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Carbohydrate Research 256 (1994) 303–317

CARBOHYDRATE RESEARCH

The structure of the lipopolysaccharide O antigen from *Yersinia ruckeri* serotype 01 *

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(Received August 13th, 1993; accepted October 15th, 1993)

Abstract

The O antigen obtained from the lipopolysaccharide of *Yersinia ruckeri* serotype 01, by mild acid hydrolysis, is composed of a branched tetrasaccharide repeating unit containing 2-acetamidino-2,6-dideoxy-L-galactose (L-FucAm), 2-acetamido-2-deoxy-D-glucose (D-GlcNAc), and 7-acetamido-3,5,7,9-tetradeoxy-5-(4-hydroxybutyramido)-D-*glycero*-L-*galacto*-nonulosonic acid (L-Sug).

Partial hydrolysis of the O antigen with 0.1 M HClafforded a trisaccharide and a tetrasaccharide having nonulosonic acid at their reducing ends. Cleavage of the O antigen with anhydrous methanolic hydrogen fluoride afforded the methyl glycoside derivatives of a trisaccharide and a tetrasaccharide. ^{1}H and ^{13}C NMR analysis, including ^{1}H–^{13}C heteronuclear multiple bond correlation spectroscopy to locate the *N*-acyl substituents, together with mass spectrometric analysis of the above oligosaccharides, allowed the structure of the O-specific polysaccharide to be assigned as:

$$\begin{array}{l} \rightarrow 3)\text{-}\alpha\text{-L-Fuc}p\text{Am-}(1 \rightarrow 3)\text{-}\alpha\text{-D-Glc}p\text{NAc-}(1 \rightarrow 8)\text{-}\alpha\text{-L-Sug-}(2 \rightarrow_n \\ \qquad\qquad\qquad\quad 4 \\ \qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\quad \beta\text{-D-Glc}p\text{NAc} \end{array}$$

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SSDI 0008-6215(93)E0387-G

1. Introduction

Yersinia ruckeri, the etiological agent of enteric redmouth disease in salmonid fish, is responsible for serious economic losses in the fish farming industry. The organism was first isolated in the USA in the 1950s, but it has since spread to Europe including Great Britain and Scandinavia [1]. Recently, Davies [2] proposed a serotyping scheme, based on heat-stable lipopolysaccharide (LPS) O antigens, in which five serotypes 01, 02, 05, 06, and 07 are recognized. Serotype 01, which is more virulent than the other serotypes, accounts for 91% of the European isolates.

We now report the structure of the O-specific polysaccharide of *Y. ruckeri* serotype 01.

2. Results and discussion

Extraction of *Y. ruckeri* serotype 01 cells (527 g wet weight) by a modified phenol–water procedure [3], followed by ultracentrifugation of the dialyzed concentrated aqueous phase, afforded two LPS fractions: a solid gel-like precipitate (Fraction A, 2 g) and an upper, less viscous product (Fraction B, 1.5 g). SDS–PAGE analysis [4] of the LPS showed a banding pattern typical of S-type LPS.

Partial hydrolysis of the LPS fractions (300 mg) with 1% acetic acid gave insoluble precipitates of lipid A (Fraction A; 74 mg, Fraction B; 22mg). Gel filtration of the clear solutions on Sephadex G-50 gave, as the sole products, high-molecular-weight polymers that eluted in the void volume (K_{av} 0.0, Fraction A, 147.4 mg, Fraction B, 120 mg). ^{1}H NMR spectral analysis showed the O polysacharides from both LPS fractions to be the same; the O polymer from the LPS Fraction B was used in all further experiments.

Characterization of the O-polysaccharide.—The O-polysaccharide had $[\alpha]_D$ + 11.7° (*c* 0.6, H_2O). Anal. Found: C, 51.3; H, 7.3; N, 4.4%. Complete acid hydrolysis of the O antigen and GLC–MS of the derived alditol acetates [5] showed it to contain D-glucosamine (D-GlcN) and L-fucosamine (L-FucN) in the molar ratios 1.9 : 1.0, the absolute configurations of the residues being established from their $[\alpha]_D$ values.

The ^{13}C NMR spectrum (Fig. 1) of the native polysaccharide showed four signals in the region for anomeric carbons: 104.6, 100.9, 97.7, and 94.1 ppm. There were five signals in the region characteristic of carbons attached to nitrogen (57.2–62.6 ppm), the signal at 54.7 ppm having an intensity of two. Signals at 17.7 and 15.7 ppm resulted from the presence of two CH_3 groups of two 6-deoxyhexoses and at 23.5 (intensity 2) and 23.1 ppm from the presence of three methyl groups of *N*-acetylated sugars. Two signals at 20.7 and 167.9 ppm were assigned to a CH_3 group and a C=N group, respectively, of an *N*-acetimidoyl substituent [6]. In addition, the low-field region of the spectrum also contained signals at 172.6 ppm for COOH, 174.7 ppm for C=O of two NAc substituents, and 177.3 for C=O of another acetamido substituent. A DEPT spectrum showed only three signals for

