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Note

Rhamnogalacturonan I from xylem differentiating zones of *Cryptomeria japonica*

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Abstract

Rhamnogalacturonan I (RG-I) was isolated from the pectin of the cell wall of xylem differentiating zones of sugi (*Cryptomeria japonica* D. Don, gymnosperm) by digesting with endo-α-1,4-polygalacturonase followed by gel-permeation chromatography. Structure of RG-I was characterized by glycosyl composition and glycosyl linkage analyses, partial acid hydrolysis, and lithium (Li)-treatment in ethylenediamine. The oligosaccharides produced by partial acid hydrolysis showed the backbone of RG-I to be a disaccharide repeating unit of →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→. Arabinan and oligogalactosyl side chains were attached at O-4 of rhamnosyl residues of the backbone. Glycosyl composition and glycosyl linkage analyses suggested that the structures and/or the distribution of the arabinan sidechains of sugi RG-I were different from those of sycamore (angiosperm) RG-I.

Keywords: *Cryptomeria japonica*; Cell wall polysaccharides; Pectin; Rhamnogalacturonan I; Xylem differentiating zone

1. Introduction

Primary cell wall consists of three different polysaccharides, namely, pectin, hemicellulose, and cellulose [1]. Keegstra et al. [2] and O'Neill et al. [3] have extensively studied the pectic and hemicellulosic polysaccharides of the suspension-cultured sycamore (*Acer pseudoplatanus*) cells and characterized RG-I, rhamnogalacturonan II (RG-II), arabinogalactan, xylan and xyloglucan. Furthermore, they demonstrated that the oligosaccharides derived from primary cell wall polysaccharides such as homogalacturonan and xyloglucan regulated plant cell growth [4,5]. However, biologically active oligosaccharides were not obtained from RG-I and RG-II. RG-II reportedly inhibited the uptake of [^{14}C] leucine into suspension-cultured tomato cells [6]. Therefore, biologically active oligosaccharides could be prepared from RG-I and RG-II. As the oligosaccharides of RG-I and RG-II produced by partial acid hydrolysis were structurally complicated in comparison with those of xyloglucan [7], it was difficult to purify the oligosaccharides enough for bioassay. Consequently, structural characterization could play an important role in detection of biologically active oligosaccharides from RG-I and RG-II.

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Lau et al. [8] reported that the backbone of sycamore (angiosperm) RG-I had a repeating unit of → 4)-α-D-Gal*p*A-(1 → 2)-α-L-Rha*p*-(1 → and that arabinan and galactan side chains linked to O-4 of the rhamnosyl residues of the backbone. On the other hand, there are few reports on the pectin of gymnosperms. Thomas et al. [9] showed that the cell walls of suspension-cultured Douglas fir (*Pseudotsuga menziesii*, gymnosperm) contained both RG-I and RG-II. In previous papers, we reported that the pectin extracted from the cell wall of xylem differentiating zones of sugi was composed of RG-I, RG-II and homogalacturonan [10,11]. Isolation and structural characterization of RG-I from the cell wall of xylem differentiating zones of sugi is now reported.

2. Results and discussion

The pectin fractions isolated from the cell walls of xylem differentiating zones of sugi with 0.05 M *trans*-1,2-cyclohexanediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) and 0.05 M Na_2CO_3 were digested with endo-1,4-α-polygalacturonase. The enzymic di-

Table 1
Glycosyl linkage composition of RG-I isolated from CDTA and Na_2CO_3 extracts of sugi xylem differentiating zones

