Carbohydrate Research, 218 (1991) 211–222
Elsevier Science Publishers B.V., Amsterdam

Structural characterization of two oligosaccharide fragments formed by the selective cleavage of rhamnogalacturonan II: evidence for the anomeric configuration and attachment sites of apiose and 3-deoxy-2-heptulosaric acid

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(Received December 10th 1990; accepted March 1st 1991)

ABSTRACT

Evidence for the anomeric configurations and attachment sites of 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA) and apiosyl residues has been obtained through the characterization of two oligoglycosyl fragments isolated from rhamnogalacturonan II (RG-II). One of the oligoglycosyl fragments, a pentaglycosyl aldonic acid generated by Smith degradation of RG-II, was composed of four D-galactopyranosyluronic acid residues, a DHA residue, and a threonic acid residue (derived from a D-galactopyranosyluronic acid residue). The structural analysis of the pentaglycosyl aldonic acid established the β-D-configuration for the DHA residue. Furthermore, it established that a previously identified diglycosyl side chain, 5-*O*-(β-L-arabinofuranosyl)-DHA was directly attached to O-3 of a D-galactopyranosyluronic acid residue in the backbone of RG-II. The second oligoglycosyl fragment, a peralkylated diglycosyl hex-1-enitol, was generated by hex-5-enose degradation of permethylated and carboxyl-reduced RG-II. The structure of the peralkylated diglycosyl hex-1-enitol, β-L-Rha*p*-(1→3′)-β-D-Api*f*-(1→5)-hex-1-enitol, was determined by a combination of glycosyl-linkage composition analysis and n.m.r. spectroscopy. The n.m.r. data indicated the β-configuration for the D-apiosyl residue. The isolation and characterization of the diglycosyl hex-1-enitol also established that a previously identified heptaglycosyl side chain was directly attached to O-2 of a D-galactopyranosyluronic acid in the backbone of RG-II.

INTRODUCTION

Rhamnogalacturonan II (RG-II) is a complex acidic polysaccharide present in the primary cell walls of higher plants. RG-II has been isolated from the walls of suspension-cultured sycamore cells[1] by treatment with α-1,4-endopolygalacturonase[2]. RG-II has also been isolated from Douglas fir[3], from rice cell walls[4], and from Pectinol AC[5,6], and evidence has been obtained for its presence in the primary cell walls of all higher plant cell walls that have been examined. Pectinol AC is a commercially available mixture of plant cell wall enzymes obtained from the culture filtrate of the fungus, *Aspergillus niger*, when the fungus is grown on cell-wall residues as the carbon source. The RG-II isolated from sycamore and rice cell walls are structurally indistinguishable[7].

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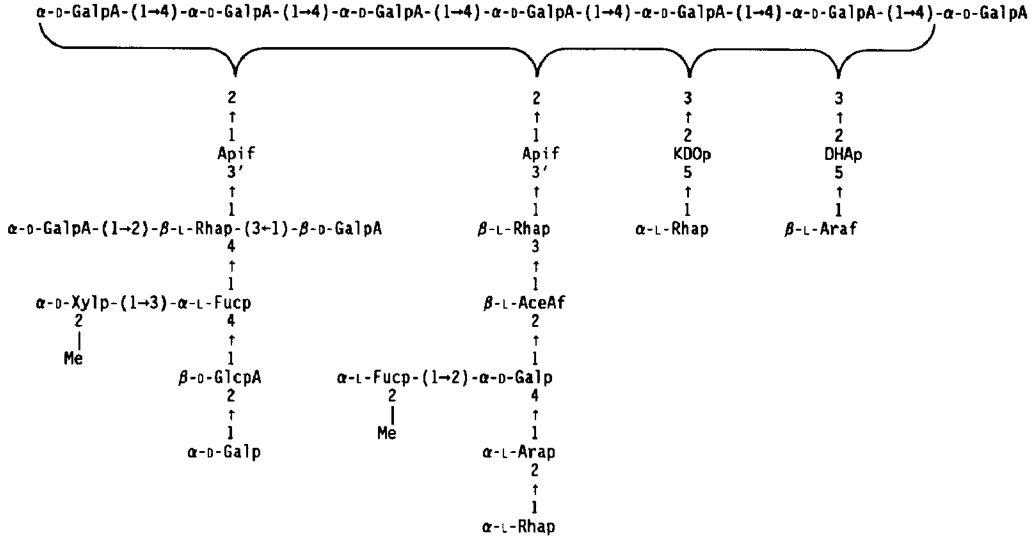


Fig. 1. Previously characterized oligoglycosyl fragments of rhamnogalacturonan II. The particular backbone Gal*p*A residue to which each oligoglycosyl side chain is attached has not been determined. AceA*f*: 3-*C*-carboxy-5-deoxy-L-xylose (aceric acid); DHA: 3-deoxy-D-*lyxo*-2-heptulosaric acid.

