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Stereochemistry of *erythro*- and *threo*-syringylglycerol-*8-O-4′*-(sinapyl alcohol) ethers and their enzymatic formation with optical activity in *Eucommia ulmoides*

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Abstract: In this study an enzymatic system, horseradish peroxidase (HRP)-hydrogen peroxide was used as catalysts for the enzymatic formation of enantiospecific syringulglycerol-8-*O*-4′ (sinapyl alcohol) ethers (SGSEs) from enzyme preparations of *Eucommia ulmoides* with sinapyl alcohol (SA) as a monolignol precursor. Reversed phase HPLC analysis of the *erythro*- and *threo*-SGSE showed that the ratio of *erythro*: *threo* was 47: 53. Both isomers were isolated by HPLC and their structural confirmation was done by ^{1}H NMR spectra. Chiral column HPLC analysis of the *erythro*- and *threo*-SGSE showed that their enantiomeric compositions were as follows: (+)-*erythro*: (-)-*erythro* = 46.7:53.3 (6.6% e.e), and (+)-*threo*: (-)-*threo* = 45.2: 54.8 (9.6% e.e). To elucidate the stereochemistry of *erythro* and *threo*- SGSEs, we have determined absolute configurations of the four stereoisomers, (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-SGSEs as (7*R,* 8*S*), (7*S,* 8*R*), (7*S,* 8*S*), and (7*R,* 8*R*), respectively, by Mosher's method through the ^{1}H NMR spectroscopy of (+)-(*R*)-α-methoxy- α- trifluoromethylphenylacetate (MTPA) esters of α, γ and γ' positions.

Key words: Stereochemistry, SGSE, *erythro*, *threo***,** MTPA, soluble enzyme, *Eucommia ulmoides*

الكيمياء المجسمة لاثيرات erythro- and threo-syringylglycerol-8-O-4′-(sinapyl alcohol) وتركيبها الإنزيمي بواسطة النشاط الضوئي في نوع يوكوميا المويديس Eucommia ulmoides.

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الملخص:تم في هذه الدراسة استخدام منظومة انزيمية ل horseradish peroxidase (HRP)-hydrogen peroxide كوسطاء للتكوين إثيرات enantiospecific syringulglycerol-8-*O*-4′ (SGSEs) (sinapyl alcohol) من تحضيرات إنزيم ال*Eucommia ulmoides* باستخدام كحول ال sinapyl alcohol (SA) كبادئ للmonolignو . أظهر التحليل الكروماتوجرافي العكسي بالضغط العالي (HPLC) لل *erythro*- and *threo*-SGSE أن نسبة *erythro*: *threo* كانت 47: 53.تم اتخلاص كل من مشابهي الانزيم بواسطة التحليل الكروماتوجرافي بالضغط العالي (HPLC) وتم تأكيد تركيبهم باستخدام التحليل الطيفي ^{1}H NMR spectra. أظهر تحليل *erythro*- and *threo*-SGSE بال Chiral column HPLC أن تركيبهما كما يلي: (+)-*erythro*: (-)-*erythro* = 46.7:53.3 (6.6% e.e), and (+)-*threo*: (-)-*threo* = 45.2: 54.8 (9.6% e.e). لتحقيق التركيب الكيميائ المجسم ل *erythro*- and *threo*-SGSEs حددنا التركيب المطلق للأربع مشابهات (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-SGSEs كونه (7*R,* 8*S*), (7*S,* 8*R*), (7*S,* 8*S*), and (7*R,* 8*R*) بالترتيب بطريقة Mosher's باستخدام التحليل الطيفي ^{1}H NMR spectra للأسترات (+)-(*R*)-α-methoxy- α- trifluoromethylphenylacetate (MTPA) ذات المواقع α, γ and γ'.

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Introduction

Arylglycerol-*β*-aryl ethers are one of the most important substructures in lignin as well as lignans and neolignans. The biosynthesis of lignin has been advanced; phenolic phenylpropanoides occur lignin via oxidative processes catalyzed by enzymes such as peroxidase, which exchange the phenols into phenoxy radicals by an electron abstraction which is followed by C-C and carbon-oxygen bond formation (Ayres and Loike, 1990). Formation of chiral centers has been occurring in this circumstance. In addition biosynthesis of neolignans, especially syringyl 8-O-4'has a little known in contrast to guaiacyl one. Enantiomerically pure, (±)-α-oxo-guaiacylglycerol- *β-(*vanillic acid) ether which formed from bio-degradation and α-ketonised racemate guaiacylglycerol- *β-(*vanillic acid) ethers have been reported by Katayama and others (Katayama et al., 1986; Katayama and Sogo, 1989). Recently Katayama et al. (2005) 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 (Dale et al., 1969; Dale and Mosher, 1973; Yamaguchi, 1985). They suggested that (+)-*erythro*-GGCE was formed by the selective water addition to the (8*R*)-enantiomer of the racemic quinonemethide. Lignans and neolignans (except for 9,9′-deoxy ones) are biosynthesized from coniferyl alcohol (which contains 4-hydroxy-3-methoxyphenyl group, so-called guaiacyl group) or sinapyl alcohol (which contains 4-hydroxy-3,5-dimethoxyphenyl group, so-called syringyl group). Biosynthesis of guaiacyl lignans/neolignans from coniferyl alcohol have been studied much more than that of syringyl lignans/neolignans derived from sinapyl alcohol. Especially there were no studies on syringyl-8-*O*-4′ neolignans information. Therefore, Lourith et al. (2005) found the formation of optically active *erythro* and *threo*-SGSEs by feeding experiments using radiolablled SA and the excised shoots of *E. ulmoides* as well as by the incubation of a mixture of SA and CA with an insoluble enzyme preparation from the plant. Recently, it have been reported (Alam et al., 2009) that incubation of SA with a cell-free extract (a soluble enzyme preparation) of *E. ulmoides* in the presence of hydrogen peroxide (H_2O_2) gave (-)-*erythro*- and (-)-*threo*-SGSEs, whereas incubation of SA with a cell-wall residue (an insoluble enzyme preparation) in the absence of H_2O_2 (+)-*erythro*- and (-)-*threo*-SGSEs. Tuning the enzymatic reaction to give dimers, and the use of metal complexes which mimic the enzymatic reaction allows the observation of the stereochemical course at the stereocenters and gives information related to the mechanism of lignin formation in nature. To clarify the formation mechanism of SGSE, stereochemistry of the four stereoisomers, (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*, of SGSEs, were required. However, the absolute configurations were unknown. In this paper, we report determination of their absolute configurations by Mosher's method [^{1}H NMR spectroscopy of (*R*)-(+)-(MTPA) esters of SGSEs] and the related empirical rules (Katayama et al., 2000). In this study an enzymatic system, horseradish peroxidase (HRP)-hydrogen peroxide was used as catalysts for the enzymatic formation of enantiospecific syringulglycerol-8-O-4' (sinapyl alcohol) ethers (SGSEs) from enzyme preparations of *E. ulmoides* with sinapyl alcohol (SA) as a monolignol precursor. The (-)-*erythro* isomer was more favored than the (-)-*threo* one, with optical activity.

