Global Journal of Biotechnology & Biochemistry 6 (2): 87-94, 2011
ISSN 2078-466X
© IDOSI Publications, 2011

Stereochemical Formation of 8-*O*-4′ Neolignans from Higher Plants

[1,2]*Md. Shameul Alam,* [1]*Toshisada Suzuki and* [1]*Takeshi Katayama*

[1]Faculty of Agriculture, Kagawa University, Kagawa, Japan
[2]Faculty of Medicine, Kagawa University, Kagawa, Japan

Abstract: To elucidate the stereochemical formation of *erythro*- and *threo*- syringylglycerol-8-*O*-4'-(sinapyl alcohol) ethers (SGSEs) with optical activity, incubation of sinapyl alcohol (SA) with the soluble and insoluble enzyme preparations were carried out in the presence and absence of H_2O_2, respectively. Diastereoselective formation of *erythro*-SGSE was formed in preference to *threo*-SGSE. Incubation of a soluble enzyme preparation of *E. ulmoides* stems with [8-^{14}C] SA in the presence of H_2O_2 gave *erythro*- and *threo*-[^{14}C] SGSEs with [^{14}C] syringaresinol (SYR). The *erythro*-SGSE was diastereoselectively formed from 2 to 60 min incubations with 63.2% d.e. at 30 min, except for formation of *threo*-SGSE (31.6% d.e) at 120 min. Both diastereomers, (-)*erythro*- and (-)*threo*-SGSEs, had optical activity with 47.4% e.e. and 21.6% e.e., respectively, at 60 min. Incubation of an insoluble enzyme preparation of *E. ulmoides* with [8-^{14}C]SA also afforded *erythro*- and *threo*-[^{14}C]SGSE with [^{14}C]SYR. The *erythro*-SGSE was diastereoselectively formed from 2 to 60 min incubations with 60.8% d.e. at 2 min, except for formation of *threo*-SGSE (8.38% d.e. at 120 min). Both diastereomers (+)-*erythro*- and (-)*threo*-SGSEs, have optical activity with 24.8% e.e. and 14.6% e.e., respectively, at 60 min. Both preparations catalyzed the diastereoselective formation of *erythro*- and *threo*-SGSEs with optical activity. Interestingly, the soluble preparation catalyzed the formation of (-)*erythro*-SGSEs, whereas the insoluble preparation did that of (+)-*erythro*-SGSEs, the opposite enantiomar.

Key words: Stereochemical formation · 8-*O*-4′Neolignan · Enzyme preparation · *Eucommia ulmoides* · Optical activity

INTRODUCTION

Lignans and neolignans are plants secondary metabolites, structurally diverse, a group of phenylpropanoids generally distributed in higher plants. They are formed primarily from ρ-hydroxycinnamyl alcohols (monolignols mainly coniferyl alcohol and sinapyl alcohol) by dehydrogenative dimeraization and distinguished by the intermonomer linkages like: lignans linked with 8-8′ bond whereas neolignans linked other than 8-8′ bond (e.g. 5-5′, 8-5′, 8-*O*-4′). The intermonomer linkages are the most abundant ones in natural products except for glycosidic linkages in carbohydrates. An 8-*O*-4′ neolignans have four stereoisomers, *erythro* and *threo* diastereomers (Fig. 1) and their enantiomers. Most of the lignans and neolignans in plants have optical activity. They have important physiological functions in plant defense, particularly for heartwood and seed-forming tissues [1]. They also display an important role on human health. Recently naphthoquinone compounds (lignan) have been reported to possess in *vitro* antiproliferative activity against panel cancer cells [2] and Surinamensin (a 9,9′-deoxy- 8-*O*-4′ neolignan) has potential anti-leishmanial activity [3]. Lignins are phenolic polymers produced through dehydrogenation of monolignols by nonselective peroxidase/H_2O_2 (and laccase/O_2) [4]. But they are optically inactive, although structurally similar to lignans and neolignans.

Stereoselective formation of the lignans and neolignans from coniferyl alcohol (CA) and/or sinapyl alcohol (SA) is not well-known, except that a dirigent protein catalyzed a regioselective and enantioselective coupling of two achiral CA radicals yielding (+)-pinoresinol. The protein was originally obtained from an insoluble enzyme preparation (a cell-wall residue) of *Forsythia* plants [5-7]. Since biosynthesis that of 8-*O*-4′ neolignans have not been advanced in contrast to lignans. Arylglycerol-8-*O*-4′-aryl ether linkages are present in lignins and 8-*O*-4′ neolignans. This laboratory has investigated the biosynthesis of 8-*O*-4′ neolignans. Present report also has been performed in continuation of our previous study on the biosynthesis and stereochemistry of 8-*O*-4′ neolignans.

Corresponding Author: Dr. Md. Shameul Alam, Faculty of Medicine, Kagawa University, Kagawa, Japan.

