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Structural studies of *Vibrio fluvialis* M-940 O-antigen polysaccharide

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ABSTRACT

The structure of the *Vibrio fluvialis* M-940 O-antigen polysaccharide has been investigated by sugar and methylation analyses, specific degradations, NMR spectroscopy, and mass spectrometry. It is proposed that it consists of a heptasaccharide unit having the following structure.

α-L-Rha*p*-(1 → 2)-α-L-Fuc*p*-(1 → 2)-α-D-Gal*p*-(1 → 2)-α-L-Fuc*p*-(1 → 3)-β-D-Glc*p*A-(1 → 4)-α-L-
-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1 →

The heptasaccharide is most probably linked to the 3-position of an α-D-galactopyranosyl residue in the core.

INTRODUCTION

Some types of *Vibrio fluvialis* cause diarrhoea and other symptoms[1], although generally not as severe as those caused by *Vibrio cholerae* O:1 (Asian cholera). One of these types was isolated from pond water in Bangladesh, identified as a *V. fluvialis*, and given the number M-940 at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) in Dhaka[2]. The lipopolysaccharide (LPS) was isolated from this strain by conventional methods, and we now report structural studies of this material.

RESULTS AND DISCUSSION

The LPS (900 mg) was treated with 1% aqueous acetic acid at 100°C and the product fractionated on a Bio-Gel P-4 column. One main fraction (180 mg) was

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TABLE I

Sugar analysis of PS, CPS, and degradation products

Sugar	Mole %				
	PS	CPS	PSH-1	PHS-2	PHS-3
L-Rha	17.5		17.9		
L-Fuc	17.5				
D-Glc	18.8	28.5	25.0	17.6	27.3
D-Gal	18.8	10.5	15.9	29.1	26.0
D-GlcN	11.2		18.0	7.2	
D,D-Hep	11.1	47.3	14.6	32.5	36.1
L,D-Hep	5.0	13.7	8.5	13.6	10.7
GlcA	+		+		

eluted shortly after the void volume, followed by a second fraction (90 mg). Sugar analysis of the first fraction, PS, by GLC–MS of the derived alditol acetates, gave L-rhamnose, L-fucose, D-glucose, D-galactose, 2-amino-2-deoxy-D-glucose, D-*glycero*-D-*manno*-heptose (D,D-Hep), and L-*glycero*-D-*manno*-heptose (L,D-Hep) (Table I). Analysis of a carboxyl-reduced[3] sample, prepared using sodium borodeuteride, revealed that it also contained D-glucuronic acid. The absolute configurations of the sugars were determined as described by Gerwig et al.[4,5], which also confirmed their identities.

The second fraction was dephosphorylated by treatment with aqueous 48% hydrofluoric acid. Sugar and methylation analyses before and after this treatment gave similar results, and the sugar analysis of the dephosphorylated material, CPS, is given in Table I. The sugar analysis of CPS gave D-glucose, D-galactose, D,D-Hep, and L,D-Hep, a composition typical of the core of an LPS from a Gram-negative bacterium, although the high proportion of D,D-Hep is unusual. The PS was not dephosphorylated, as this might have caused cleavage of some glycosidic linkages.

The ^{1}H NMR spectrum of the PS (Fig. 1) showed the presence of *N*-acetyl and *C*-methyl groups in the ratio 1:4. The anomeric region was complex with signals for ~12 anomeric protons. The ^{1}H NMR spectrum of the CPS (Fig. 2) was less complicated, but indicated that the fraction was heterogeneous. The signals below 3 ppm may be derived from modified Kdo residues.

