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STRUCTURE OF THE BACKBONE OF RHAMNOGALACTURONAN I, A PECTIC POLYSACCHARIDE IN THE PRIMARY CELL WALLS OF PLANTS*,**

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ABSTRACT

Rhamnogalacturonan I (RG-I), solubilized from the walls of suspension-cultured sycamore cells (*Acer pseudoplatanus*) by digestion of the walls with a highly purified endo-1,4-α-polygalacturonase, was found to possess a backbone consisting of the diglycosyl repeating unit →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→. This structure was established by means of l.c.-m.s., g.c.-m.s., and ^{1}H-n.m.r. spectroscopic studies of per-*O*-alkylated oligoglycosyl-alditol fragments of RG-I. Side chains are attached to O-4 of half the rhamnosyl residues. No regular pattern was found for the arrangement in the backbone of the branched and unbranched rhamnosyl residues.

INTRODUCTION

Rhamnogalacturonan I (RG-I) is a pectic polysaccharide[2] solubilized from the walls of suspension-cultured sycamore (*Acer pseudoplatanus*) cells by treatment of the walls with a highly purified endopolygalacturonase[3]. RG-I, as solubilized, is a polymer of mol. wt. ~200,000 (as determined by gel-permeation chromatography), constituting about 7% of the wall material[4]. It is composed of L-rhamnopyranosyl, D-galactopyranosyluronic acid, L-arabinofuranosyl, D-galactopyranosyl, and L-fucopyranosyl residues[4]. Approximately half the L-rhamnosyl residues are 2-linked and half are 2,4-linked[4]. The D-galactosyluronic acid residues are predominantly 4-linked[4]. D-Galactosyluronic acid residues have been shown to be glycosidically linked to O-2 of L-rhamnosyl residues in pectic polysaccharides[5,6], including RG-I. Previous studies of RG-I have suggested that 2,4-linked L-rhamnosyl residues are glycosidically attached to D-galactosyluronic acid residues[4]. It has also been suggested that interconnected L-rhamnosyl and D-galactosyluronic acid residues constitute the backbone of the polysaccharide, from which emanate

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side chains that consist largely of L-arabinosyl and D-galactosyl residues[4]. This idea led us to investigate the arrangement, in RG-I, of L-rhamnosyl and D-galactosyluronic acid residues. The experiments reported in this paper establish that the backbone of RG-I possesses a strictly alternating sequence of L-rhamnosyl and D-galactosyluronic acid residues. This structural feature of RG-I was determined by means of l.c.-m.s., g.c.-m.s., and ^{1}H-n.m.r.-spectroscopic analyses of per-*O*-alkylated oligoglycosyl-alditol fragments.

EXPERIMENTAL

Isolation of RG-I. — RG-I was solubilized from the purified walls of suspension-cultured sycamore cells by treatment of the walls with an endo-1,4-α-polygalacturonase purified from the culture medium of *Colletotrichum lindemuthianum*[3]. The solubilized polysaccharide was purified by means of ion-exchange and gel-permeation chromatography, as described[4]. The polysaccharide was then de-esterified by incubation for 2 h at pH 12 and 0°, and then treated again with the endo-1,4-α-polygalacturonase. The resulting polysaccharide was further purified by gel-permeation chromatography on a Bio-Gel P-10 column (3 × 50 cm), with 50 mM sodium acetate, pH 5.2, as the eluant. The fraction emerging from this column in the void volume constituted RG-I.

Determination of molecular weight of RG-I. — The molecular weight of RG-I was estimated by gel-permeation chromatography on an Agarose A-5m column (3 × 50 cm) with 50mM sodium acetate, pH 5.2, as the eluant[4].

Reduction of the carboxyl groups of the galactosyluronic acid residues. — The carboxyl groups of the D-galactosyluronic acid residues of RG-I were reduced to the corresponding 6,6-dideuterio-D-galactosyl residues by the method of Taylor and Conrad[7], except that sodium borodeuteride and deuterium oxide were substituted for sodium borohydride and H_2O. RG-I (40 mg) was reduced twice in this way. The resulting material is called carboxyl-reduced RG-I. The extent of reduction was determined by the formation of trimethylsilyl ether-methyl glycoside derivatives, and analysis by means of g.l.c. and g.c.-m.s.[8].

Glycosyl-composition analysis. — The glycosyl-residue composition of carboxyl-reduced RG-I (250 μg) was determined by means of g.c. and g.c.-m.s. analysis of the acetylated alditol derivatives obtained by hydrolysis (2M trifluoroacetic acid, 1 h, 120°), reduction ($NaBD_4$), and acetylation (1:1 acetic anhydride and pyridine, 25 min, 120°)[9] of the polysaccharide.

Per-O-methylation of carboxyl-reduced RG-I. — Dry, carboxyl-reduced RG-I (22 mg) was dissolved in dimethyl sulfoxide (2 mL) and the solution stirred for 1 h at room temperature. Sodium methylsulfinylmethylide (200 μL, 4M) was added, and the solution stirred for 2 h. Then methyl iodide (50 μL) was added, and the mixture was stirred overnight. Sodium methylsulfinylmethylide and methyl iodide were added two more times in the same manner, but with 75 μL of methyl iodide on the final addition[10]. The carboxyl-reduced, per-*O*-methylated RG-I was purified

on Sep-Pak C-18 cartridges (Waters Associates), as described[9]. (Note: Hereafter, carboxyl-reduced per-*O*-methylated RG-I refers to RG-I that has been first carboxyl-reduced and then per-*O*-methylated.)

