Doctoral Thesis

Stereochemistry of β-O-4 Structures in Lignin

リグニンのβ-O-4型構造の立体化学に関する研究

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2003

Nomenclature and Abbreviations

Nomenclature

Abbreviations

C_6-C_3 unit	Phenylpropane unit
GC	Gas chromatography
HPLC	High performance liquid chromatography
MS	Mass spectroscopy
NMR	Nuclear magnetic resonance
MWL	Milled wood lignin
PhOH	Phenolic hydroxyl group
DMSO	Dimethylsulfoxide
HMDS	Hexamethyldisilazane
TMCS	Trimethylchlorosilane
VG	Veratrylglycerol-β-guaiacyl ether (1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol)

Contents

List of Publications

This thesis is based on the following papers.

I Akiyama T., Magara K., Matsumoto Y., Meshitsuka G., Ishizu A., Lundquist K. 2000. Proof of the presence of racemic forms of arylglycerol-β-aryl ether structure in lignin: studies on the stereo structure of lignin by ozonation. Journal of Wood Science 46, 414-415

II Akiyama, T., Sugimoto, T., Matsumoto, Y., Meshitsuka, G. 2002. *Erythro*/*threo* ratio of β-O-4 structures as an important structural characteristic of lignin. Part 1. Improvement of ozonation method for the quantitative analysis of lignin. side-chain structure. Journal of Wood Science 48, 210-215.

III Absolute configurations of the α- and β-asymmetric carbons of β-O-4 structures in hardwood lignin. (under preparation)

IV *Erythro/threo* ratio of β-O-4 structures as an important structural characteristic of lignin. Part 3. Ratio of *erythro* and *threo* forms of β-O-4 structures in tension wood lignin. (submitted to *Phytochemistry*)

V *Erythro/threo* ratio of β-O-4 structures as an important structural characteristic of lignin. Part 4. Variation in the *erythro*/*threo* ratio in hardwood lignins and its relation to aromatic ring types. (submitted to *Holzforschung*)

Chapter 1

Introduction

1.1 Various linkage types in lignin

1.1.1 Formation of various linkage types in lignin

Radical coupling reactions between the resonance structures of monolignol radical (Fig.1.1) are involved in the formation of lignin, and the presence of multiple reaction sites in a monolignol gives the various linkage types (β-O-4, β-5, β-β, 5-5, and β-1 types etc.) in lignin. In this process (Fig. 1.2), quinone methide intermediates can be stabilized by the nucleophilic addition of water or some hydroxyl groups. Further single electron oxidation give rise to macromolecular lignins.

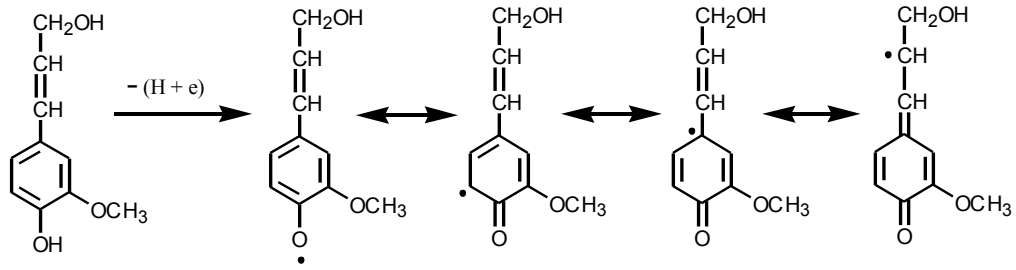


Fig. 1.1 Mesomeric forms of monolignol radical

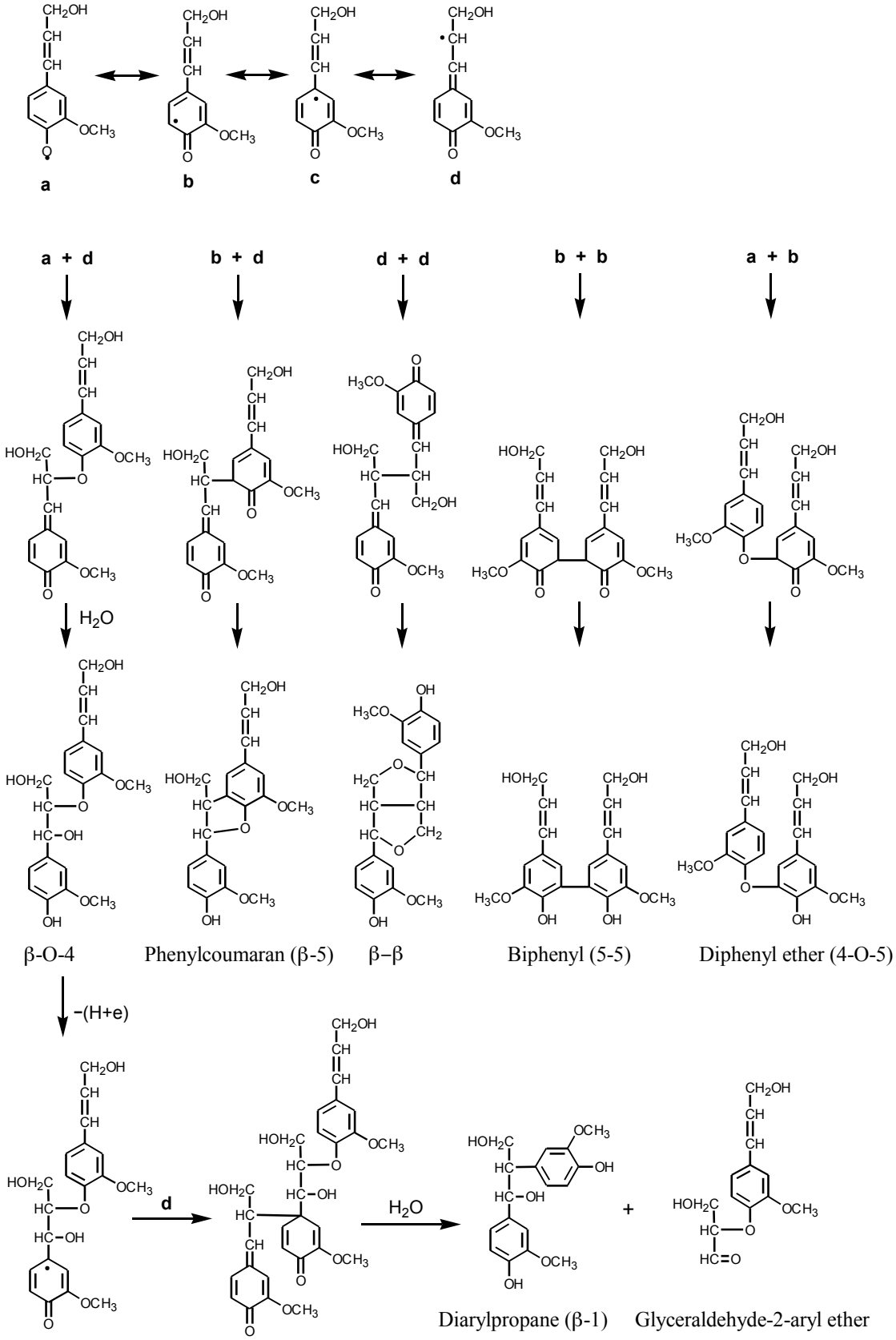


Fig. 1.2 Reactions involved in the polymerization of monolignols. The various types of dilignols were shown here.

1.1.2 Occurrence of various linkage types in lignin

Some researchers (Freudenberg, 1968; Sarkanen, 1971; Adler, 1977) have proposed the tentative estimates of the frequency of various linkage types in lignin to understand the structure of whole lignin based on the relative yields of permanganate oxidation products, acidolysis products, thioacetolysis etc. Each frequency per C_6-C_3 unit was summarized in Table 1.1.

Table 1.1 Tentative estimates of the frequencies of various linkage types per phenylpropane unit (C_6-C_3) in lignins

Author	Freudenberg (1968)	Sarkanen (1971)	Adler (1977)
Species	Spruce	Spruce	Birch
	(/ C6-C3)	(/C6-C3)	(/ C6-C3)
β-O-4	0.44	0.45	0.60
α-O-4 (non-cyclic)	0.08	0.08	0.06-0.08
Phenylcoumaran (β-5)	0.09	0.14	0.06
Biphenyl (5-5, 5-6, 5-1, 6-6, 2-6)	0.22	0.25	0.06-0.07
Diphenyl ether (4-O-5, 1-O-4, 4-O-6)	0.06	0.05	0.07
Diarylpropane (β-1, 1-6)		0.15	0.07
β-β	0.09	0.17	0.03
Glyceraldehyde-2-aryl ether			0.02

The quantitative estimates of the each linkage type have been performed by degradation methods and nuclear magnetic resonance (NMR) studies. The knowledge of some main linkage types in lignin was summarized in the following;

β-O-4 structure

Arylglycerol-β-aryl ether (β-O-4) structure is the most predominant linkage type in lignin (Adler, 1977). Quantitative estimates by ^{1}H-NMR studies with different solvents (Lundquist and von Unge, 1986; Lundquist, 1992) showed that spruce milled

wood lignin (MWL) contains 0.3-0.4 /C_6-C_3 of this structure and birch MWL contains 0.4-0.5 /C_6-C_3.

The occurrence of noncyclic benzyl aryl ether (α, β-bis-O-4) structure is still an open question in lignin structure, their existence in lignin has not been confirmed by spectroscopic or other method (Kilpeläinen et al, 1994). Since acetylated spruce MWL did not exhibit any peak at the expected δ value in its ^{1}H-NMR spectrum, Lundquist et al. suggested that the content of α, β-bis-O-4 structure is present at most few percent of β-O-4 structures (Lundquist and von Unge, 1986; Lundquist, 1992). Also in the different 2D NMR studies of birch MWL, the existence of this structure was not observed in the comparison with the model compound of the structure (Kilpeläinen et al, 1994; Ede and Ralph, 1996).

With respect to the diastereomeric forms (*erythro* and *threo* froms) of β-O-4 structure, the present knowledge will be described in Chapter 1.3.

β-5 structure

Phenylcoumaran (β-5) type structure is one of the main structures in lignin, and its content in spruce lignin was estimated to be 0.09-0.12 by an acidolysis (Adler and Lundquist, 1963). Based on the ^{1}H NMR studies, β-5 linkage was estimated to be 0.07/ C_6-C_3 in spruce MWL and few percent of C_6-C_3 units in birch MWL (Lundquist, 1992). Later, β-5 linkage was estimated to be 0.07/ C_6-C_3 in spruce MWL by acidolysis with 0.1M HBr (Li and Lundquist, 1999). Ozonation analysis of spruce MWL showed that the main configuration of the β-5 structures is the *trans* form (Habu et al., 1988).

β-1 structure

Diarylpropane (β-1) type structure has been considered to be one of the main structures in lignin in the early studies (in Table 1.1), but this of type structure does not

seem to be main structure any more in the recent studies.

In the ^{1}H-NMR studies, β-1 linkage was estimated to be 0.01-0.02/ C_6-C_3 in spruce MWL and 0.05/ C_6-C_3 in birch MWL based on the signal of Hβ (Lundquist, 1987). By ozonation analysis, β-1 linkage was estimated to be 0.02/ C_6-C_3 in spruce (Habu et al., 1990), which was in agreement with the estimates of glycealdehyde-2 aryl ether structure (0.016/C_6-C_3) in spruce by ozonation (Matsumoto et al., 1984).

With respect to the diastereomeric forms, the presence of both *erythro* and *threo* forms of β-1 structures were reported. (Ede and Ralph, 1996; Ede et al., 1996).

Recently, Ralph et al. proposed new type of β-1 structure with a 6-membered di-α-ether ring and with the original side chain migrated (Ralph et al., 1998; Peng et al., 1999). The presence of β-1 structure as "masked form", suggested by Lundquist, is not completely denied, and the presence as such a form could be the reason why β-1 structure is often observed in degradation products of lignin.

β-β structure

Pinoresinol structures were estimated to be present at 0.05-0.10/C_6-C_3 in softwood lignin based on the yields of dilactones obtained by nitric acid oxidation (Ogiyama and Kondo 1968). Quantitative estimates by the acidolysis of birch lignin indicated that the total of resinol structures (syringaresinol, pinoresinol, and their analogous structures) may be at the order of 10% of C_6-C_3 units (Lundquist, 1973).

The resinol structures in birch MWL were estimated to be 0.07/C_6-C_3 by ^{1}H NMR studies (Lundquist, 1991).

1.2 Optical activity of lignin

In this section, the present knowledge about the absolute configuration of β-asymmetric carbons in lignin was reviewed.

It has long been assumed that lignin is optically inactive, and that radical reactions involved in lignin formation are not stereochemically controlled by enzyme. When dilignols are produced by simple radical coupling from monolignols without the effect of any asymmetric carbon, the products must be racemic. Freudenberg and Rasenack (1953) showed that dimmers, dehydro-diconiferylalcohol (β-5), pinoresinol (β-β), and the analogous compound (β-β) obtained by the enzymatic oxidation of coniferyl alcohol *in vitro*, were optically inactive. This finding provided a fundamental concept for the optically inactive lignin.

Freudenberg et al. (1965) for the first time isolated optically inactive β-β linkage type compounds. They isolated the crystals of (±)-pinoresinol from spruce and of (±)-syringaresinol from beech by a mild hydrolysis with MeOH containing 0.5% of HCl at 20°C for 48h. Ogiyama and Kondo also showed the evidence for β-β linkage type (Ogiyama and Kondo, 1966). (±)-*Cis*-di-γ-lactone of α,β-bis-(hydroxymethyl)-succinic acid ($[\alpha]_D^{20}$ =0 in $CHCl_3$) originated from pinoresinol type (β-β) was isolated in a crystalline form by the nitric acid oxidation of dioxane-lignin prepared from the exhaustive ethanol-benzene extraction of Japanese cedar wood meal.

However, the influence of the presence of some kinds of asymmetric carbons on this process should be taken into the consideration. For example, when a monolignol radical combines with an oligomeric structure which has been already formed, the formation of a β-asymmetric carbon could be affected by other asymmetric carbons already present in oligomer parts. Furthermore, when the radical coupling reaction is taking place in the cell wall, the presence of optical active carbohydrates may cause the enrichment of one enantiomeric form of certain lignin substructure.

Recently, the stereoselective radical coupling of a chiral ferulic acid amide was performed by horseradish peroxidase/H_2O_2. Following elimination of amine and subsequent reduction resulted in β-5 linkage type dimmers with the excess of one enantiomer (Bolzacchini et al. 1998; Orlandi et al. 2001). This is an example of the synthesis of β-asymmetric carbon using radical coupling under the effect of the other asymmetric carbon. And in the recent rapid progress of the studies of lignan which is also formed by radical coupling reaction, a new type protein (dirigent protein) was discovered. This protein does not have peroxidase activity but assists, as an indispensable factor, the stereoselective formation of (+)-pinoresinol from coniferyl alcohol by peroxidase/H_2O_2 (Davin et al. 1997). This finding seemed to re-create an interest in the investigation of the optical activity of lignin, although lignin has been generally considered to be optically inactive (Hatfield and Vermerris 2001).

The structural evidence would be required for the resolution of this question. Recently, Ralph 's group obtained the optically inactive compounds by DFRC method. Ralph et al. showed that β-5 and β-β type dimers obtained from pine lignin by DFRC (derivatization followed by reductive cleavage) method are optically inactive. In addition, they showed that no optical activity could be detected by the circular dichroism (CD) of isolated lignins from pine, kenaf, or maize (Ralph et al., 1999). Thus the proof of the non-stereoselective radical coupling in lignin has been performed by the structural evidences of some linkage types in lignin

1.3 Diastereomeric forms of β-O-4 structures

About equal amounts of *erythro* and *threo* forms of β-O-4 structures are present in softwood, which was determined by ^{1}H-NMR studies of spruce MWL (Lundquist 1980), and ozonation analysis of spruce wood meal (Matsumoto et al 1986). On the other hand, the *erythro* form of β-O-4 structures was shown to be dominant in hardwood without exception, which was determined by ^{1}H-NMR studies of birch MWL (Lundquist, 1979; Hauteville et al., 1986) and of chestnut, oak, alder, maple, sycamore, and cherry MWLs (Tollier et al., 1986), by ozonation analysis of birch wood meal and beech MWL (Matsumoto et al., 1993), and by ^{31}P-NMR studies of cherry MWL (Saake et al., 1996).

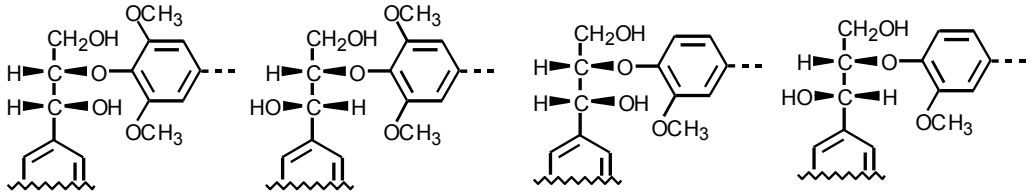


Fig. 1.3 The *erythro* and *threo* forms of β-syringyl ethers, and those of β-guaiacyl ethers

Relationship between the *erythro*/*threo* ratio and aryroxyl group types (B-ring) has been suggested. In ^{13}C-NMR studies of hardwood lignins, β-O-4 structures were separated to four categories by the difference of aryroxyl group types (syringyl and guaiacyl): *erythro* forms of β-syringyl ethers, *threo* forms of β-syringyl ethers, *erythro* forms of β-guaiacyl ethers and *threo* forms of β-guaiacyl ethers (Fig. 1.3). The *erythro* form of β-syringyl ether type structures was found to be the predominant linkage type in hardwood by ^{13}C-NMR studies of beech MWL (Nimz et al 1984). Bardet et al (1986) applied 2D-INADEQUATE experiment to ^{13}C-enriched lignin

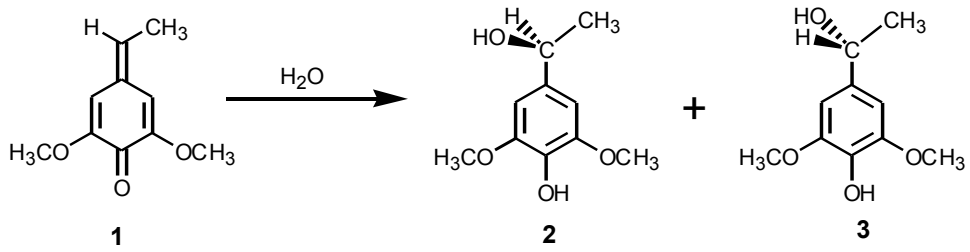
(MWL) from aspen wood which grew in $^{13}CO_2$ enrichment air. It was clearly elucidated that the *erythro* form of β-syringyl ether predominates in the four structures, and, for the first time, their proportion was estimated from the ^{13}C-NMR 2D-INADEQUATE spectrum that the *erythro* forms of β-syringyl type was ca. 55% in four structural types, other types were about almost equal amount (Bardet et al 1998).

1.4 Possible factors controlling the ratio of the *erythro* and *threo* forms of β-O-4 structures

1.4.1 Mechanism of the diastereo-differentiating water addition to a quinone methide

According to the widely accepted theory of lignin biosynthesis, the α-asymmetric carbon of arylglycerol-β-aryl ether (β-O-4) structure is formed by the water addition to the quinone methide intermediate which was produced by the coupling reaction of phenoxy radicals. In this section, the effect of β-asymmetric carbon on the stereoselective water addition to quinone methide will be discussed. Its reaction mechanism was based on the assumption that the proportion of *erythro* and *threo* forms of β-O-4 structure in lignin is given by the difference of the reactivity of *Re* and *Si* face of quinone methide rather than by the result of the equilibrium state.

Quinone methide undergoes addition reactions with hydroxyl groups at the electron-deficient α-carbon position. In the water addition to **1** which produces **2** and **3** (Fig. 1.4), the enantiomer **2** would arise by addition from above the plane of drawing (*Si* face), and **3** by addition from below (*Re* face). Since the mirror plane relationship between the products (**2** and **3**) is retained in the transition states, the reactions which produce **2** and **3** must be exactly equal in rate, giving racemic mixture.



1 **2** **3**

Figure 1.4 Non stereoselective water addition to quinone methide

However, a reaction which can produce two possible stereoisomeric products is capable of producing one in excess. Major case for such a reaction is that a new stereocenter is formed in a molecule which already contains one stereocenter. In the

case of the reaction of quinone methide **4** to produce **5** and **6** (Fig. 1.5), since the products are diastereomeric, the transition states which lead to each of products are themselves diastreomeric. Their stabilities can be different and then the diastereomeric products (**5** and **6**) can be formed in different amounts.

The conformation of quinone methide **4** also can cause the uneven proportion of β-O-4 structures **5** and **6**. If Cα-Cβ bond freely rotate without a stable conformation, *Re* and *Si* face of quinone methide will be attacked by water in the same frequency. On the other hand, when the conformation of **4** is stabilized in a certain position, the frequency of being attacked by water molecule to the *Re* and *Si* faces can be different and the diastereomeric products (**5** and **6**) can be formed in different amounts.

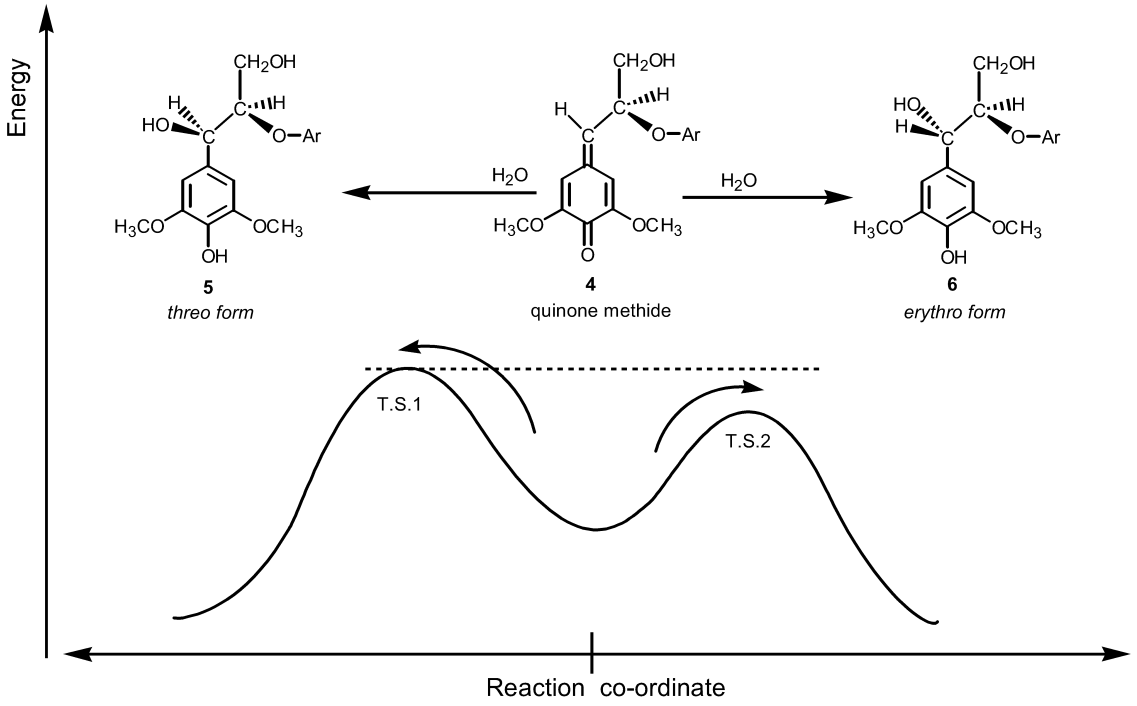


Fig. 1.5 Diastereo-differentiating water addition to quinone methide

In addition to the β-asymmetric carbon of quinone methide, the presence of other asymmetric carbons can make this process diastereomeric. The α- and β-asymmetric carbons in already grown lignin structure and asymmetric carbons in cellulose and

hemicellulose should be taken into consideration (Fig 1.6). Such asymmetric carbons can make the water addition diastereomeric without participation of the adjacent β-asymmetric carbon in the quinone methide. When such a reaction mainly occurs, the configuration of α-asymmetric carbon will be independent of that of β-asymmetric carbon. In this case, the *erythro*/*threo* ratio of β-O-4 structure with Cβ*R* configuration does not have to be the same as that with Cβ*S* configuration.

Such an asymmetric carbon also can indirectly control the formation of α-asymmetric carbon by changing the conformation of β-asymmetric carbon of quinone methide. For example, an asymmetric carbon apart from α-carbon of quinone methide in the same oligomer lignin might control the water addition in a manner that the asymmetric carbon transmits its effect to β-asymmetric carbon of quinone methide indirectly by changing the conformation of the neighboring substituents.

Thus, the effect of asymmetric carbons are separated into two categories, one is stereocontrol originating from β-asymmetric carbon of quinone methide, another is stereocontrol originating from the asymmetric carbons outside of quinone methide.

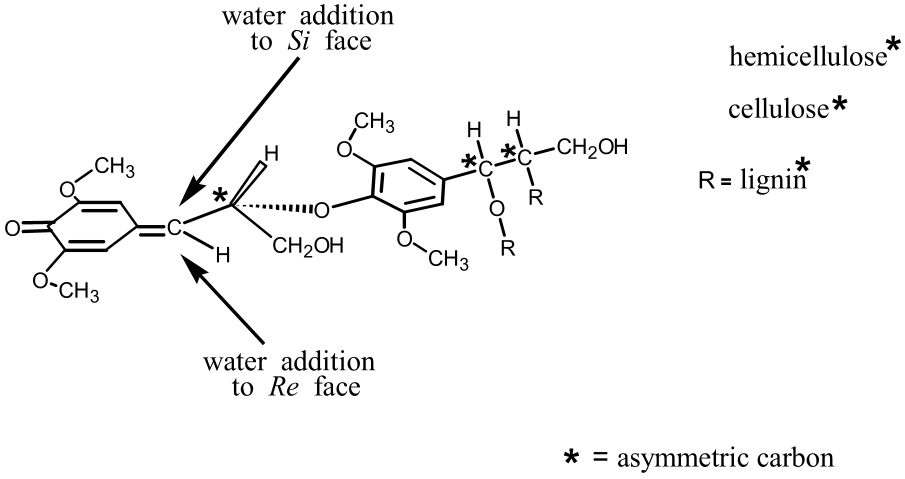


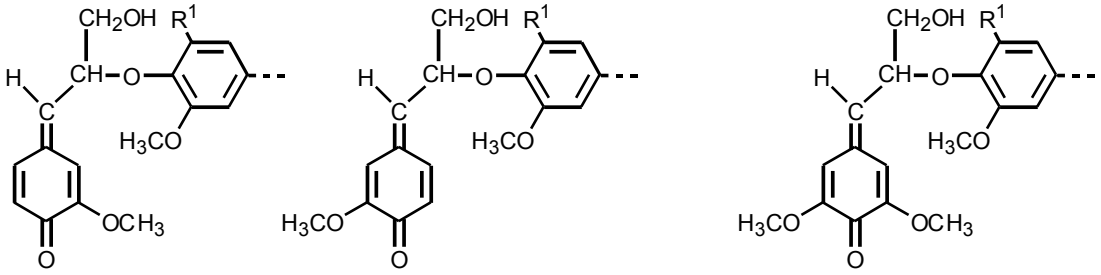
Fig. 1.6 Various types of asymmetric carbon

1.4.2 Possible factors controlling the ratio of the *erythro* and *threo* forms of β-O-4 structures

Quinone methids of β-O-4 structure

In this section, the present knowledge of model experiment, the crystal structures of β-O-4 dimer model compounds, and the characteristics of quinone methides are summarized, and the discussion was focused on the possible factors which control the formation of the *erythro* and *threo* forms of β-O-4 structures.

The various types of the quinone methides of β-O-4 structures would be formed in the process of lignin formation due to the difference of the aromatic ring types (guaiacyl and syringyl). As shown in Fig. 1.7, the quinone methides of β-O-4 dimer model compounds consist of GG, GS, SG, and SS types. Furthermore, GG and GS types in these 6 types were separated to *syn* and *anti* isomers. Because each quinone methide can cause the diastereoface-differentiating water addition in different manners, the different values of *erythro*/*threo* ratio are expected in lignin.



