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Formation of Optically Active *Erythro*-Syringylglycerol-8-*O*-4'-(Sinapyl Alcohol) Ethers, from Achiral Monolignols with Enzyme Reparations of Higher Plant

Md. Shameul Alam, Toshisada Suzuki, Takeshi Katayama

Faculty of Agriculture, Kagawa University, Japan

Abstract: Continuation of our neolignan biosynthesis, to clarify the stereochemistry on the formation of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ether (SGSE) from sinapyl alcohol (SA) in *Eucommia ulmoides*, enzymatic reactions were carried out. Incubation of a soluble enzyme preparation of *Eucommia ulmoides* with sinapyl alcohol (SA) in the presence of hydrogen peroxide (H_2O_2) produced (–)-*erythro* and (–)-*threo*-syringylglycerol-8-*O*-4'-(sinapyl alcohol) ethers (SGSEs). Conversely, incubation of an insoluble enzyme preparation of this plant with SA in the absence of hydrogen peroxide afforded (+)-*erythro*- and (–)- *threo*-SGSEs. Both preparations catalyzed the diastereoselective formation of *erythro*-SGSEs with optical activity within 60 min.

Key words: Sinapyl alcohol · SGSE · Neolignan · *Eucommia ulmoides* · *Plant metabolites*

INTRODUCTION

Lignans and neolignans in plants have optical activity. They have important physiological functions in plant defense and human health. Recently, its have been reported that Surinamensin (8-*O*-4′ neolignan) has potential anti-leishmanial activity. Arylglycerol- (8-*O*-4′)-aryl ether linkages are present in lignins and 8-*O*-4′ neolignans. The intermonomer linkages are the most abundant ones in natural products except for glycosidic linkages in carbohydrates.

Eucommia ulmoides (Fig. 1) is a higher plant known to produce *trans*-polyisoprene [1] native to China. Its roasted bark (Eucommia *c*ortex) has been used a crude drug in china since ancient times and its leaves are used as a tochu tea in Japan. Many of bioactive compounds are presents in this medicinal plant. It's have pharmacological effects on blood pressure [2], antihypertensive [3], reinforce muscle and lung, inhibitory on oxidative damage in biomolecules. There are plenty of lignans and neolignans are present in Tochu. Deyama and coworkers [4] first isolated guaiacylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (GGSE) from *Eucommia ulmoides*. Recently discovered that a "dirigent protein" which catalyzes formation of (+)-pinoresinol was obtained from an insoluble residue of *Forsythia* plant young shoots. Moreover, an 8-*O*-4′ neolignans that have four stereoisomers {*erythro* and *threo* diastereomers (Fig. 2) and their enantiomers}.In contrast to lignans, biosynthesis of 8-*O*-4′ neolignans have not been advanced. Previously, our research group has investigated the biosynthesis of 8-*O*-4′ neolignans.

Katayama and Kado found for the first time that incubation of cell-free extracts from *Eucommia ulmoides* with coniferyl alcohol (CA) in the presence of H_2O_2 gave (+)-*erythro*- and (-)-*threo*-guaiacylglycerol-8-*O*-4'-(coniferyl alcohol) ether (GGCE) and that the *erythro* isomer was preferred to the *threo* one. Absolute configuration of the four isomers of GGCE, (+)-*erythro*, (-)-*erythro*, (+)-*threo* and (-)-*threo* were determined as (7*R*, 8*S*), (7*S*, 8*R*), (7*S*, 8*S*) and (7*R*, 8*R*) by Mosher's method [5]. Our recent study that also found for the first time a novel enzyme activity which catalyzes the stereoselective addition of water to the (8*R*)- 8-*O*-4′-quinonemethide from two coniferyl alcohols giving (7*R*, 8*S*)- (+)-*erythro*-GGCE. The water attacked from *re* face of the quinonemethide. Non-enzymatic addition of water to the (8*S*) - 8-*O*-4′-quinonemethide gave (7*S*, 8*R*) - (-)-*erythro*-and (7*R*, 8*R*)-(-)-*threo*-GGCE in the almost same ratio.

