Carbohydrate Research, 173 (1988) 255–262
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURE OF THE GROUP G STREPTOCOCCAL POLYSACCHARIDE*

DAVID G. PRITCHARD†, BRENDA P. RENER,
Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294 (U.S.A.)

RAYMOND L. FURNER,
Neurosciences Program, University of Alabama at Birmingham, Birmingham, Alabama 35294 (U.S.A.)

DEE HUA HUANG, AND N. RAMA KRISHNA‡
Comprehensive Cancer Center, and the Departments of Medicine and Biochemistry, University of Alabama at Birmingham, Birmingham, Alabama 35294 (U.S.A.)

(Received February 2nd, 1987; accepted for publication, March 23rd, 1987)

ABSTRACT

The structure of the group-specific polysaccharide of group G *Streptococcus* was determined by means of methylation analysis and selective chemical degradations. The anomeric configurations and conformations of the sugar residues were studied by ^{1}H- and ^{13}C-n.m.r. spectroscopy. The tetrasaccharide repeating unit, →3)-α-D-Gal*p*-(1→2)-[α-L-Rha*p*-(1→3)-β-D-Gal*p*NAc-(1→4)]-α-L-Rha*p*-(1→, was determined.

INTRODUCTION

Group G streptococci have become recognized during the past fifteen years as a significant cause of opportunistic and nosocomial infections[1]. Group G streptococci, like other beta-hemolytic streptococci, are classified by the serological detection of a group-specific polysaccharide. Strains possessing the group G-specific polysaccharide were first described by Lancefield and Hare[2] in 1935. The polysaccharide was subsequently reported to be composed of rhamnose, galactose, and *N*-acetylgalactosamine[3]. This report describes the complete structural determination of the group G streptococcal polysaccharide.

EXPERIMENTAL

Chemicals and reagents. — Sugars and alditols were obtained from Sigma Chemical Co. (St. Louis, MO 63178). High-purity methanol, hexane, dimethyl

*This investigation was supported by grant DMB 85-10963 from the National Science Foundation, and by grant CA-13148 from the National Institutes of Health.
†To whom correspondence should be addressed.
‡Leukemia Society of America Scholar.

0008-6215/88/$ 03.50

sulfoxide, and *N,N*-dimethylformamide were purchased from Burdick & Jackson Laboratories Inc. (Muskegon, MI 49442). *N*-Methylbis(trifluoroacetamide) (MBTFA) was obtained from Pierce Chemical Co. (Rockford, IL 61105). (*R*)-(−)-2-Butanol,(*S*)-(+)-2-butanol, and acetyl chloride were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI 53233).

Methanolic HCl was prepared by passing dry HCl gas through methanol until the concentration was 1.4M (determined by titration with standard base). Glass ampules of the reagent were stored at −70°. Butanolic HCl was prepared by adding acetyl chloride (0.5 mL) to the ice-cold chiral alcohol (4.5 mL), and allowing the mixture to remain at room temperature for 2 h before use. A 10% (w/v) solution of MBTFA in *N,N*-dimethylformamide was prepared weekly. The trifluoroacetylation reagent was prepared immediately before use by adding pyridine (10 μL) to the 10% MBTFA solution (100 μL).

Analytical procedures. — The rhamnose content of column fractions was routinely monitored by the procedure of Dische and Shettles[4]. Monosaccharides were analyzed with a modification of the highly sensitive, gas-chromatographic procedure described previously[5]. Polysaccharide samples of 0.2 μg were routinely analyzed. They were dried in 90 × 0.8 mm capillary tubes. 1.4M Methanolic HCl (~10 μL) was added to each capillary tube by means of a glass micropipet. The capillaries were sealed and placed in a heating block for 24 h at 80°, after which they were opened and the samples rapidly dried *in vacuo*. Derivatization was accomplished by adding the trifluoroacetylation reagent (5 μL) to each capillary and allowing it to react for at least 2 h at room temperature.

The absolute configurations of the sugar residues was determined essentially as described by Leontein *et al.*[6] by g.l.c. of the (*S*)-(+)-2-butyl glycosides. However, HCl instead of trifluoroacetic acid was used as the catalyst and, rather than using acetate derivatives as in the original procedure, trifluoroacetate derivatives were prepared as described above. The retention times of the uncommon optical isomers of the sugars were determined by chromatographing the (*R*)-(−)-2-butyl glycosides of the common form of the sugars.

