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Stereoselective formation of β–O–4 structures mimicking softwood lignin biosynthesis: Effects of solvent, and the structures of quinone methide lignin models

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KEYWORDS: Lignification, para-quinone methide, radical coupling, *erythro* and *threo*, gymnosperm, *p*-hydroxyphenyl, guaiacyl

ABSTRACT

p-Quinone methide (QM) is formed as an intermediate during lignin biosynthesis. The aromatization of the QM by the attack of a nucleophile at the α-position of its side-chain generates a phenolic hydroxy group in a growing polymer and creates stereoisomeric forms in the side-chain. A series of β–O–4-aryl ether QMs was reacted with water at 25 °C to replicate the formation of *p*-hydroxyphenyl (H) and guaiacyl (G) β–O–4 structures in plant cell walls. Water addition occurred in 3-methoxy-substituted QMs (G-type QMs) with half-lives ($t_{1/2}$) between 13 and 15 min, at pH 7, in 50% water solution (dioxane-water, 1:1). The rate increased as the water concentration increased to 99% ($t_{1/2}$, 1.2–1.4 min). Similar solvent effects were observed for more reactive nonsubstituted QMs (H-type QMs with $t_{1/2}$ of <1 min). Consequently, $t_{1/2}$ of the H-type QMs was shorter than that of the G-type QMs under every solvent condition. Upon increasing the water concentration, the variation in the *erythro*/*threo*

ratios of the four dimeric β–O–4 products increased. Interestingly, the effect of pH on the stereo-preference, which was observed in 50%-water solution, was small and became imperceptible as the water concentration increased to 99%, suggesting that the effect of the solvent as well as the effect of the pH, play an important role in understanding the reaction condition in cell walls during lignin biosynthesis. The *threo* isomer was preferably formed in the four dimeric β–O–4 structures, which is inconsistent with the structural features of compression wood lignin rich in H-units. However, the *erythro*-selective formation was attained in an H-type QM at every pH studied (pH 3.5–7) by introducing a biphenyl structure into the β-etherified ring moiety.

INTRODUCTION

Lignins are aromatic polymers that are deposited in vascular-plant cell walls, providing rigidity and structural reinforcement to the cell walls. They are primarily derived from three monolignols, *p*-coumaryl, coniferyl, and sinapyl alcohols that give rise to *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, respectively, in the lignin structures.[1-3] Lignins differ greatly in their chemical structures, which is largely due to the compositions of the monolignols, and their polymerization mechanisms during lignin biosynthesis.

p-Quinone methides (QMs) are formed as transient intermediates during lignin biosynthesis. They are generated by a β–O–4, β–β, β–5, or β–1 coupling between two radicals arising from monolignols or the phenolic end of the growing polymer. The QMs are rearomatized by the addition of water at the exocyclic methylene-group site. The aromatization, i.e. the regeneration of a phenolic hydroxy group, is essential for lignin polymerization. A phenolic hydroxy group is regenerated by the aromatization to form a phenolic end in the growing lignin, thereby being prepared for further oxidation into a phenoxyl radical that couples with another radical to elongate the lignin chain.

The structural variation of QMs and their chemical properties are thought to influence the polymerization efficiency and the structure of the resulting lignins.[4-8] Kobayashi et al.[9] reported in their study of monolignols oxidizability, that coniferyl alcohol (G-type) was consumed faster than *p*-coumaryl alcohol (H-type) in a peroxidase–H_2O_2 system. The

oxidation potentials determined by cyclic voltammetry indicated that *p*-coumaryl alcohol can be less oxidized than coniferyl alcohol.[9] Therefore, *p*-coumaryl alcohol does not seem to have an advantage over coniferyl alcohol in driving the lignin-polymerization cycle efficiently. In contrast, the results from our previous model study[8] indicated that water addition occurred much faster in nonsubstituted QMs (H-type QMs) than in 3-methoxy-substituted QMs (G-type QMs) when the water addition experiments were conducted in dioxane-water mixture (1:1, v/v), at pH 3–7 and 25 °C. This result suggested that once a radical-coupling reaction occurs, generating a β–O–4-bonded QM at the growing end of a lignin chain, the subsequent aromatization proceeds faster in the H-type QMs than in the G-type QMs. Therefore, we postulated that the rapid aromatization in H-type QMs might provide an advantage over G-type QMs for efficiently driving the lignin-polymerization cycle, which may possibly play a certain role in the rapid lignin deposition, and the development of highly lignified compression wood in response to growing stress.

Water addition to the β–O–4-aryl ether QM is a stereo-differentiating reaction, which cause an increase in the structural variety of lignins. During the formation of arylglycerol-β-aryl ether structures (β-O-4 structures), a β–O–4-linkage is formed by a radical coupling reaction between a monolignol and the phenolic end of a growing lignin to produce a QM intermediate, which houses a chiral center: the β-carbon atom (Scheme 1).[10] The β–O–4-aryl ether QM bearing the β-asymmetric carbon is aromatized by the water addition at the prochiral α-carbon, affording either the *erythro* or *threo* forms of the β–O–4 structure. The generation of *erythro* or *threo* forms depends on which face of the QM reacts faster with water.[4,6,11] The *erythro*/*threo* ratio is well known to influence the lignin degradation efficiency under alkaline delignification,[12-15] and enzymatic and chemical oxidation.[16-19] Therefore, the *erythro*/*threo* ratio of β–O–4 structures have been recognized as an important structural and chemical characteristic of lignins.

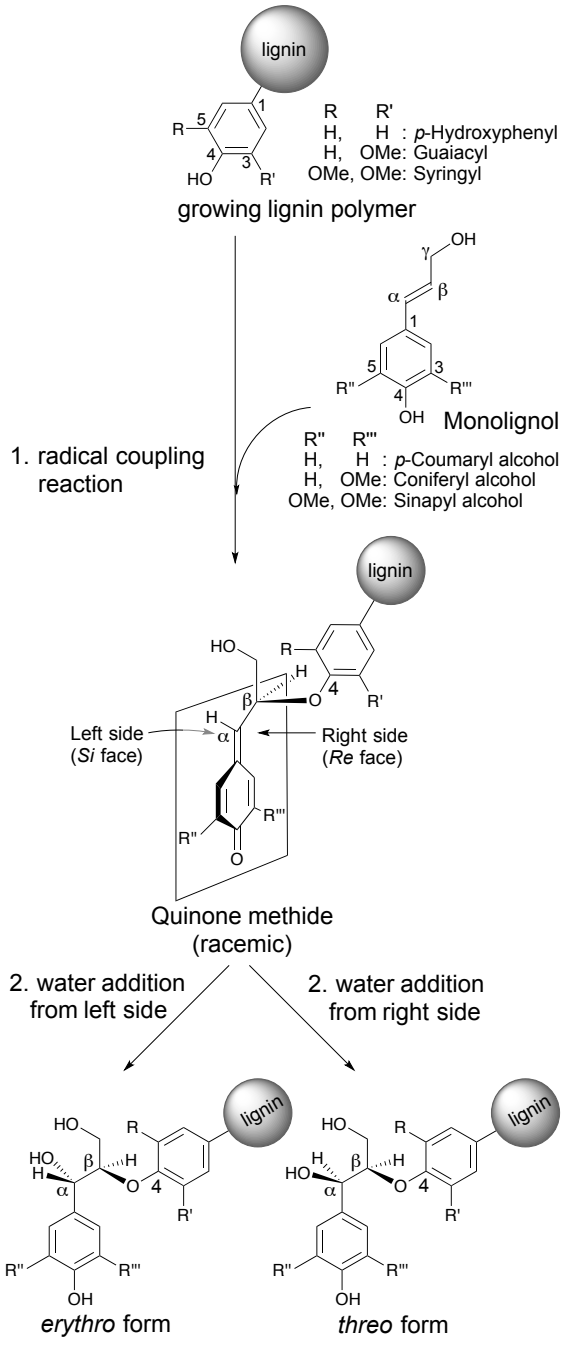
The proportion of the *erythro* form to the *threo* form of β–O–4 structures was nearly 50:50 in softwood lignins mostly composed of G-units, i.e., the proportion of *erythro* form determined by ozonation method was within the 50.0 ± 0.6% range for five gymnosperm species.[20] In contrast, the *erythro* form dominates in the β–O–4 structures in hardwood lignins primarily composed of G- and S-units.[20-24] A positive relationship between the *erythro*/*threo* ratio and S/G ratio has been found among various hardwood species.[20,25-27]

Brunow et al.[6] conducted an in vitro water addition reaction to G-type and 3,5-dimethoxy-substituted (S-type) QMs for replicating the formation of β–O–4 structures in hardwood lignin biosynthesis; they revealed that an *erythro*-preferential reaction occurred for the formation of arylglycerol-β-syringyl ethers at pH 3 (GS, and SS), whereas the stereopreferential formation of *erythro* isomers was not observed for a β-guaiacyl ether (GG) at the same pH.

Lignin structural studies of the reaction wood in softwood revealed that the *erythro*-preferential formation occurs in the compression wood, of which lignins contain a substantial amount of H-units. The β–O–4 structures in the compression wood lignin of softwoods were slightly, but clearly dominantly in the *erythro* form.[26,28] A positive correlation was found between the *erythro*/*threo* ratio and H/G ratio in the compression wood lignins.[26] This result implied that the *erythro*-selective formation reaction can occur under the influence of the H-units in lignins.

Previously, as described above, we conducted water addition experiments similar to those by Brunow's group,[6] for mimicking the formation of H-type β–O–4 structures in compression wood lignin biosynthesis.[8] The *threo*-preferential water addition was observed both for H- and G-types QMs examined at pH 3.5–7. An *erythro*-preferential reaction was not observed in the model experiments, whereas the *erythro*/*threo* ratio of the β–O–4 structures in compression wood, which contains a substantial amount of H-units, is slightly but clearly higher than 1.0.[26,28]

Herein, we investigated the gap between the model experiment and the lignin structures in actual wood cell walls. Water addition experiments were conducted focusing on the effect of the solvent to advance our understanding of the reaction condition that facilitates *erythro*-preferential reactions with H-type QMs. In addition to previously reported dimeric QM models, a novel trimeric QM compound bearing a biphenyl structure on the β-etherified ring moiety was examined (Scheme 2). Dehydrogenation polymers (DHPs) were also prepared from *p*-coumaryl alcohol in an in vitro peroxidase–H_2O_2 system under various conditions. The DHPs were characterized by SEC and ozonation method[29] to investigate the relationship between their molecular weights and the *erythro*/*threo* ratio of the β–O–4-structures.



β-O-4 structures in lignin

Scheme 1. Formation of arylglycerol-β-aryl ether (β–O–4) structures during lignin biosynthesis.

