Carbohydrate Research, 252 (1994) 223–233
Elsevier Science B.V., Amsterdam

Structural elucidation of the specific capsular polysaccharide of *Rhodococcus equi* serotype 7 *

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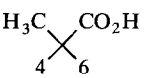
(Received March 22nd 1993; accepted June 18th, 1993)

ABSTRACT

Structural analysis of the specific capsular polysaccharide produced by *Rhodococcus equi* serotype 7 indicated it to be a high-molecular-weight polymer consisting of equal molar amounts of D-galactose, D-mannose, L-rhamnose, and pyruvic acid. By employing a combination of chemical and NMR techniques, it was established that the polysaccharide is composed of the linear repeating trisaccharide units,



→ 3)-α-D-Gal*p*-(1 → 3)-α-D-Man*p*-(1 → 3)-α-L-Rha*p*-(1 → ,

in which the cyclic pyruvic acid acetal groups bridging the O-4 and O-6 positions of the α-D-Man*p* residues have the *S*-configuration. The ^{1}H and ^{13}C NMR spectra of the native and pyruvic acetal-free polysaccharides were fully assigned.

INTRODUCTION

Rhodococcus equi is a heavily encapsulated gram-positive bacterial pathogen which causes serious and often fatal respiratory diseases in foals[1]. The chronic nature of these infections suggest that foals are exposed to the pathogen weeks before the onset and diagnosis of disease[2,3]. The prevalence of disease in foals is thought to be related to the number of organisms in the environment as well as exposure to particularly virulent strains[4]. Capsular polysaccharide (CPS) is a potential virulence determinant of this organism. Serological studies have indicated considerable antigenic heterogeneity among the capsular antigens exhibited by strains of *R. equi*, and at least seven capsular serotypes are recognized[5,6]. Although strains belonging to two capsular serotypes are most frequently isolated

* Issued as NRCC Publication No. 34330

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SSDI 0008-6215(93)E0252-V

from diseased foals, the relationships between capsular serotypes and pathogenicity of the organism is not known[5,7].

Information on the structures of *R. equi* CPS antigens can provide the molecular framework for identifying important epitopes involved in pathogenesis and thereby lead to better methods for detection and prevention of diseases. We have undertaken a detailed chemical study of the *R. equi* CPS antigens. To date, the CPS of four serotypes (serotypes 1, 2, 3, and 6) have been fully characterized[8–11]. These serotype-specific polysaccharides were each found to be high-molecular-weight heteroglycans composed of tetrasaccharide repeating units containing D-glucuronic acid residues and immunologically significant acidic substituents (pyruvic acid acetals and/or lactic acid ethers). In this study we report the structure of the specific CPS of *R. equi* serotype 7 that contains cyclic pyruvic acid groups, but lacks D-glucuronic acid residues.

RESULTS AND DISCUSSION

Isolation and purification.—The specific capsular polysaccharide produced by *R. equi* serotype 7 was isolated from plate-grown cells by suspension of the cellular material into 2 M sodium chloride. CPS was separated from coextracted nucleic acid and protein by precipitation from cold aqueous ethanol and then purified by ion-exchange chromatography on DEAE-Sephacel from which it was obtained as a discrete band at the beginning of the sodium chloride gradient. The polysaccharide eluted as the void volume fraction on a Sephadex G-50 gel-filtration system indicating it to be a high-molecular-weight polymer.

Composition.—Complete acid hydrolysis of the serotype 7 capsular polysaccharide revealed three glycose residues that were identified as their alditol acetate derivatives by GLC[12]. L-Rhamnose, D-mannose, and D-galactose were detected in the molar ratios of 0.7:0.8:1.0, respectively. The absolute configurations of the three glycoses were determined from the GLC retention times and mass spectra of their acetylated (*R*)-2-butyl glycoside derivatives[13]. In addition, pyruvic acid was identified by high-performance anion-exchange chromatography (HPAEC)[8,14].

The pyruvic acid moiety could be readily released from the polysaccharide by an autohydrolysis procedure (pH ~ 4, 80°C, ca. 16 h), indicating that the substituent was linked to a glycosyl residue as a cyclic acetal[15]. Correspondingly, the ^{1}H and ^{13}C NMR spectra of the native polysaccharide showed signals characteristic of acetal-linked pyruvic acid substituents[16].

