Carbohydrate Research, 121 (1983) 243–255
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE STRUCTURE OF THE REPEATING OLIGOSACCHARIDE UNIT OF THE PNEUMOCOCCAL CAPSULAR POLYSACCHARIDE TYPE 18C

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(Received March 18th, 1983; accepted for publication, April 12th, 1983)

ABSTRACT

The structure of the repeating oligosaccharide of the pneumococcal capsular polysaccharide type 18C has been investigated. The repeating oligosaccharide, isolated from an aqueous hydrofluoric acid hydrolyzate of the polysaccharide, was shown, by fast atom bombardment–mass spectrometry, to have a molecular weight of 928, and to contain an *O*-acetyl group and a glycerol residue. Information about the sequence in the per-*O*-methylated oligosaccharide was derived from electron-impact mass spectrometry. Supporting data were obtained from methylation analysis, periodate and chromium trioxide oxidations, and enzymic and acid hydrolyses of the oligosaccharide. These studies indicated that the polysaccharide consists of the following pentasaccharide repeating unit.

$$\begin{array}{l}\alpha\text{DGlc}p(1\rightarrow4)\alpha\text{DGal}p(1\rightarrow4)\beta\text{DGlc}p(1\rightarrow3)\text{-}\alpha\text{LRha}p(1\rightarrow1)\text{glycerol}\\ \qquad\qquad\qquad\qquad\quad 2\\ \qquad\qquad\qquad\qquad\quad \uparrow\\ \qquad\qquad\qquad\qquad\quad 1\\ \qquad\qquad\qquad\quad \alpha\text{DGlc}p\\ \qquad\qquad\qquad\qquad\quad 3\\ \qquad\qquad\qquad\qquad\quad |\\ \qquad\qquad\qquad\qquad\quad \text{OAc}\end{array}$$

INTRODUCTION

Historically, interest in the pneumococcal polysaccharides can be traced to the discovery of a mucin-like substance on cells of *Diplococcus pneumoniae* by Friedländer[1] in 1883. Since then, chemical and immunochemical investigations have resulted in mixture formulations of these capsular polysaccharides for prophylaxis of pneumococcal pneumonia[2]. The pneumococcal vaccine used at present in the U.S.A. is a broad mixture of capsular polysaccharide antigens of 14 different serotypes, including type 18C (Danish nomenclature). Immunochemical studies of these compounds have led to structural investigations of the capsular

0008-6215/83/$ 03.00

polysaccharides. If increased protection against disease could be obtained through modification of the composition of the vaccine, an intimate knowledge of the chemical structure of these polysaccharides would be of immense importance.

Pneumococcal capsular polysaccharide type 18C was first isolated by Brown[3] in 1939. Heidelberger and co-workers determined the composition and relative mole ratios of the type 18C polysaccharide to be three mol of D-glucose, one mol of D-galactose, one mol of L-rhamnose, one mol of glycerol, one mol of *O*-acetyl, and one mol of phosphate[4–6]. Herein, we report some structural studies of this capsular polysaccharide, and propose a structure for the repeating oligosaccharide unit thereof.

EXPERIMENTAL

Reagents. — The various chemicals used in this work were A.C.S-certified, reagent-grade materials, or the equivalent, and were used without purification. Dimethyl sulfoxide (Sigma Chemical Company, St. Louis, MO) was dried by distillation from calcium hydride, and stored over molecular sieves. Pyridine and acetic anhydride (Fisher Scientific Co., Fair Lawn, NJ) were redistilled, and stored over molecular sieves. Hydrofluoric acid (60%) was prepared by distillation of 60 mL of hydrogen fluoride (Matheson, Dorsey, MD) into a Teflon vessel (HF-reaction apparatus type II, Peninsula Laboratories, Inc., San Carlos, CA) containing 40 mL of water, and the resulting solution was stored at −20°.

