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Occurrence of 2,4-dihydroxy-3,3,4-trimethylpyroglutamic acid as an *N*-acyl substituent in the O-polysaccharide chain of the lipopolysaccharide of *Vibrio anguillarum* V-123 *

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ABSTRACT

A new and highly branched amino acid was found as an *N*-acyl substituent of the O-polysaccharide chain obtained from the lipopolysaccharide of *Vibrio anguillarum* V-123 (serogroup JO-2) and evidence is presented to support the structure as 2,4-dihydroxy-3,3,4-trimethylpyroglutamic acid. Acid hydrolysis of the O-polysaccharide gave the lactone of 2,4-dihydroxy-3,3,4-trimethylglutamic acid, together with 2-amino-2-deoxy-D-galacturonic acid, 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine), and 4-amino-4,6-dideoxy-D-glucose (D-viosamine). Degradation of the O-polysaccharide with hydrogen fluoride yielded a fragment (H1) that was indicated by the ^{1}H-NMR data to be 4-amino-4,6-dideoxy-D-glucose *N*-acetylated with 2,4-dihydroxy-3,3,4-trimethylpyroglutamic acid. The configuration of the amino acid was not determined.

INTRODUCTION

The Gram-negative bacterium, *Vibrio anguillarum*, is an important pathogen of marine and estuarine fish that causes haemorrhagic septicemia[1,2]. In fish culture, this micro-organism is a major cause of diseases. *V. anguillarum* has been classified tentatively into 2 or 3 groups on the basis of the serological properties of somatic antigens. The appearance of antibiotic-resistant *V. anguillarum* strains is a severe problem in the control of fish disease and there has been a trial of vaccines[3]. However, little attention has been paid to the structures and antigenic properties of *Vibrio* cell-surface components. Bonoub et al.[4] proposed a structure for the O-polysaccharide chain isolated from the LPS of *V. anguillarum* strain ST-40 and indicated 3-acetamido-3,6-dideoxy-4-*O*-methyl-L-glucose to be the immunodeter-

* Studies of the Structure of the Lipopolysaccharide of *Vibrio anguillarum* V-123, Part I.
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minant. Ezura et al.[5] reported that the majority of causative organisms of fish vibrios in Japan is *V. anguillarum* and assigned 190 isolated strains to one of four groups (JO-1/3 and unclassified) on the basis of their serological properties. Eleven strains belonging to serogroup JO-2 were isolated from *Pleocaglossus altivalis* and *Anguilla* sp.

We now report the characterisation of 2-amino-2-deoxy-D-galacturonic acid, 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine), and 4-amino-4,6-dideoxy-D-glucose (D-viosamine) *N*-acylated with 2,4-dihydroxy-3,3,4-trimethylpyroglutamic acid as constituents of the LPS of *V. anguillarum* V-123 (serogroup JO-2). The structure of the O-polysaccharide is reported in the following paper[6].

EXPERIMENTAL

Preparation of LPS and O-polysaccharide.—*V. anguillarum* strain V-123 (serogroup JO-2)[5], kindly provided by Dr. Y. Ezura (Faculty of Fisheries, Hokkaido University, Hakodate), was cultivated to the late exponential phase in a medium containing 1.5% NaCl, 0.5% polypeptone, 0.5% yeast extract, 0.5% meat broth, 0.2% D-glucose, and 0.2% KH_2PO_4 (pH 7.6). Harvested cells (~ 444 g, wet weight) from a 135-L culture were defatted by successive extraction with acetone and $CHCl_3$. The LPS was extracted[7] from the defatted cells (100 g) with aq 45% phenol and thoroughly dialysed to give the crude LPS. A portion of the aqueous solution was centrifuged at 105 000*g* for 4 h and the LPS recovered from the pellet was the purified preparation. Capsular polysaccharides in the supernatant solution were isolated by treatment with aq 1% Cetavlon and EtOH precipitation.

The crude LPS (9 g) was hydrolysed with aq 2% acetic acid (750 mL) at 100° for 2 h. The hydrolysate was extracted with $CHCl_3$ in order to remove lipids and leave the crude O-polysaccharide (4 g). This crude preparation was subjected to gel chromatography on a column (2.5 × 100 cm) of Sephadex G-50 by elution with 50 mM $(NH_4)_2CO_3$. Most of the hexosamine-containing polysaccharides were excluded, and they were combined, dialysed against deionised water, and subjected to ion-exchange chromatography on a column (1.5 × 4 cm) of DEAE-Sephacel equilibrated with 5 mM ammonium acetate (pH 7.5). The column was eluted with a linear gradient of 0 → 0.4 M NaCl. Two hexosamine-containing polysaccharides (PS-1 and PS-2) were isolated. PS-1 (377 mg) was used as the O-polysaccharide preparation after thorough dialysis against deionised water. PS-2 (435 mg) appeared to have been derived from capsular materials.

