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A polysaccharide produced by a mucoid strain of *Moraxella nonliquefaciens* with a 2-acetamido-2-deoxy-5-*O*-(3-deoxy-β-D-*manno*-octulopyranosyl)-β-D-galactopyranosyl repeating unit

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ABSTRACT

A capsular polysaccharide, isolated from the mucoid *Moraxella nonliquefaciens* strain 3828/60, has been investigated by component analyses, periodate oxidation, methylation analyses, mass spectrometry, ^{1}H and ^{13}C NMR spectroscopy, and hydrolysis to give a disaccharide that was isolated and characterised. The results showed that the polysaccharide has the repeating unit → 3)-β-D-Gal*p*NAc-(1 → 5)-β-Kdo*p*-(2 →, with ~ 40% of O-8 of Kdo being acetylated.

INTRODUCTION

Capsular polysaccharides (CPSs) of Gram-negative pathogenic bacteria are often negatively charged, simple structures with repeating units of only one or two sugars. The acidic character is due to phosphate or carboxyl groups, often of *N*-acetylneuraminic acid (Neu5Ac) or of Kdo. These features are characteristic, for example, of several CPSs of *Escherichia coli*[1,2] and most CPSs of *Neisseria meningitidis*[2,3].

Although *Moraxella nonliquefaciens* is not usually encapsulated, several strains express a homopolysaccharide of Neu5Ac that is chemically and immunologically identical to the specific CPS of group B meningococci and the CPS K1 of *E. coli*[4]. We now report on another CPS (designated Mn2) of *M. nonliquefaciens* (strain

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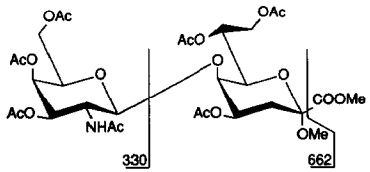
3828/60) with a repeating unit [→ 3)-β-D-Gal*p*NAc-(1 → 5)-β-Kdo*p*-(2 →] different from those of the CPS from *N. meningitidis* 29E [→ 3)-α-D-Gal*p*NAc-(1 → 7)-β-Kdo*p*-(2 →] (ref 5) and of *E. coli* CPS K14 [→ 6)-β-D-Gal*p*NAc-(1 → 5)-β-Kdo*p*-(2 →] (ref 6).

RESULTS AND DISCUSSION

Chemical analysis and mass spectrometry.—Sugar analysis of Mn2 and determination of the absolute configuration revealed D-GalNAc and Kdo to be present in equimolar amounts. Of the Kdo moieties, ~ 40% carried acetyl groups (see below). On a weight basis, these constituents accounted for ~ 90% of Mn2.

O-Deacetylated Mn2 consumed 1.2 mol of periodate per mol of Kdo during 35 h, but no distinct plateau was reached, even after 100 h, which is suggestive of overoxidation. GLC–MS of *O*-deacetylated, periodate-oxidised, and reduced Mn2 (methanolysed and trifluoroacetylated) revealed approximately equimolar amounts of derivatives of D-GalNAc and a presumed degradation product of Kdo. The mass spectrum of the latter contained abundant fragments with *m/z* 465, 351, and 237. These fragments correspond to the Kdo fragments[7] of *m/z* 591, 477, and 363, with a mass difference of 126 ($CHOCOCF_3$) which indicated elimination of C-8 of Kdo on periodate oxidation. The compound is assumed to be 3-deoxy-D-*lyxo*-heptulosonic acid, described[8] as a Kdo-degradation product of the CPS K14. Similarly treated CPS K14 provided the same sugar, whereas CPS 29E yielded only D-GalNAc, as expected[8] from the 7-linked Kdo. Hence, the resistance of the



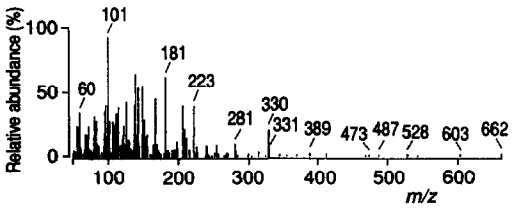
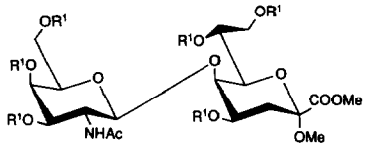


Fig. 1. EI-Mass spectrum of the disaccharide derivative **2**.