EXPERIMENTAL

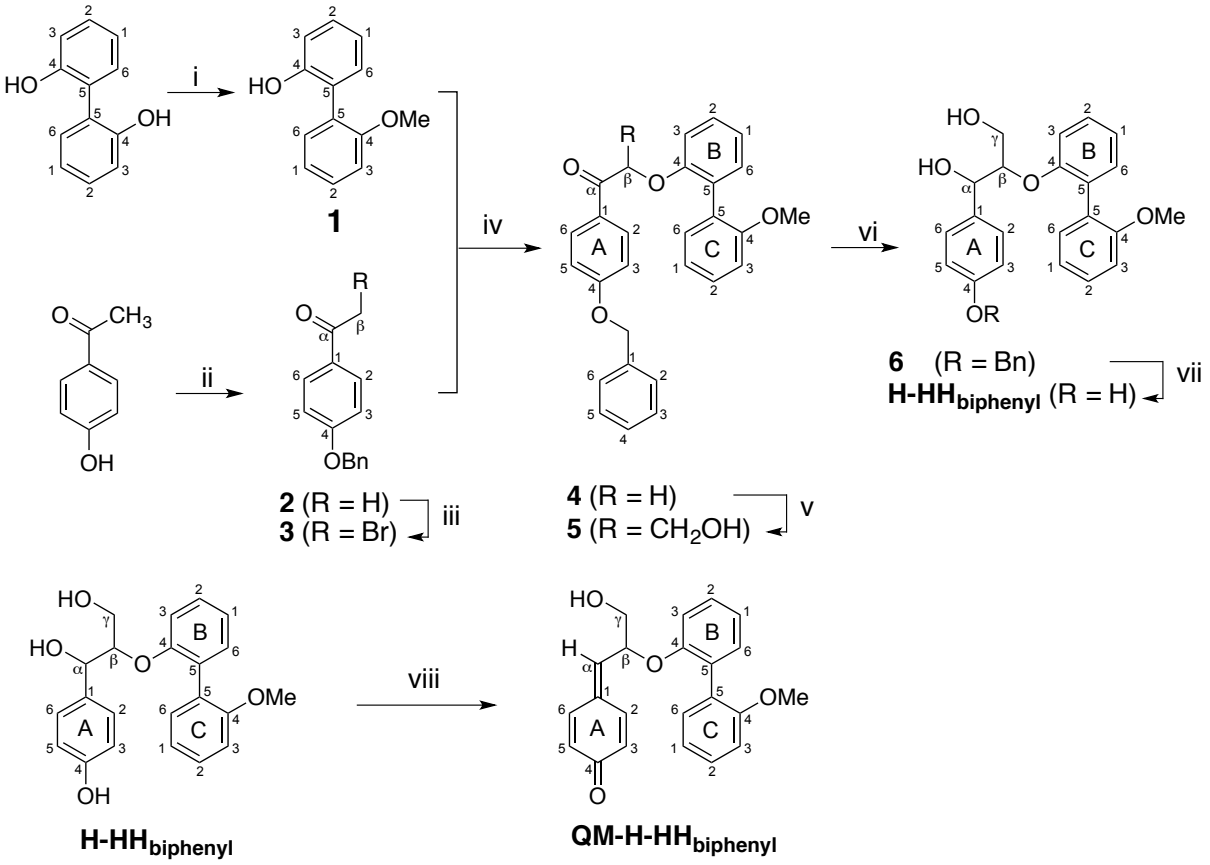
General. The reagents and solvents used were purchased from Fujifilm Wako Pure Chemical Co. (Osaka, Japan), Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), or Sigma-Aldrich (Tokyo, Japan). The pH levels of the buffers were recorded with a Horiba F-52 pH meter equipped with a JF15 electrode (Horiba, Kyoto, Japan). The UV spectra were recorded on a UV–visible spectrometer (Jasco V-660, Jasco International Co. Ltd., Tokyo, Japan) equipped with a circulating water bath (cooling-circulator CB-15, Iuchi Seieido Co. Ltd., Japan). The NMR spectra were recorded with a JEOL JNM-A500 500 MHz spectrometer for structural elucidation, and assignment of newly synthesized compounds. The standard JEOL programs of one- and two-dimensional (proton, carbon, DEPT-135, COSY, HSQC, and HMBC) NMR experiments were used. The central peak of the residual solvent was used as an internal reference (δ_H 7.26, δ_c 77.0 ppm for $CDCl_3$; δ_H 2.04, δ_c 29.8 ppm for acetone-d_6). The traditional numbering system for lignins[1,30] was used rather than the systematic IUPAC numbering system.

Synthesized model compounds.

Four β–O–4-type lignin model compounds, viz. guaiacylglycerol-β-guaiacyl ether (**GG**), guaiacylglycerol-β-*p*-hydroxyphenyl ether (**GH**), *p*-hydroxyphenylglycerol-β-guaiacyl ether (**HG**), and *p*-hydroxyphenylglycerol-β-*p*-hydroxyphenyl ether (**HH**), bearing H- and/or G-type nuclei on two aromatic rings (Scheme 3), were synthesized according to Adler's method.[31] The *erythro* and *threo* isomers of the β–O–4 model (**GG**, **GH**, **HH**, or **HG**) were separated from each other,[32-34] and were used as the authentic compounds for the structural determination of products in the water addition experiment. NMR data were described in our previous study,[8] together with the configuration of each isomer, determined by the ozonation method.[29] *p*-Coumaryl alcohol and coniferyl alcohol were prepared according to the method of Quideau and Ralph.[35]

A β–O–4-biphenyl ether compound (H-HH$_{biphenyl}$).

As a trimeric β–O–4 model, *p*-hydroxyphenylglycerol-β-biphenyl ether (**H-HH$_{biphenyl}$**), bearing a 5–5-linked biphenyl in its β-etherified structural moiety, was prepared as shown in Scheme 2. This β–O–4 model was synthesized from 2,2'-biphenol and acetoguaiacone by Alder's method[31] as described below.



Scheme 2. Synthetic route of β–O–4 model compound **H-HH$_{biphenyl}$** and its quinone methide, **QM-H-HH$_{biphenyl}$**: (i) MeI, K_2CO_3; (ii) BnCl, KI, K_2CO_3; (iii) Br_2; (iv) K_2CO_3; (v) HCHO, K_2CO_3; (vi) $NaBH_4$; (vii) H_2, Pd/C; and (viii) 1, TMSiBr and 2, $NaHCO_3$.

Compound 1 (2'-methoxy-biphenyl-2-ol). To a solution of 2,2'-biphenol (12.02 g, 64 mmol) in acetone (dried over $MgSO_4$, 200L), methyl iodide (10.98 g, 77 mmol) and potassium carbonate (10.67 g, 77 mmol) were added. The reaction suspension was stirred at room temperature for 20 h. The reaction was monitored by thin-layer chromatography [TLC, hexane-EtOAc, 2:1; Rf: 0.34 (starting material) and 0.38 (target compound)]. The mixture was partially concentrated under reduced pressure and extracted with dichloromethane. The

organic layer was washed with water and saturated NaCl, successively, and dried over Na_2SO_4. After filtration, the filtrate was concentrated under vacuum pressure. The residue was separated by silica gel chromatography with hexane-EtOAc (2:1, v/v) to afford 2'-methoxy-biphenyl-2-ol **1** in syrup form (11.3 g, 88% yield). ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.91 (3H, s, OMe), 6.24 (0.9H, s, B4-OH), 7.04 (1H, broad-td, *J* = 7, 1 Hz, B1), 7.06 (1H, br-dd, *J* = 7, 1 Hz, B3), 7.07 (1H, br-dd, *J* = 7, 1 Hz, C3), 7.14 (1H, br-td, *J* = 7, 1 Hz, C1), 7.28 (1H, br-dd, *J* = 7, 2 Hz, B6), 7.32 (1H, br-td, *J* = 7, 2 Hz, B2), 7.36 (1H, br-dd, *J* = 7, 2 Hz, C6), 7.41 (1H, br-td, *J* = 7, 2 Hz, C2). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 56.2 (OMe), 111.6 (C3), 117.4 (B3), 120.9 (B1), 122.2 (C1), 126.2 (B5), 127.1 (C5), 129.2 (B2), 129.3 (C2), 131.3 (B6), 132.5 (C6), 153.7 (B4), 155.5 (C4).

Compound 2 (4-benzyloxyacetophenone). Potassium iodide (2.32 g, 14 mmol), potassium carbonate (38.70 g, 280 mmol), 4-hydroxyacetophenone (27.23 g, 200 mmol), and 200 mL of dimethylformamide (dried over $MgSO_4$) were placed in a 500-mL round-bottom flask equipped with a reflux condenser. The mixture was heated to 90 °C in an oil bath while stirring. Benzyl chloride (35.44 g, 280mmol) was added in small portions. The reaction mixture was continuously stirred at 90 °C for an additional 1.5 h. The reaction progress was monitored by TLC [hexane-EtOAc, 2:1; Rf: 0.17 (starting material) and 0.37 (target compound)]. The reaction mixture was cooled to room temperature, poured onto crushed ice (approx. 150 g), and stirred until the ice melted. The white solid formed was isolated by suction filtration, after which it was washed with water, and once with hexane. The solid was crystalized from EtOH to afford 4-benzyloxyacetophenone (40.6 g, 91% yield). ^{1}H NMR (acetone-d_6, 500 MHz), δ_H: 2.50 (3H, s, β), 5.21 (2H, s, PhCH_2), 7.09–7.96 (9H, m, Ar). ^{13}C NMR (acetone-d_6, 125 MHz), δ_C: 26.5 (β), 70.7 (PhCH_2), 115.5, 128.6, 128.9, 129.4 and 131.3, (Ar, -CH), 131.6, 137.8 and 163.6, (Ar, quaternary C), 196.4 (α).

Compound 3 (4-benzyloxy-α-bromoacetophenone). The compound, 4-benzyloxyacetophenone **2** (12.32 g, 54 mmol), was dissolved in EtOH (390 mL) while stirring under nitrogen atmosphere. Bromine (10.55 g, 66 mmol) was quickly added to the stirred mixture at room temperature. The reaction was monitored by TLC [hexane-EtOAc, 2:1; Rf: 0.44 (starting material) and 0.52 (target compound)]. After 25 min of continuous stirring, the color of the solution faded from dark red to pale yellow accompanied by the disappearance of the starting material. The reaction mixture was poured onto approx. 200 g of

ices and a white solid substance began to form during stirring; the mixture was stirred until the ice melted. The solid was collected by suction filtration, after which it was washed with water, and once with hexane. The solid was crystalized from EtOH to afford 4-benzyloxy-α-bromoacetophenone (16.5 g, 98% yield). ^{1}H NMR (acetone-d_6, 500 MHz), δ_H: 3.88 (2H, s, β), 4.46 (2H, s, PhCH_2), 6.36–7.25 (9H, m, Ar). ^{13}C NMR (acetone-d_6, 125 MHz), δ_C: 32.7 (β), 70.9 (PhCH_2), 115.8, 128.66, 129.0, 129.5 and 132.1 (Ar, -CH), 12.2, 137.7 and 164.3, (Ar, quaternary C), 196.4 (α).