Isolation of the repeating oligosaccharide unit. — The polysaccharide (74 mg) was depolymerized by hydrolysis for 3.5 h with hydrofluoric acid (60%) by the method previously described[7,8]. After neutralization of the acid, the hydrolyzate was delivered to a column (2 by 200 cm) of Bio-gel P-2, 400 mesh (Bio-Rad Laboratories, Richmond, CA)[9]. The column was eluted with water, 4.6-mL fractions were collected, and portions (10 μL) of the fractions were assayed for the presence of carbohydrate by using the anthrone–sulfuric acid reagent[10].

Periodate oxidation. — Samples (2–4 mg) of the oligosaccharide were each dissolved in 1 mL of 0.02M sodium metaperiodate, pH 4.5, and kept in the dark for 18 h at 4°. Ethylene glycol was then added to decompose the excess of periodate, and the resulting mixtures were kept in the dark for an additional 4 h at 4°, diluted with ethanol (1 mL), filtered, and the filtrates evaporated to dryness under diminished pressure. The oxidized carbohydrates were reduced as described later. After oxidation, reduction, and hydrolysis with 0.5M hydrochloric acid for 18 h at 100°, the samples were analyzed for neutral sugars by the method of Boykins and Liu[11], and, in a separate experiment, subjected to methylation analysis.

Methylation analyses. — Potassium methylsulfinylmethide (dimsyl potassium) was prepared as described[12,13], and used for permethylation of samples[13–15]. Methylation analyses of the samples were performed as previously described[16,17].

Gas–liquid chromatography–mass spectrometry. — Mass spectra were recorded with an LKB 2091 gas chromatograph–mass spectrometer equipped with an

LKB 2130 data system (DEC PDP 11/34 minicomputer employing the RT-11 disk-based, operating system). Electron-impact (e.i.) mass spectra were scanned from samples introduced *via* a 25-m, fused-silica capillary column, wall-coated with SE-30 as the stationary phase (Hewlett–Packard). A 2-μL sample was injected onto the column through a glass-lined splitter at 250°, set at a 1:20 split ratio. The column temperature was initially set at 80° for a 2-min isothermal period, and then elevated at 8°/min to a final temperature of 250°. The linear velocity of helium through the column was 40 cm/s. The single-stage, jet separator was at 225°, and the ion-source temperature was 180°; the ionizing potential was 70 eV, and the ionizing current was 50 μA.

High-field, mass spectrometry. — Mass spectra were recorded with a Kratos MS-50 mass spectrometer fitted with a high-field magnet[18] and equipped with a Kratos DS-55 data system (Data General Nova 4X minicomputer, employing the RDOS operating system). Electron-impact mass spectra were scanned from samples introduced directly into the ion source on a ceramic-tipped, sample-probe shaft (Vacumetrics, Inc., Ventura, CA). The ion-source temperature was initially set at 100°, and then raised to 200°; mass spectra were scanned every 25 s. The accelerating potential was 8 kV, the ionizing potential was 70 eV, and the ionizing current was 400 μA.

Fast-atom bombardment (f.a.b.), mass spectra[19,20] were scanned from samples introduced directly into the ion source as colloidal suspensions in a glycerol sample-matrix[21] (Alfa Products, Danvers, MA) placed on a gold-plated copper, sample probe-tip; bombardment was with a 40-μA beam of 9 keV xenon (Matheson, Dorsey, MD) fast atoms generated in a Saddle-Field neutral-beam gun[22] (Ion Tech Ltd., Teddington, England). Some mass-spectral feature-enhancements were observed by addition to the sample matrix of a sodium chloride–potassium chloride mixture[23,24] or of small organic compounds[20,25].

Anomeric analysis. — The presence of β-anomeric linkages was determined by the method of Hoffman *et al.*[26], followed by analysis for neutral sugars[11].