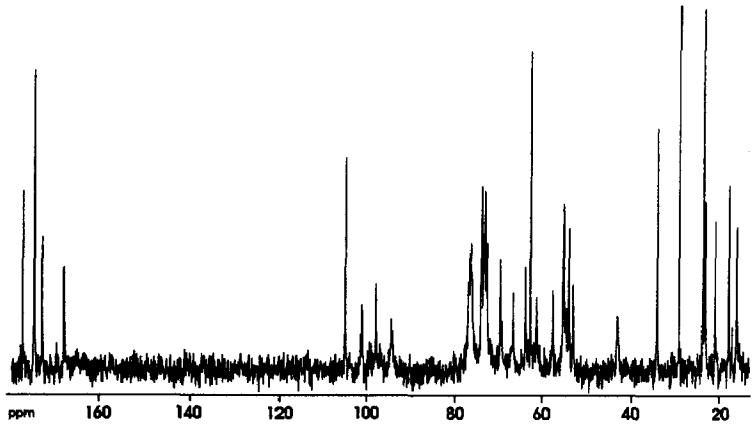


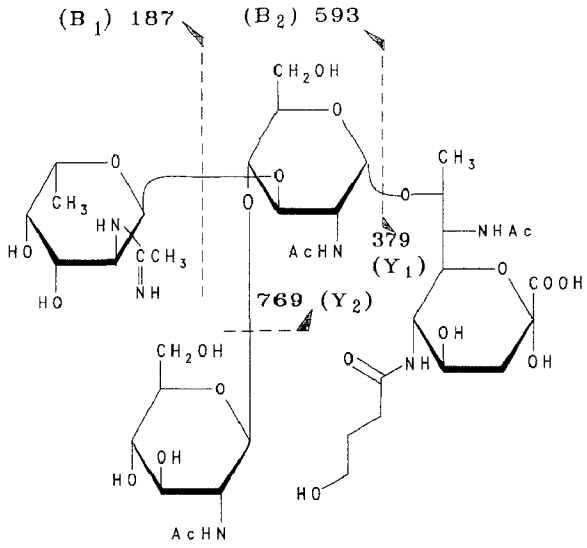
Fig. 1. The ^{13}C NMR spectrum of *Y. ruckeri* serotype 01 polysaccharide (180–15 ppm).

anomeric carbon atoms, and, hence, the signal at 104.6 ppm in the ^{13}C NMR spectrum (Fig. 1) was assigned to a quaternary ketose carbon. In addition, the DEPT spectrum contained signals for CH_2 at 42.8 ppm (deoxy sugar), at 60.8 and 63.4 ppm (CH_2OH of two hexopyranose residues), and at 28.9, 33.9, and 62.4 ppm. The assignments of these latter resonances were made via COSY and ^{13}C–^{1}H chemical shift correlation experiments made on the native polysaccharide. A ^{1}H spin system was identified in the COSY spectrum arising from a substituent that had signals at 2.35 (2 H), 1.87 (m, 2 H) and 3.67 ppm (t, 2 H). This led to the assignment of corresponding resonances in the ^{13}C and DEPT spectra and thus the identification of the substituent as a 4-hydroxybutyramido moiety. The connectivity pathway representative of the C-3 to C-9 carbon framework of a 3,9-dideoxynonulosonic acid was also identified in the COSY spectrum. This residue was further defined by NMR analysis of key oligosaccharide fragments (see below).

Methylation analysis of the polysaccharide, by GLC–EIMS of the derived methylated alditol acetates, indicated the presence of a L-Fuc$_p$N residue linked through position O-3, a terminal D-Glc$_p$N residue, and a branched D-Glc$_p$N residue linked through positions O-3 and O-4, although under methylation of the amino functions of all three residues was a persistent problem. A minor amount (ca. 9%) of a D-Glc*p*N residue linked through position O-3 was also present, suggesting that some repeating units lacked the terminal D-Glc*p*N residue and also indicating that the terminal D-Glc*p*N sugar is linked to position O-4 of the branched D-Glc*p*N residue.

The combined analytical results indicated the polysaccharide to be composed of a branched tetrasaccharide repeating unit of which one residue was a deoxyketose moiety. Furthermore, the repeating unit contains two acetamido and a 4-hydroxybutyramido substituent and one of the amino sugar residues carries an *N*-acetimidoyl group.

Partial hydrolysis of the polysaccharide with 0.1 M HCl cleaved the acid-labile ketose residue and gave oligosaccharides **1** and **2**. FABMS of underivatized **1**, in



1

Fig. 2. The structure of oligosaccharide **1** showing fragment ions obtained by FABMS in the positive-ion mode. The fragment ions at m/z 187 (B_1) and 379 (Y_1) indicate that the nonreducing and reducing sugars are the FucAm and ulosonic acid residues, respectively. The nomenclature of Domon and Costello [7] was used for the fragment ions.

the positive-ion mode, gave a protonated molecular ion at 972 and *inter alia* fragment ions at m/z 187 (B_1), 593 (B_2), 379 (Y_1), and 769 (Y_2) [7] (Fig. 2). In the positive-ion mode, underivatized **2** gave a $[M+H]^+$ ion at m/z 768, and an abundant fragment ion at m/z 187 (B_1). A CID FABMS–MS experiment on the $[M-H]^-$ ion (m/z 766) showed diagnostic daughter ions at m/z 580 (Y_2), 377 (Y_1), and 202 ($B_{2/1}$) (Fig. 3). These results indicate that **1** is a tetrasaccharide and **2** is a trisaccharide, both having nonreducing terminal L-Fuc_pAm residues (m/z 187). The fragments observed in the positive-ion mass spectrum for **1** at m/z 379, and at m/z 377 in the negative-ion mass spectrum of **2** indicate that the ketose residue forms the reducing terminal sugar and that it carries the 4-hydroxybutyramido substituent in both **1** and **2**.