Compound 4 (1-(4-benzyloxyphenyl)-2-(2'-methoxy-[1,1'-biphenyl]-2-yloxy)ethanone). Biphenol monomethylated **1** (8.20 g, 41 mmol), bromoacetophenone **3** (11.02 g, 36 mmol), powdered potassium carbonate (6.03 g, 44 mmol), and 180 mL of acetone (dried over $MgSO_4$) were placed in a 500-mL round-bottom flask. The mixture was heated at 50 °C for 15 h while stirring. The reaction was monitored by TLC [hexane-EtOAc, 2:1; Rf: 0.44 (target compound) and 0.48 (starting material)]. The mixture was partially concentrated under reduced pressure, and extracted with dichloromethane. The organic layer was washed with 0.1 M NaOH (× 2), water, and saturated NaCl, successively, and dried over Na_2SO_4. After filtration, the filtrate was concentrated under vacuum pressure. The crude product was purified by crystallization from EtOH to afford compound **4** (14.2 g, 93% yield). ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.71 (3H, s, OMe), 5.08 (2H, s, β), 5.13 (2H, s, PhCH_2), 6.93 (2H, d, J = 8.9 Hz, A3 and A5), 6.93 (2H, broad-d, J = 7 Hz, C3 and B3), 6.99 (1H, br-td, J = 7, 1 Hz, C1), 7.05 (1H, br-td, J = 7, 1 Hz, B1), 7.27 (1H, br-dd, J = 7, 2 Hz, B6), 7.28 (1H, br-td, J = 7, 2 Hz, B2), 7.29 (1H, br-dd, J = 7, 2 Hz, C6), 7.32 (1H, br-td, J = 7, 2 Hz, C2), 7.36 (1H, m, Bn4), 7.42 (2H, m, Bn3 and Bn5), 7.42 (2H, d, J = 8.6 Hz, Bn2 and Bn6), 7.89 (2H, d, J = 8.9 Hz, A2 and A6). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 55.6 (OMe), 70.1 (PhCH_2), 72.3 (β), 110.9 (C3), 113.1 (B3), 114.6 (A3 and 5), 120.2 (C1), 121.5 (B1), 127.4 (Bn2 and Bn6), 127.5 (A1 and C5), 128.1 (B5), 128.2 (Bn4), 128.6 (B2), 128.7 (C2), 128.7 (Bn3 and Bn5), 130.9 (A2,6), 131.6 (C6), 131.7 (B6), 136.1 (Bn1), 155.7 (B4), 157.0 (C4), 162.9 (A4), 193.8 (α).

Compound 5 (1-(4-benzyloxyphenyl)-3-hydroxy-(2'-methoxy-[1,1'-biphenyl]-2-yloxy)propan-1-one). To a solution of compound **4** (9.67 g, 23 mmol) in THF (115 mL), a 37 wt% aqueous solution of formaldehyde (6.84 g, 229 mmol), and potassium carbonate (3.16 g, 23 mmol) were added

while stirring. The mixture was heated at 40 °C, and continuously stirred for 16 h. The reaction was monitored by TLC [hexane-EtOAc, 2:1; Rf: 0.31 (target compound) and 0.46 (starting material)]. The reaction mixture was neutralized with 1M HCl, and extracted with dichloromethane. The organic layer was washed with water and saturated NaCl, successively, and dried over Na_2SO_4. After filtration, the filtrate was concentrated under vacuum pressure. The crude product was purified by silica gel chromatography with hexane-EtOAc (2:1, v/v) to afford compound **5**, a pale-yellow syrup (8.86 g, 86% yield). ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.76 (3H, s, OMe), 3.85 (1H, broad-dd, *J* = 12, 7 Hz, γ_1), 3.91 (1H, br-dd, *J* = 12, 3 Hz, γ_2), 5.12 (2H, s, Ph*C*H_2), 5.37 (1H, br-dd, *J* = 7, 3 Hz, β), 6.77 (1H, br-d, *J* = 8 Hz, C3), 6.93 (2H, d, *J* = 8.9 Hz, A3 and A5), 7.02 (1H, br-d, *J* = 8 Hz, B3), 7.03 (1H, br-t, *J* = 8 Hz, C1), 7.07 (1H, br-t, *J* = 8 Hz, B1), 7.23 (1H, br-td, *J* = 8, 2 Hz, C2), 7.24 (1H, br-dd, *J* = 8, 2 Hz, C6), 7.27 (1H, br-dd, *J* = 8, 2 Hz, B6), 7.37 (1H, br-td, *J* = 8, 2 Hz, B2), 7.34-7.42 (5H, m, *Ph*CH_2), 7.95 (2H, d, *J* = 8.9 Hz, A2 and A6). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 56.5 (OMe), 63.5 (γ), 70.1 (Ph*C*H_2), 83.4 (β), 112.2 (B3), 113.1 (C3), 114.7 (A3 and A5), 121.3 (B1), 121.7 (C1), 127.4, 127.8 (A1), 128.2 (C5), 128.3 (Bn4), 128.6 (B5), 128.7, 128.8 (C2), 129.0 (B2), 131.4 (A2 and A6), 131.6 (B6 and C6), 136.0 (Bn1), 154.7 (B4), 156.7 (C4), 163.1 (A4), 195.0 (α).

Compound 6 (1-(4-benzyloxyphenyl)-2-(2'-methoxy-[1,1'-biphenyl]-2-yloxy)propane-1,3-diol). To a stirred solution of compound 5 (10.29 g, 22.76 mmol) in THF-EtOH (180 mL, 1:1, v/v), excess $NaBH_4$ (1.72 g, 45.51 mmol) was added slowly over 5 min. The reaction suspension was stirred at room temperature for 11 h. The completion of the reaction was monitored by TLC [hexane-EtOAc, 1:1, v/v; Rf: 0.39 (*erythro*, target compound), 0.48 (*threo*, target compound), and 0.53 (starting material)]. To deplete the excess $NaBH_4$, 1 M HCl was added dropwise. The reaction mixture was extracted with dichloromethane, washed twice with H_2O, once with saturated NaCl, and dried over Na_2SO_4. After filtration, the filtrate was concentrated under vacuum pressure to afford compound **6** (9.74 g, 94% yield) as a mixture of *erythro* and *threo* isomers (**6e** and **6t**, respectively). Each isomer was separated from the mixture by silica-gel chromatography (hexane-EtOAc, 9:1 to 3:7). Compound **6e** (*erythro* isomer). ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.60 (1H, m, γ_1), 3.72 (1H, broad-dd, *J* = 12, 6 Hz, γ_2), 3.74 (3H, s, OMe), 4.42 (1H, m, β), 4.90 (1H, t, *J* = 4.0 Hz, α), 5.04 (2H, s, Ph*C*H_2), 6.87 (2H, d, *J* = 8.6 Hz, A2 and A6), 7.02 (1H, br-d, *J* = 7 Hz, B3 and C3), 7.06 (1H, br-td, *J* = 7, 1 Hz, B1), 7.07 (1H, br-td, *J* = 7, 1 Hz, C1), 7.11 (2H, d, *J* = 8.6 Hz, A3 and A5), 7.21 (1H,

br-dd, *J* = 7, 2 Hz, C6), 7.25 (1H, br-dd, *J* = 7, 2 Hz, B6), 7.32 (1H, br-td, *J* = 7, 2 Hz, B2), 7.33 (1H, m, Bn4), 7.39 (1H, br-td, *J* = 7, 2 Hz, C2), 7.39 (2H, br-t, *J* = 7 Hz, Bn3 and Bn5), 7.43 (2H, br-d, *J* = 7 Hz, Bn2 and Bn6). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 56.5 (OMe), 60.3 (γ), 70.1 (Ph*C*H_2), 72.3 (α), 82.2 (β), 112.0 (C3), 113.9 (B3), 114.7 (A2 and A6), 121.2 (C1), 121.5 (B1), 127.3 (A3 and A5), 127.4 (Bn2 and Bn6), 127.9 (Bn4), 128.3 (C5), 128.6 (Bn3 and Bn5), 128.8 (B2), 129.0 (C2), 129.4 (B5), 131.5 (C6), 131.5 (B6), 132.2 (A1), 136.9 (Bn1), 154.7 (B4), 156.7 (C4), 158.2 (A4). Compound **6t** (*threo* isomer). ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.34 (1H, broad-dd, *J* = 12, 3 Hz, γ_1), 3.64 (1H, br-dd, *J* = 12, 3 Hz, γ_2), 3.78 (3H, s, OMe), 4.37 (1H, m, β), 4.75 (1H, d, *J* = 8.0 Hz, α), 5.06 (2H, s, Ph*C*H_2), 6.94 (2H, br-d, *J* = 9 Hz, A2 and A6), 7.05 (1H, br-d, *J* = 7 Hz, B3 and C3), 7.07 (1H, br-td, *J* = 7, 1 Hz, B1), 7.09 (1H, br-td, *J* = 7, 1 Hz, C1), 7.24 (1H, br-dd, *J* = 7, 2 Hz, C6), 7.25 (1H, br-dd, *J* = 7, 2 Hz, B6), 7.29 (2H, br-d, *J* = 9 Hz, A3 and A5), 7.32 (1H, br-td, *J* = 7, 2 Hz, B2), 7.33 (1H, br-t, *J* = 7 Hz, Bn4), 7.39 (2H, br-t, *J* = 7 Hz, Bn3 and Bn5), 7.39 (1H, br-td, *J* = 7, 2 Hz, C2), 7.43 (2H, br-d, *J* = 7 Hz, Bn2 and Bn6). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 56.4 (OMe), 60.3 (γ), 70.0 (Ph*C*H_2), 73.2 (α), 83.5 (β), 111.9 (C3), 113.5 (B3), 114.9 (A2 and A6), 121.4 (C1), 121.5 (B1), 127.4 (Bn2 and Bn6), 127.9 (Bn4), 128.3 (A3 and A5), 128.5 (Bn3 and Bn5), 128.9 (B2), 129.0 (C2), 129.1 (C5 and B5), 131.4 (C6), 131.6 (B6), 131.7 (A1), 136.9 (Bn1), 155.1 (B4), 156.6 (C4), 158.7 (A4).

