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Studies on electro-oxidation of lignin and lignin model compounds. Part 2: *N*-Hydroxyphthalimide (NHPI)-mediated indirect electro-oxidation of non-phenolic lignin model compounds

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Abstract

The *N*-hydroxyphthalimide (NHPI)-mediated indirect electro-oxidation of non-phenolic lignin model compounds has been investigated for selective C_α-carbonylation of lignin. A cyclic voltammogram of NHPI in 0.1 M $LiClO_4/CH_3CN$ with 2,6-lutidine interpreted that NHPI can act as a mediator in the indicated process in the range 0.5–0.8 V vs. Ag/Ag^+. The corresponding C_α-carbonyl compounds was obtained in high yields (85–97%) in the case of the monomer 1-(4-ethoxy-3-methoxyphenyl) ethanol in 0.1 M $LiClO_4/CH_3CN$ or 0.1 M $LiClO_4/(CH_3CN/H_2O=7/3)$ with a small amount of 2,6-lutidine at 0.7 V vs. Ag/Ag^+. The processing of the dimeric lignin model compound (4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether) also gave the corresponding C_α-carbonyl compound in high yield (88–92%). The reaction proceeds through hydrogen atom transfer in the NHPI-mediated electro-oxidation. On the other hand, the direct electro-oxidation and indirect electro-oxidation mediated by ABTS [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate)] of the dimeric compound preferentially gave the corresponding C_α-C_β cleavage product in low or moderate yields (5–40%). The conclusion is that NHPI is an excellent mediator for selective C_α-carbonylation of non-phenolic β-O-4 structures in lignin in electronic mediator system.

Keywords: C_α-carbonylation; cyclic voltammetry (CV); hydrogen atom transfer; *N*-hydroxyphthalimide (NHPI); indirect electro-oxidation; lignin.

Introduction

The introduction of C_α-carbonyl groups (C_α-carbonylation) into non-phenolic β-O-4 substructure is one of the targets of the pre-treatment for kraft pulping, because the alkali cleavage of non-phenolic β-O-4 linkages is significantly accelerated in the presence of C_α-carbonyl groups (Gierer and Norén 1982; Ljunggren and Olsson 1984). The electrochemical oxidation is known to be a suitable mean to this purpose (Limosin et al. 1985, 1986). The electrolytic mediator system (EMS) (Figure 1a) is also promising for such reactions, because it does not require expensive and/or hazardous reagents, and it has two advantages over laccase-mediator system (LMS): (1) a wide range of pH and temperature conditions can be selected, and (2) a mediator may have a higher redox potential compared with LMS.

It is well known that C_α-carbonylation is in competition with C_α-C_β cleavage in the oxidation of lignin both in EMS and LMS (Pardini et al. 1991). However, there is little knowledge for C_α-carbonylation in the former. There are only two basic studies concerning the oxidation of non-phenolic β-O-4 lignin dimers in EMS. Pardini et al. (1991) applied *tris*-(4-bromophenyl) amine as a mediator, and the prevailing C_α-C_β cleavage yielded a product in low amount (10%). In the paper of Rochefort et al. (2002a), electro-oxidation with 1-hydroxybenzotriazole (HBT) and with 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS) afforded a $C_\alpha=O$ product in 15% and 10% yields, respectively, and C_α-C_β cleavage gave rise to product in 8% and 32.5% yields, respectively. The influence of the mediator is obvious. On the other hand, there are also several reports about pulp delignification by electrolysis with violuric acid (VA) (Kim et al. 2001; Mickel et al. 2003) and with $K_4[Mo(CN)_8]$ or iron(II) *tris*-bipyridyl complexes (Rochefort et al. 2002b). The role of mediators was not discussed in detail in these papers.

The mediators which are proposed for LMS – such as ABTS, HBT, VA, *N*-hydroxyphtalimide (NHPI), 2,2′,6,6′-tetramethylpiperidine-*N*-oxyl (TEMPO) (Galli and Gentili 2004; Morozova et al. 2007; González Arzola et al. 2009) – are also candidates for EMS, although EMS and LMS gave different results with the same mediator (Rochefort et al. 2002a). According to Fabbrini et al. (2002), the LMS oxidation of 1-(3,4-dimethoxyphenyl)- 2,2-dimethyl-1-propanol with HBT and NHPI afforded only the corresponding α-ketone in 30% and 50% yields, respectively. Annunziatini et al. (2005) oxidized 1-(3,4-dimethoxyphenyl)-2-phenoxyethanol in LMS with NHPI and found the corresponding α-ketone in 13.1% yield.

In the present study, NHPI was the mediator in EMS for C_α-carbonylation, considering the results in preliminary

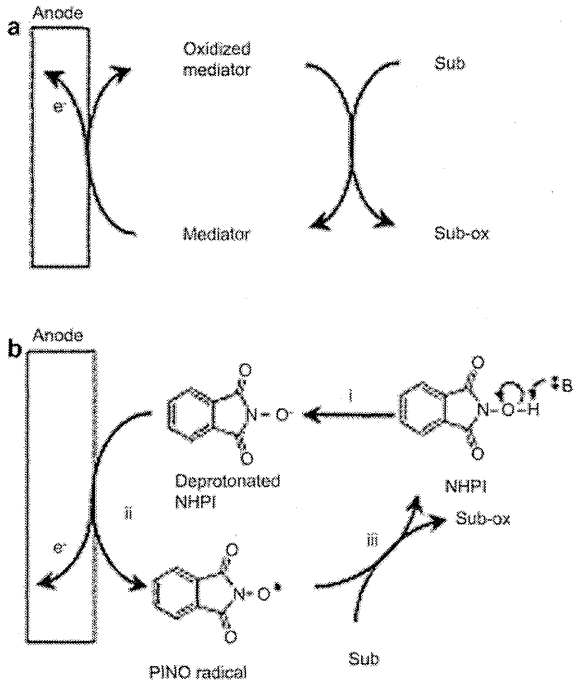

Figure 1 Indirect oxidation with a redox catalyst. (a) The principle of the reaction and (b) the catalytic cycle of NHPI mediated electro-oxidation.

screening experiments. NHPI is known to be a powerful and common catalyst for organic oxidation reactions (Coseri 2009). However, there is only one report concerning the electro-oxidation of lignin model compound in EMS with NHPI, in which the oxidation of veratryl alcohol was studied by cyclic voltammetry (Kishioka and Yamada 2005). This paper describes the results in EMS/NHPI of non-phenolic lignin model compounds concerning the selective C_α-carbonylation.

Materials and methods

Non-phenolic lignin model compounds 1G, 1S, 1P, and 3G were prepared as described by Shiraishi et al. (2011). Other chemicals were used as obtained from Nacalai Tesque Inc. (Kyoto, Japan).

^{1}H-NMR spectroscopy: Varian INOVA 300 FT-NMR (300 MHz) spectrometer (solvent: $CDCl_3$, internal standard: TMS).

Cyclic voltammetry (CV) measurements were performed according to the method in the previous paper (Shiraishi et al. 2011). Bulk electrolyses of monomers (1G, 1S, or 1P) were performed using NHPI (0.1–0.2 eq. of the substrate) at 0.7 V vs. Ag/Ag^+. Other reaction conditions were described in the previous paper (Shiraishi et al. 2011).

Bulk electrolysis of compound 3G (0.05 mmol) with NHPI and 2,6-lutidine was carried out at 0.7 V vs. Ag/Ag^+ in 0.1 M $LiClO_4$ in CH_3CN (10 ml) or CH_3CN/water (7/3, v/v) (10 ml) in a divided cell until the current dropped to about 1 mA. Electrodes: 1.5×2.0 cm^2 carbon felt working electrode; an Ag/Ag^+ reference electrode; a platinum wire counter electrode. The reaction mixture was treated at 0 V vs. Ag/Ag^+ for 2 min for reduction of the oxidized mediator.

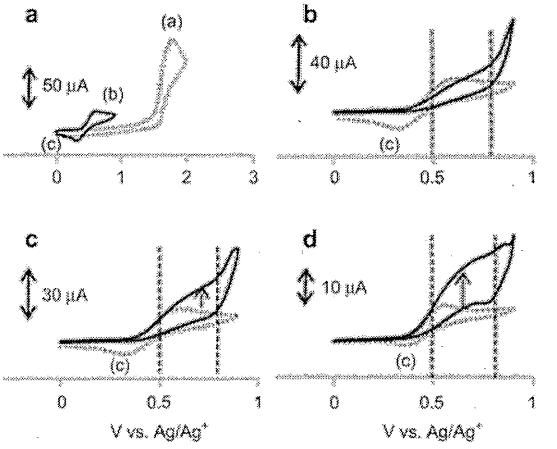

Figure 2 Cyclic voltammograms of NHPI. (a) in the system without a catalyst in 0.1 M $LiClO_4/CH_3CN$ (dotted line) with 2,6-lutidine (solid line); scan rate 0.1 V s^{-1} (b–d) in the system with compound 1G (solid line) and without substrate (dotted line); scan rate 0.1 V s^{-1} (b), 0.05 V s^{-1} (c), 0.01V s^{-1} (d).

For quantitative study, CH_3CN (0.5 ml) containing 18 μmol benzophenone was added. The anolyte was extracted by the conventional method to give a colorless oil. The product was silylated with *N,O*-bis(trimethylsilyl)acetamide/pyridine, and subjected to GC. Conditions: fused silica capillary column (DB-1, 30 m×0.25 mm i.d., coated with 0.25 μm 100% dimethylpolysiloxane, J & W Scientific, Folsom, CA); temp. program: 180°C (10 min)→10°C min^{-1}→250°C (60 min); injector temp. 270°C; detector temp. 270°C; carrier gas He (0.1 MPa).

For structure determination, the anolyte was evaporated, extracted with EtOAc. The organic layer was washed with 0.1 M HCl, saturated $NaHCO_3$ solution and distilled water; drying over Na_2SO_4. The concentration gave a colorless oil, which was recrystallized from EtOAc to afford compound 4G, which was identified by NMR spectroscopy (Crestini and D'Auria 1997).

Results and discussion

Cyclic voltammetry (CV) of NHPI

The CV of NHPI in 0.1 M $LiClO_4/CH_3CN$ was studied to determine the applied oxidation potential of the electro-oxidation (EO). $LiClO_4$ as supporting electrolyte can be easily removed after electrolysis. Figure 2a shows the results without a catalyst. Oxidation peak (a) at 1.8 V was found, which is thought to be the peak by several electrons withdrawing, because a shoulder peak at 1.3 V is present in CV of NHPI in 0.1 M tetrabutylammonium perchlorate (TBAP)/CH_3CN (data not shown), and a broad peak at 1.3 V was reported in CV of NHPI in 0.1 M TEAP/CH_3CN (Kishioka and Yamada 2005). The oxidation potentials (1.3–1.8 V) are higher than those of non-phenolic lignin model compounds (for example, 1.1 V for compound 1G, see Shiraishi et al. 2011). Accordingly, NHPI does not work as a mediator in the system without a catalyst.

