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ISOLATION AND IDENTIFICATION OF 3-DEOXY-D-*threo*-HEXULOSONIC ACID AS A CONSTITUENT OF THE LIPOPOLYSACCHARIDE OF *Vibrio parahaemolyticus* SEROTYPES O7 AND O12*

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ABSTRACT

Treatment of the lipopolysaccharide of *Vibrio parahaemolyticus* serotypes O7 and O12 with mild acid gave a compound which, on the basis of chemical analysis, g.l.c.–m.s., and ^{1}H and ^{13}C-n.m.r. spectroscopy, was characterized as 3-deoxy-D-*threo*-hexulosonic acid and shown to be present as a terminal non-substituted pyranose unit.

INTRODUCTION

The halophilic, marine Gram-negative bacterium *Vibrio parahaemolyticus* is responsible for food poisoning associated with sea products. Based on differences in serological properties of their heat-stable somatic antigens (lipopolysaccharide, LPS), these *Vibrio* strains have been divided into twelve O-serotypes[1–3], the sugar compositions of the O-antigenic LPS of which have been reported[4–7]. 3-Deoxy-D-*manno*-2-octulosonic acid (KDO), which is a common constituent of enterobacterial LPS, was not detectable in the LPS of most *V. parahaemolyticus* species by the thiobarbituric acid (TBA) assay using the mild conditions of acid hydrolysis [0.1M acetate (pH 4.4), 1 h, 100°] which are conventional[8] for the detection and quantification of KDO in enterobacterial LPS.

However, the LPS of *V. parahaemolyticus* serotypes O7 and O12 gave an intense reaction in the TBA assay, and the chromophore of the substance had λ_{max} (549 nm) identical to that obtained from β-formylpyruvate. High-voltage paper electrophoresis (h.v.p.e.) of LPS hydrolysates revealed[4] a TBA-positive "KDO-like" compound migrating faster than KDO. We now describe the identification of this compound as 3-deoxy-D-*threo*-hexulosonic acid (**1**).

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EXPERIMENTAL

Bacterial strains and lipopolysaccharides. — *V. parahaemolyticus* serotype O7 (strain Ta 11, K19) was provided by Drs. M. Ohasi and K. Ohta (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan) and serotype O12 (strain OP204, K19) was provided by Dr. T. Miwatani (Institute of Microbial Diseases, Osaka University, Osaka, Japan). Cultivation of the bacteria and extraction of the LPS by the hot phenol–water procedure[9] have been described[4].

Isolation and purification of 3-deoxyhexulosonic acid **1**. — A suspension of the LPS (1 g) of serotype O12 in acetic acid (5%, 100 mL) was kept for 45 min at 100°, then dialysed extensively against distilled water. The diffusates were combined and concentrated, and water was evaporated several times from the residue in order to remove traces of acetic acid. A solution of the residue in water (2 mL) was added to a column (100 × 2.0 cm) of Sephadex G-50 (super fine) which was eluted with water. Fractions (5.0 mL) were subjected[8] to the TBA assay. TBA-positive fractions were combined and freeze-dried to give a syrup (140 mg), a solution of which in mM acetic acid (1.3 mL) was transferred to a column (25 × 2.5 cm) of AG 1-X8 (AcO^- form, Bio-Rad). After elution with water (200 mL), **1** was eluted by a linear gradient of 0 → 2M acetic acid (total volume, 1,200 mL). Combining the TBA-positive fractions of the major peak yielded **1** (15 mg, 56% of the TBA-positive material in the LPS of serotype O12). A minor TBA-positive fraction (0.5 mg) was also obtained which had the same mobility (M_{KDO} 1.2) as **1** in h.v.p.e. This substance was not investigated further.