A single terminal rhamnosyl residue is missing in the RG-II extracted from Pectinol AC[7]. The terminal rhamnosyl residue was probably removed by a rhamnosidase known to be present in Pectinol AC.

RG-II contains at least eleven different monosaccharides, including the following seldom-observed sugars: 3-deoxy-D-*manno*-2-octulosonic acid (Kdo)[6], 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA)[8], apiose, and aceric acid (3-*C*-carboxy-5-deoxy-L-xylose)[5], 2-*O*-methylfucose, and 2-*O*-methylxylose. The backbone of RG-II is composed of at least seven 1,4-linked α-D-galactopyranosyluronic acid residues[9]. A variety of oligosaccharide side chains are attached to the backbone[7]. The carboxyl groups of some of the D-galactopyranosyluronic acid residues are esterified with methyl groups[9]. Partial acid hydrolysis of both native and *O*-permethylated RG-II have led to the isolation and structural characterization of oligosaccharide fragments of RG-II containing all or almost all of its glycosyl residues (see Fig. 1)[6–11]. The characterized oligosaccharide side chains (Fig. 1) were generated by selective cleavage of the acid-labile glycosidic linkages of apiose, Kdo, and DHA. The specific sites of attachment of the apiosyl, Kdo, and DHA residues need to be confirmed, and their anomeric configurations remain to be established.

We have been investigating the utility of Smith degradation[12] and hex-5-enose degradation[13,14] procedures to generate oligosaccharide fragments from RG-II containing the glycosidic linkages of apiofuranosyl, Kdo, and DHA residues. The structural characterization of such oligosaccharides could also provide information about the particular D-galactopyranosyluronic acid residues in the RG-II backbone to which each side chain is attached. This paper describes the structural characterization of a pentaglycosyl aldonic acid generated by Smith degradation of RG-II and a diglycosyl hex-1-

enitol derivative generated by the hex-5-enose degradation of methylated RG-II, which respectively contain glycosidically linked DHA and apiofuranose residues.

RESULTS AND DISCUSSION

Smith degradation of RG-II. — Glycosyl-linkage composition analysis of RG-II (data not shown[7]) showed that the apiosyl residues are only substituted at C-1 and at C-3′. Thus, periodate treatment of RG-II should lead to the oxidation of the apiosyl residues because they possess vicinal free hydroxyl groups at C-2 and C-3. Therefore oligosaccharides with intact apiosidic linkages cannot be obtained by Smith degradation of RG-II. The DHA and Kdo residues in RG-II are substituted at C-2 and C-5[6,8]. Thus, there are no vicinal hydroxyl groups on these residues except those on C-7 and C-8 of Kdo. Oxidative scission between C-7 and C-8 would convert Kdo, following reduction of the carbonyl group formed by the oxidation, to the corresponding heptulosonic acid. Therefore oligosaccharides containing glycosidically linked heptulosonic acid and others containing glycosidically linked DHA should be generated by Smith degradation of RG-II. The backbone of RG-II contains two 4-linked Gal*p*A residues somewhere in the chain that are susceptible to periodate oxidation and, following Smith degradation, would be modified to yield a 3-*O*-substituted threonic acid residue. These 4-linked Gal*p*As are the only points in the backbone of RG-II that would be cleaved by Smith degradation.

RG-II was oxidized with periodate, reduced with borodeuteride, and the resulting oxidized–reduced polysaccharide desalted by dialysis. The oxidation–reduction sequence was repeated to ensure that all the periodate-susceptible glycosyl residues were oxidized. Glycosyl-composition analysis of the oxidized–reduced material showed that the apiosyl, 2-*O*-methyl-D-xylosyl, and D-glucopyranosyluronic acid residues were completely destroyed. Aceryl acid, fucosyl, and DHA residues were not oxidized by the periodate (see preceding paragraph). Glycosyl residue composition analysis established that a heptulosonic acid residue had been generated by periodate cleavage of Kdo (see above). Approximately 70% of the 2-*O*-methylfucosyl, 23% of the arabinosyl, 52% of the rhamnosyl, 57% of the galactosyl, and 80% of the galacturonosyl residues survived the periodate oxidation. The quantities of periodate-resistant rhamnosyl, galactosyl, and galacturonosyl residues recovered are within the range expected by consideration of the glycosyl-linkage composition of RG-II[7]. The resistance of 2-*O*-methylfucosyl and arabinosyl residues to periodate oxidation was unexpected since these glycosyl residues are known to be present in RG-II at the non-reducing termini. *O*-Deacylation of RG-II with base prior to periodate oxidation did not alter the periodate susceptibility of these glycosyl residues. The most likely explanation for their resistance to periodate oxidation is that the 2-*O*-methylfucosyl and arabinosyl residues of RG-II are sterically protected against periodate oxidation.

