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Structural studies of the O-polysaccharide from the lipopolysaccharide of *Moraxella* (*Branhamella*) *catarrhalis* serotype A (strain ATCC 25238)

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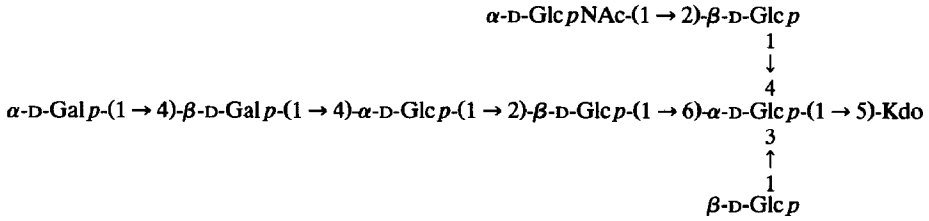
Abstract

The polysaccharide of the *Moraxella* (*Branhamella*) *catarrhalis* serotype A lipopolysaccharide was prepared by mild acid hydrolysis followed by gel permeation chromatography. The structure was established by methylation analysis, mass spectrometry, and NMR spectroscopy. It is concluded that the O-antigenic polysaccharide has the following structure.



Methylation analysis of the intact lipopolysaccharide showed that the lipid A portion consisted of 6-substituted glucosamine residues. Methylation followed by methanolysis showed that two Kdo residues were present, one terminal and one 4,5-substituted residue. A terminal Kdo thus substitutes the branch-point Kdo in the 4-position.

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1. Introduction

Moraxella (*Branhamella*) *catarrhalis* has been increasingly recognised as a major pathogen in childhood otitis media and a frequent cause of maxillary sinusitis [1]. It also causes respiratory diseases in patients with chronic bronchitis and in immunocompromized hosts [2]. The bacterium is found with a frequency of ca. 50% in the oropharyngeal cavity in healthy children [3]. In adults, however, the frequency is below 5% [4].

The lipopolysaccharide (LPS) of *Moraxella catarrhalis* lacks the extended O-antigenic side chains characteristic of enteric pathogens, thus being similar in general structure to the LPSs of *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Bordetella pertussis* [5–8]. Serological typing of *Moraxella catarrhalis* based on LPS antigens has been described [9]. Three major antigenic types were distinguished, accounting for 95% of strains isolated from different sources. It was shown that D-glucose, D-galactose, D-glucosamine, and 3-deoxyoct-2-ulosonic acid (Kdo), but no heptose, were present [6,10,11]. The qualitative composition of the different sugar constituents was similar in all strains tested, but differences in their relative amounts were recorded. In preliminary studies, antigenic differences were demonstrated between LPSs from different strains by an immunoblot assay [7,8]. Similar findings were reported by Storm Fomsgaard et al. [11]. The structure of *Moraxella catarrhalis* LPS has not been previously described. In this paper, we report the structure of the polysaccharide part of the LPS from a *Moraxella catarrhalis* serotype A.

2. Results and discussion

Moraxella catarrhalis, like most Neisseria species, secretes complex vesicle structures (blebs) that modulate or deflect the immune system. The vesicles contain significant amounts of LPS, which is identical to the LPS of the cell wall [6]. These vesicles are supposed, but not proved, to be of great importance as a virulence factor, in infections with *Moraxella catarrhalis*.

Cells of *Moraxella catarrhalis* were grown in brain heart infusion broth containing yeast extract, and the bacteria were collected by centrifugation. Extraction of the freeze-dried pellet with phenol–chloroform–petroleum ether yielded a purified LPS. A low UV absorption indicated the absence of nucleic acids, and no protein contamination was detected by Bio-Rad protein assay. A significant portion of the LPS preparation, however, consisted of phospholipids as evident from fatty acid analysis and TLC.

SDS-polyacrylamide gel electrophoresis of the LPS showed a single band upon silver staining. The molecular weight was estimated by comparison of the migration distance with those from *Salmonella* LPSs of known molecular weight, and was ~ 4200 (Fig. 1). Treatment of the LPS in a 0.1 M acetate buffer of pH 4.4, containing 0.1% of SDS, for 2 h at 100°C yielded the polysaccharide (PS). Subsequent work-up gave a lipid-free solution of the PS which was purified by gel

Fig. 1. SDS-polyacrylamide gel electrophoresis of *Moraxella catarrhalis* serotype A (lane 5), together with reference standards from *Salmonella typhimurium* R mutants. Lane 1, SL1102 (R_e); lane 2, SL3769 (Rd_1); lane 3, SL1181 (Rd_2); lane 4, SL805 (Rc); lane 6, TV119 (Ra); lane 7, TV161 (Rb_2); lane 8, SL733 (Rb_1); lane 9, TV148 (Rb_3).

filtration. The PS eluted as a single peak in the region for large oligosaccharides. A hydrolysate of the PS contained glucose, galactose, and glucosamine in the proportions 4.8:2.0:1.0 as shown by analysis of the corresponding alditol acetates. GLC analysis of the LPS showed glucose and galactose in the same proportions and double the amount of glucosamine. The absolute configurations of the sugars except Kdo were determined using the trimethylsilylated (*S*)-2-butyl glycosides [12,13] and were all in the D configuration. The Kdo residue was assumed to have the D-*manno* configuration.

Analysis of the molecular weight of the PS by positive FABMS showed a pseudomolecular ion $(M + H)^+$ at m/z 1598.5 indicating that it consisted of seven hexoses, one 2-acetamido-2-deoxyhexose, and one Kdo-Na residue, i.e., a nonasaccharide. An ion at 1580.4 was also present in almost equal amounts, possibly originating from dehydration.