Thirteen methylated sugars were found on methylation analysis of the PS, with carboxyl reduction of the methylated product (Table II). Of these, 2,4-di-*O*-Me-D-Glc was not observed when the carboxyl reduction was omitted, and is thus derived from 3-linked D-glucuronic acid. The methylated sugars were identified by GLC–MS of their alditol acetates and, when necessary, by comparison with authentic samples. The alditol acetate from 2,3,4,6-tetra-*O*-Me-D,D-Hep had the same retention time as an authentic sample. Those from 2,3,4,6,7-penta-*O*-Me-D,D-Hep and 3,4,6,7-tetra-*O*-Me-L,D-Hep had different retention times than the corresponding authentic L,D- and D,D-derivatives, respectively. The two remaining heptose deriva-

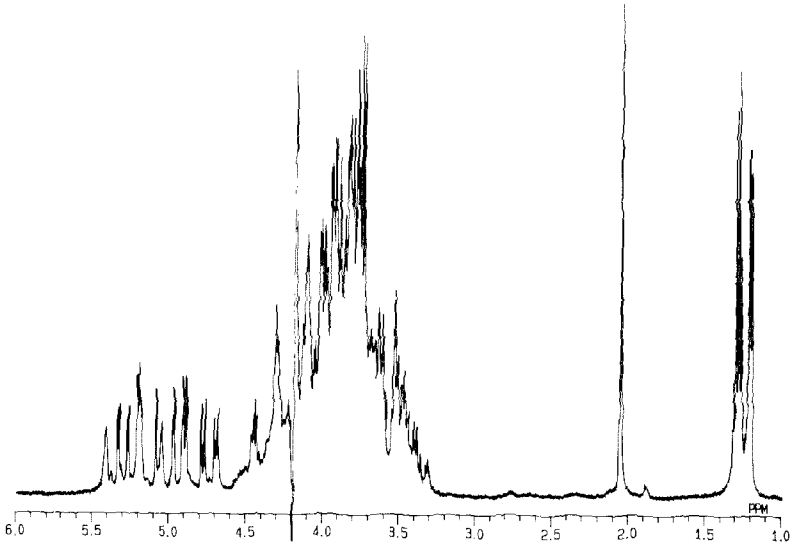

Fig. 1. ^{1}H NMR spectrum of the *V. fluvialis* M-940 O-antigen polysaccharide.

tives were tentatively assumed to be D,D-Hep derivatives, as this is in agreement with the sugar analysis. Seven methylated sugars were observed on methylation analysis of the CPS (Table II), six of which were also found in the former analysis. As the CPS is most probably heterogeneous, it is not possible to draw quantitative

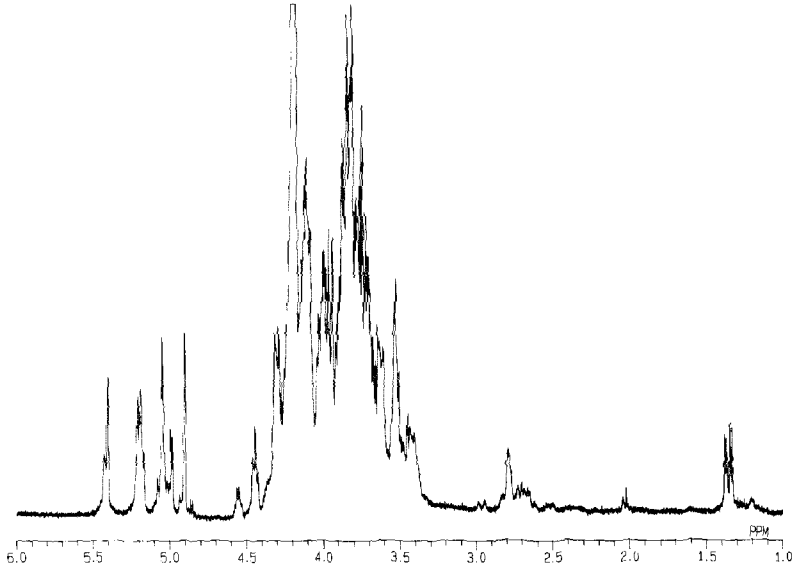

Fig. 2. ^{1}H NMR spectrum of the *V. fluvialis* M-940 core oligosaccharide.