Solvolysis of the polysaccharide with anhydrous HF in methanol gave two oligosaccharides **3** and **4**, which were obtained by gel filtration. Underivatized **3** and **4** were analysed by FABMS in the positive- and negative-ion modes. Oligosaccharide **3** gave a $[M+H]^+$ ion at m/z 985 and a $[M+Na]^+$ at m/z 1007, together with a major fragment ion at m/z 782 (Y_3) (Fig. 4). In the negative-ion mode, the molecular ion $[M-H]^-$ at m/z 983 gave rise to the corresponding fragment ion at m/z 780 (Y_3). Oligosaccharide **4** gave a $[M+H]^+$ ion at m/z 782 and a $[M+Na]^+$ ion at m/z 804 together with fragment ions at m/z 579 (Y_2) and 219 (Y_1) (Fig. 5). These results are consistent with **3** and **4** being the methyl glycoside derivatives of a tetra- and tri-saccharide, respectively, both having a

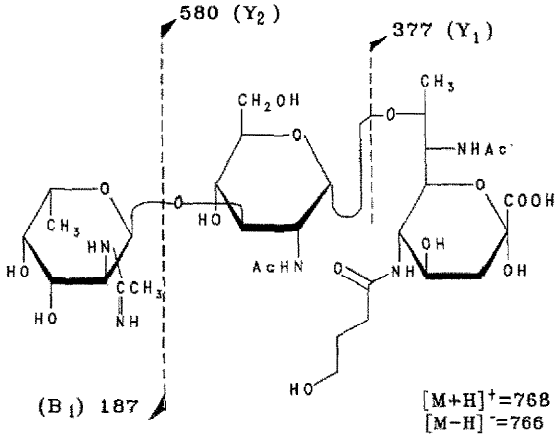


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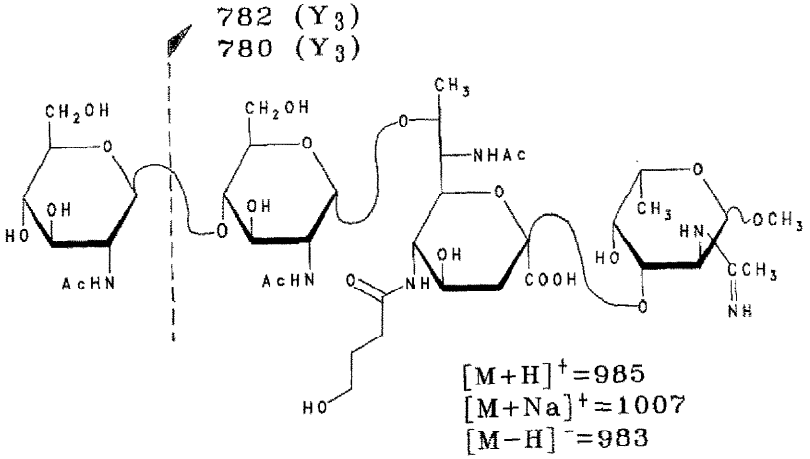
Fig. 3. The structure of oligosaccharide **2** showing fragment ions obtained by FABMS in the positive-ion mode [m/z 187 (B_1)] and by CID FABMS–MS in the negative-ion mode [m/z 580 (Y_2) and m/z 377 (Y_1)]. The latter fragment ions demonstrate the sequential loss of FucAm and GlcNAc from the nonreducing terminus.

terminal nonreducing D-Glc$_p$NAc residue. The fragment ion at m/z 219 indicates that **4** has an L-Fuc$_p$Am at its reducing end (Fig. 5).

The structure of the tetrasaccharide repeating unit of the polysaccharide was



3

Fig. 4. The structure of oligosaccharide **3** showing fragment ions obtained by FABMS in the positive-[m/z 782 (Y_3)] and negative-ion [m/z 780 (Y_3)] mode, indicating the loss of the nonreducing terminal GlcNAc.

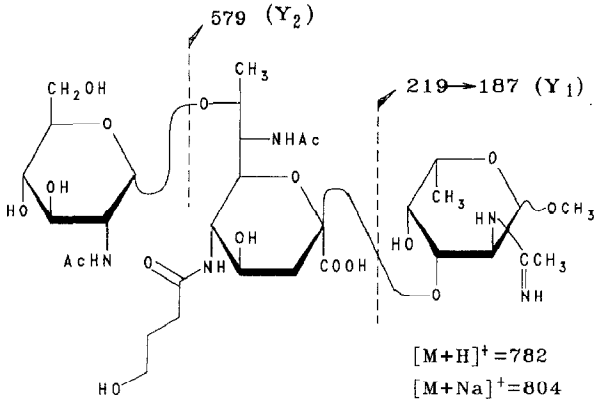


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Fig. 5. The structure of oligosaccharide **4** showing fragment ions obtained by FABMS in the positive-ion mode arising from the sequential loss of the GlcNAc [*m*/*z* 579 (Y_2)] and ulosonic acid residues [*m*/*z* 219 (Y_1)] from the nonreducing terminus.

determined from the results of the analysis of these four oligosaccharides using both 1D and 2D NMR spectroscopic methods.