Methylation analysis of the PS gave the sugars listed in Table 1. The results indicated that the PS is composed of one residue each of terminal, 4-substituted, and 3,4,6-substituted glucose, two residues of 2-substituted glucose, one residue each of terminal and 4-substituted galactose, and one residue of terminal glucosamine. The Kdo residue was later shown by NMR spectroscopy to be substituted through the 5-position. Additional evidence on the Kdo region of the LPS was obtained through methylation of the intact LPS followed by methanolysis which, *inter alia*, yielded the partially methylated Kdo methyl ester methyl glycosides. The GLC–MS results demonstrated that the LPS has two Kdo residues, one terminal and one 4,5-substituted. The yields were low but no other Kdo-derivatives were found. The mass spectra were identical with those obtained from the Salmonella Rd_2 core [14,15] and are shown in Fig. 2. The terminal Kdo thus

Table 1
Methylation analysis of *Moraxella* serotype A LPS and PS

Sugar	T [a]	Detector response (%)	
		LPS	PS
2,3,4,6-Glc [b]	1.00	13	14
2,3,4,6-Gal	1.04	11	12
3,4,6-Glc	1.20	22	23
2,3,6-Gal	1.22	13	13
2,3,6-Glc	1.24	13	14
2-Glc	1.77	13	15
2,3,4,6-GlcNAc	1.86	9	9
2,3,4-GlcNAc	1.92	5	

[a] Retention time relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol (1.00) and hexa-*O*-acetylglucitol (2.00) on an HP-5 capillary column, using the temperature program 180°C (1 min) to 250°C at 3°C min. [b] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methylglucose.

substitutes the branch-point Kdo in the 4-position. In addition, the lipid A portion could be shown to consist of only 6-substituted glucosamine residues.

The ^{1}H NMR spectrum of the PS in D_2O obtained at 500 MHz (Fig. 3a) confirmed that the glucosamine was *N*-acetylated, as indicated by a singlet at 2.18 ppm. In some preparations, one or both of the signals from 3-deoxy protons of Kdo were absent, although the pD was neutral. It has been reported for *N*-acetylneuraminic acid that both 3-deoxy protons can be exchanged in D_2O, but only above pD 7 [16]. The axial proton was exchanged up to 25 times faster than the equatorial. It seems reasonable to assume that Kdo should behave similarly. This also demonstrates that the Kdo is the reducing sugar in the PS. The 3-deoxy protons appeared at 1.88 and 2.04 ppm, indicating that the reducing sugar had predominantly the α configuration. Model compounds with the β configuration have a much larger chemical shift difference [17]. The anomeric region (Fig. 3b) of the PS contained signals for eleven protons. Of these, three could not be anomeric and it was later shown that, in addition to the signal for H-5 (δ 4.37) of the terminal α-D-galactosyl group, signals for H-3 (δ 4.53) and H-5 (δ 4.62) in the 3,4,6-substituted D-glucosyl residue overlap with the signals for two β anomers at 4.54 and 4.62 ppm, respectively. At low field, signals for four α-linked residues are observed ($J \sim 4$ Hz) and also signals for two β-anomeric protons at an unusually low field, 5.08 and 5.20 ppm. The ^{13}C NMR spectrum, obtained at 100 MHz, was well resolved and displayed separate signals for all carbons except two. Two large signals could, however, be assigned to two carbons each; thus, the spectrum accounts for all the carbons in the polysaccharide.

With the aid of 2D COSY, TOCSY, and broad-band decoupled HMQC (Fig. 4) spectra, almost all of the ^{1}H and ^{13}C NMR signals could be assigned (Tables 2 and 3, respectively). The $^1J_{C\text{-}1,H\text{-}1}$ coupling constants were determined from a coupled HMQC spectrum. One anomalous value of 166 Hz was observed, see below. The hexose and hexosamine residues were designated **A–H**, and the Kdo residue was designated Kdo. Assignments were made as described in the Experimental section.

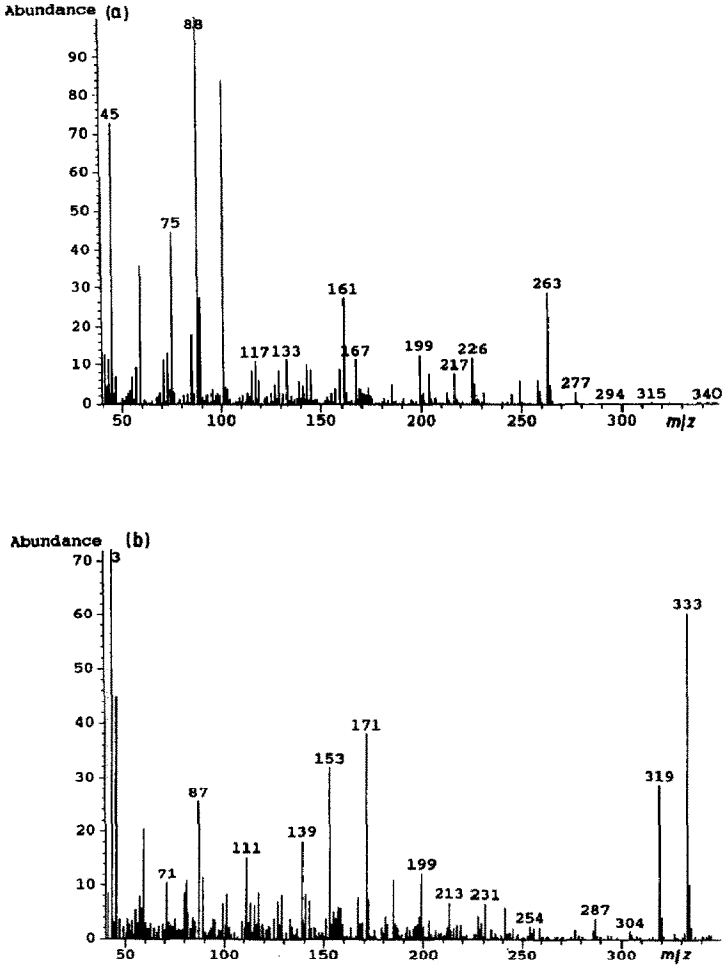


Fig. 2. Mass spectra of the methyl glycoside methyl esters of 4,5,7,8-tetra-*O*-methyl- (a) and 4,5-di-*O*-acetyl-7,8-di-*O*-methyl-Kdo (b).

