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Structural elucidation of polysaccharide part of glycoconjugate from *Treponema medium* ATCC 700293

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Glycoconjugates are distributed on the cell surfaces of some small-sized treponemes and have been reported to be completely different from lipopolysaccharides. We separated a glycoconjugate fraction from *Treponema medium* ATCC 700293, a medium-sized oral spirochete, to assess its immunobiological activities and elucidate the chemical structure of its polysaccharide part using phenol/water extraction, hydrophobic chromatography, and gel filtration. The glycoconjugate showed negligible or weak endotoxic and immunobiological properties. The chemical structure of the polysaccharide part was shown by two-dimensional NMR and MALDI-TOF-MS to be a tetrasaccharide backbone with two amino acids:

[→4)β-D-Glc*p*NAc3NAcA(1→4)β-D-Man*p*NAc3NA Orn(1→3)β-D-Glc*p*NAc(1→3)α-D-Fuc*p*4NAsp(1→]

where GlcNAc3NAcA is 2,3-diacetamido-2,3-dideoxyglucuronic acid, ManNAc3NAOrn is N^{δ}-(2-acetamido-3-amino-2,3-dideoxymannuronyl)ornithine, and Fuc4NAsp is 4-(α-aspartyl)amino-4,6-dideoxygalactose.

Keywords: glycoconjugate; MALDI-TOF-MS; NMR; *Treponema medium*.

Treponemal species are anaerobic bacteria with a typical helical shape that have been implicated in the induction of chronic human diseases, such as syphilis caused by *Treponema pallidum* [1] and periodontal diseases caused by *Treponema denticola* [2]. Such chronic diseases are characterized by an inflammatory reaction induced by pathogens followed by extensive tissue loss. Cell surface components released by treponemal species have been shown to mediate the synthesis of inflammatory mediators by host cells, such as macrophages. In a review of reports regarding the lipoprotein of *Escherichia coli* [3], treponemal species lipoproteins were shown to have potent virulence, i.e. the lipoprotein of *T. pallidum* [4] induced NF-κB translocation in monocytes [5] and that of *T. denticola* [6] activated macrophages [7]. Although some researchers have suggested that lipopolysaccharide (LPS) of treponemal species is responsible for the activation of host cells, the presence of LPS in spirochetes remains controversial, as a genome analysis of *T. pallidum* revealed the absence of LPS synthesis genes [8]. Recently, novel glycolipids were extracted from the small-sized spirochetes *T. denticola*, *Treponema maltophilum*, and *Treponema brennaborense* [9,10], and characterized as activators of mitogen-activated protein kinase and NF-κB [11,12], which may be potent virulent factors of treponemal species. Further, though the chemical structures of glycolipids have not been fully elucidated, they are known to be different from that of LPS [9,10].

Treponema medium is a medium-sized oral spirochete found in subgingival plaque from patients with adult periodontitis [13]. As *T. medium* seems to be associated with chronic inflammation [14], its cell surface components are expected to possess immunostimulating activities like other treponemal species. We previously demonstrated that the outer membrane extract of *T. medium* activates epithelial cells [15] and may be responsible for periodontal diseases, however, it has not been shown that a carbohydrate-containing component of *T. medium* acts as a virulence factor. In the present study, we separated a glycoconjugate from *T. medium*, and then elucidated the chemical structure as well as its endotoxic and immunobiological properties.

Materials and methods

Bacteria and preparation of glycoconjugate

T. medium ATCC 700293 was grown anaerobically in tripticase-yeast extract-gelatin-volatile fatty acids-rabbit serum broth containing 5% rabbit serum, as described previously [16]. The cells were subjected to phenol/water extraction [17], and then the extract was subjected to enzymatic digestion with DNase and RNase followed by proteinase K. To remove contaminated proteins, the digested portion was further subjected to phenol/water extraction to yield a glycoconjugate preparation, which was designated as Tm-Gp. Tm-Gp was then subjected to hydrophobic interaction chromatography. Tm-Gp was dissolved in 0.1 M acetate buffer (pH 4.5) containing 15% 1-propanol and applied to an Octyl Sepharose CL-4B column (36 × 2.5 cm) (Amersham Bioscience, Piscataway, NJ, USA). The column

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Abbreviations: CID, collision induced dissociation; HMBC, heteronuclear multiple bond connectivity; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MS/MS, tandem MS.

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was eluted using the buffer with the linear gradient of 1-propanol (15–60%). Six mililiter fractions were collected, after which they were monitored by measuring phosphorous, hexose, and amino group contents. The eluates were then combined, dialyzed using a Spectra/Por 7 (MWCO 3500; Spectrum Laboratories Inc., Dominguez, CA, USA), and lyophilized to yield pass-through (OS-P) and retained fractions. The retained fraction was designated as Tm-GC and used as a glycoconjugate fraction, while the OS-P portion was dissolved in water, applied to a Sephacryl S-200 HR column (81 × 1.5 cm) (Amersham Bioscience), eluted with water, and 2.5 mL fractions were collected and monitored as above. Finally, the eluates were combined, dialyzed, and lyophilized to obtain a polysaccharide fraction, which was designated as Tm-PS.