D-GalNAc of Mn2 to periodate indicated it to be 3- or 4-linked, and the formation of 3-deoxy-D-*lyxo*-heptulosonic acid indicated the Kdo to be 4- or 5-linked.

Mn2 consumed considerably less periodate than *O*-deacetylated Mn2 and the subsequent component analysis revealed Kdo, 3-deoxy-D-*lyxo*-heptulosonic acid, and D-GalNAc. The ratio of Kdo to the heptulosonic acid was ~1:1, which confirmed that ~50% of the Kdo residues of Mn2 were protected, and NMR spectroscopy revealed C-8 to be acetylated (see below).

Treatment of Mn2 with hydrazine at 80°C removed the *O*- and *N*-acetyl groups. Methanolysis of the product followed by acetylation and HPLC gave a disaccharide derivative (**2**), *O*-deacetylation of which gave **1**. CI(ammonia)MS of **2** gave ions with m/z 722 $[M + H]^+$ and 739 $[M + NH_4]^+$ indicating a MW of 721 (see Fig. 1). EIMS gave a characteristic ion at m/z 662 $[M - COOMe]^+$ and a fragment ion at m/z 330 (terminal D-GalNAc). These data accord with the structure methyl [methyl 5-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-galactopyranosyl)-4,7,8-tri-*O*-acetyl-3-deoxy-α-D-*manno*-2-octulopyranosid]onate for **2**.



1 R¹ = H

2 R¹ = Ac

Methylation analysis.—Methylation of Mn2 followed by hydrolysis, reduction with borohydride, and acetylation gave (GLC) a single alditol acetate derivative, EIMS of which gave abundant fragment ions at m/z 158 and 116, diagnostic of 2-amino-2-deoxy sugars[9]. The formation of primary fragments with m/z 274 (C-1/4) and 161 (C-4/6) is compatible with a 3-linked D-GalNAc moiety. The lack of products that originated from Kdo accords with results from other Kdo-containing polysaccharides[8,10,11].

Methanolysis of methylated Mn2 followed by acetylation gave a mixture that contained (GLC) six major components, three being derived from each of Kdo and D-GalNAc. The Kdo-derived peaks, characterised by the fragment with m/z 291 $(M - COOCH_3)^+$, were identical (retention times and mass spectra) with those obtained by similar treatment of CPS K14 and suggested the Kdo to be 5-linked. The three peaks of the methyl glycoside of D-GalNAc were superimposable with those obtained from CPS 29E.

Anomeric configuration.—The ^1H NMR spectrum of Mn2 exhibited a slightly resolved doublet at δ 4.66 (data not shown) and that of **1** at δ 4.646, $J_{1,2}$ 8.4 Hz (Table I), which corresponded to the H-1 signal of the 3-linked β-D-GalNAc in CPS K14 (δ 4.7, $J_{1,2}$ 8.6 Hz) (ref 6). Corresponding values for α-D-GalNAc are δ 4.7 and $J_{1,2}$ 3.6 Hz (ref 12). These data, together with the observed ^{13}C resonance

TABLE I

^{1}H NMR data [a] (δ in ppm, J in Hz) of the disaccharide derivatives **1** [b] and **2** [c] isolated from the polysaccharide Mn2 and reference compounds [c]

Atom	**1**		β-D-Gal*p*NAc-OMe		**2**		β-D-Gal*p* NAc-OMe (acetylated)	
	δ	J	δ	J	δ	J	δ	J
GalpNAc								
H-1	4.646	8.4	4.304	8.4	5.342	8.2	4.337	8.4
H-2	3.894	10.5	3.811	10.8	3.091	11.4	4.141	11.0
H-3	3.610	3.6	3.631	3.3	6.093	3.4	5.294	3.5
H-4	3.814	0.8	3.855	0.8	5.468	0.8	5.484	1.0
H-5	3.562	5.3	3.609	4.4	3.397	5.8	3.502	6.8
H-6a	3.563	11.4	3.686	11.7	3.904	11.1	4.217	10.8
H-6b	3.711	4.2	3.742	7.8	3.968	7.2	4.160	6.6
NH-2					4.544	7.1	4.414	9.6
NHAc	1.958				1.423		1.477	
			α-Kdo-OMe[27]				α-Kdo-OMe[28] (acetylated)	
Kdo								
H-3*ax*	1.878	12.2, 11.4 [d]	1.775	12.8, 12.2 [d]	2.257	12.2, 12.3 [d]	2.25	12.8
H-3*eq*	1.985	5.0	2.015	4.8	2.426	4.7	2.29	5
H-4	4.105	2.8	4.027	3.1	5.415	2.6	5.56	3
H-5	4.151	0.7	4.010	0.6	4.257	1.2	5.62	1.5
H-6	3.560	9.4	3.556	8.9	3.876	5.8	3.89	9.7
H-7	4.064	5.5	3.944	7.0	5.626	6.0	5.52	2.5
H-8a	3.633	11.3	3.665	12.5	4.385	12.1	4.67	12.5
H-8b	3.845	2.7	3.936	3.0	5.125	2.2	4.14	5
OMe	3.177				3.154			
COOMe	3.761				3.332			