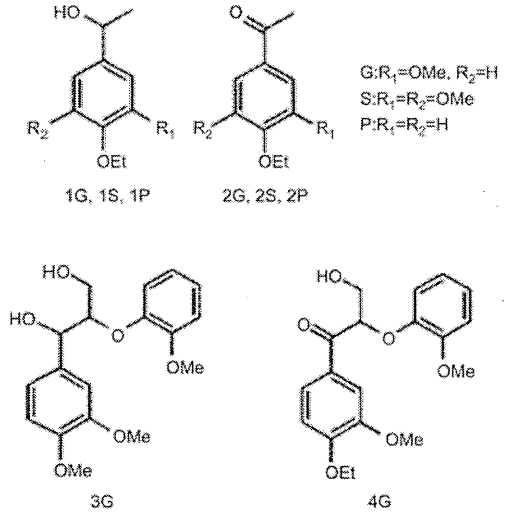

Figure 3 Non-phenolic lignin model compounds investigated in the present study.

The anodic peak potential of *N*-hydroxylamine derivatives, such as NHPI, is known to be shifted in a negative direction in the presence of a base because of their acidic nature (Serve 1975; Masui et al. 1983; Gorgy et al. 1998; Kishioka and Yamada 2005, 2006). Figure 2a shows the CV of NHPI in 0.1 M $LiClO_4/CH_3CN$ with 2,6-lutidine (5.0 eq of NHPI) as a base. The oxidation peak (b) at 0.5 V and the reduction peak (c) at 0.4 V, which are corresponding to the single-electron exchange between the deprotonated NHPI (>N-O$^-$) and phthalimide-*N*-oxyl (PINO) radical (>N-O$^\bullet$) (Figure 1b, reactions ii), can be observed.

The oxidation peak current of mediators is enhanced in the presence of a substrate (Ueda et al. 1987; Bourbonnais et al. 1998; Gorgy et al. 1998; Kishioka and Yamada 2005; González Arzola et al. 2009). Figure 2b–d display the CVs of NHPI in the presence of compound 1G (2.0 eq of NHPI) and 2,6-lutidine (5.0 eq of NHPI) in 0.1 M $LiClO_4/CH_3CN$ at various scan rate. The current in the potential 0.5–0.8 V range is enhanced in the CV at 0.05 V s^{-1} or 0.01 V s^{-1} (Figure 2c and d), although remarkable enhancement cannot be detected in the CV at 0.1 V s^{-1} (Figure 2b). The reduction peak of PINO radical to deprotonated NHPI (corresponding to peak c in Figure 2a) was not observed in the CVs at any

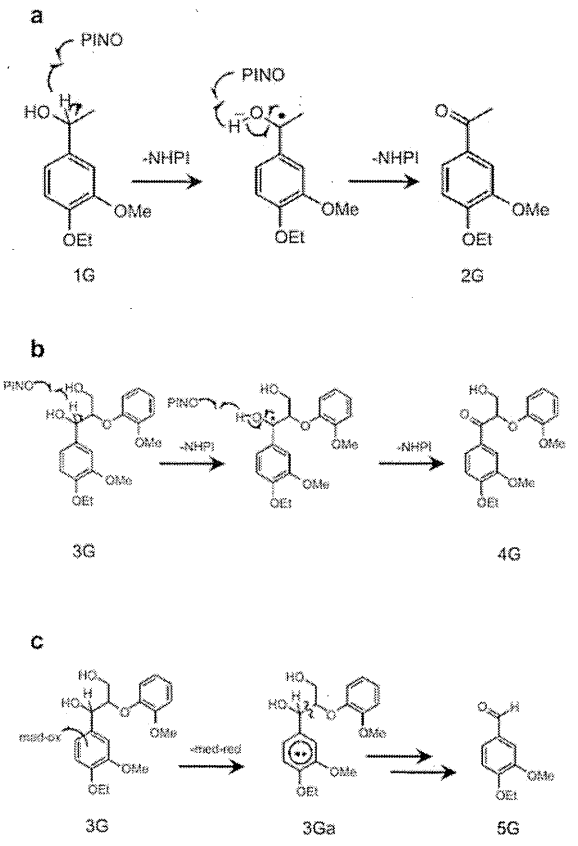

Figure 4 Proposed reaction mechanism of the indirect electro-oxidation (EO). (a) Reaction mechanism of NHPI-mediated indirect EO of compound 1G via H atom transfer route. (b) Reaction mechanism of NHPI-mediated indirect EO of compound 3G via H atom transfer route. (c) Reaction mechanism of C_α-C_β cleavage of compound 3G via electron transfer route.

scan rate, indicating that PINO radical was consumed rapidly. The enhancement of anodic current can be explained by the efficiency of the reactions presented in Figure 1b, as PINO radical reacts with compound 1G to regenerate NHPI (reaction iii) and the regenerated NHPI reacts again (reactions i and ii). Based on a time scale of CV at 0.1 V s^{-1}, it is probably that NHPI was scarcely regenerated, because of the slow rate of reactions i and iii. It can be concluded that

Table 1 Bulk electrolyses of 1-(4′-ethoxyphenyl) ethanol. See model compound 1G, 1S, and 1P in Figure 3.

Substrate	NHPI (eq)	Solvent	Potential (V vs. Ag/Ag$^+$)	2,6-Lutidine (eq)	Yield[a] (%)	Electricity (F mol^{-1})	CE[b] (%)
1G	0.2	MeCN	0.7	2	94.1	2.08	90.5
1G	0.2	MeCN	0.7	5	97.2	2.18	89.3
1S	0.2	MeCN	0.7	2	92.2	1.93	95.5
1S	0.2	MeCN	0.7	5	96.3	1.97	85.3
1P	0.2	MeCN	0.7	5	84.8	2.28	74.3
1G	0.2	MeCN/H_2O (7/3)	0.7	5	94.1	2.23	84.2
1S	0.2	MeCN/H_2O (7/3)	0.7	5	98.3	2.43	80.9
1P	0.2	MeCN/H_2O (7/3)	0.7	5	94.2	2.46	76.5

[a]Yields were evaluated by GC with benzhydrol as IS; [b]current efficiency.

Table 2 Bulk electrolyses of 4-ethoxy-3-methoxyphenylglycerol-β-guaiacyl ether (3G in Figure 3).

Mediator	(eq)	Solvent	Pot. (V vs. Ag/Ag^+)	2,6-Lutidine (eq)	Yield[a] (%) 4G	5G	Electricity ($F\ mol^{-1}$)	CE[c] (%)
NHPI	0.1	MeCN	0.70	5	61.3	n.d.[b]	1.58	77.5
NHPI	0.2	MeCN	0.70	5	88.3	n.d.[b]	2.21	79.8
NHPI	0.2	MeCN/H_2O (7/3)	0.70	5	92.3	n.d.[b]	2.23	82.7
ABTS	0.2	MeCN/H_2O (7/3)	0.75	5	2.8	5.5	1.03	1.3

[a]Yields were evaluated by GC with benzophenone as IS; [b]not detected; [c]current efficiency.

NHPI has a high potential to be a good mediator in EMS of non-phenolic lignin model compounds in the potential range of 0.5–0.8 V.

Results with non-phenolic monomers

The non-phenolic monomers 1G, 1S, 1P (Figure 3) were submitted to EO in 0.1 M $LiClO_4/CH_3CN$ in the presence of NHPI (0.2 eq of the substrate) and 2,6-lutidine (5.0 eq of the substrate) at 0.7 V vs. Ag/Ag^+, at which the monomers are not oxidized by direct electrolysis in the anolyte. The results are listed in Table 1. The corresponding C_α-carbonyl compounds 2G, 2S, and 2P arose in 97.2, 96.3, and 84.8% yields, respectively. These yields are similar but higher than those in direct EO (Shiraishi et al. 2011). In other words, C_α-carbonylation in NHPI-mediated indirect EO is more efficient. A small amount of 2,6-lutidine (5.0 eq) was enough to promote this type of reaction, whereas 20.0 eq 2,6-lutidine was required for direct EO. In the indirect EO, the reaction proceeds through two hydrogen atom transfer steps (Cantarella 2003), in which PINO radical directly withdraws hydrogen atom radical at C_α-H from the substrate, leading to an easy C_α-carbonylation (Figure 4a).

Direct EO of aromatic compounds in an aqueous medium is inefficient at high potentials, at which electrolysis of water itself occurs (Weinberg and Weinberg 1968). Thus, the NHPI-mediated indirect EO of the monomers 1G, 1S, 1P was performed in 0.1 M $LiClO_4/(CH_3CN/H_2O = 7/3$ by vol). The indicated mixture ratio was needed for the solution of dimer 3G. C_α-carbonyl compounds 2G, 2S, and 2P were also obtained in excellent yields with good current efficiency.

Results with a non-phenolic dimer 3G

The non-phenolic β-O-4 dimeric model 3G was submitted to the reaction indicated in 0.1 M $LiClO_4/CH_3CN$ in the presence of 2,6-lutidine at 0.7 V vs. Ag/Ag^+. The NMR spectrum of the resulting product is in agreement with that of authentic sample with $C_\alpha = O$ compounds 4G (Crestini and D'Auria 1997). The quantitative results are listed in Table 2. The compound 4G arose in 88.3% yield, and 4-O-ethyl vanillin (a C_α-C_β cleavage product, 5G) was not detected. In the paper of Shiraishi et al. (2011), the direct EO of compound 3G led to 5G in ca. 40% yield, and compound 4G was not detected. Accordingly, NHPI-mediated indirect EO is better suited for C_α-carbonylation of the monomeric and dimeric model compounds with good current efficiency. Probably, the reaction of 3G proceeded via hydrogen atom transfer depicted in Figure 4b, whereas the direct oxidation occurs via electron transfer (Figure 4c). Fabbrini et al. (2002) interpreted their results in a similar way.

The reaction discussed here led to $C_\alpha = O$ compound 4G in 92.3% yield. Thus, an aqueous medium does not affect the indirect oxidation. On the other hand, ABTS-mediated indirect EO of compound 3G under the same conditions afforded compounds 4G and 5G in 2.8% and 5.5% yields, respectively. Probably, the reaction proceeded via electron transfer.

