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Structural studies of the *Vibrio mimicus* W-26768 O-antigen polysaccharide

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ABSTRACT

The structure of the *Vibrio mimicus* W-26768 O-antigen polysaccharide has been investigated by sugar and methylation analyses, Smith degradation, and NMR spectroscopy. It is proposed that it is composed of chains of β-(1 → 4)-linked 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucopyranosyl residues (D-Qui*p*3NR), ~ 60% of which are substituted in the 2-position with 2-acetamido-2-deoxy-α-D-galactopyranosyl groups. The polysaccharide does not seem to be composed of oligosaccharide repeating units but has a less regular structure, schematically indicated below.

→ 4)-β-D-Qui*p*3NR-(1 → 4)-β-D-Qui*p*3NR-(1 → 4)-β-D-Qui*p*3NR-(1 →
2 2
↑ ↑
1 1
α-D-Gal*p*NAc α-D-Gal*p*NAc

INTRODUCTION

A bacterium, identified as *Vibrio mimicus* and given the number W-26768, was isolated from diarrhoeal patients at the treatment center of the International Center for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) in Dhaka[1]. The lipopolysaccharide (LPS) was isolated from this strain by conventional methods[2], and we now report structural studies of this material.

RESULTS AND DISCUSSION

The LPS was treated with aqueous 1% acetic acid at 100°C and the product, after conventional work-up, was fractionated on a column of Bio-Gel P-4. One fraction (PS) was eluted shortly after the void volume, followed by a second (CPS).

TABLE I

Sugar analysis of polysaccharide fractions from *Vibrio mimicus* W-26768

Sugar	Mole %				
	PS [a]	PS [b]	PSHF [b]	CPS [a]	CPSHF [a]
D-Qui3NAc	21.1				
D-Glc	4.9	5.5		38.2	39.4
D-GalNAc	38.3	39.0	46.5		
L,D-Hep	9.3	9.0		61.8	60.6
D-Qui3NR	26.5	46.5	53.5		

[a] Conventional sugar analysis. [b] Analysis after solvolysis in liquid hydrogen fluoride.

Sugar analyses of these fractions (Table I), by GLC of the derived alditol acetates, showed the presence of D-glucose and L-*glycero*-D-*manno*-heptose (L-D-Hep) in the CPS, and these sugars, 2-amino-2-deoxy-D-galactose, and two further components in the PS. The results indicate that the first fraction consists of the O-antigen linked to the core, and that the second is the core. The absolute configurations of the sugars were determined as devised by Gerwig et al.[3,4]. GLC–MS of the two unknown alditol acetates demonstrated that one was a 3-acetamido-3,6-dideoxyhexitol tetraacetate and the other an analogous 3-acetoxybutyramido derivative.

After solvolysis, in liquid hydrogen fluoride, of a product (PSHF, see below) in which the O-antigen part of the PS had been enriched, 2-acetamido-2-deoxy-D-galactose and another component were isolated. On borohydride reduction and acetylation, the latter gave the 3-amino-3,6-dideoxyhexitol derivatives mentioned above. As amide linkages are not cleaved during the solvolysis, these results demonstrate that the 2-amino-2-deoxy-D-galactose in the PS is *N*-acetylated and the 3-amino-3,6-dideoxyhexose is *N*-acylated by a hydroxybutyric acid. A sugar analysis of the PS, using solvolysis in liquid hydrogen fluoride instead of hydrolysis with acid (Table I), confirmed this result.

The acid was isolated from a hydrolysate of the PS by extraction with ethyl acetate. The ^{1}H NMR spectrum was identical with that given by authentic 3-hydroxybutyric acid, with signals at δ 4.15 (m, 1 H), 2.35 (m, 2 H), and 1.20 (d, 3 H). The acid was transformed into the 3-*O*-trifluoroacetyl derivative of its methyl ester, which had the same retention time in GLC on an FS-LIPODEX A column as the authentic (*R*) derivative, but was well separated from the corresponding (*S*) form.

The ^{1}H NMR spectrum of the 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]hexose was fully assigned (Table II), and from the coupling constants it is evident that the sugar has the *gluco* configuration. The value for the specific rotation, $[\alpha]_D$ +15° (*c* 0.3, H_2O), is not very accurate but demonstrates that the sugar is a D-glucose derivative, namely, 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-D-glucose (D-Qui3NR).