[a] Additional OAc signals from **2**, 1.999, 1.951, 1.868, 1.7747, and 1.743 ppm; and from β-D-Gal*p*NAc-OMe (acetylated), 1.735, 1.648, and 1.635 ppm. [b] In CD_3OD (internal Me_4Si). [c] In C_6D_6 (internal Me_4Si). [d] $J_{3ax,3eq}$, $J_{3ax,4}$.

of C-1 [103.44 ppm (Table II); cf. 100.2 ppm for α-D-GalNAc (ref 5)], indicated the Gal*p*NAc in Mn2 to be β.

The ^{1}H resonances for Mn2 at δ 2.4 and 1.7 were assigned to H-3*eq* and H-3*ax* of β-Kdo (cf. $\delta \sim 2.0$ for both H-3*eq* and H-3*ax* in α-Kdo[13–15]). The corresponding signals for **1** (methyl α-ketoside) were δ 1.878 (H-3*ax*) and 1.985 (H-3*eq*) (Table I). Also, the C-1 signal (175.2 ppm) of *O*-deacetylated Mn2 (Table II) and the slightly downfield-shifted C-3 signal (35.9 ppm for β-Kdo, cf. 34.6 ppm for α-Kdo) indicate[5,6,16] the Kdo residue in Mn2 to be β.

Linkage analysis.—Since only the exocyclic part of Kdo (*O*-deacetylated) was susceptible to periodate oxidation, the Kdo moiety must be 4- or 5-linked. As seen from Table II, the ^{13}C signals of Kdo C-4 were in the same range as those for Mn2 (68.8 ppm), **1** (66.2 ppm), and β-Kdo-OMe (66.8 ppm). The corresponding Kdo C-5 signals were 74.5 ppm for Mn2 and 74.8 ppm for **1** (cf. 67.1 ppm for β-Kdo-OMe), indicative of 5-linked Kdo.

TABLE II

^{13}C NMR data (δ in ppm) [a] of the polysaccharide Mn2 [b], the disaccharide derivative **1** [c], and reference compounds [c]

Atom	Mn2	Mn2 *O*-deacetylated	**1**	Reference compound
*Gal*p*NAc*				β-D-Gal*p*NAc-OMe
C-1	103.4	103.6	103.9	103.3
C-2	51.8	51.7	54.2	53.2
C-3	75.2	75.2	71.7	72.0
C-4	68.8	68.8	68.6	68.7
C-5	75.9	76.0	72.2	76.0
C-6	61.8	61.8	61.9	61.8
NAc	23.5	23.5	23.0	23.0
Kdo				β-Kdo-OMe
C-1	172.6	175.2	170.5	176.2
C-2	103.4	103.6	100.2	101.2
C-3	35.6	35.9	34.6	34.9
C-4	68.8	68.8	66.2	66.8
C-5	74.5	74.6	74.8	67.1
C-6	74.3	74.1	75.8	72.1
C-7	69.8	69.7	71.7	70.1
C-8	64.5	64.8	63.1	63.9
C-8	66.0			

[a] Additional signals from Mn2: OAc 21.2 ppm; and from **1**: COOMe 53.5 and OMe 52.0 ppm. [b] In D_2O (external 1,4-dioxane, 125 MHz). [c] In CD_3OD (internal MeCN, 90.6 MHz).