Individual samples (1–2 mg) of the oligosaccharide were also subjected to enzymic digestion[27] with β-D-glucosidase at pH 4.8, for 3 h at 37°, and the digests analyzed for the presence of liberated glucose. The samples were then reduced with sodium borohydride, the products hydrolyzed with M hydrochloric acid, and the neutral sugar compositions determined[11]. Samples of the oligosaccharide were also treated with methanolic sodium methoxide for 1 h at room temperature to effect *O*-deacetylation, and the resulting oligosaccharide was similarly subjected to enzymic digestion and analysis.

RESULTS AND DISCUSSION

Isolation and composition of the oligosaccharide. — Pneumococcal capsular polysaccharide type 18C has been shown to contain phosphorus[3–6], and this was confirmed by elemental analysis, the results most probably indicating the presence

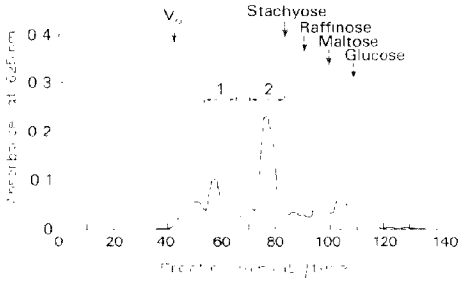


Fig. 1. Fractionation of the hydrolyzate (60% aqueous hydrofluoric acid) of pneumococcal capsular polysaccharide type 18C on a column (2 × 200 cm) of Bio-Gel P-2. A portion (0.01 mL) of each fraction was analyzed by reaction with the anthrone–sulfuric acid reagent.

of phosphoric diester linkages. Depolymerization of the polysaccharide by treatment with 60% aqueous hydrofluoric acid[7,8] was followed by chromatography[9] on Bio-Gel P-2, to give a single, dephosphorylated oligosaccharide whose retention index indicated a molecular weight corresponding to ~5 sugar units (Peak 2 in Fig. 1). Absence of carbohydrate from the other peaks, and other fractions along the baseline, of the P-2 elution profile was confirmed by analysis for neutral sugar(s)[10]. G.l.c.–m.s. analysis of the per-*O*-acetylated, hydrochloric acid hydrolyzate[28] of the oligosaccharide revealed penta-*O*-acetylhexopyranose, tetra-*O*-acetyl-6-deoxyhexopyranose, and tri-*O*-acetylglycerol in the ratios of 4.1:1.0:0.25. The hydrochloric acid hydrolyzate of the oligosaccharide was found to contain D-glucose, D-galactose, and L-rhamnose in the ratios of 2.8:1.0:1.2, as determined by the method of Boykins and Liu[11].

F.a.b.–m.s. of the repeating oligosaccharide. — Although a few reports of fast atom bombardment-mass spectrometry of carbohydrates have recently appeared[19,20,29,30], the technique has not yet sufficiently matured to permit routine, structural analysis of carbohydrates with a high degree of confidence. The major issues of concern have been (*1*) the quantity of material required for obtaining meaningful mass-spectral data, and (*2*) the changes in the mass spectrum after addition of inorganic salts and small organic molecules to the glycerol sample-matrix. To address these problems, we briefly investigated the f.a.b.–m.s. of cellobiose, raffinose, and stachyose, three oligosaccharides that contain only neutral sugars, as in the oligosaccharide of type 18C.

The f.a.b.–mass spectra of the di-, tri-, and tetra-saccharide were obtained from glycerol suspensions of these compounds that were deposited on a copper sample-probe tip supplied by the manufacturer. Initially, a fast-atom beam of argon neutrals (8 keV) was used for bombardment. The change to using a fast-atom beam of xenon neutrals (9 keV) for bombardment greatly lessened the undesirably high levels of fragmentation observed with argon[31]. Protonated molecular