R_1 = H (GG type quinone methide)
= OCH_3 (GS type quinone methide)

R_1 = H (SG type quinone methide)
= OCH_3 (SS type quinone methide)

Fig. 1.7 Six kinds of quinone methide of β-O-4 structure

Model experiment for diastereo-differentiating water addition to quinone methides

In the model studies on the water addition to the quinone methide of β-O-4 dimer, reaction conditions (effect of solvent and acidity) and the aromatic ring type (guaiacyl

and syringyl units) of A- and B-rings have been shown to affect the *erythro/threo* ratio of β-O-4 structures.

Nakatsubo et al. (1976) showed that the *erythro*/*threo* ratio of guaiacylglycerol-β-guaiacyl ether (GG) obtained from the corresponding quinone methide was 1.1 in chloroform with the catalytic amount of acid, 0.5 in dioxane-water (9:1 v/v), and 0.4 in dioxane-water (1:1 v/v). These results indicate the effect of solvent. Such an excess of *threo* form of β-O-4 structure have not been reported in softwood as well as hardwood, and implies the possibilities of uneven *erythro*/*threo* ratio in softwood which is usually 1:1.

Brunow et al. (1993) showed that the *erythro*/*threo* ratio of GG, and GS obtained from the corresponding quinone methides increased from 0.5 to 1.0, and from 0.8 to 2.0, respectively, with the decrease of pH (7 to3) in dioxane-water (1:1 v/v). These results indicate the effect of acidity. In addition to these results, higher *erythro*/*threo* ratio (3.0) of SS was obtained at pH3. These results indicate not only the type of quinone methide structure (A-ring) but also the type of aromatic ring (B-ring) could affect the ratio of *erythro* and *threo* forms.

The crystal structure of β-O-4 dimeric model compounds

The crystal structure of the quinone methide of β-O-4 model compound has hardly been reported, but those of β-O-4 dimer models as the products of water addition have been investigated in detail. The crystal structures of β-O-4 dimer models reported were summarized in Figure 1.8 (Stomberg et al., 1988; Stomberg and Lundquist, 1994; Lundquist et al. 1996; Langer and Lundquist, 2001; Langer et al., 2002a and 2002b).

A specific conformation was found in β-syringyl ethers. The α-OH group is gauche to γ-OH group and β-aryroxyl group both in *erythro* and *threo* forms. Especially, in *erythro* form of syringylglycerol-β-syringyl ether (*erythro* 4-methoxy SS in Fig 1.8), both oxygen atoms of two methoxyl groups in B-ring form hydrogen bondings with

both γ-OH and α-OH groups.

With respect to the conformation of A- and B-rings in β-syringyl ethers, the A-ring is antiperiplanar to B-ring in *erythro* form, and, A-ring is gauche to B-ring in *threo* forms. Interestingly, in β-guaiacyl ethers, the conformations of *erythro* and *threo* forms are opposite to those in β-syringyl ethers.

If the conformations of transition states during water addition to quinone methides are similar to those in crystal structures of β-O-4 model compounds, the presence of hydrogen bondings possibly play an important role in the stability of the transition state, although only one of the stable conformation in products of water addition is discussed here. In addition, inter molecular hydrogen bondings in crystal forms should be taken into consideration. Further information will be required to discuss the transition states and the participation of hydrogen bondings.

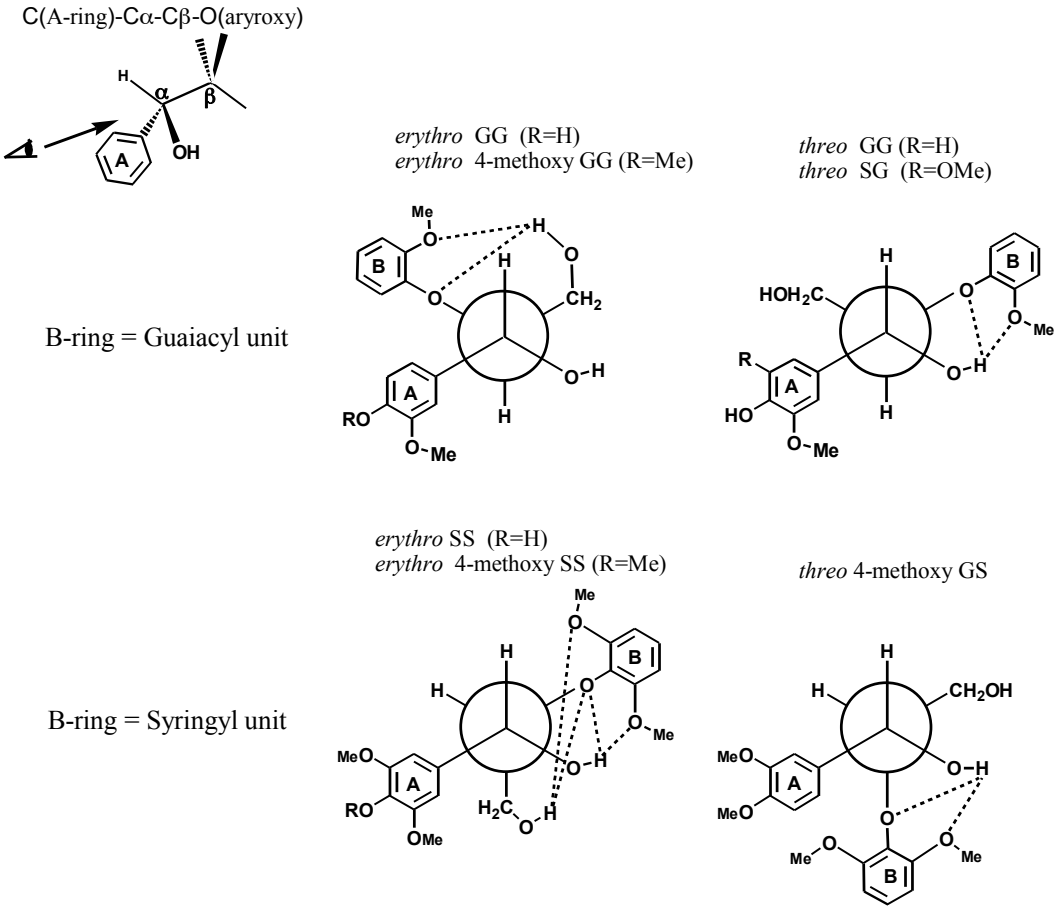


Fig. 1.8 Crystal structures of β-O-4 dimer model compounds

Syn and anti isomers of quinone methide

Only when A-ring is guaiacyl type, *syn* and *anti* isomers are present due to the lack of methoxyl group at 5-position (Fig. 1.9). The effect of this type of isomers on *erythro*/*threo* ratio should be taken into consideration, if the excess of one diastereomeric form is found in softwood. The stability of quinone methides and the reactivity with nucleophile have been indicated to be different in two isomers as follows;

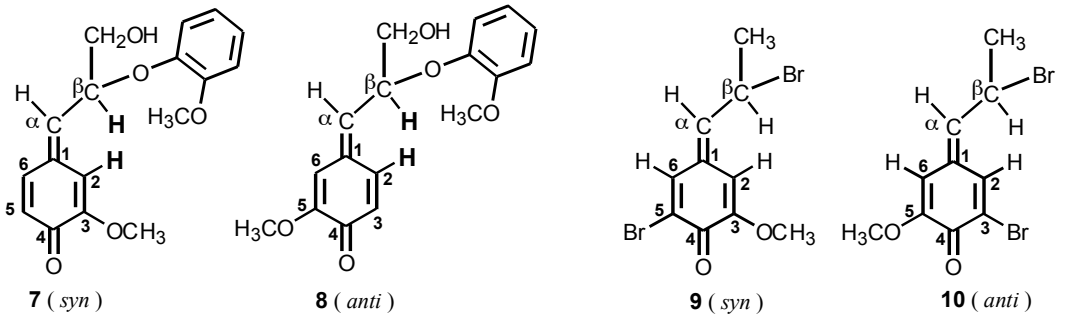


Fig. 1.9 GG type of quinone methide and brominated quinone methide

Quinone methides of GG dimer models **7** and **8** were synthesized from the different guaiacylglycerol-β-guaiacyl ether derivatives by the elimination of α-functional group, and the ratio of *syn*/*anti* isomers obtained were 2:1 in all cases (Ede et al., 1990). The *syn* isomer of brominated quinone methide **9** was shown to isomerize to *anti* isomer **10** in $CHCl_3$ or benzene solution. The ratio of *syn*/*anti* isomer became 1.8:1 after keeping the solution overnight at room temperature (Li et al. 1995). These results indicate the difference of the stabilities of *syn* and *anti* isomers.

The difference of the reactivity of the *syn* and *anti* isomers was reported (Ede et al., 1990). In the addition of amines (RNH_2: R = $-CH_2CH_2OH$ or –*t*Bu) to GG type quinone methide and its analogue, the *anti* isomer reacted slightly faster than *syn* isomer.

According to the explanation proposed by Ede, the *anti* isomer was under greater

steric strain than the *syn* isomer, which is suggested by the results of the NMR observation that the internuclear distance between Hβ and H2 in *anti* form **8** is smaller than that between Hβ and H2 *syn* form **7** (Ede et al., 1990). An enlargement of angles, C(2)-C(1)-C(α)[123.6˚] and C(1)-C(α)-C(β)[122.5˚] was shown in *syn* form **9** (Li et al. 1995).

The *erythro*/*threo* ratio obtained from *syn* isomer and that from anti isomer might be expected to different due to the uneven water addition to each diastereomeric faces, *Re* and *Si* faces of *syn* isomer, and *those* of *anti* isomer.

1.5 Ozonation of lignin

1.5.1 Cleavage of carbon-carbon double bond: Criegee mechanism

According to the generally accepted Criegee mechanism in Fig 1.10 (Bailey, 1978), an carbon-carbon double bond **11** attacked by ozone forms a primary ozonide **12** by 1,3-dipolar cycloaddition or via electrophilic addition. An unstable primary ozonide **12** is cleaved to a carbonyl oxide (or zwitterion) **13** and an aldehyde or ketone **14**. There are several ways for stabilizing the carbonyl oxide **13**: a) reaction with an aldehyde or ketone to give an ozonide **15** or polymeric ozonide; b) dimerization or polymerization give to dimeric peroxide **16** or polymeric peroxides; c) reaction with hydroxyl group, if such a group is present in reaction system, to give a peroxide **17**. For the purpose of the isolation of an ozonide, non-protic solvent has been used at low temperature. In the case of ozonation in protic solvent, the formation of the peroxide **17** would be involved.

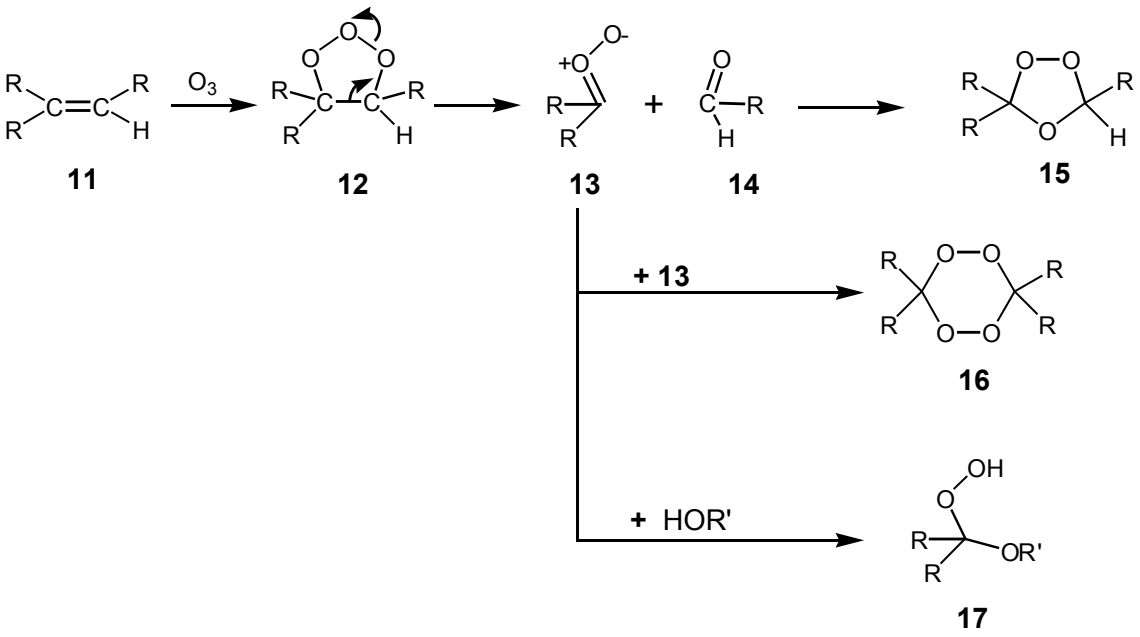


Fig 1.10 The cleavage of carbon-carbon double bond by ozone

1.5.2 Cleavage of aromatic ring in lignin

Benzene ring is less reactive toward ozone than an olefinic double bond. For example, the ozonation of isoeugenol has been performed for the synthesis of vanillin (Bailey, 1982). However, benzene ring can be attacked by ozone to undergo the cleavage of its carbon-carbon double bonds. The kinetic study on the ozonation of benzene and substituted benzenes have been investigated, and the various types of substituted benzenes with electron donating groups are known to be more reactive toward ozone than benzene.

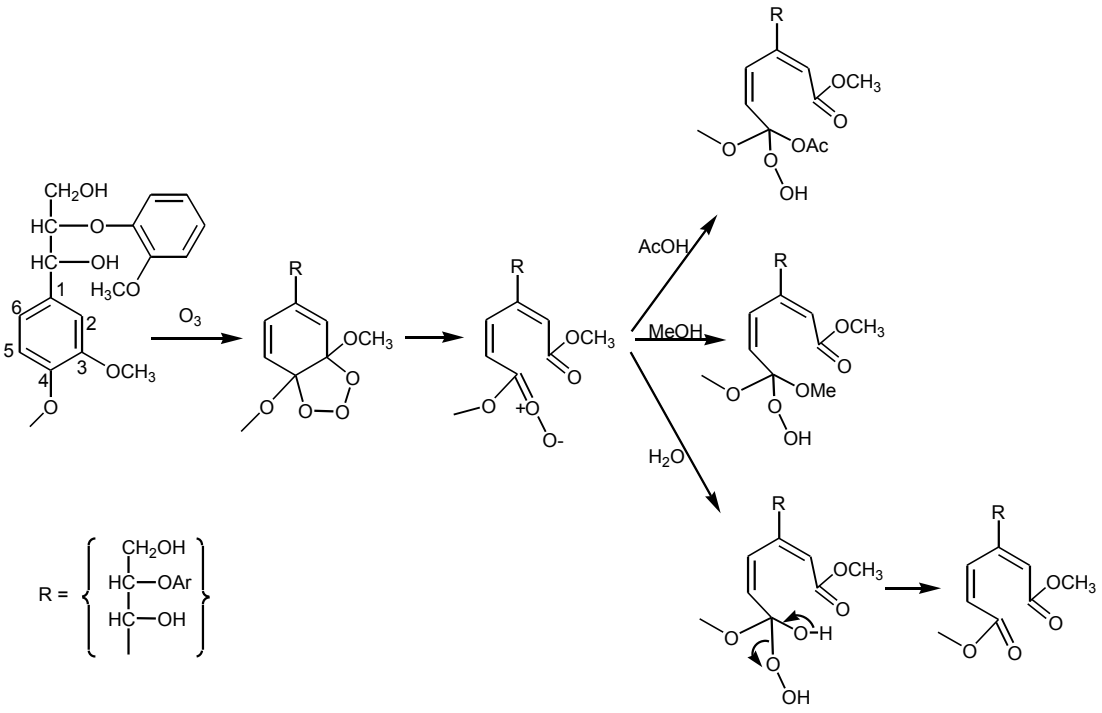


Fig 1.11 The cleavage of aromatic ring by ozone

With respect to the reactivity of guaiacyl and syringyl type lignin model compounds with ozone, the initial attack by ozone seems to occur on the double bond between carbons at 3- and 4- positions with electron donating groups (Fig 1.11). As a result of this initial attack, muconic acid derivatives are produced by the cleavage of the double bond between carbons at 3- and 4- positions (Kaneko et al., 1983). The ozonation of

vanillyl alcohol and veratryl alcohol in AcOH-AcONa at pH4 gave the δ-lactone of the monomethyl ester of β-hydroxymethyl muconic acid, which was obtained as a crystal form (Hatakeyama et al., 1967; Kaneko et al., 1979). By the reaction of guaiacylglycerol- β-guaiacyl ether (GG, 200mg) with ozone in MeOH, a muconic acid derivative,7-hydroxy-4-methoxycarbonyl-methylene-6-(2-methoxyphenoxy)-2-hepten-5 -olide, was obtained as diastereomers in relatively high yields (20 and 30% of starting diastereomeric materials), and one diastereomer (45mg) was obtained as a crystal form (Kaneko et al., 1979).

In the case of the α-carbonyl type of GG dimer model, the aromatic ring conjugated to the α-carbonyl group was more stable against ozone than the aromatic ring of β-aryl ether (Kaneko et al., 1981). And the kinetic study on the various types of lignin related compounds showed that α-carbonyl type compounds reacted slower than the corresponding benzyl alcohol types (Kaneko et al., 1983).

1.5.3 Cleavage of carbon-hydrogen bonds

The carbon-hydrogen (C-H) bonds of acetal **18**, hemiacetal **19**, aldehyde **20**, and alcohol can be attacked by ozone (Bailey, 1982; Deslongchamps, 1983). The ozonation of C-H bonds have been considered to start from the formation of hydrotrioxide intermediates **21** by the 1, 3-dipolar insertion mechanism (Fig. 1.12).

The carbon-hydrogen (C-H) bonds of glycosides can be attacked by ozone to give the corresponding aldonic acids. Deslongchamps et al. showed that acetylated methyl-β-D-glucopyranoside reacts with ozone, but the corresponding α-anomer does not react (Deslongchamps and Moreau, 1971; Deslongchamps et al., 1972 and 1974).

Although these C-H bonds can react with ozone, the reactivity of ozone with cellulose and hemicellulose seem to be less than lignin. Eriksson and coworkers showed that methyl-β-D-glucopyranoside reacts with ozone much slower than various types of lignin related compounds in MeOH (Eriksson and Gierer, 1985a), and in 0.1M phosphate buffer of pH 3.1 (Eriksson and Gierer, 1985b). The difference in the reactivity of lignin and cellulose with ozone, and the difference in reactivity of C-H bonds (acetal > secondary alcohol > primary alcohol) would predict that the formation of erythronic and threonic acids (ozonation products of β-O-4 structure described below) from glycosides such as cellulose and hemicellulose is not comparable with that from lignin. The formation of these acids from carbohydrates will be discussed in the Chapter 2.3.6.

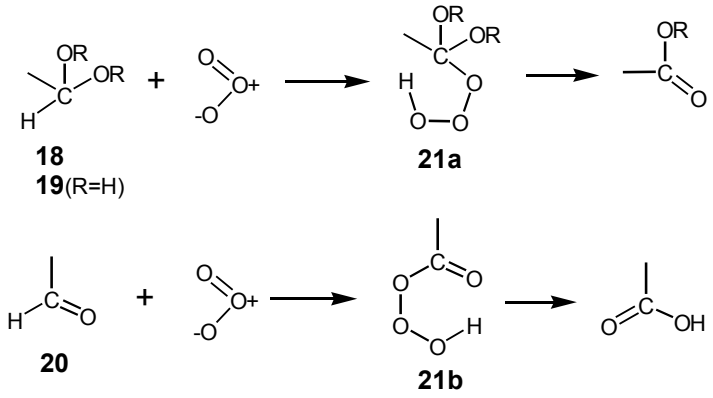


Fig. 1.12 The cleavage of carbon-hydrogen bond by 1,3-dipolar insertion of

ozone

1.5.4 Ozonation for the configurational proof purpose

The exhaustive ozonation of compounds containing an aromatic ring has been commonly performed for synthetic or structural and configurational proof purpose (Bailey 1982). The usual procedure is the treatment of the aromatic compound in an acetic acid solution with a large excess of ozone. The ozonation of the various linkage types of lignin was considered to give low-molecular-weight compounds that retain the original stereo structures of lignin side-chains (Fig. 1.13)

With respect to the study of stereostructure of lignin related compound, a phenylcoumaran-type compound obtained by dehydrogenative condensation of isoeugenol was first application to ozonation (Aulin-Erdtman et al., 1963). Later, the ozonation analysis for the configurational study of lignin has been developed by Matsumoto and co-workers (1981).

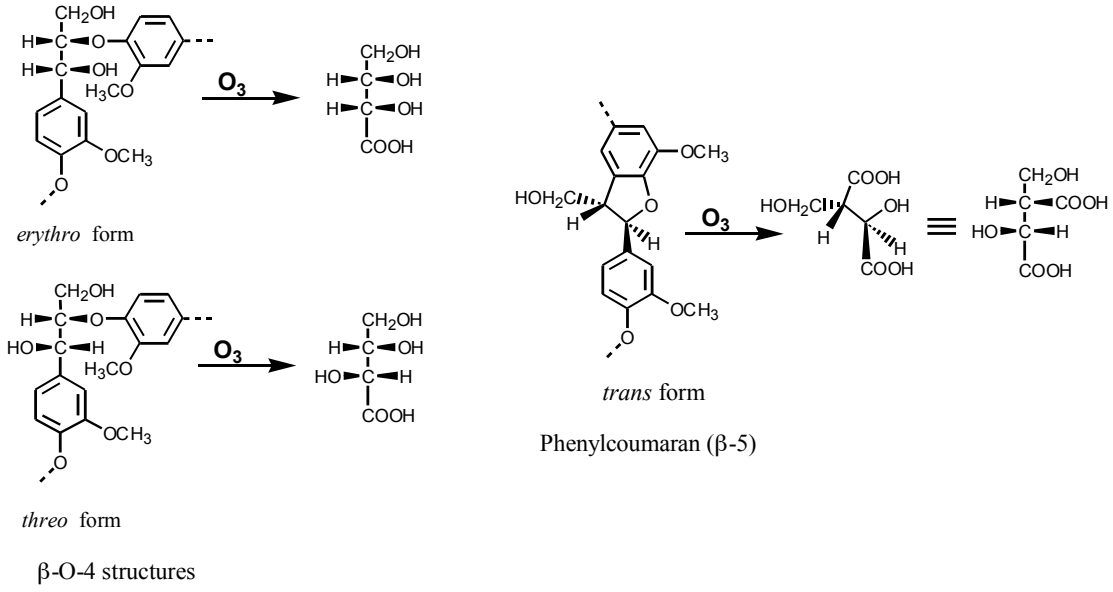


Fig. 1.13 Ozonation of *erythro* and *threo* forms of β-O-4 structures and *trans* form of phenylcoumaran structure

The many applications of this method have been reported, and some important chemical characteristics of side-chain structures in lignin were discussed. The

erythro/*threo* ratios of β-O-4 structure in spruce lignins prepared from different methods (MWL, dioxane lignin, Klason lignin, soda lignin) were compared (Matsumoto et al. 1986). The presence of about equal amount of *erythro* and *threo* forms in spruce MWL was suggested from the ratio of erythronic and threonic acids in ozonation products. On the other hand, the lower *erythro*/*threo* ratio was obtained in soda lignin, which indicated the stereoselective reaction in alkaline solution. The main configuration of the phenylcoumaran (β-5)-type structure in spruce MWL was determined to be the *trans* form (in Fig. 1.13) (Habu et al., 1988). The diarylpropane (β-1)-type structure, if present, and glyceraldehyde-2-aryl ether structure was shown to be minor structures in spruce lignin (Matsumoto et al. 1981,1984; Habu et al. 1990). Tsutsumi et al. (1990) compared this method with acidic permanganate oxidation, and showed that yields of erythronic and threonic acids by ozonation of lignin were quite higher than that by acidic permanganate oxidation. In this report, the modified scheme of trimethylsilylation as free acid from of these acids was proposed. Recently, ozonation method was applied to the lignin-carbohydrate complex model compound of the benzylic ether type (Karlsson et al. 2000), which suggests a wide application of this method.

1.6 Objectives of this work

The formation of the various types of asymmetric carbons is involved in the polymerization of monolignols to form high molecular lignin. It will be an interesting topic to investigate the absolute configuration of these asymmetric carbons, because the investigation of the stereochemical characteristics could contribute to understanding of mechanism of lignin formation. The elucidation of the stereochemical structure of arylglycerol-β-aryl ether (β-O-4) structure is quite important among many linkage types of lignin because this structure is the most predominant one in lignin.

The objectives of this work are investigation of the absolute configuration of the asymmetric carbons of β-O-4 structure in lignin, and the improvement of the ozonation method for the analysis of the stereo structure.

Targets of this work are as following;

1. To investigate the reproducibility of the yields and the quantitativeness of the scheme of the ozonation method as the analytical tool of β-O-4 structure, and to improve this method (Chapter 2).

2. To investigate the absolute configuration of β-asymmetric carbon of β-O-4 structure, and to elucidate whether or not the formation of the β-asymmetric carbon is stereochemically controlled in lignin formation (Chapter 3).

3. To investigate the relationship between the diastereomeric forms of β-O-4 structure and the aromatic ring types in lignin, and to discuss the effect of the difference of aromatic ring types on the absolute configuration of α-asymmetric carbon of β-O-4 structure in lignin formation (Chapter 4).

Chapter 2

Improvement of Ozonation Method as Analytical Tool for β-O-4 Structure

2.1 Introduction

Selective degradation of the aromatic nuclei of lignin by ozone gives low-molecular-weight compounds that retain the original stereo structures of the side-chain. Ozonation analysis has been applied to the analysis of some linkage types in lignin and their stereochemical characteristics of the side-chain have been reported (Chapter 1.5).

This method is applicable not only to a soluble lignin but also to an insoluble one, such as lignin in wood meal or pulp, highly polymerized dehydrogenation polymer (DHP), Klason lignin. This applicability to insoluble samples is a great advantage over other spectroscopic methods applying for isolated soluble lignins because the isolation usually cannot be performed quantitatively and is always accompanied by chemical modification.

The *erythro/threo* ratio of β-O-4 structures is one of the most important information obtained by this method. Formerly, determination of the *erythro/threo* ratio was calculated based on the ratio of the erythronolactone/threonolactone (Matsumoto et al., 1986). Ozonation method has to be improved in order to make quantitative discussion on the content of the β-O-4 structure because, as is shown below, erythronic and threonic acids formed during ozonation cannot be recovered quantitatively, and the reproducibility of the yields of these acids relative to the internal standard are not always satisfactory. Therefore, it became necessary to develop a suitable procedure for the quantitative analysis.

In this study, post-treatment procedures after ozone treatment were examined in detail, and two modifications are proposed. The modified experimental scheme for the ozonation method is shown in Fig. 2.1. One is the reductive post-treatment of ozonation products with sodium thiosulfate. Another is the trimethylsilylation of ammonium salts without forming lactones for gas chromatography (GC) (Tsutsumi et al., 1990; Hyppänen et al. 1983). We aimed to improve the ozonation method for the

quantitative analysis of the β-O-4 structure in lignin.

