Carbohydrate Research, 167 (1987) 295–300
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

Isolation and identification of a rhamnosyl-rhamnosyl-3-deoxy-D-*manno*-octulosonic acid trisaccharide from the lipopolysaccharide of *Acinetobacter calcoaceticus* (10303 NTCC London)*

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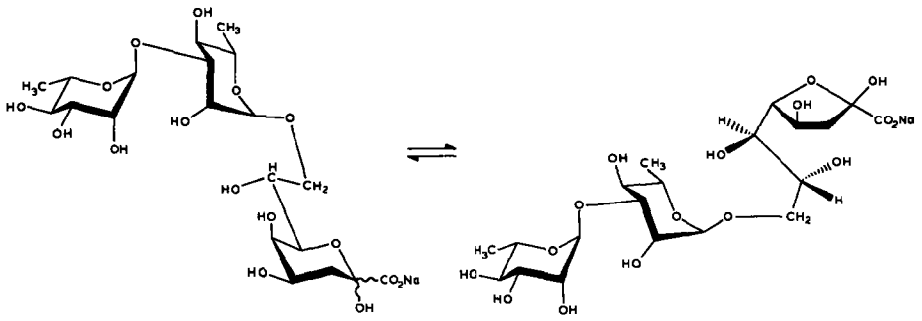
(Received August 6th, 1986; accepted for publication, November 29th, 1986)

Recently, we reported[1] on the occurrence of a normal mouse serum protein which binds specifically to the heptose/3-deoxy-D-*manno*-octulosonate (KDO) region of all lipopolysaccharides (LPS) studied. Immunochemical investigations indicated that the minimal carbohydrate structure required for binding included KDO and a neutral sugar attached thereto. The stereochemistry of the neutral sugar did not seem to contribute to the specificity of binding, since LPS containing L-*glycero*-D-*manno*-heptopyranose[1,2], D-mannopyranose[2], and D-glucopyranose[3,4] were equally active in the interaction with the above-mentioned protein. However, the neutral sugar was always linked[5] to position 5 of KDO.

During these studies, we found an LPS in a strain of *Acinetobacter calco-*



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*Presented at the XIIIth International Carbohydrate Symposium, Ithaca, 10–15 August, 1986.
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0008-6215/87/$ 03.50

aceticus which did not bind the mouse serum protein but which contained KDO and neutral sugars, and therefore the structure of the KDO region was investigated.

Hydrolysis (pH 4.4, 100°, 1 h) of the LPS followed by gel-permeation chromatography gave a trisaccharide which, on the basis of the following results, was identified as 3-deoxy-8-*O*-(3-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl)-D-*manno*-octulosonate (**1**).

Analysis showed that **1** contained rhamnose and KDO in the molar ratio 2:1. The thiobarbiturate assay for KDO became negative after borohydride reduction of **1** and gave the same values with and without hydrolysis prior to the periodate-oxidation step. Hence, KDO was the reducing unit in **1**.

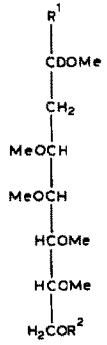
Carbonyl-reduction of **1** with sodium borodeuteride followed by methylation yielded **2**, which was a mixture of the D-*glycero*-D-*galacto* and D-*glycero*-D-*talo* isomers of 3-deoxyoctonate although g.l.c. gave only one peak (*T* 31.0 min). C.i. (ammonia)-m.s. of **2** gave a peak at *m/z* 705 for $[M + NH_4]^+$, indicating a mol. wt. of 687 (spectrum not shown). E.i.-m.s. of **2** gave fragment ions at *m/z* 189, 157, and 125 derived from the terminal rhamnopyranosyl residue, and peaks at *m/z* 308, 276, 244, and 212 attributed to the alditol chain after cleavage of the glycosidic bond. Peaks at *m/z* 162 and 130 (162 − 32), 206 and 174 (206 − 32), and 218 (250 − 32) represent the C-1/4, C-1/5, and the C-1/6 moieties, respectively, and their corresponding sub-fragments after loss of methanol.