Isolation of a pentaglycosyl aldonic acid. — The acid-labile acyclic acetals that are formed by periodate oxidation and reduction of susceptible glycosyl residues can be selectively hydrolyzed by mild acid treatment (Smith degradation). To accomplish this, oxidized–reduced RG-II was treated with 0.1N TFA at room temperature for 24 h.

Glycosyl-linkage composition analysis of the resulting products indicated that appreciable depolymerization of the oxidized–reduced RG-II did not occur under these conditions. The acyclic acetals derived from periodate-susceptible glycosyluronic acid residues are known to be resistant to the mild hydrolytic conditions that we used[15], and higher temperatures have been found to give better yields of the depolymerization products. Thus, the oxidation–reduction product of RG-II was treated with 0.1N TFA at 60° for 1 h, which gave appreciable quantities of oligomeric depolymerization products. This treatment also resulted in the total cleavage of heptulosonic acid (data not shown); therefore, no oligosaccharides containing the glycosidically linked heptulosonic acid could be isolated. However, the relative stability of the DHA[8] linkage to acidic conditions allowed us to isolate an oligoglycosyl aldonic acid containing glycosidically linked DHA.

Anion-exchange chromatography of the products derived from Smith degradation of RG-II afforded an oligoglycosyl aldonic acid. Glycosyl-composition analysis by the Stevenson procedure[8] (see below) showed that it contained GalA, DHA, and [^{2}H]-labelled threonic acid. In the Stevenson procedure[8], the acidic sugars released by hydrolysis are lactonized, reduced with NaBD$_4$, and acetylated to yield the alditol acetates of the corresponding neutral sugars. [^{2}H]-Labelled threonic acid, located at the reducing terminus of the oligosaccharide, is the product expected from Smith degradation of 4-Gal*p*A. Threonic acid cannot be generated by hydrolysis of a D-galactopyranosyluronic acid residue glycosidically linked at C-1. Due to the low recovery of DHA after acid hydrolysis, its proportion of the glycosyl residues in the oligoglycosyl threonic acid could not be determined. However, the negative-ion f.a.b.-mass spectrum of the oligoglycosyl threonic acid contained ions at *m/z* 1044 and 1066 corresponding to [M –

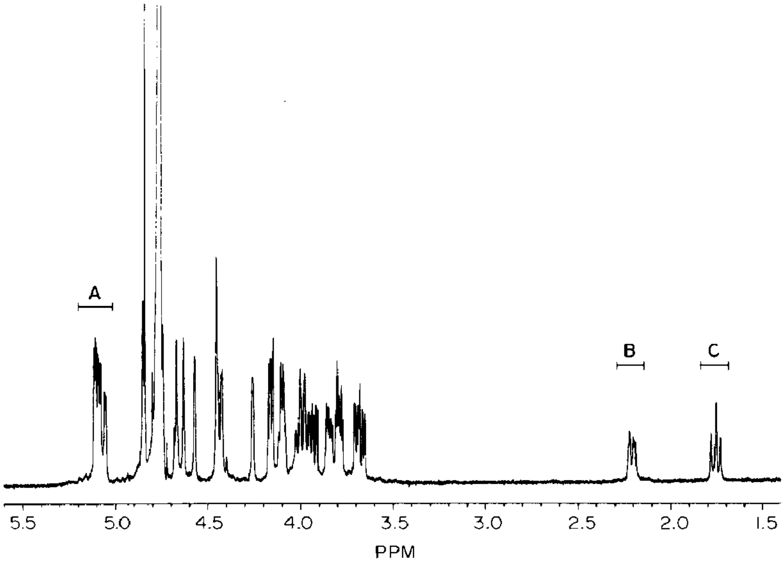


Fig. 2. The ^{1}H-n.m.r. spectrum (500 MHz) of the pentaglycosyl aldonic acid in D$_2$O at 27°. (A) H-1 of four α-D-Gal*p*A residues; (B) H-3e of β-D-DHA residue; (C) H-3a of β-D-DHA residue.

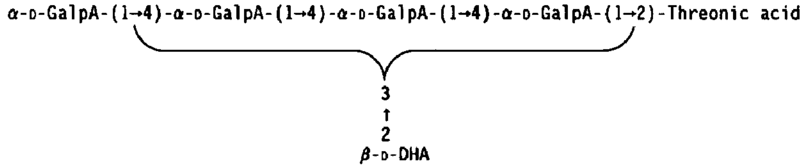
H]$^-$ and [M − 2H + Na]$^-$ ions, respectively, of a pentaglycosyl threonic acid derivative containing four D-GalA residues, one DHA residue, and one [^{2}H]-labelled threonic acid.