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 (0.25mm and 0.5mm thickness, respectively). NMR spectra (600 MHz) were measured on a JEOL JNM – DELTA – 600 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. 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.

Plant materials

Eucommia ulmoides (Tochu) plants (Figure 1) were obtained from Sanyo Nouen Inc. and maintained at Faculty of Agriculture, Kagawa University, Japan.

Figure 1. *Eucommia ulmoides* young shoots with leaves.

Chemical synthesis

Monolignol (sinapyl alcohol) is the major precursor of syringyl lignans and neolignans (SGSE) was synthesized by the literature methods (Lourith et al., 2005; Tanahashi et al., 1976) and their diastereomer (*erythro* and *threo* SGSEs) were separated by TLC, subsequently quantification was done by high-performance liquid chromatography (HPLC) and their structures were determined by ^{1}H NMR, ^{13}C NMR. Enantiomeric separation of *erythro* and *threo* SGSEs has been done by chiral column HPLC as (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-SGSEs for transformed there to (+)-MTPA esters derivatives. Diazomethane (CH_2N_2) has been prepared by the method of

Bore and Backer (Bore and Backer, 1963) using carbitol with *ρ*-tolylsulfonyl meththyl nitrosamide in ether (Et_2O). Methylation of the phenolic hydroxyl group of SGSEs and tri-(R)-MTPA esters were prepared by a method described in the literature (Dale and Mosher, 1973; Katayama et al., 2000).

Preparation of (+)-*erythro*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ethers

To a stirred solution of (+)-*erythro*-SGSE (4.6 mg, 10.5384µmol) in MeOH (0.5ml), a yellow color of freshly prepared diazomethane (CH_2N_2) solution (3ml) was added at 0^o C temperature with a small size of staraba. Reaction condition was observed by analytical TLC [benzene/acetone = 1:1 (x1)] after 30min interval. The reaction was quenched by the addition of a small amount of granulated Na_2SO_4. The reaction mixture was then filtered over Na_2SO_4 with short column and wash by CH_2Cl_2. The filtered solution was then evaporated to dryness in vacuo. The residue was purified by preparative TLC [benzene/acetone = 1:1 (x1)] to give (+)-*erythro*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (3.48 mg, yield 96.90%). Structure was determined by ^{1}H NMR spectra. Other stereoisomers [(-)-*erythro,* (+)-*threo* and (-)-*threo*-SGSEs] also converted to 3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ethers by the similarly to above one and confirmed by ^{1}H NMR spectra .

4-*O*-methyl ether of (+)-*erythro*-SGSE

^{1}H NMR delta ($CDCl_3$): 3.923 (s, 7-OH), 3.871 (s, 9-OH), 3.830 (s, 9′-OH), 3.850 [(exchangeable), 6H, *s*, 3 & 5-OCH_3], 3.907 [(exchangeable), 6H, *s*, 3′ & 5′-OCH_3], 3.822 (3H, s, 4-OCH_3), 4.123 (1H, m, 8-H), 5.040 (1H, d, *J*=8.82, 7-H), 6.590 (1H, *d*, *J*=15.96, 7′-H), 6.365 (1H, m, 8′-H), 4.357 (2H, d, *J*=4.68, 9′-H), 6.569 [(exchangeable), 2H, s, 2 & 6-H], 6.687 [(exchangeable), 2H, *s*, 2′ & 6′-H].

