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Biosynthesis of a syringyl 8-*O*-4′ neolignan in *Eucommia ulmoides*: formation of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ether from sinapyl alcohol

Abstract To investigate the biosynthesis and stereochemistry of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ether (SGSE), a syringyl 8-*O*-4′ neolignan, feeding experiments and enzyme assays using *Eucommia ulmoides* were carried out. Diastereoselective formation of *erythro*-SGSE was found. When [8-^{14}C]sinapyl alcohol was administered to excised shoots of *E. ulmoides*, ^{14}C was incorporated into free SGSE and SGSE glucosides. In stems, incorporation into (+)-*erythro*-[^{14}C]SGSE (0.037%) with 9.1% enantiomeric excess (% e.e.) was found; incorporation into the *threo* isomer was not detectable. *Erythro*-[^{14}C]SGSE glucosides (0.047%) dominated over *threo* forms (0.007%) with 74.0% diastereomeric excess (% d.e.); both diastereomers were levorotatory with 32.0% e.e. and 18.3% e.e., respectively. In leaves, higher incorporation into (−)-*erythro*-[^{14}C]SGSE (0.500%, 15.9% e.e.) than into the *threo* isomer (0.206%, 7.4% e.e.) was observed (41.6% d.e.). (−)-*Erythro*-[^{14}C]SGSE glucosides (1.692%, 25.0% e.e.) were produced at higher rates than *threo* isomers (0.177%, 16.4% e.e.) with 81.0% d.e. In incubations of a mixture of [8-^{14}C]sinapyl and [8-^{14}C]coniferyl alcohols with an insoluble enzyme preparation from stems of *E. ulmoides*, *erythro*-SGSE was preferentially produced. The highest % d.e. (82.8) was observed at 60 min with the (+)-*erythro* isomer (21.4% e.e.) and the (−)-*threo* form (4.3% e.e.).

Key words Biosynthesis · 8-*O*-4′ Neolignan · Sinapyl alcohol · *Eucommia ulmoides* · Diastereomer

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Introduction

Lignans and neolignans are typically dimeric phenylpropanoids, which are widely distributed in higher plants. Compounds of this class that have an oxygen atom at the C_9 position are generated by dehydrogenative dimerization of *p*-hydroxycinnamyl alcohols termed monolignols (mainly coniferyl alcohol and sinapyl alcohol). Lignans and neolignans are distinguished by the type of intermonomer linkages: lignans have an 8-8′ bond while neolignans might show any type other than the 8-8′ bond. Coupling of mesomeric monolignol radicals leads to several intermonomer linkages; coupling of different monolignol radicals gives rise to cross-coupling products. Most lignans and neolignans isolated from plants are optically active. Recently, a "dirigent protein" was found to catalyze a regioselective as well as enantioselective coupling of two coniferyl alcohol radicals that yielded (+)-pinoresinol,[1–3] an 8-8′ coupling product. On the other hand, lignins, the cell wall polymers in wood, are structurally similar to lignans and neolignans, but they are optically inactive, racemate-like polymers. Lignins are formed through dehydrogenation of monolignols by nonselective peroxidase/H_2O_2 (and laccase/O_2) and subsequent nonenzymatic coupling of the mesomeric radicals.[4] Their main intermonomer linkages are 8-*O*-4′ bonds.[5]

In contrast to lignans, the biosynthesis of 8-*O*-4′ neolignans has not been studied intensively and awaits clarification. This investigation has been performed in continuation of our previous works[6,7] on the biosynthesis and stereochemistry of 8-*O*-4′ neolignans.

The bark (Eucommia Cortex) of *Eucommia ulmoides* Oliv. (Eucommiaceae) has been used as a crude drug in China since ancient times, and its leaves today are used to prepare a popular tea in Japan. It is well known that it has effects on blood pressure[8] and has an antihypertensive activity.[9] Recently, antimutagenic effects[10] and inhibitory effects on oxidative damage in biomolecules[11] have been reported. From this plant, syringaresinol,[12] an 8-8′ dimer of sinapyl alcohol, and citrusin B,[13] an 8-*O*-4′ neolignan con-

sisting of coniferyl and sinapyl alcohol moieties, have been isolated. We therefore assumed that this plant is able to utilize sinapyl alcohol as a monolignol for biosynthetic processes involving 8-O-4′ coupling, and selected it for this study. Recently, Katayama and Kado[6] discovered that incubation of cell-free extracts of *E. ulmoides* with coniferyl alcohol in the presence of hydrogen peroxide resulted in the formation of (+)-*erythro*- as well as (−)-*threo*-guaiacylglycerol-8-O-4′-(coniferyl alcohol) ethers (GGCEs), optically active 8-O-4′ neolignans, and in the diastereoselective formation of *erythro*-GGCE. We also found diastereoselective formation of *erythro*-guaiacylglycerol-8-O-4′-(sinapyl alcohol) ether (GGSE), following administration of a mixture of coniferyl and sinapyl alcohols to excised shoots of *E. ulmoides*.[7] Here we describe biosynthesis, feeding experiments and an enzymatic reaction, of a syringyl 8-O-4′ neolignan, syringylglycerol-8-O-4′-(sinapyl alcohol) ether (SGSE).

