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Carbohydrate Research 301 (1997) 187–192

CARBOHYDRATE RESEARCH

Structure of the O18 antigen from *Acinetobacter baumannii*

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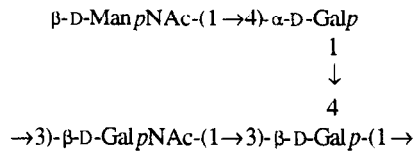
Received 11 December 1996; accepted 6 March 1997

Abstract

The polymeric O antigen was obtained from lipopolysaccharide extracted from isolated, defatted cell walls of the reference strain for *Acinetobacter baumannii* serogroup O18. Monosaccharide analyses and NMR spectra established that the polymer had a regular structure with a repeating unit based on residues of D-galactose (2), *N*-acetyl-D-galactosamine (1), and *N*-acetyl-D-mannosamine (1). Further interpretation of the NMR spectra, combined with the results of methylation analysis and a Smith degradation, showed that the repeating unit had the following structure.



Keywords: *Acinetobacter baumannii*; Lipopolysaccharide; O18 Antigen, structure

1. Introduction

There is much current interest in both the delineation (by phenotype and by genotype) and the differentiation of strains belonging to the bacterial species *Acinetobacter baumannii* [1–3], partly because of clinical interest in the organism as an opportunistic pathogen [4]. Although not widely used for epidemiological purposes, the possibility of serological typing of strains by their heat-stable O antigens has been demonstrated [5–7], and useful progress in chemical characterisation of the relevant lipopolysaccharide (LPS)-derived polymers has been made [8–15]. All of the structures established to date have branched repeating-units containing *N*-acylamino sugars. In some cases, e.g. the O11 [12], O16 [13], and O22 [8] antigens, the component sugars are commonplace, in others, e.g. the O2 [10,14], O5 [11,14], and O23 [15] antigens, unusual sugars and/or acyl groups are present. Although most polymers seem to be neutral, some contain sugar acids, e.g. 2-acetamido-2-deoxy-D-galacturonic acid in the O5

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0008-6215/97/$17.00
PII S0008-6215(97)00095-5

antigen [11,14]. Here we describe the structure of the O18 antigen.

2. Results and discussion

LPS was recovered from the aqueous phase after extraction of defatted cell walls from the O18 reference strain of *A. baumannii* by the aqueous phenol method (yield, ~ 40%). The polysaccharide component was isolated by gel-permeation chromatography (Sephadex G-50) after mild hydrolysis (1% acetic acid, 100 °C, 2 h) of the LPS (yield, 52%). An impression of heterogeneity given by the elution profile was confirmed by NMR and monosaccharide analyses of the early and late fractions of the eluate. The material of higher M_r gave a clean ^{1}H NMR spectrum (*vide infra*), while Gal, GalN, and ManN (each as the D isomer) were identified after acid hydrolysis. The material corresponding to the tail of the main peak was very similar but contained small proportions of Rha and GlcN as additional components. Structural studies were confined to the material of higher M_r.

The ^{1}H NMR spectrum of the major polymer (Fig. 1) contained five signals in the anomeric region with δ 4.91 (2 H, unresolved), 4.74 (1 H, $J_{1,2}$ 7.7 Hz), 4.59 (1 H, d, J 4.6 Hz), 4.51 (1 H, m, unresolved), and 4.46 (1 H, d, $J_{1,2}$ 7.4 Hz). It was clear from an HMQC spectrum (Fig. 2) that the signals with δ 4.59

