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Structural analysis of the specific capsular polysaccharide of *Rhodococcus equi* serotype 2*

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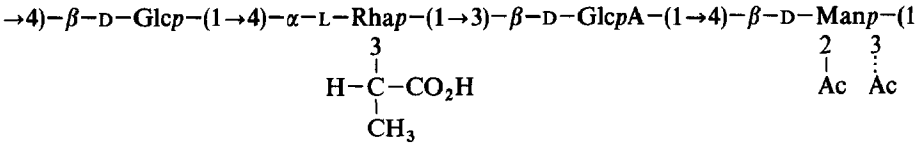
ABSTRACT

The specific capsular polysaccharide produced by *Rhodococcus equi* serotype 2 is a high-molecular-weight acidic polymer composed of D-glucose, D-mannose, D-glucuronic acid and 3-*O*-[(*S*)-1-carboxyethyl]-L-rhamnose in equimolar proportions. Structural analysis, employing a combination of chemical and n.m.r. techniques, established that the polysaccharide is composed of linear repeating tetrasaccharide units,



in which the β-D-mannose residues carry *O*-acetyl groups at O-2 and O-3 to the extent of 1.7 mol equivalents. Unequivocal determination of the absolute chirality of the 3-*O*-[(*S*)-1-carboxyethyl]-α-L-rhamnose residues was achieved by chemical correlation with an authentic synthetic sample. The ^{1}H and ^{13}C-n.m.r. resonances of the native and *O*-deacetylated serotype 2 polysaccharides were fully assigned by homo- and hetero-nuclear chemical-shift correlation methods.

INTRODUCTION

Rhodococcus equi[1] is a bacterial pathogen that causes a serious, and often fatal, bronchopneumonia of foals of between 4 and 12 weeks of age[2]. The chronic nature of the infection suggests that the foals are exposed to the disease weeks before diagnosis[3], and antibiotic treatment[4] at this time is largely ineffectual. Limited success in the control of *R. equi* pneumonia has been achieved with the use of crude, whole-cell vaccines[5], however, the efficacy of these vaccines is uncertain.

Seven seroptypes of *R. equi* are recognized, of which types 1 and 2 constitute 60 and 26% of the disease isolates respectively[6]. The occurrence of capsule has been confirmed by electron microscopy[7] and serotype specificity is reported[6] to be associated with the polysaccharide capsular antigens. Because the development of protective vaccines and diagnostics to *R. equi* are of considerable interest as a means of controlling

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disease, our laboratory has undertaken detailed chemical studies of the specific capsular polysaccharide antigens. The serotype 1 specific capsular polysaccharide has been fully characterized[8]. We now report the structure of the specific capsular polysaccharide produced by *R. equi* serotype 2.

RESULTS AND DISCUSSION

Isolation and purification. — The specific capsular polysaccharide produced by *R. equi* serotype 2 was isolated from plate-grown cells by mechanical dissociation from the cellular material into 4M sodium chloride solution. Following enzymic digestion, with ribonuclease, deoxyribonuclease, and proteinase K, the polysaccharide was purified *via* its insoluble cetyltrimethylammonium salt[9]. After dissolution in 4M sodium chloride solution, serotype 2 specific polysaccharide was recovered by a sequence of precipitation with ethanol, dialyses, deionization, and lyophilization.

Pure capsule was obtained, by ion-exchange chromatography on DEAE-Sephacel, as a discrete band at the beginning of the sodium chloride gradient and was judged to be free from nucleic acids and proteins since neither ribose nor amino acids were detectable in its acid hydrolysis products. The polysaccharide had $[\alpha]_D$ $-50.7°$ (*c* 1.4, H_2O), *Anal.* found: C, 40.14; H, 5.25; N, 0.00%, and gave a single sharp precipitin line in immunodiffusion against rabbit antisera to *R. equi* serotype 2 cells[6]. All subsequent analyses were made on the DEAE-Sephacel purified product.

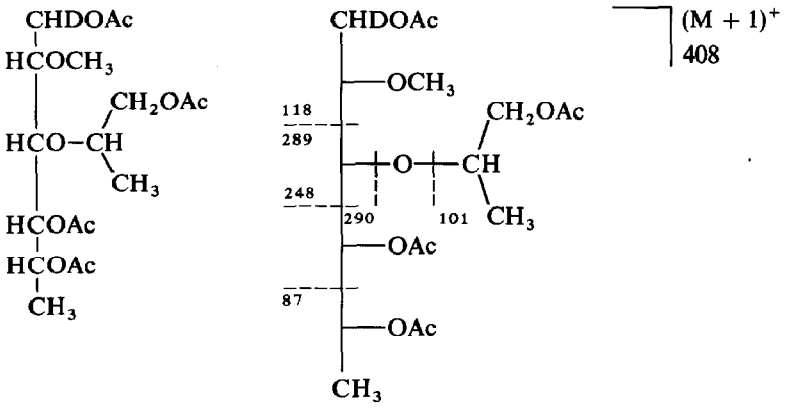
Composition. — The serotype 2 capsular polysaccharide eluted as a sharp band at the void volume (K_{av} 0.0) of a Sephadex G-200 gel-filtration system, indicating it to be a polymer of high molecular weight. Qualitative analysis of the eluant revealed that the polysaccharide contained both neutral[10] and hexuronic acid[11] components.

Analysis of the polysaccharide hydrolysis products (2M trifluoroacetic acid, 16 h, 100°) by h.p.l.c.[12] (eluant *A*) indicated the neutral glycoses to be, glucose (R_{Glc} 1.0) and mannose (R_{Glc} 1.1), while glucuronic acid (R_{Glc} 3.9) and a fourth component, residue **a** (R_{Glc} 2.4), were identified, using eluant *B*, as the acidic constituents of the hydrolyzate.

G.l.c. analysis (program *A*) of the reduced and acetylated hydrolysis products[13], derived from the *R. equi* serotype 2 polysaccharide, indicated that the neutral glycosyl residues, which were identified as the hexaacetates of glucitol (T_{GA} 1.0) and mannitol (T_{GA} 1.1), were present in equimolar proportions. Methanolysis and treatment of the polysaccharide with sodium borohydride was employed to effect carboxyl-group reduction. Subsequent hydrolysis and formation of the alditol acetates indicated the presence of mannose, glucose and residue **a** (T_{GA} 1.2) in the ratio of 1:2:1. The glucuronic acid component was identified by m.s. as 1,2,3,4,5,6-hexa-*O*-acetylglucitol-6,6-d_2 when carboxyl reduction was carried out with sodium borodeuteride, and it constituted 48% of the derived D-glucitol hexaacetate. The c.i.–m.s. fragmentation pattern of the carboxyl-reduced ($NaBH_4$) alditol acetate derived from the residue **a** exhibited a molecular ion at *m/z* 435. An intense peak observed at *m/z* 101 is indicative of an acetoxypropyl group[14] and the shift of this fragment by two mass units to *m/z* 103 when carboxyl reduction was effected with $NaBD_4$ indicated it to be derived from a carboxyethyl moiety. These data

suggested that the acidic fragment of residue **a** is a lactic acid residue etherifing a deoxyhexose.

The methylated[15], reduced[16] ($NaBH_4$), and hydrolyzed polysaccharide afforded products that, after reduction ($NaBD_4$) and acetylation[17], furnished valuable mass-spectral data on the component glycosyl residues. The e.i.–m.s. fragmentation pattern proposed for the derivatized residue **a** is depicted here (**1**). The primary fragment of *m/z* 101 confirms the presence of the acetoxypropyl moiety, and peaks at *m/z* 87, 118, 246, 289, and 290 place this substituent at the 3-position of a 6-deoxyhexopyranose. The combined results taken together with subsequent n.m.r. data (see later) establish that the residue **a** is 3-*O*-(1-carboxyethyl)rhamnose.



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Absolute configuration. — The absolute configurations of the unsubstituted glycoses were established from the characteristic g.l.c. retention times of their acetylated (*R*)-2-butyl glycosides[18]. In this way the glucosyl, mannosyl, and glucuronic acid residues were all assigned to the D-series.