Carboxyl-reduction of **2** followed by methylation afforded **3**, g.l.c. of which gave two peaks (*T* 30.02 and 30.18 min). C.i.(ammonia)-m.s. of **3** gave a peak at

TABLE I

CHARACTERISTIC E.I. MASS-SPECTRAL FRAGMENT IONS OF **2–5**

Compound	m/z *(% of base peak)*
2	45 (10.2), 59 (12.2), 71(16.1), 75 (35.9), 85 (13), 88 (100), 89 (17.9), 101 (89.7), 125 (10.6), 130 (95.8), 157 (30), 162 (17.6), 174 (22.3), 189 (68.6), 205 (20.3), 206 (7.8), 212 (6.1), 244 (5.2), 276 (0.5), 308 (5.4)
3	45 (11.6), 59 (20.5), 71 (14.1), 75 (27.8), 85 (12.1), 88 (100), 89 (19.5), 90 (30.7), 101 (96.8), 116 (62.7), 148 (11.6), 157 (23.1), 160 (6.3), 189 (56.5), 192 (12.2), 198 (2.9), 204 (2.6), 205 (16), 230 (2), 294 (1.7)
4	45 (9.8), 60 (13.3), 88 (9.4), 90 (55.1), 101 (65.7), 116 (100), 117 (34.2), 128 (17.4), 148 (18.2), 160 (5.7), 161 (18.9), 192 (8.2), 204 (1.1)
5	45 (10.8), 70 (24.6), 75 (15.2), 88 (13.8), 101 (100), 102 (23.2), 114 (22.9), 117 (56), 118 (76.3), 128 (8.6), 144 (31), 146 (6.5), 161 (21.1), 173 (4.7), 188 (1.3), 220 (5.5), 232 (1.3)



2 R^1 = CO_2Me, R^2 = 2,4-di-*O*-methyl-3-*O*-(2,3,4-tri-*O*-methyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl

3 R^1 = CH_2OMe, R^2 = 2,4-di-*O*-methyl-3-*O*-(2,3,4-tri-*O*-methyl-α-L-rhamnopyranosyl)-α-L-rhamnopyranosyl

4 R^1 = CH_2OAc, R^2 = Ac

5 R^1 = CH_2OMe, R^2 = Ac

m/z 691 for $[M + NH_4]^+$, indicating a mol. wt. of 673 in accord with the replacement of COOMe in **2** by CH_2OMe in **3** (spectrum not shown). The e.i. mass spectrum of **3** contained peaks for fragment ions derived from the rhamnopyranosyl residues as for **3**, but those belonging to the 3-deoxyoctonate chain were shifted to lower masses by 14 a.m.u. The fragment ion at *m/z* 90 represents the C-1/2 moiety, which accords with the rules proposed[6] for fragmentation of 3-deoxyoctitol derivatives. The characteristic fragment ions and their abundances are listed in Table I.

On methylation analysis[6], **2** yielded 1,5-di-*O*-acetyl-6-deoxy-2,3,4-tri-*O*-methyl-L-mannitol, 1,3,5-tri-*O*-acetyl-6-deoxy-2,4-di-*O*-methyl-L-mannitol, and 1,8-di-*O*-acetyl-3-deoxy-2,4,5,6,7-penta-*O*-methyl-D-*glycero*-D-*galacto*/*talo*-(2-^{2}H)-octitol (**4**) in the molar ratios 1:1:1, whereas **3** yielded 8-*O*-acetyl-1,2,4,5,6,7-hexa-*O*-methyl-D-*glycero*-D-*galacto*/*talo*-(2-^{2}H)-octitol (**5**) instead of **4**. Compounds **4** and **5** were identified by e.i.-m.s. in comparison with other derivatives of partially methylated and acetylated derivatives of 3-deoxyoctitol[6]. The characteristic fragment ions are listed in Table I.

