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Carbohydrate Research 319 (1999) 204–208

CARBOHYDRATE RESEARCH

Note

Structure of the O-specific polysaccharide for *Acinetobacter baumannii* serogroup O1

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Received 22 March 1999; accepted 28 May 1999

Abstract

A polymeric fraction containing D-galactose, *N*-acetyl-D-galactosamine, and *N*-acetyl-D-glucosamine was isolated from the lipopolysaccharide produced by the reference strain for *Acinetobacter baumannii* serogroup O1. By means of NMR spectroscopy, methylation analysis, and chemical degradation, the repeating unit of the polymer was identified as a branched trisaccharide of the following structure.

α-D-Gal*p*
1
↓
6
→3)-α-D-Glc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→

Keywords: *Acinetobacter baumannii*; Lipopolysaccharide; O1 Antigen, structure

1. Introduction

Acinetobacter baumannii is a Gram-negative, opportunistic pathogen responsible for outbreaks of nosocomial infection [1–4]. Strains may be differentiated serologically by their heat-stable O antigens, apparently corresponding to the polymeric side chains of the lipopolysaccharides (LPSs) produced [5–8]. Structural studies of these polymers in this laboratory and elsewhere ([9–11] and references cited therein) have probed the chemical diversity that underlies their serological specificity, and have shown that the polymers often have branched repeating units constructed from common sugars, with amino sugars as major components. Here we report a further example of such a polymer, derived from the LPS of the reference strain for *A. baumannii* serogroup O1 [5].

2. Results and discussion

LPS was extracted from defatted cell walls of the reference strain for *A. baumannii* O1 by treatment with hot aqueous phenol. The product (yield, ~40%) was recovered from the aqueous phase by exhaustive dialysis and

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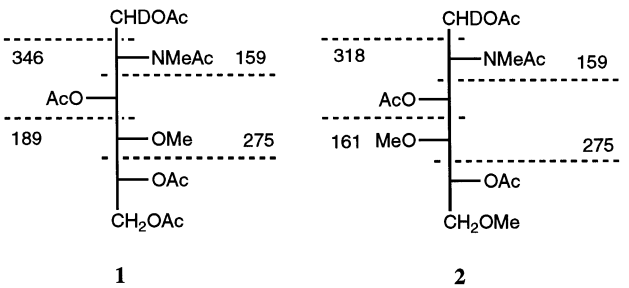
PII: S0008-6215(99)00144-5

freeze-drying. Mild acid hydrolysis of the LPS, followed by GPC of the water-soluble products on Sephadex G-50, provided a polymeric fraction containing D-Gal, D-GalN, and D-GlcN. Hydrolysates (2 M CF_3CO_2H, 98 °C, 16 h) also contained a basic, ninhydrin-positive compound with a mobility similar to that of the hexosamines on paper electrophoresis (pH 5.3) but with $R_{GlcN} \sim 0.8$ on PC (solvent A). As NMR spectra for the polymer (vide infra) did not provide evidence for components additional to the sugars named, it was assumed that the unidentified product was a minor contaminant or a disaccharide from incomplete hydrolysis.

The ^{1}H NMR spectrum of the polymer showed signals for anomeric protons at δ 5.00 (2 H, not resolved) and 4.60 (1 H, $J_{1,2}$ 7.7 Hz) and two *N*-acetyl methyl groups (δ 2.05 and 2.02) inter alia. The ^{13}C NMR spectrum (Fig. 1) consisted of 22 discrete signals, including those for three anomeric carbons (δ 101.99, 99.54, and 94.42), two *N*-acetyl groups (δ 175.01, 174.88, 23.81, and 23.44), two carbons attached to nitrogen (δ 53.02 and 52.23), and two unsubstituted hydroxymethyl carbons (δ 62.22 and 62.01). It could therefore be inferred that the polymer was based on a trisaccharide repeating-unit of Gal*p*, Gal*p*NAc, and Glc*p*NAc residues, that one of the residues was 6-substituted, and that only one had the β configuration. Gal*p*NAc could be identified as the β-linked residue from the relatively large chemical shift for both nitrogen-linked carbons, irrespective of assignment [12].