The complete assignment of the ^1H NMR spectrum of each oligosaccharide was achieved via a series of COSY experiments (Table 1) from which the pyranose ring systems of L-Fuc$_p$Am, D-Glc$_p$NAc and 3,5,7,9-tetradeoxynonulosonic acid residues were readily identified. The respective ^{13}C resonances were assigned from ^{13}C–^1H chemical shift correlation experiments (Table 2).

The ^1H and ^{13}C NMR data (Tables 1 and 2) are consistent with **1** and **2** being tetra- and tri-saccharide units, respectively. Comparison of the ^{13}C chemical shifts for C-3 of the α-D-Glc$_p$NAc of **2** (76.3 ppm) and **4** (73.5 ppm) show this residue to be substituted at O-3 in **2**. The C-4 resonance of the α-D-Glc$_p$NAc residue of **1** (73.7 ppm) is deshielded in comparison to that of **2** (68.5 ppm), demonstrating that the terminal β-D-Glc$_p$NAc residue is linked via position O-4 to the α-D-Glc$_p$NAc residue of **1**. The chemical shift data for the α-L-Fuc$_p$Am residues of both **1** and **2** are consistent with this residue being the nonreducing terminal of both oligosaccharides [8].

The NMR data are consistent with **3** being a mixture of the anomers of the methyl glycoside (CH_3O at 57.0 ppm) representative of the tetrasaccharide repeating unit of the polysaccharide with L-Fuc$_p$Am forming the reducing terminal (C-1: α-L-Fuc$_p$Am, 98.1 ppm; β-L-Fuc$_p$Am, 102.8 ppm). The C-3 resonances of both α-L-Fuc$_p$Am (76.0 ppm) and β-L-Fuc$_p$Am (78.5 ppm) are deshielded in comparison with that of the C-3 resonance of the unsubstituted α-L-Fuc$_p$Am residue of **1** (68.1 ppm) and **2** (68.3 ppm), indicating substitution at this position. The relatively deshielded value for the C-4 resonance (79.8 ppm) of the α-D-Glc$_p$NAc residue of **3** compared to that of the trisaccharide **4** (70.9 ppm) is consistent with this residue being substituted at the O-4 position. The chemical shifts of the resonances from

the β-D-Glc$_p$NAc of **3** and the α-D-Glc$_p$NAc of **4** are consistent with these residues being terminal, nonreducing units [9].

The ^{1}H (Table 1) and ^{13}C (Table 2) NMR data show that the ketose residue has a 9-carbon skeleton and that the deoxy functions are located at C-3, C-5, C-7, and C-9. The chemical shifts for C-5 (53.3–54.0 ppm) and C-7 (53.6–55.5 ppm) indicate that these carbons carry nitrogen atoms. In both **3** and **4** the ulosonic acid was glycosylated at O-8 (C-8, 73.6–74.0 ppm and C-9, 15.1–16.0 ppm). In the unsubstituted sugar C-8 resonates at 70.0 ppm, and C-9 resonates at 20.0 ppm [6].

The relatively large coupling constants for the vicinal protons of the ulosonic acid in the ^{1}H spectra of **4** ($J_{3a,4}$ 10, $J_{4,5}$ 9.8, and $J_{5,6}$ 10.4 Hz) is indicative of a pyranosyl ring form (C-2–C-6) in which H-4, H-5, and H-6 occupy axial orientations, while the small coupling constant ($J_{6,7}$ 2.4 Hz) would suggest the *threo* configuration for C-6 and C-7 (ref 6). The C-7–C-8–C-9 fragment of the ulosonic acid is similar to the C-2–C-3–C-4 fragment of *N*-acetylthreonine. Using α-D-galactopyranosyl-*N*-acetyl-D- and L-threonine (and D- and L-*allo*threonine) Pavia and Lacombe [10] observed that the chemical shifts for the anomeric and threonine methyl carbons could be correlated to the absolute configurations at C-1 and the aglyconic (C-3′) chiral carbon centres. Particularly noteworthy is the pronounced shielding of the C-1 chemical shifts in compounds in which these centres have the same absolute configuration, i.e., in compounds α-D-Gal-D-Thr [C-1 (*S*); C-3′ (*S*)] and β-D-Gal-L-Thr [C-1 (*R*); C-3′ (*R*)]. The anomeric signals are shifted upfield by as much as 5.5 and 3.6 ppm, respectively, relative to the corresponding methyl glycosides [10]. Similar effects were also found for the methyl carbon. For the *Y. ruckeri* polysaccharide, the upfield shifts of C-1 (94.1 ppm) of α-D-GalNAc (compared to methyl α-D-GlcNAc, 98.9 ppm [9]) and the methyl carbon (15.7 ppm) of the ulosonic acid (compared to D- or L-threonine, 20.3 ppm [10]) give values of 4.8 and 4.6 ppm, respectively. As the anomeric configuration of the α-D-GalNAc residue could be established from the $J_{1,2}$ values for oligosaccharides **1**–**4** (Table 1), this data would suggest that the ulosonic acid is homomorphic to D-threonine or L-*allo*threonine. The chemical shift for C-9 (15.7 ppm) of the ulosonic acid in the native polysaccharide is in good agreement with that of C-4′ in α-D-Gal-D-Thr (15.3 ppm) and differs from that in α-D-Gal-L-*allo*Thr (13.2 ppm) [10], suggesting the C-7–C-8 fragment has the D-*threo* configuration and the ulosonic acid is of the D-*glycero*-L-*galacto* configuration.