Analytical procedures

Phosphorous contents were determined according to the method of Bartlett [18] and hexose contents were measured using the anthrone/sulfuric acid method [19]. Amino group contents were analyzed using ninhydrin. Samples (100 μL) were added to 500 μL of ninhydrin reagent (ninhydrin/collidine/acetic acid/ethanol = 0.6 : 10 : 75 : 250, w/v/v/v) and heated at 100 °C for 5 min, after which the absorbance at 570 nm was measured.

Analysis of the sugar constituents in the sample was performed using the alditol acetate method [20] and the absolute configurations of sugars were determined using *R*-(+)-2-butanol [21]. Estimation of 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) was performed by the thiobarbiturate method [22], and fatty acids were analyzed according to the method of Ikemoto *et al.* [23].

For amino acids analysis, the samples were hydrolyzed with 6 M hydrochloric acid for 24 h at 110 °C. The hydrolysate was analyzed by the *o*-phthalaldehyde/*N*-acetylcysteine method [24] using an LC-10AD amino acids analysis system (Shimadzu, Kyoto, Japan). The absolute configurations of amino acids were determined using 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide [25].

SDS/PAGE was performed with 10% polyacrylamide gels according to the method of Laemmli [26] and visualized using the silver staining method [27].

Endotoxic and immunobiological assays

Lethal toxicity in D-GalN-sensitized C57BL/6 mice, cytokine production in human peripheral blood mononuclear cells, and mitogenicity of mononuclear cells from C3H/HeN and C3H/HeJ mice were assayed as described previously [28]. Animals received humane care in accordance with our institutional guidelines and the legal requirements of Japan.

Chemical degradation and separation

Tm-GC was treated with 0.6% AcOH at 100 °C for 2 h, 0.1 M NaOH at 37 °C for 2 h, or 48% aqueous HF at 4 °C for 24 h, and the liberated hydrophobic products were extracted using chloroform/methanol (2 : 1, v/v). Tm-PS was hydrolyzed with anhydrous trifluoromethanesulfonic acid at 0 °C for 1 h and the reaction mixture was neutralized with ammonia solution [29]. The hydrolysate was subjected to gel filtration chromatography using a Toyopearl HW-40S (Tosoh, Tokyo, Japan) to give oligosaccharides.

NMR spectroscopy and mass spectrometry

^{1}H and ^{13}C NMR spectra were measured at 500 and 126 MHz, respectively, using a JMN-LA500 spectrometer (JEOL, Tokyo, Japan) equipped with an indirect detection gradient probe, IDG500-5VJ (Nanorac Cryogenics, Martinez, CA, USA). Spectra were obtained at 310 K in D_2O or 10% D_2O/H_2O. The chemical shifts were expressed as δ values with water (δ 4.7) as the internal standard for ^{1}H NMR spectra and with dioxane (δ 67.4) as the external standard for ^{13}C NMR spectra.

MALDI-TOF-MS was measured using an Ultraflex instrument (Bruker Daltnics, Bremen, Germany). Samples were dissolved in water, combined with 2,5-dihydroxybenzoic acid as a matrix, and placed on a sample plate, then spectra were obtained in positive ion reflector mode, and tandem MS (MS/MS) spectra were obtained in positive ion TOF/TOF mode. ESI-MS was measured using a Q-TOF instrument (Micromass. Manchester, UK). Spectra were obtained in negative reflector and collision induced dissociation (CID) modes.

Results and discussion

Separation of glycoconjugate from *T. medium*

Extraction of a glycoconjugate from *T. medium* was performed using standard hot phenol/water extraction followed by chromatographic separations. The bacteria were subjected to phenol/water extraction, nuclease and proteinase digestion, and re-extraction with phenol/water to yield a glycoconjugate preparation, Tm-Gp (5.3%). As shown in Fig. 1, a ladder-like pattern was observed in the SDS/PAGE profile of Tm-Gp, indicating the presence of a glycoconjugate with repeating units. During phenol/water extraction of bacteria, capsular and extracellular polysaccharides as well as glycoconjugates are simultaneously extracted in the aqueous phase [30], thus, we further separated the glycoconjugate fraction using hydrophobic interaction chromatography [31,32]. Tm-Gp was subjected to hydrophobic interaction chromatography to yield a pass-through fraction, OS-P (50% based on Tm-Gp), and a retained fraction, Tm-GC (14%). The glycoconjugate in Tm-GC was confirmed by SDS/PAGE (Fig. 1). OS-P was further subjected to gel filtration

Fig. 1. SDS/PAGE profiles of Tm-Gp and Tm-GC. SDS/PAGE was performed using a 10% gel and visualized by silver staining.

chromatography to yield a polysaccharide fraction, Tm-PS (65% based on OS-P), that contained no material visualized by SDS/PAGE, demonstrating the absence of the glycoconjugate.

Tm-Gp lacked lethal toxicity in mice at a dose of up to 100 μg per mouse, and stimulated no or very weak IL-1β and IL-6 production in human peripheral blood mononuclear cells at concentrations up to 100 μg per well. Further, Tm-Gp also exhibited no mitogenic activity towards spleen cells from C3H/HeN and C3H/HeJ mice at the same concentrations. These results indicate that the glycoconjugate from *T. medium* possessed a negligible or very weak endotoxin-like acute phase activity. Schröder *et al.* [11] and Opitz *et al.* [12] have reported that glycolipids extracted from small-sized spirochetes stimulated activation in mitogen-activated protein kinases and NF-κB in monocytic cells at similar concentrations. The different immunobiological activity seen in the present study may have been caused by differences in the structure of the glycoconjugate.