Glycosyl-linkage-composition analysis. — The glycosyl-linkage composition of four aliquots (250 μg each) of carboxyl-reduced, per-*O*-methylated RG-I was determined by means of g.c. and g.c.-m.s. analyses of the partially *O*-acetylated, partially *O*-methylated alditol derivatives, as described[11,12].

Preparation of carboxyl-reduced, per-O-alkylated oligoglycosyl-alditols. — Carboxyl-reduced, per-*O*-methylated RG-I (12 mg) was partially hydrolyzed with 88% formic acid (16 mL, 80 min, 70°). The formic acid was removed by rotary evaporation in the presence of toluene. The oligosaccharides formed by partial hydrolysis were then reduced with sodium borodeuteride. The resulting partially *O*-methylated oligoglycosyl-alditols were desalted on a Dowex 50-W X-12 (H^+) column (4 mL) eluted with 1:1 ethanol–water. The eluate was evaporated to dryness under a stream of filtered air and the residue was further dried *in vacuo* at 40°. The dried, partially *O*-methylated oligoglycosyl-alditols were then dissolved in dimethyl sulfoxide (2 mL), and the solution was stirred for 1 h before the addition of 4M sodium methylsulfinylmethylide (200 μL). This solution was stirred for 2 h, pentadeuterioethyl iodide (100 μL) was added, and the solution was stirred overnight. The carboxyl-reduced, partially *O*-pentadeuterioethylated, partially *O*-methylated oligoglycosyl-alditols were purified on Sep-Pak C-18 cartridges as described[9].

L.c. fractionation of the per-O-alkylated oligoglycosyl-alditol fragments and c.i.-m.s. analysis. — The mixture of carboxyl-reduced, per-*O*-alkylated oligoglycosyl-alditols, generated by means of partial hydrolysis, reduction, and pentadeuterioethylation of carboxyl-reduced, per-*O*-methylated RG-I, was fractionated by reversed-phase l.c. on an Altex ultrasphere ODS column (5 μm, 4.6 mm × 25 cm) at 3.5 MPa (0.5 mL/min) with a gradient of 50 to 75% acetonitrile in water (45 min), and then a gradient of 75 to 100% acetonitrile in water (5 min). Detection and molecular-weight determination of the eluted per-*O*-alkylated oligoglycosyl-alditols were accomplished by means of c.i.-m.s. analysis of 3% of the l.c. effluent, as described[13].

G.c.-m.s. of the per-O-alkylated oligoglycosyl-alditols that had been partially separated by l.c. — Fractions (0.25 mL) of the remaining 97% of the l.c. effluent were collected, evaporated to dryness, and analyzed by g.c.-m.s. (e.i.) on a DB-1 (J and W Scientific Co.) capillary column (25 m × 0.32 mm i.d.) with a temperature program of 2 min at 140°, 30°/min to 220°, and 6°/min to 340°, as described[13,14].

^{1}H-N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra of selected carboxyl-reduced, per-*O*-alkylated oligoglycosyl-alditols were recorded with a Bruker WM-250 Fourier-transform n.m.r. spectrometer, operated at 250 MHz. Samples were dissolved in hexadeuterioacetone (99.997% D). Chemical shifts are reported relative to pentadeuterioacetone (δ 2.04).

Glycosyl-linkage-composition analysis of the per-O-alkylated oligoglycosyl-al-

ditols. — The glycosyl-linkage compositions of the per-*O*-alkylated oligoglycosyl-alditols were determined by means of g.c. and g.c.-m.s. analyses of their partially *O*-acetylated, partially *O*-methylated, partially *O*-pentadeuterioethylated alditols derived by hydrolysis, reduction, and acetylation, as described[13,15].

RESULTS AND DISCUSSION

The glycosyl and glycosyl-linkage compositions of RG-I. — When RG-I was purified and studied previously[4] it was not de-esterified and was not treated a second time with endopolygalacturonase (see Experimental section). Treating RG-I with endopolygalacturonase, after de-esterification with base, yielded two fractions. One fraction was eluted at the included volume of the Bio-Gel P-10 column and contained only D-galactosyluronic acid residues, most likely as mono-, di-, and trisaccharides, which are the expected products of the endopolygalacturonase digestion of uninterrupted stretches of D-galactosyluronic acid residues[3].

TABLE I

GLYCOSYL-LINKAGE COMPOSITION OF CARBOXYL-REDUCED RG-I

Residue	*Mole %*	
	Present data	*Previously reported*[4]
T-Gal*p*A[a]	1.6	1.8
4-Gal*p*A[a]	15.2[b]	33.0
2,4-Gal*p*A[a]	1.0	tr
T-Rha*p*	1.8	0.5
2-Rha*p*	7.8	7 0
2,4-Rha*p*	8.0	6.0
2,3,4-Rha*p*	0.6	tr.
T-Ara*f*	9.5	9.4
2-Ara*f*	2.2	0.4
3-Ara*f*	2.2	0 8
5-Ara*f*	11.2	8 0
2,5-Ara*f*	1.0	1 6
3,5-Ara*f*	3.5	2.0
T-Fuc*p*	1.4	1.6
T-Gal*p*	6.3	4.0
2-Gal*p*	0.6	0.8
3-Gal*p*	2.7	1 6
4-Gal*p*	8.4	9.0
6-Gal*p*	7.5	4.0
2,4-Gal*p*	0.5	tr.
2,6-Gal*p*	1.2	0 8
3,6-Gal*p*	1.2	0.8
4,6-Gal*p*	2 4	tr.
T-Ara*p* & T-Xyl*p*[c]	2.0	0.0