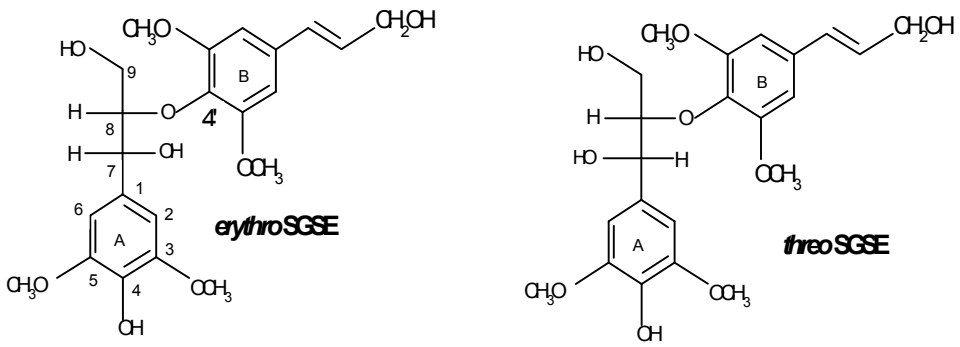

Fig. 1: The configuration of *erythro* and *threo*-syringylglycerol-8-O-4'-(sinapyl alcohol) ethers (SGSE)

Eucommia ulmoides Oliver (Eucommiaceae) is a higher plant known to produce *trans*-polyisoprene [8] native to China, known as "Gutta-percha tree". Tochu in Japanese (Du-Zhong in Chinese), is a medicinal woody plant whose bark is used as a crude drug in China and whose leaves are used as tea (Tochu tea) in Japan. Tochu tea has pharmacological activity on blood pressure [9], pain relief, diuresis, antihypertensive activity [10], Recently, water extracts of Tochu leaves have been reported as a potent antioxidant [11] and anti-mutagenic effects [12], inhibitory effect against human low-density lipoprotein [13], anti-diabetic [14], inhibitory effect on oxidative damage in biomolecules [15] and ability in the prevention of oxidative gastric injury that precedes carcinogenesis [16]. The bark of *E. ulmoides* has diverse bioactive lignans and neolignanas. Deyama and coworkers [17] first isolated guaiacylglycerol-8-*O*-4′-(sinapyl alcohol) ether (GGSE), from this medicinal plant. Subsequently, an 8-8′ dimer of SA, syringaresinol, an 8-*O*-4′ neolignans consisting of CA and SA moieties, has been isolated. Therefore we have been selected this plant for our present study to clarify the stereochemical formation of 8-*O*-4′ neolignans using SA as a monolignol precursor. Recently, Katayama and Kado [18] found enzymatic formation of (+)-*erythro*- and (-)-*threo*-guaiacylglycerol-8-*O*-4'-(coniferyl alcohol) ethers (GGCEs) by the incubation of CA with cell-free extracts (soluble preparations) of *E. ulmoides* in the presence of H_2O_2. Katayama and Tabuchi [19] has determined absolute configuration of the four stereoisomers, (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-GGCEs as (7*R,* 8*S*), (7*S,* 8*R*), (7*S,* 8*S*), and (7*R,* 8*R*), respectively, by Mosher's method. They suggested that (+)-*erythro*-GGCE was formed by the selective water addition to the (8*R*)-enantiomer of the racemic quinonemethide. Lourith *et al* [20] found the stereoselective formation of *erythro* and *threo*-syringylglycerol-8-*O*-4'-(sinapyl alcohol) ethers (SGSEs) with optical activity by feeding experiments using this plant as well as by incubation of a mixture of SA and CA with an insoluble enzyme preparation from this one. In this study, to elucidate formation of *erythro*- and *threo*-SGSEs with optical activity, incubation of SA with the soluble and insoluble enzyme preparations were carried out in the presence and absence of H_2O_2, respectively.

Chemical synthesis of SGSE was done by the method of Tanahashi *et al*. [21] Because dehydrogenation of sinapyl alcohol by peroxidase/H_2O_2 in aqueous solution mainly produce syringaresinol with little amount of SGSE, on the other hand, Tanahashi *et al* found that SGSE was the main product when dehydrogenation of sinapyl alcohol with $FeCl_3$ in a dioxane-water (10:1). However the separations of the diastereomers (*erythro* and *threo* forms) were done by preparative TLC and identified them by nuclear magnetic resonance (NMR) spectroscopy.

MATERIALS AND METHODS

Instrumentation and Chromatography Materials: All reagents and solvents were reagent grade. Analytical and preparative thin-layer chromatography (TLC) was done by using of plates precoated with Merck silica gel 60 F-254 (0.25 mm and 0.5 mm thickness, respectively). NMR spectra (400 MHz) were measured on a JEOL α-400 FT-NMR spectrometer with tetramethylsilane (TMS) as an internal standard. Chemical shifts and coupling constants (J) were expressed as δ (in ppm) and Hz, respectively. Column chromatography was conducted on Merck silica gel 60 (230-400 mesh ASTM). Analytical high performance liquid chromatography (HPLC) was carried out on a Jasco PU-2089 equipped with a Jasco UV-2075 plus Intelligent UV/VIS detector and a Shimadzu chromatopac C-R7A plus using a reversed phase column (TSK-GEL, ODS-80Ts, column No E9479). Compounds were separated at a flow rate of 1.0 ml/min using the following linear gradient solvent system: MeOH with 3% AcOH in H_2O (v/v) starting with isocratic elution at 25:75 (or 23:77) which was

held for 10 min, and then linearly increased to 32:68 (or 28:72) within 5 (or3) min. This elution condition was then held for the remainder of the analysis. Chiral analysis was performed on a Daicel Chiralcel OD (H) column (250 x 46 mm) eluted with EtOH/n-hexane (23:77) (v/v) at a flow 1.0ml/min (for *erythro* SGSE) rate and of 0.8 ml/min (for *threo*-SGSE). All detection condition was at 280nm.

