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STRUCTURAL STUDIES OF THE O-ANTIGEN FROM *Vibrio cholerae* O:21*

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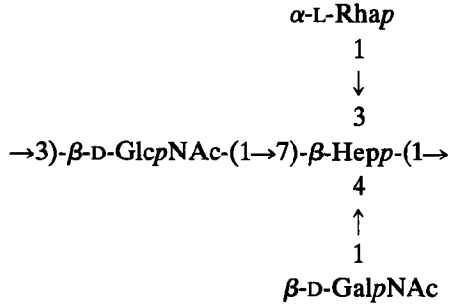
ABSTRACT

The O-antigen from *Vibrio cholerae* O:21 has been investigated, using n.m.r. spectroscopy, methylation analysis, and Smith degradation as the main methods. It is concluded that the O-antigen is composed of tetrasaccharide repeating-units having the following structure (in which Hep = D-*glycero*-D-*manno*-heptose).



INTRODUCTION

The species *Vibrio cholerae* is divided into several serogroups on the basis of their O-antigens. The disease Asiatic cholera is caused only by strains belonging to serogroup O:1. In addition, there are at least 72 additional serogroups of *V. cholerae,* collectively known as non-O:1 *V. cholerae.* In the past, the non-O:1 *V. cholerae* have been inappropriately designated[1] as nonagglutinating (NAG) or noncholera vibrios (NCV).

Non-O:1 *V. cholerae* have caused sporadic cases of gastrointestinal illness and have occasionally been isolated from persons with extraintestinal disease[2]. A

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cholera-like enterotoxin having biological and immunological properties identical to those of cholera toxin, but with different physiochemical properties, has been isolated from some non-O:1 *V. cholerae*[3]. We now report structural studies of the O-antigen from *V. cholerae* O:21.

RESULTS AND DISCUSSION

Treatment of the *V. cholerae* O:21 LPS with aqueous acetic acid of pH 3.1 at 100° for 6 h gave lipid A and a polysaccharide PS. These hydrolysis conditions are more severe than those required for most LPS, but similar to those used[4] for *V. cholerae* O:1.

On acid hydrolysis, the PS yielded approximately equimolar amounts of L-rhamnose, 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, and a heptose. The amino sugars are *N*-acetylated in the PS, as shown by n.m.r. data (see below). The absolute configurations of the first three sugars were determined using the procedure devised by Gerwig *et al.*[5]. The alditol acetate of the heptose had the same retention time in g.l.c. as that obtained from D-*glycero*-D-*manno*-heptose, but was well separated from the corresponding L-*glycero*-D-*manno* derivative. The heptose was transformed into the methyl glycoside penta-acetate by methanolysis followed by acetylation. The ^{1}H-n.m.r. spectrum of this compound demonstrated that the aglycon was axial and that the configuration of C-2–C-5 was *manno*, which was confirmed by comparison with data for methyl α-D-mannopyranoside tetra-acetate (Table I). The acetylated glycoside had $[\alpha]_{578}^{24}$ +74° (*c* 0.1, chloroform). This value is not very accurate because of the small amount of substance available, but the positive value demonstrates that the glycoside is an α-D-*manno* derivative. From the combined evidence, it is concluded that the glycoside is methyl D-*glycero*-α-D-*manno*-heptopyranoside and, consequently, that the sugar is D-*glycero*-D-*manno*-heptose.

TABLE I

^{1}H-N.M.R. DATA FOR FULLY ACETYLATED METHYL D-*glycero*-α-D-*manno*-HEPTOPYRANOSIDE (A) AND METHYL α-D-MANNOPYRANOSIDE (B)

Compound	*Chemical shifts (δ)*									
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*H-7*	*H-7'*	*OCH$_3$*
A	4.684	5.198	5.302	5.274	3.969	5.194	—	4.270	4.439	3.393
B	4.723	5.244	5.339	5.284	3.985	4.125	4.294	—	—	3.414
	Coupling constants (Hz)									
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$	$J_{5,6'}$	$J_{6,6'}$	$J_{6,7}$	$J_{6,7'}$	$J_{7,7'}$
A	1.7	3.4	9.8	9.2	2.9	—	—	3.4	6.5	12.1
B	1.7	3.4	10.0	10.0	2.7	5.4	12.2	—	—	—