The PS was treated with aqueous 48% hydrogen fluoride for 24 h at 4°C, and the product was reduced with sodium borohydride and then fractionated on a

TABLE II

^{1}H NMR chemical shifts (ppm) and coupling constants (Hz) for 3,6-dideoxy-3-[(*R*)-3-hydroxybutyramido]-α- and -β-D-glucose

	H-1	H-2	H-3	H-4	H-5	H-6	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
α Anomer	5.19	3.61	4.07	3.17	3.95	1.26	3.4	7.0	10.4	9.9	6.2
β Anomer	4.72	3.27	3.87	3.17	3.58	1.29	7.5	8.0	10.2	8.2	6.3

column of Bio-Gel P-2. A main fraction (PSHF) was eluted shortly after the void volume, followed by a smaller fraction (CPSHF). Sugar analyses of these fractions (Table I), using solvolysis with liquid hydrogen fluoride, showed that PSHF was composed of the two amino sugars, and CPSHF of D-glucose and L-D-Hep. A comparison of the ^{1}H NMR spectrum of PSHF (Fig. 1) with that of PS showed that the typical signals assigned to the amino sugars were essentially unaffected by the treatment with aqueous 48% hydrogen fluoride, but that signals caused by other sugar residues were considerably reduced. The O-antigen polysaccharide was consequently released from the core by this treatment.

The results of the methylation analyses of PS and PSHF (Table III), performed using solvolysis of the methylated polysaccharides in liquid hydrogen fluoride, show that the O-antigen polysaccharide is composed of terminal 2-acetamido-2-deoxy-D-galactopyranosyl groups, D-Qui3NR residues linked through O-4, and residues of the same sugar linked through O-2 and O-4, NMR evidence (see below) shows that all residues are pyranosidic. The molar proportions, estimated from the flame-ionisation detector response and use of effective carbon response factors[6], are not expected to be very accurate for these structurally different derivatives. An unusually high percentage of the amino sugars were not *N*-methylated in these analyses.

TABLE III

Methylation analysis of polysaccharide fractions from *Vibrio mimicus* W-26768

Sugar [a]	t_R [b]	Mole %		
		PS	PSHF	CPS
2,3,4,6-Glc	1.00	17.7		42.5
2,3,4,6,7-Hep	1.42			21.8
3,4,6-GalNAc	1.77	25.8	30.5	
2,3,4,6-GalNAc	2.00	10.8	17.7	
2,6-Hep	2.13	8.2		35.7
2,3-QuiNR	2.43	10.8	11.8	
2-QuiNR	2.48	5.2	6.4	
3-QuiNR	2.66	21.5	33.7	

[a] 2,3,4,6-Glc = 2,3,4,6-tetra-*O*-methyl-D-glucose, etc. [b] Retention time of the corresponding alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on a DB-225 fused-silica capillary column at 190°C.

