{\underset{O}{}}>CHCH_3$), 3.27-3.8 (2H, m, $-O\underline{CH_2}CH_3$), 3.80 (3H, s, Ar-OCH_3), 3.86 (3H, s, Ar-OCH_3), 3.90 (3H, s, Ar-OCH_3), 4.03 (2H, m, γ -CH_2-), 4.40 (1H, d, J = 11.8, α "-$C<^{H}_{H}$), 4.53 (1H, d, J = 11.8, α "-$C<^{H}_{H}$), 4.55-4.65 (1H, m, β -CH-), 4.77 (1H, q, J = 5.4, $\overset{O}{\underset{O}{}}>CHCH_3$), 5.25 (1H, d, J = 7.4, α -CH-), 5.59 (1H, s, Ar-OH), 6.62-7.03 (7H, m, Ar-A-H, -C-H, and -B-C_5-H), 7.24-7.37 (2H, Ar-B-C_2- and C_6-H), 9.80 (1H, s, α '-CHO). ^{13}C-NMR: (Table 3-2).

Threo-76: ^{1}H-NMR ($CDCl_3$): δ 1.19 (3H, t, J = 7.0, $-OCH_2\underline{CH_3}$), 1.32 (3H, d, J = 5.3, $_O>CHC\underline{H}3$), 3.26-3.9 (2H, m, $-O\underline{CH_2}CH_3$), 3.60-3.75 (2H, m, γ -CH_2-), 3.79 (3H, s, Ar-OCH_3), 3.84 (3H, s, Ar-OCH_3), 3.87 (3H, s, Ar-OCH_3), 4.38 (1H, d, J = 11.8, α "-$C<^{H}_{H}$), 4.50 (1H, d, J = 11.8, α '-$C<^{H}_{H}$), 4.55-4.75 (1H, m, β -CH-), 4.75 (1H, q, J = 5.4, $\overset{O}{\underset{O}{}}>C\underline{H}CH_3$), 5.38 (1H, d, J = 6.5, α -CH-), 5.72 (1H, s, Ar-OH), 6.66-7.04 (7H, m, Ar-A-H, -C-H, and -B-C_5-H), 7.37-7.46 (2H, Ar-B-C_2- and C_6-H), 9.86 (1H, s, α '-CHO). ^{13}C-NMR: (Table 3-2).

Guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68).

To a stirred solution of 24.1 mg (0.043 mmol) of erythro-76 in 1 ml of THF was added 0.5 ml of 1N HCl at room temperature. After 40 min the reaction solution was partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC (2 % MeOH in CH_2Cl_2) to give 14.9 mg (71.2 % yield) of erythro-68. Threo-68 also was synthesized similarly.

Erythro-68: ^{1}H-NMR ($CDCl_3$): δ 3.80 (3H, s, Ar-OCH_3), 3.85 (3H, s, Ar-OCH_3), 3.90 (3H, s, Ar-OCH_3), 4.03 (2H, s, γ -CH_2-), 4.57 (2H, s, α"-CH_2-), 4.48-4.75 (1H, m, β -CH-), 5.26 (1H, d, J = 7.4, α -CH-), 5.62 (1H, s, Ar-OH), 6.64-7.03 (7H, m, Ar-A-H, -C-H, and -B-C_5-H), 7.23-7.36 (2H, Ar-B-C_2- and -C_6-H), 9.80 (1H, s, α'-CHO). ^{13}C-NMR: (Table 3-2). MS m/z (%): 330 (13.4), 312 (100), 300 (92.1), 297 (43.7), 211 (65.3), 161 (97.2), 154 (41.9), 152 (66.9), 151 (76.7), 137 (78.5).

Triacetate of erythro-68: ^{1}H-NMR ($CDCl_3$): δ 1.96 (3H, s, alcoholic-OAc), 2.07 (3H, s, alcoholic-OAc), 2.26 (3H, s, Ar-OAc), 3.76 (3H, s, Ar-OCH_3), 3.82 (3H, s, Ar-OCH_3), 3.85 (3H, s, Ar-OCH_3), 4.61 (1H, d, J = 4.5, γ -CH_2-), 4.82-5.02 (1H, m, β -CH-), 4.98 (2H, s, α"-CH_2-), 5.38 (1H, d, J = 6.5, α -CH-), 6.70-7.37 (9H, Ar-H), 9.82 (1H, s, α'-CHO). MS m/z (%): 415 (9.6), 373 (2.4), 355 (11.2), 331 (3.3), 327 (3.1), 313 (33.5), 281 (13.8), 222 (17.3), 179 (21.7), 162 (15.1), 152 (20.9), 151 (28.9), 137 (19.9), 131 (17.3), 119 (19.1), 43 (100).

Threo-68: ^{1}H-NMR ($CDCl_3$): δ 3.71 (2H, m, γ -CH_2-), 3.79 (3H, s, Ar-OCH_3), 3.85 (3H, s, Ar-OCH_3), 3.87 (3H, s, Ar-OCH_3), 4.54 (2H, s, α"-CH_2-), 4.65 (1H, m, β -CH-), 5.39 (1H, d, J = 6.6, α -CH-), 5.66 (1H, s, Ar-OH), 6.65-7.04 (7H, m, Ar-A-H, -C-H, and B-C_5-H), 7.38-7.46 (2H, Ar-B-C_2- and C_6-H), 9.87 (1H, s, α'-CHO). ^{13}C-NMR: (Table 3-2). MS m/z (%): 330 (16.7), 312 (93.4), 300 (73.5), 297 (39.9), 271 (12.7), 211 (51.0), 161 (82.7), 154 (100), 152 (53.5), 151 (62.7), 137 (83.8).

3.2 Degradation of an Arylglycerol-α,β-Diaryl Ether

3.2.1 INTRODUCTION

The mechanism of lignin biodegradation has been investigated using major substructure models in lignin. However, no investigations have been reported on degradation of a non-cyclic benzyl aryl ether (α-O-4).

In the previous section, guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68) which is an adequate α-O-4 model compound for lignin biodegradation research was synthesized. Present section describes the degradation of 68 by F. solani M-13-1.

3.2.2 EXPERIMENTAL

Microorganism, substrate, and culture conditions

F. solani M-13-1 was used as the microorganism[44]. Composition of the basal mineral-salts medium and the nutrient medium (both pH 6.0) were the same as described in section 1.2. The fungus was incubated as described in section 1.4, and its mycelia were washed with distilled water and added to a biodegradation culture.

Guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68) was used as a substrate. The biodegradation culture in a test tube (1.5 cm X 18 cm) contained 2 mg of 68, 14 mg of the washed mycelia (dry weight), and 4 ml of the basal medium. The substrate was added to the culture as a solution of 0.02 ml of DMF. Five cultures (10 mg of

68) were used. Two control cultures without mycelia or the substrate were prepared similarly. All cultures were shaken at 28℃.

Analysis of catabolic products

Degradation of the substrate and the formation of catabolic products were monitored by UV spectroscopy and TLC of the culture filtrate.

Mycelia were filtered off and washed with distilled water. The filtrate and the washings were combined and extracted three times with CH_2Cl_2. The aqueous layer was then acidified to pH 2-3 with 1N HCl and extracted three times with EtOAc. The EtOAc extracts were methylated for 140 min with an excess of diazomethane in MeOH at 0℃ and then submitted to preparative TLC. The isolated compounds were identified by ^{1}H-NMR and MS. Synthetic authentic samples were used as references for identification.

Chromatography and spectrometry

Analytical and preparative TLC, and column chromatography were the same as described in section 1.4.

^{1}H-NMR and UV spectra were taken by the same instruments as described in section 1.4. Mass spectra were determined by a Shimadzu LKB 9000 gas chromatograph-mass spectrometer and a JEOL JMS DX-300 mass spectrometer with a direct inlet system at ionizing voltage of 70 eV.

Syntheses of compounds

Synthesis of guaiacylglycerol-α-(vanillyl alcohol)-

β-vanillin diether (68) was described in section 3.1.

Synthesis and ^{1}H-NMR data of 3-hydroxy-1-(3,4-dimethoxy phenyl)-2-(2-methoxy-4-(methoxycarbonyl)phenoxy)-1-propanone (88') are described in section 4.1. MS: Fig. 3-5.

Vanillyl alcohol (89) was obtained by the reduction of vanillin with $NaBH_4$ in MeOH at 0℃. MS m/z (%): 154 (64.7, M^+), 137 (34.4), 136 (93.2), 135 (34.6), 125 (22.4), 122 (20.4), 107 (100), 106 (59.8), 105 (49.2), 93 (40.4), 90 (17.3), 78 (56.3), 77 (31.6), 65 (87.8).

3.2.3 RESULTS AND DISCUSSION

Fig. 3-4 shows that UV absorption at 280 and 311 nm of the culture filtrates decreased gradually during incubation. TLC of the EtOAc extracts after 70, 96, and 120 hr incubations gave similar patterns showing a main spot near the starting point (developed with 5 % MeOH in CH_2Cl_2). Thus, these extracts were combined. The main product gave dark blue color with $FeCl_3$-$K_3Fe(CN)_6$ but did a negative coloration with 2,6-dichloroquinone-4-chloroimide-1N NaOH, indicating the presence of a phenolc hydroxyl group and the absence of a p-hydroxybenzyl alcohol group. TLC analysis of the extracts after methylation indicated that the methylated derivative of the main product was identical with synthetic 3-hydroxy-1-(3,4-dimethoxyphenyl)-2-(2-methoxy-4-(methoxycarbonyl)phenoxy)-1-propanone (88').

Mass spectrum of the product isolated by TLC (2% MeOH in CH_2Cl_2, X 3) was the same as that of synthetic 88' (Fig. 3-5). The molecular ion peak at m/z 390 and the fragment

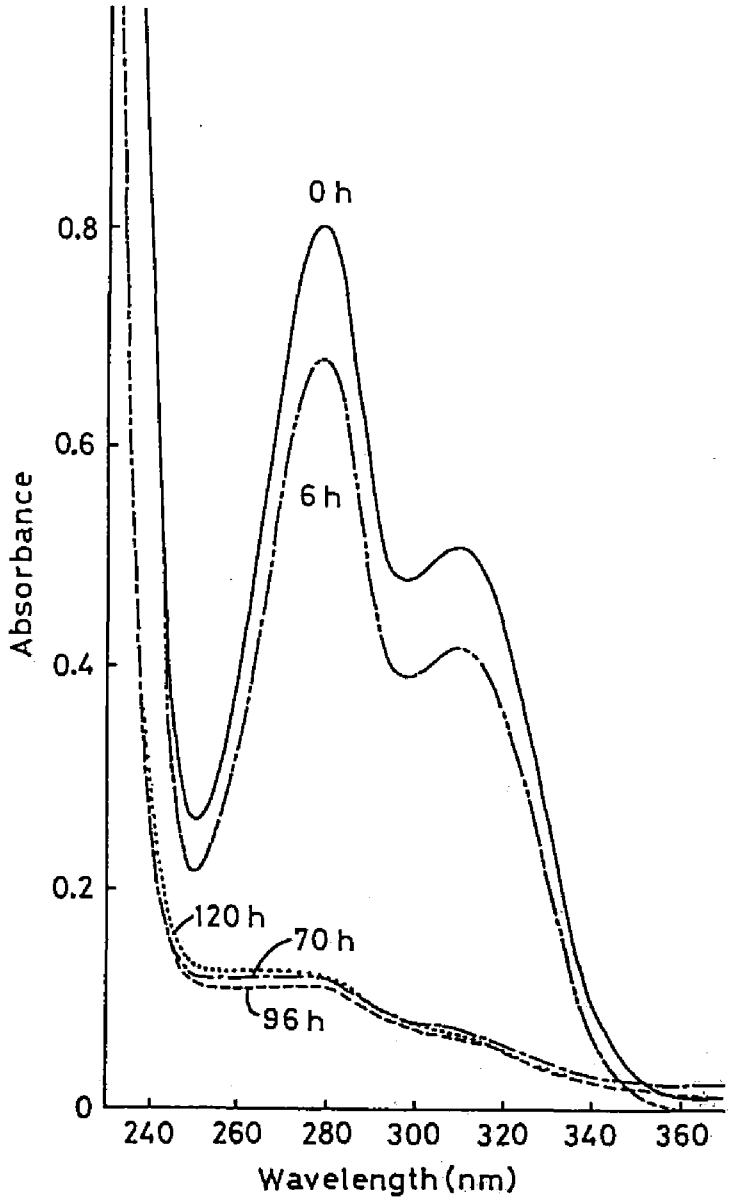


Fig. 3-4 Changes in the UV absorption of culture filtrates containing guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68) during incubation with Fusarium solani M-13-1

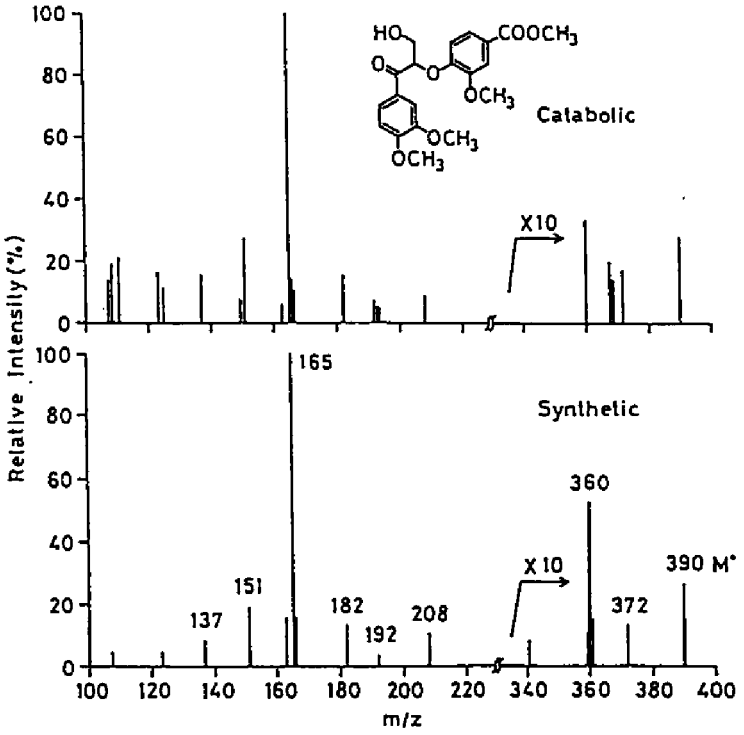


Fig. 3-5 Mass spectra of catabolic and synthetic 3-hydroxy-1-(3,4-dimethoxyphenyl)-2-(2-methoxy-4-methoxycarbonylphenoxy)-1-propanone (88')

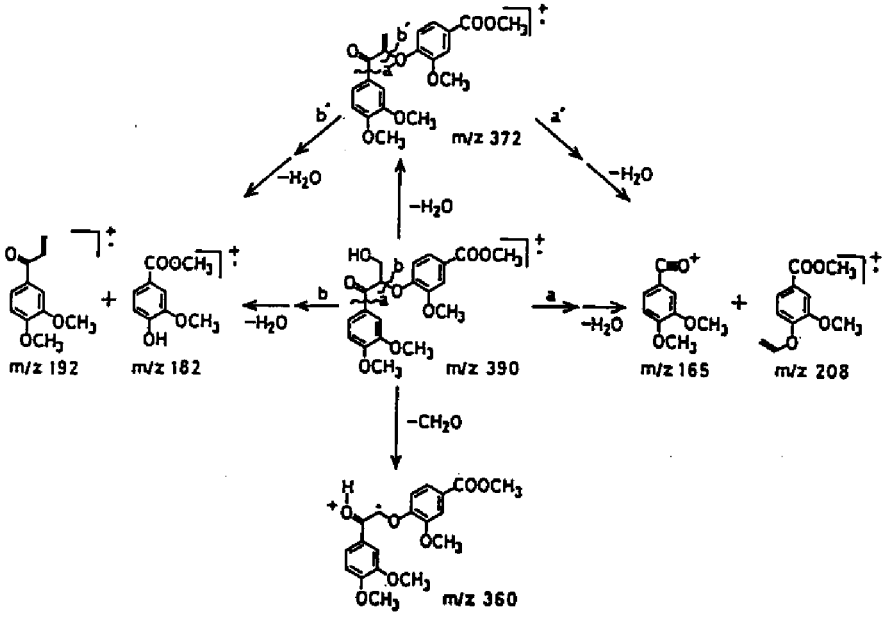


Fig. 3-6 Mass fragmentation of 3-hydroxy-1-(3,4-dimethoxyphenyl)-2-(2-methoxy-4-methoxycarbonylphenoxy)-1-propanone (88')

ion peaks at 372 (M^+-H_2O), 360 (M^+-CH_2O), and 165 (Ar(A)-$C\equiv O^+$, base peak) are characteristic of the α-ketone derivative of arylglycerol-β-aryl ethers as shown in Fig. 3-6. Because the amount of the product was very small, its ^{1}H-NMR gave only four signals at δ 3.871, 3.898, 3.906, and 3.944 (peak heights were similar to each other; number of acquisition is 11805). The synthetic 88' gave one $-COOCH_3$ and three Ar-OCH_3 signals at δ 3.869, 3.894, 3.907, and 3.945 which are identical with the above four signals. Therefore, the catabolic product was identified as 2-(4-carboxy-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (88).