Residues **A**, **C**, **E**, and **F** were assigned to α-linked residues from their $J_{H\text{-}1,H\text{-}2}$ coupling constants of 3.7, 4.4, 4.1, and 3.4 Hz, respectively, and $J_{C\text{-}1,H\text{-}1}$ coupling constants of 175, 172, 174, and 171 Hz, respectively. Similarly, residues **B**, **D**, **G**, and **H** were assigned to β-linked residues from their $J_{H\text{-}1,H\text{-}2}$ coupling constants of 7.8, 7.3, 7.8, and 6.9 Hz. The $J_{C\text{-}1,H\text{-}1}$ coupling constants were 166, 163, 163, and 164 Hz, respectively. The first value is close to what is considered to be the value for an α-linked residue, but occasionally high values are observed for β-linked residues and the $J_{H\text{-}1,H\text{-}2}$ value clearly indicates a *trans*-diaxial disposition of H-1 and H-2. Residue **E** was assigned to the terminal 2-acetamido-2-deoxy-D-glucosyl group from the chemical shift of the C-2 signal, 54.4 ppm, typical of nitrogen-bearing carbons. Residues **F** and **H** were assigned to galactose derivatives from the low value of $J_{H\text{-}3,H\text{-}4}$ (< 5 Hz) and, from the large glycosylation shift for the C-4

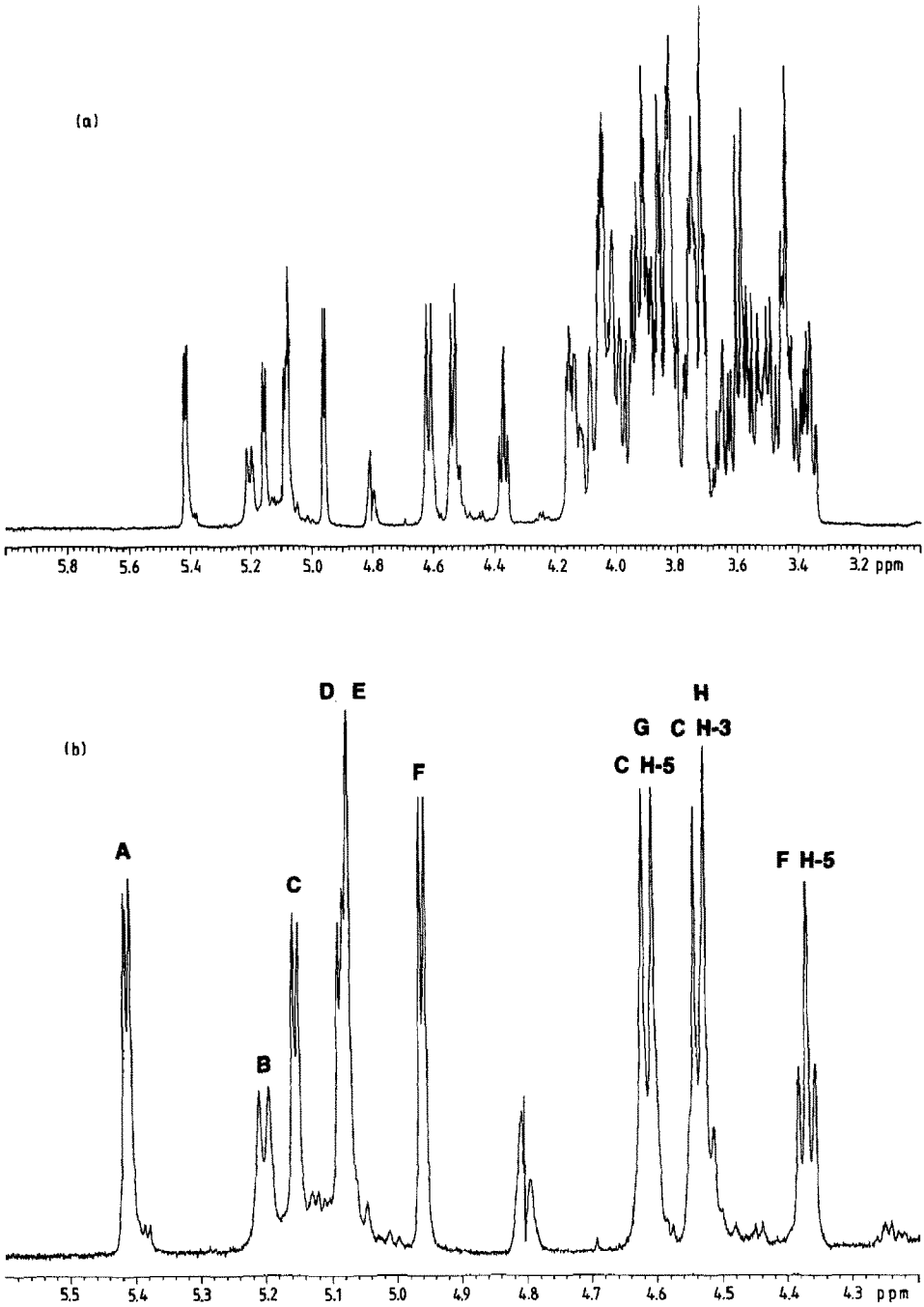


Fig. 3. (a) The 3–6 ppm region in the ^{1}H NMR spectrum of *Branhamella catarrhalis* serotype A oligosaccharide. (b) The anomeric region of the ^{1}H NMR spectrum of *Branhamella catarrhalis* serotype A polysaccharide.

