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ORIGINAL ARTICLE

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Erythro/threo ratio of β-*O*-4 structures as an important structural characteristic of lignin. I: Improvement of ozonation method for the quantitative analysis of lignin side-chain structure

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Abstract Ozonation as a quantitative tool to analyze the stereo structures of arylglycerol-β-aryl ether linkages was examined using 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). The procedure was improved. When mild postreduction 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 postreduction. A decrease in the recovery of these two acids under prolonged ozonation treatment was successfully suppressed by postreduction. The *erythro/threo* ratio of VG determined by the ozonation method with postreduction is in good agreement with the ratio determined by ^{1}H-nuclear magnetic resonance. Excellent reproducibility of the yield was obtained by adopting a procedure that included trimethylsilylation of ammonium salts of ozonation products using a dimethylsulfoxide-hexamethyldisilazane-trimethylchlorosilane mixture and subjecting it to gas chromatography analysis. It was concluded that arylglycerol-β-aryl ether structures comprise at least 35% of the C_3-C_6 structure in birch wood meal, with an *erythro/threo* ratio of 2.8.

Key words Lignin · Arylglycerol-β-aryl ether structure · Ozonation · *Erythro* · *Threo*

Introduction

Selective degradation of the aromatic nuclei of lignin by ozone gives low-molecular-weight compounds that retain the stereo structures of the original stereo side-chain structures of lignin. This method was first applied by Aulin-Erdtman et al.[1] for configuration analysis of a phenylcoumaran-type compound obtained as a major product by dehydrogenative condensation of isoeugenol. Later, this method was developed in our laboratory to analyze various side-chain structures of lignin including β-*O*-4 structures (Fig. 1). Many applications of this method have been reported, and some important chemical characteristics of side-chain structures in various lignins were discussed: The main configuration of the phenylcoumaran (β-5)-type structure in spruce milled wood lignin (MWL) was confirmed to be the *trans* form[2]; the diarylpropane (β-1)-type structure, if present, and glyceraldehyde-2-aryl ether structure are not the major structures in spruce lignin.[3–5] Recently, use of the ozonation method for analysis of lignin–carbohydrate bonds of the benzylic ether type has been reported,[6] which suggests a wide application for this method.

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

The *erythro/threo* (E/T) ratio of arylglycerol-β-aryl ether (β-*O*-4) structures is one of the most important information obtained by this method. Formerly, determination of the E/T ratio by the ozonation method was based on the ratio of the erythronolactone/threonolactone obtained.[7] We, however, thought that quantitative discussion of the content of the β-*O*-4 structure should be avoided because, as is shown below, erythronic and threonic acids formed during ozonation cannot be recovered quantitatively, and the reproducibility of the yields of erythronic and threonic acids

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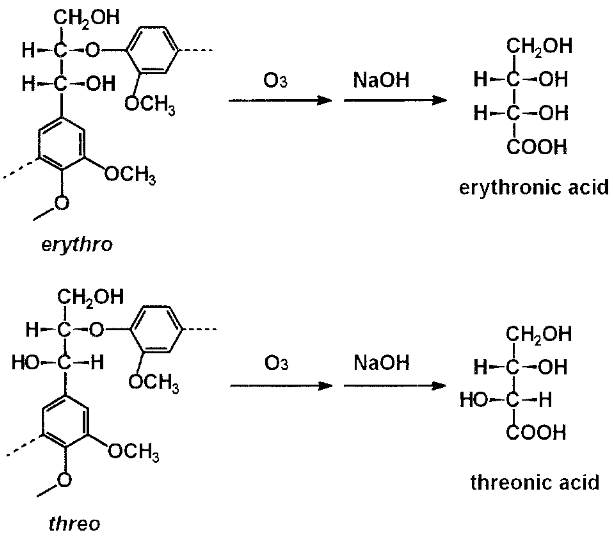

Fig. 1. Formation of two tetronic acids from side chains of arylglycerol-β-aryl ether structures by ozonation