Compound H-HH$_{biphenyl}$-*erythro* (*erythro* isomer of 1-(4-hydroxyphenyl)-2-(2'-methoxy-[1,1'-biphenyl]-2-yloxy)propane-1,3-diol). A stirred suspension of compound **6e** (1.65 g, 3.6 mmol) and 10% Pd/C (0.17 g) in THF (73 mL) was fitted with a hydrogen-gas-filled balloon. The reaction mixture was continuously stirred overnight, at room temperature. The completion of the reaction was monitored by TLC [hexane-EtOAc=1:1; Rf: 0.35 (target compound) and 0.45 (starting material)]. After the reaction, Pd/C was passed through a glass filter using celite and washed with acetone. The filtrate was concentrated under vacuum pressure. The residue was purified by silica gel chromatography (hexane-EtOAc, 1:1) to afford the *erythro* isomer of compound **H-HH$_{biphenyl}$** in syrup form (1.3 g, 98% yield). The configuration of the compound was determined by ozonation in AcOH-H_2O-MeOH (80:15:5, v/v/v) solvent, which afforded only erythronic acid. ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.55 (1H, broad-dd, *J* = 12, 3 Hz, γ_1), 3.69 (1H, br-dd, *J* = 12, 5 Hz, γ_2), 3.71 (3H, s, OMe), 4.39 (1H, m, β), 4.84 (1H, d, *J* = 4.3 Hz, α), 6.62 (2H, br-d, *J* = 8 Hz, A2 and A6), 6.98 (2H, br-d, *J* = 8 Hz, A3 and A5), 6.99 (1H, br-d, *J* = 8 Hz, B3),

7.01 (1H, br-d, *J* = 8 Hz, C3), 7.05 (1H, br-t, *J* = 8 Hz, B1), 7.05 (1H, br-t, *J* = 8 Hz, C1), 7.19 (1H, br-dd, *J* = 8, 2 Hz, C6), 7.23 (1H, br-dd, *J* = 8, 2 Hz, B6), 7.30 (1H, br-td, *J* = 8, 2 Hz, B2), 7.36 (1H, br-td, *J* = 8, 2 Hz, C2). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 56.5 (OMe), 60.1 (γ), 72.2 (α), 82.0 (β), 112.2 (C3), 114.0 (B3), 115.2 (A2 and A6), 121.4 (C1), 121.6 (B1), 127.4 (A3 and A5), 128.3 (C5), 128.9 (B2), 129.0 (C2), 129.4 (B5), 131.3 (A1), 131.5 (C6), 131.6 (B6), 154.6 (B4), 155.3 (A4), 156.7 (C4).

Compound H-HH$_{biphenyl}$-*threo* (*threo* isomer of 1-(4-hydroxyphenyl)-2-(2'-methoxy-[1,1'-biphenyl]-2-yloxy)propane-1,3-diol). Following the procedure described above for the *erythro* isomer of **H-HH$_{biphenyl}$-*erythro***, compound **6t** (5.57 g, 12.2 mmol) was converted to the title compound (**H-HH$_{biphenyl}$-*threo***) in syrup form (4.4 g, 98% yield). Ozonation of this compound in AcOH-H_2O-MeOH (80:15:5, v/v/v) solvent afforded only threonic acid. The Rf values on TLC (hexane-EtOAc, 1:1, v/v) were 0.42 (target compound) and 0.56 (starting material). ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.29 (1H, broad-dd, *J* = 13, 3 Hz, γ_1), 3.61 (1H, br-dd, *J* = 13, 3 Hz, γ_2), 3.74 (3H, s, OMe), 4.36 (1H, m, β), 4.68 (1H, d, *J* = 8.0 Hz, α), 6.63 (2H, d, *J* = 8.6 Hz, A2 and A6), 7.04 (1H, br-d, *J* = 8 Hz, B3), 7.05 (1H, br-d, *J* = 8 Hz, C3), 7.06 (1H, br-t, *J* = 8 Hz, B1), 7.08 (1H, br-t, *J* = 8 Hz, C1), 7.13 (2H, d, *J* = 8.6 Hz, A3 and A5), 7.22 (1H, br-dd, *J* = 8, 2 Hz, C6), 7.23 (1H, br-dd, *J* = 8, 2 Hz, B6), 7.32 (1H, br-td, *J* = 8, 2 Hz, B2), 7.38 (1H, br-td, *J* = 8, 2 Hz, C2). ^{13}C NMR ($CDCl_3$, 125 MHz), δ_C: 56.6 (OMe), 60.3 (γ), 73.4 (α), 83.4 (β), 112.3 (C3), 113.4 (B3), 115.6 (A2 and A6), 121.6 (C1 and B1), 128.4 (B5 and C5), 128.5 (A3 and A5), 129.0 (B2), 129.1 (C2), 130.6 (A1), 131.4 (C6), 131.7 (B6), 155.1 (B4), 156.0 (A4), 156.6 (C4).

Preparation of QMs. Dimeric β–O–4-aryl ether QM compounds (**QM-HH**, **-HG**, **-GH**, and **-GG**) were synthesized from the corresponding dimeric β–O–4 model compounds (**HH**, **HG**, **GH**, and **GG**, respectively) by mild alkaline treatment of the benzyl bromides,[4] which were prepared using trimethylsilyl bromide (TMSiBr).[5, 36] The detailed procedure was described in our previous report.[8] The β–O–4-biphenyl ether QM (**QM-H-HH$_{biphenyl}$**) was also prepared according to this method, as described below.

The β-O-4 compound **H-HH$_{biphenyl}$** (0.1 mmol) was dissolved in chloroform (1 mL) in a 4-mL vial with a screw cap. TMSiBr (0.2 mmol) was added to this solution and shaken for 90 s at room temperature to generate benzyl bromide. The reaction mixture was shaken with

saturated $NaHCO_3$ (1 mL) for 10 s. The collected organic layer was washed with saturated NaCl and dried over Na_2SO_4. The resulting **QM-H-HH$_{biphenyl}$** (pale-yellow) was diluted with dioxane into desired concentrations (approx. 0.1 mM or 8 mM). These QM solutions were used for the subsequent water addition experiments without further purification. For NMR structural determination, the QM was prepared in a separate experiment, the same way, using deuterated chloroform as a reaction solvent. The chloroform-*d* solution of the crude QM was transferred into an NMR tube and kept in liquid N_2 before the NMR measurement. No residual peak from the β–O–4 dimer was found on the ^{1}H NMR spectrum of **QM-H-HH$_{biphenyl}$** (Figure S1) as well as on those of the other **QMs**.[8]

Compound QM-H-HH$_{biphenyl}$. ^{1}H NMR ($CDCl_3$, 500 MHz), δ_H: 3.68 (2H, m, γ), 3.77 (3H, s, C4-OMe), 5.25 (1H, m, β), 6.33 (1H, d, *J* = 7.2 Hz, α), 6.37 (1H, broad-dd, *J* = 10, 2 Hz, A3), 6.40 (1H, br-dt, *J* = 10, 2 Hz, A5), 6.84 (1H, br-d, *J* = 8 Hz, B3), 7.00 (1H, br-dd, *J* = 10, 2 Hz, A2), 7.04 (1H, br-d, *J* = 8 Hz, C3), 7.08 (2H, br-t, *J* = 7 Hz, B1 and C1), 7.23 (1H, br-dd, *J* = 7, 2 Hz, C6), 7.26 (1H, br-dd, *J* = 7, 2 Hz, B6), 7.28 (1H, m, B2), 7.38 (1H, br-td, *J* = 7, 8, 2 Hz, C2), 7.46 (1H, br-dd, *J* = 10, 2 Hz, A6). ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C: 56.5 (C4-OMe), 64.6 (γ), 77.4 (β), 112.2 (C3), 114.1 (B3), 121.4 (C1), 122.2 (B1), 128.0 (B5 and C5), 128.9 (A3), 129.1 (B2 and C2), 130.4 (A5), 131.5 (C6), 131.8 (B6), 133.0 (A1), 133.1 (A6), 141.5 (A2), 144.9 (α), 154.7 (B4), 156.6 (C4), 186.9 (A4).

Water addition to QMs in different solvent conditions at pH 5 or 7. A dioxane solution of QM prepared as described above (approx. 8 mM × 75 μL, which originates from 0.6 μmol of β-O-4 dimer), was further diluted with a certain volume of dioxane (0–3 mL) in a small vial with a screw cap; after mixing with a buffer, the volume ratio of dioxane-water (v/v) became 1:79, 1:7, 1:5, 1:1.5, or 1:1 with a total volume of approx. 6 mL (Therefore, water addition reaction was conducted at the concentration of approx. 0.1 mM QM). The water addition reaction was initiated by the addition of the buffer to the dioxane solution of QM. This marked the reaction time of 0 s. After the immediate shaking of the reaction mixture for a few seconds, an aliquot of the mixture (approx. 3 mL) was transferred into a quartz UV cell (1 cm square, TOS-UV-10; Toshin Riko Co., Ltd., Japan), which was placed in a UV–visible spectrometer equipped with a circulating water bath. The reaction mixture was maintained at 25 °C, and monitoring of the reaction progress began at 20 s. The disappearance of QMs and their half-life ($t_{1/2}$) in the buffered dioxane–water mixture was determined on the basis of the

decrease in UV absorbance.[8,37] Absorbance data at 304 nm was collected every 0.2 s (or 1 s) from the reaction time of 20 s to 10 min (or 120 min) depending the reaction rates of **QMs**. The collected data were fitted with an exponential function to calculate the pseudo-first-order reaction rate constants (k_{obs}) for the disappearance of the QMs (Table S1). The citrate–phosphate buffer at pH 4.8, 4.5, 4.4, 3.8, or 3.2 was used to prepare the 1:79, 1:7, 1:5, 1:1.5, or 1:1 ratio of dioxane-water mixture, respectively; after mixing with dioxane, the resulting buffered dioxane–water mixture indicated a pH of 5.0. Similarly, the buffer at pH 6.8, 6.6, 6.4, 5.5, or 5.3 was used to conduct the water addition experiment at pH 7.0 in the dioxane–buffer mixtures (1:79, 1:7, 1:5, 1:1.5, or 1:1, v/v). The buffer at pH 3.4 was used for the water addition experiment in dioxane–water mixture (1:79), at pH 3.5.

The ^{1}H NMR spectra of the QMs indicated that each crude QM solution was essentially pure and contained only small amounts of unknown products. Additionally, the ^{1}H NMR spectra of **QM-H-HH$_{biphenyl}$** (Figure S1) and of the other **QMs**[8] did not exhibit any residual peak of the substrate (β–O–4 model). The β–O–4 yield was expressed as the yield of the succeeding two reaction steps consisting of the QM formation (from a β–O–4 model) and water addition (from QM to the β–O–4 products).