TLC analysis indicated that two major products and minor starting material 68 were present in the extract from the control solution after 96 hr incubation, although their yields were not determined. The products were isolated by TLC (5% MeOH in CH_2Cl_2) and identified as vanillyl alcohol (89) and guaiacylglycerol-β-vanillin ether (1) by comparison with the authentic samples (MS and TLC).

The degradation pathway of guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin ether (68) shown in Fig. 3-7 is proposed based on the compounds identified. The α-ketone 88 could be formed by two modes, (I) cleavage of the benzyl aryl ether linkage of 68 mediated by a phenol oxidizing enzyme, and (II) a non-enzymic hydrolysis of the benzyl aryl ether of 68 followed by the oxidation of 1 mediated by the phenol oxidizing enzyme.

Mode (I) is analogous to the degradation mechanism of a phenolic phenylcoumaran (chapter 2) and dl-syringaresinol[101]

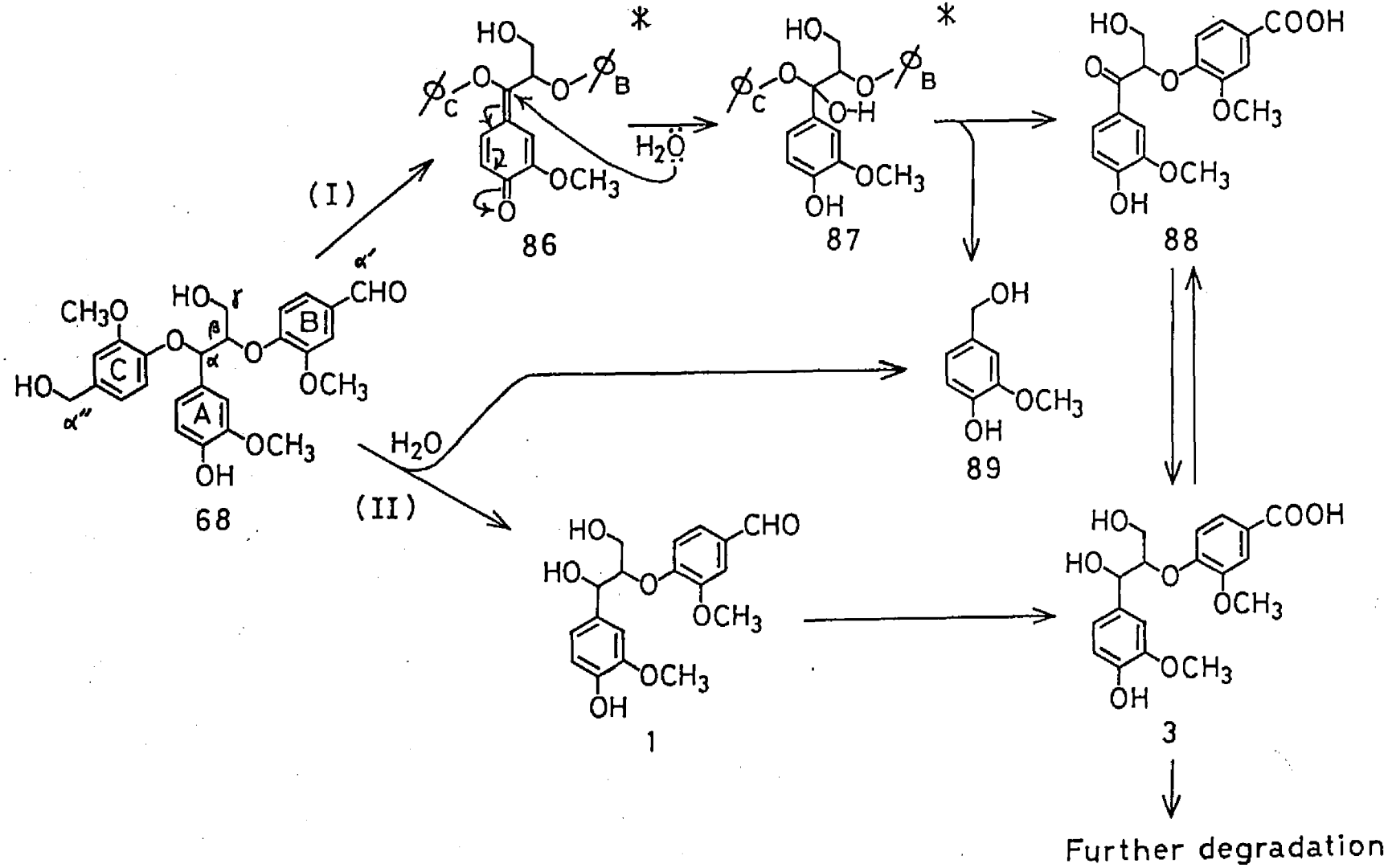


Fig. 3-7 Degradation pathways of guaiacylglycerol-α-(vanillyl alcohol)-β-vanillin diether (68) by Fusarium solani M-13-1 (* assumed compound)

which contain a cyclic benzyl aryl ether and a cyclic benzyl alkyl ether, respectively. Dehydrogenation of the phenolic hydroxyl group of 68 by the phenol oxidizing enzyme and subsequent disproportionation of the phenoxy radicals could give a quinonemethide derivative 86. The nucleophilic addition of water to the Cα position in 86 results in a hemiketal 87 which can be split readily to yield the α-ketone 88 and vanillyl alcohol (89). Iwahara[94] found that a laccase purified from Fusarium sp. degraded pinoresinol and syringaresinol oxidatively.

Mode (II) is described as follows: compounds 1 and 89 were isolated as main components from the control solution without mycelia. This suggests that the non-cyclic benzyl aryl ether of 68 was cleaved by non-enzymic hydrolysis similar to that reported previously[98,99]. Oxidation of the α'-aldehyde of 1 to the α'-carboxylic acid 3 was described in section 1.2. Cα-oxidation of 3 may give 88. A laccase-type enzyme catalyzed oxidation of p-hydroxybenzyl alcohol to the corresponding α-ketones[80,102]. It was reported that mycelial suspension of F. solani M-13-1 oxidized syringylglycerol-β-syringaresinol ether to the corresponding α-ketonic compound[87]. However, neither an α-ketone derivative such as 88 nor glyceric acid-2-(vanillic acid) ether (33) have been detected as the catabolite of guaiacylglycerol-β-aryl ethers such as 1 and 3 by the fungus, and glycerol-2-(vanillic acid) ether (29) was a major degradation product (section 1.3).

Further study is necessary to clarify which is the main mode, (I) or (II). Anyway, the benzyl aryl ether linkage of

68 was initially cleaved.

In chapter 4, F. solani M-13-1 was found to reduce the α-ketone 88 to 3 which was further degraded to glycerol-2-(vanillic acid) ether (29) and methoxy-p-benzoquinone (37). Thus, 1 may be degraded to 29 and 37 via 3. Vanillyl alcohol (89) may be catabolized via vanillic acid[84].

CHAPTER 4

DEGRADATION AND STEREOSELECTIVE REDUCTION OF AN α-CARBONYL DERIVATIVE OF AN ARYLGLYCEROL-β-ARYL ETHER BY FUSARIUM SOLANI M-13-1

4.1 Degradation and Reduction of an α-Carbonyl Derivative

4.1.1 INTRODUCTION

α-Ketonic phenylpropane structure is one of the characteristic feature of biodegraded lignin. The structure also occurs in native lignin in a small amount[5]. Phenolic and non-phenolic β-O-4 dilignols are oxidized to their corresponding ketones by laccase[22] and a lignin-degrading enzyme[104] from white-rot fungi, respectively. Previous investigation by Kamaya et al.[87] showed that Fusarium solani M-13-1 transformed syringylglycerol-β-syringaresinol ether to its α-ketone derivative. It was additionally found that phenolic dilignols with the cyclic α-ether bond such as syringaresinol[102] and syringylcoumaran (chapter 2) were partly oxidized by the fungus to the corresponding α-ketone structure. Furthermore, a trilignol with a non-cyclic p-hydroxybenzyl aryl ether was degraded oxidatively to give a ketone, 2-(4-carboxy-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (88) by the fungus (chapter 3). In this section the degradation of an α-ketone derivative, 2-(4-formyl-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (90), an analog of 88, by F. solani M-13-1 was described.

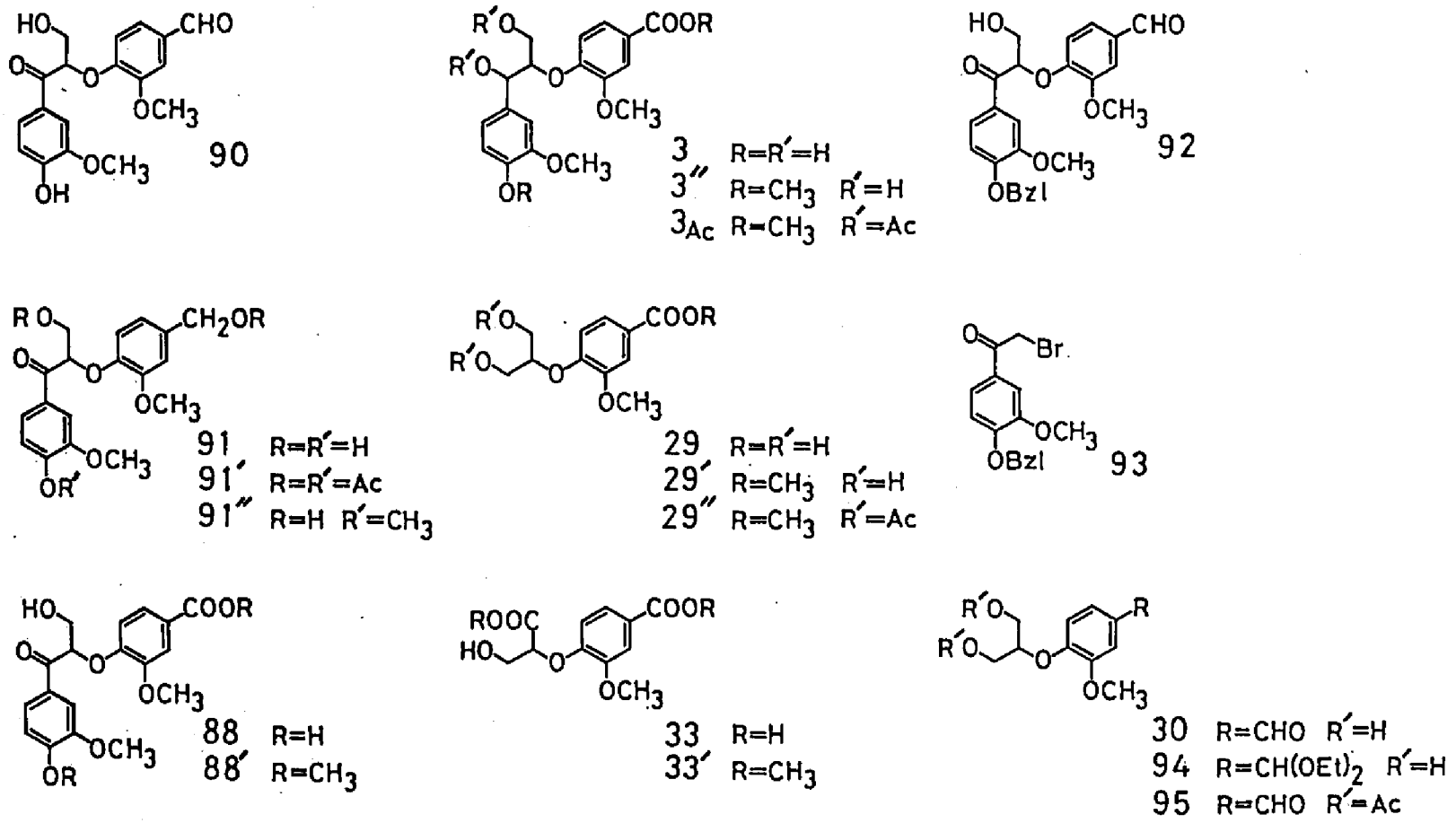


Fig. 4-1 Structures of compounds synthesized (section 4.1)

It has hitherto been suggested that the phenolic α-ketone derivative is directly cleaved between Cα-C_{aryl} bond by a phenol oxidizing enzyme to give a glyceric acid derivative and methoxy-p-benzoquinone. However, it was found that the α-ketone 90 is stereoselectively reduced by F. solani M-13-1 to the alcohol and then the cleavage of the Cα-C_{aryl} linkage occurs.

4.1.2 EXPERIMENTAL

Microorganism and preparation of fungal mycelia

Fusarium solani M-13-1 was used[44]. Composition of the basal inorganic medium and nutrient medium (both pH 6.0) were the same as described in section 1.2. Fungal mycelia were prepared as described in chapter 2.

Biodegradation

α-ketone 90 was used as a substrate. The substrate (50 mg) was dissolved in 0.5 ml of DMF. The DMF solution and 350 mg (dry weight) of the mycelial suspension in 10 ml of the basal medium were added to 90 ml of the basal medium in a 500 ml of Erlenmeyer flask. Degradation experiments were carried out three times (part Ⅰ, Ⅱ, and Ⅲ). The number of the culture at part Ⅰ, Ⅱ, and Ⅲ were ten (500 mg of 90), eight (400 mg), and twelve (600 mg), respectively. Two contol flasks without mycelia or the substrate in the medium were prepared. All flasks were shaken on the rotary shaker at 28℃.

Analysis of catabolic products

Degradation of the substrate and formation of catabolic products were monitored by TLC and UV spectroscopy of the culture filtrate. Mycelia were removed from the culture by filtration and washed with distilled water. The filtrate and the washings were combined and extracted twice with CH_2Cl_2. In the first and second experiments (part I and II), the aqueous layer was acidified to pH 2 with concentrated HCl and extracted three times with EtOAc. Both extracts were washed with saturated brine, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue of the EtOAc extracts was methylated for 12 hr with an excess of diazomethane in MeOH.

In third experiment (part III), the aqueous layer after extraction with CH_2Cl_2 was freeze-dried without acidification. The residue was extracted with MeOH and the MeOH soluble parts were methylated with diazomethane.

Those fractions were then submitted to preparative TLC and compounds isolated were identified by NMR and MS.

Examination of isomerization of erythro-guaiacylglycerol-β-(vanillic acid) ether (3) in an aqueous solution

A solution of 50 mg of erythro-3 in 100 ml of the basal medium was prepared and shaken for 30 hr by the same condition as control without mycelia. Compound 3 was recovered by EtOAc extraction after acidification, methylated with diazomethane for 12 hr, and purified by TLC (MeOH-CH_2Cl_2 = 5:95). The resulting veratrylglycerol-β-(methyl vanillate) ether (3") was characterized by ^{13}C-NMR spectrum.

Determination of a diastereomeric ratio of veratrylglycerol-β-(methyl vanillate) ether (3")

The ratio of erythro to threo form of 3", dimethylated derivative of guaiacylglycerol-β-(vanillic acid) ether (3), was determined by integrating the two peaks of β-carbon in the ^{13}C-NMR, which gave the best resolution between erythro and threo peaks of β-carbon (Fig. 4-3).

Chromatography and spectrometry

Chromatography and spectrometry were the same as described in section 3.1.

Syntheses of compounds

2-(4-Formyl-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (90)

Compound 90 was synthesized via 1-(4-benzyloxy-3-methoxyphenyl)-2-(4-formyl-2-methoxyphenyl)-3-hydroxy-1-propanone (92) prepared by a modification of the method of Adler and Eriksoo[101]: a) α-bromo-4-benzyloxy-3-methoxyacetophenone (93)/ vanillin/ K_2CO_3/ KI/ DMF/ r.t. (88 %); b) $(CH_2O)_n$/ DMSO/ r.t. (70%). ^1H-NMR ($CDCl_3$): δ 3.90 (3H, s, $-OCH_3$), 3.92 (3H, s, $-OCH_3$), 4.16 (2H, broad, γ $-CH_2-$), 5.23 (2H, s, $-CH_2Ph$), 5.57 (1H, t, J = 5.2, β $-CH-$), 6.82-6.96 (2H, Ar-C_5-H), 7.26-7.45 (7H, Ar-A-$C_{2,6}$-H and $-CH_2Ph$), 7.60-7.75 (2H, Ar-B-$C_{2,6}$-H), 9.82 (1H, s, -CHO).

