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Stereochemical aspects of the antigenic determinants of bacterial polysaccharides: the *Rhodococcus equi* capsular polysaccharides

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The antigenic specificity of bacterial capsular polysaccharides has been extensively investigated. The origin of this specificity arises from the diversity of the oligosaccharide epitopes of the polysaccharide. Nuclear magnetic resonance spectroscopy provides a very powerful tool for probing the structure of the oligosaccharide epitopes. Using this technique in conjunction with chemical methods, the structures of the serotype-specific capsular polysaccharide antigens of *Rhodococcus equi* have been investigated. These studies have led to the identification of several structural features which can be related to serologic type specificity. This review will focus on some stereochemical aspects of the antigen determinants. Nuclear magnetic resonance based methods for determination of bacterial polysaccharide primary structure and for stereochemical analysis of chiral acidic substituents are presented.

INTRODUCTION

Carbohydrates associated with the bacterial cell surface have long been known for their immunological properties (Jennings, 1990). These include the extracellular polysaccharides of encapsulated bacteria and the lipopolysaccharides of the Gram-negative cell wall. These carbohydrate-containing polymers are antigenic; they extend out and away from the bacterial cell surface (Kenne & Lindberg, 1983; Wicken, 1985) and are associated with the virulence of pathogenic bacteria (Jann & Jann, 1990; Moxon & Kroll, 1990). The capsular polysaccharide (CPS) antigens of organisms such as *Escherichia coli*, *Klebsiella pneumoniae* and *Streptococcus pneumoniae* have been extensively investigated (Nimmich, 1968; Jann & Jann, 1990; Lee, 1987; van Dam *et al.*, 1990) and are related to serologic-type specificity. The origin of this specificity arises from the structural diversity of the antigenic polysaccharides which are high molecular weight polymers composed of repeating oligosaccharide units (Kenne & Lindberg, 1983; Jennings, 1990). They are immunogenic in animals and humans, and are readily isolated in their immunogenic high-molecular-weight form (Kabat & Bezer, 1958; Jennings, 1990). The effectiveness of CPS-based vaccines against bacterial infections has been clearly demonstrated. Polysaccharide vaccines are currently available for prevention of pneumococcal pneumonia and meningitis caused by *Neisseria meningititis* and type B *Haemophilus influenzae* (Jennings, 1990, 1983).

The antigenic determinants of CPS can be associated with particular structural features (Kenne & Lindberg, 1983; Kasper, 1986; Jennings, 1990). Recent developments in nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) have provided powerful tools for probing the structure of oligosaccharide epitopes (van Halbeek, 1990; Hellerquist & Sweetman, 1990). In an attempt to understand at the molecular level the specificity exhibited by these antigens, the fine structures of the CPS from a number of pathogenic bacteria of human and veterinary importance are currently being investigated. In several instances these antigenic CPS have been found to carry chiral acidic non-carbohydrate components that are related to their immunological specificities (Lindberg, 1990). This review will focus on stereochemical aspects of some commonly encountered chiral antigenic determinants and will highlight structural studies on the specific CPS of *Rhodococcus equi*.

STRUCTURAL FEATURES OF BACTERIAL CPS ANTIGENS

The complete characterization of carbohydrate epitopes of bacterial polysaccharides involves: (1) determination

of the primary structure of the oligosaccharide repeating unit; (2) identification of the mode of polymerization of the repeating unit; (3) determination of conformation or secondary structure.

The first two aspects will be dealt with here. For characterization of primary structure, the identity of the component monosaccharide residues, and their arrangement within the oligosaccharide unit, including the position and stereochemistry of the glycosidic linkages must be determined. In addition, the presence of any non-carbohydrate component needs to be established.

The oligosaccharide units can be joined together either in a head-to-tail fashion which involves only glycosidic linkages to give a sugar backbone, or through phosphate diester linkages. Examples of the different modes of polymerization are shown in Table 1.

Pasteurella haemolytica and *Actinobacillus pleuropneumoniae* are major causes of pneumonia in domestic animals and, based on differences in their capsular polysaccharide antigens, several serologically distinct types of these Gram-negative pathogens are recognized (Adlam, 1989; Perry *et al.*, 1990). Several of these CPS antigens have been found (Perry *et al.*, 1990; Richards & Leitch, 1990) to be polymers of repeating oligosaccharide units joined by phosphate diester groups. The CPS of *P. haemolytica* serotype 3 is composed of partially acetylated galactosyl-glycerol-phosphate units to form a teichoic acid like structure (Richards & Leitch, 1990). *A. pleuropneumoniae* serotype 12 CPS contains 1,3-linked *N*-acetyl-D-glucosamine residues to form a trisaccharide unit in which one of the glucosamine residues is α-glycosidically linked to phosphate thereby affording polymerization of the unit (Beynon *et al.*, 1991).

R. equi is a heavily encapsulated Gram-positive bacterium which causes a serious and often fatal respiratory disease in foals (Woolcock & Mutimer, 1978). To date, seven serotypes have been recognized which differ in the nature of their CPS antigens (Prescott, 1981). The structures of these serotype specific CPS antigens have recently been determined (Leitch & Richards, 1990; Severn & Richards, 1990; Severn & Richards, 1992; Masoud & Richards, 1994; Richards & Leitch, unpublished; Taylor & Richards, unpublished). These polysaccharides were each found to be high-molecular-weight heteroglycans composed of repeating oligosaccharide units joined in a head-to-tail fashion involving only glycosyl linkages. In addition, chiral acidic non-carbohydrate components were identified that appeared to be important to the serological specificities of the polysaccharide antigens. For example, the *R. equi* serotype 5 polysaccharide consists of a linear trisaccharide repeating unit of α-1,3-linked D-mannose, (D-Man), D-glucose (D-Glc) and D-glucuronic acid (D-GlcA) residues. This polysaccharide contains amino acid residues (L-glutamic acid) which are amide-linked to the 6-position of the D-GlcA residues (Table 1).

The capsular polysaccharides of serotypes 1, 6 and 7 *R. equi* contain β-D-mannopyranosyl (β-D-Man*p*) or α-D-galactopyranosyl (α-D-Gal*p*) residues in which the *O*-4 and *O*-6 positions are bridged by 1-carboxyethylidene (pyruvic acid acetal) groups, while the serotype 2 polysaccharide carries biosynthetically related 1-carboxyethylidene (pyruvic acid acetal) groups, while the serotype 2 polysaccharide carries biosynthetically related 1-carboxyethyl (lactic acid) ether substituents at *O*-3 of α-L-rhamnopyranosyl (α-L-Rhap) residues (Leitch & Richards, 1990; Masoud & Richards, 1994; Taylor & Richards, unpublished). Both pyruvic acid and lactic acid substituents occur in the serotype 3 polysaccharide (Severn & Richards, 1992): the 1-carboxyethylidene groups linked to *O*-2 and *O*-3 of β-D-glucopyranosyl uronic acid residues and the 1-carboxethyl groups are etherified at *O*-4 of β-D-mannopyranosyl residues. The structures of these polysaccharide repeating units are shown in Table 2.

Pyruvic acid acetals commonly occur in bacterial polysaccharides (Kenne & Lindberg, 1983; Lindberg, 1990) and are recognized (Heidelberger *et al.*, 1970) as immunodominant features. Although less frequently encountered, lactic acid ether substituted sugars have been reported as components of both lipopolysaccharides and extracellular polysaccharides. The structural diversity of these antigenic determinants is influenced by: (1) the nature of the substituted monosaccharide residue; (2) the position(s) of substitution; and (3) the chirality of the substituent. These features of bacterial polysaccharide antigens can be determined by primary structure analysis.

