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Occurrence of [→3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→]$_n$ units in the antigenic polysaccharides from *Leptospira biflexa* serovar patoc strain Patoc I

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Abstract

In this study, we isolated three kinds of antigenic polysaccharide components (tentatively designed as AP-1 ~ 3) from cells of *Leptospira biflexa* serovar patoc strain Patoc I (*L. biflexa* patoc Patoc I) by the hot phenol–water procedure, followed by treatment with mild acid and column chromatography. Two of them (AP-1 and AP-2) were recovered from the phenol-soluble fraction whereas another (AP-3) was recovered from the aqueous fraction. All of them reacted toward an anti-*L. biflexa* serum and also cross-reacted in similar extents toward most of the other leptospiral antisera tested. Such immunoreactions were specifically inhibited by a β-(1→4)-linked mannobiose, but were not by any mono- and oligosaccharide tested. From their structural analyses including ^{1}H and ^{13}C NMR spectrometry, Smith degradation and methylation analysis, it was revealed that all of these antigenic polysaccharides had the same disaccharide unit →3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→ in their major polysaccharide parts, but they differed in the acyl substituents. Therefore it is most likely that such mannobiose unit is a candidate for the antigenic epitopes of *L. biflexa* polysaccharides.

Keywords: *Leptospira biflexa*; Genus-specific antigen; Antigenic polysaccharide; Leptospiral lipopolysaccharide

1. Introduction

Leptospires, helically coiled Gram-negative bacteria, are causative agents of leptospirosis, which is an acute and febrile illness. This disease is still a problem in developing countries and regions. Several serological methods such as the microscopic agglutination test (MAT) [1], ELISA (specific for leptospirosis) [2,3] and the indirect fluorescent-antibody test [4] have been reported to be useful for the detection of anti-*Leptospira* antibodies in sera from patients infected with leptospirosis and the diagnosis of leptospirosis. More recently, on the basis of findings that a variety of sera from leptospirosis patients are cross-reactable with antigens from non-pathogenic *Leptospira biflexa* patoc Patoc I [5,6], a much more convenient LEPTO dipstick assay has been devel-

Abbreviations: HPAEC-PAD, high-performance anion-exchange chromatography-pulsed amperometric detection; COSY, homonuclear shift correlation spectroscopy; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; HMQC, heteronuclear multiple-quantum coherence spectroscopy; ELISA, enzyme-linked immunosorbent assay.

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oped by using antigens bound to nitrocellulose membrane [7,8]. Such heat-stable antigens are presumably candidates for leptospiral genus-specific antigens. There are several reports on the isolation and occurrence of genus-specific antigens including LPS-like components from *L. biflexa* [9,10] and leptospiral polysaccharide antigens from a variety of leptospires [11–14], however, the structural characterization remains to be completed.

This paper describes the isolation and structural analysis of three antigenic polysaccharide components derived from *L. biflexa* patoc Patoc I.

2. Results and discussion

Isolation and sugar composition of antigenic polysaccharides.—The hot phenol–water method [15] is often applied to the isolation of antigenic components from leptospires although the antigenic components have been reported to be recovered variously in the aqueous or phenol-soluble fraction or both, depending on strains of leptospires used or culture conditions [14,16–18]. In this study after the same extraction, dialysis and removal of proteinaceous and nucleic acid components, both the phenol-soluble and aqueous fractions from *L. biflexa* patoc Patoc I were checked for their antigenicity by ELISA using an anti-*L. biflexa* serum. Chemical composition and immunogenicity of materials obtained on the purification steps are summarized in Table 1. From the defatted cells (10 g) immunoreactive and hexose-containing components were recovered in the phenol-soluble (60 mg) and aqueous fractions (45 mg). SDS-PAGE of the phenol-soluble fraction showed a ladder pattern typical for LPSs, although that of the aqueous fractions did not show any silver-stain positive bands. In contrast, chemical composition analysis revealed that both fractions did not contain typical components of LPSs such as heptose and Kdo in significant amounts, suggesting the occurrence of hexose-containing materials in a form different from typical enterobacterial LPSs. After treatment with mild acid, followed by gel chromatography of the resulting water-soluble components on Sephadex G-50, the phenol-soluble fraction gave two hexose-containing components (tentatively designated as antigenic polysaccharides, APs), which were recovered in the elution positions corresponding to molecular sizes of >10 kDa (AP-1, 13 mg) and 5–6 kDa (AP-2, 6 mg). Both showed strong immunoreactivities toward anti-*L. biflexa* serum in ELISA (Table 1). Therefore AP-1 and AP-2 were further investigated after purification by successive chromatography on DEAE-Sepharose and CM-Sephadex. On the other hand, the aqueous fraction gave three hexose-containing components [A-1 (>10 kDa, 2 mg), A-2 (5–6