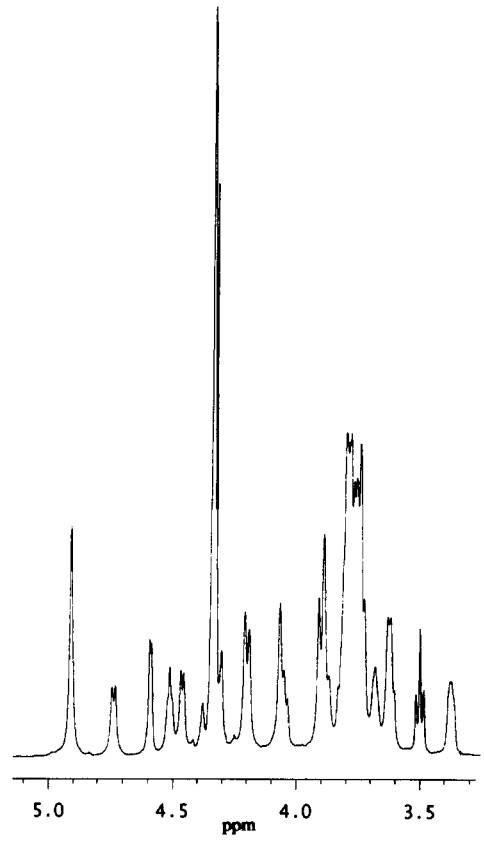


Fig. 1. ^{1}H NMR spectrum of the O18 polymer. The spectrum for the sample in D_2O was recorded at 600 MHz and 70 °C with acetone (δ 2.20) as the internal reference. In addition to the signals shown, the spectrum contained those for two *N*-acetyl groups (δ 2.06 and 2.01).

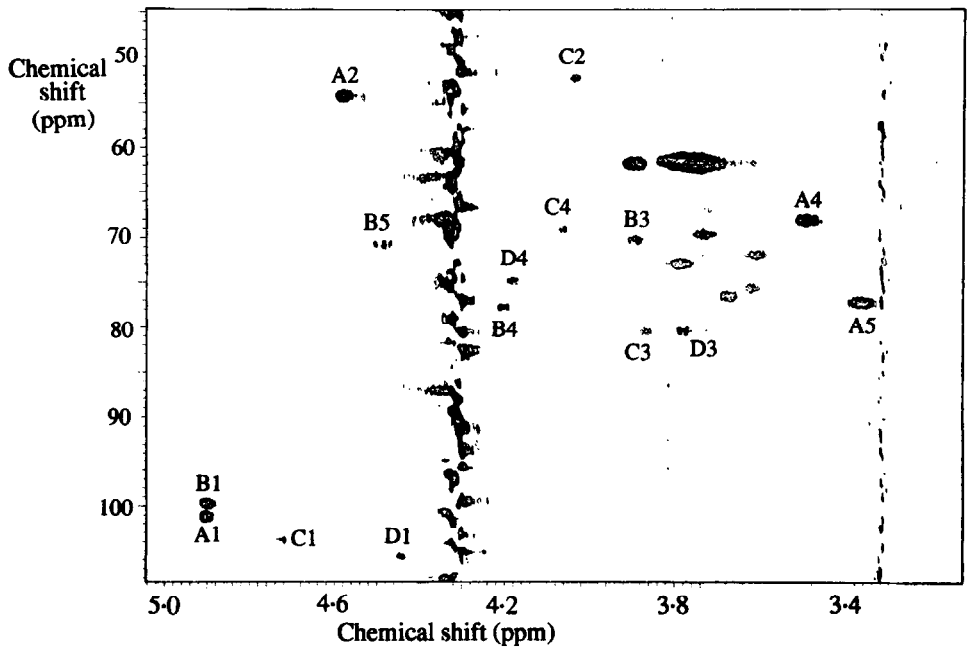


Fig. 2. HMQC spectrum for the O18 polymer. The spectrum for the sample in D_2O was recorded at 70 °C with a Varian INOVA600 spectrometer and acetone (δ_H 2.20, δ_C 31.05) as the internal reference. Signals for the *N*-acetyl groups are not shown.