[a]Analyzed as 6,6-dideuteriogalactosyl residues; T = nonreducing terminal. [b]Includes 0.7 mol% of 6,6-dideuteriogalactosyl residues undermethylated at O-6. [c]Partially acetylated, partially methylated alditol derivatives of T-Ara*p* and T-Xyl*p* co-elute on DB-1 column

The other fraction was eluted at the void volume of the Bio-Gel P-10 column and was composed of L-rhamnosyl (16%), D-galactosyluronic acid (17%), L-arabinosyl (32%), D-galactosyl (31%), and L-fucosyl (2%) residues. It had less D-galactosyluronic acid, the same neutral glycosyl-residue composition, and approximately the same size as RG-I before de-esterifying and re-treating with endopolygalacturonase[4]. This fraction constituted "purified" RG-I. Since neither the size of RG-I nor the neutral glycosyl-residue composition of RG-I was detectably altered by treatment with base and endopolygalacturonase, it is likely that the only structural change produced by these treatments was the cleavage of exterior, linear stretches of D-galactosyluronic acid residues. Another possibility is that these treatments resulted in the hydrolysis of polygalactosyluronic acid chains that had been co-purified with, but were not covalently attached to, RG-I.

The carboxyl groups of the D-galactosyluronic acid residues of RG-I were reduced to the corresponding 6,6-dideuterio-D-galactosyl residues (see Experimental section). The extent of reduction, as determined by g.c.-m.s. analysis of the trimethylsilylated methyl glycosides, was 82% after the first reduction and virtually complete (no uronic acids were detected) after the second reduction. Thus, the galactosyluronic acid residues were converted into deuterium-labeled D-galactosyl residues in order to mark the positions of the D-galactosyluronic acid residues during subsequent analyses, and the potentially troublesome carboxyl groups were eliminated[16].

Carboxyl-reduced RG-I was methylated by a procedure involving three additions of sodium methylsulfinylmethylide interspersed with three additions of methyl iodide. The glycosyl-linkage composition of carboxyl-reduced per-*O*-methylated RG-I (Table I), determined by hydrolysis, reduction, and acetylation, differed from that reported previously[4]. The most notable difference was a decrease, in the present case, in the proportion of 4-linked D-galactosyluronic acid analyzed as 6,6-dideuterio-D-galactosyl residues, undoubtedly due to the de-esterification and retreatment of RG-I with endopolygalacturonase. The proportions of 2- and 2,4-linked L-rhamnosyl residues are approximately equal and the sum of these two is approximately equal to the proportion of 4-linked D-galactosyluronic acid in the purified RG-I (Table I).

Preparation of partially O-methylated, partially O-pentadeuterioethylated oligoglycosyl-alditols. — The sequence of glycosyl residues in oligo- and polysaccharides can be elucidated by forming overlapping di-, tri-, tetra-, and pentasaccharide fragments of the polymer, and then separating and structurally characterizing these fragments[15]. After conditions for the production of tri-, tetra-, and pentasaccharide fragments of the L-rhamnosyl- and 6,6-dideuterio-D-galactosyl-rich regions of RG-I were optimized, carboxyl-reduced, per-*O*-methylated RG-I was partially hydrolyzed. The optimal partial hydrolysis conditions, determined as described[15], caused hydrolysis of the glycosidic linkages of 42% of the 2-linked L-rhamnosyl residues, 33% of the 2,4-linked L-rhamnosyl residues, and 27% of the 4-linked 6,6-dideuterio-D-galactosyl residues. These conditions resulted in exten-

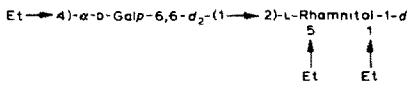
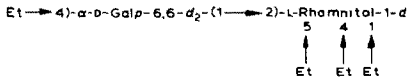
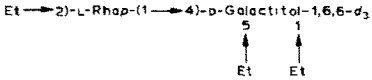
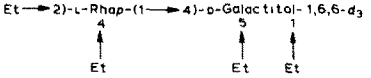
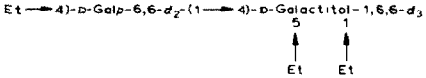
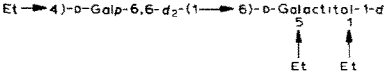
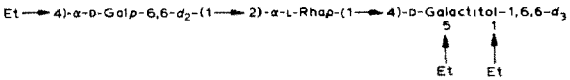
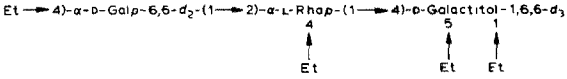
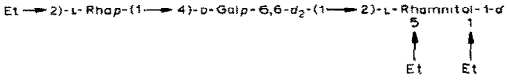
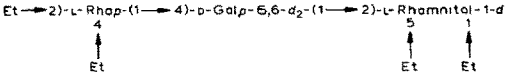
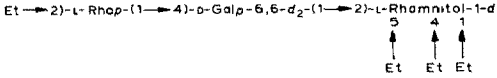
sive hydrolysis of other glycosidic linkages in RG-I (~95% T-Ara*, ~70% 5-Ara, ~65% T-Gal, ~50% 6-Gal). The more extensive hydrolysis of the glycosidic linkages of α-L-arabinofuranosyl residues was expected, because they are known to be more susceptible to acid-catalyzed hydrolysis than are the glycosidic linkages of α-D-galactopyranosyl and α-L-rhamnopyranosyl residues[17]. Hydrolysis of the glycosidic linkages of the β-D-galactopyranosyl residues was expected to be more extensive than that of the glycosidic linkages of the α-D-galactopyranosyl and α-L-rhamnopyranosyl residues[17]. This differential hydrolysis resulted in removal of the L-arabinosyl- and D-galactosyl-rich regions and formation of large numbers of oligosaccharides composed only of L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues.