Compound 90 was obtained by transformation of 92 via the following steps: c) $CH(OCH_3)_3$/ MeOH-THF/ p-TsOH/ r.t.; d) H_2/ 10% Pd-C/ MeOH/ r.t.; e) 1N HCl/ dioxane/ r.t.. The

product was purified by column chromatography (EtOAc-n-hexane = 3:1) and subsequent recrystallization from EtOAc-n-hexane (73% from 92). ^{1}H-NMR ($CDCl_3$): δ 3.90 (3H, s, Ar-OCH_3), 3.92 (3H, s, Ar-OCH_3), 4.16 (2H, broad d, J = 5, γ -CH_2-), 5.61 (1H, t, J = 5.0, β -CH-), 6.28 (1H, broad s, Ar-OH), 6.86 (1H, J = 8.0, Ar-A-C_5-H), 6.95 (1H, d, J = 8.1, Ar-B-C_5-H), 7.32 (1H, dd, J = 8.1, J = 1.8, Ar-B-C_2-H), 7.42 (1H, d, J =1.8, Ar-A-C_2-H), 7.59 (1H, d, J = 1.8, Ar-B-C_2-H), 7.70 (1H, dd, J = 8.3, J = 1.9, Ar-B-C_6-H), 9.81 (1H, s, -CHO). ^{13}C-NMR ($CDCl_3$): δ 55.9 (Ar-OCH_3), 56.0 (Ar-OCH_3), 63.6 (γ -CH_2OH), 82.8 (β -CH-), 110.2 (Ar-B-C_2), 110.7 (Ar-A-C_2), 114.26 (Ar-B-C_5), 114.34 (Ar-A-C_5), 123.9 (Ar-A-C_6), 126.1 (Ar-B-C_6), 127.1 (Ar-A-C_4), 131.0 (Ar-B-C_4), 147.0 (Ar-A-C_3), 149.9 (Ar-B-C_3), 151.5 (Ar-A-C_4), 152.1 (Ar-B-C_4), 190.6 (α'-CHO), 193.4 (α -C=O). MS m/z (%): 346 (0.4, M^+), 328 (0.8), 316 (9.5), 151 (100), 137 (7.1), 123 (9.6).

Diacetate was prepared by treatment of 90 with Ac_2O and pyridine. ^{1}H-NMR ($CDCl_3$): δ 2.06 (3H, s, γ -OAc), 2.33 (3H, s, Ar-OAc), 3.84 (3H, s, Ar-OCH_3), 3.89 (3H, s, Ar-OCH_3), 4.53 (1H, dd, J = 12.0, J = 6.9, γ -C<^H_H), 4.73 (1H, dd, J = 12.1, J = 3.9, γ -C<^H_H), 5.79 (1H, dd, J = 7.1, J = 3.9, β -CH-), 6.88-7.85 (6H, Ar-H). MS m/z (%): 430 (0.02, M^+), 400 (0.03), 370 (1.9), 328 (11.5), 151 (100), 43 (44).

3-Hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(4-hydroxymethyl-2-methoxyphenoxy)-1-propanone (91).

Compound 90 (6.4 mg) was dissolved in a mixture of 2 ml of MeOH and 1 ml of THF, and 6.4 mg of 10% Pd-C was added to the solution. The mixture was stirred for 45 min under

hydrogen. The catalyst was filtered off and washed with MeOH. The filtrate and the washings were combined and evaporated *in vacuo*. The residue was purified by TLC (5% MeOH in CH_2Cl_2) to give 5 mg of 91. ^{1}H-NMR (acetone-d_6): δ 3.80 (3H, s, Ar-OCH_3), 3.89 (3H. s, Ar-OCH_3), 4.05 (2H, d, J = 4.6, γ -CH_2-), 4.49 (2H, s, α'-CH_2-), 5.48 (1H, t, J = 4.7, β -CH-), 6.70-7.15 (4H, Ar-B-H and Ar-A-C_5-H), 7.52-7.72 (2H, Ar-A-C_2- and C_6-H), 8.0-8.7 (1H, broad, Ar-OH).

Compound 91 was converted to its triacetate (91') by treatment with Ac_2O and pyridine. ^{1}H-NMR ($CDCl_3$): δ 2.04 (3H, s, alcoholic-OAc), 2.07 (3H, s, alcoholic-OAc), 2.32 (3H, s, Ar-OAc), 3.76 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 4.50 (1H, dd, J = 11.9, J = 6.8, γ -$C<^{H}_{H}$), 4.65 (1H, dd, J = 12.0, J = 4.3, γ -$C<^{H}_{H}$), 5.00 (2H, s, α'-CH_2-), 5.60 (1H, dd, J = 6.8, J = 4.2, β -CH-), 6.80-7.00 (3H, Ar-B-H), 7.12 (1H, d, J = 8.7, Ar-A-C_5-H), 7.76 (1H, d, J = 2.0, Ar-A-C_2-H), 7.77 (1H, dd, J = 8.7, J =2.0, Ar-A-C_6-H).

3-Hydroxy-1-(3,4-dimethoxyphenyl)-2-((2-methoxy-4-methoxycarbonyl)phenoxy)-1-propanone (88').

To a stirred solution of 191 mg (0.487 mmol) of erythro veratrylglycerol-β-(methyl vanillate) ether (3") in 8 ml of dioxane was added 442 mg (1.95 mmol) of DDQ at room temperature. After stirring for 30 min the precipitate in the reaction mixture was removed by filtration and washed with dioxane. The filtrate and the washings were combined and evaporated *in vacuo*. The residue was purified by TLC (EtOAc-*n*-hexane = 3:1) to give 181 mg (95%) of 88'. ^{1}H-NMR ($CDCl_3$): δ 3.87, 3.89, 3.91, and 3.95 (four 3H, four s, -$COOCH_3$ and

three Ar-OCH_3), 4.13 (2H, broad t, J = 6, γ -CH_2OH), 5.53 (1H, t, J = 5.1, β -CH-), 6.82 (1H, d, J = 8.9, Ar-A-C_5-H), 6.89 (1H, d, J = 8.4, Ar-A-C_6-H), 7.54 (1H, dd, J = 8.9, J = 2.0, Ar-A-C_6-H), 7.55-7.61 (2H, Ar-A- and Ar-B-C_2-H), 7.74 (1H, dd, J = 8.4, J = 2.0, Ar-B-C_6-H). MS m/z (%): 390 (2.6, M^+), 372 (1.3), 360 (5.3), 208 (10), 192 (3.6), 182 (13), 165 (100), 151 (18), 137 (8.1).

Veratrylglycerol-β-(methyl vanillate) ether (3")

Erythro-3" and threo-3" were synthesized by the similar procedure described in section 1.1 and 1.3. Erythro-3", ^{1}H-NMR ($CDCl_3$): δ 3.8-3.9 (2H, γ -CH_2-), 3.86 (6H, s, two -OCH_3), 3.89 (3H, s, -OCH_3), 3.91 (3H, s, -OCH_3), 4.20-4.42 (1H, m, β -CH-), 4.98 (1H, d, J = 4.8, α -CH-), 6.80-7.00 (4H, Ar-A-H and B-C_5-H), 7.52-7.67 (2H, Ar-B-C_2- and C_6-H). MS m/z (%): 392 (0.9, M^+). Diacetate of erythro-3" (3_{Ac}), ^{1}H-NMR ($CDCl_3$): δ 2.01 (3H, s, -OAc), 2.05 (3H, s, -OAc), 3.86 (3H, s, two -OCH_3), 3.875 (3H, s, -OCH_3), 3.883 (3H, s, -OCH_3), 4.23 (1H, dd, J = 11.9, J = 4.6, γ -$C<^{H}_{H}$), 4.38 (1H, dd, J = 11.9, J = 5.8, γ -$C<^{H}_{H}$), 4.86 (1H, ddd, J = 5.8, J = 5.2, J = 4.6, β -CH-), 6.00 (1H, d, J = 5.2, α -CH-), 6.74-7.06 (4H, Ar-A-H and Ar-B-C_5-H), 7.50-7.64 (2H, Ar-B-C_2- and C_6-H). MS m/z (%): 476 (9.1, M^+), 445 (0.6), 416 (3.9), 356 (29), 341 (18), 267 (12), 236 (16), 225 (12), 209 (17), 193 (40), 182 (40), 167 (100), 151 (69), 43 (78).

Threo-3", ^{1}H-NMR ($CDCl_3$): δ 3.5-3.7 (2H, broad, γ -CH_2-), 3.87 (6H, s, two -OCH_3), 3.90 (3H, s, -OCH_3), 3.95 (3H, s, -OCH_3), 4.10-4.32 (1H, m, β -CH-), 4.98 (1H, d, J = 7.5, α -CH-), 6.83 (1H, d, J = 8.7, Ar-A-C_6-H), 6.98 (1H,

dd, J = 8.7, J = 1.8, Ar-A-C_5-H), 6.98 (1H, d, J = 1.9, Ar-A-C_2-H), 7.12 (1H, d, J = 8.9, Ar-B-C_5-H), 7.57-7.70 (2H, Ar-B-C_2-H and C_6-H). Diacetate of threo 3" (3_{Ac}), ^{1}H-NMR ($CDCl_3$): δ 1.99 (3H, s, -OAc), 2.00 (3H, s, -OAc), 3.86 (6H, s, two $-OCH_3$), 3.89 (3H, s, $-OCH_3$), 3.90(3H, s, $-OCH_3$), 4.05 (1H, dd, J = 12.0, J = 6.2, γ $-C<^{H}_{H}$), 4.27 (1H, dd, J = 11.8, J = 3.9, γ $-C<^{H}_{H}$), 4.79 (1H, ddd, J = 6.7, J = 6.2, J = 3.9, β -CH-), 6.06 (1H, d, J = 6.7, α -CH-), 6.72-7.07 (4H, Ar-A-H and Ar-B-C_5-H), 7.52-7.70 (2H, Ar-B-C_2- and C_6-H). MS m/z (%): 476 (11, M^+), 445 (0.9), 416 (3.8), 356 (36), 341 (22), 267 (14), 236 (15), 225 (12), 209 (19), 193 (32), 182 (44), 167 (100), 151 (75), 43 (75).

The mixture of erythro and threo isomers of 3" was obtained by reduction of 3" with $NaBH_4$ in MeOH at 0℃. The product was purified by TLC (MeOH-CH_2Cl_2 = 5:95), but the isomers were not separated. The erythro/threo ratio was 9:10. ^{13}C-NMR ($CDCl_3$): δ 52.0 (E+T, $-COOCH_3$), 55.8 and 55.9 (E+T, Ar-OCH_3), 61.2 (E+T, γ $-CH_2-$), 73.1 (E, α -CH-), 73.4 (T, α -CH-), 84.8 (E, β -CH-), 86.6 (T, β -CH-), 109.9 (E, Ar-A-C_2), 110.1 (T, Ar-A-C_2), 111.1 (E+T, Ar-B-C_2), 112.8 (T, Ar-A-C_5), 112.9 (E, Ar-A-C_5), 116.6 (E, Ar-B-C_5), 117.0 (T, Ar-B-C_5), 118.7 (E, Ar-A-C_6), 119.2 (T, Ar-A-C_6), 123.4 (E, Ar-B-C_6), 123.5 (T, Ar-B-C_6), 124.1 (E, Ar-B-C_1), 124.3 (T, Ar-B-C_1), 132.4 (T, Ar-A-C_1), 132.9 (E, Ar-A-C_1), 148.4 (E, Ar-A-C_3), 148.7 (T, Ar-A-C_3), 148.8 (E, Ar-B-C_3), 148.9 (T, Ar-B-C_3), 149.8 (T, Ar-A-C_4), 149.9 (E, Ar-A-C_4), 151.2 (E, Ar-B-C_4), 151.8 (T, Ar-B-C_4), 166.4 (E+T, $-\underline{C}OOCH_3$)

Glycerol-2-(vanillic acid) ether (29)

Previous synthetic method of 29 (section 1.3) was modified, since glycerol-2-vanillin ether (32), an synthetic intermediate, was unstable in the solution. Diethyl acetal of glycerol-2-vanillin ether (93) was used for the following synthesis without any purification.

To a stirred solution of the acetal 94 (ca. 4 mmol) in 6.47 ml (80 mmol) of pyridine was added 3.81 ml (40 mmol) of Ac_2O at room temperature. After 12 h, the reaction solution was evaporated in vacuo. The residue was dissolved in 10 ml of dioxane, and 0.5 ml of 1N HCl was added to the solution. After stirring for 15 min the reaction solution was poured into EtOAc. The solution was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was chromatographed on silica gel column (EtOAc-n-hexane = 1:4) to give 1.08 g (87%) of diacetate of glycerol--2-vanillin ether (95).

To a stirred solution of 1.08 g (3.48 mmol) of 95 in 15 ml of dioxane was added a solution of 825 mg (5.22 mmol) of $KMnO_4$ in 15 ml of water. After 30 min, 5 ml of MeOH was added to the reaction mixture and the stirring was continued for additional 30 min. The resulting precipitate of MnO_2 was filtered off and washed with MeOH and hot water, successively. The filtrate and the washings were combined, acidified to pH 2 with 1N HCl and extracted with EtOAc. The extract was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was dissolved in 20.9 ml (20.9 mmol) of 1N NaOH at room temperature. After stirring for 2 hr, the reaction solution was

acidified to pH 2 with concentrated HCl and extracted six times with EtOAc. The extracts were combined, washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. Recrystallization of the residual powder from EtOAc gave 645 mg of 29 (77%) as colorless needles. MS m/z (%): 242 (14, M^+), 168 (100), 153 (42), 125 (7.2). The methyl ester of 29 (29') and its diacetate (29") were prepared in the usual way. Their ^{1}H-NMR and MS were identical with those previously described (section 1.3).

Glyceric acid-2-(vanillic acid) ether (33)

Compound 33 and its dimethyl ester 33' which was prepared in section 1.3 were used in this investigation.

4.1.4 RESULTS

Degradation of 2-(4-formyl-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (90) (part I and II).

Fig. 4-2 shows that UV absorption at 280 and 312 nm of the culture filtrate which contained 90 as substrate decreased gradually during incubation. In the first experiment (part I) ten cultures and control run were extracted after 30 hr (the rate of decrease at 280 nm was 41%). Compound 3 and 91 were isolated and identified from EtOAc and CH_2Cl_2 extract, respectively, as follows.