The relative configuration of the 3-*O*-(1-carboxyethyl)rhamnosyl residue, **a**, was determined by comparison of its chromatigraphic properties with those of synthetic samples of the diastereoisomeric acids[19]. Treatment of the polysaccharide with anhydrous methanol–HCl (16 h, 100°) afforded predominantly the methyl ester of the methyl α-glycoside (**2**) which, after carboxyl reduction and acetylation, exhibited a g.l.c. retention time coincident with that observed for the peracetylated derivative of methyl 3-*O*-[(*S*)-1-acetoxypropyl]-α-L-rhamnopyranoside (Table I). This result suggested that the polysaccharide contains either a L-rhamnopyranose residue 3-*O*-substituted with lactic acid having the *S*-configuration or the enantiomeric D-rhamnopyranose substituted by the *R*-lactic acid.

A distinction between the two enantiomeric forms was achieved by transglycosylation of the carboxyl-reduced, methyl 3-*O*-[1-(methoxycarbonyl)ethyl]-α-rhamnopyranoside (**2**), derived from the polysaccharide, into the peracetylated (*R*)-2-butyl glycoside[18]. The g.l.c. retention time of this derivative was identical with that of the corresponding derivative from the 3-*O*-[(*S*)-1-hydroxypropyl]-α-L-rhamnopyranoside (Table I), establishing its absolute chirality.

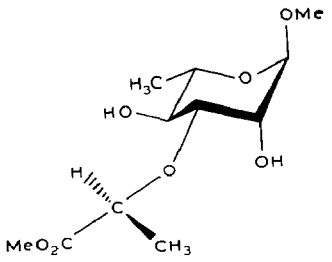

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TABLE I

Comparison of the chromatographic properties of the peracetylated methyl and 2-butyl glycosides of 3-*O*-(1-hydroxypropyl)-α-L-rhamnopyranosides

Derivative	*T*
Methyl glycosides	
Carboxyl-reduced residue **a**	1.01[a]
Methyl 3-*O*-[(*S*)-1-hydroxypropyl]-α-L-rhamnopyranoside	1.01
Methyl 3-*O*-[(*R*)-1-hydroxypropyl]-α-L-rhamnopyranoside	1.06
2-Butyl glycosides	
Carboxyl-reduced residue **a**[b]	1.33[c]
(*R*)-2-Butyl 3-*O*-[(*S*)-1-hydroxypropyl]-α-L-rhamnopyranoside	1.33
(*S*)-2-Butyl 3-*O*-[(*S*)-1-hydroxypropyl]-α-L-rhamnopyranoside[d]	1.31

[a] Retention time relative to that of methyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, using g.l.c. program *A*. [b] As the (*R*)-2-butyl glycoside. [c] Retention time relative to that of (*R*)-2-butyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranoside, using g.l.c. program *A*. [d] Chromatographic properties are identical to those of the enantiomeric (*R*)-2-butyl 3-*O*-[(*R*)-1-hydroxypropyl]-α-D-rhamnopyranoside.

TABLE II

Methylation analysis data for the *R. equi* serotype 2 capsular polysaccharide

Derivative	T_{GM}	*Mol ratio*
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-mannitol-*1-d*	0.98[a]	1.20
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol-*1-d*	1.00	1.00
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl-D-glucitol-*1-d*	1.44	0.77
1,4,5-Tri-*O*-acetyl-2-*O*-methyl-3-*O*-[(*S*)-1-acetoxypropyl]-L-rhamnitol-*1-d*	1.71	0.55

[a] Retention times relative to that of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol-*1-d*, using g.l.c. program *A*.

Methylation analysis. — The results from methylation analysis of the *R. equi* S2 capsular polysaccharide are presented in Table II. The polysaccharide was methylated and following reduction of the resulting carboxyl methyl esters ($NaBH_4$), hydrolysis gave the partially methylated free sugars. From g.l.c.–m.s. analysis of the corresponding alditol acetate derivatives it can be inferred that the capsular polysaccharide is com-

posed of →4)-D-Glc*p*-(1→, →4)-D-Man*p*-(1→, →3)-D-Glc*p*A-(1→ and →4)-3-*O*-[(*S*)-1-carboxyethyl]-L-Rha*p*-(1→ residues which form a linear tetrasaccharide unit.

Determination of the ring size and anomeric configurations of the component sugar residues was achieved by employing high-resolution ^{1}H- and ^{13}C-n.m.r. techniques, which also served to establish the glycose sequences and the location of the *O*-acetyl groups.

Assignment of the ^{1}H- and ^{13}C-n.m.r. spectra. — The ^{1}H- and ^{13}C-n.m.r. spectra of the native polysaccharide were complex owing to partial substitution by *O*-acetyl groups. However, treatment of the native material with dilute ammonia gave an *O*-deacetylated polysaccharide, $[\alpha]_D$ −3.04° (*c* 1.25, H_2O), *Anal.* found: C, 37.79; H, 4.85; N, 0.00%. Examination of the ^{1}H-n.m.r. spectrum of the latter revealed anomeric ^{1}H resonances corresponding to the four glycosyl residues in the low-field region of the spectrum at 5.14 (s, 1 H), 4.74 (s, ~1 H), 4.73 (d, ~1 H, $J_{1,2}$ 8.2 Hz) and 4.48 p.p.m. (d, 1 H, $J_{1,2}$ 7.0 Hz). Furthermore, characteristic high-field resonances at 1.36 (d, 3 H, *J* 6.6 Hz) and 1.28 p.p.m. (d, 3 H, *J* 6.1 Hz) corresponded to the methyl protons of the carboxyethyl group and the 6-deoxyhexose.

In accord with the compositional analysis, the ^{13}C-n.m.r. spectrum of the *O*-deacetylated polysaccharide exhibited four discrete anomeric carbon resonances at 103.73, 103.48, 101.32, and 101.20 p.p.m., indicating the polymer to be composed of a regular repeating tetrasaccharide unit. Diagnostic ^{13}C resonances were observed at 20.12 and 18.22 p.p.m. from the methyl carbons of the carboxyethyl group and 6-deoxyhexose residues, and two signals at low field, 177.85 and 175.40 p.p.m., indicated the presence of the two carboxyl groups within the repeating unit. A ^{13}C-DEPT experiment[20] showed the intense signal at 61.75 p.p.m. to have arisen from the two hydroxymethyl groups.

Assignment of the proton resonances was made from two-dimensional homonuclear shift correlation (COSY[21] and Relayed COSY[22]) experiments. Thus, by analysis of the connectivities defined by the cross-peaks, the ^{1}H resonances were grouped into five subspectra[23] corresponding to the four glycosyl ring-systems, labelled **a**–**d** according to the decreasing order of the chemical shifts from their H-1 resonances, and the carboxyethyl substituent labelled **e**. In the full scale COSY and Relayed COSY spectra (see experimental section) many of the cross-peaks in the region 3.3 to 4.2 p.p.m. were close to the diagonal and not well-resolved. However, when a phase-sensitive COSY[24] experiment was recorded using a narrow sweep-width (1200 Hz) and a larger data-set, much better digital resolution was obtained (Fig. 1), which permitted assignment of all the proton resonances associated with the four glycosyl residues. The respective subspectra were then identified from a comparison of the chemical-shift data and the vicinal ^{1}H–^{1}H coupling constants with values reported in the literature[25,26].