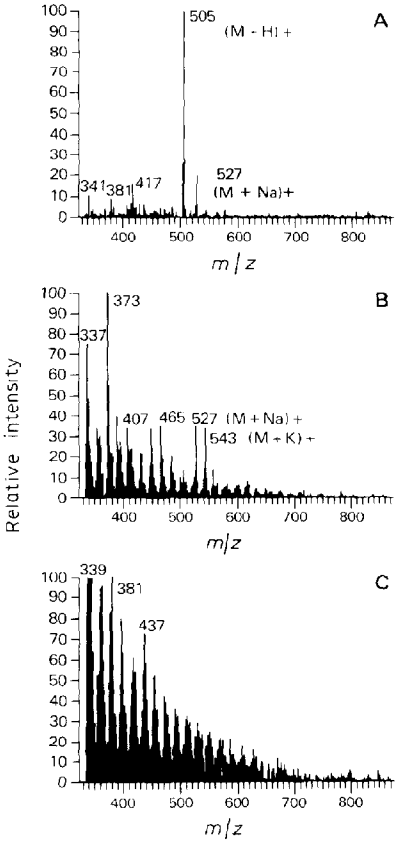


Fig. 2. F.a.b.–mass spectrum of (A) raffinose, (B) raffinose with an equimolar mixture of sodium chloride and potassium chloride (0.05% by weight) added to the sample matrix; and (C) raffinose with an equimolar mixture of sodium chloride and potassium chloride (5% by weight) added to the sample matrix.

ions $(M + H)^+$, were observed for each oligosaccharide. However, attempts to determine a standard dilution-curve revealed linearity in a very narrow range of concentrations, generally between 0.2 and 1.0 mM, in the glycerol sample-matrix. Ions attributable to the samples were not discernible from background at concentrations lower than this.

The addition of such small organic molecules as 1-thioglycerol[20], oxalic acid[25], or mercaptoacetic acid[32] allowed this curve to be extended to about 0.1μM in the glycerol matrix; however, these additives contributed to an increased background in the mass spectra. The addition of small amounts of such inorganic salts as sodium chloride or potassium chloride gave rise to $(M + Na)^+$ and $(M + K)^+$ ions, but also began to suppress the intensities of ions belonging to the samples (see Figs. 2A and 2B). Even at modest concentrations of the salt, complete suppression of the molecular-ion species, as well as of the alkali metal-attachment species, was observed (see Fig. 2C). Similar results were obtained on using a silver sample probe-tip[32]. However, the addition of certain inorganic salts to the sample matrix should not be ignored, as important information can be extracted from the mass spectrum under carefully controlled circumstances. For example, a protonated molecular ion $(M + H)^+$ was observed for raffinose at *m/z* 505 (see Fig. 2A). An ion of lesser intensity appears at *m/z* 527, corresponding to the sodium-attachment, molecular ion of raffinose. Its presence indicates residual sodium in either the sample, or the water used to dissolve the sample, or both. It is quickly perceived that the quality of the f.a.b.–mass-spectral data will be dependent upon careful exclusion of such unwanted impurities, as sodium salts, in order that interpretation of the data proceeds unhindered.

Conversely, controlled inclusion of such impurities may, under certain circumstances, serve to aid in the interpretation of the data. The addition of a small proportion (0.05%, by weight) of an equimolar mixture of sodium chloride and potassium chloride to the sample matrix gave rise to ions corresponding to sodium- and potassium-attachment, molecular ions of raffinose at *m/z* 527 and 543 (see Fig. 2B). The feature to note is that the protonated molecular-ion, although still present, is substantially lower in intensity in the presence of the added salts. Furthermore, the appearance of ions at *m/z* 527 and 543, which are 22 and 38 amu greater than the protonated molecular-ion, would indicate the attachment of sodium and potassium, respectively, to a single, molecular species. If approached as an unknown, the molecular weight of raffinose could be inferred, as the two ions, separated by 16 amu, would most probably correspond to sodium- and potassium-attachment ions of a single species.