TABLE II

Methylation analysis of PS, carboxyl-reduced PS, and CPS

Sugar [a]	T [b]	Mole%		
		PS	Carboxyl-reduced PS	CPS
2,3,4-Rha	0.63	5.2	4.9	
2,3-Rha	0.88	11.6	8.1	
3,4-Fuc	0.95	14.7	9.8	
2,3,4,6-Gal	1.05	4.3	3.6	15.2
3,4,6-Gal	1.36	7.9	7.1	
2,4,6-Gal	1.38	8.1	8.1	
2,3,4-Glc	1.40	9.2	8.6	21.2
2,3,4,6,7-D,D-Hep	1.48	3.6	4.7	9.4
2,4-Glc	1.67		3.7	
2,3,4,7-D,D-Hep	1.90			10.0
3,4,6,7-L,D-Hep	1.96	8.2	11.0	16.3
2,3,4,6-D,D-Hep	2.03	11.3	12.7	10.7
6,7-D,D-Hep	2.49	6.2	6.9	17.2
2,4,6-GlcNAc	2.76	9.9	10.8	

[a] 2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, 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.

conclusions from these results. The results indicate, however, that the O-antigen polysaccharide, in addition to L-fucose, L-rhamnose, 2-amino-2-deoxy-D-glucose, and D-glucuronic acid, also contains D-galactose, linked through the 2- or 3-position. The presence of 2,3,4,6-tetra-*O*-methyl-D-galactose as the only D-galactose derivative in the methylation analysis of CPS may further indicate that the O-antigen polysaccharide is linked to the D-galactose residue in the core.

In order to obtain further information on the structure of the O-antigen polysaccharide, the PS was subjected to hydrolysis with acid under mild conditions (0.2 M trifluoroacetic acid, 80°C, 4 h), and the hydrolysate was reduced with sodium borohydride and fractionated by chromatography on Bio-Gel P-2. In addition to a high molecular weight fraction, PSH-1, three oligosaccharide-alditols (OA-1, OA-2, and OA-3, eluted in that order) were obtained. Sugar analysis of PSH-1 (Table I) showed that, in addition to the core sugars, it contained L-rhamnose and 2-amino-2-deoxy-D-glucose. Carboxyl reduction showed that it also contained D-glucuronic acid. Therefore, it was hydrolysed again under the same conditions and worked up as above. Two high molecular weight fractions (PSH-2 and PSH-3) and a fourth oligosaccharide-alditol (OA-4) were obtained. Sugar analysis of PSH-2 and PSH-3 (Table I) showed that they contained the same sugar components as the CPS, and that PSH-2 further contained 2-amino-2-deoxy-D-glucose. All the oligosaccharides were purified by reversed phase chromatography on a C18 column.

Hydrolysis of OA-3, followed by reduction with sodium borodeuteride, yielded L-fucitol and D-galactitol-1*d*. Methylation analysis yielded 2,3,4,6-tetra-*O*-methyl-

D-galactose and 1,3,4,5-tetra-*O*-methyl-L-fucitol, demonstrating that the L-fucitol was linked through the 2-position. This was further confirmed by GLC–MS (EI) of methylated OA-3, which gave the expected fragments[6]. The ^{1}H NMR spectrum of OA-3 showed, *inter alia*, one signal in the region for anomeric protons, at δ 5.14, $J_{1,2}$ 2.9 Hz. OA-3 consequently has structure **1**.

α-D-Gal*p*-(1 → 2)-L-Fucitol
1

Analogous studies of OA-2 gave L-fucitol and L-rhamnitol-*1d*, and 2,3,4-tri-*O*-methyl-L-rhamnose and 1,3,4,5-tetra-*O*-methyl-L-fucitol, respectively, demonstrating that it was a disaccharide-alditol in which L-rhamnose was linked to the 2-position of L-fucitol. This was confirmed by GLC–MS of methylated OA-2 as above. The ^{1}H NMR spectrum of OA-2 showed, *inter alia*, one signal in the region for anomeric protons, at δ 5.05, $J_{1,2}$ 1.8 Hz. OA-2 consequently has structure **2**.