The methylated EtOAc extract gave five bands (fr-A1-A5) by TLC (MeOH-CH_2Cl_2 = 2:98). TLC analysis indicated fr-A2 was pure. Yield was 133 mg (23.5% from 500 mg of 90). The

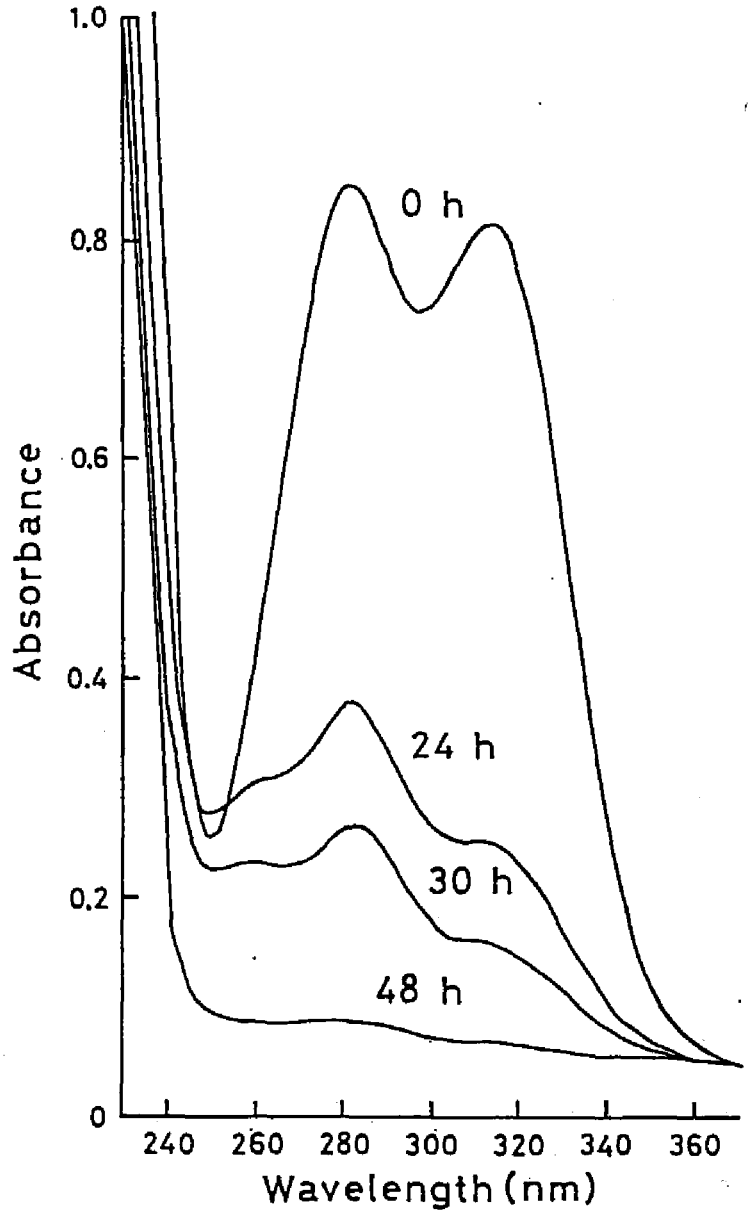


Fig. 4-2 Changes in the UV absorption of culture filtrates containing 2-(4-formyl-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (90) during incubation of Fusarium solani M-13-1

^{1}H-NMR spectrum showed a doublet at δ 4.98, four $-OCH_3$ signals, and the absence of aldehydic proton. All signals were assigned as follows: ($CDCl_3$) δ 3.75-3.95 (2H, γ $-CH_2-$), 3.86 (6H, s, two $-OCH_3$), 3.89 (3H, s, $-OCH_3$), 3.91 (3H, s, $-OCH_3$), 4.1-4.42 (1H, m, β -CH-), 4.98 (1H, d, J = 5.6, α CH-), 6.82-7.00 (4H, Ar-A-H and -B-C_5-H), 7.53-7.67 (2H, Ar-B-C_2- and -C_6-H). ^{13}C-NMR spectrum (Fig. 4-3) of the compound showed the presence of methyl ester and α -carbon, and the absence of α -ketone and α'-aldehyde. Major peaks in the spectrum were identical with those of erythro-veratryl-glycerol-β -(methyl vanillate) ether (3"). Minor peaks (1/3.5-1/4, in peak height) near the major peaks were identical with those of threo-3". All peaks were assigned as follows: ($CDCl_3$) δ 52.0 (E+T, $-COO\underline{C}H_3$), 55.8 and 55.9 (E+T, Ar-OCH_3), 61.1 (E+T, γ $-CH_2-$), 73.1 (E, α -CH-), 73.4 (T, α -CH-), 84.9 (E, β -CH-), 86.7 (T, β -CH-), 109.8 (E, Ar-A-C_2), 110.0 (T, Ar-A-C_2), 111.0 (E+T, Ar-B-C_2), 112.9 (E+T, Ar-A-C_5), 116.7 (E, Ar-B-C_5), 117.1 (T, Ar-B-C_5), 118.7 (E, Ar-A-C_6), 119.2 (T, Ar-A-C_6), 123.4 (E, Ar-B-C_6), 123.5 (T, Ar-B-C_6), 124.2 (E, Ar-B-C_1), 124.3 (T, Ar-B-C_1), 132.3 (T, Ar-A-C_1), 132.9 (E, Ar-A-C_1), 148.5 (E, Ar-A-C_3), 148.7 (T, Ar-A-C_3), 148.8 (E, Ar-B-C_3), 148.9 (T, Ar-B-C_3), 149.8 (T, Ar-A-C_4), 149.9 (E, Ar-A-C_4), 151.2 (E, Ar-B-C_4), 151.8 (T, Ar-B-C_4), 166.4 (E+T, $-COOCH_3$). Off resonance decoupling experiment supported the assignment. The ^{1}H-NMR spectrum was identical with those of synthetic erythro-3" except for four minor peaks, which were identical with those of synthetic threo-3". Peaks of catabolic threo-3" were found to be overlapped with those of erythro-3".

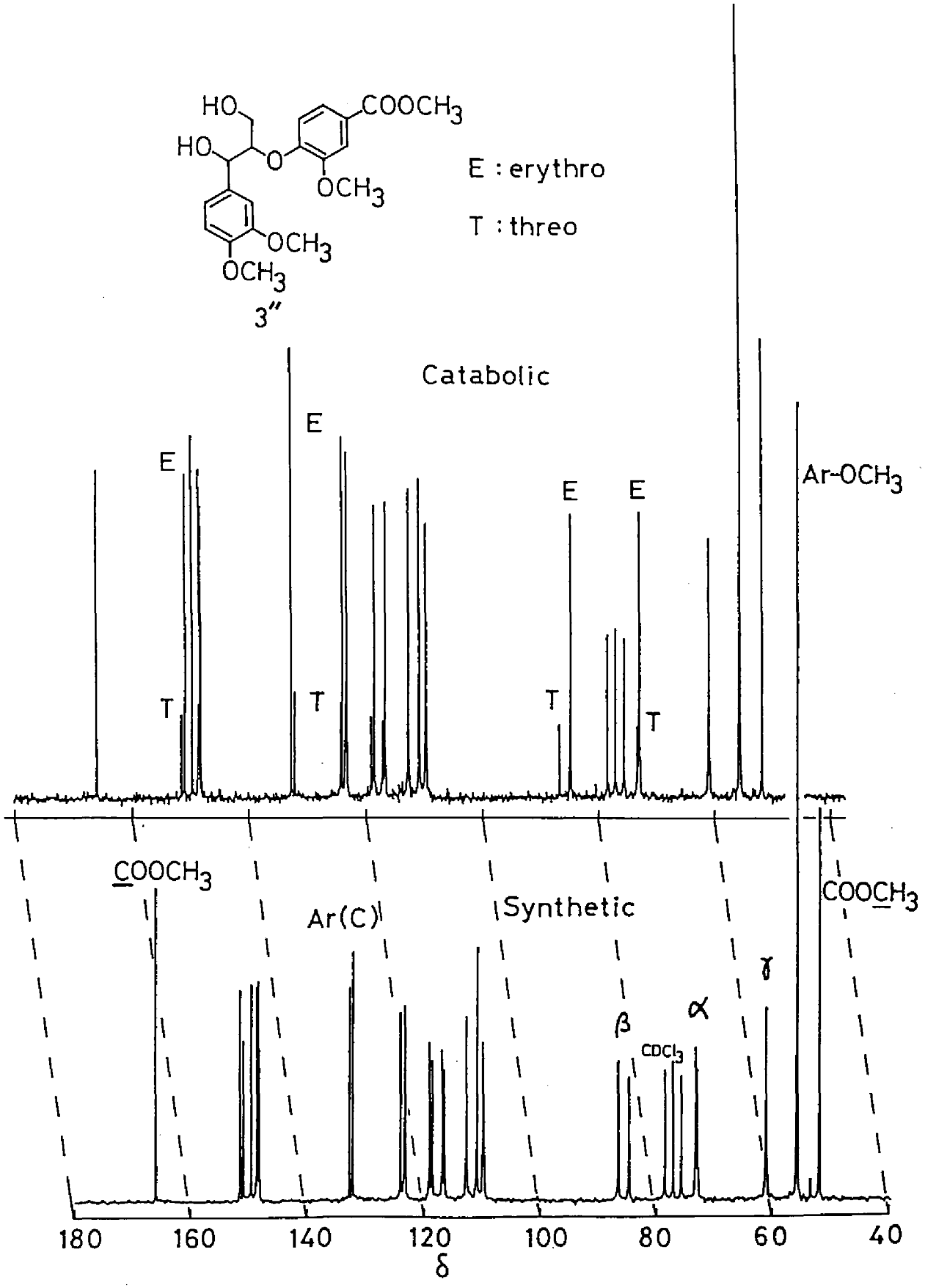


Fig. 4-3 ^{13}C-NMR spectra of catabolic and synthetic veratryl-glycerol-β-(methyl vanillate) ether (3"). Synthetic 3" is a mixture of erythro and threo forms.

The erythro/threo ratio in the catabolic 3" was 4:1.

The MS of the compound afforded the following peaks: MS m/z (%): 392 (0.74, M^+), 374 (1.4, M^+-H_2O), 361 (0.77, M^+-OCH_3), 360 (0.99), 356 (8.6), 344 (96, $374-CH_2O$), 329 (18), 315 (12), 283 (9), 256 (16), 208 (63), 182 (32), 167 (33), 166 (34), 165 (34), 151 (100). These peaks were identical with those of synthetic 3". High resolution mass spectrum of the compound gave 392.1483 (M^+, $C_{20}H_{24}O_8$; calcd. 392.14705) and 344.1233 ($M^+-H_2O-CH_2O$, $C_{19}H_{20}O_6$; calcd. 344.12594).

The compound was acetylated with acetic anhydride and pyridine for further identification and separation of the two diastereomers. ^{1}H-NMR spectrum of the acetate 3_{Ac} showed two major singlets of acetyl groups in erythro form at δ 2.01 and 2.05, minor signals of acetyl groups in threo form at δ 1.98-2.01 and the signals of γ $-CH_2-$ at δ 4.0-4.5 (the signal of γ $-CH_2-$ in erythro-3" was overlapped with its $-OCH_3$ signals). Careful separation of the acetate (41.2 mg) by TLC (a precoated plate, 2 mm X 20 cm X 20 cm, MeOH-CH_2Cl_2 = 1:99, X 12) gave the erythro and the threo forms. Each ^{1}H-NMR spectrum was identical with that of synthetic acetate (3_{Ac}).

Separation of fr-A-3 (6.4 mg) by TLC (Et_2O-n-hexane = 5:1, X 4) gave three bands: fr-A-31 (less 1 mg), fr-A-32 (less 1 mg), fr-A-33 (2.9 mg). Fr-A-33 was tentatively identified by ^{1}H-NMR as monomethyl ether of veratrylglycerol-β-(methyl vanillate) ether.

Separation of fr-A-1 (16.4 mg) by TLC (EtOAc-n-hexane = 5:1, X 3) gave three bands: fr-A-11 (1.7 mg), fr-A-12 (3.3

mg), fr-A-13 (5.2 mg). Fr-A-13 was identified as guaiacyl-glycerol-β-(methyl vanillate) ether (3') by comparison with synthetic compound (^{1}H-NMR and TLC) (section 1.1). Further methylation of fr-A-13 with diazomethane for 12 hr showed it to be identical with 3".

Fr-A-11 was identified as 3-hydroxy-2-(4-hydroxymethyl-2-methoxyphenoxy)-1-(3,4-dimethoxyphenyl)-1-propanone (91") by ^{1}H-NMR spectrum: ($CDCl_3$) δ 3.87, 3.92 and 3.94 (three 3H, three s, three $-OCH_3$), 4.07 (2H, d, J = 5.3, γ $-CH_2-$), 4.61 (2H, s, α' $-CH_2-$), 5.39 (1H, t, J = 5.3, β $-CH-$), 6.88-7.00 (4H, Ar-A-C_5-H and Ar-B-H), 7.62(1H, d, J = 2.0, Ar-A-C_2-H), 7.75 (1H, dd, J = 8.4, J = 2.0, Ar-A-C_6-H). A part of catabolic 91 remained in the aqueous layer after CH_2Cl_2 extraction, was extracted with EtOAc and methylated giving 91".

The CH_2Cl_2 extract after 30 h incubation was separated into five fractions (fr-N-1 - N-5) by TLC (MeOH-CH_2Cl_2 = 2:98, X 5). Substrate 90 (52 mg) was recovered from fr-N-4 (R_f 0.36-0.49). Further purification of fr-N-2 (R_f 0.13-0.20) by TLC (MeOH-CH_2Cl_2 = 5:95, X 3) gave a colorless syrup (R_f 0.40-0.46) which gave the following spectrum, ^{1}H-NMR: ($CDCl_3$) δ 3.83 (3H, s, Ar-OCH_3), 3.92 (3H, s, Ar-OCH_3), 4.06 (2H, d, J = 5.2, γ $-CH_2-$), 4.59 (2H, s, α' $-CH_2-$), 5.39 (1H, t, J = 5.1, β $-CH-$), 6.17 (1H, broad s, Ar-OH), 6.75-7.00 (3H, Ar-B-H), 6.93 (1H, d, J = 8.0, Ar-A-C_5-H), 7.61 (1H, d, J = 2.0, Ar-A-C_2-H), 7.69 (1H, dd, J = 8.2, J = 2.0, Ar-A-C_6-H). MS m/z (%): 348 (2.0, M^+), 330 (1.2, M^+-H_2O), 318 (14, M^+-CH_2O), 302 (1.1), 300 (0.7), 180 (4), 151 (100), 137(12), 123 (11). The compound was acetylated with acetic anhydride and pyridine for further identification.

^{1}H-NMR: ($CDCl_3$) δ 2.04 (3H, s, alcoholic-OAc), 2.07 (3H, s, alcoholic-OAc), 2.32 (3H, s, Ar-OAc), 3.76 (3H, s, Ar-OCH_3), 3.88 (3H, s, Ar-OCH_3), 4.50 (1H, dd, J = 11.9, J = 4.2, γ-$C<^{H}_{H}$), 4.65 (1H, dd, J = 11.9, J = 4.2, γ-$C<^{H}_{H}$), 5.00 (2H, s, α'-CH-), 5.60 (1H, dd, J = 6.8, J = 4.2, β-CH-), 6.81-6.89 (3H, Ar-B-H), 7.12 (1H, d, J = 8.7, Ar-A-C_5-H), 7.78 (1H, d, J = 1.9, Ar-A-C_2-H), 7.79 (1H, dd, J = 8.7, J = 1.9, Ar-A-C_6-H). MS m/z (%): 474 (0.8, M^+), 432 (0.2), 414 (12), 372 (4.7), 312 (28), 279 (37), 151 (100), 137 (34). These data and the R_f values of TLC were identical with those of synthetic 3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-2-(4-hydroxymethyl-2-methoxyphenoxy)-1-propanone (91) and its triacetate 91'.

On the basis of the yield and the structure of the isolated products, it was evident that after 24-30 hr the accumulation of 3 ($\lambda^{H_2O}_{max}$ = 254 and 282 nm) resulted in decrease of relative absorbance at 312 nm to 280 nm in the UV spectrum (Fig. 4-2), and followed by the appearance of absorption maximum at 255 nm.

In the second experiment (part II), eight cultures were extracted after 96 hr. Purification of the methylated EtOAc extract (52 mg) by TLC (MeOH-CH_2Cl_2 = 2:98, X 2) gave a main band at R_f 0.13. TLC analysis indicated that the compound was pure (yield, 3.7 mg). Its ^{1}H-NMR spectrum was assigned as follows: ($CDCl_3$) δ 3.86-3.95 (4H, $-CH_2-$), 3.90 (3H, s, $-COOCH_3$), 3.93 (3H, s, Ar-OCH_3), 4.33 (1H, quintet, J = 4.7, -CH-), 7.04 (1H, d, J = 8.4, Ar-C_5-H), 7.55-7.73 (2H, Ar-C_2- and C_6-H). The compound was converted to its diacetate whose ^{1}H-NMR spectrum ($CDCl_3$) showed a singlet of the acetyl

groups s at δ 2.06 (6H). Other peaks were assigned as follows: 3.90 (6H, s, $-COOCH_3$ and $Ar-OCH_3$), 4.35 (4H, d, J = 5.0, $-CH_2-$), 4.71 (1H, quintet, J = 5.0, -CH-), 7.05 (1H, d, J = 8.3, $Ar-C_5-H$), 7.56-7.70 (2H, $Ar-C_2-$ and C_6-H). These spectra and R_f values on TLC were identical with those of synthetic glycerol-2-(methyl vanillate) ether (29') and its diacetate 29". It is thus evident that the product 29 is formed by cleavageof the $C\alpha-C_{aryl}$ bond of the substrate 90. Several minor compounds were seen by TLC but not characterized further. The CH_2Cl_2 extract (9 mg) gave several spots on TLC plate but these were not enough for further characterization.

Methyl glycerate-2-(methyl vanillate) ether (33') was not detected from the EtOAc extract after both 30 hr (part I) and 96 hr (part II) incubation. None of condensation products were obtained.