The yields of *erythro* and *threo* β–O–4 products were determined by HPLC analyses. After the complete consumption of QM, an aliquot of the reaction mixture (1 mL) was mixed with a MeOH solution of 3,4-dimethoxyacetophenone (0.05 μmol), which was used as an internal standard (IS). The mixture was passed through a hydrophilic PTFE membrane filter (Millex-LG, 0.2 μm, Millipore), and analyzed by a high-performance liquid chromatograph (HPLC, LC-10A, Shimadzu Co., Kyoto, Japan) equipped with an SPD-M10A detector (280 nm, Shimadzu Co.).

The conditions for the HPLC analyses of the β–O–4 dimers **GG**, **GH**, **HG** and **HH** were described in our previous report.[8] The conditions for the HPLC analysis of the β–O–4 product **H-HH$_{biphenyl}$** were as follows: column, Luna 5u C18 (2) 100 A (150 mm × 4.6 mm; Phenomenex, Inc., Torrance, CA, USA); oven temperature, 40 °C; flow rate, 1.0 mL min^{-1}. The *erythro* and *threo* diastereomers of compound **H-HH$_{biphenyl}$** in the reaction products mixture were separated using a binary gradient system consisting of CH_3OH and H_2O initially at 15:85 ratio (v/v), and subsequently increased linearly over 7.5 min to 45:55, and again over

27.5 min to 75:25 (35 min total), following an equilibration step. The retention times of the products were 13.2 min for 3,4-dimethoxyacetophenone (IS), and 21.7 and 22.8 min for **H-HH$_{biphenyl}$** (*erythro* and *threo* isomers, respectively). The calibration curves of **H-HH$_{biphenyl}$** were as follows: Y = 1.93X + 0.008 (for the *erythro* isomer) and Y = 1.83X + 0.026 (for the *threo* isomer). Y is the molar ratio of each isomer to IS, and X is the peak area of each isomer to IS.

Water addition to QM-H-HH$_{biphenyl}$ at different pH levels in dioxane-water (1:1, v/v). The effect of pH on the *erythro*/*threo* ratio of β–O–4 products in the water addition to **QM-H-HH$_{biphenyl}$** was examined using the same procedure as that used in our previous study on **QM-HH**, **QM-HG**, **QM-GH**, and **QM-GG**, in which the water addition reaction was conducted at a concentration of approx. 0.05 mM QM. A dioxane solution of QM prepared as described above (approx. 0.1 mM × 3 mL, which originates from 0.3 μmol of β–O–4 dimer) was placed in a small vial with a screw cap. The reaction was initiated by mixing with the same volume of citrate–phosphate buffer at pH 2.4, 3.2, 3.9, 4.5, or 5.3 (3 mL). Therefore, the water addition reaction was conducted at the concentration of approx. 0.05 mM QM. This marked the reaction time of 0 s. The citrate–phosphate buffers mentioned above were prepared by mixing 0.01 M citric acid and 0.02 M disodium phosphate in different proportions; after mixing with an equal volume of dioxane, the resulting buffered dioxane–water mixture (1:1, v/v) indicated the desired pH values (3.5, 4.5, 5.5, 6.0, and 7.0). After the immediate shaking of the reaction mixture for 2–3 s, an aliquot of the mixture (approx. 3 mL) was transferred into a quartz UV cell, and UV absorbance data was measured using the same procedure as that in "Water addition to QMs in different solvent condition at pH 5 or 7" described above.

Preparation of DHP of *p*-coumaryl alcohol (H-DHP) by dropwise addition (Zutropfverfahren[38]). *p*-Coumaryl alcohol (200 mg in 100 mL of dioxane-water (3:7, v/v)), hydrogen peroxide (1.77 mmol in 20 mL water), and horseradish peroxidase (3.3 mg in 20 mL of water; 454 units/mg, Oriental yeast CO., LTD) were separately added dropwise over 25 h, using three syringe pumps (YSP-202, YMC, Japan), to a 500-mL three-necks flask containing a 0.5 M citrate-phosphate buffer at pH 3.5, 4.5, 5.0, 6.0 or 7.0 (100 mL), while stirring at room temperature. Therefore, the proportion of dioxane in the reaction mixture was changed during the addition, and the final proportion of dioxane-water was 1:7 when the

addition was complete. The flask was covered with aluminum foil to limit light penetration during the reaction. When the addition was finished, the reaction was brought to a stop by the addition of an aqueous sodium thiosulfate solution (1 mmol) and NaCl powder (36 g). Subsequently, the insoluble part was collected by filtration with a hydrophilic PTFE membrane filter (ADVANTEC, 0.2 μm), washed with distilled water, and dried under reduced pressure to afford light-brown colored powder (H-DHPs, 132–202 mg, 66–101 wt% yield). A part of the H-DHP (20 mg) was acetylated with acetic anhydride (0.5 mL) and pyridine (1.5 mL), at room temperature overnight. The reaction mixture was concentrated under reduced pressure at 45 °C. The remaining solvent was removed by repetition of the evaporation with EtOH to afford acetylated DHP.

Preparation of DHP of coniferyl alcohol (G-DHP) by dropwise addition (Zutropfverfahren[38]). Coniferyl alcohol (200 mg in 100 mL of dioxane-water (3:7, v/v)), hydrogen peroxide (1.77 mmol in 20 mL water), and horseradish peroxidase (3.3 mg in 20 mL of water; 454 units/mg) were separately added dropwise over 25 h using three syringe pumps to a 500 mL three-necks flask containing a 0.5 M citrate-phosphate buffer at pH 6.0 (100 mL), while stirring at room temperature. The same work-up procedure as that of the DHPs mentioned above was used to collect an insoluble part from the reaction suspension (G-DHP-6, 151 mg, 76 wt% yield). A part of G-DHP-6 (20 mg) was acetylated with acetic anhydride and pyridine.

Preparation of Zulauf-DHPs by one-time addition (Zulaufverfahren[38]). Monolignol (200 mg of *p*-coumaryl alcohol (H-type monolignol) or coniferyl alcohol (G-type monolignol) in 100 mL of dioxane-water (3:7, v/v)), hydrogen peroxide (1.77 mmol in 20 mL water), and horseradish peroxidase (3.3 mg in 20 mL of water; 454 units/mg) were added simultaneously to a 0.5 M citrate-phosphate buffer at pH 6.0 (100 ml), and stirred for 1 h at room temperature. The same work-up procedure as that of the DHPs mentioned above was used to collect an insoluble part from the reaction suspension (H-Zulauf-DHP, 174 mg, 87 wt% yield; G-Zulauf-DHP, 120 mg, 60 wt%). A part of the Zulauf-DHP (20 mg) was acetylated with acetic anhydride and pyridine.

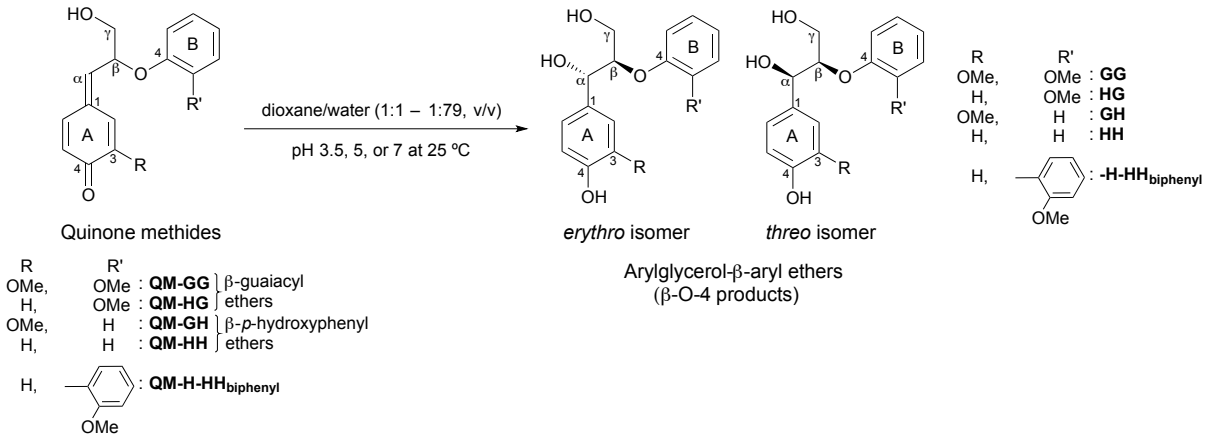
Ozonation method. The stereo configurations of the synthesized β–O–4 model compounds and the β–O–4 structures in DHPs were determined by the ozonation method according to

Akiyama et al.[29] A β–O–4 compound (10 μmol) or DHP (20 mg) was treated with ozone in AcOH-H_2O-MeOH solvent (80:15:5, v/v/v). The erythronic acid (E) and threonic acid (T) obtained as ozonation products were determined by gas chromatography as trimethylsilyl derivatives. The *erythro*/*threo* ratio was defined as the E/T ratio.

Size-exclusion chromatographic (SEC) analyses. SEC analyses of the acetylated DHP samples were performed on a Shimadzu HPLC/GPC system with an LC-20AD pump and an SPD-20A UV-Vis detector. The conditions for the SEC analyses were as follows: column, Shodex GPC KF-802 and KF-802.5 (Showa Denko, Japan) in series; column oven temperature, 40 °C; eluent, THF; flow rate, 1.0 mL min^{-1}. The retention time of the column was calibrated with polystyrene standards (Shodex Standards, SL-105, Showa Denko, Japan) in the MW range of 482–0.94 × 10^3 g mol^{-1}, propylbenzene (Mw, 120), and benzene (Mw, 78). Approximately 2.5 mg of acetylated DHPs (H-DHP-3.5, -4.5, and -5, H-zulauf-DHP-6, and G-zulauf-DHP-6) were dissolved in THF (5 mL) and kept overnight. The solution was passed through a 0.45-μm filter, and 20 μL of the solution was injected into the columns. Other acetylated DHPs (H-DHP-6, and -7, and G-DHP-6) were only partially dissolved in THF and could not be analyzed. Shimadzu LC solution (ver. 5.73) software was used for the data acquisition and the data processing at 280 nm to obtain the peak MW (*M*p), weight average MWs (*M*n and *M*w, respectively), and polydispersity (*M*w/*M*n).

