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The structure of the specific capsular polysaccharide of *Rhodococcus equi* serotype 4☆

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Abstract

The specific capsular polysaccharide produced by *Rhodococcus equi* serotype 4 was found to be a high-molecular-weight acidic polymer composed of D-glucose, D-mannose, pyruvic acid and a previously unidentified 5-amino-3,5-dideoxynonulosonic (rhodaminic) acid in the proportions 2:1:1:1. Structural analysis, employing a combination of microanalytical methods, nuclear magnetic resonance spectroscopy, and mass spectrometric techniques, established that the polysaccharide consisted of linear repeating tetrasaccharide units having the sequence of residues shown below.

In the native polysaccharide, the rhodaminic acid residues were present as their acetamido derivatives (RhoANAc) and carried 1-carboxyethylidene groups that bridged the O-7 and O-9 positions. Treatment of the capsular polysaccharide with dilute acetic acid and/or anhydrous hydrogen fluoride under hydrolytic/solvolytic conditions, resulted in the formation of four different oligosaccharide species. The ^{1}H and ^{13}C NMR resonances of these oligosaccharide fragments and of the native serotype 4 capsular polysaccharides were fully assigned by homo- and heteronuclear chemical shift correlation methods. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Rhodococcus equi*; Structure; 5-Amino-3,5-dideoxynonulosonic acid; Rhodaminic acid; NMR spectroscopy

1. Introduction

The bacterial horse pathogen *Rhodococcus equi* [1] elaborates a serotype-specific capsular polysaccharide (CPS) that has been identified as a potential virulence factor [2]. Currently, seven serotypes are recognised [3,4], of which the CPSs of serotypes 1, 2, 3, 6 and 7 have been fully characterised [5–9]. All were found to be acidic high-molecular-weight heteroglycans composed of linear saccharide units carrying either a 1-carboxyethylidene (pyruvic acid) acetal group (serotypes 1, 6 and 7), or the biosynthetically related [10] 1-carboxyethyl (lactic acid) ether substituent (serotype 2). The *R. equi* serotype 3 capsular polysaccharide contains both the pyruvic acid and lactic acid moieties. In the present investigation, we iden-

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tify the first *R. equi* serotype in which a nonulosonic acid residue is a component of the CPS.

2. Results and discussion

Isolation and purification.—Crude specific CPS, obtained by mechanical dissociation from plate-grown *R. equi* serotype 4 cells, was enzymically treated with ribonuclease, deoxyribonuclease and proteinase K, and recovered via its insoluble cetyltrimethyl ammonium salt [6]. Purification was performed by ion-exchange chromatography on DEAE-Sephacel from which the capsule eluted as a discrete band at the beginning of the sodium chloride gradient. The polysaccharide had $[\alpha]_D$ +6.78° (*c* 1.2, water), Anal. Found: C, 42.61; H, 6.04; N, 2.39; ash, 2.11%, and gave a single sharp precipitin line in immunodiffusion against rabbit antisera to *R. equi* serotype 4 cells [4]. All subsequent analyses were made on the DEAE-Sephacel purified product. The native serotype 4 CPS eluted as a sharp band at the void volume (K_{av} 0.00) of a Sephacryl S-500 gel-filtration system, indicating it to be a high-molecular-weight polymer. Qualitative analysis of the eluant revealed that the polysaccharide contained neutral glycose [11] and 5-amino-3,5-dideoxynonulosonic acid residues [12].

Glycosyl constituent analysis.—The CPS obtained from *R. equi* serotype 4 was completely depolymerised by a two-step process involving acid hydrolysis (AcOH, 2%, 100 °C, 2 h) and HF solvolysis (HF in MeOH, 20 °C, 2 h). Following reduction of the resulting methyl esters with $NaBH_4$, the free glycoses were released with a second treatment of anhydrous HF (HF, 20 °C, 2 h). GLC analysis of the corresponding alditol acetates [13] indicated that the neutral glycose residues, which were identified as the 1,2,3,4,5,6-hexa-*O*-acetyl derivatives of glucitol (T_{GA} 1.0) and mannitol (T_{GA} 0.9), were present in a ratio of 2:1. An additional pair of peaks of equal intensity and giving similar mass spectra was observed at T_{GA} 1.96 and 2.44. Mass spectral analysis of these peaks, employing chemical ionisation (CIMS), revealed a molecular ion $(M+1)^+$ at *m/z* 593. Primary fragment ions at *m/z* 303 and 360, obtained by electron impact ionisation (EI-MS), were indicative of an acetylated 3-deoxynonositol having an acetamido substituent at the 5-position (Fig. 1). A shift of the $(M+1)^+$ ion by two mass units to *m/z* 595 when carboxyl group reduction was effected with $NaBD_4$ provided evidence that these compounds were the two epimeric products derived from a 5-amino-3,5-dideoxynonulosonic acid.

Glycosyl sequence determination.—The sequence of glycosyl residues within the *R. equi* serotype 4 capsular polysaccharide was established using a range of high-resolution NMR techniques. Complete assignment of the ^1H NMR spectra (Fig. 2(a)) was achieved by 2D homonuclear double quantum filtered chemical shift correlation (DQCOSY) experiments [14,15] (Fig. 3). Heteronuclear $^1H-^{13}C$ chemical shift correlation (HMQC) experiments [16] permitted unambiguous assignment of all the ^{13}C resonances within the CPS by reference to the determined ^1H assignments. The NMR spectra of the CPS were recorded at 42 °C in D_2O at pD 2.0 and the ^1H and ^{13}C chemical shifts are given in Tables 1 and 2, respectively.

The 1D ^1H NMR spectrum (Fig. 2(a)) of the CPS exhibited resonances corresponding to the anomeric resonances of three glycosyl residues in the low-field region of the spectrum at 5.03 (s, ca. 1 H, $J_{1,2}$ 3.2 Hz), 4.76 (d, ca. 1 H, $J_{1,2}$ 0.6 Hz) and 4.50 ppm (d, ca. 1 H, $J_{1,2}$ 8.0 Hz). Methylene proton resonances observed at 1.86 (dd, 1 H), and 2.75 ppm (m, 1

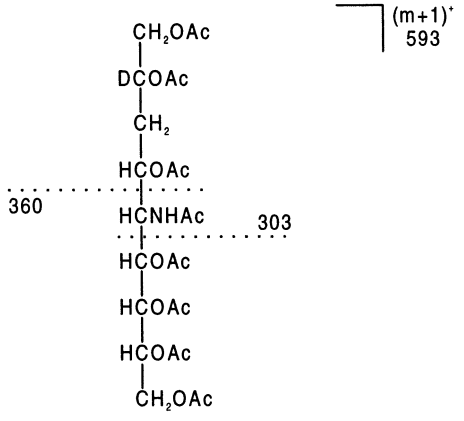


Fig. 1. Mass spectral fragmentation pattern of the carboxyl-reduced, acetylated alditol derivative of the 5-amino-3,5-dideoxynonulosonic acid residue.