The ^{13}C- and ^{1}-H-n.m.r. spectra of the PS revealed that it contained *O*-acetyl groups, the ratio between *N*-acetyl and *O*-acetyl being ~2:0.2. In order to obtain simpler n.m.r. spectra, the PS was *O*-deacetylated. The ^{1}H- and ^{13}C-n.m.r. spectra together with a two-dimensional C,H chemical shift-correlated spectrum then contained, *inter alia,* signals for four anomeric carbons and protons at δ 101.96 ($J_{C,H}$ 162 Hz), 4.413 ($J_{1,2}$ 8.1 Hz); 101.93 ($J_{C,H}$ 160 Hz), 4.607 ($J_{1,2}$ 8.2 Hz); 101.38 ($J_{C,H}$ 160 Hz), 4.657 ($J_{1,2}$ not resolved, $\nu_{1/2}$ 3.7 Hz); and 97.28 ($J_{C,H}$ 170 Hz), 5.005 ($J_{1,2}$ not resolved, $\nu_{1/2}$ 3.1 Hz). A fifth signal in the anomeric region at δ 4.596 (m) was assigned to H-5 of the L-rhamnose residue by a COSY spectrum. The ^{13}C-n.m.r. spectrum further showed signals for two *N*-acetyl groups (δ 175.48, 175.34, 23.24, 23.20), two *N*-substituted carbons (δ 55.36, 53.78), and a *C*-methyl group (δ 16.35). The ^{1}H-n.m.r. spectrum further showed signals for two *N*-acetyl groups (δ 2.072 and 2.051) and a *C*-methyl group (δ 1.289, $J_{5,6}$ 6.5 Hz). From the results discussed above, it is concluded that the PS is composed of tetrasaccharide repeating-units containing one residue each of 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, L-rhamnose, and D-*glycero*-D-*manno*-heptose. The two amino sugars and one of the sugars with the *manno* configuration are β-pyranosidic, and the fourth sugar is α-pyranosidic.

Methylation analysis of the PS (Table II, column A) demonstrated that the L-rhamnopyranosyl and 2-acetamido-2-deoxy-D-galactopyranosyl residues are terminal, that the 2-acetamido-2-deoxy-D-glucopyranosyl residue is linked through O-3, and that the heptopyranosyl residue is linked through O-3, O-4, and O-7.

The terminal sugars in the PS were eliminated by Smith degradation[6] (periodate oxidation, borohydride reduction, and hydrolysis with acid under mild conditions). Methylation analysis of the product (Table II, column B) indicated that it is a linear polysaccharide, in which the heptose is linked through O-7. The ^{1}H-n.m.r. spectrum of the product showed, *inter alia,* signals for two anomeric protons at δ 4.669 ($J_{1,2}$ 7.4 Hz) and 4.655 ($J_{1,2}$ 1.6 Hz), demonstrating that both

TABLE II

METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED *V. cholerae* O:21 POLYSACCHARIDE

Sugar[a]	T[b]	*Detector response (%)*[c]		
		A	B	C
2,3,4-Rha	0.46	27		10
2,3,4,6-Hep	3.8		55	8
2,3,4,6-GalNAc	5.3	17		28
2,3,6-Hep	6.3			21
2,4,6-GlcNAc	7.8	24	45	21
2,6-Hep	8.8	32		12

[a]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.*; Hep = D-*glycero*-D-*manno*-heptose. [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 an SE-54 fused-silica capillary column at 150°. [c]*A*, PS; *B*, Smith-degraded PS; *C*, PS partially degraded by treatment with acid.

sugars are β-linked, as in **1**. The ^{13}C-n.m.r. spectrum showed, *inter alia,* signals at δ 102.08, 101.63 (anomeric carbons), 55.93 (C-N), 175.19, and 23.13 ($OCCH_3$). As the heptosyl residue is β-linked, the L-rhamnopyranosyl group in the original PS is consequently α-linked.

→3)-β-D-Glc*p*NAc-(1→7)-β-Hep*p*-(1→
1

The PS was treated with 0.2M trifluoroacetic acid in deuterium oxide at 70° and the reaction monitored by ^{1}H-n.m.r. spectroscopy. When the signal at δ 5.005, assigned to H-1 of the α-L-rhamnopyranosyl group, was considerably reduced, the polymeric material was recovered and subjected to methylation analysis (Table II, column C). The decrease of 2,3,4-tri-*O*-methyl-L-rhamnose and 2,6-di-*O*-methylheptose, as compared to the analysis of the original PS, and the appearance of 2,3,6-tri-*O*-methylheptose, demonstrate that the α-L-rhamnopyranosyl group is linked to O-3 of the heptosyl residue. This could also be inferred from the ^{13}C-n.m.r. spectrum of the PS. The signal for C-1 of the α-L-rhamnopyranosyl group appears at high field (δ 97.28), which demonstrates that it is linked to O-3 of a sugar having the D-*manno* configuration[7,8].