The oligosaccharide mixture produced by partial hydrolysis was reduced with sodium borodeuteride, dried, and dissolved in dimethyl sulfoxide. The unsubstituted hydroxyl groups (those freed by partial hydrolysis and those formed by reduction) were pentadeuterioethylated to yield a mixture of partially *O*-methylated, partially *O*-pentadeuterioethylated oligoglycosyl-alditols.

The *O*-pentadeuterioethyl labeling group was chosen instead of an *O*-ethyl group in order to forestall any ambiguities in the mass-spectrometric analysis of the oligoglycosyl-alditols. A problem could have arisen because the mass of rhamnosyl residues is 14 AMU higher than that of arabinosyl residues, and an *O*-ethyl labeling group would impart a mass gain of 14 AMU over the corresponding *O*-methylated derivative. The pentadeuterioethyl group imparts a mass gain of 19 AMU over a corresponding methyl group, so an arabinosyl residue carrying one pentadeuterioethyl group can be distinguished from an otherwise similarly substituted rhamnosyl residue.

L.c. fractionation of the mixture of per-O-alkylated oligoglycosyl-alditols. — An aliquot (5 mg) of the mixture of per-*O*-alkylated oligoglycosyl-alditols was fractionated by means of l.c. The components in the effluent were located by c.i.-m.s. analysis. In this technique, approximately 3% of the l.c. effluent is analyzed by means of c.i.-m.s. and ~97% of the effluent is collected in fractions and retained for further analyses. All oligoglycosyl-alditols eluted from the l.c. column were analyzed for their content of L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues. This analysis was accomplished by computer-assisted calculation of all possible M + 1 ions of per-*O*-alkylated oligoglycosyl-alditols that could contain L-rhamnosyl or 6,6-dideuterio-D-galactosyl residues of the type that were shown to be present in RG-I by glycosyl-linkage analysis (Table I) and, then, by computer-assisted checking of reconstructed selected-ion mass chromatograms for those ions, as described[13]. The analysis was simplified because, as a result of the hydrolysis conditions selected, almost all the per-*O*-alkylated oligoglycosyl-alditols produced in abundance contained L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues.

*T = nonreducing terminal.

Numerical designation in this paper	Per-*O*-alkylated oligoglycosyl-alditol
1	
2	
3	
4	
5	
6	
7	
8	
9	
10	
11	