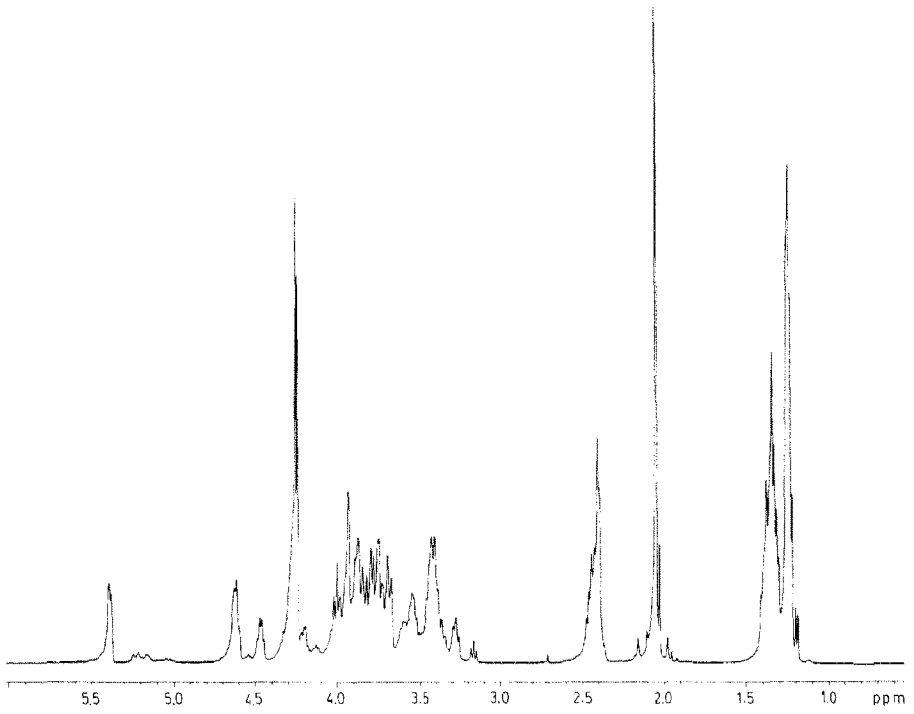

Fig. 1. 500-MHz ^{1}H NMR spectrum of the *V. mimicus* W-26768 O-antigen polysaccharide (PSHF).

PSHF was subjected to a Smith degradation[7]. It was evident from the ^{1}H NMR spectrum of the polymeric product that it contained solely D-Qui3NR residues. A single signal in the region for anomeric protons, at δ 4.47, $J_{1,2}$ 7.3 Hz, demonstrated that these residues are β-linked. Methylation analysis of the product showed that they are linked through O-4.

The ^{1}H NMR spectrum of PSHF (Fig. 1 showed, *inter alia*, signals for anomeric protons at δ 5.39 (1 H), 4.61 (1.2 H), and 4.46 (0.5 H); for methylene groups at δ 2.40 (3.4 H); for *N*-acetyl groups at δ 2.05 (2.8 H); and for *C*-methyl groups at δ 1.33 (5.9 H) and 1.23 (5.9 H). The ^{13}C NMR spectrum (Fig. 2) was in qualitative agreement with these results and showed, *inter alia*, signals for carbonyl groups at δ 175.21 and 174.83; for anomeric carbons at δ 103.55, 103.04, and 95.84; for a hydroxymethyl group at δ 62.06; for carbons linked to nitrogen at δ 56.15, 54.66, and 50.13; for C-2 of the 3-hydroxybutyryl group at δ 46.24; for *N*-acetyl at δ 23.01; and for *C*-methyl groups at δ 18.32 and 18.05. Most of the signals in the ^{1}H NMR spectrum could be assigned by COSY and relayed COSY experiments (Table IV). The signals for anomeric protons and carbons were correlated from an HMQC experiment, and the $J_{C\text{-}1,H\text{-}1}$ values determined. The results of the NMR experiments show that D-Gal*p*NAc is α-linked and D-Qui*p*3NR β-linked.

Bacterial polysaccharides are generally composed of oligosaccharide repeating units, but several signals in the ^{1}H NMR spectrum of PSHF were broad or

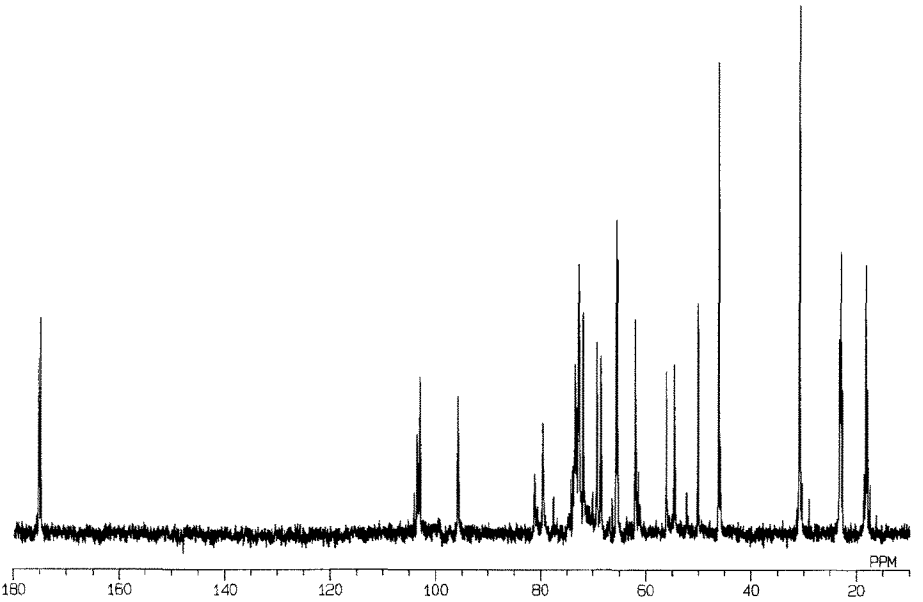

Fig. 2. ^{13}C NMR spectrum of the *V. mimicus* W-26768 O-antigen polysaccharide (PSHF).