Residue **a** was identified as a rhamnopyranose unit because of (*i*) the observed small magnitude of the vicinal couplings, $J_{1,2}$ (~0.5 Hz) and $J_{2,3}$ (3.2 Hz), characteristic of the *manno* configuration, and (*ii*) the high-field methyl doublet (1.28 p.p.m., $J_{5,6}$ 6.1 Hz), which could be readily associated with the corresponding subspectrum through cross-peaks to the H-5**a** resonance (4.07 p.p.m.) in the full-scale COSY spectrum. In

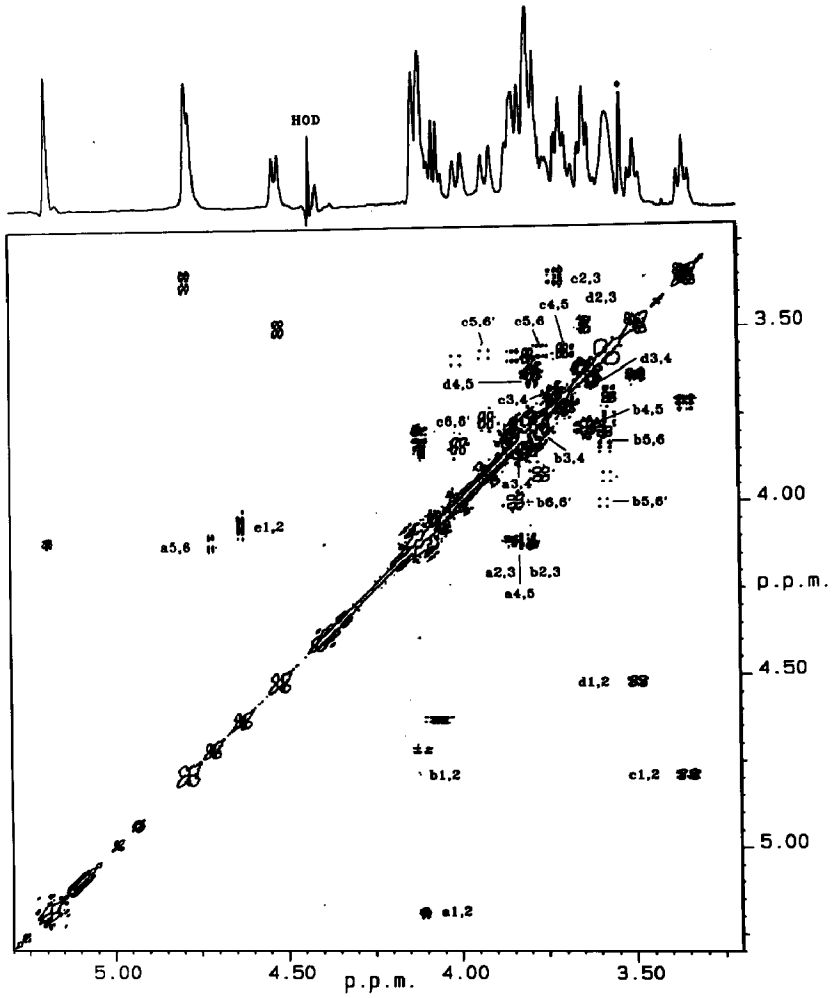


Fig. 1. Partial phase-sensitive COSY contour map of the *O*-deacetylated *R. equi* S2 capsular polysaccharide recorded at 62°, showing correlations of the ring protons from 3.3–4.2 p.p.m. Cross-peak assignments are indicated where **a**5,6 and **e**1,2 arise from the folding-in of the high field methyl doublets. The asterisk denotes an impurity of tris(hydroxymethyl)methylamine.

addition, the A_3X spin-system associated with the carboxyethyl substituent of residue **a** was identified from cross-peaks in the full-scale COSY spectrum relating the methyl doublet (J 6.6 Hz) at 1.36 p.p.m. to the lactic acid H-2 quartet at 4.02 p.p.m. (compare Fig. 1).

The anomeric resonances arising from residues **b** and **c** were coincident, however, the respective connectivity pathways were readily discernible and, on the basis of vicinal ring-coupling constants, residue **b** could be identified as also having the *manno* configuration and is attributed to the D-mannopyranosyl residue. The low indensity of the cross-peaks relating H-1 to H-2 of residues **a** and **b** (Fig. 1) is attributed to the small values (~ 1–3 Hz) for the scalar coupling, $J_{1,2}$, as noted previously[16].

Seven ^{1}H resonances were associated with the subspectrum from the residue **c** and, from the large ^3J-values ($J_{1,2} \simeq J_{2,3} \simeq J_{3,4} \simeq J_{4,5} \simeq 10$ Hz), it could be identified as a β-D-glucopyranosyl unit. Analogously, the subspectrum arising from residue **d** could be attributed to a pyranosyl residue having the *β-gluco* configuration, however, this spin-system comprised only five ^{1}H resonances, indicating it to correspond to the D-glucopyranosyluronic acid residue.

Spin simulations of the ^{1}H subspectra were performed to verify the assignments for each glycosyl residue (Fig. 2) and the refined chemical shifts and ^{1}H–^{1}H couplings are given in Table III.

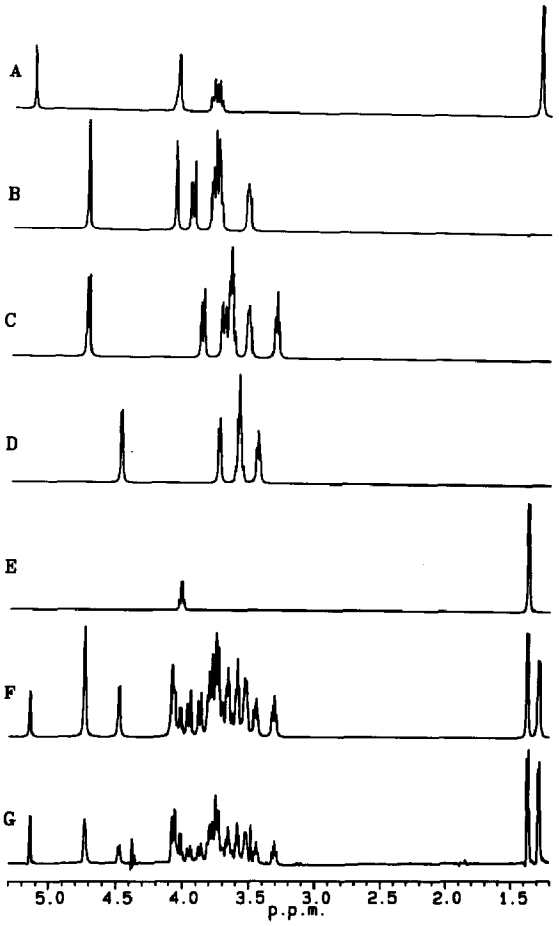


Fig. 2. Experimental and simulated ^{1}H-n.m.r. spectra of the *O*-deacetylated *R. equi* S2 capsular polysaccharide: A, subspectrum from the α-L-rhamnopyranosyl residue, **a**; B, subspectrum from the β-D-mannopyranosyl residue, **b**; C, subspectrum from the β-D-glucopyranosyl residue, **c**; D, subspectrum from the β-D-glucopyranosyluronic acid residue, **d**; E, subspectrum from the rhamnosyl carboxyethyl substituent, **e**; F, composite simulated spectrum from A–E; and G, observed spectrum.

TABLE III

Proton chemical shifts and coupling constants (Hz) for the *O*-deacetylated *R. equi* serotype 2 capsular polysaccharide[a]

Residue	*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'* ($J_{5,6'}J_{6,6'}$)	*Lactic acid residue* $-CH_3$	$-CH$ (J_{CH-CH3})
a	→4)-α-L-Rha*p*-(1→ 3 CH CH_3 CO_2H	5.144 (0.5)	4.059 (3.2)	3.816 (10.0)	3.754 (10.0)	4.069	1.280 (6.1)		1.360	4.018 (6.6)
b	→4)-β-D-Man*p*-(1→	4.737 (2.4)	4.080 (3.6)	3.741 (9.7)	3.772 (9.4)	3.529	3.950 (2.1)	3.896 (5.0, 12.4)		
c	→4)-β-D-Glc*p*-(1→	4.734 (8.2)	3.308 (9.3)	3.670 (9.6)	3.645 (9.5)	3.525	3.869 (2.0)	3.715 (5.0, 12.0)		
d	→3)-β-D-Glc*p*A-(1→	4.479 (7.0)	3.448 (9.6)	3.602 (9.6)	3.580 (9.9)	3.738				

[a] Observed first-order chemical shifts and coupling constants (Hz) measured at 62° in D_2O (pD ~2) and refined by spectral simulation

The anomeric configurations of the pyranosyl residues having the *manno* configuration (**a** and **b**) were determined from the magnitude of their one-bond ^{13}C–^{1}H coupling constants, $^{1}J_{C\text{-}1,H\text{-}1}$ measured from a ^{1}H-coupled ^{13}C-n.m.r. spectrum[27]. Heteronuclear ^{13}C–^{1}H chemical shift correlations[28] (Fig. 3) permitted unambiguous assignment of the carbon-13 resonances associated with the glycose residues within the repeating unit of the *O*-deacetylated polysaccharide by reference to the detemined ^{1}H assignments and ^{13}C data from the literature[29] (Table IV). The $^{1}J_{C,H}$ value for the anomeric carbon resonance (C-1) of the L-rhamnopyranosyl unit, residue **a**, (172 Hz) was characteristic of the α configuration, whereas the smaller coupling-constant observed for the D-mannopyranosyl unit (residue **b**, 159 Hz) was indicative of the β configuration[30].