Isolation of the components of an acid hydrolysate of PS-1.—PS-1 (20 mg) was hydrolysed in 4 M HCl 92 mL) at 100° for 1 h. The hydrolysate was lyophilised and the residue was subjected to gel chromatography on a column (1 × 145 cm) of Cellulofine GCL-25-m in 50 mM pyridine–acetic acid–water buffer (pH 5.0), giving 4 hexosamine-containing fractions. The components of the third fraction were fractionated further by ion-exchange chromatography on a column (0.6 × 20 cm) of Dowex 50W (H^+) resin with 0.33 M HCl into hexosamine-containing (A3-1)

and ninhydrin-positive (A3-2) components. A3-1 (1.2 mg) and A3-2 (3.1 mg) were 2-amino-2-deoxygalacturonic acid (GalNA) and X, respectively. Using a similar procedure, 4-amino-4,6-dideoxyglucose [viosamine (VioN), Y, 2.0 mg] and quinovosamine (QuiN, 1.3 mg) were isolated from the fourth fraction, a trisaccharide (Al, 1.8 mg) from the first fraction, and a disaccharide (A2, 9.9 mg) from the second fraction.

Isolation of an amino acid-containing fragment from the hydrogen fluoride hydrolysate of PS-1.—PS-1 (50 mg) was treated with aq 55% HF (5 mL) at 25° for 24 h. After removal of the HF in a stream of air, the hydrolysate was subjected to gel chromatography on Cellulofine GCL-25-m under the conditions described above. Several hexosamine-containing components were isolated, one of which was eluted near to the position of 2-acetamido-2-deoxy-D-glucose used as a standard and was purified further by ion-exchange chromatography on Dowex 50W (H^+) resin to give a neutral fragment (H1).

Analytical methods.—Amino sugars and amino acids were analysed with a Sibata amino acid analyser, using a column (0.5 × 100 cm) of Aminex A-5 (Bio-Rad) under the conditions described[8]. In column chromatography, amino sugars and amino acids were monitored by the ninhydrin reaction[9]. Total hexosamine was determined by the method of Tsuji et al.[10] with 2-amino-2-deoxy-D-glucose as the standard after hydrolysis (2 M HCl, 100°, 1 h). Paper electrophoresis (30 V/cm) was carried out on Toyo No. 50 paper in pyridine–acetic acid–water (35:5:960, pH 6.0) and 50 mM formic acid–acetic acid (pH 1.9). Amino sugars and amino acids were detected with ninhydrin. Methylation was performed according to the method of Ciucanu and Kerek[11]. ^{1}H- (internal sodium 3-trimethylsilylpropanesulfonate) and ^{13}C-NMR spectra (external acetone) were obtained at 25° for solutions in 99.96% D_2O or $CDCl_3$ as appropriate with Jeol FX-500 and FX-100 spectrometers, respectively. GLC and GLC–MS were carried out as described[12]. Optical rotations were measured with a JASCO DIP-360 polarimeter on aq 1% solutions at 25°. Other materials were as described[12,13].

RESULTS

Analysis of the O-polysaccharide.—The crude LPS (9 g, ~ 18%), isolated from the defatted cells of *V. anguillarum* V-123 (50.7 g) by extraction with hot aqueous phenol, contained typical core and lipid A components such as 3-deoxy-2-octulosonic acid, heptose, 2-amino-2-deoxyglucose, glucose, and phosphorus, in addition to large proportions of O-polysaccharide components. Mild hydrolysis of the crude LPS with aqueous 2% acetic acid followed by gel chromatography on Sephadex G-50 afforded water-soluble polysaccharides that were separated into 2 hexosamine-containing components (PS-1 and PS-2) by ion-exchange chromatography on DEAE-Sephacel (Fig. 1). The apparent molecular weights of PS-1 and PS-2 were ~ 17000 and ~ 20000, respectively, on the basis of the results of gel filtration on Sephacryl S-200. Thus, two surface polysaccharides, different in