4-*O*-methyl ether of (–)-*erythro*-SGSE

^{1}H NMR delta ($CDCl_3$): 3.923 (s, 7-OH), 3.871 (s, 9-OH), 3.490 (o, 9′-OH), 3.851 [(exchangeable), 6H, *s*, 3 & 5-OCH_3], 3.907 [(exchangeable), 6H, s, 3′ & 5′-OCH_3], 3.821 (3H, s, 4-OCH_3), 4.128 (1H, m, 8-H), 5.039 (1H, d, *J*= 8.52, 7-H), 6.510 (1H, d, *J*=16.04, 7′-H), 6.344 (1H, m, 8′-H), 4.345 (2H, dd, *J* = 12.24, 7.50, 9′-H), 6.568 [(exchangeable) 2H, s, 2 & 6-H], 6.687 [(exchangeable) 2H, s, 2′ & 6′- H]

4-*O*-methyl ether of (+)-*threo*-SGSE

^{1}H NMR delta ($CDCl_3$): 2.691 (s, 7-OH), 2.612 (s, 9-OH), 2.656 (d, *J* = 3.54, 9′-OH), 3.872 [(exchangeable), 6H, *s*, 3 & 5-OCH_3], 3.925 [(exchangeable), 6H, s, 3′ & 5′-OCH_3], 3.830 (3H, s, 4-OCH_3), 4.314 (1H, m, 8-H), 5.037 (1H, d, J=8.52, 7-H), 6.568 (1H, d, *J* =15.66, 7′-H), 6.321 (1H, dt, *J* = 15.81, 5.22, 8′-H), 4.352 (2H, d, *J*=4.38, 9′-H), 6.665 [(exchangeable), 2H, s, 2 & 6-H], 6.703 [(exchangeable), 2H, *s*, 2′ & 6′-H].

4-*O*-methyl ether of (–)-*threo*-SGSE

^{1}H NMR delta ($CDCl_3$): 2.690 (s, 7-OH), 2.611 (s, 9-OH), 2.655 (d, *J*=5.22, 9′-OH), 3.871 [(exchangeable), 6H, s, 3 & 5-OCH_3], 3.924 [(exchangeable), 6H, s, 3′ & 5′-OCH_3], 3.830 (3H, s, 4-OCH_3), 4.3103 (1H, m, 8-H), 5.039 (1H, d, *J*=8.52, 7-H), 6.567 (1H, d, *J*=15.90, 7′-H), 6.320 (1H, dt, *J*=15.90, 5.41, 8′-H), 4.347 (2H, d, *J*=5.22, 9′-H), 6.664 [(exchangeable), 2H, s, 2 & 6-H], 6.703 [(exchangeable), 2H, s, 2′ & 6′-H].

Preparation of (+) -*erythro*-tri-(R)-MTPA esters of 3,4, 5-trimethoxyphenylglycerol-8-O-4′-(SA) ether

To a stirred solution of (+)-*erythro*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (3.48mg, 7.66µmol) in 2ml of dry CH_2Cl_2 with DCC (18.96mg, 91.88µmol), (R)-MTPA esters (16.96mg, 71.90µmol) and DMAP (7.94mg, 65.00µmol) in 3ml of dry CH_2Cl_2 were added at 35^oC temperature. After stirring for 15h under N_2 atmosphere reaction mixture was cold at room temperature. The mixture was filtered by KIRIYAMA filter, washed with CH_2Cl_2 and then concentrated under reduced pressure by rotary evaporator. The resulting residue was purified by preparative TLC (100% CH_2Cl_2) to afford (+) -

erythro-tri-(*R*)-MTPA esters of 3,4,5-trimethoxyphenylglycerol-8-O-4′-(SA) ether (4.1mg,48.43%). Other stereoisomers also converted to tri-(R)-MTPA esters of 3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ethers by the similarly to above one and determination was done by ^{1}H NMR spectra.

(+) -*Erythro*-tri-(*R*)-MTPA esters of 3, 4, 5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether

^{1}H NMR delta (Chloroform D+TMS): 3.6779 (3H, s, 7-MTPA-OCH_3), 3.4229 (3H, s, 9-MTPA-OCH_3), 3.5834 (3H, *d*, *J*=0.84, 9′-MTPA-OCH_3), 3.6157 [(exchangeable),6H, *s*, 3 & 5 -OCH_3], 3.6422 [(exchangeable),6H, *s*, 3′ & 5′ -OCH_3], 3.7951 (3H, s, 4-OCH_3), 4.86315 (1H, *dd*, *J*=11.94, 6.72 , 9a-H), 4.41125 (1H, *dd*, *J*=12.12, 3.30, 9b-H), 4.7312 (1H, *m*, 8-H), 6.1613 (1H, *d*, *J*=3.24, 7-H), 6.2045(1H, *dt*, *J*=15.66, 6.60, 8′-H), 6.5927(1H, *d*, *J*= 15.66, 7′-H), 4.9665 (2H, *d*, *J*=6.6, 9′-H), 6.2826 [(exchangeable),2H, *s*, 2 & 6 -H], 6.5311 [(exchangeable),2H, *s*, 2′ & 6′-H].

(-) -*Erythro*-tri-(*R*)-MTPA esters of 3, 4, 5-trimethoxyphenylglycerol-8-O-4′-(SA) ether

^{1}H NMR delta (Chloroform D+TMS): 3.3699(3H, *s*, 7-MTPA-OCH_3), 3.5489 (3H, *s*, 9-MTPA-OCH_3), 3.5741 (3H, *s*, 9′-MTPA-OCH_3), 3.5951 [(exchangeable),6H, *s*, 3 & 5 -OCH_3], 3.7645 [(exchangeable),6H, *s*, 3′ & 5′ -OCH_3], 3.8144 (3H, *s*, 4-OCH_3), 4.6255 (1H, *dd*, *J*=11.94, 3.42, 9a-H), 4.3910 (1H, *dd*, *J*=11.91, 4.53, 9b-H), 4.8043 (1H, *m*, 8-H), 6.12405 (1H, *d*, *J*=6.3, 7-H), 6.1435 (1H, *dt*, *J*=15.36,6.6,8′-H), 6.52985 (1H, *d*, *J*= 12.36, 7′-H), 4.9400 (2H, *d*, *J*=6.54, 9′-H), 6.4154 [(exchangeable),2H, *s*, 2 & 6 -H], 6.6136 [(exchangeable),2H, *s*, 2′ & 6′-H].