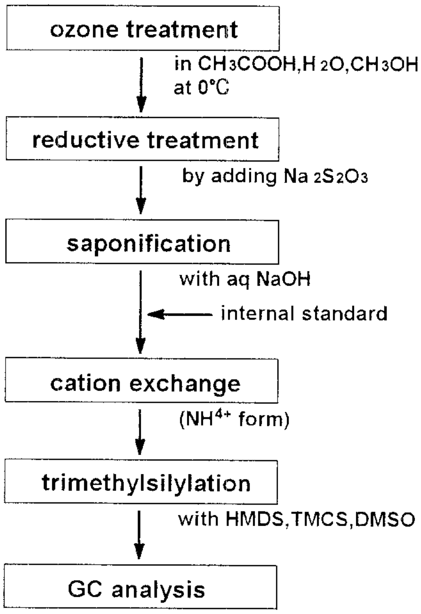

Fig. 2. Experimental scheme for modified ozonation method. aq. aqueous; HMDS, hexamethyldisilazane; TMCS, trimethylchlorosilane; DMSO, dimethylsulfoxide; GC, gas chromatography

relative to the internal standard are not always satisfactory. Therefore, it became necessary to develop a suitable procedure for quantitative analysis.

In this study, posttreatment 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. One is the reductive posttreatment of ozonation products with sodium thiosulfate. Another is the trimethylsilylation of ammonium salts without forming lactones for gas chromatography (GC).[8,9] We aimed to improve the ozonation method for quantitative analysis of the β-*O*-4 substructure in lignin.

Experiment

Materials

Model compounds. D-Erythronic and L-threonic acid ammonium salts were prepared from commercial D-erythronolactone and L-threonic acid calcium salt as follows.[9] D-Erythronolactone and L-threonic acid calcium salt were 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.[10]

Milled wood lignin. MWL 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[11] except that the milling was conducted without dispersing in toluene.

Wood meal. An 80-mesh-pass birch (*Betula maximowiczii* Regel) wood meal was extracted with ethanol/benzene (1:2, v/v) for 6 h. The lignin content determined by acetyl bromide was 21%.

$NaClO_2$-delignified wood meal. A 5-g sample of wood meal was treated with 2 g of $NaClO_2$ in diluted acetic acid aqueous solution at 70°–80°C for 1 h. This treatment was repeated five times. The treated wood meal was extracted with 1 M NaOH solution at room temperature.

Ozonation

Samples (200 mg of wood meal, 10–30 mg of MWL, and 0.02 mmol of erythronic and threonic acid ammonium salts) were suspended or dissolved in 30 ml of ozonation solvent consisting of acetic acid/water/methanol (16:3:1 v/v/v). Oxygen containing ca. 3% ozone was bubbled into the solution at the rate of 0.5 l/min for 5, 10, 20, 30, 40, 60, 120, 180, and 240 min at 0°C with stirring. The ozone generator used was a Nippon Ozone type ON-3-2.

Posttreatment steps

After ozone treatment, the residual ozone was removed by oxygen bubbling. The reaction mixture was reduced with 300 μl of 0.1 M sodium thiosulfate (reductive posttreatment). The solvent was evaporated under reduced pressure at 40°C. The trace of acetic acid was removed by repetition of evaporation with water. The ozonation products were saponified with 20 ml of 0.1 M NaOH at room temperature for 12 h. A 1 ml of 10.0 mM erythritol was added as an internal standard. The solution was then passed through a column filled with 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 80 ml. A part of the eluent (1 ml) was 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 for 30 min with 300 μl of dimethylsulfoxide (DMSO), 200 μl of hexamethyldisilazane (HMDS), and 100 μl of trimethylchlorosilane (TMCS) at 60°C.[12] The reaction mixture was separated into two phases. The upper layer consisted of aimed derivatives and the excess of silylating agents. The silylation of erythronolactone was carried out with pyridine instead of DMSO.

Gas chromatography and ^{1}H-nuclear magnetic resonance

The trimethylsilylated compounds were subjected to gas chromatography (GC) analysis on a Shimadzu 17A gas chromatograph equipped with a flame ionization detector under the following conditions.

Column: GL science NB1 fused-silica capillary column (30 m, 0.25 mm i.d)
Column temperature: held for 5 min at 120°C, raised by 4°C/min to 170°C and by 10°C/min to 280°C
Injection port temperature: 250°C
Detector port temperature: 280°C
Column flow: 1.9 ml helium/min

The E/T ratio of VG acetylated with acetic anhydride and pyridine was determined by ^{1}H-nuclear magnetic resonance (NMR) according to the chemical shift of Hα.[13] The spectrometer used was a Bruker AC 300, and the solvent was $CDCl_3$.

