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ORIGINAL ARTICLE

**Olov Karlsson · Tsutomu Ikeda · Takao Kishimoto
Kengo Magara · Yuji Matsumoto · Shuji Hosoya**

Isolation of lignin–carbohydrate bonds in wood. Model experiments and preliminary application to pine wood

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Abstract A novel method for analysis of benzylic ether type lignin–carbohydrate bonds has been developed by using model compounds. Four diastereomers of model compound 3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-3-(methyl β-D-glucopyranoside-6-O-yl)-1-propanol (GGMGP), were ozonized in acetic acid/water/methanol 16:3:1 for 1 h at 0°C. The product from ozonation of each diastereomer was saponified and the corresponding α-etherified tetronic acid (TAMGP) was isolated using ion exchange chromatography. Minor amounts of methyl β-D-glucopyranoside (MGP) and small amounts of a gluconic acid etherified with tetronic acid (TAGLCA), tetronic acid, gluconic acid, and glyceric acid were detected in the product mixture of ozonated benzylic ether type model compounds. The results suggest that a benzyl ether bond between lignin and carbohydrate is rather stable during the ozone treatment. Acid treatments with sulfuric acid or trifluoroacetic acid of the derived TAMGP led to cleavage of the glucosidic bond but only a small amount of products (tetronic acid and glucose) resulting from cleavage of the α-ether bond were formed. The successful chemical treatments were used for studies of benzylic ether bonds in Japanese red pine. The results suggest the presence of benzylic ether bonds to polysaccharides in the wood.

Key words Ozone · Lignin–carbohydrate bond · LCC model compound · Benzylic ether · Threonic/erythronic acid

O. Karlsson[1] · T. Ikeda · T. Kishimoto[2] · K. Magara · S. Hosoya (✉)
Wood Chemistry Laboratory, Department of Chemical Utilization, Forestry and Forest Products Research Institute, P.O. Box 16, Tsukuba Norin Kenkyu-Danchi Nai, Ibaraki 305-8687, Japan
Tel. +81-298-73-3211; Fax +81-298-74-3720
e-mail: hosoya@ffpri.affrc.go.jp

S. Hosoya
Institute of Agricultural and Forest Engineering, University of Tsukuba, Ibaraki 305-8572, Japan

Y. Matsumoto
Laboratory of Wood Chemistry, Department of Biomaterial Sciences, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657, Japan

Present addresses:
[1] LTU, Skellefteå Campus, Skellefteå S-931 87, Sweden
[2] Laboratory of Wood Chemistry, Research Group of Bioorganic Chemistry, Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8598, Japan

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Introduction

The presence of lignin–carbohydrate (LC) bonds may play an important role in determining the mechanical and chemical properties of wood such as strength and the isolation of individual wood polymers. In the case of kraft pulps, studies of the molecular weight of the wood polymers in the pulp suggest that a considerable part of the residual lignin is bonded to polysaccharides.[1,2] The α-ether bond in structural elements of benzylic ether type **1** has been proposed as a linkage between lignin and polysaccharides in wood (Fig. 1). The α-ether bond in the nonphenolic type benzylic ether LC model compound is stable under pulping conditions.[3] The presence of benzyl ether bonds is also considered to be important during bleaching.[4] To find evidence for the presence of phenolic and nonphenolic benzylic ether bonds between lignin and polysaccharides, techniques are needed that can degrade the wood polymers (lignin and polysaccharides) selectively without cleavage of the LC bonds. Lignin can be selectively degraded by ozonation in a solvent mixture consisting of acetic acid/water/methanol 16:3:1 at 0°C.[5] Arylglycerol-β-aryl ethers constitute the dominant structural element in lignin. Ozonation of such structures and subsequent saponification lead to the formation of tetronic acid (2,3,4-trihydroxybutanoic acid) via degradation of their aromatic nuclei.[5] If the α-ether bond in benzylic ether **1** is stable during the ozone treatment and saponification, corresponding etherified tetronic acid **2** may

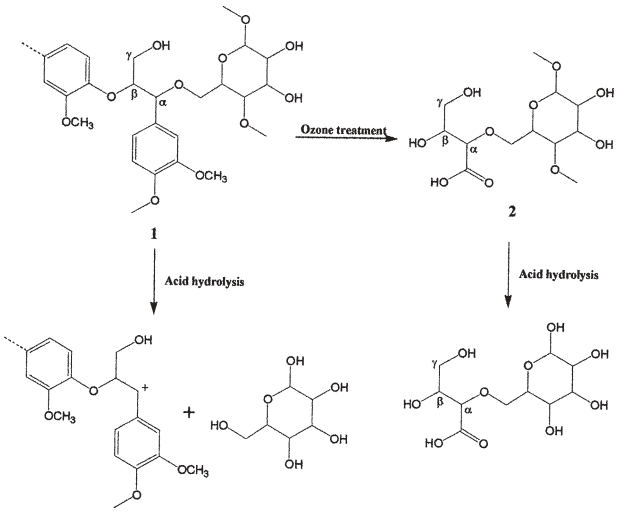

Fig. 1. Chemical treatments (ozonation and acid hydrolysis) of a benzylic ether of the lignin–carbohydrate complex (LCC) type **1**

be formed (Fig. 1). By using model compounds of the benzylic ether type, we found that the α-ether bond is fairly stable during the ozonation (Fig. 1).[6] Treatment with acid can be used to depolymerize polysaccharides and analyze the resulting monosaccharides in wood. Cleavage of the benzylic ether bonds can also be accomplished by acid hydrolysis.[7] However, we found that the α-ether bond in ether **2** was stable under the acid hydrolysis conditions employed (Fig. 1).[6]

In this article, further details from the studies on the stability of the α-ether bond in model compounds of benzylic ether type **1** during ozonation and subsequent acid hydrolysis are presented. The successive chemical treatments were used prior to isolation and analysis techniques to study the benzylic ether bonds between lignin and carbohydrates in wood.