Table 1
^{1}H NMR chemical shifts and coupling constants (Hz) for the *R. equi* S4 polysaccharide and derived oligosaccharides [a]

Residue	Monosaccharide unit	Saccharide	H-1 ($J_{1,1'}$ $J_{1,2}$)	H-1′ ($J_{1',2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-3′ ($J_{3,3'}$ $J_{3',4}$)	H-4 ($J_{4,5}$)	H-5 ($J_{5,6}$)	H-6 ($J_{5,6}$) or ($J_{6,7}$)	H-6′ ($J_{5,6'}$ $J_{6,6'}$)	H-7 ($J_{7,8}$)	H-8	H-9 ($J_{8,9}$)	H-9′ ($J_{8,9'}$ $J_{9,9'}$)	CH_3
A	→4)-α-D-Glc*p*-(1→	**1** (polysaccharide) [b]	5.032 (3.2)		3.568 (10.2)	3.716 (9.8)		3.645 (10.0)	4.122	3.852 (2.0)	3.932 (6.5, −9.0)					
	→4)-α-D-Glc*p*-(1→	**2** (tetrasaccharide) [c]	5.019 (3.8)		3.544 (9.8)	3.726 (9.8)		3.625 (9.2)	4.119	4.066 (2.0)	3.865 (7.2, −12.6)					
	→4)-D-Glcol	**3** (trisaccharide) [c]	3.842 (−11.9, 3.5)	3.710 (6.5)	4.018 (6.2)	3.907 (2.0)		3.807 (7.5)	3.980	3.938 (1.8)	3.807 (9.0, −12.3)					
B	→3)-β-D-Man*p*-(1→	**1**	4.763 (0.6)		4.187 (3.0)	3.850 (9.8)		3.649 (9.8)	3.471	3.743 (1.8)	3.919 (6.5, −12.0)					
	β-D-Man*p*-(1→	**2**	4.744 (0.6)		4.055 (2.8)	3.649 (9.8)		3.591 (10.0)	3.421	3.929 (1.8)	3.939 (4.0, −12.4)					
	β-D-Man*p*-(1→	**3**	4.818 (0.6)		4.124 (3.2)	3.718 (9.8)		3.650 (9.6)	3.488	3.801 (3.2)	3.915 (7.0, −12.4)					
	→3)-D-Manol	**4** (disaccharide − pyr) [c]	3.673 (−11.7, 4.0)	3.680 (6.7)	4.132 (4.5)	4.102 (1.0)		3.729 (9.2)	3.645	3.841 (2.3)	3.640 (6.3, −11.8)					
	→3)-D-Manol	**5** (disaccharide + pyr) [c]	3.673 (−11.7, 4.0)	3.754 (6.7)	4.132 (4.5)	4.102 (1.0)		3.729 (9.2)	3.806	3.841 (2.3)	3.640 (6.3, −11.8)					
C	→4)-β-D-Glc*p*-(1→	**1**	4.502 (8.0)		3.398 (9.8)	3.698 (9.8)		3.705 (9.8)	3.587	3.676 (2.0)	3.922 (6.5, −11.0)					
	→4)-β-D-Glc*p*-(1→	**2**	4.515 (8.0)		3.390 (10.0)	3.698 (9.8)		3.718 (9.8)	3.584	3.929 (2.0)	3.741 (6.8, −12.6)					
	→4)-β-D-Glc*p*-(1→	**3**	4.664 (8.0)		3.447 (9.6)	3.752 (9.6)		3.768 (9.8)	3.629	3.822 (2.0)	4.006 (7.2, −12.6)					
D	→4)-α-RhoANAc*p*-(2→	**1**				1.855 [d] (12.7)	2.747 [e] (4.6, −13.0)	3.962 (3.5)	4.447 (1.5)	3.864 (8.5)		4.062 (4.5)	4.054	3.707 (3.0)	3.726 (6.0, −12.0)	
	→4)-β-RhoANAc*p*	**2**				1.945 (12.0)	2.056 (4.6, −12.7)	4.246 (3.5)	4.520 (1.5)	4.126 (9.5)		3.576 (4.5)	3.866	3.646 (2.8)	3.632 (5.8, −10.0)	
	α-RhoANAc*p*-(2→	**4**				1.849 (13.2)	2.593 (4.6, −12.7)	4.112 (3.0)	4.408 (1.5)	3.691 (8.5)		4.001 (4.5)	4.021	3.961 (5.0)	3.649 (5.8, −11.5)	
	α-RhoANAc*p*-(2→	**5**				1.799 (13.2)	2.533 (4.6, −12.7)	3.960 (3.0)	4.423 (1.5)	4.098 (8.5)		3.915 (4.5)	4.490	3.797 (5.0)	3.649 (5.8, −11.5)	
E	Pyruvate	**1**														1.529
		5														1.513
F	*N*-acetyl	**1**														2.079
		2														2.078
		4														2.133
		5														2.113

[a] Observed first-order chemical shifts and coupling constants (Hz) refined by spectral simulation.
[b] Measured at 42 °C in D_2O (pD ~ 2).
[c] Measured at 27 °C in D_2O (pD ~ 2).
[d] H-3 axial.
[e] H-3 equatorial.

Table 2
^{13}C NMR chemical shifts and $^{1}J_{C,H}$ values (Hz) for the *R. equi* S4 polysaccharide and derived oligosaccharides [a]

Residue	Monosaccharide unit	Saccharide	C-1 ($J_{C-1,H-1}$)	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	CH_3	CO_2H
A	→4)-α-D-Glc*p*-(1→	**1** (polysaccharide) [b]	98.9 (171)	71.8	72.0	79.1	71.8	61.0					
	→4)-α-D-Glc*p*-(1→	**2** (tetrasaccharide) [c]	97.5	71.6	72.0	79.3	71.3	60.6					
	→4)-D-Glcol	**3** (trisaccharide) [c]	63.1	72.5	69.5	79.4	71.7	62.6					
B	→3)-β-D-Man*p*-(1→	**1**	101.5 (159)	72.1	79.0	65.2	74.4	62.3					
	β-D-Man*p*-(1→	**2**	100.7	71.3	73.5	67.4	77.1	61.6					
	β-D-Man*p*-(1→	**3**	100.5	71.2	73.2	67.0	76.5	61.5					
	→3)-D-Manol	**4** (disaccharide − pyr) [c]	61.8	71.9	72.9	69.6	67.8	61.5					
		5 (disaccharide + pyr) [c]	62.8	71.7	72.0	69.3	68.4	60.9					
C	→4)-β-D-Glc*p*-(1→	**1**	103.3 (162)	73.8	74.8 [d]	79.9 [d]	75.6	62.0					
	→4)-β-D-Glcol	**2**	101.1	73.6	74.8	79.3	75.3	61.0					
	→4)-β-D-Glc*p*-(1→	**3**	102.5	74.3	74.5	79.4	74.6	60.5					
D	→4)-α-RhoANAc*p*-(2→	**1**	172.8	103.9 [e]	34.1	72.5	47.5	72.0	74.4 [d]	68.5 [d]	63.2		
	→4)-β-RhoANAc*p*	**2**	173.0	103.9 [e]	32.9	71.8	48.9	70.3	69.0	70.5	63.8		
	α-RhoANAc*p*-(2→	**4**	172.8	102.5 [e]	34.5	73.9	47.3	71.7	69.1	70.0	61.3		
	α-RhoANAc*p*-(2→	**5**	173.5	102.8 [e]	34.5	73.7	47.3	73.3	72.5	79.3	60.6		
E	Pyruvate	**1**	99.8									22.6	174.2 [e]
		5	110.3									24.0	178.2 [e]
F	*N*-acetyl	**1**										25.8	175.6 [e]
		2										24.9	175.2 [e]
		4										22.3	175.6 [e]
		5										22.0	175.8 [e]

[a] Assignments were determined by ^{13}C–^{1}H chemical shift correlation (HMQC) unless otherwise indicated.
[b] Measured at 42 °C in D_2O (pD ~ 2).
[c] Measured at 27 °C in D_2O (pD ~ 2).
[d] Assigned by HMQC-TOCSY experiment.
[e] Identified by long-range ^{13}C–^{1}H chemical shift correlation (HMBC).