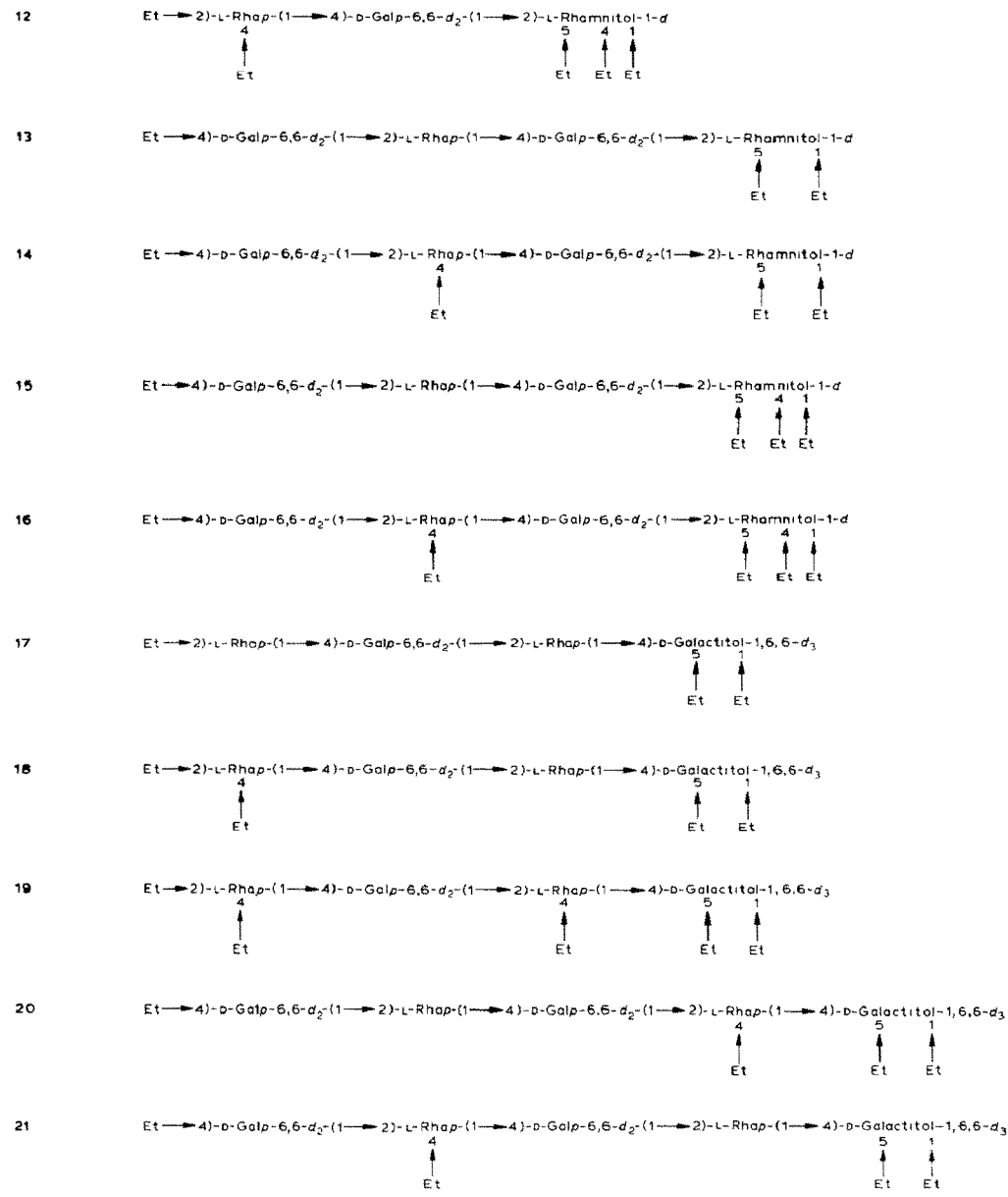


Fig. 1. List of structurally characterized partially-*O*-methylated, partially-*O*-pentadeuterioethylated oligoglycosyl-alditols. Any oxygen atom not substituted with an ethyl group or involved in a glycosidic bond or in ring formation is substituted with a methyl group. Anomeric configurations are indicated when ^{1}H-n.m.r. determinations have been completed. Et = pentadeuterioethyl

TABLE II

DIAGNOSTIC C I -M S AND E I -M S IONS OF THE PER-*O*-ALKYLATED MONOGLYCOSYL-ALDITOLS DERIVED FROM RG-I

Per-O-alkylated oligoglycosyl-alditol	*R.t. (l.c.)*[b]	*C.i.-m.s. ions*[a]					*R.t. (g.l.c.)*[c]	*E.i.-m.s. ions*[a]						
		M + 1	aJ_2	$aJ_2 + H_2O$	bA_1	bA_2		aJ_1	aJ_2	bA_1	bA_2	*Alditol*		
1	10.23	501	244	262	240	208	7.5	304	244	240	208	422	378	
		(38)	(23)	(7)	(10)	(3)		(0.25)	(100)	(25)	(50)	(1)	(2)	
2	15.10	520	263	281	240	208	7.8	323	263	240	208	141		
		(51)	(29)	(9)	(11)	(12)		(1)	(75)	(40)	(60)	(35)		
3	11.28	501	276	294	208	176	7.02	336	276	208	176	391		
		(5)	(1)	(100)	(1)	(2)		(3)	(20)	(50)	(2)	(2)		
4	16.02	520	276	294	227	195	7.2	336	276	227	176	153		
		(18)	(5)	(51)	(15)	(3)		(1)	(5)	(16)	(2)	(1)		
5	8.9	533	276	294	240	208	8.25	336	276	240	208	379	423	
		(3.1)	(3.6)	(2.5)	(1)	(1)		(10)	(50)	(40)	(80)	(1)	(1)	
6	11.83	531	274	292	240	208	9.8	334	274	240	208	197	333	377
		(7.7)	(2.5)	(1)	(1.3)	(1)		(3.4)	(100)	(8.5)	(64.4)	(3)	(1)	(2.9)

[a]Numbers in parentheses indicate ion intensity; dashes refer to ions not detected. [b]L.c. retention time (min) on the ODS column described in the Experimental section. [c]G.l.c. retention time (min) on the DB-1 column described in the Experimental section.

TABLE III

DIAGNOSTIC C I -M S IONS FOR PER-*O*-ALKYLATED DIGLYCOSYL-ALDITOLS DERIVED FROM RG-I[a]

Per-O-alkylated oligoglycosyl-alditol	*R.t. (l.c.)*[b]	*M + 1*	*Elim 1*	aJ_2	$aJ_2 + H_2O$	cA_1	cA_2	cbA_1	cbA_2
7	13.28	707	533	276	294	240	208	414	382
		(15)	(10)	(5)	(100)	(21)	(20)	(13)	(20)
8	18.93	726	533	276	294	240	208	433	401
		(11)	(13)	(14)	(100)	(42)	(40)	(8)	(27)
9	15.40	675	469	244	262	208	176	—	—
		(19)	(10)	(18)	(45)	(92)	(55)		
10	21.62	694	488	244	262	227	195	433	401
		(4)	(7)	(14)	(51)	(43)	(20)	(1)	(6)
11	21.62	694	488	263	281	208	176	414	382
		(4)	(7)	(24)	(6)	(11)	(31)	(1)	(2)
12	31.57	713	507	263	281	227	195	433	401
		(3)	(3)	(7)	(13)	(18)	(10)	(2)	(3)

[a]As in Table II. [b]As in Table II.

