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STRUCTURAL STUDIES OF RHAMNOGALACTURONAN II, A PLANT CELL WALL PECTIC POLYSACCHARIDE

by

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ABSTRACT

STRUCTURAL STUDIES OF RHAMNOGALACTURONAN II,

A PLANT CELL WALL PECTIC POLYSACCHARIDE

By

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This dissertation describes the elucidation of part of the primary structure of the plant cell wall pectic polysaccharide rhamnogalacturonan II. Rhamnogalacturonan II may be the most structurally complex plant polysaccharide ever isolated. Rhamnogalacturonan II is composed of at least ten different monosaccharides interconnected by at least 20 different glycosidic linkages.

A branched-chain acidic sugar, 3-C-carboxy-5-deoxy-L-xylose, was isolated by anion-exchange chromatography of acid-hydrolyzed rhamnogalacturonan II. The sugar was shown to be a carboxyl-branched deoxy-pentose by nuclear magnetic resonance spectroscopy and mass spectrometry of the isolated sugar and of several derivatives. X-ray crystallography of one of the derivatives revealed that the sugar had the xylo configuration. The sugar was shown to have the L absolute configuration by the identification of L-lactic acid as the major product of periodate oxidation of the anomeric mixture of methyl glycosides. This is the first report of a naturally occurring branched-chain acidic monosaccharide. "Aceric acid" has been proposed as a trivial name for 3-C-carboxy-5-deoxy-L-xylose.

Selective acid hydrolysis of apiosyl glycosidic linkages of

rhamnogalacturonan II was used to generate a mixture of oligosaccharides for structure analysis. A heptasaccharide was purified to homogeneity by gel-filtration and anion-exchange chromatography of partially hydrolyzed rhamnogalacturonan II. Apiose was at the reducing end of the heptasaccharide. The heptasaccharide was found to contain, in addition to 3^1-linked apiose, terminal L-rhamnopyranosyl, terminal (2-O-methyl)-L-fucopyranosyl, 2-linked L-arabinopyranosyl, 2,4-linked D-galactopyranosyl, 2-linked L-acerofuranosyl, and 3-linked L-rhamnopyranosyl residues. The sequence of these glycosyl residues was determined by forming and characterizing overlapping partially O-deuteriomethylated, partially O-deuterioethylated oligosaccharide-alditols from the heptasaccharide. Some of the heptasaccharide molecules were found to be mono- and di-O-acetylated.

All aspects of the primary structure of the heptasaccharide were determined except the absolute configuration of the apiose and the point of attachment of O-acetyl esters. Comparison of the glycosyl-linkage composition of the heptasaccharide to that of intact rhamnogalacturonan II revealed that a molecule of rhamnogalacturonan II probably contains more than one heptasaccharide unit. It is postulated that the heptasaccharide units occur in rhamnogalacturonan II as side chains attached to a galactosyluronic acid-rich backbone.

To Jody and to my parents

for their love, support, and patience

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Figure Page

CHAPTER I

CHAPTER II

INTRODUCTION

A major goal of plant cell wall research is to determine the structures of the macromolecular components of the cell wall and, from this knowledge, to attempt to describe the structure of the cell wall. The experiments described in this dissertation were aimed at elucidating part of the primary structure of one such macromolecule, the pectic (galactosyluronic acid-rich) polysaccharide rhamnogalacturonan II (RG-II).

RG-II is an extremely complex, yet structurally well-defined, polysaccharide released from primary cell walls of suspension-cultured sycamore (Acer pseudoplatanus) cells by incubation of the walls with a purified endopolygalacturonase. RG-II yields at least 10 different monosaccharides upon acid hydrolysis, and appears to be the most structurally complex plant polysaccharide that has been isolated to date.

Chapter I of this dissertation describes the isolation and characterization of 3-C-carboxy-5-deoxy-L-xylose, a branched-chain acidic glycosyl residue of RG-II. This is the first report of a naturally occurring branched-chain acidic sugar. Chapter II describes the isolation and the elucidation of the primary structure of a heptasaccharide released from RG-II by selective acid hydrolysis. This heptasaccharide was found to contain six different glycosyl residues,

including aceric acid. The structure of this heptsaccharide has provided insight into the primary structure of RG-II.

The two chapters are preceded by a literature review, which is divided into two sections. The first section provides an overview of the research on plant cell wall structures, with emphasis on pectic polysaccharides. The second section reviews current approaches to structural analysis of complex carbohydrates.

LITERATURE REVIEW

THE PLANT CELL WALL

Introduction

The plant cell wall, which surrounds every plant cell and which is composed primarily of polysaccharides and glycoproteins, has long been the object of research because it is involved in so many aspects of plant growth and development. Several excellent reviews are available on this subject.[1-4]

Plant cell walls are sufficiently rigid to provide support for the plant and to protect the plant cells against osmotic shock, but the walls of growing cells must also be capable of enlarging to allow cell growth and development. The wall presents a physical barrier to invading pathogens, and thus plays a role in host-pathogen interactions in plants. Recent reports that oligosaccharide fragments of the plant cell wall can function as regulatory molecules in plant-pathogen interactions[5-8] and in plant growth and development[7,9] have added new impetus to plant cell wall research.

Plants contain both primary and secondary cell walls. Primary cell walls are the thin, flexible walls synthesized by undifferentiated cells that are still growing. The primary cell wall is a dynamic substance, capable of loosening as the cells elongate.

Secondary cell walls are synthesized by cells that are no longer growing; these walls are thicker and more rigid than primary walls. RG-II, the subject of this dissertation, is a primary cell wall polysaccharide. Therefore, this literature review will be limited to components of the primary cell wall and will not consider biopolymers (for example, lignin) that are present exclusively in secondary cell walls.

There has been a great deal of research on the biosynthesis of cell wall polymers and on the ultrastructure of plant cell walls. Both topics have been reviewed[10-12], and will not be considered here.

Classification of the Major Types of Primary Cell Wall Polymers

Early workers divided cell wall polymers into three major types: pectic polysaccharides, the hemicelluloses, and cellulose. This classification was made primarily on the basis of solubility. Pectic fractions were extracted from the wall by hot water, ammonium oxalate, weak acid, or chelating reagents; hemicelluloses were extracted by strong alkali; and cellulose remained as an insoluble residue after alkali extraction[3].

Pectic polysaccharides are now classified as those in covalent association with D-galactosyluronic acid residues[3]. Cellulose is known to consist of unbranched chains of 4-linked β-D-glucosyl residues, and the hemicelluloses are those polysaccharides that are non-covalently bound to cellulose[3]. Two other classes of primary cell wall polymers have also been identified. These are the non-cellulosic glucans and the hydroxyproline-rich glycoproteins[3].

Cellulose[13,14], the hemicelluloses[3,15,16], the hydroxyproline-rich glycoproteins[17], and the chemistry of non-cellulosic glucans[2-4] have all

been the subjects of reviews. The pectic polysaccharides have also been considered in reviews[18-21], but they will be discussed in detail here because rhamnogalacturonan II, the subject of this dissertation, is a pectic polysaccharide.

Pectic polysaccharides

Introduction. The pectic substances are a heterogeneous mixture of polysaccharides that are characterized by a relatively large proportion (10-100%) of D-galactosyluronic acid residues[3,4]. These polysaccharides are found as constituents of all plant cell walls, and are localized in the primary cell wall[22].

Many neutral sugars, in addition to galactosyluronic acid, have been recognized as components of pectic polysaccharides. These sugars are L-rhamnose[23-25], D-galactose[23-25], D-xylose[23,24], L-arabinose[26], L-fucose[24], D-glucose[23], D-apiose[27-30], 2-O-methyl fucose[23,24,30], and 2-O-methyl xylose[23,24,30]. The carboxyl groups of the galactosyluronic acid residues of pectic polysaccharides are frequently methyl-esterified[31,32]. O-Acetyl esters have also been reported in pectic polysaccharides[4,33].

Early workers established that the predominant feature of pectin was a linear chain of 4-linked-α-D-galactosyluronic acid residues in which some of the carboxyl groups were methyl-esterified[1,18,19,31]. It was believed that pectins consisted of a triad of homopolymers[18,19]; isolations of pure galacturonan[34], araban[26,35,36], and galactan[37] were reported. However, it eventually became apparent that the idea of a triad of homopolymers was an oversimplification[1,4], and it now seems likely that most of these "homopolymers" were artifacts caused by acid-[1,4] or base-[38,39] catalyzed degradation during extraction of the

polysaccharides from the cell walls. There are a few well-documented instances of homopolymeric arabans[40-42], galactans[43], and galacturonans[44,45] being isolated under such mild conditions that degradation was unlikely. However, the polyuronide is found far more often to contain covalently attached neutral sugar components[46-51].

Isolation of the aldobiuronic acid 2-O-(α-D-galactosyluronic acid)-L-rhamnose[24,47-49] provided the first direct evidence for covalent attachment between neutral sugar residues and galactosyluronic acid residues. It is now accepted that the basic structural units of pectins are rhamnogalacturonans in which the 4-linked α-D-galacturonan chains are interrupted at intervals by the insertion of 2-linked and 2,4-linked α-L-rhamnosyl residues[3,4]. Most neutral glycosyl residues are thought to occur in side chains attached to rhamnosyl residues of the rhamnogalacturonan backbone[2,3,4].

Rhamnogalacturonans from primary cell walls of suspension-cultured sycamore cells. In 1973, Albersheim and co-workers published the results of an analysis of structural polymers of cell walls of suspension-cultured sycamore cells[52-54]. In this study, cell wall preparations were obtained from suspension-cultured sycamore cells[55] that had been transferred and harvested in the logarithmic phase of growth, thus providing a source of homogeneous primary cell walls[56]. Structural polymers were solubilized by incubating these walls with purified hydrolytic enzymes, such as an endopolygalacturonase purified from the culture filtrate of the fungus Colletotrichum lindemutheanum[57]. This approach was an improvement over earlier cell wall structural studies, because polymers were solubilized in a defined manner from homogeneous cell wall preparations.

Pectic polysaccharides account for approximately 30% of the primary cell wall of suspension-cultured sycamore cells[2]. Digestion of the wall with endopolygalacturonase solubilizes approximately 16% of the cell wall material[52]. Two pectic polysaccharides, rhamnogalacturonan I[58,59] and rhamnogalacturonan II[30], have been isolated from the material released by endopolygalacturonase digestion of cell walls isolated from suspension-cultured sycamore cells.

Rhamnogalacturonan I (RG-I) comprises approximately 7% of the cell wall of suspension-cultured sycamore cells and, therefore, represents 20-25% of the pectic material. The molecular weight of RG-I is estimated to be approximately 200,000, as determined by gel-filtration chromatography[58].

Some aspects of the structure of RG-I are known. RG-I is thought to have a backbone that is made up of alternating rhamnosyl and galactosyluronic acid residues. About half of the rhamnosyl residues are 2-linked, while the other half are 2,4-linked. Side chains, with an average size of six glycosyl residues, are attached to O-4 of the 2,4-linked rhamnosyl residues. The side chains are rich in L-arabinosyl and D-galactosyl residues[58]. Thus, isolation of RG-I side chains could explain many of the early reports of araban and galactan homopolymers.

RG-I is not a homogeneous polysaccharide as isolated[58], but instead appears to be a family of related polysaccharides[59]. It has been found that seven differently linked glycosyl residues are attached to O-4 of 2,4-linked rhamnosyl residues of RG-I[59]. These seven glycosyl residues are, therefore, the first residues of at least seven different side chains of RG-I. These results give an indication of the great structural complexity of RG-I.

The other pectic polysaccharide that has been isolated from cell walls of suspension-cultured sycamore cells is rhamnogalacturonan II (RG-II)[30]. RG-II comprises 3-4% of the primary cell wall of suspension-cultured sycamore cells and, therefore, 10-15% of the pectic material. RG-II is a size-homogeneous polysaccharide with a molecular weight of approximately 10,000. Acid hydrolysis of RG-II yields at least ten different monosaccharides, including galactosyluronic acid, glucosyluronic acid, rhamnose, fucose, arabinose, galactose, glucose, apiose, 2-O-methyl fucose, and 2-O-methyl xylose. With the exception of xylose, all of the neutral sugars that have been reported to be constituents of pectin[4] are found in RG-II. The isolation of RG-II provided the first indication that the minor glycosyl constituents of pectins were located in a single small (50-60 glycosyl residues) polysaccharide[30].

RG-II is fundamentally different from RG-I. Galactosyluronic acid and rhamnosyl residues are abundant glycosyl residues of both RG-I and RG-II, but the rhamnosyl residues of RG-II are mostly terminal and 3-linked[30], while those of RG-I are 2-linked and 2,4-linked[57]. Therefore, RG-II cannot contain the rhamnogalacturonan backbone present in RG-I[58]. The other glycosyl residues of these two polysaccharides are also very dissimilar[30,58].

RG-II has been found in all dicots that have been examined[3,30,60]. In addition, a polysaccharide with the glycosyl-residue composition of RG-II has been isolated from the walls of suspension-cultured gymnosperm cells[61]. Small amounts of the unusual sugars that characterize RG-II have also been found in monocots[3,30]. It is interesting that such a structurally complex polysaccharide should be so widely distributed in the plant kingdom.

STRUCTURAL ANALYSIS OF COMPLEX CARBOHYDRATES

Introduction

Seven characteristics of a complex carbohydrate must be elucidated before its complete primary structure can be known[62]. These characteristics are

1) the glycosyl residue composition of the complex carbohydrate
2) the absolute configuration, D or L, of each glycosyl residue
3) the glycosyl-linkage composition of the complex carbohydrate
4) the ring form, furanoid or pyranoid, of each glycosyl residue
5) the sequence of the variously linked glycosyl residues
6) the anomeric configuration, α or β, of each glycosyl residue
7) the identity and points of attachment of any noncarbohydrate moieties.

The techniques that are available to determine these features are described below. This review is not intended to be all-inclusive, but will, instead, introduce techniques used in this dissertation.

Glycosyl-residue composition

A sugar residue glycosidically linked through its own reducing carbon (C-1) is called a glycosyl residue. The glycosyl-residue composition of a complex carbohydrate describes the types and ratios of glycosyl residues that comprise the complex carbohydrate. For example, a complex carbohydrate could be composed of 50% glucosyl residues and 50% galactosyl residues. Several methods are available for acquiring this information. One method is to acid-hydrolyze the complex carbohydrate, reduce the resulting monosaccharides to the corresponding alditols, and form the volatile O-acetyl derivatives called alditol

acetates[63]. The mixture of alditol acetates is then resolved and quantitated by gas-liquid chromatography (g.l.c.). Another widely used method involves depolymerizing the complex carbohydrate by methanolysis[64] and the subsequent synthesis of the trimethylsilyl derivatives[65] of the resulting methyl glycosides. The O-trimethylsilyl derivatives of the methyl glycosides are then resolved and quantitated by g.l.c.

Absolute configuration of the glycosyl residues

Both D sugars and L sugars are found in nature. In fact, different optical isomers of the same glycosyl residue have been found in the same complex carbohydrate[66]. For example, porphyran is a polysaccharide that is made up of alternating D-galactosyl and L-galactosyl residues[67]. Therefore, the absolute configurations of all glycosyl residues in a complex carbohydrate must be determined.

The most versatile methods for determining absolute configuration utilize solvolysis reactions in which the solvent is an optically active alcohol[68,69,70]. When enantiomers are derivatized with an optically active reagent, the products are diastereomers that can be resolved by g.l.c.

Glycosyl-linkage composition

The glycosyl-linkage composition of a complex carbohydrate describes, for each glycosyl residue, the positions to which other glycosyl residues are attached. For example, a 4-linked galactosyl residue is linked to another glycosyl residue through C-1 and has a glycosyl residue linked to it at C-4. A 2,4-linked galactosyl residue is linked to another glycosyl residue through C-1 and has glycosyl residues linked to C-2 and C-4. A terminal galactosyl residue is linked

to another glycosyl residue through C-1 and has no glycosyl residues linked to other carbons. The glycosyl-linkage composition also describes the ratios of the variously linked glycosyl residues within the complex carbohydrate. For example, a complex carbohydrate could be composed of 50% 4-linked galactosyl residues, 25% 2,4-linked galactosyl residues, and 25% terminal galactosyl residues.

The most widely used technique for determining glycosyl-linkage composition is the formation and analysis of partially O-methylated alditol-acetate[71] derivatives of the glycosyl residues that comprise the complex carbohydrate. The intact complex carbohydrate is per-O-methylated by treatment with sodium dimethylsulfinyl anion and methyl iodide[72,73]. This reaction does not depolymerize the complex carbohydrate, but, instead, converts all free hydroxyl groups into methyl ethers. The per-O-methylated complex carbohydrate is then depolymerized by acid-catalyzed hydrolysis or formolysis, yielding a mixture of partially-O-methylated monosaccharides that are then reduced to partially-O-methylated alditols. The free hydroxyl groups of each partially O-methylated alditol mark those carbon atoms to which other glycosyl residues were attached in the complex carbohydrate and those carbon atoms involved in forming the ring (C-1 and C-5 for a residue in the pyranoid ring-form). The mixture of partially O-methylated alditols is per-O-acetylated, yielding partially-O-methylated alditol acetates[74]. The partially-O-methylated alditol acetates are analyzed by g.l.c.-mass spectrometry (g.l.c.-m.s.). The electron-impact (e.i.) mass spectral fragmentation patterns of partially-O-methylated alditol acetates have been well characterized[75,76] and reveal the positions of O-methyl and O-acetyl groups on each partially O-methylated alditol

acetate. The glycosyl linkage of each residue is deduced from this information. The ratios of the variously linked glycosyl residues are determined by flame ionization g.l.c. of the mixture of the derived partially O-methylated alditol acetates. This gives the glycosyl-linkage composition of the complex carbohydrate.

Ring form of each glycosyl residue

Most glycosyl residues can exist in furanoid (5-membered) or pyranoid (6-membered) rings. The ring form of a glycosyl residue can often be determined from the positions of O-methyl groups on the derived partially methylated alditol acetate[62]. For example, a partially O-methylated alditol acetate with an O-methyl group at C-4 and an O-acetyl group at C-5 must be derived from a glycosyl residue in the pyranoid ring-form. If a partially methylated alditol acetate has O-acetyl groups at both C-4 and C-5, the ring form cannot be determined from the methylation analysis. Two methods have been developed [77,78] for resolving this possible ambiguity; one of the methods is described in Chapter II of this dissertation.

Sequence of glycosyl residues

Determining the sequence of glycosyl residues in a complex carbohydrate is more difficult than, for example, determining the sequence of amino acids in a peptide. This is because glycosyl residues can be interconnected by a great variety of linkages, and the linkage information must be retained in any glycosyl-residue sequencing procedure. For example, a sequence in which a 4-linked galactosyl residue is linked to C-4 of a glucosyl residue is different from a sequence in which a 2-linked galactosyl residue is linked to C-4 of a glucosyl residue. A general method for determining the sequence of the

variously linked glycosyl residues in complex carbohydrates has been developed in the laboratory of Professor Peter Albersheim at the University of Colorado[62,77]. This sequencing method is described in Chapter II, and will not be discussed here.

Anomeric configurations

C-1 of a sugar that is glycosidically linked is an asymmetric carbon and can exist in either of two configurations. These are the two possible anomeric configurations of glycosyl residues and are designated α and β. Proton nuclear magnetic resonance (^{1}H-n.m.r.) spectroscropy is the most versatile technique for determining the anomeric configuration of each glycosyl residue of a complex carbohydrate[79-81]. Both the coupling constant between H-1 and H-2 ($J_{1,2}$) and the chemical shift of the anomeric proton can give information about the anomeric configuration of a glycosyl residue that is in the pyranoid ring-form.

The coupling constant depends upon the dihedral angle between H-1 and H-2[82,83]. A coupling constant of 7-8 Hz is usually observed with a 1,2-*trans*-diaxial relationship for H-1 and H-2, whereas a 2-4 Hz coupling constant is observed when H-1 and H-2 are in *gauche* orientation. For most glycopyranosyl residues, H-1 is axial. Therefore, these residues will exhibit $J_{1,2}$ of 7-8 Hz when the anomeric proton is axial, and $J_{1,2}$ of 2-4 Hz when H-1 is equatorial. This information can be related to the anomeric configuration of a glycosyl residue. If, for example, the anomeric proton resonance of a D-glucopyranosyl residue has a 7 Hz coupling constant, then H-1 of this residue is axial, and the residue has the β anomeric configuration. In the case of a glycosyl residue (such as rhamnose) in which H-2 is equatorial, the coupling constant cannot be used to determine anomeric configuration. This is

because H-1 and H-2 are in gauche orientation in both anomers. The anomeric configuration of such a glycosyl residue can usually be determined from the chemical shift of the anomeric proton. Equatorial anomeric protons typically have chemical shifts less than 4.9 p.p.m., and axial protons have chemical shifts greater than 4.9 p.p.m[84].

Non-carbohydrate moieties

Pectic polysaccharides are known to contain endogenous O-acetyl esters[4,33] These substituents, which are readily detected by ^{1}H-n.m.r. spectroscopy of the underivatized polysaccharide, are base-labile and are, therefore, removed by the strong base (sodium dimethylsulfinyl anion) routinely used in determining glycosyl-linkage composition of polysaccharides. Several chemical methods[85,86,87] are available for determining the locations of base-labile substituents. These methods involve derivatizing the free hydroxyl groups of the complex carbohydrate under conditions (neutral or mildly acidic pH) that do not remove the base-labile substituents.

Summary

The techniques described above permit the elucidation of the complete primary structure of most complex carbohydrates, starting with milligram quantities of material. The greatly improved methods now available for complex carbohydrate structure determination have made it feasible to attempt the elucidation of the primary structures of extremely complex polysaccharides such as RG-II.

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CHAPTER I

ISOLATION AND CHARACTERIZATION OF THE FIRST NATURALLY OCCURRING BRANCHED-CHAIN ACIDIC MONOSACCHARIDE

ABSTRACT

A branched-chain acidic monosaccharide, 3-C-carboxy-5-deoxy-L-xylose, has been identified for the first time as a component of plant cell walls. This sugar was first observed as a constituent of rhamnogalacturonan II, a primary cell wall pectic polysaccharide. The sugar was shown to be a 3-C-carboxy-5-deoxypentose by nuclear magnetic resonance spectroscopy and mass spectrometry of the underivatized sugar and of several derivatives. X-ray crystallography of one of the derivatives confirmed these structural features and established that the sugar has the xylo configuration. The absolute configuration of the sugar was elucidated by the identification of L-lactic acid as the major product of periodate oxidation of the methyl glycoside. "Aceric acid" has been proposed as a trivial name for this, the first branched-chain acidic sugar to be found as a natural product.

INTRODUCTION

Rhamnogalacturonan II (RG-II) is a pectic polysaccharide that is released from primary cell walls of suspension-cultured sycamore (Acer pseudoplatanus) cells by incubation with an endopolygalacturonase purified from the culture fluid of the fungus Colletotrichum lindemuthianum. RG-II is an extremely complex polysaccharide that comprises approximately 3% of the plant cell wall and contains at least 10 different monosaccharides, including apiose (3-C-[hydroxymethyl]-D-glycerotetrose), 2-O-methyl fucose, and 2-O-methyl xylose. The glycosyl residues of RG-II are interconnected by a great variety of glycosyl linkages[1].

The presence in RG-II of two unidentified glycosyl residues has been reported[1]. These residues were detected by gas-liquid chromatography (g.l.c.) analysis after hydrolysis of RG-II, reduction of the resulting aldoses to alditols, and acetylation. One of the unidentified components was subsequently shown to be an underacetylated derivative of apiose (W. S. York, M. McNeil, A. G. Darvill, and P. Albersheim, unpublished results).