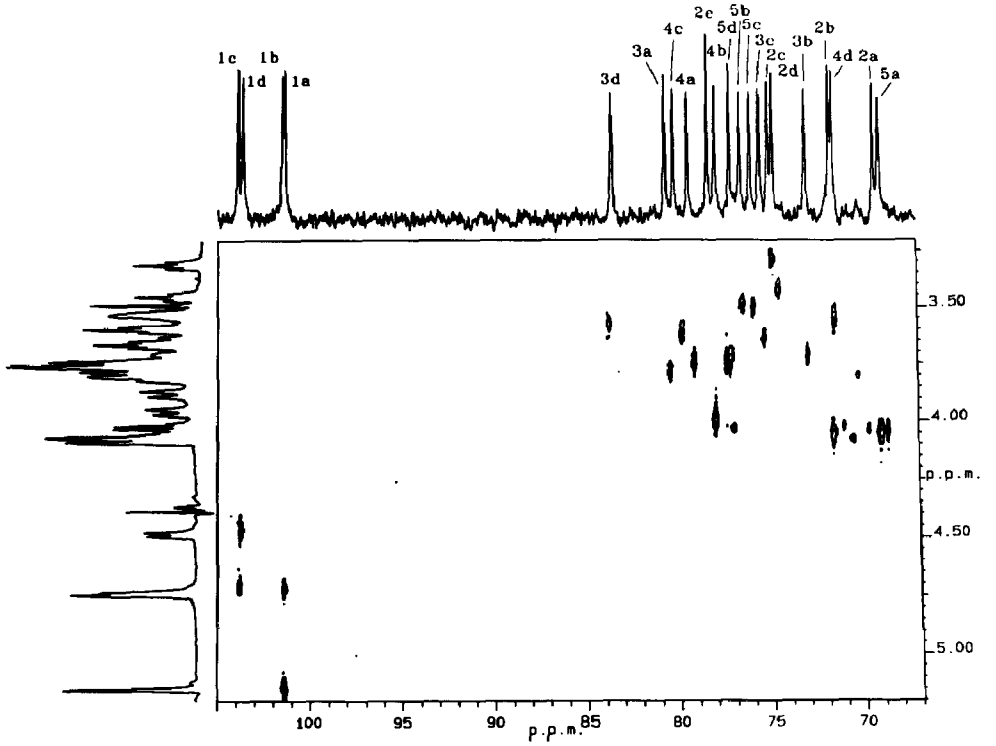


Fig. 3. Part of the ^{13}C–^{1}H chemical-shift correlation map of the *O*-deacetylated *R. equi* S2 capsular polysaccharide. The 1D ^{13}C- and ^{1}H-n.m.r. spectra are displayed above and at the side respectively; the ^{13}C assignments are indicated, where **2e** denotes C-2 of the lactic acid substituent.

Sequence of glycosyl residues. — The arrangement of four glycosyl residues within the repeating unit was determined from interresidue ^{1}H–^{1}H n.O.e. measurements[31], which also served to confirm the positions of the glycosidic linkages. N.O.e. measurements were made by difference spectroscopy[32] (Fig. 4).

Irradiation of the anomeric-proton resonance of the L-rhamnopyranosyl unit (Fig. 4A) showed a single intraresidue n.O.e. at H-**2a** (4.06 p.p.m.) together with an interresidue n.O.e. across the glycosidic linkage at H-**3d** of the D-glucopyranosyluronic acid residue (3.60 p.p.m.), indicating that the L-rhamnosyl unit was α-linked to O-3 of

TABLE IV

Carbon-13 chemical shifts and $^{1}J_{C,H}$ values (Hz) for the *O*-deacetylated *R. equi* serotype 2 capsular polysaccharide[a]

Residue	*Glycosyl residue*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*Lactic* $-CH_3$	*Acid* $-CH$	*Residue* $-CO_2H$
a	→4)-α-L-Rha*p*-(1→ 3 | CH / \\ CH_3 CO_2H	101.20 (172)	69.20	80.55	79.31	68.86	18.22	20.12	78.23	177.85[b]
b	→4)-β-D-Man*p*-(1→	101.32 (159)	71.56	72.88	77.81	76.45	61.75[c]			
c	→4)-β-D-Glc*p*-(1→	103.73 (164)	74.91	75.39	80.09	75.91	61.75[c]			
d	→3)-β-D-Glc*p*A-(1→	103.48 (162)	74.67	83.43	71.40	76.99	175.40[b]			

[a] Measured at 62° in D_2O (pD ≃2) and, unless indicated, assignments were determined by ^{13}C–^{1}H chemical shift correlation. [b] Assignments may be reversed.
[c] Indentified by DEPT experiment.

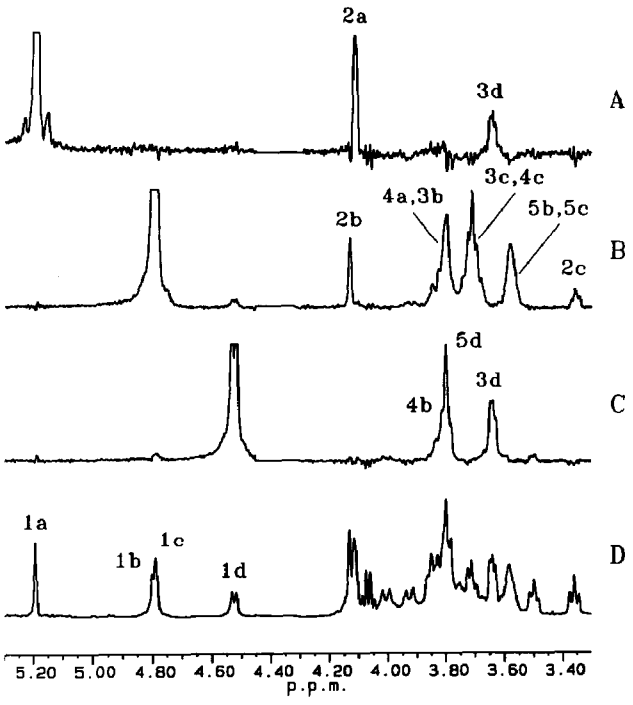


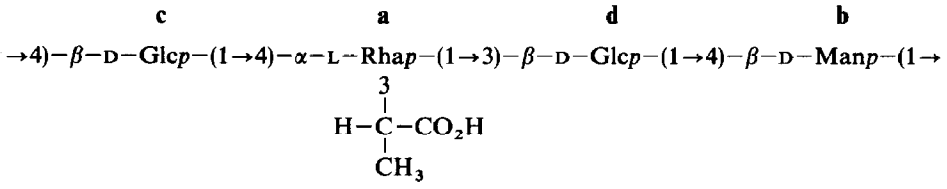
Fig. 4. N.O.e. difference spectra obtained on the *O*-deacetylated *R. equi* S2 capsular polysaccharide at 62° for irradiation of: A, H-1 of the rhamnosyl unit, **a**; B, combined H-1 of the mannosyl unit, **b** and H-1 of the glucosyl unit, **c**; C, H-1 of the glucuronic acid unit, **d**; and D, the off-resonance control spectrum.

the D-glucuronic acid residue. The occurrence of an interresidue n.O.e. between H-1**d** of the D-glucuronic acid unit and H-4**b** of the D-mannopyranosyl unit (3.77 p.p.m.) (Fig. 4C) indicates that the D-glucuronic acid is in turn O-4-linked to the D-mannosyl residue. Intraresidue n.O.e. observed between H-1**d** (4.48 p.p.m.) and H-3**d** (3.60 p.p.m.), and between H-1**d** and H-5**d** (3.74 p.p.m.) (Fig. 4C) were indicative of the assigned β configuration of the D-glucopyranosyluronic acid residue. The absence of any corresponding intraresidue 1,3-*syn*-axial effects following saturation of H-1**a** (5.14 p.p.m.) (Fig. 4A) was consistent with the L-rhamnopyranosyl unit having the α configuration. These data indicate the partial sequence **a**→**d**→**b** within the polysaccharide.