Very recently Lourith *et al.* [6] determined the relative configuration of guaiacylglyerol-8-*O*-4'-(sinapyl alcohol) ether (GGSE) isolated from the plant by Deyama *et al.* as *erythro* form and found the stereoselective formation of *erythro*-GGSE and *erythro*-syringylglycerol-8-*O*-4'-(sinapyl alcohol) ether (SGSE) with optical activity by feeding experiments.

Corresponding Author: Dr. Md. Shameul Alam, 2444-2 Ikenobe, Miki-cho, Kagawa 761-0701, Japan.
E-mail: shameulalam@yahoo.com.

Fig. 1: *Eucommia ulmoides* young shoots with leaves

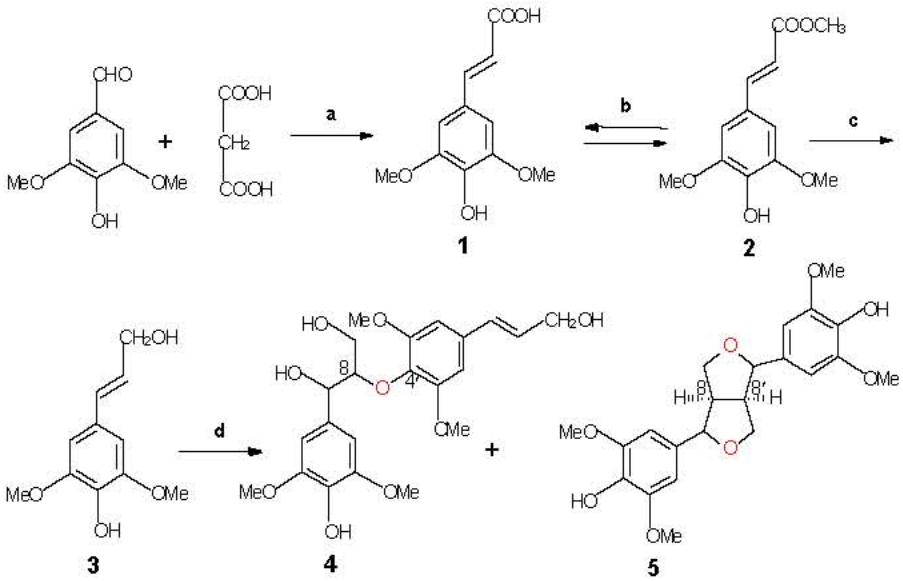

Fig. 2: Synthetic steps of SGSE and syringaresinol (SYR) with their chemical structures a; $C_5H_5N/C_5H_{11}N/C_6H_5NH_2$ (55-60°C), b; CH_3OH/H_2SO_4 (reflux), c; DIBALH/Toluene, 0°C, $FeCl_3.6H_2O/1$, d; 4-dioxen H_2O (10:1).

In this study, to clarify stereochemistry on the formation of SGSE from SA in *E. ulmoides*, enzyme reactions were carried out. Incubation of a soluble enzyme preparation with [8-^{14}C] SA in the presence of H_2O_2 gave [^{14}C] SGSE. The (-)-*erythro* isomer was more favored than the (-)-*threo* one. Incubation of an insoluble enzyme preparation with [8-^{14}C] SA also gave [^{14}C] SGSE. The (+)-*erythro* isomer was more favored than the (-)-*threo* one. Interestingly, the soluble preparation catalyzed the formation of (-)-*erythro* SGSE, whereas the insoluble preparation did (+)-*erythro* one. Both preparations catalyzed the diastereoselective formation of *erythro*- and *threo*-SGSEs with optical activity.

Dehydrogenation of SA by peroxidase/ H_2O_2 in aqueous solution gave mainly syringaresinol but little SGCE [7] Tanahashi *et al.* [8] achieved a satisfactory yield of SGSE by dehydration of sinapyl alcohol with $FeCl_3$ in dioxane-water (10:1) system in an appropriate yield. However, the diastereomers (*erythro/threo*) were not distinguished. Therefore, diastereomeric composition of SGSE was synthesized by a one-step reaction.

