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Structure of the O-7 antigen from *Acinetobacter baumannii*

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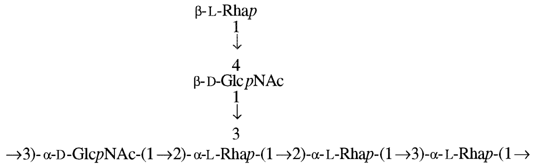
Abstract

The polymeric O-antigen was isolated from the lipopolysaccharide of the reference strain for *Acinetobacter baumannii* serogroup O-7. Both the lipopolysaccharide and the isolated polymer reacted with the homologous antiserum. Monosaccharide analyses and NMR spectra showed that the polymer had a hexasaccharide repeating unit constructed from residues of L-rhamnose (4) and *N*-acetyl-D-glucosamine (2). The following structure for the repeating unit was established by means of detailed interpretation of the NMR spectra, methylation analysis, and chemical degradations. The tetrasaccharide backbone is identical to that for the O-10 antigen of *A. baumannii*, which has α-D-Man*p*NAc as the lateral substituent in place of the disaccharide present in the O-7 antigen.



$$\begin{array}{rcl} & \beta\text{-L-Rha}p & \\ & 1 & \\ & \downarrow & \\ & 4 & \\ & \beta\text{-D-Glc}p\text{NAc} & \\ & 1 & \\ & \downarrow & \\ & 3 & \\ \rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 2)\text{-} & \alpha\text{-L-Rha}p & \text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \end{array}$$

Keywords: Acinetobacter baumannii; Lipopolysaccharide; O-7 antigen, structure

1. Introduction

The demonstration [1–3] that strains of *Acinetobacter baumannii* were amenable to serotyping by their heat-stable antigens prompted us to question the existing impression [4] that *Acinetobacter* spp. produced only *R*-type lipopolysaccharides (LPS), i.e., lacking an O-specific polymer. Examination in these laboratories of 13 reference strains revealed that all produced *S*-type LPS, and structures have been determined for polymers isolated from 10 of them [5–12]. Parallel studies in another laboratory of the O-anti-

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gens of strains from *A. baumannii* and other genospecies of *Acinetobacter* have confirmed and extended these findings [13–17]. Here, we report the structure of the O-antigen from the reference strain for *A. baumannii* serogroup 7.

2. Results and discussion

Water-soluble LPS was obtained by treatment of isolated cell walls with hot, aqueous phenol (yield, 30%). Mild acid hydrolysis of the LPS, followed by gel-permeation chromatography (Sephadex G-50), gave the polymeric, side-chain fraction (yield, 30%). The monosaccharide components were identified as L-Rha and D-GlcN (molar ratio, ~ 2:1). 1D NMR spectra for the polymer were consistent with a hexasaccharide repeating unit constructed from Rha*p* (4) and Glc*p*NAc (2) residues. Thus, the ^{1}H NMR spectrum contained signals (each 1 H) at δ 5.13, 4.99, 4.91, and 4.86 (all unresolved, Rha H-1), 4.95 ($J_{1,2}$ 2.9 Hz, α-GlcNAc H-1), and 4.74 ($J_{1,2}$ 7.2 Hz, β-GlcNAc H-1), as well as methyl singlets at δ 2.05 and 2.04 (*N*-acetyl), and four methyl doublets ($J_{5,6}$ ~ 6 Hz, Rha H-6) in the range δ 1.27–1.32. The ^{13}C NMR spectrum (Fig. 1) contained signals for six anomeric carbons at δ 103.62, ~ 101.7, 101.67, 101.49, 100.09 and 96.86, two *N*-acetyl groups (δ 175.33, 174.59, 23.21, and 22.91), two carbons linked to nitrogen (δ 56.94 and 54.06), C-6 of four Rha residues (δ 17.63, 17.58, 17.55, and 17.40), and two unsubstituted hydroxymethyl carbons (δ 62.05 and 61.40). Although the anomeric configurations of the GlcNAc residues were clear from the ^{1}H NMR spectrum, the corresponding assignments for Rha residues had to await more detailed interpretation of the NMR spectra (vide infra); extensive overlap of signals in a proton-coupled (gated) ^{13}C NMR spectrum limited assignments based on $^{1}J_{CH}$ values. Methylation analysis of the polymer, monitored by GLC and MS, identified the following residues (relative peak areas on GLC of the methylated alditol acetates): unsubstituted Rha*p* (0.23), 2-substituted Rha*p* (1.02), 3-substituted Rha*p* (0.81), 2,3-disubstituted Rha*p* (1.00), 4-substituted Glc*p*NAc (0.58), and 3-substituted Glc*p*NAc (0.46). The repeating unit of the O-7 antigen is, therefore, branched, with Rha residues at both the branch point and the non-reducing terminus.