From the results discussed above, it is evident that the O-antigen of *Vibrio cholerae* O:21 is composed of tetrasaccharide repeating-units with the structure **2** (Hep = D-*glycero*-D-*manno*-heptose).

α-L-Rha*p*
1
↓
3
→3)-β-D-Glc*p*NAc-(1→7)-β-Hep*p*-(1→
4
↑
1
β-D-Gal*p*NAc
2

In the ^{1}H-n.m.r. spectrum of the original PS, the signals for *O*-acetyl groups appeared at δ 2.211 and 2.185. The *O*-acetyl group giving the stronger signal (δ 2.211) was located at O-4 of the α-L-rhamnopyranosyl group, as the signal for H-6 at δ 1.289 ($J_{5,6}$ 6.5 Hz) was partially shifted to δ 1.170 ($J_{5,6}$ 6.3 Hz). The corresponding shifts for H-6 of methyl α-D-glucopyranoside on acetylation at O-2, O-3, or O-4 were +0.02, +0.02, and −0.16 p.p.m., respectively. A small signal in the ^{1}H-n.m.r. spectrum at δ 1.300, which overlapped the signal at δ 1.289, indicated that some α-L-rhamnopyranosyl groups were acetylated at position 3. It is expected that the *O*-acetyl groups should be distributed between positions 2, 3, and 4 of the rhamnosyl group because of *O*-acetyl migration. In agreement with this conclusion,

the signal for C-6 of this residue at δ 16.35 was partially shifted to δ 16.15. Small negative shifts (0.1–0.3 p.p.m.) for C-6 were also observed on monoacetylation of methyl α-D-glucopyranoside at O-2, O-3, or O-4.

D-*glycero*-D-*manno*-Heptose, which is an intermediate in the biosynthesis of L-*glycero*-D-*manno*-heptose, is sometimes incorporated in the core of LPS. This, however, seems to be the first observation of its presence as a residue in the repeating unit of a bacterial antigen.

On treatment of an LPS with acid under mild conditions, it is split into lipid A and a polysaccharide because of the acid-labile glycosidic linkage of a 3-deoxy-D-*manno*-octulosonic acid (KDO) residue. The conditions used to split the *V. cholerae* O:1 LPS are more severe than for most other LPS, and it has been assumed[9] that this LPS does not contain KDO. However, the 5-phosphate of KDO has recently been isolated after acid hydrolysis of *V. cholerae* O:1 LPS[10,11]. The reason why the glycosidic linkage of KDO is more resistant to acid hydrolysis in this and some other LPS is not well understood.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath) or at room temperature by flushing with air. For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separations of alditol acetates and partially methylated alditol acetates were performed on SE-54 fused-silica capillary columns either isothermally or using a temperature gradient of 150→220° at 2°/min. G.l.c.–m.s. was performed with a Hewlett–Packard 5970 instrument, using the above conditions. Identifications from mass spectra were unambiguous and will not be discussed.

Methylation analyses were performed essentially as previously described[12]. Products were recovered by reversed phase chromatography on Sep-Pak C_{18} cartridges[13]. The sample was diluted with an equal volume of water and applied to the cartridge. This was washed with water and acetonitrile–water (15:85), and the sample was then eluted with acetonitrile.

N.m.r. spectra of solutions in deuterium oxide were determined at 70° (^{13}C) or 85° (^{1}H) using a JEOL GX-400 instrument. Chemical shifts are reported in p.p.m., using internal 1,4-dioxane (δ 67.4; ^{13}C) and sodium 3-trimethylsilylpropanoate-d_4 (^{1}H) as references.

Preparation of LPS and PS. — *V. cholerae* 109-68 (O:21) was cultivated in an aerated, stirred, 12-L fermentor at 37° and at a constant pH of 7.2, using a tryptone–yeast extract medium[14,15]. LPS was extracted from the bacteria by the hot phenol–water method[16] and purified by high-speed centrifugation as described earlier[15].

The LPS (500 mg) in aqueous acetic acid of pH 3.1 was kept at 100° for 6 h, cooled, centrifuged, and freeze-dried. The product was fractionated on a column (90 × 3 cm) of Sephadex G-50 that was irrigated with water. A void fraction

consisted of glycogen and the following fraction of PS (48 mg).

The PS was *O*-deacetylated by treatment with 2M aqueous ammonia for 14 h, followed by concentration and freeze-drying. It had $[\alpha]_{578}^{24}$ −54° (*c* 0.5, water).