Structural elucidation of polysaccharide part

Because treponemal species are associated with chronic diseases in humans, they have a mechanism that allows escape from the host defense system. With many bacteria, capsular polysaccharide renders the organisms resistant to phagocytosis, the major clearance system in hosts lacking specific antibodies [33]. Although the location of Tm-GC/Tm-PS on the cell has not been elucidated, this component may act as a protective factor. Thus, we examined the structure of the polysaccharide part.

The chemical composition (Table 1) and ^{1}H-NMR spectrum (Fig. 2) of Tm-PS was found to be similar to those of Tm-GC, except for the fatty acids, suggesting that the fundamental structure of the polysaccharide part in both is the same. Further, it is reasonable to assume that Tm-PS is a dephosphorylated or deacylated component of Tm-GC therefore we elucidated the structure of the polysaccharide part using Tm-PS.

Compositional analysis showed that Tm-PS contained mainly glucosamine (GlcN), aspartic acid (Asp), and ornithine (Orn) (Table 1). The absolute configuration of GlcN was determined as D, while the absolute configurations of the amino acids were determined as D for Asp and L for Orn.

The ^{1}H and ^{13}C NMR spectra of Tm-PS are shown in Figs 2 and 3. Four anomeric signals (at δ 4.63, δ 4.78, δ 5.05, and δ 5.13 for ^{1}H, and δ 98.8, δ 99.8, δ 100.4, and δ 102.0 for ^{13}C) were mainly observed, and the corresponding sugars were designated as **a** to **d** in order of ^{13}C NMR chemical shift. Eight signals at δ 50.3, δ 51.0, δ 53.7, δ 53.8, δ 54.0, δ 54.2, δ 54.5, and δ 54.7 in the ^{13}C NMR spectrum were assigned to carbons substituted by the amino group. Six amide proton signals at δ 7.92, δ 8.14, δ 8.22, δ 8.27, δ 8.47, and δ 8.71 were observed in the ^{1}H NMR spectra measured in 10% D_2O. The ^{1}H NMR signals were assigned using the DQF-COSY, TOCSY, and HSQC-TOCSY spectra, while the ^{13}C NMR signals were assigned using the HMQC, HSQC-TOCSY, and HMBC spectra (Table 2). The coupling constants were estimated from a one-dimensional spectrum and DQF-COSY. Based on the assignment, one 4-amino-4,6-deoxyhexose for residue **a**, two 2,3-diamino-2,3-dideoxyhexuronic acids each for residues **b** and **c**, one 2-amino-2-deoxyhexose for residue **d**, Asp, and Orn were found.

Table 1. Chemical composition (μmol·mg^{-1}) of Tm-GC and Tm-PS.

	Tm-GC	Tm-PS
D-GlcN	1.06	0.71
D-Asp	0.83	0.64
L-Orn	1.06	0.92
Phosphate	0.02	0.08
Fatty acids		
14:0	0.02	0
13-Me 14:0	0.02	0
16:0	0.06	0
17:0 $^{\Delta 9,10}$	0.02	0
18:1 ^{c9}	0.02	0

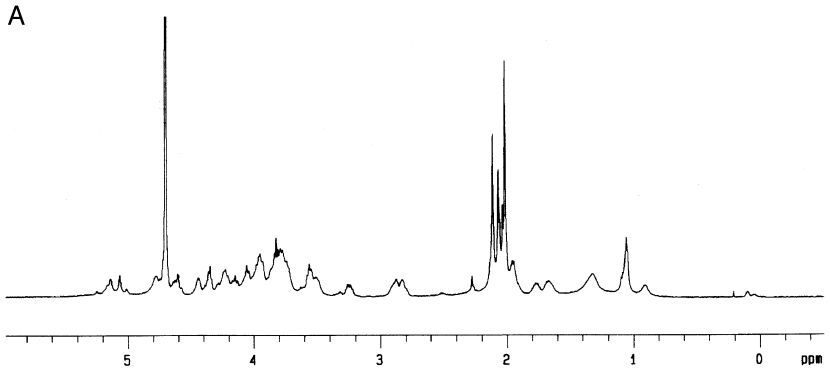
Residue **d** was assigned to 2-amino-2-deoxy-β-glucopyranose (β-Glc*p*N). The coupling constants for the anomeric protons, $^3J_{1,2}$ of 8.0 Hz, showed a β-configuration. Although the coupling constant $^3J_{2,3}$ could not be determined, $^3J_{3,4}$ and $^3J_{4,5}$ of ≈ 10 Hz indicated the glucopyranosyl configuration. The chemical shift of δ 54.7 for C2-**d** was indicative of a 2-amino-2-deoxy structure. Intraresidual ROESY couplings from H1-**d** to H3-**d** and H1-**d** to H5-**d** (Fig. 4) as well as the $^1J_{C,H}$ value for the anomeric atoms (160.7 Hz) determined from the nondecoupling DEPT spectrum [34] supported a β-configuration.

