Aromatic ring cleavage in degradation of β-*O*-4 lignin substructure by *Phanerochaete chrysosporium*

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The degradation of a β-*O*-4 lignin substructure model dimer, 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (I), by the white-rot fungus *Phanerochaete chrysosporium* was investigated. The guaiacyl aromatic ring of the dimer (I) was first cleaved to give the cyclic carbonate of 4-ethoxy-3-methoxyphenylglycerol (II) which was then converted to 4-ethoxy-3-methoxyphenylglycerol (III). The carbonate carbon of (II) was found to be derived from the guaiacyl group of (I) based on tracer experiments with ^{13}C.

Aromatic ring cleavage *Lignin degradation* *Cyclic carbonate* *β-O-4 substructure* *Arylglycerol* *White-rot fungus*

1. INTRODUCTION

In the metabolism of the β-*O*-4 (arylglycerol-β-aryl ether) lignin substructure models, the most frequent substructure of lignin [1], by white-rot fungi β-*O*-4 bond cleavage to give arylglycerol as a metabolite was established [2–5]. We previously proved, as for the β-*O*-4 bond cleavage and arylglycerol formation by the white-rot fungus *Phanerochaete chrysosporium*, that the β-*O*-4 ether bond which is an alkyl aryl ether bond was cleaved between the ether oxygen and C_4 of the aryl group, suggesting initial fungal attack on the aryl group etherified to the C_β position prior to β-*O*-4 bond cleavage [6].

Here, initial aromatic ring cleavage was found to be involved in arylglycerol formation based on ^{13}C tracer experiments. This is the first report on the isolation and definite identification of an aromatic ring cleavage product derived from the β-*O*-4 substructure.

2. MATERIALS AND METHODS

Cultures (20 ml/300 ml Erlenmeyer flask) of *P. chrysosporium* Burds. (ME-446) were grown at 39°C without agitation in a nitrogen-limiting, glucose, dilute mineral salts medium [3,7]. 4-Ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (I) was added to 6-day-old cultures as a solution of *N,N*-dimethylformamide (DMF) (3.44 mg in 0.1 ml of DMF/20 ml culture, 5 cultures) and the flasks were flushed with O_2 and incubated for an additional 93 h at 39°C without agitation [8]. Metabolites were extracted with ethyl acetate from whole cultures acidified to pH ~2 with 1 N HCl. The extract was acetylated (Ac_2O/pyridine = 1:1, v/v, room temp., 24 h) and separated twice by preparative TLC (Kiesel-gel 60 F_{254}, Merck) (developing solvent: 1st, CH_2Cl_2/*n*-hexane = 1:1, v/v, once then CH_2Cl_2, 4 times; 2nd, EtOAc/*n*-hexane = 1:2, v/v, once). Purified metabolites were analyzed by ^1H-NMR and MS spectrometry. 4-Ethoxy-3-methoxyphenylglycerol-β-[*U-ring*-^{13}C]guaiacyl ether (I′) and cyclic carbonate of 4-ethoxy-3-methoxyphenylglycerol (II) were incubated [(I′), 90 h; (II), 32 h], and the cultures were extracted and acetylated as above except that the cultures were not acidified before ethyl acetate extraction. Both acetates of the extracts were analyzed by GC-MS.

[*U-ring*-^{13}C]Guaiacol was synthesized from [*U-*

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ring-^{13}C]aniline (CEA, minimum isotopic purity: 90 atom%) by the method of Kratzl and Vierhapper [9]. (I) and (I′) were prepared from guaiacol and [*U-ring*-^{13}C]guaiacol, respectively, by the method reported previously [4]. (I′): MS (acetate) *m/z*(%), 438(6.8), 437(4.3), 436(1.2), 435(0.2), 434(0), 433(0), 432(0), 250(6.6), 249(5.5), 223(11.0), 215(12.1), 207(27.6), 206(25.9), 181(100). (II) was synthesized from 1-(4-ethoxy-3-methoxyphenyl)-propane-1-one-2,3-diol which was prepared previously [4] via the following steps: (1) 1,1′-carbonyldiimidazole/benzene/reflux (28.2%) [10], (2) $NaBH_4$/methyl alcohol/CH_2Cl_2/0°C (64.3%). Acetate of (II), (II-Ac), was prepared by treating (II) with Ac_2O and pyridine at room temp. (II-Ac), ^{1}H-NMR ($CDCl_3$) δ(ppm): 1.46(3H,t,J=7.00,-O-C-CH_3), 2.14(3H,s,-OAc), 3.89(3H,s,-OCH_3), 4.09(2H,q,J=6.93,-O-CH_2-C), 4.15(1H,dd,J=8.99,J=6.35,γ-H), 4.33(1H,dd,J=8.99,J=8.34, γ-H), 4.96(1H,m,β-H), 5.83(1H,d,J=6.22,α-H), 6.8–7.0(3H,m,aromatic). The mass spectrum of (II-Ac) is shown in fig.1. Acetate of 4-ethoxy-3-methoxyphenylglycerol (III-Ac) was prepared previously [4]. ^{1}H-NMR and MS spectra were obtained with a Varian XL-200 FT-NMR spectrometer (TMS as an internal standard) and a Shimadzu-LKB 9000 gas chromatograph-mass spectrometer (EI-MS, 70eV).