Results and discussion

The quantitativeness during the posttreatment procedure of the ozonation method was investigated. The loss of ozonation products by the sample drying procedure before trimethylsilylation was suspected to cause poor reproducibility. Figure 3 shows the influence of the drying time in vacuo on the yield of erythronolactone. The peak area of erythronolactone by GC analysis decreased with increasing drying time, whereas that of erythritol used as the internal standard was fairly stable. 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 and to exclude the influence of Na salts and NaOH on silylation, erythronic and threonic acids were converted to their trimethylsilyl ethers directly from their ammonium salts without lactonization and then were subjected to GC analysis.[9]

The calibration curves of erythronic and threonic acids with erythritol as an internal standard are shown in Fig. 4. When ammonium salts were silylated with DMSO, HMDS, and TMCS, the calibration curves of both acids fit well with straight lines. The absolute peak area of erythritol was reproducible (1805 ± 169) when the same volumes of sample solutions with the same erythritol concentration were in-

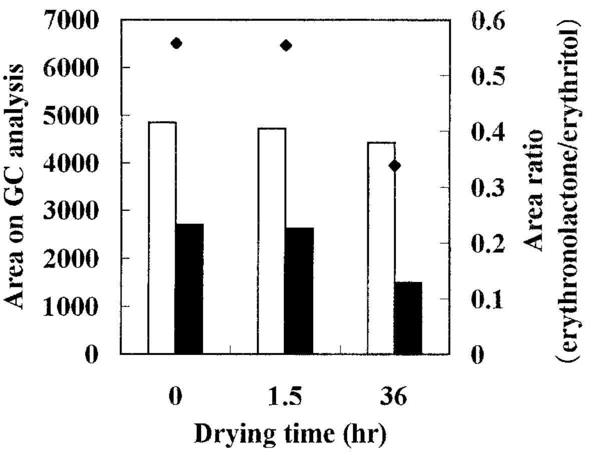

Fig. 3. Influence of drying time in vacuo on the loss of erythronolactone. *Open bars*, area on GC analysis of erythritol; *filled bars*, area on GC analysis of erythronolactone; *diamonds*, area ratio (erythronolactone/erythritol)

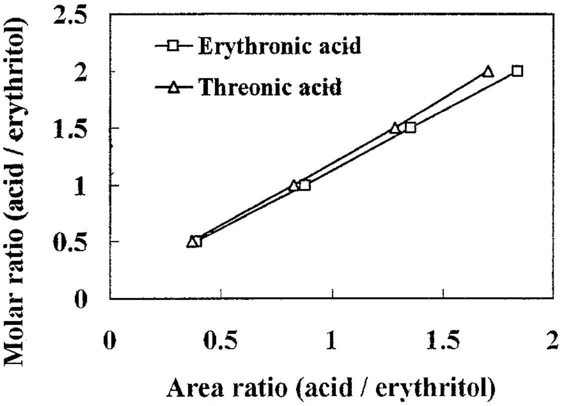

Fig. 4. Calibration curves of erythronic and threonic acids with erythritol. Erythronic acid: $Y = 1.036X + 0.096$ ($R^2 = 0.999$); Threonic acid: $Y = 1.123X + 0.077$ ($R^2 = 0.999$)

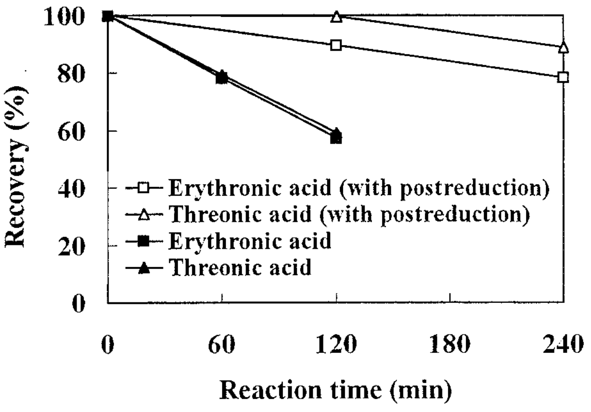

Fig. 5. Stability of erythronic and threonic acids with ozonation with and without postreduction