Dehydrogenation of sinapyl alcohol by peroxidase/H_2O_2 in aqueous solution gave mainly syringaresinol but little SGSE.[14] On the contrary, Tanahashi et al.[15] found that SGSE was a main product when dehydrogenation of sinapyl alcohol with $FeCl_3$ in a dioxane–water (10:1) system was done. However, the separation of the diastereomers (*erythro* and *threo* forms) and their detailed identification were not reported. Therefore, we prepared *erythro*- and *threo*-SGSEs separately by stepwise chemical synthesis and identified them by nuclear magnetic resonance (NMR) spectroscopy of their acetonide derivatives, which are intermediates in the synthesis.

Experimental

Instrumentation and chromatography

All reagents and solvents were reagent grade. Column chromatography was conducted on Merck silica gel 60 (230–400 mesh ASTM). Analytical and preparative thin-layer chromatography (TLC) were performed by using plates precoated with Merck silica gel 60 F-254 (0.25 and 0.5 mm thickness, respectively). NMR spectra (400 MHz) were determined on a JNM Alpha 400 FT-NMR spectrometer. Chemical shifts and coupling constants (J) were expressed as δ (in ppm) and Hz, respectively. Mass spectra were acquired on a JMS-SX102A mass spectrometer [electron impact (EI), 70 eV]. Analytical high performance liquid chromatography (HPLC) was carried out on a Hitachi L-6200 equipped with a Hitachi L-4200 UV/Vis detector (280 nm) and a Shimadzu chromatopac C-R7A plus, using a reversed-phase column (Waters, Nova-Pak C_{18}, 150 × 3.9 mm, stainless steel). Compounds were separated at a flow rate of 1.0 ml/min using the following linear gradient solvent system: MeOH–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 (or 3) min. This elution condition was then held for the remainder of the analysis. For feeding experiments, the ratios and time in the parentheses were used. Chiral analysis was performed on a Daicel Chiralcel OD column eluted with EtOH/n-hexane (23:77; v/v) at a flow rate of 0.8 ml/min. Radioactivity of the samples was measured in liquid scintillation cocktail consisting of scintiblender-II/toluene/polyethylene glycol mono-p-isooctylphenyl ether (6:54:40; v/v/v) (Nacalai Tesque) using a liquid scintillation counter (LSC-1000, Aloka).

Chemical syntheses

1-(4-Benzoyloxy-3,5-dimethoxyphenyl)-2-[4-(2-formylvinyl)-3,5-dimethoxyphenoxy] ethanone (**1**). To a stirred solution of sinapaldehyde (199.8 mg, 0.961 mmol) and 1-(4-benzoyloxy-3,5-dimethoxyphenyl)-2-bromoethanone (α-bromoacetosyringone-4-benzoate) (364.3 mg, 0.961 mmol) in 10 ml of *N,N*-dimethylformamide (DMF), powdered K_2CO_3 (132.5 mg, 0.959 mmol) and powdered KI (81.6 mg, 0.492 mmol) were added successively. The stirring was continued for 110 min at room temperature under nitrogen atmosphere. The reaction mixture was filtered and the precipitates were washed with Et_2O. The filtrate and the washings were combined and then partitioned between Et_2O and H_2O. The aqueous layer was extracted twice with Et_2O. The Et_2O solutions were combined and washed twice with saturated brine, dried over anhydrous Na_2SO_4, and evaporated to dryness in vacuo to afford **1** (499.7 mg, 102.8%) as a syrup which was used in the next step without further purification.

1-(4-Benzoyloxy-3,5-dimethoxyphenyl)-2-[4-(2-formylvinyl)-3,5-dimethoxyphenoxy]-3-hydroxy-1-propanone (**2**). To a stirred solution of **1** (470.2 mg, 0.928 mmol) in dimethyl sulfoxide (DMSO) (6 ml), a solution of powdered (80%) paraformaldehyde (41.9 mg, 1.12 mmol) in DMSO (4 ml), and powdered K_2CO_3 (32.8 mg, 0.276 mmol) were added. The reaction mixture was stirred at ambient temperature for 4 h 30 min under nitrogen atmosphere and then filtered. The precipitates were washed with EtOAc. The filtrate and washings were combined and partitioned between EtOAc and brine. The organic layer was washed with saturated brine, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue (513.7 mg) was purified by column chromatography (EtOAc/n-hexane 2:1) to give **2** (370.5 mg, 74.4%). Yield from sinapaldehyde was 71.9%.

1-(4-Benzoyloxy-3,5-dimethoxyphenyl)-2-[4-(3,3-dimethoxy-1-propenyl)-2,6-dimethoxyphenoxy]-3-hydroxy-1-propanone (**3**). To a stirred solution of **2** (300.4 mg, 0.560 mmol) in a mixture of tetrahydrofuran (THF, 8 ml) and MeOH (5 ml), methyl orthoformate (600 μl, 5.82 mg, 5.48 mmol) and a solution of *p*-toluenesulfonic acid (1.6 mg, 0.0084 mmol) in MeOH (1.6 ml) were added. The reaction solution was stirred at room temperature for 24 min under argon atmosphere, and then neutralized by the addition of powdered $NaHCO_3$. The mixture was filtered and the salts were washed with EtOAc.