The ^{13}C NMR data of the Gal*p*NAc moieties (Table II) revealed a downfield shift of the signal assigned to C-3 of Mn2 (75.2 ppm) compared to those of **1** (71.7 ppm) and β-D-Gal*p*NAc-OMe (72.0 ppm), indicating a Kdo-(2 → 3)-GalNAc linkage. Furthermore, the signal (51.8 ppm) assigned to GalNAc C-2 of Mn2 deviated from those of **1** (54.2 ppm), β-D-GalNAc[5] (53.2 ppm), and also of the β-D-GalNAc of CPS K14 (ref 6) (54.2 ppm). An upfield displacement of the C-2 signal of this magnitude from 3-substituted β-D-GalNAc is likely to occur[5,17]. Thus, the Gal*p*NAc moiety in Mn2 is 3-linked.

Assignment of O-acetyl groups.—The ^{13}C signal for OAc in Mn2 (21.2 ppm) was allocated to Kdo O-8 on the basis of the increase in the intensity of the ^{13}C signal at 64.8 ppm and the disappearance of the signal at 66.0 ppm on *O*-deacetylation (Table II). The presence of an OAc group was verified by the ^1H signal at δ 2.15 for Mn2 which disappeared on *O*-deacetylation (data not shown). The intensity of this signal compared to that for NAc indicated ~ 40% *O*-acetylation.

The foregoing data show that the Mn2 has the structure

→ 3)-β-D-Gal*p*NAc-(1 → 5)-β-Kdo*p*-(2 →

with ~ 40% of O-8 of Kdo being acetylated.

EXPERIMENTAL

β-D-GalNAc-OMe was prepared and purified by standard procedures. The α-Kdo-OMe was a gift from Paul Kosma (Universität für Bodenkultur, Wien, Austria). The CPS from *N. meningitidis* 29E was prepared as described[18,19] and CPS K14 of *E. coli* was obtained from K. Jann (Max-Planck-Institut für Immunbiologie, Freiburg-Zähringen, Germany).

Bacteria and cultivation.—The mucoid *M. nonliquefaciens* strains 3828/60 and 672/58 were isolated from nose and sputum, respectively, in cases of ozaena[20]. Preliminary investigations indicated that the CPSs from these strains were identical, and the CPS from strain 3828/60 was used. *N. meningitidis* strain 4-II was identified as group 29E by component analysis and group-specific antisera[4]. Bacteria were grown on solid Müller–Hinton medium (Difco) with 0.5% of yeast extract (Difco) in 20 Roux bottles for 24 h at 34°C in a 5% CO_2/air atmosphere.

Isolation of the CPS.—Cells of *M. nonliquefaciens* were harvested by flushing the agar surface with saline, then scraping the cells off the surface. All procedures were carried out at 4°C. To the bacterial suspension was added 1 vol of aq 0.2% cetyltrimethylammonium bromide (Cetavlon) followed by centrifugation at 10 000*g* for 20 min. 2 M $CaCl_2$ was added to the supernatant solution to a final concentration of 0.05 M, and Mn2 was then precipitated with 4 vol of EtOH[10]. Further purification was achieved by extraction with cold buffered phenol[10,18], followed by two cycles of dissolution in $CaCl_2$ and precipitation with EtOH (yield, 267 mg). Final purification[10] was effected by elution from a column (15 × 1.5 cm) of DEAE Sephadex A50 with 0.2 M sodium acetate buffer (pH 5.0), which contained 2 M NaCl. The eluate was dialysed against distilled water, freeze-dried, and stored at −20°C.

Isolation of the disaccharide derivatives 1 and 2.—Mn2 (40 mg) was *O*- and *N*-deacetylated by treatment with water-free hydrazine (5 mL, Kodak) in sealed ampoules at 80°C for 14 h. The mixture was poured into cold 2-butanol (30 mL) at 4°C followed by centrifugation (16 000*g*, 1 h). A solution of the sediment in water was dialysed, neutralised with Amberlite IR-120 (H^+) resin, and dried in a vacuum desiccator over P_2O_5 overnight. The product was methanolysed (M HCl–MeOH, 100°C, 4 h), then acetylated (Ac_2O–pyridine), and subjected to HPLC on Nucleosil 50-5 (Macherey & Nagel, two connected columns, each 25 × 0.7 cm), by elution with $CHCl_3$–MeOH (99:1) at 2.5 mL/min. Fractions (1 mL) were monitored by GLC and combined as appropriate to give the acetylated disaccharide derivative **2** (5.3 mg), $[\alpha]_D$ +20° (*c* 0.4, $CHCl_3$).