Conclusion

NHPI-mediated indirect electro-oxidation of non-phenolic monomers 1G, 1S, and 1P with a small amount of 2,6-lutidine gave the corresponding C_α-carbonyl compounds 2G, 2S, and 2P in high yield as well as the direct electro-oxidation. In the NHPI-mediated electro-oxidation of non-phenolic β-O-4 dimer 3G, C_α-carbonylation exclusively proceeded to give the corresponding C_α-carbonyl compound 4G in excellent yield, whereas in the direct electro-oxidation and in ABTS-mediated indirect electro-oxidation, C_α-C_β cleavage product was preferentially obtained in low yield. These results might be explained by the differences of oxidation mechanism (hydrogen atom transfer system for the former and electron transfer system for the two latter). It can be hypothesized that NHPI-mediated indirect electro-oxidation is a promising EMS for C_α-carbonylation of non-phenolic β-O-4 structures in lignin.

Acknowledgements

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Fractionation and characterization of lignin carbohydrate complexes (LCCs) of *Eucalyptus globulus* in residues left after MWL isolation. Part I: Analyses of hemicellulose-lignin fraction (HC-L)

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Abstract

The residual wood meal left after milled wood lignin (MWL) isolation (MWR) was extracted with the cellulose solvent lithium chloride/*N,N*-dimethylacetamide (LiCl/DMAc) to obtain a soluble fraction (C-L) and an insoluble fraction (C-L-residue). The C-L-residue was further extracted with the hemicellulose solvent 3 M NaOH to give a soluble fraction named hemicellulose-lignin fraction (HC-L) with 21.3% yield based on MWR. It was found that HC-L was composed of xylan, cellulose and lignin with abundant S-type β-O-4 substructures. HC-L lignin was bonded to HC-L cellulose or HC-L hemicelluloses or both. The method, which comprised acetylation for hardwood xylan (by acetic anhydride/pyridine/formamide) and extraction with chloroform, was found to be effective for selective xylan acetate fractionation. HC-L was further fractionated by the same method and subsequent deacetylation to give a xylan-lignin fraction (X-L) in 11.3% yield based on HC-L. X-L was composed mainly of xylan and lignin with abundant S-type β-O-4 substructures, and bonded to X-L xylan. X-L is considered as a promising fraction for elucidation of the structure of lignin-carbohydrate linkages.

Keywords: *Eucalyptus globulus*; lignin; lignin-carbohydrate complex (LCC); lignin-carbohydrate linkages; size exclusion chromatography (SEC); xylan; xylan acetate.

Introduction

The moiety of the cell wall in which lignins and polysaccharides are covalently bonded can be isolated as lignin-carbohydrate complexes (LCCs) (Lawoko et al. 2005; Li et al. 2011). The lignin-carbohydrate (LC) linkages prevent a complete separation of the macromolecular components of the wood.

The main LC linkages are phenyl glycosidic bonds, esters, and benzyl ethers (Koshijima and Watanabe 2003; Balakshin et al. 2007). The elucidation of the structure of LC linkages in native samples is still a challenge, although many experiments with model compounds have given a clear idea of their possible structure (Koshijima and Watanabe 2003; Barakat et al. 2007; Kishimoto 2009). There are two approaches to LC analysis: (1) direct analysis of native samples and fractions from them by means of ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy; (2) isolation of degradation products containing the original LC linkages from a native sample. The former method was recently applied to the qualification of the LC linkages (Balakshin et al. 2011). Overlapping of signals in the NMR spectra complicates their interpretation, because it is difficult to isolate the less complex fractions that contain the original LC linkages. As to the latter method, there are few reports concerning such degradation products (Karlsson et al. 2004). The TIZ method (Katahira et al. 2003) and γ-TTSA method (Ando et al. 2012) have the potential to obtain the relevant degradation products, as they degrade β-O-4 linkages while keeping the α-linkages intact and permit a qualitative analysis of the fractions obtained. Fractionation of the degradation products is also attractive for NMR spectroscopy because of their simple composition.

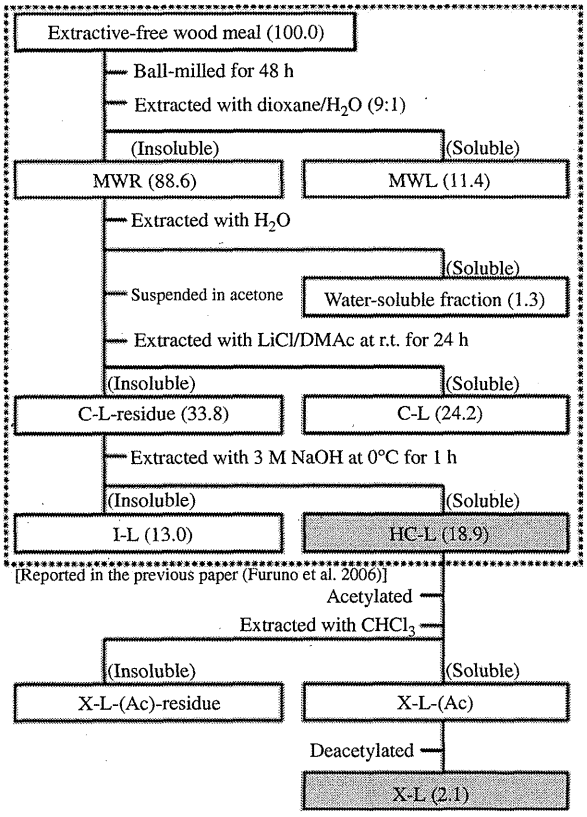
Our group also proposed a fractionation method based on the cellulose solvent lithium chloride/*N,N*-dimethylacetamide (LiCl/DMAc) and the hemicellulose solvent 3 M NaOH aqueous solution (Hirosawa et al. 2002). After milled wood lignin (MWL) extraction of *Eucalyptus globulus,* fractionation of the residue (MWR) gave three major fractions: the cellulose-lignin fraction (C-L), the hemicellulose-lignin fraction (HC-L), and the insoluble lignin fraction (I-L), as illustrated in Figure 1. The first major fraction, C-L, was found to be composed mainly of cellulos with small amounts of hemicelluloses and lignin, although it contained the LC linkages between lignin and xylan (Furuno et al. 2006).

This paper describes the characterization of the second major fraction HC-L and its further fractionation into fractions that consist mainly of lignin and xylan.

Materials and methods

Materials

Wood meal of 5-year-old *Eucalyptus globulus* (obtained from the experimental forest of Kyoto University in Japan; 60-mesh pass) was



( )= Yield based on extractive-free wood meal

Figure 1 Fractionation scheme of extractive-free wood meal.

investigated. The wood meal was fractionated by the conventional method to give crude MWL and MWR in 11.4% and 88.6% yields, respectively (Furuno et al. 2006). Xylan (from oats spelts, Tokyo Chemical Industry, Tokyo, Japan), cellulose microcrystalline (Avicel, Merck, Darmstadt, Germany), glucomannan (Wako Chemical Co., Osaka, Japan), were used in the model experiments for the selective xylan acetate fractionation as described below. Cellulase (Celluclast 1.5L FG, 84 000 U ml^{-1}, from *Trichoderma reesei*) was supplied by Novozymes Japan, Chiba, Japan). It was diluted 100-fold with distilled water before use. Xylanase (2 U ml^{-1}, from *Bacillus subtillis*) was supplied by Oji Paper Co. (Tokyo, Japan). The enzymes were used without further purification. All other chemicals were purchased from commercial sources and used as received.

Preparation of hemicellulose-lignin fraction (HC-L)

MWR (30.1 g) was stirred in distilled water (1350 ml) at room temperature (r.t.) for 24 h and centrifuged (10 000 rpm, 10 min). The precipitate was washed with acetone and stirred in acetone (450 ml) at r.t. for 24 h. The suspension was centrifuged (10 000 rpm, 10 min). The precipitate was washed with DMAc and stirred in DMAc (1200 ml) at r.t. for 3 h. LiCl (90 g) was added. The suspension was stirred at r.t. for 24 h and centrifuged (10 000 rpm, 10 min). The residue was washed twice with DMAc and five times with distilled water to remove DMAc, and lyophilized to give an amorphous powder (C-L-residue; 11.5 g, 38.2% yield based on MWR).

C-L-residue (10.4 g) was stirred in distilled water (225 ml) at r.t. over night. 6 M NaOH aqueous solution (225 ml) was added to the suspension at 4°C. The mixture was stirred at 0°C for 1 h and centrifuged (10 000 rpm, 10 min). The precipitate was washed twice with 1 M NaOH solution. The combined supernatant and washings were acidified with excess acetic acid until precipitation occurred. The precipitate was collected by centrifugation (10 000 rpm, 10 min), washed several times with distilled water until the filtrate was at pH 6 and lyophilized to give an amorphous powder (HC-L; 6.4 g, 55.7% yield based on C-L-residue).

Model experiments for fractionation of xylan acetate

The sample [xylan, or cellulose, or glucomannan (1 mM)] was stirred in formamide (6 ml) at r.t. overnight. Pyridine (8 ml) and acetic anhydride (Ac_2O) (1 ml) were added to the suspension at 0°C. After stirring for 7 h, Ac_2O (1 ml) was added again. The reaction mixture was kept at r.t. for 24 h, and then added dropwise to 2% HCl solution at 0°C. The resulting precipitate was collected by centrifugation (10 000 rpm, 10 min), washed several times with distilled water and lyophilized to give the corresponding acetylated product [weight percent gain (WPG) of the acetlylated product (the acetlylated product/starting material, w/w): xylan 106.8%, cellulose 105.6%, and glucomannan 55.0%].

The acetylated product was suspended in $CHCl_3$ (10 ml) at r.t. for 24 h, and filtered with $CHCl_3$ to remove an insoluble fraction of the acetylated product. The combined filtrate and washings were evaporated to give a soluble fraction of the acetylated product [yield of the soluble fraction (the soluble fraction/the acetylated product, w/w): xylan 54.0%, cellulose 4.1%, and glucomannan 1.6%].

Preparation of xylan-lignin fraction (X-L)

HC-L (2.7 g) was stirred in formamide (60 ml) at r.t. overnight. Pyridine (80 ml) and Ac_2O (10 ml) were added to the suspension at 0°C. After stirring at 0°C for 7 h, Ac_2O (10 ml) was further added at r.t. and the mixture was kept for 24 h, and then added dropwise to 2% HCl solution at 0°C. The resulting precipitate was collected by centrifugation (10 000 rpm, 10 min), washed five times with distilled water and lyophilized to give an amorphous powder (3.6 g). This was stirred in $CHCl_3$ (20 ml) for 24 h and centrifuged (18 000 rpm, 10 min). The precipitate was washed three times with $CHCl_3$ to give acetate of xylan-lignin residue (X-L-(Ac)-residue; 2.92 g). The combined supernatant and washings were concentrated to give acetate of xylan-lignin fraction (X-L-(Ac); 0.68 g).