Experimental

Chromatography and spectroscopy

High performance liquid chromatography (HPLC). HPLC columns: Discovery C18 4.6 × 150 mm and Zorbax octadecyl dimethyl silyl (ODS) 21.2 × 250 mm. Flow rate: 0.5 ml/min (analytical), 7 ml/min (preparative). Column temperature: 35°C. Eluent: acetonitrile/water 18:82. UV-detector (280 nm). Anion-exchange chromatography. Preparative Asahipak NH2P-90 21F column. Eluent: 2 M acetic acid. Flow rate: 6 ml/min. Column temperature: 35°C. RI-detector.

Gas chromatography (GC). GC column: 0.2 mm i.d., 25 m long, HP-1 (crosslinked methyl siloxane). Column temperature: 150°–250°C (initial time 1 min, gradient: 8°C min^{-1}). Carrier gas: Helium. D-Erythronic acid-γ-lactone was used as internal standard. A DB-1 column (15 m long) and column temperature of 80°–200°C were used for the GC-MS experiments.

Mass spectrometry (MS). Electron impact (EI) and chemical ionization (CI) in the presence of ammonia gas were performed. The mass spectrometer was calibrated with perfluorokerosene. Before analysis with GC and GC-MS, the sample was trimethylsilylated with 0.2 ml hexamethyldisilazane and 0.1 ml trimethylchlorosilane in 1 ml dimethyl sulfoxide (DMSO).

Nuclear magnetic resonance spectroscopy (NMR). The ^{1}H NMR and ^{13}C NMR spectra were recorded with a JEOL JNM Lambda 400 FT-NMR spectrometer in acetone-d_6 or D_2O with tetramethylsilane (TMS) or 3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid sodium salt (TSP-d_4) as internal standard, respectively. Some chemical shift assignments were made by correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond connectivity (HMBC). Chemical shifts (δ) and coupling constants (J) are given in δ values (ppm) and Hz, respectively.

Preparation of benzylic ether model compounds

3-(4-Hydroxy-3-methoxyphenyl)-2-(2-methoxyphenoxy)-3-(methyl β-D-glucopyranosid-6-O-yl)-1-propanol (GGMGP). Synthesis of GGMGP (Fig. 2) was performed by applying the procedure for the synthesis of the corresponding lignin–carbohydrate complex (LCC) model compounds carrying methyl α-D-glucopyranoside.[3] Quinonemethide of guaiacylglycerol-β-guaiacyl ether (GG) in $CHCl_3$ and methyl β-D-glucopyranoside (MGP) in dry DMSO (10 times excess of MGP) were mixed. The mixture was kept at 4°C for 2 days. Water was added to the reaction mixture and the whole was extracted with $CHCl_3$ to remove unreacted GG. The aqueous phase was extracted with ethyl acetate. The organic fraction from the ethyl acetate extraction was removed by evaporation under reduced pressure. After fractionation of the residue by HPLC with a preparative ODS column (Fig. 3,4), diastereomers GGMGP-**a**, -**b**, -**c**, and -**d** were isolated. The diastereomers from the ethyl acetate phase were present in higher yield and were purer than those from the $CHCl_3$ phase.

GGMGP-**a** (in acetone-d_6). ^{1}H NMR: δ 3.13 (1H, t, J = 7.8, C_2-H), 3.22–3.37 (2H, m, C_3-H and C_4-H), 3.41 (3H, s, OCH_3), 3.44–3.53 (3H, m, C_γ-H, C_5-H and C_6-H), 3.61 (1H, m, C_γ-H), 3.76–3.83 (1H, m, C_6-H), 3.84 (6H, s, OCH_3), 4.13 (1H, d, J = 7.8, C_1-H), 4.32 (1H, m, C_β-H), 4.72 (1H, d, J = 5.8, C_α-H), 6.80–7.20 (7H, aromatic protons). ^{13}C NMR: δ 56.2 (1C, OCH_3), 56.3 (1C, OCH_3), 56.7 (1C, OCH_3), 62.2 (1C, C_γ), 69.8 (1C, C_6), 71.7 (1C, C_4), 74.7 (1C, C_2), 76.7 (1C, C_5), 78.0 (1C, C_3), 82.9 (1C, $C\alpha$), 86.3 (1C, C_β), 105.0 (1C, C_1). Aromatic carbons at δ 112.0, 113.4, 115.3, 119.2, 121.4, 121.9, 122.8, 131.1, 147.2, 148.3, 150.1, 151.6.

GGMGP-**b** (in acetone-d_6). ^{1}H NMR: δ 3.17 (1H, t, J = 7.8, C_2-H), 3.44 (3H, s, OCH_3), 3.84 (6H, s, OCH_3), 4.14 (1H, d,

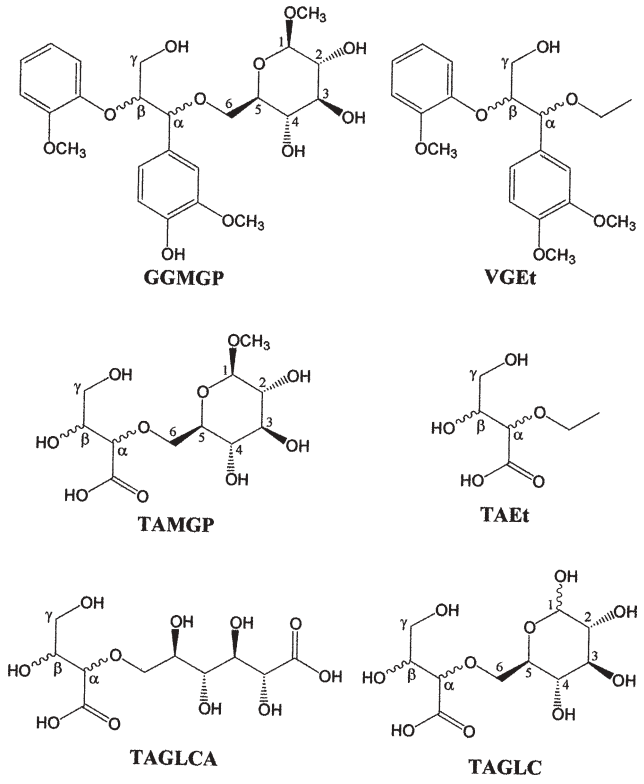

Fig. 2. Benzylic ethers as reactants and reaction products. *GGMGP*, 3-(4-hydroxy-3-methoxyphenyl)-2-(2-methoxy-phenoxy)-3-(methyl β-D-gloucopyranosid-6-O-yl)-1-propanol; *VGEt*, 3-(3,4-dimethoxyphenyl)-3-ethoxy-2-(meth-oxyphenoxy)-1-propamol; *TAMGP*, 3,4-dihydroxy-2-(methyl β-D-glucopyranosid-6-O-yl)butanoic acid; *TAEt*, 3,4-dihydroxy-2-ethoxybutanoic acid; *TAGLCA*, D-gluconic acid 6-O-(3,4-dihydroxybutanoic acid-2-yl)ether; *TAGLC*, 3,4-dihydroxy-2-(D-glucopycranose-6-O-yl)butanoic acid