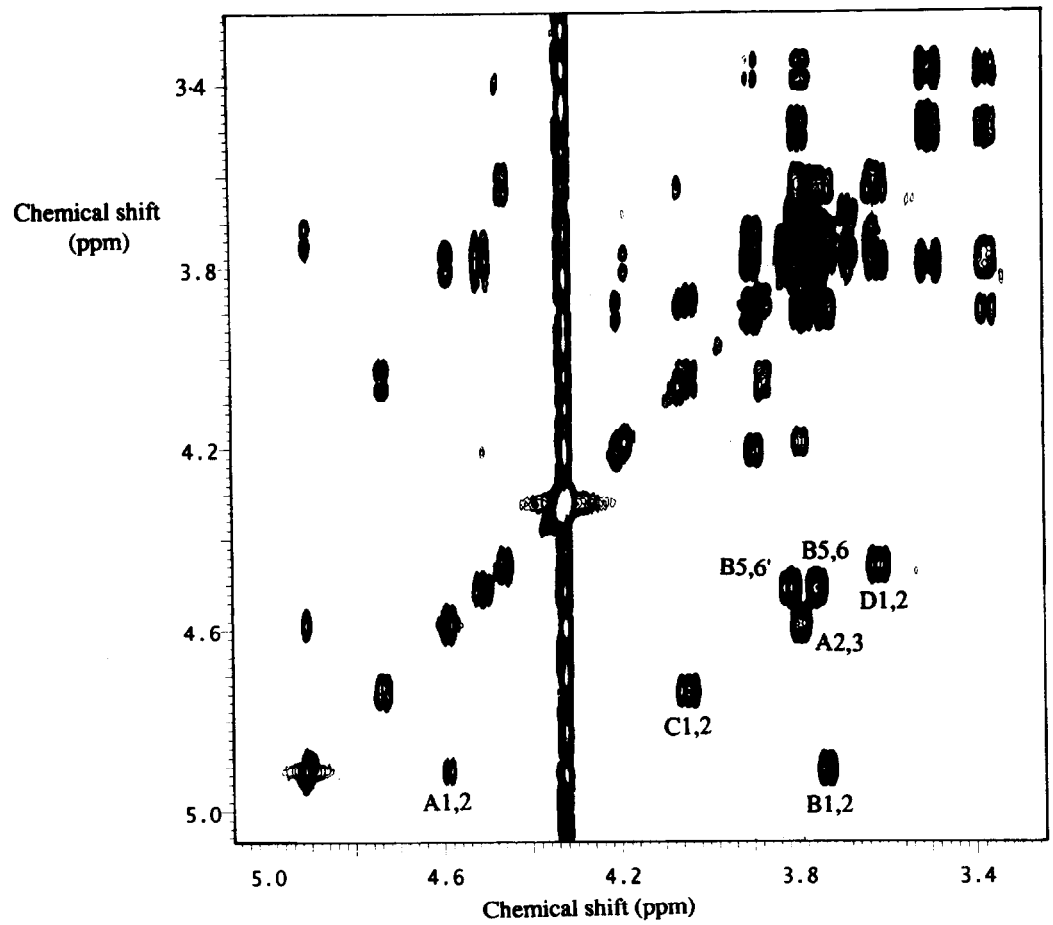


Fig. 3. COSY spectrum for the O18 polymer. Operating parameters were the same as for Fig. 1.

and 4.51 were not attributable to anomeric protons: a COSY spectrum (Fig. 3) showed coupling between the former signal and one of the protons with δ 4.91, indicating that these protons could be assigned to H-2 and H-1, respectively, of ManNAc [16]. The presence in the spectrum of methyl singlets with δ 2.06 and 2.01 showed that both hexosamines occurred as the *N*-acetyl derivatives, and this inference was supported by the presence of signals for two acetamido groups (δ 176.30, 175.31, 54.14, 52.43, 23.26, and 22.92) in the ^{13}C NMR spectrum. The ^{13}C NMR spectrum contained 27 discrete signals (that at δ 80.24 corresponding to 2 C), including 4 anomeric signals (δ 105.40, 103.58, 100.99, and 99.48), consistent with a tetrasaccharide repeating-unit containing residues of GalNAc (1), ManNAc (1), and Gal (2). The presence of four signals corresponding to hydroxymethyl carbons (δ 61.92, 61.68, 61.49, and 61.07) showed that no sugar residues were 6-substituted. The results of methylation analysis, monitored by GLC and MS of the methylated alditol acetates, showed that the polymer was branched and constructed from 4-substituted Gal, 3,4-disubstituted Gal, unsubstituted Man*p*NAc, and 3-substituted Gal*p*NAc residues (ratio of peak areas on GLC, 0.7:1.0:0.3:0.4).