As judged by the coupling constant, residue a was 4-amino-4,6-dideoxy-α-galactopyranose (α-Fuc*p*4N). The coupling constants, $^3J_{2,3}$ of ≈ 11 Hz, $^3J_{3,4}$ of ≈ 5 Hz, and a characteristically small $^3J_{4,5}$ determined from the TOCSY spectrum showed the galactopyranosyl configuration. The coupling constant for the anomeric proton, $^3J_{1,2}$ of 4.4 Hz, and the $^1J_{C,H}$ value of the anomeric atoms (172.3 Hz) indicated an α-configuration. The chemical shift of δ 54.0 for C4-**a** was indicative of a 4-amino-4-deoxy structure, while δ 15.7 for C6-**a** showed a 6-deoxy structure.

Residue **b** was assigned to 2,3-diamino-2,3-dideoxy-β-mannopyranuronic acid (β-Man*p*N3NA). The coupling constants of residue **b**, characteristically small (≈ 3 Hz) $^3J_{2,3}$, and large (≈ 11 Hz) $^3J_{3,4}$ and $^3J_{4,5}$, as well as a singlet like signal of H-1**b** were typical for a mannopyranosyl configuration. Intraresidual ROESY couplings from H1-**b** to H3-**b** and H1-**b** to H5-**b** along with the $^1J_{C,H}$ value (163.0 Hz) indicated a β-configuration. The chemical shifts of δ 50.3 for C2-**b** and δ 53.7 for C3-**b** were indicative of a 2,3-diamino-2,3-dideoxy structure, and δ 168.7 for C6-**b** showed a uronic acid structure.

Residue **c** was determined to be 2,3-diamino-2,3-dideoxy-β-glucopyranuronic acid (β-Glc*p*N3NA). The coupling constants for the anomeric proton, $^3J_{1,2}$ of 8.0 Hz, indicated a β-linkage, while the coupling constants $^3J_{2,3}$ of ≈ 10 Hz, $^3J_{3,4}$ of ≈ 8 Hz, and $^3J_{4,5}$ of ≈ 11 Hz indicated a glucopyranosyl configuration. The chemical shifts of δ 53.8 for C2-**c** and δ 54.2 for C3-**c** were indicative of a 2,3-diamino-2,3-dideoxy structure, and δ 174.5 for C6-**c** showed a uronic acid structure.

The sequences of the sugar and amino acid residues were determined using ROESY, NOESY, and HMBC spectra. Four glycosidic linkages were established from results of



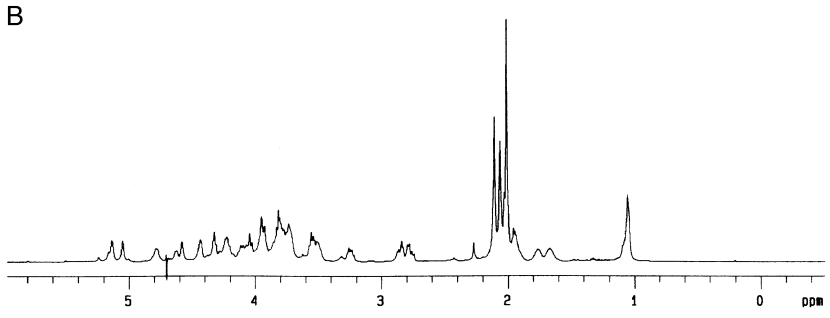


Fig. 2. 500 MHz ^{1}H NMR spectra of Tm-GC (A) and Tm-PS (B). Spectra were obtained at 310 K in D_2O. The spectrum in (B) was recorded using a DANTE sequence for water suppression.

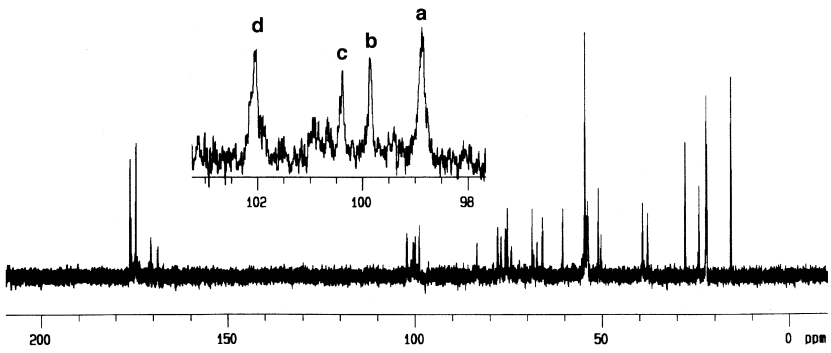


Fig. 3. 126 MHz ^{13}C NMR spectra of Tm-PS. Spectra were obtained at 310 K in D_2O.

the ROESY (Fig. 4) and HMBC experiments. Interresidual couplings from H1-**a** to H4-**c** indicated that residue a was linked to O4 of residue **c**, while those from H1-**b** to H3-**d** showed that residue **b** was linked to O3 of residue **d**, those from H1-**d** to H3-**a** indicated that residue **d** was linked to O3 of residue **a**, and those from H1-**c** to H4-**b** showed that residue **c** was linked to O4 of residue **b**. Long-range HMBC couplings from H1-**c** to C4-**b**, H1-**b** to C3-**d**, H1-**d** to C3-**a**, and H1-**a** to C4-**c** were observed (data not shown), which supported the above linkages.