In the ^{1}H NMR spectrum, the resonance arising from the pyruvate methyl protons occurred at 1.54 ppm (Fig. 1A); this signal was absent from the spectrum of the polymeric autohydrolysis product (Fig. 1B). The native and the pyruvic acetal-free polysacccharides both showed three signals of equal signal area in the region characteristic of anomeric protons (4.5–5.5 ppm), together with high-field resonances at ~ 1.3 ppm (d, J ~ 6 Hz), indicative of the methyl protons for the L-rhamnose residues (Fig. 1).

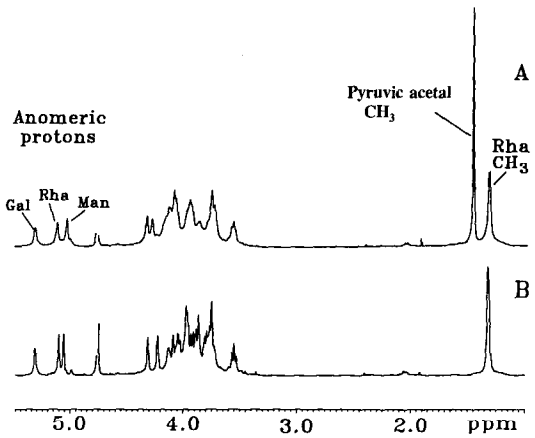


Fig. 1. ^{1}H NMR spectra for A. native and B. pyruvic acetal-free *R. equi* serotype 7 capsular polysaccharide.

The ^{13}C NMR spectrum of the pyruvic acetal-free polysaccharide showed three resonances for the anomeric carbons of the constituent glycosyl residues (101.5, 102.9, and 97.1 ppm), clearly indicating the polymer to be composed of regular trisaccharide repeating units. A fourth signal was observed in same region of the ^{13}C NMR spectrum (95–105 ppm) of native CPS (100.7 ppm), which was identified as a quaternary ^{13}C resonance from an HMQC experiment[17] and therefore assigned to the pyruvic acid acetal carbon. In addition, resonances for the carboxyl and methyl carbons of the pyruvic acetal occurred at 176.1 and 25.5 ppm, respectively, and for the L-rhamnose methyl carbon at 17.5 ppm.

Methylation[18] of the native and pyruvic acetal-free polysaccharides, followed by hydrolysis, reduction and acetylation[19], afforded derivatives which by GLC–MS analysis gave the positions of the linkages of the component glycosyl residues (Table I). For both polysaccharides, the data indicated that the L-rhamnose and D-galactose residues were substituted at the O-3 positions. The presence of 2-*O*-methyl-D-mannose in the hydrolysis products from the methylated native CPS indicated that the D-mannose residues are substituted at the O-3, O-4, and O-6 positions. Since the products derived from the methylated pyruvic acetal-free polysaccharide contained 2,4,6-tri-*O*-methyl-D-mannose instead of the 2-*O*-methyl analogue, established that the pyruvic acetal groups are linked to the O-4 and O-6 positions of $\rightarrow$3)-D-Man*p*-(1$\rightarrow$ units in the native polymer. This substitution pattern has also been identified for the pyruvic acetal-substituted D-mannose residues present in the *R. equi* serotype 1 CPS[8].

The combined analytical results indicated that the *R. equi* serotype 7 CPS is composed of linear repeating trisaccharide units containing 1,3-linked D-Gal*p*, L-Rha*p*, and D-Man*p* component glycosyl residues.

Sequence analysis.—The sequence of the glycosyl residues within the polysaccharide was determined by high-resolution NMR spectroscopy. This required

TABLE I

Methylation analysis data for the native and pyruvic acetal-free *R. equi* serotype 7 capsular polysaccharides

Derivative	T_G [a]	Molar ratio [b]	
		Native Polysaccharide	Pyruvic acetal-free polysaccharide
1,3,5-Tri-*O*-acetyl-2,4-di-*O*-methyl-L-rhamnitol-1-*d*	0.68	0.7	0.4
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol-1-*d*	1.00	1.0	1.0
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-mannitol-1-*d*	0.98		1.0
1,3,4,5,6-Penta-*O*-acetyl-2-*O*-methyl-D-mannitol-1-*d*	1.39	0.3	

[a] Retention time relative to that of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol. [b] Uncorrected detector response expressed relative to that of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-galactitol.

initial complete assignment of the ^1H and ^{13}C resonances and this was achieved by employing 2D homo- and hetero-nuclear chemical shift correlation techniques.