α-L-Rha*p*-(1 → 2)-L-Fucitol
2

Sugar analysis of OA-1 gave equal parts of L-fucose, L-rhamnose, D-galactose, and L-fucitol. FAB–MS, in the positive mode, gave the pseudomolecular ions $[M + H]^+$ (*m/z* 621.5) and $[M + Na]^+$ (*m/z* 643.3). This agrees with the molecular weight 620.6, calculated for a tetrasaccharide-alditol composed of these sugars. Methylation analysis gave 2,3,4-tri-*O*-methyl-L-rhamnose, 3,4-di-*O*-methyl-L-fucose, 3,4,6-tri-*O*-methyl-D-galactose, and 1,3,4,5-tetra-*O*-methyl-L-fucitol. FAB–MS of methylated OA-1 gave the pseudomolecular ions $[M + H]^+$ (*m/z* 789.9) and $[M + Na]^+$ (*m/z* 811.9), in agreement with the calculated value for the molecular weight, 788.7. EIMS gave, *inter alia*, the aA_1 ion (*m/z* 189), the baA_1 ion (*m/z* 363), and the dA_1 ion (*m/z* 205), as indicated in **3**, thus establishing the sequence of the sugar residues.

Rha–	–O–Fuc–	–O–Gal–O–	–Fucitol
189	363		205

3

The ^{1}H NMR spectrum of OA-1 showed, *inter alia*, signals for anomeric protons at δ 5.40, $J_{1,2}$ 1.5 Hz; δ 5.12, $J_{1,2}$ 3.7 Hz; and δ 5.09, $J_{1,2}$ 1.5 Hz. Thus, all the sugar residues are α-linked and consequently OA-1 has the structure **4**.

α-L-Rha*p*-(1 → 2)-α-L-Fuc*p*-(1 → 2)-α-D-Gal*p*-(1 → 2)-L-Fucitol
4

The ^{1}H NMR spectrum of OA-4 showed, *inter alia*, one signal in the region for anomeric protons, at δ 4.59, $J_{1,2}$ 7.5 Hz, indicating that it was a disaccharide-alditol. Sugar analysis gave only L-rhamnitol, but, after carboxyl reduction, D-glucose was also obtained. Methylation analysis of OA-4, with carboxyl reduction of the methylated product, yielded 2,3,4-tri-*O*-methyl-D-glucose and 1,2,3,5-tetra-*O*-methyl-L-rhamnitol, demonstrating that OA-4 has structure **5**. As neither D-

glucuronic acid nor L-rhamnose was found in the CPS, it is concluded that OA-4 is derived from the O-antigen part of the PS.

β-D-Glc*p*A-(1 → 4)-L-Rhamnitol

5

In order to obtain oligosaccharides containing 2-amino-2-deoxy-D-glucose, the PS was *N*-deacetylated by treatment with sodium hydroxide and thiophenol in a mixture of water and dimethyl sulfoxide[7]. The product, the ^{1}H NMR spectrum of which did not contain signals for *N*-acetyl groups, was hydrolysed with 2 M trifluoroacetic acid at 120°C for 2 h and the hydrolysate fractionated by chromatography on a column of Dowex 50 (H^+). Neutral components were eluted with water and basic components with M trifluoroacetic acid. The latter fraction was reduced with sodium borodeuteride, acetylated, *O*-deacetylated by treatment with aqueous ammonia, and purified by chromatography on Bio-Gel P-2, giving oligosaccharide-alditol OA-5. Sugar analysis of OA-5 gave 2-amino-2-deoxy-D-glucose and D-galactitol-*1d*, and methylation analysis gave 1,2,4,5,6-penta-*O*-methyl-D-galactitol-*1d* and 2-deoxy-3,4,6-tri-*O*-methyl-2-*N*-methylacetamido-D-glucose. The ^{1}H NMR spectrum showed, *inter alia*, signals for one anomeric proton at δ 4.62, $J_{1,2}$ 8.0 Hz, and for one *N*-acetyl group at δ 2.06. OA-5 consequently has structure **6**.