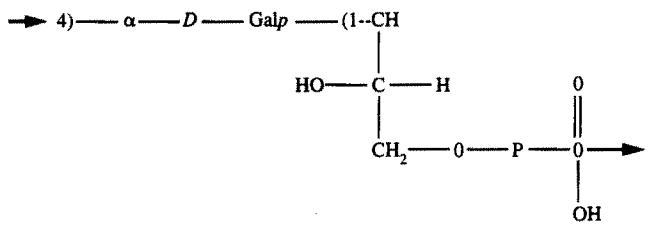
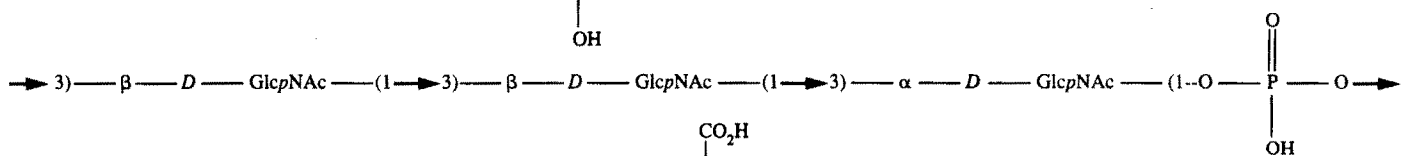
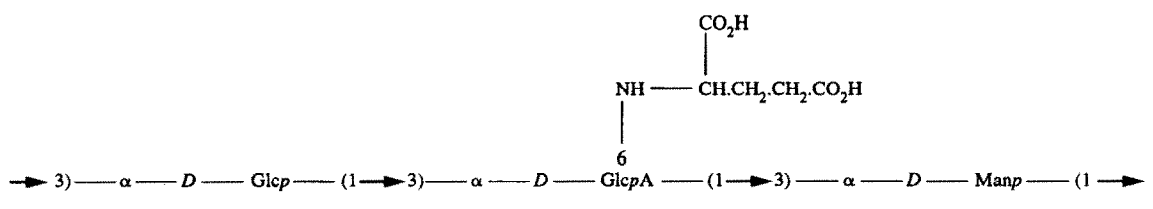
DETERMINATION OF POLYSACCHARIDE PRIMARY STRUCTURE

The approach to polysaccharide structure analysis described here involves the integeration of classical chemical methods and the more recently developed instrumental techniques. These include micro-analytical chemical methods for determination of composition (Hellerquist & Sweetman, 1990), specific chemical degradations (Lindberg *et al.*, 1975), mass spectrometry (Dell, 1987) and high-field ^{1}H- and ^{13}C-NMR methods (van Halbeek, 1990).

Application of NMR methods to primary structure analysis

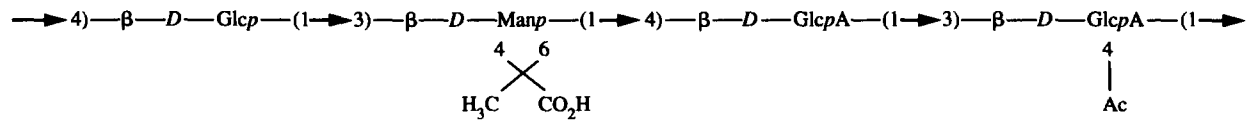
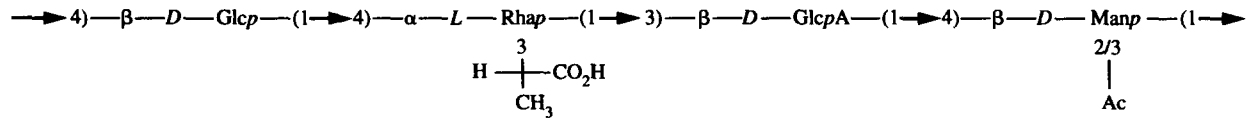
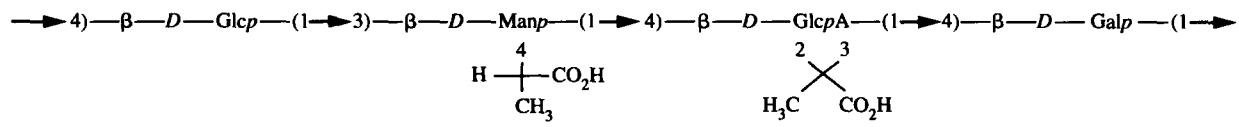
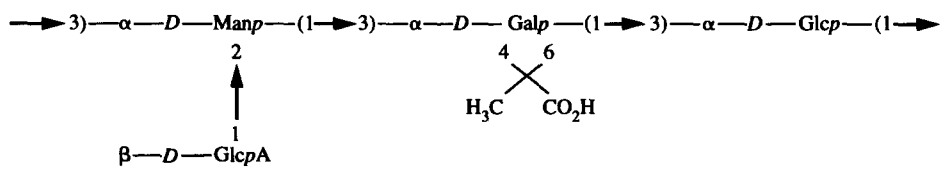
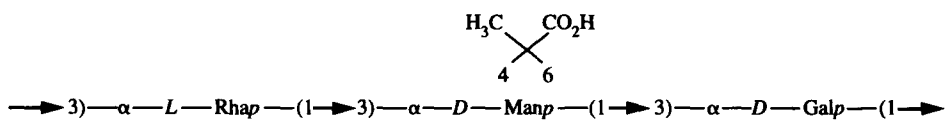
NMR spectroscopy has proved to be a very powerful technique that can provide both primary and secondary structural information on the intact polysaccharide. The successful application of this approach requires complete unequivocal assignment of the ^{1}H and ^{13}C resonances from which the relative stereochemistry, ring

Table 1. Mode of polymerization of oligosaccharide units of selected bacterial capsular polysaccharides

Type of linkage	Bacterial CPS	Repeating unit
Phosphate diester	*Pasteurella haemolytica* serotype 3[a]	
Phosphate diester	*Actinobacillus pleuropneumoniae* serotype 12[b]	
Glocosyl	*Rhodococcus equi* serotype 5[c]	

Structures are from [a] Richards and Leitch (1990) (partial *O*-acetylation of Gal*p* not indicated); [b] Beynon *et al* (1991); [c] Richards and Leitch (unpublished).

Table 2. Structures of *Rhodococcus equi* capsular polysaccharides containing pyruvic acid acetals and lactic acid ether substituents

Serotype	Repeating unit
1[a]	
2[b]	
3[c]	
6[d]	
7[e]	

Structures are from [a] Leitch & Richards, (1990); [b] Severn & Richards (1990); [c] Severn & Richards (1992); [d] Taylor & Richards, (unpublished); and [e] [e] Masoud & Richards (1994).

size and anomeric configuration of the component glycoses can be identified, as well as the presence and location of any non-carbohydrate substituents. Assignment of the ^{1}H NMR spectrum from a bacterial polysaccharide can be achieved through the use of two-dimensional (2D) homonuclear chemical shift correlation techniques, such as COSY and relayed COSY (Richards *et al.*, 1989). Spectral editing techniques, such as TOCSY (Braunschweiler & Ernst, 1983), are also of considerable value for ^{1}H-resonance assignment (van Halbeek, 1990). The carbon-13 resonances can then be assigned by heteronuclear chemical shift correlation between pairs of directly attached ^{1}H and ^{13}C nuclei by using the 1D CHORTLE pulse sequence (Leitch & Richards, 1990), by 2D NMR via ^{13}C-detection (Richards *et al.*, 1989; Severn & Richards, 1990), or by using very sensitive ^{1}H-detected methods (Beynon *et al.*, 1992).

These experiments provide information about proton and carbon-13 nuclei which show through-bond scalar couplings, referred to as *J*-coupling connectivities. In the homonuclear experiments, the information is transferred via the geminal (^2J) or vicinal (^3J) ^{1}H-^{1}H couplings; in the heteronuclear experiments, connectivities are defined by the one-bond ^{1}H-^{13}C coupling constant ($^1J_{C,H}$). Applications of these experiments to the structural analysis of complex carbohydrates and polysaccharides have recently been reviewed (Bush, 1988; van Halbeek, 1990).

Figure 1 shows the COSY contour plot for a depyruvylated sample of the *R. equi* serotype 1 CPS. The pyruvate acetal was removed from the native polysaccharide by treatment with dilute acid (Leitch & Richards, 1990). In this experiment, the chemical shift information is spread out over two dimensions essentially leading to deconvolution of the complex one-dimensional ^{1}H spectrum which is shown above. The diagonal peaks in the 2D spectrum are representative of this ^{1}H-NMR spectrum, while the off-diagonal or cross-peaks arise from protons that are related through the carbon framework of the molecule by ^{1}H-^{1}H coupling.