TABLE IV

DIAGNOSTIC C I -M S IONS FOR PER-*O*-ALKYLATED TRIGLYCOSYL-ALDITOLS DERIVED FROM RG-I[a]

Per-O-alkylated oligoglycosyl-alditol	*R.t. (l.c.)*[b]	*M + 1*	*Elim 1*	*Elim 2*	aJ_2	$aJ_2 + H_2O$	dA_1	dA_2	dcA_1	dcA_2
13	18.52	881	675	501	244	262	240	208	414	382
		(1)	(7)	(3)	(28)	(46)	(44)	(35)	(10)	(31)
14	26.20	900	694	501	244	262	240	208	433	401
		(2)	(3)	(3)	(21)	(33)	(28)	(25)	(5)	(18)
15	27.17	900	694	520	263	281	240	208	414	382
		(1)	(0.4)	(0.5)	(4)	(7)	(7)	(11)	(2)	(4.4)
16	36.53	919	713	520	263	281	240	208	433	401
		(1)	(1.1)	(0.5)	(8)	(9)	(9)	(11)	(3)	(7)
17	18.82	881	707	501	276	294	208	176	414	382
		(1.2)	(1)	(2)	(5)	(86)	(46)	(32)	(5)	(13)
18	26.43	900	—	520	276	294	227	195	433	401
		(1.3)		(0.5)	(3.8)	(13.6)	(8.7)	(4.0)	(4 5)	(13.9)
19	35.73	919	726	520	276	294	227	195	433	401
		(1)	(0.5)	(0.6)	(8)	(9)	(16)	(10)	(3)	(4)

[a]As in Table II. [b]As in Table II.

Determination of the glycosyl sequences of the per-O-alkylated oligoglycosyl-alditols. — The glycosyl sequences of each of 19 per-*O*-alkylated oligoglycosyl-alditols that contained L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues were determined in part by c.i.-m.s. analysis of these components in the l.c. effluent. For each component, this gave the M + 1 ion, the informative c.i.-elimination ions[18], and diagnostic c.i.-m.s. fragment-ions[19] (Fig. 1; Tables II–IV). The complete

TABLE V

DIAGNOSTIC E I -M S IONS FOR PER-*O*-ALKYLATED DIGLYCOSYL-ALDITOLS DERIVED FROM RG-I[a]

Per-O-alkylated oligoglycosyl-alditol	*R.t. (g.l.c.)*[b]	aJ_1	aJ_2	abJ_1	abJ_2	cA_1	cA_2	cbA_1	cbA_2
7	13.93	336 (2)	276 (10)	510 (2)	450 (1)	240 (10)	208 (50)	414 (2)	382 (3)
8	13.95	336 (10)	276 (40)	529 (5)	—	240 (40)	208 (100)	433 (1)	401 (4)
9	14.12	304 (1)	244 (50)	510 (1)	—	208 (30)	176 (3)	414 (1)	382 (5)
10	14.48	323 (2.3)	263 (82)	529 (3.1)	—	208 (52)	176 (26)	414 (1.5)	382 (2.2)
11	14.57	304 (14)	244 (100)	510 (6.3)	—	227 (54)	195 (2)	433 (0.2)	401 (7)
12	13.05[c]	323 (0.6)	263 (15)	529 (1)	469 (0.5)	227 (15)	195 (5)	—	401 (1)

[a]As in Table II. [b]As in Footnote *c* of Table II. [c]Starting temp. of the g.l.c. program was 150°; all other parameters were the same as given in Experimental.

TABLE VI

DIAGNOSTIC E I -M.S IONS FOR PER-*O*-ALKYLATED TRIGLYCOSYL- AND TETRAGLYCOSYL-ALDITOLS DERIVED FROM RG-I[a]

Per-O-alkylated oligoglycosyl-alditol	*R.t. (g.l.c.)*[b]	aJ_1	aJ_2	abJ_1	abJ_2	cA_1	cA_2	cbA_1	cbA_2	
13	21.35	304 (1)	244 (100)	510 (1)	—	240 (10)	208 (50)	414 (2.5)	382 (10)	
		aJ_1	aJ_2	abJ_1	abJ_2	eA_1	eA_2	edA_1	edA_2	
20[c]	24.00	336 (6)	276 (51)	529 (1)	469 (12)	240 (57)	208 (100)	414 (1)	382 (17)	
21[c]	24.03	336 (6)	276 (51)	510 (4.6)	450 (1.1)	240 (57)	208 (100)	433 (3)	401 (1)	
		abJ_1	$abcJ_1$	$abcJ_2$	$abcdJ_1$	$abcdJ_2$	$edcA_1$	$edcA_2$	$edcbA_1$	$edcbA_2$
20[c,d]	24.00	529 (84)	735 (11.1)	—	909 (7.8)	849 (3.8)	—	588 (9.5)	—	781 (3)
21[c,d]	24.03	510 (100)	716 (9.8)	—	909 (4.0)	849 (3.5)	639 (4.3)	607 (10.1)	—	781 (3)

[a]Same as in Table II. [b]As in Footnote *c* of Table II. [c]Mass spectra of tetraglycosyl-alditols were obtained after g.l.c. separation (no l.c. separation was performed) of an aliquot of the oligoglycosyl-alditol mixture. [d]High-mass fragment-ions were obtained by a second analysis of tetraglycosyl-alditol-containing samples with the mass spectrometer scanning from 500 to 1000 amu.