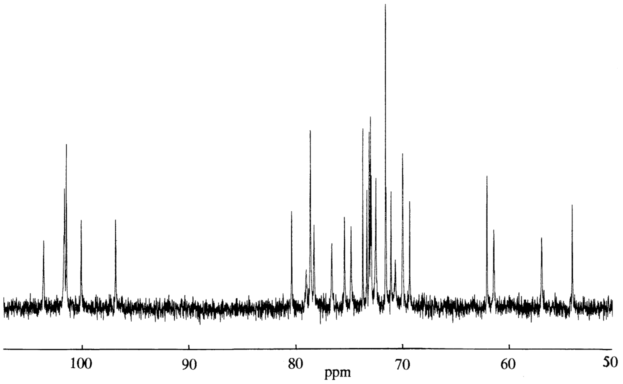


Fig. 1. ^{13}C NMR spectrum of the O-7 polymer. The spectrum for the sample in deuterium oxide was recorded at 100 MHz and 70 °C with acetone (δ 31.07) as the internal reference. In addition to the signals shown, the spectrum contained those for two *N*-acetyl groups (δ 175.33, 174.59, 23.21, 22.91) and C-6 of four Rha residues (δ 17.63, 17.59, 17.55, 17.40).

Almost all of the signals in the ^{1}H and ^{13}C NMR spectra could be assigned (Table 1) with the aid of COSY, relayed COSY, TOCSY, NOESY, and HMQC experiments. For this purpose, the monosaccharide residues were coded **A–F**, in order of decreasing chemical shift for the anomeric protons. Residues **C** and **F** were readily recognised as the GlcNAc residues (α and β, respectively) from the coupling constants for the anomeric protons and the chemical shifts for C-2. The anomeric configurations were confirmed by the intra-residue contacts in the NOESY spectrum between H-1 and H-2 (**C**), or H-1, H-3 and H-5 (**F**). The α configuration was similarly assigned to the Rha residues **A**, **B**, and **D** (contacts between H-1 and H-2 only) as well as from the relatively low-field location of the signal for H-5 in each case [18]. Evidence for the β configuration of the fourth Rha residue (**E**) was provided by the NOESY contacts of H-1 with H-3 and H-5, the location of H-5 (δ ~ 3.39), and the close fit overall of the proton sub-spectrum with that for unsubstituted β-Rha*p* [19]. Although the superposition of the signal for **E** H-2 with that for **A** H-2 made the assignments of the respective C-2 signals in the HMQC spectrum problematic, the spectral data (Table 1) point to **E** as the non-reducing terminal Rha residue. Substitution patterns for the remaining residues were clearcut from the large downfield glycosylation shifts at C-2 (**A**), C-3 (**C** and **D**), C-4 (**F**), and both C-2 and C-3 (**B**) compared with the parent monosaccharides [19].

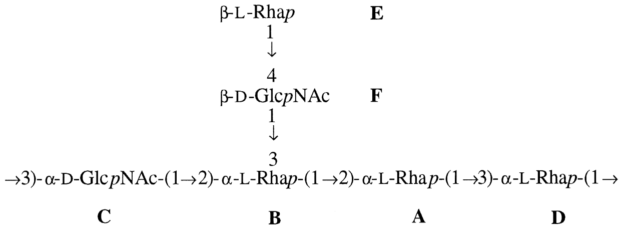
Information about the location and sequence of residues within the repeating unit was provided by inter-residue contacts in the NOESY spectrum. Thus, the disaccharide elements present in the backbone sequence of structure **1** $(\rightarrow \mathbf{A} \rightarrow \mathbf{D} \rightarrow \mathbf{C} \rightarrow \mathbf{B} \rightarrow)$ were apparent from the following contacts (Fig. 2): **A**

