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Enzymatic Synthesis of a Dihydrobenzofuran Neolignan by Oxidative Coupling

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The oxidative dimerization of ferulic acid has been carried out using horse-radish peroxidase as catalyst to give a dihydrobenzofuran neolignan (**1**), the structure of which was elucidated as (2*SR*,3*RS*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-*n*-butoxycarbonyl-5-(2*E*-carboxyethenyl)-7-methoxybenzofuran by spectroscopic analyses. This compound showed more potent cytotoxicity against several tumor cell lines than the starting material.

Key words: Oxidative dimerization, Peroxidase, Ferulic acid, Dihydrobenzofuran neolignan, Cytotoxicity

INTRODUCTION

Plants induce the hypersensitive defense responses toward pathogens or mechanical damages. These responses include the production of phytoalexins and the secretion of hydrolytic enzymes such as chitinase and β-glucanase, as well as the deposition of extracellular molecular barriers such as lignin and hydroxyproline-rich glycoprotein (Bowles, 1990). Lignin and lignin-like polymers resulting from the oxidative polymerization of phenolic phenylpropenoids within the plant cell wall form covalent crosslinks with proteins and polysaccharides, and render cell walls highly resistant to mechanical and enzymatic disruption (Vance *et al.*, 1980). Plant peroxidases have been proposed to catalyze the last enzymatic step of lignin biosynthesis, namely the conversion of phenolic phenylpropenoids into their free-radical forms at the expense of H_2O_2, followed by the formation of carbon-carbon and carbon-oxygen bonds (Higuchi, 1985). Although the role of peroxidases in the defense responses is not clear, the accumulation of lignin-like materials associated with the increase of peroxidase activity in infected plant tissues is a widely reported phenomenon (Bruce and West, 1989; Smit and Dubery, 1997; Vance *et al.*, 1980).

Similary, it has recently been reported that the horse-radish peroxidase (HRP)-catalyzed oxidative coupling of simple phenolic compounds produces the polyaromatics with novel structures and properties (Kobayashi *et al.*, 1998; Schmitt *et al.*, 1998; Uyama *et al.*, 1996). Since the HRP is commercially available and has high potential for oxidative coupling reactions, this enzymatic reaction system has been applied to produce the polymeric materials which are often difficult to synthesize by conventional methods (Chioccara *et al.*, 1993; Donnelly *et al.*, 1987). These findings suggested that HRP could convert a variety of plant phenolics into biologically active compounds.

In continuation of our search for bioactive natural compounds, we now report that the HRP-catalyzed oxidative dimerization of ferulic acid yielded a dihydrobenzofuran neolignan (**1**), which showed more potent cytotoxicity against several tumor cell lines than the starting material.

MATERIALS AND METHODS

Horse-radish peroxidase (HRP, EC 1.11.1.7) and ferulic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and employed without further purification. Fetal calf serum, RPMI 1640, penicillin and streptomycin were obtained from GIBCO Lab. (Grand Island, NY, USA). Column chromatography was performed using silica gel (230~400 mesh, Merck) and Sephadex LH-20 (bead size 20~100 μm, Sigma Chemical Co.). Thin-layer chromatography was performed on precoated silica gel $60F_{254}$ plate (Merck). Melting point was determined on a Büchi 535 melting point apparatus and is uncorrected. Optical rotation was measured on a JASCO DIP-370 digital polarimeter. Infrared spectrum was recorded on a Perkin Elmer 1710 FT-IR spectrometer. Mass spectrum was obtained with a VG Trio-2 spectrometer. UV spectrum

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was measured on a Beckman DU 650 spectrometer. ^{1}H- and ^{13}C-NMR spectra were recorded on a JEOL GSX 400 spectrometer with reference to the residual solvent signals.

Enzymatic oxidative dimerization of ferulic acid

The reaction system consisted of 18 mmol of ferulic acid, 18 mmol of hydrogen peroxide, and 10 mg of HRP in 2000 ml of 0.1M phosphate buffer (pH 6.7). The reaction mixture was kept at 25°C for 24 h, and then adjusted to pH 2 with 2N HCl soln.

