Carbohydrate Research, 245 (1993) 353–359
Elsevier Science Publishers B.V., Amsterdam

Note

Identification of oligosaccharides consisting of D-glucuronic acid and L-*glycero*-D-*manno*- and D-*glycero*-D-*manno*-heptose isolated from *Vibrio parahaemolyticus* O2 lipopolysaccharide

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(Received September 21st, 1992; accepted January 26th, 1993)

Vibrio parahaemolyticus, a halophilic marine vibrio responsible for food poisoning, is now divided serologically into 12 (or 13) O-serotypes[1–4] based on differences in the serological specificities of its O-antigen [lipopolysaccharide (LPS)]. In our previous structural analysis[5] of the sugar chain isolated from the LPS of *V. parahaemolyticus* serotype O12, it was shown that D-glucuronic acid (GlcA) is β-linked to O-2 of L-*glycero*-D-*manno*-heptose (L,D-Hep) which is linked to O-5 of 3-deoxy-D-*manno*-octulosonate (Kdo). Compositional sugar analysis of LPSs isolated from twelve O-serotypes[6,7] revealed that uronic acid was distributed in the LPSs of all serotypes; however, two kinds of heptose, i.e., L,D-Hep and D-*glycero*-D-*manno*-heptose (D,D-Hep), were detectable only in the LPS of serotype O2. These results suggest that the core region, in particular the inner core region, of O2 LPS is structurally different from that of O12 LPS. Acid hydrolysis of O2 LPS released two oligosaccharides, one consisting of uronic acid and L,D-Hep and the other consisting of uronic acid, L,D-Hep, and D,D-Hep[6]. In the present study, we describe the isolation and identification of these oligosaccharides by means of GLC–MS and ^1H and ^{13}C NMR spectroscopy.

Disaccharide **2** and trisaccharide **5** were isolated from *V. parahaemolyticus* O2 LPS (500 mg) after a two-step hydrolysis (acetic acid and CF_3CO_2H), methanolysis in hydrochloric acid, and peracetylation, HPLC purification afforded pure **2** (2.4 mg) and **5** (24.8 mg). GLC and GLC–MS of *O*-acetylated (*S*)-(+)- and (*R*)-(−)-*sec*-butyl glycosides[8] of the glucose (Glc) obtained from carboxyl-reduced **2** and **5** gave retention times (t_R 17.25 and 17.52 min, respectively) and EI-mass spectra

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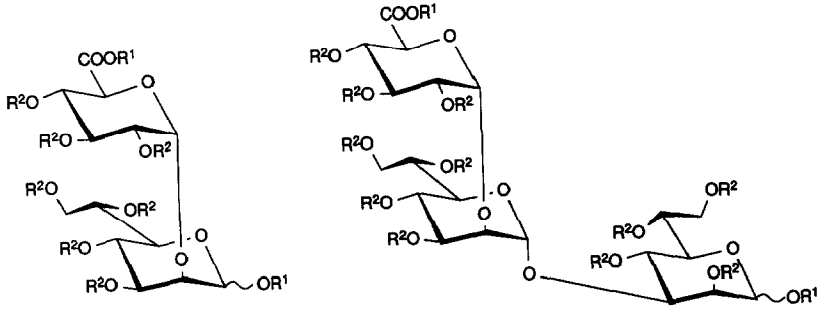


1 R^1=R^2=H
2 R^1=Me, R^2=Ac
3 R^1=R^2=Me

4 R^1=R^2=H
5 R^1=Me, R^2=Ac
6 R^1=R^2=Me

(data not shown) identical to those obtained for the respective derivatives of standard D-Glc, indicating that the Glc, and consequently the GlcA present in **2** and **5**, had the D configuration.

Results obtained by ^{1}H and ^{13}C NMR analysis of **2** and **5** are shown in Tables I and II, respectively. The $J_{1,2}$ value of 3.67 Hz for Glc*p*A in both oligosaccharides indicates an α-glycosidic linkage, which is further confirmed by ^{13}C NMR data (Table II, $J_{C\text{-}1,H\text{-}1}$ 173.96 and 175.48 Hz)[9]. The heteronuclear coupling constant of L,D-Hep in **5** was determined as 173.16 Hz, indicating an α-glycosidic linkage. The signals for H-2 of the L,D-Hep residues in **2** and **5** (Table I, 3.94 and 4.02 ppm, respectively) are shifted upfield by ~ 1 ppm as compared with H-2 (5.191 ppm) of unsubstituted and fully acetylated methyl L-*glycero*-α-D-*manno*-heptopyranoside[10], indicating that the L,D-Hep residues in **2** and **5** are substituted at position 2. Similarly, the signal for H-3 of the D,D-Hep residue (4.24 ppm) in **5** was shifted upfield by ~ 1 ppm as compared with unsubstituted H-3 (5.302 ppm)[10,11]. This indicates substitution at position 3 of the D,D-Hep residue in **5**. These interpretations were further confirmed by ^{13}C NMR analysis. The signal for C-2 of L,D-Hep was shifted downfield by ~ 6 ppm in **2**, whereas that of C-3 in D,D-Hep showed a similar shift, as compared with unsubstituted C-2 and C-3 of L,D-Hep[12].