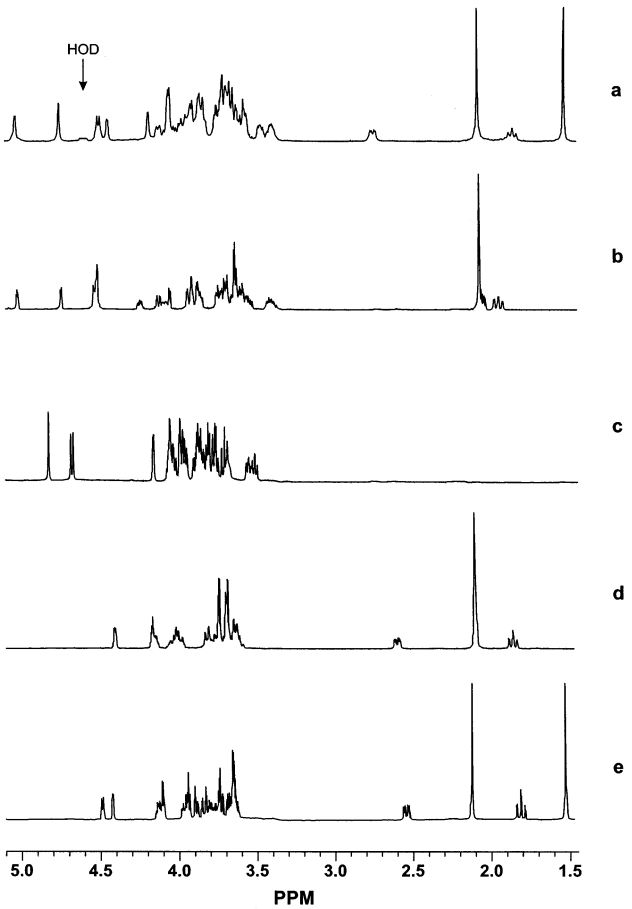


Fig. 2. ^{1}H NMR spectra of the oligosaccharides derived from the *R. equi* serotype 4 capsular polysaccharide: (a) native polysaccharide, (b) tetrasaccharide, (c) reduced trisaccharide, (d) reduced disaccharide without the pyruvate acetal and (e) reduced disaccharide with the pyruvate acetal.

H) were characteristic of the respective signals associated with the axial and equatorial H-3 protons of the α-linked 3-deoxynonosulonic acid residue [17], rhodaminic acid (RhoA). The signal at 2.08 ppm was assigned to the methyl group in the acetamido substituent, and the signal at 4.44 ppm was assigned to the proton of the nitrogen-bearing carbon of the parent RhoA residue. A diagnostic high-field resonance at 1.53 ppm corresponded to the methyl protons of the carboxyethylidene group. The anomeric resonances and the residues from which they came were labelled as **A**–**C** in decreasing order of the chemical shifts (Table 1), while the RhoA residue and its NMR resonances were labelled as **D**. The carboxyethylidene group and the *N*-acetyl substituent were labelled **E** and **F**, respectively.

Five discrete ^{13}C resonances were observed within the 90–110 ppm region of the 1D ^{13}C NMR spectrum of the native polysaccharide. The three resonances of approximately equal intensity at 103.3 ($^{1}J_{C,H}$ 162 Hz), 101.5 ($^{1}J_{C,H}$ 159 Hz) and 98.9 ppm ($^{1}J_{C,H}$ 171 Hz) were assigned to the anomeric carbons of the constituent hexose residues **C**, **B**, and **A**, respectively. The signals observed at 103.9, 34.1 and 47.5 ppm were attributed to the resonances from the quaternary (C-2), methylene (C-3) and nitrogen-bearing carbons (C-5) of the RhoA residue. The carboxyethylidene group exhibited quaternary and methyl carbon resonances at 99.8 and 22.6 ppm and methyl resonance at 25.8 ppm suggested that the amino group was present as its acetamido derivative [18]. The low-field signals at 172.8, 174.2 and 175.6 ppm indicated the presence of the three carboxyl groups within the repeating unit.

The ^{1}H and ^{13}C NMR signals within the spin system of residue **A** were fully assigned. A small observed ring proton coupling constant for the anomeric proton (5.03 ppm, $^{3}J_{H-1,H-2}$ 3.2 Hz), the large $^{3}J_{H,H}$ values (>8 Hz) of the signals for all other sugar ring protons and a proton–carbon coupling constant $^{1}J_{C-1,H-1}$ of 171 Hz are consistent with the α-D-*gluco* configuration. Typically, coupling constants above 170 Hz are indicative of an α-anomeric configuration [19]. The signals for C-4 (79.1) and H-4 (3.65) are shifted 8.5 and 0.36 ppm downfield of the corresponding chemical shift for α-D-glucose [20], indicating that **A** is a 4-*O*-substituted α-glucopyranosyl residue.

The subspectrum arising from residue **C** could also be assigned to a glucopyranosyl residue; however, the chemical shift of the H-1 signal (4.50 ppm) and the $^{1}J_{C-1,H-1}$ and $^{3}J_{H-1,H-2}$ values (162 and 8 Hz, respectively) are consistent with this residue possessing the β-anomeric configuration. Despite the proton resonances arising from H-3**C** and H-4**C** being nearly coincidental, their respective carbon resonances were readily discernible in the HMQC-TOCSY experiment. This heteronuclear experiment takes advantage of the chemical shift dispersion in the carbon domain, and by employing an appropriate mixing time after the HMQC step, the TOCSY sequence can

be tuned for the transfer of magnetisation from the directly attached proton to its nearest neighbours. In the HMQC spectrum the overlapping signals between 3.69 and 3.71 ppm (H-3/4**C**) gave connectivities to ^{13}C resonances at 74.8 and 79.9 ppm. The cross-section through the HMQC-TOCSY at 74.8 ppm (Fig. 4(b)) exhibited the proton resonances from H-2**C** (3.40) and H-3/4**C** (3.70 ppm), clearly indicating this carbon to be C-3**C**. Similarly the proton resonances corresponding to H-3/4**C** (3.70) and H-5**C** (3.59 ppm) were observed in the HMQC-TOCSY cross section at 79.9 ppm, allowing this carbon resonance to be assigned to C-4**C** (Fig. 4(d)). The exact positions of the H-3**C** (3.70 ppm) and H-4**C** (3.71 ppm) resonances were ascertained from the proton signals observed in the cross sections through the HMQC-TOCSY spectrum at C-2**C** (73.8 ppm) and C-5**C** (75.6 ppm), respectively (Fig. 4(a and c)). The shielded chemical shift values of the C-4 (79.9 ppm) and H-4 signals (3.71 ppm) are typical for β-glucopyranosyl residues substituted at O-4 [20].