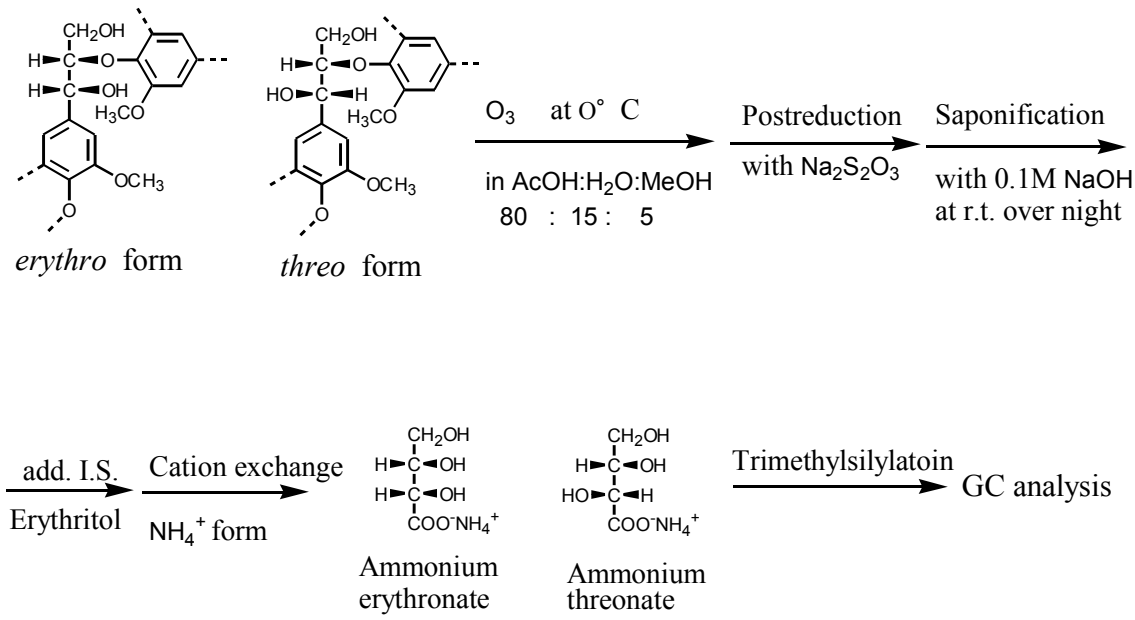


Fig. 2.1 Experimental scheme for the modified ozonation method

2.2 Experimental

2.2.1 Materials

Model compounds

D-Erythronic and L-threonic acid ammonium salts were prepared from D-erythronolactone (Tokyo Kasei) and L-threonic acid calcium salts (Aldrich) by the following procedure (Hyppänen et al. 1983); D-Erythronolactone or L-threonic acid calcium salt was kept at pH 10 with NaOH overnight at room temperature, and each solution was then passed through a column filled with cation-exchange resin (NH_4^+ form).

1-(3,4-Dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (veratrylglycerol-β-guaiacyl ether, VG) was synthesized according to the method of Adler et al. (1952).

Milled wood lignin (MWL)

Milled wood lignin was prepared from ethanol/benzene-preextracted beech (*Fagus crenata* Bl.) wood meal. The wood meal was milled for 24 h according to Björkman's method (1956) except that the milling was conducted without dispersing in toluene.

Wood meal

Birch (*Betula maximowiczii* Regel) wood meal (80-mesh-pass) was extracted with ethanol/benzene (1:2, v/v) under reflux for 6 h by a Soxhlet apparatus. Lignin content (Klason lignin and acid soluble part) was 25.4%.

$NaClO_2$-delignified wood meal

Ethanol/benzene-preextracted birch wood meal (5g) was treated with sodium chlorite ($NaClO_2$, 2g) in diluted acetic acid aqueous solution at 70-80 ºC for 1 h. This treatment was repeated 5 times. The treated wood meal was extracted with 1M NaOH solution at room temperature.

2.2.2 Ozonation

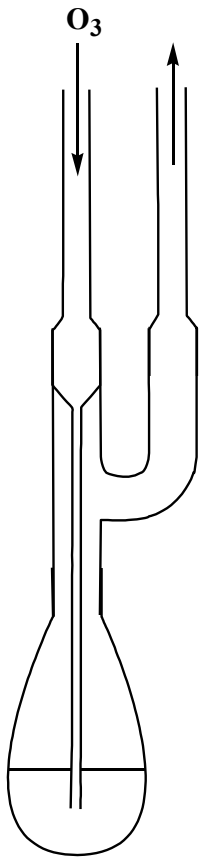


Fig. 2.2 Reaction vessel

Ozone treatment

Samples (Table 2.1) were dissolved or suspended in 30ml of ozonation solvent consisting of AcOH-H_2O-MeOH (80:15:5 v/v/v). Oxygen containing ca. 3% ozone was bubbled into the solution at the rate of 0.5 L/min for 5-240 min at 0ºC with stirring. The ozone generator used was type ON-3-2 (Nippon ozone).

Post-treatment

After ozone treatment, the residual ozone was removed by oxygen bubbling. The reaction mixture was reduced with 300 μl of 0.1M sodium thiosulfate (post-reduction). The solvent was removed under the reduced pressure at 40°C. The trace of acetic acid was removed by the repetition of evaporation with small amount of water (ca. 1ml, 3 times).

The ozonation products were saponified with 0.1M NaOH (20ml) at room temperature overnight. Erythritol (Table 2.1) was added as an internal standard. After that, in the case using wood meal or delignified one as a sample, the insoluble residue was removed by filtration. The solution was then passed through a column filled with 10-15 ml of cation-exchange resin (Dowex-50W-X4, NH_4^+ form), and the column was washed with water until the pH of eluent was 7-8, obtaining a total volume of 100ml. An aliquot of the eluent (1-4 ml) was concentrated and dried *in vacuo* at 40°C.

Preparation of trimethylsilyl derivatives

Ozonation products containing ammonium salts of organic acids were subjected to trimethylsilylation, which was carried out with 300 μl of dimethylsulfoxide (DMSO), 200 μl of hexamethyldisilazane (HMDS), and 100μl of trimethylchlorosilane (TMCS) at 60 °C for 30 min (Verhaar et al., 1969). The reaction mixture was separated into two phases. The upper layer consisted of target derivatives and the excess of silylating agents. The silylation of erythronolactone was carried out with pyridine instead of DMSO.

GC analysis

The upper layer was analyzed with gas chromatography (GC-FID: Shimadzu 17A) for quantitative analysis and gas chromatography-mass spectrometry (GC-MS: Shimadzu 17A, QP-5000) for identification of degradation products. A fused-silica capillary column (GL Science NB1, 0.25 mm i.d. ×30 m) was used. The analytical conditions were as follows;

GC-FID: Oven temperature program 1: 120°C, 5min —4 °C/min→170 °C—10 °C/min→280 °C
program 2: 80°C, 5min —4 °C/min→170 °C—10 °C/min→280 °C
Injection temp. 250°C; Detector temp. 280°C; Column flow rate of He gas 1.9 ml/min; Splitting ratio 60:1; Injection volume 1 μl.

GC-MS: (Shimadzu QP-5000).
Oven temperature program: 120°C,5min —5 °C/min→190 °C—10 °C/min→270 °C
Ionization mode EI, 70eV; Injection temp. 250°C; Interface temp. 270°C; Column flow rate of He gas 0.7 ml/min; Splitting ratio 60:1; Injection volume 1-2 μl.

Table 2.1 Samples and reaction conditions for ozonation in this chapter

Section	Sample	Quantity of sample	Erythritol I.S. (mmol)	Reaction time (min)	Post-reduction with $Na_2S_2O_3$
2.3.2	Erythronolactone and Threonolactone	0.02 mmol each	0.01	0-120	—
	Erythronic and Threonic acids as NH_4^+ salts	0.02 mmol each	0.01	0-240	—
2.3.3	Erythronic and Threonic acids as NH_4^+ salts	0.02 mmol each	0.01	0-240	+
	Veratrylglycerol-β-guaiacyl ether (VG)	0.04 mmol	0.01	10-120	—
				5-240	+
2.3.4	Beech MWL	10 mg	0.005	5-120	+
		30 mg	0.01	120	+
	Birch wood meal	200 mg	0.04	30-240	+
		50 mg	0.01	120-240	+
2.3.5	Cedar wood meal	50 mg	0.01	120	+
2.3.6	Delignified wood meals	200 mg	0.005 or 0.04	120	+
	Aldoses or their glycosides	30 mg	0.001	120	+

2.2.3 ^{1}H-NMR measurement

Erythronolactone or erythronic acid ammonium salt was dissolved in CD_3COOD-D_2O-CD_3OD (80:15:5 v/v/v), and ^{1}H-NMR spectrum were measured with 300-MHz Bruker AC 300 spectrometer. ^{1}H-NMR spectrum of erythronic acid: δ 3.77 (2H, d, J = 5.5 Hz), 4.05 (1H, td, J = 4.4 and 5.5 Hz), 4.34 (1H, d, J = 4.4 Hz). ^{1}H-NMR spectrum of erythronolactone: δ 4.34 (1H, d, J = 10.8 Hz), δ 4.45 (1H, dd, J = 2.9 and 10.8 Hz), δ 4.57 (1H, dd, J = 2.9 and 4.9 Hz), δ 4.65 (1H, d, J = 4.8 Hz).

The *erythro*/*threo* ratio of VG acetylated with acetic anhydride and pyridine was determined by ^{1}H-NMR based on the chemical shift of Hα at δ 6.02 (*erythro*) and δ 6.08 (*threo*) (Lundquist, 1979). Solvent was $CDCl_3$.

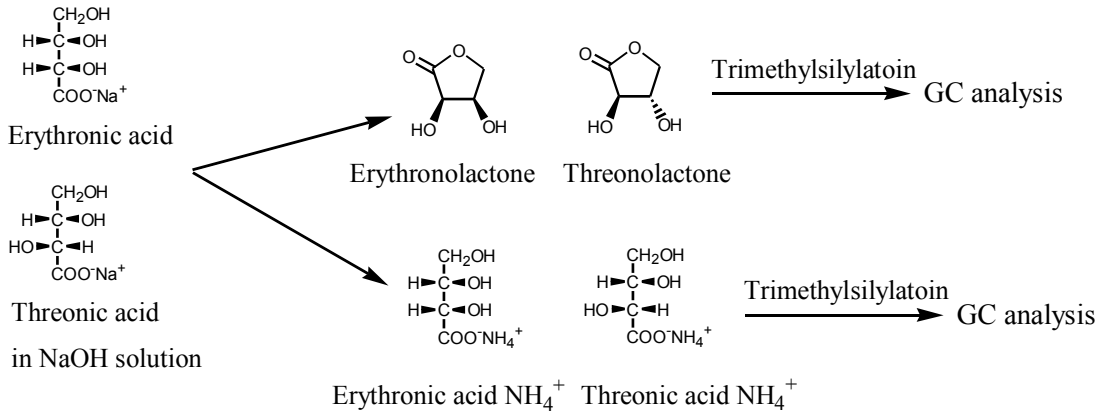
2.2.4 Neutral sugar analysis

Neutral sugar analysis was performed according to the method of Borchardt and Piper (1970). Sample (100mg of Wood meal, $NaClO_2$-delignified wood meal, and

$NaClO_2$-delignified wood meal with successive extraction by NaOH solution) were kept for 2 hr in 2 ml of 72% H_2SO_4 at room temperature. The hydrolyzate was diluted to 3% H_2SO_4 with 56ml of water and heated in an autoclave for 1hr at 121°C. After cooling, the suspension or solution was diluted to 100ml. After decantation, the 20 ml of the supernatant was mixed with inositol as an internal standard (4.0 mg for the analysis of glucitol and xylitol acetates, and 0.3 mg for rhamnitol, arabinitol, mannitol and galacitol acetates). The solutions were neutralized to pH 5.5 with a saturated $Ba(OH)_2$, and centrifuged. Sodium borohydride ($NaBH_4$, 10-15 mg) was added to the supernatants and kept overnight at room temperature. The excess of $NaBH_4$ was degraded with AcOH until gas evolution ceased. After being concentrated by evaporation under reduced pressure, the residue was subjected to evaporation from methanol solution (3ml) 3 times. The residue was dried *in vacuo* at 40°C. The acetylation of alditols was performed by Ac_2O (4ml) for 3 hr at 120°C, and analyzed with a Shimadzu 17A gas chromatograph (GC) equipped with a flame ionization detector (FID) and a fused-silica capillary column (GL science TC-17, 0.25 mm i.d. ×30 m). The oven temperature was held for 20min at 180°C, raised at 2 °C/min to 190 °C and held for 20min. The injector and detector temperatures were 250 and 280 °C, respectively. The injection volumes were 1 μl for glucitol and xylitol acetates, and 2 μl for other alditol acetates. The splitting ratio was 60:1, and the flow-rate of He carrier gas was 1.9 ml/min. The calibration curves of alditol acetates were obtained by subjecting known amount of corresponding aldoses and inositol to GC determination after $NaBH_4$ reduction and acetylation.

2.3 Results and discussion

2.3.1 Quantitativeness during the post-treatment procedure



The quantitativeness during the post-treatment procedure of the ozonation method was investigated. The loss of the ozonation products during sample drying procedure before trimethylsilylation was suspected to cause poor reproducibility of the results. Figure 2.3 shows the influence of the period of drying *in vacuo* at 40°C on the recovery of erythronolactone. The peak area of erythronolactone by the GC analysis decreased with an increase of drying time, whereas that of erythritol used as the internal standard was fairly constant. The loss of erythronolactone confirmed here must have resulted in the poor reproducibility of the relative molar responses of erythronolactone to the internal standard. To avoid this, erythronic and threonic acids were converted to their trimethylsilyl ethers directly from their ammonium salts without lactonization and then were subjected to GC analysis (Hyppänen et al., 1983). Trimethylsilylation from Na salts is often used for the analysis of organic acids, but the use of Na salts was not employed in this procedure because of the formation of insoluble precipitates during trimethylsilylation which disturbed quantitative reaction and handling (data was not shown).

The calibration curves of erythronic and threonic acids with erythritol as an internal

standard are shown in Fig. 2.4. When ammonium salts were silylated with mixture of DMSO, HMDS and TMCS, the calibration curves of both acids fit well with straight lines. And the absolute peak area of erythritol on GC chromatogram was reproducible (1805±139) when the same volumes of sample solutions with the same erythritol concentration were injected. From these results, the quantitativeness of this derivatizing procedure was confirmed.

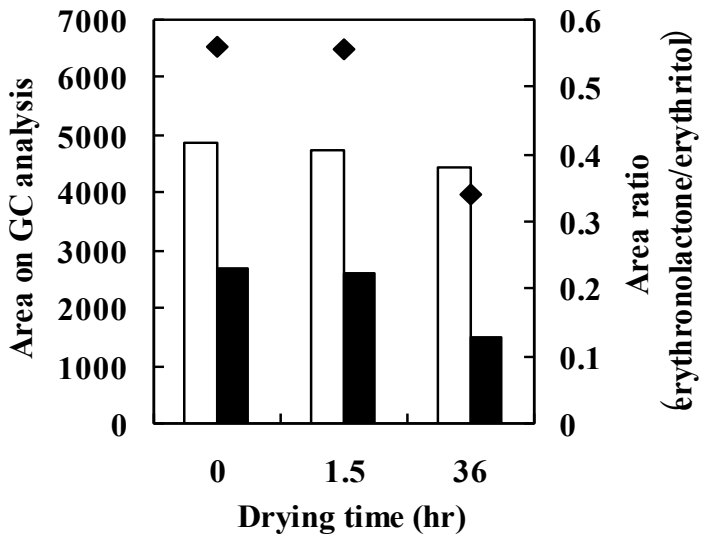


Fig. 2.3 Influence of drying time in vacuo on the loss of erythronolactone.

□: area of erythritol on GC chromatogram
■: area of erythronolactone on GC chromatogram
◆: area ratio (erythronolactone/erythritol) on GC chromatogram

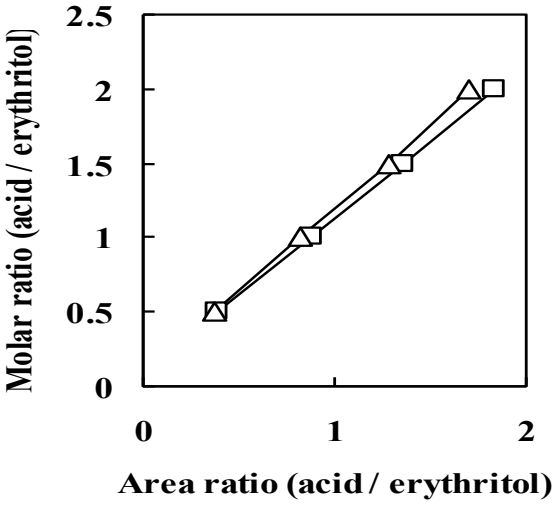
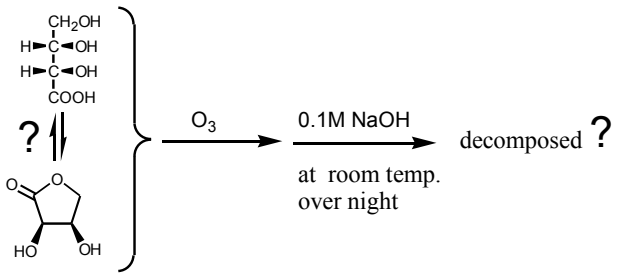


Fig. 2.4 Calibration curves of erythronic and threonic acids with erythritol as internal standard

□:erythronic acid; Y=1.036X+0.096 (R^2=0.999)

△:threonic acid; Y=1.123X+0.077 (R^2=0.999)

2.3.2 Stabilities of erythronic and threonic acids toward ozone



in AcOH-H_2O-MeOH (80:15:5) at O° C

The both free acid and lactone forms of erythronic and threonic acids (Table 2.1) were subjected to ozonation analysis to investigate stabilities of these acids toward ozone. Lactone forms of these acids (erythronolactone and threonolactone) were rather stable towards prolonged ozone treatment (Fig. 2.5). However, the free acid forms of these acids (erythronic and threonic acids) were unstable, the recoveries decreased to 57% and 61%, respectively, at 2hr of ozonation. Surprisingly, the recoveries raised to 83% and 86%, respectively, by prolonged ozone treatment for 4 hr. Although the reproducibility of this phenomena was confirmed by repeated experiments, it is difficult to explain this phenomena at the moment.

Possible species which are involved in the degradation of erythronic and threonic acids are peroxides in various forms. If peroxides play a role in the degradation of erythronic and threonic acids, it could decompose those acids during the post-treatment in alkaline solution as well as during ozone treatment. As such a peroxide, peracetic acid, peroxides in degradation products of erythronic and threonic acids, and also per-erythronic and per-threonic acids are postulated, the latter two of which may return to erythronic and threonic acid, respectively, by reduction. If aldehydes (i.e. glycealdehyde and glycolaldehyde in Fig. 2.6 generated from a part of erythronic and threonic acids by prolonged ozone treatment (3-4hr) can reduce the peroxides, recoveries of erythronic and threonic acids may rise to some extent.

Since lactone forms of erythronic and threonic acids were more stable towards ozone than the free acid forms (Fig. 2.5), the possibility of lactonization of these acids during

ozonation was examined under the same condition of ozone treatment. Erythronic acid (ammonium salts) dissolved in CD_3COOD-D_2O-CD_3OD (80/15/5 v/v/v) was kept at room temperature. As shown in Fig. 2.7, ^{1}H-NMR spectrum showed that small amount of lactonization was observed after 3.3 hr, although the half of erythronic acid was changed to erythronolactone after 15days. From this result, it was indicated that the lactonization hardly occur in the conventional ozone treatment condition (1-4hr in AcOH-H_2O-MeOH at 0 °C).

In this section, it was shown that considerable amounts of erythronic and threonic acids are decomposed during ozonation procedure, and the participation of peroxides in the decomposition of erythronic and threonic acids during saponification step was implied. In the following section, the effect of reductive post-treatment on the recoveries of those acids will be discussed.

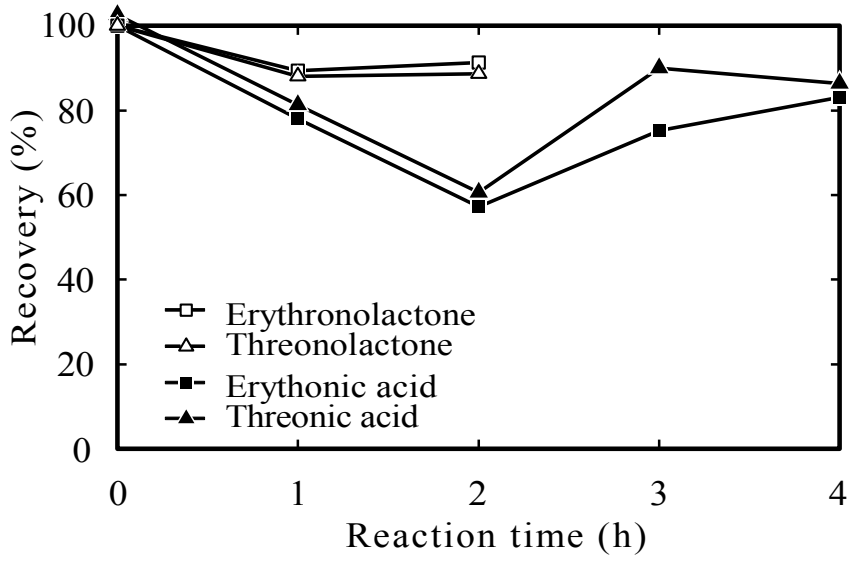


Fig. 2.5 Stability of erythronic and threonic acids toward ozone

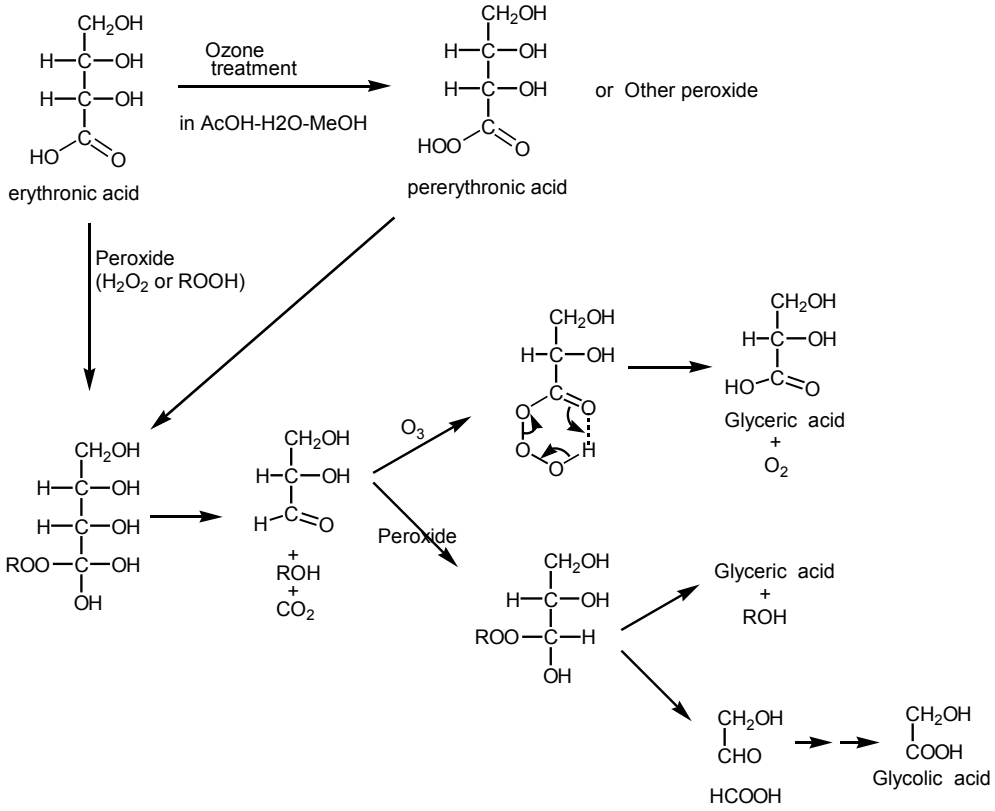
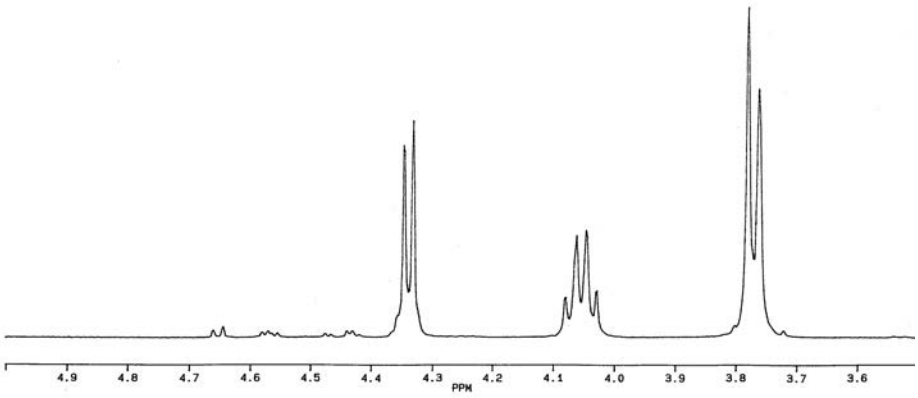
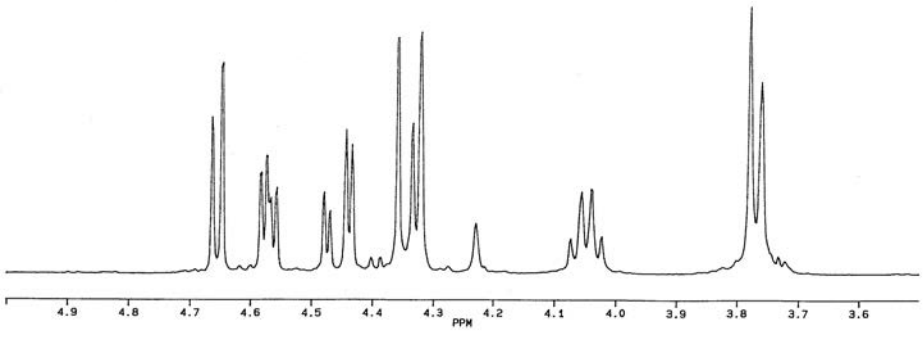


Fig. 2.6 Possible reactions for degradation of erythronic acid by ozonation

a) Erythronic acid NH_4^+ salt after 3.3 hr



b) Erythronic acid NH_4^+ salt after 15 days



c) Erythronolactone

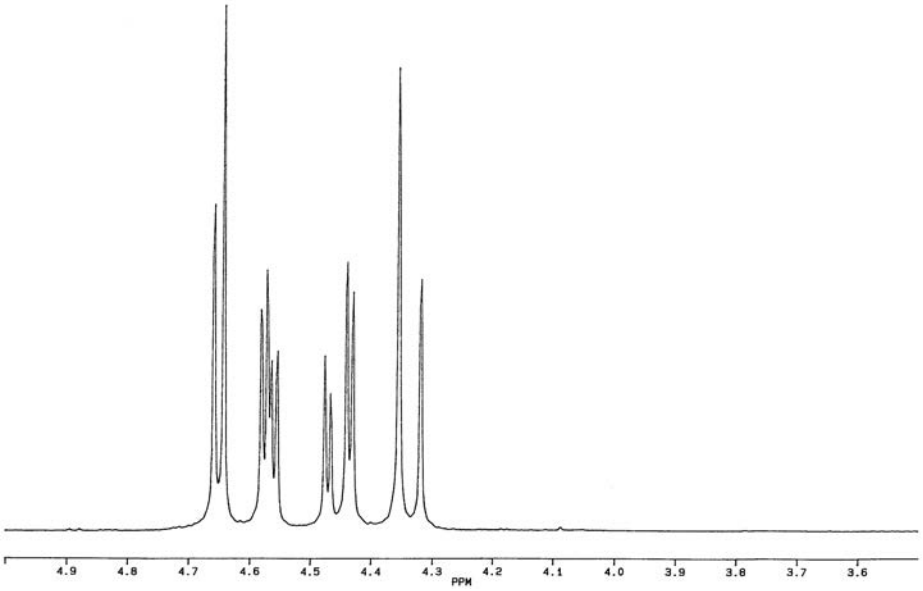
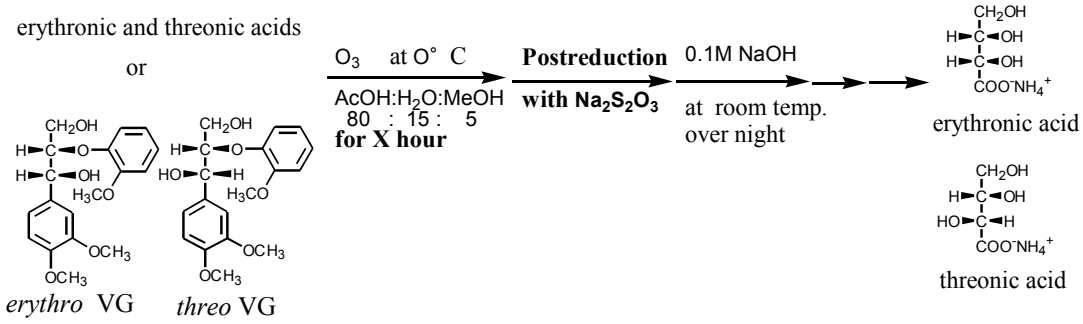


Fig. 2.7 ^{1}H-NMR spectrum of erthronic acid in CD_3COOD-D_2O-CD_3OD (80:15:5, v/v/v).