Glycosyl residue	Linkage	CDTA-I[a]	CDTA-II[a]	Na_2CO_3-I[a]	Na_2CO_3-II[a]	Sycamore	Douglas fir
		RG-I[b]	RG-I[b]	RG-I[b]	RG-I[b]	RG-I[c]	RG-I[d]
		Mol %					
Rhamnosyl	Terminal	0.2	0.2	0.2	–	–	–
	2-Linked	9.6	9.9	5.7	+	7.8	1.1
	2,3-	3.4	–	0.9	–	–	–
	2,4-	9.1	9.8	9.9	7.6	8.0	2.8
	2,3,4-	+	+	+	+	0.6	0.5
Fucosyl	Terminal	0.6	0.6	0.5	–	1.4	–
Arabinosyl	Terminal	5.6	11.8	11.9	10.9	9.5	13.4
	2-Linked	–	–	–	–	2.2	–
	3-	+	+	+	+	2.2	17.2
	5-	24.6	26.2	25.7	20.8	11.2	19.8
	2,5-	3.7	2.9	4.5	4.3	1.0	6.8
	3,5-	–	–	–	–	3.5	7.4
Xylosyl	Terminal	–	–	0.2	–	2.0	–
	4-Linked	0.1	–	0.1	–	–	–
Galactosyl	Terminal	11.4	11.5	12.8	14.5	6.3	15.1
	2-Linked	–	–	–	–	0.6	0.8
	3-	4.4	3.1	4.0	4.5	2.7	4.2
	4-	3.6	2.4	4.4	4.0	8.4	4.9
	6-	0.5	0.4	1.1	–	7.5	1.0
	2,4-	1.0	0.7	–	–	6.3	15.1
	2,6-	–	–	–	–	1.2	–
	3,6-	1.5	–	0.2	–	1.2	1.2
	4,6-	–	–	–	–	2.4	1.1
	2,4,6-	0.7	0.3	0.2	–	–	–
Glucosyl	Terminal	–	–	–	–	–	1.6
	4-Linked	–	0.7	–	–	–	–
	2,4-	–	–	1.0	–	–	–
Gal A	Terminal	1.3	1.1	0.7	2.8	1.6	nd
	4-Linked	12.7	16.2	14.3	26.7	15.2	nd
	2,4-	2.1	0.6	0.8	4.0	1.0	nd
	3,4-	2.0	1.0	0.8	–	–	nd
Glc A	4-Linked	1.7	0.3	–	–	–	nd

Notes: [a]The extraction procedure was described in Section 3.
[b]The RG-I purified from each extract.
[c]York et al. [19].
[d]Thomas et al. [9].
+ = present but not determined.
nd = not determined.

Table 2
Diagnostic CIMS ions for the per-*O*-alkylated oligoglycosyl alditols prepared from partially hydrolyzed sugi RG-I

Per-*O*-alkylated oligoglycosyl alditols[a]	CIMS ions				
	$(M+H)^+$	$aldA_1$	$aldA_1 + H_2O$	aA_1	aA_2
IV	529	274	292	238	206
	[100]	[15]	[49]	[58]	[66]
V	552	276	294	259	227
	[100]	[4]	[70]	[29]	[22]

Notes: [a]Deduced structures were shown in Fig. 1. Compounds I, II, III and VI were previously identified by Lau et al. and their MS data are reported in Ref. [8].
[b]The nomenclature is based on Lau et al. [8] and Kováčik et al. [13,14].
Numbers in square brackets indicate ion intensities.

gests were separated on a Bio-Gel P-30 column to give RG-I, RG-II and oligogalacturonides. The RG-Is isolated from both 0.05 M CDTA and Na_2CO_3 extracts had quite similar glycosyl linkage compositions (Table 1). Major glycosyl linkages were 2- and 2,4-linked rhamnosyl, terminal and 5-linked arabinosyl, terminal galactosyl and 4-linked galacturonic acid residues. The contents of the glycosyl linkages constituting the backbone, namely, 2-linked and 2,4-linked rhamnosyl residues were similar between sugi and sycamore RG-I. However, the RG-Is from gymnosperms, namely, sugi and Douglas fir, contained greater amount of 5-linked arabinosyl residues than sycamore RG-I. Lerouge et al. [12] showed that sycamore RG-I contained arabinan side chains composed of more than 20 arabinosyl residues. The struc-