Table 1
NMR data[a] for the O-7 polymer

Atom		Residue					
		→ 2)-α-Rha*p*-(1 →	→ 2,3)-α-Rha*p*-(1 →	→ 3)-α-Glc*p*NAc-(1 →	→ 3)-α-Rha*p*-(1 →	β-Rha*p*-(1 →	→ 4)-β-Glc*p*NAc-(1 →
		A	**B**	**C**	**D**	**E**	**F**
1	H	5.13	4.99	4.95	4.91	4.86	4.74
	C	101.67	100.09	96.86	~ 101.7	101.49	103.62
2	H	4.07	4.24	4.04	3.85	4.07	3.71
	C	78.60	76.60	54.06	71.51	71.56	56.94
3	H	~ 3.91	3.96	3.78	~ 3.80	3.58	~ 3.71
	C	71.04	78.98	80.37	78.60	72.46	74.79
4	H	3.44	3.58	3.64	3.55	3.38	3.63
	C	73.30	73.68	69.28	72.46	72.97	78.26
5	H	~ 3.75	3.74	4.12	4.04	3.39	3.51
	C	69.95	70.64	72.90	69.95	73.09	75.40
6	H	1.30	1.30	~ 3.9	1.27	1.32	~ 3.82; ~ 3.94
	C	17.58[b]	17.55[b]	61.40	17.40[b]	17.63	62.05

[a] Values for chemical shift relative to acetone (δ_H 2.22; δ_C 31.07) recorded at 70 °C. The *N*-acetyl signals had δ_H 2.05 and 2.04, δ_C 175.33, 174.59, 23.21, and 22.91. [b] Set of values which may be interchanged.

H-1 with **D** H-3, **D** H-1 with **C** H-3, **C** H-1 with **B** H-2, and **B** H-1 with **A** H-2. The additional cross-peaks (Fig. 2) for **B** H-1 with **A** H-1 (minor), and **C** H-1 with **B** H-1 (major), are also consistent with substitution of the α-L-Rha residues (**A** and **B**) at position 2 [20]. The attachment of the disaccharide branch (**E** → **F** →) to position 3 of the Rha residue **B** was similarly clear from the contacts of **E** H-1 with **F** H-4, and **F** H-1 with **B** H-3 (Fig. 2).



$$\begin{array}{c}\beta\text{-L-Rha}p \qquad \mathbf{E}\\ 1\\ \downarrow\\ 4\\ \beta\text{-D-Glc}p\text{NAc} \qquad \mathbf{F}\\ 1\\ \downarrow\\ 3\\ \rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 2)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow\\ \mathbf{C} \qquad\qquad \mathbf{B} \qquad\qquad \mathbf{A} \qquad\qquad \mathbf{D}\end{array}$$

1

The spectral evidence for structure **1** as the repeating unit of the O-7 antigen was supported by the results of degradative studies. The expected outcome of a Smith degradation of the polymer was depolymerisation as a consequence of oxidation of the 2-substituted Rha residue **A**, and concomitant removal of the terminal Rha residue **E**. In accord with this, methylation analysis of the degradation product (SD1) yielded mainly the derivatives of the following residues: unsubstituted Rha*p*, 2,3-disubstituted Rha*p*, unsubstituted Glc*p*NAc, and 3-substituted Glc*p*NAc. However, although the ^{1}H NMR spectrum contained methyl signals for two Rha residues (δ 1.31 and 1.26) and two *N*-acetyl groups (δ 2.07 and 2.05), the anomeric region was complex and gel-permeation chromatography showed that depolymerisation was incomplete. Both the polymeric and oligomeric fractions of SD1 gave similar ^{1}H NMR spectra and methylation data. Side reactions (including the protection of residues through hemiacetal formation during the oxidative step, and transacetalation during the hydrolytic step) are well-documented problems in Smith degradations [21]. Nevertheless, the unpurified SD1 was subjected to a second Smith degradation to give SD2. Methylation analysis of oligomeric SD2 showed the presence of unsubstituted GlcNAc and 2-substituted Rha, in accord with the trisaccharide–