Synthetic reference compounds. — 3-Deoxy-D- (**1**) and DL-*threo*-hexulosonic acid were synthesized according to Portsmouth[10] by condensation of D- (276 mg, 8 mmol) or DL-glyceraldehyde (27.6 mg, 0.8 mmol) with oxalacetate (2.64 g, 20 mmol; and 264 mg, 2 mmol; respectively). The desired products were separated from minor amounts of 3-deoxy-D- (**2**) or -DL-*erythro*-hexulosonic acid by anion-exchange chromatography as described above for the isolation of **1**. Synthetic **1** (2.3 mg, sodium salt) had $[\alpha]_D^{20}$ −2° (*c* 0.2, water).

Methyl (methyl 3-deoxy-α-D-*erythro*-hexulopyranosid)onate[11] (**5**) was a gift from Dr. D. Charon (Châtenay-Malabry, France).

Methyl (methyl 4,5-di-O-acetyl-3-deoxyhexulopyranosid)onate (**6**). — Compound **1** (15 mg) was converted into **4** using 0.5M HCl in methanol (2 mL) at 80°. The reaction was followed by t.l.c. on Kieselgel 60 F_{254} (Merck), using chloroform–methanol–water (10:10:4) (R_F values: **3**, 0.76; **4**, 0.82) and was complete in 1 h. The solution was then concentrated to dryness in a stream of nitrogen and the residue was treated with 10:1 pyridine–acetic anhydride (2.0 mL) for 14 h at room temperature in the presence of 4-dimethylaminopyridine as catalyst. The mixture was concentrated to dryness and the product was purified by h.p.l.c. on a column (30 cm × 7 mm, Bischoff, Leonberg) packed with Nucleosil 50, 5 μm (Macherey & Nagel, Düren) by elution with toluene–ethyl acetate–ethanol (900:100:1) at 2.5 mL/min. The eluate was monitored by t.l.c. (toluene–ethyl acetate, 3:1). The fractions containing **6** (R_F 0.34) were concentrated to yield a product (4.0 mg) having $[\alpha]_D^{21}$ +8° (*c* 0.4, chloroform).

Methyl [(−)-sec-butyl 4,5-di-O-acetyl-3-deoxyhexulopyranosid]onate (**8**). — Treatment[12] of **6** (0.1 mg) with M HCl in (−)-2-butanol (0.2 mL) for 2 h at 86° afforded **8**, which was *O*-acetylated and subjected to g.l.c. and g.l.c.–m.s.

G.l.c.–m.s. — Compounds were methylated according to Hakomori[13] with modifications as described[14]. G.l.c. was carried out on a Varian gas-chromatograph (model 3700) equipped with a fused-silica capillary column (25 m × 0.22 mm i.d.) with chemically bonded SE-54 (film thickness, 0.35 μm) and a temperature programm of 3 min at 130° then to 300° at 3°/min. G.l.c.–m.s. was performed as described[15].

N.m.r. spectroscopy. — ^{1}H-N.m.r. spectra were recorded on a Bruker model WM-360 instrument; ^{1}H-, proton-decoupled ^{13}C-, and ^{1}H,^{13}C-COSY n.m.r. spectra of **6** and **7** were recorded for solutions (0.5 mL) in benzene-d_6 with Me_4Si (^{1}H) and acetonitrile (^{13}C, 1.70 p.p.m.) as internal references.

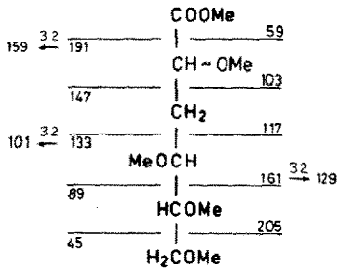
Other analytical methods. — Neutral sugars were determined by g.l.c. as alditol acetates after hydrolysis in 0.1M HCl for 48 h at 100°. Amino sugars were determined by an amino acid analyser (4151 Alpha Plus, LKB) after hydrolysis in 4M HCl for 16 h at 100°. The TBA assay was performed as described[8]. High-voltage paper electrophoresis (h.v.p.e.) was carried out in pyridine–formic acid–acetic acid–water (1:2–3:10:90) at pH 2.8 and 50 V/cm for 90 min. Substances were detected using TBA[16], alkaline silver nitrate[17], or molybdate[18]. Optical rotations were determined with a Perkin–Elmer 243 polarimeter.