Table 3
Diagnostic EIMS ions and FABMS ions for per-*O*-alkylated oligoglycosyl alditols prepared from partially hydrolyzed sugi RG-I

Per-*O*-alkylated oligoglycosyl alditols[a]	Retention time[c] (min)	EIMS ions					FABMS[b]
		$aaldA_1$	$aldA_1$	aA_1	aA_2	Alditols	$(M+Na)^+$
IV	15.2	334	274	238	206	483	551
		[46]	[73]	[30]	[41]	[3]	
V	15.3	336	276	259	227	442	574
		[41]	[60]	[6]	[28]	[16]	

Notes: [a]Deduced structures were shown in Fig. 1. Compounds I, II, III and VI were previously identified by Lau et al. and their MS data are reported in Ref. [8].
[b]Ions detected by FABMS.
[c]Retention time (min) on a DB-1 GC column.
Numbers in square brackets indicate ion intensities.

Table 4
Diagnostic CIMS ions for the per-*O*-methylated oligoglycosyl alditols prepared from the degradation products of sugi RG-I by lithium-treatment in ethylenediamine

Per-*O*-alkylated oligoglycosyl alditols[a]	CIMS ions				
	$(M+H)^+$	$aldA_1$	$aldA_1 + H_2O$	aA_1	aA_2
1-a	383	191	209	175	143
	[12]	[4]	[100]	[57]	[64]
2	411	205	223	189	157
	[4.8]	[3]	[48]	[100]	[27]
3	427	191	209	219	187
	[87]	[16]	[100]	[50]	[83]

Notes: [a]Deduced structures were shown in Fig. 2. Compounds previously identified by Lau et al. [17] are omitted.
[b]Nomenclature based on Lau et al. [8] and Kováčik et al. [13,14].
Numbers in square brackets indicate ion intensities.

Numerical designation	Retention time (min)	Per-*O*-alkylated oligoglycosyl alditols
I*	13.6	Et→4)-L-Rha*p*-(1→4)-D-Galactitol-1,6,6-d_3 (2-Et, 5-Et, 1-Et)
II*	14.2	Et→4)-D-Gal*p*-6,6-d_2-(1→2)-L-Rhamnitol-1-d (5-Et, 4-Et, 1-Et)
III*	14.3	Et→4)-D-Gal*p*-6,6-d_2-(1→2)-L-Rhamnitol-1-d (5-Et, 1-Et)
IV	15.2	Et→4)-D-Gal*p*-(1→4)-D-Galactitol-1-d (5-Et, 1-Et)
V	15.3	Et→4)-D-Gal*p*-6,6-d_2-(1→4)-D-Galactitol-1,6,6-d_3 (?-Et, 5-Et, 1-Et)
VI*	20.5	Et→4)-D-Gal*p*-6,6-d_2-(1→2)-L-Rha*p*-(1→4)-D-Galactitol-1,6,6-d_3 (4-Et, 5-Et, 1-Et)

Fig. 1.

ture and/or the distribution of the side chains of gymnosperm RG-I would be different from those of angiosperm RG-I.

To characterize the backbone structure of sugi RG-I, the RG-I from the Na_2CO_3 extract was partially hydrolyzed with acid and the oligosaccharides

Table 5
Diagnostic EIMS ions for the per-*O*-methylated oligoglycosyl alditols prepared from the degradation products of sugi RG-I by lithium-treatment in ethylenediamine

Per-*O*-methylated oligoglycosyl alditols[a]	Retention time[b] (min)	EIMS ions				Alditols		
		$aaldA_1$	$aldA_1$	aA_1	aA_2			
1-a	16.8	251 [62]	191 [24]	175 [100]	143 [68]			
2	17.4	265 [100]	205 [21]	189 [86]	157 [53]			
3	19.0	219 [87]	187 [80]	251 [100]	191 [73]	381 [10]	337 [22]	293 [10]

Notes: [a]Deduced structures shown in Fig. 2. Compounds identified by Lau et al. are omitted.
[b]Retention time (min) on a DB-1 GC column.
Numbers in square brackets indicate ion intensities.