The filtrate and the washings were combined, and then partitioned between EtOAc and saturated NaHCO$_3$ solution. The EtOAc layer was washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and evaporated in vacuo to afford **3** (319.0 mg, 97.9%) as a syrup which was used in the next step without further purification.

1-(4-Benzoyloxy-3,5-dimethoxyphenyl)-[4-(3,3-dimethoxy-1-propenyl)-2,6-dimethoxyphenoxy]-1,3-propanediol (4). A stirred solution of **3** (318.9 mg, 0.546 mmol) in a mixture of THF (2 ml) and MeOH (2 ml) was cooled to 0°C, and NaBH$_4$ (148.3 mg, 3.920 mmol) was added to the cold solution under argon atmosphere. After stirring for 45 min, the reaction solution was partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and then evaporated to dryness to afford **4** (319.5 mg, 99.9%) as a syrup which was used in the next step without further purification.

Syringylglycerol-8-O-4'-sinapaldehyde ether 4-benzoate (5). The crude **4** was dissolved in 90% AcOH (1.5 ml) and the solution was stirred for 30 min at room temperature, and then partitioned between EtOAc and saturated brine. The organic layer was washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and evaporated in vacuo to give **5** (305.8 mg, 95.5%) as a syrup which was used in the next step without further purification.

Syringylglycerol-8-O-4'-sinapaldehyde ether 4-benzoate 7,9-O-isopropylideneketal (6) (acetonide derivative). To a stirred solution of **5** (305.8 mg, 0.524 mmol) in 2 ml of acetone, 2,2-dimethoxypropane (2.85 ml, 23.3 mmol) and camphorsulfonic acid (2.03 mg, 8.74 μmol) were added at ambient temperature. After stirring for 28 h under argon atmosphere, the solution was neutralized by the addition of powdered NaHCO$_3$. The mixture was filtered and the salts were washed with EtOAc. The filtrate and the washings were combined and partitioned between EtOAc and saturated NaHCO$_3$ solution. The EtOAc layer was washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and then concentrated under reduced pressure. The resulting residue was purified by column chromatography (EtOAc/n-hexane 1:2) to afford *erythro*-**6** (65.5 mg, 20.3%) and *threo*-**6** (175.8 mg, 54.4%). *Erythro*-acetonide: ^{1}H NMR (CDCl$_3$): δ 1.50 (3H, s, C-CH$_3$), 1.67 (3H, s, C-CH$_3$), 3.73 (6H, s, B-OCH$_3$), 3.75 (6H, s, A-OCH$_3$), 4.06 (1H, dd, J = 11.71, 5.61, H-9a), 4.11 (1H, dd, J = 11.71, 8.29, H-9b), 4.33 (1H, ddd, J = 11.71, 8.42, 5.61, H-8), 4.93 (1H, d, J = 9.02, H-7), 6.60 (1H, dd, J = 15.85, 7.56, H-8'), 6.67 (2H, s, H-2', H-6'), 6.75 (2H, s, H-2, H-6), 7.34 (1H, d, J = 15.86, H-7'), 7.50 (2H, t, J = 7.81, BzH-3, BzH-5), 7.62 (1H, tt, J = 7.32, 1.38, BzH-4), 8.22 (2H, dd, J = 8.29, 1.22, BzH-2, BzH-6), 9.66 (1H, d, J = 7.81, H-9'). *Threo*-acetonide: ^{1}H NMR (CDCl$_3$): δ 1.59 (3H, s, C-CH$_3$), 1.66 (3H, s, C-CH$_3$), 3.74 (6H, s, B-OCH$_3$), 3.77 (6H, s, A-OCH$_3$), 4.09 (1H, dd, J = 12.93, 2.20, H-9a), 4.15 (1H, dd, J = 12.93, 1.96, H-9b), 4.40 [1H, d (with shoulder), J = 1.95, H-8], 5.11 (1H, d, J = 1.46, H-7), 6.60 (1H, dd, J = 15.85, 7.80, H-8'), 6.70 (2H, s, H-2', H-6'), 6.82 (2H, s, H-2, H-6), 7.35 (1H, d, J = 15.85, H-7'), 7.49 (2H, t, J = 7.81, BzH-3, BzH-5), 7.61 (1H, t, J = 7.44, BzH-4), 8.21 (2H, dd, J = 7.32, 1.22, BzH-2, BzH-6), 9.65 (1H, d, J = 7.60, H-9').

Syringylglycerol-8-O-4'-sinapaldehyde ether 4-benzoate (5). *Erythro*-**6** (65.5 mg, 0.113 mmol) was dissolved in 90% AcOH (1.5 ml). The solution was stirred at room temperature for 5 h 20 min under argon atmosphere. The reaction solution was partitioned between EtOAc and saturated brine. The organic layer was washed with saturated NaHCO$_3$ solution and brine (twice), dried over anhydrous Na$_2$SO$_4$, and evaporated in vacuo to give *erythro*-**5** (61.2 mg, 100%). *Threo*-**6** (175.8 mg, 0.304 mmol) was transformed similarly into *threo*-**5** (145.9 mg, 89.2%). Both isomers were used for the next reaction without further purification.