β-D-Glc*p*NAc-(1 → 3)-D-Galactitol-*1d*

6

All of the five oligosaccharide-alditols isolated contain at least one component that is present in the PS but not in the CPS, and which, consequently, should be derived from the O-antigenic side-chain. The results of the sugar and methylation analyses of the PS also indicate that all structural elements present in the O-antigenic side-chain are accounted for by these oligosaccharide-alditols. As this side-chain contains terminal L-rhamnose, a structural element corresponding to OA-1 should occupy a terminal position. The presence of terminal D-galactose in the CPS but 3-linked D-galactose in the PS further indicates that a structural element corresponding to OA-5 represents the linkage between the O-antigenic side-chain and the core. The disaccharide element corresponding to OA-4 should therefore be enclosed by those corresponding to OA-1 and OA-5. In the ^{1}H NMR spectrum of the PS, there are signals for two anomeric protons at δ 4.70, $J_{1,2}$ 7.6 Hz, and δ 4.78, $J_{1,2}$ 8.4 Hz (Table III), accounted for by the β-D-Glc*p*A and β-D-Glc*p*NAc residues in OA-4 and OA-5. The other signals in the region for anomeric protons were all at lower fields and had small coupling constants. The signals for the corresponding anomeric carbon atoms, at δ 104.7 and 103.3, as determined by an HMQC experiment, showed $J_{C\text{-}1,H\text{-}1}$ values of 163 and 165 Hz, respectively (Table III), typical for β-pyranosidic residues. All the other $J_{C\text{-}1,H\text{-}1}$ values were larger, between 169 and 174 Hz, as expected for α-pyranosidic residues. The two residues above are thus the only β-pyranosidic residues in the PS. From the combined evidence, it is therefore proposed that the O-antigenic

TABLE III

^{1}H NMR and ^{13}C NMR data for the *V. fluvialis* M-940 PS

H-1		C-1	
δ (ppm)	$J_{1,2}$ (Hz)	δ (ppm)	$J_{C\text{-}1,H\text{-}1}$ (Hz)
5.41	n.r. [a]		
5.33	3.6	98.75	171.8
5.27	3.8	100.40	174.4
5.20	4.0	97.90	170.6
5.09	1.3	98.60	170.6
5.05	n.r. [b]	102.50	169.4
4.97	3.3	99.90	169.6
4.91	1.6	100.90	169.6
4.89	1.6	102.40	169.4
4.78	8.4	103.25	164.6
4.70	7.6	104.70	163.4

[a] Not resolved, half-width of 6.6 Hz (400 MHz). [b] Not resolved, half-width of 8.0 Hz (400 MHz).

side-chain of the *Vibrio fluvialis* M-940 LPS is a heptasaccharide with the structure **7**. This is most probably linked to the 3-position of an α-D-galactopyranosyl residue in the core. Several LPS contain D-*glycero*-D-*manno*-heptose in their core, but the high proportion of this sugar in the present LPS in unusual.

α-L-Rha*p*-(1 → 2)-α-L-Fuc*p*-(1 → 2)-α-D-Gal*p*-(1 → 2)-α-L-Fuc*p*-(1 → 3)-β-D-

-Glc*p*A-(1 → 4)-α-L-Rha*p*-(1 → 3)-β-D-Glc*p*NAc-(1 →

7

EXPERIMENTAL

General methods.—Evaporations were performed under diminished pressure at < 40°C or at room temperature by flushing with air. For GlC, a Hewlett–Packard 5830 instrument, with a flame-ionisation detector, and a DB-225 fused-silica capillary column were used. Effective carbon response factors[8] 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. A JEOL SX-102 instrument was used for the FAB–MS. Ions were produced by a beam of Xe atoms (4–6 keV), using a matrix of 1 : 1 glycerol–thioglycerol.

NMR spectra of solutions in D_2O were recorded at 70°C (^{13}C) and 85°C (^{1}H), using a 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 ^{1}H as internal references. The HMQC experiment was performed using standard pulse sequences available in the JEOL software.

Fractionations on Bio-Gel, using aq 1% 1-butanol as irrigant, were monitored with a differential refractometer. The oligosaccharides were purified by reversed

phase chromatography, performed on a Shimadzu LC6A HPLC system including a UV detector. The C18 column (μBondapak) used was irrigated with 3:97 acetonitrile–water.