Architectural details for the repeating unit of the O18 polymer were determined through interpretation of the ^{1}H and ^{13}C NMR spectra (Table 1) with the aid of COSY, relayed COSY, TOCSY, NOESY, and HMQC experiments. For this purpose, the sugar residues were coded **A–D** in order of decreasing chemical shift for the anomeric protons. The complete proton spin system for residue **A** was readily traced and the NMR data (Table 1) were consistent with its identification as unsubstituted β-D-Man*p*NAc [16]. The β-linked residue **C** was also readily identified as the 3-substituted D-Gal*p*NAc from the values (δ 52.43 and 80.24) of the chemical shifts for C-2 and C-3, respectively. The proton spin system (H-1 to H-4) and HMQC correlations for residue **B**

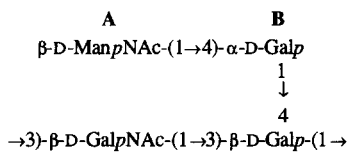
Table 1
NMR data for the O18 polymer [a]

Atom		Residue			
		β-ManNAc-(1 → A	→ 4)-α-Gal-(1 → B	→ 3)-β-GalNAc-(1 → C	→ 3,4)-β-Gal-(1 → D
1	H	4.91	4.91	4.74	4.46
	C	100.99 [b]	99.48 [b]	103.58	105.40
2	H	4.59	3.74	4.05	3.61
	C	54.14	69.56	52.43	71.83
3	H	3.79	3.90	3.88	3.78
	C	72.83	70.17	80.24	80.24
4	H	3.50	4.21	4.07	4.19
	C	68.03	77.80	69.12	74.83
5	H	3.39	4.51	3.63	3.68
	C	77.05	70.69	75.49	76.36
6	H	3.90	3.76	–	–
		3.79	3.82		
	C	61.07 [c]	61.49 [c]	61.68 [c]	61.92 [c]

[a] Values for chemical shift relative to acetone (δ_H 2.20; δ_C 31.05) as internal reference. The *N*-acetyl signals had δ_H 2.06, 2.01 and δ_C 176.30, 175.31, 23.26, 22.92.
[b,c] Assignments may be interchanged within each set.

led to its characterisation as 4-substituted D-Gal*p*: the α configuration was apparent from the NMR data for H-1 (δ 4.91, low value for $J_{1,2}$) and C-5 (δ 70.69), in particular. Finally, it was clear from the signals for the H-1 and C-1, and the glycosylation effects for C-3 and C-4, that residue **D** corresponded to 3,4-disubstituted β-D-Gal*p*.

The assignments of the anomeric configurations were confirmed by the intraresidue contacts at H-2 (for **B**) or H-3 and H-5 (**A**, **C**, and **D**) in the NOESY spectrum. Clear inter-residue contacts between H-1 of **C** and H-3 of **D**, and between H-1 of **D** and H-3 of **C**, showed that the backbone of the polymer was based on a disaccharide repeating-unit of these 3-substituted residues. Further NOE contacts involving H-1 of **A** and **B**, and H-4 of **B** and **D**, confirmed that a disaccharide branch (**A** → **B** →) was attached to position 4 of the branch-point residue **D**, as shown in structure **1**.



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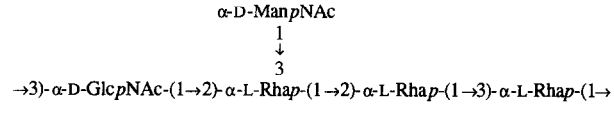

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Information which could be obtained in support of structure **1** for the O18 antigen through a Smith degradation was limited due to the water-insolubility of the β-1,3-linked, linear glycan produced, which prevented characterisation by NMR. However, methylation analysis of the product gave mainly the derivatives from 3-substituted Gal *p* and 3-substituted Gal*p*NAc, reinforcing the evidence for the location of the lateral disaccharide in the parent polymer.