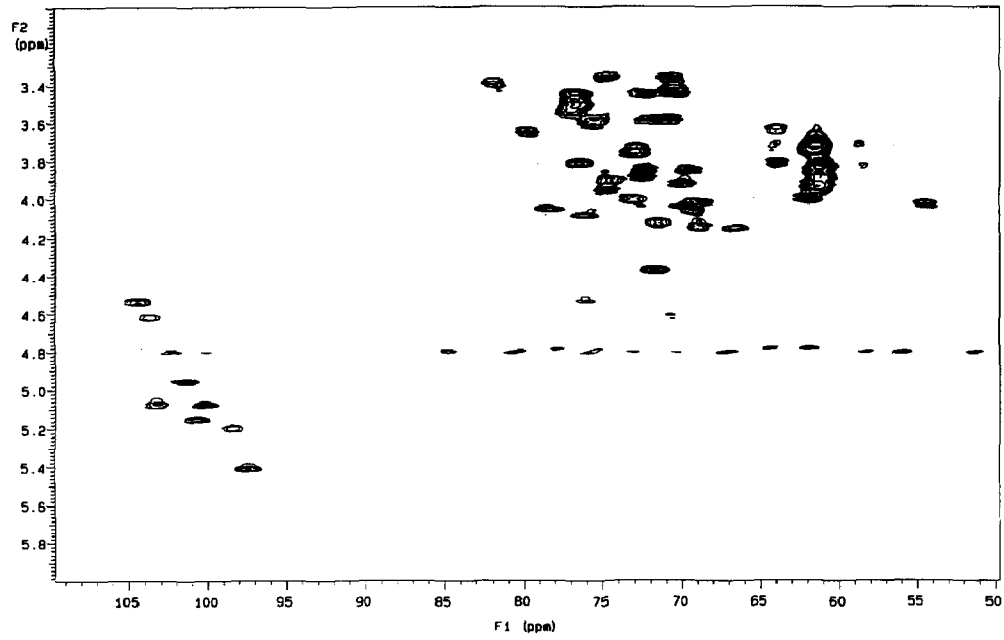


Fig. 4. Decoupled carbon–proton HMQC spectrum of *Branhamella catarrhalis* serotype A polysaccharide.

signal in the latter, they were assigned as terminal and 4-substituted, respectively. It is a general observation that large glycosylation shifts follow from substitution. From the large glycosylation shift for the C-2 signal for **B**, it could be assigned to the 2-substituted D-glucose residue. Residues **D** and **G** have relatively small ^{13}C NMR glycosylation shifts but, from the absence and presence of NOE contacts (Table 4) to these residues, it was evident that **D** was terminal glucose and that **G** was 2-substituted glucose. From the chemical shift differences of the signals from C-3 and C-4 of residue **A** and C-3, C-4, and C-6 of residue **C**, it is evident that the former is the 4-substituted and the latter the 3,4,6-substituted residue. The glycosylation shift of the C-5 signal of the Kdo residue, 8.7 ppm, demonstrates that it is 5-substituted. From the methylation analysis data and the absence of low-field C-1 and C-4 ^{13}C NMR signals, it is evident that all the residues are pyranoid. The data thus suggest a doubly branched polysaccharide, where the branch-point residue is 3-, 4-, and 6-substituted.

The sequence of sugars could partly be established with NOESY experiments (Table 4). For all residues, interresidual NOE should be observed between the anomeric proton of one residue and the proton on the linkage carbon of a neighbouring residue only, because no neighbouring equatorial protons are present, as, e.g., in the disaccharide element α-D-Hex*p*-(1 → 3)-D-Gal*p*, for which the NOE from the anomeric proton to H-4 should be substantial [18,19]. Exceptions were, however, observed as discussed below. A tetrasaccharide branch could be established starting from residue **F**, the terminal α-D-Gal residue, which has an

Table 2
^{1}H NMR data at 25°C for the oligosaccharide isolated from *Moraxella* (*Branhamella*) *catarrhalis* Serotype A (ATCC 25238)

Sugar residue	Chemical shifts [a] (δ)							
	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b	NAc
→4)-α-D-Glc*p*-(1→	5.41 [3.7]	3.45	3.88	3.64	4.12			
A	(0.18)	(−0.09)	(0.16)	(0.22)	(0.28)			
→2)-β-D-Glc*p*-(1→	5.20 [7.8]	3.39	3.60	3.44	3.51			
B	(0.56)	(0.14)	(0.10)	(0.02)	(0.05)			
→3,4,6)-α-D-Glc*p*-(1→	5.16 [4.4]	3.90	4.53	3.95	4.62	4.02	4.15	
C	(−0.07)	(0.36)	(0.81)	(0.53)	(0.78)	(0.26)	(0.31)	
β-D-Glc*p*-(1→	5.08 [7.3]	3.36	3.55	3.36	3.47			
D	(0.44)	(0.11)	(0.05)	(−0.06)	(0.01)			
α-D-Glc*p*NAc-(1→	5.08 [4.1]	4.03	3.74	3.58	3.85			2.18
E	(−0.13)	(0.15)	(−0.01)	(0.09)	(−0.02)			(0.12)
α-D-Gal*p*-(1→	4.96 [3.4]	3.85	3.92	4.04	4.37	3.71	3.71	
F	(−0.26)	(0.07)	(0.11)	(0.09)	(0.32)	(0.02)	(0.02)	
→2)-β-D-Glc*p*-(1→	4.62 [7.8]	3.49	3.58	3.44	3.44			
G	(−0.02)	(0.24)	(0.08)	(0.02)	(0.05)			
→4)-β-D-Gal*p*-(1→	4.54 [6.9]	3.59	3.76	4.06	3.81			
H	(0.01)	(0.14)	(0.17)	(0.17)	(0.16)			
	H-3*ax*	H-3*eq*	H-4	H-5	H-6	H-7	H-8a	H-8b
→5)-Kdo*p*	2.04	1.88	4.15	4.08	3.59	4.00	3.81	3.64
Kdo	(0.25) [b]	(−0.15)	(0.11)	(0.06)	(0.03)	(0.04)	(−0.14)	(−0.04)