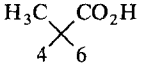
The ^1H NMR spectra of the native and pyruvic acetal-free polysaccharides were fully assigned from COSY experiments from which subspectra corresponding to each of the three glycosyl residues were fully delineated. The anomeric proton resonances served as the starting point for spectral analysis (Fig. 1). From the chemical shift[20] and vicinal proton coupling constant[21] values, ^1H spin-systems typical of an *α-galacto-* and two *α-manno*pyranosyl units were identified. A distinction between the spin systems from the α-D-Man*p* and α-L-Rha*p* residues was evident from the large high-field value for the H-6 methyl protons of the latter residue. The observed chemical shifts and coupling constants are recorded in Table II.

Assignment of the ^{13}C resonances was effected by correlation with the ^1H resonances of the directly attached protons by $^1H-^{13}C$ HMQC experiments[17] (Table III). For the pyruvic acetal-free polysaccharide, the magnitude of the heteronuclear one-bond $^1H-^{13}C$ coupling constants was found to be ca. 170 Hz for each of the anomeric carbon resonances, confirming that all linkages possess the α-anomeric configuration[22]. The ^{13}C resonances for C-4 and C-6 of the α-D-Man*p* residues showed upfield shifts of 8.3 and 3.9 ppm, respectively, following depyruvulation of the native CPS, which provided further evidence for substitution by the pyruvic acetals at the corresponding positions[22]. The chemical shift value for the pyruvic methyl carbon (25.5 ppm) is indicative of an equatorially disposed methyl group in the cyclic pyruvic acetal[16]. This corresponds to the *S*-configuration at the pyruvic acetal carbon. In agreement with the methylation analysis data, significant deshielding of the C-3 resonances (Table III) clearly indicated the respective positions as the sites of the glycosyl linkages[22]. The $^1H-^{13}C$ chemical shift correlation map for the ring region of the pyruvic acetal-free polysaccharide is shown in Fig. 2.

The sequence of the glycosyl residues within the repeating trisaccharide units was determined from transglycosidic NOE connectivities observed between

TABLE II

^{1}H NMR chemical shifts (ppm) and coupling constants [a] (Hz) for native and pyruvic free *R. equi* serotype 7 capsular polysaccharides

Glycosyl residue	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′ ($J_{5,6'}$, $J_{6,6'}$)	Pyruvic acid residue CH_3
Native								
→3)-α-D-Gal*p*-(1→	5.35	3.92	4.03	4.07	4.09	3.77	3.73	
	(4.7)	(9.5)	(3.8)	(~1)		(7.6)	(3.8, 11.3)	
→3)-α-L-Rha*p*-(1→	5.10	4.26	3.94	3.55	3.85	1.31		
	(<3)	(4.4)	(9.5)	(10.4)		(5.5)		
								
→3)-α-D-Man*p*-(1→	5.03	4.28	4.21	4.01	4.04	4.16	3.74	1.54
	(<3)	(4.7)	(10.4)	(9.1)		(4.4)	(9.4, 11.4)	
Pyruvic acetal-free								
→3)-α-D-Gal*p*-(1→	5.31	3.95	4.03	4.08	4.13	3.77	3.73	
	(4.1)	(10.3)	(3.8)	(~1)		(7.5)	(3.8, 11.7)	
→3)-α-L-Rha*p*-(1→	5.10	4.30	3.96	3.54	3.86	1.31		
	(<1)	(4.1)	(10.3)	(10.3)		(5.9)		
→3)-α-D-Man*p*-(1→	5.05	4.22	4.05	3.91	3.79	3.97	3.87	
	(<1)	(3.8)	(9.4)	(9.8)		(2.8)	(4.7, 11.3)	

[a] First-order chemical shifts and coupling constants (±0.5 Hz) measured at 27°C in D_2O (pD 7.0).