Isolation of compound 1

The reaction mixture was extracted with *n*-BuOH and then concentrated *in vacuo*. The *n*-BuOH extract (3.3 g) was subjected to silica gel column chromatography using gradient elution with toluene-MeOH-AcOH (100:2:1) to give ten fractions. The fraction 3 (0.5 g) was further purified by Sephadex LH-20 column chromatography and then recrystalized with MeOH to afford compound **1** (132 mg).

Compound 1: White amorphous powder from MeOH, mp 174~177°C, $[\alpha]_D$+10.0° ($CHCl_3$, *c* 0.03), IR ν_{max}(KBr) cm^{-1}: 3440 (OH), 1740, 1690 (C=O), 1640 (olefinic C=C), 1600, 1520, 1505 (aromatic C=C), 1280, 1150 (C-O); UV_{max} (MeOH) nm: 289, 315; EIMS *m/z* (rel. int.) 442 (M^+, 63), 368 (100), 350 (42), 341 (13), 297 (9), 165 (14), 151 (17), 77 (8); ^{1}H-NMR (400 MHz, acetone-d_6) δ 0.92 (3H, *t*, *J*=8.0 Hz, H-15), 1.41 (2H, *sextet*, *J*=8.0 Hz, H-14), 1.68 (2H, *quintet*, *J*=8.0 Hz, H-13), 3.83 (3H, *s*, 3'-OCH_3), 3.92 (3H, *s*, 7-OCH_3), 4.22 (2H, *m*, H-12), 4.45 (1H, *d*, *J*=7.8 Hz, H-3), 6.03 (1H, *d*, *J*=7.8 Hz, H-2), 6.39 (1H, *d*, *J*=16.0 Hz, H-9), 6.83 (1H, *d*, *J*=7.8 Hz, H-5'), 6.91 (1H, *d*, *J*= 7.8 Hz, H-6'), 7.08 (1H, *s*, H-2'), 7.29 (1H, *s*, H-4), 7.31 (1H, *s*, H-6), 7.63 (1H, *d*, *J*=16.0 Hz, H-8); ^{13}C-NMR (100 MHz, acetone-d_6) δ 12.9 (C-15), 18.8 (C-14), 30.4 (C-13), 55.1 (C-3), 55.3 (3'-OCH_3), 55.5 (7-OCH_3), 65.0 (C-12), 87.2 (C-2), 109.7 (C-2'), 112.6 (C-6), 114.8 (C-5'), 115.7 (C-9), 117.8 (C-4), 119.2 (C-6'), 126.7 (C-3a), 128.5 (C-5), 131.1 (C-1'), 144.6 (C-8), 144.8 (C-7), 147.0 (C-4'), 147.6 (C-3'), 149.9 (C-7a), 167.1 (C-10), 170.2 (C-11).

Cytotoxicity assay

The *in vitro* cytotoxic activity of ferulic acid and compound **1** was evaluated according to the standard protocols developed by the National Cancer Institute (Monks *et al.*, 1991). The tumor cell lines used for cytotoxicity test were as follows: $P388D_1$ (mouse leukemia), HL60 (human leukemia), A549 (human lung carcinoma), HCT15 (human colon adenocarcinoma), and DLD1 (human colon adenocarcinoma). Each cell was maintained in RPMI 1640 medium supplied with 5% heat-inactivated fetal calf serum and incubated at 37°C in a humidified atmosphere at 5% CO_2.