2.3.3 Effect of reductive post-treatment



In the previous section 2.3.2, it was implied that peroxides participated in the decomposition of erythronic and threonic acids during ozone treatment and post-alkaline step. Such a peroxide was assumed to be generated during ozone treatment and it will be more reactive in post-alkaline step. The decomposition of these acids during the post-alkaline step could be suppressed by the reduction of peroxides. For this purpose, the addition of $Na_2S_2O_3$ as a reducing agent after ozone treatment (post-reductive treatment) was examined.

Effect of post-reductive treatment on recoveries of erythronic and threonic acids

Figure 2.8 shows the recovery of erythonic and threonic acids by ozonation of these acids in free acid form with and without the post-reductive treatment. As described in section 2.3.2, erythronic and threonic acids were unstable toward ozone treatment, and their recoveries decreased to 57% and 61%, respectively, at 2hr of ozonation. They, however, were raised to 85% and 93%, respectively, by adding $Na_2S_2O_3$ after ozone treatment. In this thesis, this post-reduction is performed in order to diminish the possible action of peroxides during the alkali treatment, and is distinguished from that in general "ozonolysis" procedure, in which a reductant such as $NaBH_4$ reduces ozonides and/or peroxides to the corresponding aldehydes and/or ketones or alcohol.

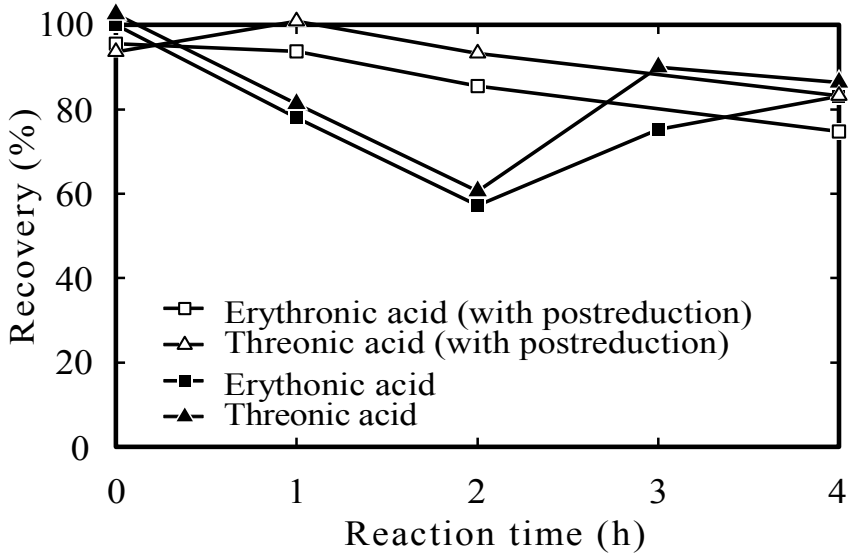


Fig. 2.8 Stability of erythronic and threonic acids toward ozonation with and without post-reduction

Effect of post-reductive treatment on the yields of erythronic and threonic acids obtained from a lignin model compound

A lignin model compound, Veratrylglycerol-β-guaiacyl ether (VG), was subjected to ozonation with and without the post-reductive treatment (Table 2.1). In the case of ozonation without post-reductive treatment, the total yields of erythronic and threonic acids reached 59% at 120 min (Fig. 2.9), and the *erythro*/*threo* ratio based on the yield of these two acids was 1.8 (*erythro*:*threo* =64:36), which was slightly lower than the 2.3 (*erythro*:*threo* =70:30) determined by ^{1}H-NMR of acetylated VG (Table 2.2). The total yield of these acids was increased by the post-reductive treatment, which was 74% at 120 min (Fig. 2.10). The yields of both acids were maintained at a similar level during the ozonation for 40-120 minutes, and the *erythro*/*threo* ratio determined by ozonation analysis (*erythro*:*threo* =69:31) was in good agreement with that determined by ^{1}H-NMR (Table 2.2).

Concerning about the degradation of these acids during ozone treatment and post-alkaline treatment, it was suggested that the latter degradation was suppressed by the reductive post-treatment with $Na_2S_2O_3$. One possible explanation for the effect of

post-reductive treatment was shown in Fig. 2.11, which includes the formation of glyceric acid detected as ozonation products (data was not shown).

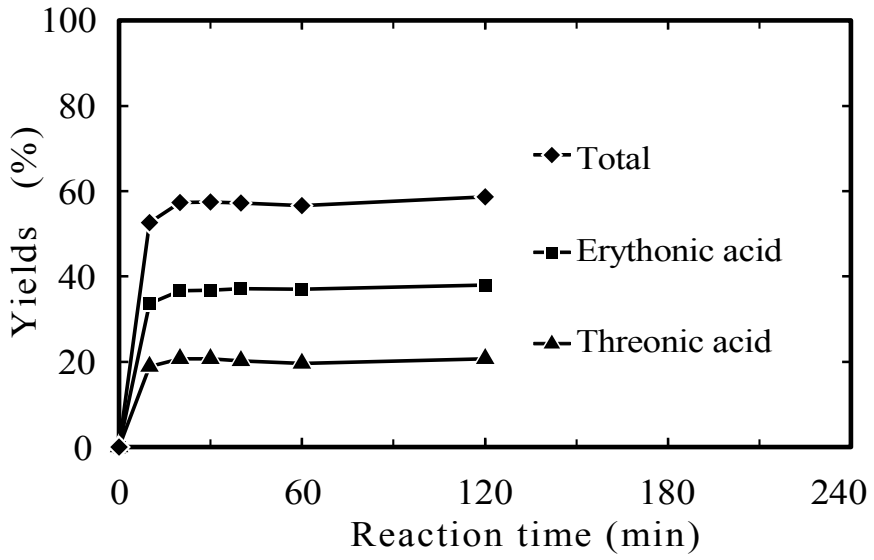


Fig. 2.9 Yields of ozonation products from veratrylglycerol-β-guaiacyl ether (VG) without post-reduction

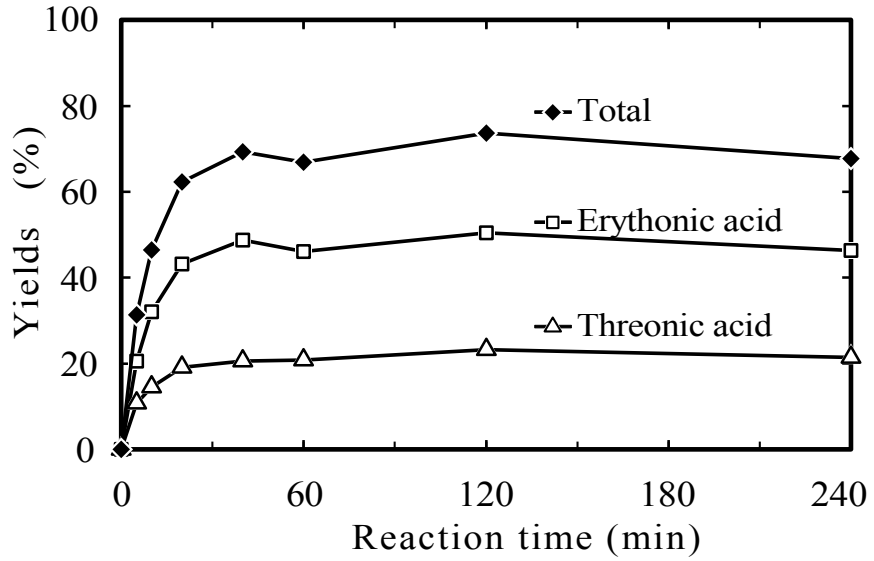


Fig. 2.10 Yields of ozonation products from VG with post-reduction

Table 2.2 *Erythro*/*threo* ratio of VG determined by the ozonation method with and without post-reduction

Method		*erythro*: *threo*	*erythro/threo* ratio
^{1}H-NMR		70:30	2.3
Ozonation without post-reduction			
Reaction time (min)	10	64:36	1.78
	20	64:36	1.77
	30	64:36	1.78
	40	65:35	1.84
	60	65:35	1.88
	120	65:35	1.84
Ozonation with post-reduction			
Reaction time (min)	5	66:34	1.91
	10	69:31	2.21
	20	69:31	2.26
	40	70:30	2.37
	60	69:31	2.21
	120	69:31	2.18
	240	68:32	2.17

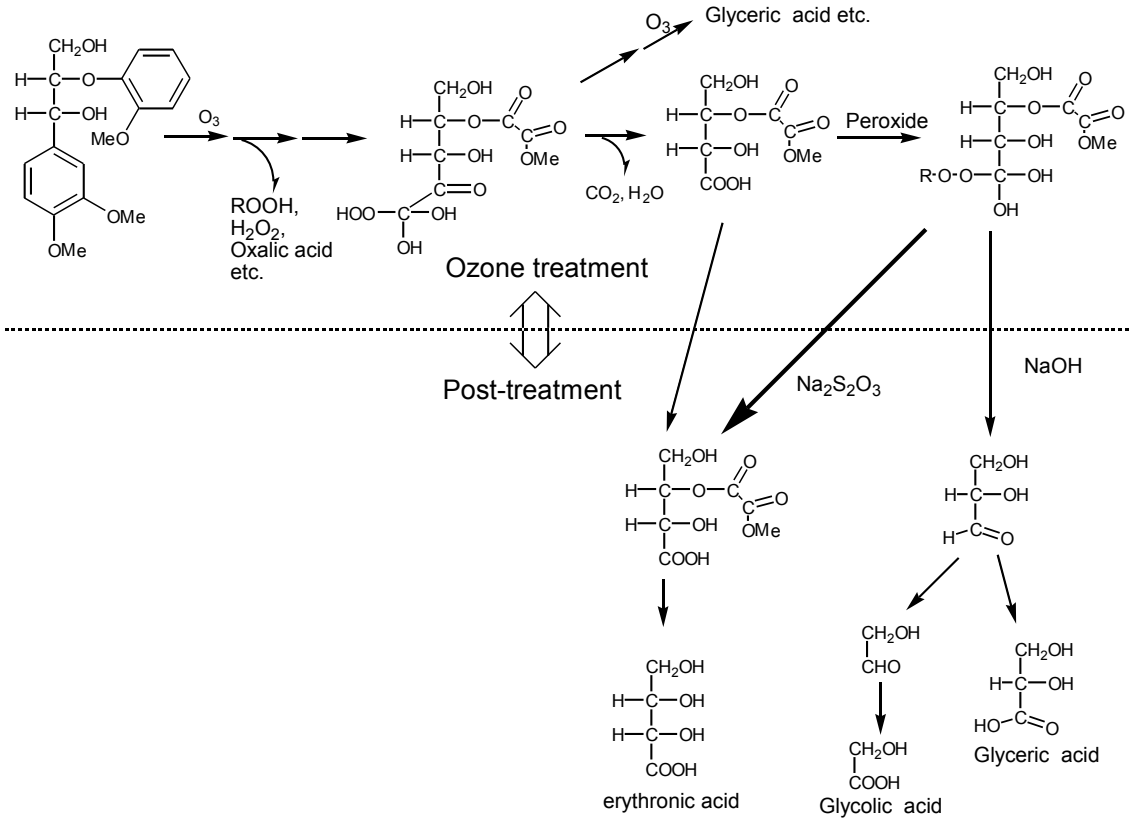
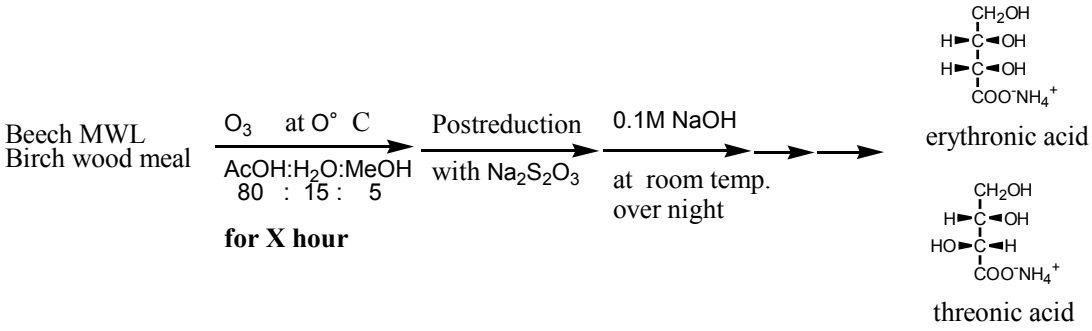


Fig. 2.11 One possible explanation for the effect of reductive post-treatment with $Na_2S_2O_3$ on the degradation of erythronic and threonic acids.

2.3.4 Application to wood meal and MWL



The improved ozonation method (section 2.3.3) was applied to beech MWL and birch wood meal (Table 2.1), and the reproducibility of the yields of erythronic and threonic acids was investigated.

The effect of sample amount on the yields of these acids was investigated. When 200 mg of wood meal was subjected to the improved ozonation method for 2 hr, the mean total yields of these acids from three trials was 0.32±0.02 mmol/g-wood meal, and the mean value of *erythro*/*threo* ratio was 2.79±0.05. Change of the sample weight from 200 mg to 50 mg did not cause significant change (Fig. 2.13, dotted line). In the case of MWL (10 mg and two 30 mg samples), the mean yield and *erythro*/*threo* ratio were 1.08±0.02 mmol/g-MWL and 1.68±0.02, respectively (Fig. 2.12). Those data confirmed the reproducibility of this improved ozonation method even when the sample amount varied.

In both cases of MWL and wood meal (Fig. 2.12 and 2.13), the yields of erythronic and threonic acids significantly increased until 40-60 min of reaction time. After 60 min the yields increased only gradually and the *erythro*/*threo* ratio became almost constant (Table 2.3). Based on the results in Figs. 2.10, 2.12 and 2.13, it appears that at least 1 hr of ozonation is required for the determination of the *erythro*/*threo* ratio of lignin samples, though the size of insoluble samples such as wood meal may affect the rate of ozonation.

In those figures, the yields of erythronic and threonic acids were expressed as the molar yields calculated based on the assumption that an equivalent molecular weight of

one unit of lignin is 200. The total yield of these two acids from MWL was 22.1% (mol% per one C_6-C_3 unit) at 120 min (Fig. 2.12), and that from wood meal which contains 25.4% of lignin (Klason method) was 29.1% at 240 min (Fig. 2.13). It was thus concluded that β-O-4 structures includes at least 22.1% of the C_6-C_3 structure in beech MWL, and at least 29.1% of the C_6-C_3 structure in birch wood meal. Those values are expressed without taking any factor such as the yields of erythronic and threonic acids from a model compound into consideration.

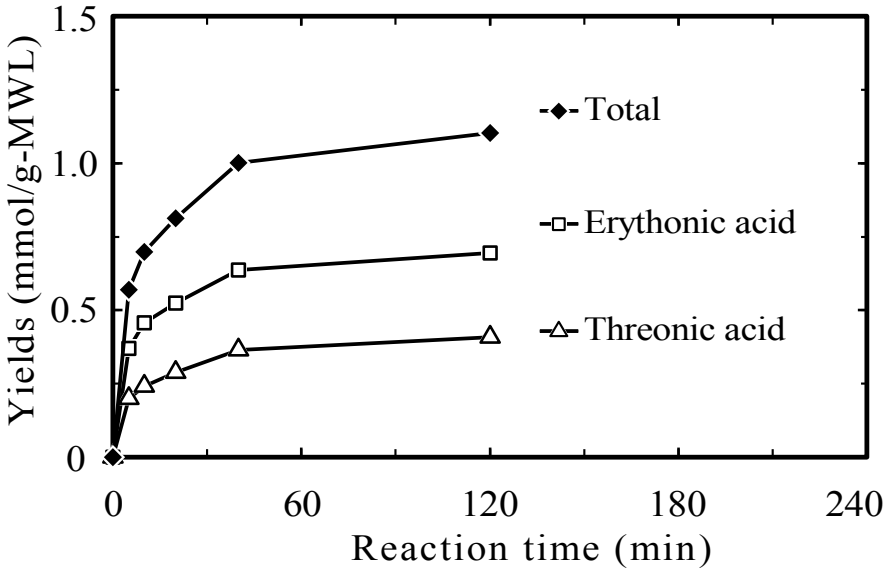


Fig. 2.12 Yields of products from beech MWL by ozonation with post-reduction

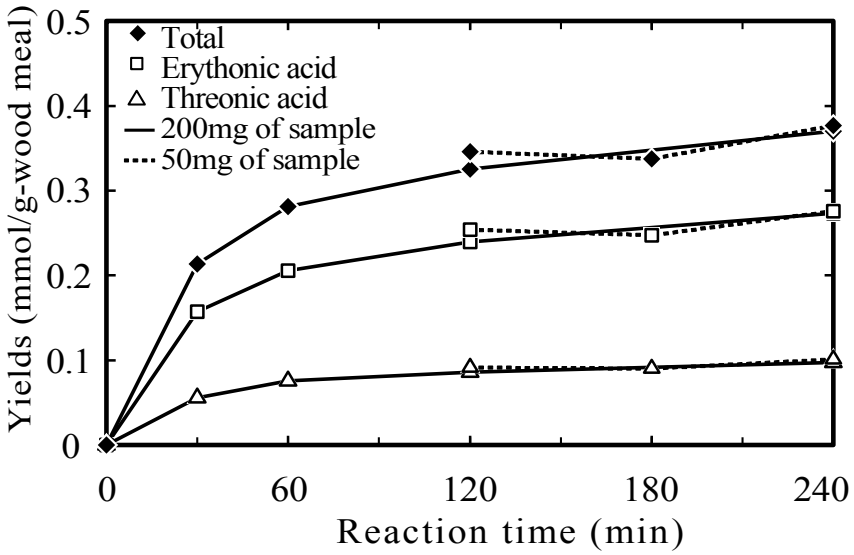
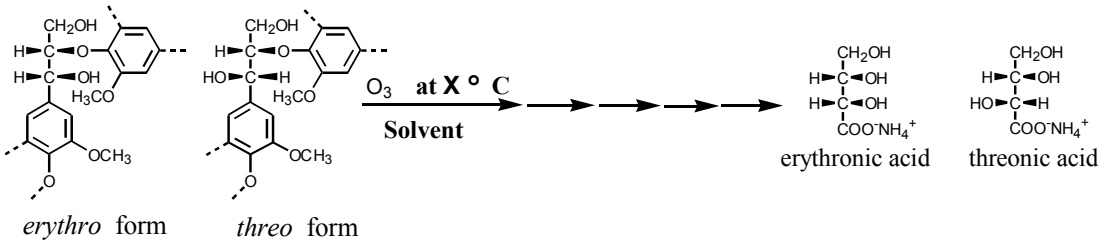


Fig. 2.13 Yields of products from birch wood meal by ozonation with post-reduction.

Table 2.3 *Erythro*/*threo* ratio of lignin samples determined by the ozonation method with pos-treduction

Samples		*erythro*: *threo*	*erythro*/*threo* ratio
Beech MWL			
ozoantion treatment (min)	5	65:35	1.86
	10	65:35	1.89
	20	65:35	1.82
	40	64:36	1.75
	120	63:37	1.70
Birch wood meal			
ozoantion treatment (min)	30	74:26	2.83
	60	73:27	2.73
	120	74:26	2.79
	240	74:26	2.81

2.3.5 Effects of reaction temperature and solvent



erythro form *threo* form

Japanese cedar wood meal (*Cryptomeria japonica*) was subjected to ozonation in different solvents and at different reaction temperature to investigate these effects on the yields of erythronic and threonic acids. The same volume of the solvent (30ml) was used.

In all conditions employed, no important change of the *erythro*/*threo* ratio was found (Table 2.4). With respect to the total yields of these two acids, the influence of reaction temperature was not found at 0-40 °C. The difference of solvent, however, caused the significant change of the yields. On the discussion about the effect of solvent on the reactivity of a substrate with ozone, the solubility of ozone was taken in consideration, because the solubility of ozone in AcOH is about 9 times lager than that in H_2O (Battino, 1981). The total yield of these two acids increased when a part of AcOH was replaced by H_2O (AcOH-H_2O), even though the solubility of ozone in AcOH-H_2O must be lower than that in AcOH. The result suggested that H_2O was involved in the reaction of lignin with ozone. The yield, furthermore, increased when a part of H_2O in AcOH-H_2O was replaced by MeOH (AcOH-H_2O-MeOH 80/15/5). Further experiments, such as ozonation under different ratio of MeOH at constant volume of H_2O (i.e. AcOH-H_2O-MeOH 75/20/5) and under the absence of H_2O, will be required to investigate whether or not MeOH was involved in the reaction of lignin with ozone.

Table 2.4 Effects of temperature and solvent of ozonation on the total yield of erythronic and threonic acids obtained from wood meal (*Cryptomeria japonica*)

Solvent	Reaction Temp.	O °C	21 °C	40 °C
AcOH: H_2O: MeOH	Total yield/C_6-C_3 *	26.2%	26.7%	25.6%
80 : 15 : 5	*Erythro* : *threo***	49:51	49:51	48:52
AcOH: H_2O	Total yield/C_6-C_3	16.3%	16.4%	-----
80 : 20	*Erythro* : *threo*	49:51	49:51	
AcOH	Total yield/C_6-C_3	Under m.p.	11.5%	-----
100%	*Erythro* : *threo*	of AcOH	46:54	
Ac_2O	Total yield/C_6-C_3	< 1%	-----	-----
100%	*Erythro* : *threo*			
Ac_2O***	Total yield/C_6-C_3	ca. 5%	-----	-----
100%	*Erythro* : *threo*			

*Total yield (mol%) of erythronic and threonic acids was calculated based on the assumption that Mw of lignin unit = 200g/mol, lignin content = Klason lignin +Acid soluble part. ** Ratio of erythronic and threonic acids. *** Acetylated wood meal was used as the ozonation sample.

The stabilizing step of carbonyl oxide by the addition of solvent seems to be important to understand the effect of solvent. Fig. 2.14 shows one possible reaction scheme for producing the erythronic acid from arylglycerol. The carbonyl oxide 1 would be stabilized by the addition of protic solvent (AcOH, H_2O, or MeOH) to give peroxides **2, 2', or 2"**. The reactivity of a carbon-carbon double bond with ozone might be different in these peroxides (**2, 2', or 2"→4**). And the carbonyl oxides produced from primary ozonide might **4** be preferably led to the formation of erythronic and threonic acids depending on the kind of solvent which stabilize the carbonyl oxides (**4→5**). The radical scavenging effect of methyl group in the solvents (methanol, acetic acid) could also contribute to the yield gain by preventing ozonation products from further degradation.

In this experiment, the combination of AcOH-H_2O-MeOH at the ratio of 80:15:5 (v/v/v) was the most effective for ozonation of wood meal. Further experiment using model compounds will be required to elucidate the "solvent effect" in more detail, and, by the regulation of the reaction of peroxides, the yields of erythonic and threonic acids might increase further.

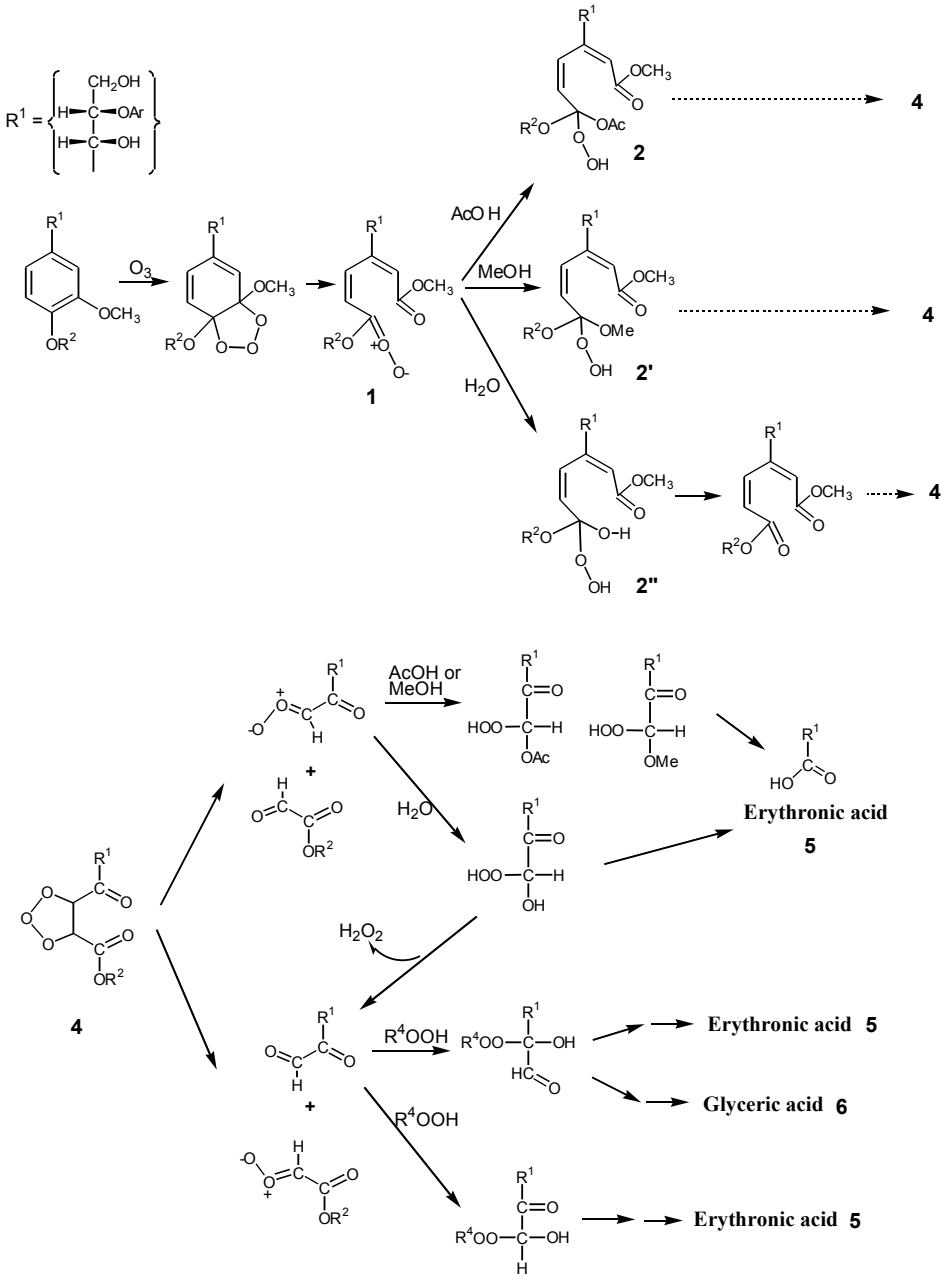
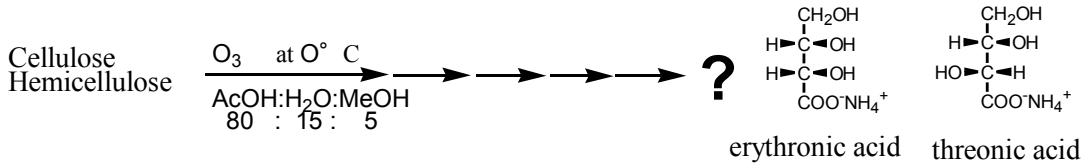


Fig. 2.14 Possible reactions for stabilizing the carbonyl oxide by the participation of solvent

2.3.6 Influence of Cellulose and Hemicellulose



erythronic acid threonic acid

Wood meal and the delignified wood meal (Table 2.1) were subjected to ozonation to evaluate the participation of cellulose and hemicellulose on the formation of erythronic and threonic acids.

The delignified wood meal was prepared by delignification of birch wood meal with $NaClO_2$ and the successive extraction with 1M NaOH at room temperature. The neutral sugar analysis of this sample, $NaClO_2$-delignified wood meal with NaOH extraction, gave alditol acetates in quite high yields, 96.8 wt% as the total yield of anhydrosugars (Table 2.5). This high yield indicated that the sample contains substantial amount of cellulose and hemicellulose as components with little lignin, although the ratio of xylose to glucose was lower than untreated wood meal.