Table 1
Chemical composition and immunogenicity of materials obtained from *L. biflexa* patoc Patoc I on purification steps

Preparation	Hexose (nmol mg^{-1})	Hexosamine (nmol mg^{-1})	Phosphorus (nmol mg^{-1})	Protein (nmol mg^{-1})	Fatty acyl [a]	Antigenicity [c]
Phenol-soluble fraction	2470	150	90	5	nd [b]	0.653
Aqueous fraction	1550	290	12	5	nd	0.520
AP-1	3100	250	80	2	large	0.872
AP-2	4900	380	<2	<0.1	negligible	0.830
A-1	3660	1200	<2	12	nd	0.076
AP-3 (A-2)	4800	370	<2	<0.1	negligible	0.805
A-3	2010	390	5	<0.1	nd	0.030
D-AP-1	4860	350	<2	<0.1	nd	0.786

[a] Based on the integral values of proton signals ranging within 0.8–1.8 ppm (characteristic for acyl groups) in ^{1}H NMR spectra of APs.

[b] Not determined.

[c] Average absorbance from three separated ELISA data.

kDa, 7 mg) and A-3 (1–2 kDa, about 20 mg)] upon gel chromatography of Sephadex G-50. Since A-2 alone exhibited strong antigenicity (Table 1), it was further purified by successive chromatography on DEAE-Sepharose and CM-Sephadex and tentatively designated as AP-3 (about 5 mg).

AP-2 and AP-3 showed nearly same contents of sugar and phosphorus and were free from protein (Table 1). AP-1 showed lower contents of sugars and higher contents of phosphorus and protein, in addition to the presence of large amounts of acyl residues. Acid hydrolyzates of AP-1, AP-2 and AP-3 were shown to contain mannose (Man), glucose (Glc), galactose (Gal) and glucosamine (GlcN) in nearly similar molar ratios (about 27:3:1:3) by analysis with HPAEC-PAD (without derivatization) and GLC (as their alditol acetates), suggesting the similarity of their polysaccharide structures. In addition, the configuration of mannose was determined to be in D-stereoisomer by GLC analysis after conversion of the isolated mannose component into peracetylated (+)-2-octyl-mannoside.

Immunoreactions of antigenic polysaccharides with antisera against L. biflexa *and other leptospiral strains.*—As shown Table 1, AP-1, AP-2 and AP-3 exhibited strong immunoreactivities toward anti-*L. biflexa* serum in ELISA. To find out which structures are involved in the immunoreactions and whether a variety of rabbit antisera against other leptospira strains can react with these APs, competitive and cross-reaction assays were carried out. In competitive ELISA, the immunoreaction between AP-2 and anti-*L. biflexa* serum was not inhibited by either monosaccharide relevant to APs (Man, Glc, Gal or GlcNAc) or irrelevant (GalNAc, ManNAc or GlcN) or several oligosaccharides [α-(1→4)-mannobiose, α-(1→3)-mannobiose, maltose, cellobiose, lactose, melibiose or chitobiose], even at high concentrations (above 200 μg mL^{-1}, the respective absorbance showing more than 0.550; without inhibitor, 0.550). On the contrary, the immunoreaction was specifically inhibited by β-(1→4)-mannobiose [50% inhibition, at 1 μg mL^{-1}; full inhibition, at 10 μg mL^{-1} (the absorbance, 0.040 or less)]. Therefore, it is most likely that the anti-*L. biflexa* serum preparation contains, at least, an antibody specific for a β-D-Man*p*-(1→4)-D-Man*p* unit.