TABLE VII

GLYCOSYL LINKAGE ANALYSIS OF PER-*O*-ALKYLATED OLIGOGLYCOSYL-ALDITOLS DERIVED FROM RG-I

Per-O-alkylated oligoglycosyl-alditol	*Residue*	*Positions of O-methyl groups*	*Positions of O-pentadeuterio-ethyl groups*	*Positions of O-acetyl groups*
1	Rhamnitol-1-*d*	3,4	1,5	2
	Gal-6,6-d_2	2,3,6	4	1,5
2	Rhamnitol-1-*d*	3	1,4,5	2
	Gal-6,6-d_2	2,3,6	4	1,5
5	Galactitol-1,6,6-d_3	2,3,6	1,5	4
	Gal-6,6-d_2	2,3,6	4	1,5
6	Galactitol-1-*d*	2,3,4	1,5	6
	Gal-6,6-d_2	2,3,6	4	1,5
7	Galactitol-1,6,6-d_3	2,3,6	1,5	4
	Rha	3,4		1,2,5
	Gal-6,6-d_2	2,3,6	4	1,5
8	Galactitol-1,6,6-d_3	2,3,6	1,5	4
	Rha	3	4	1,2,5
	Gal-6,6-d_2	2,3,6	4	1,5
9	Rhamnitol-1-*d*	3,4	1,5	2
	Gal-6,6-d_2	2,3,6		1,4,5
	Rha	3,4	2	1,5

sequences were determined from the e.i.-m.s. fragment-ions generated during g.c.-m.s. analysis of the per-*O*-alkylated oligoglycosyl-alditols present in fractions of the l.c. effluent[13,20] (Tables II, V, and VI) and, in some instances, by means of glycosyl-linkage analysis[13,15] (Table VII). Whenever both L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues were present in the per-*O*-alkylated oligoglycosyl-alditols, they were arranged in a strictly alternating sequence. Since glycosyl-linkage analysis (Table I) showed that RG-I contains equal proportions of 2- and 2,4-linked L-rhamnosyl residues, one might predict that the branched L-rhamnosyl residues would possess, in the chain, unbranched L-rhamnosyl residues as nearest neighbors. However, some oligoglycosyl-alditols were found to contain either two unbranched L-rhamnosyl residues as nearest neighbors (**9, 13,** and **17**) or two branched L-rhamnosyl residues as nearest neighbors (**12, 16,** and **19**). Therefore, with respect to each other, no regular pattern could be detected in the arrangement of the 2- and 2,4-linked L-rhamnosyl residues.

D-Galactosyluronic acid residues, analyzed as 6,6-dideuterio-D-galactosyl residues, were found in two per-*O*-alkylated oligoglycosyl-alditols that lacked L-rhamnosyl residues (**5** and **6**). In **5**, a 4-linked 6,6-dideuterio-D-galactosyl residue was attached to O-4 of another 4-linked 6,6-dideuterio-D-galactosyl residue. In **6**, a 4-linked 6,6-dideuterio-D-galactosyl residue was attached to O-6 of a 6-linked D-galactosyl residue. Both per-*O*-alkylated oligoglycosyl-alditols could have arisen from the L-arabinosyl- and D-galactosyl-rich regions (side chains) of RG-I. They were found in proportions that were small (<15%) compared to the proportion of

TABLE VIII

^{1}H-N.M.R. CHEMICAL SHIFTS AND COUPLING CONSTANTS OF THE ANOMERIC PROTONS OF REPRESENTATIVE PER-*O*-ALKYLATED OLIGOGLYCOSYL-ALDITOLS

Per-O-alkylated oligoglycosyl-alditol	*Chemical shift (p.p.m. δ)*	$J_{1,2}$ *(Hz)*	*Assignment*
1	5.24	2.88	α-D-Gal
2	5.27	2.74	α-D-Gal
7	5.04	2.0	α-L-Rha
	4.98	2.7	α-D-Gal
8	5.04	1.88	α-L-Rha
	4.99	2.72	α-D-Gal

an analogous backbone disaccharide (**1**) in which a 4-linked 6,6-dideuterio-D-galactosyl unit is attached to O-2 of a 2-linked L-rhamnosyl residue.

The results of hydrolysis, reduction, and acetylation of representative per-*O*-alkylated oligoglycosyl-alditols obtained from the l.c. effluent fractions (Table VII) showed that the L-rhamnosyl residues were invariably linked to 6,6-dideuterio-D-galactosyl residues at O-4 and that the 6,6-dideuterio-D-galactosyl residues were invariably linked to L-rhamnosyl residues at O-2. Pentadeuterioethyl labeling groups were found to be at O-4 in the branched 2-linked L-rhamnosyl residues, thereby indicating the point of attachment of structures other than D-galactosyluronic residues, presumably the L-arabinosyl- and D-galactosyl-rich portions of RG-I.