The ^{1}H-n.m.r. spectrum of the pentaglycosyl threonic acid (Fig. 2) contained signals for four anomeric protons between δ 5.1 and 5.3 ($J_{1,2}$ 3.5–3.9 Hz), which were assigned to four α-linked Gal*p*A residues. Signals at δ 1.76 (dd, 1 H, $J_{3a,3e}$ 12.8 $J_{3a,4}$ 12.5 Hz, H-3a) and 2.20 (dd, 1 H, $J_{3e,3a}$ 12.8, $J_{3e,4}$ 4.5 Hz, H-3e) were assigned to the resonance of the H-3a and H-3e protons, respectively, of DHA. The anomeric configurations of ketosidic linkages in complex carbohydrates (*e.g.*, Kdo and sialic acid residues) have been assigned on the basis of the chemical shift differences between the H-3a and H-3e resonances[16]. The chemical shift difference of H-3a and H-3e is, in model compounds and polysaccharides, between 0.25 and 0.6 p.p.m. for β-linked Kdo structures. In contrast, the chemical shift differences of H-3a and H-3e is usually between 0.0 and 0.25 p.p.m. for α-linked Kdo structures[16]. We assume that DHA, a structural homologue of Kdo, will exhibit very similar differences in the chemical shifts of the H-3a and H-3e resonances. Thus, the observed chemical shift difference of 0.44 p.p.m. between the H-3a and H-3e signals in the ^{1}H-n.m.r. spectrum of the pentaglycosyl aldonic acid is consistent with the DHA residue being β-linked.

Glycosyl-linkage composition analysis of the pentaglycosyl threonic acid fragment. — Free DHA is destroyed by the acidic conditions used to cleave most glycosidic linkages. Therefore, a modified procedure that was developed by Stevenson *et al.*[8] was used to determine the glycosyl-linkage composition of the pentaglycosyl threonic acid. The pentaglycosyl threonic acid was *O*-permethylated and then carboxyl-reduced with $LiB(OEt)_3D$. The resulting partially methylated oligosaccharide was hydrolyzed under mild acidic conditions (0.1N TFA, 60°, 0.5 h) to cleave the ketosidic linkage of partially methylated, carboxyl-reduced DHA. This was followed by reduction with $NaBD_4$ to form a DHA-ol derivative which was stable to the acidic conditions (2N TFA, 120°, 1 h) used to cleave the remaining glycosidic linkages of the pentaglycosyl threonic acid. The resulting partially methylated monosaccharides were reduced to their corresponding alditols with $NaBD_4$, acetylated, and analyzed by g.l.c.–m.s. Glycosyl-linkage composition analysis yielded the derivatives expected of T-Gal*p*A, 4-Gal*p*A, 3,4-Gal*p*A, T-DHA and 3-linked [^{2}H]-labelled threonic acid. Recoveries of the partially methylated derivatives of T-DHA and 3-linked [^{2}H]-labelled threonic acid were low due to the acid lability of the former and high volatility of the latter derivatives. The glycosyl-linkage composition analysis data of the pentaglycosyl aldonic acid will be discussed in the next section in light of results obtained from an experiment designed to determine the attachment site of DHA.

The site of attachment of DHA. — The ketosidic linkage of DHA was selectively cleaved by treating the pentaglycosyl threonic acid with 0.1N TFA at 100° for 1 h. The resulting tetraglycosyl threonic acid was *O*-permethylated and carboxyl reduced. The glycosyl-linkage composition analysis of the tetraglycosyl threonic acid showed T-Gal*p*A, 4-Gal*p*A, and 3-linked [^{2}H]-labelled threonic acid. These results established that the tetraglycosyl threonic acid is composed of linear 4-linked Gal*p*A residues attached at its reducing end to a 3-linked threonic acid. Comparing these data with those obtained prior to removal of DHA showed that removal of DHA resulted in the disappearance of 3,4-Gal*p*A and the appearance of 4-Gal*p*A. Thus, DHA is linked in the pentaglycosyl threonic acid, to the 3-position of a 4-linked Gal*p*A residue. This arrangement agrees with results of Stevenson *et al.*[7] obtained by an indirect approach. However, Stevenson *et al.* did not determine if DHA was directly attached to O-3 of a 3,4-linked Gal*p*A residue; some other component with an acid-labile glycosidic linkage could have been interspersed between DHA and O-3 of the 3,4-linked Gal*p*A residue. The molecular weight of the pentaglycosyl threonic acid (see above) established that DHA is directly linked to O-3 of a Gal*p*A.