Ozonation of the $NaClO_2$-delignified wood meal with NaOH extraction gave erythronic and threonic acids in only 0.0033 mmol/g of sample (Table 2.6). This value was about 1% of the yield from the same amount of wood meal. When $NaClO_2$-delignified wood meal without NaOH extraction was subjected to ozonation method, a considerable amount of these two acids was produced; the yield was 42.2% of that from the same amount of wood meal. These results suggested that a degradation method performed under acidic condition, such as $NaClO_2$ treatment, cannot remove all the degraded lignin fragments, especially those derived from the side-chain parts, and that those fragments will still act as precursors of erythronic and threonic acids when subjected to ozonation.

Table 2.5 The yields of alditol acetates obtained from the delignified wood meals by neutral sugar analysis.

aldose unit / Samples		Glu	Xyl	Rha	Ara	Man	Gal	Total
Wood meal **	mg/g*	385.4	180.7	3.4	3.8	11.4	5.5	590.2
	mmol/g*	2.38	1.37	0.026	0.029	0.070	0.034	3.91
$NaClO_2$-delignified wood meal	mg/g	473.9	211.4	3.4	2.9	10.7	4.8	707.2
	mmol/g	2.93	1.60	0.026	0.022	0.066	0.030	4.67
$NaClO_2$-delignified wood meal with NaOH extraction	mg/g	891.8	51.5	0.9	1.8	19.3	3.0	968.4
	mmol/g	5.50	0.39	0.007	0.014	0.119	0.019	6.05

*Values are expressed as weight of each aldose unit or molar yields per sample weight.
Weight yields (mg/g-sample) of aldose units were calculated as following:
Mw of one aldose unit = Mw of the corresponding aldose – 18 (Mw of H_2O)
Values were expressed without any factor (i.e. survival factor during hydrolysis)
**lignin content of wood meal was 25.4 wt% (Klason lignin + the acid soluble part)

Table 2.6 The yields of erythronic and threonic acids obtained from the delignified wood meals by ozonation method.

Sample	Erythronic acid (μmol/g)	Threonic acid (μmol/g)	Total (μmol/g)	*Erythro*:*threo*
Wood meal	239	86	325	74:26
$NaClO_2$-delignified wood meal	110	27	137	80:20
$NaClO_2$-delignified wood meal with NaOH extraction	2.28	0.99	3.27	70:30

Some aldoses and their glycosides were subjected to ozonation, and the yields of erythronic and threonic acids were investigated. As shown in Table 2.7, D-glucopyranoside, D-xylopyranoside, and D-mannopyranoside gave these two acids in ca. 5, 26, and 5 mol%, respectively, but the corresponding methyl-β-glycosides gave these two acids only in minor yields. Because the yields were quite low compared with 74mol% yield from lignin model compound VG, it was concluded that the influence of cellulose and hemicellulose in wood sample on the yields of erythronic and threonic acids from wood meal is small, unless a sample containing polysaccharide with many reducing terminal residues is used.

Table 2.7 The yields of erythronic and threonic acids obtained from aldose and acetals by ozonation method.

Products / Samples		Erythronic acid	Threonic acid	Total
Me-β-D-Glucopyranoside	mol%	0.8	not detected	0.8
Me-β-D-Xylopyranoside	mol%	0.1	1.1	1.2
D-Glucopyranoside	mol%	5.2	0.2	5.4
D-Xylopyranoside	mol%	1.8	ca 24	ca. 26
D-Mannopyranoside	mol%	4.7	0.1	4.8

2.4 Conclusions

Ozonation method as the analytical tool of the stereo structures of arylglycerol-β-aryl ether (β-O-4) linkage was examined by the use of wood meal, milled wood lignin and a lignin model compound, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (veratrylglycerol-β-guaiacyl ether (VG)), and the procedure was improved. When mild post-reduction was applied to the ozonation products, the total yield of erythronic and threonic acids from this model compound was 74%, which is 15% higher than the yield without post-reduction. Decrease in the recovery of these two acids under prolonged ozonation treatment was successfully suppressed by the post-reduction. The *erythro/threo* ratio of VG determined by ozonation method with post-reduction was in good agreement with the ratio determined by ^{1}H-NMR. Excellent reproducibility of the yield was obtained by the adoption of trimethylsilylation of ozonation products as ammonia salts form by DMSO-HMDS-TMCS mixture when subjecting to gas chromatography analysis. It was concluded that β-O-4 structures include at least 29.1% of the C_3-C_6 unit in birch wood lignin, and the *erythro*/*threo* ratio is 2.8.

Chapter 3

Absolute Configurations of the α- and β-Asymmetric Carbons of β-O-4 Structures: Evidence for the presence as the racemic forms

3.1 Introduction

Lignin contains two kinds of asymmetric carbons, α- and β-asymmetric carbons, in the three carbons side chain. Based on the widely accepted hypothesis of lignin biosynthesis, the β-asymmetric carbon is formed by the coupling reaction of phenoxy radicals, and the presence of multiple coupling sites in the phenoxy radical gives various linkage types such as β-O-4, β-5, β-β, and β-1 types in lignin structure. In the absence of any asymmetric carbon, the radical coupling reaction must give the racemic forms of the products containing β-asymmetric carbons with *R* and *S* configurations. Some asymmetric carbons, however, are present in an already grown lignin structure and in a polysaccharide in this process, and such an asymmetric carbon possibly may cause enantioselective radical coupling to form the excess of the β-carbon with *R* or *S* configuration.

As described in Chapter 1.2, no optical activities have been shown in the isolated pinoresinol and syringaresinol (β-β) (Freudenberg et al., 1965; K. Ogiyama and Kondo, 1966), and in β-5 and β-β derivatives obtained by DFRC method (J. Ralph et al 1999). However, no information is available on the absolute configuration of β-asymmetric carbon of β-O-4 structure. It will be desired to determine the proportion of the enantiomeric forms of β-O-4 structures (Fig. 3.1), because this structure is the most predominant linkage type in lignin.

Ozonation analysis is the only way for the resolution of this question as to my knowledge. The proportion of the β-carbons with *R* or *S* configuration in β-O-4 structures can be estimated from the proportion of four stereoisomers of tetronic acids obtained by ozonation of β-O-4 structures in lignin (Fig. 3.2).

In addition to β-asymmetric carbon, the absolute configuration of α-asymmetric carbon can be also discussed based on the results of ozonation analysis. The formation of the α-asymmetric carbon is related to the determination of the ratio of *erythro* and *threo* forms of β-O-4 structure in lignin biosynthesis. The excess of *erythro* form of

β-O-4 structure in hardwood will be caused by a diastereo-differentiating water addition to the quinone methide intermediate (Fig. 3.1). If the diastereo-differentiating water addition is controlled only by the adjacent β-asymmetric carbon of a quinone methide, both quinone methides with different configurations (**2** and **2'** in Fig. 3.1) should give the same value of the *erythro*/*threo* ratio of β-O-4 structures (**3*e*/3*t*** and **3'*e*/3'*t***)(Chap 1.4.1 for the reaction mechanism).

In this chapter, the proportion of four stereoisomeric forms in hardwood lignin, **3*e***, **3*t*, 3'*e***, and **3'*t***, was investigated by ozonation analysis. Based on the proportion, the stereoselectivity of α-and β-asymmetric carbons in lignin formation was discussed.

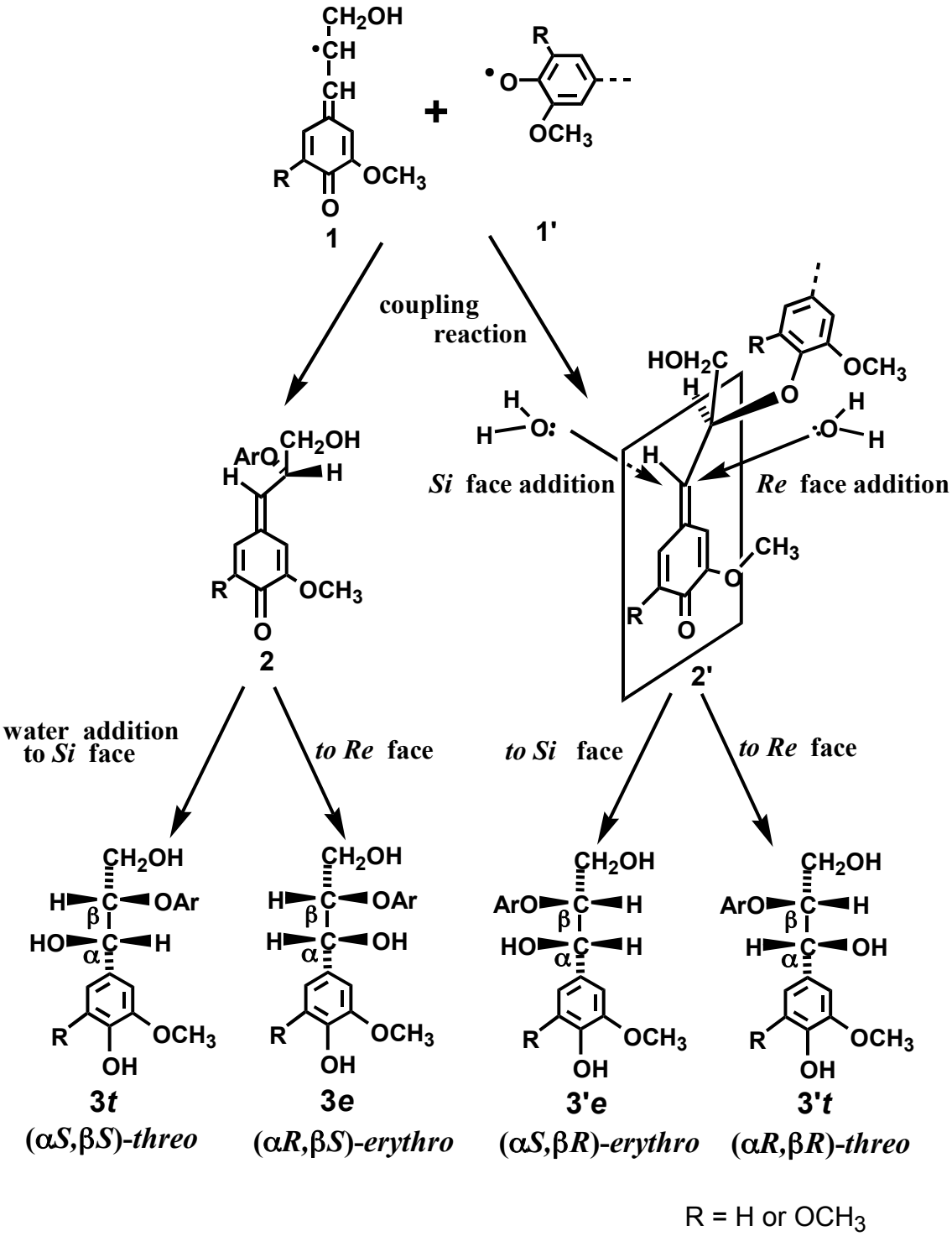


Fig. 3.1 Formation of α- and β-asymmetric carbons of arylglycerol-β-aryl ether structures in lignin biosynthesis

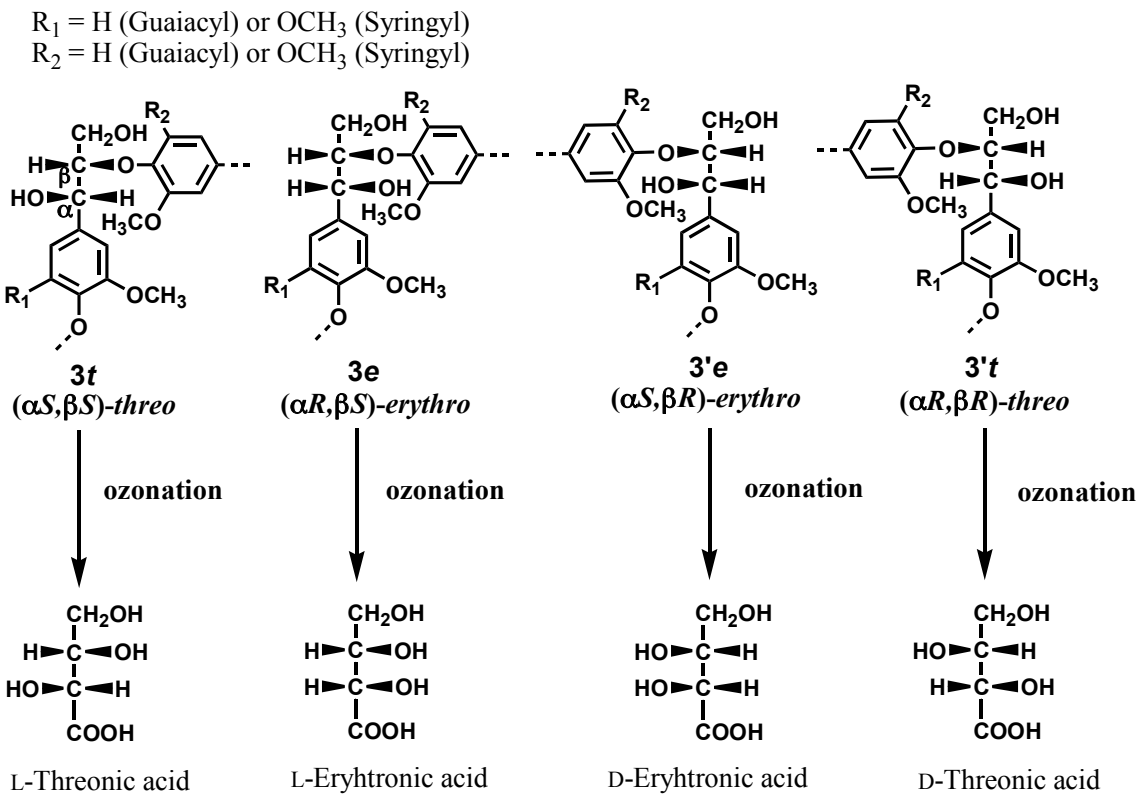


Fig. 3.2 Ozonation products of four stereoisomeric forms of β-O-4 structure

3.2 Experimental

3.2.1 Materials

Chemicals

D-erythronic and L-threonic acid ammonium salts were prepared from D-erythronolactone (Tokyo Kasei) and L-threonic acid calcium salt (Aldrich) as described in chapter 2.2.1. Ammonium (NH_4^+) form of cation exchange resin was prepared from H^+ form of the resin (Dowex-50W-X4, 50-100mesh). Acetate (CH_3COO^-) form of anion exchange resin was prepared from the Cl^- form of the resin (Muromac-1X8, 100-200mesh). All other chemicals were reagent grade.

Wood meal and MWL

Birch wood meal and beech MWL used were the same as those in chapter 2.2.1.

3.2.2 HPLC analysis

HPLC was performed on non-chiral column (cation exchanger with polystyrene/divinyl benzene matrix) with an on-line refractive index or an optical rotation detector. The former detector was used for separation of erythronic and threonic acids from ozonation products and for the determination of the concentrations. The latter was used for determination of the optical activities. They were performed under the following conditions.

Column: BIO-RAD HPX87H and Yokogawa CHA-E11 connected in series
Eluent 1: 0.01M sulfuric acid (used for analysis of beech MWL, Chapter 3.2.4)
Eluent 2: 0.1M acetic acid (used for analysis of birch wood meal, Chapter 3.2.5)
Flow rate: 0.3ml/min
Detector: Refractive index detector (Shimadzu RID-10A) or optical rotation detector (Jasco OR-990, HgXe lump:350-900nm)

3.2.3 Ozonation

Beech MWL (30mg) or birch wood meal (200mg) was subjected to ozonation

according to the method of Chapter 2.2.2 except for reaction time of ozonation (2 h). Yields of erythronic and threonic acids were determined by GC analysis of their trimethylsilyl derivatives using erythritol as an internal standard.

3.2.4 Measurement of optical activities of ozonation products of MWL: preliminary experiment

The solution of the saponified ozonation products obtained from totally 60mg of beech MWL (30mg ×2) was neutralized with AcOH at pH 8, and was passed through a column filled with 40ml of anion exchange resin (CH_3COO^- form), and then the eluents of H_2O (60ml) and 5M-AcOH (300ml) were obtained. The AcOH eluent (monocarboxylic acids fraction) was concentrated under the reduced pressure and saponified with 0.1M-NaOH again. The solution was passed through an ammonium form cation exchange resin to convert organic acids to ammonium salts. The ozonation products were subjected to HPLC with a non-chiral column, and their optical activities were measured by an on-line optical rotation detector. The condition of the HPLC analysis was described in detail as above (section 3.2.2).

3.2.5 Measurement of optical activities of isolated erythronic and threonic acids from ozonation products of wood meal

Isolations

The saponified ozonation products obtained from totally 4g of birch wood meal (200mg ×20) were combined. The solution was separated from residual wood meal by centrifugation (15000rpm, 30min) and filtration (pore size: 0.45μm). The solution was neutralized with AcOH to pH 8, and passed through a column filled with 120ml of anion exchange resin (CH_3COO^- form). The eluent of 1150ml of H_2O and 1050ml of 1M-AcOH were obtained. The AcOH eluent (monocarboxylic acids fraction) was evaporated under the reduced pressure, and saponified with 0.1M-NaOH again. The solution was passed through an ammonium form cation exchange resin to convert

organic acids to ammonium salts.

The monocarboxylic acid fraction was subjected to HPLC separation (eluent: 0.1M acetic acid), and erythronic acid and threonic acid rich fractions were obtained. The fractions were purified by the repetition of HPLC separation in the manner that the first half of the peak of erythronic acid and the second half of the peak of threonic acid were separately collected. The collected erythronic acid fraction was converted to the lactone form by repeating of concentration under reduce pressure with diluted aqHCl, and then further HPLC separation was performed by collecting the erythronolactone peak (retention time: 60min). With respect to threonic acid, the collection as lactone form cannot be performed because threonolactone was easily converted to free acid form under the HPLC condition (eluent: 0.1M AcOH).

Optical activities

The purified erythronic and threonic acids (free acid form) were independently subjected to HPLC with non-chiral column. The optical activities were measured with on-line optical rotation detector. The condition of the HPLC analysis was described as above (section 3.2.2).

3.3 Results and discussion

3.3.1 Evidence for racemic forms of *erythro* type of β-O-4 structure in lignin by ozonation of beech MWL

The optical activities of β-O-4 structures in lignin were evaluated by the optical activities of erythronic and threonic acids of ozonation products from beech MWL.

Yield of erythronic and threonic acids obtained by ozonation of birch wood meal

The total yield of erythronic and threonic acids from MWL was 23.6% (per lignin unit) at 120 min ozone treatment based on the assumption that equivalent of molar weight of lignin unit is 200. And the *erythro/threo* ratio was 1.68 (*erythro*:*threo* =63:37).

Optical activities of ozonation products

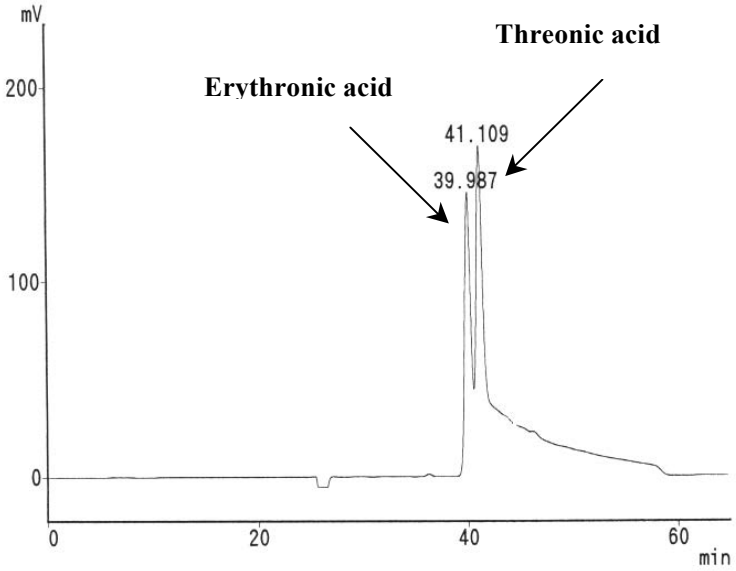
Ozonation products were subjected to HPLC analysis to investigate the optical activities of erythronic and threonic acids. Two kinds of detector, a refractive index (RI) detector or an optical rotation detector was connected to HPLC.

Figure 3.3 shows HPLC profiles obtained by an on-line RI detector. The ozonation products of MWL contained 45mM erythronic acid and 27mM threonic acid (Fig. 3.3a), which were determined by GC analysis. The MWL ozonation products and the authentic samples adjusted to 45mM D-erythronic acid and 27mM L-threonic acid showed almost the same height of the peaks on the HPLC profiles (Fig. 3.3a and b). It was confirmed that the concentrations of these acids in the MWL ozonation products were the same as those of the authentic samples, although the separation of erythronic acid from threonic acid was not perfect.

Figure 3.4 shows HPLC profiles of the same samples obtained by an on-line optical rotation detector. If these acids in the ozonation products do not show optical activity,

it would be concluded that β-O-4 structure is present as racemic forms. Although D-erythronic acid showed an enough peak to detect its optical rotation (Fig. 3.4a), L-threonic acid showed only a small peak (Fig. 3.4b). Only the optical rotation of erythronic acid can be discussed because of the small peak of threonic acid due to the lower concentration and the lower specific rotation of the compound, and the incomplete separation of these two acids. The optical rotation profiles were obtained for MWL ozonation products and the authentic samples, which include the same concentration of 45mM erythronic and 27mM threonic acids. As shown clearly, the optical rotation profile of ozonation products was almost flat at the retention time corresponding to these acids (Fig 3.4d) compared with that of the authentic samples (Fig. 3.4c). Even when a small fluctuation in the baseline was taken into consideration, excess of one enantiomer was not found in a detectable range for erythronic acid. The erythronic acid in MWL ozonation product was confirmed to be almost optically inactive. It was suggested that the *erythro* form of β-O-4 structure would be present as racemic form in lignin. With respect to the *threo* form of β-O-4 structure, a clear conclusion could not be reached because L-threonic acid shows only a small peak compared with that of D-erythronic acid. In the next section, a large amount of ozonation products was obtained from wood meal, and the isolations of erythronic and threonic acids were examined.

a)



b)

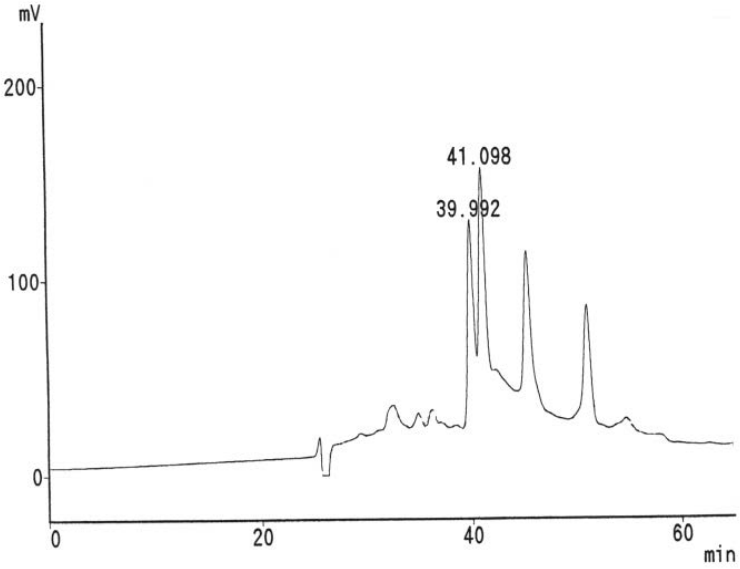


Fig. 3.3 HPLC profiles obtained by a refractive index detector:
a) ozonation products obtained from beech MWL,
b) 45mM D-erythronic and 27mM L-threonic acids

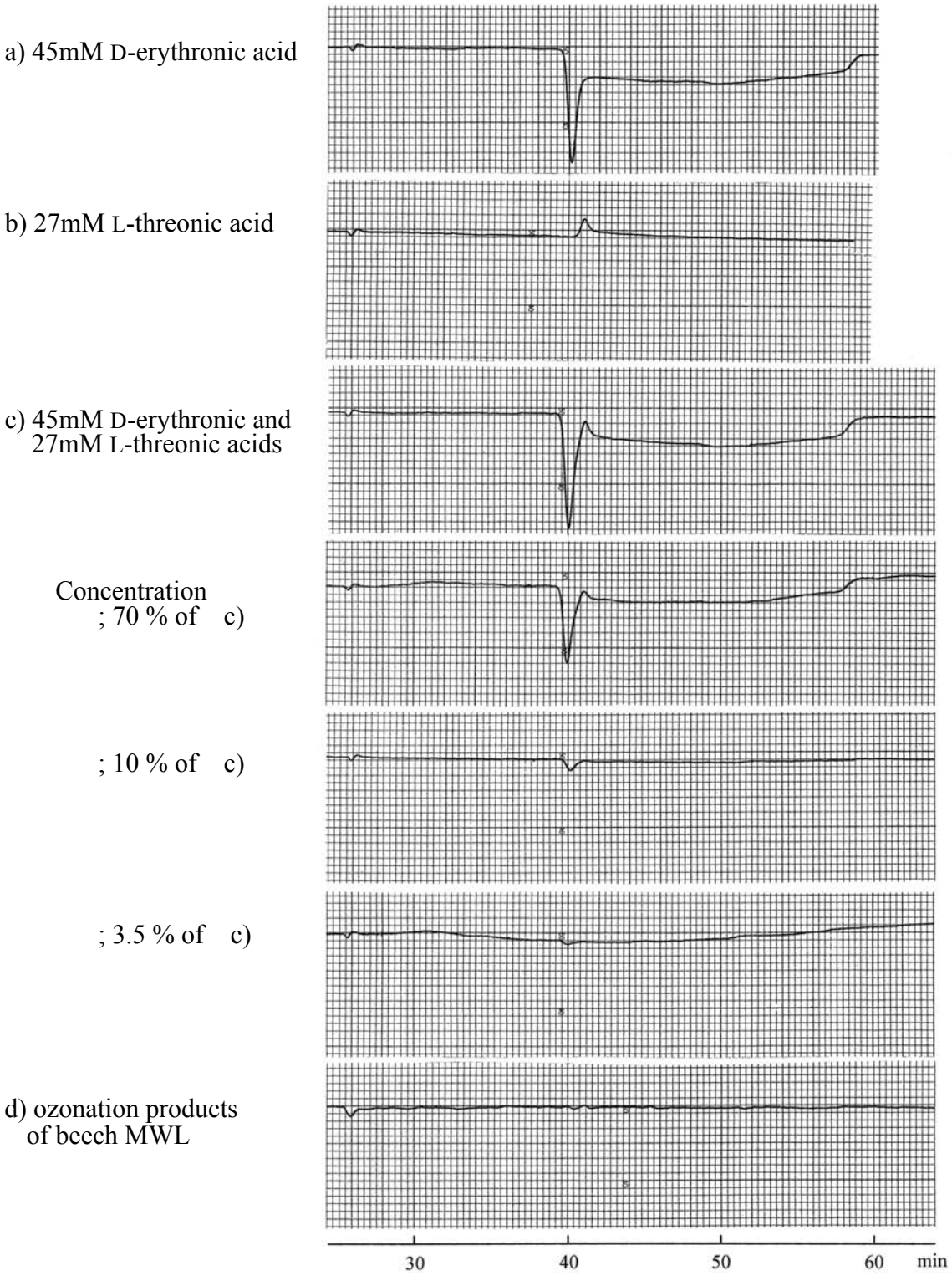


Fig. 3.4 HPLC profiles by an optical rotation detector for ozonation products obtained from beech MWL and the same concentrated authentic samples: 45mM D-erythronic and/or 27mM L-threonic acids, and those diluted ones

3.3.2 Evidence for racemic forms of β-O-4 structure in lignin by ozonation of birch wood meal

The proportion of four stereoisomeric forms of β-O-4 structures including the enantiomeric forms (Fig. 3.2) was determined based on the ratio of erythronic and threonic acids in the ozonation products of birch wood meal (*erythro*/*threo* ratio) and the optical activities of these acids. The *erythro*/*threo* ratio was determined by GC analysis of ozonation products. Isolations of erythronic and threonic acids from ozonation products were performed by HPLC separation, and their optical activities were determined with an online optical rotation detector (Fig. 3.5).