Residue **B** had the β-anomeric configuration as determined from the chemical shift value for the H-1 signal (4.76 ppm) and the small $^{1}J_{C\text{-}1,H\text{-}1}$ value (159 Hz). The $^{3}J_{H,H}$ values measured from the subspectrum of residue **B** were consistent with this residue being a hexopyranose possessing the *manno* configuration, and

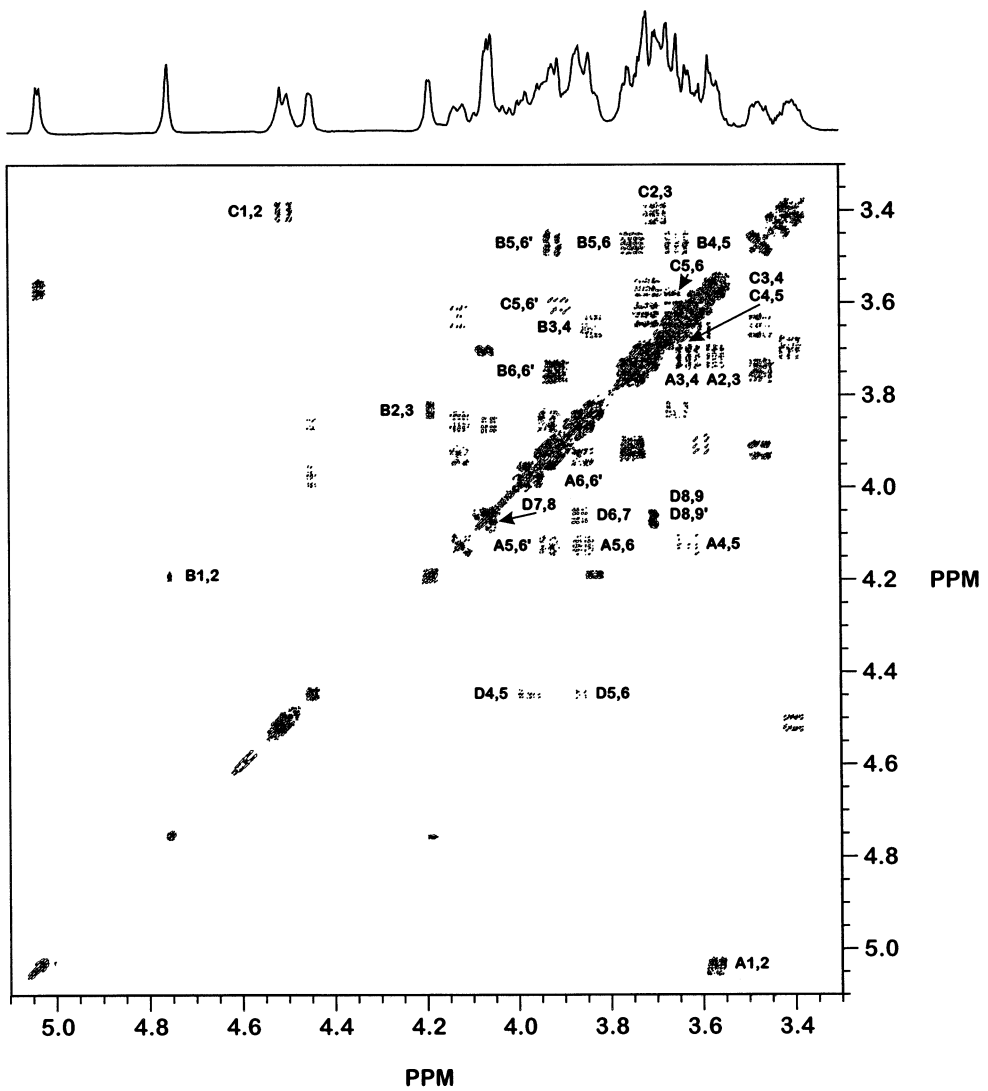


Fig. 3. Partial phase-sensitive COSY contour map of the native *R. equi* serotype 4 capsular polysaccharide recorded at 42 °C, showing correlations of the ring protons from 3.3 to 5.1 ppm.

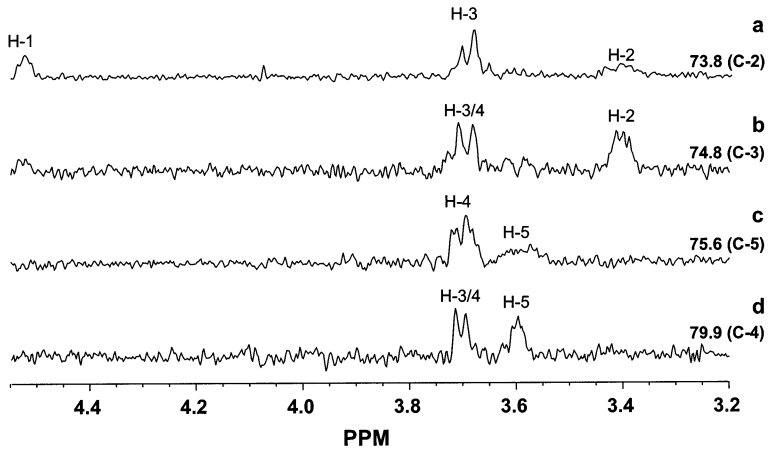


Fig. 4. Slices through the HMQC-TOCSY spectra showing $^{1}H-^{13}C$ correlations after the TOCSY step at ^{13}C resonances corresponding to (a) C-2 (73.8), (b) C-3 (74.8), (c) C-5 (75.6), and (d) C-4 (79.9 ppm).

the downfield shifts signals arising from C-3 and H-3 (5.7 and 0.32 ppm, respectively) are indicative of **B** being a 3-*O*-substituted β-D-mannopyranosyl residue [20].