The results obtained from glycosyl-residue composition analysis, ^{1}H-n.m.r. spectroscopy, f.a.b.-m.s., and glycosyl-linkage composition analyses before and after removal of DHA allow us to propose structure **1** for the pentaglycosyl threonic acid. The exact location of DHA has not been determined, but the glycosyl-linkage data established that the DHA is attached to one of the internal Gal*p*A residues (see **1**).



$$\alpha\text{-D-GalpA-}(1\rightarrow4)\text{-}\underbrace{\alpha\text{-D-GalpA-}(1\rightarrow4)\text{-}\alpha\text{-D-GalpA-}(1\rightarrow4)\text{-}\alpha\text{-D-GalpA-}}_{\substack{3\\ \uparrow\\ 2\\ \beta\text{-D-DHA}}}(1\rightarrow2)\text{-Threonic acid}$$

1

The four 1,4-linked α-D-Gal*p*As and the threonic acid (derived from a 4-Gal*p*A) in the pentaglycosyl threonic acid must have originated from the backbone of RG-II since it is the only part of RG-II with contiguous Gal*p*A residues[9,11]. The four Gal*p*A residues in the pentaglycosyl threonic acid probably had glycosyl residues attached in intact RG-II which rendered them resistant to periodate oxidation. This fact indicates that, of the approximately seven Gal*p*A residues in the backbone, there are four in a row that are branched. This result agrees with glycosyl-linkage composition analysis of RG-II which indicated that only two of the seven Gal*p*A residues are unbranched[7]. The Gal*p*A residue from which the threonic acid was derived must have been unbranched and 4-linked. No information has been previously obtained regarding which of the Gal*p*A residues in the backbone are not substituted with at least one side chain.

Hex-5-enose degradation of methylated RG-II. — Aspinall and co-workers developed a procedure for the selective cleavage of glycopyranosyluronic acid linkages in permethylated polysaccharides[13,14]. In their procedure, primary hydroxyl groups formed by reduction of the glycopyranosyluronic acid residues in the permethylated polysaccharide are converted into the corresponding 6-deoxy-6-iodo derivatives. Treatment of the iodinated polysaccharides with Zn causes selective chain cleavage at the former glycopyranosyluronic acid residues. This results in the generation, from the modified glycopyranosyluronic acid residues, of hex-5-enose derivatives with concomitant liberation of aglycone (see Fig. 3). The reaction conditions in the hex-5-enose degradation procedure do not cleave neutral glycosidic linkages in a permethylated polysaccharide. Therefore, the hydroxyl groups exposed by the hex-5-enose degrada-

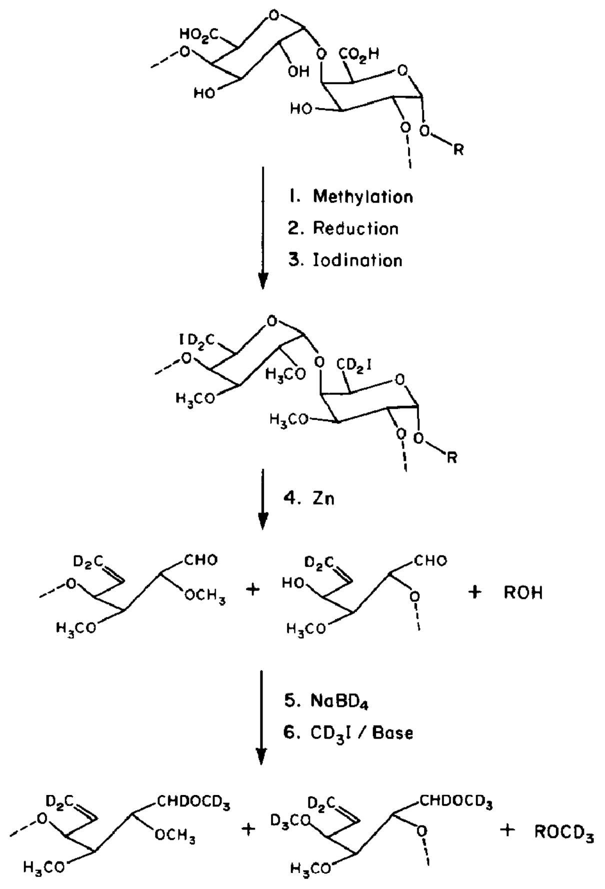
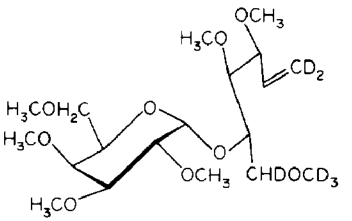


Fig. 3. Cleavage of modified glycopyranosyluronic acid residues of permethylated polysaccharides by the hex-5-enose procedure.

tion procedure indicate former attachment sites of glycosyluronic acid residues in the intact polysaccharide. The hydroxyl groups exposed by the degradation can be labelled by trideuteriomethylation.

The backbone of RG-II is composed of seven 1,4-linked α-D-Gal*p*A residues to which various side chains are attached[7-11] (see Fig. 1). Two terminal, non-reducing Gal*p*A residues and an internal 2-linked Glc*p*A residue have previously been shown to be part of an octaglycosyl side chain of RG-II[11]. The glycosyluronic acid residues in the backbone as well as in the side chain are likely sites for modification and cleavage by the hex-5-enose procedure. RG-II was subjected to the hex-5-enose procedure to obtain oligoglycosyl hex-1-enitol derivatives that contain intact apiofuranosidic linkages. Such oligomers have not previously been isolated from RG-II.