released were analyzed. Prior to the partial acid hydrolysis, galacturonic acid residues in the RG-I were reduced to the corresponding 6,6′-dideuteriogalactosyl residues to determine the linkages of galacturonic acid residues. The carboxy-reduced RG-I was per-O-methylated and partially hydrolyzed. The hydroxy groups exposed by partial acid hydrolysis were further per-O-pentadeuterioethylated. The resulting partially O-pentadeuterioethylated, partially O-methylated oligoglycosyl alditols were analyzed by FABMS and GC–MS. The FABMS spectrum had the quasimolecular ions $(M + Na)^+$ at *m/z* 523, 542, 551, 574 and 748, which correspond to derivatives from disaccharides and trisaccharide, respectively. From the MS fragmentation patterns (Tables 2 and 3) and methylation analysis results, the glycosyl sequences of five per-O-alkylated monoglycosyl alditols and one per-O-alkylated diglycosyl alditol were determined (Fig. 1). The nomenclature developed by Lau et al. [8] and Kováčik et al. [13,14] was used in Tables 2–5. The glycosyl composition and glycosyl linkage analyses of sugi RG-I revealed that the deoxyhexosyl, hexosyl and 6,6-dideuteriohexosyl residues identified by GC–MS were rhamnosyl, galactosyl and galacturonic acid residues, respectively. As compounds I, II, III and VI were previ-

Numerical designation	Retention Time (min)	per-*O*-methylated mono- and diglycosyl alditol
1-a	16.8	Ara→Araol
2	17.4	Rha→Rha
1-b*	17.5	Ara-(1→5)-Araol
3	19.0	Gal-(1→5)-Araol
4*	20.2	Gal-(1→4)-Rhaol
5*	21.4	Gal→Galol
6-a*	26.6	Ara→Ara→Araol
6-b*	27.1	Ara→Ara→Araol
6-c*	27.3	Ara→Ara→Araol
6-d*	27.7	Ara→Ara→Araol
7*	29.1	Ara-(1→3)→Gal-(1→4)-Rhaol

Fig. 2.

ously identified by Lau et al. [8], their MS data were omitted in Tables 2 and 3. Compounds I, II, III and VI were major products, suggesting that the backbone of sugi RG-I was mainly composed of such units. As rhamnosyl residue were linked to O-4 of galacturonic acid residue and galacturonic acid residues to O-2 of rhamnosyl residues, the backbone of sugi RG-I was supposed to be a disaccharide repeating unit of →4)-D-Gal*p*A-(1→2)-L-Rha*p*-(1→. Furthermore, pentadeuteroethylated groups were attached at O-4 of rhamnosyl residues, showing that the side chains were connected at O-4 of rhamnosyl residues. Other minor compounds, IV and V were probably derived from →4)-D-Gal*p*-(1→4)-D-Gal*p*-(1→ and →4)-D-Gal*p*A-(1→4)-D-Gal*p*A-(1→, respectively. Compound IV was galactobiose, which was found also in the products of lithium-treatment described later (Fig. 2). As the treatment was carried out in order to structurally analyze the side chains, compound IV was probably derived from the side chains. Compound V was definitely derived from homogalacturonan contaminated with RG-I. These results indicate that the major structure of the backbone of sugi RG-I is a disaccharide repeating of →4)-D-Gal*p*A-(1→2)-L-Rha*p*-(1→ and that the side chains are attached to O-4 of rhamnosyl residues of the backbone.

Anomeric configurations of rhamnosyl and galacturonic acid residues in RG-I were determined by ^{13}C NMR spectroscopy (data not shown). The signals appeared at 105.5 ppm and 100.5 ppm, which were assigned to C-1 of α-L-rhamnosyl and C-1 of α-D-galacturonic acid residues, respectively [15]. This result shows that the backbone structure of sugi RG-I had a disaccharide repeating unit of →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→, which is equal to that of sycamore RG-I.