RESULTS AND DISCUSSION

In our previous study,[8] water addition experiments were conducted in 50% water solution (dioxane/water, 1:1, v/v) to investigate the reactivity of QMs and the stereoselectivity of the formation reaction of β–O–4 structures from the QMs. We followed the experimental conditions developed by Nakatsubo's group[4] and Brunow's group,[6] and the experiments were conducted at a wide pH range, under neutral and mildly acidic conditions (pH 3.5–7), to cover the possible pH conditions assumed in cell walls since the pH condition at the lignification site remains unknown. Similarly, the solvent conditions or circumstances that model the surroundings of the quinone methide structure, located at the end of growing lignins in the cell walls where cellulose and hemicellulose are already present, remains unknown. Nakatsubo et al.[4] conducted water addition experiments to **QM-GG** and found that the *erythro*/*threo* ratio of the β–O–4 product **GG** was 0.4 in dioxane/water (1:1, v/v), and 0.5 in dioxane/water (1:9, v/v), respectively. In the present study, we investigated the details of the solvent effect. The water concentration of buffered dioxane-water solvent was changed from 50 vol% to approximately 99 vol% (dioxane/water, 1:1 to 1:79, v/v), and the water addition experiments were conducted for the various types of QMs at 25 °C (Scheme 3).



Scheme 3. Water addition experiments to replicate the formation of H- and G-type β–O–4-structures from the quinone methides (QMs) during lignin biosynthesis.

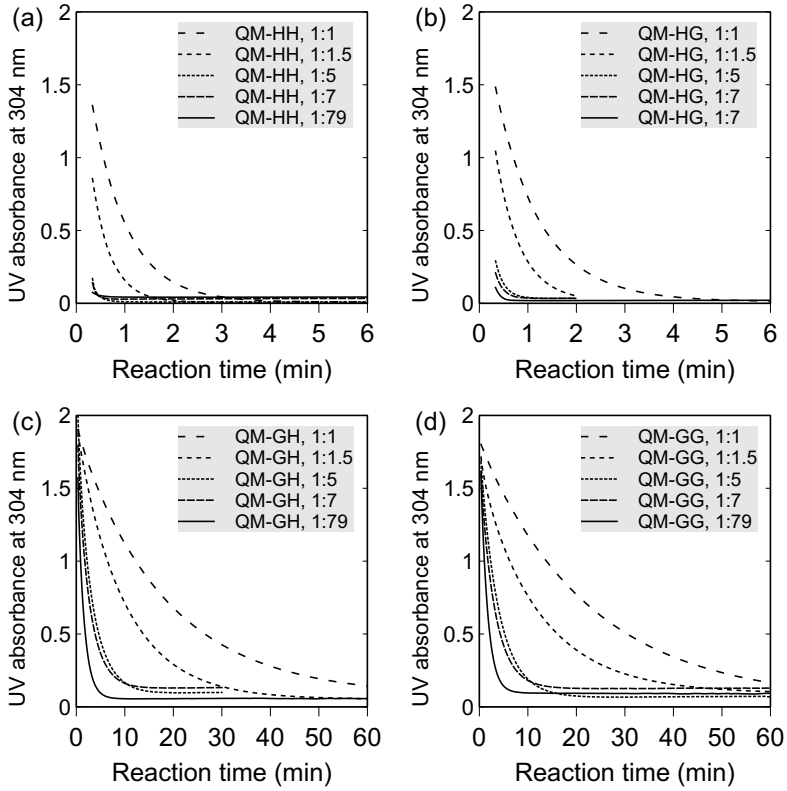
481

482

483 **Table 1.** Half-lives of quinone methides (QMs)[a] in buffered dioxane-water solutions[b] at 25 °C
484 and the yields of *erythro* and *threo* stereoisomers of β–O–4-products.

Entry	Dioxane/water solvent (v/v)	Half-life ($t_{1/2}$)		Product	Yield (mol%)		*erythro*/*threo*	
		pH 5	pH 7		pH 5	pH 7	pH 5	pH 7
QM-HH	1:79	< 20 s	< 20 s	**HH**	88	87	20:80	19:81
	1:7	< 20 s	24 s	**HH**	86	86	21:79	20:80
	1:5	25 s	24 s	**HH**	87	87	22:78	21:79
	1:1.5	32 s	36 s	**HH**	86	82	25:75	23:77
	1:1	45 s	51 s	**HH**	87	78	26:74	23:77
QM-HG	1:79	28 s	26 s	**HG**	85	84	31:69	30:70
	1:7	29 s	28 s	**HG**	89	90	30:70	29:71
	1:5	29 s	29 s	**HG**	84	87	30:70	28:72
	1:1.5	36 s	41 s	**HG**	83	85	31:69	27:73
	1:1	49 s	59 s	**HG**	84	79	31:69	27:73
QM-GH	1:79	77 s	72 s	**GH**	81	86	23:77	23:77
	1:7	126 s	123 s	**GH**	77	83	25:75	24:76
	1:5	130 s	133 s	**GH**	79	83	26:74	24:76
	1:1.5	398 s	426 s	**GH**	75	68	28:72	26:74
	1:1	759 s	795 s	**GH**	74	66	29:71	27:73
QM-GG	1:79	81 s	84 s	**GG**	73	76	44:56	43:57
	1:7	146 s	150 s	**GG**	63	71	40:60	39:61
	1:5	157 s	182 s	**GG**	66	80	40:60	38:62
	1:1.5	448 s	501 s	**GG**	67	60	39:61	35:65
	1:1	839 s	978 s	**GG**	75	74	38:62	34:66
	(1:79, pH3.5)[c]	(47 s)[c]	---	**GG**	(86)[c]	--	(49:51)[c]	--
QM-H-HH[d]	1:79	nd[e]	nd[e]	**H-HH**[d]	65	64	55:45	54:46
	1:7	nd[e]	nd[e]	**H-HH**[d]	78	78	58:42	55:45
	1:5	nd[e]	nd[e]	**H-HH**[d]	78	82	59:41	56:44
	1:1.5	nd[e]	nd[e]	**H-HH**[d]	87	83	61:39	56:44
	1:1	nd[e]	nd[e]	**H-HH**[d]	85	78	60:40	54:46

485 [a]See Table S1 for the pseudo-first-order-reaction-rate constants (k_{obs}) for the disappearance of
486 the QMs. [b]Dioxane-water ratio of 1:79, 1:7, 1:5, 1:1.5, and 1:1; pH 3.5, 5, and 7.
487 [c]Dioxane-water ratio of 1:79 at pH 3.5. [d]**QM-H-HH$_{biphenyl}$** and **H-HH$_{biphenyl}$**. [e]Not determined.



488

489 **Figure 1.** Effect of solvent on the disappearance of quinone methides (QMs) in pH 7 buffered
490 dioxane-water solutions, at 25 °C. Changes in the absorbance at 304 nm for (a) **QM-HH**, (b)
491 **QM-HG**, (c) **QM-GH**, and (d) **QM-GG**. Dioxane—water ratio of 1:1 to 1:79 (v/v). See
492 Figure S2 for the UV spectra of the QMs under at pH 7 condition.

493

494

Reactivities of QMs with water.

The effect of the solvent on the half-lives of QMs was investigated. The water addition reaction proceeded fast in the order of **QM-HH** > **-HG** > **-GH** > **-GG** in the dioxane-water (1:1, v/v) solvent, at both pH 5 and 7 (Table 1). These results confirmed the reproducibility of our previous result obtained in 50% water solution[8]. As shown in Table 1 and Figure 1, the reaction rate of QMs increased when the proportion of the water in a dioxane-water (1:1, 1:1.5, 1:5, 1:7, and 1:79, v/v) solvent was increased from 50 % to approx. 99 %. For example, **QM-GG** had the longest half-life ($t_{1/2}$) of 978 s in the 50%-dioxane solution (dioxane-water, 1:1, v/v) at pH 7. Increasing the proportion of water shortened the $t_{1/2}$ by up to 12-times ($t_{1/2}$: 84 s in 99 %-water) at the same pH. The solvent effect was observed for another G-type QM (**QM-GH**). More so, the reaction rate of the less stable H-type QMs (**QM-HH** and **-HG**) increased with the proportion of the water (e.g., $t_{1/2}$ of QM-HG at pH 7: 59 s and 26 s in 50%- and 99%-water solutions, respectively). Consequently, $t_{1/2}$ was always short in the order of **QM-HH** < **-HG** < **-GH** < **-GG**, regardless of the solvent conditions. Moreover, this order of $t_{1/2}$ observed at pH 7 in different solvent systems did not change at pH 5.

The half-life of **QM-H-HH$_{biphenyl}$** could not be measured because its UV absorbance peak intensively overlapped with the shoulder of the peak of the β–O–4 products H-HH (Figure S2-c). However, it was clear that **QM-H-HH$_{biphenyl}$** disappeared within 4 min in dioxane-water (1:1, v/v) at pH 7 (Figure S2-c). This period (4 min) was even shorter than the half-life of **QM-GH** and **-GG** ($t_{1/2}$, 795 s for **QM-GH**, and 978 s for **QM-GG**) under the same reaction condition. In addition, **QM-H-HH$_{biphenyl}$** afforded the β–O–4 products, **H-HH$_{biphenyl}$**, in high yields (82% as a total of *erythro* and *threo* isomers), suggesting that the water addition reaction was the main reaction in this experiment. Therefore, it can be safely concluded that the reaction rate of **QM-H-HH$_{biphenyl}$** with water was higher than that of methoxy-substituted QMs (**QM-GH** and **-GG**), and that rapid rearomatization occurred to generate the β–O–4 structure similar to other non-substituted QMs (**QM-HH** and **-HG**).

Previously,[8] as an implication for the lignin biosynthesis, we proposed that when a new β–O–4-linkage is introduced at the end of a growing lignin by a radical-coupling reaction with a monolignol, a nonsubstituted QM moiety (H-type), derived from *p*-coumaryl alcohol,

will undergo rearomatization faster than the methoxy-substituted QM (G-type) from coniferyl alcohol will. This proposal was based on the results of $t_{1/2}$ values obtained for QM models in the 50% water system. The results obtained in the present study using different solvent condition also supported this proposal.

Effect of solvent on the stereopreferential formation of the β–O–4 structure

We previously reported[8] that the four kinds of QMs bearing a guaiacyl or a *p*-hydroxyphenyl nuclei in their β-etherified ring (**QM-HH**, **-HG**, **-GH**, and **-GG**) showed *threo*-preferential water addition for the formation of dimeric β–O–4-products. The resultant *erythro*/*threo* ratio was always high in the order of **GG** > **HG** > **GH** > **HH**, at every pH condition within the range of 3.5–7, when the water addition experiments were conducted in the 50%-water solvent (dioxane-water, 1:1, v/v). The solvent effect on the *erythro*/*threo* ratio was examined at pH 5 and 7 in the present study. As shown in Figures 2a1, and a2 and Table 1, the order did not change when the water content in the solvent changed from 50 to 99% (*erythro*/*threo* ratio: **GG** > **HG** ≥ **GH** > **HH**).