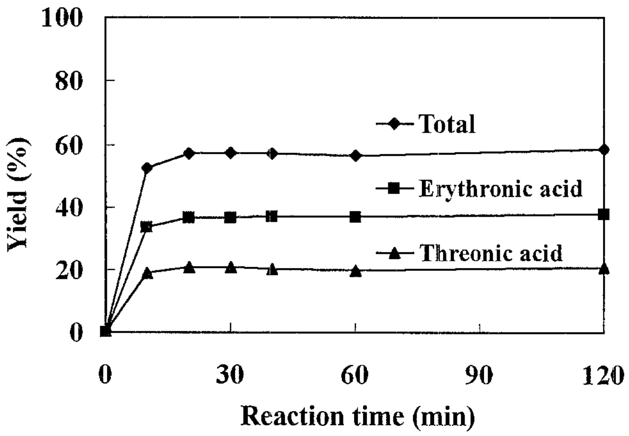

Fig. 6. Yields of ozonation products from veratryl glycerol-β-guaiacyl ether (VG) without postreduction

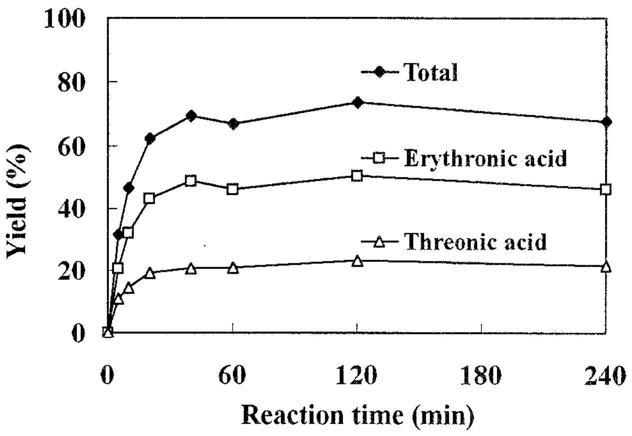

Fig. 7. Yields of ozonation products from VG with postreduction

Table 1. *Erythro*/*threo* ratio of VG determined by the ozonation method without postreduction

Method	E/T ratio
^{1}H-NMR	2.3
Ozonation (min)	
10	1.78
20	1.77
30	1.78
40	1.84
60	1.88
120	1.84

VG, veratrylglycerol-β-guaiacyl ether; E/T, *erythro*/*threo* ratio; NMR, nuclear magnetic resonance

Table 2. *Erythro*/*threo* ratio of VG determined by the ozonation method with postreduction

Method	E/T ratio
^{1}H-NMR	2.3
Ozonation (min)	
5	1.91
10	2.21
20	2.26
40	2.37
60	2.21
120	2.18
240	2.17

jected. From these results, the quantitativeness of this derivatizing procedure was confirmed.

The stability of erythronic and threonic acids toward ozone was examined in their free acid forms. As shown in Fig. 5, although erythronolactone was fairly stable with prolonged ozone treatment,[14] free acids of erythronic and threonic acids were unstable, with the recoveries falling to 57% and 59%, respectively, after ozonation for 120min. However, they increased to 89% and 99%, respectively, by adding sodium thiosulfate immediately after ozone treatment (postreduction).

As a lignin model compound, VG was subjected to ozonation with and without postreduction. Figure 6 and Table 1 show the results without postreduction. Total yields of erythronic and threonic acids reached 59% at 120min, and the E/T ratio based on the yield of these two acids obtained by ozonation was 1.8, which was smaller than the 2.3 determined by ^{1}H-NMR of acetylated VG. On the other hand, as shown in Fig. 7 and Table 2, the ozonation of VG with postreduction gave these acids in a total yield of 69% at 40min and 74% at 120min. These yields were maintained at a similar level during ozonation for 40–120min, and the E/T ratio determined by this method was in good agreement with that derived by ^{1}H-NMR. Based on these results obtained for model compounds, it was demonstrated that the postreduction and trimethylsilylation of ozonation products in the form of ammonium salts ensures the quantitativeness of the ozonation method for the β-*O*-4 structure.

This improved ozonation method was applied to wood meal and MWL. The reproducibility of the yields of erythronic and threonic acids from MWL and wood meal was examined. The results obtained for 10mg of beech MWL and 200mg of birch wood meal are illustrated in Figs. 8 and 9, respectively. When 200mg of wood meal was subjected to the improved ozonation method for 2h, the mean total yield (per gram of sample) of these two acids for three trials was 0.318 ± 0.018mmol, and that of the E/T ratio was 2.79 ± 0.05. When the sample weight was changed from 200mg to 50mg, almost the same results were obtained (Fig. 9, dotted line). In the case of MWL (10mg and two 30-mg samples), the mean yield and E/T ratio were 1.078 ± 0.02mmol and 1.68 ± 0.02, respectively. Those data confirmed the reproducibility of this improved ozonation method even when the sample amount varied.