Syringylglycerol-8-O-4'-(sinapyl alcohol) ether 4-benzoate (7). A stirred solution of *erythro*-**5** (61.2 mg, 0.114 mmol) in a mixture of THF (0.3 ml) and MeOH (0.5 ml) was cooled to 0°C, and then NaBH$_4$ (128.7 mg, 3.402 mmol) was added into the cold solution. Stirring was continued at the same temperature for 30 min. The reaction mixture was partitioned between EtOAc and brine. The organic layer was washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and then evaporated in vacuo to give *erythro*-**7** (56.8 mg, 92.5%). Similarly, *threo*-**5** was transformed into *threo*-**7** (145.4 mg, 99.3%). Both isomers were used for the next reaction without further purification.

Syringylglycerol-8-O-4'-(sinapyl alcohol) ether (8). To a stirred solution of *erythro*-**7** (56.8 mg, 0.105 mmol) in benzene (1.5 ml, freshly distilled over anhydrous CaCl$_2$), n-butylamine (520 μl, 5.26 mmol) was added under argon atmosphere. After stirring at room temperature for 70.5 h, the reaction solution was partitioned between EtOAc and 3% HCl solution. The organic layer was washed twice with saturated brine, dried over anhydrous Na$_2$SO$_4$, and concentrated in vacuo. The residue was further purified by preparative TLC (EtOAc/n-hexane 4:1, ×2) to give *erythro*-**8** (30.2 mg, 65.8%). The benzoyl group of *threo*-**7** (145.4 mg, 0.269 mmol) was similarly removed and the product was purified by means of column chromatography (EtOAc/n-hexane 10:1) to afford *threo*-**8** (61.8 mg, 52.6%). *Erythro*-SGSE: ^{1}H NMR (acetone-d_6 + D$_2$O): δ 3.49 (1H, dd, J = 11.96, 3.42, H-9a), 3.82 (6H, s, A-OCH$_3$), 3.887 (1H, dd, J = 11.95, 5.61, H-9b), 3.89 (6H, s, B-OCH$_3$), 4.20–4.24 [1H, overlap (o), H-8], 4.23 (2H, dd, J = 5.12, 1.46, H-9'), 4.99 (1H, d, J = 4.63, H-7), 6.39 (1H, td, J = 15.86, 5.25, H-8'), 6.57 (1H, td, J = 15.86, 1.46, H-7'), 6.73 (2H, s, H-2, H-6), 6.82 (2H, s, H-2', H-6'). EI-MS m/z (%): 436 (4.3) [M]$^+$, 418 (5) [M − H$_2$O]$^+$, 210 (100), 93 (3.7), 77 (9.6). *Threo*-SGSE: ^{1}H NMR (acetone-d_6 + D$_2$O): δ 3.35 (1H, dd, J = 12.20, 3.41, H-9a), 3.70 (1H, dd, J = 12.20, 3.66, H-9b), 3.81 (6H, s, A-OCH$_3$), 3.92 (6H, s, B-OCH$_3$), 4.00 (1H, td, J = 7.07, 3.54, H-8), 4.22 (2H, dd, J = 5.36, 1.34, H-9'), 4.99 (1H, d, J = 7.07, H-7), 6.39 (1H, td, J = 15.86, 5.25, H-8'), 6.56 (1H, td, J = 16.00, 1.47, H-7'), 6.78 (2H, s, H-2, H-6), 6.82 (2H, s, H-2', H-6'). EI-MS m/z (%): 436 (3.5) [M]$^+$, 418 (3.9) [M − H$_2$O]$^+$, 210 (100), 93 (3), 77 (6).

Preparation of **8** by dehydrogenation of sinapyl alcohol by FeCl$_3$.[15] 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$_2$O (25.8 mg, 0.095 mmol) in H$_2$O (0.3 ml) was added dropwise 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 dropwise 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$_2$SO$_4$, and then evaporated to dryness in vacuo. The residue was purified by preparative TLC (5% MeOH in CH$_2$Cl$_2$) to give SGSE (25.8 mg, 28.8%) as a mixture of the *erythro* and *threo* isomers. 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 (×5)] to give *threo*-SGSE (R_f 0.27, 2.60 mg) and *erythro*-SGSE (R_f 0.25, 2.63 mg). The diastereomeric identification was achieved by comparison of ^{1}H NMR spectra and HPLC chromatograms with those of the *erythro* and *threo* SGSEs (**8**) synthesized as before. *Erythro*-SGSE: ^{1}H NMR (acetone-d_6): δ 3.38–3.42 (1H, o, 9-OH), 3.46 (1H, dd, $J = 7.08$, 3.44, H-9a), 3.81 (6H, s, A-OCH$_3$), 3.81–3.84 (2H, o, 9′-OH, H-9b), 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, H-6), 6.82 (2H, s, H-2′, H-6′), 7.07 (1H, s, 4-OH). EI-MS *m/z* (%): 436 (3.9) [M]$^+$, 418 (11.1) [M−H$_2$O]$^+$, 210 (100), 93 (3.7), 77 (9). *Threo*-SGSE: ^{1}H NMR (acetone-d_6): δ 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-OCH$_3$), 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, H-6), 6.82 (2H, s, H-2′, H-6′), 7.10 (1H, s, 4-OH). EI-MS *m/z* (%): 436 (3.9) [M]$^+$, 418 (12) [M−H$_2$O]$^+$, 210 (100), 93 (4), 77 (6).