Because of overlapping H-1**b** and H-1**c** resonances, interpretation of the resulting n.O.e. difference spectra was difficult. However, n.O.e.s from saturation of these resonances (~4.74 p.p.m.) were observed at the resonances assigned to H-2**b**, H-3**b**, and H-5**b**, and to H-3**c** and H-5**c** (Fig. 4B), which could be attributed to specific interactions within the respective β-pyranose ring systems of residues **b** and **c**. By comparison of the ^{1}H resonance peak shapes observed in the difference spectrum (Fig. 4B) with those calculated for the individual subspectra (compare Fig. 2), additional interresidue effects at H-4**c** and H-4**a** could be identified from which the partial sequence **b**→**c**→**a** can be inferred as part of the repeating unit. Furthermore, methylation analysis indicated the

polysaccharide to have a linear tetrasaccharide repeating-unit which would position residue **c** between residues **b** and **a**, therefore establishing the linear sequence of glycoses as **c**→**a**→**d**→**b**→.

In agreement with the foregoing results and those from methylation analysis (Table I), the relatively deshielded values for the ^{13}C-n.m.r. resonances from C-3**d** (83.43 p.p.m.), C-4**b** (77.81 p.p.m.), C-4**c** (80.09 p.p.m.), and C-4**a** (79.31 p.p.m.) would indicate that the respective positions are glycosylated[29]. Correspondingly, the low-field position of C-3**a** (80.55 p.p.m.) is consistent with substitution at that position by the lactate ether. The accumulated evidence from compositional, methylation, and n.m.r. analyses permit the repeating unit of the *O*-deacetylated *R. equi* S2 polysaccharide to be assigned the structure **3**.



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Periodate oxidation. — The foregoing structural conclusions were supported by analysis of the oligosaccharide fragment produced upon periodate oxidation of the *O*-deacetylated capsular polysaccharide. Subsequent reduction ($NaBH_4$) and Smith-type hydrolysis[33] with dilute acetic acid followed by purification on Sephadex G-15 gave an oligosaccharide **4** (K_{av} 0.65), which had $[\alpha]_D$ −55.94 (*c* 1.2, water). Methanolysis followed by quantitative analysis of the carboxyl-reduced ($NaBD_4$) acid hydrolysis products showed **4** to be composed of approximately equimolar proportions of 3-*O*-[(*S*)-1-carboxyethyl]-L-rhamnose, D-glucuronic acid, and erythritol.

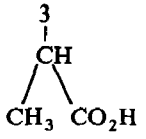
Structural information was obtained from the ^{1}H- and ^{13}C-n.m.r. spectra of the oligosaccharide and the mass-spectral fragmentation pattern of the corresponding methylated derivative.

The ^{1}H- and ^{13}C-n.m.r. spectra of the oligosaccharide were fully assigned by the COSY[21] and CHORTLE[34] techniques, respectively, and the n.m.r. data are presented in Tables V and VI. Characteristic ^{1}H-n.m.r. signals from the two anomeric protons of the trisaccharide were observed at 5.10 ($J_{1,2}$ 1.8 Hz) and 4.59 p.p.m. ($J_{1,2}$ 8.1 Hz). As expected, the ^{13}C-n.m.r. spectrum contained 19 signals, with diagnostic high-field ^{13}C resonances at 18.54 and 17.13 p.p.m. from the two methyl carbons of the 3-*O*-[(*S*)-1-carboxyethyl]-α-L-rhamnopyranosyl residue. From a ^{13}C-DEPT experiment the signals at 62.90 and 61.63 p.p.m. could be attributed to the two hydroxymethyl groups of the erythritol residue; the latter assignment facilitated assignment of the corresponding methylene protons *via* chemical-shift correlation.

G.l.c.–m.s. of the methylated oligosaccharide gave a quasimolecular ion (M + NH_4^+) at *m/z* 660 in the c.i. spectrum and fragmentation patterns (c.i. and c.i.)

TABLE V

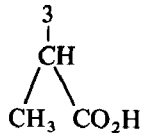
Proton chemical shifts and coupling constants (Hz) for the trisaccharide obtained by periodate oxidation and Smith type hydrolysis of the *O*-deacetylated *R. equi* serotype 2 capsular polysaccharide[a]

Glycosyl residue	*H-1* ($J_{1,2}$)	*H-2* ($J_{2,3}$)	*H-3* ($J_{3,4}$)	*H-4* ($J_{4,5}$)	*H-4'* ($J_{3,4}$-$J_{4,4'}$)	*H-5*	*H-6* ($J_{5,6}$)	*Lactic acid residue* $-CH_3$	$-CH$ (J_{CH-CH3})
α-L-Rha*p*-(1→ 	5.10 (1.8)	4.18 (3.5)	3.63 (9.1)	3.52 (10.3)		4.01	1.25 (5.0)	1.49	4.37 (6.6)
→3)-β-D-Glc*p*A-(1→	4.59 (8.1)	3.44 (9.1)	3.63 (9.1)	3.59 (9.5)		3.95			
→2)-Erythritol	3.79[b] (3.6)	3.87 (6.4)	3.65 (7.3)	3.67	3.79 (3.4, 11.8)				

[a] Observed first-order chemical shifts and coupling constants (Hz) measured at 62° in D_2O (pD ~2). [b] The methylene protons at C-1 of the erythritol residue have identical chemical shifts.

TABLE VI

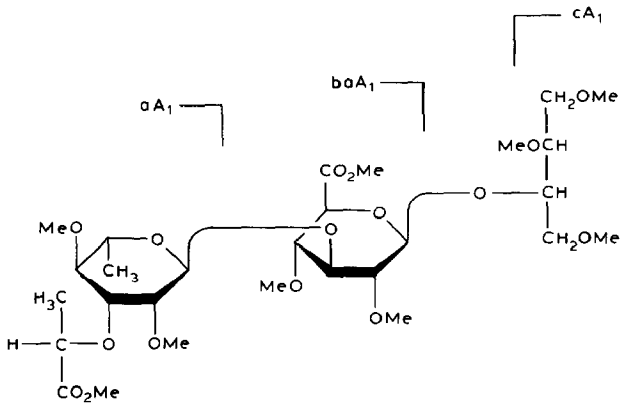
Carbon-13 chemical shifts for the trisaccharide obtained by periodate oxidation and Smith-type hydrolysis of the *O*-deacetylated *R. equi* serotype 2 capsular polysaccharide[a]

Glycosyl residue	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*Lactic* $-CH_3$	*Acid* $-CH$	*Residue* $-CO_2H$
α-L-Rha*p*-(1→	101.48	69.09	79.30	71.95	69.49	17.13	18.54	75.87	178.10[b]
									
→3)-β-D-Glc*p*A-(1→	102.97	74.11	79.35	70.50	75.51	176.20[b]			
→2)-Erythritol	61.63[c]	71.26	70.50	62.90[c]					

[a] Measured at 62° in D_2O (pD ≃ 2) and, unless indicated, assignments were determined by ^{13}C–^{1}H chemical shift correlation. [b] Assignments may be reversed. [c] Indentified by DEPT experiment.

TABLE VII

Mass-spectral fragmentation data for the trisaccharide obtained from periodate oxidation and Smith hydrolysis of the *O*-deacetylated *R. equi* serotype 2 polysaccharide



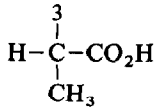
m/z		Assignment[a]
c.i.	*e.i.*	
660		$(M + NH_4^+)$
479		baA_1
447		baA_2
	375	ab-lactic acid
261	261	aA_1
229	229	aA_2
	207	bcJ_1
	202	a-CO_2Me
	197	aA_3
	157	a-lactic acid
147	147	cA_1
115	115	cA_2

[a] Nomenclature is as used in ref. 35.

consistent with the presence of a terminal erythritol and non-reducing 3-*O*-[(*S*)-1-carboxyethyl]-α-L-rhamnopyranosyl residues (Table VII).