Two amide linkages were established by the NOESY (Fig. 5) and HMBC experiments. Couplings from the amide proton at the 4-position of residue a (NH4-**a**) to H2-Asp indicated that the carboxyl group of the 1-position in Asp was attached to N4 of residue a by an amide linkage. Couplings from NH5-Orn to C5-**b** indicated that the amino group at the 5-position of Orn was attached to the 6-position of residue **b** by an amide linkage. Long-range HMBC couplings from H4-**a** to C1-Asp and H5-Orn to C6-**b** (data not shown) supported the amide linkages. These results showed the sequence, [→4)β-GlcN3NA(1→4)β-ManN3NA6Orn(1→3)β-GlcN(1→3)α-Fuc4NAsp(1→].

To elucidate the amidation pattern of Tm-PS, the pD dependence of ^{1}H NMR chemical shifts was studied. The signals for H5-**c** and H5-**b** were shifted downfield by +0.15 and +0.02, respectively, with a decrease of pD from 7 to 2, indicating a free carboxyl group in residue **c**, however, not in residue **b**. The signals for H3-Asp and H2-Orn were shifted downfield by +0.23–0.28 and +0.19, respectively, with a decrease of pD from 7 to 2, indicating a free carboxyl group of the 4-position in Asp and the 1-position in Orn. The signals for H2-**b**, H3-**b**, and H4-**b** were shifted upfield by −0.34, −0.69, and −0.38, respectively, with an increase of pD from 7 to 13, indicating a free amino group at the 3-position of residue **b**. The signals for H2-Asp and H2-Orn were shifted upfield by −0.55 and −0.54, respectively, with an increase of pD from 7 to 13, indicating a free amino group at the 2-position of Asp and the 2-position of Orn.

To determine the relative absolute configuration of the constituent monosaccharids, glycosylation effects [35] in the

Table 2. ^{1}H and ^{13}C-NMR data for *T. medium* glycoconjugate. Spectra were measured at 310K. Chemical shifts are expressed as δ values.

	Residues					
Atoms	α-Fuc*p*4N (**a**)	β-Man*p*N3NA (**b**)	β-Glc*p*N3NA (**c**)	β-Glc*p*N (**d**)	Asp	Orn
H1	5.13	5.05	4.63	4.78		
H2	4.23	4.58	3.84	3.74	4.33	3.81
H3	4.08	3.73	4.22	3.78	2.77, 2.86	1.95
H4	4.43	4.12	3.94	3.55		1.66, 1.76
H5	4.23	4.04	3.96	3.49		3.24, 3.53
H6	1.06			3.78, 3.93		
C1	98.8	99.8	100.4	102.0	170.5	175.2
C2	67.3	50.3	53.8	54.7	51.0	54.5
C3	77.0	53.7	54.2	83.4	37.8	27.8
C4	54.0	72.8	74.3	68.7	176.1	24.1
C5	65.9	75.8	77.9	75.3		39.2
C6	15.7	168.7	174.5	60.5		
NH2		8.22	7.92	8.16		
NH3			8.27			
NH4	8.47					
NH5						8.71