Some of X-L-(Ac) (0.49 g) was suspended in 20% MeOH/$CHCl_3$ (10 ml). The rest of X-L-(Ac) (0.19 g) was kept for analysis. 28%-NaOMe in MeOH (a few drops by disposal pipette) was added to the solution. The reaction mixture was stirred at r.t. overnight, neutralized with acetic acid, and dialyzed in a dialysis tube [Spectra/Por dialysis membrane from Spectrum Japan, Shiga, Japan; molecular-weight cutoff (MWCO) = 1000) against distilled water]. The dialysate was freeze dried to give an amorphous powder (xylan-lignin fraction (X-L); 0.22 g).

Enzymatic treatment of the fractions

The treatment conditions are summarized in Table 1. The fraction (HC-L or X-L) was added to 0.1 M phosphate buffer solution (pH=6.5). After the suspension was stirred at r.t. for 30 min, the enzyme was added. The mixture was stirred at 50°C for 5 days, filtered, washed several times with distilled water, and lyophilized to give the corresponding residue (ct-HC-L-R, or xt-HC-L-R, or xt-X-L-R).

Table 1 Results of enzymatic treatments of the fractions.

	Enzymatic treatment conditions[a]						Analysis of the residue							
							Neutral sugars in hydrolysate (%)[b]						Lignin	
No.	Sample (mg)		Enzyme (ml)		Buffer (ml)	Residue	Yield (%)	Rha	Ara	Xyl	Man	Gal	Glc	content (%)[c]
A-1	HC-L	971.7	Cellulase	6.1	101	ct-HC-L-R	76.2	0.3	1.4	41.1	-	3.0	54.2	32.1 (59)
A-2	HC-L	485.3	Xylanase	3.0	51	xt-HC-L-R	76.3	0.6	1.1	3.7	1.7	2.0	91.0	30.2 (55)
B-1	X-L	175.5	Xylanase	1.1	18	xt-X-L-R	42.0	0.9	0.9	10.1	7.0	2.2	78.9	38.9 (39)

[a]Conditions: 0.1 M phosphate buffer (pH=6.5); temperature 50°C; time 5 days. [b]Relative amount (sum of neutral sugars=100%). [c]Determined by the acetyl bromide method. Values in parentheses are percentage of lignin based on the fractions before enzymatic treatment. HC-L, hemicellulose-lignin fraction; X-L, xylan-lignin fraction; Rha, rhamnose; Ara, arabinose; Xyl, xylan; Man, mannose; Gal, galactose; Glc, glucose.

Characterization of the fractions

Neutral sugars were determined by the alditol-acetate method (Borchardt and Piper 1970). The lignin contents were determined as Klason residue or by the acetyl bromide method (280 nm, absorption coefficient 20.091 g^{-1} cm^{-1}) (Iiyama and Wallis 1988). Alkaline nitrobenzene oxidation was conducted according to a modification by Katahira and Nakatsubo (2001). Fourier-transform infra-red (FT-IR) spectroscopy was carried out using a Shimadzu 8600 PCs FT-IR spectrophotometer (Shimadzu Corporation, Kyoto, Japan) (KBr pellet method; resolution: 4.0; number of scans: 100).

The fractions (HC-L, ct-HC-L-R, xt-HC-L-R, xt-X-L-R) were acetylated by the method reported by Lu and Ralph (2003) before ^{1}H-NMR and size-exclusion chromatography (SEC) measurements. For example, HC-L (840 mg) was stirred in dimethyl sulfoxide/*N*-methylimidazole (DMSO/NMI) (2:1, vol./vol., 24 ml) at r.t. for 1.5 h. Acetic anhydride (4.8 ml) was added to the suspension. The reaction mixture was stirred at r.t. for 1.5 h, and then added dropwise to distilled water. The resulting precipitate was collected by centrifugation (15 000 rpm, 10 min), washed five times with distilled water and lyophilized to give acetate of HC-L (HC-L-(Ac); 1000 mg). X-L was also re-acetylated by the same procedure to give acetate of X-L (X-L-(Ac)-2). ^{1}H-NMR spectroscopy was carried out using a Varian INOVA300 FT-NMR (300 MHz) spectrometer (Varian Medical Systems, Palo Alto, CA, USA) [tetramethylsilane (TMS) as the internal standard in $CDCl_3$]. Size-exclusion chromatography (SEC) was carried out using a Shimadzu LC-10 system equipped with a Shimadzu UV-Vis detector (SPD-10Avp) and a Shimadzu refractive index detector (RID- 10A) (Shimadzu Corporation) (columns: Shodex K-802, K-802.5, K-805 in series (Showa Denko K.K., Tokyo, Japan); column temperature: 40°C; eluent: $CHCl_3$; flow rate: 1.0 ml min^{-1}; polystyrene (Shodex) standards; UV detection at 280 nm).

Results and discussion

Preparation of HC-L

Milled wood lignin (MWL) was isolated from 5-year-old *E. globulus* wood and the residue left behind afforded MW residue (MWR) with 88.6% yield. The MWR was further fractionated according to the method of Furuno et al. (2006) as illustrated in Figure 1. That is, fractionation was performed with water, and with the cellulose solvent LiCl/DMAc and resulted in an insoluble fraction (C-L residue) with 38.2% yield based on MWR (or 33.8% yield based on extractive-free wood meal). The C-L residue was further fractionated with the hemicellulose solvent 3 M NaOH to produce a soluble fraction (HC-L) with 55.7% yield based on C-L residue (or 18.9% yield based on extractive-free wood meal).

Characterization of HC-L

As shown in Table 2, HC-L is composed of xylan, cellulose, and lignin. These data are supported by ^{1}H-NMR spectrum of HC-L-(Ac) shown in Figure 2a. The signals were assigned on the basis of published data (Evtuguin et al. 2003; Heinze and

Table 2 Results of fraction analyses.

		Neutral sugars in hydrolysates (%)[a]						Lignin analysis		
No.	Sample	Rha	Ara	Xyl	Man	Gal	Glc	Content (%)	SA/VA[b]	SA+VA (%)[b]
A	HC-L	0.5	1.3	35.9	1.0	1.8	59.4	39.4[c]/41.6[d]	5.2	55.3
B	X-L	1.1	0.7	75.4	–	2.3	20.5	41.8[d]	6.5	37.3
		(28)	(7)	(27)	–	(16)	(4)	(11)	–	–
C	Extr.-free WM	1.0	0.9	24.2	1.4	1.6	70.9	22.1[c]	4.2	54.8
D	MWL	6.1	6.1	66.7	12.1	6.1	3.0	95.9[c]	2.5	37.7
E	MWR	0.6	0.9	20.1	1.5	1.7	75.2	20.1[c]	5.5	47.2
F	C-L residue	1.0	2.1	73	1.0	3.9	18.9	32.5[c]	4.9	57.2

[a]Relative amount (sum of neutral sugar=100%). [b]By nitrobenzene oxidation. [c]Klason lignin. [d]By acetyl bromide method. X-L data in parentheses are percentages of the compounds based on the level in HC-L. C-L, cellulose-lignin fraction; HC-L, hemicellulose-lignin fraction (see Figure 1); Extr.-free WM, extractive-free wood meal; MWL, milled wood lignin; MWR, milled wood residue; SA, syringaldehyde; VA, vanillin; X-L, xylan-lignin fraction (see Figure 1).

Liebert 2004; Kishimoto et al. 2008). The signals at 5.0, 4.7, 4.4 and 3.3 p.p.m. are assigned to fractions H-3, 2, 1 and 5a of xylan acetate, respectively, and the signals at 5.0, 4.8, 4.4, 4.1, and 3.5 p.p.m. are assigned to fractions H-3, 2, 1, 6a, 6b, and 5 of cellulose acetate, respectively. The signals from cellulose acetate H-4 and xylan acetate H-4, H-5b seem to overlap with the signal at 3.7 p.p.m. from the methoxy group of lignin. The signals at 6.6 and 6.0 p.p.m. are attributable to aromatic protons in syringyl units and H-α of the β-O-4 substructure, respectively. In Table 2, the ratios of syringaldehyde/vanillin (SA/VA) and the sum of SA+VA of HC-L are higher than those of MWL. These results show that HC-L is made up of more non-condensed S units, significantly differing from MWL. The band at 1740 cm^{-1} in the spectrum of C-L residue (Figure 3a) is the result of C=O stretching of esters, and had disappeared in the spectrum of HC-L (Figure 3b) because the ester-type LC linkages were lost during preparation.

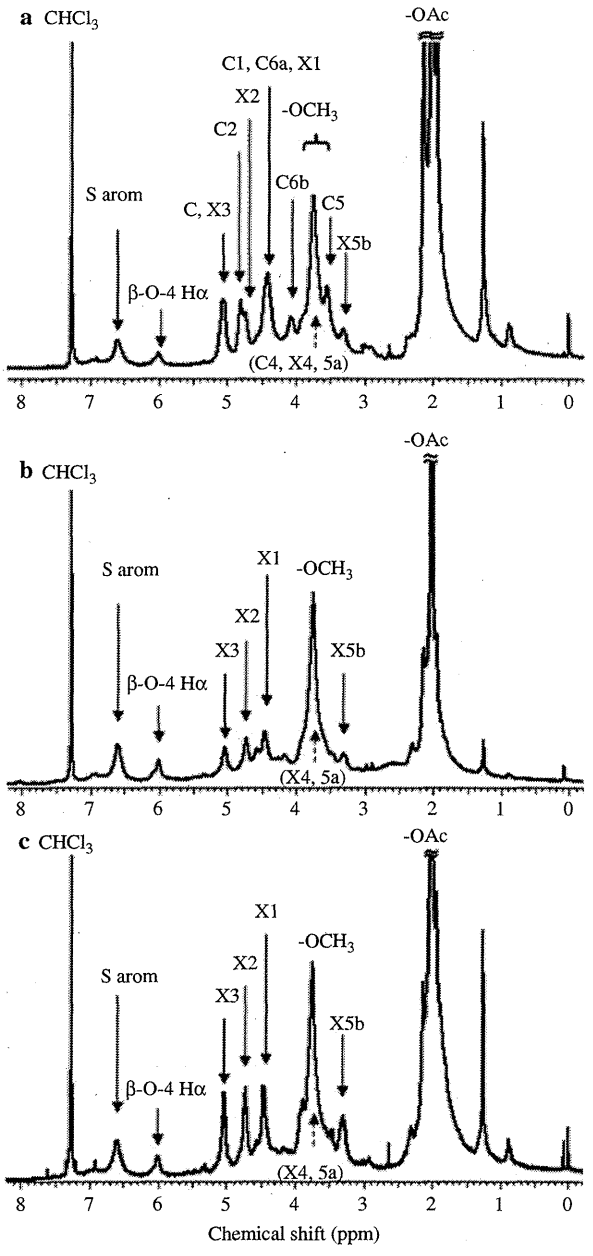


Figure 2 ^{1}H nuclear magnetic resonance (NMR) spectra of a) HC-L-(Ac) b) X-L-(Ac) and c) X-L-(Ac)-2; C, cellulose; X, xylan.