The results of GLC–MS analysis of **2**, **3**, **6**, **7**, and **8** are shown in Table III. The molecular weight of each derivative was determined based on the peak at *m/z* for $(M + H)^+$ in CIMS. EI-fragments of **2** at *m/z* 317 and 375 were assigned to the tri-*O*-acetylated methyl ester of GlcA and 1-*O*-methyl-tetra-*O*-acetyl-L,D-Hep residues, respectively. EI-fragments at *m/z* 233 and 263 of **3** and **6** originated from the GlcA residue and heptose residue (L,D-Hep, **3**; D,D-Hep, **6**). Upon EIMS of **6**, fragment ions *m/z* 481 and 449 (481 − 32) were detected at relatively high intensity and assigned to the GlcA–L,D-Hep disaccharide moiety in **6**. EIMS of **8** revealed the presence of fragments at *m/z* 643 and 687 originating from a

TABLE I

^{1}H NMR data of **2** and **5** (400 MHz, C_6D_6) [a]

Assignment [b]	**2**		**5**	
	δ	J (Hz)	δ	J (Hz)
D,D-Hep				
H-1			4.38	$J_{1,2}$ 1.46
H-2			5.47	$J_{2,3}$ 3.48
H-3			4.24	$J_{3,4}$ 9.52
H-4			5.63	$J_{4,5}$ 10.07
H-5			3.90	$J_{5,6}$ 2.57
H-6			5.57	$J_{6,7b}$ 7.10
H-7a			4.55	$J_{7a,6}$ 7.87
H-7b			4.78	$J_{7a,7b}$ 11.90
L,D-Hep				
H-1	4.72	$J_{1,2}$ 1.83	5.27	$J_{1,2}$ 1.83
H-2	3.94	$J_{2,3}$ 3.94	4.02	$J_{2,3}$ 2.38
H-3	5.50	$J_{3,4}$ 10.07	5.56	$J_{3,4}$ 9.89
H-4	5.63	$J_{4,5}$ 10.07	5.77	$J_{4,5}$ 10.70
H-5	3.83	$J_{5,6}$ 1.83	4.60	$J_{5,6}$ 3.85
H-6	5.56	$J_{6,7b}$ 6.60	5.70	$J_{6,7b}$ 6.68
H-7a	4.30	$J_{7a,6}$ 7.88	4.62	$J_{7a,6}$ 5.36
H-7b	4.40	$J_{7a,7b}$ 11.35	4.62	$J_{7a,7b}$ 11.77
GlcA				
H-1	5.40	$J_{1,2}$ 3.67	5.33	$J_{1,2}$ 3.67
H-2	4.90	$J_{2,3}$ 10.26	4.90	$J_{2,3}$ 10.44
H-3	5.95	$J_{3,4}$ 9.89	5.92	$J_{3,4}$ 9.89
H-4	5.59	$J_{4,5}$ 9.53	5.52	$J_{4,5}$ 9.71
H-5	4.69	$J_{5,4}$ 10.25	4.73	$J_{5,4}$ 10.25

[a] Other signals: $COCH_3$ and $COOCH_3$, δ 1.6–3.4. [b] Assignment by ^{1}H,^{1}H COSY NMR.

3-substituted D,D-Hep moiety by preferential cleavage at the C-4–C-5 and C-5–C-6 bonds[13], respectively.

Methylation analysis of **2** gave methyl 2,6-di-*O*-acetyl-3,4,5-tri-*O*-methylgulonate (10.06 min, from terminal GlcA) and 1,2,5-tri-*O*-acetyl-3,4,6,7-tetra-*O*-methyl-L-*glycero*-D-*manno*-heptitol (15.07 min, from 2-substituted L,D-Hep). Besides these two compounds, 1,3,5-tri-*O*-acetyl-2,4,6,7-tetra-*O*-methyl-D-*glycero*-D-*manno*-heptitol (14.90 min, from 3-substituted D,D-Hep) was obtained from trisaccharide **5**. These results were compatible with those obtained by ^{1}H and ^{13}C NMR spectroscopy of the peracetylated di- (**2**) and tri-saccharide (**5**), respectively.