Radioactive samples were analyzed in liquid scintillation cocktail consisting of scintilationblende –II/toluene/polyethylene glycol mono-*p*-isooctyl phenyl ether (6/54/40; v/v/v) (Nacalai Tesque) and measured using a liquid scintillation counter (LSC-1000, Aloka).

Protein content in the enzyme samples was determined (Shimadzu spectrophotometer: UV-1600 at 595nm.) by the Bio-Rad micro assay procedure using bovine serum albumin as standard [22].

Plant Materials: *Eucommia ulmoides* (Tochu) plants were obtained from Sanyo Nouen Inc. were maintained at Faculty of Agriculture, Kagawa University.

Chemical Synthesis: Monolignol (sinapyl alcohol) is the major precursor of syringyl lignans and neolignans (SGSE) was synthesized through following steps and identified by thin-layer chromatography (TLC) and purified by the column chromatography. SGSE was synthesized by the method of Tanahashi *et al*. [21] and their diastereomer were quantified by high-performance liquid chromatography (HPLC) and their structures were determined by ^{1}H NMR, ^{13}C NMR.

Sinapic Acid Preparation: Malonic acid (1.1398g) and syringaldehyde (975.9mg) were dissolved in pyridine (5ml), to which was added piperidine (0.1ml) and aniline (0.1ml). The reaction solution was stirred for 23h at 55°C. After cooling to room temperature, the reaction solution was concentrated and acidified with 2N HCl solution. The whole was then extracted three times with ethyl acetate (EtOAc) and washed with saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The yield was dried in vacuum sample drying apparatus.

Methyl Sinapate Preparation: A soxhlet extractor containing anhydrous $CaSO_4$ was used for this reaction. The crude sinapic acid (516.0mg) was dissolved in methanol (MeOH, 20ml) in the flask, and 80ml of MeOH was added from the extractor. To the reaction solution in the flask was added 0.2ml of concentrated H_2SO_4. The solution was refluxed with the refluxing MeOH dried continuously. After 3h the reaction mixture was cooled to room temperature and neutralized (pH 5-6) by the addition of $NaHCO_3$. The whole was then filtered by KIRIYAMA filter and the salts were washed with MeOH. The filtrate and the washings were combined and concentrated in vacuo. The residue was partitioned between dichloromethane (CH_2Cl_2) and sodium hydrogen carbonate ($NaHCO_3$). The organic layer was washed three times with saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness in vacuo to give methyl sinapate crude.

Sinapyl Alcohol and [8-^{14}C] Sinapyl Alcohol [21]: A solution of methyl sinapate (332.0mg, 1.32mmol) in toluene (15ml, freshly distilled), was cooled to 0°C in an ice-water bath. To the stirred cold solution, a solution (1.5M, 3.5ml) of diisobutylaluminium hydride (DIBAL-H, Aldrich) in toluene was drop wisely added with syringe over ca. 5 min under N_2 and then the stirring was continued for additional 40 min. The reaction mixture was then carefully quenched with ethanol (3ml) at the same temperature. The solvents were partially removed in vacuo at 40°C. Water (10ml) was added, and the aqueous layer, containing a gelatinous precipitate of aluminum salts, was extensively extracted with ethyl acetate (EtOAc) (3 × 50ml). The combined organic layers were dried over anhydrous sodium sulfate (Na_2SO_4) and evaporated to dryness in vacuo at 35-38°C under nitrogen (balloon) to give syrupy sinapyl alcohol (231.54mg, 69% yield) which was immediately placed to the freezer because of its unstable property. Crude sinapyl alcohol was purified by light-shielded column chromatography and recrystallization. [8-^{14}C] sinapyl alcohol (5.0 MBq/mmol) was prepared by the same method as above except for the use of [2-^{14}C] Malonic acid (9.25 MBq/mmol, Moravek Biochemical's Inc.), and its chromatographic behavior was identical with that of unlabeled sinapyl alcohol.

Preparation of SGSE [23]: To a stirred solution of sinapyl alcohol (43.0 mg, 0.205 mmol) in 1,4-dioxane (3 ml), a solution of $FeCl_3.6H_2O$ (25.8 mg, 0.095 mmol) in H_2O (0.3ml) was added drop wise at room temperature over a period of 5 min. After a drop of the aqueous solution was added, the light yellow color of the original reaction was changed to light green, then the solution became clearly original color again, and then the drop wise addition of the reagent was further continued, and this procedure was repeated. The reaction was quenched by the addition of a small amount of granulated NaCl. The reaction mixture

was then extracted three times with EtOAc. The EtOAc solution was combined, washed with saturated brine, dried over anhydrous Na_2SO_4 and then evaporated to dryness in vacuo. The residue was purified by preparative TLC (7% MeOH in CH_2Cl_2) to give SGSE (15.22 mg, yield 17.0%) as mixture of *erythro* and *threo* isomer (Fig.1). Diastereomeric ration of this SGSE sample was quantified by reverse phase HPLC. Diastereomeric separation of SGSE was carefully carried out by preparative TLC [benzene/acetone = 2:1 (x5)] to give *threo*-SGSE (R_f 0.27, 2.60 mg) and *erythro*-SGSE (R_f0.25, 2.63 mg) successfully.