[8-^{14}C]Coniferyl alcohol and [8-^{14}C]sinapyl alcohol. Both radiolabeled compounds were synthesized by literature methods.[16–18]

Plant materials

Eucommia ulmoides plants obtained from Sanyo Nouen were maintained at the Faculty of Agriculture, Kagawa University.

Feeding experiments

Excised young shoots of *E. ulmoides* were administered 25 mM [8-^{14}C]sinapyl alcohol (6.65 MBq/mmol) in K-Pi buffer (0.1 M, pH 7) (140 µl each) and then allowed to metabolize for 3 h at 25°C in an environment-controlled room. Leaves and stems were divided, immediately frozen in liquid nitrogen, individually freeze-dried, reduced into small pieces (~2 mm) by means of scissors, and extracted five times with MeOH at 65°C. The MeOH solutions were combined, concentrated to small volume (one-tenth), and to this was added water (×5 of the residual volume). The whole was centrifuged (200 *g*, 20°C for 20 min) and the supernatant was partitioned between EtOAc (containing a mixture of unlabeled *erythro*- and *threo*-SGSE from the chemical synthesis as cold carriers) and water. The aqueous layers were twice extracted with EtOAc. The EtOAc solutions were combined, washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and evaporated to dryness in vacuo. This fraction was named as "organic layer."

The aqueous layer was freeze-dried and the resulting powder was treated with a mixture of cellulase [(700 mg) (Wako; from *Trichoderma viride*, 1000 units/mg)] and β-glucosidase [(200 mg) (Oriental Yeast; from sweet almond, 34 units/mg)] in sodium acetate buffer (20 mM, pH 4.5) for 24 h at 50°C under nitrogen atmosphere.[19] The whole was extracted with EtOAc (containing the mixture of unlabeled *erythro*- and *threo*-SGSE from the chemical synthesis as cold carriers), the aqueous layers were washed twice with EtOAc. The combined EtOAc solution was washed with saturated brine, dried over anhydrous Na$_2$SO$_4$, and evaporated in vacuo. This fraction was named as "aqueous layer."

Both of the EtOAc extracts from the organic and aqueous layers were reconstituted in MeOH (500 µl) and filtered. An aliquot (10 µl) of the filtrate was subjected to reversed-phase (C$_{18}$) HPLC. The eluate was collected in scintillation vials every 30 s for liquid scintillation counting. Furthermore, larger aliquots were repeatedly applied to the C$_{18}$ reversed-phase column in order to separate *erythro*- and *threo*-SGSE on a preparative scale. MeOH solubles of the two fractions were then subjected to chiral column HPLC and the eluate was collected at 1-min intervals for liquid scintillation counting.

Enzyme preparation

Insoluble enzyme was prepared by the method of Davin et al.[20] All enzyme preparations were carried out at 4°C unless otherwise stated. Defoliated young shoots of *E. ulmoides* (20–40 cm long, 137 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 1-liter beaker containing K-Pi buffer (50 mM, pH 7, 500 ml) and Triton X-100 (1%). After 4 h of stirring, the homogenate was filtered through one layer of nylon cloth, and the insoluble residue was rinsed with cold distilled water (1.5 l) and squeezed to remove excess fluid. The insoluble residue was then extracted with 0.5 M NaCl (500 ml) for 16 h, filtered through one layer of nylon cloth, rinsed with 2 l cold distilled water, and squeezed as before. The moist residue was reground

with a mortar and pestle to afford an insoluble stem residue (99 g). This insoluble enzyme preparation (free of soluble and ionically bound enzymes) was assayed immediately.

Enzyme assay

Each assay consisted of the insoluble enzyme preparation (3.5 g) suspended in K-Pi buffer (50 mM, pH 7, 12 ml). The reaction was initiated by addition of solutions of 15 mM [8-^{14}C]coniferyl alcohol (4.53 MBq/mmol) and 15 mM [8-^{14}C]sinapyl alcohol (4.03 MBq/mmol) in K-Pi buffer (50 mM, pH 7, 0.7 ml). After normal incubation at 30°C for various time intervals, glacial AcOH (0.7 ml) was added. The assay mixture was then extracted with EtOAc (30 ml) containing the mixture of unlabeled *erythro-* and *threo-*SGSEs from dehydrogenation as cold carriers (100 μg). 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 [benzene/acetone 2:1 (×5)] to isolate [^{14}C]SGSE, which was then reconstituted in MeOH (100 μl) and filtered. An aliquot (10 μl) of the filtrate was applied to C_{18} column reversed-phase HPLC. The eluate was collected in scintillation vials every 30 s for liquid scintillation counting. Diastereomeric separation and enantiomeric analyses were similarly performed as described above.