The absolute configurations of the monosaccharide constituents of **1** were determined by using Klyne's rule[7]. Thus, **1** had $[\alpha]_D^{20}$ −39° (*c* 0.5, water), which is in good agreement with that (−24.7°) calculated {KDO, $[\alpha]_D^{23}$ +40.3° (ref. 8); methyl α-L-rhamnopyranoside, $[\alpha]_D$ −67.2° (ref. 9)}.

The ^{1}H-n.m.r. spectrum of **1** contained resonances for H-3 of KDO at 1.88 (H-3*a*-*ap*, *J* 5.0 and 11.5 Hz), 2.01 (H-3*e*-*ap*, *J* 11.5 and 11.5 Hz), 2.09 (H-3*a*-*f*, *J* 2.5 and 14.0 Hz), and 2.60 p.p.m. (H-3*e*-*f*, *J* 6.5 and 14.0 Hz), which are in the range expected for reducing KDO[10]. The signals [5.05 (s), 4.80 (s), and 1.30 p.p.m. (d, *J* 6.5 Hz)] for H-1 and H-6 of the α-L-rhamnopyranosyl residues corresponded to those of α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→6)-D-galactopyranose[11].

TABLE II

^{13}C-N.M.R. RESONANCES OF **1** AND RELATED COMPOUNDS[a]

	1	*A*		**1**	*B*
1″	103.28	103.20	1*αp*	177.40	177.46
2″	71.08	70.97	2*αp*	97.31	97.33
3″	71.08	70.97	3*αp*	34.70	34.60
4″	72.95	72.86	4*αp*	67.18	67.09
5″	70.01	69.88	5*αp*	67.56	67.57
6″	17.58	17.36	6*αp*	71.80	72.18
1′	101.40 101.25	101.20	7*αp*	69.09	70.32
2′	70.85	70.63	8*αp*	70.01	63.97
3′	79.15 79.20	79.00	1*βf*	178.29	178.26
4′	72.35	72.17	2*βf*	105.28	105.13
5′	69.73	69.63	3*βf*	45.64	45.54
6′	17.54	17.36	4*βf*	73.55	73.47
			5*βf*	86.49	86.57
			6*βf*	71.71*	71.93*
			7*βf*	71.32*	72.66*
			8*βf*	69.89	63.97

[a] *A* represents the α-L-Rha-(1→3)-α-L-Rha-(1→6) portion of **18** in ref. 13, and *B* represents the α-pyranosidic and β-furanosidic forms of the ammonium salt of KDO. For C-1/8 of *B*, the α-furanosidic signals appear at 177.69, 104.06, 44.52, 71.93, 86.21, 70.99*, 72.62*, and 63.83 p.p.m., and the β-pyranosidic signals at 176.27, 98.23, 36.11, 68.51, 66.39, 74.63, 70.13, and 64.68 p.p.m. The assignment of signals marked * may be interchanged.

The assignment of the resonances (Table II) in the ^{13}C-n.m.r. spectrum of **1** was facilitated by the clustering of the signals into three groups corresponding to two rhamnosyl residues and the pyranose and furanose forms of KDO with ratios of intensities of ~1:0.6:0.3. The resonance of C-1 at 103.3 p.p.m. is characteristic[11,12] of a glycosidically linked, unsubstituted α-rhamnopyranose. The signals at 101.3 and 101.4 p.p.m. (ratio 1:2, similar to the pyranose–furanose ratio of KDO) together with the signals at 79.2 and 17.5 p.p.m. show the internal rhamnose to be substituted at O-3, since substitution at O-2 would cause a β-shift at C-1 (observed values 101.3 and 101.4 p.p.m.) and substitution at O-4 a downfield shift at C-6 (17.5 p.p.m.). The rhamnose resonances are similar to those of *O*-α-L-rhamnopyranosyl-(1→3)-*O*-α-L-rhamnopyranosyl-(1→6)-D-galactopyranose[13], and therefore the anomeric configuration can be assigned empirically to the rhamnopyranosyl residues (Table II). The resonances of the ring-carbons of the KDO unit are similar to those reported[10–14]. The fact that no signals were observed in the range 60–65 p.p.m., where signals corresponding to unsubstituted C-8 of KDO would be expected, proves that the KDO unit is substituted at C-8. As suggested by McNicholas *et al.*[15], the anomeric configuration of the furanoid tautomer of KDO is β.