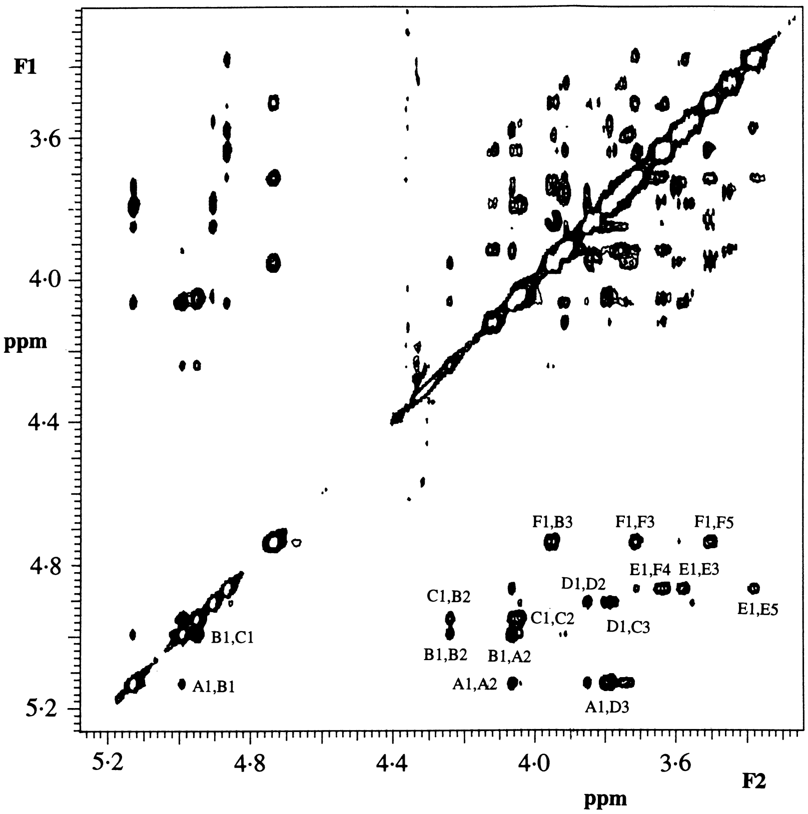


Fig. 2. NOESY spectrum of the O-7 polymer. The spectrum for the sample in deuterium oxide was recorded at 600 MHz and 70 °C with acetone (δ 2.22) as the internal reference.

alditol structure **2** derived from residues **C**, **B**, and **A** of the original polymer.

α-D-Glc*p*NAc-(1→2)-α-L-Rha*p*-(1→2)-Gro

2

Further evidence for structure **1** for the repeating unit of the O-7 polymer was obtained by *N*-deacetylation–deamination–reduction. When *N*-deacetylation of the reduced ($NaBH_4$) polymer was carried out under relatively mild conditions [22], the major product (DA1a) was polymeric and the ^1H NMR spectrum contained four signals for anomeric protons (each 1 H) at δ 5.17, 5.01, and 4.91 (each unresolved) and 4.97 ($J_{1,2} \sim 3$ Hz), as well as three methyl doublets in the range δ 1.23–1.35 ($J_{5,6} \sim 6$ Hz) and a methyl singlet (δ 2.05). These results are consistent with the formation of a polymer with the repeating sequence $\rightarrow \mathbf{A} \rightarrow \mathbf{D} \rightarrow \mathbf{C} \rightarrow \mathbf{B} \rightarrow$ by removal of the disaccharide branch $\mathbf{E} \rightarrow \mathbf{F} \rightarrow$, without *N*-deacetylation and deamination of the 3-substituted α-GlcNAc residue **C**. The minor deamination product (DA1b) was identified as the rhamnosyl derivative of 2,5-anhydro-D-mannitol produced from the $\mathbf{E} \rightarrow \mathbf{F} \rightarrow$ unit by its ^1H NMR spectrum (signals for H-1 and H-6 of β-Rha*p* at δ 4.78 and 1.31, respectively) and by FABMS of the permethylated compound, giving pseudomolecular ions with *m/z* 395 ($[M+H]^+$), 417 ($[M+Na]^+$), and 433 ($[M+K]^+$) and fragment ions with *m/z* 189 (aA_1 and bA_1) and 249 (abJ_1) [23].

N-Deacetylation of the reduced O-7 polymer under more drastic conditions [24], followed by deamination and reduction ($NaBH_4$), led to the formation of two oligomeric products, which were partially resolved by HPLC. The product with the higher M_r (DA2a) could be identified as the tetrasaccharide–alditol of structure **3**, derived from the backbone of the O-7 polymer, from the following data: (a) the ^1H NMR spectrum contained three major signals for anomeric protons (δ 5.17, $J_{1,2} \sim 1.2$ Hz; δ 4.97, $J_{1,2} \sim 1.2$ Hz; δ 4.85, $J_{1,2} \sim 1.5$ Hz) and three methyl doublets (Rha H-6) in the range δ 1.2–1.4; (b) FABMS of the permethylated derivative gave a pseudomolecular ion with *m/z* 765 ($[M+Na]^+$) and the fragment ion with *m/z* 189 as base peak; (c) methylation analysis showed the presence of unsubstituted, 2-substituted, and 3-substituted Rha as well as 3-substituted 2,5-anhydromannitol residues. The ^1H NMR data for the anomeric protons are in accord with those for the identical product derived from the O-10 antigen of *A. baumannii* [6]. The deamination product of lower M_r (DA2b) was the rhamnosyl-2,5-anhydromannitol (DA1b) already described.