Degradation of compound 90 (part III)

After 20 hr seven cultures were extracted. The catabolic product 3, which gave a main spot on TLC in the MeOH extract, afforded a bright blue color with 2,6-dichloroquinone-4-chloroimide in ethanol-1N NaOH, indicating the presence of a p-hydroxybenzyl alcohol moiety (α-OH group). Compound 91 (6.4 mg, 1.8 %) and 3' (109.3 mg, 27.6 %) were obtained from CH_2Cl_2 extract and methylated MeOH extract, respectively. ^{13}C-NMR spectrum of the catabolic 3" was identical with that of the catabolic 3" in part I. As shown in Fig. 4-4, no difference was found in the erythro/threo ratio, 4:1, of the compound between the two extraction

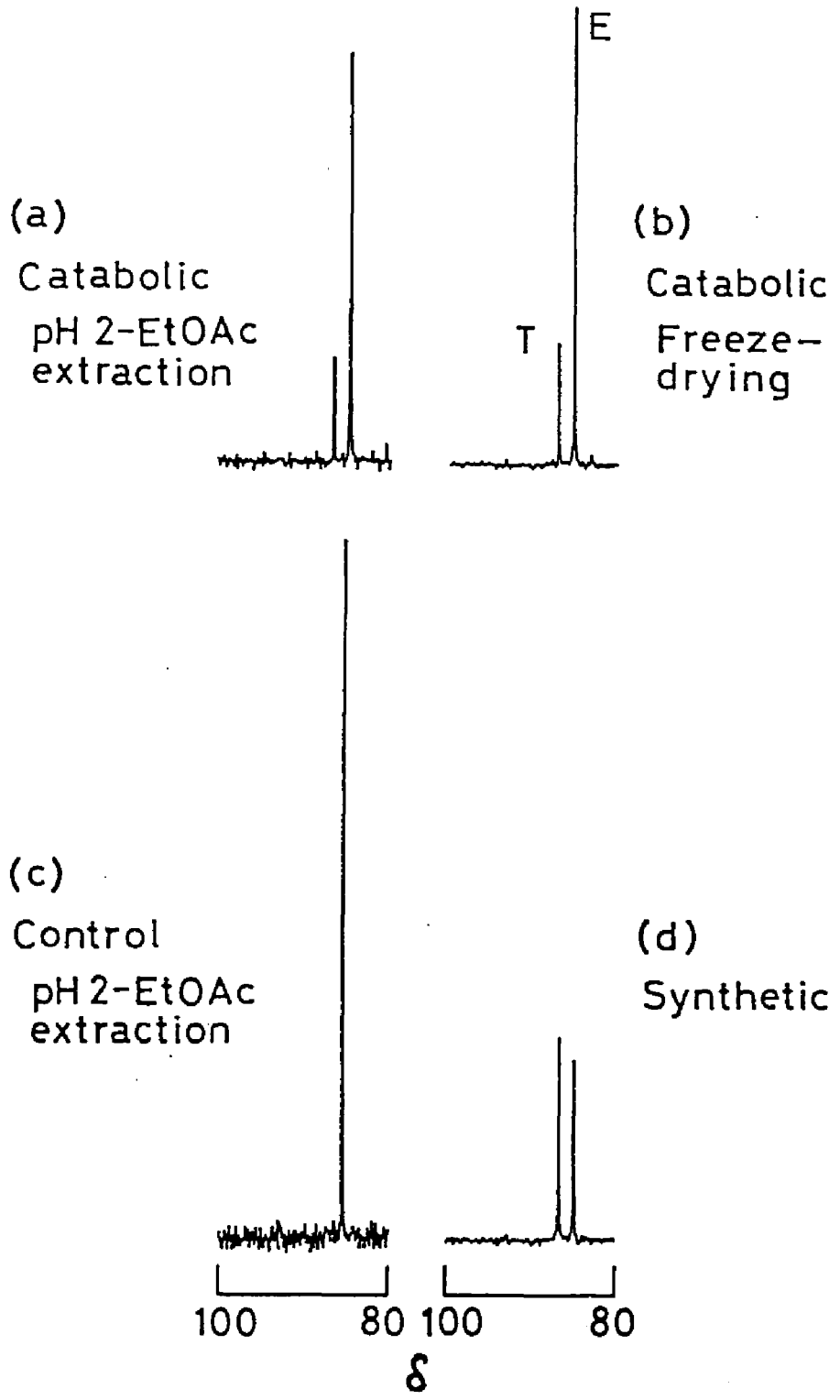


Fig. 4-4 ^{13}C-NMR spectra of veratrylglycerol-β-(methyl vanillate) ether (3"), showing β-carbon. (a) Catabolic product obtained by acidification of the culture filtrate and subsequent extraction with EtOAc. (b) Catabolic product obtained by freeze-drying of the culture filtrate and subsequent extraction with MeOH. (c) Recovered compound from control solution of erythro-guaiacylglycerol-β-(vanillic acid) ether (3) without mycelium. (d) Synthetic compound.

methods.

Examination of isomerization of erythro-guaiacylglycerol-β-(vanillic acid) ether (3)

Erythro-3 incubated in the basal medium (pH 6.0) without mycelia was recovered by EtOAc extraction after acidification. The recovered compound showed no corresponding peak to the β-carbon of threo-3" (Fig. 4-4). It is evident that isomerization of erythro form to threo form did not occur during shaking and extraction.

4.1.4 DISCUSSION

The α-ketone derivative 90 was completely degraded by F. solani M-13-1. The degradation pathway based on the catabolic products is shown in Fig. 4-5. α'-Aldehyde group of 90 is oxidized and/or reduced by the fungus to give 88 and 90. Such oxidation and reduction were found in many types of dilignols (phenolic β-O-4 (chapter 1), non-phenolic β-O-4 (chapter 1), and β-5 (chapter 2)) and veratraldehyde[92]. The α'-oxidation is a major reaction in the catabolism, since the yield of α'-oxidation products such as 3 and 29 was high. The result is in agreement with previous results (chapter 1 and 2). The fact that 88 was not detected is probably due to its rapid reduction to 3.

Reduction of α-ketone to secondary alcohol of oligolignols was found for the first time in the present investigation. Fenn and Kirk[105] showed that reduction of the α-ketone derivative of 4-ethoxy-3-methoxyphenylglycerol-β-

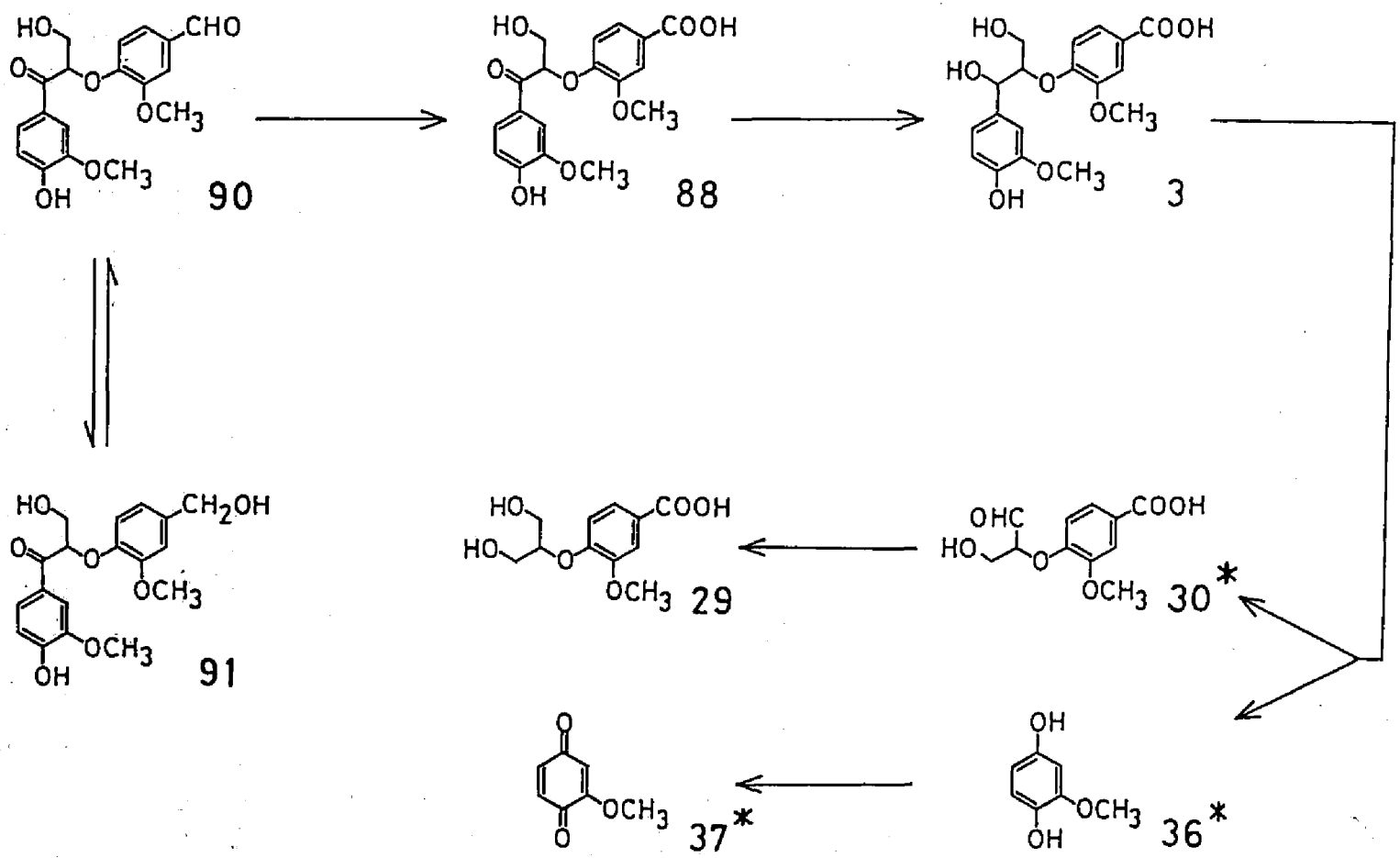


Fig. 4-5 Proposed pathway for the degradation of 2-(4-formyl-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (90) by Fusarium solani M-13-1 (* assumed compound)

guaiacyl ether did not occur by ligninolytic culture of Phanerochaete chrysosporium although its reverse reaction was prominent in the white-rot fungus.

Glycerol-2-(vanilic acid) ether (29), a degradation product of the reduced product 3, was isolated, but methoxyhydroquinone (36) and methoxy-p-benzoquinone (37) were not isolated as in section 1.3. It seems that a phenol oxidizing enzyme catalyzes the degradation of 3 to give glyceraldehyde-2-(vanillic acid) ether (40) and methoxyhydroquinone (36). The former is reduced to 29, and the latter is further degraded via ring cleavage found by Ander et al.[106] or oxidized to methoxy-p-benzoquinone (38).

Glyceric acid-2-(vanillic acid) ether (33), a product of direct oxidative cleavege between Cα-C_{aryl} bond in the ketone such as 90 and 88, was not detected from the culture filtrate in spite of co-chromatographic search with synthetic 33 and 33'. As described in section 1.3, 33 was stable but 29 was rapidly degraded in the culture condition. Hence, it is concluded that the reduction of the α-ketone and subsequent cleavage of Cα-C_{aryl} bond are main reactions in F. solani M-13-1.

It was reported that syringylglycerol-β-syringaresinol ether was converted by F. solani M-13-1 to the corresponding α-ketone derivative, which was cleaved oxidatively to give a glyceric acid derivative but the degradation of the glyceric acid derivative was much slower than that of the glycerol derivative[87]. This is consistent with the above conclusion, althugh the reduction of the α-ketone derivative of syringylglycerol-β-syringaresinol ether was not

examined.

In this investigation guaiacyl substructure model was used but a 5-5' condensation product (biphenyl type) was not detected in contrast to the earlier work with white-rot fungi by Kirk et al.[21].

When erythro-guaiacylglycerol-β-guaiacyl ether was treated with 0.2 N HCl in dioxane-H_2O (= 9:1) at 50℃, the erythro/threo ratio became 9:1 after 1 hr and it completely isomerized after 12 hr (1:1)[107]. However, isomerization of guaiacylglycerol-β-(vanillic acid) ether (3) did not occur in this culture condition and extraction procedure. A minor amount of threo-3 in the catabolic product is not due to the isomerization of erythro-3.

4.2 Optical Activity and Enantiomeric Purity of an α-Reduction Product

4.2.1 INTRODUCTION

Technical development of reactions of lignin bioconversion and elucidation of mechanism of lignin biodegradation are essential for biochemical utilization of lignins and lignocellulose materials. However, on the stereochemistry in lignin biodegradation little information has been obtained.

Lignin peroxidase and laccase non-stereospecifically attacked lignin model compounds[107,108]. A degradation product of a 1,2-diarylpropane-1,3-diol (β-1) substructure model by Phanerochaete chrysosporium revealed optical activity, but its enantiomeric purity was only 16%[108]. The fungus oxidized an allyl alcohol side chain of methyl dehydrodiconiferyl alcohol, a phenylcoumaran (β-5) substructure model, to the corresponding glycerol structure which has two asymmetric carbons. However, the circular dichroism (CD) spectrum of the obtained glycerol derivative showed no optical activity[89].

Previous section showed that an α-carbonyl group of 2-(4-formyl-2-metoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (90) was reduced by F. solani M-13-1 to the corresponding alcohol, yielding guaiacylglycerol ol-β-(vanillic acid) ether (3), followed by the cleavage of the Cα-C_{aryl} linkage. The α-reduction product 3 was a mixture of erythro and threo forms, with the ratio 4:1.

In the present investigation, both erythro and threo forms of the α-reduction product 3 were found to be optically active and pure, and which demonstrated stereoselectivity of the α-reduction.

4.2.2 EXPERIMENTAL

Isolation of α-reduced products 3

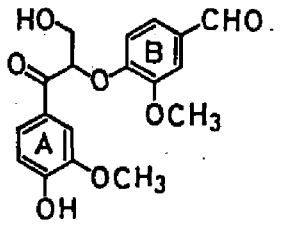
Extraction, isolation, and identification of the α-reduction product 3 were described in section 4.1.

Derivatization of catabolic 3" to separate its erythro and threo forms.

(a) Acetylation

A part of catabolic 3" was acetylated with 0.5 ml of Ac_2O and 0.5 ml of pyridine in 1.5 ml of EtOAc at room temperature for over night. The reaction solution was evaporated *in vacuo* and the residue was purified by TLC (2 mm X 15 cm X 20 cm, EtOAc-n-hexane = 2:3, X 5) to give 41.2 mg of the diacetate of 3" (3_{Ac}) which was a mixture of erythro and threo forms. The mixture was carefully separated by TLC (2 mm X 20 cm X 20 cm, two plates, 1 % MeOH in CH_2Cl_2, X 11) to give 30 mg of pure erythro-3_{Ac} and 8 mg of threo-3_{Ac}. The latter was further purified by TLC to remove small amounts of erythro-3".

^{1}H-NMR and MS of the catabolic erythro-3_{Ac} and threo-3_{Ac} were identical with those of synthetic compounds described in section 4.1.



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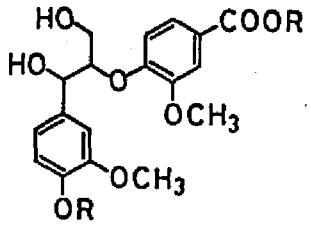
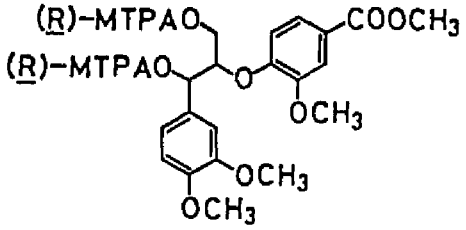


3 R=H

3″ R=CH_3



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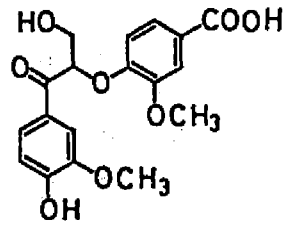

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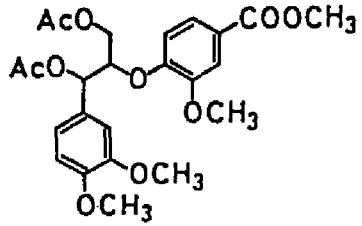
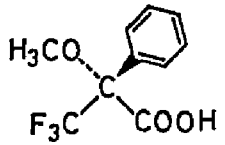
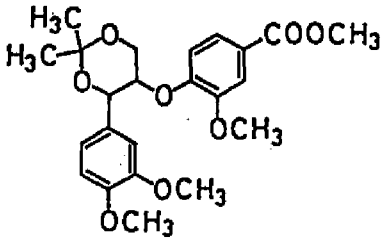


3Ac



(R)-MTPA



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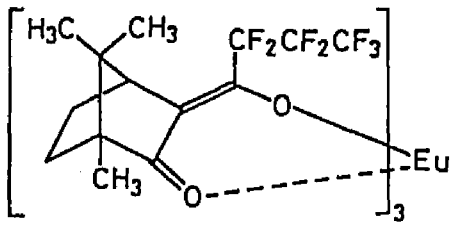

$Eu(hfc)_3$

Fig. 4-6 Structures of compounds synthesized, (R)-MTPA, and $Eu(hfc)_3$. (section 4.2)

(b) Formation of acetonide (isopropylidene acetal)

To a stirred solution of 25.9 mg (0.060 mmol) of catabolic 3" in 1.5 ml of of acetone were added 0.32 ml (138 mg, 2.6 mmol) of 2,2-dimethoxypropane and 0.3 mg of d-camphor-10-sulfonic acid (CSA), successively, at room temperature. After 5 hr, the reaction solution was neutralized by the addition of solid $NaHCO_3$ and the stirring was continued for additional 15 min. The solid $NaHCO_3$ was filtered off and washed with EtOAc. The filtrate and the washings were combined and partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by TLC (EtOAc-n-hexane = 1:2, X 3) to give 21.0 mg (73%) of erythro-96 and 5.5 mg (19%) of threo-96.