In previous reports [4–9], a large number of antisera from leptospirosis patients were shown to cross-react with the antigens prepared from *L. biflexa*, suggesting a possibility of occurrence of common antigenic materials among *Leptospira*. Thus, cross-reactivity of AP-2 against various leptospira strains was confirmed by using rabbit antisera against different leptospires. The cross-reaction assay revealed that almost 85% of rabbit anti-sera raised against whole cells of leptospira strains were also reactive to AP-2 (Table 2), regardless of their serovars or serogroups. Leptospires belonging to serogroups Australis, Pomona and Sejroe did not exhibit any cross-reactivity with AP-2. It was reported that antisera against these serogroups also cross-reacted with the antigen prepared from *L. biflexa* [4,7,9]. Thus, it needs to reconfirm whether there is no cross-reactivity between these serogroups and AP-2 to be true using more serum samples. The cross-reactions were also specifically inhibited by β-(1→4)-mannobiose. Similar results were obtained for AP-1 and AP-3 (data not shown). These results suggest that a β-D-Man*p*-(1→4)-D-Man*p* unit may be present as one of the antigenic epitopes in AP-2 as well as in AP-1 and AP-3 and that cell surface components of most leptospira strains tested may contain such antigenic epitope or a closely related one.

Structural characterization of AP-2.—The ^1H NMR spectra of AP-1, AP-2 and AP-3 (Fig. 1) were nearly identical to each other, except that the spectrum of AP-1 showed additional signals between 0.8 and 1.8 ppm characteristic of methyl (0.8 ~ 1.0 ppm) and methylene protons (1.1 ~ 1.8 ppm) of fatty acyl residues. Fatty acyl analysis of AP-1 with GLC revealed the presence of hexadecanoic acids as a major acyl residue (see below). The similarities in the sugar compositions and proton signal patterns among AP-1, AP-2 and AP-3 strongly suggest that they may have main mannose-containing sequences with the same or similar structures and minor sugar components, Glc, Gal and GlcNAc. Further structural studies were performed on AP-2.

Table 2
Cross-reactivity of AP-2 with various rabbit antisera to leptospira strains [a]

Leptospira	Serogroup	Cross-reactivity [c]
L. biflexa patoc Patoc I	Semaranga	+[1.612]
L. interrogans autumnalis Akiyami A	Autumnalis	+[1.702]
L. interrogans australis Ballico	Australis	−[0.152]
L. interrogans canicola Galtoni	Canicola	+[0.380]
L. interrogans canicola HondUtrecht IV	Canicola	−[0.125]
L. interrogans canicola Malaya	Canicola	+[0.686]
L. interrogans canicola Moulton	Canicola	+[1.437]
L. interrogans copenhageni Shibaura	Icterohaemorrhagiae	+[0.287]
L. interrogans hebdomadis Hebdomadis	Hebdomadis	+[1.524]
L. interrogans icterohaemorrhagiae Okinawa	Icterohaemorrhagiae	+[0.254]
L. interrogans icterohaemorrhagiae CF-1	Icterohaemorrhagiae	+[1.237]
L. interrogans icterohaemorrhagiae RGA	Icterohaemorrhagiae	+[0.344]
L. interrogans kremastos Kyoto	Hebdomadis	+[0.532]
L. interrogans naam Naam	Icterohaemorrhagiae	+[1.247]
L. interrogans pomona Pomona	Pomona	−[0.195]
L. interrogans smithi Smith	Icterohaemorrhagiae	+[0.763]
L. interrogans wolffi 3705	Sejroe	−[0.161]
L. kirschneri kabura Kabura	Hebdomadis	+[1.473]
L. kirschneri kambale Kambale	Hebdomadis	+[1.557]
L. santarosai beye Beye	Mini	+[1.078]
L. santarosai borincana Borincana	Hebdomadis	+[1.557]
L. santarosai maru Maru	Hebdomadis	+[1.612]
L. weilii samin Sarmin	Sarmin	+[0.289]
L. borgpetersenii worsfoldi (*L. weilii*) Worsford	Hebdomadis	+[0.596]
jules Jules [b]	Hebdomadis	+[0.478]
ndahmbukuje Ndahmbukuje [b]	Icterohaemorrhagiae	+[0.798]
nona Nona [b]	Hebdomadis	+[1.423]
perameles Perameles [b]	Mini	+[1.215]
Control		[0.088]

[a] Absorbance is shown in square brackets.
[b] Species are unclassified or not determined [30].
[c] Samples that gave absorbance values higher than twice that of the control value were judged to be positive antisera (+).

Methylation analysis of AP-2 showed equimolar amounts of 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylmannitol and 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylmannitol from 3- and 4-*O*-substituted D-mannopyranose, respectively.