Erythro-SGSE: ^{1}H NMR delta (acetone-d_6): 3.46 (1H, dd, J = 7.08, 3.44, H-9a), 3.38-3.42 (1H, o, 9-OH), 3.84-3.88 (2H, o, 9´-OH and H-9b), 3.81 (6H, s, A-OCH_3), 3.90 (6H, s, B-OCH_3) 4.24 (2H, dt, J = 5.32, 1.54, H-9´), 4.17 (1H, m, H-8), 4.38 (1H, d, J = 4.14, 7-OH), 4.99 (1H, t, J = 4.27, H-7), 6.39 (1H, td, J = 15.85, 5.13, H-8´), 6.57 (1H, td, J = 15.86, 1.55, H-7´), 6.72 (2H, s, H-2 and 6), 6.82 (2H, s, H-2´ and 6´), 7.07 (1H, s, 4-OH). EI=MS m/z (%): 436 (3.9) $[M]^+$, 418 (11.1) $(M-H_2O)^+$, 210 (100), 93 (3.7), 77(9).

Threo-SGSE: ^{1}H NMR delta (acetone-d_6): 3.33 (1H, m, 9-OH), 3.52 (1H, dd, J = 7.92, 4.76, H-9a), 3.66 (1H, m, H-9b), 3.81 (6H, s, A-OCH_3), 3.88-3.90 (1H, o, 9´-OH), 3.92 (6H, s, B-CH_3) 3.94-3.96 (1H, o, H-8), 4.23 (2H, dt, J = 4,76, 1.50, H-9´), 4.38 (1H, d, J= 2.93 7-OH), 4.97 (1H, dd, J = 7.44, 2.81, H-7), 6.39 (1H, td, J = 15.86, 5.12, H-8´), 6.56 (1H, dt, J = 15.85, 1.59, H-7´), 6.77 (2H, s, H-2 and 6), 6.82 (2H, s, H-2´ and 6´), 7.10 (1H, s, 4-OH).EI=MS m/z (%): 436 (3.9) $[M]^+$, 418 (12) $(M-H_2O)^+$, 210 (100), 93 (4), 77(6).

Enzyme Preparations: A soluble enzyme preparation [cell-free extracts with potassium phosphate (K-Pi) buffer] was prepared by the method of Katayama and Kado [9]. All manipulations were carried out at 4°C unless otherwise stated. Defoliated young shoot of *E ulmoides* (12-44 cm height, 58 g) were washed by tap water and distillation water wiped, sectioned (1-2 mm), frozen in liquid nitrogen, and reduced to a powder by means of a mortar and pestle. The resulting powder was homogenized with (polyvinyl polypyrilidon) PVPP (11.44 g), acid washed sea sand (22.88 g) and K-Pi buffering containing 10 mM (dithiothreitol) DTT (150 ml). The homogenate was filtered (Whatman GFA glass fiber filter). The filtrate (2.5 ml) was applied onto PD-10 column (Pharmacia, Sephadex G-25 M) equilibrated with K-Pi buffer. The excluded fraction (3.5 ml) was collected and used as crude enzyme. Protein content in the enzyme samples was determined by the Bio-Rad micro assay procedure using bovine serum albumin as standard.

An insoluble enzyme preparation (cell wall residue removing soluble and ionically bound enzymes) was prepared by the method of Davin *et al*. [7]. All enzyme preparations were carried out at 4°C unless otherwise stated. Defoliated young shoots of *E ulmoides* (16-45 cm long, 54.8 g) were washed with tap and distilled water, sectioned (1-2 mm), frozen in liquid nitrogen, and reduced to a powder by means of a mortar and pestle. The powder was transferred to a 1L beaker containing K-Pi buffer (50 mM, pH 7, 200 ml) and triton X-100 (1%). After 4 h stirring, the homogenate was filtered through one layer of nylon cloth, and the insoluble residue was ringed with cold distilled water (600ml) and squeezed to remove excess fluid. The insoluble residue was then extracted with 0.5 M NaCl (200 ml) for 16 h, filtered through one layer of nylon cloth, ringed with 800ml cold distilled water, and squeezed as before. The moist residue was reground with a mortar and pestle to afford an insoluble stem residue (25.23 g). This insoluble enzyme preparation (free of soluble and ionically bound enzymes) was assayed immediately.

Enzyme Assay

Soluble Enzyme: Each assay mixture (5.75ml × 2) consisted of the soluble enzyme (5.0 ml) from defoliated young shoots of *E. ulmoid*es, 0.43 mM of H_2O_2 (10 mM, 0.25 ml), and 2.6 mM of [8-^{14}C]SA (5.0 MBq/mmol, 30 mM, in 0.5ml of K-Pi buffer). After incubation at 30°C for various time intervals, glacial AcOH (0.5 ml) was added. The assay mixture was then extracted with EtOAc (30 ml) containing the unlabelled *erythro*- and *threo*- SGSEs from dehydrogenation as cold carrier (100µg). The aqueous layer was further extracted twice with EtOAc (30 ml). The EtOAc solutions were combined, washed twice with saturated brine, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure. The resulting EtOAc extract was subjected to preparative TLC (7% MeOH in CH_2Cl_2 × 2) to isolate [^{14}C] SGSE (a mixture of *erythro* and *threo* forms), which was then reconstituted in MeOH (100µl) and filtered through filter. An aliquot (10µl) of the filtrate was applied to C_{18} column reversed-phase HPLC. The eluate was collected in scintillation vials every 30s for liquid scintillation counting (LSC). The *erythro* and *threo* diastereomers (Fig. 1) were quantified by reverse-phase column HPLC and LSC, and isolated by the HPLC. Each diastereomer was then subjected to chiral column HPLC and LSC to quantify the enantiomers