The products from methylation analysis of the polymer, examined by GLC–MS of the methylated alditol acetates (labelled with deuterium at C-1), were those derived from unsubstituted Gal*p*, a monosubstituted Hex*p*NAc (diagnostic fragment ion with *m*/*z* 318, containing C-2 to C-6), and a disubstituted Hex*p*NAc (diagnostic fragment ion with *m*/*z* 346, containing C-2 to C-6). The derivative from the disubstituted Hex*p*NAc also gave primary fragments with *m*/*z* 275 and 189 (see **1**, diagnostic for 3,6-substitution) and had the retention time of the Glc*p*NAc derivative [13]. Identification of the derivative from the monosubstituted Hex*p*NAc (Gal*p*NAc) was less clearcut. However, the virtual absence of primary fragment ions with *m*/*z* 233 and 203, and the presence of minor ions with *m*/*z* 275 and 161 (see **2**), indicated substitution at position 3 rather than 4.



From the results of the methylation analysis, it could be inferred that the O1 polymer

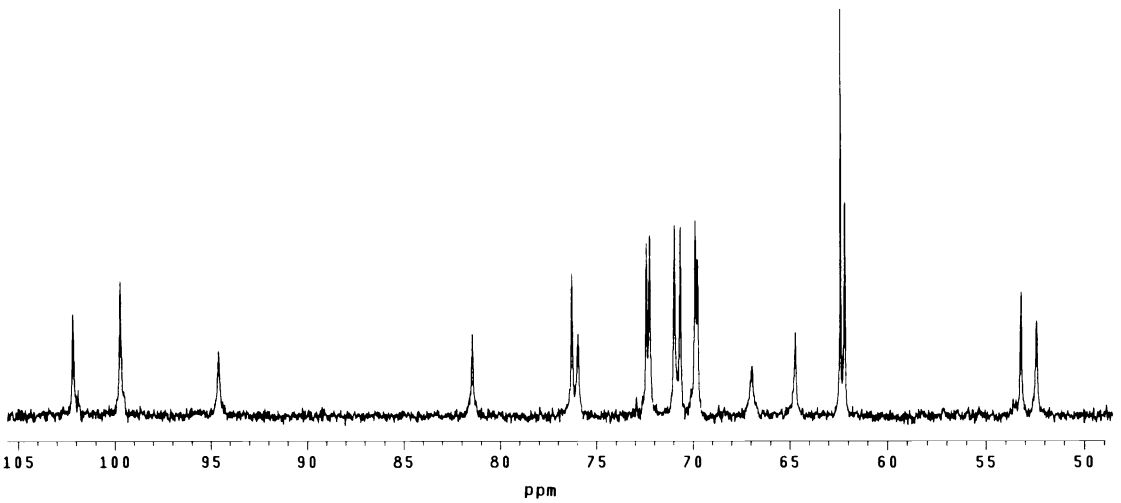


Fig. 1. ^{13}C NMR spectrum of the O1 polymer. The spectrum for the sample in D_2O was recorded at 70 °C and 150 MHz. In addition to the signals shown, the spectrum contained those for two *N*-acetyl groups (δ 175.01, 174.88, 23.81, 23.44).

Table 1
NMR data [a] for the O1 polymer

Atom		Residue		
		→3,6)-α-Glc*p*-NAc-(1→ **A**	α-Gal*p*-(1→ **B**	→3)-β-Gal*p*-NAc-(1→ **C**
1	H	5.00	5.00	4.60
	C	94.42	99.54	101.99
2	H	4.10	3.86	4.04
	C	53.02	69.70	52.23
3	H	3.75	3.89	3.83
	C	81.26	70.78	75.78
4	H	3.96	4.03	4.11
	C	72.07	70.48	64.54
5	H	~3.74 [b]	3.74 [b]	3.67
	C	69.59 [b]	72.24 [b]	76.10
6	H	4.03; 3.75	~3.76	~3.80
	C	66.77	62.22	62.01

[a] Values for chemical shifts relative to internal acetone (δ_H 2.22) or sodium 4,4-dimethyl-4-sila-2,2,3,3-($^2H_4$)pentanoate (δ_C 0.00). *N*-Acetyl signals (δ_H 2.05 and 2.02; δ_C 175.01, 174.88, 23.81 and 23.44) were also present.
[b] Tentative assignment.

was based on a branched trisaccharide unit, with Gal*p* as a lateral substituent and Glc*p*-NAc at the branch point. Repeated attempts to establish the outstanding structural details via a Smith degradation were thwarted by the low solubility of the product in water or dimethyl sulfoxide, whether it was recovered by rotary evaporation or lyophilization, and even after prolonged sonication, although sufficient material for an NMR spectrum (^1H) was recovered from one degradation. The spectrum contained the expected signals for two anomeric protons [δ 5.00 ($J_{1,2}$ ~3 Hz) and 4.59 ($J_{1,2}$ ~8 Hz)] and two *N*-acetyl groups (δ 2.02 and 2.01), consistent with removal of the α-Gal*p* units.