The Smith degradation product of AP-2 was shown to be 2-*O*-D-mannopyranosyl-D-erythritol. Thus, it is concluded that the main part of AP-2 consists of 3- and 4-substituted mannose residues and has the repeating unit →3)-D-Man*p*-(1→4)-D-Man*p*-(1→. This conclusion was also supported by assignment of the ^{1}H and ^{13}C NMR spectra for AP-2 (Fig. 2). From a comparison of the ^{13}C NMR spectral data reported for mannan and manno-oligosaccharides [19–21], all the anomeric and other signals in the ^{13}C NMR spectra were assigned as shown in Table 3. Complete signal assignment of the ^{1}H NMR spectrum (Table 3) was also carried out in combination with the spectral data of the COSY, HOHAHA (Fig. 3) and HMQC (Fig. 4) techniques. The chemical shifts for two anomeric protons (4.72 and 4.85 ppm) and carbons (98.7 and 101.7 ppm) and $J_{H\text{-}1,\ C\text{-}1}$ values (160 and 161 Hz) were consistent with for β-mannoside [20–25], suggesting that all the mannose residues are linked through β-configuration. From the results of chemical analysis and NMR spectroscopy, the main structural part of AP-2 is concluded to have a repeating disaccharide →3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→, which may also be included in the main structure parts of AP-1 and AP-3. The presence of this proposed unit in AP-2 as well as in AP-1

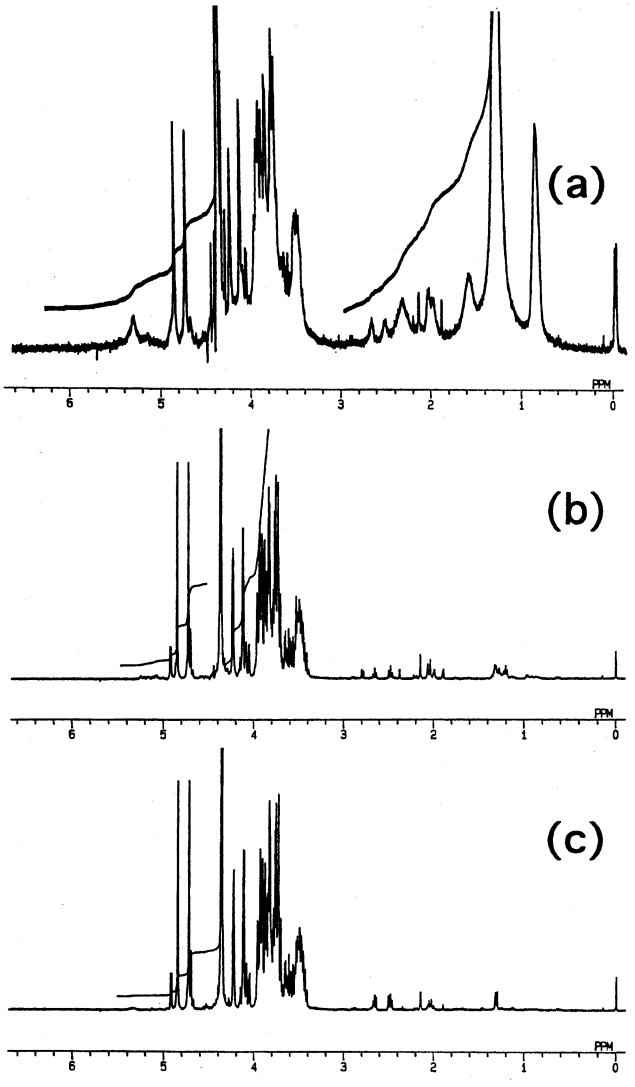

Fig. 1. ^{1}H NMR spectra of AP-1 (a), AP-2 (b) and AP-3 (c). The spectra were recorded at 400 MHz in D_2O at 65 °C. A large signal at 4.36 ppm was derived from HOD.

and AP-3 is consistent with the above finding that the immunoreactions of these polysaccharides with anti-*L. biflexa* serum were specifically inhibited by β-(1→4)-mannobiose.