O-Permethylated RG-II was treated with $LiB(OEt)_3D$ to convert the methyl esters of carboxylic acids into dideuteriolabelled primary hydroxyl groups. The primary hydroxyl groups were iodinated and the modified RG-II cleaved by treatment with Zn. The newly formed carbonyl groups were reduced with $NaBD_4$, and the hydroxyl groups exposed by depolymerization and by $NaBD_4$ reduction were alkylated with CD_3I. The resulting mixture of peralkylated oligoglycosyl derivatives was separated by reversed-phase h.p.l.c., and two components (**2** and **3**) that each contained a single oligoglycosyl-hex-1-enitol were isolated. Both components were shown to be homogeneous by t.l.c. and by g.l.c.-m.s. These oligoglycosyl-hex-1-enitols were characterized by n.m.r. spectroscopy, by g.l.c.–m.s., and by glycosyl-linkage composition analysis as described below.



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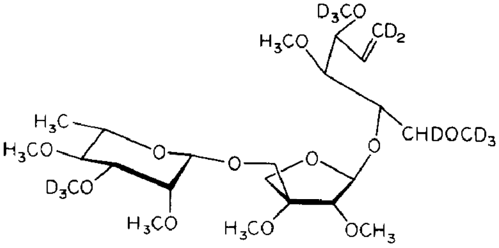

3

Monoglycosyl hex-1-enitol **2**. Glycosyl-composition analysis of *O*-peralkylated oligoglycosyl hex-1-enitol **2** gave T-Gal*p* and 5-linked 1,2-dideoxy-hex-1-enitol. A trideuteriomethyl group was located on O-6 of the hex-1-enitol. This was expected as each hex-1-enitol residue, regardless of its origin, must contain exposed C-6 hydroxyl groups available for trideuteriomethylation (generated by the reduction of the corresponding hex-5-enose residue, see Fig. 3). The ^{1}H-n.m.r. spectrum of *O*-peralkylated monoglycosyl-hex-1-enitol **2** contained a signal for an anomeric proton at δ 5.10 (d, $J_{1,2}$ 2.9 Hz) indicating that the T-Gal*p* residue was in the α-D configuration. G.l.c.–c.i.-m.s. analysis of **2** established its molecular weight to be 414, which together with e.i.-m.s. fragmentation, ^{1}H-n.m.r. data, and its glycosyl-residue composition is consistent with structure **2** shown for peralkylated 5-*O*-(α-D-galactopyranosyl)-hex-1-enitol. In previous studies, a terminal 2-*O*-(α-D-galactopyranosyl)-D-glucopyranosyluronic acid unit was identified in an octaglycosyl side chain of RG-II[11]. The peralkylated D-galactopyranosyl-hex-1-enitol generated by hex-5-enose degradation of methylated RG-II must have been derived from that terminal 2-*O*-(α-D-galactopyranosyl)-D-glucopyranosyluronic acid because there are no other T-Gal*p* residues in RG-II. Thus our present data confirm the anomeric configuration and site of attachment of the terminal Gal*p* residue in RG-II[11].

Diglycosyl hex-1-enitol **3**. Glycosyl-composition analysis of oligoglycosyl hex-1-enitol **3** showed the presence of T-Rha, 3′-Api*f*, and 5-linked 1,2-dideoxy-hex-1-enitol. Trideuteriomethyl groups were found on O-3 of the T-rhamnosyl residue and O-3 and O-6 of the hex-1-enitol residue (see below for explanation). G.l.c.–c.i.-m.s. analysis of the oligoglycosyl hex-1-enitol **3** established its molecular weight to be 550 which, together with its e.i.-m.s. fragmentation pattern and glycosyl-composition indicate that it was a peralkylated diglycosyl hex-1-enitol with structure **3**.

The trideuteriomethyl group at O-6 of hex-1-enitol arises, again as expected, from alkylation of the OH group that was formed by reduction of the hex-5-enose derivative. The trideuteriomethyl group at O-3 established that the 5-linked hex-1-enitol was derived from a 2,4-linked glycopyranosyluronic acid residue of RG-II, which had been substituted at C-4 with another glycopyranosyluronic acid residue (see Fig. 3). These results show that the diglycosyl hex-1-enitol **3** must have been generated from a 2,4-linked Gal*p*A residue in the backbone of RG-II, which contains the only contiguous and only branched galactopyranosyluronic acid residues. The presence of a trideuteriomethyl group at O-3 of T-Rha established that the glycosidic linkage of the aceryl acid residue attached to O-3 of a rhamnosyl residue in the heptaglycosyl side chain of RG-II was cleaved, either during the hex-5-enose or the permethylation procedures. We believe that the latter probably is the cause for the cleavage of aceryl acid residue that was attached to O-3 of the rhamnosyl residue; the aceryl acid residue in the heptaglycosyl side chain of RG-II had previously been shown to degrade and cleave during methylation, resulting in the exposure of the 3-OH group of the rhamnosyl residue[10]. Thus, peralkylated diglycosyl hex-1-enitol **3** is derived from the first two residues of the heptaglycosyl side chain and a Gal*p*A residue of the backbone to which the heptaglycosyl side chain is attached (Fig. 1).