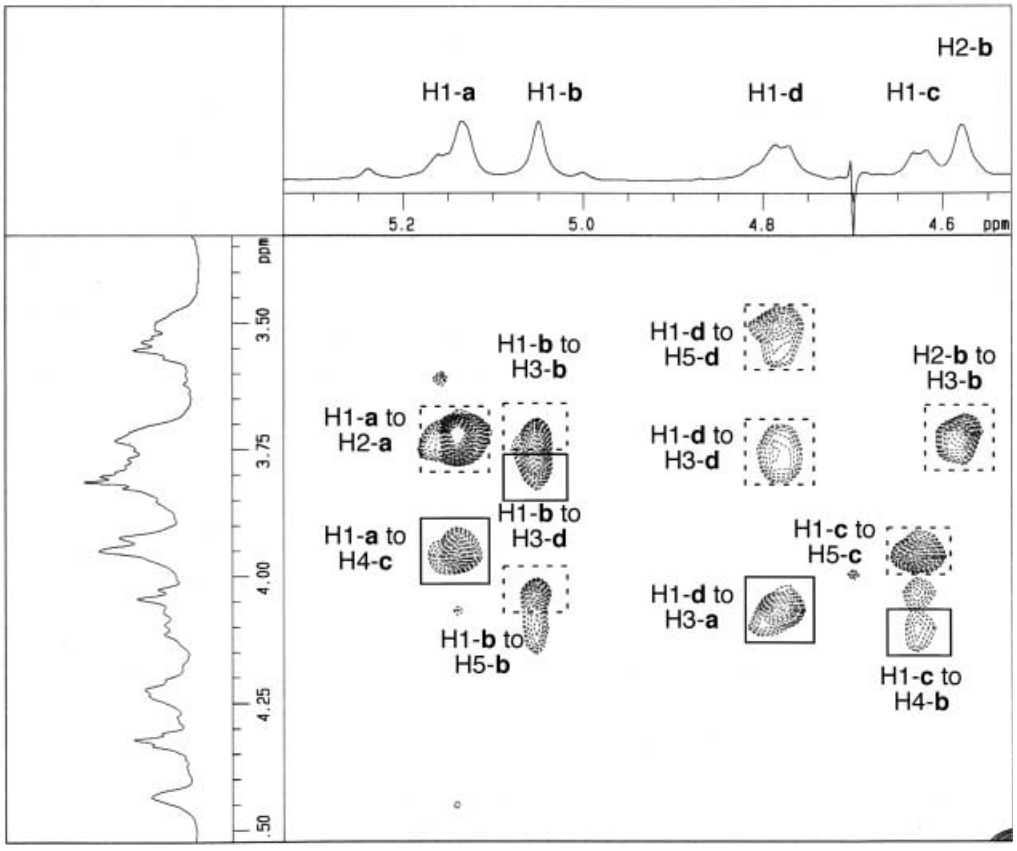


Fig. 4. ROESY spectra of Tm-PS. Spectra were obtained in phase sensitive mode with DANTE water suppression at 310 K in D_2O. The mixing time was 200 ms. Solid and dashed squares represent interresidual and intraresidual couplings, respectively.

^{13}C NMR spectrum of Tm-PS were analyzed. For the β-linked disaccharide **d** → **a**, β-GlcN(1 → 3)Fuc4N, the observed values were +6.9 for the α-effect of C1-**d**, +8.4 for that of C3-**a**, and −0.4 for the β-effect of C4-**a**, as compared with those for standard GlcNAc and Fuc4NAc [36]. The expected effects on C1-**d**, C3-**a**, and C4-**a** were +8.0 ± 0.4, +9.1 ± 1.3, and −0.4 ± 0.4, respectively, for the same and +2.9 ± 0.9, +5.4 ± 1.6, and

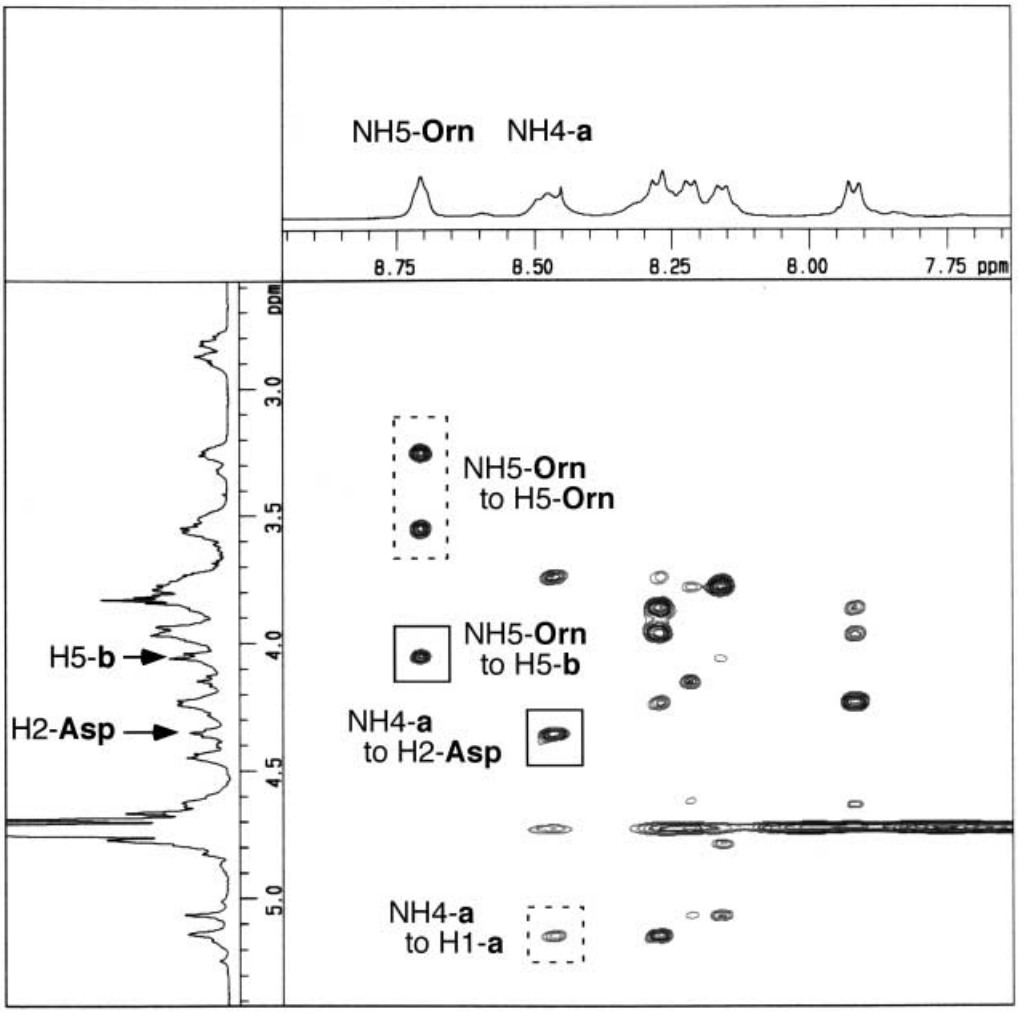


Fig. 5. NOESY spectra of Tm-PS. Spectra were obtained in phase sensitive mode with DANTE water suppression at 310 K in 10% D_2O. The mixing time was 490 ms. Solid and dashed squares represent interresidual and intraresidual couplings, respectively.