(+) -*Threo*-tri-(*R*)-MTPA esters of 3, 4, 5-trimethoxyphenylglycerol-8-*O*-4′-(SA) ether

^{1}H NMR delta (Chloroform D+TMS): 3.4582(3H, *s*, 7-MTPA-OCH_3), 3.4916(3H, *s*, 9-MTPA-OCH_3), 3.5772 (3H, *d*, *J*=1.08 9′-MTPA-OCH_3), 3.6010 [(exchangeable),6H, s, *3* & 5 -OCH_3], 3.7667 [(exchangeable),6H, *s*, 3′ & 5′ -OCH_3], 3.8546 (3H, *s*, 4-OCH_3), 3.9211 (1H, *dd*, *J*=12.24, 3.96, 9a-H), 4.5168 (1H, *dd*, *J*=12.09, 3.57, 9b-H), 4.7845 (1H, *dt*, *J*=3.84, 6.3,8-H), 6.2966 (1H, *dt*, *J*=6.3, 7-H), 6.1718 (1H, *dt*, *J*= 15.96, 6.6, 8′-H), 6.5635 (1H, *d*, *J*= 15.66, 7′-H), 4.9523 (2H, *dd*, *J*=6.6, 1.08, 9′-H), 6.4739 [(exchangeable),2H, *s*, 2 & 6 -H], 6.6428 [(exchangeable),2H, *s*, 2′ & 6′-H].

(-) -*Threo*-tri-(*R*)-MTPA esters of 3, 4, 5-trimethoxyphenylglycerol-8-*O*-4′-(SA) ether

^{1}H NMR delta (Chloroform D+TMS): 3.7178 or 3.6409 (3H, *s*, 7-MTPA-OCH_3), 3.6409 or 3.4999 (3H, *s*, 9-MTPA-OCH_3), 3.5851 (3H, *s*, 9′-MTPA-OCH_3), 3.5068 [(exchangeable),6H, *s*, 3 & 5 -OCH_3], 3.6643 [(exchangeable),6H, *s*, 3′ & 5′ -OCH_3], 3.8057 (3H, *s*, 4-OCH_3), 4.7625-4.7052 (3H, *m*, 8-H,9a-H & 9b-H), 6.3179 (1H, *d*, *J*=8.76, 7-H), 6.2070 (1H, *dt*, *J*=15.72, 6.51, 8′-H), 6.5983 (1H, *d*, *J*= 15.66, 7′-H), 4.9675 (2H, *d*, *J*=6.6, 9′-H), 6.3710 [(exchangeable),2H, *s*, 2 & 6 -H], 6.5394 [(exchangeable),2H, *s*, 2′ & 6′-H].

Enzyme preparations

A soluble enzyme preparation [cell-free extracts with potassium phosphate (K-Pi) buffer] was prepared by the method of Katayama and Kado (Katayama and Sogo, 1989). All manipulations were carried out at 4^0C 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.

Enzyme assay

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 SA (30 mM, in 0.5ml of K-Pi buffer). After incubation at 30^0C for various time intervals, glacial AcOH (0.5 ml) was added. The assay mixture was then extracted three times with EtOAc (30 mlX3). 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 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 *erythro* and *threo* diastereomers (Figure 2) were quantified as well as separated by reverse-phase column HPLC. Each diastereomer was then subjected to chiral column HPLC to quantify the enantiomers of SGSEs.

