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The structure of the O-antigenic chain of the lipopolysaccharide of *Rhizobium trifolii* 4s

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Abstract

The structure of the O-antigen chain of the lipopolysaccharide (LPS) of *Rhizobium trifolii* 4s has been determined by a combination of chemical and spectroscopic methods. The glycosyl components were found to be L-rhamnose, *N*-acetyl-D-glucosamine, and *N*-acetyl-D-mannosamine in 3:1:1 molar proportion, as determined by gas chromatography and gas chromatography–mass spectrometry of alditol acetate and persilylated (*R*)-2-hydroxybutyl glycoside derivatives. The linkage positions and configurations of the glycosyl residues were obtained by 1D and 2D NMR spectroscopy. The polymer has a pentasaccharide repeating-unit containing rhammose and *N*-acetylglucosamine in the main chain and *N*-acetylmannosamine as the sole-side chain component. This latter residue is linked to a main-chain rhamnose residue. This result was suggested by NMR spectroscopy and confirmed by periodate oxidation. The sequence was deduced by 1D and 2D NMR NOE experiments and by partial hydrolysis studies. The repeating unit of the polysaccharide is shown. This constitutes the first complete structure of an O-antigenic chain of the lipopolysaccharide of any strain of *Rhizobium trifolii*.

$$\begin{array}{l} \qquad\qquad\qquad\quad \alpha\text{-D-ManNAc} \\ \qquad\qquad\qquad\quad \downarrow \\ \qquad\qquad\qquad\quad 2 \\ \rightarrow 3)\text{-}\alpha\text{-L-Rha-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Rha-}(1 \rightarrow 4)\text{-}\beta\text{-D-GlcNAc-}(1 \rightarrow 3)\text{-}\alpha\text{-L-Rha-}(1 \rightarrow \end{array}$$

1. Introduction

The lipopolysaccharides of *Rhizobium* are known to be involved in critical aspects of bacteroid development and nodule occupancy. Several years ago it was

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demonstrated [1–3] that bacteria having defects in lipopolysaccharide structure (as judged by compositional analyses or electrophoretic properties) also have defects in their ability to successfully complete the earlier aspects of nodule invasion. Such bacteria were not released from infection threads and the nodules formed from their infection were invariably unoccupied. More recently, a more-defined structural basis for the inability of these "rough" bacterial mutants to form viable nodules was advanced when it was demonstrated that such mutants contained lipopolysaccharides that had no O-antigen and had truncated or different core components [4–6].

Although there has been much progress in elucidating the structures of the R-core components of the lipopolysaccharides of the *Rhizobiacae* [7–9], there has been considerably less success in elucidating the structures of the O-antigen components. No complete structures have yet been proposed for the O-antigen of any of the "fast growing" strains. Here we describe the complete structure of the O-antigen chain of *R trifolii* strains 4s. This is a much studied strain for which there is a considerable amount of biochemical and physiological data [10,11]. These studies will facilitate biochemical studies of the roles of the O-antigen of this strain in bacteroid development. Questions about whether this specific O-antigen structure is formed *in planta* can now be answered.

2. Materials and methods

O-Antigen isolation.—*Rhizobium trifolii* 4s was grown at 30°C in modified Bergensen's (BIII) medium. The LPS was isolated as described previously [12]. A sample (10 mg) of LPS was hydrolyzed with 2 mL of 1% AcOH for 3 h at 100°C and then extracted several times with an equal volume of 5:1 $CHCl_3$–MeOH. The aqueous fraction containing the polysaccharide was dried under a stream of N_2. The low-molecular-weight components derived from the R-core were selectively removed by acetylating the carbohydrate mixture with 1:1 pyridine–Ac_2O. This effectively removed the low-molecular-weight core oligosaccharides without altering the O-antigenic polysaccharide, as the latter is insoluble in this acetylating mixture. This polymer was found by chromatographic analyses to be one homogeneous component and was used for all of the subsequent studies.

Compositional analysis.—The polysaccharide was converted into alditol acetate derivatives. A sample of polysaccharide (0.1 mg) was treated with 3 mL of 1% HCl in MeOH. The solution was sonicated for 1 min, heated for 15 min at 68°C, and then kept overnight at room temperature. The solution was then dried under a stream of N_2. To the dried sample was added 5 mg of $NaBD_4$ and 0.3 mL of 1:1 MeOH–water. The solution was kept overnight at room temperature and then dried under N_2 and treated with 2 mL of 2 M CF_3CO_2H for 1.5 h at 115°C. After cooling to room temperature, the solution was concentrated to dryness under N_2. A few drops of water were added and the solution was dried again under N_2 with heating in a water bath (50°C). This addition of water and drying was repeated twice in order to remove traces of acid. The sample was then dissolved in 0.2 mL