−3.3 ± 0.6, respectively, for different absolute configurations. These results showed that residues **d** and **a** had the same absolute configuration. For the β-linked disaccharide **b** → **d**, β-ManN3NA(1 → 3)GlcN, the observed value was −1.4 for the β-effect of C4-**d**, as compared with standard GlcNAc. The expected effects were −1.4 ± 0.2 for the same and −0.2 ± 0.4 for different absolute configurations. This finding also suggested that residues **d** and **a** had the same absolute configuration. For the α-linked disaccharide **a** → **c**, α-Fuc4N(1 → 4)GlcN3NA, the observed value was −0.1 for the β-effect of C3-**c** as compared with the nonreducing terminal GlcNAc3NAc (54.3 p.p.m. for C3) of the oligosaccharides described below. The expected effect was +0.3 ± 0.6 for the same and −1.3 ± 0.4 for different absolute configurations. Thus, residues **a** and **c** were found to have the same absolute configuration. Therefore, all the sugars had the same absolute configuration as D.

To confirm its structure, Tm-PS was subjected to solvolysis with trifluoromethanesulfonic acid [29]. The MALDI-TOF-MS of the resulting oligosaccharides showed pseudomolecular ions $[M + H]^+$ at *m*/*z* 1070.5 and 810.3, and their dehydrated ions at *m*/*z* 1052.4 and 792.2. The ions at *m*/*z* 1070.5 represented a tetrasaccharide composed of amino-deoxyhexose (HexN), amino-dideoxyhexose (dHexN), and two diamino-dideoxyhexuronic acids (HexNNA), with Asp, Orn, and four acetyl groups. The tetrasaccharide could be obtained by the selective cleavage of the 4-aminofucosidic linkage of Tm-PS and the MS/MS spectrum of the ion at *m*/*z* 1070 (Fig. 6) indicated the sequence (HexNNA + 2Ac) → (HexNNA + Ac + Orn) → (HexN + Ac) → (dHex + Asp). The ions at *m*/*z* 810.3 represented a trisaccharide composed of a single HexN and two HexNNA, with Orn and four acetyl groups. The trisaccharide could be yielded by a double cleavage of the 2-aminoglucosidic and 4-aminofucosidic linkages of Tm-PS. The results of solvolysis agreed with the structure established by NMR analysis.

From our results, we proposed a chemical structure of Tm-PS and the polysaccharide part of Tm-GC, as shown in Fig. 7, which is the first known structural elucidation of a polysaccharide obtained from a treponemal species. Although the structure is novel, the constituents, including the unusual aminosugar and diaminouronic acids, have been previously found in O-specific, capsular, and

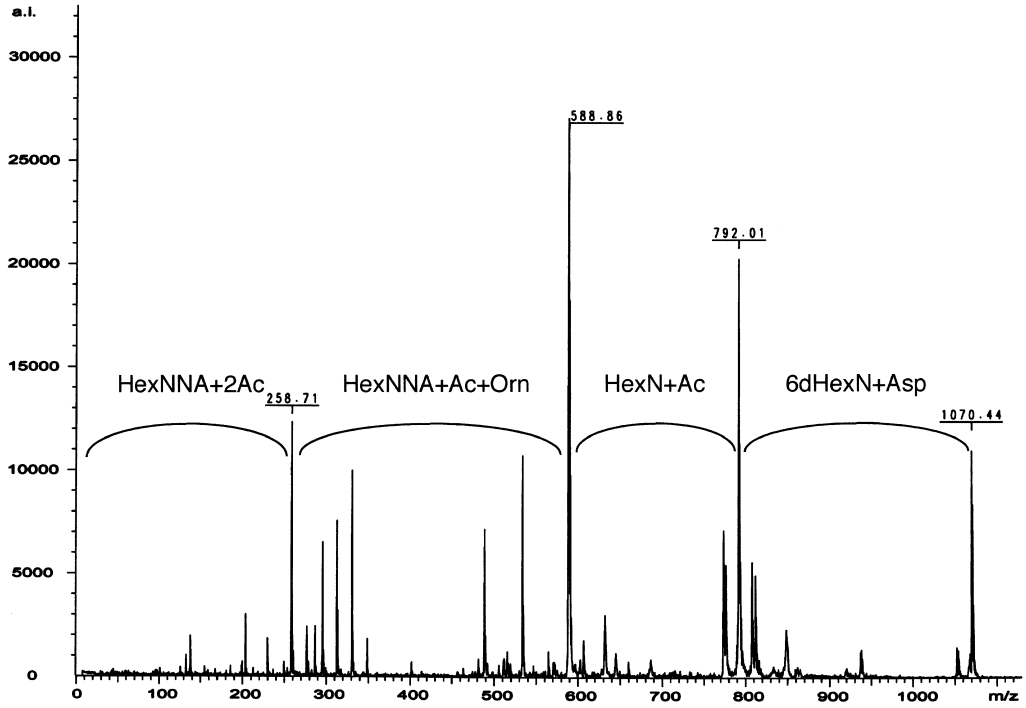


Fig. 6. MALDI-TOF-MS/MS spectrum of the parent ion at *m/z* 1070 in TfOH-hydrolysate of Tm-PS. The spectrum was obtained in positive ion TOF/TOF mode using 2,5-dihydroxy benzoic acid as a matrix.