D,D-Hep has been reported to be present in some LPSs of Vibrionaceae strains such as non-O1 *Vibrio cholerae, Vibrio fluvialis, Aeromonas sobria, Aeromonas hydrophila*, and *Photobacterium phosphoreum*[11,14–18]. In the LPSs of non-O1 *V. cholerae* O21[11] and O3[15], D,D-Hep has been shown as a constituent of their O-polysaccharide moieties, while it is present in the core region of the LPS of *A. hydrophila*[17]. In the present study, it has been demonstrated that D,D-Hep carries L,D-Hep at O-3, suggesting that the D,D-Hep is involved in the inner core region of

TABLE II

^{13}C NMR data of **2** and **5** (100.6 MHz, C_6D_6) [a]

Assignment [b]	**2**	**5**
	δ	δ
D,D-Hep		
C-1		98.87 ($J_{C\text{-}1,H\text{-}1}$ 171.62 Hz) [c]
C-2		70.81
C-3		73.82
C-4		69.06
C-5		70.91
C-6		70.45
C-7		62.19
L,D-Hep		
C-1	99.76 ($J_{C\text{-}1,H\text{-}1}$ 170.18 Hz) [c]	100.16 ($J_{C\text{-}1,H\text{-}1}$ 173.16 Hz) [c]
C-2	75.67	77.05
C-3	71.23	70.56
C-4	65.80	65.51
C-5	69.30	70.34
C-6	67.24	67.43
C-7	62.80	62.04
GlcA		
C-1	97.23 ($J_{C\text{-}1,H\text{-}1}$ 173.96 Hz) [c]	97.74 ($J_{C\text{-}1,H\text{-}1}$ 175.48 Hz) [c]
C-2	71.10	70.89
C-3	69.30	69.30
C-4	69.99	69.99
C-5	69.69	69.66
C-6	167.73	167.78

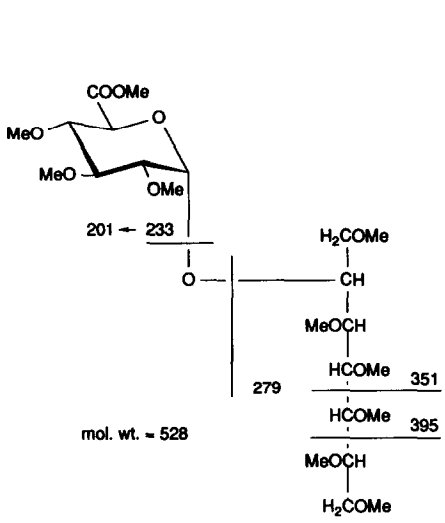
[a] Other signals: $COCH_3$ and $COOCH_3$, δ 20–21; $COCH_3$, δ 169–171. [b] Assignment by ^1H,^{13}C COSY NMR spectroscopy. [c] $J_{C\text{-}1,H\text{-}1}$ values were determined by gated ^{13}C NMR experiments.

TABLE III

GLC MS data for derivatives **2**, **3**, **6**, **7**, and **8**

Derivative	Mol wt [a]	Characteristic fragments (intensity, %) [b]
2	708	127(77.2) 155(97.9) 169(27.1) 197(42.0) 215(34.5) 257(84.9) 317(100.0) 374(3.8) 375(2.8) 389(5.8) 487(2.8) 589(3.7) 649 (2.5)
3	512	75(59.1) 89(21.5) 101(91.0) 111(12.4) 141(10.4) 169(8.3) 201(100.0) 233(20.1) 263(8.2) 319(13.1) 323(15.4) 394(7.2)
6	760	75(31.4) 89(18.9) 101(54.9) 145(23.4) 171(11.5) 201(100.0) 233(13.2) 263(18.6) 323(14.6) 375(3.6) 449(10.2) 481(8.9)
7	528	75(50.2) 89(50.6) 101(100.0) 111(26.3) 133(16.7) 145(41.2) 177(11.3) 187(11.3) 201(45.7) 221(4.9) 233(7.9) 279(11.8) 293(3.6) 395(4.2)
8	776	89(38.8) 101(85.1) 115(20.5) 145(20.0) 201(100.0) 233(9.7) 279(43.4) 339(2.3) 449(8.5) 481(5.4) 643(1.3) 687(1.0)

[a] Determined by CIMS, based on peaks at m/z for $(M+H)^+$. [b] Determined by EIMS at 70 eV.



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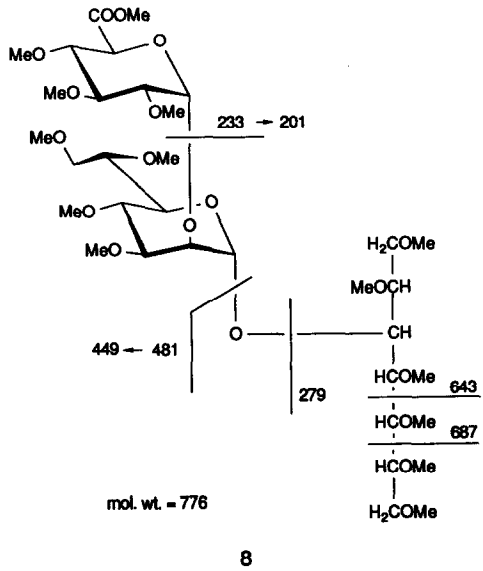

8

O2 LPS. Among the LPSs isolated from 13 O-serotypes of *V. parahaemolyticus*, D,D-Hep is detectable only in O2 LPS, indicating that the structure of O2 LPS is different from that of LPSs of the other serotypes, most likely in their inner core regions.