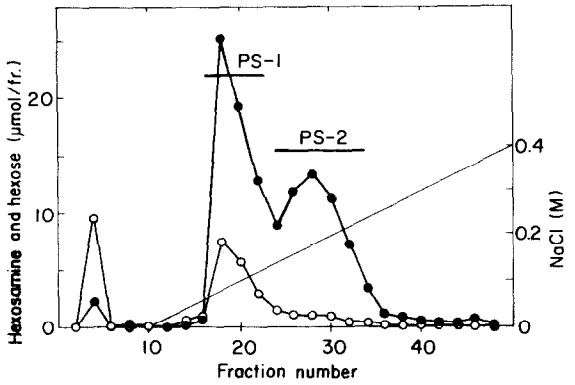


Fig. 1. Ion-exchange chromatography of the crude O-polysaccharide (91 mg, see Experimental) obtained from *V. anguillarum* V-123 LPS on a column (1.5×4 cm) of DEAE-Sephacel equilibrated with 5 mM ammonium acetate (pH 7.5). The column was eluted with the same buffer and then with a linear gradient of NaCl. Fractions (4 mL) were assayed for hexosamine (●) and hexose (○). Fractions indicated by bars were combined to give PS-1 and PS-2.

charge and molecular size but similar in chemical composition, may be present in the crude LPS.

When a portion of the crude LPS was subjected to ultracentrifugation, hexosamine-containing polysaccharides were found in the supernatant solution (capsular polysaccharide) and pellet (LPS) in the ratio 1:4. Mild treatment of the LPS with acid gave a product with chromatographic behavior similar to that of PS-1 and the capsular polysaccharide PS-2. Thus, PS-1 and PS-2 appear to be derived from the LPS and capsular materials, respectively. PS-1 was regarded as the O-polysaccharide.

Acid hydrolysis (4 M HCl, 100°, 4 h) of PS-1 gave unidentified ninhydrin-positive products (X–Z) together with GalNA and QuiN (Fig. 2). Successive chromatography of the hydrolysate on Cellulofine GCL-25-m and Dowex 50W (Fig. 3) gave components A1, A2, A3-1, A3-2, A4-1, and A4-2. Use of the amino acid analyser identified A3-2 as X, A2 as Z (GalNA-QuiN), A3-1 as GalNA, A4-1 as Y (VioN), and A4-2 as QuiN. In addition, Al seemed to be a trisaccharide consisting of GalNA and QuiN in the ratio 2:1. Details of the structure of the O-polysaccharide are described in the following paper[6].

Absolute configuration of sugar components.—The $[\alpha]_D$ values indicated the 2-amino-2-deoxygalacturonic acid (+84°; lit.[14] +84.5°), quinovosamine (+55°; lit.[14] +47.9°), and viosamine (+27°; lit.[15] +20.1°) to be D sugars; X had $[\alpha]_D$ −21°.

Further characterisation of Y.—Because standard 4-amino-4,6-dideoxy-D-glucose (VioN) was not available, the assigned structure of Y was confirmed by EI-mass spectrometry and by ^{1}H-NMR spectroscopy. Compound Y was *N*-acetylated, reduced with $NaBD_4$, and methylated. The EI-mass spectrum of the resulting methylated alditol acetate contained a primary fragment at *m/z* 278 and a series

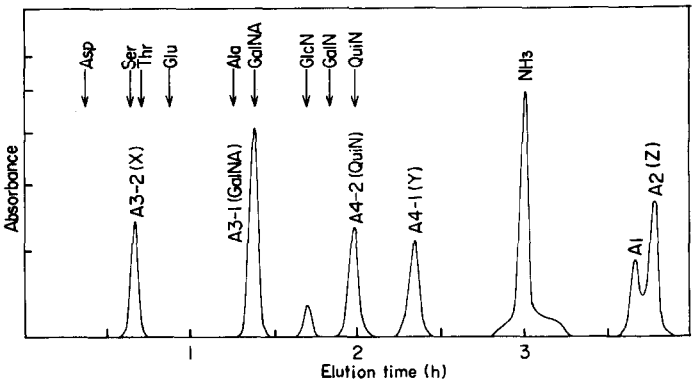


Fig. 2. Analysis of the acid hydrolysate (4 M HCl, 100°, 4 h) of PS-1 (0.5 mg) on an amino acid analyser (Sibata). Arrows show the elution positions of standard amino acids and amino sugars.