The anomeric proton resonances are readily identified in the low-field region of the spectrum from their chemical shift values and their single proton couplings (Bock & Thøgersen, 1982); these resonances provide a convenient starting point for spectral analysis. By

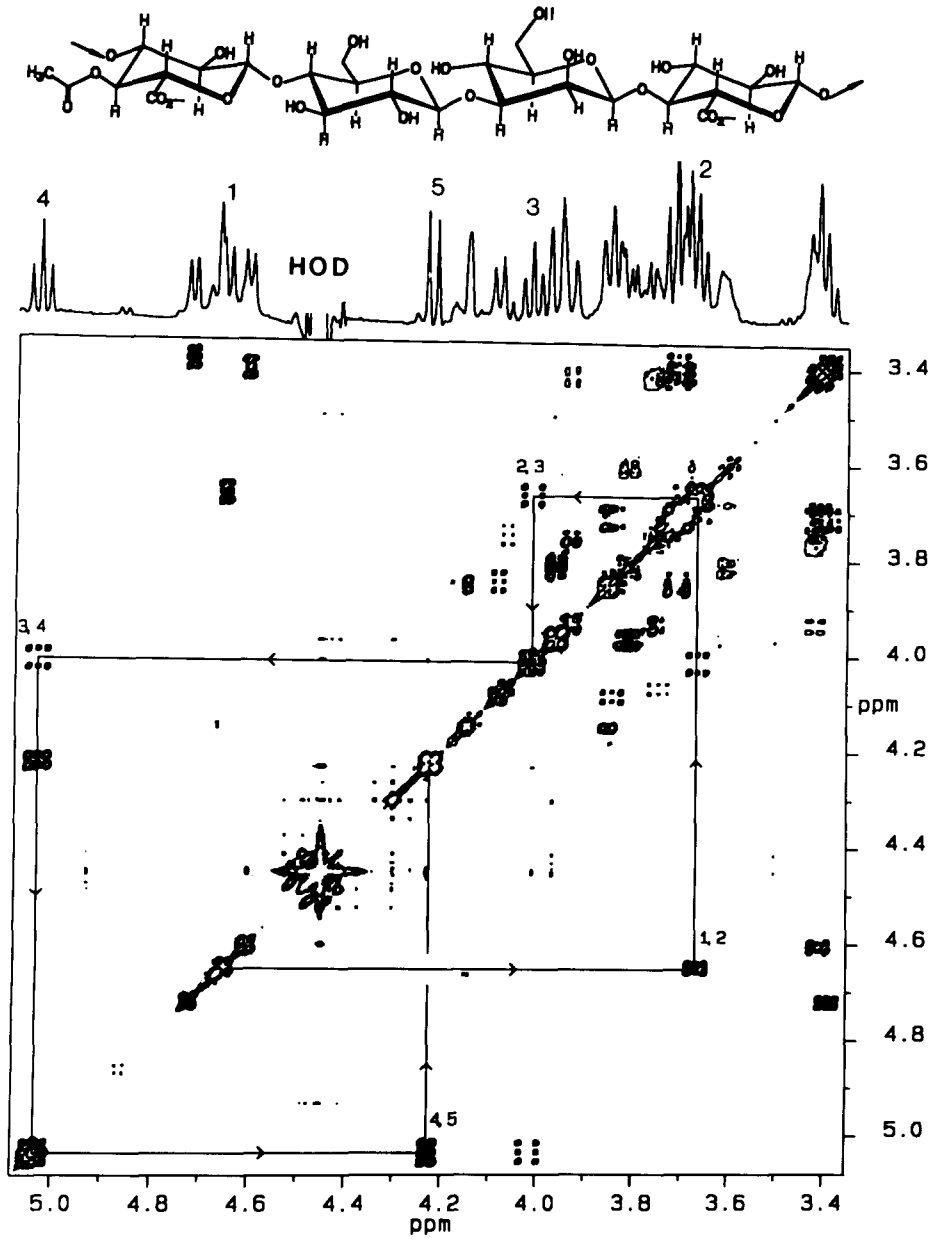


Fig. 1. Contour map of part of the COSY spectrum of the *Rhodococcus equi* serotype 1 depyruvylated CPS (Leitch & Richards, 1990). The 1D ^{1}H-spectrum and the structure of the repeating tetrasaccharide unit are shown above. The cross-peak assignments and connectivity pathway are indicated for the 4-*O*-acetyl-D-GlcA residue. This spectrum was obtained on a sample in D_2O on a Bruker AM-500 spectrometer.

tracing through the connectivity pathways defined by the cross-peaks in the COSY spectrum, the individual proton resonances can be identified and grouped into subspectra corresponding to each of the monosaccharide residues in the repeating unit. In Fig. 1, the connectivity pathway is indicated for a glucuronic acid residue in the repeating unit. Since this subspectrum was found to be comprised of five vicinally related ^{1}H resonances (H-1–H-5), it could be assigned to one of the uronic acid residues that was identified by chemical analysis (Fig. 2). From the observed large values of the vicinal proton coupling constants ($^3J \approx 10$ Hz) (Altona & Haasnoot, 1980), the residue could be identified as having the *β-gluco* configuration. The low-field value of the H-4 resonance (~5·0 ppm) of this residue suggested (Jansson *et al.*, 1987) that it carried an *O*-acetyl substituent at the C-4 position; this influence was supported by the large upfield ^{1}H-resonance shift which accompanied removal of the substituent. The presence of acetyl groups was indicated from the characteristic high-field methyl proton resonance (CH_3CO_2, ~2·0 ppm) observed in the full-scale ^{1}H-NMR spectrum of the native polysaccharide.

This approach to glycosyl residue identification is

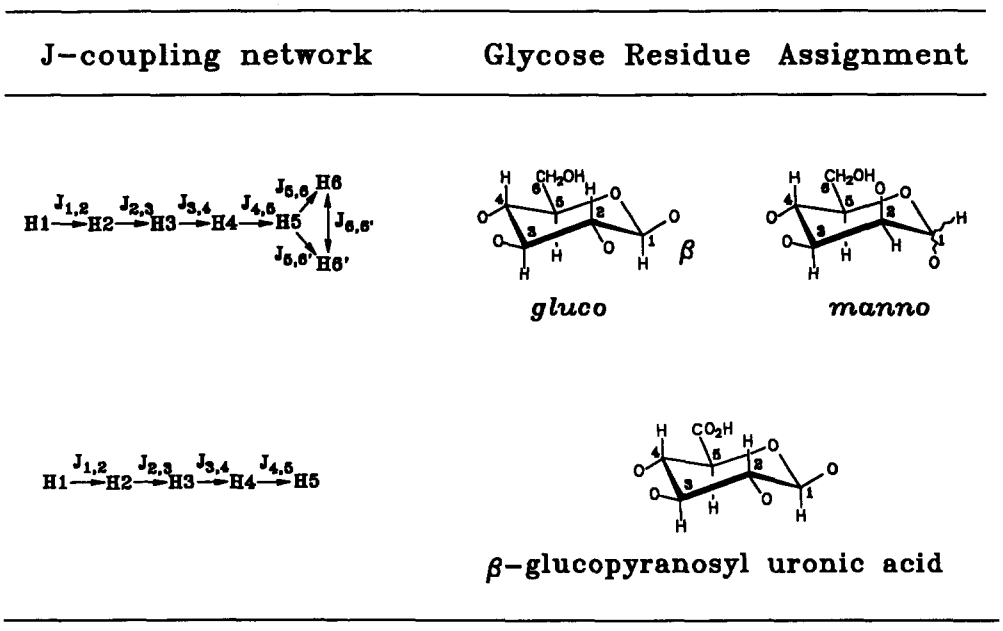


Fig. 2. Schematic representation of the ^{1}H-NMR approach used for identification of glycosyl residues from the *J*-coupling networks in the *Rhodococcus equi* serotype 1 polysaccharide. This method is of general applicability.

shown schematically in Fig. 2. By using this analysis, relative stereochemistry, ring-size and anomeric configuration of the other monomeric components were determined. For determination of absolute configuration of the glycose residues a chemical approach was used (see below) from which it was determined that the four component monosaccharides in the *R. equi* serotype 1 CPS belonged to the D-series. Subspectra corresponding to two β-D-glucopyranosyl uronic acids, a β-D-glucopyranose and a D-mannopyranose were identified from the *J*-coupling networks. In the case of the mannose residue the anomeric configuration was not evident from the value of the vicinal coupling constant, $J_{1,2}$, due to the axial disposition of the C-2 hydroxyl. The anomeric configuration of this type of residue can be determined from the magnitude of the one-bond ^{1}H-^{13}C coupling constant ($^1J_{H,C}$) (Block & Pedersen, 1974) or from proton nuclear Overhauser effect (NOE) measurements (Richards & Perry, 1988).