Figure 4a shows the molecular mass distribution (MMD) of HC-L-(Ac). In SEC analysis, the refractive index (RI) detector is sensitive to all compounds, whereas the ultraviolet (UV) detector is specific for lignin. The RI and UV elution curves have nearly identical elution profiles. This can be interpreted to mean that LC linkages are present, i.e., that HC-L lignin is probably covalently bonded to HC-L cellulose or HC-L xylan, or both. LC linkages were also shown to be present by the conventional technique of treatment with carbohydrate-degrading enzyme followed by SEC analysis (Karlsson et al. 2001). First, HC-L was treated with cellulase under the conditions set out in Table 1 to give a residue (ct-HC-L-R) with 76.2% yield. The cellulase treatment did not change significantly the neutral sugar composition (Table 1), but D-xylose was removed to some extent with the removal of D-glucose. Removal of D-xylose is the result of xylanase activity in cellulase, which was demonstrated in a preliminary experiment. However, 41% of HC-L lignin was solubilized. Figure 4b shows the MMD of ct-HC-L-R-(Ac). The RI and UV elution curves of HC-L-(Ac) were shifted to the region of low MM after cellulase treatment. These results prove that HC-L lignin (especially the solubilized lignin) is bonded to HC-L cellulose or HC-L xylan or both. Next, HC-L was treated with xylanase under the conditions described in Table 1, which led to a xylanase treatment residue (xt-HCL-R) with 76.3% yield. The analysis results, in terms of neutral sugar and lignin of xt-HC-L-R, are also shown in Table 1. As for xylanase treatment, most of the D-xylose was selectively removed. The characteristic signals from xylan had disappeared from the ^{1}H-NMR spectrum of xt-HC-L-R-(Ac) (data not shown). It was also confirmed in a preliminary experiment that the xylanase did not

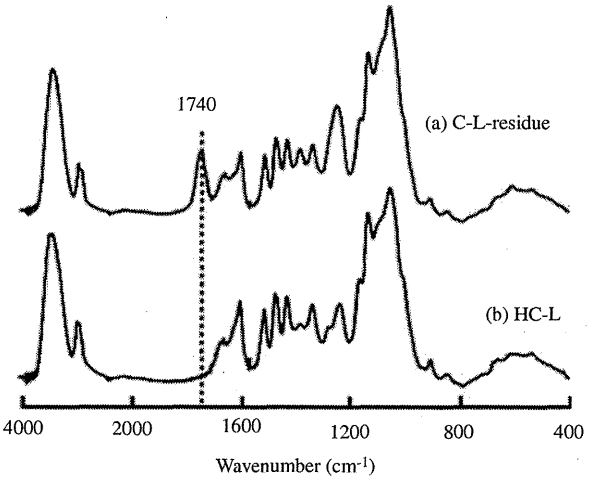


Figure 3 Fourier-transform infra-red (FT-IR) spectra of a) cellulose-lignin (C-L) residue and b) hemicellulose-lignin fraction (HC-L).

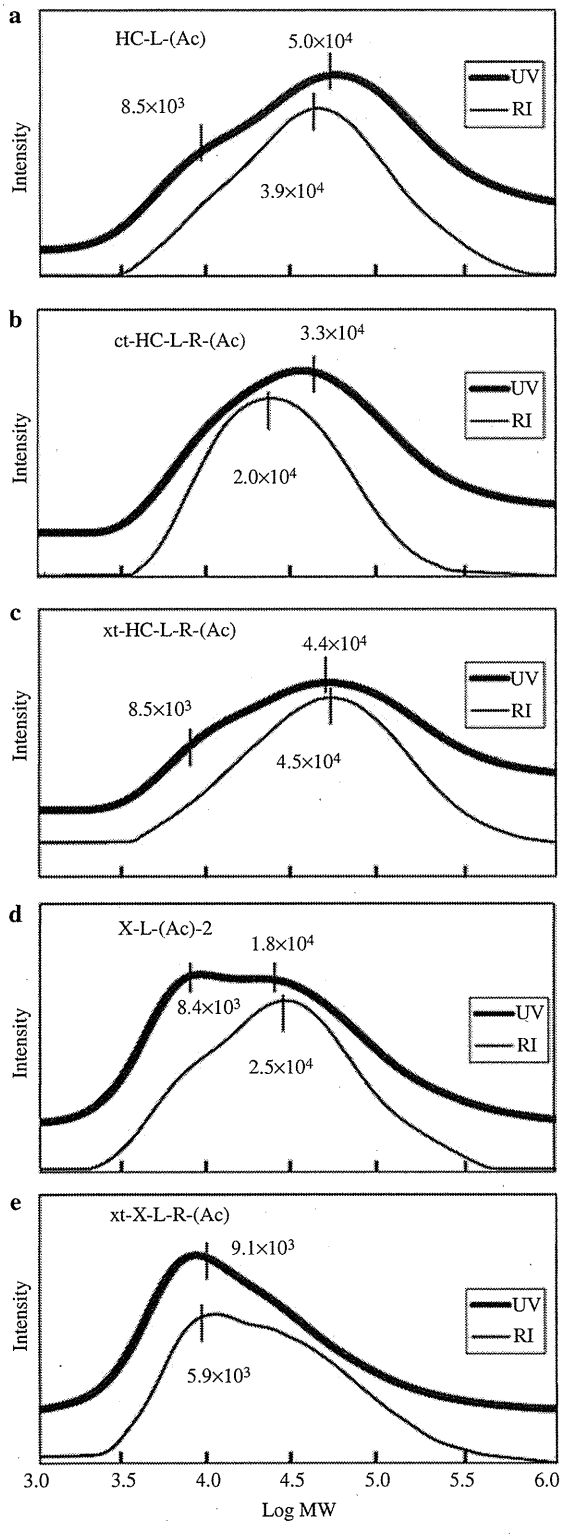


Figure 4 Molecular mass distribution of fractions a) acetate of HC-L [HC-L-(Ac)] b) acetate of HC-L residue after cellulase treatment [ct-HC-L-R-(Ac)] c) acetate of HC-L residue after xylanase treatment [xt-HC-L-R-(Ac)] d) acetate of xylan-lignin (X-L) [X-L-(Ac)-2] and e) acetate of X-L residue after xylanase treatment [xt-X-L-R-(Ac)].

have cellulase activity. However, approximately half of HC-L lignin was solubilized, showing that the solublized HC-L lignin is bonded to HC-L xylan. Figure 4c shows the MMD of xt-HC-L-R-(Ac). The RI and UV elution curves of xt-HC-L-R-(Ac) have nearly identical elution profiles, and did not change significantly after xylanase treatment. However, clear information about residual HC-L lignin after xylanase treatment was not obtained.

In summary, it was found that HC-L is composed of xylan, cellulose and lignin containing abundant syringyl lignin with abundant β-O-4 linkages, and that HC-L has LC linkages except that of the ester type.

New selective xylan acetate fractionation method

The next task was to selectively remove the cellulose component from HC-L. A cellulase treatment is an alternative method, but the cellulase had xylanase activity as described above. We tested a method combining acetylation for hardwood xylan (Koshijima and Timell 1966) (conditions: Ac_2O/pyridine/formamide, 0°C for 7 h and then r.t. for 24 h) and extraction with $CHCl_3$. First, xylan was acetylated leading to the acetylated product in 106.8% WPG. The subsequent extraction with $CHCl_3$ gave rise to the soluble and insoluble fractions in 54.0% and 46.0% yields (based on the acetylated product), respectively. Figure 5a shows FT-IR spectra of the fractions. The soluble fraction is a product with high degree of substitution of acetyl groups (DS_{Ac}), whereas the insoluble fraction has low DS_{Ac}. This contradicts published reports that glucuronoxylan can be fully acetylated within a 90-min reaction time (Grödahl et al. 2003). The degree of polymerization (DP) of the soluble fraction is similar to that of the insoluble fraction (data not shown). Therefore the various DS_{Ac} of the fractions can be ascribed to differences in solubility of the xylan fractions.

Next, cellulose was acetylated by the same procedure and resulted in 105.6% WPG. The extraction with $CHCl_3$ of the product gave the soluble fraction in only 4.1% yield. Figure 5b shows FT-IR spectra of the soluble and insoluble fractions. The soluble fraction of cellulose, like the soluble fraction from xylan, is a product with high DS_{Ac}. Finally, acetylation of glucomannan led to 55.0% WPG. Approximately half of the glucomannan was lost in the work-up process (as a result of washing with distilled water). The extraction with $CHCl_3$ of the product gave the soluble fraction in only 1.6% yield. The FT-IR spectrum of the insoluble fraction indicates that the fraction is a product with low DS_{Ac} (Figure 5c). The DS_{Ac} of the glucomannan seems to be significantly influenced by the solubilities of the fractions. In summary, model experiments reveal that the combination method can be applied for selective xylan acetylation. Further investigation is needed concerning the range of the applicability of this method.

Further fractionation of HC-L (preparation of X-L)

The new fractionation method was applied to HC-L to give an acetate of the xylan-lignin fraction [X-L-(Ac)] in 25.2%

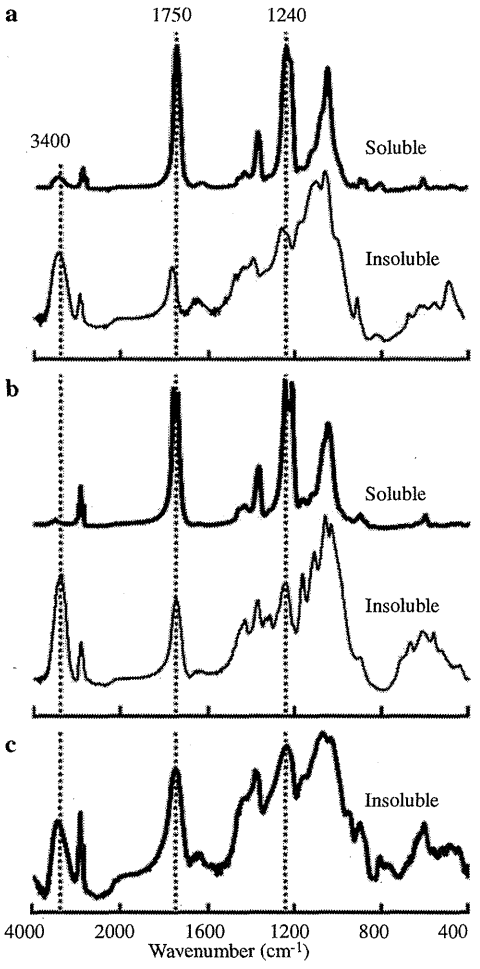


Figure 5 FT-IR spectra of a) soluble and insoluble fractions from xylan in $CHCl_3$ b) soluble and insoluble fractions from cellulose in $CHCl_3$ and c) insoluble fraction from glucomannan in $CHCl_3$.