Gas-liquid chromatographic analyses. — A Hewlett-Packard Model 5830A gas chromatograph, equipped with a splitless, capillary-inlet system and a ^{63}Ni linear, electron-capture detector was used. A fused silica WCOT column [0.32 mm (i.d.) × 30 m] coated with a 0.6-μm film of OV-105 was obtained from Chrompack Inc. (Bridgewater, NJ). Hydrogen at an inlet pressure of 26.7 kPa was used as the carrier gas (38 cm/s linear flow), and for the septum (3 mL/min) and inlet (60 mL/min) purges. Argon–methane (19:1) was used for the detector make-up gas at a flow rate of 60 mL/min. The injector temperature was 195°, and the detector was operated at 280°. The chromatograph was held at 80° for 1 min, and the temperature was then programmed to 90° at 0.5°/min and to 200° at 5°/min. A 0.2-μL sample, followed by 1 μL of hexane was injected, and the inlet purge-gas flow was delayed for 18 s.

Methylation analysis. — The methylation analysis procedure of Stellner *et*

al.[7] was employed, but with potassium methylsulfinylmethanide in dimethyl sulfoxide rather than the sodium compound. The resulting, partially methylated alditol acetates were separated on a 12.5-m fused silica WCOT column (0.2 mm i.d.), coated with a 0.33-μm film of cross-linked dimethylsilicone (Hewlett–Packard) in a Hewlett–Packard 5985A combined gas chromatography–mass spectrometry system. Electron-impact mass spectra were obtained at an ionizing voltage of 70 eV.

Nuclear magnetic resonance studies. — N.m.r. experiments were performed at the n.m.r. Core Facility of the Comprehensive Cancer Center at the University of Alabama at Birmingham. A Bruker WH-400 (9.4 T) spectrometer operating in the F.t. mode was employed, and all spectra were recorded at ambient temperature (24°), for a solution of the polysaccharide in D_2O; ^{1}H-chemical shifts are referenced to the HOD signal of the solvent (δ 4.8). For the ^{13}C-n.m.r. spectra, dimethyl sulfoxide (5 μL) was added to the solution to provide an internal reference (δ 39.5). ^{1}H-Signals were assigned by two dimensional J-resolved spectroscopy, chemical shift-correlated spectroscopy (2D–COSY), relayed-coherence-transfer (RCT) experiments, and phase sensitive NOESY[8]. ^{1}H-Decoupled, ^{13}C-n.m.r. spectra were obtained by pulsed decoupling, and ^{1}H-signals directly coupled to the ^{13}C-nuclei were identified by single frequency heteronuclear decoupling. ^{1}H–^{13}C Coupling constants were measured in the ^{1}H-coupled spectra recorded with the decoupler frequency offset by 2000 Hz from the proton-spectral region.

Smith degradation. — Group G polysaccharide (100 mg) was dissolved in 40mM $NaIO_4$ in 0.1M sodium acetate buffer, pH 4.0 (10 mL), and kept for 48 h at 4° in the dark. Progress of the oxidation was monitored by measuring the absorbance at 223 nm of a 1:500 dilution of the reaction mixture. Excess periodate was reduced by the addition of 1,2-ethanediol and the mixture desalted on a column of Bio-Gel P-2, eluted with 1% acetic acid. The oxidized polysaccharide was reduced with $NaBH_4$ (20 mg/mL in 0.2M NH_4OH) for 48 h at 4°. The sample was acidified with acetic acid, again desalted on a Bio-Gel P-2 column, and lyophilized. The resulting material was hydrolyzed with 0.5M trifluoroacetic acid for 24 h at room temperature. The reaction mixture was loaded directly onto a column of Bio-Gel P-2 and eluted with 1% (w/v) acetic acid, and the peak of carbohydrate-containing material, eluted in the void volume of the column, was pooled and lyophilized.

Preparation of an acid-resistant disaccharide. — The group G polysaccharide was *N*-deacetylated essentially as described by Erbing *et al.*[9]. In this procedure, the polysaccharide (50 mg) was dissolved in M sodium thiophenolate (1 mL). The solution was cooled in ice and 2M potassium methylsulfinylmethanide in dimethylsulfoxide (5 mL) was added. The mixture was heated for 18 h at 100°, cooled, diluted with water, and made acidic with acetic acid. It was extracted with hexane (4 × equal vol.), and the aqueous phase was clarified by centrifugation and desalted on a Bio-Gel P-2 column. The material eluted in the void volume was pooled and lyophilized.