3. RESULTS

After *P. chrysosporium* was incubated with (I) for 93 h, acetate of cyclic carbonate [(II-Ac), about 40 μg, 0.26% (mol product formed/mol initial substrate) × 100] was isolated from the acetate of the culture extract and identified by comparison of the ^{1}H-NMR and MS spectra with those of synthesized authentic compound. To prove that the carbonate carbon atom was derived from the aromatic ring carbon of the guaiacyl group of (I), (I′) was synthesized and metabolized under the same condition. GC-MS analysis [1% OV-1 on chromosorb W (AW-DMCS), glass column (2 m × 0.3 cm, i.d.), carrier gas (He, 31 ml/min), column temp. (210°C)] of the acetate of the metabolites showed that the mass spectrum of the acetate of cyclic carbonate from (I′) exhibited a molecular ion peak higher by one mass unit than unlabeled authentic compound as shown in fig.1. Since the mass spectrum of (III-Ac) from (I′) was the same

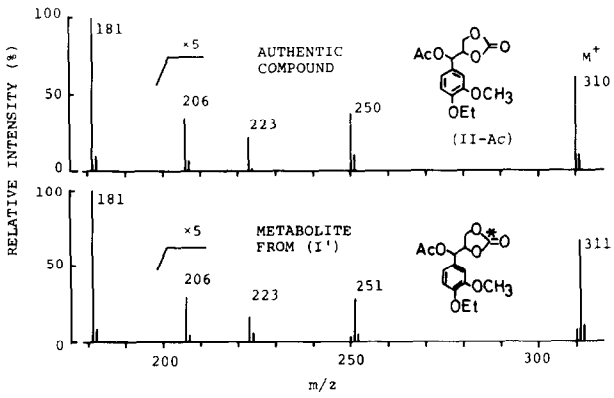

Fig.1. Mass spectra of the cyclic carbonate of 4-ethoxy-3-methoxyphenylglycerol (acetate) (II-Ac). Upper, synthesized authentic compound; lower, metabolite from 4-ethoxy-3-methoxyphenylglycerol-β-[*U-ring*-^{13}C]guaiacyl ether (I′). * ^{13}C.

as that of unlabeled authentic compound and since the pathway (II)⟶(III) was established in the present investigation, the excess ^{13}C was found to be located in the carbonate carbon. The acetyl groups of (II-Ac) and (III-Ac) were derived from the artificial acetylation, because GC-MS analysis showed that the culture extract from (I) before the acetylation did not contain the acetates. The acetate of the extract of the culture incubated with (II) for 32 h was analyzed by GC-MS, and the triacetate of arylglycerol (III-Ac) was identified by comparison of the mass spectrum with that of authentic compound. However, (III-Ac) was also detected in the acetate of the extract of non-inoculated control culture incubated with (II) for 32 h. Thus, the pathway (I)⟶(II)⟶(III) was established.

4. DISCUSSION

As for arylglycerol formation in the metabolism of (I) by *P. chrysosporium*, we suggested that initial attack on the guaiacyl aromatic ring occurred prior to β-*O*-4 bond cleavage and was followed by arylglycerol formation [6]. Chen et al. [11] also suggested aromatic ring cleavage of the β-*O*-4 substructure by *P. chrysosporium*. The present results prove for the first time that the guaiacyl aromatic ring of (I) was cleaved and that the aromatic ring cleavage product, cyclic carbonate

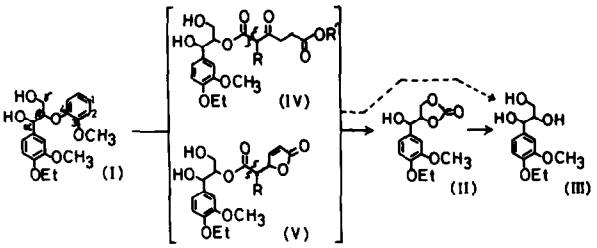

Fig.2. Aromatic ring cleavage in the degradation of 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (I) by the ligninolytic culture of *P. chrysosporium*. The pathway (I)⟶(II)⟶(III) was established. (IV) and (V) are hypothetical intermediates. R, H or OCH_3; R', H or CH_3.

(II), was a precursor for the arylglycerol (III) (fig.2).

At present there is no evidence which explains the mechanism of formation of the cyclic carbonate (II). However, a speculative mechanism might be pointed out. *cis,cis*-Muconates, muconolactones and 3-oxoadipate are well known metabolic intermediates of phenols by bacteria and fungi [12]. So, one possibility is that the precursor of the cyclic carbonate (II) is a 3-oxo ester or an ester which has a leaving group at the 3-position to the ester carboxyl group (e.g., a 3-oxoadipate (IV) or an ester of muconolactone (V)). As shown in fig.2, these compounds may be decarboxylated to give (II) and/or directly hydrolyzed to give (III).

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