Sugar analysis. — A solution of PS (1 mg) in 2M aqueous trifluoroacetic acid (0.5 mL) was kept in a closed vial at 120° for 1 h. The sugars in the hydrolysate were then converted into alditol acetates by conventional methods. The relative g.l.c. retention times at 170° were L-Rha 0.32, D-Glc 1.00, D-GlcNAc 14.2, D-GalNAc 16.2, and Hep 18.4.

Smith degradation of the PS. — A solution of the PS (45 mg) and sodium metaperiodate (100 mg) in 0.1M acetate buffer (pH 6, 7 mL) was kept in the dark at 5° for 56 h. Excess of periodate was then reduced with ethylene glycol, and the solution was dialysed overnight and freeze-dried. A solution of the product in water (3 mL) was treated with sodium borohydride (20 mg) for 4 h, and the polyalcohol was recovered by dialysis and freeze-drying. Sugar analysis of a small sample showed that all of the L-rhamnosyl and 2-acetamido-2-deoxy-D-galactosyl residues had been oxidised. A solution of the polyalcohol in 0.5M aqueous trifluoroacetic acid (7 mL) was kept at room temperature for 36 h, then diluted with water (25 mL), and freeze-dried. The modified PS, purified by chromatography on a Sephadex G-50 column, had $[\alpha]_{578}^{24}$ −31° (*c* 0.5, water).

Partial acid hydrolysis of the PS. — A solution of the PS (12 mg) in deuterium oxide, which was 0.2M with respect to trifluoroacetic acid, was kept in an n.m.r. tube at 70°. When the ^{1}H-n.m.r. signal at δ 5.005 had been considerably reduced, the solution was diluted with water and freeze-dried. Fractionation on a Sephadex G-25 column gave a polymeric fraction and L-rhamnose.

Characterisation of the heptose. — The PS obtained on Smith degradation was treated with methanolic M hydrogen chloride at 80° for 16 h. The acid was removed by repeated co-distillations with methanol, and a solution of the methanolysate was passed through a column of Dowex 50 (H^+) resin which removed the amino sugar. The eluate was concentrated, the residue was acetylated, and the product was purified by chromatography on a column of silica gel irrigated with ethyl acetate–toluene (3:7).

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REFERENCES

1 R. SAKAZAKI, K. TAMURA, C. Z. GOMES, AND R. SEN, *Jpn. J. Med. Sci. Biol.*, 23 (1970) 13–20.
2 P. A. BLAKE, *Annu. Rev. Microbiol.*, 34 (1980) 341–367.
3 K. YAMAMOTO, Y. TAKEDA, T. MIWATANI, AND J. P. CRAIG, *Infect. Immun.*, 41 (1983) 896–901.

4 L. KENNE, B. LINDBERG, P. UNGER, B. GUSTAFSSON, AND T. HOLME, *Carbohydr. Res.*, 100 (1982) 341–349.
5 G. J. GERWIG, J. P. KAMERLING, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 77 (1979) 1–7.
6 I. J. GOLDSTEIN, G. W. HAY, B. A. LEWIS, AND F. SMITH, *Methods Carbohydr. Chem.*, 5 (1965) 361–370.
7 N. K. KOCHETKOV, O. S. CHIZHOV, AND A. S. SHASHKOV, *Carbohydr. Res.*, 133 (1984) 173–185.
8 H. BAUMANN, P.-E. JANSSON, AND L. KENNE, *J. Chem. Soc., Perkin Trans. 1*, to be submitted.
9 G. D. F. JACKSON AND J. W. REDMOND, *FEBS Lett.*, 13 (1971) 117–120.
10 H. BRADE, C. GALANOS, AND O. LÜDERITZ, *Eur. J. Biochem.*, 131 (1983) 195–200.
11 H. BRADE, *J. Bacteriol.*, 161 (1985) 795–798.
12 P.-E. JANSSON, L. KENNE, B. LINDBERG, H. LIEDGREN, AND J. LÖNNGREN, *Chem. Commun., Univ. Stockholm*, (1976) 1–75.
13 T. J. WAEGHE, A. G. DARVILL, M. MCNEIL, AND P. ALBERSHEIM, *Carbohydr. Res.*, 123 (1983) 281–304.
14 T. HOLME, S. ARVIDSON, B. LINDHOLM, AND B. PAVLU, *Proc. Biochem.*, 5 (1970) 62–66.
15 B. GUSTAFSSON, T. HOLME, AND A. ROSÉN, *Infect. Immun.*, 38 (1982) 449–454.
16 O. WESTPHAL AND K. JANN, *Methods Carbohydr. Chem.*, 5 (1965) 83.