The location of the 4-hydroxybutyramido group was determined for **4** from one-bond (HMQC) and three-bond (HMBC) ^{1}H–^{13}C chemical shift correlation experiments. In the HMBC experiment the carboxyl carbon resonance at 177.3 ppm correlated with both the H-2 (2.30 ppm) and H-3 (1.83 ppm) of the 4-hydroxybutyramido substituent and thus was assigned to C-1 of this group. This resonance also showed a cross-peak at H-5 (3.74 ppm) of the ulosonic acid residue, thus locating the substituent at C-5 of this residue. Moreover, the two overlapping *N*-acetyl carbonyl resonances (174.7 ppm) correlated to both H-2 of α-D-Glc*p*NAc (3.89 ppm) and H-7 of the ulosonic acid (3.89 ppm). This latter result provided confirmation of the location of the acetamidoyl group at C-2 of the L-FucN

Table 1
^{1}H NMR chemical shift data for oligosaccharides **1–4** [a]

Residue/ Compound	^{1}H NMR chemical Shift [b]									
	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5}$)	H-5	H-6 ($J_{5,6}$)	H-6′ ($J_{5,6'}$) ($J_{6,6'}$)	H-7 ($J_{6,7}$)	H-8 ($J_{7,8}$)	H-9 ($J_{8,9}$)
α-L-Sug[c,d]										
1			1.80a 2.21e	3.90	3.76	4.05		3.92	3.79	1.11
				(10.3)		(10.3)		(>1.0)	(–) [e]	(6.5)
2			1.81a 2.22e	3.88	3.76	4.04		3.93	3.84	1.12
				(9.9)		(9.5)		(2.0)	(10.2)	(7.2)
3			1.68a 2.65e	3.50	3.76 3.73	4.06 4.11		3.91	3.72	1.32
4			1.67a 2.65e	3.50	3.74 3.73	4.06 4.11		3.89	3.74	1.32 (–) [e]
				(9.8)		(10.4)		(2.4)		(6.3)
α-D-GlcNac										
1	4.84 (3.6)	4.14 (9.6)	3.70 (9.1)	3.89 (10.3)	3.63	3.89 (–) [e]	3.77 (–) [e]			
2	4.86 (4.4)	4.06 (10.2)	3.68 (10.2)	3.57 (–) [e]	3.59	3.79 (–) [e]	3.85 (3.9) (12.7)			
3	4.97 (3.4)	3.93 (9.0)	3.63 (9.8)	3.69 (10.3)	3.58	3.85 (4.6)	3.73 (–) [e] (12.1)			
4	4.97 (~1.0)	3.89 (10.7)	3.49 (9.0)	3.57 (9.0)	3.51	3.88 (3.6)	3.83 (12.5)			

α-L-FucAm							
1	5.13	3.84	4.17	3.85	4.81	1.32	
	(4.1)	(10.9)	(2.3)	(< 1.0)		(6.9)	
2	5.17	3.86	4.03	3.84	4.42	1.21	
	(3.9)	(10.6)	(3.2)	(< 1.0)		(7.1)	
3	4.85	3.97	4.08	3.98	4.08	1.27	
	(–) [e]	(9.2)	(–) [e]	(–) [e]	(–) [e]	(7.0)	
4	4.81	3.94	4.07	3.98	4.07	1.27	
	(3.7)	(10.5)	(4.6)	(< 1.0)		(7.5)	
β-L-FucAm							
3	4.52	3.65	3.85	3.93	3.82	1.30	
	(7.5)	(9.4)	(–) [e]	(–) [e]		(–) [e]	
4	4.52	3.63	3.84	3.92	3.81	1.30	
	(8.4)	(10.7)	(3.6)	(–) [e]		(–) [e]	
β-D-GlcNAc							
1	4.56	3.72	3.50	3.22	3.44	4.02	3.57
	(8.2)	(9.2)	(9.2)	(9.2)		(–) [e]	(–) [e]
							(11.5)
3	4.60	3.78	3.57	3.49	3.52	3.94	3.75
	(8.3)	(8.4)	(10.3)	(10.1)		(–) [e]	(–) [e]
						(–) [e]	
4-Hydroxybutyramido							
1		2.32	1.84	3.65			
2		2.31	1.83	3.64			
3		2.31	1.83	3.65			
4		2.30	1.83	3.65			

[a] Measured at 27°C relative to internal acetone (δ 2.225 ppm). [b] Coupling constants in hertz in parentheses. Additional signals: 3.52 and 3.47 ppm for OCH_3 (**1** and **2**); ~2.32–2.27 ppm for *N*-acetamidoyl; ~2.10–1.90 ppm for NAc. [c] L-Sug = 7-acetamido-3,5,7,9-tetradeoxy-5-(4-hydroxybutyramido)-D-*glycero*-L-*galacto*-nonulosonic acid. [d] $J_{3a,4}$ ~10–13 Hz, $J_{3e,4}$ ~4.5 Hz, and $J_{3a,e}$ ~12.5 Hz. [e] Unresolved.