WPG [X-L-(Ac)/HC-L, w/w]. In the ^{1}H-NMR spectrum of X-L-(Ac) (Figure 2b), the signals from cellulose acetate have disappeared, and the signals from xylan acetate are readily visible; therefore most of cellulose moiety in HC-L was removed. Signals from aromatic protons in the syringyl units and H-α of the β-O-4 substructure are observable, in addition to signals from HC-L-(Ac).

X-L-(Ac) was further deacetylated with 28%-NaOMe in MeOH to achieve a xylan-lignin fraction (X-L), because it was not suitable to hydrolysis to obtain neutral sugar and lignin analyses and enzymatic treatments. The yield of X-L from HC-L was calculated to be 11.3%. Deacetylation was confirmed by the disappearance of the band at 1751 cm^{-1} (acetyl group) in the FT-IR spectrum of X-L (data not shown). X-L was reacetylated by the method suited for ball-milled plant material [Ac_2O/dimethyl sulfoxide/*N*-methylimidazole (Ac_2O/DMSO/NMI)] according to Lu and Ralph (2003) to obtain re-acetylated product (X-L-(Ac)-2). The ^{1}H-NMR spectrum of this product was nearly identical to that of X-L-(Ac) (Figure 2b and 2c). Accordingly, the chemical structure of X-L-(Ac) was not affected by deacetylation except the ester linkages.

Characterization of X-L

As shown in Table 2, X-L is composed mainly of xylan and lignin, with a small amount of cellulose. The D-glucose content is significantly reduced, i.e., a considerable amount of cellulose was removed during preparation, as expected. This finding is also supported by the disappearance of characteristic signals of cellulose acetate in the ^{1}H-NMR spectrum of X-L-(Ac)-2 (Figure 2c). In Table 2, the SA/VA ratio of X-L is higher than that of HC-L, whereas the SA+VA yield of XL is lower than that of HC-L. These observations prove that X-L lignin is made up of more condensed S units.

The MMD of XL-(Ac)-2 is presented in Figure 4d. The UV elution curve of X-L-has a bimodal profile with two maxima: one for a high MM fraction (1.8×10^4) and another for a low one (8.4×10^3), whereas the RI elution curve shows only one maximum (2.5×10^4). Obviously, there are two types of X-L lignin concerning the MM, and the high MM fraction is bonded to the X-L xylan in contrast to the low MM fraction. Then X-L was treated with xylanase (conditions in Table 1) and analyzed by SEC. The xylanase treatment residue (xt-X-L-R) was obtained with 42.0% yield. As can be seen in Table 1, most of the D-xylose was removed, and approximately 60% of the X-L lignin was solubilized. This can be interpreted to mean that solubilized X-L lignin is bonded to X-L xylan.

Figure 4e illustrates the MMD of xt-XL-R-(Ac). After xylanase treatment, the UV elution curve was shifted to a region of low MM as the case for the RI elution curve. The high MM fraction of XL-(Ac)-2 (1.8×10^4) had clearly disappeared after xylanase treatment. These results indicate that X-L lignin with high MM (1.8×10^4) is bonded to X-L xylan. However, this finding is not proof of the existence of LC linkages in X-L lignin with low MM (8.4×10^3). In summary, X-L is mainly composed of xylan and lignin, with a small amount of cellulose, and approximately half of X-L lignin is bonded to X-L xylan via various covalent linkages, except of the ester type.

Conclusion

The extraction residue left behind after isolation of milled wood lignin (MWL), designated as MWR, was fractionated with the cellulose-solvent LiCl/DMAc. A soluble fraction (C-L) and an insoluble fraction (C-L residue) were obtained. The latter was extracted with the hemicellulose solvent 3 M NaOH to give a soluble fraction (HC-L; 21.3% yield based on MWR). HC-L is composed of xylan, cellulose, and lignin. The lignin part is rich in syringyl units linked mostly with β-O-4 linkages. HC-L lignin differs significantly from MWL and it is bonded to HC-L cellulose or HC-L xylan or both.

HC-L was further fractionated by a novel selective xylan acetate fractionation method, which comprises an acetylation step developed for hardwood xylan and an extraction step

with $CHCl_3$. After deacetylation, a new fraction (X-L) was obtained with 11.3% yield (based on HC-L). X-L is composed mainly of xylan and lignin, with a small amount of cellulose. X-L lignin is covalently bonded to X-L xylan. Therefore, X-L seems to be the favorable fraction for application of the described lignin degradation methods and for NMR for the detailed analysis of the LC linkages.

Acknowledgments

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ORIGINAL PAPER

Synthesis and thermoreversible gelation of diblock methylcellulose analogues via Huisgen 1,3-dipolar cycloaddition

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Abstract A novel synthetic method to link acetylated cellulose derivatives with methylated cellulose derivatives via Huisgen 1,3-dipolar cycloaddition was developed to produce 1,2,3-triazole-linked diblock copolymers consisting of hydrophilic cellobiose or low-molecular-weight cellulose and a hydrophobic 2,3,6-tri-*O*-methyl-cellulose. Huisgen 1,3-dipolar cycloaddition had the advantage over glycosylation reaction of being able to connect a hydrophilic block having higher molecular weight than cellobiose with a hydrophobic 2,3,6-tri-*O*-methyl-cellulose block. As a consequence, 2.0 wt% aqueous solutions of the 1,2,3-triazole-linked diblock methylcellulose analogues exhibited the thermoreversible gelation in water at around 25 °C as same as that of β-(1 → 4)-linked diblock methylcellulose. Differential scanning calorimetry measurements of 2.0 wt% aqueous solutions of the diblock copolymers revealed that an important structural factor for its thermoreversible gelation was not a β-(1 → 4)-glycosidic linkage between hydrophilic and hydrophobic blocks of diblock methylcellulose, but a sequence of anhydro 2,3,6-tri-*O*-methyl-glucopyranosyl units and that of unmodified glucopyranosyl ones.

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Introduction

This paper describes the important structural factor for thermoreversible gelation via the self-assembly of the amphiphilic diblock copolymers consisting of only saccharide chains in both hydrophilic and hydrophobic blocks. We have focused on the chemical structure of crosslinking points of a methylcellulose (MC) gel, and synthesized cooligomers and copolymers having blocky functionalization patterns as models of industrially produced MC (Kamitakahara et al. 2006, 2007, 2008, 2012; Kamitakahara and Nakatsubo 2010; Nakagawa et al. 2011a, b, c).

The cross-linking points of MC play an important role in thermoreversible gelation (Hirrien et al. 1998). It is of academic significance to clarify the nature and structure of the reversible cross-linking points responsible for the solution behavior of MC to tune the properties of the gel. To gain insight into the structure of the cross-linking points, light scattering and X-ray scattering studies have been undertaken on industrially produced MCs with heterogeneous functionalization pattern and laboratory-produced MCs with relatively uniform functionalization pattern (Bodvik et al. 2010; Zhou et al. 2008). Kato et al. (1978) have reported that the crystalline regions consisting of 4–8

of 2,3,6-tri-*O*-methyl-glucopyranosyl units act as 'crosslinking loci' on heating. However, isolation of well-defined MC fragments containing such a sequence of 2,3,6-tri-*O*-methyl-glucopyranosyl units from MC with heterogeneous functionalization pattern were very difficult since they represent only a small fraction of total MC (Adden et al. 2006).

Based on our model synthesis strategy, a diblock MC copolymer consisting of hydrophilic cellobiosyl and hydrophobic 2,3,6-tri-*O*-methyl-cellulosyl blocks (GG-236MC) was synthesized via glycosylation reaction of 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-α-D-glucopyranosyl 2,2,2-trichloroacetimidate with methyl 2,3,6-tri-*O*-methyl celluloside having a hydroxyl group at C-4 of non-reducing end and subsequent deacetylation to investigate thermal aggregation behavior and thermoreversible gelation of industrially produced MC (Nakagawa et al. 2011b). As a result, 2.0 wt% aqueous solution of the diblock copolymer, GG-236MC, formed a hydrogel at room temperature, accompanied by the formation of hydrophobic environment (Nakagawa et al. 2011a). Our synthesis approach to the structure–property relationships achieved the results that a sequence of 2,3,6-tri-*O*-methyl-glucopyranosyl units caused thermoreversible gelation of MC, and that the length of hydrophobic block influenced the gelation temperature. However, the diblock copolymers having unmodified cello-oligosaccharides with higher *DP* than cellobiose still remain important synthetic targets to investigate the influence of hydrophilic block on gelation.

It is a key determinant for synthesis of diblock copolymer through glycosylation method whether a glycosyl donor is actually available. Glycosylation of the glycosyl donors having higher molecular weight than cellobiose remains difficult. We therefore planned to synthesize the diblock copolymers having linkages other than glycosidic bond between hydrophilic and hydrophobic blocks, and investigated the influence of the linkage on the hydrogelation of the diblock copolymers.

Several cellulosic diblock copolymers have been synthesized by introduction of long alkyl groups into the reducing end of cellulose. Kamitakahara et al. have reported the synthesis of a cellulosic diblock copolymer by introduction of long alkyl chain via amide linkage, which was stable under alkaline condition for removal of acetyl groups of cellulose triacetate block (Kamitakahara and Nakatsubo 2005; Kamitakahara et al. 2005). In this synthetic route for the cellulosic diblock copolymer, cellulose triacetate having an azide group at C-1 of reducing end has been synthesized as a key synthetic intermediate by stepwise modification of cellulose (Enomoto-Rogers et al. 2009, 2012).

