Bacterial lipopolysaccharide from *Escherichia coli* O9 (O9 LPS) has various characteristic biological activities other than endotoxic activities. The biological activities exhibited depend on the structure of the O-antigen. The O-antigen region of O9 LPS is composed of the mannose homopolysaccharide (MHP). This structure was reported previously, but not all its proton and carbon signals were assigned. In the present study, we completely assign all proton and carbon signals of the O-antigen of O9 LPS using 1H- and 13C-NMR spectroscopy, including the DQF-COSY, TOCSY, NOESY, HSQC, H2BC, HSQC-TOCSY and HMBC methods.

Key words *Escherichia coli* O9; lipopolysaccharide; O-antigen; NMR; high mannose type

In the present study, we report on the structure of the O-antigen of O9 LPS, and completely assign all protons and carbons using 1D-, 2D-NMR spectroscopy including the DQF-COSY, TOCSY, NOESY, HSQC, H2BC, HSQC-TOCSY, and HMBC methods.

**Experimental**

**Bacterial Strain and Conditions for Growth** *E. coli* ATCC23505 was obtained from American Type Culture Collection (ATCC). The strain was grown in Luria Bertani (LB) medium.

**Preparation of LPS** O9 LPS was extracted from *E. coli* ATCC23505 (O9-K98-H12) by the phenol water method. The acetone-dried cells (10 g) cultured in LB medium were suspended in 175 ml of distilled water (preheated at 65 °C), and then 175 ml of 90% liquid phenol (preheated at 65 °C) was added under vigorous stirring and the mixture was stirred for 20 min at 65 °C. This suspension was then allowed to cool to 4 °C. After centrifugation, the upper aqueous layer was carefully collected. The residual layer was re-extracted by the methods described above. The combined aqueous phase was re-heated to 65 °C, an equal volume of 90% liquid phenol was added, and the mixture was incubated for 20 min under vigorous stirring. This water extract was dialyzed against distilled water. The dialyzed solution was concentrated at 40 °C in vacuo to a volume of about 50 ml. After centrifugation for the removal of traces of insoluble material, the water solution was lyophilized. The lyophilized crude extract was dissolved in water to give a 3% solution, which was centrifuged for 8 h at 105000g. The sediment was suspended in water, and the suspension was recentrifuged 3 times at 105000g for 3 h each. The final sediment was taken up in a minimum amount of water and lyophilized (yield 1.23%).

**Isolation of O-Antigen** The LPS was hydrolyzed with 1% H2OAc for 1 h at 100 °C, and a water-soluble carbohydrate portion was fractionated on a TOYOPEARL HW-65F (1.5×100 cm, equilibrated with water) column to give the O-specific polysaccharide.

**NMR Spectroscopy** Exchangeable protons were removed by dissolving the O-antigen in D,O and lyophilized. This exchange process was repeated three times. All NMR spectra were recorded in D2O at 308 K using a Bruker Avance 500 spectrometer equipped with a TXI xyz-three gradient probe for 1H detection or BBO z-gradient probe for 13C detection. Chemical shifts are reported in ppm relative to acetone-d6 as an internal standard (δH = 2.189 ppm, δC = 31.45 ppm). Data processing was performed using XWinNMR software. The 1D-1H experiment was performed using a Bruker standard pulse sequence with 1947 Hz in 64 K complex data points. The relaxation delay was used 5 T1, in order to calculate accurate signal integrations. Prior to Fourier Transformation, 4 times zero filling was used, and noise was reduced using the Trafication function. The 1D-13C (Power gated and Gated decoupling) experiments were performed using a Bruker standard pulse sequence with 30581 Hz in 64 K complex data points. Prior to Fourier Transformation, 4 times zero filling was used, and noise was reduced using