The ^{1}H-NMR spectra of native and *O*-deacetylated samples of the serotype 1 *R. equi* CPS were assigned similarly, by homonuclear chemical shift correlation. Assignment of the ^{13}C-NMR spectra of the respective polysaccharides was then made by heteronuclear chemical shift correlation experiments (Leitch & Richards, 1990). From a comparison of the NMR data from the native and pyruvate-free polysaccharides, it was established that the pyruvic acid acetal is substituted at the *O*-4 and *O*-6 positions of the Man*p* residue; the chemical shift values indicating an equatorially disposed methyl group in the cyclic pyruvate acetal.

The arrangement of the glycosyl residues within the repeating unit of the polysaccharide can be determined from transglycosidic ^{1}H-^{1}H NOE measurements (Bernstein & Hall, 1982; Prestegard *et al.*, 1982). For this application, NOEs are employed qualitatively to establish short (≤3 Å) through-space connectivities between anomeric and aglyconic protons on adjacent glycosidically linked residues. NOE connectivities can be measured in the 1D mode using difference spectroscopy (Severn & Richards, 1990) or by 2D NMR spectroscopy (NOESY experiment) (Leitch & Richards, 1990).

Figure 3 shows the 1D ^{1}H-NMR spectrum of an *O*-deacetylated sample of the *R. equi* serotype 1 polysaccharide. The NOE difference spectrum obtained by saturation of the anomeric proton of the D-mannopyranosyl residue is shown in the inset. The occurrence of an NOE across the glycosidic linkage to H-4 of the β-D-glucuronic acid residue establishes the indicated partial sequence. In addition, the occurrence of NOEs to H-3 and H-5 within the mannopyranosyl ring system served to establish the β-anomeric configuration for this residue.

The complete sequence of the residues within the CPS was achieved by measuring interresidue NOE connectivities on samples of the *O*-deacetylated and depyruvylated polysaccharides. Observed transglyosidic NOE connectivities are indicated in Fig. 4 and the indicated linkage positions are in agreement with results obtained chemically by methylation analysis (Hellerquist & Sweetman, 1990).

This NMR-based approach to polysaccharide primary structure analysis has been successfully applied to all the serotype-specific CPS of *R. equi*. The structure

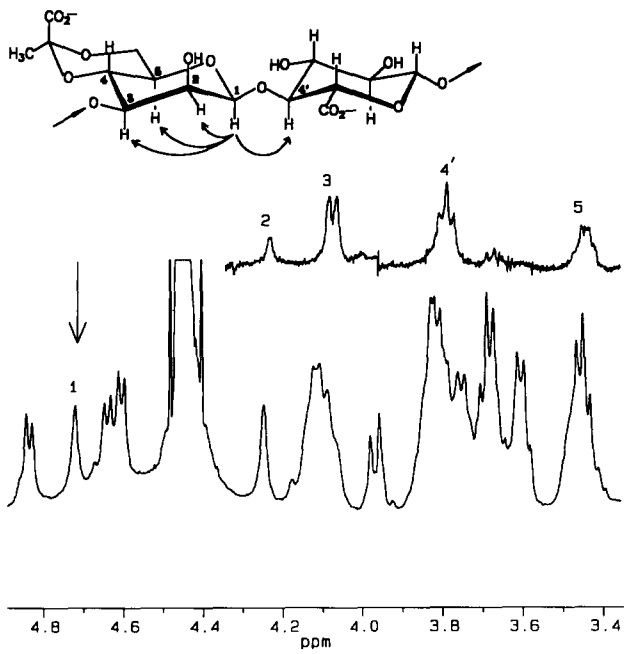


Fig. 3. Part of the ^{1}H-NMR spectrum of an *O*-deacytylated sample of the serotype 1 *Rhodococcus equi* CPS. The NOE difference spectrum obtained by saturation of the D-Man*p* H-1 resonance and the structure of the partial sequence deduced from the observed NOEs are shown above.

of the repeating trisaccharide unit of the serotype 5 *R. equi* CPS is shown in Fig. 5 (Richards & Leitch, unpublished). From the detection of transglycosidic NOEs, the sequence of the three glycose residues within the repeating unit was readily determined. The location of the glutamic acid residue was unambiguously established from a long-range, through-bond ^{13}C-^{1}H connectivity between the GlcA carboxyl carbon and the glutamic acid α-proton. A 1D selective INEPT experiment was employed for detection of the long range $^3J_{C,H}$ coupling (Bax *et al.*, 1984). Long-range ^{13}C-^{1}H shift-correlation spectroscopy has also been successfully applied to sequencing oligosaccharides (Bax *et al.*, 1984; van Halbeek, 1990) and polysaccharides (Bush, 1988; Richards & Leitch, 1989). A very useful approach involves the use of ^{1}H-detected 2D heteronuclear multiple bond correlation (HMBC) methods for providing the linkage positions and sequence of glycosyl residues (van Halbeek, 1990; Bush, 1988).

STEREOCHEMICAL ASPECTS OF POLYSACCHARIDE PRIMARY STRUCTURE

Determination of component glycose absolute stereochemistry

The absolute stereochemistry of the glycose residues can be determined by measurement of optical rotation, application of specific enzymes or microanalytical chemical methods. The latter method which involves initial acid hydrolysis of the polysaccharide to liberate the monosaccharide components has been extensively employed in this study. The monomeric components are then reacted with a chiral derivatizing reagent followed by chromatographic separation of the derived diastereometric derivatives and comparison with standards of known configuration. A very successful approach involves glycosidation of the glycose residues with enantiomerically pure (*R*)-2-butanol or (*S*)-2-octanol and gas-liquid chromatography (GLC) analysis of the peracetylated or tetramethylsilyated glycoside derivatives (Gerwig *et al.*, 1978; Leontein *et al.*, 1978).

Application of this approach to the *R. equi* serotype 5 CPS is illustrated in Scheme 1. Treatment of the hydrolysis products from this polysaccharide with (*R*)-2-butanol afforded a mixture of the (*R*)-2-butyl glycosides of Glc, Man and GlcA which were each identified as belonging to the D-series by GLC of the acetylated derivatives. During this reaction sequence, the glutamic acid residues underwent cyclization to give the monoester of the *N*-acetylated lactam 1, which was identified by comparison of its mass spectrum and GLC retention time with a sample derived from D-glutamic acid. The

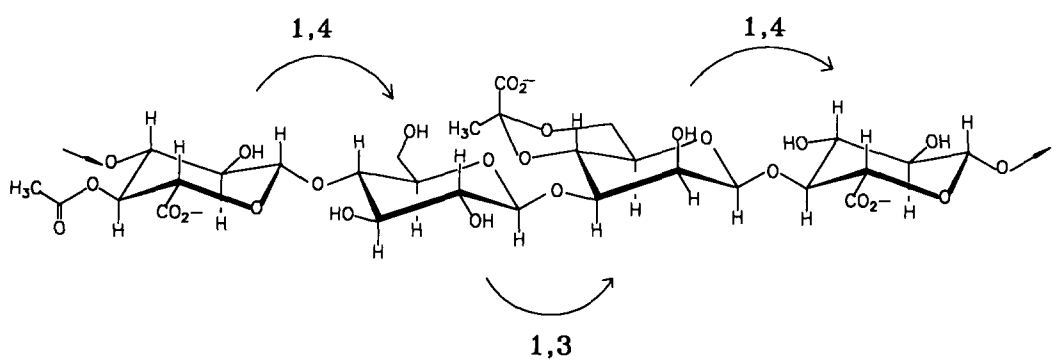


Fig. 4. Structure of the tetrasaccharide repeating unit of the serotype 1 *Rhodococcus equi* CPS showing the transglycosidic NOE connectivities.