For the identification, quantification, and modification of carbohydrates, there are many chemical reactions that use copper or silver, or their oxides, as reagents. In view of these circumstances, it would then be most appropriate to use a sample probe-tip that would present an inert surface to the sample matrix. We therefore chose to plate the sample probe-tip with gold. On re-examination of the oligosaccharides, it was found that the standard-dilution curve could be obtained with excellent linearity to a concentration of $<10\mu$M in the glycerol, without the addition of small organic compounds or inorganic salts.

The underivatized oligosaccharide isolated from the hydrofluoric acid hydrolyzate of the pneumococcal capsular polysaccharide type 18C was then examined by f.a.b.–m.s.[19]. It had already been determined that the composition of the

oligosaccharide was Glc:Gal:Rha in the ratios of 3:1:1. Also, it was found that the oligosaccharide was only partially glycerated (~25%), reflecting, perhaps, an acid-sensitive linkage in the original compound. It could then be expected that a simple mixture of various forms of the oligosaccharide would be examined. A reasonable expectation would be the observation of a protonated molecular-ion corresponding to the oligosaccharide (without covalently bonded glycerol) at *m/z* 813, and of a protonated molecular-ion, corresponding to the oligosaccharide with covalently bonded glycerol, at *m/z* 887. However, on the basis of observations regarding the f.a.b.–mass spectra of raffinose, it was decided to add, to the sample matrix containing the oligosaccharide, a small proportion (0.05% by weight) of an equimolar mixture of sodium chloride and potassium chloride, in order to aid in the interpretation of the resulting f.a.b.–mass spectrum. It was therefore predicted that, in addition to observation of a small ion at *m/z* 813, corresponding to the oligosac-

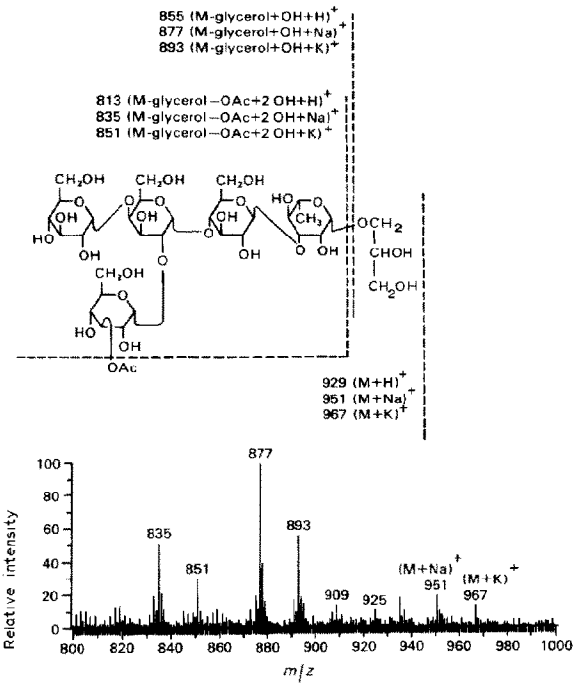


Fig. 3. F.a.b.–mass spectrum of the oligosaccharide isolated from the hydrolyzate (60% aqueous hydrofluoric acid) of pneumococcal capsular polysaccharide type 18C.

charide without covalently bonded glycerol, the sodium- and potassium-attachment ions, at *m/z* 835 and 851, should also be present. Similarly, it was predicted that ions at *m/z* 909 and 925, corresponding to the sodium- and potassium-attachment ions of the oligosaccharide with covalently bonded glycerol, should also be present.