MATERIALS AND METHODS

Instrumentation and Chromatography Materials: All reagents and solvents are reagent grad. Analytical and preparative thin-layer chromatography (TLC) was done by using of plates precoated with Mereck silica gel 60 F-254 (0.25 and 0.5 mm thickness, respectively). 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: 30% MeOH-3% AcOH in H_2O (v/v). Chiral analysis was performed on a Daicel Chiracel OD column (250 x 46 mm,) eluted with EtOH/n-hexane = 23:77 (v/v) at a flow rate of 0.8 ml/min (for *threo*-SGSE) and 1.0 ml/min (for *erythro* SGSE) with a Jasco OR-990 chiral detector. Protein contents of soluble and insoluble both preparations were determined by Shimadzu spectrophotometer (UV-1600) at 595nm.

Chemical Syntheses: Sinapyl alcohol was synthesized by the literature method [9, 10] through following steps (Figure 2) and identified by thin-layer chromatography (TLC) and purified by the column chromatography. SGSE was synthesized by the method of Tanahashi *et al.* [8] and their diastereomer were quantified by high-performance liquid chromatography (HPLC) and their structures were determined by ^{1}H NMR.

Sinapic Acid Preparation (1a, Step a): Malonic acid (1.10 g) and syringaldehyde (941.82 mg) were dissolved in pyridine (5 ml), to which was added piperidine (0.1 ml) and aniline (0.1 ml). The reaction solution was stirred for 23 hrs 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 the combined ethyl acetate solutions were washed with saturated NaCl solution. The organic layer was dried over anhydrous Na_2SO_4 and evaporated.

Methyl Sinapate Preparation (2a, Step b): A Soxhlet extractor containing anhydrous $CaSO_4$ was used for this reaction. The crude sinapic acid (500.0 mg) was dissolved in methanol (MeOH, 20 ml) in a flask of the extractor and 80ml of MeOH was added from the extractor. To the reaction solution in the flask was added 0.2 ml of concentrated H_2SO_4. The solution was refluxed with the refluxing MeOH dried continuously. After 3 hrs the reaction mixture was cooled to room temperature and neutralized (pH 5-6) by the addition of solid $NaHCO_3$. The whole was then filtered by a 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 5% 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 (3a, Step c): A solution of methyl sinapate (332.0 mg, 1.32 mmol) in toluene (15 ml, freshly distilled), was cooled to 0°C in an ice-water bath. To the stirred cold solution, a solution (1.5M, 3.5 ml) 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 (3 ml) at the same temperature. The solvents were partially removed in vacuo at 40°C. Water (10 ml) was added and the aqueous layer, containing a gelatinous precipitate of aluminum salts, was extensively extracted with ethyl acetate (EtOAc) (3 X 50 ml). 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 (231.5 mg, 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.

PreparationofSyringylglycerol-8-*O*-4'-(SinapylAlcohol) Ether (Sgse), (4a, Step d): To a stirred solution of sinapyl alcohol (86.0 mg, 0.41 mmol) in 1,4-dioxane (5 ml), a solution of $FeCl_3$•$6H_2O$ (51.6 mg, 0.19 mmol) in H_2O (0.6 ml) 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 solution changed to light green, then the original color of the solution returned. The drop wise addition of the reagent was continued. 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 solutions were 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 (5% MeOH in CH_2Cl_2) to give SGSE (49.2 mg, 27.8%) as a mixture of (±)-*erythro*- and (±)-*threo*-isomers, in preference of (±)-syringaresinol (SYR) (5a, step d). The diastereomeric ratio of this SGSE

was quantified by reversed-phase HPLC and then diastereomeric separation was carefully carried out by preparative TLC [benzene/acetone 2:1 (X5)] to give *threo*-SGSE (R_f 0.38, 4.8 mg) and *erythro*-SGSE (R_f 0.35, 8.8 mg). The diastereomeric identification was achieved by comparison of ^{1}H NMR spectra (data not shown).