of water, and a solution of 2 mg of $NaBH_4$ in 0.1 mL of water was added dropwise. The mixture was kept for 2 h at room temperature and then 2.4 M HCl was added to the solution dropwise until effervescence ceased. The solution was dried under N_2 and to the residue was then added 1 mL of 2%AcOH–MeOH. This solution was then evaporated under N_2 again. This last step was repeated 5 times. The dried product was peracetylated by treatment with 1 mL of pyridine, followed by 2 min of sonication and then 1 mL of Ac_2O. The solution was heated for 1 h at 70°C with occasional sonication and then dried under N_2. The acetylated product was partitioned between 1 mL of sat aq NaCl and 3 mL of $CHCl_3$. The $CHCl_3$ fraction was removed and dried under N_2 with no heat and subjected to GC analysis on a Hewlett–Packard 5890 gas chromatograph using a DB225 capillary column with He as the carried gas. A temperature program with an initial temperature of 180°C holding for 2 min, then increasing to 230°C at the rate of 2°C/min with a final hold of 60 min was employed. GC–MS analysis was then performed on a Jeol 505 mass spectrometer system using electron-impact ionization (70 eV) and detecting in the positive mode.

Determinination of D *and* L *configuration.*—The monosaccharides were converted into their trimethylsilylated (−)-2-butyl glycoside derivatives [14], and these were subjected to GC analysis. A sample of polysaccharide (0.2 mg) was treated with 2 mL of 2 M CF_3CO_2H for 1.5 h at 120°C. The hydrolyzed product was then dried under a stream of N_2 and acetylated as already described. The acetylated product was then dried under N_2 and treated with 0.2 mL of (−)-*R*-2-butanol–HCl, which was made with (−)-*R*-2-butanol and AcCl in a ratio of 10:1 (v/v). After butanolysis for 9 h at 80°C, the solution was neutralized with Ag_2O. The mixture was kept for 0.5 h at room temperature to allow the silver salts to precipitate. The supernatant solution was removed and dried under N_2, and then treated with 5.0 μL of 1.5 mequiv/mL *N*-trimethylsilylimidazole in silylation-grade pyridine for 15 min at 60°C. The trimethylsilylated mixture was injected onto a GC column directly. The GC analysis was performed on a Hewlett–Packard 5890 gas chromatograph equipped with a DB1 capillary column with He as the carrier gas. The temperature was programmed from 150 to 300°C at 3°C/min. GC–MS analysis was performed with the same instrument as already described.

Periodate oxidation.—A sample of polysaccharide (0.1 mg) was treated with 0.5 mL of 0.2 M aq $NaIO_4$ in the dark for 48 h at 10°C. The solution was then desalted on a TSK 2000 gel filtration HPLC column, using water as eluant and refractive-index detection. Fractions corresponding to peaks were collected and the sole carbohydrate-containing fraction was identified by the phenol–H_2SO_4 assay [13]. The oxidized polysaccharide was hydrolyzed with 0.2 M CF_3CO_2H for 10 min at 55°C. A sample of the polysaccharide thus oxidized and purified was oxidized again with $NaIO_4$ as already described. Both oxidized polysaccharide products were converted into alditol acetate derivatives and analyzed on GC as already described.

Partial hydrolysis.—A sample of polysaccharide (0.1 mg) was treated with 2 mL of 44% HCO_2H overnight at 70°C. The solution was dried under N_2 and then ~ 50 mL of water was added. The solution was dried under N_2 again to ensure

complete removal of HCO_2H. The partially hydrolyzed products were then peracetylated as already described and subjected to fast-atom-bombardment mass spectrometric (FABMS) analysis. 4-Nitrobenzyl alcohol was used as the matrix.

NMR spectroscopy.—All NMR spectra were measured in D_2O at 500 MHz for ^1H or 125 MHz for ^{13}C with a Varian VXR500 spectrometer. For the HMQC experiments [15], a spectral width of 25740 Hz was employed for the ^{13}C dimension. A total of 64 transients were required at 1024 points each. A total of 256 data sets were acquired. Double quantum filtered *J*-correlated 2-dimensional spectrum (phase sensitive mode) [16] was obtained using a total of 256 data sets (32 transients at 2048 data points each). Data for the HOHAHA experiments [17] were obtained using similar acquisition and processing conditions. A mixing time of 100 ms was used for both the HOHAHA and NOESY experiments. The NOESY spectrum [18] was obtained using a total of 600 data sets with 32 transients at 2048 data points each. The spectrum was obtained over a spectral width of 4329 Hz. The NMR sample of the polysaccharide was purged with He for 0.5 h before the NOESY experiment was performed. The water line was suppressed by presaturation. All 1D and 2D proton NMR spectra were recorded at 70°C and the proton chemical-shifts were referenced to the water line at 4.25 ppm. This was established using an external reference. The HMQC spectrum was recorded at 50°C and the ^{13}C and ^{13}C-DEPT spectra were recorded at room temperature. The carbon chemical shifts were referred to an external standard $CDCl_3$ signal at 77.0 ppm.