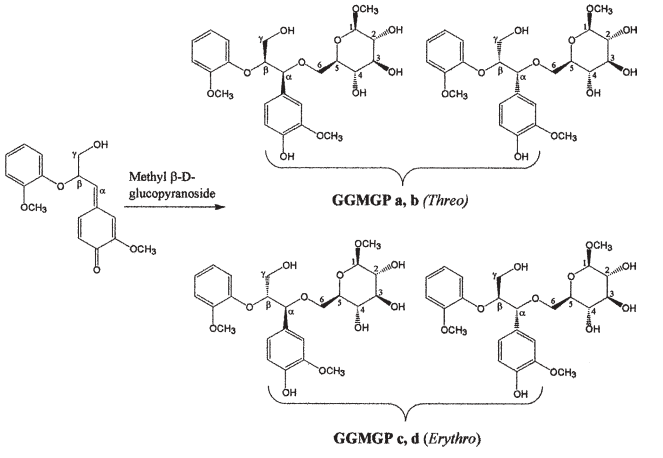

Fig. 3. Formation of diastereomers GGMGP-**a**, -**b**, -**c**, and -**d** from reaction between a quinine methide of guaiacylglycerol-β-guaiacyl ether and methyl β-D-glucopyranoside (MGP)

J = 7.8, C_1-H), 4.29 (1H, m, C_β-H), 4.70 (1H, d, J = 5.8, C_α-H), 6.80–7.20 (7H, aromatic protons). ^{13}C NMR: δ 56.2 (1C, Ar-OCH_3), 56.3 (1C, Ar-OCH_3), 56.7 (1C, OCH_3), 62.1 (1C, C_γ), 69.5 (1C, C_6), 71.7 (1C, C_4), 74.8 (1C, C_2), 76.1 (1C, C_5), 78.1 (1C, C_3), 82.4 (1C, C_α), 86.4 (1C, C_β), 105.1 (1C, C_1).

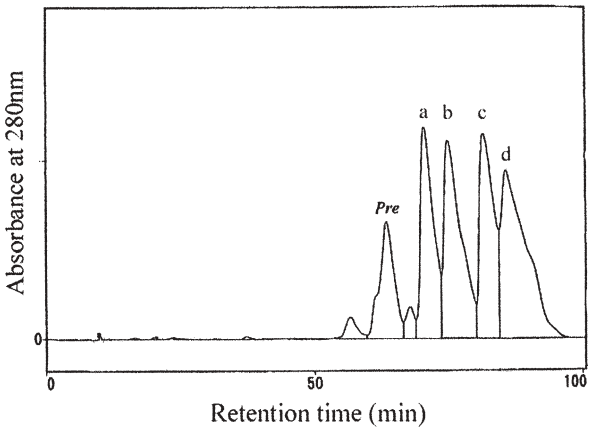

Fig. 4. HPLC of diastereomers of benzyl ethers GGMGP-**a**, -**b**, -**c**, and **d**. *Pre*, structural isomers of GGMGP

Aromatic carbons at δ 111.9, 113.5, 115.3, 119.6, 120.8, 121.9, 122.9, 130.9, 147.2, 148.3, 150.0, 151.7.

GGMGP-**c** (in acetone-d_6). ^{1}H NMR: δ 3.17 (1H, t, J = 8.3, C_2-H), 3.47 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.83 (3H, s, OCH_3), 4.20 (1H, d, J = 7.8, C_1-H), 4.30 (1H, m, C_β-H), 4.66 (1H, d, J = 6.3, C_α-H), 6.80–7.20 (7H, aromatic protons). ^{13}C NMR: δ 56.3 (1C, Ar-OCH_3), 56.3 (1C, Ar-OCH_3), 56.8 (1C, OCH_3), 62.3 (1C, C_γ), 69.8 (1C, C_6), 71.6 (1C, C_4), 74.8 (1C, C_2), 76.5 (1C, C_5), 77.9 (1C, C_3), 82.9 (1C, C_α), 85.3 (1C, C_β), 105.2 (1C, C_1). Aromatic carbons at δ 112.2, 113.7, 115.2, 119.1, 121.7, 121.7, 123.0, 131.4, 147.1, 148.1, 149.1, 151.8.

GGMGP-**d** (in acetone-d_6). ^{1}H NMR: δ 3.15 (1H, t, J = 7.8, C_2-H), 3.46 (3H, s, OCH_3), 3.77 (3H, s, OCH_3), 3.82 (3H, s, OCH_3), 4.15 (1H, d, J = 7.8, C_1-H), 4.33 (1H, m, C_β-H), 4.62 (1H, d, J = 6.3, Cα-H), 6.80–7.20 (7H, aromatic protons). ^{13}C NMR: δ 56.2 (1C, OCH_3), 56.3 (1C, OCH_3), 56.7 (1C, OCH_3), 61.9 (1C, C_γ), 69.4 (1C, C_6), 71.6 (1C, C_4), 74.7 (1C, C_2), 75.9(1C, C_5), 78.0 (1C, C_3), 82.1 (1C, C_α), 85.5 (1C, C_β), 105.1 (1C, C_1). Aromatic carbons at δ 112.2, 113.6, 115.2, 119.2, 121.7 (2C), 123.0, 131.3, 147.0, 148.2, 149.1, 151.8.