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

Enzyme Preparations and Enzyme Assay: A soluble enzyme preparation [cell-free extracts with potassium phosphate (K-P) buffer] was prepared by the method of Katayama and Kado [6]. 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.* [11].

Soluble enzyme assay: Each assay mixture (5.75ml) 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 SA (30 mM, in 0.5ml of K-Pi buffer). After 60 min incubation at 30°C, glacial AcOH (0.5 ml) was added. The assay mixture was then extracted with EtOAc. SGSE (a mixture of *erythro* and *threo* forms) was isolated by preparative TLC (7% MeOH in CH_2Cl_2). The *erythro* and *threo* diastereomers 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 assay: Each assay consisted of the insoluble residue (3.5 g) suspended in K-Pi buffer (50 mM, pH 7, 12 ml) and the solution of 1.7 mM SA (30 mM, in 0.7 ml of K-P buffer). The assay mixture was then extracted and separated as above and stereoisomers of the resulting SGSE were analyzed similar to the soluble enzyme assay.

RESULTS AND DISCUSSION

Incubation of a soluble enzyme preparation of *Eucommia ulmoides* with sinapyl alcohol (SA) in the presence of H_2O_2 gave *erythro*- and *threo*-syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSEs) (Figure 3). The identification was achieved by 1H NMR and HPLC. *Erythro*-SGSE: 1H NMR (acetone-d6): δ 3.71 (1H, s, H-9a), 3.77 [1H, overlapping (o), 9-OH], 3.82 (6H, s, A-OCH3), 3.83-3.88 (2H, o, 9′-OH & H-9b), 3.90 (6H, s, B-OCH3), 4.22 (2H, dd, J = 5.61, 4.15, H-9′),4.72 (1H, d, J = 4.39, 7-OH), 4.87 (1H, m, H-8), 4.99 (1H, d, J = 4.64, H-7), 6.36 (1H, dt, J = 15.85, 5.29, H-8′),

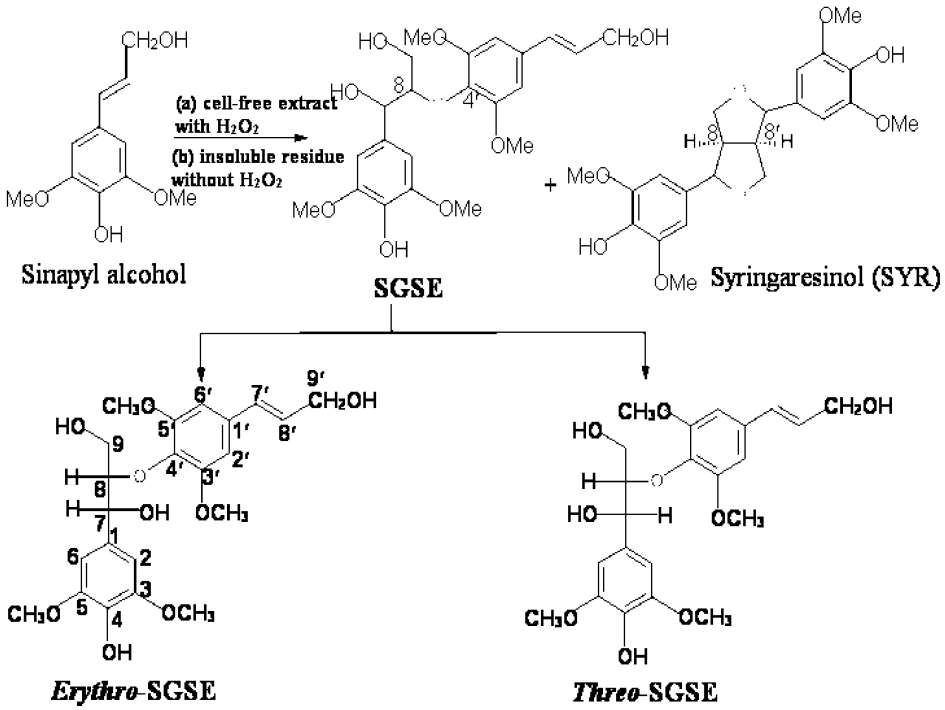

Fig. 3: The configuration of *erythro* and *threo*-syringylglycerol-8-*O*-4'-(sinapyl alcohol) ethers (SGSE), that obtained by soluble and insoluble enzyme preparations from E. ulmoides with sinapyl alcohol at 60 min incubation