Lithium-treatment in ethylenediamine degrades uronic acid residues in RG-I to release the side chains consisting of neutral sugar residues [16,17]. Structural analysis of the products obtained by such treatment gives useful information on the side chains of RG-I. Glycosyl composition analysis of the Li-treated RG-I showed that over 90% of the galacturonic acid residues in RG-I were degraded (data not shown). Glycosyl linkage analysis showed that the arabinosyl and galactosyl residues in RG-I were stable toward the Li-treatment (data not shown). The major glycosyl residues of the Li-treated products were terminal,

m/z [(M+Na)$^+$]	Composition consistent with pseudomolecular ion
565	$(pent)_3$
579*	$(pent)_2$ deoxyhex
623*	pent hex deoxyhex
667*	$(hex)_2$ deoxyhex
725	$(pent)_4$
783*	$(pent)_2$ hex deoxyhex
885	$(pent)_5$
943*	$(pent)_3$ hex deoxyhex
1045*	$(hex)_3$ $(deoxyhex)_2$

Fig. 3.

3- and 5-linked arabinosyl, and terminal galactosyl units. Per-O-methylated glycosyl alditols of the Li-treated products were analyzed by GC–MS to elucidate the glycosyl sequences. Based on the MS fragment patterns (Tables 4 and 5), the glycosyl sequences of 11 compounds were identified (Fig. 2). The compounds previously identified by Lau et al. [17] were omitted in the tables. From the glycosyl composition of the Li-treated product, pentosyl, deoxyhexosyl and hexosyl units tentatively assigned by GC–MS were identified to be arabinosyl, rhamnosyl and galactosyl residues, respectively. Oligosaccharides obtained by partial acid hydrolysis showed that the side chains were linked to O-4 of the rhamnosyl residues of the backbone (Fig. 1). Presence of compound 7 (Fig. 2) indicates that arabinosyl units were linked to the backbone through galactosyl residue. Furthermore, the compounds lacking rhamnosyl residues such as 2-a, -b, 3, 5, 6-a, -b, -c, -d would be attached to galacturonic acid residues directly or be produced by cleavage of neutral glycosyl linkages under Li-treatment. Presence of compounds 2-a, -b, 6-a, -b, -c, -d indicates that different types of arabinan oligosaccharides interconnected at O-3 and/or O-5 of arabinosyl residues existed in the side chains. FABMS of the Li-treatment products gave the quasimolecular molecular ions $(M + Na)^+$ at *m/z* 565, 783 and 885, suggesting the existence of long arabinan oligosaccharides (Fig. 3). These arabinan oligosaccharides would be derived from long arabinan side chains linked to rhamnosyl residue of the backbone directly or through galactosyl residues. FABMS further gave the quasimolecular ions at *m/z* 783 and 943 corresponding to $(pent)_2$-hex-deoxyhex and $(pent)_3$-hex-deoxyhex, respectively. The reducing end residue could not be determined only by FABMS. However, based on structures determined by GC–MS (Figs. 1 and 2), deoxyhexoses were supposed to be the reducing end residues of the compounds if present. The glycosyl sequences determined by GC–MS indicate that the side chains of arabinan oligosaccharides were attached to the rhamnosyl residues of the backbone through galactosyl residues (7 in Fig. 2). But, the presence of the ion at *m/z* 579, which corresponded to {$(pent)_3$-deoxyhex}, suggests that some side chains of arabinan oligosaccharides were linked to the backbone directly. The presence of compound 5 (Fig. 2) shows that the oligosaccharides consisting of galactose residues also existed in the products. The FABMS ions at *m/z* 667 and 1045 corresponded to $(hex)_2$-deoxyhex and $(hex)_3$-deoxyhex, respectively, which imply that the side chains containing galactosyl residues were attached to the backbone directly (Fig. 3). Presence of compound 1 (Fig. 2) suggests that the side chains contained rhamnosyl residues as minor components. Compound 3 implies that galactosyl residues were attached to the side chains composed of arabinosyl residues. These results show that the side chains of sugi RG-I consisted mainly of arabinosyl and galactosyl residues and had considerably complicated structures.

