An Unambiguous Assignment and Structural Analysis Using Solution NMR Experiments of O-Antigen from *Escherichia coli* ATCC23505 (Serotype O9)

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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 ^{1}H- and ^{13}C-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

Bacterial lipopolysaccharides (LPSs) are major surface-exposed structural components of the outer membrane of Gram-negative bacteria. LPSs consist of lipid A, a core region, and an O-antigen region in many bacteria.[1)] LPSs play an important role in the pathogenicity of Gram-negative infections. In experimental animals, purified LPSs induce numerous pathophysiological activities that can lead to shock and death, a condition called "Sepsis".[2)] Most of these biological activities, including the induction of inflammatory cytokines, are expressed by the lipid A moiety, a ubiquitous component of LPSs that is structurally similar and serologically cross-reacting among Enterobacteriaceae, *via* toll-like receptor 4 (TLR4).[3—5)]

However, numerous recent studies have revealed that LPSs possessing the mannose homopolymer (MHP) as the O-antigen region, such as the LPS from *Escherichia coli* (*E. coli*) O9 (O9 LPS), exhibit several characteristic immunological activities differ from those of the well-known endotoxic shock. In addition, we previously described that the mannan moieties of *Candida albicans* water-soluble fraction, CAWS, from fungi shows similar immunological properties to Gram-negative bacterial O9 LPS.[6)] These characteristic activities should be exhibited by recognizing mannose residues *via* lectins, such as mannose-binding lectin (MBL).[7,8)] Therefore, analyzing the structure of O-antigens, especially mannose-rich regions, is important for understanding some of the biological activities of LPSs.

The O-antigen structure of O9 LPS has been studied using methylation analysis, smith degradation, optical rotation and enzymatic methods,[9—11)] however, a precise assignment by NMR spectroscopy has not been conducted.

In general, focusing on the anomeric proton as the starting point for assignment, TOCSY and HSQC-TOCSY experiments are useful for the analysis of individual sugar components in polysaccharides.[12)] However, in some cases, the assignment can be empirically based due to signals that heavily overlap and small *J* coupling constants. For the reasons mentioned above, we attempted to apply recently developed pulse sequences, the heteronuclear 2-bond correlation (H2BC) experiments[13—15)] in order to achieve an unambiguous assignment of the high mannose type polysaccharide, the O-antigen from O9 LPS.

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:K9B:H12) by the phenol water method.[16)] 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 reheated 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 105000 ***g***. The sediment was suspended in water, and the suspension was recentrifuged 3 times at 105000 ***g*** 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% HOAc 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) to give the O-specific polysaccharide.