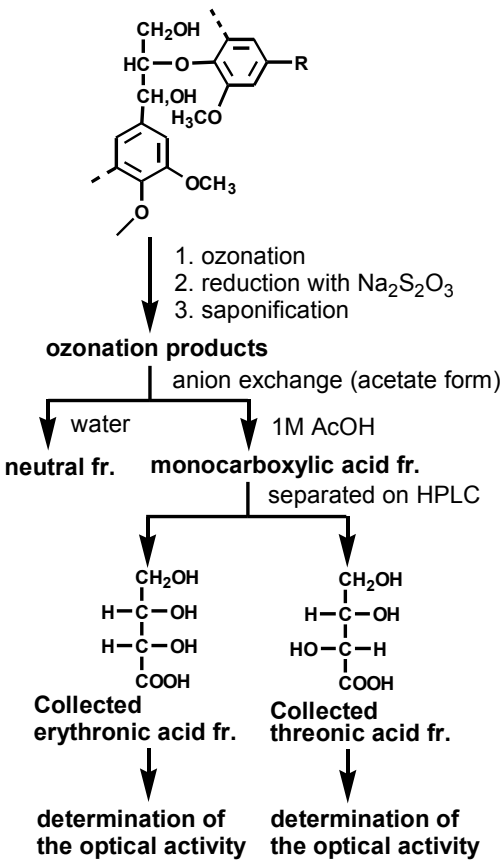


Figure 3.5 Outline of the scheme for the determination of optical activities of erythronic and threonic acids obtained by ozonation of birch wood meal.

Yield of erythronic and threonic acids obtained by ozonation of birch wood meal

The total yield of erythronic and threonic acids from wood meal which contains 25.4% of lignin (Klason method) was calculated to be 25.6% (per lignin unit) at 120minutes ozone treatment based on the assumption that equivalent of molar weight of lignin unit is 200. *Erythro/threo* ratio was 2.78 (*erythro*: *threo* =74:26).

Isolation of erythronic and threonic acids from ozonation products

Figure 3.6-a shows the HPLC profile of monocarboxylic acid fraction of the ozonation products detected by a refractive index detector. Because the separation of these two peaks was incomplete, the isolation of erythronic and threonic acids was tried by the repetition of collecting the separated peak by HPLC (see section 3.2.5 for the purification method). Figures 3.6-b and -c show the HPLC profile of collected erythronic and threonic acids fractions, respectively. In Figure 3.7, these peaks were compared with the peaks of authentic D-erythronic and L-threonic acids, respectively, at the same concentration (110mM D-erythronic acid and 186mM L-threonic acid) by the refractive index detector. The HPLC profile of the collected erythronic acid fractions was in agreement with that of authentic 110mM D-erythronic acid at around the retention time of erythronic acid, and the purity was ensured (Fig. 3.7a). For the collected threonic acid fraction, as shown in Fig. 3.7b, the contamination of erythronic acid was considered to be negligible degree compared with the peak height of threonic acid for the measurement of the optical activity, although a shoulder peak might be present at the retention time of the second half of peak of threonic acid.

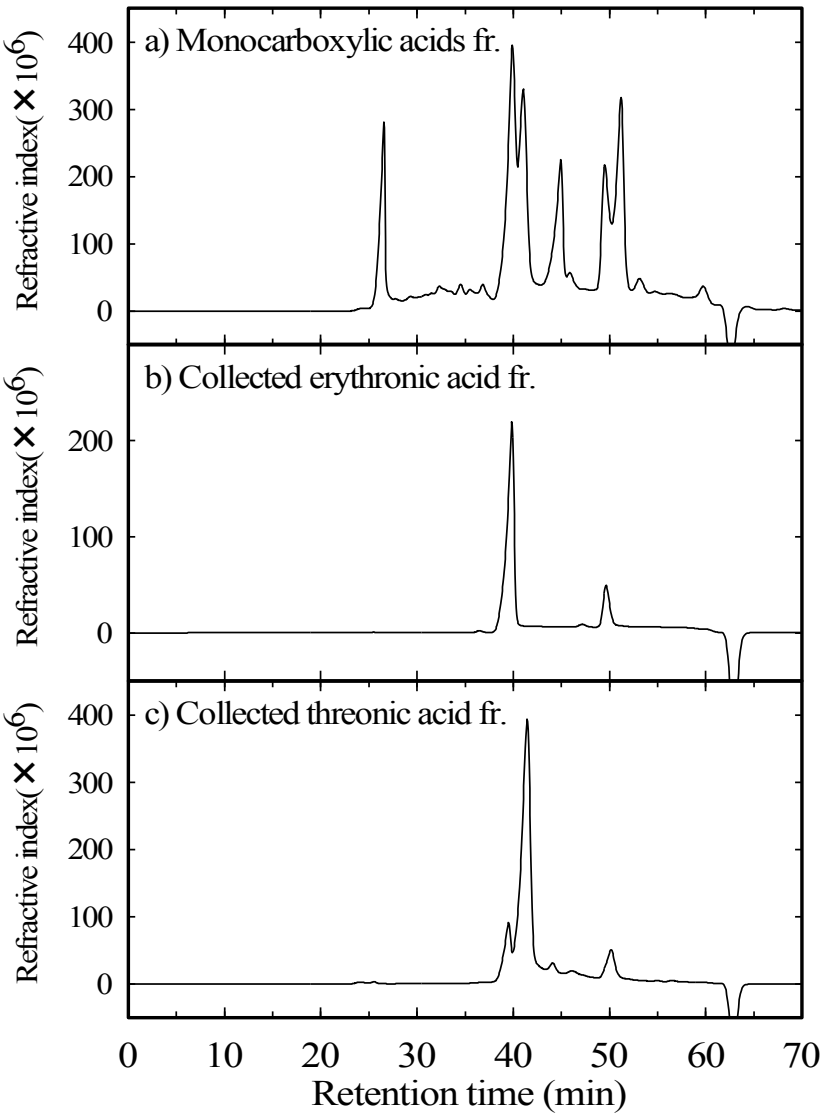


Fig. 3.6 HPLC profiles (r.t. 0-70 min) obtained by a refractive index detector for ozonation products of birch wood meal: a) HPLC profile of monocarboxylic acids fraction, b) collected erythronic acid fraction, c) collected threonic acid fraction.

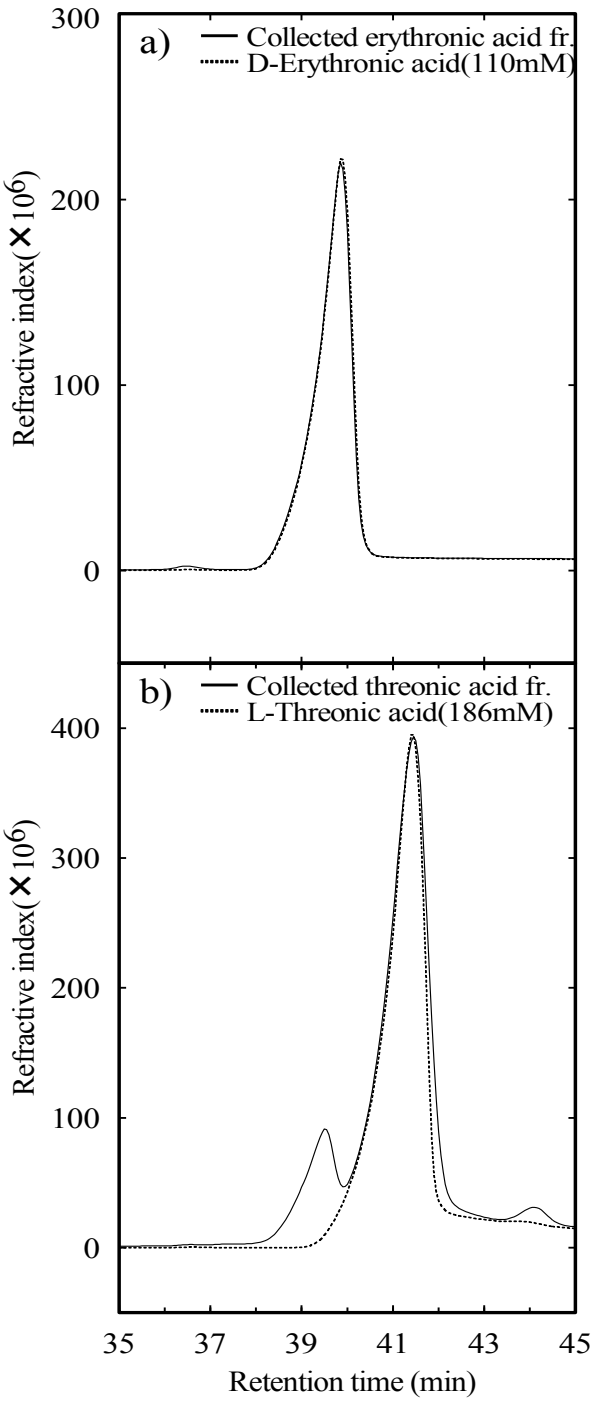


Fig. 3.7 HPLC profiles (r.t. 35-45 min) obtained by a refractive index detector for ozonation products of birch wood meal (solid lines) and authentic samples (dotted lines): a) collected erythronic acid fraction and 110mM D-erythronic acid, b) collected threonic acid fraction and 186mM L-threonic acid

Optical activities of erythronic and threonic acids from ozonation products

The HPLC profiles by an optical rotation detector were obtained for the collected erythronic and threonic acids fractions to investigate whether the peak indicating optical activity is present.

As shown in Fig. 3.8, the erythronic acid fraction did not show a peak in a detectable degree at the retention time (40.17min) for the top peak of the authentic sample (110mM of D-erythronic acid). An un-identified peak (r.t. 40.48min) suggesting the optical activity was detected at near the peak of erythronic acid. This small peak or inflexion, however, was not likely to originated from the optical activity of erythronic and threonic acid, because of the following reason. As shown in Figure 3.10, glycerol (authentic sample) showed the small peak in HPLC profile by an optical rotation detector, even though the compound is optically inactive. Enantiomeric excess (e.e.) of erythronic acid obtained from the ozonation products was calculated to be less than 3.0 %, even if the un-identified peak was counted in the estimation of e.e. This suggested that the *erythro* forms of β-O-4 structure consist of almost equal amounts of (β*R*)- and (β*S*)-enantiomeric forms (**3e** and **3'e** in Fig. 3.11).

With respect to the threonic acid obtained from the ozonation products (Fig. 3.9), similarly to the erythronic acid fraction, an un-identified peak (r.t. 42.08min) existed at near the peak of threonic acid (r.t. 41.65min). Even if this peak was counted in the estimation, enantiomeric excess (e.e.) of threonic acid obtained from the ozonation products was less than 8.0 %. This suggested that the *threo* forms of β-O-4 structure consist of approximately equal amounts of (β*R*)- and (β*S*)-enantiomeric forms (**3*t*** and **3'*t*** in Fig. 3.11). The significant excess was not found for the configuration of β-asymmetric carbon in β-O-4 structures in birch lignin.

Configurational studies on the asymmetric carbons of other structures have been already reported for β-β structures (Freudenberg et al., 1965; K. Ogiyama and Kondo, 1966) and β-5 structures (J. Ralph et al 1999). The present study is the first report on

the absolute configuration of α and β-asymmetric carbons of β-O-4 structures in lignin. It should be emphasized that the conclusion was drawn based on the degradation products obtained in high yield. Those studies represent a strong support for the generally accepted theory that the radical coupling involved in lignin formation is not stereochemically controlled.

The proportion of four stereoisomeric forms of β-O-4 structure was estimated based on the *erythro*/*threo* ratio (*erythro*: *threo* =74:26) and the enantiomeric excess of erythronic acid (e.e. <3%) and threonic acid (e.e. <8%) obtained by the ozonation of birch wood meal. Results of this estimation are summarized in Fig. 3.11. The proportion of four stereoisomeric forms of β-O-4 structures was: (α*S*,β*S*)-*threo*: (α*R*,β*S*)-*erythro*: (α*S*,β*R*)-*erythro*: (α*R*,β*R*)-*threo* = **3*t***: **3*e***: **3'*e***: **3'*t*** = 12-13%: 36-37%: 37-38%: 13-14%. The β-O-4 structures consist of approximately equal amounts of β-asymmetric carbons with *R* and *S* configurations ((β*R*)-β-O-4: (β*S*)-β-O-4 = **3*t***+**3*e***: **3'*e***+**3'*t*** = 50-52: 48-50). Furthermore, this proportion of four stereoisomeric forms indicated that the configurations of β-asymmetric carbon and the adjacent α-asymmetric carbon are not independent of each other, since the *erythro*/*threo* ratios were almost the same in both β-O-4 structures with *R* and *S* configuration at β-position (**3*e*/3*t*** and **3'*e*/3'*t***). This conclusion is related to the topic of the next chapter, in which the factors for the determination of the *erythro/threo* forms in lignin biosynthesis will be discussed.

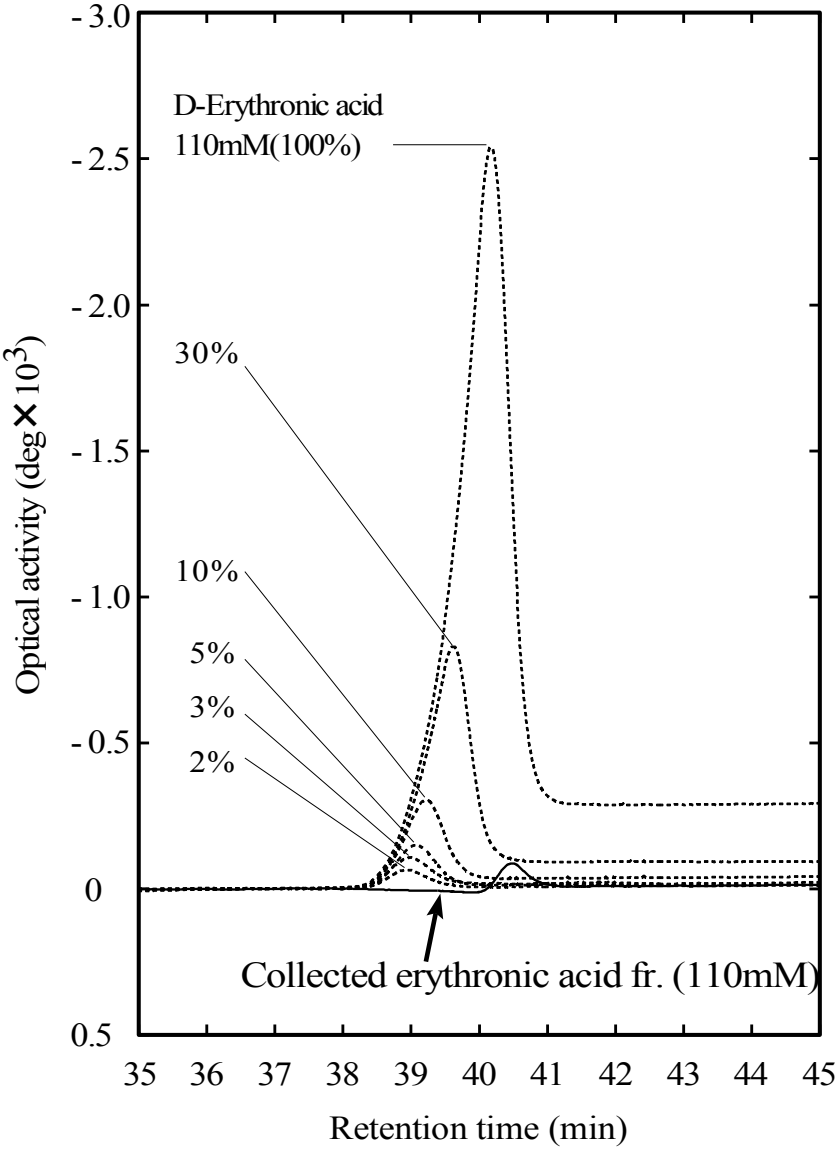


Fig. 3.8 HPLC profiles obtained by an optical rotation detector for the collected erythronic acid fraction from ozonation products of birch wood meal and 110mM D-erythronic acid (authentic sample)

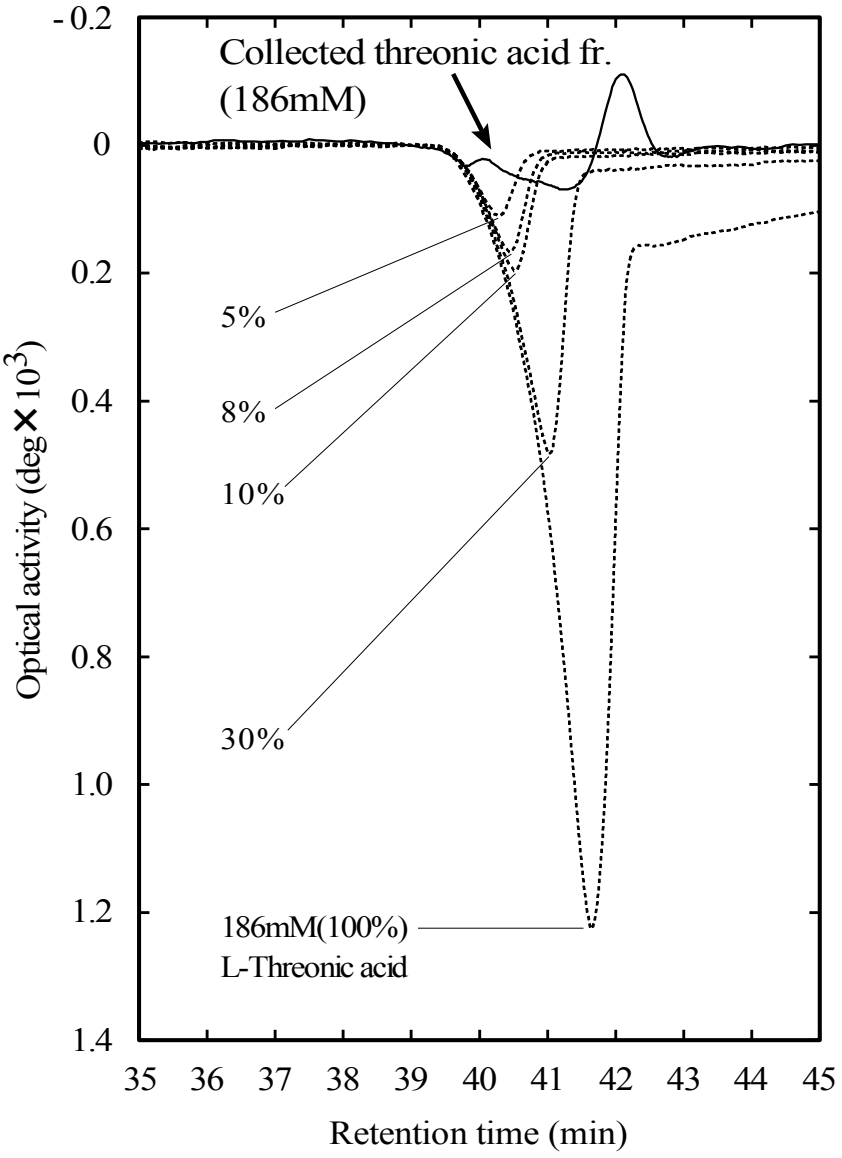


Fig. 3.9 HPLC profiles obtained by an optical rotation detector for the collected threonic acid fraction from ozonation products of birch wood meal and 186mM L-threonic acid (authentic sample)

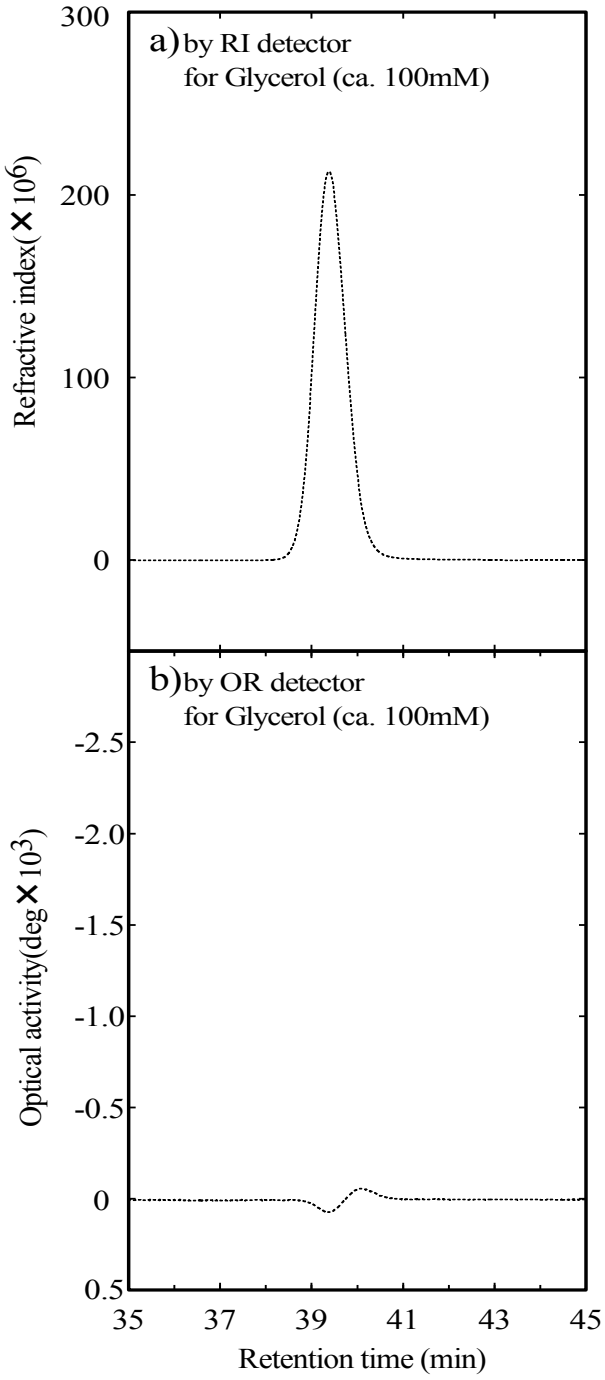
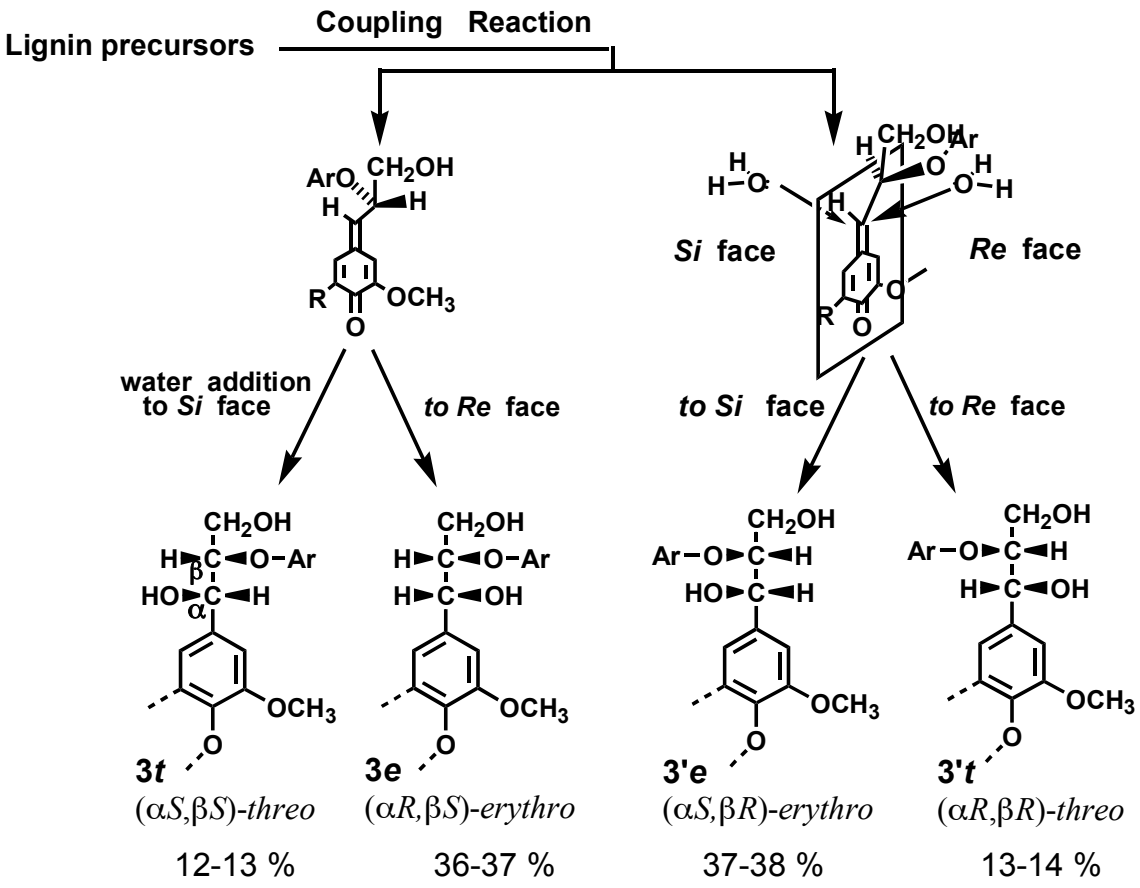


Fig. 3.10 HPLC profiles of glycerol (authentic sample): a) glycerol detected by refractive index (RI) detector, b) glycerol detected by an optical rotation (OR) detector



Absolute configurations of carbon at β-position of β-O-4 structures

(β*S*)-β-O-4 : (β*R*)-β-O-4 = 48-50 % : 50-52 %

Fig. 3.11 The proportion of the stereoisomeric forms of β-O-4 structure in birch lignin estimated by ozonation analysis

3.4 Conclusions

The distribution of the absolute configuration of α- and β-asymmetric carbons of β-O-4 structure in hardwood lignin was estimated based on the *erythro*/*threo* ratio and optical activities of erythronic and threonic acids obtained by ozonation of birch wood meal. The proportion of four stereoisomeric forms of β-O-4 structures was: (α*S*,β*S*)-*threo*: (α*R*,β*S*)-*erythro*: (α*S*,β*R*)-*erythro*: (α*R*,β*R*)-*threo* = 12-13%: 36-37%: 37-38%: 13-14%. The following conclusions were obtained.

1) The proportion indicated that β-O-4 structures consist of approximately equal amounts of β-carbons with *R* and *S* configurations ((β*R*)-β-O-4 structure: (β*S*)-β-O-4 structure =50-52:48-50). This result was agreement with the generally accepted theory of lignin formation that the radical reactions are not stereochemically controlled.

2) The proportion indicated that both β-O-4 structures with *R* and *S* configurations at β-position have almost the same value of the *erythtro*/*threo* ratio (*erythro*:*threo* =ca. 37:13). Concerning about the determination of the *erythro*/*threo* ratio by water addition to quinone methide intermediate in lignin formation, it was suggested that the formation of α-asymmetric carbon is affected by the configuration of the adjacent β-asymmetric carbon.

Chapter 4

Ratio of the *Erythro* and *Threo* Forms of β-O-4 Structures and Its Controlling Factor in lignin formation

4.1 Introduction

The *erythro* form of β-O-4 structure dominates in *angiosperms* lignin (Lundquist, 1979; Hauteville et al., 1986; Tollier et al., 1986; Matsumoto et al., 1993; Saake et al., 1996), while the about equal amounts of *erythro* and *threo* forms are present in *gymnosperms* lignin (spruce lignin) (Lundquist, 1980; Matsumoto et al., 1986).

How was the ratio of the *erythro* and *threo* forms of β-O-4 structure controlled in lignin biosynthesis? In particular, what a kind of chemical structures control the diastereo-differentiating water addition to *Re* or *Si* face of the quinone methide intermediate (in Fig. 4.1)? Since the participation of any enzyme and other protein has not been reported in the process of the water addition, there has been interest in how this stereo selective formation occurs in lignin biosynthesis.