However, the effect of the change in the solvent water content on the *erythro*/*threo* ratio appeared in an opposite way between the β–O–4-guaiacyl ether QMs (**QM-GG**, and **-HG**) and the β–O–4-*p*-hydroxyphenyl ether QMs (**QM-GH**, and **-HH**). The *erythro*/*threo* ratio was high for the formation of β–O–4-products, **GG** and **HG**, with an increase in the water concentration, whereas the *threo* isomers of **GH** and **HH** were more selectively formed under a high water concentration condition. This trend was observed at both pH 5 and 7 (Figures 2a1 and a2). Consequently, the variation in the *erythro*/*threo* ratios of the four dimeric β–O–4-products obtained in 99%-water solution was much wider than that in 50%-water solution (the proportion of *erythro* isomer obtained at pH 7: 19–43% (Δ24%) in 99%-water solution, and 23–34% (Δ11%) in 50%-water solution). In our previous study, a wider variation in the *erythro*/*threo* ratio was obtained under a more acidic condition, in 50%-water solution (proportion of *erythro* isomer: 30–64% (Δ26%) at pH 3.5). More so, under the neutral pH condition, a comparatively wide variation in the *erythro*/*threo* ratio was observed as the water concentration increased to 99% (Figure 2a2).

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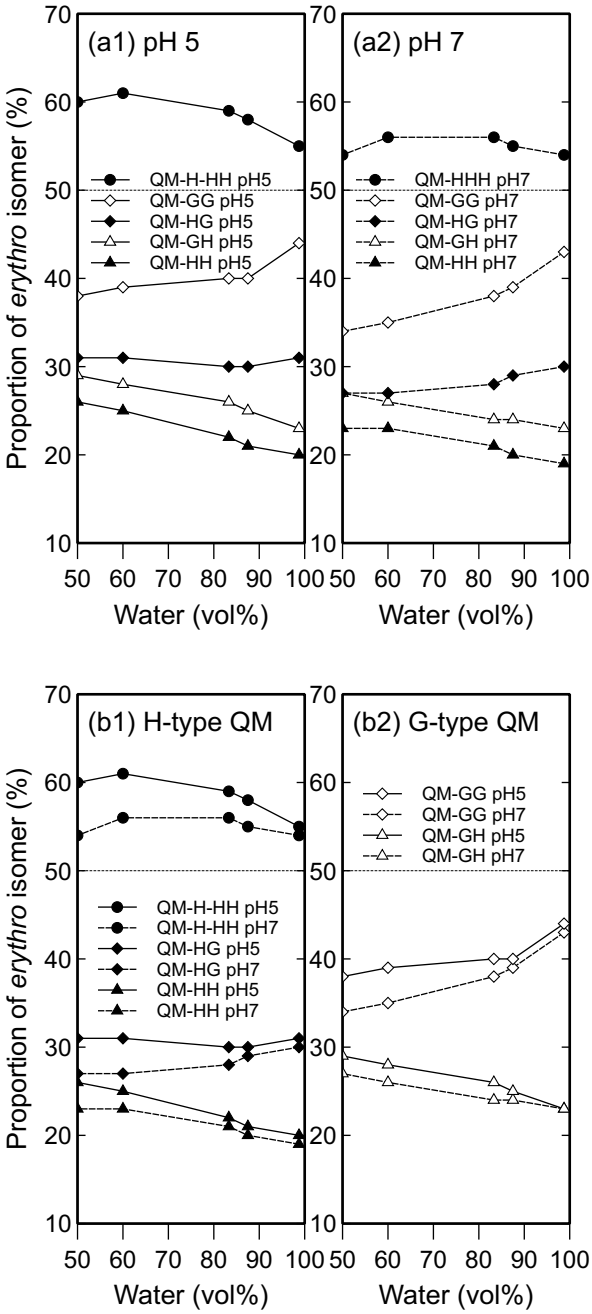

555

556 **Figure 2** Effect of solvent on the *erythro*/*threo* ratio of the β–O–4-products obtained by water
557 addition to quinone methides (QM) in dioxane-water solutions, at 25 °C, at pH 5 and 7. The
558 proportion of *erythro* isomer observed for (a1) all QMs at a pH 5, (a2) all QMs at pH 5, (b1)
559 H-type QMs, (b2) G-type QM. Water (vol%): proportions of water in the dioxane–water

560 solutions (1:1, 1:1.5, 1:5, 1:7, and 1:79, v/v). *erythro* isomer (%): proportion of *erythro*
561 isomer to the total of *erythro* and *threo* isomers.

562 In a 50%-water solution (dioxane-water, 1:1, v/v), the *erythro*/*threo* ratio observed
563 at pH 5, (solid lines in Figures 2b1 and 2b2) was significantly higher than that observed at pH
564 7 (dotted lines). The reproducibility of the pH dependence observed in our previous study was
565 confirmed by this result. However, interestingly, the difference in the *erythro*/*threo* ratios
566 caused by the difference in pH gradually ceased as the solvent water concentration increased.
567 Almost no difference was found between the *erythro*/*threo* ratios obtained at pH 5 and 7, in
568 the 99%-water solvent (dioxane-water, 1:79). This trend was observed for all five QMs
569 examined here, regardless of whether the *erythro*/*threo* ratios increase or decrease as the
570 water proportion in the dioxane-water solvent increased. In addition, as shown in Table 1, a
571 relatively high *erythro*/*threo* ratio of **GG** was obtained under a more acidic condition, in the
572 order of pH $3.5 > 5 > 7$, in 99%-water solution. The same order was observed in the
573 50%-water solution in the previous study;[8] however, the range of the *erythro*/*threo* ratio was
574 narrower in 99%-water solution than in the 50%-water solution (the proportion of *erythro*
575 isomer of **GG** at pH 3.5–7: 43–49% (Δ6%) in 99%-water solution, and 35–50% (Δ15%) in
576 50%-water solution). Apparently, the effect of pH on the stereo-preference of the water
577 addition reaction was smaller in the 99%-water solution than in 50%-water solution.

578 The increase or decrease in the *erythro*/*threo* ratio with the increase in the water
579 proportion depends on the chemical structures of QMs. The trend of the *erythro*/*threo* ratio
580 seems to be largely dependent on the β-etherified ring-type. As shown in Figures 2a1 and a2,
581 the *erythro*/*threo* ratio of β–O–4 products obtained from β-*p*-hydroxyphenyl ether QMs
582 (**QM-HH** and **-GH**) decreased as the water proportion in the solvent increased. This trend
583 was observed at both pH 5 and 7. In contrast, for β-guaiacyl ether QMs, the *erythro*/*threo*
584 ratio obtained for **QM-HG** did not significantly change, while that of **QM-GG** increased as
585 the water proportion increased (Figures 2a1 and a2), indicating that, as well as the structures
586 of β-etherified ring, the type of quinone methide moiety also influences, to some extent, the
587 *erythro*/*threo* ratio as the water proportion in the solvent increases.

588 **The *erythro*-preferential formation of β–O–4 structure**

The *erythro*/*threo* ratio of β–O–4 structures in compression wood lignin are slightly but clearly higher than 1.0, which was determined by the ozonation analyses in the reaction woods of Loblolly pine (*Pinus taeda*)[28] and Sumatran pine (*Pinus merkusii*).[26] The *erythro*/*threo* ratio was 50:50 in the upper side of the leaning stem and increased towards the lower side, i.e., the compression wood side with an increased content of H-units (the *erythro* form 49.7% and 53.5% in the upper and lower side, respectively).[26] However, as described above, the water addition to the QMs stereo-preferentially afforded the *threo* isomer during the formation of all the H- and G-type dimeric β–O–4-products, although the variation in the *erythro*/*threo* ratio widened as the water concentration increased.

Conversely, Brunow et al.[6] conducted a model study using β-syringyl ether QMs to replicate the hardwood lignin biosynthesis and evidenced that *erythro*-selective formation reaction occurs *in vitro* water addition to QMs. To investigate whether *erythro*-selective formation reaction occurs in the absence of the syringyl unit, we examined an H-type QM bearing a biphenyl structure, which serves to increase the steric bulkiness of the aryl group etherified at the β-position of QM, which can possibly affect the preferable stereo conformation of the functional groups attached to the β-asymmetric carbon of the QM, and may influence the stereo-preference of the water addition reaction. We synthesized an H-type QM-β-biphenyl ether compound (**QM-H-HH$_{biphenyl}$** in Scheme 2) from the corresponding trimeric β–O–4-compound (**H-HH$_{biphenyl}$**) that is composed of only H-units. This structure can potentially be formed during biosynthesis, although no structural evidence has been obtained (Scheme 3). The HH-type biphenyl present in the structure was reported to be that of dibenzodioxocin structure,[39,40] however, this 8-membered ring structure can be formed by the intramolecular cyclization of a β-O-4-biphenyl ether QM, instead of the water addition reaction. The phenolic hydroxy group was protected by methylation in the **QM-H-HH$_{biphenyl}$** to avoid the cyclization reaction and to make the water addition reaction possible.

As shown in Table 2, the *erythro*/*threo* ratios of the β–O–4 products were higher than 1.0 under all the condition examined. It was evidenced that *erythro*-preferential water addition can occur to a quinone methide composed of only H-units. Notably, an *erythro*-preferential formation was observed even under neutral conditions in a water addition experiment to a lignin-related QM, although the mechanism of the stereo-preference remains unknown.

620

621 **Table 2.** Effect of pH on the *erythro*/*threo* ratio of the β–O–4-product (**H-HH$_{biphenyl}$**) obtained
622 by the water addition reaction to **QM-H-HH$_{biphenyl}$**, in buffered dioxane-water solutions (1:1),
623 at 25 °C.

Entry	pH	Product	Yield (mol%)	*erythro*/*threo*
QM-HH$_{biphenyl}$	3.5	**H-HH$_{biphenyl}$**	93	70:30
	4.5	**H-HH$_{biphenyl}$**	82	66:34
	5.5	**H-HH$_{biphenyl}$**	76	57:43
	6	**H-HH$_{biphenyl}$**	75	56:44
	7	**H-HH$_{biphenyl}$**	82	56:44

624

625 **The *erythro*/*threo* ratio of the β–O–4 structures in DHPs**

626 In the water addition experiments, the *threo* preference observed in the H- and
627 G-type dimeric β–O–4-products turned to *erythro* preference in the trimeric biphenyl ether β–
628 O–4-products, implying that an increase in the steric bulkiness of the structure etherified at
629 the β-position of QM induced the *erythro* preference. Dehydrogenation polymers (DHPs)
630 were prepared from *p*-coumaryl alcohol by enzymatic oxidative coupling reaction with
631 horseradish peroxidase and hydrogen peroxide, and the *erythro*/*threo* ratios of the β–O–4
632 structures in the DHP were analyzed by ozonation method. We attempted to investigate the
633 size effect of the structure attached to the β-etherified ring of QM on the stereopreference in
634 the water addition reaction.