[a] Chemical shift differences compared to the corresponding monomers are given in parentheses and $^3J_{H,H}$ values [Hz] are given in square brackets.
[b] Chemical shift differences compared to α-methyl Kdo are given in parentheses [29].

Table 3
^{13}C NMR data at 25°C for the oligosaccharide isolated from *Moraxella* (*Branhamella*) *catarrhalis* Serotype A (ATCC 25238)

Sugar residue	Chemical shifts [a] (δ)							
	C-1	C-2	C-3	C-4	C-5	C-6	NAc	C=O
→ 4)-α-D-Glc*p*-(1 →	97.0 [175]	72.3	72.3	79.6	71.3			
A	(4.0)	(0.2)	(0.5)	(8.9)	(−1.1)			
→ 2)-β-D-Glc*p*-(1 →	98.1 [166]	81.9	75.5	70.5	76.5			
B	(0.8)	(6.7)	(−1.3)	(−0.2)	(−0.3)			
→ 3,4,6)-α-D-Glc*p*-(1 →	100.3 [172]	74.3	75.9	74.5	70.5	68.6		
C	(7.3)	(−0.2)	(2.4)	(3.9)	(−2.0)	(6.8)		
β-D-Glc*p*-(1 →	103.0 [163]	74.6	76.9	70.5	76.8			
D	(6.3)	(−0.7)	(0.1)	(−0.2)	(0.0)			
α-D-Glc*p*NAc-(1 →	99.6 [174]	54.4	72.9	70.7	72.1		23.5	175.1
E	(7.8)	(−0.6)	(1.2)	(−0.6)	(−0.3)		(0.6)	(0.0)
α-D-Gal*p*-(1 →	101.1 [171]	69.4	69.9	69.7	71.6			
F	(7.9)	(0.6)	(0.2)	(−0.6)	(0.3)			
→ 2)-β-D-Glc*p*-(1 →	103.3 [163]	76.3	75.3	70.4	76.5			
G	(6.5)	(1.1)	(−1.5)	(−0.3)	(−0.3)			
→ 4)-β-D-Gal*p*-(1 →	104.1 [164]	71.7	73.0	78.1	76.2			
H	(6.2)	(−1.3)	(−0.8)	(8.4)	(0.3)			
	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
→ 5)-Kdo*p*	177.3	97.2	34.9	66.5	75.9	72.7	73.0	63.9
Kdo	(1.3) [b]	(−4.1)	(0.0)	(−0.3)	(8.7)	(0.4)	(2.8)	(0.2)

[a] Chemical shift differences compared to monomers are given in parentheses and $^1J_{C,H}$ values [Hz] are given in square brackets. [b] Chemical shift differences compared to α-methyl Kdo are given in parentheses [29].

NOE contact to H-4 in residue **H**, at 4.06 ppm, and an intraresidual NOE to H-2 at 3.85 ppm. No NOE was observed from any other residue to residue **F**, further demonstrating that **F** is terminal. Residue **H** (-4-β-D-Gal-) has an NOE contact to H-4 in residue **A**, in addition to internal NOEs to H-2, H-3, and H-5. The anomeric proton of residue **A** (-4-α-D-Glc-) has an interresidue NOE to H-2 in **G**, as well as to H-2 internally. This makes up the tetrasaccharide **F–H–A–G**. A disaccharide branch could be constructed from residues **E** (α-D-GlcNAc) and **B** (-2-β-D-Glc-) as an interresidue NOE between H-1 in **E** and H-2 in **B** at 3.39 ppm was observed, establishing a (1 → 2) linkage and the disaccharide element **E–B**. Residue **E** was also devoid of other NOEs, but for the internal to H-2, corroborating that it was terminal. For residue **B**, internal NOEs from H-1 to H-3 and H-5 were observed, thus demonstrating the β configuration.

The remaining points to be established are which of the residues **B**, **D**, and **G** substitute the branch-point residue **C** at each of the positions 3, 4, and 6. The NOE contacts observed do, however, not give a straightforward answer to the substitution pattern. H-1 in **B** has an NOE to a signal at 4.53 ppm. This corresponds to H-3 in residue **C** but, is also very close to the chemical shift of H-1 in **H**. The latter is however already shown to be linked to **A**. The observed

Table 4
NOE data for the oligosaccharide isolated from *Moraxella* (*Branhamella*) *catarrhalis* Serotype A (ATCC 25238), obtained at 500 and 600 MHz, using mixing times of 350 and 200 ms, respectively, at 25°C