appeared as pairs, with small differences in chemical shifts between them. This is typical for a less regular structure. The values of the integrated signals in the ^1H NMR spectrum also indicate ~0.6 α-D-Gal*p*NAc groups per one β-D-Qui*p*3NR residue.

From the combined result, it is thus concluded that the O-antigen polysaccharide from the *Vibrio mimicus* W-26768 LPS is composed of chains of β-(1 → 4)-linked 3,6-dideoxy-3-[(*R*)-hydroxybutyramido]-D-glucopyranosyl residues, ~60% of which are substituted in the 2-position with 2-acetamido-2-deoxy-α-D-galactopyranosyl groups. The results are not consistent with a structure composed of repeating units, but with a less regular structure schematically represented below.

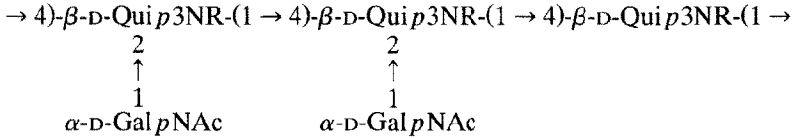

TABLE IV

Proton chemical shifts (ppm) and $J_{C\text{-}1,H\text{-}1}$ (Hz) values for the sugar residues in the *Vibrio mimicus* W-26768 O-antigen polysaccharide

Residue	H-1	H-2	H-3	H-4	H-5	H-6		C-1	$J_{C\text{-}1,H\text{-}1}$
α-D-Gal*p*NAc-(1 →	5.39	4.19	3.68	3.9	4.15	3.86	3.79	95.8	178
→ 2,4)-β-D-Qui*p*3NR-(1 →	4.61	3.43	4.11	3.66	3.63	1.34		103.0	165
→ 4)-β-D-Qui*p*3NR-(1 →	4.46	3.28	3.92	3.47	3.69	1.34		103.6	160
$-COCH_2CH(OH)CH_3$		2.40	4.21	1.23					

The cleavage of the O-antigen polysaccharide from the core on treatment with aqueous 48% hydrogen fluoride was fortuitous, but there is no indication of the type of linkage that was actually broken during this treatment.

EXPERIMENTAL

General methods.—Concentrations were performed under diminished pressure at $< 40°C$ or at room temperature by flushing with air. For GLC, a Hewlett–Packard 5830 instrument, a flame-ionisation detector, and a DB-225 fused-silica capillary column were used. Effective carbon response factors[6] were used for calculation of the molecular proportions of alditol acetates and partially methylated alditol acetates. GLC–MS was performed on a Hewlett–Packard 5970 instrument.

NMR spectra of solutions in D_2O were recorded at 70°C (^{13}C) and 85°C (^1H), using a Varian Unity 500, JEOL GX-400, or GX-270 instrument. Chemical shifts are reported in ppm, using acetone (δ 31.07) for ^{13}C and sodium 3-trimethylsilylpropanoate-d_4 (δ 0.00) for ^1H as internal references. The 2-D experiments were performed using standard pulse sequences available in the JEOL software.

Fractionations on Bio-Gel, using water containing 1% of 1-butanol as irrigant, were monitored with a differential refractometer. Optical rotations were measured at room temperature ($\sim 22°C$) with a Perkin–Elmer 241 instrument.

Preparation of the PS.—The LPS (600 mg) in aq 1% acetic acid (400 mL) was kept for 90 min at 100°C, cooled, and centrifuged. The supernatant solution was extracted with diethyl ether, freeze-dried, and fractionated on a column (90×2.6 cm) of Bio-Gel P-10. Two fractions, PS (200 mg) and CPS (50 mg), were obtained.