RESULTS

The LPS of *V. parahaemolyticus* serotypes O7 and O12 contained D-glucose, D-galactose, L-*glycero*-D-*manno*-heptose, 2-amino-2-deoxy-D-glucose, 2-amino-2-deoxy-D-galactose, 3-amino-3,6-dideoxy-D-glucose, uronic acid, and a TBA-positive substance in amounts comparable to those reported[4–7]. In addition, after mild acid hydrolysis [0.1M acetate buffer (pH 4.4), 1 h, 100°], 0.12 and 0.15 μmol, respectively, of a TBA-positive substance were detected per mg of LPS of serotypes O7 and O12, using 3-deoxy-D-*manno*-octonate (KDO) as reference. These values refer to a molar ratio of ~1:2 for KDO:2-amino-2-deoxy-D-glucose (0.25 μmol/mg). Since 2-amino-2-deoxy-D-glucose was the only amino sugar in the amino sugar-containing disaccharide of the lipid A backbone, the LPS contains 1 mol of a TBA-positive substance.

In h.v.p.e., no KDO was found, but a TBA-positive substance with a higher mobility (M_{KDO} 1.2) was present which also stained with alkaline silver nitrate but not with the molybdate reagent, indicating that it was not a phosphorylated sugar. As shown below, this substance was identified as 3-deoxy-D-*threo*-hexulosonic acid (**1**). Treatment of the LPS (1 g) with aqueous 5% acetic acid for 45 min at 100° released >90% of **1**. Purification by anion-exchange chromatography yielded **1** and a minor fraction which was not studied further because of the small quantity.

G.l.c.–m.s. of **1**. — Both synthetic **1** and **1** from the LPS (each 1 mg) were



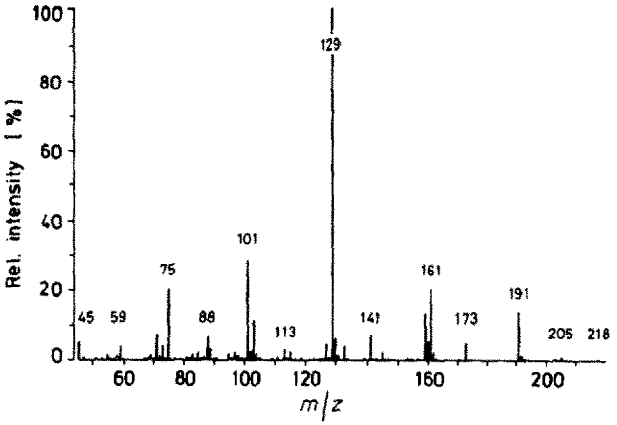


Fig. 1. E.i.-mass spectrum (70 eV) and fragmentation pattern of carbonyl-reduced ($NaBH_4$) and methylated 3-deoxyhexulosonate **10** isolated from the LPS of *V. parahaemolyticus* serotype O12.

carbonyl-reduced ($NaBH_4$) and then methylated. In g.l.c.–m.s., two peaks with similar intensities were detected at T 5.87 and 6.45 min. C.i. (ammonia)-m.s. of the material in each peak gave the same pseudomolecular ions [m/z 251 $(M + 1)^+$ and 268 $(M + NH_4)^+$] corresponding to a mol. wt. of 250 and compatible with the structures **9** and **10**. The formation of diastereomers was expected[19]. The e.i.-mass spectra of these isomers were identical (Fig. 1). The fragmentation pattern (Fig. 1) was similar to that[20] for carbonyl-reduced and methylated KDO. Synthetic **1** and **1** from the LPS had identical retention times and mass spectra.