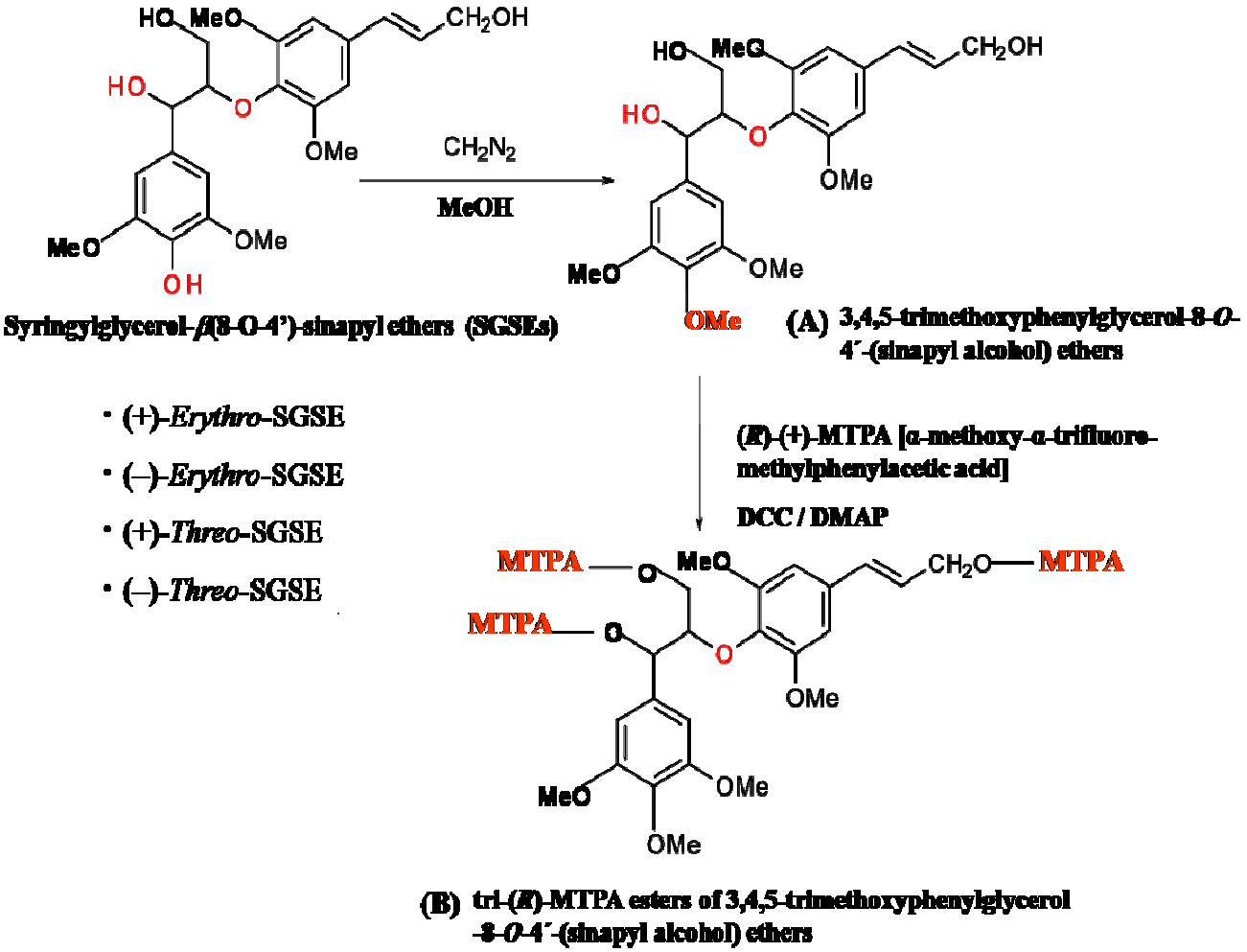


Figure 2. (A) Preparation of 4-*O*-methyl ether of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSEs) (B) Preparation of 7,9,9′-tri-(*R*)-MTPA esters of 4-*O*-methyl ether of syringylglycerol -8-*O*-4′-(sinapyl alcohol) ethers (SGSEs).

Results and Discussion

A mixture of (±)-*erythro*- and (±)-*threo*-SGSEs was prepared by dehydrogenative dimerization of SA with $FeCl_3$ in dioxane-H_2O (10:1) and purified by preparative TLC. (±)-*Erythro*-SGSE and (±)-*threo*-SGSE were separated carefully by preparative TLC and identified by ^{1}H NMR acetone-d_6 (data not shown) and HPLC using authentic samples (Lourith et al., 2005). The diastereomeric ratio (*erythro*:*threo* = 6:4) was determined by reverse phase HPLC. Their enantiomers were separated by chiral column HPLC to afford (+)- and- (-)-*erythro*-SGSEs, and (+)-and (-)-*threo*-SGSEs. Each phenolic hydroxyl group of the four stereoisomers was methylated with diazomethane (Figure 2A) separately. The resulting four stereoisomers were converted to 7, 9, 9′-tri-(*R*)-MTPA esters of 3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) (Figure 2B) ethers and their structures were confirmed by ^{1}H NMR spectra. According to the definition of *erythro* and *threo* diastereomers, absolute configuration of *erythro* SGSE and its 4-*O*-methyl ether is (7*R*, 8*S*) or (7*S*, 8*R*), and that of *threo* SGSE and its 4-*O*-methyl ether is (7*R*, 8*R*) or (7*S*, 8*S*).

According to the Mosher's method (Dale et al., 1969; Dale et al., 1973; Yamaguchi et al., 1985), because a preferred conformation of the MTPA esters has α-CH_3, the >C=O of the MTPA ester, and the benzyl C-H in an eclipsed arrangement, the (*R*)-MTPA-OCH_3 of (*R*)-MTPA ester of an (7*S*)-secondary benzyl alcohol [(*R*, 7*S*)-MTPA ester] is located on aromatic (e.g. 3,4,5-trimethoxyphenyl) ring, and the C8-H of the X group is on the benzene ring of the MTPA moiety, that is, the (*R*)-MTPA-OCH_3 and the C8-H receive shielding effects. In contrast, the (*R*)-MTPA-OCH_3 of (*R*)-MTPA ester of an (7*R*)-secondary benzyl alcohol [(*R*, 7*S*)-MTPA ester] is located not on the aromatic ring but on the C8-H, and the benzene ring is on the aromatic ring, that is the (*R*)-MTPA-OCH_3 and the C8-H have no shielding effect. Therefore, the ^{1}H NMR chemical shift (δs) of the (*R*)-MTPA-OCH_3 of the (*R*, 7*S*)-MTPA ester is upfield relative to that (δ_R) of the (*R*)-MTPA-OCH_3 of the (*R*, 7*R*)-MTPA ester. The Δδ value in the Mosher's method was defined as the absolute value of the difference in the ^{1}H chemical shifts of the peak between the diastereomers, $|\delta_S - \delta_R|$. [Note: the relations between (*R*)- and (*S*)-isomers and between (+)- and (–)-isomers are enantiomers, whereas the relations between (*R*)-MTPA ester of (*R*)-isomer and that of (*S*)-isomer and between (*R*)-MTPA ester of (+)-isomer and that of (–)-isomer are diastereomers.