Starting from the signal at 1.86 ppm, corresponding to H-3_{ax}, the nine proton resonances making up the subspectrum from residue **D** were delineated from the DQCOSY spectrum. The H-3 methylene proton resonances at 2.75 and 1.86 ppm exhibited an ABX pattern typical of an α-linked 3-deoxynonulosonic acid residue [17]. The geminal AB coupling of 13.0 Hz and the two vicinal couplings, $J_{3a,4}$ 12.7 and $J_{3e,4}$ 4.6 Hz, are consistent with a pyranose ring form and confirm the axial stereochemistry at the neighbouring proton, H-4. Carbon atoms C-4 and C-6–C-8 have signals in the region characteristic of secondary carbons substituted with hydroxy or glycosyloxy groups [C-4 (72.5), C-6 (72.0), C-7 (74.4), and C-8 (68.5 ppm)], whereas the chemical shifts for the signals at 37.6 (C-3) and 63.2 ppm (C-9) were typical, respectively, of a methylene carbon and a carbon bearing a primary hydroxy functionality. The signal corresponding to C-5 (47.5 ppm) appeared in the region for nitrogen-bearing carbons, and the discrete position of H-5 in the low-field (4.45 ppm), as well as the small magnitude of the vicinal couplings observed for $J_{H-4,H-5}$ (3.5 Hz) and $J_{H-5,H-6}$ (1.5 Hz), indicate the acetamido substituent at C-5 and the side chain at C-6 to have axial and equatorial dispositions, respectively. Thus, the C-4–C-6 fragment of residue **D** was deduced to have the *lyxo* configuration. Pseudaminic acid (5,7 - diamino - 3,5,7,9-tetradeoxy - L - *glycero* - L - *manno* - nonulosonic acid) is the only other nonulosonic acid in which the *lyxo* configuration has been identified for the C-4–C-6, [21–23]. However, unlike pseudaminic acid, the C-9 of rhodaminic acid is a methylene carbon bearing a primary hydroxyl group, thus making this sugar unique amongst nonulosonic acids. The pseudaminic acid residue in the *P. aeruginosa* O-5 (immunotype 6) O-antigen is substituted at the O-4 position and exhibits a ^{13}C resonance for C-4 at 72.4 ppm. The corresponding value in a nonsubstituted pseudaminic acid residue is 66.4 ppm [21]. Thus, the chemical shift of 72.5 ppm for C-4**D** in the *R. equi* CPS is indicative of glycosyl substitution at O-4.

The positions of the quaternary carbon resonances were established from the long-range $^{1}H-^{13}C$ connectivities identified in the proton-detected HMBC experiment [24] (Fig. 5). Thus, a cross-peak at 1.86 ppm (H-3$\mathbf{D}_{ax}$) led to identification of the resonances from the ketose and carbonyl carbon atoms, C-2**D** (103.9 ppm) and C-1**D** (172.8 ppm). The quaternary and carbonyl carbon resonances, at 99.8 and 174.2 ppm, respectively, within the carboxyethylidene group (**E**) were established by correlation with the methyl protons (1.53 ppm), and in a similar fashion, the remaining carbonyl resonance in the low-field region (175.6 ppm) was assigned to the *N*-acetyl carbonyl group.

From these data, the glycoses within the *R. equi* serotype 4 capsular polysaccharide were

identified as α-Glc*p* (**A**), β-Glc*p* (**C**), β-Man*p* (**B**) and 5-acetamido-3,5-dideoxy-α-nonulosonic acid (**D**) residues, and the ^{1}H and ^{13}C chemical shifts and vicinal proton coupling constants (Tables 1 and 2) are in agreement with typical published data for such residues [17,20,25].

The sequences of the four monosaccharide residues and the positions of the glycosidic linkages within the repeating unit of the *R. equi* serotype 4 capsular polysaccharide were established by measuring inter-residue $^{1}H-^{1}H$ NOEs [26] and long-range $^{1}H-^{13}C$ couplings. NOEs were measured using 2D NOESY experiments [27] and were employed qualitatively to establish short (≤ 3 Å) through-space connectivities between the anomeric proton of each of the hexoses with the aglyconic protons of the adjacent glycosidically linked residue. The long-range $^{1}H-^{13}C$ couplings across the glycosidic linkages, obtained by a proton detected HMBC experiment [24], allowed the sequence of residues to be determined independently. The HMBC spectrum is shown in Fig. 5 and data obtained from both NOESY and HMBC experiments are presented in Table 3.

Thus, the anomeric proton resonance of the α-Glc*p* residue **A** (5.03 ppm) showed a single intra-residue NOE at H-2**A** (3.57 ppm), together with inter-residue NOEs at H-3$\mathbf{D}_{eq}$ (2.75), H-3$\mathbf{D}_{ax}$ (1.86), and H-4**D** of the non-

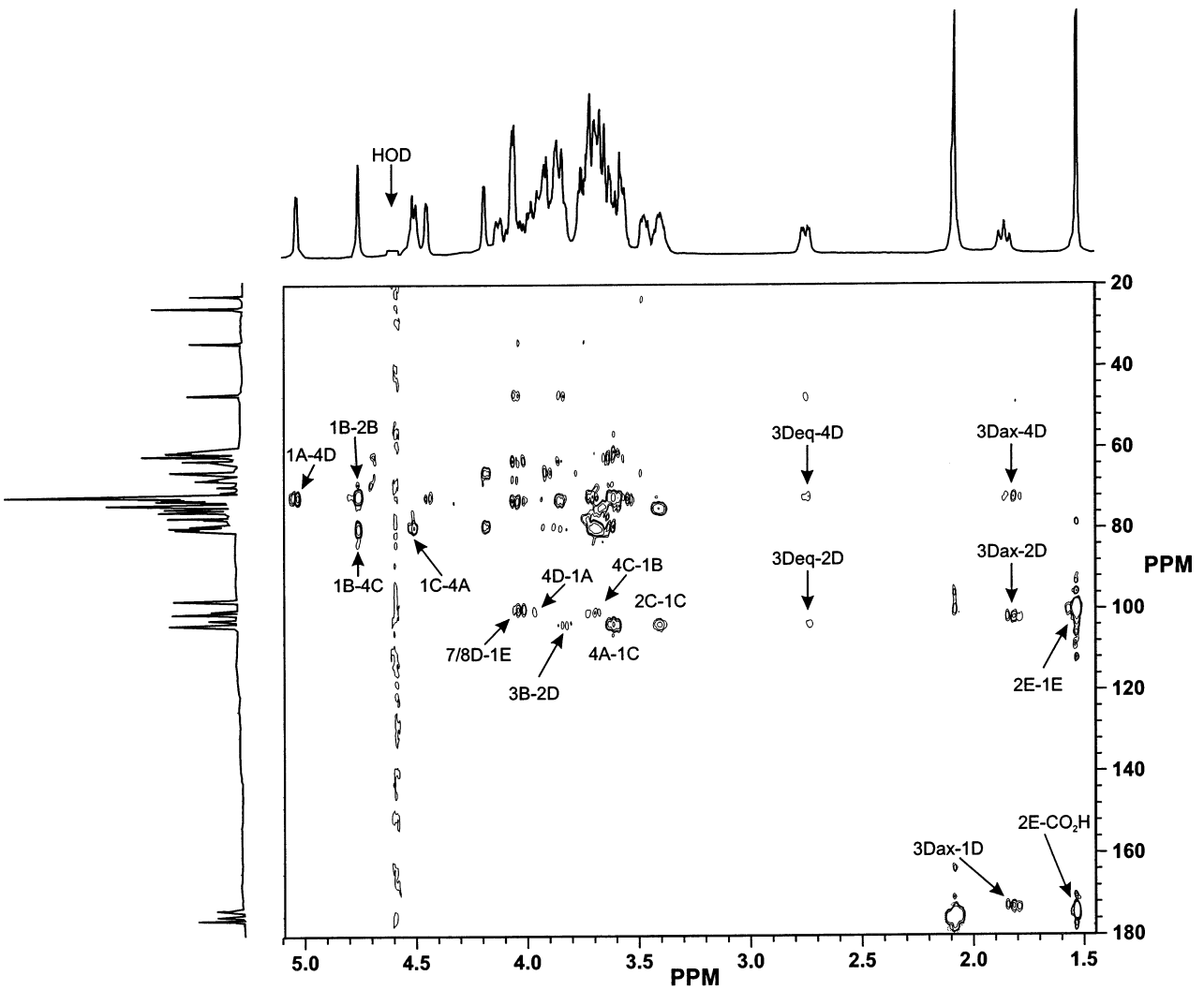


Fig. 5. HMBC spectra of the *R. equi* serotype 4 capsular polysaccharide showing some of the observed long-range heteronuclear shift correlations.