Therefore, an aqueous solution of the oligosaccharide (20 μg) was added to glycerol that contained sodium chloride and potassium chloride (0.05% by weight). The sample mixture was placed on a gold-plated copper sample-stage, introduced into the mass spectrometer, and bombarded with 9 keV xenon atoms. Fig. 3 displays the mass range corresponding to the region of the molecular ion expected for a pentasaccharide. The sample was scanned to *m/z* 3000; however, there were no ions larger than *m/z* 1000 that possessed a signal-to-noise ratio >2:1. The previous predictions of particular ions were, indeed, found to be valid. However, attention was immediately drawn to the intense ions at *m/z* 877 and 893. The mass difference of 16 amu between these intense ions would indicate the presence of a single species that contains an added sodium atom (*m/z* 877), or an added potassium atom (*m/z* 893). The metal-free species would, therefore, be 854 amu, and this most probably represents a pentasaccharide composed of four hexoses, one deoxyhexose, and one *O*-acetyl group. Similarly, the ions at *m/z* 951 and *m/z* 967 imply a single species of 928 amu that contains an added sodium atom (*m/z* 951), or an added potassium atom (*m/z* 967) and most probably represents a pentasaccharide composed of one glycerol, one deoxyhexosyl, four hexosyl and residues, and one *O*-acetyl group.

Determination of the monosaccharide linkages — Methylation analysis[16,17] of the oligosaccharide showed four different, partially methylated alditol acetates, and further supported the conclusion that the sugar residues are pyranosidic. Direct comparison by g.l.c.–m.s. of authentic 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol to the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol found in the methylation-analysis mixture showed them to be identical. The presence of a 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol indicated a branch point in the oligosaccharide. The other two alditols were identified as a 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-6-deoxyhexitol and a 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylhexitol.

Determination of the monosaccharide sequence — The oligosaccharide was permethylated, and analyzed directly by electron-impact mass spectrometry[33–37]. The symbols[38] A–K, with lower-case letters a, b, c, and d to designate the monosaccharide units (counting from the reducing end), are used to denote the fragment ions[39]. The portion of the mass spectrum in the range at *m/z* >125 is displayed in Fig. 4. The largest ion-intensity in that region was *m/z* 187, the aA_2 ion of a hexitol at a nonreducing terminus, and was assigned an arbitrary value of 100%, against which, all other ion-intensities were normalized. Based on the data from the compositional analysis and f.a.b.–m.s., it was expected that *m/z* 1124 and 1036 could be present, representative of the per-*O*-methylated oligosaccharide with and without covalently bonded glycerol. However, ions corresponding to

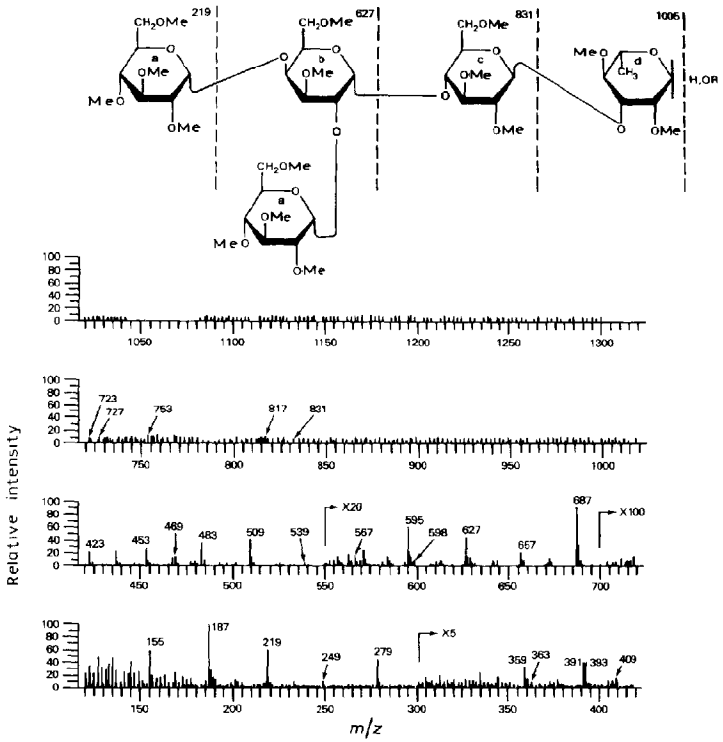


Fig. 4 Electron-impact, mass spectrum of the per-*O*-methylated oligosaccharide isolated from the hydrolyzate (60% aqueous hydrofluoric acid) of pneumococcal capsular polysaccharide type 18C. (A schematic representation of simple cleavage-fragmentations is indicated on the structural formula.)

either the molecular ions (M^+), or to simple losses from a molecular ion such as M − MeOH or M − CH_2OMe, were of such low intensity as to be indistinguishable from the background.