TABLE III

^{13}C NMR chemical shift (ppm) for native and pyruvic acetal-free *R. equi* serotype 7 capsular polysaccharide [a]

Glycosyl Residue	C-1	C-2	C-3	C-4	C-5	C-6	Pyruvic acid residue		
							$-CH_3$	-CH	-COOH
Native									
→ 3)-α-D-Gal*p*-(1 →	100.9	68.8	77.8	69.9	72.3	62.0			
→ 3)-α-L-Rha*p*-(1 →	102.7	67.3	75.8	71.0	69.9	17.5			
H_3C CO_2H 4 6									
→ 3)-α-D-Man*p*-(1 →	98.3	71.0	75.5	75.0	63.9	65.5	25.5	100.7	176.1
Pyruvic acetal-free									
→ 3)-α-D-Gal*p*-(1 →	101.5 (174)	68.9	77.9	70.0	72.4	62.1			
→ 3)-α-L-Rha*p*-(1 →	102.9 (171)	67.0	75.1	71.2	70.0	17.6			
→ 3)-α-D-Man*p*-(1 →	97.1 (169)	70.9	79.4	66.7	73.6	61.6			

[a] Measured at 27°C in D_2O (pD 7). $^1J_{C\text{-}1,H\text{-}1}$ values are indicated in parentheses.

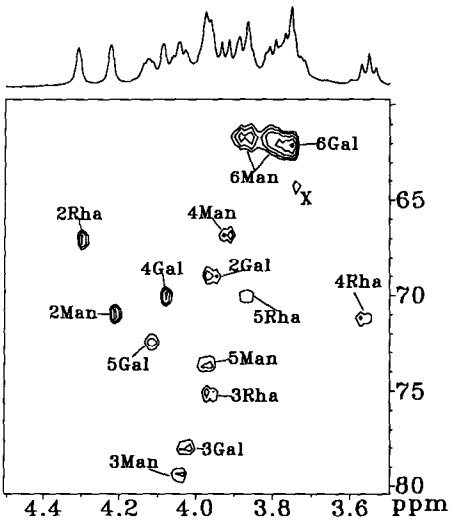


Fig. 2. Contour map of the glycosyl ring-region of the $^{1}H-^{13}C$ chemical shift correlation experiment on the pyruvic acetal-free *R. equi* serotype 7 polysaccharide. Assignments are indicated; contour indicated by *X* is due to a minor impurity in the sample.

anomeric and aglyconic protons on contiguous residues in the polysaccharide. NOE measurements were made on the pyruvic acetal-free polysaccharide in the 2D mode by using a NOESY experiment.

In accord with the assigned α-anomeric configurations of the three component glycosyl residues, strong intraresidue NOEs were observed between the resonances for H-1 and H-2 within each pyranose ring system. A strong transglycosidic NOE observed between the α-D-Gal*p* H-1 (5.31 ppm) and the α-D-Man*p* H-3 (4.05 ppm) resonances was indicative of the partial disaccharide sequence, α-D-Gal*p*-(1 → 3)-α-D-Man*p*. The anomeric proton resonance of the α-D-Man*p* (5.05 ppm) showed a strong NOE connectivity across the glycosidic linkage to both the H-2 (4.30 ppm) and H-3 (3.96 ppm) resonances of the α-L-Rha*p* residue establishing the sequence of the trisaccharide unit. Since the methylation analysis results (Table I) indicated that the L-Rha*p* residues are substituted at the O-3 position, it follows that the trisaccharide unit of the pyruvic acetal-free polysaccharide has the structure,

→ 3)-α-D-Gal*p*-(1 → 3)-α-D-Man*p*-(1 → 3)-α-L-Rha*p*-(1 → .

Moreover, transglycosidic NOE connectivities were observed between the resonances for α-L-Rha*p* H-1 (5.10 ppm) and α-D-Gal*p* H-3 (4.03 ppm), establishing the contact between adjacent trisaccharide units within the polymer. The observed NOE connectivities are shown in Fig. 3.