Insoluble Enzyme:

Each assay consisted of the insoluble enzyme preparation (3.5 g) suspended in K-Pi buffer (50mM, pH 7, 12ml). The reaction was initiated by addition of solutions of 15mM [8-^{14}C]coniferyl alcohol (4.53MBq/mmol) and 15mM [8-^{14}C]SA (5.0MBq/mmol) in K-Pi buffer (30mM, pH 7, 0.7ml). After incubation at 30°C for various time intervals, glacial AcOH (0.5 ml) was added. Those assay mixtures were then extracted with EtOAc containing the mixture of unlabeled *erythro*- and *threo*-SGSEs as cold carriers (100µg) and stereoisomers of the resulting [^{14}C] SGSE were analyzed similar to the soluble enzyme assay.

RESULTS AND DISCUSSION

Enzymatic formation (soluble and insoluble enzyme preparations from *E. ulmoides)* of *erythro*-and *threo*-syringylglycerol-8-*O*-4´-(sinapyl alcohol) ethers (SGSEs) and their formation mechanism.

Formation Mechanism of 8-*O*-4´-sgses Catalyzed by a Soluble Enzyme Preparation: Enzymatic formation of 8-*O*-4´ neolignans, especially syringyl 8-*O*-4´ neolignan is noteworthy to investigate to clarify its formation mechanism from SA with enzyme preparations of *E. ulmoides*. Because, *In vitro* investigations were undertaken in addition to the feeding experiments and suggested that formation of syringyl 8-*O*-4´ neolignans with optical activity from two sinapyl alcohols [20]. In this aspect, in *vivo* investigations were carried out to clarify the formation mechanism of 8-*O*-4´ neolignans. Incubations of soluble enzyme preparation from defoliated young shoots of *Eucommia ulmoides* with [8-^{14}C] SA in the presence of hydrogen peroxide gave [^{14}C] SGSE with [^{14}C] syringaresinol. During the incubation at 30°C for various time intervals (2, 10, 30, 60 and 120 min. respectively), formation of SGSE rapidly increased at the initial stage (2-10min). When its amount linearly reached at the maximum (31.7 µmol/mg protein) and increasing continue until 30min at the highest (41.7 µmol/mg protein), then it was declined rapidly until 60 min (13.8 µmol/mg protein). The formation was slightly increased again from 60 to 120 min (24.9 µmol/mg protein). When soluble enzyme preparation denatured by heating in boiling water for 10 min was incubated with the substrate for 60 min or at the same time the cell-free extract was incubated with the substrate without hydrogen peroxide, no significant formation of SGSE (3.13 and 5.69 µmol/mg protein, respectively) was observed. This result suggested that the cell-free extracts produced higher percentage of syringyl 8-*O*-4´ neolignans to utilize sinapyl alcohol individually within the incubation period of 10 to 30 minutes.

Diastereomeric composition was observed over the time course shows as Table 1. SGSE was diastereoselectively formed in *erythro* isomer from 2 to 60 min incubations with 63.2 percentage of diastereomeric excess (% d.e.) at 30 min, except for a preferential formation of *threo*-SGSE (31.6%) at 120 min. When the enantiomeric composition was examined by chiral column HPLC, soluble enzyme preparation enantioselectively produced (Table 1[a]), (-)-*erythro* and (-)-*threo*-SGSEs those have optical activity with 47.4% e.e. and 21.6 % e.e., respectively, at 60 min. This result corresponding with Lourith *et al.* [24] because soluble enzyme preparation enantioselectively produced *erythro* and *threo* isomer both levorotatory with optical activity on the other hand Lourith *et al.* [20] feeding experiments data shows the same result at 3h metabolism with incubation of [8-^{14}C]SA to excised shoots of *E. ulmoides*.

Formation of 8-*O*-4´-sgse Catalyzed by an Insoluble Enzyme Preparation: Incubations of insoluble enzyme preparation from *E. ulmoides* with [8-^{14}C] SA in the presence of H_2O_2 also gave [^{14}C] SGSE with [^{14}C] SYR. Throughout the time course, maximum amount of SGSE (24.4 nmol/mg residue) has been accumulate the initial stage at 2 min, then it was declined gradually at 10 min (18.7 nmol/mg residue) 25%, at 30 to 60 min (6.4 nmol/mg residue, 5.9 nmol/mg residue) 75%, and at 120 min (1.8 nmol/mg residue) 7% respectively. When the insoluble enzyme preparation was denatured by heating in boiling water for 10 min and then incubated with the substrate for 60 min, no significant formation of SGSE (3.4 nmol/mg residue) was observed. Amusingly, substrate was reacting with insoluble residue very quickly at the initial stage of the reaction in the absence of H_2O_2 and produce moderate amount of SGSE. The easier oxidized monolignol is the radical mediator for sinapyl alcohol oxidation [25]. This founding demonstrated that the coupling with the same oxidation potentials species (sinapyl alcohol) were easily occurred to give favored SGSE. Diastereomeric composition was further examined by reverse-phase HPLC and shows Table 2. SGSE was d iastereoselectively formed in *erythro* isomer from 2 to 60 min incubations with 60.8% of diastereomeric excess (% d.e.) at 2 min, except for a preferential formation of