3-(3,4-Dimethoxyphenyl)-3-ethoxy-2-(2-methoxyphenoxy)-1-propanol (VGEt). VGEt was synthesized according to Taneda et al.[3]

VGEt (in acetone-d_6). ^{1}H NMR: δ 1.46 (3H, t, J = 7.1, CH_3), 3.63 (2H, m, C_γ-H), 3.87 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 4.03 (1H, m, C_β-H), 4.09 (2H, q, J = 7.1, CH_2-H), 4.98 (1H, d, J = 8.3, C_α-H), 6.80–7.20 (7H, aromatic protons). ^{13}C NMR: δ 14.8 (1C, CH_3), 55.9 (1C, OCH_3), 56.0 (1C, OCH_3), 61.1 (1C, C_γ), 64.4 (1C, CH_2), 74.0 (1C, Cα), 89.6 (1C, C_β). Aromatic carbons at δ 110.2, 112.2, 112.6, 119.6, 121.1, 121.7, 124.3, 132.1, 147.7, 148.3, 149.5, 151.4. VGEt, probably in the *erythro*-form, was isolated from the product mixture of both the *threo*- and *erythro*-form by HPLC. The product mixture and diastereomerically pure VGEt were used for ozonation experiments.

Miscellaneous NMR data

The following NMR-data were obtained from the prefraction in Fig. 4. Prefraction (in acetone-d_6). ^{1}H NMR: δ 2.5–4.0 (45H, m, C_γ-H, sugar protons), 3.3–3.5 (30H, m, OCH_3), 3.7–3.9 (30H, m, OCH_3), 4.0–4.2 (5H, m, C_1-H), 4.3–4.5 (5H, m, C_β-H), 5.03, 5.20, 5.23, 5.26, 5.30 (5H, d, J = 6.8, 7.6, 7.2, 7.4, 6.4, C_α-H), 6.75–7.28 (35H, m, aromatic protons). ^{13}C NMR: δ 56.0–56.6 (OCH_3), 61.2–62.8 (C_6, C_γ), 70.3–77.1 (15C, sugar carbons), 81.7–83.2 (5C, C_α), 84.1–85.2 (5C, sugar carbons), 85.5–86.3 (5C, C_β), 104.6–105.8 (5C,C_1), 112.4–151.7 (60C, aromatic carbons).

Ozone treatment of benzylic ether model compounds

Starting material (ca. 50 mg) was dissolved with 20 ml of acetic acid/water/methanol 16:3:1 at 0°C. Ozone (3% in O_2) was purged into the solution at the rate of 0.1 l/min for 1 h. After ozonation solvents were removed by evaporation with a rotary evaporator, the sample was saponified with 0.1 M NaOH under nitrogen gas flow for 1 h. Sodium ions were exchanged with ammonium ions by passing the alkaline solution through a Dowex 50W-X8 cation-exchange column that had been treated with NH_3 (25% aqueous solution). Solvents were evaporated and the product was dried over P_2O_5 in vacuo overnight.

Acid treatments

3,4-Dihydroxy-2-(methyl β-D-glucopyranosid-6-O-yl) butanoic acid (TAMGP) (2–3 mg) was dissolved with 0.1 ml 72% sulfuric acid and left standing at room temperature for about 1 h. The solution was diluted to ca. 3% and heated in an autoclave at 120°C for about 1 h. The solution was applied to an anion-exchange column (AG 1-X8, acetate form) and was eluted with water and 1 M acetic acid. The combined fractions were evaporated and dried. In one experiment, TAMGP was hydrolysed with 2 M trifluoroacetic acid (TFA) in an autoclave. After the solvent was removed by evaporation, it was washed with water twice. The products were dried over P_2O_5 in vacuo overnight.

Identification of reaction products from GGMGP-**a**

TAMGP was isolated from the product mixture from ozone treatment of GGMGP-**a** by anion-exchange chromatography (see also Results and discussion). The following NMR data were obtained for TAMGP (ca. 15 mg/ml in D_2O). ^{1}H NMR (400 MHz): δ 3.14 (1H, dt, J = 7.3, 2.4, C_2-H), 3.33–3.39 (2H, m, C_3-H and C_4-H), 3.44 (3H, s, OCH_3), 3.40–3.50 (1H, m, C_5-H), 3.54 (1H, dd, J = 11.6, 6.7, C_γ-H), 3.60 (1H, dd, J = 11.6, 5.5, C_γ-H), 3.69–3.76 (2H, m, C_6-H), 3.88–3.92 (1H, m, C_β-H), 3.98 (1H, d, J = 3.0, C_α-H), 4.24 (1H, d, J = 8.0, C_1-H). ^{13}C NMR: δ 58.3(1C, OCH_3), 63.4 (1C, C_γ), 70.6 (1C, C_4), 70.7 (1C, C_6), 73.6 (1C, C_β), 74.2 (1C, C_2), 75.9 (1C, C_5), 76.6 (1C, C_3), 82.2 (1C, C_α), 104.4 (1C, C_1), 177.5 (1C, COOH).

CI-MS of this fraction in the presence of ammonia gas gave the base peak m/z 762 (M + NH_4^+). EI-MS of this fraction gave fragment peaks: m/z 612 (M − 132) and m/z 729 (M − 15).

D-Gluconic acid 6-O-(3,4-dihydroxybutanoic acid-2-yl) ether (TAGLCA) was identified in the trimethylsilylated product mixture of ozonated TAMGP by GC-MS (CI): m/z 908 (M + NH_4^+).

Two isomers of 3,4-dihydroxy-2-(D-glucopyranose-6-O-yl)butanoic acid (TAGLC), which have either an α or β hydroxyl group at the C1 carbon of the glucopyranose part in TAGLC, were identified in the product mixture from acid hydrolysis of TAMGP by GC-MS (CI): m/z 820 (M + NH_4^+). Characteristic proton signals were observed in the product mixture by ^{1}H NMR (in D_2O). ^{1}H NMR: δ 3.13 (1H, m, C_2-H), 3.35 (1H, m, C_3-H), 3.45 (1H, m, C_4-H), 3.97 (1H, m, C_α-H), 4.51 (1H, d, J = 8.1, C_1-H), 5.10 (1H, d, J = 3.7, C_1-H).