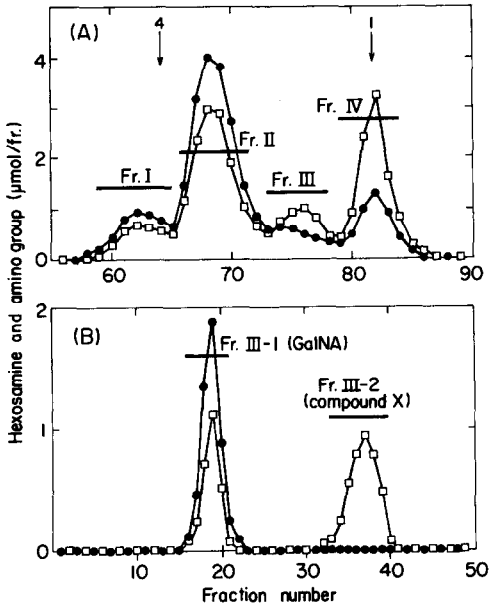


Fig. 3. Separation of X and sugar components from the acid hydrolysate of PS-1: (A) PS-1 (20 mg) was hydrolysed (4 M HCl, 100°, 1 h) and the hydrolysate was applied on a column (1×145 cm) of Cellulofine GCL-25-m in 50 mM pyridine–acetic acid. Fractions (1 mL) were assayed for hexosamine (●) and free amino groups (□). The fractions indicated by bars were combined to give I–IV. The arrows 1 and 4 indicate the elution positions of GlcNAc and $(GlcNAc)_4$. (B) Fraction III from (A) was applied to a column (0.6×20 cm) of Dowex 50W (H^+) resin and eluted with 0.33 M HCl. Fractions (1 mL) were assayed for hexosamine and free amino groups. Fractions indicated by bars were combined to give III-1 and III-2, which correspond to A3-1 (GalNA) and A3-2 (X), respectively.

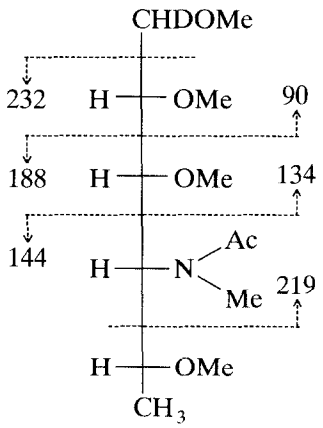
TABLE I

^{1}H-NMR data [a] (δ in ppm, J in Hz) for acetylated Y (4-amino-4,6-dideoxy-D-glucose, VioN) (see Fig. 2)

Atom	Chemical shift	Multiplicity	Coupling constant
α-Form			
H-1	6.301 (6.27)	d (d)	$J_{1,2}$ 3.91 (3.6)
H-2	5.083 (5.05)	dd (dd)	$J_{2,3}$ 10.26 (10.1)
H-3	5.251 (5.22)	t (t)	$J_{3,4}$ 10.25 (10.1)
H-4	*4.020 (4.73)*	q (ddd)	$J_{4,5}$ 9.77 (10.0)
H-5	*3.846 (4.54)*	dq (dq)	$J_{5,6}$ 5.85 (6.0)
H-6	1.243 (1.21)	d (d)	$J_{5,6}$ 5.86 (6.0)
NH	5.486 (5.50)	d (d)	$J_{NH,4}$ 10.25 (9.5)
NAc	1.962 (1.93)	s (s)	
β-Form			
H-1	5.657 (5.63)	d (d)	$J_{1,2}$ 8.31 (8.2)
H-2	5.118 (5.09)	dd (dd)	$J_{2,3}$ 9.77 (9.5)
H-3	5.047 (5.03)	t (t)	$J_{3,4}$ 10.26 (9.7)
H-4	*4.020 (4.72)*	q (ddd)	$J_{4,5}$ 9.77 (9.5)
H-5	*3.582 (4.22)*	dq (dq)	$J_{5,6}$ 6.35 (6.1)
H-6	1.286 (1.26)	d (d)	$J_{5,6}$ 6.34 (6.1)
NH	5.546 (5.64)	d (d)	$J_{NH,4}$ 9.77 (9.5)
NAc	1.962 (1.93)	s (s)	

[a] The data in brackets are for 4-acetamido-1,2,3-tri-*O*-acetyl-4,6-dideoxy-D-glucopyranose[16]. There were signals for six OAc groups in the range 2.012–2.151 ppm.

of peaks at m/z 232, 219, 134, and 90 consistent with the fragmentation shown in **1**.