Table 1: Formation of SGSE, its percent diastereomeric excess and part of enantiomeric excess, following incubation of [8-^{14}C] sinapyl alcohol with a soluble enzyme preparation in the presence of H_2O_2 at various time intervals

						Control	
	Complete (μmol / mg protein)					-H_2O_2	Boiled
SGSE							
Time (min)	2	10	30	60	120	60	60
Erythro	1.23	23.0	34.0	8.76 [(-) 47.4[a]]	8.53	2.48	2.19
Threo	0.77	8.68	7.66	5.07 [(-) 21.6[a]]	16.4	3.21	0.94
Percent diastereo-meric excess	23.0	45.0	63.2	26.7	-31.6	-12.8	39.9

[a]Percent enantiomeric excess was analyzed at 60 min

Table 2: Formation of SGSE, its percent diastereomeric excess and part of percent enantiomeric excess, following incubation of [8-^{14}C] sinapyl alcohol with an insoluble enzyme preparation in the absence of H_2O_2 at various time intervals

	Complete (nmol / mg residue)					Boiled
SGSE						
Time (min)	2	10	30	60	120	60
Erythro	19.6	7.41	3.84	3.28 [(+) 24.8[a]]	0.82	2.35
Threo	4.87	5.08	2.60	2.61 [(-) 14.6[a]]	0.97	1.30
Percent diastereo-meric excess	60.8	18.7	19.3	11.4	-3.83	28.8

Table 3: Formation of SGSE, its percent diastereomeric excess and part of percent enantiomeric excess, following incubation with a mixture of [8-^{14}C] sinapyl alcohol and [8-^{14}C] coniferyl alcohol with an insoluble enzyme preparation in the absence of H_2O_2 at various time intervals

	Complete (nmol / mg residue)					Control
SGSE						Boiled
Time (min)	2	10	30	60	120	60
Erythro	nd	0.85	4.85	5.10 [(+) 21.4[a]]	3.34	nd
Threo	nd	0.22	1.45	0.48 [(-) 4.3[a]]	0.33	nd
Percent diastereo-meric excess	-	45.0	54.0	82.8	82.0	-

[a] Percent enantiomeric excess was analyzed at 60 min.

threo-SGSE (18.7% at 10 min). The enantiomeric composition of the *erythro* and *threo* SGSEs formed was determined by chiral column HPLC is shown in Table 2. Insoluble enzyme preparation with SA enantioselectively produced (+)-*erythro* and (-)- *threo*-SGSEs those have also optical activity with 24.8% e.e. and 14.6% e.e., respectively, at 60 min. Interestingly, the soluble preparation produced (-)-*erythro*- and (-)-*threo*-SGSEs, whereas the insoluble preparation produced (+)-*erythro*- and (-)-*threo*-SGSEs. Both preparations catalyzed the diastereoselective formation of *erythro*- and *threo*-SGSEs with optical activity. On the other hand, the soluble preparation catalyzed the formation of (–)-*erythro*-SGSE, whereas the insoluble preparation did that of (+)-*erythro* one, the opposite enantiomer.

On the other hand, when cell wall residue from defoliated young shoots of *E. ulmoides* were incubated with a mixture of [8-^{14}C]CA and [8-^{14}C]SA for various time intervals. After affluent of incubation they produced a mixture of SGCE, GGCE, SGSE, and GGSE respectively. A preferential formation of *erythro*-[^{14}C]SGSE was detected after 10 min and lasted for the period of observation (Table 3). The formation rate increased from 10 to 30min. The highest % d.e. (82.8) was found at 60min, when the enantiomeric composition was examined by chiral HPLC. (+)-*Erythro*- and (-)-*threo*-[^{14}C]SGSE were formed with 21.4% e.e. and 4.3% e.e., respectively, (Table 3). No [^{14}C]SGSE formation was detectable in denatured (10 min heating in boiling water) preparations (Table 3). In addition to SGSE, other 8-*O*-4'-neolignans, GGCE,

GGSE, and syringylglycerol-8-*O*-4'-(coniferyl alcohol) ethers (SGCE), were also produced in the order of each quantity (data not shown). The predominant diastereomer of SGSE and GGCE was the *erythro* isomer and the diastereomeric excess of SGSE was higher than that of GGCE. Whereas, the predominant diastereomer of GGSE and SGCE was the *threo* isomer and the diastereomeric excess of GGSE was higher than that of SGCE. The preference on *erythro* isomer of SGSE is in accordance with those of lignin that a predominance of the *erythro* forms of β- syringyl ethers greater than that of β-guaiacyl ethers [26, 27]. This result supports our findings that *erythro* isomer composition of SGSE was the highest in favor of *threo* isomer composition of SGSE.