The ^{1}H-n.m.r. spectrum of oligoglycosyl hex-1-enitol **3** contained signals for anomeric protons at δ 5.15 (d, $J_{1,2}$ 2.9 Hz) and 4.79 (d, $J_{1,2}$ 1.5 Hz). In previous studies[10], the internal 3-linked L-rhamnosyl residue in the heptaglycosyl side chain was shown by ^{1}H-n.m.r. spectroscopy to be β-linked. Accordingly, the signal at δ 4.79 in the ^{1}H-n.m.r. spectrum of the diglycosyl hex-1-enitol derivative was assigned to the β-L-Rha residue.

The signal at δ 5.15 in the ^{1}H-n.m.r. spectrum was assigned to the anomeric proton of the apiofuranosyl residue. The anomeric configuration of the apiofuranosyl residues cannot be unambiguously determined on the basis of the H-1 chemical shift and $J_{1,2}$ value. The cyclic form of apiose has an additional asymmetric carbon at C-3 that makes it possible to have two ring forms; that is, either a ring with the D-*erythro*- or the L-*threo*-furanose configuration. Depending on the configuration at C-1, the L-*threo* and D-*erythro* ring forms give rise to a total of four possible ring structures for D-apiofuranose.

The H-1 chemical shifts and vicinal ^{1}H–^{1}H coupling constants ($J_{1,2}$) observed for apiofuranose ring structures do not allow ready discrimination between the four possible isomers[17]. The anomeric configurations of apiofuranosyl residues in complex molecules has been assigned through ^{13}C-n.m.r. data. The C-1 of β-D-*erythro*-furanosyl residues resonates at $\delta \approx 111.0$, which is 7 p.p.m. downfield to the resonance of the corresponding α-D anomer[18,19]. A ^{1}H-detected one-bond (^{1}H,^{13}C) shift-correlation experiment showed that the H-1 of the apiofuranosyl residue (δ 5.15) in the diglycosyl hex-1-enitol derivative **3** was directly coupled to a carbon signal at δ 110.2. If we assume the D-*erythro*-furanose configuration for the apiofuranosyl residue in RG-II (all naturally occurring apiosides have been found in the D-*erythro*-furanose form[20]), the n.m.r. spectroscopic data indicate that the apiofuranosyl residue in diglycosyl hex-1-enitol derivative **3** has the β-D-*erythro* configuration.

CONCLUSIONS

Rhamnogalacturonan II (RG-II), although of low molecular weight (≈5000 daltons), is one of the most complex polysaccharides known. It possesses a variety of side chains attached to a linear backbone of 1,4-linked α-D-galactopyranosyluronic acid residues.

It has previously been impossible to generate from RG-II oligosaccharide fragments that contain portions of both the backbone and a side chain. Isolation and structural characterization of such oligosaccharides is required in order to determine the anomeric configurations and specific sites of attachments of glycosyl residues that link the side chains to the backbone. In the present study, Smith degradation of RG-II led to the isolation and characterization of a pentaglycosyl aldonic acid containing a 3-deoxy-D-*lyxo*-2-heptulosaric acid (DHA) residue and four galactopyranosyluronic acid residues terminated at the reducing end with a threonic acid. Results from the structural analysis of this pentaglycosyl aldonic acid established that the DHA is β-2,3-linked to a galactopyranosyluronic acid residue in the backbone of RG-II. No other residue was interspersed between the DHA and the backbone Gal*p*A.

A diglycosyl hex-1-enitol derivative containing an intact apiofuranosidic linkage has been isolated by hex-5-enose cleavage of permethylated RG-II. Structural analysis of this diglycosyl hex-1-enitol derivative established that the D-apiofuranosyl residue of a heptaglycosyl side chain was, once again, directly linked to C-2 of a 4-linked galactopyranosyluronic acid residue in the backbone of RG-II, and that the anomeric configuration of the apiosidic linkage is β-D. The results from the present study lead us closer to elucidating the complete sequence of the glycosyl residues of RG-II.