3. Results and discussion

GC and GC–MS analyses on the alditol acetate derivatives of the polysaccharide revealed the presence of 3 glycosyl components; rhamnose, *N*-acetylglucosamine, and *N*-acetylmannosamine in 3:1:1 molar ratio. Their absolute configurations were determined to be L-rhamanose, D-glucosamine, and D-mannosamine. The ^1H NMR spectrum of the polysaccharide (Fig. 1) showed five anomeric proton resonances in the region 4.7–5.2 ppm. The doublet at 4.74 ppm (*J* 8.0 Hz) was assigned to a β-anomeric proton of *N*-acetylglucosamine in the pyranose form. The large coupling constant arises from the $J_{1,2}$ axial–axial coupling. The other four anomeric proton signals were singlets, indicating that these residues had the α configuration. Three doublets (*J* 6.5 Hz) between 1.15 and 1.25 ppm were assigned to the 6-deoxy groups of three rhamnosyl residues. Two singlets at 2.00 and 2.04 ppm were assigned to the protons of *N*-acetyl groups that arise from *N*-acetylglucosamine and *N*-acetylmannosamine residues. Similar analysis on samples that were not subjected to Ac_2O–pyridine treatment were *N*-acetylated to the same extent. Signals between 3.2–4.6 ppm were assigned to the rest of the sugar protons.

The ^{13}C NMR spectrum of the polysaccharide (Fig. 2) showed 34 signals. Two of them (at 173.8 and 174.3 ppm) corresponded to carbonyl carbons. Five anomeric-carbon signals appeared in the region of 94–102 ppm. The ^{13}C-DEPT spectrum (Fig. 3) showed the presence of 5 methyl carbons, 2 methylene carbons,

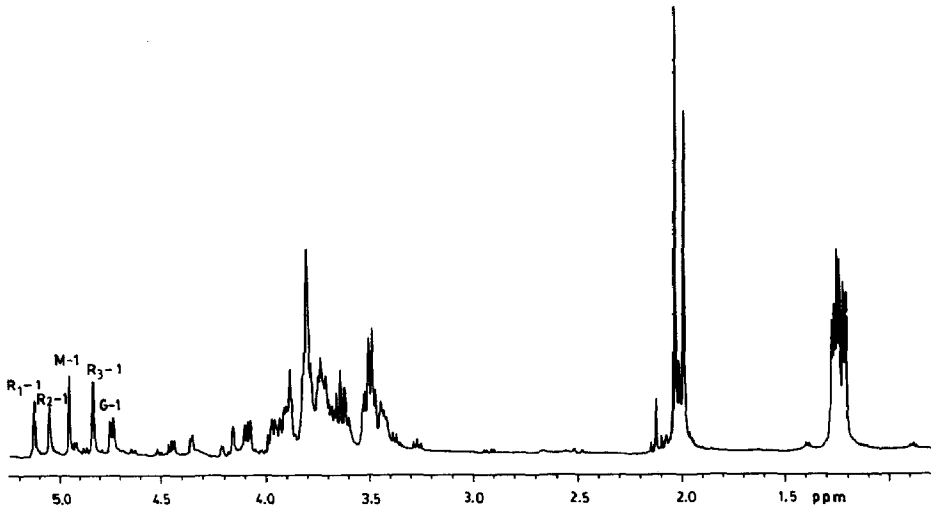

Fig. 1. ^{1}H NMR spectrum of the polysaccharide. There are five anomeric proton resonances between 4.7 and 5.2 ppm. Two singlets at 2.0 and 2.04 ppm arise from the *N*-acetyl groups of the N-acetylglucosamine and *N*-acetylmannosamine residues. Three doublets between 1.15 and 1.25 ppm are assigned to the 6-deoxy groups of the three rhamnosyl residues. Signals between 3.2 and 4.6 ppm arise from the remaining sugar protons. R_1-1 refers to the anomeric proton of the rhamnosyl residue 1. R_2-1 and R_3-1 refer to that of rhamnosyl residue 2 and 3, respectively. M-1 and G-1 refer to the anomeric proton signals of *N*-acetylmannosamine and *N*-acetylglucosamine, respectively.

and 25 methine carbons. Three methyl carbon signals at 16.0, 16.4, and 16.4 ppm were assigned to the three 6-deoxy carbons of rhamnosyl residues. Two methyl carbon signals at 21.6 and 21.8 ppm were assigned to the acetyl moieties of *N*-acetylglucosamine and *N*-acetylmannosamine. Two methine carbon signals, at 52.4 and 55.2 ppm, were assigned to the 2-positions of the amino sugars. The two methylene carbon signals at 60.0 and 60.6 ppm were assigned to the C-6 CH_2OH groups in *N*-acetylglucosamine and *N*-acetylmannosamine. Furthermore, the chemical shifts of these resonances indicated that these hydroxyl groups were

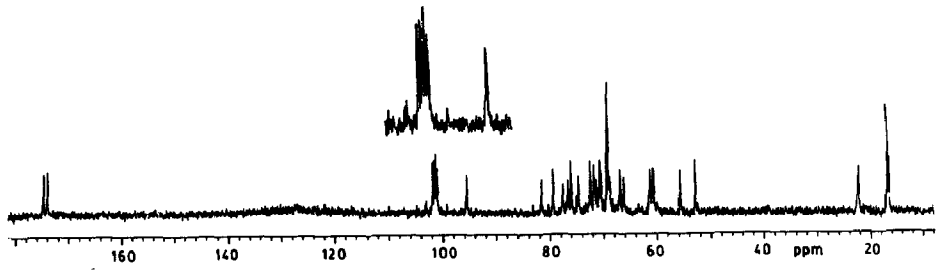

Fig. 2. ^{13}C NMR spectrum of the polysaccharide. The five anomeric-carbon signals appear in the region of 94–102 ppm. Two *N*-acetyl carbon signals are observed at 21.6 and 21.8 ppm. Three 6-deoxy carbon signals are present at 16 ppm. The five anomeric-carbon resonances are expanded in the insert.