The soluble preparation catalyzed the formation of (–)-*erythro*-SGSE, whereas the insoluble preparation did that of (+)-*erythro* one, the opposite enantiomer. These results were analogous with those of feeding experiments by Lourith *et al*. [20]. The diastereoselective formation of *erythro*-SGSE in the enzymatic reactions was consistent with that in the feeding experiments (full data not shown). *Erythro*-SGSE formed by the insoluble enzyme preparation [(-) 21.4] had the same optical activity as that of free *erythro*-SGSE derived from stems in the feeding experiments [(-) 9.1]. Furthermore, the optical activity of (-)-*threo*-SGSE in the both soluble [(-) 14.6] and insoluble assays [(-) 4.3] were in accord with that of free *threo*-SGSE derived from leaves in the feeding experiments [(-) 7.4]. Opposite optical activities of stem-derived *erythro*-SGSE between the organic (SGSE) and aqueous (SGSE glucoside) layers were observed. One reason may be that the (–)-*erythro*-SGSE, in preference to the (+)-enantiomer, was transformed into its glucosides. The selectivity of enantiomer glucosylation in leaves also remain obscure, although there is a possibility that selective formation of (–)-*erythro*-SGSE was followed by its selective transformation to (–)-*erythro*-SGSE glucoside. The observation of soluble and insoluble enzyme preparations to preferences for different enantiomers of the *erythro* isomer suggests that different enzymes regulate the 8-*O*-4′ coupling of sinapyl alcohol in *E. ulmoides*. Recently, two classes of pinoresinol-lariciresinol reductases have been identified in Western Red cedar (*Thuja pilcata*). Each class is specific for one enantiomer of the substrate [28]. This finding also supports the suggestion.

Incubation of a soluble enzyme preparation of *E. ulmoides* with [8-^{14}C] SA in the presence of hydrogen peroxide gave (–)-*erythro* and (–)-*threo*-[^{14}C] syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSEs). On the other hand, incubation of an insoluble enzyme preparation of this plant with [8-^{14}C]SA in the absence of hydrogen peroxide afforded (+)-*erythro*- and (–)-*threo*-[8-^{14}C]SGSEs. Both preparations catalyzed the diastereoselective formation of *erythro*-SGSEs with optical activity within 60 min. So far we know, this reports the first example of the soluble preparation catalyzed the formation of (–)-*erythro*-SGSE, whereas the insoluble preparation did that of (+)-*erythro* one, the opposite enantiomer. We hope this formation mechanism of 8-*O*-4′-SGSEs will be a pledge for determination of absolute configuration of 8-*O*-4′ neolignans.

REFERENCES

1. Lewis, N.G. andl L.B Davin, 1999. Lignans: biosynthesis and function. In Comprehensive Natural Products Chemistry, vol 1. Edited by Barton Sir DHR, Nakanishi K, Meth-Cohn O. Oxford: Elsevier., pp: 639-712.
2. Siripong, P., K.K. Dakul, S. Piyaviriyagul, J. Yahuafal, R. Chanpal, S. Ruchirawat and N. Oku, 2006. Antiproliferative napthoquinone esters from *Rhinacanthus nasutus* Kurz. Roots on various cancer cells., J. Trad. Med., 23: 166-172.
3. Barata, L.E.S., L.S. Santos, P.H. Ferri, J.D. Phillipson, A. Paine and S.L. Croft, 2000. Anti-leishmanial activity of neolignans from *Virola* species and synthetic analogues. *Phytochemistry*, 55: 589-595.
4. Higuchi, T. 1997. Biochemistry and molecular biology of wood. Springer, Berlin Heidelberg New York, pp: 168-181.
5. Gang, D.R., Costa, M.A., Fujita, M., Dinkova-Kostava, A.T., Wang, H.B., Burlat, V., Martin, W., Sarkanen, S., Davin, L.B. and Lewis, N.G. 1999. Regiochemical control of monolignol radical coupling: a new paradism for lignin and biosynthesis. Chem. Biol., 16: 143-151.
6. Davin, L.B. and N.G. Lewis, 2000. Dirigent proteins and dirigent sites explain the mystery of specificity of radical precursor coupling in lignin and lignin biosynthesis. Plant Physiol., 123: 453-461.
7. Davin, L.B., D.L. Bedger, T. Katayama and N.G. Lewis, 1992. On the stereoselective synthesis of (+)-pinoresinol in *Forsythia suspense* from its achiral precursor, coniferyl alcohol. *Phytochemistry*, 31: 3869-3874.
8. Jitladda, T., T. Yasuyuki, S. Ken-Ichi, K. Seiichi, S. Koji and S. Yoshihisa, 1997. Structure and biosynthesis of *trans*-polyisoprene from *E. ulmoides*, Phytochemistry, 45: 75-80.