Table 2
^{13}C NMR chemical shift data for oligosaccharides **1–4** [a]

Residue/ Compound	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9
α-L-Sug [b]									
1		97.2	40.1	68.1	53.3	70.5	53.6	70.9	14.1
2		97.2	40.6	68.2	53.5	70.8	53.8	72.0	14.6
3			42.0	69.3	53.7	73.8	55.5	73.6	15.1
4			42.8	69.8	54.0	74.0	55.4	74.0	16.0
α-D-GlcNAc									
1	92.7	54.2	73.0	73.7	72.0	60.2			
2	93.5	53.9	76.3	68.5	72.8	61.0			
3	94.0	54.5	71.6	79.8	71.5	60.7			
4	94.5	55.4	73.5	70.9	73.8	62.0			
α-L-FucAm									
1	96.1	53.5	68.1	71.8	67.3	16.4			
2	96.3	53.7	68.3	71.5	67.4	16.0			
3	98.1	52.7	76.0	72.6	67.1	17.0			
4	98.6	53.3	76.7	73.0	67.7	17.7			
β-L-FucAm									
3	102.8	55.6	78.5	72.7	71.8	17.0			
4	103.5	56.0	79.4	72.4	72.3	17.7			
β-D-GlcNac									
1	102.3	57.0	75.0	71.1	77.2	62.0			
3	100.7	56.3	75.0	71.4	76.8	62.3			
4-Hydroxybutyramido									
1		33.4	28.3	61.8					
2		33.4	28.5	61.8					
3		33.9	29.1	62.5					
4		34.6	29.8	63.0					

[a] Measured at 27°C relative to internal acetone (δc 31.07 ppm). Additional signals: ~57.0–59.0 ppm for OCH_3, ~20.0 ppm for *N*-acetimidoyl, ~22.0–24.0 ppm for NAc. [b] L-Sug = 7-acetamido-3,5,7,9-tetradeoxy-5-(4-hydroxybutyramido)-D-*glycero*-L-*galacto*-nonulosonic acid.

residue. Assignment of the carboxyl carbon of the ulosonic acid (172.6 ppm) was established from a strong correlation to the H-3ax (1.67 ppm).

The anomeric configurations of the D-Glc$_p$NAc and L-Fuc$_p$Am residues were confirmed by measurement of the J_{C1-H1} coupling constants: α-L-Fuc$_p$Am, 172 Hz; α-D-Glc$_p$NAc 171 Hz; β-D-Glc$_p$NAc 163.2 Hz. The anomeric α-L-configuration of the ulosonic acid is evident from the chemical shift of the signal for H-3e. The ^1H NMR spectra of both **1** and **2** (Fig. 6a) show shifts in the signals for the H-3e (~2.2 ppm) from those observed in the ^1H NMR spectra of the native polysaccharide, oligosaccharides **3** and **4** (2.65 ppm) (Fig. 6b). This latter value is indicative of an axial carboxyl group in the polysaccharide, oligosaccharides **3** and **4** [11,12], whereas **1** and **2** have an equatorial carboxyl group [11,13]. Thus, the ulosonic acid forms the reducing end of both **1** and **2**. Furthermore, the difference between the

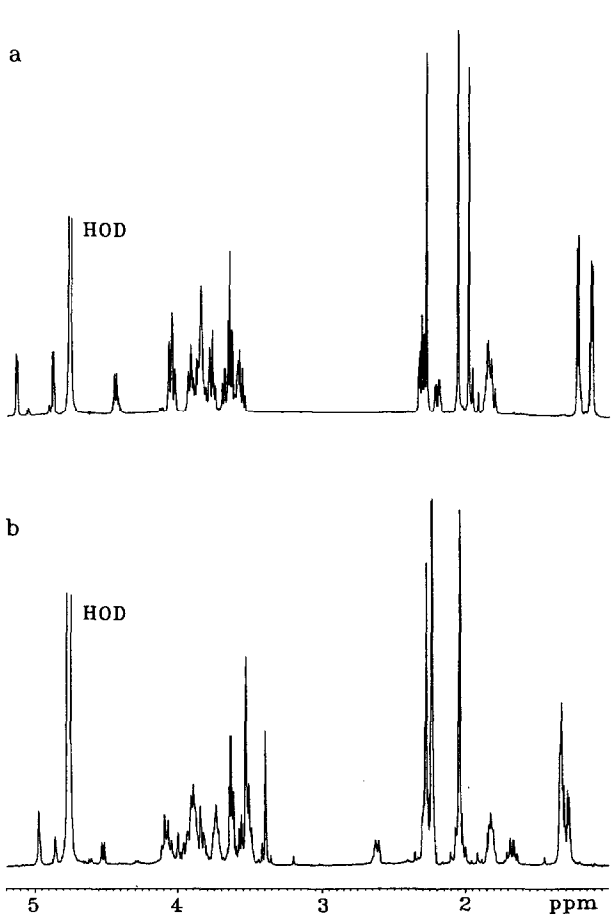

Fig. 6. The ^{1}H NMR spectra of oligosaccharide **2** (a) and oligosaccharide **4** (b) demonstrating the downfield chemical shift of H-3e from 2.22 ppm (a), indicative of an equatorial carboxyl group, to 2.65 ppm (b), typical of an axial carboxyl group.

chemical shifts of the signals for H-3e and H-3a, which in the polysaccharide is 0.97 ppm, is also typical of an axial carboxyl group, and hence O-2 has the equatorial orientation which corresponds to the α-L-configuration [6].