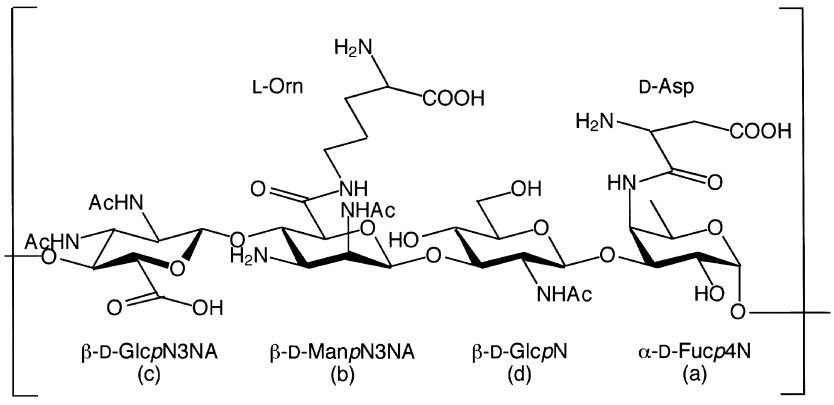


Fig. 7. Proposed chemical structure of Tm-PS.

exo-polysaccharides from other bacteria [37,38]. As many of these kind of polysaccharides seem to have antigenicity, the unusual sugars seen in the present study may be involved with antigen epitopes. Therefore, vaccination using part of the structure of Tm-GC may be useful for prevention of periodontal diseases caused by *T. medium*. As many oral treponemal species contribute to periodontal diseases [39], the structures of glycoconjugates obtained from other treponemal species should also be elucidated.

Structural features of the glycoconjugate

LPS is composed of a polysaccharide part, O-antigen and core regions, and a lipid anchor termed lipid A. Because the structure of the polysaccharide part of Tm-GC is similar to that of O-antigen of LPS, we attempted to determine whether Tm-GC is a kind of LPS. LPS usually contains characteristic components, such as heptose, β-hydroxy fatty acid, and Kdo, however, no heptose or β-hydroxy fatty acid was observed in the compositional analysis of Tm-GC, while the content of Kdo was under the detection limit of the thiobarbiturate assay. Kdo in LPS from some species does not respond in a thiobarbiturate assay, due to phosphorylation [22]. However, as the HF-treated products of Tm-GC were negative in the thiobarbiturate assay, Kdo was considered to be absent from Tm-GC.

We also characterized the hydrophobic region of Tm-GC. Alkaline treated products of Tm-GC contained no fatty acid, which showed that all fatty acids in Tm-GC are attached by ester linkages. HF-treatment liberated hydrophobic products from Tm-GC. ESI-MS of the

products showed two series of ions $[M\text{-}H]^-$ at m/z 383.2, 397.2, and 411.2 (relative intensity ratio, 0.57 : 0.40 : 1.00), and m/z 455.2, 469.2, and 483.2 (0.22 : 0.18 : 0.51), respectively. CID-MS/MS of the ion at m/z 411.2 showed daughter ions at m/z 255.2 and 155.0, and that at m/z 483.2 showed daughter ions at m/z 255.2, 227.0, and 153.0. The fragmentation patterns indicated that the former represents a lysophosphatidic acid deivative, acylglycero-fluorophosphoric acid, and the latter was lysophosphatidylglycerol. As a fluorophosphoric acid structure can be produced by the cleavage of phosphodiester linkage with HF, lysophosphatidylglycerol was considered to be the hydrophobic region of Tm-GC. Further, as lysophosphatidylglycerophosphate or its derivative were not observed, the linkage between the polysaccharide part and the hydrophobic region may be an acid labile glycosyl bond. Lipid A, which generally consists of a diglucosamine backbone with amide and ester bound fatty acids, and occasionally monoester linked phosphate, is attached to Kdo located at the reducing end of the polysaccharide of LPS, via a glycosyl bond. Therefore, the glycoconjugate from *T. medium* is likely to be different from LPS.

The present observations agreed with earlier analyses of glycolipids in the small-sized treponemal species *T. denticola* [9], *T. maltophilum*, and *T. brennaborense* [10], in which it was proposed that the structure of the glycolipids resemble lipoteichoic acid (LTA). Although the structure of the polymeric hydrophilic part is quite different, Tm-GC may be this kind of glycolipid, though the LTA-like treponemal glycolipids are known to have immunostimulating activities, whereas Tm-GC possessed no such activity. In biologically active glycoconjugates, the detailed structure of the lipophilic part is closely related to their activity, such as the acylation and phosphorylation patterns in lipid A of LPS [28], and the glycosylation position of the glycolipid in LTA [40,41]. To determine the structure–activity relationship, a detailed structural elucidation of the lipophilic part of immunobiologically active glycoconjugates from treponemal species is required.

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