Analysis of AP-1.—As shown in Fig. 1(a), large integral values of proton signals ranging between 0.8 and 1.8 ppm (0.8–1.0 ppm, about 1.5 methyl protons/Man; 1.1–1.8 ppm, about nine methylene protons/Man) indicate the presence of some fatty acyl groups in AP-1. Fatty acid analysis of AP-1 with GLC revealed the presence of hexadecanoic acid as the major acyl and four minor acyls such as tetradecanoic, hexadecenoic, octadecanoic and octadecenoic acids. When AP-1 was deacy-lated by treatment with mild alkali and the immunoreactivity of deacylated AP-1 (D-AP-1) was assayed by ELISA, D-AP-1 showed the same immunoreactivity as the intact preparation (Table 1). Thus the presence or absence of the acyl groups does not influence its immunoreactivity. Because mild acid treatment (2% acetic acid, 100 °C, 2 h) was used in the isolation procedure of AP-1 and AP-2 from the phenol-soluble fraction, there is a possibility that AP-2 may have arisen from AP-1 by partial deacylation. However, repeated treatments of AP-1 with mild acid under the same conditions did not lead to any conversion into AP-2, suggesting that AP-1 and AP-2 were derived from different origins. In addition, AP-3 shows a behavior different from these of AP-1 and AP-2 upon phenol extraction. Thus, we speculate that cells of *L. biflexa* patoc Patoc I produce three species of antigenic polysaccharides (AP-1, AP-2 and AP-3), which are derived from different cell surface components. Several problems, particularly with the types of original cell surface components, the differences of acyl substitutions in AP-1 and AP-2 and the structural characterization of minor structure of APs, remain to be answered.

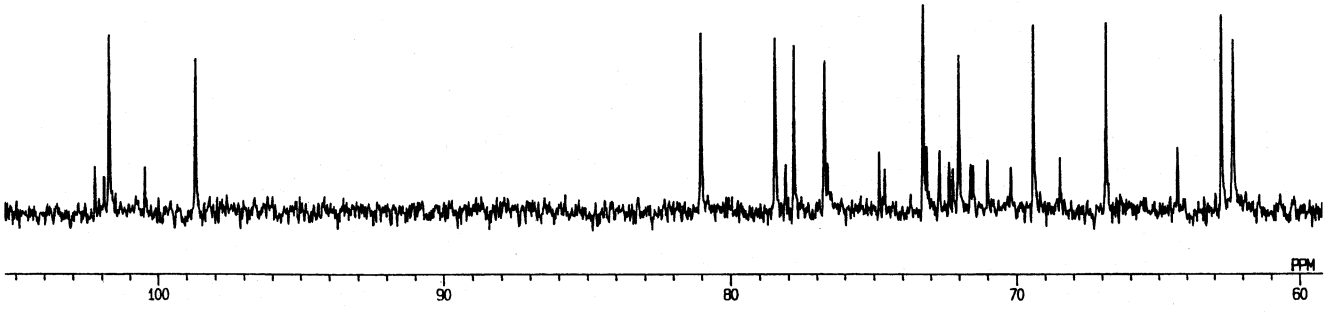

Fig. 2. ^{13}C NMR spectrum of AP-2. The spectra were recorded at 400 MHz in D_2O at 65 °C.

Table 3
Chemical shift (δ, ppm) of the signals in the ^1H and ^{13}C NMR spectra of AP-2. $J_{H\text{-}1,\ H\text{-}2}$ and $J_{H\text{-}1,\ C\text{-}1}$ values are given in Hz in square brackets

Residue	$^1H/^{13}C$						
	1	2	3	4	5	6	
→3)-β-D-Man*p*-(1→	4.72	[~0.6]	4.23	3.95	3.73	3.45	3.76/3.95
	101.7	[161]	69.3	81.0	66.8	77.8	62.7
→4)-β-D-Man*p*-(1→	4.85	[~0.5]	4.12	3.83	3.84	3.50	3.76/3.92
	98.7	[160]	72.0	73.2	78.4	76.7	62.3

Up until now, there have been a large number of reports about the occurrence of antigenic components in a variety of leptospires [9–14,16–18,26–29], although there is much confusion as to their structural identities. In the present study, we isolated three different species of antigenic polysaccharides from *L. biflexa* patoc Patoc I. From their structural characterization, the main structural parts are concluded to consist of a →3)-β-D-Man*p*-(1→4)-β-D-Man*p*-(1→ unit. Furthermore, on the basis of their immunological studies, such a structural unit or a closely related one may be widely distributed among many species of leptospires, probably as an epitope of genus-associated or genus-specific antigens.