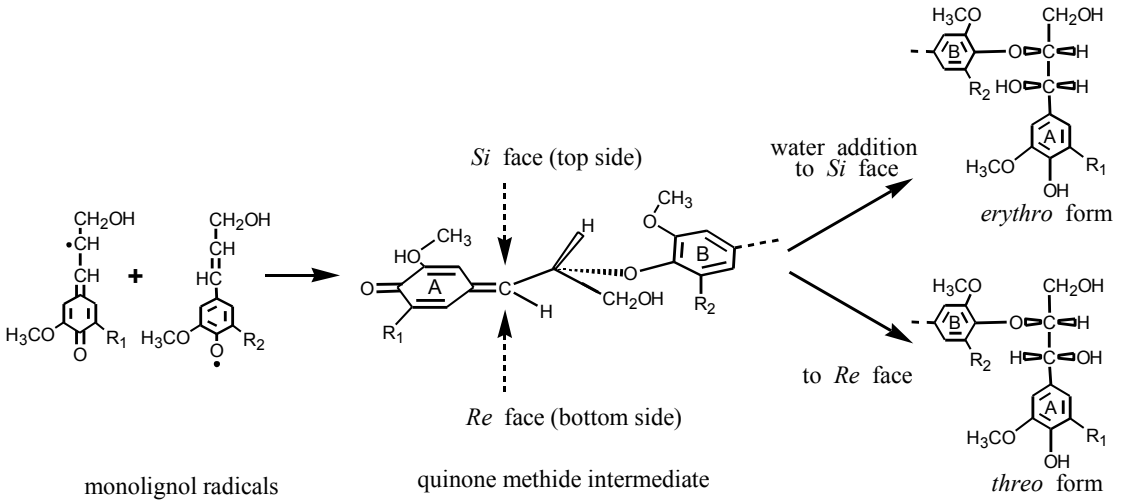


Fig. 4.1 Formation of the *erythro* and *threo* forms of β-O-4 structures by water addition to quinone methide intermediates. Only the *syn* isomer of a quinone methide intermediate (R_1=H) was illustrated here, although this structure must contain *syn* and *anti* isomers.

This process determining the absolute configuration, *R* and *S*, of α-asymmetric carbon could be affected by the mainly three types of asymmetric carbons; 1)

asymmetric carbons in carbohydrates such as cellulose and hemicellulose, 2) the adjacent β-asymmetric carbon of quinone methides, 3) α- and β-asymmetric carbons in already grown lignin molecules (Chapter 1.4.1). Based on the results of Chapter 3, both β-O-4 structures with *R* and *S* configuration at the β-asymmetric carbon have almost the same value of the *erythro*/*threo* ratio, suggesting that the β-asymmetric carbon of a quinone methide strongly affect the determination of the configuration of the adjacent α-asymmetric carbon of β-O-4 structures (Fig. 3.11).

In the model experiments designed for water addition to quinone methide intermediates, some factors affecting the *erythro*/*threo* ratio of β-O-4 structures have been shown (Nakatsubo et al. 1976; Brunow et al. 1993). Not only reaction conditions such as solvent and pH but also the type of aromatic ring, syringyl or guaiacyl, affect the *erythro*/*threo* ratio. The syringyl type of quinone methides gave higher *erythro*/*threo* ratio than the guaiacyl type, and this effect was indicated in not only the ring types of a quinone methide (A-ring) but also that of an aryroxyl group (B-ring). In this Chapter, the effect of aromatic ring type on the *erythro*/*threo* ratio was discussed as one of the factors.

Some important results about the structural study of *angiosperms* lignin suggest the effect of the type of B-ring. The ^{13}C-NMR studies of beech MWL (Nimz et al. 1984) revealed, for the first time, that the *erythro* form of β-syringyl ether structure was predominant linkage type in the four linkage types; *erythro* and *threo* forms of β-syringyl ethers, *erythro* and *threo* forms of β-guaiacyl ethers (Fig. 1.3). The proportion of the *erythro* form of β-syringyl ether was estimated to be ca. 55%, the others ca. 15% based on the ^{13}C-NMR 2D-INADEQUATE spectrum of ^{13}C-enriched aspen MWL (*Populus euramericana*) (Bardet et al., 1998). These findings indicate that the type of B-ring affects the ratio of *erythro* and *threo* forms.

In spite of these studies, the number of wood species examined has been not enough

to draw a conclusion on the relationship between the type of aromatic ring and the *erythro*/*threo* ratio of β-O-4 structures. The variation of syringyl/guaiacyl (S/G) ratio in lignin samples will be available for the investigation. An investigation about such a relationship among various lignin samples must contribute to the understanding how extent the aromatic ring type play a role as the common factor determining the *erythro*/*threo* ratio. Two experiments were designed. One is the analysis of samples taken from different parts of a single stem with different S/G ratios, another is the analysis of the different wood species.

For the former experiment, the difference of S/G ratios within a single stem is well observed in the wood with developed tension wood (Bland, 1958; Musha and Goring, 1975; Lapierre and Monties, 1986; Baba et al., 1996; Aoyama et al., 2001; Donaldson, 2001). Tension wood is produced in an upper side of a woody angiosperm leaning stem with eccentric thickening growth, and is characterized by gelatinous fiber, higher growth stress, and less lignin content than the part in the lower side which is called opposite wood. Such an experiment requires a micro scale analysis, which is one of the advantages of ozonation method (Chapter 2). The ratio of the *erythro* and *threo* forms of β-O-4 structures and methoxyl group content in lignin were investigated about wood samples taken from the stem of yellow poplar (*Liriodendron tulipifera*) with tension wood. The relationship between the type of aromatic ring and the diastereomer ratio of β-O-4 structure was discussed, and it was also discussed whether or not the distribution of the *erythro* and *threo* forms of β-O-4 structure in the stem is homogenous.

For the latter experiment, in the same way, various kinds of wood species were subjected to ozonation analysis and methoxyl group determination, and the relationship between their results was investigated.

When the quantitative relationship between *erythro* form and *threo* from of β-O-4 structure is expressed, there are two possible formulas. One is *erythro*/*threo* and

another is *erythro*/(*erythro*+*threo*). The former is often used in the literature and has been also used in our laboratory. However, if the increment of syringyl unit is simply proportional to the increment in the ratio of *erythro* form, the latter formula is more preferable because the relationships between the methoxyl content and the *erythro*/(*erythro*+*threo*) becomes linear. Based on the consideration, in order to express the quantitative relationships between *erythro* and *threo* form, the formula *erythro*/(*erythro*+*threo*) is employed in this chapter and is called "*erythro* ratio"

4.2 Experimental

4.2.1 Plant materials

Sampling

Wood blocks were prepared from the sapwood of 21 wood species (6 species of *gymnosperms* and 15 species of *angiosperms*) except for ulin (*Eusideroxylon zwageri*) and sonokeling (*Dalbergia latifolia*). Because the sapwood of ulin wood was not distinguished from the heartwood, the part being as apart from pith as possible was used. Heartwood was used for sonokeling wood. The names of wood species, and the places and years of harvest of those were listed in Table 4.1.

For yellow poplar wood with developed tension wood, the sampling of small blocks was carried out in the following manner. Fifty-one-years-old yellow poplar tree (*Liriodendron tulipifera* L.) 14 m tall and 30 cm in diameter at breast height growing in West Virginia was used (Yoshida et al., 2002; Okuyama et al., 1994). This tree showed the highest longitudinal growth stress on the upper side of the leaning stem (Okuyama et al., 1994), indicating that this side is tension wood side, though this specie does not have gelatinous fibers in the tension wood. Wood blocks consisting of 30-50th growth ring part in sapwood were taken from 13 positions along the periphery of the wood disk (Fig. 4.2).

Wood meal

The wood blocks were ground by a Wiley mill, and the wood meals obtained were extracted with ethanol/benzene (1:2, v/v) under reflux for 8 hours by a Soxhlet apparatus except for yellow poplar (*Liriodendron tulipifera*) (6 hours) and sonokeling (*Dalbergia latifolia*) (39 hours). The coloration of solvent by extraction was stopped until 3 hr except for sonokeling wood meal, for which it took ca. 30 hr. These extracted wood meals were subjected to the Klason procedure to determine lignin contents, and Klason lignins obtained were subjected to methoxyl group determination.

The more fine wood meal was prepared from the extracted wood meal using a vibratory ball mill (Retsch type MM200) for 10-15 min with vibration of 30 s^{-1} and subjected to ozonation analysis and methoxyl group determination.

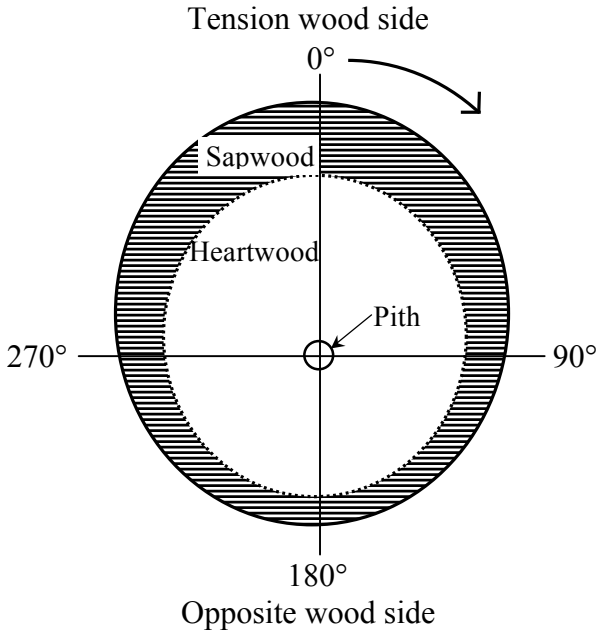


Fig. 4.2 The sampling zones in the cross section of the leaning stem of yellow poplar (*Liriodendron tulipifera*). Small wood blocks consisting of 30-50th growth ring part in sapwood were collected along the periphery of the stem.

Table 4.1 Wood species (6 species of *gymnosperms* and 15 species of *angiosperms*) used in this chapter

Category		Sample No.	Wood species *Latin name* English (e), Japanese (j), and Indonesian name (i)	Year	Place
Gymnosperms		1	*Ginkgo biloba* Maidenhair Tree (e), Ichou (j)	2001	Tokyo, Japan
	Softwood	2	*Cryptomeria japonica* Japanese Cedar (e), Sugi (j)	2000	Chiba, Japan
		3	*Abies sachalinensis* Sachalin Fir (e), Todomatsu (j)		Japan
		4	*Pinus densiflora* Japanese Red Pine (e), Akamatsu (j)	2002	Ibaraki, Japan
		5	*Agathis loranthifolia* Agathis (i)		Indonesia
		6	*Cedrus deodara* Deodara Cedar (e), Himarayasugi (j)	2001	Tokyo, Japan
Angiosperms	Hardwood	7	*Eusideroxylon zwageri* Ulin (i)	2001	Indonesia
		8	*Plumeria alba* White Frangipani (e), Kamboja (i)	2002	Bogor, Indonesia
		9	*Celtis sinensis* var. japonica Japanese Hackberry (e), Enoki (j)	2001	Tokyo, Japan
		10	*Dalbergia latifolia* Blackwood (e), Sonokeling (i)	1999	Indonesia
		11	*Acacia mangium* Acacia mangium (i)		Indonesia
		12	*Firmiana simplex* Chinese Parasol Tree (e), Aogiri (j)	2001	Tokyo, Japan
		13	*Melaleuca cajuputi* Melaleuca (i)	1996	Thailand
		14	*Hevea brasiliensis* Para Rubber Tree (e), Gomunoki (j)		Thailand
		15	*Mimusops elengi* Tanjung (i)	2002	Indonesia
		16	*Rhizophora sp.* Rhizophora (i)	2002	Tangerang, Indonesia
		17	*Fagus crenata* Japanese Beech (e), Buna (j)	2000	Chichibu, Japan
		18	*Betula maximowicziana* Monarch Birch (e), Udaikanba (j)	1998	Chiba, Japan
		19	*Eucalyptus florencia* Eucalyptus florencia (i)		Indonesia
		20	*Liriodendron tulipifera* Yellow Poplar (e), Hantenboku (j)		West Virginia, US
		21	*Avicennia sp.* Avicennia (i)	2002	Tangerang, Indonesia

4.2.2 Chemicals

The ammonium (NH_4^+) form of cation exchange resin was prepared from H^+ form of the same resin (Dowex-50W-X4, H^+ form). All other chemicals were of reagent grade.

4.2.3. Lignin contents

Lignin content was determined as the total amount of the insoluble part (Klason lignin) and the soluble part (acid soluble lignin) obtained by H_2SO_4 treatment of wood meal (Dence, 1992). The lignin content of the soluble part was determined based on the UV absorbance at 205nm using an absorption coefficient, 113 $Lg^{-1}cm^{-1}$.

4.2.4 Ozonation

The ratio of the *erythro* and *threo* forms of β-O-4 structures was determined by ozonation method according to the method of Chapter 2.2.2. except for sample weight (50 mg of wood meal), the quantity of an internal standard (10 μmol of erythritol) and the reaction time of ozonation (120 min).

4.2.5 Methoxyl group contents

The methoxyl group content was determined according to the method of Baker (1996). The procedure modified by Goto et al. (2001) was used as follows; Hydroiodic acid (57% w/w, 10ml) was sealed in a vial with Klason lignin (30 mg) or wood meal (60 mg). The vial was placed in a 130 °C oil bath for 20 min with shaking and then cooled in an ice bath. Ethyl iodide (0.1 mmol) in tetrachloromethane (CCl_4) was added as an internal standard through the septum, followed by the addition of CCl_4 (15 ml) with cooling. The vial was shaken vigorously and an aliquot of CCl_4 phase was separated into a 2 ml vial with Na_2SO_4. The yield of methyl iodide was determined by GC analysis using a Shimadzu 14B gas chromatograph, equipped with a FID and a fused-silica capillary column (Varian CP-SIL 13CB for halocarbons 0.32 mm

i.d. ×25 m). The analysis condition was as follows; Oven temp. program, 40°C, 5min —10 °C/min→180 °C; Injection temp. 200°C; Detector temp. 230°C; Column flow-rate of He gas 1.1 ml/min; Splitting ratio 55:1; Injection volume 1 μl.

4.3 Results and discussion

4.3.1 Ratio of the *erythro* and *threo* forms of β-O-4 structures in tension wood lignin.

Lignin and methoxyl group contents in tension wood

Wood meals prepared from different positions along the periphery of the wood disk (Fig. 4.2) were subjected to lignin and methoxyl group content determination. Lignin and methoxyl group contents were plotted against their peripheral position in Fig. 4.3a-b, to confirm their heterogeneity in the stem. The 0 (=360) and 180 peripheral degrees represent the tension and opposite wood sides, respectively. The stem employed did not have gelatinous fiber which is usually found in a tension wood as a typical structural characteristic, but the highest longitudinal growth stress has been confirmed in the upper side of the leaning stem (Okuyama et al., 1994). The lignin contents of tension wood were lower than those of opposite wood (Fig. 4.3a), which are in line with the characteristics of tension wood (Bland, 1958; Timell, 1969; Donaldson, 2001).

As shown in Fig. 4.3b, contrary to the distribution of lignin content, the methoxyl group contents in Klason lignin clearly decreased in the direction of tension to opposite wood. The decrease in the methoxyl group content must result from the decrease in the syringyl/guaiacyl ratio. It has been recognized that the tension wood is characterized by the lower lignin content and the higher syringyl/guaiacyl ratio than the opposite wood (Bland, 1958; Bland and Scurfield, 1964; Musha and Goring, 1975; Aoyama et al., 2001). Some studies reported the reverse trend or the absence of significant difference in those structural characteristics between the tension and opposite woods (Lapierre and Monties, 1986; Baba et al., 1996).

In the present work, methoxyl group contents in lignins were determined for

investigating the variation in aromatic ring types, namely syringyl/guaiacyl ratio, of lignins, even though some other degradation methods such as nitrobenzene oxidation (S/V ratio), thioacidolysis (S/G ratio) and permanganate oxidation was available. The value of methoxyl group content in lignin can include not only the methoxyl group

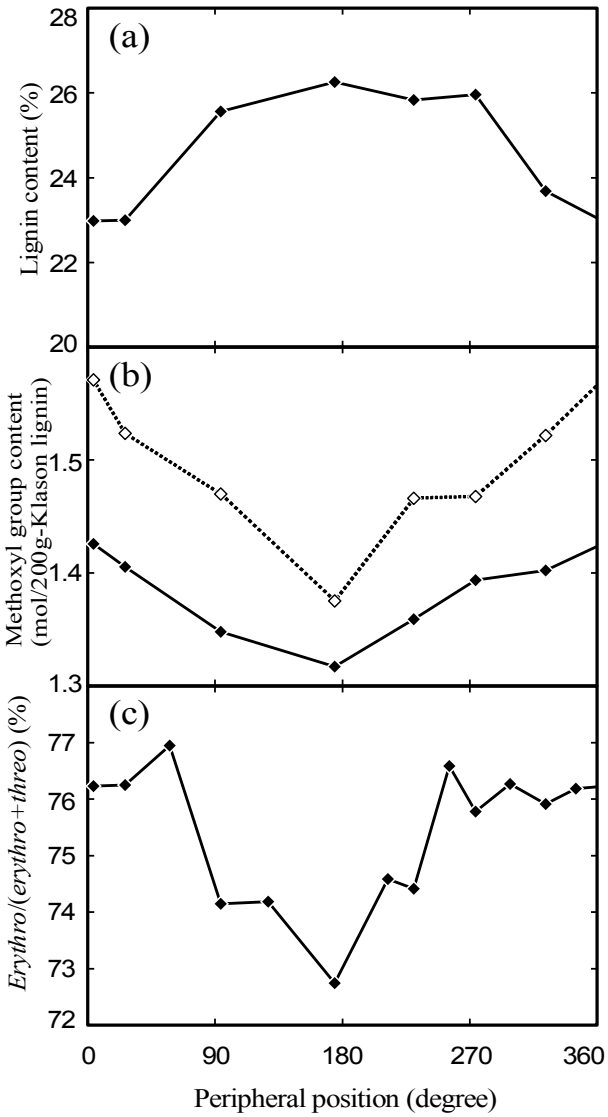


Fig. 4.3 Distributions of (a) lignin content, (b) methoxyl group content, and (c) *erythro* ratio of β-O-4 structures along the periphery of the leaning stem of yellow poplar (*Liriodendron tulipifera*), 0º (= 360º) for tension wood side, 180º for opposite wood side. The periphery degree in this figure corresponds to that in Fig. 4.2.
*(b): solid line: methoxyl group content determined for wood meal and expressed as content per lignin (Klason lignin + acid soluble lignin), dotted line: methoxyl group content determined for Klason lignin. (c): The *erythro* ratios were determined by ozonation for 120 min.

of non-condensed aromatic ring such a B-ring in Fig.4.4, but also that of condensed aromatic ring of β-O-4 unit (A-ring in Fig. 4.4) with other unit. Because the type of such an A-ring was indicated to affect the *erythro* ratio in the model experiments (Nakatsubo et al., 1976; Brunow et al., 1993), methoxyl group determination will be suitable for investigating the type of aromatic ring as the factor affecting the *erythro* ratio of β-O-4 structure.

However, the presence of methyl iodide precursors in cell wall components other than lignin is known to produce some error for this value when wood meal is analyzed (Goto et al. 2001). When wood meal is subjected to the determination, there is a possibility to overestimate the methoxyl content because of the presence of methoxyl group and some unknown structures in carbohydrates. On the other hand, the determination of the methoxyl group of Klason lignin minimizes such interference from carbohydrates but may underestimate the content because of the possible loss of methoxyl group from lignin during the Klason treatment. Because of this reason, not only wood meals but also their Klason lignins were subjected to the determination of methoxyl group, and the methoxyl content in lignin was expressed in two ways as are shown in Fig. 4.3b.

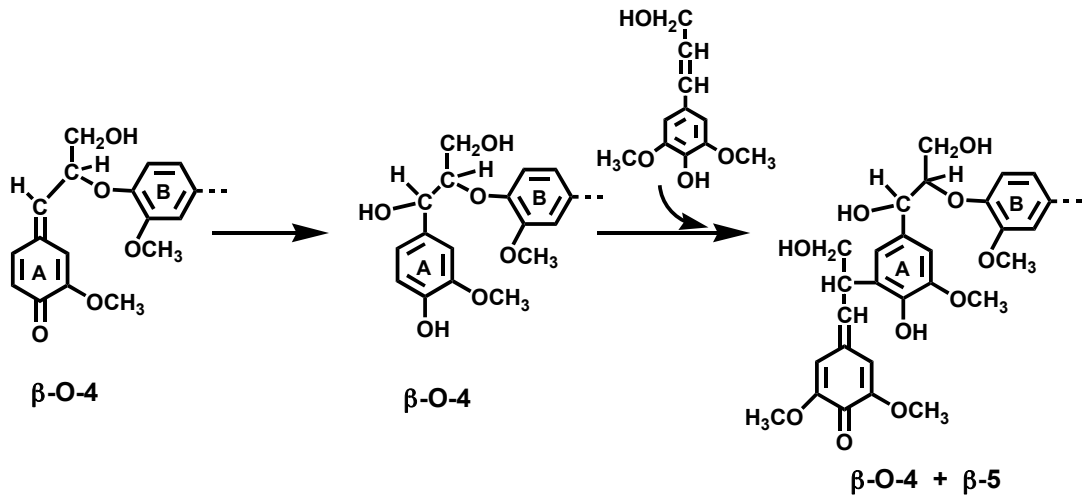


Fig. 4.4 Formation of β-O-4 structure condensed with other lignin unit.

Ratio of the erythro and threo forms of β-O-4 structures in tension wood lignin

The *erythro* ratio of β-O-4 structures was determined on the basis of yields of erythronic and threonic acids obtained by ozonation. The reaction time of 2 h was used for the determination of the *erythro* ratio because, as shown in Table 4.2, this ratio became almost constant when ozonation time exceeded 1h.

Wood meals from 13 positions along the periphery of the wood disk were subjected to ozonation analysis. As shown in Fig. 4.3c, the *erythro* ratio was as high as 76% (*erythro*:*threo*, 76:24) in the tension wood at around 0 peripheral degree. This excess of *erythro* form of β-O-4 structure (more than 50% diastereomeric excess) indicated that the significant stereoselective water addition to the quinone methide intermediates based on the widely accepted hypothesis of lignin formation. Similarly to the distribution of methoxyl group content, the *erythro* ratio in this stem showed a clear tend to decrease in the direction of tension to opposite wood, and the lowest value 73% (*erythro*:*threo*, 73:27) was obtained at 180 peripheral degree. From this result, it can be concluded that the ratio of *erythro* and *threo* forms of β-O-4 structure is variable within a single stem with developed tension wood, although the change of the *erythro* ratio as well as methoxyl content observed was not large.

The content of β-O-4 structures in lignin was evaluated as the total yield of erythronic and threonic acids in the products of 2 hr ozonation of wood meal. The total yield of these acids was 0.344 mmol per 1 gram wood meal at 0 peripheral degree (Fig. 4.5), this yield was 0.30 per one phenylpropane unit on the assumption that Mw of 1 phenylpropane unit is 200. The yields of these acids ranged from 0.26 to 0.33 per one phenylpropane unit in the different the peripheral positions, but a trend was not observed for the yields along the peripheral positions in these experiments.

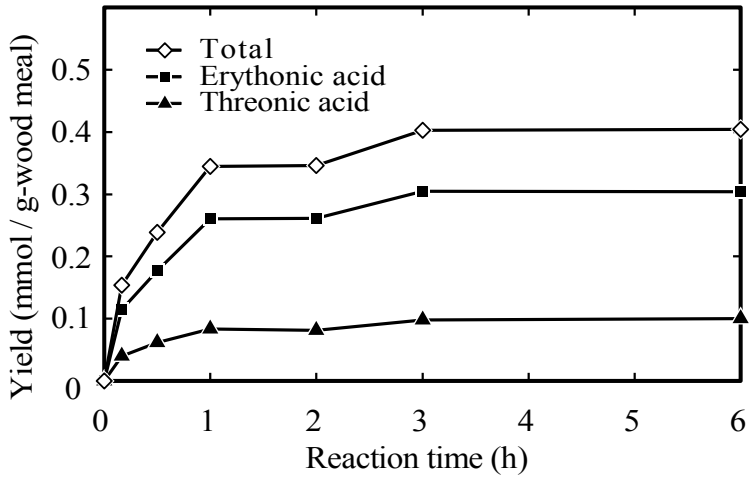


Fig. 4.5 Yields of erythronic and threonic acids obtained by ozonation of yellow poplar wood meal at 0 peripheral degree in tension wood.

Table 4.2 *Erythro*/*threo* ratio of yellow poplar at 0 peripheral degree determined by ozonation of different reaction time

ozoantion (min)	*erythro*: *threo*	*erythro*/*threo* ratio
10	74:26	2.89
30	74:26	2.86
60	76:24	3.12
120	76:24	3.20
180	76:24	3.11
360	75:25	3.03

Relationship between the erythro ratio and the methoxyl group content

In Fig. 4.6, the *erythro* ratios (*erythro*/(*erythro*+*threo*)) at different peripheral positions were plotted against the methoxyl group contents in wood meals or in their Klason lignins. The *erythro* ratio was increased with an increase of the methoxyl group content of Klason lignin (correlation coefficient, R =0.98), indicating that the lignin with a higher syringyl ratio (syringyl/(syringyl+guaiacyl)) has a higher *erythro* ratio of β-O-4 structure. The same trend with relatively high correlation coefficient (R =0.89) was obtained when wood meal was employed for the determination of the methoxyl group content in lignin (Fig. 4.6). Based on these relationships, it was

suggested that the type of aromatic ring, syringyl or guaiacyl, is one of the factors affecting the ratio of the *erythro* and *threo* forms of β-O-4 structures.

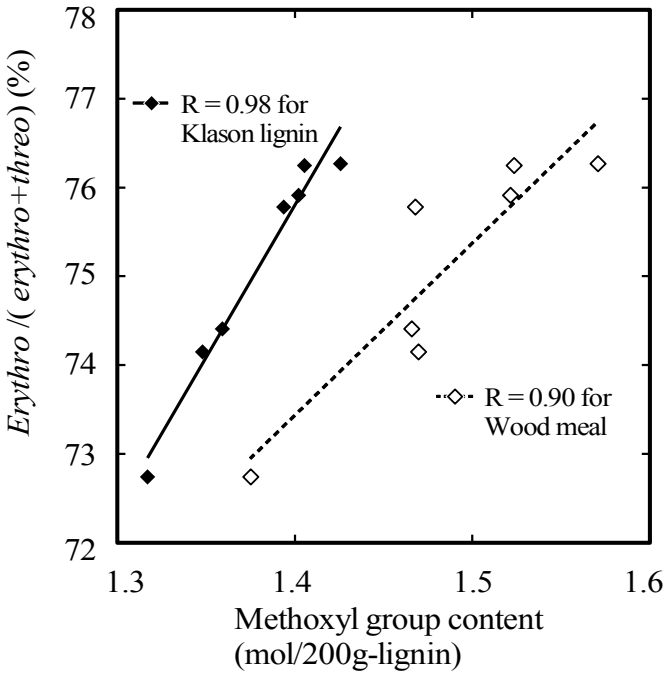


Fig. 4.6 Relationships between the *erythro* ratio of β-O-4 structures and methoxyl group contents within a single stem of yellow poplar

4.3.2 Ratios of the *erythro* and *threo* forms of β-O-4 structures in various wood species.

Lignin contents in various wood species

Wood meals of different wood species were subjected to the determination of lignin. Lignin contents were expressed as the total amount of the insoluble part (Klason lignin) and the soluble part (Table 4.3).

The lignin contents of wood species obtained in Indonesia (sample No. 7, 8, 10, 11, 13, 15 and 19 in Table 4.3) were over 30%, which were slightly higher than the usual lignin content of *angiosperms*. Especially, high lignin content was obtained for Ulin (*Eusideroxylon zwageri*; sample No.7 in Table 4.3) by Klason method. As to Ulin wood meal, there might be the participation of other substance which is not lignin and cannot be removed by EtOH-Benzene extraction. Such an influence in some tropical species has been well known (Kawamura et al. 1967; Wu et al. 1990,1992). However, the origin causing such high lignin content was not well known in detail. Further extraction such as pre-alkaline extraction of wood meal, or post-acetone extraction of Klason lignin would remove a part of lignin. Therefore, the lignin contents obtained were not corrected by any value determined by the further extraction etc.