The occurrence of significant NOE connectivities between α-D-Man*p* H-1 and both H-2 and H-3 of the α-L-Rha*p* residues can be attributed to the conformation

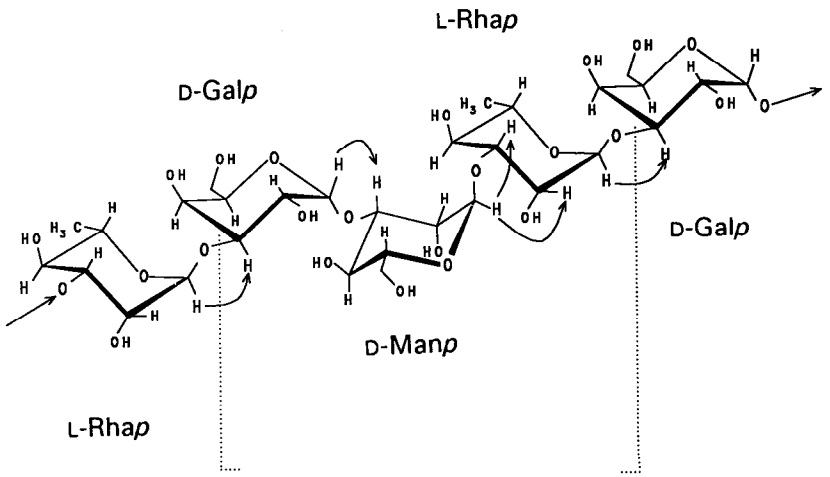
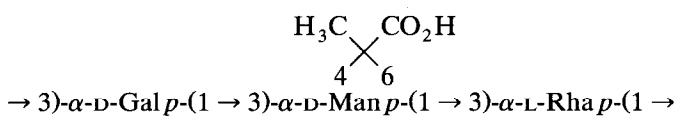


Fig. 3. Structure of the pyruvic acetal-free *R. equi* capsular polysaccharide illustrating the network of observed transglycosidic NOE connectivities used for providing the sequence of the glycosyl residues. The trisaccharide repeating unit is indicated by the dashed lines.

about the (1 → 3)-α-D-glycosidic linkage of this disaccharide unit in the polysaccharide[23].

The combined evidence from NMR spectroscopy and chemical analysis leads to the conclusion that the repeating unit of the *R. equi* serotype 7 capsular polysaccharide has the following structure,



The acidic nature of this polysaccharide is attributed solely to the pyruvic acid moieties.

The capsular polysaccharides of the *R. equi* serotypes so far investigated have all been found to carry negatively charged acid groups. It is thought that negatively charged capsules provide protection against phagocytosis and the bactericidal action of serum complement[24]. The capsular antigens of serotypes 1 and 6 contain β-D-Man*p* and α-D-Gal*p* residues, respectively, in which the O-4 and O-6 positions are bridged by pyruvic acid acetal groups[8,11], while the serotype 2 polysaccharide carries lactic acid ether substituents at the O-3 position of α-L-Rha*p* residues. Both of these acidic substituents occur in the serotype 3 polysaccharide in which the pyruvic acid acetal groups are linked to O-2 and O-3 of β-D-Glc*p*A residues and lactic acid ethers are linked to O-4 of β-D-Man*p* residues[10]. Pyruvic acid acetals, which occur widely in bacterial polysaccharides[25], are recognized as immunodominant structural features[15].

EXPERIMENTAL

Materials.—Rhodococcus equi serotype 7 (strain 97, horse lung isolate 79E 902, ATCC 33706)[5] was obtained from Dr. J.F. Prescott, University of Guelph, Guelph, Ontario. The organism was grown on plates and collected as previously described[8,9].

Isolation and purification.—The capsular polysaccharide was isolated from the bacterial growth (71.2 g wet weight) by stirring a suspension of cells in 2 M NaCl solution at ambient temperature overnight. The cellular material was removed by centrifugation (10 000 rpm, 2 h, 4°C) and was again extracted with 2 M NaCl. The combined supernatant solutions were dialyzed against distilled water and lyophilized until salt free. Crude material obtained in this way was dissolved in a minimum amount of water, treated with 4 vol of EtOH, and then kept overnight at 4°C. The precipitated CPS was obtained by centrifugation (5 000 rpm, 30 min, 4°C) and then purified by ion-exchange chromatography using DEAE-Sephacel (Pharmacia) as previously described[10].