Erythro-96: ^{1}H-NMR ($CDCl_3$): δ 1.526 (3H, s, C-CH_3), 1.638 (3H, s, C-CH_3), 3.82 (9H, s) and 3.85 (3H, s) (-$COOCH_3$ and four Ar-OCH_3), 3.77-3.93 (1H, γ -C<$^{H}_{H}$), 4.06-4.30 (1H, γ -C<$^{H}_{H}$), 4.1-4.48 (1H, m, β -CH-), 4.93 (1H, d, J = 8.8, α -CH-), 6.53 (1H, d, J = 9.0, Ar-B-C_5-H), 6.78 (1H, d, J = 8.9, Ar-A-C_5-H), 6.95-7.04 (1H, Ar-A-C_2-H), 6.97-7.11 (1H, Ar-A-C_6-H), 7.46 (1H, dd, J = 8.9, J = 1.9, Ar-B-C_6-H), 7.44 (1H, d, J =1.9, Ar-B-C_2-H). MS m/z (%): 432 (1.1, M^+), 401 (0.3), 357 (1.9), 344 (14.9), 329 (2.6), 208 (100), 193 (7.2), 179 (10.6), 177 (18.3), 166 (41.8), 151 (21.6).

Threo-96: ^{1}H-NMR ($CDCl_3$): δ 1.589 (3H, s, C-CH_3), 1.602 (3H, s, C-CH_3), 3.81, 3.83, 3.85, and 3.86 (four 3H, four s, -$COOCH_3$ and three Ar-OCH_3), 4.16-4.23 (2H, γ -CH_2OH), 4.23-4.30 (1H, m, β -CH-), 5.09 (1H, d, J = 1.7, α -CH-), 6.47 (1H, d, J = 8.9, Ar-B-C_5-H), 6.78 (1H, d, J = 8.0, Ar-A-C_5-

H), 6.96 (1H, dd, J = 8.1, J = 1.9, Ar-A-C_6-H), 7.17 (1H, d, J = 1.7, Ar-A-C_2-H), 7.43 (1H, dd, J = 8.9, J = 2.1, Ar-B-C_6-H), 7.44-7.48 (1H, m, Ar-B-C_2-H). MS m/z (%): 432 (0.6, M^+), 401 (0.7), 357 (2.3), 344 (15.7), 329 (3.0), 208 (100), 193 (6.9), 179 (12.3), 177 (21.2), 166 (45.0), 151 (17.8).

Confirmation of no ocurrence of isomerization on the formation and cleavage of the acetonide 96

Firstly, pure racemic erythro-diol 3" and threo-diol 3" were prepared as follows. Compound 3" as a mixture of erythro and threo forms was converted to its acetonide 96 by the above method. The acetonide was completely separated to erythro and threo forms by preparative TLC (EtOAc-n-hexane = 1:2, X 3). Each purity was checked by HPLC (CH_3CN:H_2O = 60:40, UV at 280 nm).

The erythro-acetonide 96 was cleaved by stirring in AcOH-H_2O (= 9:1) for 4 hr at room temperature. Then, the reaction solution was neutralized by the addition of solid $NaHCO_3$. The mixture was partitioned between EtOAc and saturated $NaHCO_3$ solution. The organic layer was washed successively with saturated $NaHCO_3$ and saturated brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was purified by preparative TLC (EtOAc-n-hexane = 2:1, X 2), giving pure racemic erythro-diol 3". The purity was checked by HPLC (CH_3CN:H_2O = 20:80, UV at 259 nm). Pure racemic threo-diol 3" was also prepared by the same method.

Then, the pure racemic erythro-3" was converted to its acetonide 96 by the same method as above. After the work-up, to detect threo 96 formed by isomerization during the

acetonide formation, the reaction residue was analyzed by HPLC without any purification. The condition used was as follows: column, Chemco Pak Finesil C_{18}-5 (4.6 mm ID X 15 cm) with a precolumn (4.6 mm ID X 5.0 cm); eluent, $CH_3CN:H_2O$ = 60:40; flow rate, 1.0 ml/min; detection, UV at 280 nm. The pure racemic threo-3" was also treated by the same method. (Fig. 4-10).

The pure racemic erythro-acetonide 96 cleaved to diol-3" by the above method. After the work-up, to detect threo-3" formed by isomerization, the reaction residue was analysed by HPLC ($CH_3CN:H_2O$ = 20:80) without any purification. The pure racemic threo-acetonide 96 was also treated by the same method. (Fig. 4-11).

Enantiomeric purities of catabolic erythro and threo 3

The following three methods were applied.

(a) ^{1}H-NMR spectra of erythro and threo 3_{Ac} were taken in $CDCl_3$ in the presence of tris(3-(heptafluoropropylhydroxymethylene)-d-camphorato), europium (III) derivative, (Eu(hfc)$_3$) (Aldrich Chemical Co., Inc).

(b) HPLC was performed using CHIRALCELL OC (4.6 mm ID X 25 cm, Daicel Chemical Industries, LTD), a column for optical resolution, with a precolumn (4.6 mm ID X 5 cm). The conditions were as follows: eluent, EtOH; flow rate, 0.5 ml/min (erythro form) and 0.2 ml/min (threo form); detection, UV at 280 nm.

(c) Erythro-3" and threo-3" were converted to their (R)-α-methoxy-α-trifluoromethylphenylacetate derivative (97) with (R)-α-methoxy-α-trifluoromethylphenylacetic acid

((R)- MTPA) (Merck & Co., Inc) by the similar method of Dale et al.[111] followed by the analysis of the derivative 97 by TLC (erythro, CH_2Cl_2-n-hexane = 3:1, X 8; threo, EtOAc-n-hexane =1:3, X 3).

Synthetic erythro-97 (high R_f value): ^{1}H-NMR (1% $CDCl_3$) δ 3.436 (3H, d, J =1.2, MTPA-OCH_3), 3.533 (3H, d, J = 1.2, MTPA-OCH_3), 3.67, 3.75, 3.85, and 3.90 (four 4H, four s, -$COOCH_3$ and three Ar-OCH_3), 4.63 (1H, dd, J = 11.4, J = 3.5, γ -C<$^{H}_{H}$), 4.61 (1H, dd, J = 11.4, J = 6.4, γ -C<$^{H}_{H}$), 4.73-4.93 (1H, m, β -CH-), 6.14 (1H, d, J = 4.2, α -CH-), 6.67-6.82 (4H, m, Ar-A-H and Ar-B-C_5-H), 7.27-7.56 (12H, m, Ar-B-C_2- and C_6-H, and two MTPA-Ar-H). MS m/z (%): 824 (5, M^+).

Synthetic erythro-97 (low R_f value): ^{1}H-NMR (1% $CDCl_3$): δ 3.384 (3H, d, J = 1.0, MTPA-OCH_3), 3.502 (3H, d, J = 1.1, MTPA-OCH_3), 3.73, 3.79, 3.86, and 3.88 (four 3H, four s, -$COOCH_3$ and three Ar-OCH_3), 4.33 (1H, dd, J = 11.9, J = 5.3, γ -C<$^{H}_{H}$), 4.48 (1H, dd, J = 11.9, J = 3.9, γ -C<$^{H}_{H}$), 4.73-4.95 (1H, m, β -CH-), 6.11 (1H, d, J = 6.1, α -H-), 6.61 (1H, d, J = 9.0, Ar-B-C_5-H), 6.80 (1H, d, J = 8.7, Ar-A-C_5-H), 6.93 (1H, dd, J = 8.6, J = 1.8, Ar-A-C_6-H), 6.95 (1H, d, J = 1.8, Ar-A-C_2-H), 7.26-7.6 (12H, m, Ar-B-C_2- and C_6-H, and two MTPA-Ar-H). MS m/z (%): 824 (5, M^+).

Synthetic threo-97 (high R_f value): ^{1}H-NMR (1% $CDCl_3$): δ 3.401 (3H, d, J = 1.1, MTPA-OCH_3), 3.585 (3H, d, J = 1.1, MTPA-OCH_3), 3.61, 3.78, 3.86, and 3.91 (four 3H, four s, -$COOCH_3$ and three Ar-OCH_3), 3.6-3.9 (1H, γ -C<$^{H}_{H}$), 4.56-4.78 (1H, γ -C<$^{H}_{H}$), 4.76-4.90 (1H, m, β -CH-), 6.19 (1H, d, J = 8.6, α -CH-), 6.66 (3H, a, Ar-A-H), 6.87 (1H, d, J = 9.0, Ar-B-C_5-H), 7.04-7.63 (12H, m, Ar-B-C_2- and C_6-H, and two

MTPA-Ar-H). MS m/z (%): 824 (4, M^+).

Synthetic threo-97 (low R_f value): ^{1}H-NMR (1% $CDCl_3$): δ 3.395 (3H, d, J = 1.0, MTPA-OCH_3), 3.438 (3H, d, J = 1.1, MTPA-OCH_3), 3.77, 3.79, 3.88, and 3.90 (four 3H, four s, -$COOCH_3$ and three Ar-OCH_3), 3.85-4.09 (1H, γ -C<$^{H}_{H}$), 4.52 (1H, dd, J = 11.9, J = 2.8, γ -C<$^{H}_{H}$), 4.83 (1H, ddd, J = 7.3, J = 4.7, J = 2.8, β -CH-), 6.19 (1H, d, J = 7.3, α -CH-), 6.75 (1H, d, J = 9.0, Ar-B-C_5-H), 6.83-6.92 (3H, Ar-A-H), 7.06-7.60 (12H, m, Ar-B-C_2- and C_6-H, and two MTPA-Ar-H). MS m/z (%): 824 (5, M^+).

Chromatography and spectrometry

Chromatography (TLC and HPLC) and spectometry (UV, ^{1}H- and ^{13}C-NMR, and MS) were the same as described in section 1.4. Optical rotation was determined by using a Jasco J-20C Automatic Recording Spectropolarimeter with 95% EtOH as a solvent at 25℃.

Biodegradation and preparation of 88

Degradation of 2-(4-carboxy-2-methoxyphenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxyphenyl)-1-propanone (88) by F. solani M-13-1 was done by the same method as in the previous section. The α -ketone 88 was prepared via following four steps: a) guaiacylglycerol-β -vanillin ether (1)/ dihydropyran / CSA/ CH_2Cl_2/ r.t./ 95%; b) $KMnO_4$/ dioxane/ r.t.; c) 1N HCl/ dioxane/ r.t.; d) DDQ/ dioxane/ r.t/ 74% (b)-d)). The α -ketone 88 was purified by TLC (15% MeOH in CH_2Cl_2) and then used as the substrate.

4.2.3 RESULTS AND DISCUSSION

In the previous section, guaiacylglycerol-β-(vanillic acid) ether (3), as a reduction product of α-ketone 88, was isolated and identified as a dimethylated derivative 3", which was a mixture of erythro and threo form. Since the ORD spectrum of the diastereomeric mixture showed an optical rotatory power, the diastereomeric mixture was separated to the respective diastereomers, and enantiomeric purities of each erythro and threo form were examined. Authentic racemic modification synthesized were used to find best condition of optical resolution. Authentic erythro- and threo-3" were individually prepared, and these were converted to diacetate derivative 3_{Ac} and acetonide derivative 95.

The diacetate derivative 3_{Ac} of synthetic erythro-3" and threo-3" were found to be resolved to their enantiomers by the ^{1}H-NMR analysis with a chiral shift reagent Eu(hfc)$_3$. However, resolution of synthetic erythro-diol 3" by the ^{1}H-NMR analysis was not successful, and that of synthetic threo-diol 3" was in low extent. The mixture of erythro and threo forms of catabolic diacetate derivative 3_{Ac} was successfully separated by multidevelopment by silica gel TLC.

Fig. 4-7 and 4-8 shows the ^{1}H-NMR spectra of the acetate 3_{Ac} with Eu(hfc)$_3$. In the case of synthetic erythro-3_{Ac} without the shift reagent, signals of three aromatic methoxyl protons and one methyl ester are overlapped on the three singlets at δ 3.855, 3.875, and 3.883. By the addition of Eu(hfc)$_3$, these peaks were shifted to downfield

giving signals a, b, c, and d, among which signals a and d were found to be resolved into a pair of enantiomers. Signals c and b did not give a good resolution.

On the other hand, the catabolic erythro-3_{Ac} gave single peak of all signals a', b', c', and d' which corresponded to the four signals (a-d). Patterns of other signals were more simplified than those of the synthetic sample.

In the case of threo-3_{Ac} (Fig. 4-8), signals of two acetyl protons are present at δ 1.988 and 1.996, and those of three methoxyl and one methyl ester protons are at δ 3.863, 3.888, and 3.995 as three singlets. By the addition of Eu(hfc)$_3$, the acetyl protons were shifted giving signals e and f, and the methoxyl and methyl ester protons were shifted giving signals g, h, i, and j. For synthetic sample, e, f, h, and j were found to be resolved into a pair of enantiomers.

On the other hand, for the catabolic threo-3_{Ac} all of the signals e', f', h', and j' gave singlets. Patterns of another signals also more simplified than those of the synthetic threo-3_{Ac}.

Secondly, optical resolution of 3" by their (R)-MTPA derivatization and subsequent TLC separation was examined. Di-MTPA ester derivative of racemic erythro-3" (synthetic sample) gave two spots on TLC (CH_2Cl_2-n-hexane = 3:1, X 8), corresponding to a pair of the resulting diastereomers. Di-MTPA ester (97) of catabolic erythro-3" gave one spot which identical with lower R_f spot of the MTPA ester (97) of the catabolic erythro-3", but another spot corresponding to

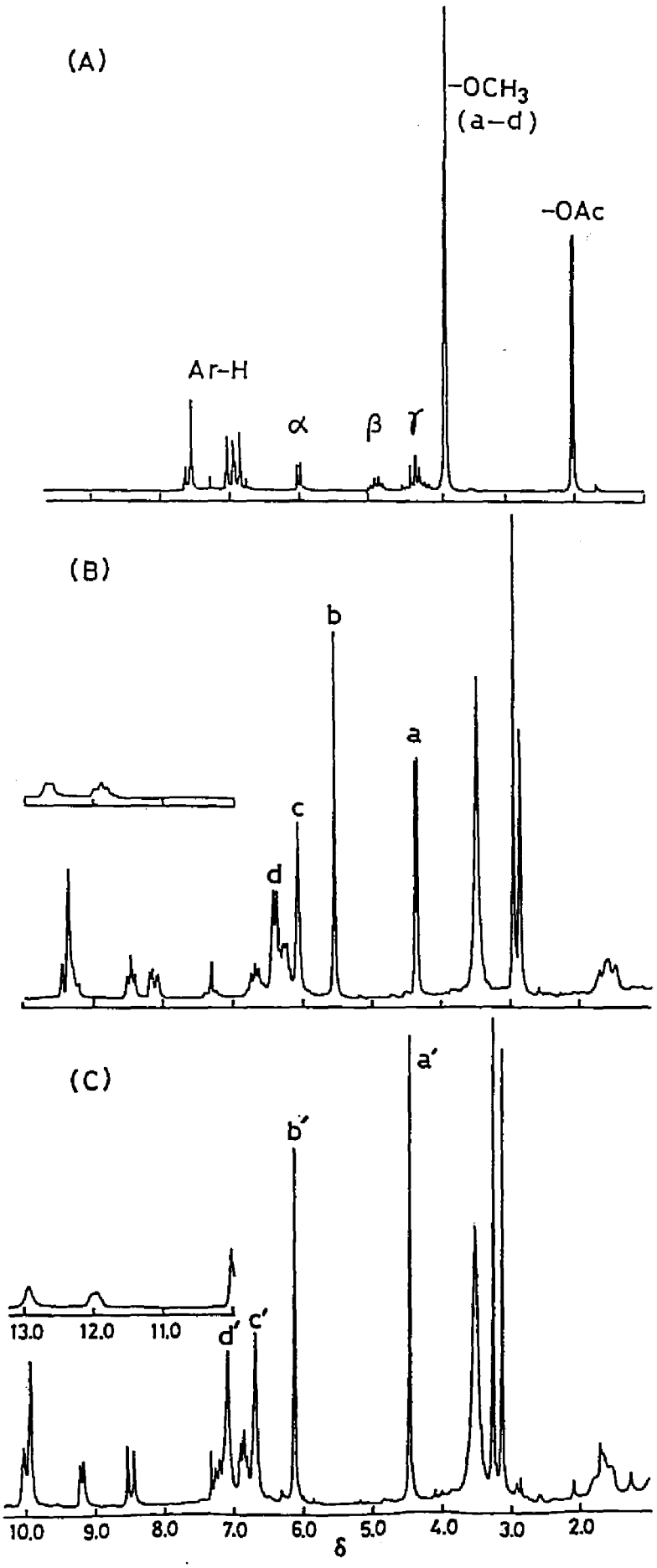

Fig. 4-7 ^{1}H-NMR spectra of (A) diacetate $\underset{\sim}{3}_{Ac}$ of erythro veratryl-glycerol-β-(methyl vanillate) ether ($\underset{\sim}{3}$"), (B) synthetic erythro $\underset{\sim}{3}_{Ac}$ in the presence of Eu(hfc)$_3$, (C) catabolic erythro $\underset{\sim}{3}_{Ac}$ in the presence of Eu(hfc)$_3$.