EXPERIMENTAL

General.—*V. parahaemolyticus* NIH 5425-62 (O2, K3) was provided by Dr. Y. Kudo, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan. Cultivation of the bacteria and extraction of LPS were previously described[6]. Methylation was done according to Hakomori[19] or Ciucanu and Kerek[20] after carboxyl-methylation with diazomethane. The absolute configuration of GlcA was determined as follows. Compounds **2** and **5** were reduced ($NaBH_4$) in 3:1 MeOH–H_2O at room temperature for 16 h and hydrolysed in 2 M CF_3CO_2H at 120°C for 60 min. The hydrolysates were treated with 2 M HCl in (*S*)-(+)- and (*R*)-(−)-2-butanol (86°C, 2 h) and *O*-acetylated according to the method of Gerwig et al.[8]. The products were analysed by GLC and GLC–MS. GLC was carried out on a Shimadzu GC 14A gas chromatograph equipped with a fused-silica capillary column (25 m × 0.25 mm) and chemically bonded HR-52 with temperature programs 3 min at 150°C then to 320°C at 5°C/min for analysis of partially methylated alditol acetates and *O*-acetylated butyl glycosides, and 3 min at 180°C then to 320°C at 5°C/min for analysis of oligosaccharides. GLC–MS analysis was performed on a DX-300 instrument (Nihon Denshi, Tokyo, Japan), using the same column and temperature programs described above. Electron impact mass spectra

(EIMS) were recorded at 70 eV and isobutane was used as the reactant gas for chemical ionisation (CIMS). *Yersinia enterocolitica* O9 LPS[21] was used as a source of standard L,D-Hep and D,D-Hep. ^{1}H NMR (400 MHz) and ^{13}C NMR (100.6 MHz) experiments were performed on a Varian VXR 400S spectrometer (5-mm probe) for solutions in benzene-d_6 at 20°C. The chemical shifts were calibrated relative to the chemical shift of internal Me_4Si. Assignments were made based on ^{1}H,^{1}H homonuclear and ^{1}H,^{13}C heteronuclear COSY NMR experiments. The anomeric configurations were determined by gated ^{13}C NMR experiments.

Degradation of the LPS.—LPS (500 mg) was hydrolysed in 5% acetic acid (50 mL) at 100°C for 2.5 h and, after removal of precipitated lipid A by centrifugation (10000*g*, 20 min), the hydrolysate was evaporated to dryness and applied to a column (2.6 cm × 100 cm) of Sephadex G-25 which was eluted with water. The eluate was monitored by a refractive index detector (Shimadzu RID-6A) and the fractions (3 mL) corresponding to the saccharide portion (Fraction I) of the LPS were combined and freeze-dried (130 mg). Fraction I (100 mg) was subjected to further hydrolysis in 2 M CF_3CO_2H at 120°C for 50 min. The hydrolysate containing **1** and **4** was evaporated, methanolysed (0.5 M HCl–MeOH at 80°C for 60 min), and acetylated in pyridine–acetic anhydride (3:1, 4 mL) at room temperature for 16 h with a catalytic amount of 4-dimethylaminopyridine, yielding crude **2** and **5**.

Purification of compounds **2** *and* **5**.—HPLC of crude **2** and **5** was performed on a column (2.0 cm × 25 cm) of Nucleosil (50 μm, Chromato-packing Center, Kyoto, Japan) by elution with 10:1 benzene–acetone (8 mL/min). The eluate was analysed by TLC (5:1 benzene–acetone) and GLC. Fractions (8 mL) containing compounds **2** and **5** were each combined and dried, yielding pure **2** (2.4 mg) and **5** (24.8 mg).

For the preparation of **6**, **5** was methylated twice (Hakomori), and the product was purified by adsorption on a SEP-PAK C-18 cartridge (Waters, USA) and, after washing with water (40 mL), eluted with MeOH (20 mL). Compounds **7** and **8** were derived from **2** and **5**, respectively, by hydrolysis (2M CF_3CO_2H, 120°C, 50 min), reduction ($NaBH_4$), and methylation (Hakomori). For methylation analysis, **2** and **5** (200 μg each) were methylated (Hakomori), acetolysed[22], reduced ($NaBH_4$), acetylated, and analysed by GLC–MS.

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