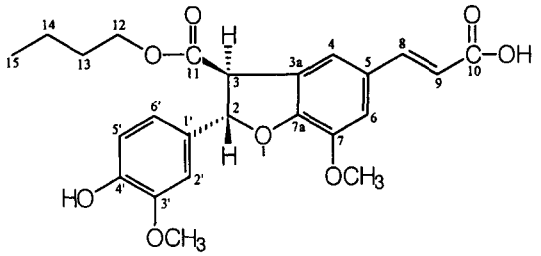

Fig. 1. Structure of compound 1

RESULTS AND DISCUSSION

Compound **1** possessed the molecular formula $C_{24}H_{26}O_8$ on the basis of EIMS and ^{13}C-NMR data, and its IR spectrum showed the absorption bands for hydroxyl (3440 cm^{-1}), carbonyl (1740 and 1690 cm^{-1}), olefinic (1640 cm^{-1}) and aromatic (1600, 1520, and 1505 cm^{-1}) groups. The ^{1}H-^{1}H COSY spectrum of **1** revealed the presence of five aromatic protons [δ 6.83, 6.91, 7.08, 7.29, and 7.31], two olefinic protons [δ 6.39 and 7.63 (each *d*, *J*=16.0 Hz)], one butoxy group [δ 0.92, 1.41, 1.68, and 4.22] and two methoxy groups [δ 3.83 and 3.92]. The correlation peak between the proton signals at δ 4.45 (1H, *d*, *J*=7.8 Hz) and 6.03 (1H, *d*, *J*=7.8 Hz) suggested the presence of a dihydrobenzofuran moiety with *trans* 2/3 configuration in **1**. This was further supported by comparison of chemical shifts and coupling constants with those of dehydrodiferulic acid methyl ester (Antus *et al.*, 1989). The chemical shifts of quaternary carbons of this compound were definitely assigned by the selective INEPT (Insensitive Nuclei Enhanced by Polarization Transfer) NMR technique (Bax, 1984), which also provided confirmation of the structure proposed for **1**. When the proton signal at δ 7.08 (H-2') was irradiated, C-2 (δ 87.2) was enhanced, along with other carbons two or three bonds distant from the irradiation site, namely, C-3' (δ 147.6), C-4' (δ 147.0), and C-6' (δ 119.2). Furthermore, the irradiation of H-2 (δ 6.03) selectively enhanced carbons C-2' (δ 109.7), C-6' (δ 119.2), and C-11 (δ 170.2). These findings indicated that a guaiacyl group was attached to the C-2 position of a dihydrobenzofuran ring. In addition, the irradiation of H-3 (δ 4.45) resulted in enhancement of carbons C-11 (δ 170.2) and C-12 (δ 65.0) as well as carbons C-1' (δ 131.1), C-7a (δ 149.9), and C-3a (δ 126.7), suggesting the placement of a butoxy group at the carboxylic acid moiety attached to the C-3 position of a dihydrobenzofuran ring. A CD (Circular Dichroism) examination of **1** showed that it was not optically active. This indicated that HRP did not induce enantioselectivity in this reaction.

Table I. *In vitro* cytotoxic activity of ferulic acid and compound **1**

Cell line	ED_{50} (μg/ml)	
	Ferulic acid	Compound **1**
$P388D_1$ (mouse leukemia)	>100	37.4
HL60 (human leukemia)	>100	38.8
A549 (human lung carcinoma)	>100	>100
HCT15 (human colon adenocarcinoma)	>100	57.5
DLD1 (human colon adenocarcinoma)	>100	46.1

From all the above data, the structure of **1** was elucidated as (2*SR*,3*RS*)-2,3-dihydro-2-(4-hydroxy-3-methoxyphenyl)-3-*n*-butoxycarbonyl-5-(2*E*-carboxyethenyl)-7-methoxybenzofuran. We doubt that *n*-butoxy moiety of this compound was unexpectedly generated during acidic extraction process with *n*-BuOH.

The cytotoxic activity of this compound against several tumor cell lines was higher than that of ferulic acid (Table I). This result proposes that the HRP-catalyzed oxidative coupling reaction system could be a useful technique for producing a new type of biologically active compound. In this respect, another attempts for a variety of phenolic compounds are in progress. In addition, the antimicrobial activity of polyaromatic compounds produced from the enzyme reaction system needs to be evaluated since the accumulation of lignin-like materials in infected plant tissue is related to the defense response of plants.

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