Consistent with the results of the methylation analysis, a nonreducing, hexosyl terminus was confirmed by the presence of the ions corresponding to the "A" series derived from fission of the glycosidic bond (aA_1, *m/z* 219; aA_2^1, aA_2^2, and aA_2^3, *m/z* 187; and aA_3^1, *m/z* 155). Compositional data for the reduced oligosaccharide had previously shown an L-rhamnosyl residue to be at the "reducing" terminus, and this was further supported by observation of the ions of the "D" series, which subsequently fragment to "J" and "A" ions (cdD_1, *m/z* 323; cdJ_1^1, *m/z* 249; and dA_1, *m/z* 189).

The results of the methylation analysis had shown the presence of a branch-point monosaccharide unit and a 1,4-disubstituted monosaccharide unit. The occurrence of the branch-point hexose necessarily indicates that two monosaccharides be present as nonreducing termini. Because the L-rhamnose residue had been established as the penultimate "reducing" end of the oligosaccharide, the only remaining monosaccharide to be assigned in the structure of the oligosaccharide was the 1,4-disubstituted monosaccharide unit. Thus, the fragment series A–K for the three possible arrangements were first predicted, and then intercompared.

Although some ambiguity exists between the three lists, the presence of certain ions, and the absence of others, allows an unequivocal determination of the remaining sugar units in the oligosaccharide. Cleavage of the glycosidic bond of sugar "b" gave rise to a set of intense "A" series ions (baaA_1, *m/z* 627; baaA_2, *m/z* 595, baA_2^1, baA_2^3, *m/z* 391; and baA_4, *m/z* 359). Additionally, the origin of other ions in the mass spectrum may be explained by "J" and "F" ions derived from the "B" series of ions evolving from the monosaccharide residue "b" (baaJ_1^1, *m/z* 483; and baaF_1^1, *m/z* 509), and by "F" ions derived from the molecular ion (baaF_1^1, *m/z* 509). Evidence that the branch-point residue and the reducing terminus were separated by a hexose unit was provided by ions corresponding to "J" and "A" ions derived from the "D" series (bcdD_1, *m/z* 527; bcdJ_1^1, *m/z* 453; and cdA_1, *m/z* 393).

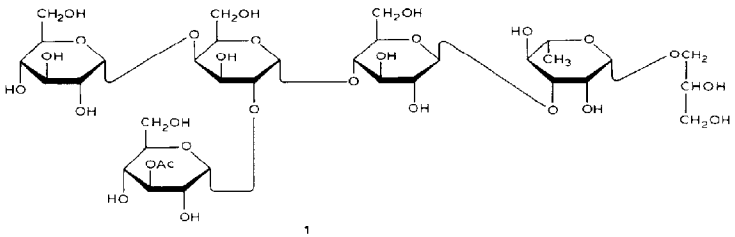
Periodate oxidation. — The oligosaccharide was oxidized with periodate. Methylation analysis of the reduced oxidation-product showed the presence of a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol and, not unexpectedly, a 1,2,4,5-tetra-*O*-acetyl-3,6-di-*O*-methylhexitol. The former suggested that the *O*-acetyl group found by f.a.b.–m.s. would most probably be situated on O-3 of the nonreducing terminal, or the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol residue. The e.i. mass spectrum of the reduced, permethylated oxidation product of the oligosaccharide contained ions of fragments indicative of an intact, 2,3,4,6-tetra-*O*-methylhexopyranosyl unit, as well as the periodate-derived remnants of a former hexopyranosyl unit. Additionally, ions were observed that corresponded to the "E", "G", and "K" series evolving from the monosaccharide residue "b", suggesting that the 2,3,4,6-tetra-*O*-methylhexopyranosyl unit (originally containing an acetyl group on O-3) is (1→2)-linked to monosaccharide unit "b" [baE_4, *m/z* 273; baG_1^1 (from baaE_1^2), *m/z* 305; and baK_1, *m/z* 264].