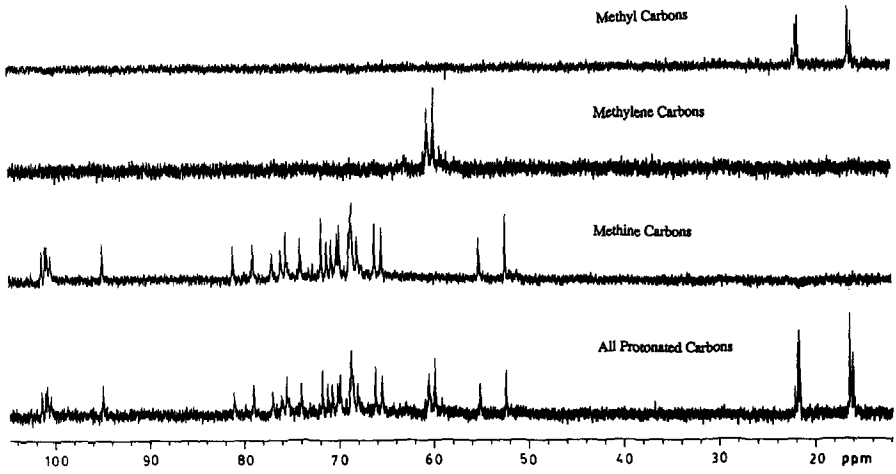

Fig. 3. ^{13}C DEPT spectrum of the polysaccharide. Note the two 6-hydroxymethyl carbons appear at 60.0 and 60.6 ppm. The C-2 resonances of the amino sugar residues are at 52.4 and 55.2 ppm.

unsubstituted. These 1D NMR results, combined with the GC data, suggested that the polysaccharide consisting of a pentasaccharide repeating-unit containing three rhamnosyl (Rha), one *N*-acetylglucosamine (GlcNAc), and one *N*-acetylmannosamine (ManNAc) residues.

The ^{1}H NMR spectrum agreed well with the ^{13}C NMR spectrum, as demonstrated by the $^{1}H-^{13}C$-HMQC NMR spectrum (Fig. 4). The five anomeric proton signals correlated with the five anomeric carbon signals. The proton signal at 4.75 ppm correlated with the ^{13}C signal at 101.5 ppm and was assigned to the anomeric proton of *N*-acetylglucosamine. The ^{13}C signal at 95.0 ppm was assigned to the anomeric carbon of *N*-acetylmannosamine. The $^{1}H-^{13}C$ correlated pair of signals at 4.95 and 95.0 ppm were thus assigned to the anomeric position of *N*-acetylmannosamine. The $^{1}H-^{13}$ correlated pair of signals at 5.13 and 101.1 ppm was assigned to the anomeric position of rhamnose (Rha1). The $^{1}H-^{13}C$ correlated pair of signals at 5.05 and 100.5 ppm was assigned to the anomeric position of another rhamnosyl residue (Rha2). The $^{1}H-^{13}C$ correlated pair of signals at 4.84 and 100.9 ppm was assigned to the other rhamnose moiety (Rha3) of the polysaccharide. The ^{13}C signals around 80 ppm were assigned to the carbons involved in substitutions. These are typically 10 ppm downfield of unsubstituted carbon signals. The five such substituted $^{1}H-^{13}C$ correlated signal pairs are as follows: 3.58 and 81.1 ppm, 3.95 and 79.1 ppm, 3.76 and 77.0 ppm, 4.22 and 76.1 ppm, and 3.42 and 75.5 ppm.

The proton signals belonging to one continuous spin system were traced from the proton HOHAHA spectrum (Fig. 5). The Rha1 anomeric-proton signal at 5.13 ppm was found to be connected to another signal at 4.16 ppm, past which there was no transfer because of the small H-1–H-2 coupling. This signal was thus assigned to H-2 of Rha1. Similarly, the ^{1}H signal at 4.22 ppm was assigned to H-2 of Rha2. This latter resonance was one that was found to be correlated with a