The electron impact-mass spectrometry (e.i.-m.s.) fragmentation patterns of the second unidentified component did not allow its identification as an alditol acetate. However, the gross features of the mass spectral data were consistent with those of an acetylated carbohydrate derivative. The e.i. mass spectrum was characterized by losses of ketene, acetic acid, and acetic anhydride[2,3]. The residue could be reduced with borohydride only after acid hydrolysis; therefore, the unknown appeared to be an internal glycosidic residue of RG-II. We now describe the isolation and characterization of this sugar.

EXPERIMENTAL

^{1}H-n.m.r. spectroscopy. - ^{1}H-N.m.r. spectra were recorded on a Bruker WM-250 Fourier-transform n.m.r. spectrometer operated at 250 MHz. Water-soluble samples were lyophilized twice from deuterium oxide (99.7 atom % D), then dissolved in deuterium oxide (99.997 atom % D). Chemical shifts were assessed relative to an external standard (capillary) of hexamethyldisilazane (δ 0.6995). The acetylated sample (compound **3**, Figure 1) was run in deuteriochloroform (99.997 atom % D), and chemical shifts were assigned relative to internal chloroform (δ 7.26).

^{13}C-n.m.r. spectroscopy. - ^{13}C-N.m.r. spectra were recorded on a Bruker WM-250 Fourier-transform n.m.r. spectrometer operated at 62.9 MHz. Samples were run in deuterium oxide (99.7 atom % D). Chemical shifts were assessed relative to internal dioxane (δ 67.4). The ^{13}C-n.m.r. spectra of compounds **1** and **6** (Figure 1) were recorded at pH 1.5. At this pH, the carboxyl resonances were sharper and more intense than those observed when the spectra were recorded at neutral pH.

Fast atom bombardment-mass spectrometry (f.a.b.-m.s.). - [Note: The f.a.b.-m.s. analyses in this dissertation were performed by Dr. Anne Dell of the Imperial College of Science and Technology, London, United Kingdom.] F.a.b.-m.s.[4] spectra were obtained using a VG Analytical HF-ZAB 1 F mass spectrometer fitted with an f.a.b. source. Samples were dissolved in 5% (v/v) aqueous acetic acid (5 to 10 µg/µL), and a 1 µL aliquot was added to a drop of glycerol on the stainless steel target. Xenon was used as the bombarding gas. Spectra were recorded on UV-sensitive chart paper, and m/z ratios were determined by manual counting.

G.l.c. and g.l.c.-m.s.. - All g.l.c. and g.l.c.-m.s. conditions were as described[5,6].

Isolation of RG-II. - Cell walls were prepared from suspension-cultured sycamore (Acer pseudoplatanus) cells[7]. Isolated cell walls were incubated with C. lindemutheanum endopolygalacturonase[8], as described[1]. The material solubilized from the cell walls by the enzyme was dialyzed against H_2O, lyophilized, and de-esterified with NaOH (pH 12, 2^o, 2 h). The pH was adjusted to 5.2 with acetic acid, and 100 units of C. lindemutheanum endopolygalacturonase were added to the de-esterified wall polymer. Thimerosal (0.1% w/v) was added as an antimicrobial agent. The solution was incubated overnight at 30^o, dialyzed against H_2O, and then lyophilized. The lyophilized material was dissolved in 5 mL of 50 mM sodium acetate buffer (pH 5.2), and chromatographed on a column of Biogel P-10, as described[1].

Isolation of 3-C-carboxy-5-deoxy-L-xylose (1) from RG-II. - Isolated RG-II (4 mg) was hydrolyzed in 2N trifluoroactic acid (TFA) (120^o, 1 h), and the TFA removed by evaporation. The hydrolyzate was applied to a column (1 x 70 cm) of AG1-X8 (acetate form) anion-exchange resin (200-400 mesh) that had been equilibrated with 0.2N acetic acid. Galactosyluronic acid and glucosyluronic acid were eluted from the column by a gradient consisting of 200 mL of 0.5N acetic acid and 200 mL of 2N acetic acid[9]. The column was then washed with 200 mL of 4N acetic acid. Compound 1, which was detected by g.l.c. and e.i.-m.s. after reduction and acetylation, eluted from the column in the 4N acetic acid wash.

Preparation of a polysaccharide-enriched fraction from Pectinol AC. - Pectinol AC (600 g) was suspended in 2.5 L H_2O, and

insoluble material was removed by filtration through GF/C glass fiber filter paper (Whatman LTD.). The filtrate was concentrated to 800 mL by rotary evaporation. Solid trichloroacetic acid (TCA) (80 g) was added, and the solution was allowed to stand 2 h at 4^{o}. Precipitated protein was removed by centrifugation.

Absolute ethanol was added to the 10% TCA supernatant to a final concentration of 63% (v/v). After 12 h at 4^{o}, the solution was centrifuged, and the precipitate discarded. The supernatant was brought to an ethanol concentration of 86% (v/v), allowed to stand 12 h at 4^{o}, and then centrifuged. The material that was soluble in 63% ethanol but insoluble in 86% ethanol represented more than 60% of the orcinol-positive[10] carbohydrate initially present in Pectinol AC.

Preparation of 3-C-carboxy-5-deoxy-L-xylitol-3^{1},1-lactone (**2**). - Sodium borohydride (1 mg) in 1N NH_4OH (0.1 mL) was added to 1 mg of isolated **1**. The solution was allowed to stand 1 h at room temperature, after which excess borohydride was destroyed by adding glacial acetic acid. The solution was evaporated to dryness under a stream of filtered air. Boric acid was removed by successive addition and evaporation of four aliquots (0.5 mL) of 10% (v/v) acetic acid in methanol and 4 aliquots (0.5 mL) of methanol[11]. The residue was dissolved in 0.5 mL H_2O and passed through a column (2 mL) of Dowex 50 cation-exchange resin (H^+ form), and the sample was lyophilized. Compound **2** (Figure 1) was recovered as a syrup.

Preparation of 3-C-carboxy-5-deoxy-2,3,4-tri-O-acetyl-L-xylitol-3^{1},1-lactone (**3**). - Compound **2** (0.25 mg) was treated for 3 h at 120^{o} with acetic anhydride (0.2 mL) containing 2 mg of sodium acetate, and excess acetic anhydride was destroyed by adding $NaHCO_3$. The sample was

partitioned between CH_2Cl_2 (0.5 mL) and H_2O (0.5 mL). The CH_2Cl_2 layer was washed with two aliquots (0.5 mL) of H_2O, and then evaporated to dryness under a stream of filtered air, yielding compound **3** (Figure 1).

Preparation of 3-C-hydroxymethyl-5-deoxy-1,2,3,3[1],4-penta-O-acetyl-D-xylitol (**5a**). - A carboxyl-reduced alditol (compound **4a**, Figure 1) was prepared by reduction of compound **2**, using the procedure of Jones and Albersheim[12]. Compound **2** (0.2 mg) was dissolved in a few drops of 10 mM sodium borate buffer (pH 7.5). Sodium borohydride (2.5 mg), dissolved in 0.25 mL of the same buffer, was added. The reaction was quenched with acetic acid after 1 h at room temperature. Boric acid was removed as described above. The sample was per-O-acetylated by treatment with 0.2 mL of pyridine/acetic anhydride (1:1) for 30 min at 120° to yield compound **5a** (Figure 1). Compound **4b** (Figure 1) was prepared by an analogous procedure, using sodium borodeuteride and 10 mM sodium borate buffer made with D_2O, and then per-O-acetylated to yield compound **5b** (Figure 1).

Preparation of 3-C-carboxy-5-deoxy-L-xylono-1,4-lactone (**6**). - Compound 1 (20 mg) was dissolved in 3 mL H_2O. Bromine (0.05 mL) was added to the solution with vigorous stirring. The solution was incubated 72 h in the dark. Excess bromine was removed by rotary evaporation. The sample was neutralized with solid $AgCO_3$, and then the insoluble AgBr was removed by filtration through GF/C glass fiber filter paper. The filtrate was lyophilized, yielding a syrup. The syrup was dissolved in 2-pentanone, and then crystallized from a mixture of 2-pentanone and $CHCl_3$[13] using vapor diffusion[14]. The sample was recrystallized twice from 2-pentanone/$CHCl_3$, yielding 15 mg (75% recovery) of crystalline compound **6** (melting point: 120 to 122°, uncorrected).

X-ray crystallography of compound **6**. - [Note: the X-ray crystallography of compound **6** was performed by Dr. Kim Henrick of the Polytechnic of North London, London, United Kingdom.] $C_6H_8O_6 \cdot H_2O$, M_r = 194.14, Monoclinic, space group $P2_1$, a = 7.697(2), b = 7.414(2), c = 7.336(2) Å, β = 101.88(2)°, Z = 2, $F(000)$ = 204, D_c = 1.567 g cm^{-3}, 787 reflections, [524; $I > 3\sigma(I)$], in the range $3 < \theta < 25°$.

A crystal with dimensions 0.14 x 0.12 x 0.12 mm was selected. The crystal data showed the systematic absences $0k0$ ($k = 2n + 1$) that established the space group as $P2_1$ (for an optically active compound). Intensity data were collected on a Philips PW1100 diffractometer with Mo-K_α radiation (λ = 0.7106 Å) and a θ-2θ scan mode. Three standard reflections were measured every 2 h during the course of data collection to monitor crystal quality and alignment; these were constant within 5%. Absorption corrections were not applied. Equivalents were averaged to give 787 unique reflections.

The structure was solved by standard multisolution tangent refinement with SHELX76[15]. Using E > 1.2, the second E map from the sign expansion (R_a = 0.073) revealed the positions of all 13 nonhydrogen atoms. The H atoms were found in a subsequent difference Fourier synthesis. The nonhydrogen atoms were refined assuming anisotropic motion, and the H atoms on isotropic motion, using full-matrix least-squares methods. Final refinement of the model in the L configuration gave a conventional R value of 0.0421, and a weighted R value of 0.0401 using a weighting factor, $w = 1/ {}^2(F_o)$. The final Hamilton R_g factor[16] was 0.0425. Neutral atom scattering factors were used[17]. A final difference map showed no significant features, with a maximum residual density of 0.22 e Å^{-3}. Final refinement of the model in the D

configuration from the same starting parameters gave identical values of $\underline{R}$, $\underline{R}_w$, and $\underline{R}_g$, making an assignment of the optical isomer impossible.

Determination of absolute configuration. - Compound **1** (2 μmole) was dried under reduced pressure over P_2O_5. The sample was treated with dry 1N methanolic HCl (1 mL) for 16 h at 85°, neutralized with solid $AgCO_3$, and centrifuged. The insoluble material was washed with 3 aliquots (1 mL) of methanol, and the supernatants were pooled and evaporated[18]. The sample was de-esterified with NaOH (pH 12, 2°, 2 h), neutralized with acetic acid, passed through a column (2 mL) of Dowex 50 (H^+ form), and then lyophilized, yielding an anomeric mixture of methyl glycosides (**7**, Figure 10).

The methyl glycosides were dissolved in 2.5 mL of 0.05M sodium acetate buffer (pH 5.2). To this was added 0.5 mL of 0.1M $NaIO_4$ in H_2O. The sample was incubated in the dark at 22° for 68 h. Excess periodate was eliminated by adding 40 μL of 1M glycerol. The sample was reduced with sodium borohydride, as described above, hydrolyzed in 0.5 mL of 1N HCl for 12 h at 22°, and neutralized with NaOH.

The content of L-lactic acid was determined enzymatically in 0.2M glycine buffer (pH 9.2) containing 0.16M hydrazine, 0.8 mg/mL nicotinamide adenine dinucleotide, and 15 units/mL beef heart L-(+)-lactic acid dehydrogenase (Sigma). Total assay volume was 1.5 mL. Substrate-dependent formation of NaDH was determined spectrophotometrically ($\underline{A}_{340}$). The content of D-lactic acid was determined in the same manner except that 20 units of Lactobacillus leichmannii D-(-)-lactic acid dehydrogenase (Sigma) were used in place of the L-(+)-lactic acid dehydrogenase. Readings of $\underline{A}_{340}$ were corrected by subtracting the $\underline{A}_{340}$ value of a solution containing all assay components except enzyme.

RESULTS & DISCUSSION

Isolation of compound 1. - Compound 1 (Figure 1) was bound by anion-exchange resins, and it could be eluted only under conditions stronger than those under which uronic acids are eluted. These results demonstrated that 1 was an acidic compound, and suggested that it could be purified by anion-exchange chromatography of the mixture of monosaccharides produced by acid hydrolysis of RG-II. Compound 1 (25-50 μg) was isolated from acid-hydrolyzed sycamore cell wall RG-II (3 mg) by chromatography in acetic acid on a column of AG1-X8 (acetate) anion-exchange resin.

Compound 1 represents about 7% of purified RG-II, and RG-II about 3% of isolated sycamore cell walls[1]. Therefore, it was impractical to use sycamore cell wall RG-II as the starting material for the isolation of sufficient 1 for structural characterization. Fortunately, a more abundant and readily accessible source of 1 was found in our laboratory. Pectinol AC (Corning Inc.), a commercial preparation of the enzymes secreted by the fungus Aspergillus niger when the fungus is grown using plant cell walls as the carbon source, was found to contain 1. Experiments (J. Thomas, W. S. York, A. G. Darvill, and P. Albersheim, unpublished results) have shown that Pectinol AC contains approximately 3% carbohydrate by weight, about 50% of which is a polysaccharide with a glycosyl-residue composition, glycosyl-linkage composition, and a size very similar to those of sycamore cell wall RG-II. The Pectinol AC RG-II contains apiose, 2-O-methyl fucose, 2-O-methyl xylose, and compound 1, the rarely seen glycosyl residues that characterize RG-II. Therefore, Pectinol AC was selected as an inexpensive, plentiful starting material from which sufficient compound 1 could be isolated to permit its structural characterization.

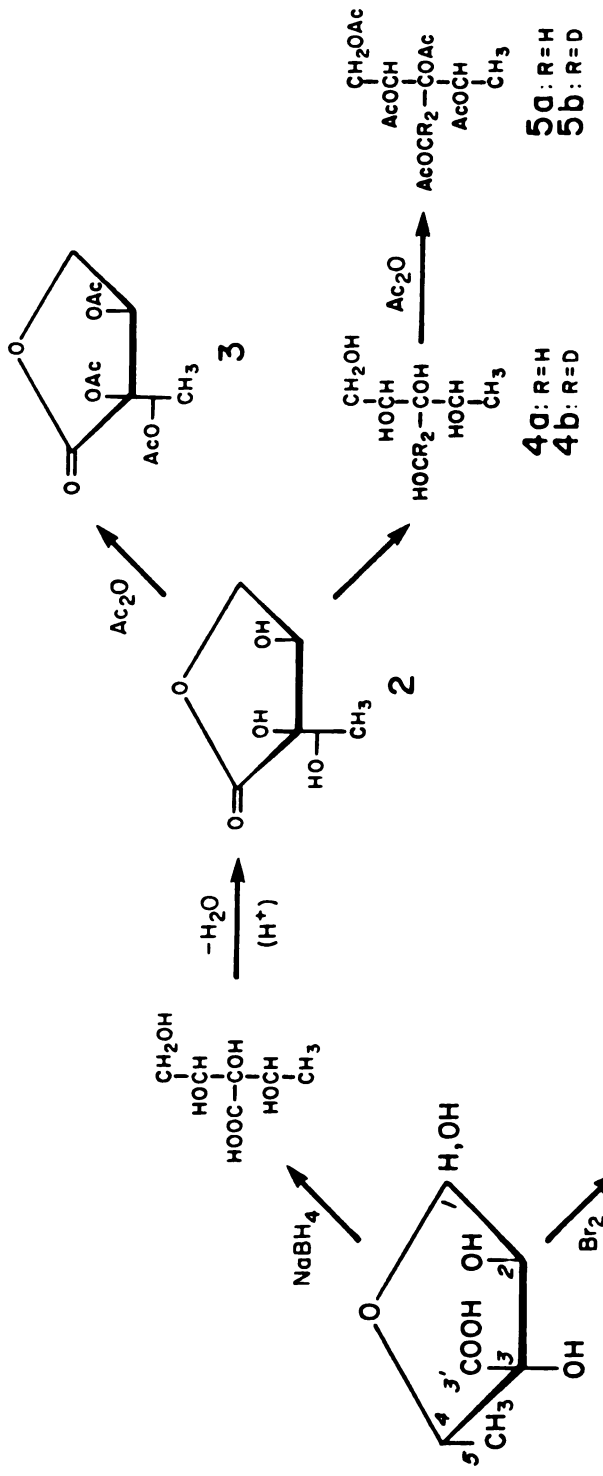

Figure 1. Summary of the derivatives prepared from compound 1. The numbering of carbon atoms indicated on compound 1 was used with all derivatives.

A polysaccharide-enriched fraction was prepared from Pectinol AC (600 g) as described in the "Experimental" section. This material was dissolved in 500 mL of 2N TFA, and then heated under reflux for 18 h (94^{o}). The TFA was removed by rotary evaporation, and the hydrolyzate was applied to a column (2 x 70 cm) of AG1-X8 (acetate form) anion-exchange resin (Figure 2). Material that adsorbed to the column was eluted with 2N acetic acid, followed by 4N acetic acid. Galactosyluronic acid (peak "a," Figure 2) and glucosyluronic acid (peak "b," Figure 2) eluted in the 2N acetic acid wash. Compound **1** was detected by g.l.c. after aliquots from fractions were reduced with borohydride and acetylated. Compound **1** eluted from the column in the 4N acetic acid wash (peak "c," Figure 2) and was recovered as a syrup (60 mg).

Comparison of **1** isolated from sycamore cell wall RG-II to **1** isolated from Pectinol AC polysaccharide. - Samples of **1** isolated from sycamore cell wall RG-II and from Pectinol AC polysaccharide were reduced and acetylated[8]. The acetylated derivatives were indistinguishable by g.l.c. on two different capillary columns (SP 2330, Supelco, and DB 1, J&W Scientific). The e.i. mass spectra of the alditol acetates were identical (Figure 3).

The procedure of Gerwig *et al.*[19] was used to demonstrate that only one optical isomer of **1** was present in the Pectinol AC polysaccharide, and that the same optical isomer of **1** was present in sycamore cell RG-II. Aliquots of **1** isolated from Pectinol AC were treated with (+)-2-butanol in HCl, and with (-)-2-butanol in HCl. Trimethylsilyl derivatives of the products of these reactions were analyzed by g.l.c. The pattern of peaks obtained with (+)-2-butanol was different from that obtained with (-)-2-butanol. In both cases, peaks that would have

Figure 2. Chromatography of the acid hydrolyzate of the Pectinol AC polysaccharide on AG1-X8 (acetate) anion-exchange resin. The column (2 x 70 cm) was equilibrated with 0.2N acetic acid. The acid-hydrolyzed Pectinol AC polysaccharide was dissolved in 1.2 L of H_2O and the pH of this solution was adjusted to 9.0 with 0.1N NaOH. After sample application, the column was washed with 500 mL of 0.2N acetic acid. The column was eluted with 2 L of 2N acetic acid, followed by 2 L of 4N acetic acid. The 2N and 4N acetic acid effluent was collected in fractions (10 mL). Aliquots of the fractions were assayed [19] for uronic acid content (A_{520}) The relative content of compound **1** was assayed as follows. Aliquots (20 µL) of the fractions were reduced with $NaBD_4$ and acetylated; this treatment converts compound **1** to compound **3** (Figure 1), which was quantitated by g.l.c. The relative content of **1** was calculated by dividing the g.l.c. peak area of compound **3** by the peak area of internal standard myo-inositol hexaacetate.

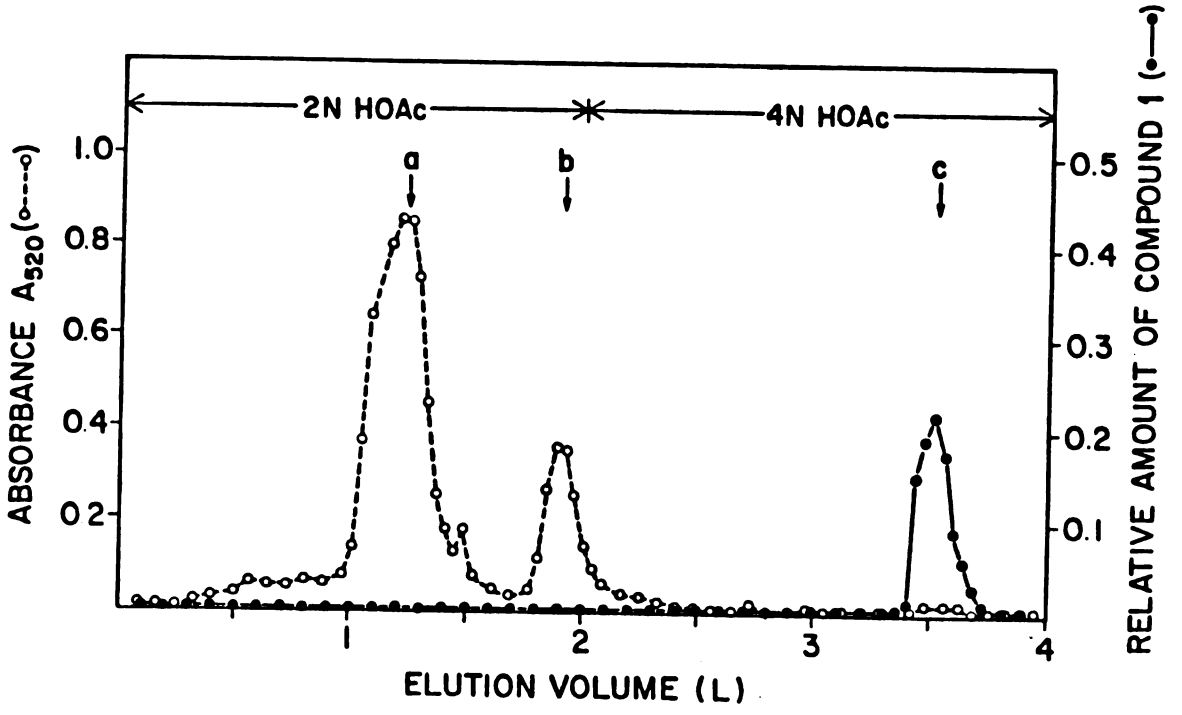
2N HOAc
4N HOAc
a
b
c
ABSORBANCE A_{520}(o----o)
RELATIVE AMOUNT OF COMPOUND 1 (•—•)
ELUTION VOLUME (L)
1.0
0.8
0.6
0.4
0.2
0.5
0.4
0.3
0.2
0.1
1
2
3
4

Figure 2

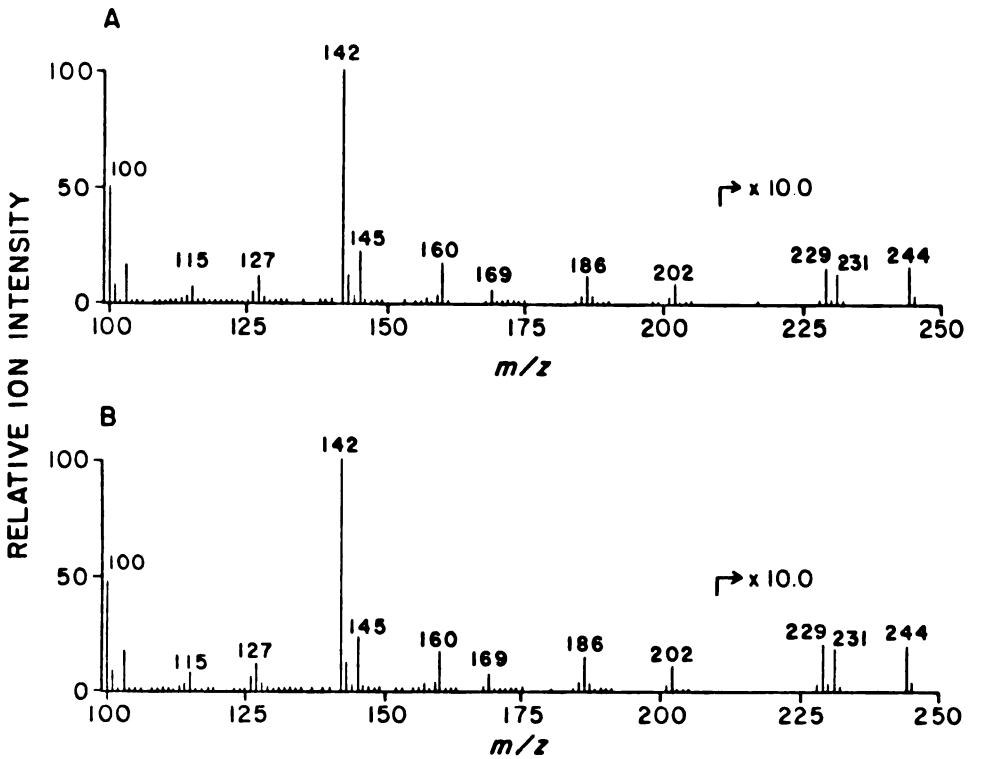

Figure 3. E.i. mass spectra of compound **3** formed by reduction and per-O-acetylation of: A) compound **1** isolated from sycamore cell wall RG-II, and B) compound **1** isolated from the Pectinol AC polysaccharide.

corresponded to the other optical isomer of **1** were not present. Therefore, it was concluded that a single optical isomer of **1** was present in Pectinol AC.