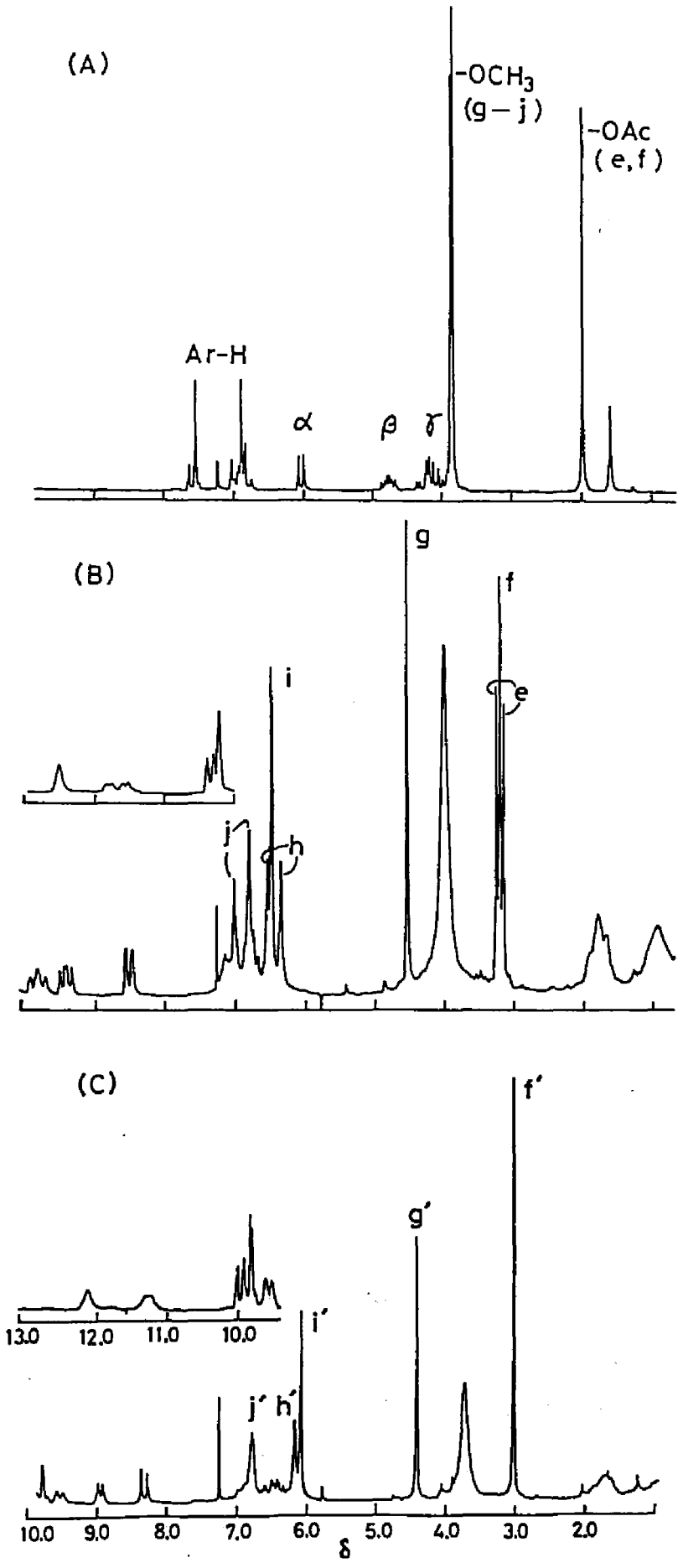

Fig. 4-8 ^{1}H-NMR spectra of (A) diacetate $\underset{\sim}{3}_{Ac}$ of threo veratryl-glycerol-β-(methyl vanillate) ether ($\underset{\sim}{3}$"), (B) synthetic threo $\underset{\sim}{3}_{Ac}$ in the presence of Eu(hfc)$_3$, (C) catabolic threo $\underset{\sim}{3}_{Ac}$ in the presence of Eu(hfc)$_3$.

the upper R_f spot was not detected. As for the threo-3", the same result was obtained.

Finally, direct optical resolution of the isomers with HPLC using a column for optical resolution was investigated. Fig. 4-9 shows that racemic erythro-96 gave two peaks whose ratio was 50:50, indicating the complete optical resolution of 96. At the same condition, catabolic erythro-96 gave a single peak whose retention time was identical with that of the second peak of the racemic erythro-96.

Racemic threo-96 also gave two peaks (50:50) (Fig. 4-9). While, catabolic threo-96 gave a single peak whose retention time was identical with that of the second peak of the racemic erythro-96.

Therefore, it was concluded that both erythro and threo α-reduction product 3 were enantiomerically pure.

Derivatization of the β-O-4 substructure models (1,3-diol) into the corresponding acetates is not suitable for separation of their erythro and threo forms. Separation of erythro-3" and threo-3" was not easy in preparative scale. Separation of diastereomeric mixtures of diacetate of syringylglycerol-β-(vanillic acid) ether was not successful, even in analytical scale (unpublished data).

However, conversion of the 1,3-diols into the corresponding six-membered ring derivatives such as the acetonide (section 1.1) and an phenylboronate[110] results in a good separation of erythro form from threo form using silica gel adsorption chromatography.

Although acidic catalysis was used for the preparation of such derivatives, isomerization at Cα and Cβ did not

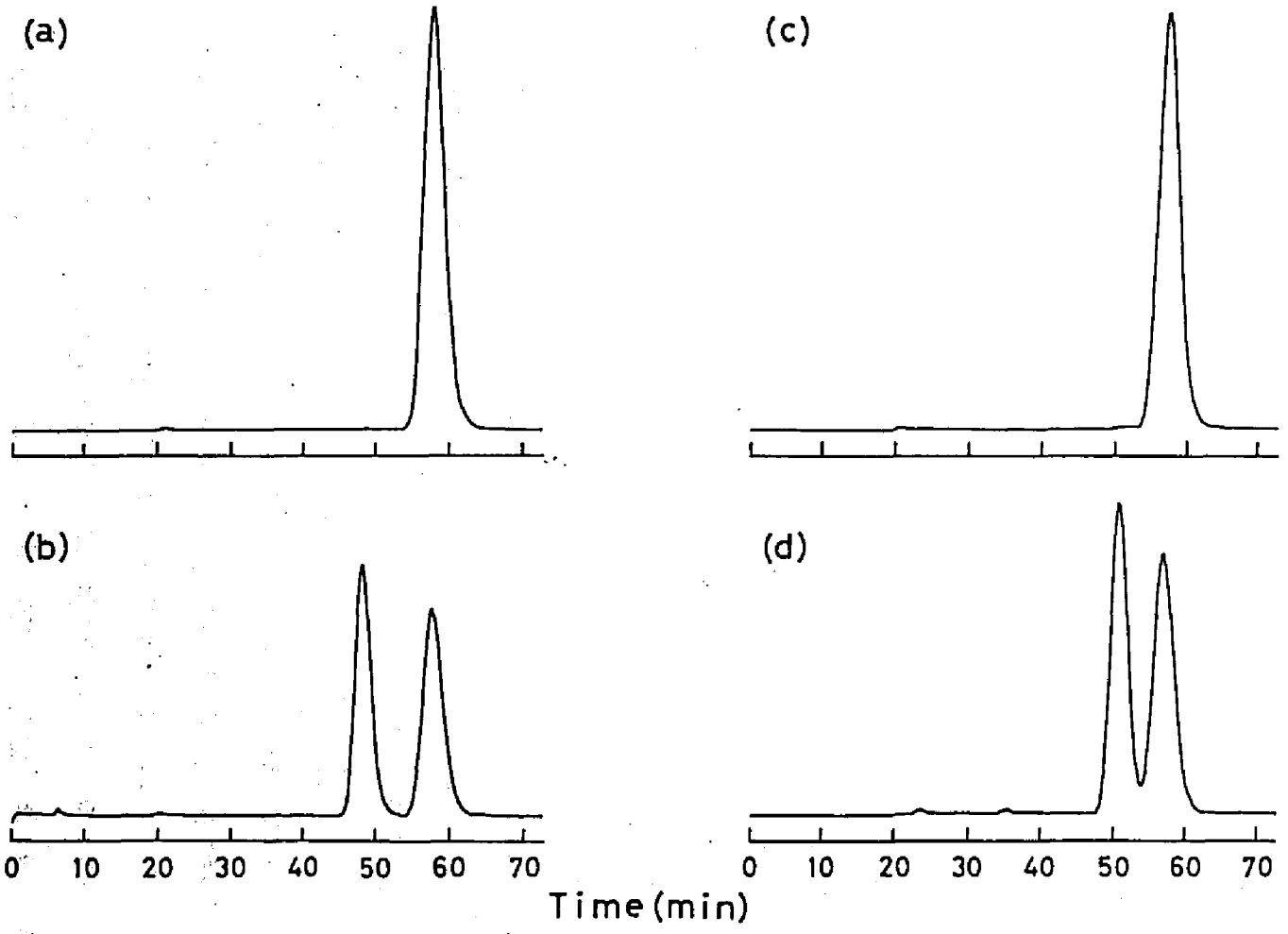

Fig. 4-9 Determination of enantiomeric purities by HPLC with a column packed with a chiral stationary phase. Samples are acetonide derivatives 95 of (a) catabolic (-)-erythro varatrylglycerol-β-(methyl vanillate) ether (3"), (b) racemic erythro 3", (c) catabolic (+)-threo 3", and racemic threo 3".

occur at this conditions. If the isomerization occurs, α-position (benzyl position) would be isomerized in preference to the β-position. If the isomerization at α-carbon occurs, threo form must be changed partly to erythro form and erythro form to threo form to give their mixture as follows.

Erythro (α R, β S) ⟶ (α S, β S): threo

Erythro (α S, β R) ⟶ (α R, β R): threo

Threo (α R, β R) ⟶ (α S, β R): erythro

Threo (α S, β S) ⟶ (α R, β S): erythro

Fig. 4-10 indicated that isomerization did not occur by conversion of both diols to the corresponding acetonide 96 at this conditions (40 eq. of 2,2-dimethoxypropane/ CSA/ acetone/ r.t.). Erythro and threo forms of the acetonide 96 were easily separated by reversed phase HPLC ($CH_3CN:H_2O$ = 60:40). Erythro-diol 3" free from threo-diol 3" was converted to its acetonide 96 and the reaction product was analyzed by HPLC. None of the corresponding threo-acetonide 95 was detected. Threo-diol 3" free from erythro-diol 3" also did not give any erythro-acetonide 96.

Fig. 4-11 also indicated that the isomerization did not occur by cleavage of both erythro- and threo-acetonide 96 to erythro- and threo-diol 3", respectively.

Catabolic erythro-96 and threo-96 were separated each other by preparative TLC. The erythro-96 showed a negative Cotton effect and its specific rotation at 589 nm $(\alpha)^{25}_{589}$ was about -75 (c = 0.125 g/100ml 95% EtOH). While, threo-96 showed a positive Cotton effect and its $(\alpha)^{25}_{589}$ was about +71 (c = 0.124). Specific rotation at 589 nm of catabolic

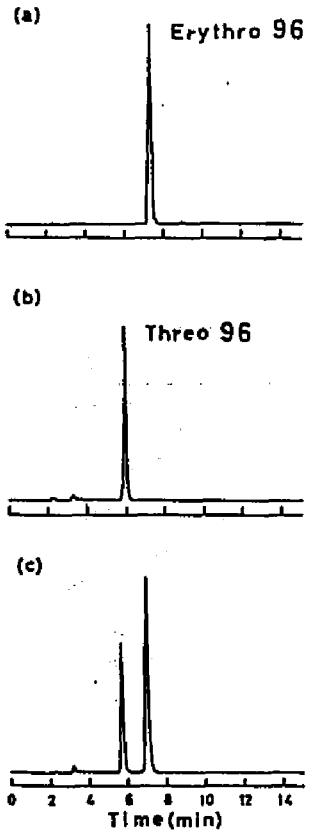

Fig. 4-10 HPLC analysis of the reaction residues obtained by the acetonide formation of (a) erythro-veratrylglycerol-β-(methyl vanillate)ether (3") and (b) threo-3", and of (c) the mixture of their residues

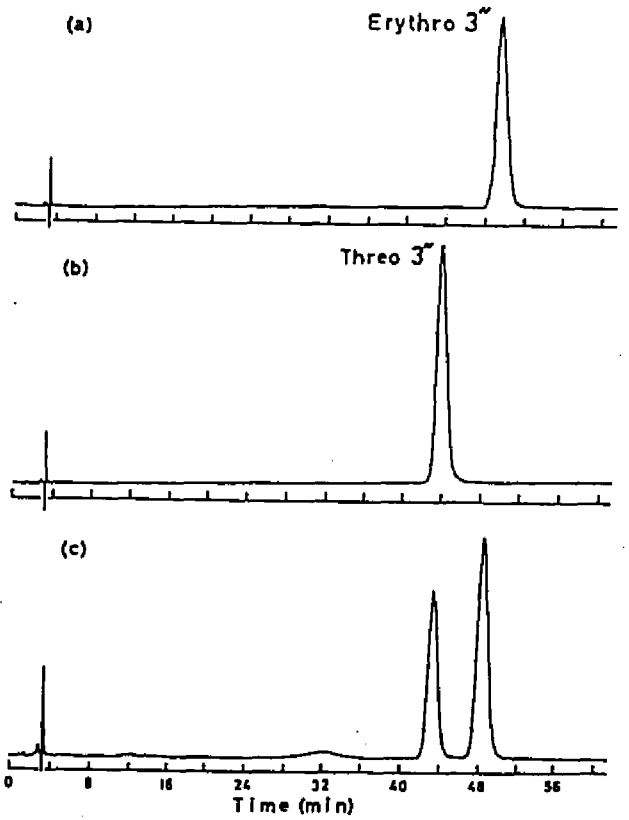

Fig. 4-11 HPLC analysis of the reaction residues obtained by the hydrolysis of (a) erythro-acetonide 96 and (b) threo-acetonide 96, and of (c) the mixture of their residues

erythro-3" was negative and that of threo-3" was positive.

The formation of optically pure erythro and threo catabolite 3 indicates that the fungal reduction of the α-ketone was stereoselective.

Both enantiomers of the racemic substrate 90 were catabolized completely and recovered substrate 90 had no optical activity, which indicating non-stereospecificity of the fungal oxidation of 90 to 88, and reduction of 88 to 3.. When racemic 88 was used as subatrste, UV absorption of the culture filtrate containing 88 decreased completely during incubation as shown in Fig. 4-12. Both enantiomers of 88 were converted to the diol 3 followed by the further degradation via glycerol-2-(vanillic acid) ether (29). It was already reported arylglycerol-β-aryl ethers were catabolized non-stereospecifically (section 1.2 and 1.3).