NMR Spectroscopy Exchangeable protons were removed by dissolving the O-antigen in D_2O, and lyophilized. This exchange process was repeated three times. All NMR spectra were recorded in D_2O at 308 K using a Bruker Avance 500 spectrometer equipped with a TXI *xyz*-three gradient probe for ^{1}H detection or BBO *z*-gradient probe for ^{13}C detection. Chemical shifts are reported in ppm relative to acetone-d_6 as an internal standard (δ_H=2.189 ppm, δ_C=31.45 ppm). Data processing was performed using XWinNMR software. The 1D-^{1}H experiment was performed using a Bruker standard pulse sequence with 1947 Hz in 64 K complex data points. The relaxation delay was used $5T_1$ 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-^{13}C (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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exponential multiplication. A 1D-TOCSY spectrum was recorded with various mixing times using the Bruker standard pulse sequence with 1947 Hz in 64 K complex data points. Prior to Fourier Transformation, 4 times zero filling was used, and noise was reduced using exponential multiplication. 1D-NOESY was performed using the Bruker standard pulse sequence with 1947 Hz in 64 K complex data points. The mixing time for NOE growth was 200 ms. Prior to Fourier Transformation, 4 times zero filling was used, and noise was reduced using exponential multiplication. The shape of the selective excitation pulse for 1D-TOCSY and 1D-NOESY was Gaussian, and the duration of the pulse was set to 80 ms for all signals. Two-dimensional ^{1}H, ^{1}H-double quantum filtered correlation spectroscopy (DQF-COSY) was performed with 512 increments of 4096 data points with 32 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 2694 Hz in each dimension. Two-dimensional total correlation spectroscopy (TOCSY) was conducted with a mixing time for TOCSY spinlock of 210 ms using the pulse sequence of Griesinger *et al.* to suppress ROE signals.[17] The spectral width was 4006 Hz in each dimension and 512 increments of 4096 data points with 16 scans per t_1 increment were recorded. Two-dimensional nuclear overhauser effect spectroscopy (NOESY) was carried out with a mixing time of 200 ms using the Bruker standard pulse sequence. The spectral width was 4006 Hz in each dimension and 512 increments of 4096 data points with 48 scans per t_1 increment were recorded. Two-dimensional ^{13}C-edited hetero nuclear single quantum coherence spectroscopy (HSQC) was conducted with 512 increments of 2048 data points with 32 scans per t_1 increment using the Bruker standard pulse sequence. The spectral width was 3501 Hz for t_2 and 12500 Hz for t_1. Two-dimensional ^{1}H, ^{13}C-hetero nuclear multiple bond coherence spectroscopy (HMBC) was performed with 256 increments of 2048 data points with 132 scans per t_1 increment using the Bruker standard pulse sequence. The delay time for evolution long range coupling was set to 62.5 ms (optimized for 8 Hz). The spectral width was 3501 Hz for t_2 and 12500 Hz for t_1. Two-dimensional hetero nuclear 2-bond correlation spectroscopy (H2BC) was recorded with 512 increments of 2048 data points with 128 scans per t_1 increment using the pulse sequence of Nyberg *et al.*[13—15] The spectral width was 5000 Hz for t_2 and 26000 Hz for t_1. All 2D experiments were zero-filled to 2k and 2k in both dimensions prior to Fourier transformation. A square sine-bell window function was applied in both dimensions, except in the H2BC experiment. The H2BC experiment was treated with a cosine window function in t_1 and a $\pi/4$ shifted sine in t_2.

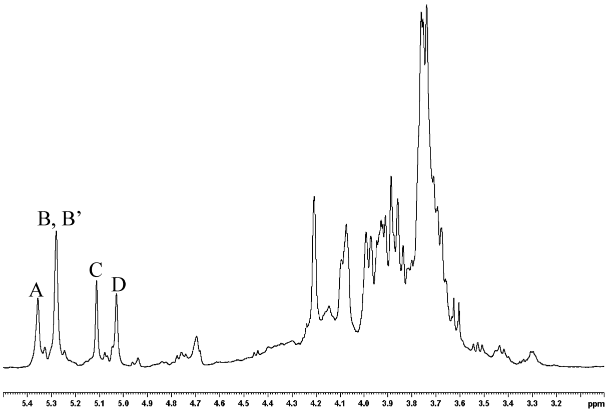


Fig. 1. 1D-^{1}H NMR Spectrum of O-Antigen from O9 LPS (20 mg/ml in D_2O) at 35 °C

The anomeric region (δ_H 4.4—5.5 ppm) of the spectrum contained five signals. These five sugar residues were arbitrarily labeled A, B, B′, C, and D as described in the figure.

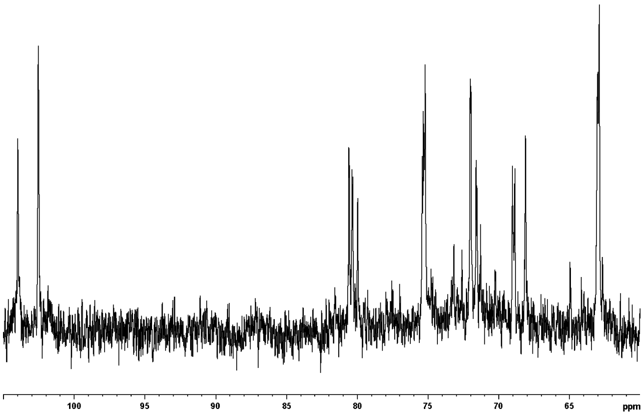


Fig. 2. Power-Gated 1D-^{13}C NMR Spectrum of O-Antigen from O9 LPS (20 mg/ml in D_2O) at 35 °C

The anomeric region (δ_C 95—110 ppm) of the spectrum contained two signals.