Capsular polysaccharide was subjected to gel filtration on a Sephadex G-50 column (2.6 × 85 cm) (Pharmacia) by eluting with pyridinium acetate (0.05 M, pH 4.5). Column eluants were monitored for changes in refractive index by a Waters R403 differential refractometer, and fractions (2 mL) were assayed colourimetrically for neutral glycoses using the phenol–H_2SO_4 assay[26].

Analytical methods.—Glycoses were identified and quantitated as their alditol acetate derivatives by GLC[12]. Samples (0.2–0.5 mg) were hydrolyzed with 2 M CF_3CO_2H for 1 h at 125°C and evaporated to dryness under a stream of N_2. The liberated glycoses were reduced ($NaBH_4$) and acetylated (Ac_2O) as previously described[12].

GLC was performed with a Hewlett-Packard model 5710A gas–liquid chromatograph fitted with a hydrogen flame-ionization detector and a model 3380 A electronic integrator using a fused-silica capillary column (0.3 mm × 25 m) containing 3% OV 17. An initial column temperature of 180°C was held for 2 min, followed by an increase to 240°C at 4°C/min.

Pyruvic acid was identified by HPAEC[8] on a Dionex BioLC system equipped with a column (4.0 × 250 mm) of Dionex CarboPac PA1 pellicular anion-exchange resin and an PA1 guard column with pulsed amperometric detection.

Absolute configurations of the glycoses were determined by GLC analysis of the corresponding acetylated (*R*)-2-butyl glycoside derivatives as previously described[8].

Methylation analysis.—Polysaccharide samples (2–5 mg) were methylated with methyl iodide in Me_2SO containing an excess of potassium methylsulfinylmethanide[18]. Excess iodomethane was evaporated under a stream of N_2, and the methylated polysaccharide was dialyzed against distilled water and lyophilized. Purified methylated polysaccharide was hydrolyzed, reduced ($NaBD_4$) and acetylated according to the acetolysis procedure of Stellner et al.[19] and analyzed using GLC–MS. Partially methylated alditol acetates were identified by EIMS on a Hewlett–Packard 5958B GLC–MS system utilizing an ionization potential of 70

eV and the temperature program, 180°C for 2 min, followed by an increase to 300°C at 5°C/min.

Depyruvylation of the polysaccharide.—The pyruvic acetal groups were removed from native capsular polysaccharide via autohydrolysis. Polysaccharide was converted to salt-free polymer using Rexyn 101 (H^+ form) ion-exchange resin, followed by lyophilization of the eluant. Deionized polysaccharide (10 mg) was dissolved in 0.5 mL H_2O (or D_2O) (pH ~ 4) and heated overnight at 80°C.

NMR spectroscopy.—NMR spectra were obtained on a Bruker AMX 500 spectrometer using standard Bruker software. Measurements were made at 27°C at concentrations of ~ 20 mg/mL D_2O subsequent to several lyophilizations with D_2O. Acetone was used as internal standard, and chemical shifts are referenced to the resonances of the methyl group (δ, 2.225 ppm for ^1H; and δ, 31.07 ppm for ^{13}C).

^1H NMR spectra were measured using a spectral width of 2.75 KHz and a 90° pulse. Broadband proton-decoupled ^{13}C NMR spectra were obtained at 125 MHz using a spectral width of 25 KHz, a 90° pulse and WALTZ decoupling[27].

Two-dimensionsal COSY[28] and NOESY[29] experiments were measured with solvent suppression employing the conventional pulse sequences as previously described[8]. A mixing time of 200 ms was employed for the NOESY experiment. Heteronuclear $^1H-^{13}C$ chemical shift correlations were measured in the ^1H-detected mode via multiple quantum coherence (HMQC)[17] as previously described. An HMQC experiment acquired without proton decoupling was employed for determining one-bond $^1H-^{13}C$ coupling constant ($^1J_{C,H}$) values.

ACKNOWLEDGMENTS

We thank Dr. J.F. Prescott, University of Guelph for providing *R. equi* serotype 7 bacterial culture, Mr. R.A. Leitch for large-scale production of cells, Mrs. A. Martin for stereochemical analysis of glycoses, and Mr. F.P. Cooper for GLC–MS analyses.

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