Anomeric proton		NOE contact to proton		
Residue	δ	δ	Intensity [a]	Residue, atom
→ 4)-α-D-Glc*p*-(1 →	5.41	3.45	s	**A** H-2
A		3.49	s	**G** H-2
		3.95	w	**C** H-4
→ 2)-β-D-Glc*p*-(1 →	5.20	3.51	s	**B** H-5
B		3.60	m	**B** H-3
		3.95	vw	**C** H-4
		4.53	s	**C** H-3
→ 3,4,6)-α-D-Glc*p*-(1 →	5.16	3.90	s	**C** H-2
C		4.08	s	Kdo H-5
β-D-Glc*p*-(1 →	5.08	3.55	s	**D** H-3
D		4.53	m	**C** H-3
α-D-Glc*p*NAc-(1 →	5.08	3.39	s	**B** H-2
E		4.03	s	**E** H-2
α-D-Gal*p*-(1 →	4.96	3.85	m	**F** H-2
F		4.06	m	**H** H-4
→ 2)-β-D-Glc*p*-(1 →	4.62	3.44	s	**G** H-5
G		3.49	m	**G** H-2
		3.58	s	**G** H-3
		3.95	vw	**C** H-4
		4.02	s	**C** H-6a [b]
		4.15	s	**C** H-6b [b]
→ 4)-β-D-Gal*p*-(1 → [c]	4.54	3.59	w	**H** H-2
H		3.64	s	**A** H-4
		3.76	s	**H** H-3
		3.81	s	**H** H-5

[a] The intensities are estimated and are given as, s = strong, m = medium, w = weak, vw = very weak. [b] Tentative assignment. See test for additional details. [c] NOE contacts assumed to derive from H-5 are omitted.

cross-peak corresponds to what should be expected from a **B** H-1/**C** H-3 cross-peak and is therefore assigned to an NOE contact between **B** and **C**. The anomeric proton of residue **D** has, in addition to an internal NOE contact to H-3, NOE contact with a proton of δ 4.53, thus possibly H-3 in **C**, or H-1 in **H**. For the same reasons as above, it is assigned to an NOE contact between **D** and **C**. The possible reason for obtaining two NOE contacts to H-3 is discussed below. Residue **G** with an H-1 chemical shift of 4.62 ppm has internal NOEs to H-2, H-3, an H-5. Correlations from the signal at 4.62 ppm, which apart from being assigned to H-1 of **G** is assigned to H-5 of **C**, are also observed to 3.95, 4.02, and 4.15 ppm, which are assigned to H-4, H-6a and H-6b, respectively, of residue **C**. It is not possible to say whether these derive from H-1 of residue **G** or H-5 of residue **C**, respectively. Thus, very little evidence is provided by NOE data to unravel the substitution pattern of residue **C**.

Table 5
Observed $^2J_{H,C}$ and $^3J_{H,C}$ connectivities in an HMBC experiment (60-ms delay) for the anomeric nuclei of the oligosaccharide isolated from *Moraxella* (*Branhamella*) *catarrhalis* Serotype A (ATCC 25238).

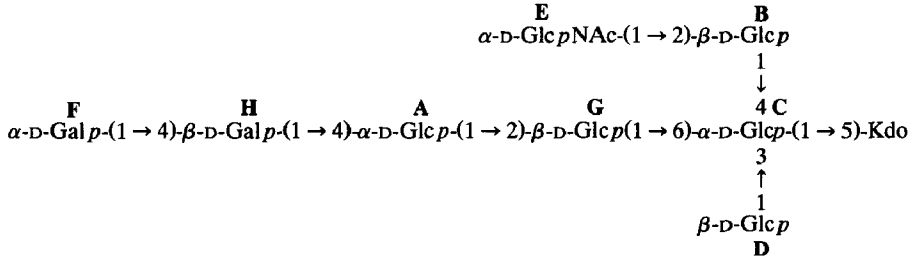
Anomeric nucleus			$J_{H,C}$ connectivities to		
Residue	$\delta(^1H)$	$\delta(^{13}C)$	$\delta(^1H)$	$\delta(^{13}C)$	Residue, atom
→ 4)-α-D-Glc*p*-(1 →		97.0	3.49		**G**, H-2
A	5.41			71.3	**A**, C-5
→ 2)-β-D-Glc*p*-(1 →		98.1	3.95		**C**, H-4
B		98.1	3.39		**B**, H-2
→ 3,4,6)-α-D-Glc*p*-(1 →	5.16			75.9	**C**, C-3, and/or Kdo, C-5
C	5.16			70.5	**C**, C-5
		100.3	4.08		Kdo, H-5
β-D-Glc*p*-(1 →		103.0	4.53		**C**, H-3
D		103.0	3.36		**D**, H-2
α-D-Glc*p*HAc-(1 →		99.6	3.39		**B**, H-2
E					
α-D-Gal*p*-(1 →		101.1	4.06		**H**, H-4
F	4.96			78.1	**H**, C-4
	4.96			71.6	**F**, C-5
	4.96			69.4	**F**, C-2
→ 2)-β-D-Glc*p*-(1 →		103.3	3.49		**G**, H-2
G	4.62 [a]			68.6	**C**, C-6
→ 4)-β-D-Gal*p*-(1 →		104.1	3.64		**A**, H-4
H		104.1	3.58		**H**, H-2
	4.54			79.6	**A**, C-4

[a] The signal of H-1 of residue **G** overlaps completely with the signal of H-5 of residue **C**.