A heptasaccharide that was isolated from sycamore cell wall RG-II and that contained **1** as an internal glycosyl residue (M. Spellman, Chapter II of this dissertation) was treated with (+)-2-butanol in HCl, then derivatized with TMS and analyzed. The g.l.c. analysis of the heptasaccharide contained, inter alia, the same pattern of peaks as was observed with compound 1 isolated from the Pectinol AC polysaccharide. The combined results demonstrated that compound **1** isolated from the Pectinol AC polysaccharide was identical to compound 1 from sycamore cell wall RG-II.

Characterization of underivatized **1**

Colorimetric assays. - Compound 1 gave a positive response in the Nelson-Somogyi[20] and the p-hydroxybenzoic acid hydrazide[21] assays for reducing sugars. It did not give a positive response in the anthrone[10], orcinol[10], or m-hydroxydiphenyl[22] assays. These results indicated that **1** was a reducing sugar that could not be transformed into a furfural derivative[10].

Fast atom bombardment-mass spectrometry. - The molecular weight of **1** was found to be 178 by fast atom bombardment-mass spectrometry (f.a.b.-m.s.). The f.a.b. mass spectrum of compound **1** did not contain the characteristic isotope ratios that would indicate the presence of silicon or sulfur in the molecule[23]. Compound **1** did not contain phosphate, as assayed by a modification of the Fisk-SubbaRow procedure[24]. Therefore, it was concluded that the acidic moiety in **1** was a carboxyl group.

^{1}H-n.m.r. spectroscopy. - The ^{1}H-n.m.r. spectrum of **1** is shown in Figure 4. This spectrum provided evidence that **1** was a 3-C-carboxy-5-deoxy-pentose. In solution, **1** existed as a mixture of its two anomers, which were present in a ratio of approximately 1.2:1. This concentration difference was large enough to allow each resonance to be assigned to one of the two anomers on the basis of its area in the n.m.r. spectrum. Each anomer gave rise to an upfield, 3-proton doublet (δ 1.27, and δ 1.34); this indicated that the molecule contained a methyl group (C-5). In each anomer, H-4 was split by the methyl protons and gave rise to a quartet (δ 4.76 and 4.64, respectively). The resonances at δ 5.56 and δ 5.27 arose from the hemiacetal protons (H-1). The hemiacetal proton of each anomer was coupled to one other proton (δ 4.25 and δ 4.13, respectively), which was not further split. The magnitude of the coupling between H-1 and H-2 of a furanose sugar can be used to distinguish the anomer in which H-1 and H-2 are cis from the anomer in which they are trans[25,26]. Therefore, the resonance at δ 5.56 ($J_{1,2}$ of 4.3 Hz) arises from H-1 of the anomer containing a cis-1,2-diol, and the resonance at δ 5.27 ($J_{1,2}$ of 2.6) arises from H-1 of the anomer containing a trans-1,2-diol.

^{13}C-n.m.r. spectroscopy. - The ^{13}C-n.m.r. spectrum of the mixture of anomers of **1** was consistent with the proposed structure (Table I). The chemical shifts of the hemiacetal carbons (C-1) of the two anomers differed by 5 p.p.m. This difference is characteristic of a furanose sugar; the hemiacetal carbon of the anomer containing a trans-1,2-diol resonates 5-6 Hz downfield of the corresponding carbon of the anomer containing a cis-1,2-diol[27]. The resonances that have been assigned as the C-3′ carbons of the anomers had chemical shifts and

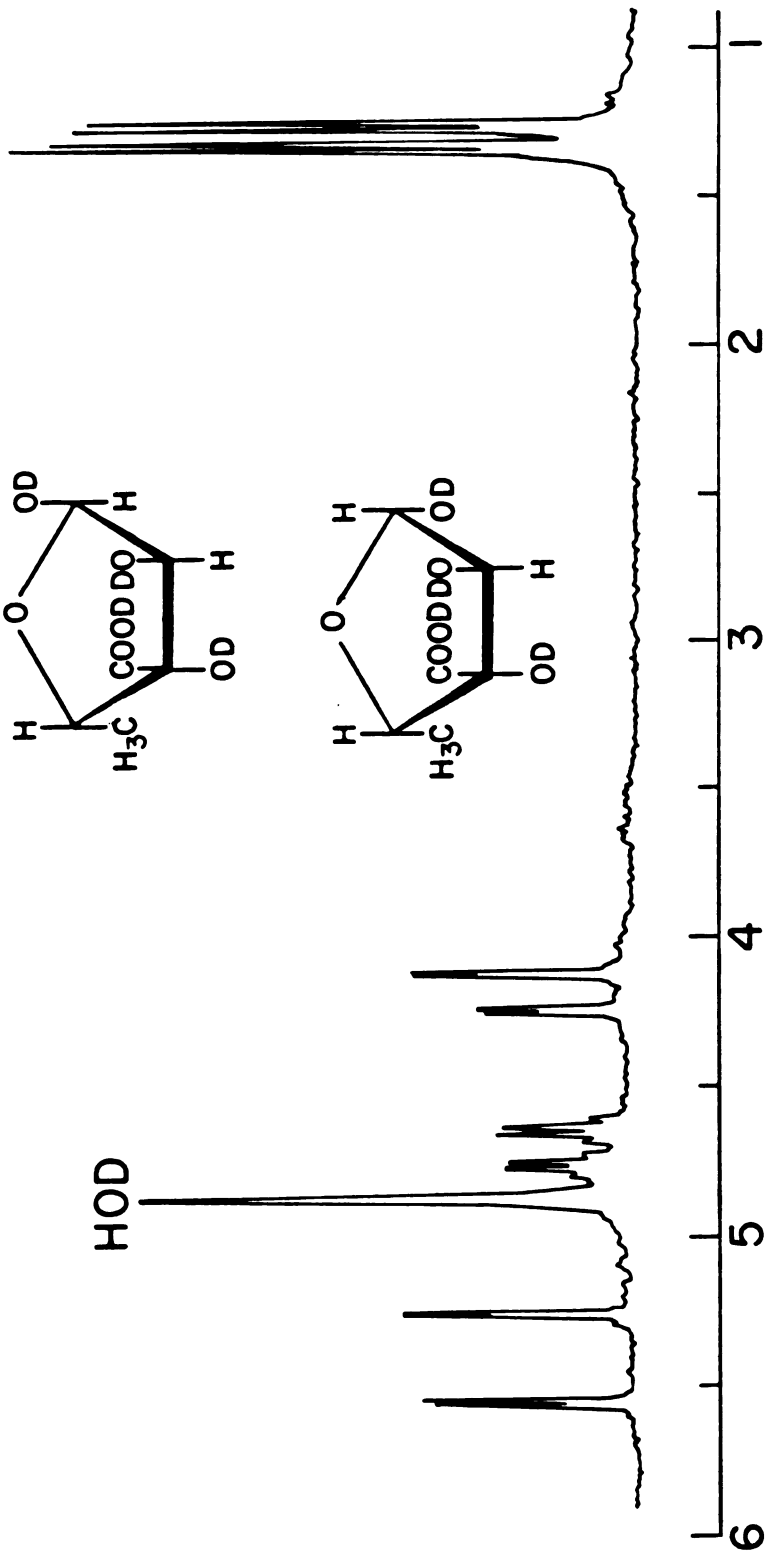

Figure 4. ^{1}H-N.m.r. spectrum of the mixture of anomers of compound **1** in D_2O. Chemical shifts were assessed relative to an external standard (capillary) of hexamethyldisilazane (δ 0.6995).

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS OF THE MIXTURE OF ANOMERS OF COMPOUND 1

	Chemical shift[a] (p.p.m.)	
	cis[b] anomer	trans[b] anomer
C-1	97.3	102.2
C-2	79.8[c]	83.5
C-3	83.6	81.0
C-3′	172.2	174.2
C-4	79.2[c]	79.7
C-5	11.4	14.6

[a] Chemical shifts were assessed relative to internal dioxane at δ 67.4. [b]The designation "cis" refers to the anomer containing a cis-1,2-diol, while "trans" refers to the anomer with a trans-1,2-diol. [c]These assignments may be reversed in the "cis" anomer.

intensities characteristic of carbonyl carbons (in this case, carboxyl). These signals were approximately 30% as intense as those arising from the methyl carbons; low signal intensity is usually observed with carbon atoms that do not have any hydrogen atoms bonded to them. The resonances assigned as C-3 carbons had chemical shifts typical of alcohol carbons, and had intensities comparable to those of the carboxyl carbons. These results indicated that C-3 of each anomer was a tertiary-alcohol carbon, and provided additional evidence for the branched-chain structure of compound **1**.

Preparation and characterization of reduction products of **1**

Compound 2. - Reduction of **1** with borohydride yielded a 3-C-carboxy-5-deoxy-pentitol, which readily formed a γ-lactone upon acidification (**2**, Figure 1). Evidence for the formation of **2** was obtained by f.a.b.-m.s. The molecular weight of borohydride-reduced **1** was found to be 162, which is the molecular weight of **2**. No ion was observed with m/z 181 that would correspond to the $(M + H^+)$ ion of the free-acid form of the hydride-reduced derivative. The ^{1}H-n.m.r. spectrum of **2** in D_2O (Figure 5) contained a 3-proton doublet (J = 6.5 Hz) at δ 1.36, and a 1-proton quartet (J = 6.5 Hz) at δ 4.11. These signals arose from the methyl protons and H-4, respectively. The other three nonexchangeable protons (2 protons on C-1, and 1 proton on C-2) gave rise to a complex 3-proton multiplet between δ 4.33 and δ 4.70. The ^{13}C-n.m.r. spectrum of **2** in D_2O contained as expected, resonances at δ 16.5 (methyl); 68.5, 68.6, 74.3 (alcohol); 79.2 (3^o alcohol); and 180.1 (carbonyl).

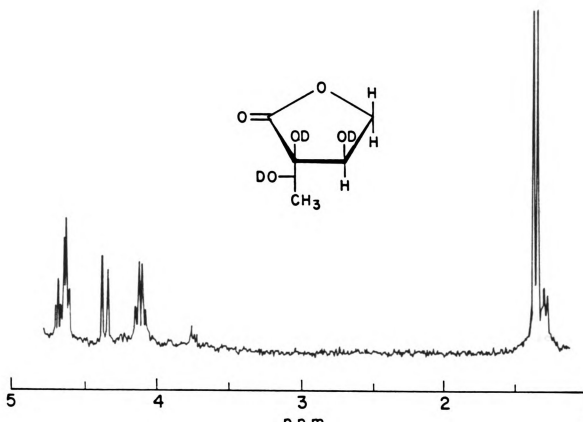

Figure 5. ^{1}H-N.m.r. spectrum of compound **2** in D_2O. Chemical shifts were assessed relative to an external standard (capillary) of hexamethyldisilazane (δ 0.6995).

Compound 3. - Acetylation of compound **2** yielded a volatile tri-*O*-acetyl lactone (**3**, Figure 1). The molecular weight of **3** was 288, as determined by c.i.-m.s. (isobutane) and f.a.b.-m.s. The molecular weight of the corresponding derivative that was per-*O*-acetylated using hexadeuterioacetic anhydride was 297. The difference of nine mass units indicated that three *O*-acetyl groups were incorporated.

The e.i. mass spectrum of **3** is shown in Figure 3. The ion with *m/z* 244 (M-44) corresponded to loss of carbon dioxide from the molecular ion. This is characteristic of a lactone[28]. The $M-CO_2$ ion sequentially eliminated two molecules of ketene to yield the ions with *m/z* 202 and *m/z* 160. The base peak, with *m/z* 142, arose from the elimination of acetic anhydride from the $M-CO_2$ ion. The ion with *m/z* 231 arose from the sequential losses of a methyl radical and ketene from the molecular ion. The other features of the mass spectrum were typical of those of alditol acetates[2,3]. The ion with *m/z* 229 resulted from the loss of an acetoxy radical from the molecular ion. This ion lost acetic acid to generate the ion with *m/z* 169, which in turn eliminated ketene to yield the ion with *m/z* 127. Loss of acetic acid from the molecular ion yielded the ion with *m/z* 228, which in turn lost ketene to yield the ion with *m/z* 186.

The ^1H-n.m.r. spectrum of **3** in $CDCl_3$ (Figure 6) contained a three-proton doublet (J = 6.3 Hz) at δ 1.38 and a one-proton quartet (J = 6.3 Hz) at δ 5.24, arising from the methyl group and H-4, respectively. The methyl protons of the *O*-acetyl groups gave rise to three-proton singlets at δ 2.04, 2.11, and 2.18. The other three non-exchangeable protons comprised an AMX spin system, and gave rise to one-proton doublets-of-doublets at δ 4.27 (J = 9.7 Hz, J = 5.5 Hz), δ 4.74 (J = 9.7 Hz, J = 8.2 Hz), and δ 5.54 (J = 5.5 Hz, J = 8.2 Hz).

Carboxyl-reduced alditols. - Borohydride reduction of lactone **2** yielded compound **4a** (Figure 1), a branched-chain deoxy alditol. Reduction of **2** with borodeuteride in borate buffer yielded compound **4b** (Figure 1), the dideuteriolabeled derivative of **4a**. Compounds **4a** and **4b** were per-O-acetylated, yielding compounds **5a** and **5b**, respectively (Figure 1), which were analyzed by g.l.c.-m.s. The e.i. mass spectrum of compound **5a** is shown in Figure 7A. The highest ions in the spectrum (m/z 317 and m/z 316) were formed by eliminating from the molecular ion an acetoxy radical and acetic acid, respectively. The other primary fragment-ions arose from fission of the alditol chain, as indicated in Figure 7A. Secondary fragment-ions were formed by eliminations of ketene, acetic acid, and acetic anhydride from the primary fragment ions. The base peak at m/z 128 and the ion at m/z 170 are present in the e.i. mass spectra of all hexa-O-acetyl hexitols and penta-O-acetyl-6-deoxy-hexitols[29], and are, therefore, of no diagnostic value.

Comparison of the e.i. mass spectrum of compound **5a** to that of compound **5b** (Figure 7B) confirmed the location and nature of the branch in compound **1**. The primary ions with m/z 317, 316, 289, and 231 in the mass spectrum of **5a** were shifted to m/z 319, 318, 291, and 233 in the mass spectrum of **5b**. The secondary ions derived from these primary fragments were also shifted by two mass units. The primary ion at m/z 145 and the secondary ion derived from it were not changed by carboxyl reduction with borodeuteride. Compound **5a** gave rise to two different primary fragment-ions with m/z 303. In the mass spectrum of compound **5b**, one of these ions had m/z 303, while the other had shifted to m/z 305. These results confirmed that compound **1** had a carboxyl branch at C-3.

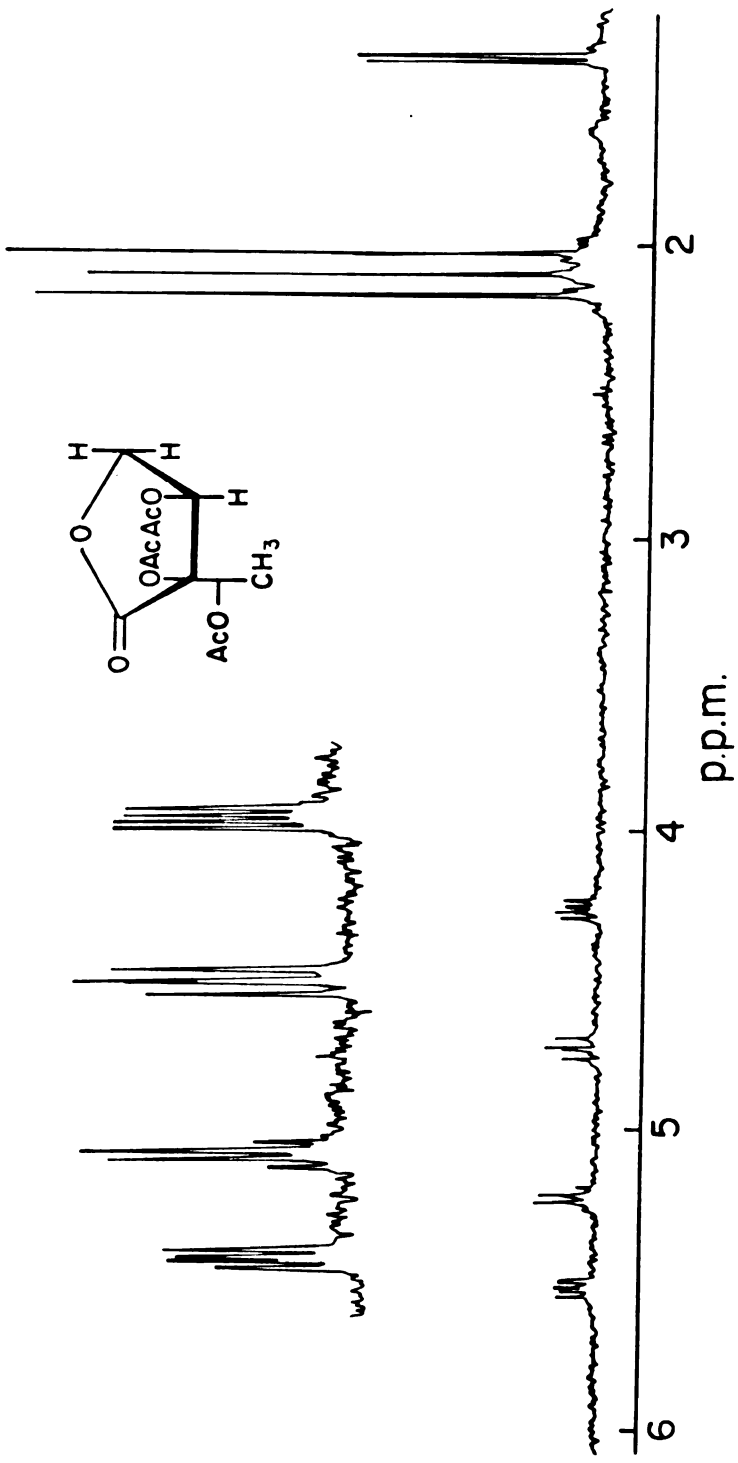

Figure 6. ^{1}H-N.m.r. spectrum of compound **3** in $CDCl_3$. Chemical shifts were assessed relative to internal chloroform (δ 7.26). The upper trace is an expanded plot of the spectral region from 4 to 5.7 p.p.m.

Figure 7. E.i. mass spectra of A) compound **5a** and B) **5b**. Secondary fragment-ions, formed by eliminations of ketene (42 mass units), acetic acid (60 mass units), and acetic anhydride (102 mass units) have been indicated.

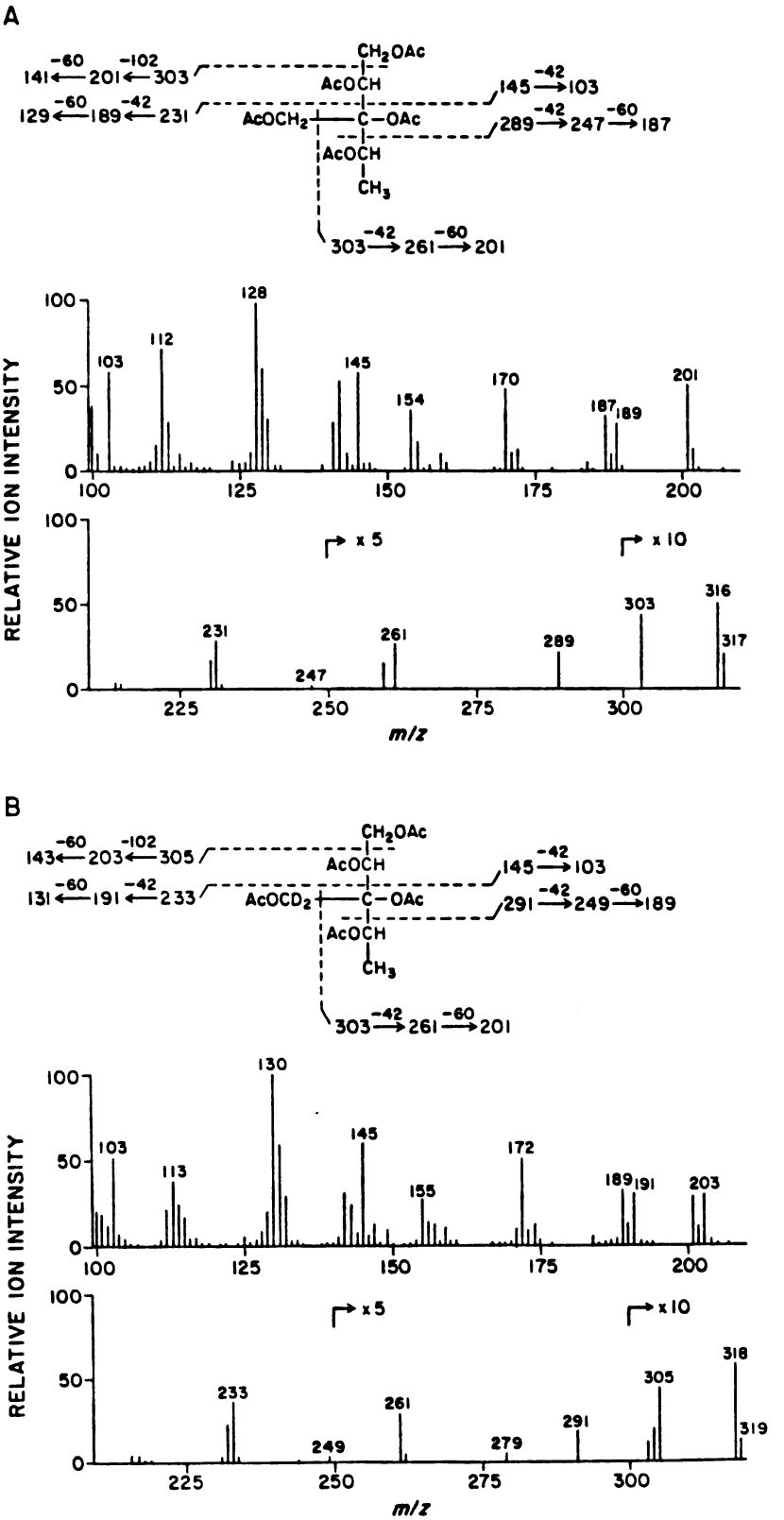
A
CH2OAc
AcOCH
AcOCH2—C—OAc
AcOCH
CH3
-60 -102
141 ← 201 ← 303
-60 -42
129 ← 189 ← 231
-42
145 → 103
-42 -60
289 → 247 → 187
-42 -60
303 → 261 → 201
RELATIVE ION INTENSITY
m/z
x 5
x 10
B
CH2OAc
AcOCH
AcOCD2—C—OAc
AcOCH
CH3
-60 -102
143 ← 203 ← 305
-60 -42
131 ← 191 ← 233
-42
145 → 103
-42 -60
291 → 249 → 189
-42 -60
303 → 261 → 201
RELATIVE ION INTENSITY
m/z
x5
x10

Figure 7

Determination of the relative configurations of the three asymmetric carbons of compound **1**. - The results presented above clearly established that compound **1** was a 3-C-carboxy-5-deoxy-pentose. The open-chain form of such a sugar has three asymmetric carbons; therefore, compound **1** could exist as one of eight stereoisomers (four L isomers and four D isomers). The relative configurations of the three asymmetric carbons were established by X-ray crystallography of compound **6** (Figure 1), an oxidation product of compound **1**.