EXPERIMENTAL

General methods. — N.m.r. spectra were recorded on a Bruker AM 500 spectrometer at 27°. The ^{1}H-detected (^{1}H,^{13}C) shift-correlation experiment (HMQC) was performed as described[21]. F.a.b.-mass spectra were recorded with a VG ZAB-SE mass spectrometer operating at low resolution (1:1000) in the negative-ion mode with an accelerating voltage of 8 kV. Aqueous solutions (1 μL) of oligosaccharides were applied to the f.a.b. probe tip together with 1 μL of 1-thioglycerol as matrix. The g.l.c. and g.l.c.–m.s conditions used for glycosyl-residue and glycosyl-linkage composition analyses were those described[7,8]. G.l.c.–c.i.-m.s. was performed using ammonia as the reagent gas. RG-II was purified from Pectinol AC as described[8]. Methylation of RG-II was carried out by the Hakomori procedure[22] using lithium methylsulfinylmethanide, which was prepared as reported[23]. Reduction of the esterified carboxyl groups in permethylated RG-II was performed with $LiB(OEt)_3D$ as described[8].

Smith degradation of RG-II. — A solution of RG-II (50 mg) in 0.015M aq. sodium metaperiodate (50 mL) was stirred for 24 h in the dark at room temperature. The consumption of periodate was monitored by measuring the u.v. absorption (222.5 nm) of aliquots of the reaction mixture. Ethylene glycol (0.1 mL) and then sodium borodeuteride (100 mg) were added, and the mixture was stirred continuously for 6 h. Excess borodeuteride was then destroyed with glacial acetic acid, and, after adjusting the pH to 5.0, the solution was dialyzed against distilled water. The oxidization and reduction procedures were repeated to ensure complete oxidation and reduction of periodate-susceptible glycosyl residues. Smith degradation was carried out by treating the oxidized and reduced polysaccharide with aqueous 0.1N TFA at room temp for 24 h or at 60° for 1 h.

Purification of Smith degradation products. — The products resulting from Smith degradation of RG-II were fractionated on a DEAE-Sephadex column. The column was eluted sequentially with H_2O (20 mL) and an aq. ammonium formate gradient (0.1–1.0M, 20 mL). Column effluents were monitored colorimetrically for hexoses[24], uronic acids[25] and Kdo[26]. Fractions that contained uronic acid and either Kdo or DHA were pooled, lyophilized, and rechromatographed on a Mono-Q column using a linear gradient of 0–0.5M aq. ammonium formate. The eluted oligosaccharide-containing fractions were individually desalted by repeated dissolution in H_2O and lyophilization, and these were analyzed by ^{1}H-n.m.r. spectroscopy, f.a.b.-mass spectrometry, and glycosyl-residue and glycosyl-linkage composition analyses.

Hex-5-enose degradation of permethylated and carboxyl-reduced RG-II. — Trifluoromethanesulfonic anhydride (50 μL) in dry dichloromethane (0.5 mL) was added to 2,6-di-*tert*-butyl pyridine (75 μL) in dichloromethane (0.5 mL) at −10°. After 10 min, carboxyl-reduced methylated RG-II (15 mg) in dichloromethane (1 mL) was added dropwise, and the mixture was stirred at −10° for 2 h. The reaction mixture was poured into ice water (2 mL), the organic phase was separated, and the aq. phase was extracted with dichloromethane. The combined organic phases were washed with cold dilute sodium hydrogencarbonate until free of base and then washed with water. The organic phase was dried over anhyd. Na_2SO_4 and evaporated to dryness to yield the polysaccharide sulfonate. LiI (60 mg) in 1:1 (v/v), oxolane–hexamethylphosphoramide (1.5 mL) was added to the polysaccharide sulfonate, and the resulting solution was heated for 15 h at 80° with stirring. The resulting iodinated polysaccharide was purified by dialysis and recovered by lyophilization. The iodinated polysaccharide in 1:1 (v/v) ethanol–water (1 mL) was heated at 90° with freshly activated Zn (60 mg). After 15 h, the reaction mixture was cooled to room temp., filtered, and the precipitate washed with methanol. The combined filtrate and the washings were concentrated, and the resulting material was reduced by the addition of $NaBD_4$ (10 mg) in 1:1 (v/v) oxolane-water (1 mL). After the usual work-up, the resulting product was peralkylated with CD_3I by the Hakomori procedure[22]. The peralkylated hex-5-enose degradation products were purified by reversed-phase h.p.l.c. on a Microsorb C_{18} column (5 μm, 4.6 mm × 25 cm). The column was eluted with 30% aq. CH_3CN for 10 min, followed by a linear gradient (30–70% aq.) over 30 min, at a flow rate of 1 mL/min. The column effluents were monitored by u.v. absorbance at 200 nm.

ACKNOWLEDGMENTS

This research supported in part by U.S. Department of Energy grant DE-FG09-85ER-13426, and by DE-FG09-87ER13810 as part of the DOE/NSF/USDA Plant Science Centers program. The authors are grateful to L. Poppe, Q. Quo, G. Impallameni, R. Carlson, C. Bergmann, M. A. O' Neill, and W. S. York for their valuable help and advice and to Karen Howard for the preparation of the manuscript.

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