Recently, a Cu(I)-catalyzed chemoselective coupling between organic azides and terminal alkynes for the formation of 1,2,3-triazoles has attracted attention owing to its convenient, quick, and quantitative reaction (Hasegawa et al. 2006). Among the multiple reactions that could be termed 'click chemistry', the Huisgen 1,3-dipolar cycloaddition has been successfully applied for the synthesis of glycosyl triazoles (Neto et al. 2010; Wilkinson et al. 2006; Chittaboina et al. 2005), oligosaccharide analogues (Marmuse et al. 2005; Hotha and Kashyap 2005), and polysaccharide derivatives (Negishi et al. 2011; Zhang et al. 2008; Elchinger et al. 2011; Liebert et al. 2006; Koschella et al. 2011; Enomoto-Rogers et al. 2012). Most of the reports regarding polysaccharides have focused on the application of 'click chemistry' to synthesize graft type copolymers of alkyne or azide compounds onto polysaccharide chains such as 6-azide-6-deoxycellulose (Negishi et al. 2011; Liebert et al. 2006; Pohl et al. 2008, 2009), 6-azide-6-deoxycurdlan (Hasegawa et al. 2006), and 3-*O*-propargyl-cellulose (Fenn et al. 2009). As far as we know, there have been no reports on the synthetic method for linking cellulose derivatives having an azide group at C-1 of reducing end and an alkyne group at C-4 of non-reducing end via 'click chemistry' (Schatz and Lecommandoux 2010). Thus, we describe here the synthesis of 1,2,3-triazole-linked diblock copolymers via 'click' reaction and their thermoreversible gelation properties in water. Essential factor for the thermoreversible gelation of 1,2,3-triazole-linked diblock MC analogues will be discussed.

Results and discussion

Basic design for diblock MC analogues

Figure 1 illustrates our synthesis strategy for preparation of diblock methylcellulose analogues via Huisgen 1,3-dipolar cycloaddition. Methyl 2,3,6-tri-*O*-methyl-celluloside having a propargyl group at C-4 of non-reducing end (**2**: **2a**, **2b**) was synthesized by propargylation of a

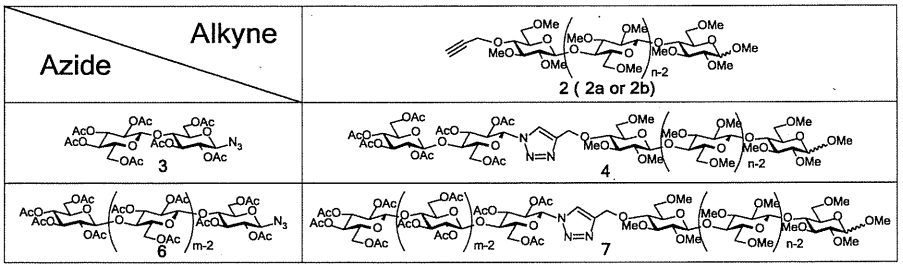


Fig. 1 Syntheses of diblock methylcellulose analogues **4** and **7** via Huisgen 1,3-dipolar cycloaddition between terminal alkyne **2** and peracetyl glycosyl azides **3** and **6**

hydroxyl group of methyl 2,3,6-tri-*O*-methyl-cellulo-side prepared according to a methanolysis published in our recent paper (Nakagawa et al. 2011b). Peracetyl *β*-cellobiosyl azide (**3**) and peracetyl *β*-cellulosyl azide (**6**) ($DP_n = 6.9$, $M_w/M_n = 1.83$) were converted from cellobiose and cellulose, respectively (Györgydeák et al. 1993; Kamitakahara and Nakatsubo 2005; Kamitakahara et al. 2005). Copper(I)-catalyzed Huisgen 1,3-dipolar cycloaddition of compounds **2a** and **2b** (terminal alkyne) to glycosyl azides **3** and **6** gave compounds **4** and **7** and subsequent removal of acetyl groups afforded 1,2,3-triazole-linked diblock MC analogues **5** and **8**, respectively, as shown in Fig. 3.

Synthesis of diblock MC analogue **5** via Huisgen 1,3-dipolar cycloaddition

Synthesis of alkyne compound 2 and azide compound 3

Propargylation of compound **1** using 3-bromopropyne in the presence of NaH in DMF gave compound **2a** in 54 % yield, as shown in Fig. 2. In the ^{1}H-NMR spectrum of compound **2a** measured in $CDCl_3$, the resonance of a methyne proton of a propargyl group appeared at 2.46 ppm as triplet. The molecular weights and polydispersity index of compound **2** (**2a**, **2b**) were summarized in Table 1. MALDI-TOF MS spectrum of compound **2a** will be shown in later section. Cellobiose octaacetate was converted into peracetyl *β*-cellobiosyl azide (**3**) by using trimethylsilylazide, according to the literature (Kamitakahara and Nakatsubo 2005) in 80 % yield in two reaction steps.

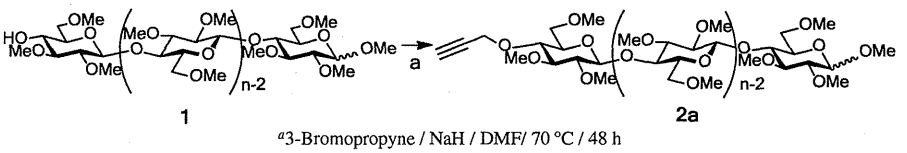
Cycloaddition of alkyne compound 2a and azide compound 3

Diblock MC analogue **5** was synthesized according to the reaction sequence, as shown in Fig. 3. The amount of peracetyl *β*-cellobiosyl azide (**3**), Cu(I)Br, sodium ascorbate, and *N*, *N*, *N′*, *N″*, *N‴*-pentamethyldiethylenetriamine (PMDETA) were calculated using the number-average molecular weight (M_n) of compound **2a** estimated by GPC analysis, since compound **2a** was a polydisperse mixture of compounds having different molecular weights.

In the case of glycosylation for the synthesis of diblock MC copolymers exemplified with methyl *β*-D-glucopyranosyl-(1 → 4)-*β*-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-methyl-celluloside, the excess amount of the glycosyl donor, 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-acety-α-D-glucopyranosyl 2,2,2-trichloroacetimidate (20 equivalents), was required to obtain a glycosylated product. In contrast, 'click' cycloaddition requires less amount of hydrophilic building block, peracetyl cellobiosyl azide (**3**), compared with glycosylation reaction. Two equivalents of peracetyl *β*-cellobiosyl azide (**3**) was reacted with compound **2a** (1.0 equivalent) in the presence of Cu(I)Br (10 equivalent), sodium ascorbate (20 equivalents), and PMDETA (10 equivalents) for 24 h at r.t. The cycloaddition product was purified by silica gel chromatography (eluent: EtOAc) to give compound **4** in 85 % yield.

The chemical structure of 'click' cycloaddition product was supported by NMR spectroscopy, as shown in Fig. 4. The characteristic resonances of a methyne proton (2.46 ppm as triplet) of compound **2a** and H-1 (4.62 ppm as doublet with coupling constant $J = 9.0$ Hz) of compound **3** disappeared in ^{1}H-NMR spectrum. The resonance corresponding to the anomeric center of the glucopyranose residue attached to the triazole unit was observed at 5.82 ppm as a doublet with $J = 9.0$ Hz. The triazole proton appeared at 7.69 ppm as a singlet, indicating the formation of a single isomer. The molecular weight and polydispersity index of compound **4** were summarized in Table 1. The DP_n of compound **4** ($DP_n = 27.3$) was higher than compound **2a** ($DP_n = 25.9$), resulting

Fig. 2 Synthetic rote for compound **2a**



1 2a

[a] 3-Bromopropyne / NaH / DMF/ 70 °C / 48 h

Table 1 Molecular weights and *DP*s of compounds **2, 4, 6** and **7**

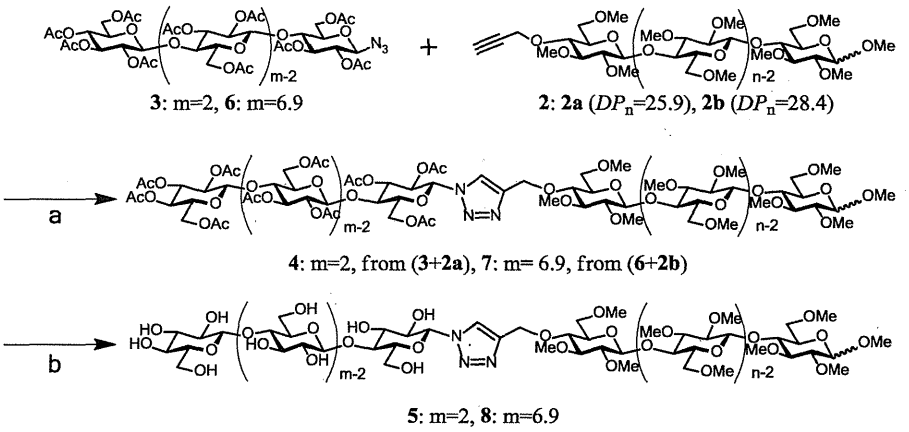
Compound	M_n ($\times 10^3$)	M_w ($\times 10^3$)	M_w/M_n	DP_n^c	m[c]	n[c]
2a (Alkyne)	5.3	8.3	1.56	25.9	–	25.9
2b (Alkyne)	5.8	8.1	1.39	28.4	–	28.4
6 (Azide)	2.0	3.7	1.85	6.9	6.9	–
4[a]	5.8	8.6	1.48	27.3	2	25.3
7[b]	7.7	11.0	1.42	35.0	6.5	28.5

[a] Peracetylated cellobiosyl azide (**3**) + **2a**, [b] **6** + **2b**, [c] calculated by ^{1}H-NMR spectroscopy

m Degree of polymerization of hydrophilic block

n Degree of polymerization of hydrophobic block

Fig. 3 Synthetic route for diblock MC analogues **5** and **8**



3: m=2, **6**: m=6.9 **2**: **2a** (DP_n=25.9), **2b** (DP_n=28.4)

4: m=2, from (**3**+**2a**), **7**: m= 6.9, from (**6**+**2b**)

5: m=2, **8**: m=6.9

[a] Cu(I)Br / Sodium ascorbate / PMDETA / 20% MeOH / CH_2Cl_2 / r.t. / 24 h; [b] 28% $NaOCH_3$ in MeOH / MeOH / THF / r.t. / 12 h.

from the connection of peracetyl cellobiosyl block with hydrophobic 2,3,6-tri-*O*-methyl-cellulosyl block.

Acetyl groups of compound **4** were removed using NaOMe in MeOH and THF at r.t. to afford compound **5** in 99 % yield. Figure 5 shows ^{1}H-NMR spectrum of compound **5** measured in D_2O. The triazole proton resonance appeared at 8.31 ppm as a singlet. In addition, the resonance corresponding to the anomeric center of the glucopyranose residue attached to the triazole unit was visible at 5.79 ppm as a doublet with $J = 9.0$ Hz, indicating that deacetylation had no influence on the resonance of H-1(**b**) proton. The ^{1}H-NMR spectrum of compound **5** revealed that acetyl groups were successfully removed without any cleavage of triazole linkage under alkali reaction condition to give a diblock MC analogue **5**.