The combined ^1H- and ^{13}C-n.m.r. evidence, together with g.l.c.–m.s. data allows the structure of oligosaccharide **4** to be identified as

α-L-Rha*p*(1→3)-β-D-Glc*p*A-(1→2)-erythritol



4

which confirms the sequence **a**→**d**→**b**→ in the repeating unit **3** of the polysaccharide.

Location of the O-acetate substituents. — Periodate oxidation and reduction ($NaBH_4$) of the native *R. equi* serotype 2 polysaccharide, followed by methanolysis and quantitative analysis of the carboxyl-reduced ($NaBD_4$) acid-hydrolysis products indicated the presence of 3-*O*-[(*S*)-1-carboxyethyl]-rhamnose, D-glucuronic acid, D-mannose, and erythritol in approximately equimolar proportions. As the 4-*O*-monosubstituted glycoses would not be expected to survive the oxidation, the presence of mannose amongst the hydrolysis products suggests that the native polysaccharide is substituted by *O*-acetyl groups at the O-2 and/or O-3 positions of the β-D-mannopyranosyl residue.

The ^{1}H.n.m.r. spectrum of the native polysaccharide (Fig. 5) exhibited signals characteristic of the methyl protons of *O*-acetyl groups in the region 2.0–2.2 p.p.m. The integrated intensities of the resonances at 2.05 and those between 2.15 and 2.25 p.p.m. (relative to that of the rhamnose H-6 signal at 1.30 p.p.m.) indicate that the polymer is substituted by *O*-acetyl groups to the extent of 1.7 mol per repeating unit and that two distinct acetate esters are present in the ratio of 0.7:1.

Complete assignment of the proton spectrum was achieved by the COSY and relayed COSY techniques. The chemical shifts observed for protons in residues **a**, **c**, and **d** were identical (within 0.1 p.p.m.) with those obtained for the *O*-deacetylated polysaccharide. On the other hand, two connectivity pathways corresponding to the β-D-mannopyranosyl spin system (residue **b**) could be identified. The integrated intensity of the low-field resonance (5.48 p.p.m., 1 H) assigned to H-2**b** suggests that the mannose residue is completely 2-*O*-acetylated[36]. Furthermore, resonances at 5.16 (~0.7 H) and 3.97 p.p.m. could both be assigned to H-3**b**, where the chemical shift of the former is indicative of the *O*-acetylation at O-3. Thus, this information taken with the foregoing

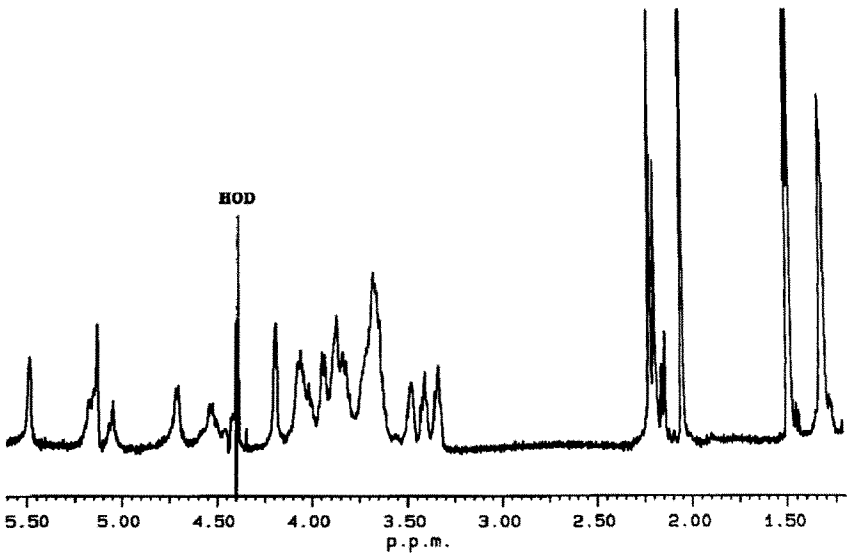
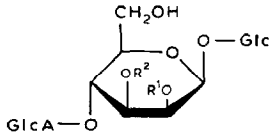


Fig. 5. ^{1}H-n.m.r. spectrum of the native *R. equi* S2 capsular polysaccharide at 62° (pD ~2).

TABLE VIII

Carbon-13 chemical shifts for the β-D-mannopyranosyl residue, **b**, of the *R. equi* serotype 2 polysaccharide



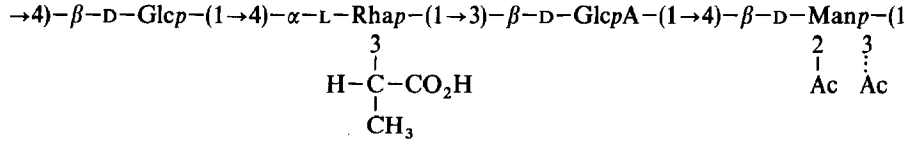
Substituent	*C-1*	*C-2*	*C-3*	*C-4*
$R^1 = R^2 = H$	101.32	71.56	72.88	77.81
$R^1 = Ac, R^2 = H$	99.50	72.80	71.41	78.08
$R^1 = R^2 = Ac$	99.52	71.17	73.06	74.90

data reveals that the *R. equi* serotype 2 capsular polysaccharide contains both 2-*O*-acetylated (59%) and 2,3-di-*O*-acetylated (41%) β-D-mannopyranosyl residues.

Comparison of the ^{13}C-n.m.r. data (Table VIII) from the native and *O*-deacetylated polysaccharides showed significant upfield shifts for the C-1 and C-3 β-D-mannopyranosyl resonances (1.8 and 1.5 p.p.m., respecitvely) (Table VIII, R^1 = Ac, R^2 = H), whereas C-2 was shifted downfield by 1.2 p.p.m., indicating[36] *O*-acetyl substitution at O-2. Consistent with the presence of di-*O*-acetylated β-D-mannopyranosyl residues (Table VIII, $R^1 = R^2$ = Ac) upfield shifts of 1.8 and 2.9 p.p.m. were observed for C-1 and C-4 respectively, whereas no significant shifts were detected for the corresponding C-2 and C-3 resonances. Empirical rules[36] for estimating the chemical-shift differences in the ^{13}C-n.m.r. spectra due to *O*-acetylation indicate that vicinal di-*O*-substitution of C-2 and C-3 would result in shielding of these carbon atoms by ~0.2 and 0.1 p.p.m., respectively.

Immunodiffusion studies suggest that the *O*-acetyl groups are part of the immunodeterminant region of the serotype 2 polysaccharide. The native polysaccharide (**5**) gave a strong positive precipitin reaction, whereas no precipitin line against serotype 2 specific rabbit antisera was obtained with the *O*-deacetylated polysaccharide.

In agreement with the foregoing structural conclusions the structure of the *R. equi* serotype 2 capsular polysaccharide (**5**) is as shown.



EXPERIMENTAL

R. equi *serotype 2 polysaccharide.* — *R. equi* serotype 2 (ATCC 33702) was obtained from Dr. J. F. Prescott, University of Guelph, Guelph, Ontario. The organism was grown on plates containing Difco brain heart infusion agar supplemented with sucrose (2% w/v) for 72 h at 25° and the cells were collected by scraping the plates and were suspended in aq. NaN_3 (0.1%, w/v). The aq. solution, containing the growth of 100 plates (109 g), was diluted with aq. NaCl (4M, 120 mL) and, after stirring overnight at 4°, the cellular material was removed by centrifugation. The supernatant was dialyzed against distilled water until salt free, digested with ribonuclease, deoxyribonuclease, and proteinase K, passed through a column of Rexyne 101 (H^+) ion-exchange resin (50 mL) and lyophilized. The crude capsular polysaccharide, so obtained, was dissolved in water (200 mL) and purified by precipitation with cetyltrimethylammonium bromide (Cetavlon) (10% w/v, 200 mL). The solution was kept overnight at 4° and the precipitated Cetavlon–polysaccharide complex was collected by centrifugation. The Cetavlon complex was redissolved in aq. NaCl (4M, 200 mL) and the polysaccharide component was recovered by precipitation with 3 volumes of 95% EtOH, followed by centrifugation. This material in water (100 mL) was exhaustively dialyzed against distilled water and lyophilized to afford the sodium salt of the serotype 2 capsular polysaccharide (396 mg, yield, 0.7%).