Furthermore, the authors' empirical rules (Katayama et al., 2000 and 2005) on the ^{1}H NMR chemical shifts of arylglycerol-β(8-*O*-4′)-aryl ethers are as below. Rule-1, the Δδ value of 7-MTPA-OCH_3s were larger than those of 9-MTPA-OCH_3s, because one diastereomer's 7-MTPA-OCH_3s are on the aromatic rings and receive the shielding effect, and the others are not on the rings nor have the shielding effect. Such shielding effect was not expected for 9-MTPA-OCH_3s. Rule-3, Katayama et.al observed that 7*S*-H of the tri-(*R*)-(+)-MTPA esters of (–)-*erythro*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether that have the shielding effect gave J = 6.30(6) (Hz), whereas 7*R*-H of the (+)-*erythro*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether that have not the effect gave J = 3.24(4), and that 7*S*-H of (+)-*threo*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether that have the shielding effect gave J = 6.30(7), 7*R*-H of the (–)-*threo*-3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether that have not the effect gave J = 8.76(8.0-8.6). Rule-4, the 9′- MTPA-OCH_3s have the smallest Δδ, almost 0, among the three (7, 9, and 9′) MTPA-OCH_3s, because the 9′- MTPA-OCH_3 groups are located farthest from the two chiral centers, 7-C and 8-C, among the three MTPA-OCH_3 groups [Δδ (7)> Δδ (9)> Δδ (9′) ~ 0].

Erythro SGSE

Figure 3 showed that, tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro*-SGSE have ^{1}H NMR peaks of MTPA-OCH_3 markedly upfield, it was suggested that the peak was due to *α*-MTPA-OCH_3 with (7*S*)-configuration, and thus tri-(*R*)-MTPA esters

and 4-*O*-methyl ether of (+)-*erythro*-SGSE have (7*R*)-configuration. The assignments in Table 1 were consistent with rules 1, 3, and 4 as follows. The Δδ values for 7-MTPA-OCH_3 in tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*erythro* and (–)-*erythro*-SGSEs ($|\delta_R - \delta_S|$) is 0.308ppm which are apparently larger than that of 9-MTPA-OCH_3: 0.126ppm in tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro* and (+)-*erythro*-SGSEs ($|\delta_S - \delta_R|$).

Table 1. ^{1}H NMR Chemical shifts (δ) of MTPA-OCH_3 peaks of tri-(*R*)-MTPA esters of 4-*O*-methyl ether of synthetic (+)- and (-)-*erythro*-, and (+)- and (-)-*threo*- syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSE) and coupling constants of their 7-H peaks.

Stereoisomers	MTPA-OCH_3 (δ)			7-H
	7(α)	9(γ)	9′(γ′)	$J_{7\text{-}8}$ (Hz)
(+)-*Erythro*	3.6779	3.4229	3.5834	3.24
(-)-*Erythro*	3.3699	3.5489	3.5741	6.30
|Δδ|	0.308	0.126	0.009	
(+)-*Threo*	3.4582	3.4916	3.5772	6.30
(-)-*Threo*	3.7178	3.6409	3.5851	8.76
|Δδ|	0.2596	0.1493	0.0079	

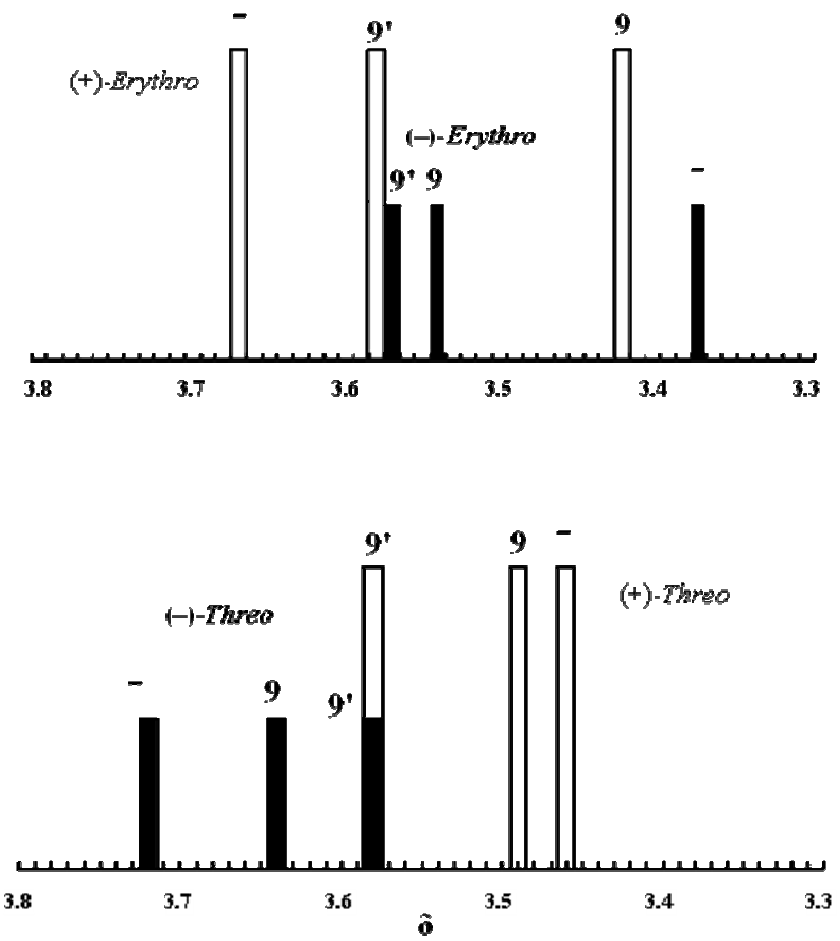


Figure 3. ^{1}H NMR chemical shifts of 7, 9, 9′-tri-(*R*)-MTPA-OCH_3 peaks of 4-*O*-methyl and 7, 9,9′-tri-(R)-MTPA ester derivatives of four stereoisomers of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSEs). The long white columns correspond to (+)-*erythro* and (+)- *threo* isomers, and short black columns correspond to (–)-*erythro* and (–)-*threo* isomers.

The tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro*–SGSEs coupling constant (J = 6.30) of Cα-H is larger than that of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*erythro*–SGSEs coupling constant (J = 3.24) of Cα-H.

On the other hand, the Δδ values for 9′-MTPA-OCH_3 in tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro* and (+)-*erythro*-SGSEs ($|\delta_S - \delta_R|$) is 0.009ppm, that mean almost 0.

Thus it was established that the 7-MTPA-OCH_3 of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro*–SGSE was effected by the shielding effect of the part of 3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether, whereas that of neither tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*erythro*–SGSE was affected. Consequently, the C7 of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro*–SGSE have an (*S*)-configuration, whereas the C7 of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*erythro*–SGSE have an (*R*)-configuration. So, the absolute configuration of (+)-*erythro*– and (–)-*erythro*–SGSEs are (7*R*, 8*S*) and (7*S*, 8*R*) respectively.

Threo SGSE

Figure 3 also showed that, tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*threo*-SGSE have ^{1}H NMR peaks of MTPA-OCH_3 markedly upfield, it was suggested that the peak was due to *α*-MTPA-OCH_3 with (7*S*)-configuration, and thus tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*erythro*-SGSE have (7*R*)-configuration. The assignments in Table 1 were consistent with rules 1, 3, and 4 as follows. The Δδ values for 7-MTPA-OCH_3 in tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*threo*-and (+)-*threo*-SGSEs ($|\delta_R - \delta_S|$) is 0.259ppm which are apparently larger than that of 9-MTPA-OCH_3: 0.149ppm in tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*threo*- and (+)-*threo*-SGSEs ($|\delta_R - \delta_S|$).

On the other hand, the Δδ values for 9′-MTPA-OCH_3 in tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*threo*- and (+)-*threo*-SGSEs ($|\delta_R - \delta_S|$) is 0.008ppm, that also, mean almost 0.

The tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*threo*-SGSEs coupling constant (J = 8.76) of Cα-H is larger than that of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*threo*-SGSEs coupling constant (J = 6.3) of Cα-H.

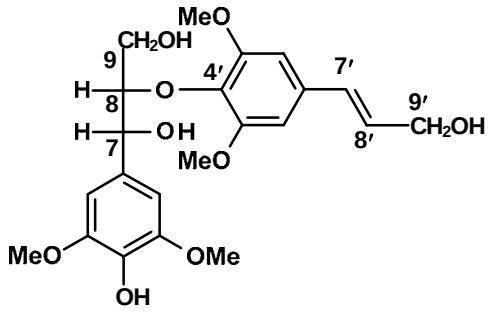
Thus it was established that the 7-MTPA-OCH_3 of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*threo*-SGSE was effected by the shielding effect of the part of 3,4,5-trimethoxyphenylglycerol-8-*O*-4′-(sinapyl alcohol) ether, whereas that of neither tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*threo*-SGSE was affected. Consequently, the C7 of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (+)-*threo*-SGSE have an (*S*)-configuration, whereas the C7 of tri-(*R*)-MTPA esters and 4-*O*-methyl ether of (–)-*threo*-SGSE have an (*R*)-configuration. So, the absolute configuration of (+)-*threo*- and (–)-*threo*-SGSEs are (7*S*, 8*S*) and (7*R*, 8*R*) respectively.

According to this assignment, it could be concluded the absolute configuration of four stereoisomer of SGSEs (Figure 4), (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-SGSEs were determined as (7*R*, 8*S*), (7*S*, 8*R*) , (7*S*, 8*S*) and (7*R*, 8*R*), respectively.

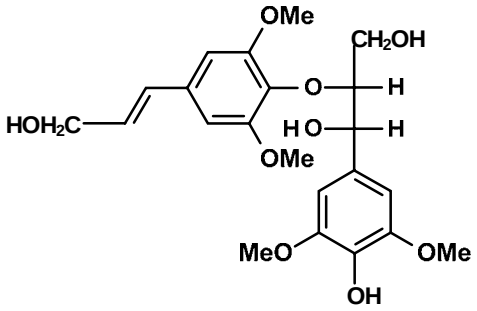
Enzymatic formation of *erythro*- and *threo*-SGSE

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) (Fig.2). 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′), 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,

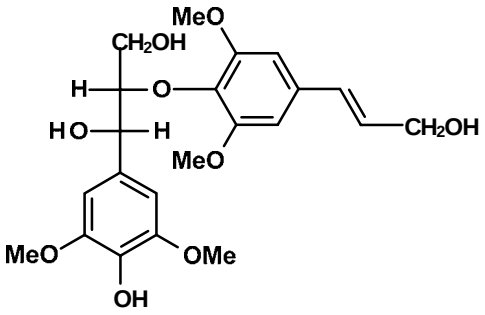
s, B-OCH3), 3.99 (2H, dd, *J* = 3.41, H-8), 4.0 (2H, dd, *J* = 6.95, 3.56, H-9′), 4.23 (1H, d, *J* = 3.66, 7-OH), 4.98 (1H, d, *J* = 6.83, H-7), 6.36 (1H, dt, *J* = 15.85, 5.29, H-8′), 6.54 (1H, d, *J* = 16.1, H-7′), 6.78 (2H, s, H-2 & 6), 6.82 (2H, s, H-2′ & 6′), 7.44 (1H, s, 4- OH). The SGSEs were diastereoselectively formed in *erythro* isomer with 53% d.e. at 60 min (Table 2). Quantification of the four stereoisomers, (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-SGSEs demonstrated that they were in a percentage of 23.8 %, 28.8 %, 22.1 %, and 25.3 %, respectively (Fig. 4). Both products, (-)-*erythro*- and (-)-*threo*-SGSEs have optical activity with 10 % e.e. and 7 % e.e., respectively, at 60 min.