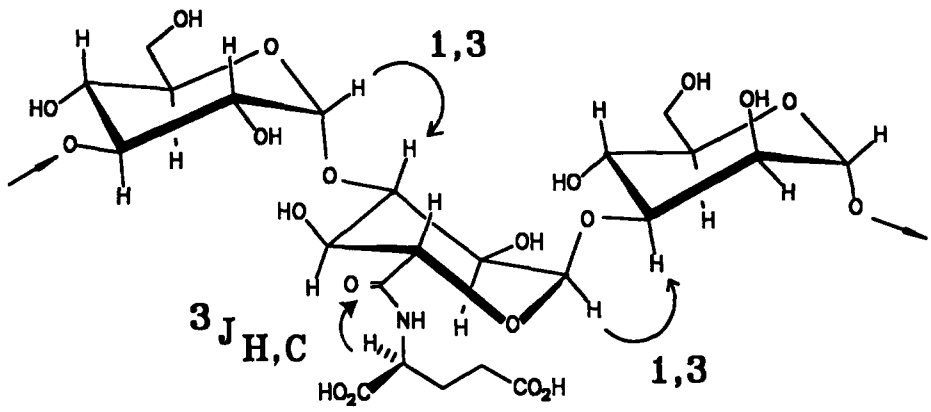
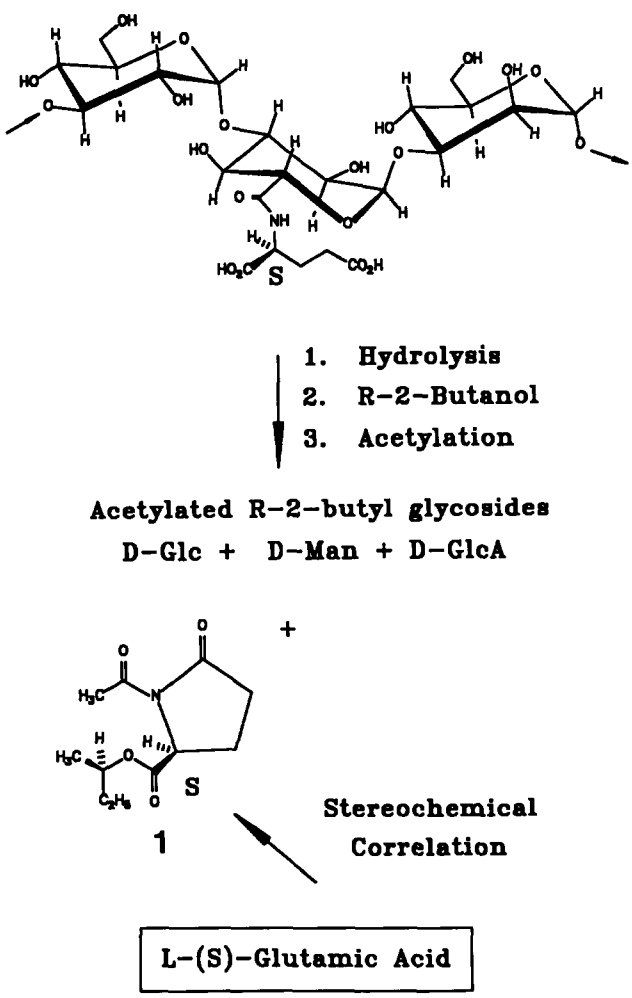


Fig. 5. Structure of the trisaccharide repeating unit of the serotype 5 *Rhodococcus equi* CPS showing the transglycosidic NOEs and a $^3J_{H,C}$ connectivity from the glutamic acid proton.



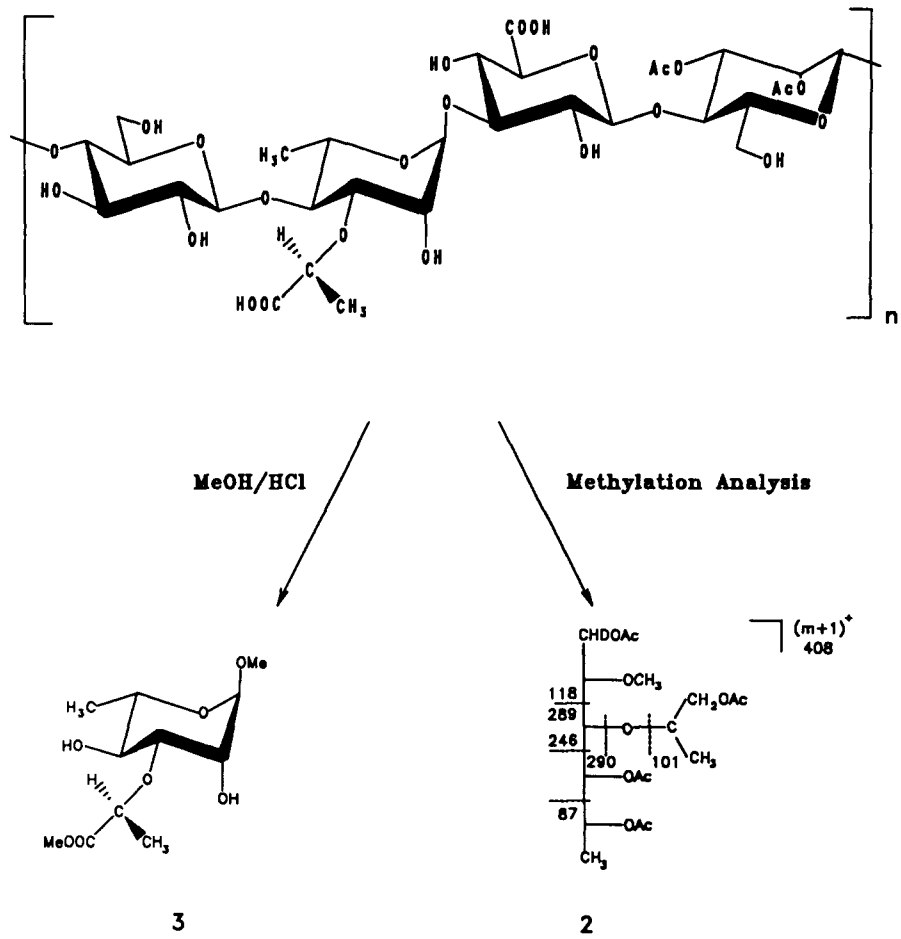
Scheme 1. Conversion of the monomeric components of the serotype 5 *Rhodococcus equi* CPS to acetylated (R)-2-butyl derivatives for stereochemical correlation.

lactam derived from L-glutamic acid had a different GLC retention time which established the absolute configuration of the glutamic acid residue in the polysaccharide as L. L-Glutamic acid amide-linked to the 6-position of a L-Glc*p*A residue has been recently reported as a component of cell-surface polysaccharides from *Klebsiella* K82 (Jansson *et al.*, 1988) and *Serratia marcescens* 023 (Oxley & Wilkinson, 1990).

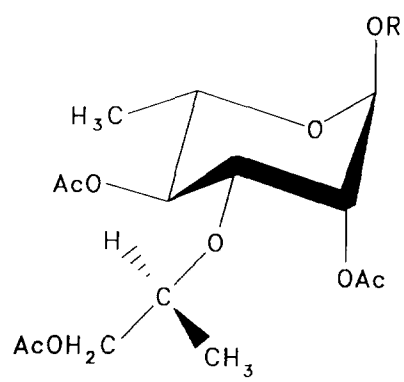
The *R. equi* serotype 2 CPS was found to carry 1-carboxyethyl groups at *O*-3 of →4)-α-L-Rha*p*-(1→ residues (Table 2). The 3-*O*-(1-carboxyethyl)-rhamnose residues were identified from the MS fragmentation pattern of the 2-*O*-methyl acetylated alditol 2 obtained from the CPS by methylation analysis. This is shown in Scheme 2 where the fragment ion observed at *m/z* 101 is diagnostic of the lactyl ether substituent. The absolute configuration of the 1-carboxyethyl sugar ether was established by stereochemical correlation of a methyl 3-*O*-(1-carboxyethyl)-α-L-rhamnopyranosyl derivative with synthetic samples of the (*R*)- and (*S*)-diasteriomers. The methyl ester of this glycose (structure 3) was readily obtained from the CPS by treatment with methanolic-HCl (Scheme 2).

The two diastereomers required for stereochemical correlation were prepared by the synthetic procedure outlined in Scheme 3 (Severn & Richards, 1993). The key step in this sequence involved the reaction of the exposed hydroxyl group of methyl 2,4-di-*O*-benzyl-α-L-rhamnopyranoside (structure 4) with (*R*)-2-chloropropionic acid to afford methyl 3-*O*-[(*S*)-1-carboxyethyl]-α-L-rhamnoside (3*S*), following removal of the benzyl protecting groups. This reaction proceeds via a S_N2-like mechanism with inversion of configuration at the asymmetric center of the 2-chloropropionic acid. Thus, when (*S*)-2-chloropropionic acid is used, the corresponding (*R*)-diasteriomer is produced (i.e. 3*R*).