Erythronic acid was identified by comparison with the erythronic acid standard which was produced by saponification from commercially available D-erythronic acid-γ-lactone. Threonic acid and gluconic acid were identified by comparing with standards produced by cation-exchange treatment of commercially available L-threonic acid calcium salt and gluconic acid calcium salt, respectively.

Etherified tetronic acids from GGMGP-**b**, -**c** and VGEt

3,4-Dihydroxy-2-ethoxybutanoic acid (TAEt) was identified in the product mixture of ozone-treated VGEt. NMR data for TAEt obtained from the mixture of ozonated VGEt (in D_2O): ^{13}C NMR: δ 15.2 (1C, CH_3), 63.4 (1C, C_γ), 67.4 (1C, CH_2), 73.5 (1C, C_β), 83.2 (1C, C_α), 178.9 (1C, COOH). Mass fragment peaks (EI) were found at m/z 248 (M − 132), m/z 291 (M − 89), m/z 335 (M − 45), and m/z 365 (M − 15).

NMR data for TAMGP obtained from mixture of ozonated GGMGP-**b** (in D_2O). ^{13}C NMR: δ 58.2 (1C, OCH_3), 63.1 (1C, C_γ), 70.3 (2C, C_4, C_6), 73.7 (1C, C_β), 74.2 (1C, C_2), 75.7 (1C, C_5), 76.7 (1C, C_3), 104.3 (1C, C_1).

NMR data for TAMGP obtained by ozonation of GGMGP-**c** (in D_2O). ^{13}C NMR: δ 58.3 (1C, OCH_3), 63.2 (1C, C_γ), 70.6 (1C, C_6), 71.0 (1C, C_4), 73.5 (1C, C_β), 74.0 (1C, C_2), 75.9 (1C, C_5), 76.7 (1C, C_3), 104.3 (1C, C_1).

Procedure for isolation and analysis of lignin–carbohydrate bonds in wood

Wood meal (<100 mesh) from red pine (*Pinus densiflora*) was Soxhlett-extracted with ethanol benzene 1:2. The extractive-free wood meal (15.0 g) was then ozonated (3.2% ozone in O_2, 0.1 l/min) in 1 l of acetic acid/water/methanol (16:3:1) with magnetic stirring at 0°C for 8 h. $Na_2S_2O_3$ (12 ml, 0.1 M) was added to the solution and the mixture was left in refrigerator overnight. The solution was filtered and the wood meal was washed with fresh reaction solvent and

dried; yield 10.93 g (73%). The yield of tetronic acid in the solution, estimated as a trimethylsilyl-derivative with GC, was 3.8 mmol (15.4% of C9-unit). Ozonated pine meal (10.0 g) was suspended in 90 ml 72% sulfuric acid and then the acid solution was diluted to 3% with water and heated in an autoclave for 1 h at 120°C. The mixture was filtered and the dark-brown precipitate was dried; 0.25 g. Soluble lignin in the filtrate was estimated to be 1.1 g by UV-spectroscopy at 280 nm. Sulfate anions were precipitated from the supernatant by addition of $Ba(OAc)_2$ (firstly as solid, then as saturated aqueous solution, total 1.02 mol). The mixture was centrifuged for 10 min and then the solution was evaporated to 50 ml. It was applied to an anion-exchange column (300 ml, 1-X8, acetate-form). The mixture was eluted with 2.3 l water and the eluted solution was evaporated (ca. 9 g). Weak acids were eluted with 1.0 M acetic acid (1.75 l + 3 l) and the two obtained fractions were evaporated and dried; yield 0.11 g and 0.38 g, respectively. The fraction from weak acids (yield 0.38 g) was trimethylsilylated and analyzed with GC and GC-MS (CI) by the same procedure as described in the previous section for model compounds.

Results and discussion

Synthesis of benzylic ethers of LCC type

GGMGP was synthesized by adding quinone methide, prepared from guaiacylglycerol-β-guaiacyl ether (GG), to a solution containing methyl β-D-glucopyranoside (Figs. 2,3). The yield of GGMGP was not determined but appeared to be rather low (10%–20%). GGMGP was obtained in a mixture of four diastereomers (Fig. 3). Each diastereomer was isolated using preparative HPLC (Fig. 4) and characterized with NMR.[7] Similar amounts of each of the diastereomers of GGMGP were present in the reaction mixture (Fig. 4). Diastereomers GGMGP-**a** and GGMGP-**b** are considered to have *threo*-configuration, while diastereomers GGMGP-**c** and GGMGP-**d** are considered to have *erythro*-configuration (Fig. 3). This assignment was based on comparison of ^{1}H NMR data of the side chain protons (H_α and H_γ).[8,9] It was also found that the relative yields of threonic acid and erythronic acid formed during ozonation of the benzylic ethers can be used for determination of the *erythro* and *threo*-forms of GGMGP (see below).

Analysis of the prefraction in Fig. 4 by NMR indicated the presence of structural isomers of GGMGP. Isolation and complete characterization of the individual isomers was not performed but from the results obtained we concluded that formation of structural isomers of GGMGP occurs in low yield (less than 25% of GGMGP). This is in accordance with previous experience in the synthesis of analogous benzyl ethers.[3,9,10] If benzylic ether bonds are formed during biosynthesis of wood they may be formed by a similar mechanism. Thus, lignin may be linked via benzyl ether bonds mostly with the primary hydroxyl groups of polysaccharides in wood.