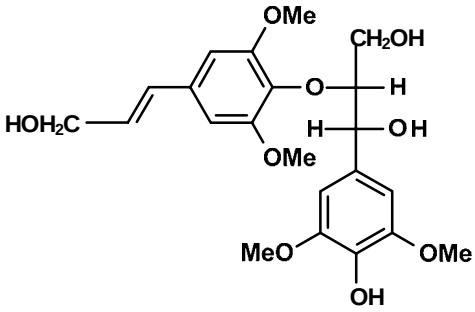
(7*R*, 8*S*)-(+)-*Erythro*



(7*S*, 8*R*)-(–)-*Erythro*



(7*S*, 8*S*)-(+)-*Threo*



(7*R*, 8*R*)-(–)-*Threo*

Figure 4. Absolute configuration of four stereoisomers of syringylglycerol-8-*O*-4′-(sinapyl alcohol) ethers (SGSEs).

Table 2. 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.

SGSE	Diastereomeric ratio (%)	Enantiomeric excess (%)	Enantiomeric ratio (%)
Erythro	53.0	(–) 10	(+)-*erythro* 16.7
			(–)-*erythro* 46.7
Threo	47.4	(–) 7	(+)-*threo* 14.4
			(–)-*threo* 22.3

The Diastereoselective formation of syringyl-8-*O*-4′-neolignans with optical activity from two sinapyl alcohols (Lourith et al., 2005). This result recognized our present study report. The observation of soluble enzyme preparation, to preference for different enantiomers of the *erythro* isomer suggests that different enzymes regulate the 8-*O*-4′ coupling of sinapyl alcohol in *E. ulmoides*.

Conclusions

The stereochemistry of four stereoisomer of SGSEs, (+)-*erythro*-, (-)-*erythro*-, (+)-*threo*-, and (-)-*threo*-SGSEs were determined as (7*R*, 8*S*), (7*S*, 8*R*), (7*S*, 8*S*) and (7*R*, 8*R*), respectively. On the other hand *erythro*-SGSE was formed more selectively from *threo*-SGSE, with soluble enzyme preparation by the incubation of SA in the presence of H_2O_2. The soluble enzyme preparation catalyzed the diastereoselective formation of *erythro*- and *threo*-SGSEs with optical activity.

References

Alam, M. S., N. Lourith, T. Suzuki and T. Katayama. 2009. Stereochemistry of the formation of arylglycerol-8-*O*-4′-aryl ethers from monolignols with enzyme preparations of *Eucommia ulmoides.* Proc. Internat. Symp. Wood, Fibre and Pulping Chemistry (ISWPFC'09), Oslo**,** Norway.

Ayres, D. C. and J. D. Loike. 1990 Lignans: Chemical, biological and clinical properties. Cambridge University Press, Cambridge, UK pp. 12-14, 90-103 (and references therein)

Bore, Th. J. de and H. J. Backer. 1963. Diazomethane. Organic Synthesis, Coll. 4:250.

Dale, J. A., D. L. Dull and H. S. Mosher. 1969. α-methoxy-α-trifluorom ethylphenyl acetic acid, a versatile reagent for the determination of enantiomeric composition of alcohols and amines. J. Org. Chem. 34:2543-2549.

Dale, J. A. and H. S. Mosher. 1973. Nuclear magnetic resonance enantiomer reagents Configuration correlations via nuclear magnetic resonance chemical shifts of diastereomeric mandelate, *O*-methylmandelate, and *α*-methoxy-*α*-trifluoromethylphenylacetates (MTPA) esters. J. Am. Chem. Soc. 95:512-519.

Katayama, T., M. Sogo and T. Higuchi. 1986. Degradation and stereoselective reduction of an *α*-ketone derivative of guaiacylglycerol-*β*-vanillin ether. A lignin substructure model, by *Fusarium solani* M-13-1. Mokuzai Gakkaishi 35:1116-1124.

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.

Katayama, T., J. Tsutsui, K. Tsueda, T. Miki, Y. Yamada and M. Sogo. 2000. Absolute configuration of arylglycerol-*β*-aryl ethers obtained by asymmetric reduction of corresponding *α*-ketonic compound with intact *Fusarium solani* cells. J. Wood Sci. 46:458-465.

Katayama, T., S. Okamoto, Y. Tabuchi and T. Suzuki. 2005. Absolute configuration of four stereoisomers of arylglycerol-β-aryl ethers and 8-O-4′ neolignans. Proc. Intl. Symp. Wood Sci. Technol., Yokohama, Japan, Vol. 1, pp 329-330.

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′-(sinapyl alcohol) ether from sinapyl alcohol. J. Wood Sci. 51:379-386.

Tanahashi, M., H. Takeuchi and T. Higuchi. 1976. Dehydrogenative polymerization of 3, 5-disubstituted *p*-coumaryl alcohols. Wood Res. 61:44-53.

Yamaguchi, S. 1985. The determination methods of chirality with NMR (in Japanese), Chem Today 168(3):14-21.