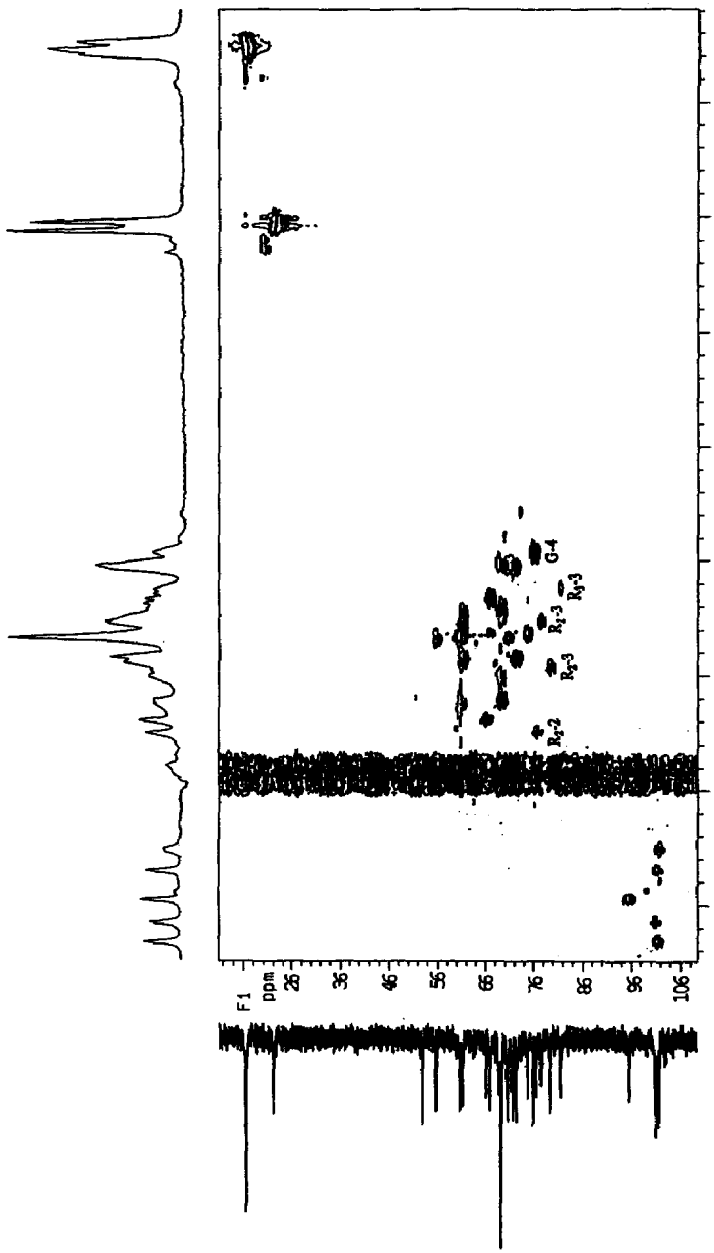

Fig. 4. $^1H-^{13}C$-HMQC spectrum of the polysaccharide. The proton signals agree well with the carbon signals. Note the assignment for the five cross-peaks corresponding to the substitution sites. R_2-2 refers to the C-2 of rhamnosyl residue 2, R_2-3 to C-3 of rhamnosyl residue 2, R_1-3 to C-3 rhamnosyl residue 1, R_3-3 to C-3 of rhamnosyl residue 3, and G-4 to C-4 of *N*-acetylglucosamine. Note that there is no such cross-peak for the *N*-acetylmannosamine residue.

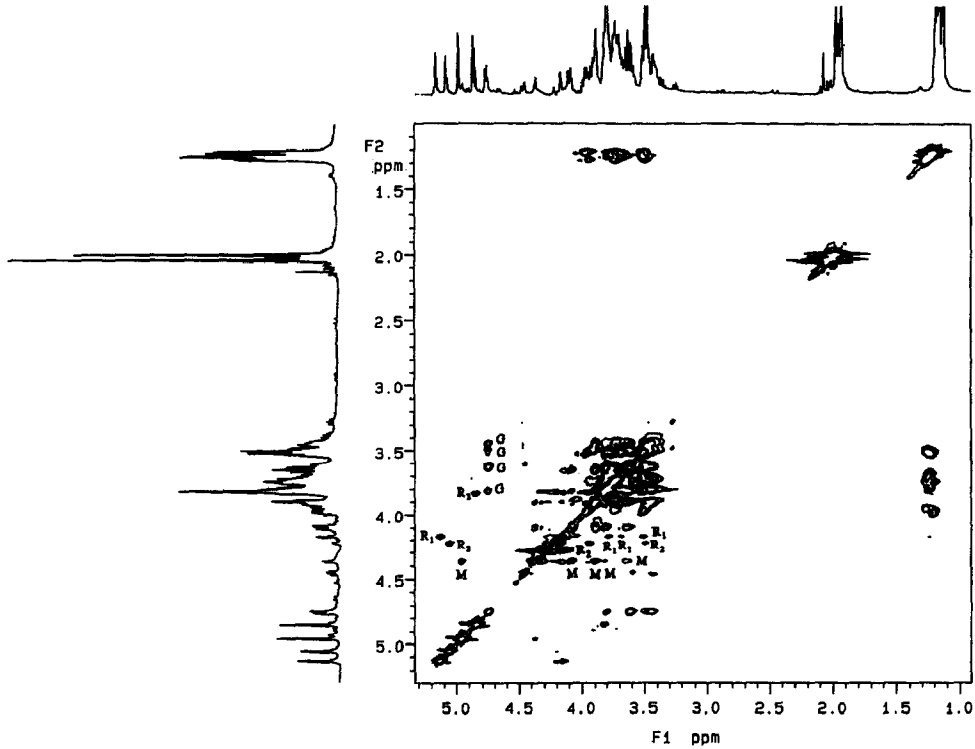

Fig. 5. HOHAHA spectrum of the polysaccharide. Note the connectivities for the amino sugar residues and rhamnosyl residues. The labels denote the glycosyl components as described in Fig. 1.