Chromatographic methods. — *(a) Ion-exchange chromatography.* Polysaccharide (20 mg) was applied to a column (1 × 40 cm) of DEAE-Sephacel (Pharmacia) and the column was eluted with 0.05M Tris-HCl buffer (pH 7.2, 50 mL) followed by a 0→1M NaCl gradient in the same buffer. Fractions (2 mL) were collected and assayed colourimetrically for neutral glycose[10] and hexuronic acid[11]. Material purified in this way (15 mg) was deionized by passing an aqueous solution (2.0 mL) through a column (0.5 × 2.0 cm) containing Rexyn 101 (H^+ form) ion-exchange resin followed by lyophilization of the eluant.

(b) Gel filtration. Polysaccharide samples (20 mg) were subjected to gel chromatography on columns (1 × 40 cm) of Sephadex G-200 (Pharmacia) eluted with pyridinium acetate (0.05M, pH 4.5) while oligosaccharides (50 mg) were separated on a column (2.5 × 70 cm) of Sephadex G-15. Column eluants were continuously monitored for changes in refractive index, by using a Waters R403 differential refractometer and fractions were assayed colorimetrically[10,11]. The gel-filtration properties of the eluted materials are expressed in terms of their distribution coefficients, $K_{av} = (V_e - V_o)/(V_t - V_o)$, where V_o 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.

(c) H.p.l.c. H.p.l.c. was performed with a Dionex BioLC carbohydrate system equipped with a column (4.6 × 250 mm) of Dionex Carbopac AS-6 pellicular anion-exchange resin, and a AG-6 guard column with pulsed amperometric detection. Isocratic elution was performed using the following eluants: *A*, 15mM NaOH or *B*, 150 mM NaOH and 150mM NaOAc, at a flow rate of 1 mL/min at ambient temperature. Retention times are quoted relative to that of D-glucose (R_{Glc}).

(d) G.l.c. G.l.c. was performed with a Hewlett–Packard model 5710A chromatograph fitted with a hydrogen-flame detector and a model 3380A electronic integrator using a fused-silica capillary column (0.3 mm × 25 m) containing 3% OV17 with the following temperature programs: *A*, 2 min at 180° and then 2°/min to 240°; *B*, 2 min at 200° and then 10°/min to 320°. The carrier gas was dry nitrogen at 30 mL/min and retention times are quoted relative to D-glucitol hexaacetate (T_{GA}) and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-D-glucitol (T_{GM}).

G.l.c.–mass spectrometry. — G.l.c.–m.s. was performed with a Hewlett–Packard 5958B system employing the g.l.c. program conditions *A* or *B* by electron impact (e.i.) with an ionization potential of 70 eV, or by chemical ionization (c.i.) with ammonia as the reagent gas.

Analytical methods. — Quantitative colourimetric methods used for the analysis of column chromatography eluants were (*i*) phenol–H_2SO_4 for neutral glycoses[10] and (*ii*) the *m*-hydroxydiphenyl–H_2SO_4 method for hexuronic acids[11].

Glycoses were determined directly by h.p.l.c.[12] or by g.l.c. of their alditol acetate derivatives[13]. Samples (0.2–0.5 mg) were hydrolyzed with trifluoroacetic acid (2M, 0.5 mL) for 15 h at 100° and evaporated to dryness *in vacuo* to obtain the free glycoses. For the g.l.c.–m.s. estimation of acidic glycoses, polysaccharide samples (0.5 mg) were treated with methanolic HCl (M, 1 mL) for 15 h at 100°. Excess methanolic HCl was removed *in vacuo* and the residues in water (1 mL) were treated with $NaBD_4$ or $NaBH_4$ (15 mg) for 6 h at 37°. Following neutralization with aq. AcOH (10% w/v), the mixtures were evaporated *in vacuo*, and borate was removed by repeated distillation of MeOH (5 × 5 mL) from the residue. The products were hydrolyzed (as stated) and the liberated glycoses were reduced ($NaBH_4$) and acetylated (Ac_2O)[17] prior to g.l.c. analysis using program *A*.

Absolute configurations of the glycoses were determined by g.l.c.–m.s. (program *A*) of the corresponding acetylated (*R*)-2-butyl-glycoside derivatives[18]. The identity of the glycose derivatives was established by comparison of their g.l.c. retention-times and mass spectra with those of authentic reference compounds.

O-Deacetylation. — A solution of the capsular polysaccharide (50 mg) in aq. NH_4OH (5% v/v, 5 mL) was stirred for 5 h at 37°. The excess of NH_3 was removed by flushing with nitrogen and the solution was lyophilized.

Methylation analysis. — Polysaccharide samples (2 mg) were methylated with MeI in Me_2SO containing an excess of sodium methylsulfinylmethanide[15]. The methylated products were recovered by partitioning the mixture between water and CH_2Cl_2, followed by evaporation of the organic phase. The methylated polysaccharide was treated with $NaBH_4$ in tetrahydrofuran for 15 h at 22° and the carboxyl-reduced products[16] were hydrolyzed, evaporated, reduced with $NaBD_4$, and acetylated[17]. Analysis was by g.l.c.–m.s. using program *A*.

Periodate oxidation. — *O*-Deacetylated capsular polysaccharide (80 mg) was treated in the dark with aq. $NaIO_4$ (0.05M, 50 mL) for 7 days at 4° and, following decomposition of periodate with ethylene glycol (0.4 mL), the oxidized polymer was reduced with $NaBH_4$ (500 mg). After stirring for 15 h at 22°, the excess of borohydride

was decomposed with aq. AcOH and the periodate-oxidized and reduced polymer (60 mg) was isolated by lyophilization after extensive dialysis. Smith-type hydrolysis[33] was effected by treating the product with aq. AcOH (2%, 10 mL) for 2 h at 100°, followed by evaporation. The Smith-degradation product was purified by gel filtration on Sephadex G-15 to give the oligosaccharide **4** (20 mg).

The oligosaccharide sample (0.5–1.0 mg) was methylated by the foregoing procedure, and the product was recovered by extraction into CH_2Cl_2 and analyzed by g.l.c.–m.s. using program *B*.

N.m.r. spectroscopy. — N.m.r. spectra were obtained with a Bruker AM-500 spectrometer equipped with an Aspect 3000 computer, operating in the pulsed F.t. mode with quadrature detection using standard Bruker software. Solutions of polysaccharide samples in D_2O (Merck, Sharp and Dohme, 99.8 atom %) were prepared at a concentration of 10–40 mg/mL subsequent to lyophilization from D_2O. Proton and carbon-13 measurements were made on solutions (0.4 mL) of samples of the polysaccharides (pD ~2) in 5-mm tubes at 62°.

^{1}H-N.m.r. spectra at 500 MHz were recorded using a spectral width of 2.5 kHz, a 16 K data block for a digital resolution of 0.3 Hz/point, an acquisition time of 3.2 s and a $\pi/2$ pulse. Resolution enhancement was achieved by using a Gaussian line-shape transformation[37] with typical values of −2.0 to −4.0 Hz for the line-broadening factor and a Gaussian-broadening factor of +0.1 or +0.2. Chemical shifts are expressed relative to internal acetone (2.225 p.p.m.).

Proton-spin simulations were performed using the Bruker PANIC program using a line width of 3 Hz.

N.O.e. difference spectra[38] were obtained using a selective low-power presaturation pulse applied to the proton resonance for 200 ms followed by an $\pi/2$ observation pulse, where each line of a multiple resonance was irradiated sequentially for 25 ms during the presaturation delay[32]. Eight transients were acquired for irradiation of each multiplet and for an off-resonance spectrum in separate files and the entire sequence was cycled to achieve a satisfactory signal-to-noise ratio.