The peracetylated (*S*)- and (*R*)-1-hydroxypropyl derivatives of the synthetic methyl α-L-rhamnopyranosides gave single peaks on GLC; the faster eluting *S*-diasteriomer (structure 5) exhibited a peak coincident with that of the corresponding derivative from the *R. equi* serotype 2 polysaccharide. The (*R*)-2-butyl glycoside of the native 3-*O*-(1-hydroxypropyl)-α-rhamnopyroside (structure 6) was observed to have a GLC retention time



Scheme 2. Isolation and characterization of the 3-*O*-(1-carboxyethyl)-rhamnose residue from the *R. equi* serotype 2 CPS. The electron-impact MS fragmentation pattern is indicated for the 2-*O*-methyl acetylated alditol 2.



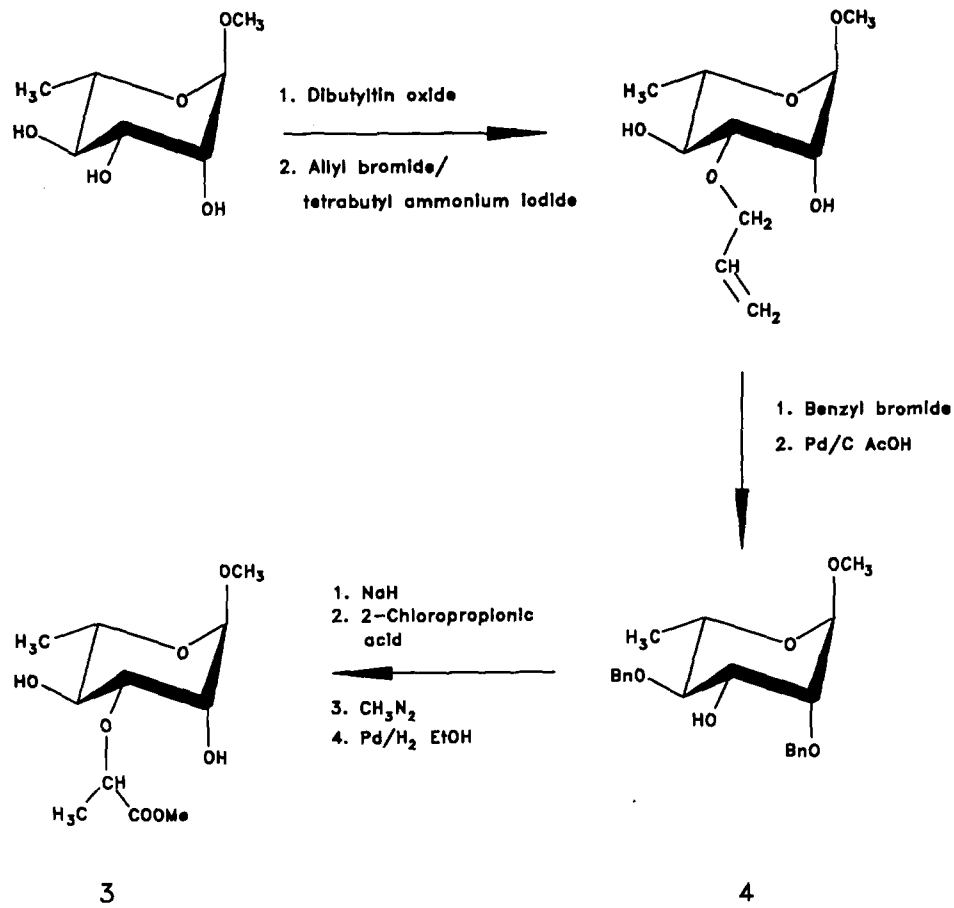
Structure 5, 6

identical to that of the corresponding derivative from 3-*O*-[(*S*)-1-hydroxypropyl]-α-L-rhamnopyranoside, establishing its absolute chirality (Severn & Richards, 1990).

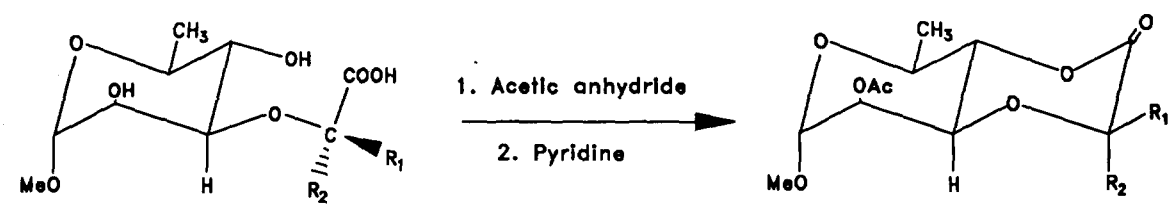
Stereochemistry of 1-carboxyethyl sugar ethers by NMR spectroscopy

Lactic acid ethers are found as substituents of a wide range of sugars (Kenne & Lindberg, 1983; Lindberg, 1990). The 3-*O*-substituted (*R*)-1-carboxyethyl derivative of 2-amino-2-deoxy-D-glucose (muramic acid) is a component of bacterial cell wall peptidoglycan. Lactic acid either sugars have also been observed as components of a number of bacterial lipopolysaccharides and extracellular polysaccharides, and both the (*R*)- and (*S*)-enantiomers of the lactyl group have been reported (Lindberg, 1990).

It is to be expected that the relative and absolute configurations of 1-carboxyethyl ether substituents of antigenic bacterial polysaccharides are of major importance in understanding their immunological reactivities. The chemical methods used to obtain this information are of limited general applicability since they rely on stereochemical correlation of 1-carboxyethyl sugar ethers with synthetic samples of defined stereochemistry (see above). A general NMR-based strategy has been developed for the determination of relative stereochemistry, in which the chirality of the lactate α-carbon is related to that of the attached sugar moiety in a conformationally rigid lactone derivative of the glycose (Severn & Richards, 1993). The lactic acid ether is a flexible substituent from which limited stereochemical information can be obtained by NMR spectroscopy.



Scheme 3. Conversion of L-rhamnose to (S)- or (R)-methyl 3-*O*-(1-carboxyethyl)-α-L-rhamnopyranoside (3S or 3R).



Scheme 4. Conversion of (R)- and (S)-methyl 3-*O*-(1-carboxyethyl)-α-L-rhamnopyranoside (3R or 3S) into the corresponding acetylated lactones (6R or 6S).

However, lactonization of the hydroxy acid constrains the carboxyethyl group within a 1,3-dioxane-4-dione ring system. This rigid conformation permits the observation of ^{1}H-^{1}H NOEs between protons of the lactone and glycose rings.

This approach was evaluated using the synthetic samples of methyl 3-*O*-(1-carboxyethyl)-α-L-rhamnopyranoside (3). The two diasteromeric lactones were prepared as outlined in Scheme 4.

Treatment of methyl 3-*O*-(1-carboxyethyl)-α-L-rhamnopyranoside (3*R* or 3*S*) with acetic anhydride in the presence of pyridine afforded the 2-*O*-acetyl derivative of the corresponding 4-*O*-lactone (6*R* or 6*S*, respectively). The ^{1}H-NMR spectra were fully assigned by homonuclear chemical shift correlation techniques. The occurrence of specific NOEs facilitated the assignment of reactive stereochemistry, as indicated in Fig. 6.

Saturation of the α-proton of the (*R*)-lactone (6*R*) resulted in an NOE at H-3 of the L-rhamnopyranosyl ring indicating the α-proton to occupy an axial position. Correspondingly, saturation of the L-rhamnosyl H-3 resulted in an NOE at H-α. Molecular modeling of the lactone system using MM2 calculations and the PC program Alchemy predicts an internuclear separation of 2·4 Å between those protons in the *R*-lactone. NOEs are generally observed for protons separated by less than 3 Å (Carver & Brisson, 1984).