resonance assigned to a carbon involved in a linkage by the HMQC experiment. The O-2 of Rha2 was thus found to be linked to another residue. The signal at 3.86 ppm was assigned to H-2 of Rha3 since no spin transfer was observed beyond this resonance. The anomeric-proton signal of *N*-acetylglucosamine showed connectivities with four other signals, at 3.79, 3.62, 3.47, and 3.42 ppm. Further proton-signal assignments were accomplished through the spin connectivities in the DQF-COSY spectrum (Fig. 6). Of the resonances for the *N*-acetylglucosamine residue, the signal at 3.79 ppm was correlated with the H-1 signal in the DQF-COSY spectrum. This signal was, therefore, assigned to H-2. The H-2 signal was also correlated with the signal at 3.62 ppm. The latter signal at 3.62 ppm was, therefore, assigned to H-3. The hydroxymethyl signal at 3.89 and 3.74 ppm (traced from HMQC cross peaks corresponding to C-6 signal at 60.0 ppm), were correlated with the signal at 3.47 ppm. This latter resonance was thus assigned to H-5 of the *N*-acetylglucosamine residue. Finally, the signal at 3.42 ppm was assigned to H-4. This position was substituted, as indicated by its large ^{13}C chemical shift from the HMQC results. Thus all of the proton signals of *N*-acetylglucosamine residue were assigned. The proton-signal assignments from HOHAHA and DQF-COSY studies, combined with HMQC results, revealed that the *N*-acetylglucosamine residue was

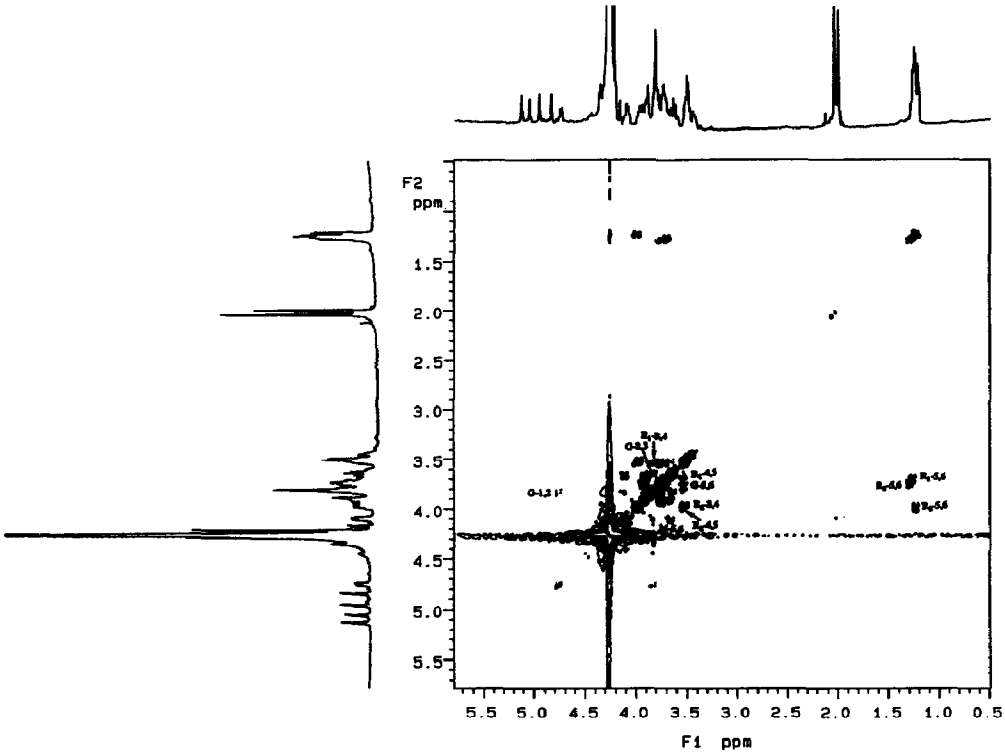

Fig. 6. DQF-COSY spectrum of the polysaccharide. Note the assignments for the various connectivities. For instance, G-1, 2 denotes the cross peak correlating the signals for H-1 and H-2 of *N*-acetylglucosamine.

substituted at the 4-position. Rha1 and Rha3 were linked at the 3-positions, and Rha2 was substituted at both the 2- and 3-positions. It was concluded that the *N*-acetylmannosamine was a side-chain substituent since it bore no substituents itself.