Evidence for the complete sequence was obtained from the HMBC spectra, which should show signals for the $^3J_{C,H}$ coupling between an anomeric proton and the linkage carbon or an anomeric carbon and the proton on the linkage carbon, in addition to less diagnostic intraresidue $^2J_{C,H}$ and $^3J_{C,H}$ couplings (Table 5). All of these $^3J_{C,H}$ connectivities are looked for but not always found. The presence of the tetrasaccharide branch **F**–**H**–**A**–**G** was thus corroborated. First, the observation of both a $^3J_{C,H}$ coupling between H-1 of **F** to C-4 of **H** as well as between C-1 of **F** and H-4 of **H** demonstrates the element **F**–**H**. The connection **H** to **A** was established by observed *J* couplings from H-1 in **H** to C-4 in **A**, and C-1 in **H** to H-4 in **A**. As the signals for both H-1 in **H** and H-3 in **C** are found at ca. 4.54 ppm, there could be ambiguities, but the observation is in accord with the other results. Residue **A** gives no interresidue correlation from H-1 but C-1 gives a coupling to H-2 in **G**, thus establishing the (1 → 2) linkage. In an analogous manner, **E** was shown to be linked to the 2-position of **B** through a long-range coupling from the anomeric carbon. The connection between residue **C** and Kdo is indicated through a correlation between H-1 in residue **C** and a signal at 75.9 which could be C-5 in Kdo, but possibly also C-3 in **C**. The correlation between C-1 in **C** and H-5 in Kdo is, however, unambiguous. Evidence of the substitution pattern of the branch-point residue **C** is obtained from residue **B**, as a correlation between the anomeric

carbon in **B** and H-4 in **C** is observed, thus establishing a (1 → 4) linkage. A correlation between the anomeric carbon in **D** and H-3 in **C** establishes the (1 → 3) linkage between these residues. The (1 → 6) linkage between residues **G** and **C** is indicated from a correlation between the anomeric proton in **G** and C-6 in **C** but, as the signal at 4.62 ppm also contains the H-5 resonance from residue **C**, it could also be a two-bond correlation between H-5 and C-6. However, it is the only possibility left, so **G** has to be linked to the 6-position.

From the combined evidence, the following structure is proposed for the polysaccharide part of the *Moraxella catarrhalis* serotype A, strain ATCC 25238 lipopolysaccharide. Another Kdo residue substitutes the 5-substituted Kdo at its 4-position.



The orientation of the tetrasaccharide **F–H–A–G** is dependent upon the rotation of the C-5–C-6 bond in the 3,4,6-substituted glucose residue. In reducing glucose, the hydroxymethyl rotamers are roughly equally distributed between the *gauche–gauche* and the *gauche–trans* conformations. In *Branhamella catarrhalis*, the hydroxymethyl group in the branch-point glucose residue is indicated to be mainly *gauche–gauche* as the $^{3}J_{H\text{-}5,H\text{-}6a}$ and $^{3}J_{H\text{-}5,H\text{-}6b}$ values are small. This is indicated from the fact that the multiplet coupling pattern between H-5, H-6a, and H-6b, as seen in the HMQC C-5–H-5 and COSY H-6a–H-6b cross-peaks, is represented by a broad doublet. This fact, as well as observed non-sequential NOEs, indicate that the overall conformation of the polysaccharide is changed compared to the conformation of its constituent disaccharides. Furthermore, anomalous chemical shifts are observed for the signals of the anomeric protons of the two β-D-glucose residues with H-1 resonances at 5.08 and 5.20 ppm. A probable reason for the large downfield shift of these anomeric signals is close proximity to oxygen atoms as a result of conformational changes.

3. Experimental

General methods.—Concentrations were performed under diminished pressure at < 40°C or by flushing with air at room temperature. For GLC, a Hewlett–Packard 5830 instrument fitted with a flame-ionisation detector was used. GLC–MS (EI) was performed on a Hewlett-Packard 5970 MSD. A Jeol SX102 instrument was used for the FABMS. Ions were produced by a beam of Xe atoms (6 keV),

using a matrix of glycerol. Fractionations of alditol acetates and partially methylated alditol acetates were performed on an HP-5 capillary column (25 m × 0.20 mm × 0.33 μm), using a temperature programme 180°C (3 min) → 250°C at 3°C/min. Gel permeation chromatography was performed on a column of Superdex 30 (Pharmacia AB), using aqueous 0.07 M pyridinium acetate buffer (pH 5.4) as irrigant, and was monitored with a differential refractometer.

NMR spectroscopy.—^{1}H and ^{13}C NMR spectra were recorded with a Jeol Alpha 400, Varian Unity 500, or Varian Unity 600 spectrometer, using standard pulse sequences. Spectra of D_2O solutions were recorded at 25°C. Samples in D_2O were lyophilised twice with D_2O. Chemical shifts are reported in ppm, using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and acetone (δ_C 31.00) as internal references. Chemical shifts were obtained from 1D spectra when possible, or from proton–proton correlated 2D (COSY) spectra, relayed COSY, or total proton–proton correlated (TOCSY) spectra, with a digital resolution of 2.3 Hz/point. Coupling constants were obtained from 1D spectra or from COSY spectra. The TOCSY experiments were recorded using mixing times of 30, 60, and 90 ms. NOESY experiments were performed using mixing times of 200 and 350 ms. Proton–carbon correlated spectra (HMQC) were obtained with or without decoupling and the long-range proton–carbon correlated spectra (HMBC) were performed using a delay time of 60 ms.

Bacteria and cultivation.—*Moraxella catarrhalis* ATCC 25238 strain obtained from American Type Culture Collection (ATCC) was used in this study. Bacteria were grown overnight on horse blood agar plates at 37°C. Bacterial cultures used for the production of LPS were grown in brain heart infusion broth (Difco laboratories, Detroit, Michigan, USA) supplemented with 0.5% (w/v) yeast extract. A 50-L fermentor containing 30 L of medium was inoculated with 5 L of late logarithmic phase culture. The bacteria were grown at constant pH 7.2 to late logarithmic or early stationary phase and treated with formaldehyde to a final concentration of 1% and kept overnight at 4°C. The cells were harvested by centrifugation, washed once with PBS, suspended in H_2O, and lyophilised. Average yield was 8.3 g/L (dry weight).