Results and discussion

Synthesis of *erythro-* and *threo-*syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSEs, **8**) and their stereochemistry

As shown in Fig. 1, SGSEs (**8**) were synthesized from α-bromoacetosyringone-4-benzoate with sinapaldehyde by the methods of Adler and Eriksoo,[21] Kawai et al.,[22] and Katayama et al.[23] Reduction of **3** gave 7,9-diol **4** as a mixture of *erythro* and *threo* isomers, which were transformed into acetonide derivatives **6**, first because the isomers of **6** can be separated on a preparative scale by silica gel column chromatography eluted with EtOAc/*n*-hexane (1:2, v/v), and second because this reaction allows the identification of the *erythro* and *threo* isomers. The protons carried by C_7 of the *erythro* and *threo* acetonide isomers have characteristic coupling constants ($J = 9.02$ and 1.46 Hz, respectively), which is due to the different conformations of *erythro-***6** and *threo-***6**. The most stable conformation of the aryl (A) and aryl (B) oxy groups of *erythro-*acetonide is the di-*equatorial* position with a H-C_7-C_8-H dihedral angle (θ) of about 180°. In contrast, the *equatorial* and *axial* positions, respectively, are favored in the *threo-*acetonide with a dihedral angle of 60°. According to the vicinal Karplus correlation,[24] a coupling constant of 9.02 Hz corresponds to $\theta = 160°$, while 1.46 Hz correspond to $\theta = 60°$. Therefore, the former C_7-H was

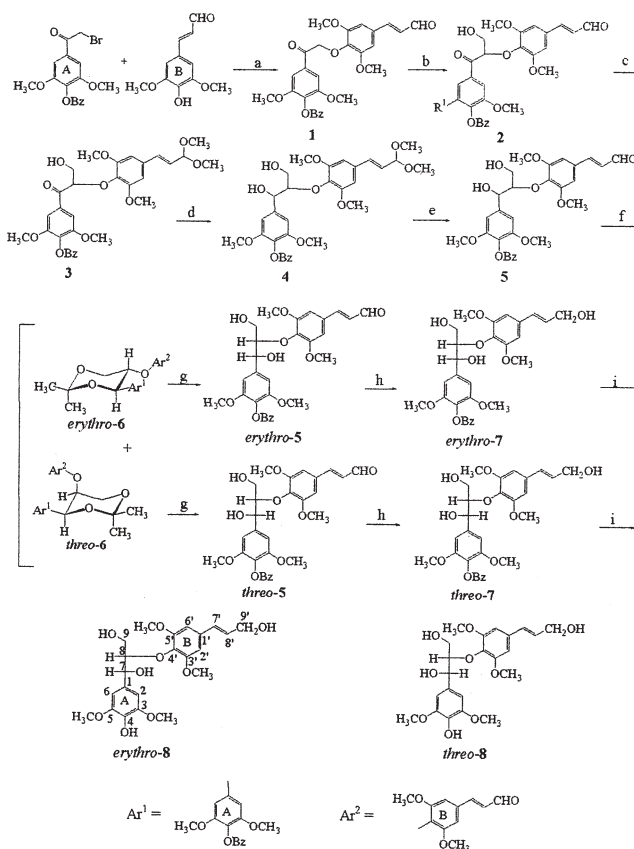**Fig. 1.** Synthetic route of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ether (SGSE). Step a K_2CO_3/KI/DMF/rt, step b-$(CH_2O)_n$-/K_2CO_3/DMSO/rt, step c $CH(OCH_3)_3$/*p*-toluenesulfonic acid/THF-MeOH/rt, step d $NaBH_4$/THF-MeOH/0°C, step e 90%AcOH/rt, step f $(CH_3)_2C(OCH_3)_2$/camphorsulfonic acid/acetone/rt/separation of diastereomers by column chromatography, step g 90% AcOH/rt, step h $NaBH_4$/THF-MeOH/0°C, step i *n*-butylamine/benzene/rt. *Bz*, (C_6H_5CO-)

identified as the *erythro* isomer and the latter as the *threo* form.

The final products, i.e., the SGSEs with 7,9-diol structures, differed from the acetonide derivatives **6** in that the coupling constants at C_7-H of *erythro-***8** ($J = 4.63$ Hz) was smaller than that of *threo-***8** ($J = 7.07$ Hz).

A mixture of *erythro-* and *threo-*SGSEs (**8**) was also obtained in preference to (±)-syringaresinol, an 8-8′ lignan, by a one-step reaction, dehydrogenative dimerization of sinapyl alcohol with $FeCl_3$ in dioxane–H_2O (10:1).[15] The diastereomers were separated on a small scale by preparative TLC [benzene/acetone 2:1 (×5)]. Two bands (R_f 0.27 and 0.25) were detected and identified as the *threo* and *erythro* isomers, respectively, by comparison with those obtained by the above chemical synthesis (^{1}H NMR and HPLC).

The diastereomeric ratio of SGSEs obtained by the two methods were compared (Table 1). At the $NaBH_4$ reduction of **3** (step d) in the synthetic route (Fig. 1), formation of *threo-***4** (72.9%) dominated over that of *erythro-***4** (27.1%). According to the Felkin-Anh model (Fig. 2), the ketone of compound **3** has two faces which hydride ions attack.