The foregoing substitution results were confirmed by two successive periodate oxidations. Gas-chromatographic analysis showed that the first periodate oxidation caused degradation of the *N*-acetylmannosamine residue, indicating that both the O-3 and O-4 positions were not substituted. As the O-6 was known to be unsubstituted (DEPT), the conclusion that *N*-acetylmannosamine was a side-chain substituent was thus confirmed. Further periodate oxidation of the first oxidation product did not cause any further degradation of the *N*-acetylglucosamine and rhamnosyl residues. This confirmed that *N*-acetylglucosamine was linked in the 4-position, and that the three rhamnosyl residues were 3-linked. This also confirmed that the *N*-acetylmannosamine was linked to the 2-position of the Rha2 residue. This conclusion could be reached because it was known from the HOHAHA and HMQC experiments that this position was substituted. The main-chain substituent must, therefore, be in the 3-position, rendering the branching residue

Table 1
The ^{1}H and ^{13}C chemical shifts of the polysaccharide. Note that the remaining unassigned ^{13}C signals are between 68.0–74.0 ppm

^{1}H (ppm)	H-1	H-2	H-3	H-4	H-5	H-6
Rha1	5.13	4.16	3.76	3.52	3.69	1.25
Rha2	5.05	4.22	3.95	3.49	3.97	1.25
Rha3	4.84	3.86	3.58	3.70		1.25
ManNAc	4.95	4.36		3.83	3.67	4.09, 3.90
GlcNAc	4.75	3.79	3.62	3.42	3.47	3.89, 3.74
^{13}C (ppm)	C-1	C-2	C-3	C-4	C-5	C-6
Rha1	101.1	65.9	77.0		68.7	16.0
Rha2	100.5	76.1	79.1		68.9	16.0
Rha3	100.9		81.1		68.8	16.0
ManNAc	95.0	52.4			68.1	60.0
GlcNAc	101.5	55.2		75.5		60.6

inert to further periodate oxidation after the side-chain *N*-acetylmannosamine residue had been removed.

The anomeric configurations and ring sizes of the rhamnosyl and *N*-acetylmannosamine residues deserved special attention. The ring sizes and positions of substitution were all consistent with the ^{13}C NMR chemical shifts of the ring carbon atoms. It is well established that ^{13}C furanose resonances are considerably downfield (often > 80 ppm) when compared to the corresponding pyranose resonances [19]. The values for the ^{13}C ring resonances are given in Table 1. It should be noted that the signals for the C-5 carbon atoms are all at ~ 68 ppm, the known value for these positions in the stated ring size and anomeric configurations [20]. Except for the anomeric carbons and the sites of substitution, none of the ring carbon resonances are downfield of 80 ppm.

The sequence of the polysaccharide was deduced by partial hydrolysis and 1D and 2D NOE experiments. The polysaccharide was partialy hydrolyzed under condition strong enough to cleave the anomeric linkages of the rhamnosyl residues but not the anomeric linkages of the acetamido sugar. The products were analyzed by FABMS. Disaccharides of GlcNAc-(1 → 3)-Rha or ManNAc-(1 → 2)-Rha were identified in the mass spectrum, but no trisaccharide species were detected. These results excluded the possible sequence of β-D-GlcNAc-(1 → 3)-α-L-Rha2-(2 → 1)-α-D-ManNAc (two acetamido residues linked to the same rhamnose residue). Two possible sequences of the repeating unit thus remained. These were: → 3)-α-L-Rha-(1 → 3)-(α-D-ManNAc-(1 → 2)-)-α-L-Rha2-(1 → 4)-β-D-GlcNAc-(1 → 3)-α-L-Rha-(1 → and → 4)-β-D-GlcNAc-(1 → 3)-α-L-Rha-(1 → 3)-[α-D-ManNAc-(1 → 2)-α-L-Rha2-(1 → 3)-α-L-Rha-(1 → . In the NOESY spectrum (Fig. 7), H-1 of the rhamnose 1 showed correlation with H-2 of Rha2 and the H-2 of itself. H-1 of the *N*-acetylmannosamine residue was observed to be spatially close to H-2 of the Rha1. This suggested the sequence: → 3)-α-L-Rha1-(1 → 3)-[α-D-ManNAc-(1 → 2)]-α-L-Rha2-(1 → . The anomeric proton of the *N*-acetylglucosamine showed an NOE effect with H-2 of Rha3, indicating the partial sequence: → 4)-β-D-GlcNAc-