Thus, mass spectrometric analysis and ^{1}H NMR data and ^{13}C NMR spectroscopic analysis of the four oligosaccharides isolated from *Y. ruckeri* serotype 01 allowed the structure of the O-specific polysaccharide to be assigned as shown in Fig. 7.

Oligosaccharides **2** and **4** are similar to trisaccharides obtained by Vinogradov et al. [8], by selective degradation of the O antigen of *Salmonella arizonae* 061, consisting of L-FucAm, D-GlcNAc and 5,7-diacetamido-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid. The main differences between the respective chemical shift data are associated with the replacement of the 3-hydroxybutyramido group in the *S. arizonae* repeating unit (C-2–C-3–C-4 at 46.5, 66.2,

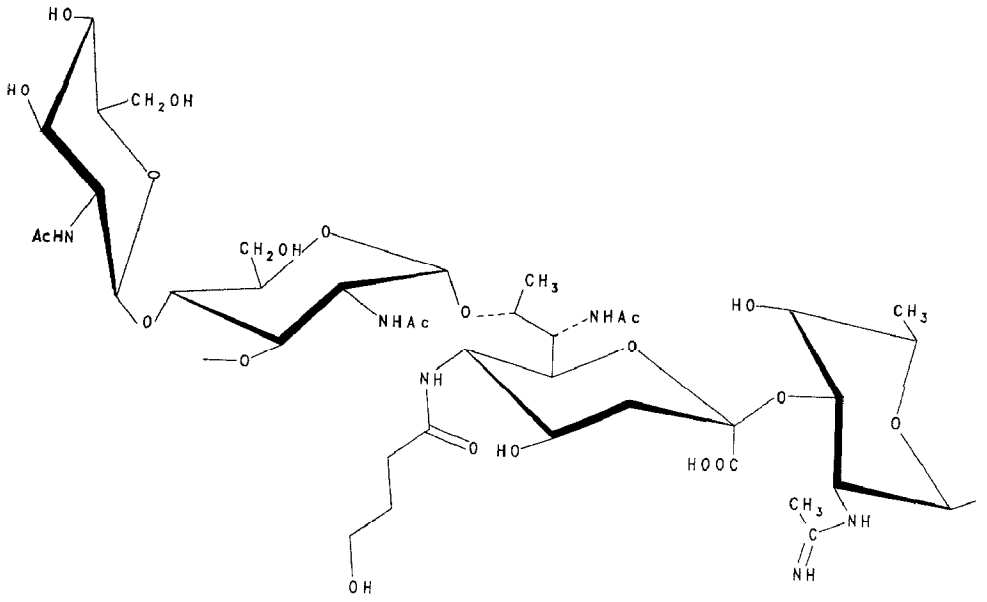

Fig. 7. The structure of the repeating unit of the O-specific polysaccharide of *Y. ruckeri* 01.

and 23.0 ppm) [8] by a 4-hydroxybutyryl substituent. This ulosonic acid residue has also been previously identified on the basis of NMR chemical shift data and coupling constants [6].

The O antigen of *Y. ruckeri* 01 is the fourth bacterial polysaccharide to be found to contain a derivative of 5,7-diamino-3,5,7,9-tetradeoxy-D-*glycero*-L-*galacto*-nonulosonic acid and is the first one to be reported to carry a 4-hydroxybutyramido group. The diacetamido derivative of this sugar has been identified as a component of the O antigens of *Pseudomonas aeruginosa* 013 [6] and *Vibrio alginolyticus* strain 945-80 [14]. It is noteworthy that the O antigens of *Y. ruckeri* serotype 01 and *S. arizonae* 016 contain a common trisaccharide unit differing only in the nature of the substituent at position O-5 of the nonulosonic acid.

3. Experimental

Production of lipopolysaccharide and O-polysaccharide.—*Y. ruckeri* (ATCC 29473) serotype 01 was grown in Bacto PPLO broth (Difco), and the LPS was isolated from the saline-washed cells (yield 527 g wet wt) by the aqueous–phenol extraction procedure [3]. Two LPS fractions [A, 2.0 g (heavy gel) and B, 1.5 g (light gel)] were obtained after ultracentrifugation (105 000g, 4°C, 12 h) of the dialysed concentrated aqueous layers. Solutions of LPS (300 mg), in 1% aqueous acetic acid, were kept at 100°C for 2 h, and after removal of insoluble lipid A by low-speed centrifugation, O polysaccharide was recovered by gel-filtration chromatography on Sephadex G-50 (Pharmacia, 2 × 90 cm) using 0.05 M pyridinium

acetate buffer (pH 4.7) as eluant. Fractions of the eluate (10 mL) were collected and analyzed colorimetrically for neutral aldoses [15], aminodeoxyglycose [16], phosphate [17], and Kdo [18].