Compound **6** was prepared by bromine oxidation of compound **1**. The general structure of compound **6** was confirmed by ^{1}H-n.m.r. and ^{13}C-n.m.r. The ^{1}H-n.m.r. spectrum of **6** in D_2O (Figure 8) contained a three-proton doublet (J = 6.6 Hz) at δ 1.40, and a 1-proton quartet at δ 4.70, arising from the methyl group and H-4, respectively. H-2 gave rise to a one-proton singlet at δ 4.62. The ^{13}C-n.m.r. spectrum of **6** contained, as expected, the following signals: δ 15.7 (methyl); 73.4 and 80.5 (alcohol); 81.0 (3^{o} alcohol); 173.9 and 175.9 (carbonyl).

Compound **6** was crystallized from a mixture of 2-pentanone and chloroform. X-ray crystallography of compound **6** confirmed all previously determined structural features, and revealed that the sugar has the xylo configuration (Figure 9)*. It was not possible to determine absolute configuration of **6** from the crystallography data.

Determination of the absolute configuration of **1**. - The absolute configuration of **1** was determined by periodate oxidation of the anomeric

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*The other results of the X-ray crystallographic analysis of compound **6** are presented in the Appendix.

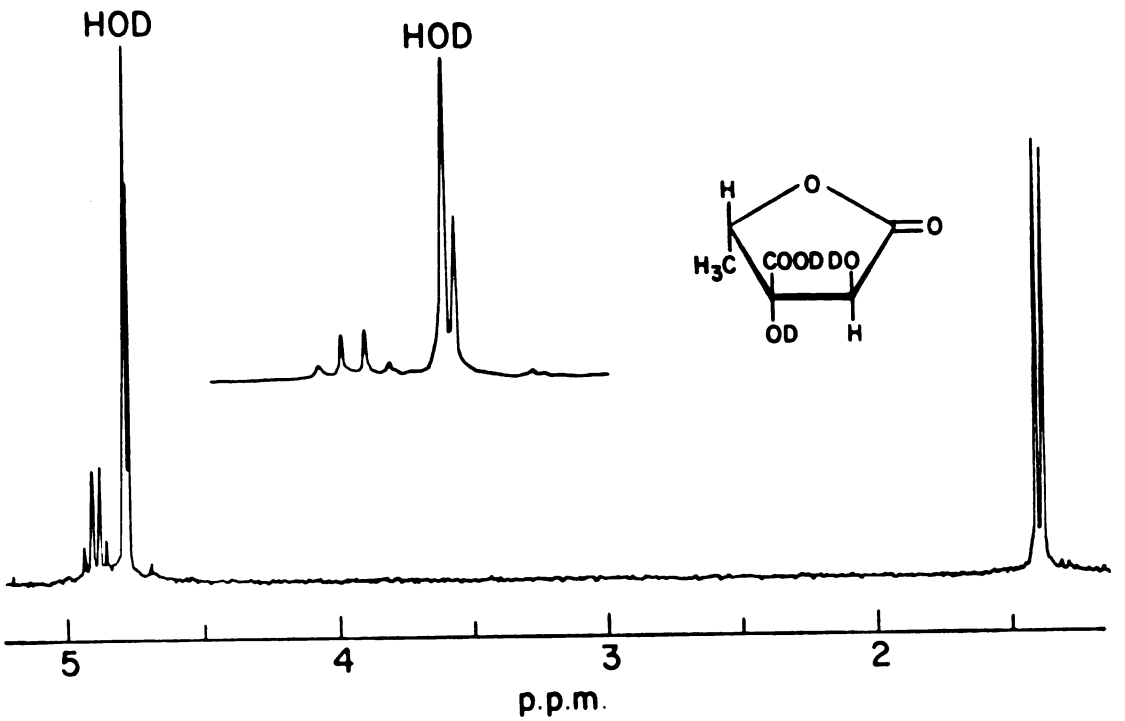

Figure 8. ^{1}H-N.m.r. spectrum of compound **6** in D_2O. Chemical shifts were assessed relative to an external standard (capillary) of hexamethyldisilazane (δ 0.6995). The upper trace is an expanded plot of the spectral region between 4.6 and 5.0 p.p.m.

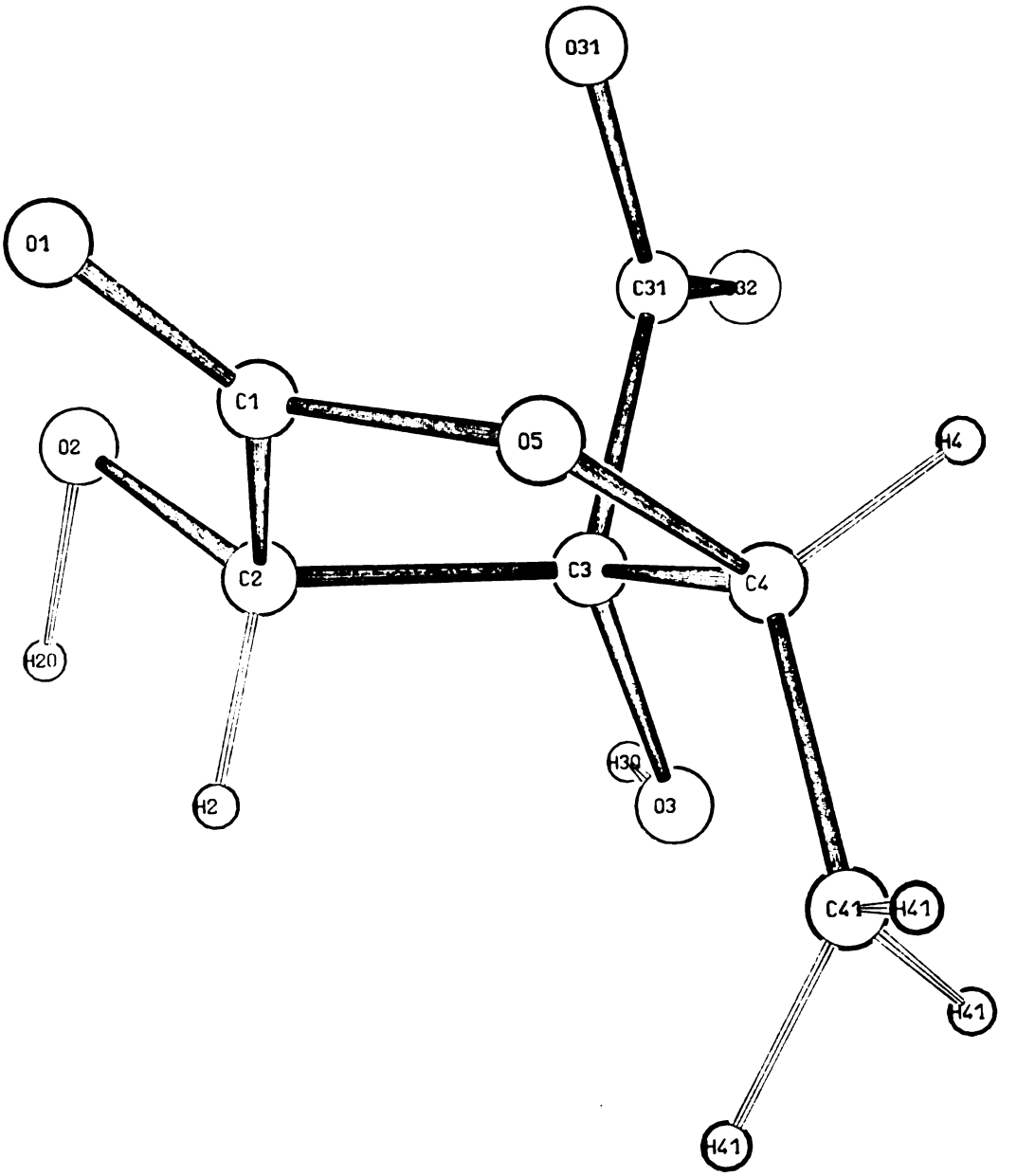

Figure 9. Structure of compound 6, as determined by X-ray crystallography.

mixture of the methyl glycosides (Figure 1). The methyl glycosides were prepared by methanolysis of **1**, followed by base de-esterification. The methyl glycosides were subjected to Smith degradation (periodate oxidation, followed by borohydride reduction and mild acid hydrolysis)[30]. The expected product of this series of reactions was D-lactic acid if **1** had the D configuration, or L-lactic acid (Figure 10) if **1** had the L configuration.

Enzymatic analysis was used to determine the concentrations of D- and L-lactic acid obtained by Smith degradation of the methyl glycosides of **1**. L-lactic acid (0.6 μmole of L-lactic acid per μmole of starting material) was the major product recovered. Therefore, it was concluded that **1** was an L sugar. A small amount of D-lactic acid (0.1 μmole/μmole starting material) was also detected. The origin of the D-lactic acid is not understood because compound **1** was shown to be optically pure by the method of Gerwig et al. (see above). A possible explanation for the formation of D-lactic acid is that some isomerization occurred during the series of reactions used to form the lactic acid.

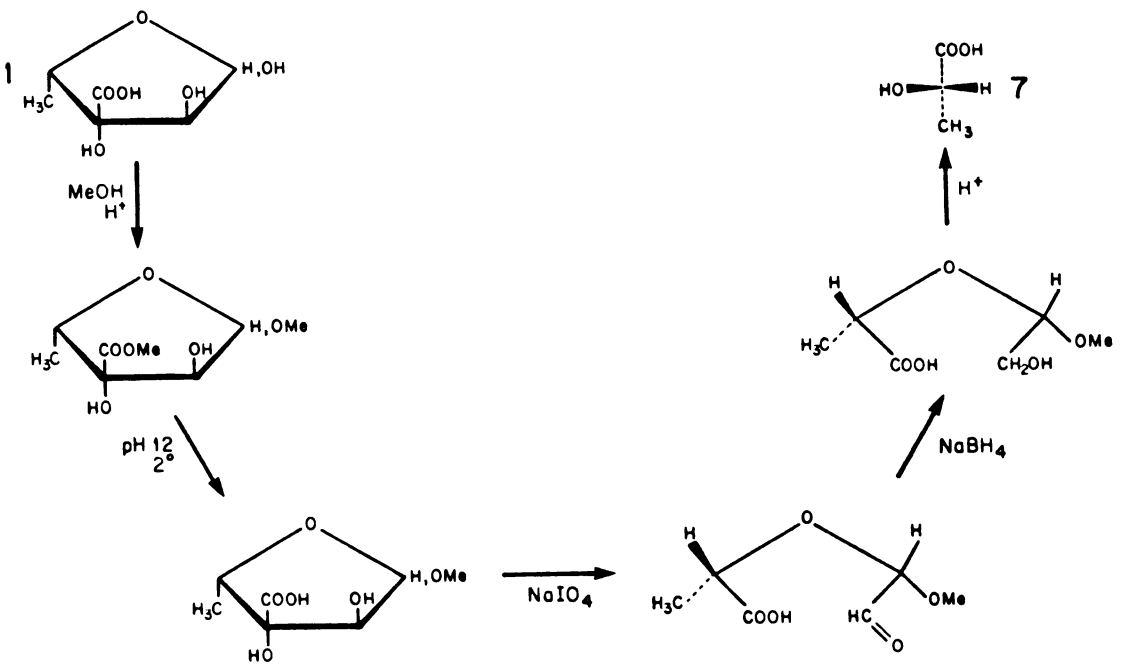

Figure 10. Reaction sequence used to determine the absolute configuration of compound 1.

GENERAL DISCUSSION

Compound 1 is 3-C-carboxy-5-deoxy-L-xylose. We propose the trivial name "aceric acid" for this sugar because it was first observed[1] as a constituent of cell walls isolated from suspension-cultured cells of Acer pseudoplatanus (sycamore). More than a dozen branched-chain sugars have been found in nature, frequently as components of antibiotics[31,32]. Streptose (3-C-formyl-5-deoxy-L-lyxose)[10], a neutral-sugar component of the antibiotic streptomycin, is the most similar of these to aceric acid, but it has a formyl rather than a carboxyl group at C-3′, and the lyxo rather than the xylo configuration. Only two other branched-chain sugars have previously been found in plant tissue[33]; these are apiose (3-C-(hydroxymethyl)-D-glycero-tetrose) and hamamelose (2-C-(hydroxymethyl)-D-ribose). Aceric acid is the first branched-chain acidic sugar to be found as a natural product.

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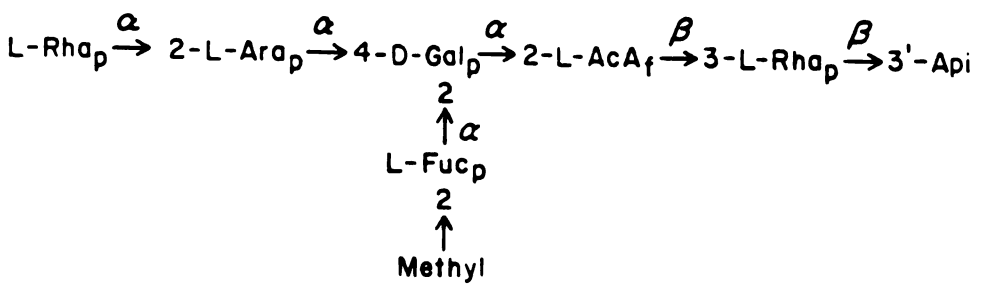
CHAPTER II

CHARACTERIZATION OF A STRUCTURALLY COMPLEX HEPTASACCHARIDE ISOLATED FROM RHAMNOGALACTURONAN II

ABSTRACT

A heptasaccharide was released from the plant cell wall pectic polysaccharide rhamnogalacturonan II by selective acid hydrolysis of the glycosidic linkages of apiosyl residues. The heptasaccharide was purified to homogeneity by gel-filtration and anion-exchange chromatography. Some of the heptasaccharide molecules were found to be mono- and di-O-acetylated; the location of the acetyl esters was not determined. The heptasaccharide was found to have the following structure (where Rha = rhamnosyl, Ara = arabinosyl, Gal = galactosyl, Fuc = fucosyl, and AcA = aceryl [3-C-carboxy-5-deoxy-L-xylosyl] residues, and Api = apiose):



INTRODUCTION

Rhamnogalacturonan II (RG-II) is a complex pectic polysaccharide that is released from the cell walls of suspension-cultured sycamore cells (Acer pseudoplatanus) by the action of an endopolygalacturonase isolated from Colletotrichum lindemutheanum. RG-II is size-homogeneous, containing about 60 glycosyl residues[1].

RG-II yields at least 10 different monosaccharides upon acid hydrolysis, including the unusual sugar apiose (Api; 3-C-[hydroxymethyl]-D-glycero-tetrose), and the unusual sugar derivatives 2-O-methyl-fucose, and 2-O-methyl-xylose[1]. A new branched-chain sugar, aceric acid (AcA; 3-C-carboxy-5-deoxy-L-xylose) has also been identified as a component of RG-II (Chapter 1). The monosaccharide constituents of RG-II are interconnected by at least 15 different glycosidic linkages[1].

The structure of intact RG-II is too complex to be characterized by conventional methods. Therefore, selective acid hydrolysis of the apiosyl glycosidic linkages of RG-II was used to generate a mixture of smaller oligosaccharides. In this paper, we report the structural characterization of a heptasaccharide that was isolated after selective acid hydrolysis of RG-II.

EXPERIMENTAL

Isolation of RG-II. - RG-II was isolated from primary cell walls of suspension-cultured sycamore cells, as described in Chapter I.

Glycosyl-composition analysis. - The glycosyl-residue composition of the heptasaccharide was determined by g.l.c. and g.l.c.-m.s. analyses after hydrolysis in 2N trifluoroacetic acid (TFA) (2 h, 120°), reduction with sodium borodeuteride ($NaBD_4$), and acetylation[2].

Preparation of 2-O-methyl-L-fucose. - A sample of 2-O-methyl-L-fucose was prepared from methyl-α-L-fucopyranoside (Sigma Chemical Co.). Methyl-α-L-fucopyranoside (25 mg) was converted to the 3,4-di-O-isopropylidene derivative by the procedure of Liptak et al.[3], and then O-methylated[4,5]. Acid hydrolysis of this product (2-O-methyl-3,4-di-O-isopropylidene-α-methyl-L-fucopyranoside) yielded 2-O-methyl-L-fucose.

Glycosyl-linkage composition of the heptasaccharide. - Isolated RG-II heptasaccharide (0.5 mg) was dissolved in 0.4 mL dimethyl sulfoxide. Sodium dimethylsulfinyl anion (75 µL of 4M) was added, and the solution stirred for 2 h. Methyl iodide (75 µL) was added, and the solution stirred for another 2 h. Per-O-methylated carbohydrate was recovered by chromatography on a Sep-Pak C_{18} cartridge (Waters Associates), as described by Waeghe et al.[6] The glycosyl linkages of the neutral residues were determined by g.l.c.-m.s. after hydrolysis, reduction with $NaBD_4$, and acetylation. The glycosyl linkage of aceric acid was determined by a modified procedure. An aliquot (0.2 mg) of the per-O-methylated carbohydrate was hydrolyzed and reduced with $NaBD_4$, as described[7], deionized by passage through a column (2 mL) of Dowex 50 (H^+

form), and then lyophilized. This procedure yielded a mono-O-methyl lactone derivative of aceric acid (compound **2**, Figure 5). The mono-O-methyl lactone was carboxyl reduced, using the procedure of Jones and Albersheim[8]. This process yielded compound **4** (Figure 5), which was analyzed by g.l.c.-m.s. after per-O-acetylation (compound **5**, Figure 5).

Per-O-deuteriomethylation. - The heptasaccharide-alditol (3 mg) was dissolved in dimethyl sulfoxide (2 mL), and the solution was stirred for 4 h. Sodium dimethylsulfinyl anion (75 µL of 4M) was added, and the mixture was stirred overnight. Trideuteriomethyl iodide (20 µL; Stohler Isotopic Chemicals) was added, and the solution was stirred for 2 h. Sodium dimethylsulfinyl anion and trideuteriomethyl iodide were added twice more; 100 µL of trideuteriomethyl iodide was used for the final addition.

L.c.-m.s. separation of the mixture of per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols generated by degradation of the aceryl residue during per-O-deuteriomethylation of the heptasaccharide-alditol. - The mixture of per-O-deuteriomethylated oligomers, generated by partial degradation of the aceryl residue of the heptasaccharide, was separated by l.c.-m.s. on a Dupont Zorbax ODS column using a linear, 45-min gradient of 50 to 75% acetonitrile. All other l.c.-m.s. conditions were as described[9].

Preparation of per-O-alkylated oligosaccharide-alditols from per-O-deuteriomethylated fragments generated by degradation of the aceryl residue of the heptasaccharide-alditol. - The fractions containing material that eluted from the Dupont Zorbax ODS column with retention times between 4 and 10 min (Figure 7) were pooled and evaporated to

dryness under a stream of filtered air. The material was partially hydrolyzed by treatment with 88% formic acid for 2 h at 50°, and the formic acid was evaporated under a stream of filtered air. The sample was reduced with $NaBD_4$, deionized by passage through a column (2 mL) of Dowex 50 (H^+ form), lyophilized, and then dried overnight in a vacuum oven at 40°. The sample was dissolved in 0.5 mL of dimethyl sulfoxide. Sodium dimethylsulfinyl anion (100 µL of 4M) was added, and the solution was stirred for 4 h. Pentadeuterioethyl iodide (100 µL; Stohler Isotopic Chemicals) was added, and the mixture was stirred for 3 h. The per-O-alkylated oligosaccharide-alditols were recovered from the reaction mixture by chromatography on a Sep-Pak C_{18} cartridge, as described[6].

G.l.c.-m.s. of per-O-alkylated oligosaccharide-alditols derived from the heptasaccharide. - Partially-O-deuteriomethylated, partially-O-deuterioethylated, oligosaccharide-alditols were analyzed by g.l.c.-m.s. on a 15-m DB1 (J&W) capillary column (0.32 mm i.d.), as described[10]

Isolation of per-O-alkylated oligosaccharide-alditols [a], [d'], and [f] (Tables II and III) by l.c. - An aliquot (2 mg) of the per-O-alkylated oligosaccharide-alditols derived from the heptasaccharide was separated by l.c. on a Dupont Zorbax ODS column using a linear, 30-min gradient of 50 to 65% acetonitrile. Per-O-alkylated oligosaccharide-alditols were detected in the l.c. effluent by g.l.c.-m.s. (e.i.) analysis [7] of aliquots of the column fractions.

^{1}H-N.m.r. - ^{1}H-N.m.r. spectra were recorded on a Bruker WM-250 Fourier-transform n.m.r. spectrometer operated at 250 MHz. Water-soluble samples were lyophilized twice from deuterium oxide (99.7 atom %

D), then dissolved in deuterium oxide (99.997 atom % D). Chemical shifts of aqueous samples were assessed relative to internal dioxane (δ 3.70). Methylated samples were run in hexadeuterioacetone (99.997 % D) and chemical shifts were assessed relative to internal pentadeuterio acetone (δ 2.04).

RESULTS AND DISCUSSION

Isolation of an oligosaccharide released by partial acid hydrolysis of RG-II. - Purified RG-II (125 mg) was treated with 0.1N TFA for 24 h at 40°, and the extent of hydrolysis of glycosyl residues was determined as described[10]. Under these conditions, approximately 35% of the apiosyl glycosidic linkages and approximately 5% of the methyl fucosyl and the arabinofuranosyl glycosidic linkages were hydrolyzed. There was no detectable hydrolysis of any other glycosidic linkage.

The partially hydrolyzed RG-II was applied to a heated (65°) column (1.5 x 85 cm) of Biogel P-10 (200-400 mesh) that had been equilibrated in 50mM sodium acetate buffer (pH 5.2). Two major peaks of carbohydrate-containing material were resolved (Figure 1). The first peak had the same approximate elution volume as untreated RG-II. The second peak (shaded) was pooled, deionized by passage through a column (1 x 6 cm) of Dowex 50 (H^+ form), and then lyophilized.