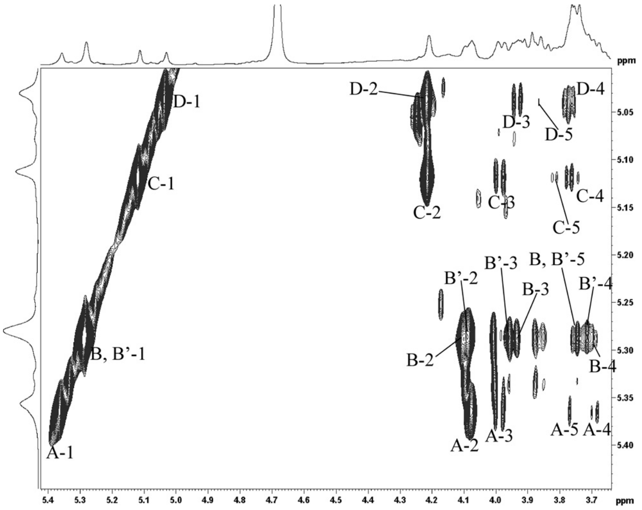


Fig. 3. Anomeric Region of the 2D-TOCSY Spectrum of O-Antigen from O9 LPS (20 mg/ml in D_2O) at 35 °C

A mixing time for TOCSY spinlock of 210 ms was used.

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_2O at 35 °C is shown in Fig. 1. The anomeric region (δ_H 4.4—5.5 ppm) of the spectrum contained five signals, two of which completely overlapped (δ_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 $^1J_{H1,C1}$ 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 (δ_H 5.36 ppm, $^1J_{H1,C1}=172.9$ Hz), residues B and B′ (δ_H 5.28 ppm, $^1J_{H1,C1}=172.3$ Hz), residue C (δ_H 5.12 ppm, $^1J_{H1,C1}=171.3$ Hz) and residue D (δ_H 5.03 ppm, $^1J_{H1,C1}=171.4$ Hz) were assigned. The 1D-^{13}C NMR spectrum in Fig. 2 showed two signals in the anomeric region (δ_C 95—110 ppm) which are assigned to residues A, B and B′ (δ_C 102.5 ppm) and residues C and D (δ_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 (δ_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-

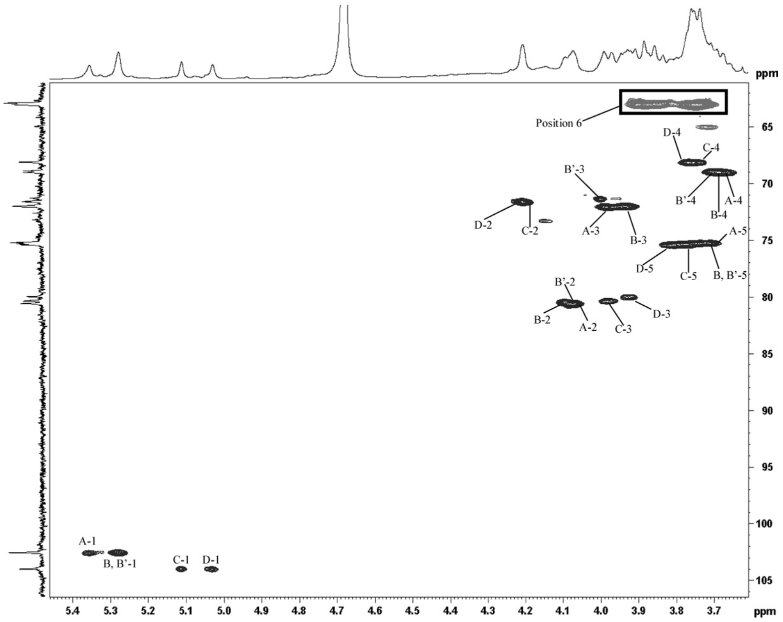


Fig. 4. ^{13}C-Edited HSQC Spectrum of O-Antigen from O9 LPS (20 mg/ml in D_2O) at 35 °C