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The ^{1}H-NMR data ($CDCl_3$) of acetylated Y shown in Table I were largely in accord with those reported[16] for 4-acetamido-1,2,3-tri-*O*-acetyl-4,6-dideoxy-D-glucose and indicated the *gluco* configuration of Y. However, there were considerable differences in the chemical shifts of the H-4 and H-5 resonances in the present and previous data, as shown by the italicised values in Table I. The sequential

relationships of H-1 to H-2 and of H-4 to H-3 and H-5 were confirmed by decoupling experiments and confirmed Y to be 4-amino-4,6-dideoxy-D-glucose (D-viosamine). Other amino sugar components of PS-1 were also characterised as 2-amino-2-deoxy-D-galacturonic acid and 2-amino-2,6-dideoxy-D-glucose (D-quinovosamine) by comparison with the respective standards[13].

Characterisation of X.—Repeated treatment of PS-1 with carbodi-imide / $NaBH_4$ effected only partial carboxyl-reduction of the uronosyl residues, probably owing to the presence of the amide form. Tentative molar ratios, 2:1:1, of GalNA, QuiN, and VioN were obtained from integration of their reporter signals at 5.36 (or 4.96), 4.81, and 4.50 ppm in the ^{1}H-NMR spectrum of PS-1. Thus, 4 amino groups were expected to be present in the repeating unit of the O-polysaccharide chain. However, in the ^{1}H-NMR spectrum of PS-1, only 3 *N*-acyl signals could be assigned (two NAc at 2.00–2.05 ppm and one NCHO at 8.12 ppm) and suggested that X could be the fourth *N*-acyl group.

On the amino acid analyser, X (A3-2) emerged near threonine or serine (Fig. 2). Upon paper electrophoresis, X was separated from threonine, lysine, or amino sugars [distance from origin (cm) at pH 1.9 and 6.0: X, −6.1 and 0.8; threonine, −7.2 and −0.5; lysine, −13.7 and −10.4; GalNA, −8.2 and −0.6; Z (GalNA-QuiN), −10.5 and −5.8; Y (VioN), −10.1 and −8.9; GalN, −9.5 and −9.2]. The electrophoretic behavior suggested that X was an amino acid-like compound with a lactone ring, a carboxyl group, and a ninhydrin-reactive amino group.

The ^{13}C-NMR data (Table II) for X revealed eight carbon atoms with signals at 171.5 and 172.0 (two COOH or COOR), 15.25, 18.00, and 19.69 (three Me), and 91.5, 76.7, and 60.9 ppm. The ^{1}H-NMR spectrum of X (Table II) contained signals at 1.62, 1.68, and 1.69 ppm (3 H each) ascribable to Me groups. Thus, all the hydrogen atoms in X are present as Me groups and the carbon atoms in the backbone chain of X are highly substituted. Furthermore, X exhibited IR absorption at 1792 cm^{-1} (lactone). When X was esterified (methanolic HCl) and then *N*-acetylated, the ^{1}H-NMR spectrum ($CDCl_3$) of the product contained signals at 6.14 (NH), 3.97 (OH), 3.82 (COOMe), and 2.19 ppm (NAc), together with signals for Me groups at 1.18, 1.59, and 1.75 ppm (Table II). Thus, X contained a hydroxyl, an amino, a carboxyl, and 3 methyl groups. Since X was a lactone, the above data indicate a highly branched structure.

Characterisation of alkali-treated X.—When treated mildly with alkali (0.1 M NaOH, 35°, 1 h), X was converted into a ninhydrin-negative product consistent with cleavage of a lactone or lactam ring and recyclisation, and supported by the ^{1}H-NMR data which showed large differences in the chemical shifts of the Me signals (Table II). Furthermore, the ^{1}H-NMR spectrum of the methylated product contained signals at 3.68 (COOMe), 3.16 and 3.38 (two OMe), 2.84 (NMe), and 1.22, 1.36, and 1.45 ppm (three Me). These data are consistent with structure **2** for X and the changes shown in Scheme 1. In addition, neither X nor alkali-treated X was oxidised by $NaIO_4$; thus, the hydroxyl groups in the latter compound (**3**) and the amino and hydroxyl groups in X (**2**) are not vicinal. Structure **2** for X was