Lyophilized peak II from the Biogel P-10 column was chromatographed on a column (1 x 2 cm) of QAE Sephadex Q-50-120 that had been equilibrated in 30mM NH_4HCO_3 (Figure 2). The column was washed with 15 bed volumes of 30mM NH_4HCO_3, then with 15 bed volumes of 250mM NH_4HCO_3. A peak of orcinol-positive[11] material (shaded region, Figure 2) was eluted from the column in the 30mM NH_4HCO_3 wash. This material, which did not give a positive response in the *m*-hydroxydiphenyl assay[12] for uronic acids, was the oligosaccharide characterized in the remainder of this paper. The 250 mM NH_4HCO_3 wash eluted material that gave positive responses in both the orcinol and the

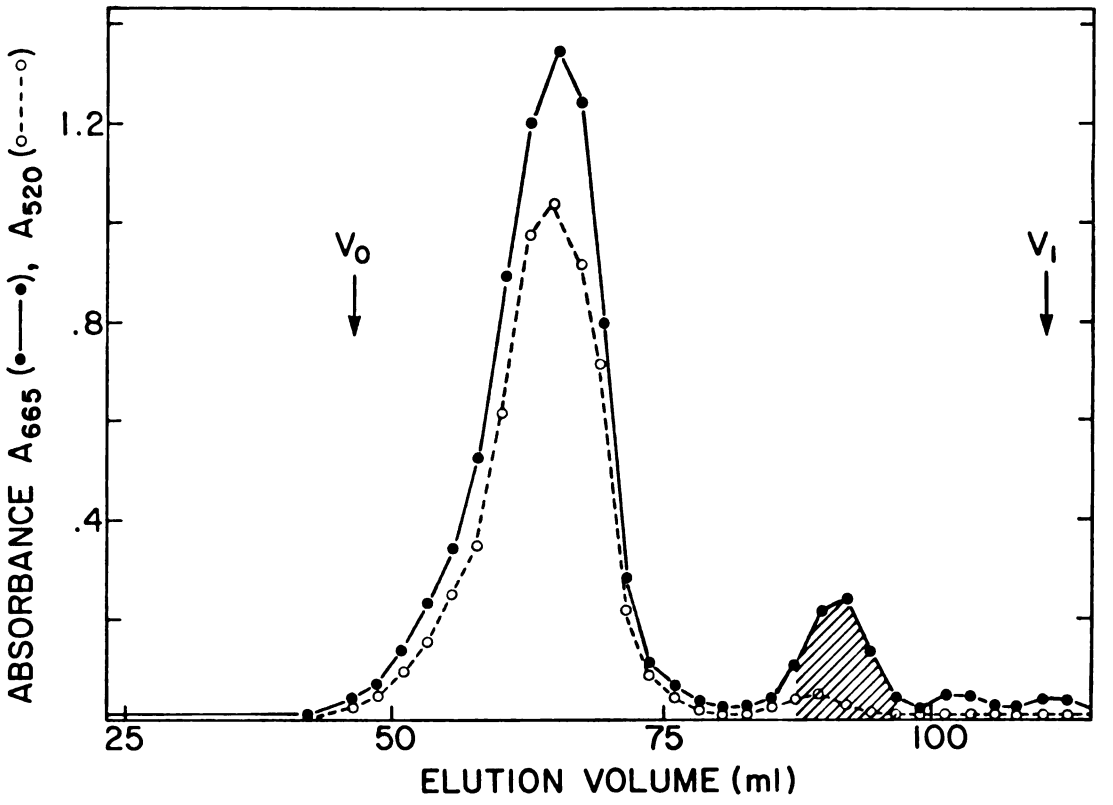


Figure 1. Chromatography of partially hydrolyzed RG-II on a heated (65° column (1.5 x 85 cm) of Biogel P-10 (200-400 mesh). The column was equilibrated in 50 mM sodium acetate buffer (pH 5.2). Collected fraction volume was 2.5 mL. Fractions were assayed for neutral-sugar content by the orcinol method[11] (A_{665}) and for uronic acid content by the m-hydroxydiphenyl method[12] (A_{520}). The shaded fractions were pooled for further purification.

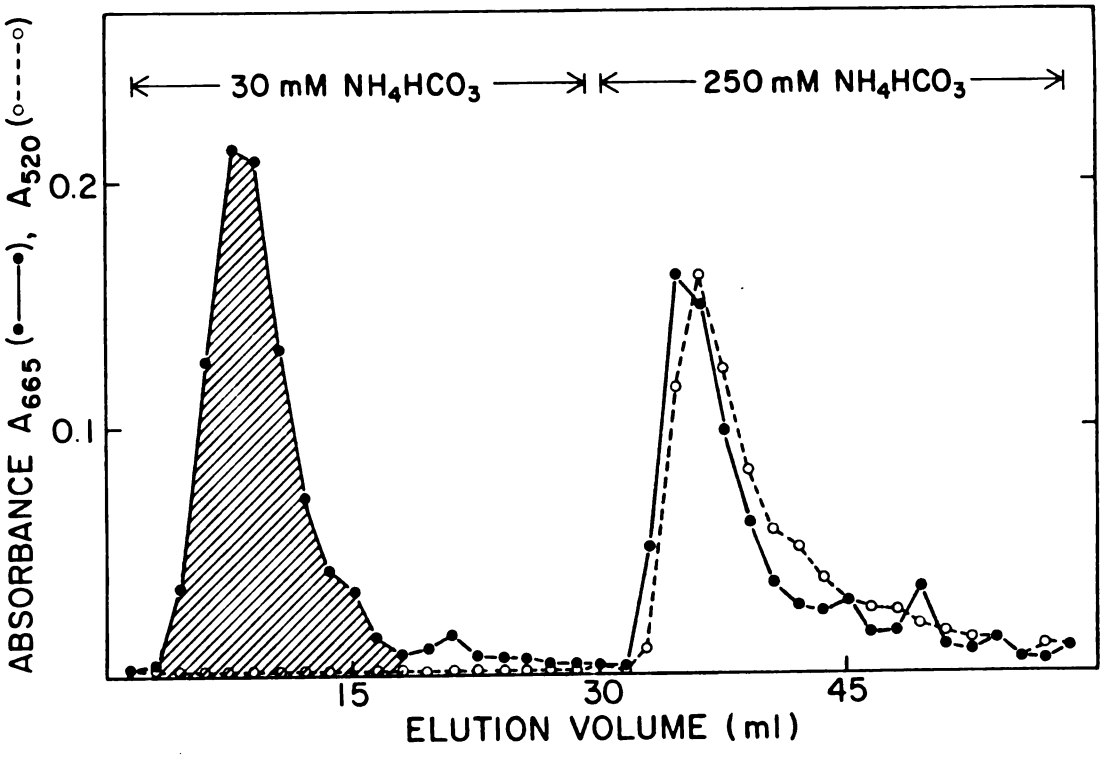


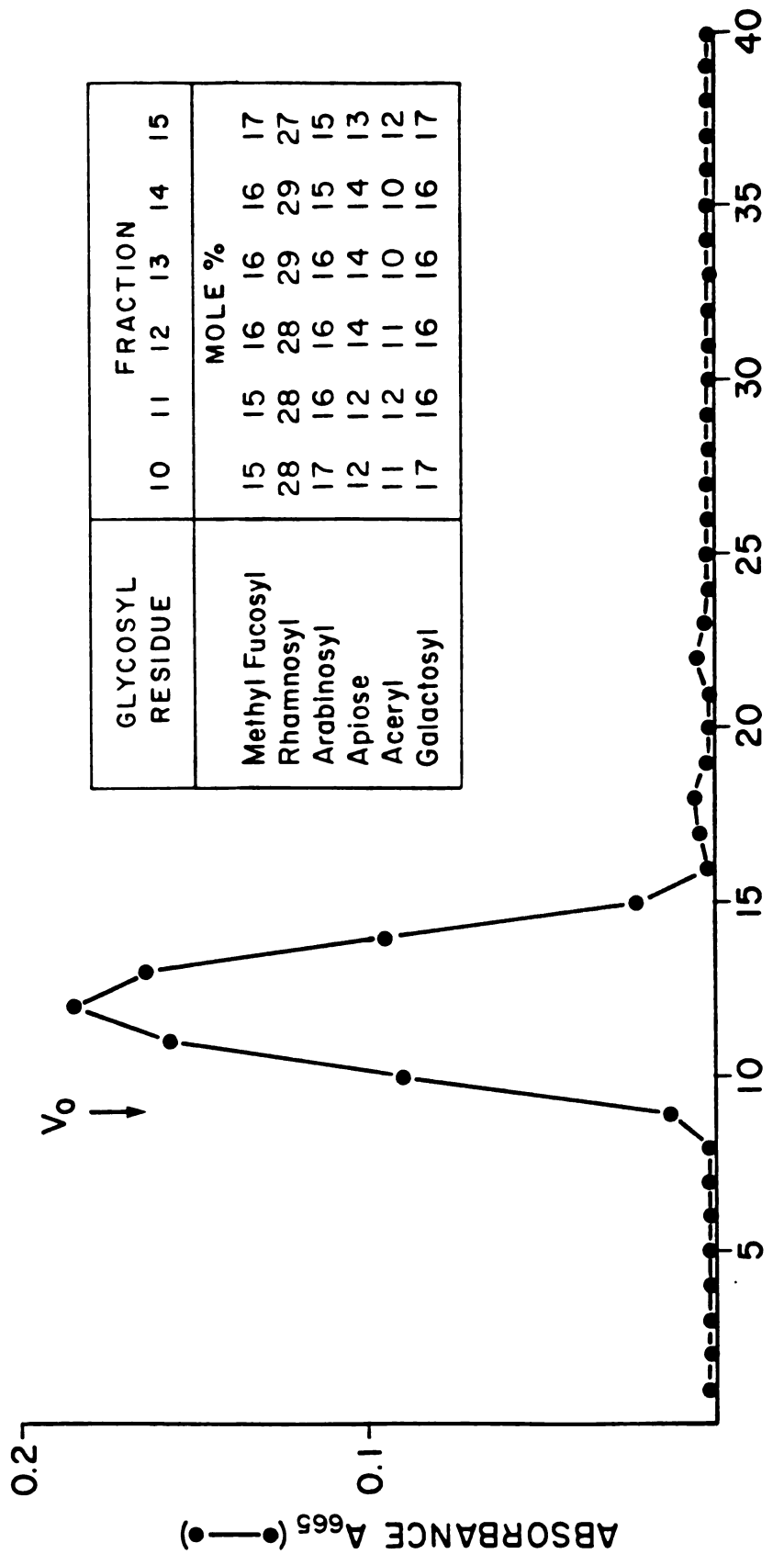
Figure 2. Chromatography, on QAE-Sephadex, of pooled material from the Biogel P-10 column. A column (1 x 2 cm) of QAE-Sephadex Q-50-120 was equilibrated with 30 mM NH_4HCO_3. After sample application, the column was washed with 15 bed volumes of 30 mM NH_4HCO_3 followed by 15 bed volumes of 250 mM NH_4HCO_3. Collected fraction volume was 1.5 mL. Fractions were assayed as described in the Figure 1 legend.

m-hydroxydiphenyl assays. This material has not been further characterized.

Determination of glycosyl composition and size of the isolated oligosaccharide. - The material that eluted from the QAE Sephadex column in 30mM NH_4HCO_3 (shaded region, Figure 2) was pooled and lyophilized. This material was chromatographed in water on a heated (65°) column (1 x 75 cm) of Biogel P-6, and eluted as a symmetrical, partially included peak (Figure 3). Aliquots were taken from the fractions comprising the peak, and the glycosyl-residue composition was determined by g.l.c. and g.l.c.-m.s. analysis of the products of reduction with borohydride, hydrolysis (2N TFA, 120°, 1 h), reduction with borodeuteride, and acetylation. The glycosyl-residue composition was constant across the peak (Figure 3, inset). The glycosyl composition data were consistent with those of a heptasaccharide. Only apiose was reducible before acid hydrolysis; this indicated that apiose was at the reducing end of the heptasaccharide, as would be anticipated from the partial hydrolysis results.

Fast atom bombardment-mass spectrometry (f.a.b.-m.s.) provided strong evidence that the isolated oligosaccharide was a heptasaccharide. The negative-ion f.a.b. mass spectrum contained ions corresponding to [M]-H (m/z 1056), [M + acetyl]-H (m/z 1097), and [M + 2 acetyl]-H (m/z 1139). The results were consistent with the glycosyl composition data, and also indicated the presence of one and of two acetyl esters on some of the heptasaccharide molecules.

The ^{1}H-n.m.r. spectrum of the underivatized heptasaccharide in D_2O (Figure 4) confirmed the presence of acetyl esters on some of the heptasaccharide molecules. The spectrum contained singlets, at δ 2.16, 2.12,



GLYCOSYL RESIDUE	FRACTION 10	11	12	13	14	15
	MOLE %					
Methyl Fucosyl	15	15	16	16	16	17
Rhamnosyl	28	28	28	29	29	27
Arabinosyl	17	16	16	16	15	15
Apiose	12	12	14	14	14	13
Aceryl	11	12	11	10	10	12
Galactosyl	17	16	16	16	16	17

Figure 3. Chromatography, on Biogel P-6, of the material that eluted from the QAE-Sephadex column in 30 mM NH_4HCO_3. A heated (65^o) column (1 x 75 cm) of Biogel P-6 (100-200 mesh) was equilibrated in H_2O. Collected fraction volume was 2.5 mL. Fractions were assayed for neutral-sugar content by the orcinol method[12]. The glycosyl-residue compositions of fractions 10-15 (inset) were determined as described in the text.

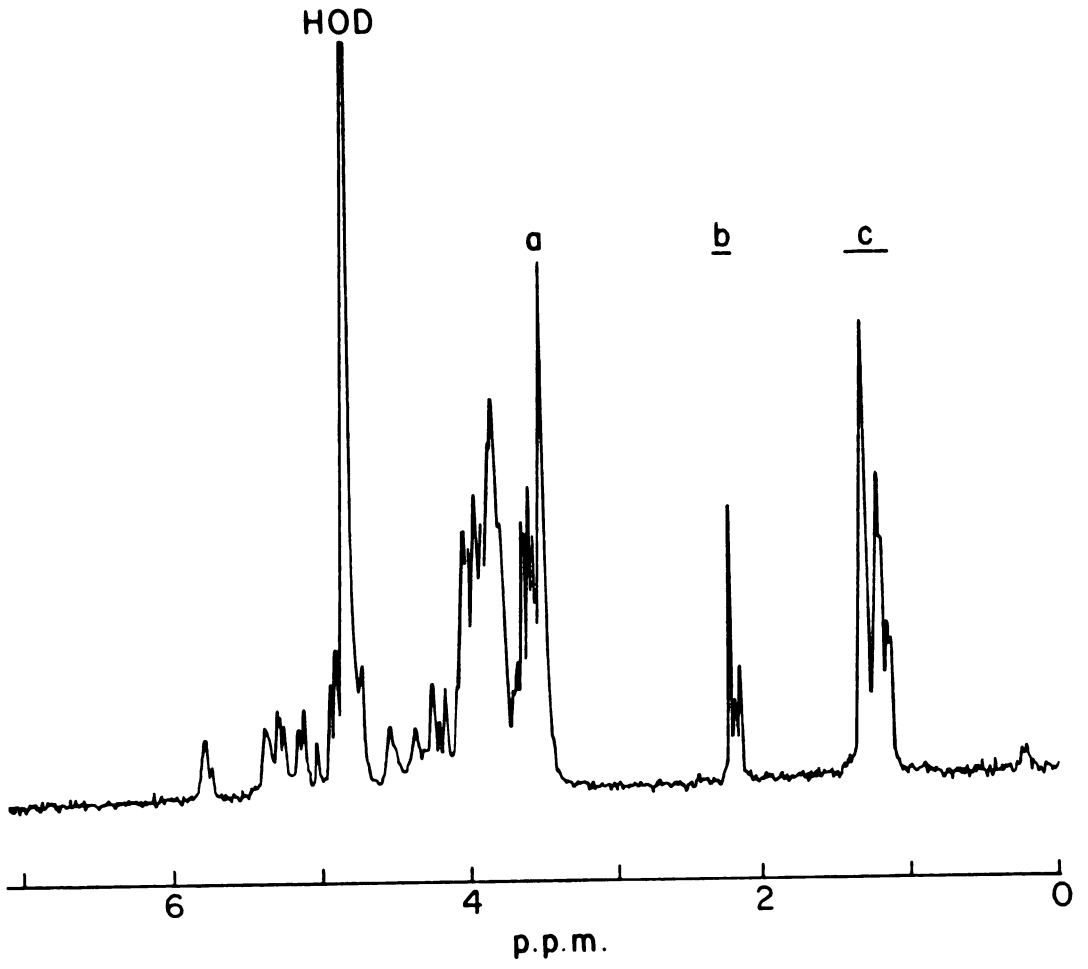


Figure 4. ^{1}H-N.m.r. spectrum of the underivatized heptasaccharide in D_2O. Chemical shifts were assessed relative to internal dioxane (δ 3.70). The resonances arising from the methoxy protons of the 2-O-methyl-fucosyl residue (a), the methyl protons of the O-acetyl esters (b), and the methyl protons of the deoxy-glycosyl residues (c) are indicated.

and 2.08, having typical chemical shifts for the methyl protons of O-acetyl esters[13]. Integration of these resonances showed that the O-acetyl esters were present in less than one mole per mole of heptasaccharide: the largest resonance (δ 2.16) corresponds to 0.6 mole acetyl ester per mole heptasaccharide; each of the other two resonances corresponds to less than 0.3 mole acetyl ester per mole heptasaccharide. It is likely that the O-acetyl esters were partially hydrolyzed by the acidic conditions used to release the heptasaccharide from RG-II. Acidic conditions are also known to cause migration of O-acetyl esters[14]. No attempt was made to determine the points of attachment of these substituents.

Determination of the absolute configurations of the glycosyl residues of the heptasaccharide. - The absolute configurations of all of the constituent sugars of the heptasaccharide except apiose were determined by the methods of Gerwig et al.[15,16] and Leontein et al.[17]. Both methods utilize solvolysis reactions in which the solvent is an optically active alcohol. When a pair of enantiomers is treated with a chiral reagent, the products are diastereomers, which can usually be resolved by g.l.c. The procedure of Gerwig et al. uses optically active butanol; the products of butanolysis are then converted into their trimethylsilyl derivatives, and analyzed by g.l.c.-m.s. The procedure of Leontein et al. is analogous, but the solvolysis reaction is run in optically active octanol, and the acetyl derivatives of the products are analyzed by g.l.c.

The galactosyl residue was shown to have the D absolute configuration, and the rhamnosyl and 2-O-methyl fucosyl residues L absolute configurations by the method of Gerwig et al. Authentic 2-O-methyl-L-

fucose was synthesized for use in these experiments, as described in the "Experimental" section. The arabinosyl residue was shown to have the L absolute configuration by the method of Leontein et al. Aceric acid was previously shown to have the L absolute configuration (Chapter 1). It was not possible to assign the absolute configuration of apiose by the method of Gerwig et al. or by the method of Leontein et al. because the diastereomeric products were not sufficiently resolved by g.l.c. The L optical isomer of apiose has not been found in nature[18]. Therefore, the apiose in the heptasaccharide has been assumed to have the D absolute configuration.

Determination of the glycosyl-linkage composition of the heptasaccharide. - The heptasaccharide was reduced with $NaBD_4$ to yield the heptasaccharide-alditol. The glycosyl-linkage composition of the heptasaccharide-alditol was determined by per-O-methylation[4,5], followed by hydrolysis, reduction with $NaBD_4$, and acetylation (Table I). The glycosyl linkage of aceric acid was determined by a modified procedure, which will be discussed separately. Prereduced apiose was recovered in low yield, presumably because some of the volatile tetra-O-methyl derivative was lost in the workup[20]. Aceric acid was partially degraded under the conditions used to methylate the heptasaccharide and consequently was also recovered in low yield. Degradation of aceric acid during the alkaline conditions of methylation became apparent from the results of experiments performed to determine the glycosyl-residue sequence of the heptasaccharide, as will be discussed later. Alkaline degradation of similar compounds has been reported[21].

TABLE I

GLYCOSYL-LINKAGE COMPOSITION OF THE HEPTASACCHARIDE

Glycosyl residue	Points of attachment of O-methyl groups	Deduced glycosidic linkage	Mole %[a]
2-O-methyl Fucosyl	2,3,4	terminal	17
Rhamnosyl	2,3,4	terminal	19
Rhamnosyl	2,4	3-linked	18
Arabinosyl	3,4	2-linked	19
Galactosyl	3,6	2,4-linked	15
Aceryl	3[b]	2-linked	6
Apiose	1,2,3,4	3[1]-linked (alditol)	5

[a] Calculated using "Effective Carbon Ratio"[20]. [b]Determined in a separate experiment, as described in text.

The glycosyl linkage of aceric acid was determined as follows. Per-O-methylation of the heptasaccharide, followed by hydrolysis, reduction, and acetylation, yielded a di-O-acetyl-mono-O-methyl lactone (compound **3**, Figure 5), which was identified by g.l.c.-m.s.(e.i.). Because compound **3** is cyclic, it was not possible to determine the position of the O-methyl group using the established fragmentation patterns of partially-O-methylated alditol acetates[20]. Therefore, compound **2** was reduced with sodium borohydride in borate buffer[8] to yield the acyclic, carboxyl-reduced alditol (compound **4**, Figure 5). This derivative was per-O-acetylated, yielding compound **5**, which was analyzed by g.l.c.-m.s. The e.i.-m.s. fragmentation pattern of compound **5** (Figure 6) clearly established that aceric acid was 2-linked in the heptasaccharide.

Determination of the sequence of glycosyl residues in the heptasaccharide. - The formation, resolution, and characterization of overlapping per-O-alkylated oligosaccharide-alditol fragments constitute a general procedure for determining the glycosyl-residue sequence of a complex carbohydrate[9,10] such as the heptasaccharide. The sequencing method was modified in the present study by using deuterated alkylating reagents in place of the normal (i.e., nondeuterated) alkylating reagents. The reason for this modification will become evident in the following discussion. The present study was complicated by degradation of the aceryl residue during per-O-deuteriomethylation of the heptasaccharide-alditol. The partial degradation of the aceryl residue made necessary an extra resolution step to recover the per-O-deuteriomethyl derivative of the intact heptasaccharide-alditol from the mixture of per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols that resulted from this degradation.

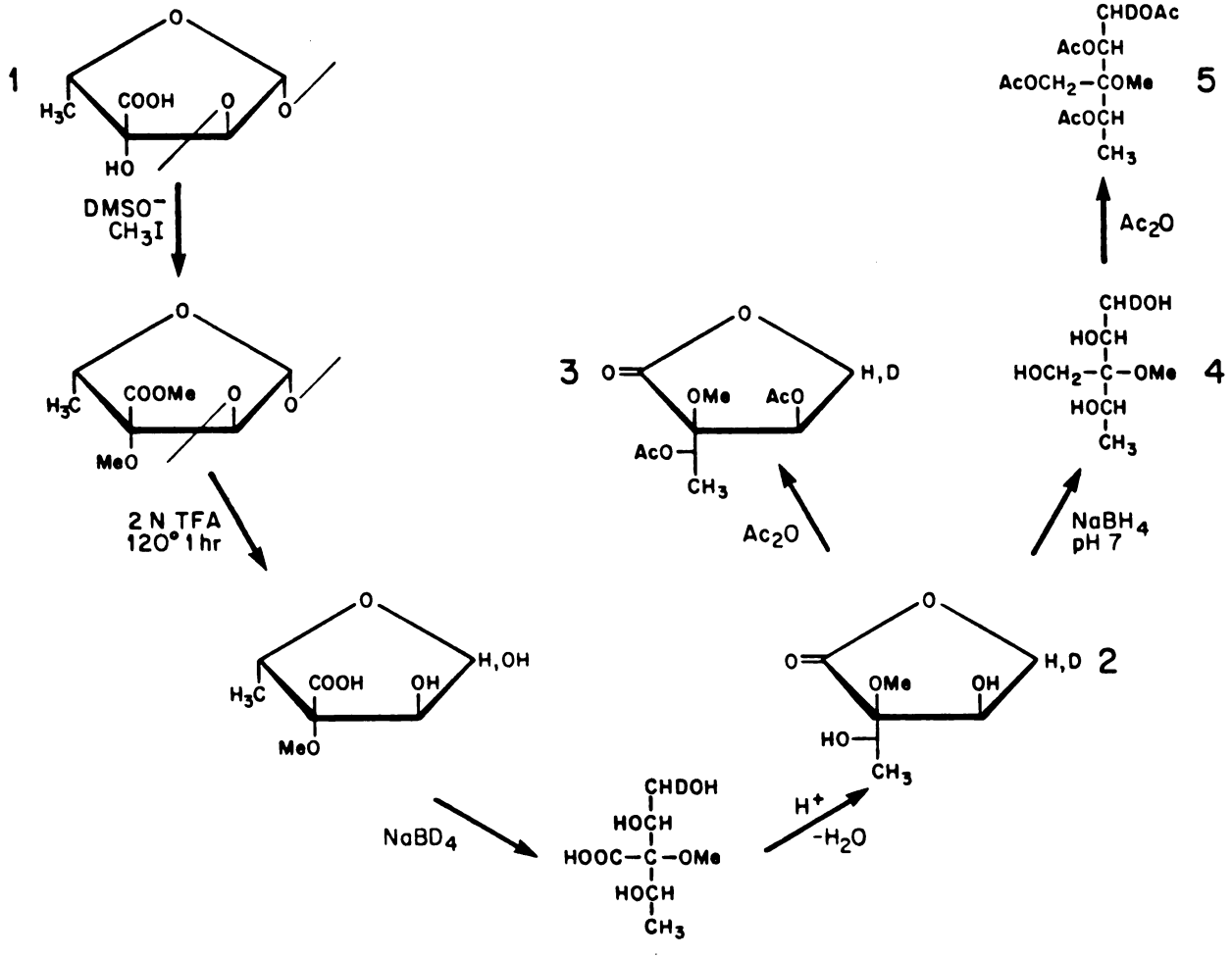


Figure 5. Reaction sequence used to determine the glycosyl linkage of the aceryl residue in the heptasaccharide-alditol. This scheme illustrates the derivatives obtained from the 2-linked aceryl residue (1).