Broad-band proton-decoupled ^{13}C-n.m.r. spectra were obtained at 125 MHz using a spectral width of 25 kHz, a 32K data block and a $\pi/2$ pulse for a digital resolution of 1.5 Hz/point, employing composite-pulse proton decoupling (WALTZ)[39]. Heteronuclear $^1J_{C,H}$ coupling-constants for the anomeric carbon atoms were measured using gated decoupling[27]. DEPT spectra[20] were obtained with broadband proton decoupling, a $3\pi/2$ proton pulse, and a 3.3 ms delay $(2J)^{-1}$ between pulses to distinguish between methine and methylene ^{13}C resonances. Chemical shifts are referenced to internal acetone (31.07 p.p.m.).

The 2D homonuclear proton chemical-shift correlation experiments, COSY[21] and Relayed COSY[22] were measured with solvent suppression employing the conventional pulse sequences. The experiments were carried out using data sets ($t_1 \times t_2$) of 256 × 1024 or 512 × 2048 points that were zero-filled in t_1 to 512 × 1024 or 1024 × 2048 points, respectively, spectral widths of either 2500 Hz (full scale experiment) or 1200 Hz (narrow sweep width experiment), and a recycle delay in the range 1.0–1.5 s. Either 32 or

96 transients were collected for each value of the incrementable delay. Resolution enhancement was implemented in both dimensions by means of a non-shifted sine-bell window function[40], and the doubly-transformed data were processed to give phase-sensitive[24] or magnitude spectra[41] with symmetrization[42].

A two-dimensional heteronuclear ^{1}H–^{13}C chemical-shift correlation experiment[28] was carried out on a concentrated solution (100 mg/mL) of the *O*-deacetylated polysaccharide. The experiment was performed with a data matrix of 128 × 1024 points and spectral widths of 12500 and 2500 Hz for the respective carbon-13 (F2) and proton (F1) chemical-shift domains. A recycle delay of 1.5 s was employed and the fixed delays, $\tau 1$ and $\tau 2$, were set at 3.2 and 1.6 ms, respectively, to select for all multiplicities. Composite broad-band proton decoupling was employed during the acquisition of 1000 transients for each of the 128 increments. The data matrix was zero-filled to 256 × 2048 points and following resolution enhancement in both dimensions by means of a Lorentz-to-Gauss transformation, the data were processed to give a power spectrum.

Heteronuclear ^{1}H–^{13}C chemical-shift correlations performed by the CHORTLE technique[34] employed 8K data sets and the spectra were acquired for 3584 transients using the following τ values, 0.16, 1.00, 2.40, and 3.20 ms and a 1.5-s recycle delay. The ^{1}H resonance offsets were calculated from the ^{13}C intensities by a nonlinear least-squares calculation, using a program developed in this division by Dr. J.-R. Brisson.

Immunodiffusion. — Polysaccharide samples were tested for serologic activity by the gel-diffusion test described by Prescott[6]. Serotype 2 *R. equi* antiserum was a gift from Dr. J. F. Prescott, University of Guelph.

General methods. — Commercial reagents and solvents were of analytical grade. Solutions were concentrated under diminished pressure at bath temperatures <40°. Optical rotations were determined at 22° in 10-cm microtubes with a Perkin–Elmer model 243 polarimeter.

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REFERENCES

1 M. Goodfellow, *Vet. Microbiol.*, 14 (1987) 205–209.
2 M. D. Barton and K. L. Hughes, *Vet. Bull.*, 50 (1980) 65–80.
3 Report of Foal Pneumonia Panel, *J. Equine Med. Surg.*, 2 (1978) 400–433.
4 J. F. Prescott and C. R. Sweeney, *J. Am. Vet. Med. Assoc.*, 187 (1985) 725–728.
5 J. B. Woolcock, M. D. Mutimer and P. M. Bowles, *Vet. Microbiol.*, 14 (1987) 215–224.
6 J. F. Prescott, *Can. J. Comp. Med.*, 45 (1981) 130–134.
7 J. B. Woolcock and M. D. Mutimer, *J. Gen. Microbiol.*, 109 (1978) 127–130; M. Nakazawa, M. Kubo, C. Sugimoto, and Y. Isayama, *Microbiol. Immunol.*, 27 (1983) 837–846.
8 R. A. Leitch and J. C. Richards, *Biochem. Cell. Biol.*, 68 (1990) 778–789.
9 J. E. Scott, *Methods Biochem. Anal.*, 8 (1960) 145–197.

10 M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
11 N. Blumenkrantz and G. Asboe-Hansen, *Anal. Biochem.*, 54 (1973) 484–489.
12 M. R. Hardy, R. R. Townsend, and Y. C. Lee, *Anal. Biochem.*, 170 (1988) 54–62.
13 S. W. Gunner, J. K. N. Jones, and M. B. Perry, *Can. J. Chem.*, 39 (1961) 1892–1899.
14 N. K. Kochetkov, B. A. Dmitriev, and V. L. Lvov, *Carbohydr. Res.*, 54 (1977) 253–259.
15 S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
16 J. C. Richards, M. B. Perry, and P. J. Kniskern, *Can. J. Chem.*, 67 (1989) 1038–1050.
17 A. B. Blakeney, P. J. Harris, R. J. Henry, and B. A. Stone, *Carbohydr. Res.*, 113 (1983) 291–299.
18 G. J. Gerwig, J. P. Kamerling, and J. F. G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357; 77 (1979) 1–7.
19 J. C. Richards and W. B. Severn, in preparation.
20 D. M. Doddrell, D. T. Pegg, and M. R. Bendall, *J. Magn. Reson.*, 48 (1982) 323–327.
21 A. Bax, R. Freeman, and G. Morris, *J. Magn. Reson.*, 42 (1981) 164–168.
22 A. Bax and G. Drobny, *J. Magn. Reson.*, 61 (1985) 306–320.
23 M. A. Bernstein, L. D. Hall, and S. Sukumar, *Carbohydr. Res.*, 103 (1982) C1–C6.
24 D. Marion and K. Wuethrich, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
25 K. Bock and H. Thøgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
26 C. Altona and C. A. G. Haasnoot, *Org. Magn. Reson.*, 13 (1980) 417–429.
27 O. A. Gansow and W. Schittenhelm, *J. Am. Chem. Soc.*, 93 (1971) 4294–4295.
28 A. Bax, *J. Magn. Reson.*, 53 (1983) 517–520; A. Bax and G. A. Morris, *J. Magn. Reson.*, 42 (1981) 501–505.
29 K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
30 K. Bock and C. Pedersen, *J. Chem. Soc. Perkin Trans. 2*, (1974) 293–297.
31 J. M. Prestegard, T. A. W. Koerner Jr., P. C. Demou, and R. K. Yu., *J. Am. Chem. Soc.*, 104 (1982) 4993–4995; M. A. Bernstein and L. D. Hall, *J. Am. Chem. Soc.*, 104 (1982) 5553–5555.
32 M. Kinnis and J. K. M. Sanders, *J. Magn. Reson.*, 56 (1984) 518–520.
33 I. J. Goldstein, G. W. Hay, B. A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1970) 361–370.
34 G. A. Pearson, *J. Magn. Reson.*, 64 (1985) 487–500.
35 N. K. Kochetkov and O. S. Chizhov, *Adv. Carbohydr. Chem. Biochem.*, 21 (1966) 39–93.
36 P.-E. Jansson, L. Kenne, and E. Schweda, *J. Chem. Soc. Perkin Trans. 1*, (1987) 377–383.
37 A. G. Ferrige and J. C. Lindon, *J. Magn. Reson.*, 31 (1978) 337–340.
38 R. Richarz and K. Wuthrich, *J. Magn. Reson.*, 30 (1978) 147–150.
39 A. J. Shaka, J. Keeler, T. Frenkiel, and R. Freeman, *J. Magn. Reson.*, 52 (1983) 335–338.
40 A. De Marco and K. Wuthrich, *J. Magn. Reson.*, 24 (1976) 201–204.
41 J. C. Lindon and A. G. Ferrige, *J. Magn. Reson.*, 36 (1979) 227–280.
42 R. Baumann, G. Wider, R. R. Ernst, and K. Wuthrich, *J. Magn. Reson.*, 44 (1981) 402–406.