Attack through the space between medium and small substituents (face a) yields the *threo* isomer, whereas the *erythro* isomer results from attack of face b. A steric hindrance between the aryl and CH_2OH groups forming part of face b explains the lower yield of the *erythro* isomer.

In contrast, the diastereomeric composition of SGSE prepared by the one-step reaction (dehydrogenation of sinapyl alcohol with $FeCl_3$ in dioxane–H_2O) showed a higher fraction of the *erythro* isomer (77.9%) than of the *threo* form (22.1%).

Biosynthesis of SGSE

[8-^{14}C]Sinapyl alcohol was administered to excised young shoots of *Eucommia ulmoides*. After 3 h, leaves and stems were divided and separately extracted with hot MeOH. The extracts were partitioned between EtOAc ("organic layer") and water ("aqueous layer"), in which free neolignans and neolignan glucosides, respectively, accumulated. The free neolignans were analyzed by HPLC. The neolignan glucosides were hydrolyzed by a mixture of β-glucosidase and cellulase to liberate neolignan aglycones, which were extracted with EtOAc and subjected to HPLC analysis. Reversed-phase radiochromatograms of all four fractions (organic and aqueous layers derived from stems and leaves) showed that [8-^{14}C]sinapyl alcohol was incorporated into free SGSE (organic layer) and into SGSE-glucosides (aqueous layer). Furthermore, it was incorporated preferentially into the *erythro* isomers of SGSE and the SGSE-glucosides (Table 2). In stems, the incorporation into free *erythro*-[^{14}C]SGSE was found even in a small amount (0.037%), whereas that into free *threo* isomer was at background level of radioactivity under this condition. *Erythro*-[^{14}C]SGSE glucosides (0.047%) dominated over the *threo* isomers [0.007%; percent diastereomeric excess (% d.e.) 74.0] in stems. In leaves, free *erythro*-[^{14}C]SGSE (0.500%) dominated over the *threo* isomer (0.206%; % d.e. 41.6) and *erythro*-[^{14}C]SGSE glucosides (1.692%) occurred at higher levels than the corresponding *threo*-forms (0.177%; % d.e. 81.0). The level of sinapyl alcohol incorporation into SGSE glucosides was higher in leaves (1.869%) than in stems (0.054%).

Therefore, it was suggested that in the plant, two molecules of sinapyl alcohol diastereoselectively gave *erythro*-
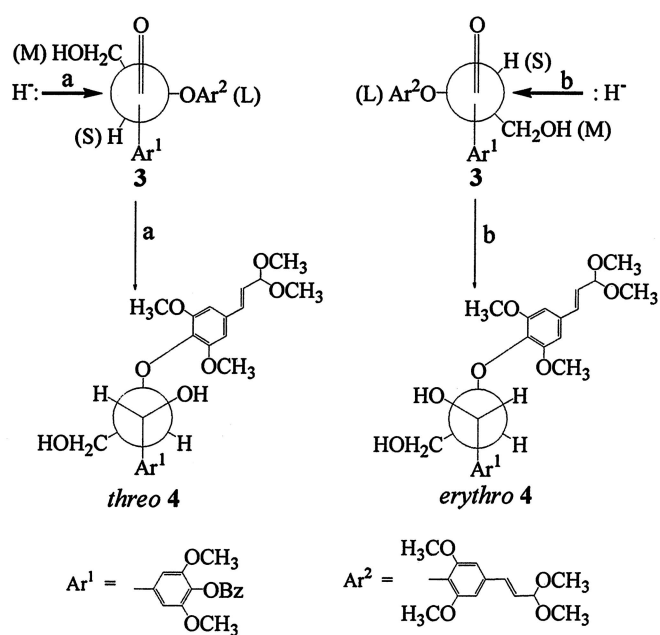
Table 1. Ratios of *erythro* and *threo* isomers of syringylglycerol-8-O-4′-(sinapyl alcohol) ether (SGSE) obtained by two preparation methods, a stepwise chemical synthesis (Fig. 1) and a one-step dehydrogenation with $FeCl_3$ in dioxane–water

Isomer	Chemical syntheses[a] (acetonide derivative, 4)	Dehydrogenation[b] (dioxane–water 10 : 1)
Erythro	27.1% (65.5 mg)	77.9%
Threo	72.9% (175.8 mg)	22.1%

[a] Gravimetry
[b] Calculated from peak area of HPLC (280 nm)

Fig. 2. Felkin-Anh model for the reduction of compound **3** (see Fig. 1) with NaBH₄ (*L*, *M*, and *S* are large, medium, and small substituents, respectively)

Table 2. Percentage incorporation, and diastereomeric and enantiomeric composition of [^{14}C]syringylglycerol-8-O-4′-(sinapyl alcohol) ether (SGSE), following administration of [8-^{14}C]sinapyl alcohol to excised shoots of *Eucommia ulmoides* and subsequent metabolism for 3 h