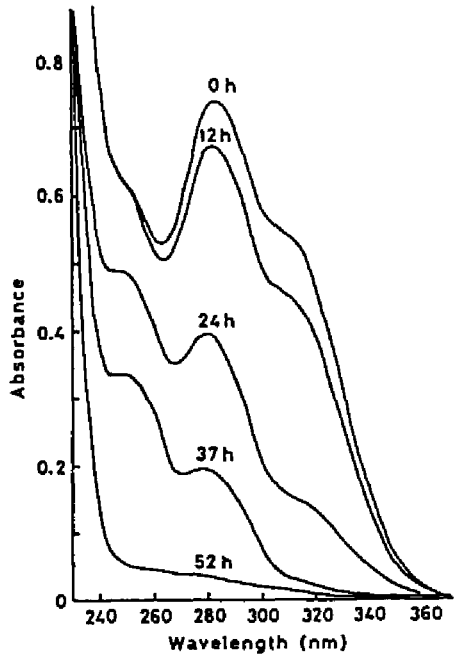

Fig. 4-12 Changes in the UV absorption of culture filtrates containing 2-(4-carboxy-2-methoxy-phenoxy)-3-hydroxy-1-(4-hydroxy-3-methoxy-phenyl)-1-propanone (88) during incubation of Fusarium solani M-13-1

P. chrysosporium mediates the formation of a new asymmetric carbon, such as the glycerol formation from allyl alcohol side chain of dehydrodiconiferyl alcohol[89] and the Cα-Cβ cleavage of a β-1 substructure model[111]. However, these reactions were found to be non-stereoselective.

Since lignin is a complex racemic polymer composed of many intermonomer linkages, lignin degradation is suggested to proceed by the mediation of non-stereospecific enzymes. In fact the enzyme isolated from P. chrysosporium by Tien and Kirk[104] catalyzed non-stereospecific oxidations in the alkyl side chain of dilignols such as β-1 and β-O-4. In white-rot fungi, even a new chiral center is introduced by the fungal non-stereoselective reaction, the racemic reaction products are converted to simple compounds by the subsequent non-stereospecific oxidative reactions.

However, the reduction of the α-ketone by F. solani M-13-1 is the first stereoselective reaction found in the catabolism of oligolignols. The fungal stereoselective reduction may be applied for the preparation of optically active aromatic compounds such as lignan.

Absolute configuration of erythro arylglycerol-β-aryl ethers is (α R, β S) or (α S, β R), and that of threo one is (α R, β R) or (α S, β S) (Fig. 4-13). The β-carbon of the racemic α-ketone is (R) and (S). If non-stereospecific and non-stereoselective reduction of the α-ketone occurs, the above four stereoisomers in an equal amount will be formed. On the other hand, stereoselective reductions of the (β R)-α-ketone will givs either (α R, β R) or (α S, β R), and of the (β S)-α-ketone either (α R, β S) or (α S,

β S).

Since, optically pure erythro-3 and threo-3 were obtained in this investigation, either pathway I or II in the stereoselective reduction (Fig. 4-13) could be examined. The pathway I lead to the formation of (α S, β R)-3 and (α S, β S)-3 from (β R)-88 and (β S)-88, respectively. The pathway II leads to the formation of (α R, β S)-3 and (α R, β R)-3 from (β S)-88 and (β R)-88, respectively. Hydrogen could be introduced from one face of the α-carbonyl group of 88. The erythro/threo ratio (4:1) may result from relatively rapid formation of erythro form and/or relatively rapid degradation of threo-3.

Very recently, catabolic erythro- and threo-3 were shown to be (α S, β R) and (α R, β S), respectively. Therefore, the fungal reduction is considered to occur by the pathway I in which hydrogen attacks to the carbonyl groups from re-face of both (β R)-88 and (β S)-88 giving (α S, β R)-erythro-3 and (α S, β S)-threo-3, respectively.

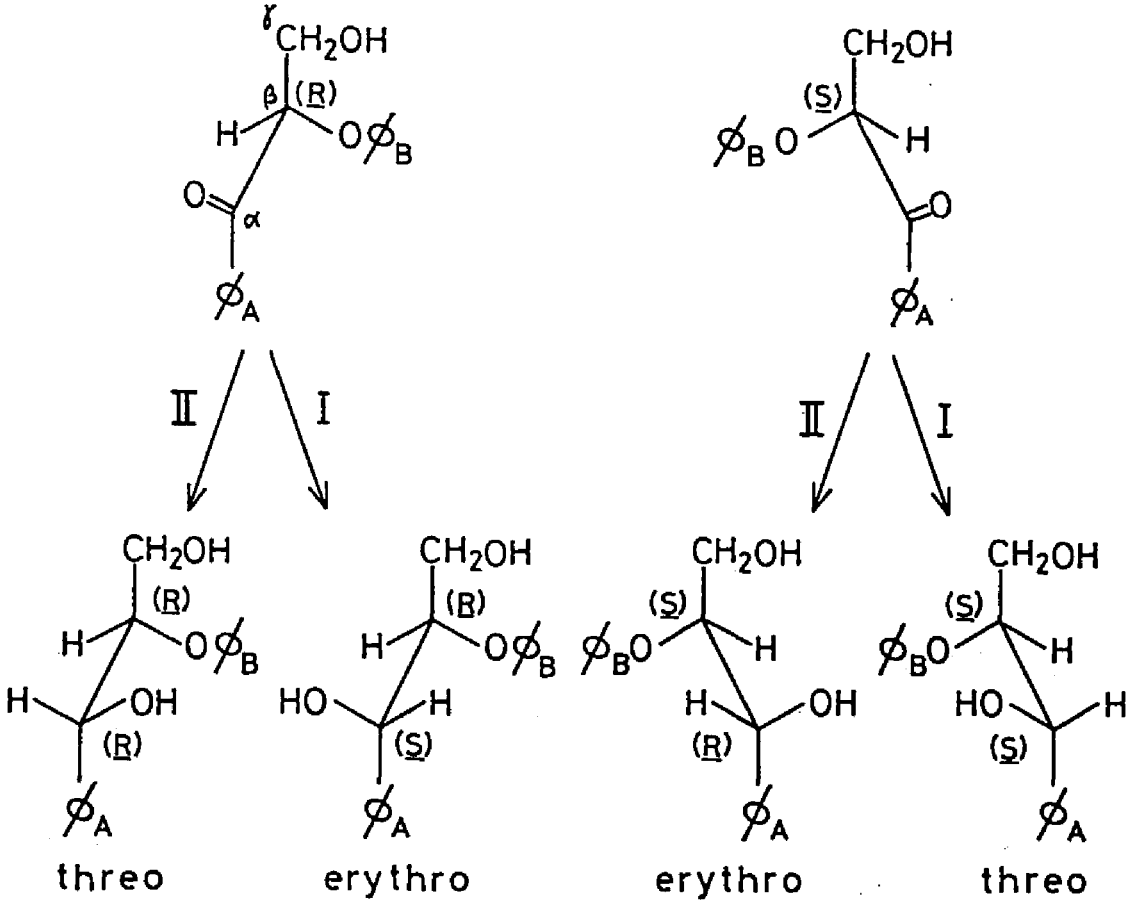

Fig. 4-13 Formation of four stereoisomers of an arylglycerol-β-aryl ether by the reduction of a racemic α-carbonyl derivative of an arylglycerol-β-aryl ether.
I : an attack on the re-face of the carbonyl group.
II : an attack on the si-face of the carbonyl group.

CONCLUSION

Degradation of lignin substructure model compounds by Fusarium solani M-13-1 was investigated. Lignin is a complex aromatic polymer comprised of several intermonomer C-C and ether linkages which are not easily hydrolyzable. To clarify the mechanism of lignin biodegradation, it is most effective to use various lignin substructure models. The catabolites must be exactly identified by comparison with authentic samples. For these purposes, the most adequate model compounds were synthesized and used as substrates, and the synthetic authentic samples were used as references for identification.

F. solani M-13-1 was isolated from soil by Iwahara et al.[44] with an enrichment technique using DHP as sole carbon source.

Firstly, degradation of arylglycerol-β-aryl ethers was investigated. Several arylglycerol-β-aryl ethers were synthesized in high yield and used as substrates for biodegradation and as references for identification of degradation products. Guaiacylglycerol-β-coniferyl ether was degraded completely by F. solani M-13-1. Its cinnamyl alcohol moiety of the terminal side chain was oxidized to the corresponding cinnamic acid group via cinnamaldehyde group. The pathway was different from that by a white-rot fungus, Phanerochaete chrysosporium. The cinnamic acid group was degraded between $C\alpha'=C\beta'$ bond to the corresponding benzoic acid via benzaldehyde. A part of the benzaldehyde was reduced to the the benzyl alcohol. In the degradative reaction pathway,

neither oxidation of the α-secondary alcohol to ketone nor cleavage of the β-O-4 linkage was observed. The results were consistent with the degradation of dehydrodiconiferyl alcohol by this fungus reported by Ohta et al.[45].

Phenolic aryiglycerol-β-(vanillic acid) ethers were found to be cleaved between the Cα-C_{aryl} linkage by F. solani M-13-1, giving glycerol-2-(vanillic acid) ether and methoxy-p-benzoquinones. However, non-phenolic veratrylglycerol-β-(vanillic acid) ether was not catabolized by the fungus at the condition used in this investigation. It was considered that a phenol oxidizing enzyme mediated the Cα-C_{aryl} cleavage. The fact was in agreement with the catabolism of other substructure models, β-5 and α-O-4 as mentioned below, and β-β'[102] and β-1'[113].

Both of erythro and threo forms of the β-O-4 model compounds undergo the fungal side chain shortening and the Cα-C_{aryl} cleavage, indicating that non-stereospecific oxidation and degradation were mediated by the fungus.

Glycerol-2-(vanillic acid) ether was completely catabolized by the fungus. Its 2-(β-) aryl ether bond could be cleaved to give vanillic acid. This was indicated by the result that glycerol-2-(3-ethoxy-4-hydroxybenzoic acid) ether was catabolized by the fungus to accumulate 3-ethoxy-4-hydroxybenzoic acid which was subsequently degraded completely, although glycerol-2-(vanillic acid) ether did not give the intermediary catabolite in detectable amounts.

A phenolic phenylcoumaran substructure model, 5-formyl-3-hydroxy-2-(4-hydroxy-3,5-dimethoxyphenyl)-7-methoxycoumaran, was catabolized by the fungus yielding many

compounds, among which 3-hydroxymethylphenylcoumarones, a 3-hydrophenylcoumarone without γ -carbon, and a 2-aryl-syringylpropanone were considered to be formed via a quinone methide intermediate. The quinonemethide could be formed by dehydrogenation of the phenolic hydroxyl group mediated by the phenol oxidizing enzyme and subsequent disproportionation of the resulting phenoxy radicals. Elimination of formaldehyde from the γ -position of the quinonemethide may afford the 3-hydrophenylcoumarone which was a new catabolite in the biodegradation of phenylcoumarans. Elimination of a β -proton of the quinonemethide and nucleophilic addition of water to α -carbon of the quinonemethide may give the 3-hydroxymethylphenylcoumarones and the syringylpropanone, respectively, in agreement with the catabolism of the phenylcoumaran by P. chrysosporium found by Umezawa et al.[90]. Further action of the phenol oxidizing enzyme to the 3-hydroxymethylphenylcoumarones may result in the formation of 3-formylphenylcoumarones. 5-Carboxyvanillic acid, syringic acid, and 2,6-dimethoxy-p-benzoquinone identified were ascribed to the cleavage of Cα -Cβ and Cα -C$_{Aryl}$ bonds in the substrate, although the mechanism of their formation is not still clear.

This is the first study on biodegradation of a trimeric non-cyclic benzyl aryl ether (α -O-4) substructure model. As an adequate model compound, guaiacylglycerol-α -(vanillyl alcohol)-β -vanillin diether was synthesized and used for biodegradation. The compound was considered to be initially cleaved by two modes, oxidative cleavage of the α -O-4 bond mediated by the phenol oxidizing enzyme, and non-enzymic

hydrolysis, because an α-ketone derivative of guaiacylglycerol-β-(vanillic acid) ether was isolated from the culture filtrate, whereas guaiacylglycerol-β-vanillin ether and vanillyl alcohol were isolated from the control solution without mycelia. In the former mode, the formation of a trimeric quinonemethide intermediate by the mediation of the phenol oxidizing enzyme was proposed.

α-Hydroxy substructure models of the β-O-4 type are degraded via 1-hydroxycyclohexadienone intermediates to glyceraldehyde derivatives and methoxyhydroquinones by Cα-C_{aryl} cleavage. The reaction would be mediated by the phenol oxidizing enzymes. On the other hand, α-ethereal substructure models such as α-O-4 and β-5 types are converted via quinonemethide intermediates to α-hemiacetal compounds or phenylcoumarones. The reactions also would be mediated by the phenol oxidizing enzymes. The α-hemiacetal compounds are converted to the α-carbonyl compounds with the cleavage of the α-ether bonds. The α-carbonyl compounds may be further degraded between Cα-C_{aryl} linkage by the mediation of the phenol oxidizing enzymes. The fact is in agreement with the result of the degradation of β-β' substructure models by F. solani M-13-1 studied by Kamaya et al.[102] and Iwahara[94]. Methoxyhydroquinones could be catabolized via aromatic ring cleavage or oxidized to the methoxy-p-benzoquinones by the phenol oxidizing enzymes.

An α-ketone derivative of phenolic guaiacylglycerol-β-aryl ether was reduced to the guaiacylglycerol-β-aryl ether which was further degraded in the same manner as

above. Direct oxidative cleavage of Cα-C_{aryl} linkage of the α-ketone derivative did not occur. Such reduction was found for the first time in the catabolism of lignin and lignin substructure models.

The reduction product from the racemic α-ketone was a mixture of erythro and threo forms, which were separated as their acetate and acetonide derivatives. Both of erythro and threo forms were found to be optically active, the former was levo-rotatory and the latter was dextro-rotatory. Their enantiomeric purities determined by the following three different methods were found to be 100%; ^{1}H-NMR analysis in the presence of a kiral shift reagent Eu(hfc)$_3$, HPLC with a column for optical resolution, and TLC and ^{1}H-NMR analyses of their (R)-MTPA derivatives. The conditions of the optical resolution were examined using racemic synthetic samples. Therefore, it was concluded that the reduction was stereoselective, and that the erythro form was formed from one enantiomer of the α-ketone, and the threo form was formed from the other enantiomer of the α-ketone.

Lignin contains 10-20% of free phenolic hydroxyl groups. F. solani M-13-1 initially attacks the aromatic moieties with phenolic hydroxyl groups, cleaves between Cα-C_{aryl} bond, and further degrades or transformes, giving newly formed phenolic hydroxyl groups which are further attacked. Phenolic arylglycerol-β-aryl ether moieties could be depolymerized by repeating a combination of Cα-C_{aryl} cleavage mediated by the phenol oxidizing enzyme and aryl ether cleavage of the resulting glycerol-2-aryl ether structure. F. solani M-13-1 also degrades oxidatively the

cinnamyl alcohol and the cinnamaldehyde moieties of the terminal side chain in lignin to the corresponding benzoic acid. Demethylation and aromatic ring cleavage in lignin polymer may not occur by F. solani M-13-1 under the present culture conditions.

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ACKNOWLEDGEMENTS

The author wishes to express his sincerest thanks to Professor Dr. Takayoshi Higuchi, Research Section of Lignin Chemistry, Wood Research Institute, Kyoto University, for his kindest guidance and encouragement during the course of this study, and also to Associate Professor Dr. Fumiaki Nakatsubo, Faculty of Agriculture, Kyoto University, for his kindest guidance and encouragement. The author thanks Professor Dr. Murao Sogo, Faculty of Agriculture, Kagawa University, for his help and encouragement.

The author is grateful to Professor Dr. Tetsuo Koshijima, Wood Research Institute, Kyoto University, and Professor Dr. Kenji Soda, Institute for Chemical Research, Kyoto University, for their valuable suggestions and critical reading of this manuscript.

Thanks are also due to all the members of Research Section of Lignin Chemistry, Wood Research Institute, Kyoto University and Laboratory of Forest Products Chemistry, Department of Agricultural Chemistry, Faculty of Agriculture, Kagawa University for their valuable discussions and kind help during the course of this study.