The MALDI-TOF MS spectra of compounds **2a**, **4**, and **5** were measured using 2,5-dihydroxybenzoic acid (DHB) as a matrix in positive linear mode, as shown in Fig. 6. The observed molecular weights of compounds were in good agreement with their calculated molecular weights, indicating that reactions proceeded as expected. The regular side peaks a and b (shown in insets in Fig. 6) could be assigned to be the mass peaks of compound **1** and methyl 2,3,6-tri-*O*-methyl-cellulloside having a methyl group at C-4 of non-reducing end derived from original 2,3,6-tri-*O*-methyl-cellulose, respectively.

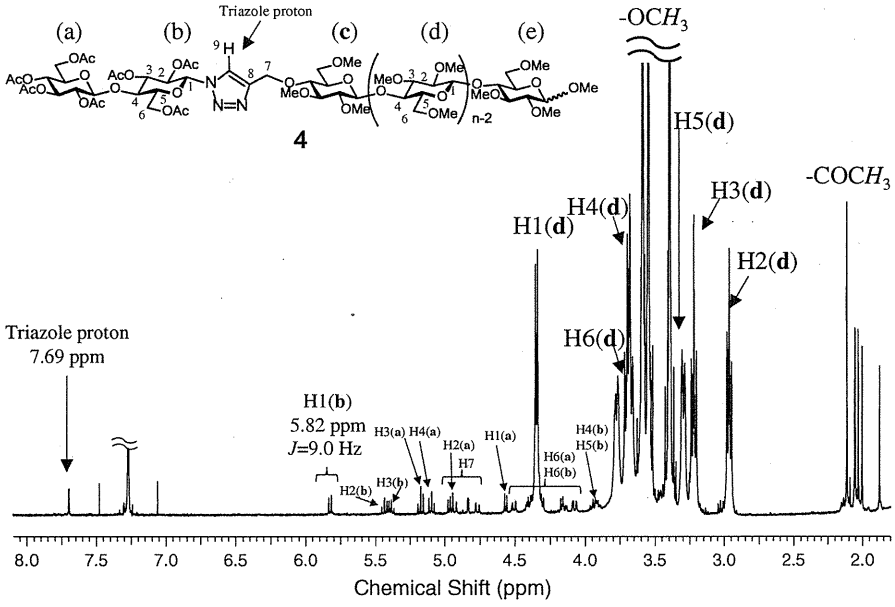


Fig. 4 ^{1}H-NMR spectrum of compound **4** taken in $CDCl_3$

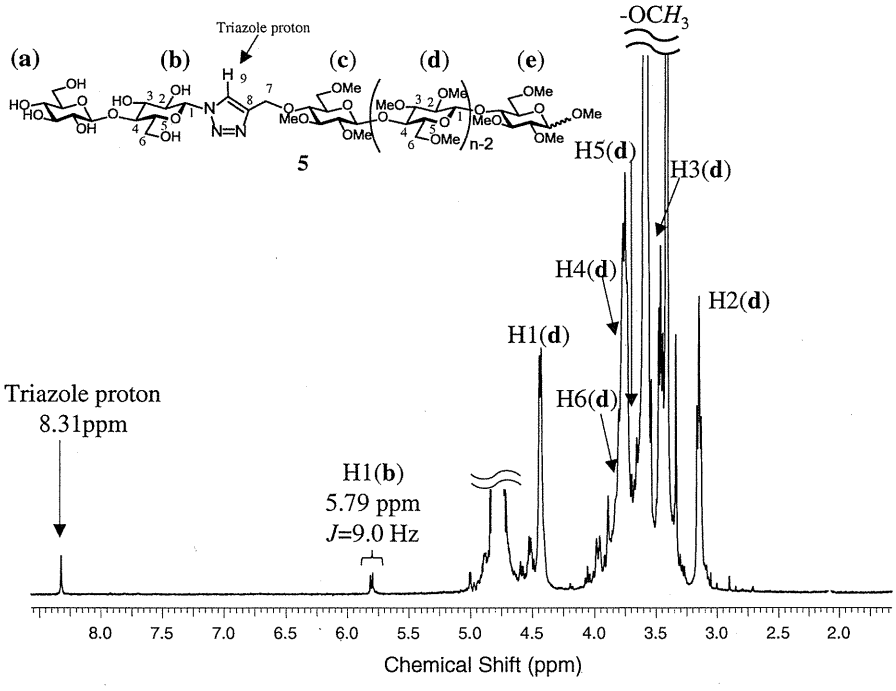


Fig. 5 ^{1}H-NMR spectrum of compound **5** taken in D_2O

*Cycloaddition of alkyne compound **2b** and azide compound **6***

Figure 3 shows synthetic route for diblock MC analogue **8** having low-molecular-weight cellulose as a hydrophilic block. Compound **7** was synthesized according to the synthetic procedure of compound **4**. Cellulose triacetate with low molecular weight synthesized by acetolysis of cellulose according to the literature (Miller et al. 1960) was converted into peracetyl β-cellulosyl azide (**6**) ($DP_n = 6.9$, $M_w/M_n = 1.39$) (Kamitakahara et al. 2005). Huisgen

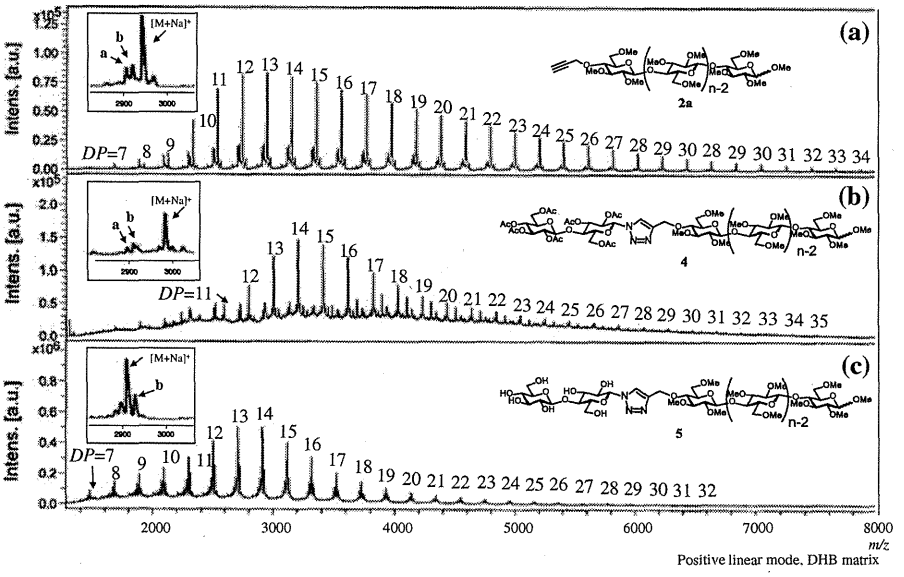


Fig. 6 MALDI-TOF MS spectra of compounds **2a** (**a**), **4** (**b**), and **5** (**c**). The *m/z* values of sodium adduct ions, $[M + Na]^+$ of compounds were observed. *DP* values of compounds **4** (**b**) and **5** (**c**) show the total *DP* (*DP* = n+2). *Inset* enlarged spectra corresponding to the mass range *m/z* 2,850–3,050. Series a: [M (compound **1**) + $Na]^+$, series b: [M (compound **1**) + 14 (Me) + $Na]^+$

1,3-dipolar cycloaddition of compound **6** with compound **2b** using Cu(I)Br (10 equivalent), sodium ascorbate (20 equivalent), and PMDETA (10 equivalent) for 24 h at r.t. and subsequent purification by silica gel chromatography (eluent: EtOAc) gave the cycloaddition product **7** in 72 % yield. Two equivalents of compound **6** to compound **2b** were at least enough to obtain the target compound **7**. In ^{1}H-NMR spectrum of compound **7** as shown in Fig. 7, the characteristic resonances of a methyne proton (2.46 ppm as triplet) of compound **2b** and H-1 (4.62 ppm as doublet) of compound **6** disappeared. The resonance corresponding to the anomeric center of the glucopyranose residue attached to the triazole unit was observed at 5.81 ppm as a doublet with $J = 9.0$ Hz. The triazole proton was visible at 7.70 ppm as a singlet.

The molecular weights, *DP*s, and the polydispersity index (M_w/M_n) of compound **7** were estimated by GPC measurements. The block lengths (m, n) of compound **7** were calculated using the resonances of H-1(c) at 5.81 ppm, H-2(e) at 2.95 ppm, and acetyl protons at 1.86–2.14 ppm. As shown in Table 1, the molecular weight of compound **7** ($M_n = 7.7 \times 10^3$) was the approximate sum of the molecular weights of compounds **2b** ($M_n = 5.8 \times 10^3$) and **6** ($M_n = 2.0 \times 10^3$), indicating that the cycloaddition of compounds **2b** and **6** proceeded successfully.

Acetyl groups of compound **7** were removed using NaOMe in MeOH and THF at r.t. to give compound **8** in 96 % yield. Figure 8 shows ^{1}H-NMR spectrum of compound **8** measured in D_2O. The triazole proton resonance appeared at 8.31 ppm as a singlet. In addition, the resonance corresponding to the anomeric center of the glucopyranose residue attached to the triazole unit was visible at 5.79 ppm as a doublet with $J = 9.0$ Hz.

Thermal property of aqueous solution of diblock methylcellulose analogues **5** and **8**

We have reported that 2.0 wt% aqueous solution of the diblock copolymer, methyl β-D-glucopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-2,3,6-tri-*O*-methyl-cellulose (GG-236MC), were soluble in water at ~0 °C and formed a gel at ~25 °C (Nakagawa et al. 2011a).

Compounds **5** and **8** were water-soluble with concentration of 2.0 wt% at ~0 °C, and were partially soluble in water with concentration of 2.0 wt% at ~25 °C. The water-insoluble [**5a** (50.3 %) and **8a** (89.3 %)] and water-soluble fractions [**5b** (49.7 %) and **8b** (10.7 %)] of compounds **5** and **8** were collected by centrifugation method at ~25 °C. The molecular weights, polydispersity index, DP_ns, and *DS* of compounds **5a**, **5b**, **8a**, **8b**, and GG-236MC were summarized in Table 2.

Figure 9 shows the photographs of 2.0 wt% aqueous solutions of compounds **5a**, **8a**, and GG-236MC. The thermoreversible gelations of compounds **5a** and **8a** could be clearly observed at around 25 °C within