The acid hydrolyzate of the reduced, oxidized oligosaccharide contained an equimolar mixture of D-galactose and D-glucose. When the oligosaccharide was pretreated with aqueous triethylamine, to effect *O*-deacetylation, the acid hydrolyzate of the reduced, oxidized oligosaccharide contained D-galactose as the preponderant component, indicating that the 1,2,4-tri-*O*-substituted hexitol discovered by the methylation analysis was derived from D-galactose, and that the 1,4-di- and 1-substituted hexitols were derived from D-glucose.

Location and substitution of glycerol. — The presence of glycerol in the dephosphorylated oligosaccharide had been demonstrated by f.a.b.–m.s. and by g.l.c.–m.s. analysis of the per-*O*-acetylated hydrolyzate of the oligosaccharide.

Both methods showed that the oligosaccharide was ~25% glycerolylated, perhaps reflecting partial hydrolysis of the glycerol during depolymerization of the polysaccharide. Analysis of the reduced, hydrolyzed oligosaccharide revealed removal of ~80% of the L-rhamnose. This served to confirm that the rhamnose was the penultimate residue at the "reducing" terminus, as previously proposed by mass-spectral analysis of the per-*O*-methylated oligosaccharide, as well as to suggest that the rhamnosyl residue was glycosidically linked to the glycerol. The oligosaccharide was per-*O*-methylated, the product hydrolyzed, the sugars acetylated, and the resulting mixture examined by g.l.c.–m.s. Comparison to synthetically prepared mixtures of partially methylated, partially acetylated glycerol revealed that the oligosaccharide contained a 1-*O*-substituted glycerol residue.

Determination of the anomeric configurations. — The per-*O*-acetylated oligosaccharide was treated with chromium(VI) oxide[26], and the product was hydrolyzed with dilute hydrochloric acid. Analysis[11] of the hydrolyzate showed that one glucose residue had been oxidized, indicating the presence of one β-linked glucose unit in the original oligosaccharide. Treatment of the oligosaccharide with β-D-glucosidase did not result in liberation of glucose, indicating that the "non-reducing" terminus was α-linked. When the oligosaccharide was treated with methanolic sodium methoxide, to effect *O*-deacetylation, and the product treated with β-D-glucosidase, liberation of glucose was not observed. These results suggest that the β-linked glucosyl unit found by chromium(VI) oxide oxidation of the oligosaccharide is the 1,4-disubstituted glucosyl residue situated between the branch-point galactose and the penultimate "reducing" rhamnosyl group.



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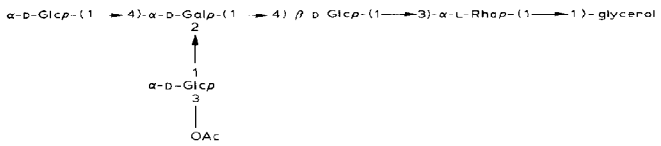

The results of these studies are summarized in **I**, the structure tentatively proposed for the repeating oligosaccharide of the pneumococcal capsular polysaccharide type 18C. A complete structure for the repeating oligosaccharide can only be suggested from the data thus far available.

ACKNOWLEDGMENTS

We thank Dr. Chi-Jen Lee for helpful discussions and suggestions, and Mr. Robert Boykins for excellent technical assistance in the sugar analyses. The polysaccharide was generously provided by Lederle Laboratories.

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