Fractions and products	Stems			Leaves		
	A	B	C	A	B	C
Organic layers						
Total SGSE	0.037			0.706		
Erythro	0.037	100% (100)	(+) 9.1	0.500	70.8% (41.6)	(−) 15.9
Threo	nd			0.206	29.2%	(−) 7.4
Aqueous layers						
Total SGSE (from glucosides)	0.054			1.869		
Erythro	0.047	87.0% (74.0)	(−) 32.0	1.692	90.5% (81.0)	(−) 25.0
Threo	0.007	13.0%	(−) 18.3	0.177	9.5%	(−) 16.4

nd, Not detected under these conditions; A, percent incorporation; B, diastereomeric composition (percent diastereomeric excess); C, percent enantiomeric excess

Table 3. Formation of [^{14}C]syringylglycerol-8-O-4′-(sinapyl alcohol) ether (SGSE) and its stereochemistry following incubation of an insoluble enzyme preparation of *Eucommia ulmoides* with a mixture of [8-^{14}C]coniferyl and [8-^{14}C]sinapyl alcohols at various time intervals

Isomer	Enzyme assay (nmol/mg residue)					
	2 min	10 min	30 min	60 min	120 min	Denatured (60 min)
Erythro	nd	0.58	4.85	5.10 (+) 21.4[a]	3.34	nd
Threo	nd	0.22	1.45	0.48 (−) 4.3[a]	0.33	nd
Diastereomeric ratio	–	2.6:1	3.34:1	10.6:1	10.1:1	–
Diastereomeric excess(%)	–	45.0	54.0	82.8	82.0	–

For a control experiment, enzymes were denatured by heating in boiling water for 10 min
[a] Percent enantiomeric excess was analyzed at 60 min when the percent diastereomeric excess reached the highest value

SGSE rather than *threo*-SGSE, and that both diastereomers were further transformed into their glucosides. The *erythro/threo* ratios of this syringyl 8-O-4′ neolignan were higher than those [1.5–3.5 (60.0%–77.8%; 20.0% d.e.–55.6% d.e.)] of arylglycerol-β-aryl ether moieties (8-O-4′ dilignol moieties) in hardwood lignin which consists of varying ratios of guaiacyl and syringyl groups (methoxyl group content 1.0–1.5).[25] Nonselective formation of SGSE and subsequent selective transformation into the glucosides appears unlikely. It remains obscure whether the dominance of glucosides of the *erythro* isomer was simply due to the greater amounts of *erythro*-SGSE available for reaction, or whether it was the result of a preference of the *erythro* form in the glucosylation. This diastereoselective formation of *erythro* isomers is consistent with our previous results concerning GGSE, the 8-O-4′ neolignan consisting of coniferyl and sinapyl alcohol moieties.[7]

The enantiomeric composition of the *erythro* and *threo* SGSEs produced was determined by chiral column HPLC; the percent enantiomeric excess (% e.e.) is shown in Table 2. In the stems, (+)-*erythro*-[^{14}C]SGSE from the organic layer was favored with 9.1% e.e., whereas in the aqueous layer (−)-*erythro*- and (−)-*threo*-[^{14}C]SGSE glucosides dominated (32.0% e.e. and 18.3% e.e., respectively). In the leaves, (−)-*erythro*- and (−)-*threo*-[^{14}C]SGSEs from the organic layer were selectively produced with 15.9% e.e. and 7.4% e.e., respectively, and the glucosides of these enantiomers in the aqueous layer predominated with 25.0% e.e. and 16.4% e.e., respectively. Opposite optical activities of stem-derived *erythro*-[^{14}C]SGSE between the organic and aqueous 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 remains obscure, although there is a possibility that selective formation of the free (−)-*erythro*-SGSE was followed by its selective transformation to (−)-*erythro*-SGSE glucoside. The observation of organ-dependent 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 plicata*). Each class is specific for one enantiomer of the substrate.[26] This finding supports our suggestion.

In vitro investigations were undertaken in addition to the feeding experiments described above. A mixture of [8-^{14}C]coniferyl and [8-^{14}C]sinapyl alcohols, as well as [8-^{14}C]sinapyl alcohol only were separately incubated with cell-free extracts[6] of *E. ulmoides*. The enzymatic reaction was initiated by the addition of H$_2$O$_2$, and the solution was incubated at 30°C for 60 min. However, [^{14}C]SGSE was not detected (data not shown).

Because these in vitro experiments using a soluble enzyme fraction did not provide useful insights, we next examined the reaction with an insoluble (cell wall) residue. Insoluble enzyme preparations from defoliated young shoots of *E. ulmoides* were incubated with a mixture of [8-^{14}C]coniferyl and [8-^{14}C]sinapyl alcohols for various periods. 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 30 min. The highest % d.e. (82.8) was found at 60 min, 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) ether (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. On the other hand, the predominant diastereomer of GGSE and SGCE was the *threo* isomer and the diastereomeric excess of GGSE was higher than that of SGCE.

The diastereoselective formation of *erythro*-SGSE in the enzymatic reactions was consistent with that in the feeding experiments. *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 enzymatic reactions [(−) 4.3] was in accord with that of free *threo*-SGSE derived from leaves in the feeding experiments [(−) 7.4].

This study reports the first example of diastereoselective formation of syringyl 8-O-4′ neolignan with optical activity from two sinapyl alcohols.

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