Assignments of the NMR data for the parent polymer were complicated by superposition of the signals for the anomeric protons in the α-linked residues, extensive overlap of signals for other ring protons, and limited correlations found in 2D NMR experiments (COSY, relayed COSY, TOCSY, and NOESY). To assign the data shown (Table 1), the residues were coded **A**, **B**, and **C** (the last being the β-GalNAc). The signals for H-2 and C-2 of GalNAc were identified from the COSY and HMQC spectra, thus permitting the corresponding assignments for GlcNAc (**A**) and Gal (**B**) by use of the same spectra and a process of elimination. Other signals in the ^{13}C NMR spectrum of immediate diagnostic value were those at δ 81.26, 76.10, 75.78, 66.77, and 64.54. The signal with δ 66.77 could be assigned (HMQC) to the 6-substituted carbon of **A**, and the magnitude of the downfield shift is consistent with α-D-glycosylation [14,15], pointing to the disaccharide unit **B**-(1→6)-**A**. The only reasonable assignment for the signal with δ 64.54 was C-4 of the β-GalNAc residue, shifted significantly upfield from its location (δ 68.55) in the free sugar [12] by α-D-glycosylation at the adjacent 3-position [16] as in **A**-(1→3)-**C**. The low-field signals in the range δ 75—82 of the ^{13}C NMR spectrum necessarily corresponded to other points of substitution [C-3 of the GlcNAc residue (**A**) and the GalNAc residue (**C**)], and C-5 of **C**, for which the δ values of the free monosaccharides [12] are 71.74, 72.01, and 75.98, respectively. As α-D-glycosylation should only give a $\Delta\delta$ shift of about +5 ppm for C-3 of **C** [16], the signal at δ 81.26 must be due to C-3 of **A**, which is subject to a larger glycosylation effect as a result of substitution by the β-D-residue **C** [16]. Differentiation of the two signals with δ ~76 (C-3 and C-5 of **C**) was based on the high-field location, appearance and coupling to H-6 (COSY) of the resolved signal for H-5. Overall, the NMR data for residue **C** are comparable with those reported [17] for a β-D-Gal*p*NAc residue in a similar stereochemical environment.

For the branch-point residue **A**, the signal corresponding to H-4, δ 3.96 (t), was clearly resolved, but H-5 could not be conclusively identified. Most of the remaining assignments (Table 1) for the unsubstituted α-D-Gal*p* residue **B** followed from the appearance (dd) of the signals for H-2 and H-3, a cross peak (COSY) between the latter and H-4, and correlations from the HMQC spectrum. Again, the data resemble those reported for such a terminal residue [18]. Further evidence for the location of the α-D-Gal*p* substituent could be drawn from the chemical shift (δ 99.54) of C-1, which corresponds to a glycosidation shift ($\Delta\delta$) of 6.36 ppm compared with the monosaccharide [12]. The expected glycosida-

tion shift for substitution at position 6 of the aglycon is ~6 ppm [14,15]. Likewise, the expected glycosidation shift for C-1 of the α-Glc*p*NAc if attached to position 3 of **C** is ~3 ppm [16] compared with the observed value of 2.65 ppm. Confirmation that the α-Gal*p* was attached to GlcNAc rather than GalNAc was obtained by N-deacetylation–deamination of the polymer. After reduction of the products, free 2,5-anhydrotalitol (but not 2,5-anhydromannitol) was identified by PC (solvent B) and by GLC–MS of the peracetate [19]. Thus, the repeating unit of the O1 polymer could be assigned the structure **3**, which shows marked similarity to that of the repeating unit (**4**) for the O antigen from *A. baumannii* strain 214 belonging to serogroup O22 [18]. Like the O1 polymer, that from strain 214 gave an insoluble product on Smith degradation [18].