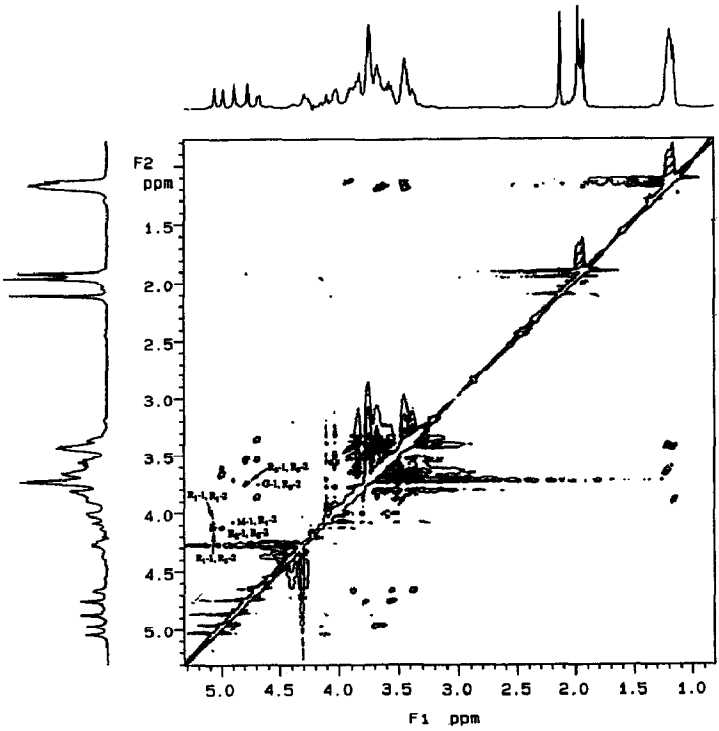

Fig. 7. NOESY spectrum of the polysaccharide. Note the correlation between each anomeric proton signal and other sugar proton signals.

(1 → 3)-α-L-Rha3-(1 → . In the 1D NOE (data not shown), the anomeric proton of *N*-acetylglucosamine showed an NOE with the *N*-acetyl protons of both *N*-acetylglucosamine and *N*-acetylmannosamine, thus indicating that these two amino sugar residues were close to each other. The structure of the repeating unit was therefore readily shown to be that in Fig. 8. The complete ^{1}H NMR assignments

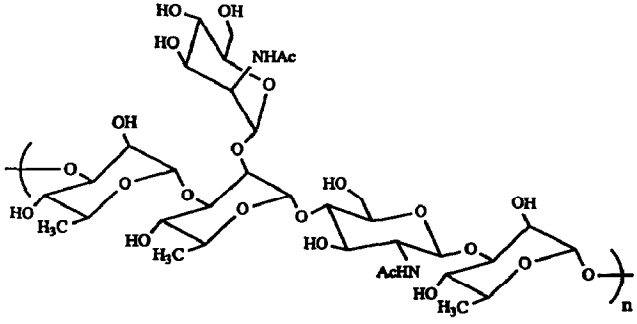

Fig. 8. Structure of the polysaccharide.

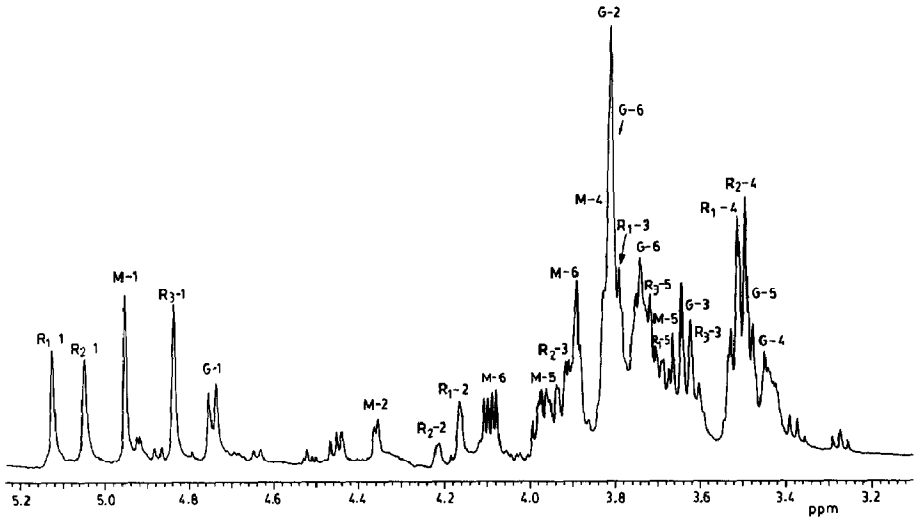

Fig. 9. Part of the ^{1}H NMR spectrum with detailed assignments.

for the carbohydrate ring-protons are given in Fig. 9. The ^{1}H and ^{13}C chemical shifts are listed in Table 1.

This study describes the complete structural characterization of the O-antigen component of the lipopolysaccharide of *Rhizobium trifolii* 4s. The structures of the capsular polysaccharide of this organism has already been determined [10]. The number of residues and their configurations were readily determined by GC, GC–MS, and NMR spectroscopy. The combination of DEPT, HOHAHA, and DQF-COSY spectroscopy allowed determination of the sites of linkages of the residues. The linkage positions were fully supported by periodate oxidation, which clearly demonstrated that the rhamnosyl residues were 3-linked and, therefore, had no vicinal diol functions that could be oxidized. The same experiment also confirmed that the *N*-acetylmannosamine residue was terminally linked as its primary hydroxyl group was unacetylated (DEPT) and the 3- and 4-positions were both free, since it was completely oxidized by periodate. Failure to remove any other residue in a second oxidation prove that the *N*-acetylmannosamine component was, in fact, the sole side-chain residue. Only one glycosyl component (a rhamnosyl residue) bore two substituent glycosyl residues (not counting the anomeric sites). The final proposed sequence was readily confirmed by partial hydrolysis and NOE measurements. The anomeric configurations were unequivocally established by the NMR chemical shifts and coupling constants of the anomeric protons.

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