From these results, the structure of sugi RG-I is now reviewed as follows. The backbone of sugi RG-I was a disaccharide repeating of unit $\rightarrow 4)$-α-D-Gal*p*A-$(1 \rightarrow 2)$-α-L-Rha*p*-$(1 \rightarrow$ and the side chains composed mainly of arabinosyl and galactosyl residues were linked to O-4 of rhamnosyl residues. The side chains were structurally quite complicated, and arabinan, one type of the side chains, linked to O-4 of the rhamnosyl residues of the backbone directly or via O-3 of 1,3-linked galactosyl residues. Though the most part of sugi (gymnosperm) RG-I was structurally similar to sycamore (angiosperm) RG-I, the structures and/or the distribution of the arabinan side chains were supposed to be different.

3. Experimental

Preparation of pectin from the cell wall of xylem differentiating zones of sugi.—The cell wall of xylem differentiating zones of sugi was prepared as described in the previous paper [10]. The cell wall material was subjected to the extraction with 50 mM CDTA (pH 6.5) and then with 50 mM Na_2CO_3 solns to solubilize pectic polysaccharides [7]. The cell wall material was treated with 50 mM CDTA solution (CDTA-I) at 20 °C for 6 h, and then at 20 °C for 2 h (CDTA-II). The residue was treated with 50 mM Na_2CO_3 soln containing 20 mM $NaBH_4$ under nitrogen gas at 1 °C for 16 h (Na_2CO_3-I) and then with 50 mM Na_2CO_3 soln under nitrogen gas at 20 °C for 3 h (Na_2CO_3-II).

Purification of pectic polysaccharides.—Each crude pectin fraction (CDTA-I, -II, Na_2CO_3-I and -II) was deesterified with dilute alkaline soln at pH 12 and 4 °C for 2 h as described [9]. The deesterified pectin was purified on a DEAE-Sepharose FAST FLOW column (80 cm × 1.5 cm i.d.) as described [10].

Separation of RG-I.—The purified pectin fractions were dissolved in 50 mM Na-acetate buffer (pH 5.2), and endo-1,4-α-polygalacturonase (EPGase) purified from pectinase of *Aspergillus niger* (Sigma, EC

3.2.1.15, 5 unit to 1 mg of sample) [18] was added. The solns were incubated at 30 °C for 48 h. The EPGase-treated pectin fraction was separated on a Bio-Gel P-30 column (80 cm × 1.5 cm i.d.) to give three fractions, namely, RG-I, RG-II and oligogalacturonides derived from homogalacturonan [10].

Glycosyl composition analysis.—Neutral and acidic glycosyl compositions were determined as Me_3Si ethers of methyl glycosides after methanolysis with 5% methanolic HCl solution at 80 °C for 16 h [19]. The derivatives were analyzed by GC using a fused Silica DB-1 capillary column (30 m × 0.25 mm i.d.) as described [19].

Glycosyl linkage analysis (methylation analysis).—Glycosyl linkage composition was determined by GC and GC–MS analysis of the partially O-methylated alditol acetates. Per-O-methylation was performed by a modification [20] of the Hakomori procedure [21]. Per-O-methylated polysaccharides were purified using a Sep-Pak C_{18} cartridge (Waters Associate) [22]. Methyl esters of per-O-methylated galacturonic acid residues in pectic polysaccharides were reduced with 1 M soln of Li-triethylborodeuteride in tetrahydrofuran (Super-Deuteride, Aldrich) at room temperature for 1 h after per-O-methylation [19]. The per-O-methylated, carboxyl-reduced polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (TFA) at 121 °C for 1 h, and were converted into their alditol acetate derivatives as described [19]. The GC analysis was performed on a GC-14A gas–liquid chromatograph (Shimazu) with a SP-2330 capillary column (30 m × 0.25 mm i.d.).