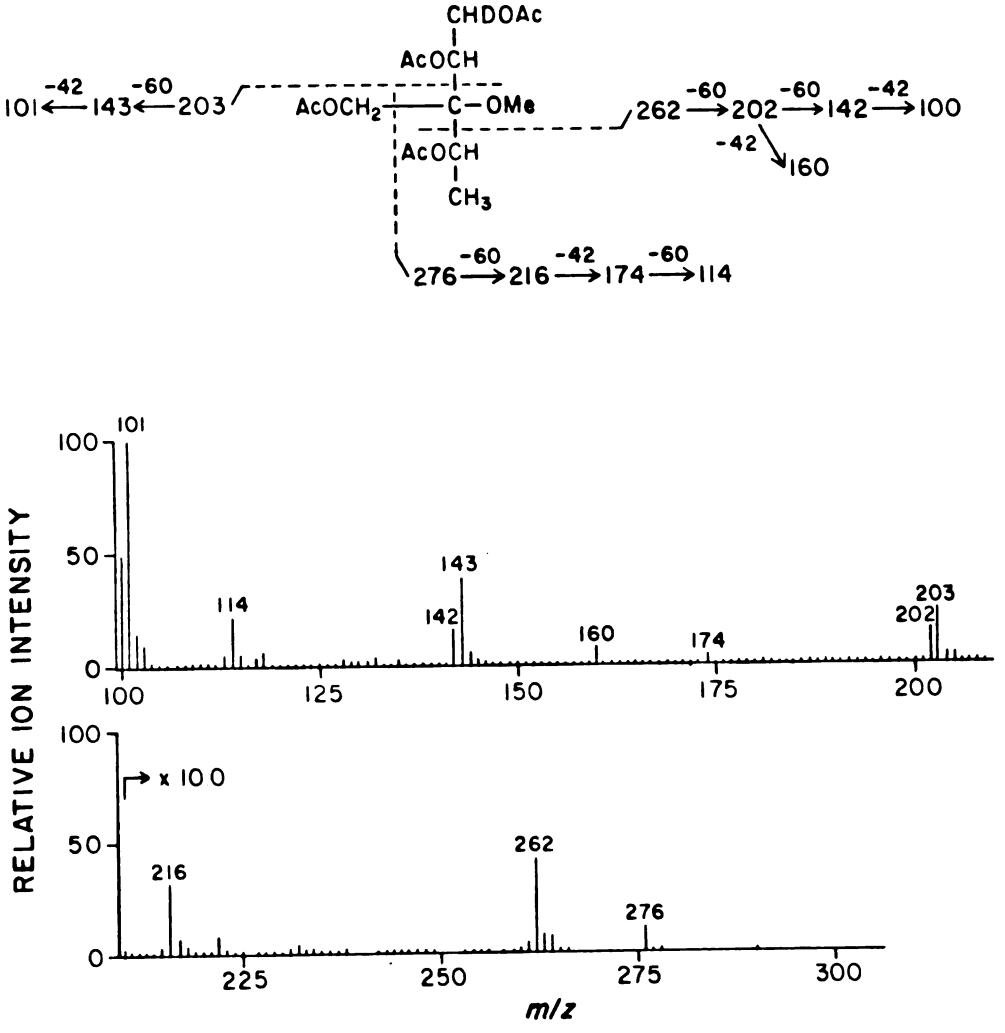


Figure 6. E.i. mass spectrum of compound **5**. Compound 5 is the per-O-acetylated branched-chain-deoxy-alditol produced from the aceryl residue of the heptasaccharide by the sequence of reactions outlined in Figure 5. Secondary fragment-ions formed by eliminations of ketene (42 mass units) or acetic acid (60 mass units) are indicated.

The first step of the sequencing procedure was to reduce the heptasaccharide with $NaBD_4$ to form the corresponding heptasaccharide-alditol, which was then per-O-deuteriomethylated . As mentioned above, the aceryl reside was partially degraded during per-O-deuteriomethylation of the heptasaccharide-alditol, generating a mixture of per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols. This mixture was partially resolved by l.c. on a Dupont Zorbax ODS column. The elution was monitored by chemical ionization (c.i.)-m.s. of 3% of the l.c. effluent (Figure 7). The remaining 97% of the l.c. effluent was collected as fractions (0.5 mL).

The material that eluted with a retention time of 11.3 min (shaded region, Figure 7) was found to be the per-O-deuteriomethyl derivative of the intact heptasaccharide-alditol. This was established by the results of hydrolysis, reduction, and acetylation of aliquots of the l.c. fractions comprising the peak (data not shown), and by f.a.b.-m.s., as will be discussed later. The fractions containing the per-O-deuteriomethylated heptasaccharide-alditol were combined and evaporated to dryness under a stream of filtered air, and saved for subsequent f.a.b.-m.s. and n.m.r. analyses.

Most features of the sequence of glycosyl residues in the heptasaccharide were elucidated by forming and characterizing overlapping per-O-alkylated oligosaccharide-alditol fragments of the heptasaccharide. The per-O-alkylated oligosaccharide-alditol fragments were produced by partial acid hydrolysis, reduction, and per-O-deuterioethylation of the mixture of per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols that resulted from degradation of the aceryl residue of the heptasaccharide (material with retention times

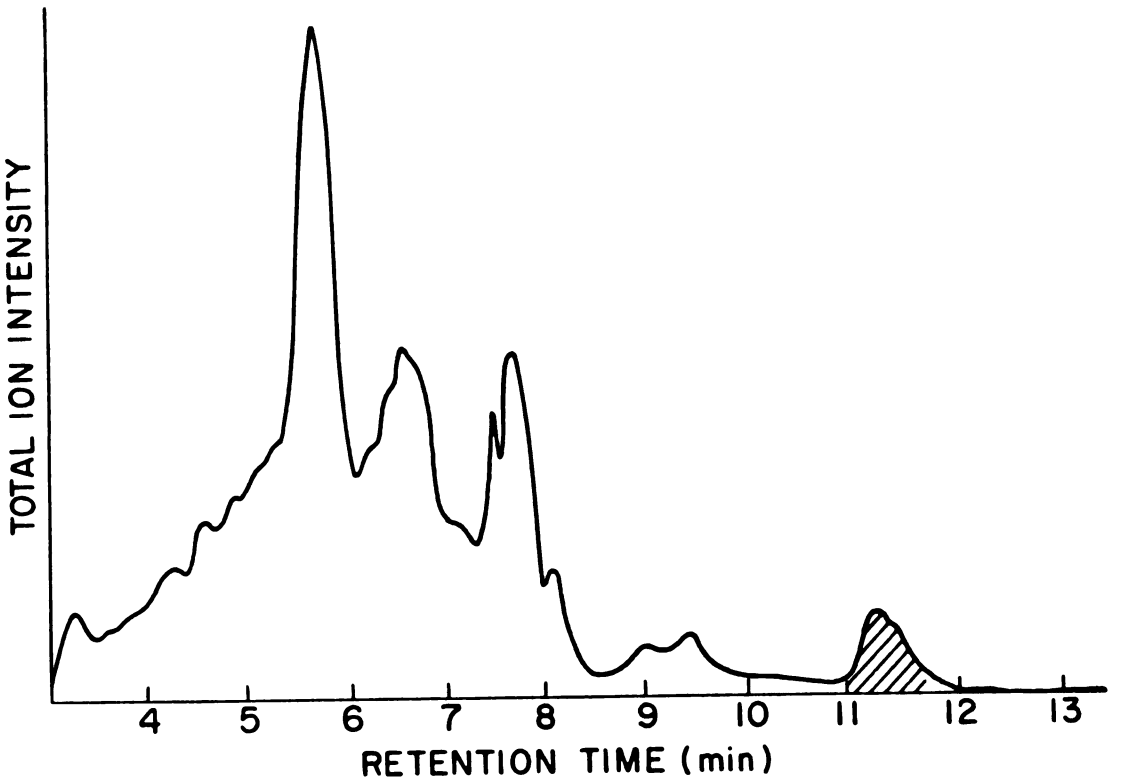


Figure 7. Reverse-phase l.c. elution profile of the per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols generated by degradation of the aceryl residue during per-O-deuteriomethylation of the heptasaccharide-alditol. The profile is the c.i.-m.s. total-ion response of 3% of the effluent from the l.c. column introduced directly into the source of the mass spectrometer. The mass spectrometer scanned from m/z 150 to 1000 once every 3 s.

between 4 and 10 min, Figure 7). These experiments are described below. The eluent fractions of the l.c. column (Figure 7) collected between 4 and 10 min were combined and evaporated to dryness. Preliminary analyses of this material were carried out as described[10] to determine conditions for further hydrolysis to form di-, tri-, and tetrasaccharide fragments. Treatment of the per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols in the 4 to 10 min eluent with 88% formic acid for 2 h at 50° was found to yield useful mixture of fragments.

The partially-O-deuteriomethylated oligosaccharide and oligosaccharide-alditol fragments generated by this partial hydrolysis were reduced with $NaBD_4$ and then per-O-deuterioethylated, yielding a mixture of partially-O-deuteriomethylated, partially-O-deuterioethylated oligosaccharide-alditols. The O-deuterioethyl groups of these derivatives mark the points of attachment of other residues in the intact heptasaccharide-alditol[10]. The term "per-O-alkylated oligosaccharide-alditols" will be used to refer to components of this mixture in the remainder of this paper.

An aliquot (200 µg) of the mixture of per-O-alkylated oligosaccharide-alditols was resolved and analyzed by g.l.c.-m.s. (Figure 8). The structures of the per-O-alkylated oligosaccharide-alditol fragments were elucidated from diagnostic ions in their e.i. mass spectra. This is illustrated in Figure 9, in which the e.i. mass spectrum of per-O-alkylated tetrasaccharide-alditol [f] is shown and the A- and J-series of fragment ions, which are typical of these molecules[22], are indicated. The A- and J-series of ions, in conjunction with the glycosyl-linkage composition of the intact heptasaccharide, established, with one

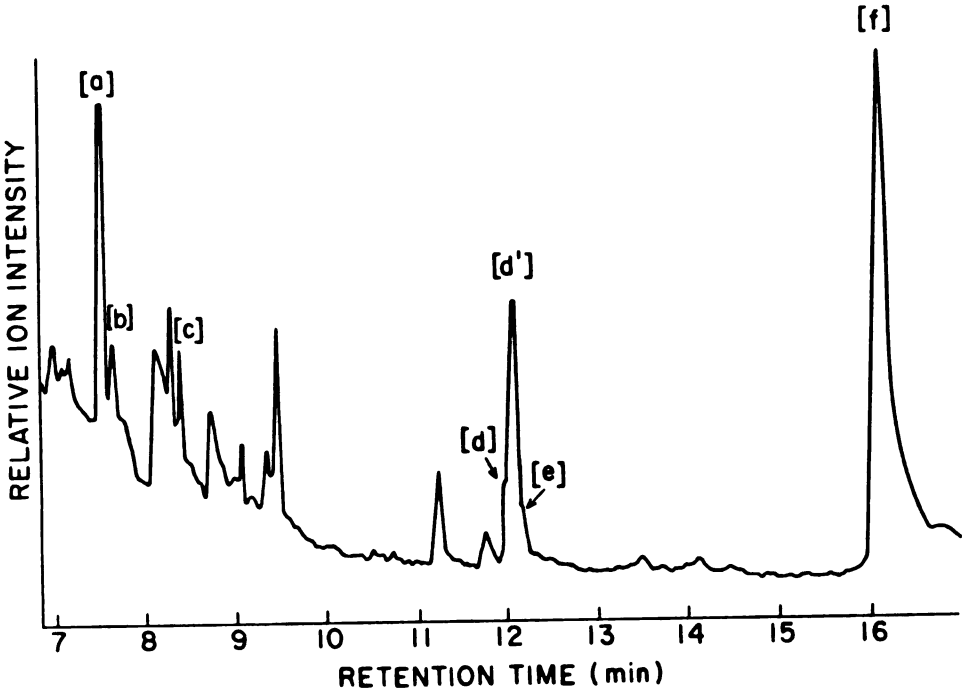


Figure 8. G.l.c.-m.s. elution profile of the per-O-alkylated oligosaccharide-alditols derived from the heptasaccharide. The per-O-alkylated oligosaccharide-alditols were produced by partial hydrolysis, reductions, and per-O-deuterioethylation of the oligosaccharide fragments that resulted from partial degradation of the aceryl residue during per-O-deuteriomethylation of the heptasaccharide-alditol. the mass spectrometer scanned from m/z 100 to 1000 every 1.5 s. Each per-O-alkylated fragment structurally characterized has been assigned a letter to show where it eluted from the g.l.c. column (Tables II, III, and IV and Figure 9).

Figure 9. E.i. mass spectrum of per-O-alkylated tetrasaccharide-alditol [f]. The g.l.c.-m.s. conditions are described in the Figure 8 legend.

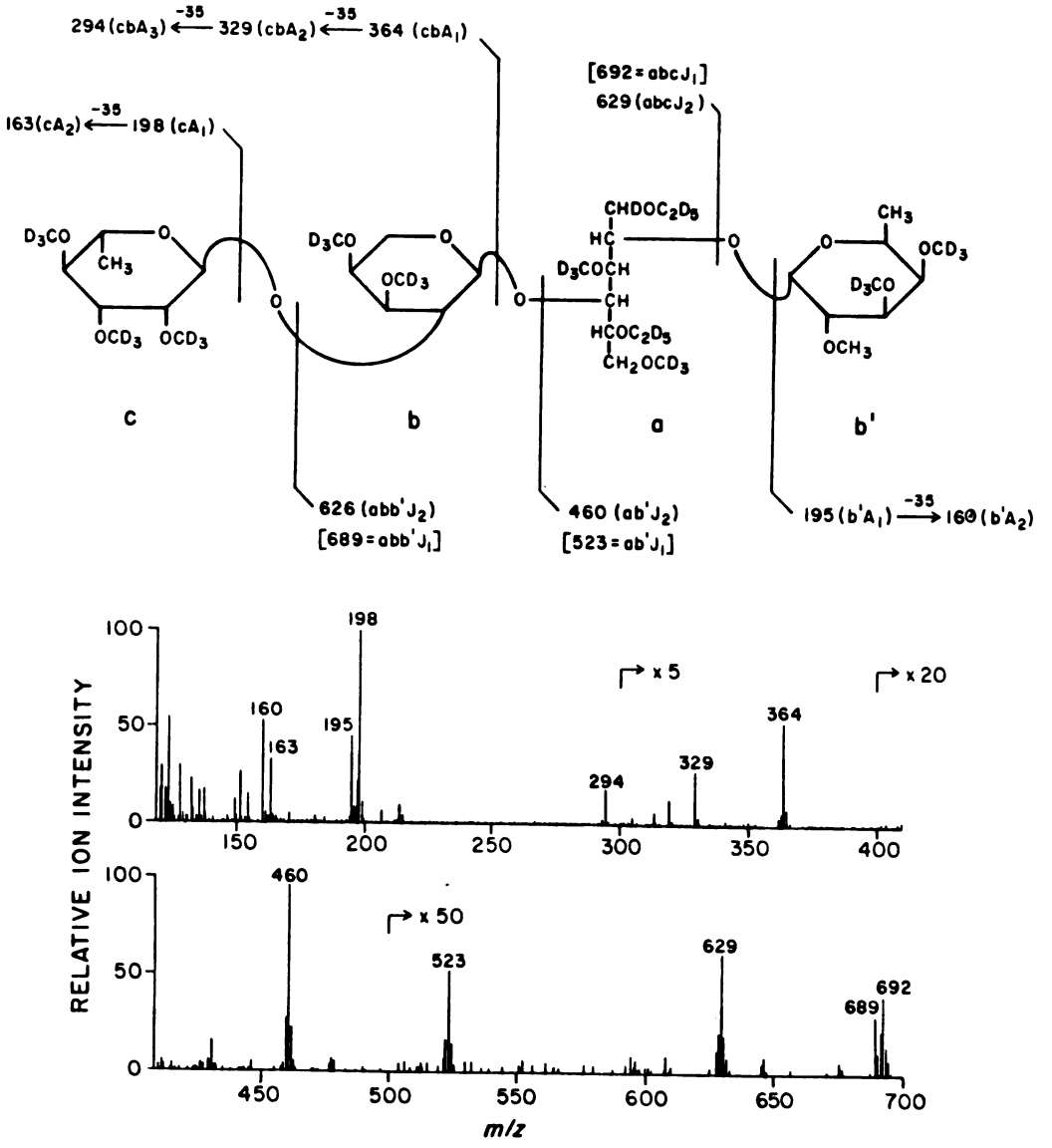

294 (cbA3) ←-35 329 (cbA2) ←-35 364 (cbA1)
163 (cA2) ←-35 198 (cA1)
[692 = abcJ1]
629 (abcJ2)
D3CO
CH3
OCD3
OCD3
D3CO
OCD3
CHDOC2D5
HC
D3COCH
CH
HCOC2D5
CH2OCD3
CH3
OCD3
D3CO
OCH3
c
b
a
b'
626 (abb'J2)
[689 = abb'J1]
460 (ab'J2)
[523 = ab'J1]
195 (b'A1) -35→ 160 (b'A2)
RELATIVE ION INTENSITY
100
50
0
198
160
163
195
294
329
364
x 5
x 20
150
200
250
300
350
400
460
523
629
689
692
x 50
450
500
550
600
650
700
m/z

Figure 9

exception, the sequence of glycosyl-residues in fragment [f]. The feature of the glycosyl-residue sequence of fragment [f] that could not be determined unambiguously from this analysis was the point of attachment to the 2,4-linked galactosyl residue of the 2-O-methyl fucosyl and the arabinosyl residues. This ambiguity was clarified in an experiment, described later.

The e.i. mass spectrum of fragment [f] also illustrates the value of using deuterated alkylating agents for the sequence analysis of the heptasaccharide. For example, the A_1 and A_2 ions with m/z 198 and m/z 163 could arise only from a terminal rhamnosyl residue. These ions were only distinguishable from the A_1 and A_2 ions arising from the terminal 2-O-methyl fucosyl residue (m/z 195 and m/z 160) because the heptasaccharide was per-O-deuteriomethylated. Had non-deuterated alkylating reagents been used, the A_1 and A_2 fragment ions of both of these residues would have had m/z 189 and m/z 154, respectively, and, thus, the terminal rhamnosyl and the terminal 2-O-methyl fucosyl residues could not have been distinguished.

Three per-O-alkylated disaccharide-alditols and three per-O-alkylated trisaccharide-alditols were identified by g.l.c.-m.s. analysis, in addition to per-O-alkylated tetrasaccharide alditol [f]. These per-O-alkylated oligosaccharide-alditols are shown in Tables II and III, and the pertinent e.i.-m.s. ions are listed. The use of deuterioethyl iodide in the second alkylation step resulted in a unique molecular weight for each of the per-O-alkylated oligosaccharide-alditols derived from the heptasaccharide. In fact, alkylation with deuterated reagents allowed the unequivocal identification from mass spectral data of the derivative of each glycosyl residue, simplifying the sequence analysis.

TABLE II

DIAGNOSTIC IONS FROM E.I.-M.S. OF PARTIALLY O-DEUTERIOMETHYLATED, PARTIALLY O-DEUTERIOETHYLATED DISACCHARIDE-ALDITOLS DERIVED FROM THE HEPTASACCHARIDE

Oligosaccharide	Fragment[a]	Electron-Impact Mass Spectral Fragment-Ions m/z (relative abundance)			
		aJ_2	aJ_1	bA_1	bA_2
Et→3Rha→3[1]Api	[a]	204(100)	283(26)	214(74)	163(21)
Rha→2Ara→	[b]	236(84)	299(28)	198(100)	163(33)
Et→4Gal→[b] (2↑Fuc, 2↑Me)	[c]	299(17)	362(4)	195(100)	160(67)

[a] See Figure 8 for location of fragment on g.l.c. trace. [b] The point of attachment of the 2-O-methyl fucosyl and arabinosyl residues to the 2,4-linked galactosyl residue was determined in a separate experiment.

TABLE III

DIAGNOSTIC IONS FROM E.I.-M.S. OF PARTIALLY O-DEUTERIOMETHYLATED, PARTIALLY O-DEUTERIOETHYLATED TRI- AND TETRASACCHARIDE-ALDITOLS DERIVED FROM THE HEPTASACCHARIDE

Oligosaccharide	Fragment	Electron-Impact Mass Spectral Fragment-Ions m/z (relative abundance)							
		aJ_2	aJ_1	abJ_2	abJ_1	cbA_1	cbA_2	cA_1	cA_2
Rha→2Ara→4Gal→[b] (2↑Et)	[d]	362(2)	299(13)	465(4)	528(1)	364(8)	329(2)	198(100)	163(37)
Rha→2Ara→4Gal→[b]	[d′]	346(2)	283(21)	449(2)	512(3)	364(6)	329(5)	198(100)	163(38)
		$ab'J_2$	$ab'J_1$	abJ_2	abJ_1	$b'A_1$	$b'A_2$	bA_1	bA_2
Ara→4Gal→[b] (2↑Et; 2↑Fuc 2↑Me)	[e]	460(4)	523(1)	465(3)	528(1)	195(100)	160(63)	200(63)	165(50)
		$ab'J_2$	$ab'J_1$	cbA_1	cbA_2	cA_1	cA_2	$b'A_1$	$b'A_2$
Rha→2Ara→4Gal→[b] (2↑Fuc 2↑Me)	[f]	460(5)	523(1)	364(9)	329(5)	198(100)	163(34)	195(43)	160(53)

[a] See Figure 8 for location of fragment on g.l.c. trace. [b] The point of attachment of the 2-O-methyl fucosyl and arabinosyl residues to the 2,4-linked galactosyl residue was determined in a separate experiment.

Fragments [d] and [d′] (Table III) had the same sequence of glycosyl residues, and differed only in the number of O-deuterioethyl groups on the alditol moiety. Each fragment had O-deuterioethyl groups at O-1 and O-5 of the alditol, showing that the galactosyl residue was reduced after the partial hydrolysis. Fragment [d] had a third O-deuterioethyl group, marking the point of attachment of the 2-O-methyl fucosyl residue in the original heptasaccharide. Fragment [d′] had an O-deuteriomethyl group instead of a third O-deuterioethyl group. The ethylation pattern indicated that fragment [d′] was derived from a hexasaccharide molecule in which the 2-O-methyl fucosyl residue was absent.