TABLE II

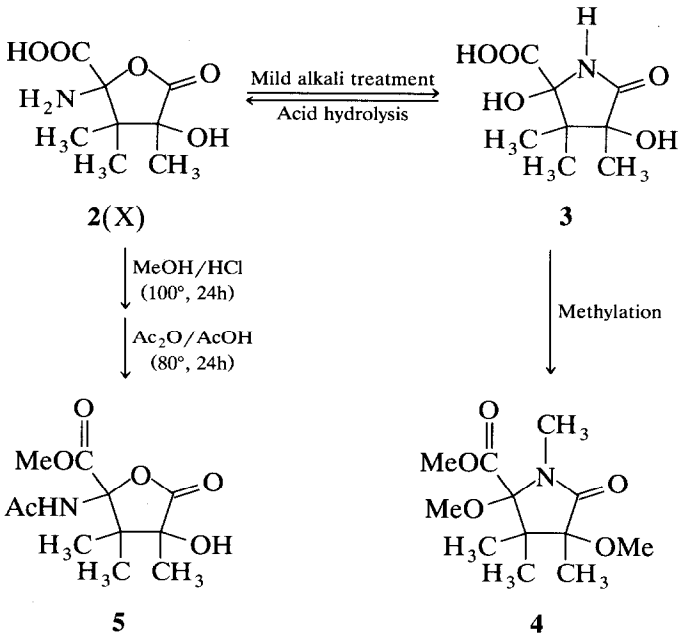
The ^{1}H- and ^{13}C-NMR data for X (**2**), alkali-treated X (**3**), and their derivatives

Derivative	Chemical shift (ppm)			Assignment
X (**2**)	^{13}C	172.0		C=O
		171.5		
		91.7		Ring carbons
		76.7		
		60.9		
		19.69		CMe
		18.00		
		15.25		
	^{1}H	1.69	(3 H)	CMe
		1.68	(3 H)	
		1.62	(3 H)	
Ester of *N*-acetylated X (**5**)	^{1}H	6.14	(1 H)	NH
		3.97	(1 H)	OH
		3.82	(3 H)	COOMe
		2.19	(3 H)	NAc
		1.75	(3 H)	CMe
		1.59	(3 H)	
		1.18	(3 H)	
Alkali-treated X(**3**)	^{1}H	1.47	(3 H)	CMe
		1.32	(3 H)	
		1.31	(3 H)	
Methylated derivative of alkali-treated X (**4**)	^{1}H	3.68	(3 H)	COOMe
		3.38	(3 H)	OMe
		3.16	(3 H)	OMe
		2.84	(3 H)	NMe
		1.45	(3 H)	CMe
		1.36	(3 H)	
		1.22	(3 H)	

supported by the NOE data for methylated **3**, which indicated the three Me and two MeO groups to be in close proximity as in structure **4**, but other combinations of substituents are possible.

High-resolution EIMS of **4** indicated a molecular weight of 259.1444 ($C_{12}H_{21}NO_5$) consistent with the structure shown. Gel chromatography of X indicated a molecular weight of ~ 200 (cf. 203 for **2**, $C_8H_{13}NO_5$). It is concluded that alkali-treated X (**3**) is a new and highly branched amino acid, 2,4-dihydroxy-3,3,4-trimethylpyroglutamic acid, and that X (**2**) has either a 2,5- or 1,4-lactone ring. However, the configurations of two asymmetric carbons in this amino acid were not determined.

Characterisation of amino acid-containing fragment.—As shown in Fig. 4, a fragment (H1) containing both X and an amino sugar was isolated from the HF hydrolysate of PS-1 in good yield (11.2 mg from 50 mg of PS-1). The acid



Scheme 1.

hydrolysate of H1 contained VioN and X in an equimolar proportion. Table III summarises the ^{1}H-NMR data for H1. All the signals ascribable to the VioN were split in the ratio 3:7, indicating the presence of α and β forms. The signals at 1.281, 1.303, and 1.451 ppm were ascribable to three Me groups of the amino acid

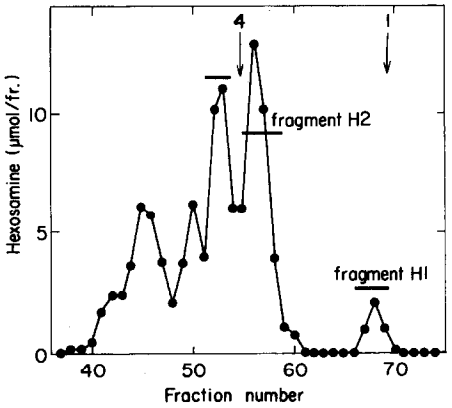


Fig. 4. Isolation of the amino acid-containing fragment (H1) from the HF hydrolysate of PS-1 (see Experimental) by gel chromatography under the conditions in Fig. 3. Fractions were assayed for total hexosamine. Fractions indicated by bars were combined.