Preparation of LPS.—LPS was extracted from lyophilised bacteria, using phenol–$CHCl_3$–petroleum ether, as described by Galanos et al. [20] with the following modification. LPS was precipitated by 6 vol of 1:5 diethyl ether–acetone [21]. No detectable amount of protein or nucleic acid was found in the LPS. The yield was 1.3 mg LPS/g (dry weight).

Sugar analysis.—The solution of LPS and PS (1 mg each) in 2 M CF_3CO_2H (0.5 mL) were kept in a closed vial at 120°C for 3 h. The sugars in the hydrolysate were then converted into alditol acetates by conventional methods.

Methylation analysis.—Methylation analyses were performed using a modification of the NaOH method [22,23]. Samples (1 mg) were dissolved in dimethyl sulfoxide (50 μL) and methylated by sequential addition of a NaOH slurry in dimethyl sulfoxide (120 mg/mL, 50μL; 20 min) and three additions of MeI (10, 10, and 20 μL) at 10-min intervals. After an additional 20 min, the methylated glycans were recovered in the organic phase after addition of $CHCl_3$ (0.5 mL × 3) and M

sodium thiosulfate (1 mL). The permethylated products were further purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge [24].

Identification of Kdo linkages.—LPS sample (2 mg) was dried in a desiccator under vacuum, methylated by following the method described earlier, and purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge. The dried permethylated LPS was subjected to methanolysis with M HCl in MeOH (0.1 mL) for 16 h at 85°C. Methanol was removed by flushing with N_2. The product was acetylated with 1:1 Ac_2O–pyridine for 30 min at 120°C. After removing the excess of pyridine and Ac_2O, the sample was used for analysis of Kdo by GLC–MS.

SDS-polyacrylamide gel electrophoresis.—LPS samples were suspended in sample buffer containing 0.1 M Tris · HCl (pH 6.8), 2% w/v SDS, 20% w/v sucrose, and 0.01% Bromophenol Blue in 1% dithiothreitol, to a concentration of 1 μg/μL, and heated at 95°C for 5 min. LPS in sample buffer was fractionated on a gel containing acrylamide (4%) in the stacking gel and acrylamide (18%) and 4 M urea in the separating gel. Each well was loaded with 10 μg of LPS preparation. SDS-PAGE was carried out at 100 V, 54 mA for the first 15 min, then 200 V, 54 mA until the front line reached the end of the gel. Bio Rad Mini Protein Electrophoresis Equipment was used. Silver staining was done as described [25] with the following modifications. The gel was oxidised with 1% periodic acid in aq 40% EtOH containing acetic acid (5%) for 25 min. Staining with $AgNO_3$ was done for 35 min. The R-type lipopolysaccharides from *Salmonella typhimurium* rough mutants were prepared as described by Galanos et al. [20].

Isolation of PS.—The LPS (100 mg) was suspended in aq 0.1 M sodium acetate (20 mL) containing 0.1% of SDS and was stirred for 5 min. Glacial acetic acid was added to pH 4.4 and the solution was kept at 100°C for 2 h [26,27]. After lyophilisation, the material was washed with EtOH (3 ×), suspended in water, and centrifuged. The supernatant solution was then applied to a Sep-Pak C18 cartridge and eluted with water. The eluate was lyophilised and purified by gel permeation chromatography. The polysaccharide was eluted at twice the void volume, and the solution was freeze-dried to yield 10 mg of the PS.

NMR assignments.—The hexose and hexosamine residues are designated **A**–**H**. The ^1H NMR chemical shifts of the residues are listed in Table 2 and the signals were assigned as described below. Chemical shifts for the hexoses [28] and for Kdo-OMe [29] were obtained from the literature. It was indicated from the $J_{H,H}$ coupling constants that all hexose rings were maintained in their normal $^4C_1$ conformation. H-1 to H-3 for residues **D** and **E**, H-1 to H-4 for residues **B**, **G**, and **H**, and H-1 to H-5 for residue **A** were unambiguously assigned using phase-sensitive double-quantum-filtered COSY and phase-sensitive TOCSY (mixing times, 30, 60, and 90 ms) experiments. These experiments also completely unravelled the proton spin systems of residues **C** and **F**. The chemical shifts for H-3 to H-5 of the Kdo residue were also assigned via the TOCSY experiments. The chemical shift for the H-5 nuclei of residues **B**, **D**, **G**, and **H** were assigned via phase-sensitive NOESY experiments (mixing times, 200 and 350 ms) using the anticipated intraresidual correlations found from the anomeric protons. Except for the primary protons, the remaining protons, i.e., **D** H-4, **E** H-4 and H-5, and Kdo H-6 and H-7,

were assigned tentatively from a reverse-detected broad-band decoupled HMQC experiment, where the experimental chemical shifts were assigned to the atoms having the closest shifts to the corresponding signals in the monosaccharides. The expected H,H scalar connectivities for H-6 and H-7 of the Kdo residue were found in the COSY experiment. The signals for the primary protons of the Kdo residue were assigned via the broad-band decoupled HMQC experiment, using the ^{13}C NMR chemical shift of the primary carbon at δ 63.9 ($\Delta\delta$ 0.2 ppm compared to the monosaccharide). The ^{13}C NMR signals were assigned via the decoupled HMQC experiment, using the proton assignments, and the chemical shifts are listed in Table 3. Carbon signals identified via tentatively assigned proton signals could, of course, only be assigned tentatively.

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