For n.m.r. spectroscopy, **1** isolated from the LPS of serotype O12 or obtained by synthesis[10] was derivatized and purified to give **6**. The ^{1}H-n.m.r. spectra (Table I) of these compounds were superimposable. The large values of $J_{3a,4}$ (10.6 Hz), $J_{4,5}$ (9.4 Hz), and $J_{5,6a}$ (9.8 Hz) accord with methyl (methyl 4,5-di-*O*-acetyl-3-deoxy-*threo*-hexulopyranosid)onate in the $^5C_2$ conformation and are assigned tentatively as α.

The ^{13}C-n.m.r. spectra (Table II) of the two derivatives **6** were identical. However, the signals for C-3,4,5 of methyl (methyl 4,5-di-*O*-acetyl-3-deoxy-α-D-*erythro*-hexulopyranosid)onate (**7**) were shifted to higher field and that for C-6 to

TABLE I

360-MHz ^{1}H-N.M.R. DATA[a] IN BENZENE-d_6 FOR METHYL (METHYL 4,5-DI-*O*-ACETYL-3-DEOXY-α-D-*threo*-HEXULOPYRANOSID)ONATE (**6**) DERIVED FROM THE LPS OF *V. parahaemolyticus* SEROTYPE O12

Assignment	*δ*	J *(Hz)*
H-3*a*	1.89 (dd)	$J_{3a,3e}$ 13.0, $J_{3a,4}$ 10.6
H-3*e*	2.69 (dd)	$J_{3e,4}$ 5.2
H-6*a*	3.49 (dd)	$J_{6a,6e}$ 11.0
H-6*e*	4.01 (dd)	$J_{5,6a}$ 9.8
H-5	5.22 (ddd)	$J_{4,5}$ 9.4
H-4	5.69 (ddd)	$J_{5,6e}$ 5.4

[a]Other signals: *δ* 1.71 (2 s, 2 OAc), 3.19 and 3.37 (s, COOMe and s, OMe).

lower field, whereas the signals for COOMe, OMe, and OAc were unchanged.

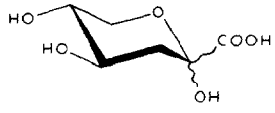
The absolute configuration of the LPS-derived **1** was determined by g.l.c.–m.s. of the methyl [(−)-*sec*-butyl 4,5-di-*O*-acetyl-3-deoxy-α-*threo*-hexulopyranosid]onate derivative (**8**) which was analyzed in parallel to the corresponding derivative of synthetic **1**. Synthetic **8** and the LPS-derived **8** each gave a single peak (*T* 15.95 ±0.03 min), whereas the synthetic racemate gave two peaks (*T* 15.98 and 16.08 ±0.03 min). Thus, the LPS-derived **8** (and **1**) have the D-*threo* configuration. This conclusion was confirmed by the finding that the LPS-derived and synthetic **6** each had $[\alpha]_D$ +8° (*c* 0.4, chloroform) and that their ^{1}H-n.m.r. spectra were superimposable. Whereas a $^5C_2$ conformation was also found for the analogous KDO derivative[11], a $^2C_5$ conformation was found[11] for **7**.

TABLE II

^{13}C-N.M.R. CHEMICAL SHIFTS[a] OF METHYL (METHYL 4,5-DI-*O*-ACETYL-3-DEOXY-α-D-*threo*-HEXULOPYRANOSID)ONATE (**6**) DERIVED FROM THE LPS OF *V. parahaemolyticus* SEROTYPE O12 AND BY SYNTHESIS AND **7**

Assignment[b]	*Signal (p.p.m.)*	
	6	**7**
$OCOCH_3$	20.4, 20.3	20.4, 20.4
C-3	37.0	32.9
OCH_3	50.7	50.9
$COOCH_3$	51.7	51.7
C-6	61.0	62.6
C-4	68.6	66.3
C-5	69.4	67.1
C-2	98.9	99.3
COOMe	167.7	167.9
$COCH_3$	169.4, 169.2	169.8, 169.3

[a]Recorded at 90.6 MHz for solutions in benzene-d_6 (internal acetonitrile 1.700 p.p.m.). [b]Assignment by ^{1}H,^{13}C-COSY n.m.r. spectroscopy.