3. Experimental

Cultivation of cells and isolation of polysaccharide components.—*L. biflexa* patoc Patoc I was grown in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium containing 10% rabbit serum [30] with gentle shaking for 8 days at 30 °C. The cells were harvested by centrifugation, washed with physiological saline and defatted with acetone. Isolation of polysaccharide components was carried out according to usual procedure [15]. Briefly, the defatted cells (10 g) were treated with 45% (v/v) hot aq phenol and the aq and phenol layers were collected separately, thoroughly dialyzed against tap water and treated with an excess amount of proteinase K. Mild acid treatment (2% (v/v) AcOH, 100 °C, 2 h), mild alkali treatment (0.2 M NaOH, 100 °C, 1 h) and treatment with 0.2% (w/v) cetyltrimetylammonium bromide (Cetavlon) were carried out depending on polysaccharide natures. The polysaccharide components were recovered by EtOH precipitation and purified by following successive chromatography on Sephadex G-50 (1.5 × 100 cm; in 50 mM $(NH_4)_2CO_3$), DEAE-Sepharose [2 mL resin bed; in 5 mM ammonium acetate (pH 7.7)] and CM-Sephadex C-25 [2 mL resin bed; in 5 mM ammonium acetate (pH 5.0)]. Hexose-containing fractions were checked their immunoreactivities by ELISA using rabbit anti-*L. biflexa* serum.

ELISA.—ELISA was performed using polystyrene plates (Nunc) precoated with 0.01% (v/v) poly-L-lysine. The wells were coated with 50 μL of each sample solution (40

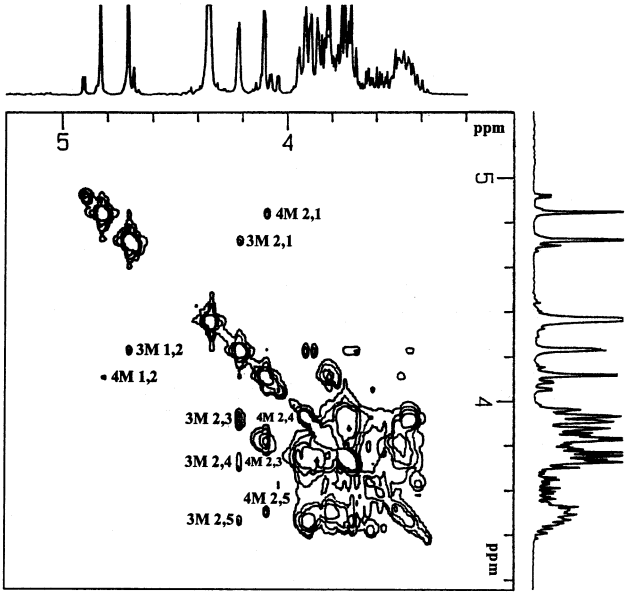

Fig. 3. Partial HOHAHA spectrum of AP-2 acquired with mixing time of 50 ms. 3 and 4 M indicate that the signals are derived from 3- and 4-*O*-substituted mannose residues, respectively.

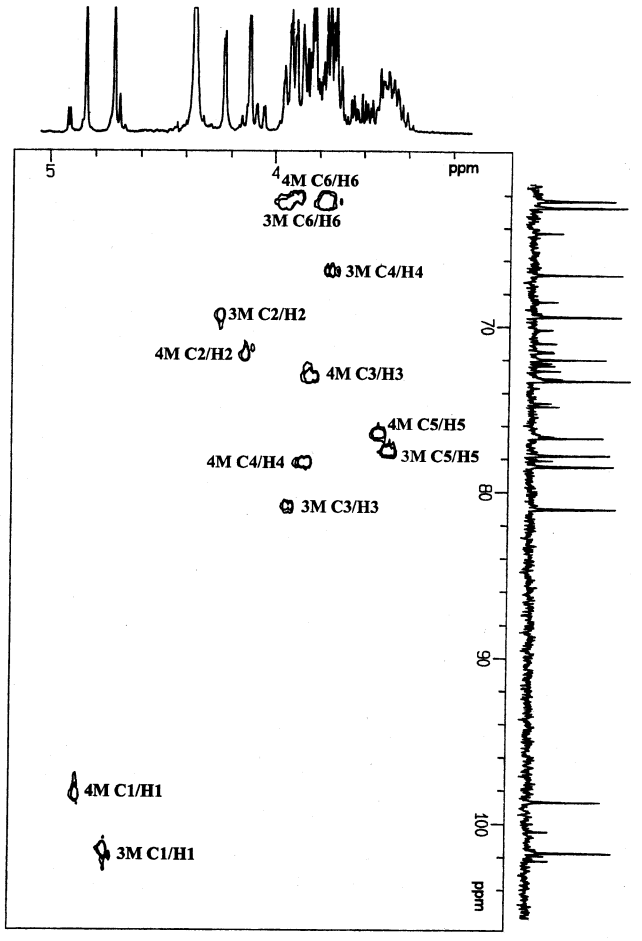

Fig. 4. HMQC spectrum of AP-2. 3 and 4M indicate that the signals are derived from 3- and 4-*O*-substituted mannose residues, respectively.