A sample of the *N*-deacetylated group G polysaccharide (10 mg) was dissolved in 1.5M methanolic HCl (0.5 mL), heated for 18 h to 80°, and then evaporated to dryness. The residue was dissolved in 5mM HCl (1 mL) and loaded onto a column containing AG 50 (H^+) resin (2 mL). The column was washed with several volumes of water and then eluted with 2M HCl. The effluent was diluted with water and lyophilized, and the residue *N*-reacetylated[10] by dissolution in methanol (0.5 mL), and addition of pyridine (150 μL) and acetic anhydride (100 μL). After 30 min at room temperature, the mixture was evaporated under vacuum.

Immunological methods. — Rabbit antisera to the group G streptococcal polysaccharide was obtained from the Centers for Disease Control in Atlanta. *Vicia villosa* lectin was obtained from E. Y. Laboratories, San Mateo, CA, and was used at a concentration of 2 mg/mL. Double-diffusion studies were carried out in 1% (w/v) agarose gels containing 0.10M Tris·HCl, pH 7.5.

Polysaccharide extraction and purification. — The group G streptococcal polysaccharide was extracted from lyophilized cells by a modified version of the nitrous acid extraction procedure of Swanson *et al.*[11]. Group G streptococcal cells, strain D166B (23 g) were suspended in water (240 mL) containing a few drops of antifoam B (Sigma Chemical Co., St. Louis, MO), 4M $NaNO_2$ (120 mL) and glacial acetic acid (120 mL) were added, and the mixture was stirred for 1 h at room temperature. The extracted cells were removed by centrifugation and the supernatant solution was rapidly desalted with a hollow-fiber device equipped with a 10 000 mol.-wt. cutoff cartridge (Amicon Corp., Danvers, MA), and then lyophilized. The resulting crude extract was purified by gel filtration on a 5 × 100 cm column of Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ), eluted with 50mM sodium phosphate buffer, pH 7.5. The single peak of rhamnose-containing material was pooled, lyophilized, desalted on a 2.5 × 25 cm column of Bio-Gel P-2 by elution with 1% acetic acid, and once again lyophilized.

RESULTS AND DISCUSSION

Gas–liquid chromatographic monosaccharide analysis of the purified group G polysaccharide revealed the presence of rhamnose, galactose, and *N*-acetylgalactosamine in a ~2:1:1 molar ratio. The D configuration was assigned to the galactosyl and the *N*-acetylgalactosaminyl residues, and the L configuration to the rhamnosyl residues on the basis of the retention times of the trifluoroacetate derivatives of their (*S*)-(+)-2-butyl glycosides.

Methylation analysis of the group G streptococcal polysaccharide gave the partially methylated alditol acetates (Table I) that indicated the presence of nonreducing terminal L-rhamnopyranosyl groups, D-galactopyranosyl and 2-acetamido-2-deoxy-D-galactopyranosyl residues linked at O-3, and branched L-rhamnopyranosyl residues linked at O-2,4. A small proportion of L-rhamnosyl residues linked at O-2 was also observed.

TABLE I

METHYLATION ANALYSIS OF THE GROUP G STREPTOCOCCAL POLYSACCHARIDE (A), THE SMITH-DEGRADED POLYSACCHARIDE (B), AND A DERIVED ACID-RESISTANT DISACCHARIDE (C)

Methylated sugars[a]	*Retention time*[b]	*Relative peak area*[c]		
		A	*B*	*C*
2,3,4-Me_3-Rha	6.02	0.89	0	0.03
3,4-Me_2-Rha	6.37	0.37	0	0
2,3-Me_2-Rha	6.42	0	0	1.00
3-Me-Rha	6.78	0.93	0.96	0
2,4,6-Me_3-Gal	7.03	1.00	1.00	0
3,4,6-Me_3-GalN(AcMe)	7.85	0	0.79	0.73
4,6-Me_2-GalN(AcMe)	8.25	0.83	0.07	0

[a]As peracetylated alditols. [b]Retention time in min on a 12.5-m cross-linked dimethylsilicone WCOT fused-silica column; temperature programmed from 50 to 270° at 15°/min. [c]Relative peak areas in the total ion chromatogram .