Ozonation of benzylic ether model compounds

Ozonolysis of arylglycerol-β-aryl ether structures have been thoroughly studied and proceed mainly via degradation of their aromatic nuclei.[5,11] Tetronic acids (erythronic acid and threonic acid) as well as acids formed by degradation of the aromatic rings are the main reaction products. The highest yield of tetronic acids reported so far include a saponification step (to cleave the ester group remaining after degradation of the β-aryloxy substituent) and addition of small amounts of an aqueous solution of sodium thiosulfate (to degrade remaining hydroperoxides).[11] From NMR studies, the purity of the *threo*-benzylic ether GGMGP-**a** sample was estimated to be about 85%–90%. Neither GG nor MGP were present. TAMGP was isolated from the product mixture obtained after ozonation of isomer GGMGP-**a** by preparative anion-exchange chromatography (Figs. 2,5). The chemical structure of TAMGP was studied with NMR and GC-MS. In the proton spectrum, relatively broad signals were observed (which makes assignment of stereochemistry difficult); however, the individual protons could be assigned by 2-D NMR. C_α and COOH could be observed in the ^{13}C NMR spectrum when the pulse delay time and the broadening factor were increased. This measurement was performed only for TAMGP-**a**, which is the reason why the Cα and COOH peaks were absent in the ^{13}C NMR spectra for TAMGP-**b**, -**c** described in the experimental section. Furthermore, cross coupling between H_α and C_α was observed in the HMQC spectrum and cross peaks between C_α-H and C_6 as well as between C_6-H and C_α were observed in the HMBC spectrum. MGP was also found in the product mixture of the ozone-treated benzylic ether GGMGP-**a** (Fig. 5). The product mixture from ozonation of benzylic ether GGMGP-**a** was analyzed with GC after trimethylsilylation (Fig. 6), and in addition to TAMGP and MGP, small amounts of other products (TAGLCA, tetronic acid, and gluconic acid) were found (Figs. 2,6). Estimated relative yields are presented in Fig. 7. Because of the presence of only small amounts of TAGLCA in the ozonation product, elucidation of its struc-

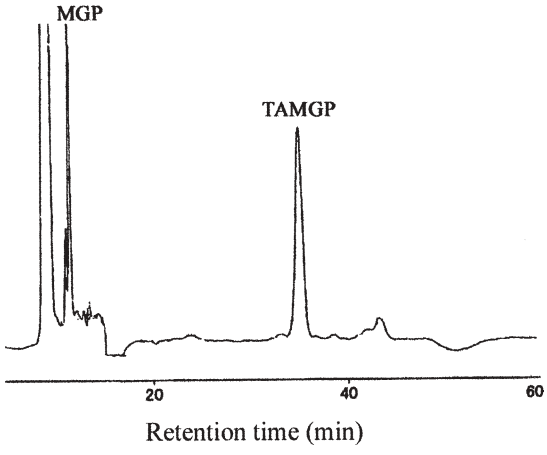

Fig. 5. Anion-exchange chromatography of product mixture from the ozone treatment of GGMGP-**a**

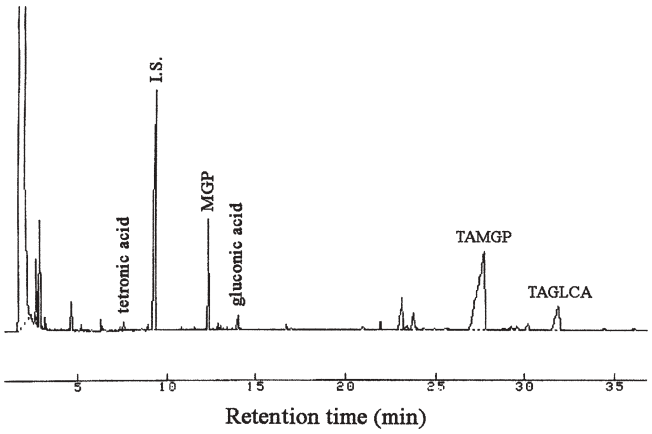

Fig. 6. GC of the trimethylsilylated product mixture from ozonation of GGMGP-**a**

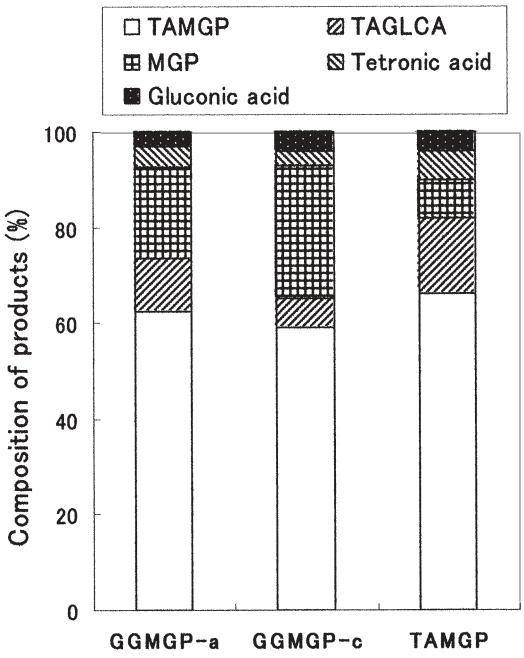

Fig. 7. Estimation of relative yields of reaction products of ozone treatment of GGMGP-**a**, -**b**, and TAMGP

ture was mainly based on GC-MS (CI) data. It is therefore not known if it consisted of only one diastereomeric form. In a separate experiment it was shown that ozonation of MGP resulted in the formation of gluconic acid. Analysis of the product mixture from ozonation of the *threo*-isomer of GGMGP-**b** by ^{13}C NMR suggested the presence of MGP and corresponding the diastereomer of TAMGP.

A similar trend in the formation of reaction products was observed when *erythro*-benzylic ether GGMGP-**c** was ozonized and the products were analyzed (Fig. 7). The cleavage of the benzylic ether bond appeared to be somewhat higher but corresponding TAMGP was still the main product (Fig. 7). The ratio between erythronic acid and threonic acid (retention time 7.25 min and 7.40 min, respectively) in the product mixtures from the ozonation of benzylic ethers can

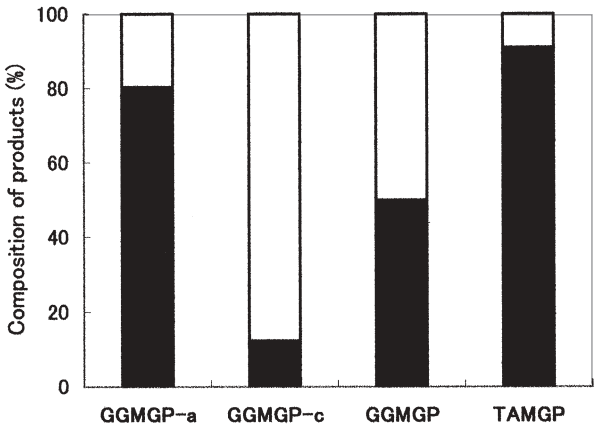

Fig. 8. Ratio between erythronic acid (*white column*) and threonic acid (*black column*) in reaction mixtures from ozonation of benzylic ethers and TAMGP

be seen in Fig. 8. It is striking that ozonation of the *erythro*-benzylic ether GGMGP-**c** leads to the formation of mostly erythronic acid while threonic acid is mostly formed by ozonation of the *threo*-isomer of GGMGP-**a**. Tetronic acid is rather stable during the ozone treatment and glyceric acid is one of the minor degradation products.[12] Isomerization of threonic acid to erythronic acid and vice versa takes place only to a low extent.[11,12] It seems therefore that the ratio between threonic acid and erythronic acid can be used to determine the *erythro* and *threo* forms of the starting benzylic ether.