Checks on the serological activity and specificity of both the whole LPS and the isolated polysaccharide, by using indirect haemagglutination tests and ELISA, revealed strong reactions with the homologous antiserum (LPS in both assays, isolated polysaccharide only by ELISA). Thus, the O18 antigen corresponds to structure **1**. A minor cross reaction between O10 and O18 serovars [5] could arise from the terminal ManNAc residue, which is also a feature of the O10 antigen although with the α configuration [9]. Other possible explanations are the presence in the O18 LPS of a minor polymer containing Rha and GlcNAc (also components of the O10 antigen, structure **2**), or even the presence of a small proportion of

the O10 polymer itself [9]. Evidence for mixtures of polymers has been reported for some other serovars of *A. baumannii* [12,15].

Structure **1** for the repeating unit of the O18 polymer of *A. baumannii* conforms to the general picture emerging for the O antigens of this species (branched, amino-sugars as components) and, like the structures for the O11 [12], O16 [13], and O22 [8] polymers, is assembled from common, neutral sugar residues. Finally, it should be noted that some of the strains so far examined may belong to the phenotypically similar genospecies 13, rather than *A. baumannii* (genospecies 2) [14,15]. This situation, which may involve the sharing of antigens by strains from different genospecies, clearly needs to be resolved.

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—The reference strain for *A. baumannii* O18 [5] was grown in Nutrient Broth no. 2 (Oxoid) for 24 h at 30 °C with aeration at 20 L/min and stirring at 300 rpm. The cells (110 g wet weight) were collected by continuous centrifugation (Sharples) with cooling, disintegrated (Dyno Mill KDL), and the cell walls were recovered and purified as in related studies [8]. LPS (2.3 g) was extracted from the defatted cell walls (5.4 g) by the standard procedure using hot aqueous phenol. The polymeric, water-soluble material released by mild hydrolysis (aq 1% HOAc, 100 °C, 2 h) of the LPS was isolated by chromatography on Sephadex G-50.

General methods.—NMR spectra (^1H and ^{13}C) for the O18 polymer in D_2O were obtained by using a Jeol-GX270 or a Varian INOVA600 spectrometer. The data reported were obtained with the Varian instrument at 70 °C with acetone (δ_H 2.20, δ_C 31.05) as internal reference. Two-dimensional spectra (COSY, relayed COSY, TOCSY, HMQC, and NOESY) were obtained by using standard pulse sequences. HPAEC utilised a CarboPac PA-100 column in a Dionex DX-300 instrument and elution with 0.1 M NaOH. GLC separations were carried out with a Carlo Erba Mega 5160 chromatograph fitted with a fused-silica capillary column (25 m) of BP1 (SGE). A Finnigan 1020B instrument was used for GLC–MS.

Determination of monosaccharide composition.—Hydrolysis conditions used for the release of neutral and amino sugars were those described for the O16 polymer [13]. Monosaccharides were identified by PC and HPAEC, and by GLC of the alditol acetates. For galactose and galactosamine, the results were confirmed by an enzymic assay [8], which also permitted assignment of the D configuration. GLC of the acetylated, diastereoisomeric but-2-yl glycosides [8,17] confirmed the D configuration for all three monosaccharides present in the O18 polymer.

Degradative methods.—Methylation analyses, monitored by GLC and MS of the methylated alditol acetates, followed standard procedures [18–20]. Conditions for the Smith degradation of the O18 polymer were those used for the O16 polymer [13], but precipitation of the degraded polymer prevented its chromatographic purification and further study by NMR spectroscopy.

Serological tests.—Procedures used for testing the O18 LPS and the isolated polysaccharide by an indirect haemagglutination assay and ELISA were those described for related studies [15,21].

Acknowledgements

We thank Drs H.M. Aucken and T.L. Pitt (Central Public Health Laboratory, London) for the reference strain of *A. baumannii* serogroup O18, and Prof. W.H. Traub for carrying out serological tests on the whole LPS and the isolated polysaccharide. We are also grateful to the S.E.R.C. for a grant to purchase the Dionex HPAEC instrument and for an allocation on the 600 MHz NMR service at the University of Edinburgh, and the University of Hull for a scholarship (S.R.H.). We are also indebted to the following: Drs J.A. Parkinson and I.H. Sadler (University of Edinburgh), and our colleagues Dr. D.F. Ewing and Mrs. B. Worthington for NMR spectra; Mr. A.D. Roberts for GLC–MS; Miss L. Galbraith for technical assistance.

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