In the cases of MWL and wood meal, the yields of erythronic and threonic acids significantly increased until 40–60min. After that the yields increased only gradually, and the E/T ratio became almost constant, as shown in Tables 3 and 4.

Based on the results in Figs. 7, 8, and 9, it appears that at least 1h of ozonation is required for determination of the

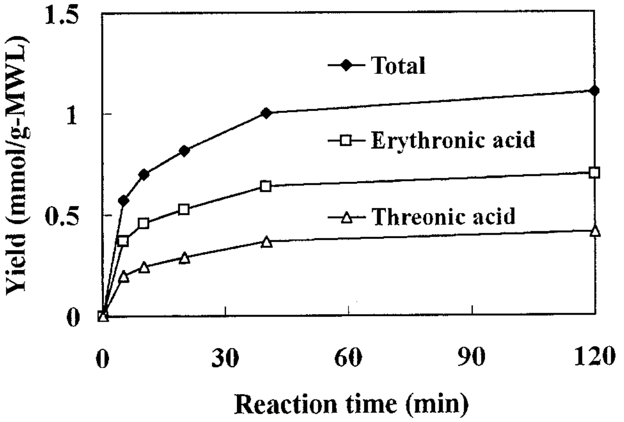

Fig. 8. Yields of ozonation products from beech milled wood lignin (*MWL*) with postreduction

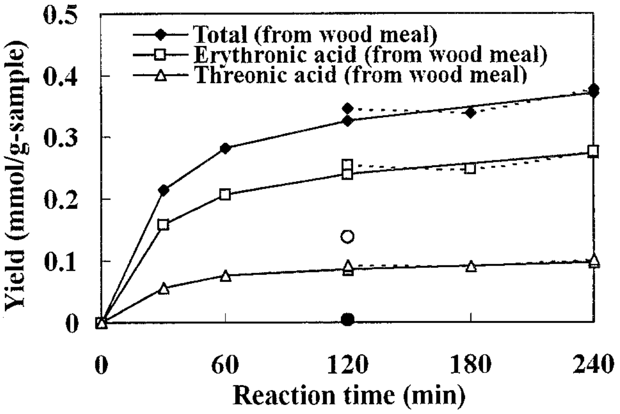

Fig. 9. Yields of ozonation products from birch wood meal and $NaClO_2$-delignified wood meal with postreduction. *Open circles*, tetronic acids obtained from $NaClO_2$-delignified wood meal; *filled circles*, tetronic acids obtained from $NaClO_2$-delignified wood meal extracted with aqueous NaOH

Table 3. *Erythro*/*threo* ratio of beech MWL determined by the ozonation method with postreduction

Reaction time (min)	E/T ratio
5	1.86
10	1.89
20	1.82
40	1.75
120	1.70

MWL, milled wood lignin

Table 4. *Erythro*/*threo* ratio of birch wood meal determined by the ozonation method with postreduction

Reaction time (min)	E/T ratio
30	2.83
60	2.73
120	2.79
240	2.81

E/T ratio of lignin samples, though the size of insoluble samples such as wood meal may affect the rate of ozonation. The ozonation of finer wood meal may give erythronic and threonic acids as rapidly as MWL.

Yields were expressed as 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% at 120min, and that from wood meal that contains 21% lignin (acetyl bromide method) was 35.3% at 240min. It was thus concluded that arylglycerol-β-guaiacyl ether and arylglycerol structures comprise at least 22% of the C_6–C_3 structure in beech MWL and at least 35.3% of the C_6–C_3 structure in birch wood meal. Those values are expressed without taking any other factors (the yields of these acids from the model compound) into consideration.

Wood meal delignified with $NaClO_2$ and successively extracted with aqueous NaOH solution gave erythronic and threonic acids at only 0.0033mmol/g of sample by ozonation. This value was about 1% of the yield from the same amount of wood meal. Interestingly, when $NaClO_2$-delignified wood meal without successive NaOH extraction was subjected to the 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 indicate that a degradation method performed under acidic conditions, such as $NaClO_2$ treatment, cannot remove all the degraded lignin fragments, especially those derived from the side chain parts, and that those fragments still act as precursors of erythronic and threonic acids when subjected to ozonation.

This improved ozonation method has already been applied to two topics, the chirality of the asymmetric carbon at the β-position of the β-*O*-4 side-chain structures[15] and the changes in the E/T ratio of the β-*O*-4 structures depending on the degree of delignification.[16]

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