Compound **2** (5 mg) was treated with NaOMe (0.1 M in MeOH, 20°C, 14 h). The mixture was neutralised with Amberlite IR-120 (H^+) resin and the product was purified by HPLC on a column (25 × 0.9 cm) of ZORBAX-NH_2 (Bischoff), using a gradient of MeCN in water (3.5 mL/min; isocratic, 90% MeCN for 5 min, then a linear decrease to 60% in 20 min). The eluate was monitored at 200 nm and fractions were collected each 0.5 min. The *O*-deacetylated disaccharide derivative

1 (1.3 mg) was eluted as the main peak (retention time, 18.15 min; 41.5% area).

Analytical and chemical methods.—GalNAc was quantified by the Elson–Morgan procedure[21], and Kdo by GLC after methanolysis[7] in 2 M HCl in MeOH at 60°C for 2 h. For determination of absolute configuration, the samples were methanolysed and the constituents were separated on a cation-exchange cartridge (Bond Elut SCX, Analytichem International). Acidic constituents were eluted with 0.1 M CF_3CO_2H and the retained D-GalN-OMe was eluted with M NH_4OH. Methyl glycoside groups were hydrolysed with M CF_3CO_2H (4 h, 100°C). Absolute configuration was established by use of (−)- and (+)-2-butyl glycosides[22] by GLC of their trifluoroacetyl derivatives in comparison with reference compounds.

O-Deacetylation of CPS K14 and 29E was carried out in dil NH_4OH (pH 11) as described[6]. These conditions proved insufficient for Mn2, which required pH 12 for 16 h at 4°C.

Periodate oxidation was carried out in 0.02 M sodium periodate in the dark for 35 and 100 h. The excess of periodate was reduced with ethylene glycol, and the samples were dialysed against distilled water, reduced with $NaBH_4$ (1 mg/mL) overnight at 4°C, neutralised with acetic acid, dialysed, and freeze-dried[6].

CPS, converted into the free acid form by use of Dowex 50 (H^+) resin in order to improve the efficiency of methylation[23], were methylated[24,25] and extracted[26] by standard techniques. The methylated products were hydrolysed in 2 M CF_3CO_2H for 2 h at 120°C, reduced, acetylated, and extracted[26], or methanolysed (2 M HCl, 85°C, 18 h) and acetylated in Ac_2O–pyridine (1 : 1) for 4 h at 60°C.

GLC of monosaccharide derivatives involved a flame-ionisation detector and a fused-silica capillary column (25 m × 0.2 mm, SE 30). The detector temperature was 300°C, and the temperature program was 90°C for 4 min and then to 280°C at 8°C/min. GLC of oligosaccharide products from methanolysis followed by acetylation was achieved with a Varian 3700 gas chromatograph equipped with a fused-silica capillary column (30 m × 0.25 mm, SPB-5) and a temperature program of 300°C for 1 min then to 320°C at 5°C/min. GLC–MS was performed with a Varian 3300 gas chromatograph connected to Finnegan Ion-Trap 700 via a heated (250°C) fused-silica transfer line, and on a Hewlett–Packard 5985 instrument equipped with a fused-silica capillary column (25 m × 0.32 mm, SE-54, Supelco) and with the same temperature program as used above for GLC. Helium served as carrier gas. EI (70 eV) and CI (ammonia) mass spectra were recorded. The ion-source temperature was 200°C.

NMR spectroscopy.—The ^1H and ^{13}C NMR spectra were recorded (Biocarb AB, Lund, Sweden) on solutions in D_2O with a Bruker AM 500 spectrometer at 25 or 70°C. Chemical shifts are expressed relative to that of internal acetone at δ 2.225 (^1H) or to external 1,4-dioxane at δ 67.4 (^{13}C). The disaccharide derivatives **1** and **2** were analysed as solutions in CD_3OD and C_6D_6, respectively, with a Bruker AM 360 spectrometer at 25°C. Chemical shifts are expressed relative to those of internal standards [Me_4Si (^1H) and MeCN (^{13}C, 1.700 ppm) for **1**; and Me_4Si (^1H) and C_6D_6 (^{13}C, 128.0 ppm) for **2**]. All 1- and 2-D homo- and hetero-nuclear

correlated spectra were run on pulse sequences using standard Bruker software DISNMR version 89 11 01.0.

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