Methoxyl group contents in various wood species

Variation in the aromatic ring types, namely syringyl/guaiacyl ratio, of lignins in different wood species were investigated. Not only wood meals but also their Klason lignins were subjected to the determination of methoxyl group because of the reason described in previous section 4.3.1, and the methoxyl group content in lignin was expressed in two ways as shown in Table 4.3. In spite of certain differences between these two ways, it was generally confirmed that the methoxyl group contents were almost the same in all *gymnosperms* lignins examined here, which were approximately

1 per phenylpropane unit when calculated based on the assumption that Mw of the unit is 200 (Table 1). In contrast, the methoxyl group contents in *angiosperm* lignin varied widely. The methoxyl group contents in Klason lignins were plotted against the lignin contents of the corresponding wood species (Fig. 4.7). The methoxyl group contents were higher when lignin contents were lower, indicating that *angiosperms* wood species with lower lignin content have higher syringyl/guaiacyl ratio. This trend was in agreement with the previous study (Fujii et al. 1987), in which the lignin contents of hardwood species were related to both S/V ratios determined by nitrobenzene oxidation and methoxyl group contents.

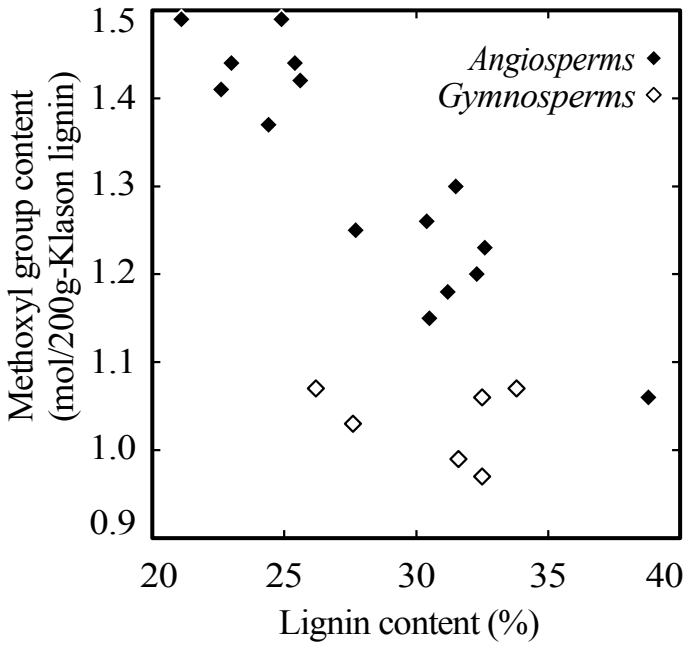


Fig. 4.7 Relationship between methoxyl group and lignin contents among different wood species

Yields of erythronic and threonic acids obtained by ozonation

The content of β-O-4 structures in lignin was evaluated on the basis of the total yield of erythronic and threonic acids obtained from wood meal by ozonation analysis for 2 hr. Total yields of these two acids from 21 species ranged from 0.22 to 0.37 per phenylpropane unit when calculated based on the assumption that Mw of one phenylpropane unit is 200 (Table 4.3). These values suggest that erythronic and threonic acids were obtained in good yields from β-O-4 structures, because the content of β-O-4 structures in lignin was determined to be 0.3-0.4 per phenylpropane unit for spruce MWL and 0.4-0.5 for birch MWL by ^{1}H-NMR (Lundquist 1991).

As shown in Fig. 4.8, the total yields of erythronic and threonic acids increased with an increase of the methoxyl group contents, though the trend was not so clear (correlation coefficient, R =0.77 only for *angiosperms*, and R =0.75 for all species). This trend implied that a lignin with higher S/G ratio is rich in β-O-4 structures, as has been suggested (Lundquist 1991).

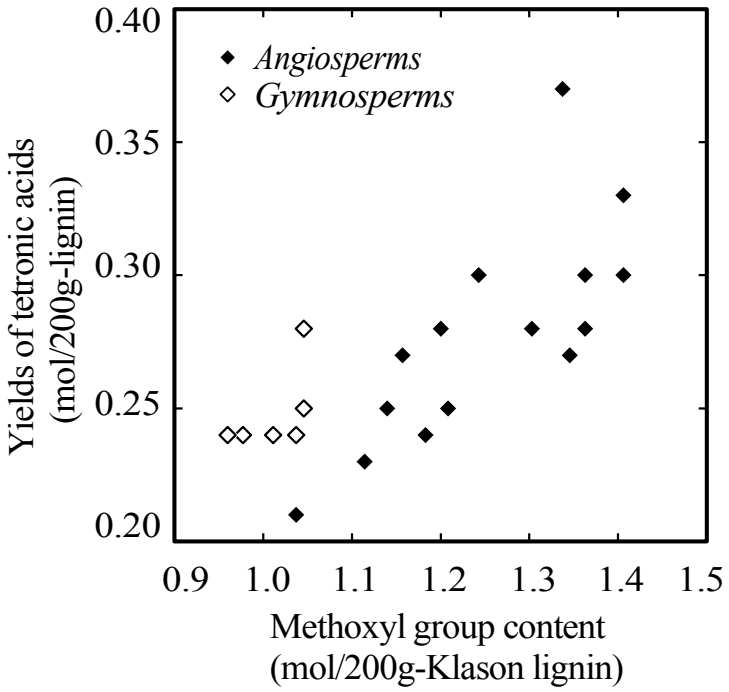


Fig. 4.8 Relationships between methoxyl group contents and the total yields of erythronic and threonic acids among different wood species

Relationship between the erythro ratio and the methoxyl group content

By ozonation, almost the same ratio of erythronic and threonic acids (*erythro*:*threo* 49-51:51-49) was obtained from all *gymnosperms* examined here (Table 4.3). In contrast, the *erythro* ratio (*erythro*/(*erythro*+*threo*)) in *angiosperms* ranged widely from 54% to 77%. Predominance of the *erythro* form over the *threo* form of β-O-4 structure was confirmed in *angiosperms*, while β-O-4 structures in all 6 species of *gymnosperms* consisted of the equal amounts of *erythro* and *threo* forms.

The *erythro* ratio was higher when the methoxyl group content of Klason lignin was higher (Fig. 4.9a). A clear trend was obtained (Correlation coefficient, R =0.80 for only *angiosperms*, and R =0.91 for all species), indicating that the lignin with higher syringyl ratio (syringyl/(syringyl+guaiacyl)) has a higher *erythro* ratio of β-O-4 structure. The same trend was shown when the methoxyl group content of wood meal was used to calculate the methoxyl group content in lignin (Fig. 4.9b).

The relationship between the syringyl ratio and *erythro* ratio within one sample has been shown by ^{13}C-NMR studies (Nimz et al. 1984; Bardet et al. 1986; Bardet et al. 1998) as described in introduction. In the present work, the relationship was confirmed beyond the difference of species, suggesting that the aromatic ring type is one of the common factors affecting the ratio of *erythro* and *threo* forms of β-O-4 structures in lignin formation.

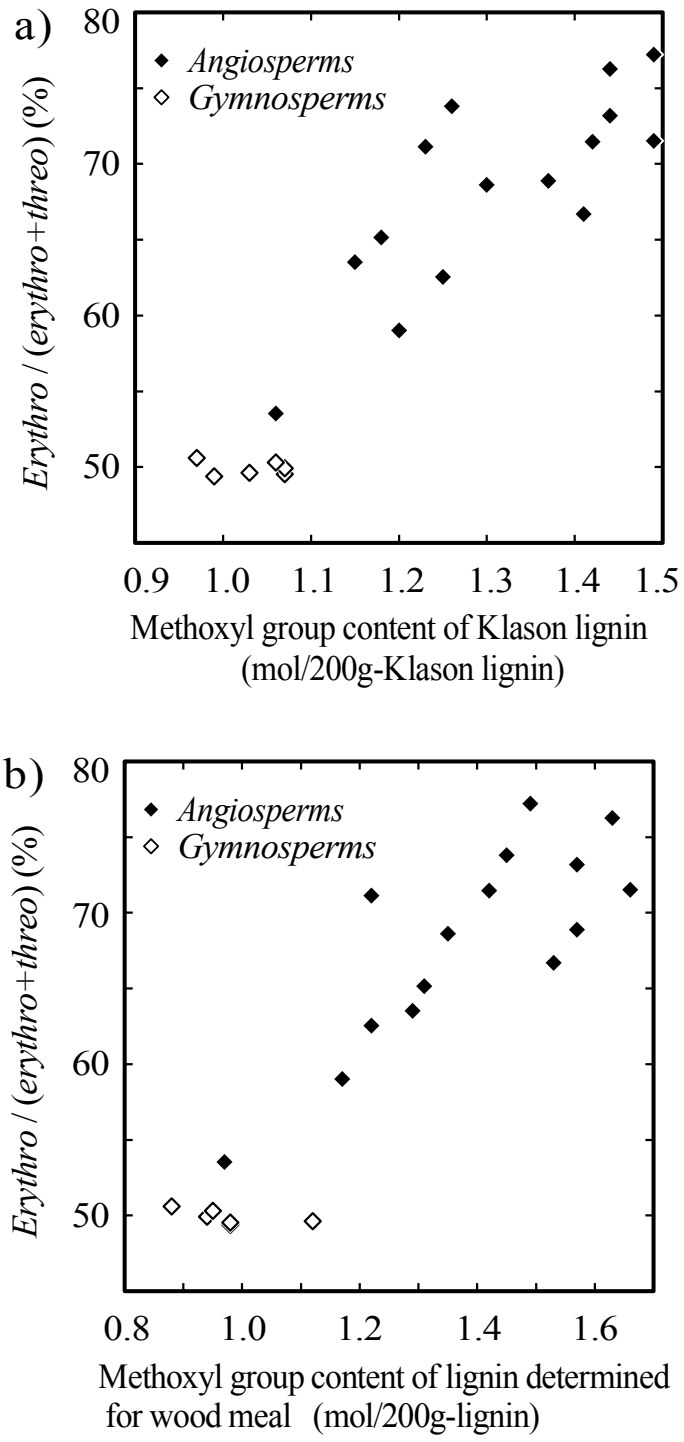


Fig. 4.9 Relationship between the *erythro* ratio of β-O-4 structures and methoxyl group content among different wood species: The *erythro* ratios were plotted against (a) methoxyl group contents of Klason lignin, and (b) methoxyl group contents of lignin determined for wood meal.

Table 4.3. Lignin contents, methoxyl group contents, and the ratios of *erythro* and *threo* forms of β-O-4 structures in various wood species

	Sample No.	Wood species*	Lignin content (wt%)**	Methoxyl group content		Ozonation	
				Klason lignin (mol/200g)	Wood meal (mol/200g-lignin)	*erythro*: *threo*	Yields (%) ***
Gymnosperms	1	*Ginkgo biloba*	31.6	0.99	0.98	49:51	23.6
	2	*Cryptomeria japonica*	33.8	1.07	0.98	50:50	27.6
	3	*Abies sachalinensis*	27.6	1.03	1.12	50:50	24.2
	4	*Pinus densiflora*	26.2	1.07	0.94	50:50	24.8
	5	*Agathis loranthifolia*	32.5	1.06	0.95	50:50	23.9
	6	*Cedrus deodara*	32.5	0.97	0.88	51:49	23.9
Angiosperms	7	*Eusideroxylon zwageri*	38.8	1.06	0.97	54:46	21.5
	8	*Plumeria alba*	32.3	1.20	1.17	59:41	27.0
	9	*Celtis sinensis (japonica)*	27.7	1.25	1.22	63:47	27.9
	10	*Dalbergia latifolia*	30.5	1.15	1.29	64:46	22.6
	11	*Acacia mangium*	31.2	1.18	1.31	65:45	24.8
	12	*Firmiana simplex*	22.6	1.41	1.53	67:33	37.0
	13	*Melaleuca cajuputi*	31.5	1.30	1.35	69:31	30.1
	14	*Hevea brasiliensis*	24.4	1.37	1.57	69:31	27.8
	15	*Mimusops elengi*	32.6	1.23	1.22	71:29	23.6
	16	*Rhizophora sp.*	25.6	1.42	1.42	71:29	27.2
	17	*Fagus crenata*	24.9	1.49	1.66	72:28	33.2
	18	*Betula maximowicziana*	25.4	1.44	1.57	73:27	28.2
	19	*Eucalyptus florencia*	30.4	1.26	1.45	74:26	24.8
	20	*Liriodendron tulipifera*	23.0	1.44	1.63	76:24	30.1
	21	*Avicennia sp.*	21.1	1.49	1.49	77:23	30.2

*Wood species were listed in the order of increasing *erythro* ratio (*erythro*/(*erythro*+*threo*)), and the sample numbers correspond to those it in Table 4.1.

** Lignin contents were expressed as the total amount of the insoluble part (Klason lignin) and the soluble part.

***Total yield of erythronic and threonic acids per one phenylpropane unit was calculated based on the assumption that Mw of 1 phenylpropane unit is 200.

4.4 Conclusions

In this chapter, effect of the methoxyl group substituents of aromatic nuclei in lignin on the ratio of *erythro* and *threo* forms of β-O-4 structures was evaluated by the ozonation analysis of samples with different syringyl/guaiacyl ratios. Two experiments were designed, and their results were obtained as follows;

1. The *erythro* ratio (*erythro*/(*erythro*+*threo*)) of β-O-4 structures was not homogeneous within a single stem of yellow poplar (*Liriodendron tulipifera*) with the development of the tension wood. The *erythro* ratio was higher in the tension wood than in the opposite wood, and the methoxyl group content showed a similar trend. They were significantly correlated (correlation coefficient R =0.98).
2. The *erythro* ratios of β-O-4 structures were investigated about over 20 wood species including both *gymnosperms* and *angiosperms*. The *erythro* ratio was about 50% in all species of *gymnosperms* examined. In contrast, the predominance of the *erythro* form was shown in all *angiosperms*, the *erythro* ratios widely ranged from 54% (*erythro*:*threo* 54:46) to 77% (*erythro*:*threo* 77:23). The *erythro* ratio was higher when the methoxyl group content was higher (correlation coefficient, R=0.91).

The relationships between the type of aromatic ring and the *erythro* ratio within a single stem with tension wood and beyond the difference of species were confirmed. As judged from this result and previous works, the ratio of syringyl and guaiacyl unit affects the ratio of the *erythro* and *threo* forms as one of the common factors during lignin formation. According to the theory for the formation of β-O-4 structures, this finding means that the presence of one additional methoxyl group in syringyl unit would play a role for producing the selectivity of the diastereo-differentiating water addition to *Si* and *Re* face of the quinone methide intermediates so that the *erythro* form is more preferably produced.

Chapter 5

Summary

Arylglycerol-β-aryl ether structures (β-O-4 structures) are predominant (linkage, structure) type in lignin, and the three carbons side chain includes two asymmetric carbons at α- and β- positions (αC and βC). According to the widely accepted hypothesis for the formation of lignin, the absolute configurations of the carbons are determined by the following two steps: (1) the formation of the β-asymmetric carbon by the coupling reaction of phenoxy radicals to give a quinone methide intermediate, (2) the formation of the α-asymmetric carbon by the addition of water to the *Si* or *Re* face of the quinone methide leading to the *erythro* or *threo* form of β-O-4 structures. In this thesis, the stereochemical characteristics of β-O-4 structures in lignin were investigated by ozonation analysis, and based on the results the process of the formation of α- and β-asymmetric carbons was discussed. In Chapter 2, the ozonation method as the analytical tool of β-O-4 structures was improved. In Chapter 3, the proportion of the four stereoisomeric forms of β-O-4 structures containing the enantiomeric forms was estimated to investigate if the radical coupling reactions in lignin (the 1st step) are stereochemically controlled, and if the absolute configurations of α- and β- carbons are independent each other (the 2nd step). In Chapter 4, the relationship between the *erythro* form of β-O-4 structure and syringyl unit was investigated, and the factors affecting the ratio of *erythro* and *threo* forms of β-O-4 structures in lignin formation (the 2nd step) were discussed.

Improvement of Ozonation Method as Analytical Tool for β-O-4 Structure (Chapter 2)

Ozonation method is a powerful tool for the analysis of the stereo structures of β-O-4 structure in lignin. The reproducibility of the yields of erythronic and threonic acids and the quantitativeness of the scheme were investigated with a lignin model compound, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (VG), milled wood lignin (MWL) and wood meals. In particular, the post-treatment procedures after ozone treatment were examined in detail, and two modifications were proposed. One is the reductive post-treatment of ozonation products with sodium thiosulfate ($Na_2S_2O_3$). Another is the trimethylsilylation of ammonium salts without forming lactones for gas

chromatography (GC) (Tsutsumi et al., 1990; Hyppänen et al. 1983)

When a mild post-reduction with $Na_2S_2O_3$ was conducted for ozonation products, the total yield of erythronic and threonic acids from this model compound was 74%, which is 15% higher than the yield without post-reduction. Decrease in the recovery of these two acids under prolonged ozonation treatment was successfully suppressed by the post-reduction. The *erythro/threo* ratio of VG determined by ozonation method with post-reduction was in agreement with the ratio determined by ^{1}H-NMR. The excellent reproducibility of the yield was obtained by the adoption of trimethylsilylation of ozonation products in ammonia salts form by DMSO-HMDS-TMCS mixture when subjecting to gas chromatography analysis. The modified ozonation method was applied to wood meal, and it was indicated that β-O-4 structures comprise at least 29.1% per phenylpropane unit in birch lignin, and the *erythro* form predominates in β-O-4 structures (*erythro*:*threo*, 74:26).

Absolute Configuration of α- and β-Asymmetric Carbons of β-O-4 Structures: Evidence for the presence as the racemic forms (Chapter 3)

The proportion of four stereoisomeric forms of β-O-4 structure in hardwood lignin was estimated based on the *erythro*/*threo* ratio and the optical activities of erythronic and threonic acids obtained by ozonation of wood meal. The following conclusions were obtained.

1) The enantiomeric excesses (e.e.) of erythronic and threonic acids obtained from ozonation products of birch wood meal were less than 3.0 and 8.0 %, respectively. This suggested that both *eythro* and *threo* forms of β-O-4 structure contain the approximately equal amounts of β-asymmetric carbons with *R* and *S* configurations. This result was agreement with the generally accepted theory that the radical reactions involved in lignin formation are not stereochemically controlled.

2) The proportion of four stereoisomers of β-O-4 structures estimated was: Cα*S*-Cβ*R*(*erythro*): Cα*R*-Cβ*R*(*threo*): Cα*S*-Cβ*S*(*threo*): Cα*R*-Cβ*S* (*erythro*) =37-38%:

13-14%: 12-13%: 36-37%. This proportion means that the values of the *erythro*/*threo* ratios were almost same in both β-O-4 structures with *R* and *S* configuration at the β-carbon. It was indicated that the formation of α-asymmetric carbon was affected by the configuration of the adjacent β-asymmetric carbon in lignin biosynthesis.

Ratio of *Erythro* and *Threo* Forms of β-O-4 Structures and Its Controlling Factor in lignin formation (Chapter 4)

The ratio of *erythro* and *threo* forms of β-O-4 structures in lignin was investigated about various samples with different syringyl/guaiacyl ratio. Based on the results of ozonation and methoxyl group determination, the effect of the methoxyl group substituents of aromatic nuclei on the ratio of *erythro* and *threo* forms in lignin formation was discussed.

The distribution of the *erythro* and *threo* forms of β-O-4 structures in the stem of Yellow poplar (*Liriodendron tulipifera*) with developed tension wood was investigated. The ratio of *erythro* and *threo* forms was variable within a single stem, and the *erythro* ratio was higher in the tension wood than in the opposite wood. The methoxyl group content showed a similar distribution, and they were significantly correlated (correlation coefficient R =0.98), suggesting that the type of aromatic ring, syringyl or guaiacyl, is one of the factors which affecting the ratio of *erythro* and *threo* forms of β-O-4 structures.

The *erythro* ratios of β-O-4 structures were investigated about 21 wood species including both *gymnosperms* and *angiosperms*. The *erythro* ratio of all 6 species of *gymnosperms* were about 50%, while the ratio of *angiosperms* widely ranged from 54% (*erythro*:*threo* 54:46) to 77% (*erythro*:*threo* 77:23). The *erythro* ratio was higher when the methoxyl group was higher. It was confirmed that the distribution of *erythro* and *threo* forms of β-O-4 structures are related to that of aromatic ring types beyond the difference of species. It was suggested that the type of aromatic ring is one of the common factors affecting the ratio of the *erythro* and *threo* forms of β-O-4 structures in lignin.

Chapter 5

Summary

Arylglycerol-β-aryl ether structures (β-O-4 structures) are predominant (linkage, structure) type in lignin, and the three carbons side chain includes two asymmetric carbons at α- and β- positions (αC and βC). According to the widely accepted hypothesis for the formation of lignin, the absolute configurations of the carbons are determined by the following two steps: (1) the formation of the β-asymmetric carbon by the coupling reaction of phenoxy radicals to give a quinone methide intermediate, (2) the formation of the α-asymmetric carbon by the addition of water to the *Si* or *Re* face of the quinone methide leading to the *erythro* or *threo* form of β-O-4 structures. In this thesis, the stereochemical characteristics of β-O-4 structures in lignin were investigated by ozonation analysis, and based on the results the process of the formation of α- and β-asymmetric carbons was discussed. In Chapter 2, the ozonation method as the analytical tool of β-O-4 structures was improved. In Chapter 3, the proportion of the four stereoisomeric forms of β-O-4 structures containing the enantiomeric forms was estimated to investigate if the radical coupling reactions in lignin (the 1st step) are stereochemically controlled, and if the absolute configurations of α- and β- carbons are independent each other (the 2nd step). In Chapter 4, the relationship between the *erythro* form of β-O-4 structure and syringyl unit was investigated, and the factors affecting the ratio of *erythro* and *threo* forms of β-O-4 structures in lignin formation (the 2nd step) were discussed.

Improvement of Ozonation Method as Analytical Tool for β-O-4 Structure (Chapter 2)

Ozonation method is a powerful tool for the analysis of the stereo structures of β-O-4 structure in lignin. The reproducibility of the yields of erythronic and threonic acids and the quantitativeness of the scheme were investigated with a lignin model compound, 1-(3,4-dimethoxyphenyl)-2-(2-methoxyphenoxy)-1,3-propanediol (VG), milled wood lignin (MWL) and wood meals. In particular, the post-treatment procedures after ozone treatment were examined in detail, and two modifications were proposed. One is the reductive post-treatment of ozonation products with sodium thiosulfate ($Na_2S_2O_3$). Another is the trimethylsilylation of ammonium salts without forming lactones for gas

chromatography (GC) (Tsutsumi et al., 1990; Hyppänen et al. 1983)

When a mild post-reduction with $Na_2S_2O_3$ was conducted for ozonation products, the total yield of erythronic and threonic acids from this model compound was 74%, which is 15% higher than the yield without post-reduction. Decrease in the recovery of these two acids under prolonged ozonation treatment was successfully suppressed by the post-reduction. The *erythro/threo* ratio of VG determined by ozonation method with post-reduction was in agreement with the ratio determined by ^{1}H-NMR. The excellent reproducibility of the yield was obtained by the adoption of trimethylsilylation of ozonation products in ammonia salts form by DMSO-HMDS-TMCS mixture when subjecting to gas chromatography analysis. The modified ozonation method was applied to wood meal, and it was indicated that β-O-4 structures comprise at least 29.1% per phenylpropane unit in birch lignin, and the *erythro* form predominates in β-O-4 structures (*erythro*:*threo*, 74:26).

Absolute Configuration of α- and β-Asymmetric Carbons of β-O-4 Structures: Evidence for the presence as the racemic forms (Chapter 3)

The proportion of four stereoisomeric forms of β-O-4 structure in hardwood lignin was estimated based on the *erythro*/*threo* ratio and the optical activities of erythronic and threonic acids obtained by ozonation of wood meal. The following conclusions were obtained.

1) The enantiomeric excesses (e.e.) of erythronic and threonic acids obtained from ozonation products of birch wood meal were less than 3.0 and 8.0 %, respectively. This suggested that both *eythro* and *threo* forms of β-O-4 structure contain the approximately equal amounts of β-asymmetric carbons with *R* and *S* configurations. This result was agreement with the generally accepted theory that the radical reactions involved in lignin formation are not stereochemically controlled.

2) The proportion of four stereoisomers of β-O-4 structures estimated was: Cα*S*-Cβ*R*(*erythro*): Cα*R*-Cβ*R*(*threo*): Cα*S*-Cβ*S*(*threo*): Cα*R*-Cβ*S* (*erythro*) =37-38%:

13-14%: 12-13%: 36-37%. This proportion means that the values of the *erythro*/*threo* ratios were almost same in both β-O-4 structures with *R* and *S* configuration at the β-carbon. It was indicated that the formation of α-asymmetric carbon was affected by the configuration of the adjacent β-asymmetric carbon in lignin biosynthesis.

Ratio of *Erythro* and *Threo* Forms of β-O-4 Structures and Its Controlling Factor in lignin formation (Chapter 4)

The ratio of *erythro* and *threo* forms of β-O-4 structures in lignin was investigated about various samples with different syringyl/guaiacyl ratio. Based on the results of ozonation and methoxyl group determination, the effect of the methoxyl group substituents of aromatic nuclei on the ratio of *erythro* and *threo* forms in lignin formation was discussed.

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Acknowledgements

This paper is based on experimental work carried out in Laboratory of Wood Chemistry, Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, during the years 1997-1998 and 2000-2002.

I am deeply grateful to Professor Gyosuke Meshitsuka, Laboratory of Wood Chemistry, The University of Tokyo, for his guidance and very helpful discussion.

I wish to express my gratitude to Professor Kenji Iiyama, Asian Natural Environmental Science Center, The University of Tokyo, for his advises and encouragement.

I wish to express my special thankful to my supervisor, Associate Professor Yuji Matsumoto, Laboratory of Wood Chemistry, The University of Tokyo, for most valuable support, advice and great encouragement during the course of this study.

I wish to express my appreciation to Assistant Professor Hiroyuki Shintani, Laboratory of Wood Chemistry, The University of Tokyo, for his helpful advises and discussion.

I would also like to express my appreciation to:

Dr. Shuji Hosoya, Dr. Kengo Magara, and Dr. Tomoko Sugimoto, Forestry and Forest Products Research Institute, for their very helpful advises and kind cooperation.

Dr. Tomoya Yokoyama, University of Tsukuba for teaching me a lot about organic chemistry during 1997-1998 and great encouragement throughout this work.

Present and former colleagues both inside and outside the Laboratory of Wood Chemistry for their friendship and discussion.

Finally, I would like to thank my family for their support and encouragement.

List of Publications

This thesis is based on the following papers.

I Akiyama T., Magara K., Matsumoto Y., Meshitsuka G., Ishizu A., Lundquist K. 2000. Proof of the presence of racemic forms of arylglycerol-β-aryl ether structure in lignin: studies on the stereo structure of lignin by ozonation. Journal of Wood Science 46, 414-415

II Akiyama, T., Sugimoto, T., Matsumoto, Y., Meshitsuka, G. 2002.
Erythro/*threo* ratio of β-O-4 structures as an important structural characteristic of lignin. Part 1. Improvement of ozonation method for the quantitative analysis of lignin. side-chain structure. Journal of Wood Science 48, 210-215.

III Absolute configurations of the α- and β-asymmetric carbons of β-O-4 structures in hardwood lignin. (under preparation)

IV Akiyama, T., Matsumoto, Y., Okuyama, T., Meshitsuka, G
Erythro/threo ratio of β-O-4 structures as an important structural characteristic of lignin. Part 3. Ratio of *erythro* and *threo* forms of β-O-4 structures in tension wood lignin. (submitted to *Phytochemistry*)

V Akiyama, T., Nawawi, Deded S., Matsumoto, Y., Meshitsuka, G.
Erythro/threo ratio of β-O-4 structures as an important structural characteristic of lignin. Part 4. Variation in the *erythro*/*threo* ratio in hardwood lignins and its relation to aromatic ring types. (submitted to *Holzforschung*)