Table 1: Formation of SGSE and its diastereomeric ratio, following incubation of sinapyl alcohol with a soluble enzyme preparation in the presence of H_2O_2 at 60 min incubation

	Complete Control (µmol / mg protein)		
SGSE Time (min)		$-H_2O_2$	Boiled
	60	60.0	60.0
Erythro (E)	34 [(-) 47[a]]	2.5	2.5
Threo (T)	7.7 [(-) 22[a]]	3.2	3.2
Diastereomeric excess (%)	63	13.0	13.0

[a]Percent enantiomeric excess was analyzed at 60 min

Table 2: Formation of SGSE and its diastereomeric ratio, following incubation of sinapyl alcohol with an insoluble enzyme preparation in the absence of H_2O_2 at 60 min incubation

	Complete Control (nmol / mg residue)	
		Boiled
SGSE Time (min)	60	60
Erythro (E)	19.6 [(+) 25[a]]	0.82
Threo (T)	4.9 [(-) 15[a]]	0.97
Diastereomeric excess (%)	61	3.83

[a]Percent enantiomeric excess was analyzed at 60 min

6.58 (1H, d, *J* = 15.85, H-7′), 6.73 (2H, s, H-2 & 6), 6.82 (2H, s, H-2′ & 6′), 7.51 (1H, s, 4-OH). *Threo*-SGSE: 1H NMR (acetone-d6): δ 3.3 (1H, s, 9-OH), 3.66 (1H, m, H-9a), 3.74 (1H, m, H-9b), 3.81(6H, s, A-OCH3), 3.82-3.88 (1H, o, 9′-OH), 3.92 (6H, s, B-OCH3), 3.99 (2H, dd, *J* = 3.41, H-8), 4.0 (2H, dd, *J* = 6.95, 3.56, H-9′), 4.23 (1H, d, *J* = 3.66, 7-OH), 4.98 (1H, d, *J* = 6.83, H-7), 6.36 (1H, dt, *J* = 15.85, 5.29, H-8′), 6.54 (1H, d, *J* = 16.1, H-7′), 6.78 (2H, s, H-2 & 6), 6.82 (2H, s, H-2′ &6′), 7.44 (1H, s, 4- OH). The SGSEs were diastereoselectively formed in *erythro* isomer with 63% d.e. at 60 min (Table 1). Both products, (-)-*erythro*- and (-)-*threo*-SGSEs have optical activity with 47% e.e. and 22% e.e., respectively, at 60 min.

On the other hand, incubation of an insoluble enzyme preparation of this plant with SA in the absence of H_2O_2 afforded the four isomers of SGSE. The SGSEs were diastereoselectively formed in *erythro* isomer at 60 min incubations with 61% d.e. (Table 2). Both products, (+)-*erythro*- and (-)-*threo*-SGSEs have optical activity with 25% e.e. and 15% e.e., respectively, at 60 min.

Syringylglycerol-8-*O*-4'-(sinapyl alcohol) ether (SGSE), containing two syringyl rings with four methoxy groups, on the other hand radical species of sinapyl alcohol bear one syringyl ring with two methoxy groups. It was found that both enzyme preparations formation of *erythro* isomer composition of SGSE was the highest in favor of *threo* isomer composition of SGSE. It assumed that both isomers were formed by the coupling with the same oxidation potential species (SA).

Diastereoselective formation of syringyl-8-*O*-4′-neolignan with optical activity from two sinapyl alcohols [12]. This result recognized our present study report. 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 [13].This finding also supports the suggestion. However, this study offers an appreciate pledge for advance improvements in syringyl-8-*O*-4′- neolignans biosynthesis as well as stereochemistry.

CONCLUSIONS

SGSE was formed more selectively with soluble enzyme preparation insoluble enzyme preparation. 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.

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