It was found that once formed TAMGP was rather stable against further ozonation (3 h). A minor amount of TAGLCA and only small amounts of MGP, gluconic acid, and tetronic acid were found in the product mixture (Fig. 7). Tetronic acid was mostly in the *threo*-form (Fig. 8).

Tetronic acid was not formed[12] or was formed to a small extent[7] during ozonation of nonphenolic benzylic ether VGEt (Fig. 2). The number of other products found in the gas chromatogram (such as glyceric acid) was low. Hence, the benzylic ether VGEt was mainly converted to the corresponding tetronic acid ethyl ether TAEt (Fig. 2).[7,12]

The results obtained by ozonation of GGMGP and VGEt suggest that the benzylic ether bond is rather stable during the ozone treatment. A small amount of TAGLCA, but not TAGLC, was found in the product mixture after ozone treatment of benzylic ethers GGMGP-**a** and GGMGP-**c** (Fig. 2). The formation of TAGLCA seems to involve oxidation of the anomeric carbon in the sugar unit although the presence of a hydrolysis mechanism cannot be ruled out as an initial step.[13] It is interesting to note that the benzylic ether bond in compounds of Ph—CH_2—OR type is easily cleaved by ozone.[14,15] An initial step may involve oxidation of the benzylic carbon. Degradation of aromatic rings by ozone is enhanced by electron-donating substituents such as hydroxyl groups and methoxyl groups.[16] Because minor benzyl ether cleavage was observed for GGMGP and VGEt, the activation of the benzylic carbon (by these groups) appears to be lower than activation of the aromatic ring. In the case of ozonation of TAMGP, which is

not activated by an aromatic ring, the ratio between TAGLCA and α-ether cleavage products (MGP and tetronic acid) is larger than the ratio of these products formed by ozonation of the benzylic ether GGMGP-**a**. The ratio between tetronic acid and MGP was also found to be higher in the case of ozonation of TAMGP than for GGMGP-**a**. This can be explained by poor activation of the α-carbon in TAMGP. Low but rather stereoselective formation of threonic acid from *threo*-benzylic ether GGMGP-**a** as well as erythronic acid from the *erythro*-benzylic ether GGMGP-**c** was observed. In addition, small amounts of threonic acid (and glucopyranose) were formed during hydrolysis of TAMGP with TFA but not with sulfuric acid (see below). Therefore, a straightforward mechanism that explains the observed stereoselectivity during ozonation is not presented at this stage.

Acid treatment of ozonated benzylic ether (TAMGP)

When TAMGP produced from GGMGP-**a** was treated with 72% sulfuric acid (room temperature for 1 h) followed by 3% sulfuric acid (120°C for 1 h), some degradation products were found (Fig. 9). Most of the starting material was consumed and two main fractions were detected in the gas chromatogram (Fig. 9). GC-MS (CI) revealed that these fractions were the α and β forms of TAGLC. Small amounts of α and β-glucopyranoses and tetronic acid were also found in the product mixture (Fig. 9). Absence of a triplet at 3.25 ppm (C_2-H) in the NMR spectrum of the product mixture also suggested low amounts of glucose. Only small amounts of other low molecular weight products were found in the chromatogram (Fig. 9). Twice as much erythronic acid than threonic acid was found in the product mixture from acid treatment of TAMGP with sulfuric acid. It was found in a separate experiment that threonic acid was stable during acid hydrolysis. Neither degradation products nor isomerization to erythronic acid was observed by analysis with GC.

Hydrolysis of sugars is usually performed not only with dilute sulfuric acid but also with TFA because it is considered to be milder. Two dominating peaks were observed when TFA was used as the hydrolyzing medium (Fig. 10). Analysis of the peaks with GC-MS (CI) strongly indicated presence of the α-anomer and β-anomer of TAGLC. In the spectrogram, a mass fragment of *m/z* 658 was observed, and a rather small amount of the lactone form of TAGLC was indicated by comparison of ^{1}H NMR data in the product mixture and ^{1}H NMR data obtained from D-erythronic acid-γ-lactone. The starting TAMGP was fully consumed (Fig. 10). Only small amounts of glucose and threonic acid were observed in the gas chromatogram. Thus, both acid treatments selectively cleave the glucosidic bond in TAMGP and the extent seems to be less in the case of TFA. Minor amounts of other compounds were observed in the product mixture from acid hydrolysis (Fig. 10); however, they could not be identified from the MS data obtained.