TABLE III

^{1}H-NMR data for H1

Residue	Chemical shift (ppm)	Integral	Coupling constant (Hz)
α-VioNAcyl			
H-1	5.202 (d)	0.3 H	3.41
H-2	3.771 (t)	0.3 H	9.76
H-3	3.634 (t)	0.3 H	10.26
H-4	3.589 (dd)	0.3 H	9.77
H-5	3.995 (dq)	0.3 H	6.35
H-6,6,6	1.135 (d)	0.9 H	6.35
β-VioNAcyl			
H-1	4.599 (d)	0.7 H	7.81
H-2	3.650 (t)	0.7 H	9.76
H-3	3.576 (m)	0.7 H	
H-4	3.554 (t)	0.7 H	9.28
H-5	3.278 (t)	0.7 H	8.30
H-6,6,6	1.171 (d)	2.1 H	5.86
N-Acyl			
CMe	1.451 (s)	3.0 H	
	1.303 (s)	3.0 H	
	1.281 (s)	3.0 H	

moiety. These spectral data were similar to those for alkali-treated X (**3**), but differed from those for X (**2**). Moreover, no *N*-acetyl, *N*-formyl, or *O*-acetyl group was detected, suggesting that the amino acid residue may be an *N*-acyl substituent of the VioN residue. The ^{1}H-NMR spectrum of reduced and methylated H1 contained signals for six OMe groups, four of which were present in the VioN-ol residue. Thus, the *N*-acyl moiety of H1 contained two OH groups, a lactam ring, and three CMe groups, i.e., structure **3**. In the ^{1}H-NMR spectrum of PS-1, three CMe signals ascribable to the amino acid moiety were similar to those arising from alkali-treated X (**3**), but were different from those arising from X (**2**, Table II), suggesting that the amino acid moieties of PS-1 are present in the lactam form.

DISCUSSION

It is concluded that the 4-amino-4,6-dideoxy-D-glucose (VioN) residues in the O-polysaccharide chain of *V. anguillarum* V-123 LPS are *N*-acylated by 2,4-dihydroxy-3,3,4-trimethylpyroglutamic acid, the configuration of which was not determined. On acid hydrolysis of the O-polysaccharide (PS-1), the amino acid residue may be converted from a lactam into a lactone form, leading to the formation of X (**2**). 3-Hydroxy-2,3-dimethyl-5-oxoproline, *N*-acetylglycine, and *N*-acetyl-L-serine have been found as *N*-acyl substituents of amino sugars in O-antigens of LPSs from *Pseudomonas fluorescens* 361[17], *Shigella dysenteriae* type 7[18], and *Escherichia coli* 0114[19], respectively. Amino acids occur as amido substituents of uronosyl

components in bacterial polysaccharides[20]; taurine, L-alanine, L-glutamic acid, L-serine, L-threonine, and L-lysine have been found in capsular polysaccharides[21,22], LPSs[23–29], and cell-wall polysaccharide[13].

Hydrolysis of PS-1 with hydrogen fluoride gave a good yield of a fragment (H1) consisting of VioN and 2,3-dihydroxy-3,3,4-trimethylpyroglutamic acid. The good yield could reflect the linking of QuiN to VioN in PS-1 because of the extreme acid lability of 6-deoxyhexosidic linkages. The isolation of H1 led to the finding that the amino acid residue is present as the *N*-acyl substituent of the VioN in the O-polysaccharide chain. In the *V. anguillarum* ST-40 LPS, propanoic acid has been reported to be an *N*-acyl substituent of 3-amino-3,6-dideoxy-L-glucose[4].

Because of the ease of interconversion of the lactam and lactone forms, the highly branched structure, and the lack of reference compounds, it is difficult to confirm the structure of the new amino acid. Thus, the proposed locations of the three CMe, two OH, and one carboxyl groups on the five-membered lactam ring are tentative at present.

Further studies of the O-polysaccharide chain of *V. anguillarum* V-123 LPS are described in the following paper[6].

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