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Results and Discussion

A sugar composition analysis showed that the O-antigen from O9 LPS was mainly composed of mannose (data not shown). A 1D-$^1$H NMR spectrum of O-antigen from O9 LPS in D$_2$O at 35 °C is shown in Fig. 1. The anomeric region ($\delta_\text{H} 4.4—5.5$ ppm) of the spectrum contained five signals, two of which completely overlapped ($\delta_\text{H} 5.28$ ppm). This overlap of anomeric signals was confirmed in signal integrity and other NMR experiments, including a COSY experiment. The five sugar residues were arbitrarily labeled A, B, B', C, and D as described in Fig. 1. On the basis of their observed chemical shifts and $J_{\text{H1,C1}}$ obtained from a $^1$H, $^{13}$C-HSQC spectrum without decoupling during acquisition (data not shown), all residues were assigned as α-hexopyranosyl residues. Namely, residue A ($\delta_\text{H} 5.36$ ppm, $J_{\text{H1,C1}} = 172.9$ Hz), residues B and B’ ($\delta_\text{H} 5.28$ ppm, $J_{\text{H1,C1}} = 172.3$ Hz), residue C ($\delta_\text{H} 5.12$ ppm, $J_{\text{H1,C1}} = 171.3$ Hz) and residue D ($\delta_\text{H} 5.03$ ppm, $J_{\text{H1,C1}} = 171.4$ Hz) were assigned. The 1D-$^{13}$C NMR spectrum in Fig. 2 showed two signals in the anomeric region ($\delta_\text{C} 95—110$ ppm) which are assigned to residues A, B and B’ ($\delta_\text{C} 102.5$ ppm) and residues C and D ($\delta_\text{C} 103.9$ ppm), as confirmed from cross-peaks in the $^{13}$C-edited HSQC spectrum described later. An attempt was made to assign all $^1$H resonances in the $^1$H-NMR spectrum by means of the DQF-COSY and TOCSY methods. In the 2D-TOCSY spectrum (Fig. 3), a series of cross-peaks between A, B, B', C, and D H-1 ($\delta_\text{H} 5.36$, 5.28, 5.28, 5.12, and 5.03 ppm, respectively) and H-2, 3, 4, and 5 were observed. These assignments were also examined using the various mixing times of 1D-TOCSY experiments (data not shown). However, $^1$H resonances of the H-6 posi-
tions of residues A, B, B’, and D, and the H-5 and H-6 positions of residue C could not be assigned. Therefore, H-6 positions of all residues were assigned using the 13C-edited HSQC and H2BC methods. In addition, the 13C-edited HSQC spectrum combined with the H2BC experiment allowed the complete assignment of the 1H resonances and corresponding 13C resonances (Fig. 4). Figure 5 shows an excerpt form the overlaid HSQC (positive peaks plotted in green, negative peaks plotted in red) and H2BC (plotted in blue) spectra showing how the entire intra-ring assignment of a residue D sugar unit from C1,H1 to C6,H6 can be traced out. This kind of assignment hinges on the two-bond correlation exclusivity of H2BC that is the key feature of the technique. Similar unequivocal assignments can be obtained with an INADEQUATE experiment which is not feasible in this study due to its inherently low sensitivity. Table 1 summarizes the 1H- and 13C-NMR spectral assignments of the O-antigen from O9 LPS. These assignments are based on DQF-COSY, TOCSY, NOESY, 13C-edited HSQC, H2BC and HMBC spectra.

Downfield displacement of the signal for C-3 of residues C and D and for C-2 of residues A, B, and B’ to δC 79—81 ppm, as compared with their positions in non-substituted α-mannopyranose, revealed the glycosylation pattern of the polysaccharide. The NOE experiments obtained from 1D-, 2D-NOESY (data not shown) showed correlations between the following transglycosidic protons: A H-1/C H-3, C H-1/D H-3, D H-1/B H-2, and A H-2/B’ H-1 at δH 5.36/3.99, 5.12/3.93, 5.03/4.10, and 4.07/5.28, respectively, and hence, an B’ → A → C → D → B fragment was present. Interresidue correlations for the one remaining H-1 proton could not be reliably assigned because of the closeness of the signals for
H-1 of residues B and B’ at δH 5.28 ppm to the signals for H-2 of residues B and B’ near δH 4.1 ppm. These results were also confirmed by the 1H, 13C-HMBC experiment (data not shown). Taking into account the linear nature of the polysaccharide, these data are sufficient for determination of the full monosaccharide sequence in the repeating unit. Therefore, the O-antigen polysaccharide of *E. coli* ATCC 23505 is a linear α-D-mannan having the structure described in Fig. 6.

These assignments of the anomeric 1H and the all 13C signals are identical to the previous reports by Parolis *et al.* and Ogawa *et al.* However, all 1H assignments except anomeric 1H are novel results obtained from recently developed NMR techniques. This information about all 1H chemical shifts is useful for investigating the O-antigen-host receptor interaction using NMR experiments because of these experiments are almost using 1H detected, e.g. transferred intramolecular nuclear Overhauser effects and saturation transfer difference technique.

In this study, we demonstrated that a H2BC experiment is useful for assigning polysaccharides, especially those containing mannose residues because of their small coupling constant, 1JH,H2. This structural information can shed light on the molecular mechanisms, for example carbohydrate ligand-protein receptor interaction analysis using NMR spectroscopy, of anaphylactoid shock induced by not only O9 LPS from Gram-negative bacteria but also *Candida* cell wall mannann from fungi.

### References


**Tables**

**Table 1. 1H and 13C Assignment of O-Antigen from O9 LPS (δ in ppm)**

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<tr>
<th>Sugar residue</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6a</th>
<th>6b</th>
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**Fig. 6. Chemical Structure of O-Antigen Polysaccharide from O9 LPS**