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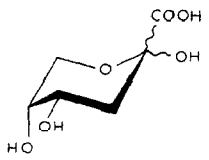

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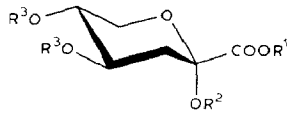
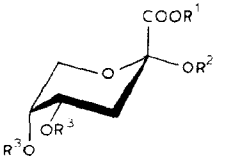
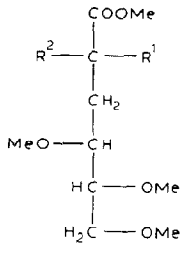


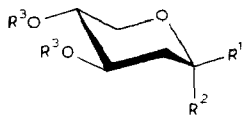
3 $R^1 = R^3 = H, R^2 = Me$
4 $R^1 = R^2 = Me, R^3 = H$
6 $R^1 = R^2 = Me, R^3 = Ac$
8 $R^1 = Me, R^2 = (-)\text{-}sec\text{-}Bu, R^3 = Ac$



5 $R^1 = R^2 = Me, R^3 = H$
7 $R^1 = R^2 = Me, R^3 = Ac$



9 $R^1 = OMe, R^2 = H$
10 $R^1 = H, R^2 = OMe$



11 $R^1 = COOMe, R^2 = OMe, R^3 = Me$
12 $R^1 = OMe, R^2 = COOMe, R^3 = Me$

Analysis of the 3-deoxyhexulosonic acid isolated from the LPS of serotypes O7 and O12 gave identical results.

In order to determine the ring size of **1** and its location in the polymer, the LPS of serotypes O7 and O12 were each methylated twice[21] and methanolysed (2M acid, 4 h, 85°). G.l.c.–m.s. (ammonia-c.i.) of the products revealed two isomers (mol. wt., 234) which gave almost identical e.i.-mass spectra (data not shown) and which were probably the α- and β-anomers **11** and **12**. One of the isomers had a retention time (T 8.07 min) and e.i.-mass spectrum that were identical with those of synthetic methyl (methyl 3-deoxy-4,5-di-*O*-methyl-α-D-*threo*-hexulopyranosid)-onate (**11**) obtained from **6** after methylation. The minor peak (T 7.80 min) was therefore the β-anomer **12**.

No furanosidic derivatives of **1** were found; thus, the 3-deoxy-D-*threo*-hexulosonic acid is present as a terminal pyranosidic unit in the LPS of each serotype.

DISCUSSION

In the present study, we have described 3-deoxy-D-*threo*-hexulosonic acid (**1**)

as a constituent of the LPS isolated from *V. parahaemolyticus* serotypes O7 and O12. Other aldulosonic acids structurally related to KDO, such as 3-deoxyheptulosonaric acid[22] and 2-octulosonic acid[15] have been reported as core sugar components of non-enterobacterial LPS. However, a 3-deoxyhexulosonic acid has not been described hitherto as a LPS constituent. 3-Deoxy-D-*threo*-hexulosonic acid is a metabolic intermediate of galactose and galactonate in *Pseudomonas saccharophila*[23] and *Gluconobacter liquefaciens*[24] and is a constituent of an extracellular polysaccharide produced by *Azotobacter vinelandii*[25]. The presence of a TBA-positive substance (different from KDO) in the LPS of *V. alginolyticus* has been reported[5]. In the TBA assay, **1**, KDO, and 3-deoxyheptulosonaric acid are oxidized to β-formylpyruvate, so that TBA-reactivity alone is not a suitable criterion for the determination of KDO in LPS.

Of the twelve serotypes of *V. parahaemolyticus* investigated[4], 3-deoxy-D-*threo*-hexulosonic acid is present only in the LPS of serotypes O7 and O12.

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