Table 3
Long-range $^{1}H–^{13}C$ and $^{1}H–^{1}H$ NOE intra- and inter-residue correlations obtained for the *R. equi* S4 capsular polysaccharide [a]

Residue	Monosaccharide unit	Observed proton	Long-range $^{1}H–^{13}C$ correlations		NOE contacts	
			Intra-residual	Inter-residual	Intra-residual	Inter-residual
A	→4)-α-D-Glc*p*-(1→	H-1	C-2**A**, C-3**A**, C-5**A**	C-4**D**	H-2**A**	H-3$\mathbf{D}_{eq}$, H-3$\mathbf{D}_{ax}$, H-4**D**
		H-4		C-1**C**		
B	→3)-β-D-Man*p*-(1→	H-1	C-2**B**	C-4**C**	H-2**B**, H-3**B**, H-5**B**	H-3/4**C**
		H-3		C-2**D**		
C	→4)-β-D-Glc*p*-(1→	H-1	C-3**C**	C-4**A**	H-2**C**, H-3**C**, H-5**C**	H-4**A**
		H-2	C-1**C**	C-1**C**		
D	→4)-α-RhoANAc-(2→	H-3a	C-1**D**, C-2**D**, C-3$\mathbf{D}_{eq}$, C-4**D**		H-3$\mathbf{D}_{eq}$	H-1**A**
		H-3e	C-2**D**, C-3$\mathbf{D}_{ax}$, C-4**D**		H-3$\mathbf{D}_{ax}$	H-1**A**
		H-4		C-1**A**		
		H-5			H-7/8**D**	H-5**A**
		H-6			H-7/8**D**	
		H-7		C-2**E**		
E	Pyruvate	H-3 (CH_3)	C-1**E**, (CO_2H), C-2**E**			H-5**D**, H-7/8**D**

[a] In addition to the long-range $^{1}H–^{13}C$ and $^{1}H–^{1}H$ NOE correlations shown in the table, the expected intra-residue contacts were observed for residues **A**, **B**, **C** and **D**.

ulosonic acid unit (3.96 ppm). The connectivity established by the transglycosidic NOE (H-1**A**/H-4**D**) indicates the partial sequence **A**-(1→4)-**D**. The absence of any corresponding 1,3-*syn*-axial correlations was consistent with the assigned α-glycosidic bond conformation for residue **A**. An inter-residue NOE at 3.70 ppm resulting from the irradiation of H-1**B** (4.76 ppm) indicated the β-Man*p* residue (**B**) to be linked to the β-D-Glc*p* residue (**C**) at either of the coincident O-3 or O-4 positions. However, a HMBC cross-peak between H-1**B** (4.76) and C-4**C** (79.9 ppm) (assigned in the HMBC-TOCSY experiment) (Fig. 5) established that the β-Glc*p* residue was glycosylated at O-4. The occurrence of cross-peaks relating H-1**C** and H-4**A** and between H-1**C** and C-4**A** in the NOESY and HMBC experiments, respectively, indicated the presence of a glycosidic linkage between C-1 of the β-Glc*p* and C-4 of the α-Glc*p* units.

Inter-residual NOEs were not observed upon irradiation of either of the two methylene H-3**D** proton resonances of the nonulosonic acid residue **D**; however, the position of the glycosidic linkage could be established through a long-range $^{1}H–^{13}C$ coupling observed between the ketose carbon C-2**D** (103.9) and H-3 (3.85 ppm) of the β-Man*p* residue (**B**) in the HMBC experiment. These results established the linear sequence of glycoses as →3)-**B**-(1→4)-**C**-(1→4)-**A**-(1→4)-**D**-(1→ and the accumulated evidence permits the basic repeating unit of the *R. equi* S4 polysaccharide to be assigned the structure **1**:

$$\underset{\mathbf{A}}{\text{Glc}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-RhoANAc-}(2\rightarrow}\underset{\mathbf{D}}{}\quad\underset{\mathbf{B}}{\rightarrow3)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow4)\text{-}\alpha\text{-D}}\underset{\mathbf{C}}{} \qquad (1)$$

Selective degradation of the capsular polysaccharide.—The *R. equi* serotype 4 CPS was depolymerised by treatment with dilute acetic acid and/or anhydrous hydrogen fluoride under mild hydrolytic/solvolytic conditions. Four different oligosaccharide species

2, **3**, **4** and **5** were generated, each of which was isolated by gel-filtration chromatography on Sephadex G25 and characterised by assignment of their ^1H and ^{13}C NMR resonances using homo- and heteronuclear chemical shift correlation methods.

Oligosaccharide **2** was generated from the CPS by the selective cleavage of the acid labile 3-deoxyaldulosonic acid glycosidic linkages with dilute acetic acid (2%, 100 °C, 2 h). These conditions also released the pyruvic acid moiety, indicating it to be linked as an acetal to one of the monosaccharide units [28].

Oligosaccharide **2** had $[\alpha]_D$ +5.0° (*c* 0.2, H_2O) and quantitative GLC analysis of the alditol acetates derived from the HF solvolysed and carboxyl-reduced products indicated the presence of glucosyl (T_{GA} 1.0), mannosyl (T_{GA} 0.9) and rhodaminic acid (T_{GA} 1.96 and 2.44) residues in an approximate ratio of 2:1:1. Diagnostic signals in the ^1H (Fig. 2(b)) and ^{13}C NMR spectra confirmed the presence of β-Man*p*, β-Glc*p*, α-Glc*p* and RhoANAc residues within the tetrasaccharide. The chemical shift of the signal arising from the equatorial proton at C-3 of the rhodaminic acid residue (2.06 ppm), being 0.69 ppm upfield of the corresponding value for H-3$\mathbf{D}_{eq}$ in the native polysaccharide (Table 1), is indicative of a β-anomeric configuration in which the C-1 carboxyl group has the equatorial disposition [29].

The value of the C-3**B** resonance in the tetrasaccharide (73.5 ppm) was observed to be shifted 5.5 ppm upfield of the corresponding carbon resonance in the native polysaccharide (79.0 ppm). This is indicative of an absence of glycosyl substitution at O-3 and confirms the placement of the mannosyl residue (**B**) at the non-reducing termini of tetrasaccharide **2**.