Determination of the anomeric configurations of the glycosyl residues by ^{1}H-n.m.r. spectroscopy. — Representative per-*O*-alkylated oligoglycosyl-alditols containing L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues were analyzed by ^{1}H-n.m.r. spectroscopy (Table VIII). Signals of the anomeric protons of β-linked L-rhamnosyl residues[21] are normally upfield of δ 4.8 and those of α-linked L-rhamnosyl residues[21] normally downfield of δ 4.8. The signals of the anomeric protons of β-linked D-galactosyl residues are upfield of δ 4.7 and possess coupling constants $J_{1,2} \geqq 5$ Hz[22]. Signals of α-linked galactosyl residues are downfield of δ 4.7 and have $J_{1,2} \leqq 3$ Hz[22]. All L-rhamnosyl and 6,6-dideuterio-D-galactosyl residues in all the per-*O*-alkylated oligoglycosyl-alditols examined by means of ^{1}H-n.m.r.-spectroscopy were unambiguously in the α-anomeric configuration.

GENERAL DISCUSSION

Previous work had suggested that L-rhamnosyl and D-galactosyluronic acid residues of RG-I form the backbone of the branched polymer[4], and that the backbone carries L-arabinosyl- and D-galactosyl-rich side chains that emanate from O-4 of the branched L-rhamnosyl residues. The results of the present study are consistent with these proposed structural features of RG-I. Indeed, the L-rham-

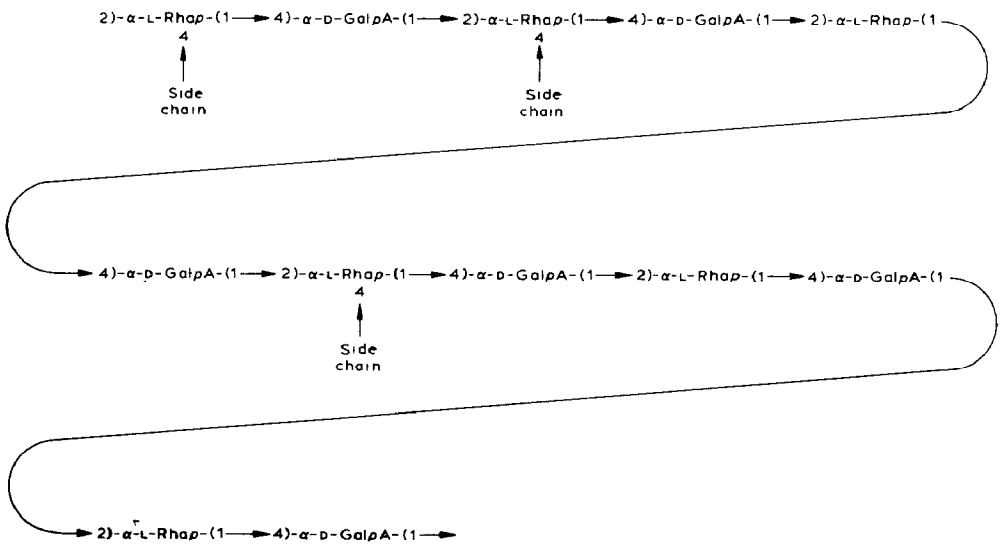


Fig. 2. Hypothetical region of the backbone of RG-I.

nosyl residues and all but a small number of the D-galactosyluronic acid residues of RG-I have now been shown to be present in a strictly alternating sequence. The length of this alternating sequence is unknown. However, since L-rhamnosyl and D-galactosyluronic acid residues comprise almost 35% of RG-I, the alternating sequence could be as long as 400 residues. About half the rhamnosyl residues in the oligoglycosyl-alditols representing this alternating sequence were found to be pentadeuterioethyl-labeled at O-4, which indicated the point of attachment of the more easily hydrolyzed α-L-arabinofuranosyl and β-D-galactopyranosyl residues.

RG-I contains a small number of D-galactosyluronic acid residues glycosidically linked to O-6 of D-galactosyl residues (**6**) (Table II). The trisaccharide fragment having a rhamnosyl residue glycosidically linked to O-4 of the galactosyluronic acid residue of **6** was specifically sought in the l.c. effluent and should have been detected if **6** were a common component of the backbone of RG-I. (The computer was used to look for the calculated M + 1 ions.) The absence of this trisaccharide indicates that these galactosyluronic acid residues are likely to be located in the side chains attached to the backbone at O-4 of the branched rhamnosyl residues. The trisaccharides →2)-L-Rha*p*-(1→4)-D-Gal*p*A-(1→4)-D-Galactitoluronic acid and →4)-D-Gal*p*A-(1→4)-D-Gal*p*A-(1→2)-L-Rhamnitol should have been detected if fragment **5**, composed of two galactosyluronic acid residues, were a common component of the backbone. These trisaccharides also were specifically sought in the l.c. effluent (as mentioned above) and were not detected. Therefore, it is also likely that fragment **5** is located in the side chains attached to the backbone at O-4 of the branched rhamnosyl residues.

This study has established that RG-I has relatively long portions of the strictly

alternating sequence →4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*(1→. No regular pattern was discernible for the arrangement of the branched and unbranched L-rhamnosyl residues. A hypothetical portion of RG-I, incorporating all of the oligosaccharides isolated from the backbone of RG-I, is presented in Fig. 2. The nature of the side chains attached to O-4 of the branched rhamnosyl residues is still in question, although at least seven differently linked[23] glycosyl residues attached to that position are known. We have yet to ascertain whether RG-I is a distinct polymer with a large number of different side chains[4] or a family of co-purifying polysaccharides, each of which possesses the same backbone but different sets of side chains.

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