Sequential periodate oxidation, borohydride reduction, and mild acid hydrolysis (Smith degradation) of the group G polysaccharide destroyed approximately one-half of the rhamnose residues and consumed 2.46 μmol of periodate per mg of polysaccharide, a result consistent with the methylation analysis data. Gel filtration of the product of Smith degradation on Bio-Gel P-2 resulted in 94% of the rhamnose present being eluted in the void volume of the column. This material no longer reacted with antisera to the group G streptococcal polysaccharide in a double diffusion experiment, but it gave a strong precipitin band with the lectin from *V. villosa,* indicating the presence of terminal 2-acetamido-2-deoxy-D-galactopyranosyl groups[12]. The results of methylation analysis of the product of Smith degradation (Table I) showed the presence of approximately equimolar amounts of terminal 2-acetamido-2-deoxy-D-galacto-pyranosyl groups, and 3-linked galactopyranosyl and 2,4-linked rhamnopyranosyl residues.

N-Deacetylation of the group G polysaccharide, followed by methanolysis, identified the residue to which the 2-acetamido-2-deoxy-D-galactopyranosyl residue was linked. An acid-resistant disaccharide was isolated from the methanolysis mixture by cation-exchange chromatography. *N*-Reacetylation of the disaccharide followed by gas–liquid chromatographic monosaccharide analysis revealed the presence of equimolar amounts of L-rhamnose and 2-acetamido-2-deoxy-D-galactose. The results of methylation analysis of the disaccharide (Table I) indicated that the amino sugar is linked to O-4 of the branching L-rhamnosyl residue in the intact polysaccharide.

The anomeric configurations and conformations of the sugar residues in both the group G polysaccharide and the Smith-degraded product were studied by ^1H- and ^{13}C-n.m.r. spectroscopy. As would be expected, the spectra of the Smith-degraded product were much simpler, and resonances more readily assigned than in the intact group G polysaccharide.

The ^{1}H-resonance assignments (Table II) were based on 2D-COSY, 2D-NOESY, and multiple-relayed-coherence-transfer experiments. For the Smith-degraded product, the signal at δ 1.32 was assigned to the methyl protons of the rhamnose unit, and H-5 of the same rhamnose unit was found to have a chemical shift of δ 3.81 in the COSY spectrum. The chemical shifts of H-3 (δ 4.01) and H-4 (δ 3.71) of the rhamnose unit were obtained from single and double-relayed-coherence-transfer experiments. The resonances of H-4, H-5, H-6, and H-6′ of the other sugar units were not assigned, owing to severe overlap of the signals between δ 3.4 and 4.

The ^{13}C-resonances for the group G polysaccharide and its Smith-degraded product were assigned by heteronuclear-decoupling experiments. The irradiation of the ^{1}H-signal at δ 3.82 resulted in the collapse of the doublet at δ 53.6 in the ^{13}C-n.m.r. spectrum, a chemical shift characteristic for C-2 of an *N*-acetylhexosamine residue[13]. Thus, the ^{1}H resonances at δ 4.67 and 3.82 were assigned to H-1 and H-2 of the 2-acetamido-2-deoxy-D-galactopyranosyl residue. The ^{13}C

TABLE II

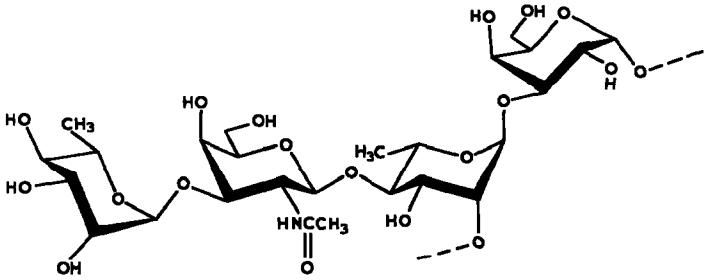
N.M.R. DATA FOR THE GROUP G STREPTOCOCCAL POLYSACCHARIDE AND THE PRODUCT OF SMITH DEGRADATION