Mass spectrometry was used to confirm the predicted structure of oligosaccharide **2**. The fast-atom bombardment mass spectra (FABMS), obtained in the positive-ion mode, gave charged $(M+1)^+$ ions at *m*/*z* 796 corresponding to a protonated molecular ion, which is in excellent agreement with the calculated mass for $C_{29}H_{49}NO_{24}$ (795 Da). The complete structure of **2** is therefore that shown below.

β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→4)-β-RhoANAc (2)
B C A D

Prolonged heating of the tetrasaccharide with dilute acetic acid (2%, 100 °C, 20 h) resulted in complete removal of the 5-amino-3,5-dideoxynonulosonic acid, and after treatment with $NaBH_4$, the resulting trisaccharide (**3**) was recovered by gel chromatography on a column of Sephadex G-25. The trisaccharide had $[\alpha]_D$ −7.2° (*c* 0.1, H_2O) and GLC analysis of the derived alditol acetates indicated the presence of glucosyl and mannosyl residues in the ratio of 2:1. NMR analysis confirmed the structure of **3** as being a trisaccharide and the ^1H and ^{13}C chemical shifts and couplings constants are given in Tables 1 and 2, respectively; the ^1H spectrum is shown in Fig. 2(c). A protonated molecular ion at *m*/*z* 507 in the FABMS was consistent with the calculated molecular mass for the trisaccharide **3** ($C_{18}H_{34}O_{16}$ = 506 Da).

β-D-Man*p*-(1→4)-β-D-Glc*p*-(1→4)-D-Glcol (3)
B C A

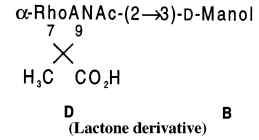
The glycosyl linkages within the trisaccharide were established by methylation analysis [30] that identified the 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylmannitol, 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 4-*O*-acetyl-1,2,-3,5,6-penta-*O*-methylglucitol derivatives in the mole ratio of 1:1:1. These analyses indicated **3** to be composed of D-Man*p*-(1→,→4)-D-Glc*p*-(1→ and →4)-D-Glcol residues, which is in accord with the sequence of glycoses determined by the HMBC experiment and by the NOE measurement performed on the intact *R. equi* serotype 4 capsular polysaccharide. The absolute configurations of the hexose residues within the polysaccharide were determined from the characteristic GLC retention times of their trimethylsilylated (*R*)-2-butyl glycosides [31] obtained from the trisaccharide **3** prior to reduction. In this way the glucosyl and mannosyl residues within the *R. equi* serotype 4 capsular polysaccharide were all assigned to the D-series.

Selective cleavage of the aldose glycosidic bonds was effected by solvolysis of the *R. equi*

S4 polysaccharide with anhydrous HF (20 °C, 2 h) and the oligosaccharides generated (**4** and **5**) were separated by high-performance anion-exchange (HPAE) chromatography after reduction with $NaBH_4$. The ^1H and ^{13}C NMR chemical shifts and coupling constants of **4** and **5** (Tables 1 and 2, respectively) were consistent with both being disaccharides comprised of single α-RhoANAc and D-mannitol residues. A comparison of the ^1H NMR spectra obtained from **4** and **5** (Fig. 2(d and e), respectively), revealed a peak in the high-field region of **5** at 1.53 ppm corresponding to the methyl protons of a pyruvic acid acetal. Analogously, the peak in the high-field region of the ^{13}C NMR spectra of **5**, at 22.6 ppm, was attributed to the methyl carbon of the carboxyethylidene group. The ketal carbon resonance (110.3 ppm) was identified by its association with the pyruvate methyl protons (1.53 ppm) in the long-range carbon–proton correlation experiment (HMBC). The absence of such resonances in the spectra of **4** indicates that the acetal had been removed during solvolysis.

Cross-peaks relating H-7 and H-9 to C-1 of the 1-carboxyethylidene group in the HMBC experiment (not shown) indicate that the pyruvate group bridges the O-7 and O-9 positions of the rhodaminic acid residue. An additional cross-peak between H-8 and the pyruvate carboxyl carbon suggests the formation of a lactone between the hydroxyl group at C-8 of RhoANAc and the carbonyl group of the pyruvic acid moiety. The formation of the lactone is corroborated by the downfield shifts of −0.43 and −10.8 ppm experienced by the H-8 (4.49) and C-8 (79.3 ppm) resonances of the RhoANAc residue in **5** when compared with the corresponding values in the polysaccharide. Furthermore, a large downfield shift (−10.5 ppm) of the signal arising from C-1**E** (110.3 ppm) was also observed in **5**. Such a significant change would be expected upon formation of the rigid bicyclic ring system produced upon lactonisation of the pyruvic acid acetal.

α-RhoANAc-(2→3)-D-Manol (4)
D B

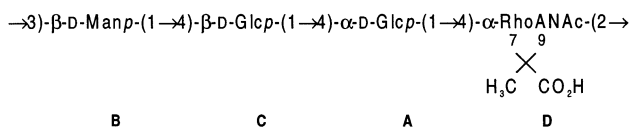


(5)

The structure of the disaccharide **4** was confirmed by FABMS, which gave a $(M-1)^-$ ion at m/z 472, in excellent agreement with the calculated mass for $C_{17}H_{31}NO_{14}$ (473 Da), thus confirming the structure to be that shown above. Disaccharide **5** gave a $(M-1)^-$ ion at m/z 524, which is 18 mass units less than the expected molecular ion for the pyruvate-containing derivative of disaccharide **4**. This result conclusively demonstrates that **5** is the lactone derivative of the structure shown above having a calculated molecular mass of 525 Da ($C_{20}H_{32}NO_{15}$).

3. Conclusions

The accumulated evidence permits the repeating unit of the native *R. equi* S4 capsular polysaccharide to be assigned the structure **6**.



(6)

4. Experimental

Isolation of the capsular polysaccharide.—*R. equi* serotype 4 (ATCC 33704), obtained from Dr J.F. Prescott, University of Guelph, Guelph, Ontario, was grown on plates as previously described [7]. Briefly, the specific CPS was isolated from plate-grown cells (109 g, wet weight) by mechanical dissociation from the cellular material into 4 M sodium chloride solution. Following enzymic digestion with ribonuclease, deoxyribonuclease and proteinase K, the crude polysaccharide was purified via its insoluble cetyltrimethyl ammonium salt [32]. After dissolution in aq NaCl (4 M), the sodium salt of the serotype 4 CPS was recovered as a white amorphous product by a sequence of precipitation with ethanol, extensive dialysis and lyophilisation (yield 520 mg, 0.5%).

Pure capsule was obtained by ion-exchange chromatography; a sample (20 mg), dissolved in water, was applied to a column (1 × 40 cm) of DEAE-Sephadex (Pharmacia) equilibrated with 0.05 M Tris–HCl buffer (pH 7.2), and the column was eluted with the same buffer (50 mL), followed by a 0–1 M NaCl gradient in Tris–HCl buffer. Fractions (2 mL) were collected and assayed colorimetrically for neutral glycose [11] and 5-amino-3,5-dideoxynonulosonic acids [12], and the appropriate fractions were pooled, dialysed and deionised with Rexyn 101 (H^+ form) ion-exchange resin before lyophilisation. Polysaccharide samples (20 mg) were subjected to gel chromatography on columns (1 × 40 cm) of Sephacryl S-500 (Pharmacia) eluted with pyridinium acetate (0.05 M, pH 4.5). Column eluants were continuously monitored for changes in refractive index by a Waters R403 differential refractometer, and fractions were assayed colorimetrically (as described above). The gel-filtration properties of the eluted materials were expressed in terms of their distribution coefficients,

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_0 is the void volume of the system, V_e is the elution volume of the specific material, and V_t is the total volume of the system.