Treatment with aqueous hydrogen fluoride.—The PS (60 mg) was dissolved in aq 48% HF (2 mL) and kept for 24 h at 4°C. Diethyl ether (6 mL) was added and the HF–diethyl ether removed by flushing with air. The procedure was repeated twice and the last traces were removed under reduced pressure. The product in water (2 mL) was treated with $NaBH_4$ (50 mg) for 2 h at room temperature. After conventional work-up, the product was fractionated on a column of Bio-Gel P-2. Two fractions, PSHF (47 mg) and CPSHF (9 mg), were obtained.

Sugar and methylation analyses.—A solution of the polysaccharide (~ 1 mg) in 2 M aq CF_3CO_2H (0.5 mL) was kept in a closed vial at 120°C for 3 h. The sugars in the hydrolysate were then converted into alditol acetates by conventional methods.

Methylation analyses were performed as previously described[8]. Products were recovered by reversed phase chromatography on Sep-Pak C_{18} cartridges[9]. The permethylated material was hydrolysed with 2 M aq CF_3CO_2H at 120°C for 3 h. The partially methylated sugars in the hydrolysate were then converted into their alditol acetates by conventional methods.

The hydrolysis of polysaccharides and methylated polysaccharides was also replaced by solvolysis with anhyd HF. The sample (~ 1 mg) was dried (P_2O_5), dissolved in anhyd HF (~ 1 mL), and kept for 3 h at room temperature. The HF

was evaporated, and the residue treated with aq 50% acetic acid for 8 h at room temperature, and concentrated.

Isolation of monomeric sugars.—In a separate experiment, PSHF (3 mg) was solvolysed as above and the resulting sugars were separated on a column (90 × 2.6 cm) of Bio-Gel P-2. Two components, identified by ^{1}H NMR spectroscopy as D-Qui3NR and D-GalNAc, were obtained.

Identification of (R)-3-hydroxybutyric acid.—The PS (10 mg) was treated with 2 M CF_3CO_2H (5 mL) for 4 h at 120°C, the solution concentrated, and the product extracted with EtOAc (5 × 3 mL). The extract was dried ($MgSO_4$), concentrated, and dissolved in M methanolic HCl. This solution, in a sealed tube, was kept at 80°C for 16 h, neutralised with Ag_2CO_3, and centrifuged. The supernatant solution was concentrated by flushing with air at 10°C, and dissolved in a mixture of trifluoroacetic anhydride (50 μL) and CH_2Cl_2 (200 μL). The mixture was kept, in a sealed tube, at 100°C for 60 min, concentrated as above, dissolved in EtOAc, and analysed by GLC on an FS-LIPODEX A column. The product had the same retention time as authentic methyl (*R*)-3-trifluoroacetoxybutyrate, but was well separated from the corresponding (*S*) derivative. The mass spectra, obtained by GLC–MS on an HP-5 column, of the product and the authentic derivative were identical.

Smith degradation.—A solution of the PSHF (5 mg) and sodium metaperiodate (45 mg) in 0.1 M sodium acetate buffer of pH 3.9 (5 mL) was kept in the dark for 30 h at 4°C. Excess of periodate was reduced with ethylene glycol (0.1 mL), $NaBH_4$ (200 mg) was added, and the solution was kept for 16 h at room temperature. After conventional work-up, the polyalcohol was isolated by chromatography on a column (90 × 2.6 cm) of Bio-Gel P-2. A solution of the polyalcohol in 0.5 M CF_3CO_2H (2 mL) was kept for 90 h at room temperature, diluted with water (25 mL), and freeze-dried. The product was fractionated on the same column, giving a polymeric (2 mg) and a low molecular weight fraction (1 mg).

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REFERENCES

1 M. Mahbubur Rahman, F. Qadri, M.J. Albert, A. Hossain, and M. Mosihuzzaman, *Microbiol. Immunol.*, 136 (1992) 327–338.
2 O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
3 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
4 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 77 (1979) 1–7.

5 H.T. Clarke, *J. Org. Chem.*, 24 (1959) 1610–1611.
6 D.P. Sweet, R.H. Shapiro, and P. Albersheim, *Carbohydr. Res.*, 40 (1975) 217–225.
7 I.J. Goldstein, G.W. Hay, B.A. Lewis, and F. Smith, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
8 P.-E. Jansson, L. Kenne, B. Lindberg, H. Liedgren, and J. Lönngren, *Chem. Commun. Univ. Stockholm*, 8 (1976) 1–75.
9 T.J. Waeghe, A.G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.