A common structural element of enterobacterial and other LPS is a 5-*O*-sub-

stituted KDO residue which links the lipid A and polysaccharide moieties and is part of the so-called inner-core region[5]. This region participates in the specific interaction of bacterial LPS with a normally occurring serum protein of mice[1]. Whereas the requirements for this interaction have been reported[1,2] to include KDO and a neutral sugar attached thereto, the structural prerequisites are not known. Therefore, we became interested in the structure of the KDO region of LPS from an *Acinetobacter* strain which did not bind to the above-mentioned mouse serum protein, but contained KDO and the neutral sugar rhamnose. The identification of the trisaccharide **1** suggests that substitution of KDO at position 8 does not fulfil the structural requirements for this interaction.

The substitution of KDO at position 8 with a neutral sugar has not been reported hitherto in bacterial LPS, but has been described[16] in the capsular polysaccharide of *Escherichia coli* K95.

EXPERIMENTAL

A. calcoaceticus strain 10303 of the NTCC (London) was grown[3] aerobically in a fermenter (14 L). LPS was extracted by a modification[3] of the phenol–chloroform–light petroleum method[17] and purified by three cycles of ultracentrifugation (100,000*g*, 4 h). The purified LPS was converted into its uniform triethylammonium salt after electrodialysis[18]. The yield was 2.8% based on dry bacteria.

Reduction with sodium borohydride or sodium borodeuteride, the removal of boric acid, and acetylation were performed by conventional methods. High-voltage paper electrophoresis was performed[19] in pyridine–acetic acid–water (10:4:86; pH 5.3) with detection for reducing sugars[20], phosphate[21], and KDO[22]. KDO was quantified by the thiobarbiturate assay[23], and rhamnose as its alditol acetate[24] by g.l.c. Optical rotations were measured with a Perkin–Elmer 141 polarimeter. The experimental conditions for methylation analysis, g.l.c., and g.l.c.–m.s. have been described[6,25]. Methylated oligosaccharides were hydrolysed with M trifluoroacetic acid (100°, 1 h). N.m.r. spectra were recorded for solutions in D_2O (external Me_4Si, 67.4 p.p.m. upfield of the signal for internal 1,4-dioxane) at 24°, using a Bruker WM 250 instrument [deuterium lock on the water signal and referenced internally to the signal of sodium 3-(trimethylsilyl)propyl-1-sulfonate]; ^1H at 250 MHz (spectral width, 2 kHz; 16 k of memory) and ^{13}C at 62.9 MHz (5-mm probe-head, spectral width, 12 kHz; 32 k of memory).

Hydrolysis of LPS. — Trisaccharide **1** was isolated from LPS (500 mg) by acid hydrolysis in 0.1M acetate buffer followed by dialysis against water (3 × 200 mL). The dialysates were then neutralised with sodium hydroxide and concentrated to dryness, and a solution of the residue in water (5 mL) was eluted from a column (120 × 2.5 cm) of Bio-Gel P2 (Bio-Rad) with 10mM sodium chloride. The eluate was monitored by the thiobarbiturate assay for KDO and with the anthrone reagent for total carbohydrates. Three peaks were obtained containing (*1*) rhamnose, KDO, and phosphate in a non-stoichiometric ratio; (*2*) **1**; (*3*) KDO. Fraction (*2*)

was re-chromatographed under the above conditions, then desalted on the same column with water as eluant, and lyophilised to give amorphous **1** (13 mg). **1** had M_{KDO} 0.6 in high-voltage paper electrophoresis, stained with alkaline silver nitrate and the thiobarbiturate reagent, and contained rhamnose and KDO in the molar ratio 2:1 but no phosphorus.

ACKNOWLEDGMENTS

The authors thank Drs. E. T. Rietschel and F. M. Unger for discussion and helpful suggestions, G. Schulz for the n.m.r. measurements, and Veronika Susott and Hermann Moll for technical assistance.

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