Polysaccharide samples were tested for serological activity by the gel-diffusion test described by Prescott [4]. Serotype 4 *R. equi* antisera were a gift from Dr J.F. Prescott, University of Guelph.

Analytical methods.—Glycoses were determined by GLC of their alditol acetate derivatives [13]. Polysaccharide samples (0.2–0.5 mg) were dried in vacuo over P_2O_5 (16 h), solvolysed with HF in MeOH (0.5 mL, 2 h, room temperature (rt)), and then evaporated to dryness under a stream of nitrogen to give saccharides as their methyl esters and/or methyl glycoside derivatives. Following treatment with $NaBD_4$ or $NaBH_4$ (15 mg) for 6 h at 22 °C, the reaction mixture was neutralised with aq AcOH (10% v/v), concentrated, and boric acid was removed by repeated distillation of MeOH (5 × 5 mL) from the residue. After deionisation by passage through a column containing Rexyn 101 (H^+ form), the aqueous solution (2 mL) of the carboxyl-reduced methyl glycosides was lyophilised and dried over P_2O_5. Following resolvolysis with anhyd HF (0.5 mL, 2 h, rt), the glycosyl fluorides were hydrolysed with aq AcOH (50%, 16 h, 40 °C), and treatment with $NaBH_4$ (as stated) and Ac_2O (0.5 mL, 2 h, 115 °C) gave the corresponding alditol acetate derivatives.

Absolute configurations of the hexoses were determined by GLC–MS of the corresponding trimethylsilyl (*R*)-2-butyl glycoside derivatives [31]. The identity of the glycose derivatives was established by comparison of their GLC retention times and mass spectra with those of authentic reference compounds.

Methylation analysis was performed on trisaccharide **3**. Samples (2 mg) with iodomethane in Me_2SO containing an excess of sodium methylsulfinylmethanide were stirred at 22 °C [30], and after 60 min the products were recovered by partitioning the reaction mixture between water and CH_2Cl_2. The organic phase was concentrated and the methylated trisaccharide was hydrolysed with trifluoroacetic acid (0.5 mL, 2 M, 16 h), concentrated to dryness, reduced with $NaBD_4$ and acetylated (as stated).

GLC was performed with a Hewlett–Packard model 5890 chromatograph fitted with a hydrogen flame detector and using a fused-silica capillary column (0.3 mm × 25 m) that contained 3% DB17. The following temperature programs were used: (A) 2 min at 180 °C then 2 °C/min to 240 °C (methylated and acetylated alditols), and (B) isothermally at 175 °C (per-*O*-trimethylsilyl 2-butyl glycosides). The carrier gas was dry nitrogen at 30 mL/min and retention times are quoted relative to D-glucitol hexaacetate (T_{GA}). GLC–MS was performed using a Jeol AX505-H mass spectrometer using the GLC program conditions A or B by electron impact (EI), employing an ionisation potential of 70 eV, or by chemical ionisation (CI) with ammonia as the reagent gas.

HPAE was carried out using a Dionex BioLC carbohydrate system with a Dionex Carbopac PA1 column (9 × 250 mm), an AG-6 guard column and pulsed amperometric detection. Elution was performed using the following gradient: 5 min with 95% 150 mM NaOH

(eluant A) and 5% 150 mM NaOH in 150 mM sodium acetate (eluant B) then 0.42% B to 30% B, at a flow rate of 2 mL/min.

Selective degradations of the capsular polysaccharide

Mild acid hydrolysis. The serotype 4 CPS (50 mg), in 2% AcOH, was kept at 100 °C for 2 h (to produce the tetrasaccharide) or 20 h (for the trisaccharide). The resulting hydrolysates were lyophilised, dissolved in pyridinium acetate buffer (0.05 M, pH 4.5) and fractionated by gel chromatography on a column (2.5 × 70 cm) of Sephadex G-25 using the same buffer as the eluant.

Hydrogen fluoride solvolysis. The native polysaccharide (50 mg), suspended in MeOH (0.2 mL), was treated with anhydrous HF (2 mL) at rt for 2 h. The excess HF was removed by evacuation under reduced pressure and the remaining traces of acid were neutralised with NH_4OH. The lyophilised products were dissolved in water and fractionated on a column of Sephadex G25.

NMR spectroscopy.—Nuclear magnetic resonance spectra were obtained on a Bruker AM 500 spectrometer equipped with an Aspect 3000 computer, using standard Bruker software. Solutions of saccharide samples in deuterium oxide (E. Merck, Sharp and Dohme, 99.8 atom%) were prepared at a concentration of 10–40 mg/mL subsequent to lyophilisation. ^1H and ^{13}C NMR measurements were made on solutions (0.4 mL) of the polysaccharides (pD ~ 2) in 5 mm tubes at 42 °C or 25 °C. ^1H NMR spectra at 500 MHz were recorded using a spectral width of 2.5 kHz, an acquisition time of 3.2 s and a 90° pulse and the chemical shifts are reported in ppm, relative to internal acetone (2.225 ppm). Proton spin simulations with a line width of 2 Hz were performed with the program LAOCN5 in the FTNMR package.

Broad-band proton decoupled ^{13}C NMR spectra were obtained at 125 MHz using a spectral width of 25 kHz and a 90° pulse, employing composite pulse proton decoupling (WALTZ) [33]. ^1H-coupled ^{13}C NMR spectra were determined by gated coupling [34], and the chemical shifts are referenced to internal acetone (31.07 ppm).

Homonuclear 2D chemical-shift-correlated spectroscopy, COSY [14], and nuclear Overhauser enhancement spectroscopy, NOESY [35], were carried out as previously described [6]. Data was acquired over the full spectrum (sweep width 2.5 kHz), or for the ring proton region (1.2 kHz). A total of 64 scans were collected for each value of t_1, and a mixing time of 200 ms was employed for the NOESY experiment.

Two-dimensional heteronuclear $^1H-^{13}C$ chemical shift correlations were measured in the ^1H-detected mode via multiple quantum coherence [$^1H(^{13}C)$ HMQC] with a Bruker 5-mm inverse broad-band probe using reverse electronics. The HMQC experiment employed the pulse sequence described by Bax and coworkers [36,37], and ^{13}C decoupling during ^1H acquisition was achieved using the GARP-1 composite pulse sequence [38]. The HMBC [24] spectrum was recorded using a delay of 60 ms for the evolution of long-range couplings, and the HMQC-TOCSY [39] experiment employed a mixing time of 22 ms.

General methods.—Commercial reagents and solvents were analytical grade. Concentrations were made under reduced pressure at bath temperatures below 40 °C or by flushing with nitrogen at rt. Optical rotations were determined at 22 °C in 10 cm microtubes using a Perkin–Elmer model 243 polarimeter.

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