When the lactate α-carbon has (*S*)-chirality (3*S*), it is the methyl group that occupies the axial position in the derived lactone (6*S*); as expected, NOEs are observed

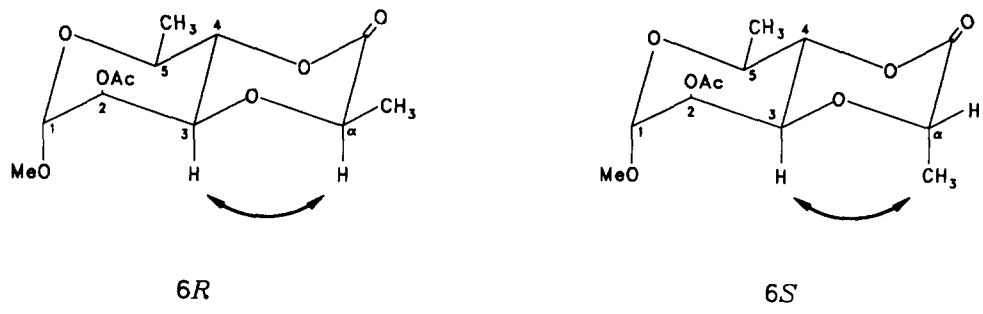
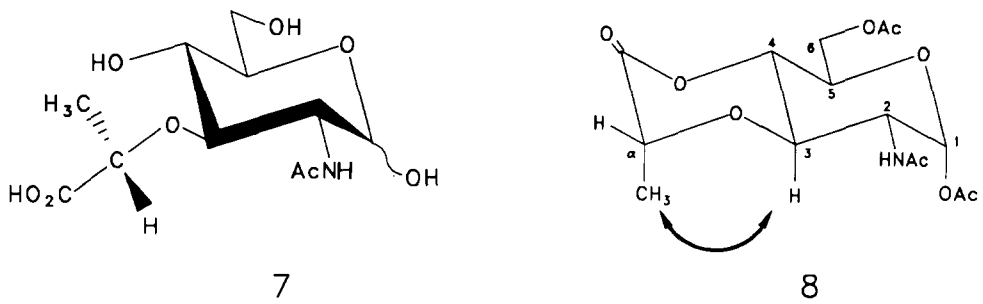


Fig. 6. Structure of the lactones obtained from (*R*)- and (*S*)-methyl 3-*O*-(1-carboxyethyl)-α-L-rhamnopyranoside (6*R* and 6*S*) in which NOEs between protons on the lactone and glycose ring systems are indicated.



Structure 7, 8

between the methyl protons and H-3 of the L-rhamnopyranosyl ring system (Fig. 6).

Interpretation of NOE and molecular modeling data will, in general, allow the assignment of the relative stereochemistry of 1-carboxyethyl-glycoses without the need for reference compounds. The general procedure involves lactonization of the free glycoses in the presence of acetic anhydride, to give the acetylated lactone derivative. Thus, application of this technique to the stereochemical analysis of *N*-acetyl muramic acid (structure 7) showed the occurrence of NOEs between the lactate methyl protons and H-3 of the 2-acetamido-2-deoxy-D-glycopyranosyl ring in the derived lactone (structure 8), a result in full agreement with the lactate moiety having the (*R*)-configuration (Lindberg, 1990).

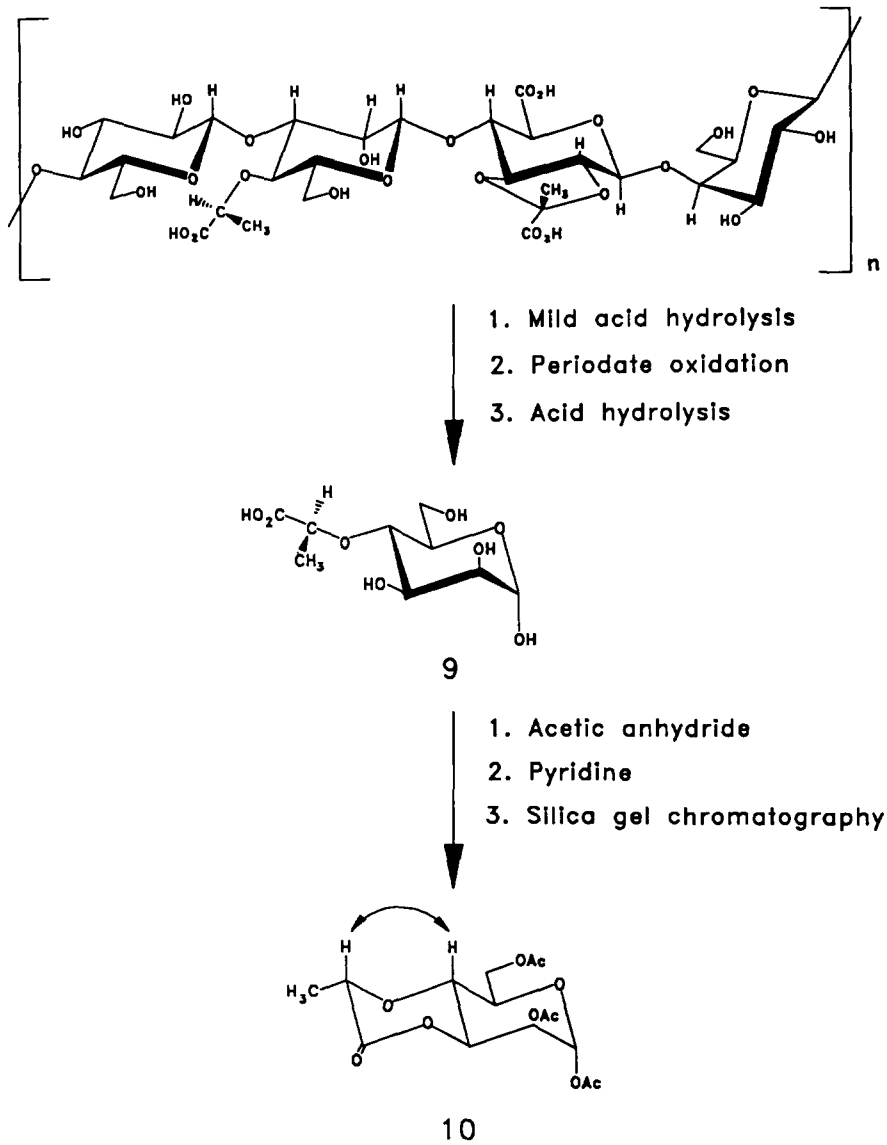
The *R. equi* serotype 3 CPS also contains a 1-carboxyethyl substituent which is ether-linked at the 4-position of a →3)-β-D-Man*p*-(1→ residue (Table 2). The 4-*O*-(1-carboxyethyl) derivative of D-mannose (structure 9) was obtained from the polysaccharide by the acid hydrolysis/periodate oxidation procedure shown in Scheme 5. The chirality of the lactate α-carbon was correlated to that of sugar residue from the observed NOEs in the acetylated lactone derivative (structure 10). Thus, the occurrence of NOEs between H-α and H-4 of the D-mannopyranose ring established that the lactate moiety has *S*-chirality (as in 10).

The absolute stereochemistry of the mannopyranosyl residue was deduced from data obtained by molecular modeling and ^{1}H-^{1}H HOE measurements of the β-D-Glc*p*-(1 → 3)-Man*p* disaccharide unit in the intact serotype 3 polysaccharide. From the minimum energy conformation of the two disaccharide units, β-D-Glc*p*-(1 → 3)-β-D-Man*p* and β-D-Glc*p*-(1 → 3)-β-L-Man*p*, calculated by the HSEA method, a NOE between Glc H-1 and Man H-2 is only predicted in the disaccharide in which both glycosyl units possess the D-configuration (internuclear separation 2·8 Å) and not in the diasteriometric D-L disaccharide (separation 3·9 Å) (Severn & Richards, 1992). A transglycosidic NOE to both those protons (see Scheme 5) established that 4-*O*-(1-carboxyethyl)-β-mannopyranose has D-absolute configuration. A similar pattern of NOE was observed for the →4)-β-D-Glc*p*-(1→3)-β-D-Man*p*-(1→ unit in the *R. equi* serotype 1 polysaccharide in which the chirality of the mannose residue was established independently by chemical correlation (Leitch & Richards, 1990).

This strategy for the determination of the relative stereochemical of 1-carboxyethyl sugar ethers is of general applicability. The absolute configuration of the glycose moiety can be achieved using conventional approaches following the dealkylation of the 1-carboxyethyl substituent (e.g. by using boron trihalide reagents (Hough & Theobald, 1963).