635 We focused on the structural differences between two types of DHPs prepared by
636 the gradual addition of a monomer to the dehydrogenation system (Zutropfverfahren), and the
637 one-time addition of the monomer (Zulaufverhahren).[38] Unless otherwise noted, the DHPs
638 were prepared by the gradual addition of the monomer. The DHP prepared by the one-time
639 addition of the monomer was named "Zulauf-DHP," in the present study (see the footnote of
640 Table 3 for the details). The yield of ozonation products (E + T) obtained from the G-type
641 DHP, prepared from coniferyl alcohol at pH 6 (G-DHP-6), was 977 μmol/g-DHP (Table 3),
642 which was not significantly lower than those obtained from softwood lignins[20] composed
643 mostly of G-unit in the previous study (E + T for six softwood species, 1180–1380
644 μmol/g-lignin, on the Klason lignin content basis). This result suggested that the β–O–4

content in the G-DHP-6 was similar to those in softwood lignins. The *erythro*/*threo* ratio of the G-DHP-6 was 55:45, which is closer to the ratios reported for softwood lignins (1:1)[20,22,41] than it is to that for H-DHPs.

Table 3. The *erythro*/*threo* ratio[a] of β–O–4-structures in dehydrogenation polymers (DHPs)[b] prepared from *p*-coumaryl or coniferyl alcohol in peroxidase-H_2O_2 system and the molecular weight distributions[c] of the DHPs.

Entry	pH[b]	Product name (DHP)[c]	Yield (wt%)	Mn[d]	Mw[d]	Mp[d]	Mw/Mn	Ozonation	
								E + T (μmol/g)	E/T
p-Coumaryl alcohol	3.5	H-DHP-3.5	66	1642	4715	1866	2.9	288	37:63
	4.5	H-DHP-4.5	98	2022	5959	2437	3.0	313	37:63
	5	H-DHP-5	99	2780	11099	2455	4.0	346	36:64
	6	H-DHP-6	100	NA[d]	NA[d]	NA[d]	NA[d]	253	36:64
	7	H-DHP-7	101	NA[d]	NA[d]	NA[d]	NA[d]	265	38:62
	6	H-Zulauf-DHP-6	87	1278	2250	1655	1.8	94	32:68
Coniferyl alcohol	6	G-DHP-6	76	NA[d]	NA[d]	NA[d]	NA[d]	977	55:45
	6	G-Zulauf-DHP-6	60	1507	2460	1839	1.6	613	65:35

[a]The *erythro*/*threo* ratio of DHP (E/T) based on the yields of erythronic acid (E) and threonic acid (T), obtained by ozonation method.[29] [b]DHP was prepared in dioxane-water systems buffered at pH 3.5, 4.5, 5, 6, or 7. [c]DHPs were prepared by the gradual addition of the monomer to the dehydrogenation system (Zutropf method) unless otherwise noted. The DHP prepared by the one-time addition of the monomer (Zulauf method) was named "Zulauf-DHP." The letters H and G are used to respectively designate the DHPs prepared from *p*-coumaryl and coniferyl alcohols. Numbers 3.5, 4.5, 5, 6, and 7 represent the pH conditions during the preparation of the DHPs. [d]Molecular weight distributions of the DHP (acetylated) measured by size exclusion chromatography. Mn and Mw (weight average MWs), Mp (peak MW), and Mw/Mn (polydispersity) were calculated using a calibration curve of polystyrene standards. NA: Not available due to the limited solubility of the DHP (acetylated).

Based on the ozonation products yields, the H-type DHP prepared from *p*-coumaryl alcohol at pH 6 (H-DHP-6), contains a much lower amount of β-O-4-structures than that contained in the G-type DHP prepared by the same method (G-DHP-6). A similar trend in the ozonation-products yields has been found between H- and G-type lignins. Namely, the yields of ozonation products were significantly lower in the compression wood containing a substantial amount of H-units (E+T, 711–930 μmol/g-lignin) than in the opposite wood containing a negligible amount of H-unit (912–1033 μmol/g-lignin).[26] The *erythro*/*threo* ratio of the H-DHP determined by ozonation method suggested that the *threo*-preferential water

addition occurred during the formation of the β–O–4-structures, similarly to the results during the formation of dimeric β–O–4-products, **HH**, by water addition to model QMs. However, the *erythro*/*threo* ratios of H-DHPs were higher than that of **HH** (the *erythro* proportion: 32-38% for all H-DHPs and H-Zulauf-DHP, and 19-30% for **HH** obtained in the present and previous studies[8]).

A significant difference in the *erythro*/*threo* ratio was also found between the H-DHP prepared by the dropwise addition method and H-Zulauf-DHP prepared by the one-time addition method. Although the H-DHPs (dropwise addition method) were prepared under different pH conditions in the pH range of 3.5–7, the variation in the *erythro*/*threo* ratio was small (*erythro* proportion: 36–38%). These *erythro*/*threo* ratios were higher than that obtained for H-Zulauf-DHP-6 (*erythro* proportion: 32%). The molecular weight distributions of acetylated samples of DHPs were measured by SEC, and it was indicated that the H-DHPs prepared at pH of 3.5, 4.5, and 5 (H-DHP-3.5, -4.5, and -5) are composed of higher molecular weight polymers relative to the polymer composition of the DHP prepared by one-time addition (H-Zulauf-DHP-6). The Mw and Mn values of the H-DHP (in acetate form) were slightly relatively high when the polymer was prepared under high pH condition (Mn and Mw: pH 5 > 4.5 > 3.5). The H-DHPs prepared at pH 6 and 7 could not be analyzed by SEC because their acetylated samples were hardly dissolved in THF solvent, which was used as a column eluent. It was suggested that the H-DHPs prepared by gradual addition method, which seem to have a higher molecular weight distribution than H-zulauf-DHP, contain β–O–4 structures with a relatively high *erythro*/*threo* ratio, although the difference in molecular weights between the two DHPs prepared by gradual-addition and one-time addition methods, under the same pH condition (H-DHP-6 and H-Zulauf-DHP-6), could not be compared. This result was in line with the results of the water addition to the H-type QMs, i.e., **QM-H-HH**$_{\mathbf{biphenyl}}$ bearing a bulky structure in its β-etherified moiety showed a higher *erythro*/*threo* ratio compared to that exhibited by simple β-*p*-hydroxylphenyl ether **QM-HH**. Moreover, the *erythro*/*threo* ratio of the H-DHP prepared by the gradual-addition method was closer to the values reported for compression wood lignins in comparison with the ratio of the H-Zulauf-DHP. Conversely, an opposite trend in the *erythro*/*threo* ratio was observed for G-type DHP, in which the G-DHP prepared by the gradual-addition method (G-DHP-6) had an *erythro*/*threo* ratio lower than that of the one prepared by Zulauf method

(G-zulauf-DHP-6), although their molecular weights could not be compared due to the low solubility of the acetylated of G-DHP sample.

In the present study, it was found that solvent and pH conditions significantly influenced the reaction rates and the stereo-preference of QM lignin models in the water addition reaction. The reaction rates of the nonsubstituted QMs were always higher than that of 3-methoxy substituted QM regardless of the reaction conditions. This result supports our previous proposal: during lignin biosynthesis, *p*-hydroxyphenyl-type QMs derived from *p*-coumaryl alcohol by radical-coupling reaction with a growing lignin chain, can undergo rearomatization faster than guaiacyl-type QMs can. On the other hand, *threo*-preferential formation observed for most QMs differed from the *erythro* preference naturally found in compression wood lignins. This model experiment may not yet perfectly mimic the water addition in lignin biosynthesis. However, it was found that an *erythro*-preferential formation was able to occur even in an H-type QM. The stereoselectivity was greatly influenced by the β-etherified aromatic ring structure. The *erythro*-preferential formation was attained by introducing a biphenyl structure into the β-etherified aromatic-ring moiety.

Solvent and pH conditions significantly influenced the stereo-preference. Interestingly, the effect of pH on the stereopreference, which was observed in the dioxane-water solvent (1:1), almost disappeared upon increasing the proportion of water to 99%. The variation of the *erythro*/*threo* ratio between the different QMs widened as the water concentration increased. The effect of solvent and also substances coexisting in the cell wall may deserve more attention in understanding and reproducing the condition for lignification in cell walls.

Supporting Information. ^{1}H NMR spectra of **H-HH$_{\text{biphenyl}}$** and **QM-H-HH$_{\text{biphenyl}}$** (Figure S1), pseudo-first-order reaction rate constants (k_{obs}) for the disappearance of β–O–4-aryl ether QMs (Table S1), the UV absorbance spectra of QMs (Figure S2), the *erythro*/*threo* ratios of β–O–4-products obtained from QMs at pH 3.7–7 (Figure S3).

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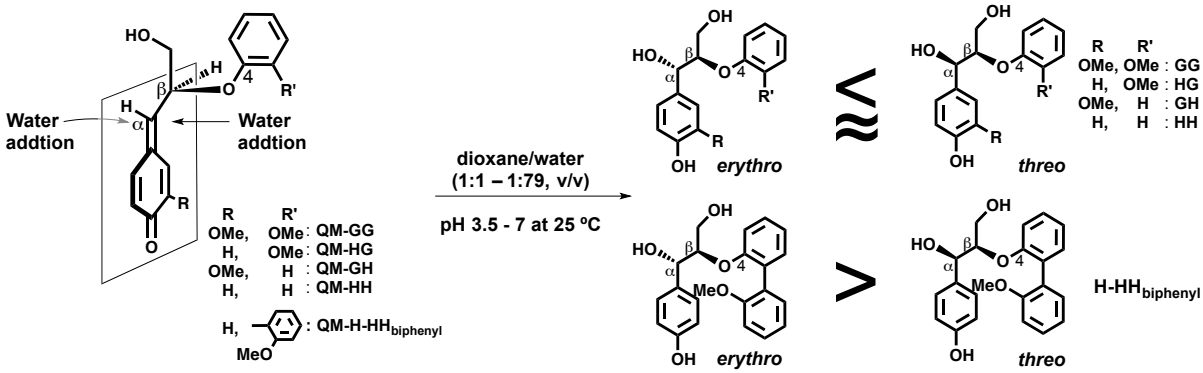

743 β-O-4-aryl ether quinone methides β-O-4 structures

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