Partial hydrolysis and preparation of per-O-alkylated oligoglycosyl alditols.—Structural analysis of the backbone of RG-I was performed according to Lau et al. [8]. The uronic acid residues in RG-I were reduced to 6,6′-dideuterio-hexosyl residues by the improved method [8] of Taylor and Conrad [23]. The carboxyl-reduced RG-I (about 10 mg) was per-O-methylated by a modification of the Hakomori procedure described above. The reduced and per-O-methylated sample was hydrolyzed with 16 mL of 88% formic acid at 70 °C for 80 min. The formic acid was co-evaporated with toluene by a stream of air. The partially O-methylated oligosaccharides released were reduced with sodium borodeuteride, Na^+ ions were removed by passing through a Dowex 50 W column (1 mL, H^+ form) and evaporated to dryness. Methanol was added and co-evaporated to remove borate. The partially O-methylated oligoglycosyl alditols were per-O-pentadeuterioethylated with pentadeuterioethyl iodide by a modification of the Hakomori procedure. The pentadeuterioethylation was used to forestall any ambiguities of mass spectrometry, because the molecular difference between ethyl group and methyl groups is equal to the difference between deoxyhexosyl and pentosyl residues. The partially O-pentadeuterioethylated and, partially O-methylated oligoglycosyl alditols were analyzed by mass spectrometry.

Lithium treatment of RG-I.—The procedure was carried out according to Lau et al. [16,17] and Ishii et al. [24]. Dried RG-I (20 mg) was dissolved in 2 mL of ethylenediamine and small pieces of lithium wire (Aldrich Chemical) were added. The soln was stirred for 60 min and kept blue by supplying additional pieces of lithium wire. Cold water (5 mL) was added to stop the reaction and the soln was co-evaporated with toluene. The products were dissolved in water and the pH of the soln was adjusted to 4.5 with acetic acid. The soln was passed through a Dowex 50 W column (10 mL, H^+ form), concentrated to small volume under reduced pressure and lyophilyzed. The lithium-treated products were reduced with $NaBH_4$ at room temperature for 3 h. After addition of a few drops of acetic acid to decompose the residual $NaBH_4$, the soln was passed through a Dowex 50 W column (H^+ form) and lyophilyzed. Methanol was added to the products and evaporated to remove borate. The obtained sample was per-O-methylated by a modification of the Hakomori procedure described above. The per-O-methylated samples were analyzed by GC–MS as described below.

Mass spectrometry.—The products prepared by the procedures shown in glycosyl composition and glycosyl linkage analyses were analyzed on a QP-2000A GC–MS spectrometer (Shimadzu) equipped with a SP-2330 capillary column (30 m × 0.25 mm i.d.) as described [10]. Per-O-methylated oligoglycosyl alditols were analyzed by capillary GC–MS. The equipment was a HP 5891J GC coupled with a JEOL JMS-DX303 mass spectrometer and a JEOL JMA-DA 5000 data system; the ionization energy was 70 eV, emission current 300 μA, acceleration voltage 3 kV and source temperature 180 °C. A DB-1 capillary column (30 m × 0.25 mm i.d.) was used in the splitless mode with a time program starting at 50 °C for 2 min, to 190 °C by a rate of 30 °C/min, followed by a rate of 6 °C/min to 340 °C.

Fast-atom bombardment (FAB)MS was measured with a JEOL DX303 mass spectrometer coupled with a JEOL JMA DA-5000 data system. Emission current was 10 mA and acceleration voltage 3 kV. Argon and *m*-nitrobenzyl alcohol were used as a bombardment gas and a matrix, respectively.

^{13}C *NMR spectroscopy.*—^{13}C NMR experiment was performed at 27 °C with a JEOL SGX-400 spectrometer. A sample was dissolved in deuterium oxide (99.96 atm % D; Aldrich). The chemical shifts were expressed in ppm relative to external d_4-MeOH (δ 49.8).

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