μg mL^{-1}) in phosphate buffered saline (PBS, pH 7.2), blocked with a solution of 0.5% (w/v) gelatin and thoroughly washed with 0.05% (v/v) Tween 20-containing PBS (Tween-PBS). In standard ELISA, 50 μL portions of dilution solution (1:1000, in Tween-PBS) of peroxidase-conjugated rabbit anti-*L. biflexa* antibody (ViroStat) were added to the above wells. After incubation at 37 °C for 30 min, and washing with Tween-PBS, 50 μL portions of peroxidase-substrate solution (TMB Peroxidase EIA Substrate Kit, BIO-RAD) were added and incubated (in the dark, room temperature, 30 min). The peroxidase reaction was stopped by addition of 50 μL of 0.5 M H_2SO_4 and the absorbance at 450 nm was measured. In competitive ELISA, saccharide inhibitors were added at various concentrations during incubation with the same antibody. The bound antibodies were determined as above. In cross-reactivity assays, a variety of rabbit antisera against whole cells of different leptospires were used instead of the anti-*L. biflexa* antibody. Amounts of antibodies bound to microtiter plate were determined by incubation with 50 μL portions of dilution solution (1:10000, in Tween-PBS) of peroxidase-labeled goat anti-rabbit IgG (H + L) (American Qualex). The serum of a healthy rabbit was used as control. Antisera against various leptospires were the same as those described previously [31].

NMR spectrometry and chemical analyses.—^1H and ^{13}C NMR measurements were performed with a JEOL EX-400 spectrometer equipped at the Center for Instrumental Analysis (Hokkaido University) by using sodium trimethylsilylpropane sulfonate as an internal standard (for ^1H NMR, δ 0.00 ppm) and acetone as an external standard (for ^{13}C NMR, δ 30.6 ppm). COSY, HOHAHA and HMQC techniques were used to confirm the assignment of complicated signals. All spectra were recorded at 65 °C.

For sugar analysis, samples were hydrolyzed with 4 M HCl at 100 °C for 3 h. The hydrolyzates were directly analyzed by HPAEC using a Dionex PA-1 column (4 × 250 mm) and the conditions reported previously [32]. Alternatively, the hydrolyzates were converted into alditol acetates and analyzed by GLC. Methylation was performed according to the method of Ciucanu and Kerek [33], and after hydrolysis (2 M CF_3COOH, 100 °C, 4 h) of the permethylation products, the partially methylated sugars were converted into their alditol acetates and analyzed by GCMS. Smith degradation was carried out as reported previously [34], and the product was isolated by gel chromatography on a Cellulofine GCL-25-m (1 × 110 cm) in 50 mM $(NH_4)_2CO_3$. GLC was carried out with a Shimadzu Gas-chromatograph GC-14B either on a capillary column of ULBON HR-17 (Shimadzu: 0.25 mm × 25 m) for analysis of alditol acetates and partially methylated alditol acetates or on a capillary column of ULBON HR-20M (Shimadzu: 0.25 mm × 25 m) for analysis of fatty acid methyl esters. GCMS analysis was carried out with a JEOL JMS-AX500 equipped at GCMS and NMR Labo-

ratory (Faculty of Agriculture, Hokkaido University). SDS-PAGE was performed according to the method of Laemmli [35], and the gel was stained with Silver Stain Plus (Bio-Rad). Total hexose was determined by the phenol–H_2SO_4 method [36], total hexosamine by the method of Tsuji et al. [37] after *N*-deacylation of samples by acid hydrolysis in 2 M HCl for 2 h at 100 °C, protein by DC protein assay (BIO-RAD), and total phosphorus by the method of Bartlett [38]. Absolute configuration of mannose was assigned by GLC analysis after conversion into peracetate of its (+)-2-octyl glycoside [39].

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