Isolation and analysis of LCC fraction from wood

Three types of LC bonds have been suggested to exist in wood: benzylic esters, benzylic ethers, and glucosidic bonds. We have focused our studies on isolation and analysis of benzyl ether type LC bond from a softwood species, Japanese red pine.[17]

After removal of extractives the wood meal was ozonized, filtered, and washed. In this way, degradation products of lignin (mostly low molecular weight acids such as oxalic acid) that are no longer bonded to wood polymers are washed away. Because ozonation of extensively milled wood (for preparation of MWL) led to relatively fast degradation of the lignin but to some losses of carbohydrates, large wood particles (<100 mesh) were used. It was found that 4 h of treatment (4% ozone in O_2 at 0.5 l/min) of 0.3 g of wood meal from red pine could degrade the lignin completely; the yield of tetronic acid was estimated to be 24%. This is in accordance with the yield reported earlier.[11,18] In a

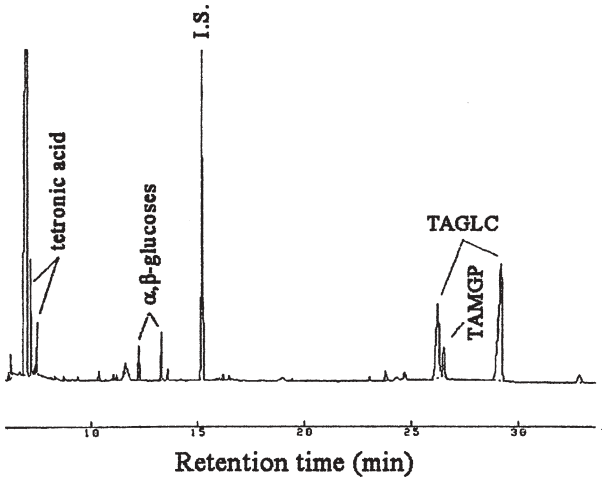

Fig. 9. GC of trimethylsilylated product mixture from treatment of TAMGP with sulfuric acid

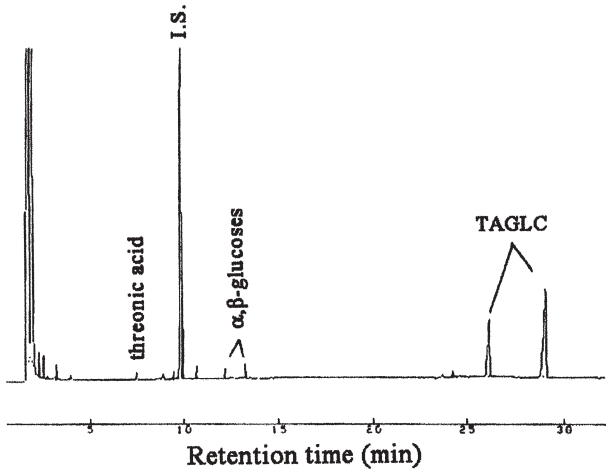

Fig. 10. GC of trimethylsilylated product mixture from treatment of TAMGP with trifluoroacetic acid (TFA)

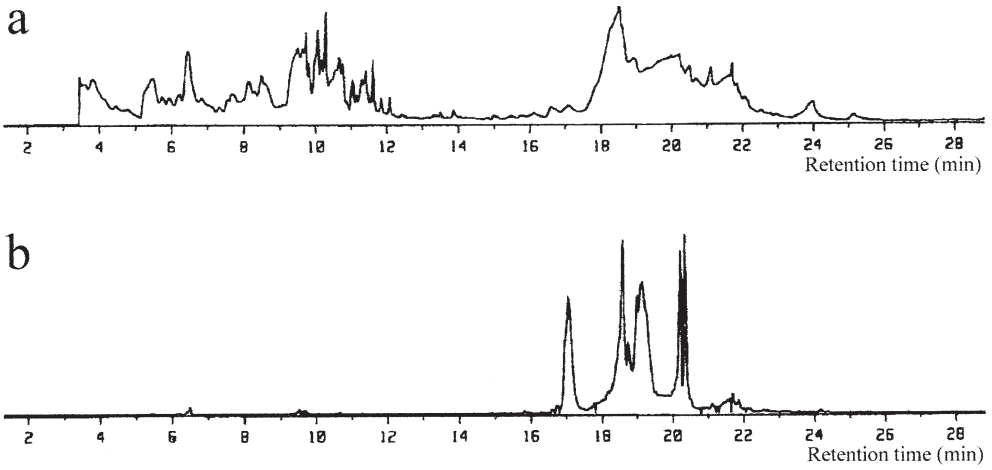

Fig. 11 a,b. GC-MS of main acid fraction isolated from red pine. **a** Total ion chromatogram. **b** Mass chromatogram at *m/z* 820

preliminary experiment, 5 mg of ozonized wood meal from pine, as well as beech, was acid hydrolysed, ion chromatographed, derivatized, and analysed by GC-MS. The results showed that the number of benzylic ether bonds was too low to be detected. When experiments were performed on a larger scale, however, the experimental procedure had to be modified because a large amount of sulfuric acid was used. The water-soluble $Ba(OAc)_2$ was found to be a good candidate to precipitate sulfate anions from the solution. Preliminary experiments using fuming TFA as a hydrolyzing reagent were not successful. The main part of the lignin was removed by ozonation of 15.0 g of red pine for 8 h. Minor amounts of Klason lignin and comparatively larger amounts of UV-absorbing (280 nm) compounds were obtained after hydrolysis of the white ozonized wood meal with sulfuric acid. Most of these UV-absorbing compounds (80%) were present in the liquor after precipitation of sulfate ions with $Ba(OAc)_2$. The liquor was concentrated by evaporation to remove some acetic acid, diluted with water, and was then applied to an anion-exchange column. Neutral components were eluted with water and then acid fractions were eluted with dilute acetic acid. Anion-exchange chromatography has been used previously for analysis of monocarboxylic acids (such as tetronic acids) in ozonized lignins.[5] In a preliminary experiment it was found that TAMGP could be easily eluted through a smaller but similar column with 1 M acetic acid. After evaporation, the main acidic fraction was trimethylsilylated and analysed by GC and GC-MS (CI). Peaks from monosaccharide derivatives and low molecular weight compounds were observed at around 16–26 min on the total ion chromatogram (Fig. 11). Figure 11 also shows the mass chromatogram at *m/z* 820. The large number of peaks on the chromatogram may be explained by the many isomers suggested to form during synthesis of benzylic ether bonds of LC type.

Conclusions

The results from ozone treatment of model compounds suggest that benzylic ether bonds between lignin and carbohydrates in wood or isolated LCC fraction are fairly stable. The ozonized product was also rather stable toward acid hydrolysis. Benzylic ether bonds between lignin and polysaccharides (hexoses and pentoses) in Japanese red pine are proposed. Benzylic ether bonds may be detected in other wood species and in other lignocellulosic materials such as kraft pulps by using the procedures described in this article. Detailed information of the bonding pattern was not obtained and needs further investigation.

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