9. Nakazawa, Y., N. Odagiri, R. Imai, T. Yoshii, E. Tagashira, C. Nakata, T. Nakamura, M. Asaumi, S. Onizuka, M. Yahara and T. Nohara, 1997. Effect of Eucommia leaf (*Eucommia ulmoides* OLIVER leaf; Du-zhong yge): Extract on blood pressure (I) effect of blood pressure in spontaneous hypertensive rats (SHR) (in Japanese). *Natural Med*., 51: 392-398.
10. Kawasaki, T., K. Uezono and Y. Nakazawa, 2000. Antihypertensive mechanism of blood for specified health use: "Eucommia leaf glycoside" and its clinical application (in Japanese). J Health Sci, 22: 26-29.
11. Park, A.S., M.S. Choi, J.U. Jung, M.K. Kim, J.D. Kim, H.M. Park, B.Y. Park and M.K. Lee, 2006. *Eucommia ulmoides* Oliver leaf extract increases endogenous antioxidant activity in Type 2 Diabetic Mice. J. Med. Food., 9: 474-479.
12. Nakamura, T., Y. Nakazawa, S. Onizuka, S. Satoh, A. Chiba, K. Sekihashi, A. Miura, N. Yasugahira and Y.F. Sasaki, 1997. Antimutagenicity of Tochu tea (an aqueous extract of (*Eucommia ulmoides* leaves): 1. The clastogen-suppressing effects of Tochu tea in CHO cells and mice. Mutation Res., 288: 7-20.
13. Gow-Chin, Y. and H. Chiu-Luan, 2002. Inhibitory effects of du-zhong (*Eucommia ulmoides* Oliv.) against low-density lipoprotein oxidative modification. Food Chem.., 77: 449-456.
14. Lee, M.K., M.J. Kim, S.Y. Cho, A.S. Park., K.K. Park, J.U. Jung, H.M. Park and M.S. Choi, 2005. Hypoglycemic effect of Du-zhong (*Eucommia ulmoides* Oliv.) leaves in streptozotocin-induced diabetic rats. Diabetes Res. Clin. Pract., 67: 22-28.
15. Hsieh, C.L. and G.C. Yen, 2000. Antioxidant actions of Du-zhong ((*Eucommia ulmoides* OLIV.) toward oxidative damage in biomolecules. Life Sci., 66: 1387-1400.
16. Yang, J., K. Kato, K. Noguchi, N. Dairaku, T. Koike, K. Iijima, A. Imatani, H. Sekine, S. Ohara, H. Sasano and T. Shimosegawa, 2003. Tochu (*Eucommia ulmoides)* leaves extract prevents ammonia and vitamin C deficiency induced gastric mucosal injury. Life Sci., 73: 3245-3256.
17. Deyama, T., T. Ikawa, S. Kitagawa and S. Nishibe, 1987. The constituents of *Eucommia ulmoides* OLIV. VI. Isolation of a new sesquilignan and neolignan glycosides. Chem Pharm Bull., 35: 1803-1807.
18. Katayama, T. and Y. Kado, 1998. Formation of optically active neolignans from achiral -10coniferyl alcohol by cell-free extracts of *Eucommia ulmoides*. J. Wood Sci., 44: 244-246.
19. Katayama, T., Y. Tabuchi and T. Suzuki, 2005. A novel Stereoselective addition of water to one enantiomer of racemic 8-*O*-4′ quinonemethide from two molecules of coniferyl alcohol. Proceedings of the International Symposium on Wood Science and Technology, Yokohama, 1: 276-277.
20. Lourith, N., T. Katayama, K. Ishikawa and T. Suzuki, 2005. Biosynthesis of a syringyl 8-O-4′ neolignan in *Eucommia ulmoides*: formation of syringylglecerol-8-O-4′-(sinapylalcohol) ether from sinapyl alcohol. J. Wood Sci., 51: 379-386.
21. Tanahashi, M., H. Takeuchi and T. Higuchi, 1976. Dehydrogenative polymerization of 3,5-disubstituted *p*-coumaryl alcohols. Wood Res., 61: 44-53.
22. Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem., 72: 248-252.
23. Nakatsubo, F., K. Sato and T. Higuchi, 1976. Enzymic dehydrogenation of *p*-coumaryl alcohol. IV. Reactivity of quinine methide. *Mokuzai Gakkaishi*, 22: 29-33.
24. Lourith, N., T. Katayama and T. Suzuki, 2005. Stereochemistry and biosynthesis of a syringyl 8-O-4′ neolignans in *Eucommia ulmoides*: diastereoselective formation of guaiacylglecerol-8-O-4′-(sinapylalcohol) ether. J. Wood Sci., 51: 370-378.
25. Sasaki, S., T. Nishida, Y. Tsutsumi and R. Kondo, 2004. Lignin dehydrogenative polymerization mechanism: a poplar cell wall peroxidase directly oxidizes polymer lignin and produces in vitro dehydrogenative polymer rich in beta-O-4 linkage. FEBS Lett., 562: 197-201.
26. Katayama, T. and M. Sogo, 1989. An optically-active compound formed by the reduction of an α-Ketonic lignin substructure model compound by *Fusarium solani* M-13-1. Mokuzai Gakkaishi, 35: 1116-1124.
27. Akiyama, T., Y. Matsumoto, T. Okuyama and G. Meshitsuka, 2003. Ratio of *erythro* and *threo* forms of β-*O*-4 structures in tension wood lignin. Phytochemistry, 64: 1151-1162.
28. Fujita, M., D.R. Gang, L.B. Davin and N.G. Lewis, 1999. Recombinanat pinoresinol-lariciresinol reductases from western red cedar (*Thuja pilcata*) catalyze opposite enantiospecific conversions. J. Biol. Chem., 274: 618-627.