The presence of fragment [d′] in the mixture of per-O-alkylated oligosaccharide-alditols is explained by hydrolysis of some of the glycosidic linkages of 2-O-methyl fucosyl residues in the partial hydrolysis that released the heptasaccharide from RG-II. The rates of hydrolysis determined for apiosyl and 2-O-methyl fucosyl glycosidic linkages support this interpretation. Under the hydrolysis conditions used to release the heptasaccharide from RG-II, 35% of the glycosidic linkages of apiosyl residues were hydrolyzed, and 5% of the glycosidic linkages of 2-O-methyl fucosyl residues were also hydrolyzed. Hydrolysis of 2-O-methyl fucosyl glycosidic linkages would have generated hexasaccharide molecules lacking the 2-O-methyl fucosyl residue. Removal of the 2-O-methyl fucosyl residue from the heptasaccharide would have had little effect on its radius of gyration and on its charge-to-mass ratio. Therefore, it is not surprising that the hexasaccharide eluted with the intact heptasaccharide during chromatography on Biogel P-10, QAE Sephadex, and Biogel P-6.

Per-O-alkylated tetrasaccharide-alditol [f] (Table III and Figure 9) and per-O-alkylated disaccharide-alditol [a] (Table II) define the sequence of six of the glycosyl residues in the heptasaccharide. No per-O-alkylated oligosaccharide-alditol containing aceric acid was characterized in this experiment. This was as expected, because the per-O-alkylated alditols were generated from the mixture of per-O-deuteriomethylated oligosaccharides and oligosaccharide-alditols that resulted from degradation of the aceryl residue during per-O-deuteriomethylation of the heptasaccharide-alditol. The 2-linked aceryl residue was the only other residue in the intact heptasaccharide, and, therefore, had to be situated between the 2,4-linked galactosyl residue and the 3-linked rhamnosyl residue. This was confirmed by f.a.b.-m.s., as described later. Each of the per-O-alkylated oligosaccharide-alditol fragments identified was consistent with the deduced glycosyl residue sequence of the heptasaccharide.

Determination of the points of attachment of the 2-O-methyl fucosyl and arabinopyranosyl residues to the 2,4-linked galactosyl residue. - The point of attachment of the 2-O-methyl fucosyl and the arabinopyranosyl residues to the 2,4-linked galactosyl residue was determined by partial acid hydrolysis of the underivatized heptasaccharide, followed by glycosyl-linkage analysis of the hydrolyzate. This experiment is described below.

An aliquot (0.2 mg) of the underivatized heptasaccharide was treated with 2N TFA for 30 min at 80°. The extent of hydrolysis of the arabinosyl glycosidic linkage was determined as described[10] to be 34%, and the hydrolysis of the 2-O-methyl fucosyl glycosidic linkages was determined to be 63%.

An identical aliquot of the heptasaccharide was partially hydrolyzed using the same conditions. This sample was then reduced with $NaBD_4$, per-O-methylated, fully hydrolyzed (2N TFA, 1 h, 120°), reduced with $NaBD_4$, and per-O-acetylated. This treatment generated a mixture of terminal, 2-linked, 4-linked, and 2,4-linked galactosyl residues (Table IV). The 4-linked galactosyl residues listed in Table IV resulted from hydrolysis of the glycosidic linkage to O-2 of the 2,4-linked galactosyl residue, whereas the 2-linked galactosyl residues resulted from hydrolysis of the linkage to O-4 of the 2,4-linked galactosyl residue. The observed ratio (51:16) of 4-linked to 2-linked galactosyl residues established that the 2-O-methyl fucosyl residue was glycosidically linked to O-2, and the arabinosyl residue to O-4, of the 2,4-linked galactosyl residue.

F.a.b.-m.s. of the per-O-deuteriomethylated heptasaccharide-alditol. - The proposed sequence of glycosyl residues in the heptasaccharide was confirmed by positive-ion f.a.b.-m.s. of the per-O-deuteriomethylated heptasaccharide-alditol (Figure 10). This material had been isolated by l.c. (retention time of 11.3 min, Figure 7), as described above. Ions corresponding to $[M + H]^+$ (m/z 1349) and $[M + Na]^+$ (m/z 1371) were observed in the f.a.b. mass spectrum. The A series of fragment ions, resulting from fragmentation from the nonreducing termini of the molecule, was also observed, as indicated in Figure 10. These fragment ions confirmed the glycosyl residue sequence of the heptasaccharide, and provided definitive evidence for the location of the aceryl residue.

Determination of the ring forms of the glycosyl residues of the heptasaccharide. - Glycosyl residues can exist in furanoid (5-membered)

TABLE IV

DETERMINATION OF THE POINT OF ATTACHMENT OF THE ARABINOSYL- AND THE 2-O-METHYL FUCOSYL RESIDUES TO THE 2,4-LINKED GALACTOSYL RESIDUE

Glycosyl residue	Positions of O-methyl groups	Deduced glycosidic linkage	Observed area %	Predicted area %[a]	
				If 2-O-methyl fucosyl residue is linked to O-2 of the 2,4-linked galactosyl residue	If 2-O-methyl fucosyl residue is linked to O-4 of the 2,4-linked galactosyl residue
Galactosyl	2,3,4,6	Terminal	21		
Galactosyl	2,3,6	4-linked	51	42	13
Galactosyl	3,4,6	2-linked	16	13	42
Galactosyl	3,6	2,4-linked	11		

[a] Calculated, based on 34% hydrolysis of arabinosyl glycosidic linkages and 63% hydrolysis of 2-O-methyl fucosyl glycosidic linkages.

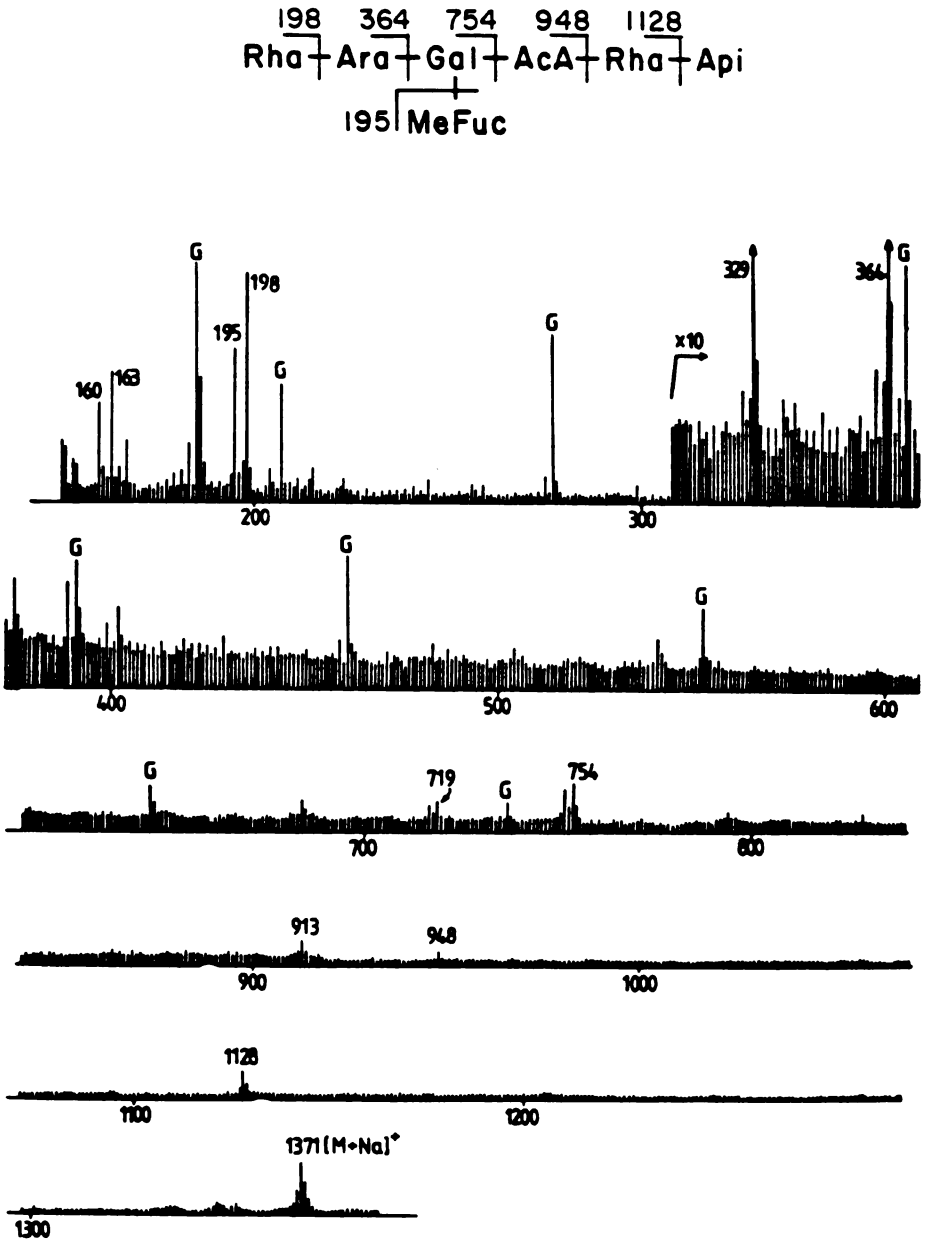


Figure 10. Positive-ion f.a.b. mass spectrum of the per-O-deuteriomethylated heptasaccharide-alditol. The signals labeled G represent glycerol polymers generated from the glycerol matrix on the f.a.b. target. The per-O-deuteriomethylated heptasaccharide-alditol was isolated by l.c. (retention time of 11.3 min, Figure 7).

or pyranoid (6-membered) rings. The apiosyl and aceryl residues of the heptasaccharide can form only furanoid rings. Methylation analysis of the heptasaccharide (Table I) established the pyranoid ring-forms of the 2-O-methyl fucosyl, arabinosyl, and the two rhamnosyl residues of the heptasaccharide because the partially-O-methylated alditol acetates derived from these residues had methyl groups at O-4.

The partially-O-methylated alditol acetate derived from the galactosyl residue of the heptasaccharide was 1,2,4,5-tetra-O-acetyl-3,6-di-O-methyl galactitol (Table I). This derivative could have arisen from a 2,4-linked galactopyranosyl residue or from a 2,5-linked galactofuranosyl residue. This ambiguity was resolved in the experiment that revealed the point of attachment of the arabinosyl and 2-O-methyl fucosyl residues to the galactosyl residue. In that experiment, the heptasaccharide was partially hydrolyzed, reduced, and per-O-methylated. The resulting fragments were then completely hydrolyzed, reduced, and acetylated. One of the partially O-methylated alditol acetates resulting from this series of reactions was 1,2,5-tri-O-acetyl-3,4,6-tri-O-methyl galactitol (Table IV). The methyl group at O-4 of this derivative established that the galactosyl residue in the heptasaccharide was in the pyranoid ring-form[23].

Determination of the anomeric configurations of the glycosyl residues of the heptasaccharide. - The anomeric configurations of the glycosyl residues of the heptasaccharide were determined by ^{1}H-n.m.r. The ^{1}H-n.m.r. spectrum of the per-O-deuteriomethylated heptasaccharide alditol (Figure 11) contained resonances from the six anomeric protons. Four of those resonances were assigned to specific glycosyl residues of the heptasaccharide with the assistance of the ^{1}H-n.m.r.

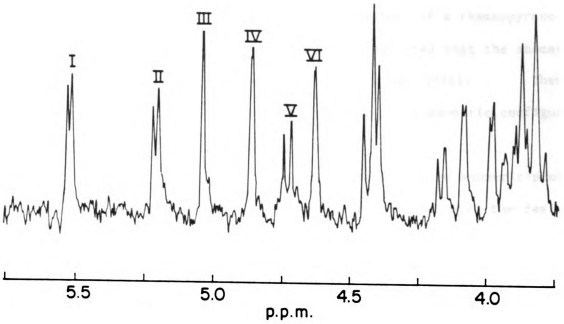


Figure 11. Spectral region from 3.75 to 5.75 p.p.m. of the ^{1}H-n.m.r. spectrum of the per-O-deuteriomethylated heptasaccharide-alditol in $CDCl_3$. Chemical shifts were assessed relative to internal hexadeuterio-acetone (δ 2.04). The resonances arising from the six anomeric protons have been designated I-IV (Table V). The per-O-deuteriomethylated heptasaccharide-alditol was isolated by l.c. (retention time of 11.3 min, Figure 7).

spectra of per-O-alkylated oligosaccharide-alditol fragments [a], [d'], and [f] (Table V). These fragments were isolated by l.c. of an aliquot (2 mg) of the mixture of per-O-alkylated oligosaccharide-alditols, and were detected by g.l.c.-m.s.(e.i.) of aliquots of the column fractions.

The ^{1}H-n.m.r. spectrum of per-O-alkylated disaccharide-alditol [a] contained an anomeric resonance, from the anomeric proton of the 3-linked rhamnosyl residue, at δ 4.70 with a 1 Hz coupling constant (Table V). A small (0-2 Hz) coupling constant is typical of a rhamnopyranosyl anomeric resonance[24], and the chemical shift indicated that the anomeric proton of the 3-linked rhamnopyranosyl residue was axial. Therefore, the 3-linked rhamnopyranosyl residue had the β anomeric configuration.

It should be noted that the chemical shift of the anomeric-proton resonance of a particular glycosyl residue may differ when the residue is situated in different oligosaccharide-alditols. For example, the chemical shift of the β-rhamnosyl residue was δ 4.64 in the per-O-deuteriomethylated heptasaccharide-alditol and δ 4.70 in fragment [a]. This was as expected, because the anomeric proton was not in an identical chemical environment in both oligosaccharide-alditols.

The ^{1}H-n.m.r. spectrum of per-O-alkylated trisaccharide-alditol [d'] contained a resonance at δ 5.34, with a coupling constant of less than 1 Hz, and another resonance at δ 4.61 with a 7 Hz coupling constant. These resonances arose from the anomeric protons of the terminal rhamnopyranosyl residue and the arabinopyranosyl residue. The resonance with a 7 Hz coupling constant could not have arisen from a

TABLE V

^{1}H-N.M.R. CHEMICAL SHIFTS AND COUPLING CONSTANTS OF THE ANOMERIC PROTONS OF THE PER-O-DEUTERIOMETHYLATED HEPTASACCHARIDE-ALDITOL AND OF DERIVED PER-O-ALKYLATED OLIGOSACCHARIDE-ALDITOLS

Per-O-deuteriomethylated heptasaccharide-alditol			Per-O-alkylated oligosaccharide-alditol			Assignment
Resonance[a]	Chemical shift[b] (p.p.m.)	$J_{1,2}$	Fragment[c]	Chemical shift[b] (p.p.m.)	$J_{1,2}$	
I	5.53	4 Hz				α-D-Galactosyl
II	5.21	4 Hz	[f]	5.21	4 Hz	α-L-(2-O-methyl)-Fucosyl
III	5.04	< 1 Hz	[d′] [f]	5.34 5.18	< 1 Hz < 1 Hz	α-L-Rhamnosyl (Terminal)
IV	4.87	2 Hz				β-L-Aceryl
V	4.73	7 Hz	[d′] [f]	4.61 4.70	7 Hz 7 Hz	α-L-Arabinosyl
VI	4.64	1 Hz	[a]	4.70	1 Hz	β-L-Rhamnosyl (3-linked)

[a]See Figure 11. [b]Assessed relative to internal pentadeuterioacetone at δ 2.04. [c]See Tables II and III.

rhamnopyranosyl residue because H-1 and H-2 of both α- and β-rhamnopyranosyl residues are in gauche orientation and exhibit coupling constants of less than 2 Hz[24]. Therefore, the resonance at δ 4.61 with a 7 Hz coupling constant was assigned to the 2-linked arabinopyranosyl residue. The chemical shift and coupling constant of this resonance indicated that the 2-linked arabinopyranosyl residue had the α anomeric configuration. The resonance at δ 5.34 with a coupling constant of less than 1 Hz must have arisen from the terminal rhamnopyranosyl residue. The chemical shift indicated that the anomeric proton of this residue was equatorial and that the residue had the α anomeric configuration.

The ^{1}H-n.m.r. spectrum of per-O-alkylated tetrasaccharide-alditol [f] contained resonances arising from the anomeric protons of the 2-O-methyl fucopyranosyl residue, the terminal rhamnopyranosyl residue, and the arabinopyranosyl residue. Two of the resonances corresponded to those identified in the spectrum of per-O-alkylated trisaccharide alditol [d']. Therefore, the third resonance (δ 5.21, with a 4 Hz coupling constant) must have arisen from the anomeric proton of the 2-O-methyl fucopyranosyl residue. The chemical shift and coupling constant of this resonance indicated that the 2-O-methyl fucopyranosyl residue had the α anomeric configuration[25].

The assignments of the 2-O-methyl fucopyranosyl and the arabinopyranosyl anomeric configurations were confirmed by a nuclear Overhauser effect (n.O.e.) difference n.m.r. experiment[26,27] that was performed with underivatized RG-II heptasaccharide in D_2O. The anomeric region of the ^{1}H-n.m.r. spectrum of the underivatized heptasaccharide (trace B, Figure 12) was more complex than the same region of the spectrum of the

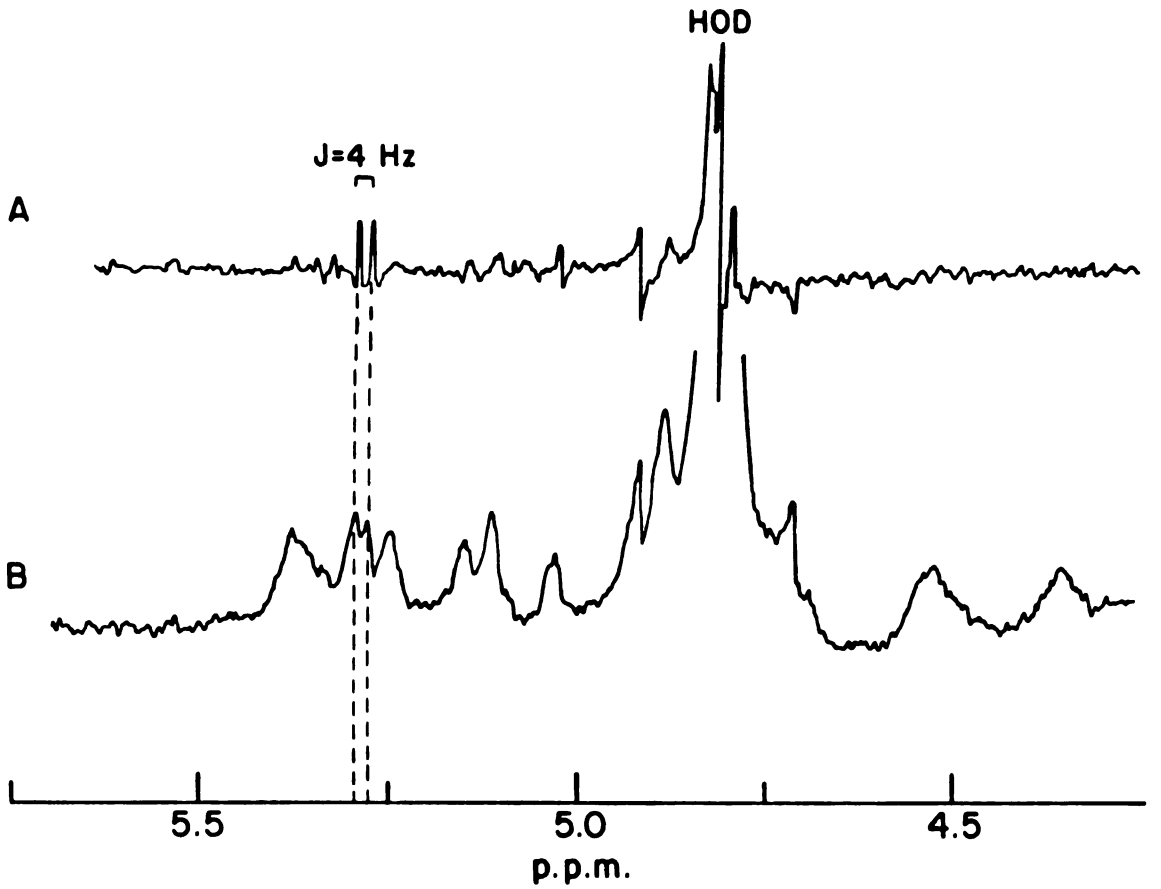


Figure 12. ^{1}H-N.m.r.-n.O.e. difference spectrum of the underivatized heptasaccharide in D_2O. Trace A: spectral region from 4.25 to 5.75 p.p.m. of an n.O.e. difference spectrum obtained with presaturation of the methoxy protons of the 2-O-methyl fucosyl residue ("a", Figure 4). Trace B: spectral region from 4.25 to 5.75 p.p.m. of the normal Fourier transform spectrum.

per-O-deuteriomethylated heptasaccharide-alditol. There were two causes for most of the increased complexity of the former spectrum: the heptasaccharide was not reduced to the heptasaccharide-alditol; therefore, some of the heptasaccharide molecules had α-apiose, while others had β-apiose, at their reducing termini. Some of the heptasaccharide molecules also contained O-acetyl esters. The inductive effect of an O-acetyl ester can cause the resonance of a non-anomeric proton to appear in the anomeric region of the spectrum.

The n.O.e. difference n.m.r. experiment was designed to discriminate between the anomeric protons of the 2-O-methyl fucosyl and arabinosyl residues by taking advantage of the O-methyl group on C-2 of the fucosyl residue. The experiment entailed presaturating the methoxy protons of the 2-O-methyl fucosyl residue (Figure 4, δ 3.5) of the underivatized heptasaccharide. Under the conditions of the experiment, any protons that were within approximately 4 Å of the presaturated protons would experience a slight (1-5%) enhancement in signal intensity. The n.O.e. enhancement is extremely distance-dependent, diminishing with increasing distance as R^{-6}[27]. Trace A in Figure 12 shows the anomeric region of the n.O.e. difference spectrum. The n.O.e. difference spectrum was generated by obtaining free induction decays with and without presaturation of the methoxy protons of the 2-O-methyl fucosyl residue and then performing a Fourier transformation on the difference between the free induction decays. The resonance at δ 5.3 with $J_{1,2}$ of 4 Hz, which was the only anomeric resonance with significant n.O.e. enhancement, was attributed to the anomeric proton of the 2-O-methyl fucosyl residue. The result confirmed the assignment of this resonance made on the basis of ^{1}H-n.m.r. analysis of fragments [d′] and [f].

Two resonances of the ^{1}H-n.m.r. spectrum of the per-O-deuteriomethylated heptasaccharide alditol (δ 5.53, 4 Hz coupling constant δ 4.87, 2 Hz coupling constant) were not assigned by ^{1}H-n.m.r. analysis of per-O-alkylated alditols [a], [d'] and [f]. These resonances were attributed to the anomeric protons of the 2,4-linked galactopyranosyl residue and the 2-linked acerofuranosyl residue because these were the only glycosyl residues whose anomeric resonances were not identified in the above experiments. A 2 Hz coupling constant is smaller than that usually observed with either anomeric configuration ofa galactopyranosyl residue, but is typical of that observed when H-1 and H-2 are trans in a furanoid ring[25,28]. Therefore, the resonance at δ 4.87 was attributed to the 2-linked acerofuranosyl residue in the β-configuration. The resonance at δ 5.53 with a 4 Hz coupling constant was in a typical range of chemical shift, with a coupling constant typical for a galactosyl residue with the α anomeric configuration. This resonance was assigned to a 2,4-linked-α-galactosyl residue. Thus, the anomeric configuration of each glycosyl residue of the heptasaccharide was determined.

GENERAL DISCUSSION

The heptasaccharide released from RG-II has the structure shown in the abstract figure. All aspects of the primary structure have been elucidated except the points of attachment of the O-acetyl groups. The heptasaccharide contains six different glycosyl residues, including aceric acid, a hitherto unobserved glycosyl residue. The complexity of this heptasaccharide is unprecedented among structures that have been elucidated for primary cell wall constituents; this finding reinforces our belief that RG-II is a well-defined, structurally complex polysaccharide.