	^{1}H-N.m.r. data			*^{13}C N.m.r. data*		
	δ^a	$J_{1,2}$ (Hz)	*Assignment*	δ^b	$J_{C\text{-}1,H\text{-}1}$ (Hz)	*Assignment*
Smith-degraded polysaccharide	5.17	1.96	H-1 of →2,4)-α-L-Rha*p*	102.8	158	C-1 of β-D-Gal*p*NAc
	4.05		H-2			
	4.01		H-3	99.6	170	C-1 of →2,4)-α-L-Rha*p*
	3.71		H-4			
	3.81		H-5	97.9	170	C-1 of →3)-α-D-Gal*p*
	1.32		H-6			
				61.8		C-6 of β-D-Gal*p*NAc and →3)-α-D-Gal*p*
	5.06	4.88	H-1 of →3)-α-D-Gal*p*			
	3.91		H-2			
	4.03		H-3			
				53.6		C-2 of β-D-Gal*p*NAc
	4.67	8.2	H-1 of β-D-Gal*p*NAc			
	3.83		H-2	17.8		CH_3 of →2,4)-α-L-Rha*p*
	3.69		H-3			
Group G polysaccharide	5.16		H-1 of →2,4)-α-L-Rha*p*	102.9	170	C-1 of α-L-Rha*p*
	5.06		H-1 of →3)-α-D-Gal*p*	102.3	164	C-1 of →3)-β-D-Gal*p*NAc
	4.85		H-1 of α-L-Rha*p*	99.6	170	C-1 of →2,4)-α-L-Rha*p*
	4.76	8.8	H-1 of →3)-β-D-Gal*p*NAc	97.9	170	C-1 of →3)-α-D-Gal*p*
				61.7		C-6 of →3)-β-D-Gal*p*NAc and →3)-α-D-Gal*p*
				52.7		C-2 of →3)-β-D-Gal*p*NAc

[a]Relative to the HOD solvent signal set at δ 4.8 relative to tetramethylsilane. [b]Relative to the signal of an internal standard of dimethyl sulfoxide set at δ 39.5 relative to the signal of tetramethylsilane.

resonances for the anomeric carbon atoms are listed in Table II. For the Smith-degraded polysaccharide, the C-1 chemical shift at δ 102.8 with a $J_{C,H}$ coupling constant of 158 Hz, and a large H-1 coupling constant, $J_{1,2}$ 8.2 Hz, of the corresponding H-1 at δ 4.67 indicated that the 2-acetamido-2-deoxy-D-galactopyranosyl residue is β-linked and exists in the $^4C_1$ (D) conformation. The ^{1}H resonance at δ 5.17 was assigned to H-1 of the rhamnose unit, based on its small $J_{1,2}$ coupling constant (<2 Hz), and the signal at δ 5.06 was assigned to H-1 of the galactose unit.

The ^{1}H–^{13}C coupling constants for the other C-1 atoms of both the group G and Smith-degraded polysaccharides are ~170 Hz. These data, along with the results of the 2D-NOESY experiment, indicated that both the 3-linked galactose and the 2,4-linked rhamnose units exist in the α configuration, with the rhamnosyl residues preponderantly in the $^1C_4$ (L) conformation, and the galactosyl residue in the $^4C_1$ (D) conformation. The aforementioned results support the conclusion that the group G streptococcal polysaccharide possesses the tetrasaccharide repeating unit **1**.



1

The small proportion of O-2-linked L-rhamnosyl units observed in the methylation analysis of the group G polysaccharide may be a procedural artifact or may correspond to positions in the backbone of the polysaccharide lacking the α-L-Rha*p*-(1→3)-β-D-Gal*p*NAc sidechains. If this is the case, it is possible that the sidechains were removed during the nitrous acid extraction of the group G polysaccharide, a reaction that would be expected to occur if any of the 2-amino-2-deoxy-D-galactopyranosyl residues were not *N*-acetylated in the native polysaccharide.

The presence of nonreducing terminal L-rhamnosyl groups in the group G streptococcal polysaccharide had been predicted several years ago on the basis of immunochemical studies[14]. It was reported that certain rabbit antisera to the type 23 pneumococcal polysaccharide cross-reacted with both the group B and the group G streptococcal polysaccharides, and immune precipitation was inhibited by free L-rhamnose. The present study of the group G polysaccharide and recent structural studies of the other two polysaccharides[15,16] suggest that the cross-reacting determinant in all three cases is a terminal α-L-rhamnopyranosyl group.

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