B

α-D-Gal*p*
1
↓
6
→3)-α-D-Glc*p*NAc-(1→3)-β-D-Gal*p*NAc-(1→

A **C**

3

α-D-Gal*p*
1
↓
6
→3)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→

4

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—The reference strain for *A. baumannii* serogroup O1 [5] was grown twice in Nutrient Broth no. 2 (Oxoid, 20 L) for 24 h at 30 °C, with aeration at 30 L/min and stirring at 300 rpm (yields of wet cells, 105 and 108 g). The cells were disintegrated (Dyno Mill KDL), and LPS (yields, 0.91 and 0.99 g) was extracted from the purified, defatted cell walls by treatment with 45% (w/w) aqueous phenol at 68 °C for 15 min [20]. The polymeric fraction was obtained by GPC (Sephadex G-50) of the water-soluble products released by mild hydrolysis of the LPS (aq 1% HOAc, 100 °C, 2 h).

General methods.—NMR spectra (^1H and ^{13}C) for the O1 polymer in deuterium oxide were obtained with a Varian DXR600S, a Jeol JNM-GX270, or a Jeol JNM-LA400 spectrometer. Spectra were routinely recorded at 70 °C with acetone (δ_H 2.22) or sodium 4,4-dimethyl-4-sila-2,2,3,3-($^2H_4$)pentanoate (δ_C 0.00) as internal reference (data cited). Standard pulse sequences were used to obtain 2D spectra (COSY, relayed COSY, TOCSY, NOESY, and HMQC). GLC separations were carried out with either a Carlo Erba Mega 5160 or a Perkin–Elmer Autosystem XL chromatograph, fitted with fused-silica capillary columns (25 m) of BP1, BP5 or BP10 (SGE). GLC–MS utilised a Finnigan MAT 1020B or GCQ instrument. Sugars were separated by HPAEC using a CarboPac PA100 column in a Dionex DX-300 system. PC and high-voltage paper electrophoresis (HVE) were carried out with Whatman no. 1 paper with solvents A, 5:5:3:1 EtOAc–pyridine–water–HOAc; B, 6:4:3 butan-1-ol-pyridine–water; C, 13:5:4 EtOAc–pyridine–water; D, 5:2:43 pyridine–HOAc–water (pH 5.3, for HVE). Detection reagents used were ninhydrin, aniline hydrogenoxalate, alkaline $AgNO_3$ and the periodate-Schiff reagents [21].

Determination of monosaccharide composition.—Samples were hydrolysed with 2 M HCl at 105 °C for 2 h (for neutral sugars), 6.1 M HCl at 105 °C for 4 h (for amino sugars), or 2 M CF_3CO_2H at 98 °C for 16 h (for all sugars). Products were identified by PC (solvents A and C), HPAEC, and by GLC and MS of the alditol acetates. Hexosamines were also examined by HVE, separated from Gal by adsorption to Dowex 50 resin (H^+ form) and resolved by cation-exchange chromatography [22]. The D configuration was determined for isolated Gal and GalN by the

combined use of D-galactose oxidase (EC 1.1.3.9) and peroxidase (EC 1.11.1.7) [23]. The configuration of GlcN was assigned by conversion to the but-2-yl glycosides [24], acetylation and GLC analysis.

Degradative methods.—Methylation analysis, monitored by GLC–MS of the partially methylated alditol acetates, was carried out by standard procedures [25–27]. For Smith degradations, samples were treated with 50 mM $NaIO_4$ at 4 °C for several days, followed by the addition of ethane-1,2-diol, reduction ($NaBH_4$), acidification (2 M HOAc), dialysis and freeze-drying. The oxidised polymer was hydrolysed with 1 M CF_3CO_2H at room temperature, and acid was removed by rotary evaporation or freeze drying. Insolubility of the product limited further analysis. Alkaline N-deacetylation of the $NaBH_4$-treated parent polymer with ~1.6 M NaOH was carried out in aq Me_2SO at 100 °C for 16 h [25] and, after dialysis, the product was deaminated and reduced [25]. The 2,5-anhydrohexitol formed was identified by PC (solvent B) and by GLC–MS of the tetra-acetate.

Acknowledgements

The authors thank the SERC for an allocation on the high-field NMR service at the University of Edinburgh. They also thank the staff of this service (Dr I.H. Sadler and Dr J.A. Parkinson) and our colleagues (Dr D.F. Ewing, B. Worthington, A.D. Roberts) for their contributions to this study. The culture of *A. baumannii* was kindly provided by Dr H.M. Aucken (Central Public Health Laboratory, London).

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