Stereochemistry of cyclic pyruvate acetals by NMR spectroscopy

The specific capsular polysaccharides from *R. equi* serotypes 1, 3, 6 and 7 contain cyclic pyruvic acid acetal



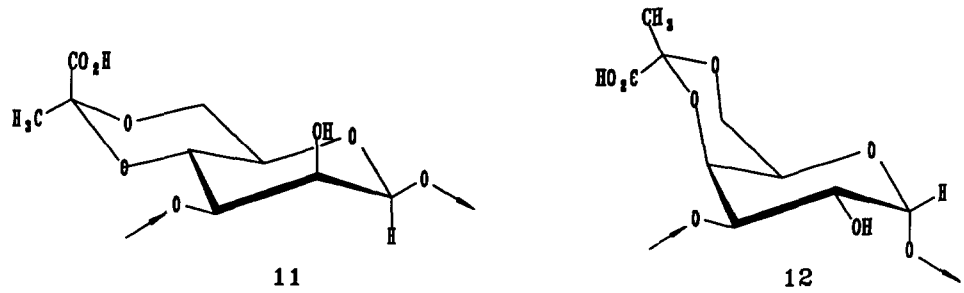
Scheme 5. Isolation of 4-*O*-(1-carboxyethyl)-α-D-mannopyranose (9) from *R. equi* serotype 3 CPS and its conversion to the acetylated lactone (10).

substituents (Table 2). As mentioned earlier, pyruvic acid acetals occur widely in bacterial polysaccharides and are recognized as immunodominant structural features (Heidelberger *et al.*, 1970; Lindberg, 1990). The stereochemistry of the pyruvate acetal moiety has been shown to be important for serological specificity (Bennett & Bishop, 1971).

1-Carboxyethylidene groups involving six membered rings are most frequently observed. The absolute configuration of the acetal carbon of cyclic pyruvate acetals involving 1,3-dioxane ring systems linked to the *O*-4 and *O*-6 positions of hexopyranosyl residues (Glc*p*, Man*p* and Gal*p*), can be readily determined by comparison of ^{13}C-NMR chemical shifts of the pyruvate methyl resonance with those of model compounds (Garegg *et al.*, 1980). For the *R. equi* serotypes 1, 6 and 7 CPS, the chemical shift values of the pyruvate methyl carbons (25–26 ppm) were indicative of equatorially disposed methyl groups in the respective dioxane ring systems. This corresponds to *S*-chirality for the serotypes 1 and 7 1-carboxyethylidene substitutes, and *R*-chirality for that of serotype 6, since the Man (structure 11) and Gal (structure 12) residues to which they are attached each has the D-configuration.

A comparison of the ^{13}C-NMR data from the pyruvate-free and the native *R. equi* serotype 3 polysaccharides indicated that the β-D-glucopyranosyl uronic acid residues were substituted by the pyruvic acid moieties at the *O*-2 and *O*-3 positions. The low-field value of the acetal carbon (≈110 ppm) was indicative of the pyruvic acid acetal forming a five membered ring system (Severn & Richards, 1992).

Assignment of the absolute configuration of cyclic pyruvate acetals involving 1,3-dioxolane ring systems is



11 12

Structure 11, 12

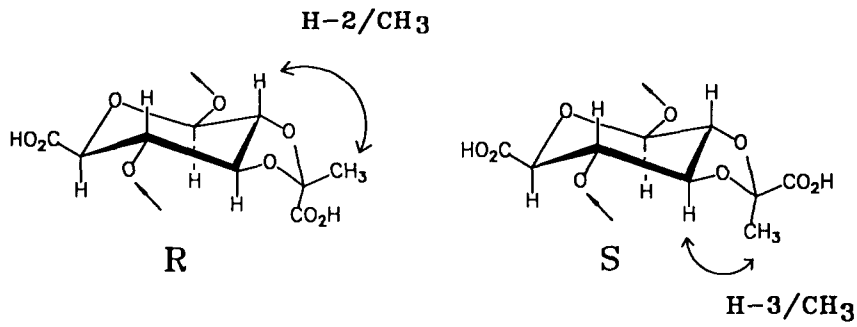


Fig. 7. Predicted NOEs for the two stereoisomers of 2,3-*O*-(1-carboxyethylidene)-D-glucopyranosyl uronic acid.

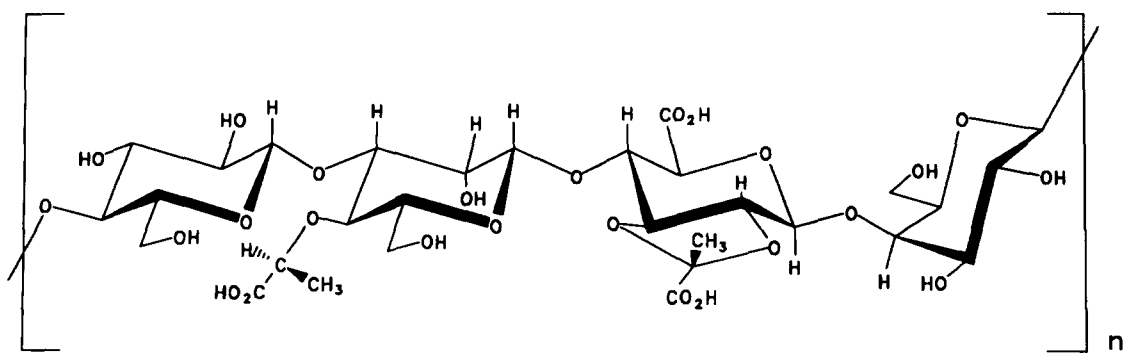


Fig. 8. Structure of the tetrasaccharide repeating unit of the serotype 3 *Rhodococcus equi* CPS showing the absolute stereochemistry of the chiral acidic substituents.

not straightforward. In systems where the 1-carboxyethylidene substituent is linked to the *O*-3 and *O*-4 positions of D-Gal*p*, no significant difference is observed for ^{13}C (or ^{1}H) chemical shifts of the methyl resonances in the (*R*)- and (*S*)-acetals (Garegg *et al.*, 1980). By analogy, the methyl resonances from the (*R*)- and (*S*)-2,3-(1-carboxyethylidene) derivatives of D-GlcA would not be expected to show significant stereochemical shift differences. Furthermore, suitable reference compounds are not readily available, and chemical correlation is further complicated by the extreme acid liability of the *trans*-fused 1,3-dioxolane ring system (Erbing *et al.*, 1976; Dutton & Savage, 1980). However, NOE measurements on the intact polysaccharide can provide a diagnostic indication of the orientation of the pyruvate methyl group, and can thereby lead to the desired stereochemical assignment (Severn & Richards, 1992). This approach has recently been applied to the stereochemical analysis of pyruvate acetals substituted at the *O*-2 and *O*-3 positions of D-Gal residues (Jones, 1990).

Considering the two stereoisomers for the cyclic pyruvate acetals shown in Fig. 7, in the (*R*)-enantiomer, the methyl group is on the same side of the pyranosyl ring system as H-2; an NOE would be expected between H-2 and the methyl protons. In the *S*-enantiomer, the methyl group is on the other side of the ring system; in this case an NOE would be expected between H-3 and the methyl protons.

Saturation of the pyruvate methyl resonance in the native *R. equi* serotype 3 polysaccharide resulted in a NOE at H-2 of the D-glucopyranosyl uronic acid residue; no detectable NOE was observed at H-3 reso-

nances. This result is consistent only with the pyruvate acid acetal having the (*R*)-configuration in which the methyl group occupies a position on the same face of the D-glycopyranosyl ring system as the axial H-2.

The structure of the repeating unit of the serotype 3 CPS is shown in Fig. 8.

CONCLUSIONS

Over the last few years, very sensitive methods have emerged which are applicable to the structural and stereochemical analysis of bacterial polysaccharides. Recent developments in NMR instrumentation and methodology give rise to several strategies for the determination of both primary and secondary structural features. This review has highlighted some applications of NMR spectroscopy, as well as microanalytical chemical techniques for probing the structural diversity of the capsular polysaccharide antigens of *Rhodococcus equi*. The major emphasis has been placed on the analysis of chiral antigenic substituents by new methods, which involve the detection of through-space NOE connectivities in either the intact polysaccharide or on conformationally-rigid glycose derivatives. It is expected that detailed structural studies of this type will provide the molecular basis for the rational development of serologically-based diagnostic probes and improved strategies for the production of protective vaccines.

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