α-L-Rha*p*-(1→2)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-2,5-Anhydro-D-mannitol

3

Checks for serological activity and specificity of whole LPS and the isolated O-7 polymer were carried out by an indirect haemagglutination assay (IHA) and by enzyme-linked immunoabsorbant assay (ELISA) [25]. LPS gave strong positive reactions with the homologous antiserum in both assays, whereas the isolated polymer was only recognised in ELISA, as found in previous studies of antigens from *A. baumannii* [7–9,11,12].

The O-7 antigen of *A. baumannii* is structurally very similar to the O-10 antigen [6]. Both are based on the same, linear tetrasaccharide ($\rightarrow \mathbf{A} \rightarrow \mathbf{D} \rightarrow \mathbf{C} \rightarrow \mathbf{B} \rightarrow$) of Rha (3) and GlcNAc (1) units. In the case of the O-10 polymer, the branch consists of a single α-D-Man*p*NAc substituent at the same position (O-3) of the Rha residue (**B**) as the disaccharide branch in the O-7 polymer. The absence of serological cross-reactions between O-7 and O-10 indicates that the branch constitutes the specific epitope. Curiously, the tetrasaccharide backbone present in the O-7 and O-10 polymers occurs in several other bacterial O-antigens. The unsubstituted (linear) polymer constitutes the O-antigen in *Serratia marcescens* O-22 [26] and several strains of *Ralstonia* (*Burkholderia* or *Pseudomonas*) *solanacearum* [27–29]. A related polymer with branching at the same point as the O-7 and O-10 antigens of *A. baumannii* and α-L-Rha*p* as the substituent has been described for *R. solanacearum* [28], and structurally similar polymers occur as O-antigens in diverse Gram-negative bacteria [30].

3. Experimental

Growth of bacteria, and isolation and fractionation of the LPS.—The reference strain for *A. baumannii* serogroup O-7 [1] was grown in Nutrient Broth no. 2 (Oxoid, 20 L) for 24 h at 30 °C with aeration at 20 L/min and stirring at 300 rpm. The cells (105 g wet weight) were collected (Sharples) and disintegrated (Dyno Mill KDL). LPS (1.35 g) was extracted from purified, defatted cell walls (4.5 g) by treatment with hot, aqueous PhOH [31] and was recovered from the aqueous phase. The water-soluble products released by mild hydrolysis of the LPS (aqueous 1% HOAc, 100 °C, 2 h) were fractionated by gel-permeation chromatography on Sephadex G-50.

General methods.—NMR spectra (^{1}H and ^{13}C) for the O-7 polymer in deuterium oxide were recorded with a Bruker WH-400 or a Varian DXR600S spectrometer at 70 °C with acetone (δ_H 2.22, δ_C 31.07) as internal reference. Standard pulse sequences were used to obtain 2D spectra (COSY, relayed COSY, TOCSY, NOESY, and HMQC). ^{1}H NMR spectra of degradation products from the O-7 polymer were recorded with a Jeol JNM-GX270 spectrometer. Methods and equipment used for PC, GLC, HPLC, HPAEC, EIMS and high-voltage paper electrophoresis (HVE) were those summarised in a related study [10].

Determination of monosaccharide composition.—The O-7 polymer was hydrolysed with 2 M CF_3CO_2H at 98 °C for 16 h [32]. The products were examined by PC, HPAEC, and GLC. After separation by HPLC on HPX-87P, the individual monosaccharides (Rha and GlcNAc) were converted into but-2-yl glycosides [33] (using racemic butan-2-ol or individual enantiomers) and acetylated to establish their absolute configuration by GLC of the disastereoisomeric products.

Degradative methods.—Methylation analyses, monitored by GLC and EIMS of the methylated alditol acetates, were carried out by standard procedures [24,34,35]. In Smith degradations, samples were treated with 0.05 M $NaIO_4$ for several days at 4 °C in the dark. After the addition of ethane-1,2-diol, reduction ($NaBH_4$), acidification (2 M HOAc), and dialysis or gel-permeation chromatography, the solution was freeze-dried. Products were routinely treated with 1 M CF_3CO_2H for 16 h at room temperature, followed by further reduction ($NaBH_4$) and fractionation by HPLC (TSKgel G-Oligo-PW). In attempts to improve the cleavage of acyclic acetals, treatments with 2 M CF_3CO_2H for 48 h at room temperature were also tried.

Alkaline *N*-deacetylation of reduced ($NaBH_4$) O-7 polymer was attempted under relatively mild [22] and more drastic [24] conditions, followed by deamination, reduction ($NaBH_4$), and fractionation by gel-permeation chromatography (Sephadex G-15 or TSKgel G-Oligo-PW), to give two sets of products (DA1a,b and DA2a,b, respectively).

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