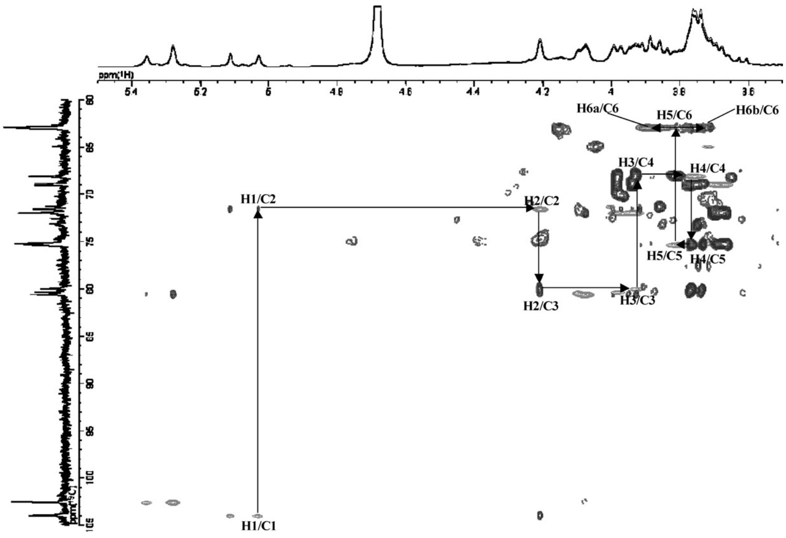


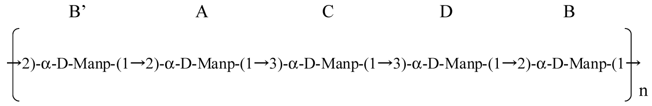
Fig. 5. Example of the Assignment of the Complete Spin System for a Residue D Based on the Overlaid ^{13}C-Edited HSQC (Positive Peaks Plotted in Green, Negative Peaks Plotted in Red) and H2BC (Plotted in Blue) Spectra Starting from the Anomeric Proton/Carbon 5.03 ppm/103.9 ppm Correlation in the HSQC Spectrum *via* H2BC to H2/C2 4.21 ppm/71.55 ppm and all the Way to Position 6

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 ^{13}C-edited HSQC and H2BC methods. In addition, the ^{13}C-edited HSQC spectrum combined with the H2BC experiment allowed the complete assignment of the ^{1}H resonances and corresponding ^{13}C 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 ^{1}H- and ^{13}C-NMR spectral assignments of the O-antigen from O9 LPS. These assignments are based on DQF-COSY, TOCSY, NOESY, ^{13}C-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 δ_C 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 δ_H 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

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

Sugar residue	^{1}H/^{13}C						
	1	2	3	4	5	6a	6b
A	5.36	4.07	3.99	3.68	3.77	3.90	3.78
	102.5	80.60	72.02	69.03	75.28	62.90	
B	5.28	4.10	3.94	3.70	3.75	3.90	3.78
	102.5	80.37	71.97	68.87	75.41	62.90	
B′	5.28	4.08	3.96	3.73	3.75	3.90	3.78
	102.5	80.56	71.29	68.87	75.41	62.90	
C	5.12	4.21	3.99	3.74	3.82	3.93	3.78
	103.9	71.61	80.32	68.11	75.20	63.01	
D	5.03	4.21	3.93	3.77	3.84	3.93	3.75
	103.9	71.55	79.97	68.11	75.34	63.01	



$$\left[\overset{\text{B'}}{\rightarrow 2)\text{-}\alpha\text{-D-Man}p}\text{-}(1\rightarrow \overset{\text{A}}{2)\text{-}\alpha\text{-D-Man}p}\text{-}(1\rightarrow \overset{\text{C}}{3)\text{-}\alpha\text{-D-Man}p}\text{-}(1\rightarrow \overset{\text{D}}{3)\text{-}\alpha\text{-D-Man}p}\text{-}(1\rightarrow \overset{\text{B}}{2)\text{-}\alpha\text{-D-Man}p}\text{-}(1\rightarrow\right]_n$$

Fig. 6. Chemical Structure of O-Antigen Polysaccharide from O9 LPS

H-1 of residues B and B′ at δ_H 5.28 ppm to the signals for H-2 of residues B and B′ near δ_H 4.1 ppm. These results were also confirmed by the ^{1}H, ^{13}C-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 ^{1}H and the all ^{13}C signals are identical to the previous reports by Parolis *et al.* and Ogawa *et al.*[10,11] However, all ^{1}H assignments except anomeric ^{1}H are novel results obtained from recently developed NMR techniques. This information about all ^{1}H chemical shifts is useful for investigating the O-antigen-host receptor interaction using NMR experiments because of these experiments are almost using ^{1}H 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, $^{1}J_{H1,H2}$. 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 mannan from fungi.

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