Isolation of the PS.—A subculture of *V. fluvialis* M-940 was grown overnight on a gelatin agar plate at 37°C. A single colony was selected and was incubated overnight in a Richardson's broth medium. The broth was spread over gelatin agar plates and incubated overnight at 37°C. The bacteria were collected and the LPS isolated from the freeze-dried cells by the phenol–water method[9].

The LPS (900 mg) in aq 1% acetic acid was kept at 100°C for 90 min, 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-4. Two main fractions, PS (180 mg) and CPS (90 mg), were obtained.

Dephosphorylation of CPS.—A solution of CPS (30 mg) in aq 48% HF (2 mL) was kept for 24 h at 4°C. Diethyl ether (6 mL) was added and the HF removed by flushing with air and then under reduced pressure. The procedure was repeated to remove all of the HF. The oligosaccharide was reduced with $NaBH_4$ (30 mg) and purified on a column (90 × 2.6 cm) of Bio-Gel P-2 irrigated with water. A phosphate analysis[10] demonstrated that the product did not contain phosphate.

Hydrolysis with acid under mild conditions.—A solution of the PS (100 mg) in 0.2 M CF_3CO_4H (5 mL) was kept at 80°C for 4 h, then concentrated to dryness. The residue was dissolved in aq M NH_4OH (2 mL), $NaBH_4$ (30 mg) was added, and the solution was kept at room temperature for 2 h. After conventional work-up, the product was fractionated on a column (90 × 2.6 cm) of Bio-Gel P-2. Fractions PSH-1 (35 mg), OA-1 (25 mg), OA-2 (10 mg), and OA-3 (6 mg) were collected. Each fraction was further purified on a reversed phase C18 column.

Fraction PSH-1 (30 mg) was hydrolysed using the same conditions as before and the hydrolysate worked up as above, yielding fractions PSH-2 (7 mg), PSH-3 (13 mg), and OA-4 (5 mg).

Sugar analysis.—The solutions of PS, CPS, PSH, and oligosaccharides (1 mg each) in 2 M aq CF_3CO_2H (0.5 mL) were kept in closed vials at 120°C for 3 h. The sugars in the hydrolysate were then converted into alditol acetates by conventional methods.

Methylation analysis.—Methylation analyses were performed as previously described[11]. Products were recovered by reversed phase chromatography on a Sep-Pak C18 cartridge[12]. Carboxyl-reduction of methylated PS (1.5 mg) in tetrahydrofuran (0.2 mL) was performed by treatment with M lithium triethyl borodeuteride in tetrahydrofuran (0.2 mL) for 1 h at 0°C. 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.

N-Deacetylation and hydrolysis of the PS.—A solution of the PS (5 mg) and NaOH (40 mg) in a mixture of thiophenol (10 μL), water (0.4 mL), and Me_2SO (1.5 mL) was kept at 80°C for 4 h. The cooled solution was neutralised with M

HCl, then concentrated to dryness, and the product was purified by chromatography on a column (50 × 1.6 cm) of Bio-Gel P-2. The product (3 mg) was treated with 2 M CF_3CO_2H (2 mL) at 120°C for 2 h, the solution was concentrated to dryness, and a solution of the residue in water was added to a column (3 × 0.5 cm) of Dowex 50 (H^+) resin, which was irrigated first with water and then with M CF_3CO_2H. The acidic eluate was concentrated to dryness and then reduced with $NaBH_4$ (10 mg) in water (2 mL). After conventional work-up, the material was acetylated (1:1 Ac_2O–pyridine, 1 mL for 2 h at 40°C), and the product *O*-deacetylated by treatment with aq 25% ammonia (2 mL) in 1,4-dioxane (0.5 mL) for 16 h at room temperature. Excess of ammonia was removed by repeated codistillation with water (3 × 2 mL) and the product was fractionated on a column (50 × 1.6 cm) of Bio-Gel P-2, yielding OA-5 (0.5 mg).

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