SDS–PAGE analysis.—LPS samples (1 μg) were analysed in 14% polyacrylamide gels by electrophoresis in the presence of 2% SDS. Bands were detected using the silver-staining method of Tsai and Frasch [4].

Analytical methods.—For analysis of constituent sugars, samples (1 mg) of polysaccharide were hydrolysed with 2 N H_2SO_4 for 3 h at 100°C (ref. 19). The solutions were neutralised with 20% *N,N*-dioctylmethylamine in CH_2Cl_2 (2 mL), and the aqueous fractions were concentrated to dryness. The glycoses were determined by GLC–EIMS of their derived alditol acetates [5]. For the determination of the absolute configurations of GlcN and FucN, the polysaccharide was hydrolysed with 6 N HCl for 2 h at 100°C, and the monosaccharides were obtained by preparative (ethyl acetate–pyridine–water, 8:2:1). Optical rotations were measured at 20°C using a Perkin–Elmer model 243 polarimeter, the strongly positive and negative rotations obtained for D-GlcN and L-FucN, respectively, being indicative of the assigned configurations.

Methylation of samples (2 mg) was carried out using the method of Ciucanu and Kerek [20]. Methylated samples were dialysed to remove low-molecular-weight impurities. Methylated products were hydrolysed (4 M trifluoroacetic acid, 1 h at 125°C) and were analysed by GLC–EIMS of their derived acetylated alditol derivatives.

Analytical GLC–MS was performed using a Hewlett–Packard model 5958B gas chromatograph fitted with an OV-17 fused silica capillary column (Quadrex Corp), in the electron-impact (EI) mode using an ionization potential of 70 eV. The following temperature programs were employed: *A* (for alditol acetates), 180°C for 2 min, then 4°C min to 240°C; *B* (for partially methylated alditol acetates) 180°C for 2 min then 2°C min to 240°C.

FABMS analyses were carried out in the positive- and negative-ion mode using a Joel AX505-H mass spectrometer. An accelerating voltage of 3 kV and a mass resolution of 1500 was employed. A Xe atom beam of 6 kV was used to sputter and ionize the sample. CID FABMS–MS experiments on $[M + H]^+$ and $[M - H]^-$ ions were performed by linked scanning using He as the collision gas. Linked scans at constant B/E were generated by the Joel Compliment data system.

NMR spectroscopy.—All measurements were made on solutions in D_2O, at 27°C (oligosaccharides) or 72°C (polysaccharide), using Bruker AMX 500 or AM 200 spectrometers.

Proton spectra were obtained using a spectral width of 2.3 kHz and a 90° pulse. Chemical shifts are expressed relative to internal acetone (δ_H, 2.225 ppm).

Broad-band decoupled ^{13}C spectra were obtained at 125 MHz with a spectral width of 31 KHz and a 90° pulse employing WALTZ decoupling [21]. Chemical shifts are expressed relative to internal acetone (δ_C, 31.07 ppm). DEPT spectra were obtained at 50 MHz as previously described [22].

Two-dimensional homonuclear chemical shift correlated (COSY) experiments were carried out as previously described [23]. The heteronuclear ^{13}C–^1H chemical

shift correlations were measured in the ^{1}H-detected mode via multiple quantum coherence (^{1}H{^{13}C}HMQC) with a Bruker 5-mm inverse broad-band probe using reverse electronics as previously described [23]. The 2D heteronuclear multiple bond correlated ^{1}H–^{13}C (^{1}H{^{13}C}HMBC) experiment on **4** was carried out using the pulse sequence described by Bax and Summer [24].

Selective degradations of the O antigen.—(1) Mild acid hydrolysis. O Polysaccharide (230 mg) in 0.1 M HCl was heated for 5 h at 95°C. The excess acid was removed by evaporation under reduced pressure, and the remaining traces were neutralized with NH_4OH. The lyophilized product was dissolved in water and fractionated on a Bio-Gel P2 column. The aminodeoxyglycose positive fractions were pooled and rechromatographed on a Bio-Gel P2 column. Two pure oligosaccharides were obtained: **1** (K_{av} 0.14, 3.0 mg); **2** (K_{av} 0.19, 7.0 mg).

(2) Hydrogen fluoride solvolysis.

The native polysaccharide (50 mg), suspended in MeOH (0.2 mL), was treated with anhyd HF (3 mL) at room temperature for 3 h. The excess HF was removed by evacuation under reduced pressure, and the remaining traces of acid were neutralized with NH_4OH. The lyophilized product was dissolved in water and partitioned on a column of Bio-Gel P2. Fractions (1 mL) were collected, analyzed using the Elson–Morgan assay for aminodeoxyglycoses [16], and the positive fractions were pooled and lyophilized. Two pure oligosaccharide were obtained: **3** (K_{av} 0.17, 1 mg); **4** (K_{av} 0.20, 2 mg).

4. Acknowledgments

We thank Mr. Fred Cooper for the expert GLC–MS and FABMS analyses, Dr. R.L. Davies for the strain of *Y. ruckeri* serotype 01, and Mr. D.W. Griffith for production of bacterial cells.

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