The glycosyl-linkage composition of RG-II is compared to that of the heptasaccharide in Table VI. The value given in Table VI for the 2-linked aceryl residue is an estimate because this residue cannot be quantitated reliably after methylation. The glycosyl residues that comprise the heptasaccharide are present in approximately equal numbers in intact RG-II, and, with the exception of the 4-linked galactosyluronic acid residues, are the most abundant glycosyl residues of RG-II. The data in Table VI also reveal that a molecule of RG-II probably contains more than one heptasaccharide unit.

Three heptasaccharide units would account for approximately 30%, and four heptasaccharide units for more than 40% of the glycosyl residues of RG-II. Release of the heptasaccharide(s) does not change significantly the P-10 elution volume of the remainder of the molecule (unpublished results). This suggests that the radius of gyration of RG-II is not changed significantly by release of the heptasaccharide, and implies that the heptasaccharide units may be side chains on a 4-linked galactosyluronic acid-rich backbone. RG-II is believed to be linked to

TABLE VI

COMPARISON OF THE GLYCOSYL-LINKAGE COMPOSITION OF INTACT RG-II TO THAT OF THE HEPTASACCHARIDE

Glycosyl residue	Linkage	Number per RG-II molecule[a]	Number per heptasaccharide molecule
Galactosyluronic acid	4-linked	12	
	Terminal	2	
	3,4-linked	1	
Rhamnosyl	Terminal	4	1
	3-linked	4	1
	2,4-linked	1	
	3,4-linked	1	
	2,3,4-linked	1	
Arabinosyl	2-linked(pyranose)	4	1
	Terminal (furanose)	2	
Galactosyl	2,4-linked	5	1
	Terminal	2	
	3-linked	1	
Apiosyl	3^1-linked	4	1
Aceryl	2-linked	(4)[b]	1
2-O-Methyl Fucosyl	Terminal	3	1
Fucosyl	3-linked		
	3,4-linked	1	
2-O-Methyl Xylosyl	Terminal	2	
Glucosyluronic Acid	2-linked	2	
Glucosyl	4-linked	2	
	TOTAL	60	

[a] The values given for intact RG-II are calculated, using the data of Darvill *et al.*[1] [b]The value given for the 2-linked aceryl residue is an estimate because this residue cannot be quantitated reliably after methylation.

other cell wall polysaccharides by such a 4-linked galactosyluronic acid chain[1].

The structure of the heptasaccharide provides the first information about the locations of the 2-O-methyl fucosyl and apiosyl residues in the primary cell wall of dicots. Methyl fucose has been long recognized as a minor constituent of pectic polysaccharides[29,30], and was identified as a component of RG-II[1]. The heptasaccharide is the first plant cell wall oligosaccharide characterized that contains a 2-O-methyl fucosyl residue.

Apiose-containing polysaccharides from monocots have been partially characterized. The best-characterized of these is the apiogalacturonan of Lemna[31,32], which consists of a chain of 4-linked α-D-galactosyluronic acid residues, with apiobiose units attached to O-2 or O-3 of some of the galactosyluronic acid residues[31,32]. Zosterin, an apiose-containing polysaccharide isolated from Zosteraceae[33], is structurally more complex than the Lemna apiogalacturonan, but is degraded by pectinase to a molecule very similar to the Lemna apiogalacturonan[34]. The apiosyl residue in the RG-II heptasaccharide occurs in a chain with other neutral glycosyl residues, the first such example. It is possible that the apiosyl residues of RG-II are gycosidically linked to 4-linked galactosyluronic acid residues. If this is true, then RG-II could be structurally related to zosterin and to apiogalacturonans.

The heptasaccharide molecule has many hydrophobic functional groups: four of the glycosyl residues are deoxy sugars and contain methyl groups instead of hydroxymethyl groups at C-6; the arabinosyl residue is in the pyranoid ring-form and contains no hydroxymethyl group; and the heptasaccharide has one endogenous O-methyl group and at

least two endogenous O-acetyl esters. The hydrophobicity and structural complexity of the heptasaccharide are important because recent evidence indicates that the interactions between carbohydrates and their protein receptors are governed by hydrophobic bonding[35,36]. These properties of the heptasaccharide are made more significant by recent reports that oligosaccharide fragments of the cell walls function as regulatory molecules in plant-pathogen interactions and in plant growth and development[37,38].

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APPENDIX

APPENDIX

Crystal Structure of 3-C-Carboxy-5-Deoxy-L-Xylono-1,4-Lactone

This appendix contains the results of the X-ray crystallographic analysis of 3-C-carboxy-5-deoxy-L-xylono-1,4-lactone (compound **6**, Chapter I). Experimental details were included in the "Experimental" section of Chapter I.

TABLE AI

FRACTIONAL ATOMIC COORDINATES AND ANISOTROPIC THERMAL PARAMETERS (Ueq) FOR THE NONHYDROGEN ATOMS OF 3-C-CARBOXY-5-DEOXY-L-XYLONO-1,4-LACTONE·H_2O

Atom[a]	Fractional	Atomic	Coordinates[b]	Ueq[c]
	x	y	z	
C-1	175(10)	5385(13)	702(10)	29(4)
C-2	1610(8)	6378(14)	2031(9)	28(4)
C-3	3271(8)	5235(14)	1952(9)	25(4)
C-4	2868(8)	4590(13)	-108(9)	25(4)
O-5	901(6)	4597(12)	-630(6)	34(4)
O-1	-1376(6)	5281(13)	689(7)	40(3)
O-2	1203(6)	6483(12)	3826(6)	34(3)
O-3	4865(5)	6229(13)	2340(6)	28(3)
C-31	3377(8)	3649(14)	3270(9)	26(4)
O-32	4649(5)	3353(12)	4513(7)	38(3)
O-31	1901(6)	2663 13)	2930(7)	37(3)
C-41	3579(10)	5814(15)	-1403(10)	44(5)
O-1w	-1794(5)	4944(13)	4768(6)	35(3)

[a] The numbering system is shown in Figure 9, Chapter I. [b]Values are $x10^4$. Estimated standard deviations, in parentheses, refer to the least-significant digit. [c]Values are $A^2 \times 10^4$. Estimated standard deviations, in parentheses, refer to the least-significant digit.

TABLE AII

FRACTIONAL ATOMIC COORDINATES AND ISOTROPIC THERMAL PARAMETERS (U_{iso}) FOR THE HYDROGEN ATOMS OF 3-C-CARBOXY-5-DEOXY-L-XYLONO-1,4-LACTONE·H_2O

Atom[a]	Fractional Atomic Coordinates[b]			U_{iso}[c]
	x	y	z	
H-2	182(7)	760(10)	135(8)	4(2)
H-2o	190(7)	746(10)	427(8)	12(2)
H-3o	512(7)	641(10)	357(8)	5(2)
H-31o	195(8)	166(10)	339(8)	9(2)
H-4	332(7)	323(9)	-20(8)	1(2)
H-41a	305(7)	543(10)	-268(8)	3(2)
H-41b	487(8)	587(10)	-110(8)	4(2)
H-41c	358(7)	720(10)	-145(8)	9(2)
H-1ow	-106(7)	536(11)	392(8)	7(2)
H-2ow	-291(7)	528(10)	445(8)	12(2)

[a] The numbering system is shown in Figure 9, Chapter I. [b]Values are x10^3. Estimated standard deviations, in parentheses, refer to the least-significant digit. [c]Values are A^2 x 10^3. Estimated standard deviations, in parentheses, refer to the least-significant digit.

TABLE AIII

BOND LENGTHS OF 3-C-CARBOXY-5-DEOXY-L-XYLONO-1,4-LACTONE·H_2O

Bond[a]	Length[b]	Bond[a]	Length[b]
C-1--C-2	1.508(9)	C-1--O-5	1.357(9)
C-1--O-1	1.195(9)	C-2--C-3	1.545(11)
C-2--O-2	1.422(9)	C-3--C-4	1.560(10)
C-3--O-3	1.409(10)	C-3--C-31	1.517(13)
C-4--O-5	1.483(7)	C-4--C-41	1.499(12)
C-31--O-31	1.331(10)	C-31--O-32	1.215(7)
C-2--H-2	1.06(7)	C-4--H-4	1.08(7)
O-2--H-2o	.92(6)	O-3--H-3o	.90(6)
O-31--H31o	.81(7)	C-41--H-41a	.99(6)
C-41--H-41b	.98(6)	C-41--H-41c	1.03(7)
O-1w--H-1ow	.98(6)	O-1w--H-2ow	.88(5)

[a] The numbering system is shown in Figure 9, Chapter I.

[b] Values are in A. Estimated standard deviations, given in parentheses, refer to the least-significant digit.

TABLE AIV

BOND ANGLES FOR 3-C-CARBOXY-5-DEOXY-L-XYLONO-1,4-LACTONE·H_2O

Angle[a]	Degrees[b]	Angle[a]	Degrees[b]
O-5--C-1--C-2	108.9(6)	O-1--C-1--C-2	129.3(7)
O-1--C-1--O-5	121.8(6)	C-3--C-2--C-1	102.0(7)
O-2--C-2--C-1	111.0(6)	O-2--C-2--C-3	114.1(6)
C-4--C-3--C-2	101.8(5)	O-3--C-3--C-2	113.5(8)
O-3--C-3--C-4	110.4(6)	C-31--C-3--C-2	109.7(6)
C-31--C-3--C-4	111.0(8)	C-31--C3--O-3	110.2(5)
O-5--C-4--C-3	103.8(5)	C-41--C-4--C-3	114.0(7)
C-41--C-4--O-5	108.6(6)	C-4--O-5--C-1	111.4(5)
O-31--C-31--C-3	112.0(6)	O-32--C-31--C-3	123.5(8)
O-32--C-31--O-31	124.5(9)	H-2--C-2--C-3	104(3)
H-2--C-2--C-1	106(3)	H-4--C-4--C-3	110(3)
H-2--C-2--O-2	118(3)	H-4--C-4--C-41	111(3)
H-4--C-4--O-5	108(3)	H-30--O-3--C-3	106(4)
H-2o--O-2--C-2	99(4)	H-41a--C-41--C-4	107(4)
H-31o--O-31--C-31	117(4)	H-41b--C-41--H-41a	115(5)
H-41b--C-41--C-4	111(4)	H-41c--C-41--H-41a	105(5)
H-41c--C-41--C-4	129(4)	H-2ow--O-1w--H-1ow	114(5)
H-41c--C-41--H-41b	88(5)		

[a] The numbering system is shown in Figure 9, Chapter I. [b]Estimated standard deviations, given in parentheses, refer to the least-significant digit.

TABLE AV

OBSERVED AND CALCULATED STRUCTURE FACTORS FOR 3-C-CARBOXY-5-DEOXY-L-XYLONO-1,4-LACTONE·H_2O

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
2	0	0	57	57	1	5	0	116	119	0	1	1	371	392	-5	4	1	114	114	4	7	1	81	81
3	0	0	329	328	2	5	0	236	235	1	1	1	205	212	-4	4	1	41	41	5	7	1	55	52
4	0	0	123	122	3	5	0	32	31	2	1	1	26	33	-1	4	1	79	75	-2	8	1	62	49
5	0	0	142	143	4	5	0	63	66	3	1	1	185	187	0	4	1	125	124	-1	8	1	44	53
6	0	0	193	199	5	5	0	65	62	4	1	1	119	113	1	4	1	88	86	0	8	1	50	47
1	1	0	306	312	6	5	0	64	60	5	1	1	57	51	2	4	1	123	130	1	8	1	102	94
2	1	0	261	262	7	5	0	44	50	6	1	1	161	161	3	4	1	131	130	-8	0	2	53	52
3	1	0	163	160	0	6	0	136	134	7	1	1	96	91	4	4	1	68	64	-7	0	2	131	129
4	1	0	231	223	3	6	0	136	133	-7	2	1	42	41	5	4	1	104	103	-5	0	2	51	54
5	1	0	71	74	5	6	0	53	58	-6	2	1	38	33	6	4	1	86	80	-4	0	2	50	47
6	1	0	52	55	3	7	0	48	48	-5	2	1	115	113	-6	5	1	72	64	-3	0	2	221	216
7	1	0	52	49	0	8	0	81	81	-4	2	1	77	81	-5	5	1	43	36	-2	0	2	58	54
8	1	0	63	64	1	8	0	66	63	-3	2	1	35	38	-4	5	1	51	46	-1	0	2	52	51
0	2	0	450	447	3	8	0	58	59	-2	2	1	299	296	-3	5	1	153	154	0	0	2	114	116
1	2	0	212	209	-7	0	1	137	131	-1	2	1	205	200	-2	5	1	76	71	1	0	2	466	474
2	2	0	72	76	-6	0	1	61	60	0	2	1	385	375	-1	5	1	58	53	2	0	2	143	140
3	2	0	190	183	-5	0	1	123	117	1	2	1	329	337	0	5	1	57	58	3	0	2	153	152
4	2	0	91	94	-4	0	1	137	137	2	2	1	298	303	1	5	1	91	89	5	0	2	74	72
5	2	0	71	72	-3	0	1	252	253	3	2	1	93	92	3	5	1	104	105	6	0	2	126	125
6	2	0	76	74	-2	0	1	84	88	4	2	1	149	145	5	5	1	45	46	8	0	2	46	46
7	2	0	40	39	-1	0	1	456	492	5	2	1	122	126	-6	6	1	60	66	-8	1	2	48	42
8	2	0	70	61	1	0	1	92	98	6	2	1	114	118	-5	6	1	61	58	-7	1	2	116	121
1	3	0	244	236	2	0	1	47	43	-7	3	1	42	37	-4	6	1	87	87	-6	1	2	71	75
2	3	0	185	184	3	0	1	248	250	-3	3	1	186	175	-3	6	1	86	90	-5	1	2	130	132
3	3	0	100	97	4	0	1	43	44	-2	3	1	207	203	-2	6	1	82	83	-4	1	2	81	81
4	3	0	251	238	5	0	1	40	38	-1	3	1	238	225	-1	6	1	57	52	-3	1	2	51	50
5	3	0	94	96	7	0	1	66	57	0	3	1	293	288	0	6	1	67	73	-2	1	2	95	98
6	3	0	85	84	8	0	1	60	56	1	3	1	239	233	1	6	1	59	62	-1	1	2	233	244
7	3	0	76	77	-9	1	1	52	67	2	3	1	116	117	2	6	1	96	97	0	1	2	190	195
0	4	0	125	121	-8	1	1	61	63	3	3	1	246	248	4	6	1	91	99	1	1	2	111	105
1	4	0	238	240	-6	1	1	55	53	4	3	1	46	42	-5	7	1	43	35	2	1	2	63	64
2	4	0	173	171	-5	1	1	156	153	5	3	1	80	86	-3	7	1	47	53	3	1	2	129	129
3	4	0	30	31	-4	1	1	109	116	6	3	1	76	90	-1	7	1	106	107	4	1	2	166	165
4	4	0	33	36	-3	1	1	124	129	7	3	1	64	66	0	7	1	111	107	5	1	2	166	165
5	4	0	97	98	-2	1	1	325	317	-7	4	1	48	52	2	7	1	88	89	6	1	2	106	113
6	4	0	86	89	-1	1	1	245	251	-6	4	1	75	69	3	7	1	57	51	-7	2	2	120	121

TABLE AV (cont'd.)

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
-5	2	2	155	151	-6	5	2	58	61	-3	1	3	36	33	4	3	3	105	106	-2	0	4	106	108
-4	2	2	103	107	-5	5	2	74	80	-2	1	3	193	192	5	3	3	102	102	-1	0	4	147	149
-3	2	2	251	245	-4	5	2	109	116	-1	1	3	394	403	6	3	3	57	52	0	0	4	90	88
-2	2	2	252	253	-3	5	2	47	55	1	1	3	206	210	7	3	3	59	48	1	0	4	28	30
-1	2	2	350	346	-2	5	2	50	53	2	1	3	129	125	-5	4	3	135	141	2	0	4	33	39
0	2	2	392	400	-1	5	2	166	168	3	1	3	96	99	-4	4	3	68	69	4	0	4	109	113
1	2	2	259	255	0	5	2	89	90	4	1	3	62	70	-3	4	3	85	84	6	0	4	63	65
2	2	2	61	62	1	5	2	126	128	5	1	3	105	107	-2	4	3	55	61	-8	1	4	43	40
3	2	2	152	148	2	5	2	65	74	6	1	3	60	60	-1	4	3	118	115	-6	1	4	94	93
4	2	2	68	67	3	5	2	86	89	7	1	3	52	53	0	4	3	81	76	-5	1	4	51	48
5	2	2	35	39	4	5	2	127	136	-8	2	3	69	64	1	4	3	103	108	-4	1	4	44	50
7	2	2	53	43	-6	6	2	72	71	-7	2	3	73	68	2	4	3	121	125	-3	1	4	134	131
-7	3	2	65	55	-5	6	2	65	66	-6	2	3	38	36	3	4	3	54	57	-2	1	4	175	174
-6	3	2	52	50	-4	6	2	50	59	-4	2	3	122	123	4	4	3	40	36	-1	1	4	81	82
-5	3	2	95	94	-3	6	2	132	139	-3	2	3	111	110	6	4	3	39	30	0	1	4	116	117
-4	3	2	126	127	-2	6	2	102	100	-2	2	3	200	195	-4	5	3	82	82	1	1	4	100	103
-3	3	2	43	36	0	6	2	84	86	-1	2	3	137	136	-3	5	3	77	74	2	1	4	73	68
-2	3	2	124	120	3	6	2	106	109	0	2	3	149	144	-2	5	3	84	81	5	1	4	94	100
-1	3	2	236	228	-1	7	2	93	93	1	2	3	119	122	-1	5	3	135	127	6	1	4	48	42
0	3	2	188	186	0	7	2	60	58	2	2	3	147	147	0	5	3	150	156	7	1	4	45	40
1	3	2	114	113	1	7	2	79	76	3	2	3	144	143	1	5	3	41	43	-7	2	4	64	74
2	3	2	36	34	0	8	2	71	79	4	2	3	70	75	3	5	3	92	88	-6	2	4	54	57
3	3	2	29	23	2	8	2	83	75	5	2	3	87	83	4	5	3	46	37	-5	2	4	99	106
4	3	2	42	44	-9	0	3	54	49	6	2	3	126	120	6	5	3	66	62	-4	2	4	101	97
6	3	2	72	63	-6	0	3	71	75	7	2	3	46	61	-6	6	3	61	56	-3	2	4	38	33
-6	4	2	61	54	-3	0	3	84	79	-7	3	3	50	53	-5	6	3	72	71	-2	2	4	122	121
-5	4	2	85	86	-2	0	3	173	177	-6	3	3	103	108	-4	6	3	44	33	-1	2	4	166	159
-4	4	2	103	100	-1	0	3	86	85	-5	3	3	64	57	-3	6	3	69	70	0	2	4	85	82
-3	4	2	68	61	1	0	3	357	369	-4	3	3	118	130	3	6	3	45	44	1	2	4	110	110
-2	4	2	155	158	2	0	3	49	51	-3	3	3	89	85	-4	7	3	60	56	2	2	4	58	59
-1	4	2	80	78	3	0	3	73	73	-2	3	3	96	93	-1	7	3	68	63	3	2	4	52	56
0	4	2	164	161	5	0	3	113	112	-1	3	3	208	201	0	7	3	44	50	4	2	4	64	56
2	4	2	108	105	6	0	3	53	50	0	3	3	31	31	2	7	3	51	56	5	2	4	89	92
3	4	2	86	87	-7	1	3	101	106	1	3	3	77	75	-6	0	4	191	200	-8	3	4	53	54
4	4	2	66	69	-5	1	3	69	80	2	3	3	92	92	-4	0	4	127	123	-7	3	4	107	108
6	4	2	48	47	-4	1	3	106	106	3	3	3	62	62	-3	0	4	245	243	-6	3	4	62	64

TABLE AV (cont'd.)

H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC	H	K	L	10FO	10FC
-5	3	4	51	45	4	6	4	46	51	-4	3	5	74	74	-5	1	6	47	39	1	5	6	51	40
-4	3	4	66	71	-3	7	4	52	57	-3	3	5	97	95	-4	1	6	66	69	-1	6	6	47	48
-3	3	4	105	109	-2	7	4	49	53	-1	3	5	35	40	-3	1	6	102	96	1	6	6	57	52
-2	3	4	116	118	0	7	4	97	95	0	3	5	114	115	-2	1	6	76	77	-3	0	7	35	32
-1	3	4	109	106	2	7	4	54	49	1	3	5	114	118	-1	1	6	37	45	-2	0	7	116	116
0	3	4	71	72	-4	0	5	55	53	2	3	5	99	103	0	1	6	155	153	-1	0	7	71	68
1	3	4	83	82	-2	0	5	142	130	3	3	5	48	44	1	1	6	41	34	0	0	7	71	62
2	3	4	85	90	-1	0	5	149	155	5	3	5	41	44	2	1	6	49	48	1	0	7	65	62
3	3	4	76	84	2	0	5	109	106	-7	4	5	54	49	3	1	6	47	52	-6	1	7	68	65
4	3	4	111	110	5	0	5	108	115	-5	4	5	49	53	-7	2	6	58	64	-5	1	7	52	57
-7	4	4	54	52	-6	1	5	133	137	-4	4	5	61	62	-6	2	6	87	97	-3	1	7	41	51
-5	4	4	57	56	-5	1	5	116	120	-3	4	5	129	135	-3	2	6	67	62	-1	1	7	67	60
-4	4	4	178	185	-4	1	5	89	85	-1	4	5	111	112	-2	2	6	83	83	0	1	7	48	55
-2	4	4	81	81	-3	1	5	57	56	0	4	5	93	101	-1	2	6	113	107	2	1	7	57	64
-1	4	4	69	74	-2	1	5	89	86	1	4	5	63	72	0	2	6	61	61	-5	2	7	39	29
0	4	4	58	62	-1	1	5	188	186	3	4	5	129	127	1	2	6	105	108	-3	2	7	51	47
1	4	4	66	62	0	1	5	79	82	-6	5	5	58	58	3	2	6	41	44	-2	2	7	40	45
2	4	4	134	141	1	1	5	95	91	-5	5	5	48	56	4	2	6	46	42	-1	2	7	70	69
3	4	4	63	63	2	1	5	93	93	-2	5	5	48	43	-6	3	6	66	60	0	2	7	92	83
4	4	4	62	57	3	1	5	86	89	-1	5	5	69	72	-4	3	6	44	40	3	2	7	64	61
-7	5	4	54	58	4	1	5	43	43	0	5	5	54	54	-3	3	6	65	70	0	3	7	37	14
-4	5	4	53	50	-6	2	5	90	84	1	5	5	48	57	-1	3	6	41	47	1	3	7	53	50
-3	5	4	60	55	-5	2	5	51	50	2	5	5	48	52	0	3	6	76	77	-1	4	7	47	44
-2	5	4	57	60	-4	2	5	68	70	-5	6	5	53	57	1	3	6	51	49	-2	5	7	44	29
-1	5	4	103	113	-3	2	5	88	84	-2	7	5	48	33	2	3	6	45	39	0	5	7	72	65
0	5	4	62	75	-2	2	5	149	145	-1	7	5	78	84	3	3	6	44	43	-2	0	8	42	46
1	5	4	54	66	-1	2	5	141	144	-3	0	6	93	89	-4	4	6	59	57	-1	0	8	44	48
2	5	4	60	58	0	2	5	97	95	-2	0	6	129	126	-3	4	6	62	59	0	0	8	39	34
3	5	4	55	59	1	2	5	32	26	1	0	6	66	67	-2	4	6	81	79	0	1	8	79	71
4	5	4	56	53	3	2	5	76	76	2	0	6	97	99	-1	4	6	66	63	-1	2	8	58	51
-3	6	4	40	39	4	2	5	70	68	3	0	6	60	58	2	4	6	55	56	0	2	8	42	36
-1	6	4	73	69	-6	3	5	67	61	5	0	6	55	55	-5	5	6	61	56	-2	3	8	46	46
0	6	4	56	52	-5	3	5	74	77	-6	1	6	75	71	-3	5	6	53	63					