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Note

Isolation and identification of 3-deoxy-7-*O*-α-D-galactopyranosyl-D-*manno*-2-octulopyranosonate from the inner core region of the lipopolysaccharide of *Escherichia coli* EH100*

OTTO HOLST, EVA RÖHRSCHEIDT-ANDRZEJEWSKI, HANS-PETER CORDES, AND HELMUT BRADE†
Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Parkallee 22, D-2061 Borstel (F.R.G.)

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Lipopolysaccharides (LPS) are common constituents of the cell walls of Gram-negative bacteria and consist of a heteropolysaccharide linked to lipid A, which is responsible for the endotoxic activity[1]. The polysaccharide comprises the O-specific chain and the core oligosaccharide. The latter contains 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and heptoses in its inner core region[2]. The inner core region, the structure of which has been elucidated[3–7], involves a single KDO residue, which links the polysaccharide and lipid A moieties in all LPS except that of a strain of *Acinetobacter calcoaceticus* in which KDO is replaced by D-*glycero*-D-*talo*-2-octulosonic acid[8]. The linking KDO residue is usually 5-substituted by a heptosyl residue, but substituents such as KDO[4,9], L-rhamnose[10], 4-amino-4-deoxy-L-arabinopyranose[11], glucuronic acid[12], galacturonic acid[13], galactose[14], phosphate[15,16], and phosphorylethanolamine[17] may be present at positions 4, 5, 7, or 8 in the LPS of certain bacterial strains.

In 1972, Hämmerling *et al.* reported[14] the occurrence of a galactosyl-KDO disaccharide as a component of the inner core region in the LPS of *E. coli* 0100, but its structure was not determined. We now report the isolation, purification, and structure of the title disaccharide **1**.

Hydrolysis of the LPS released the core constituents which were analysed by high-voltage paper electrophoresis (p.e.). One product (M_{KDO} 0.63), present in the diffusate of the products of hydrolysis, was isolated and purified, using ion-exchange and gel-permeation chromatography and preparative p.e., and shown to be **1**. Chemical analysis indicated **1** to contain KDO and galactose in the molar ratio ~1:1. The galactosyl residue was shown to be D by treatment with β-D-galactose dehydrogenase and by g.l.c. of the acetylated (−)-2-butyl glycoside.

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†Author for correspondence.

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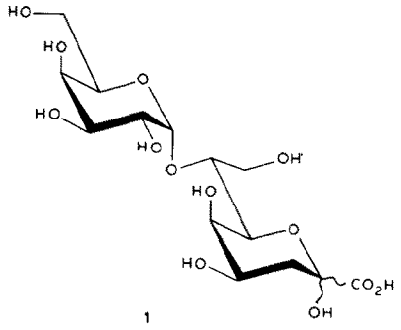


1

The following derivatives of **1** were prepared[7,18] for analysis by g.l.c.–m.s. Reduction of **1** with sodium borodeuteride followed by methylation gave **2**, which was carboxyl-reduced to yield **3**. Methylation analysis (hydrolysis in M trifluoroacetic acid for 1 h at 120° followed by reduction and acetylation) of **3** yielded equimolar amounts of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (**5**) and 1,7-di-*O*-acetyl-3-deoxy-2,4,5,6,8-penta-*O*-methyl-D-*glycero*-D-*galacto*/*talo*-(1,1,2-$^2H_3$)octitol (**7**). Likewise, **4** (obtained from **3** by methylation) yielded equimolar

TABLE I

G.L.C.–M.S. DATA FOR **2**, **4**, AND **7**

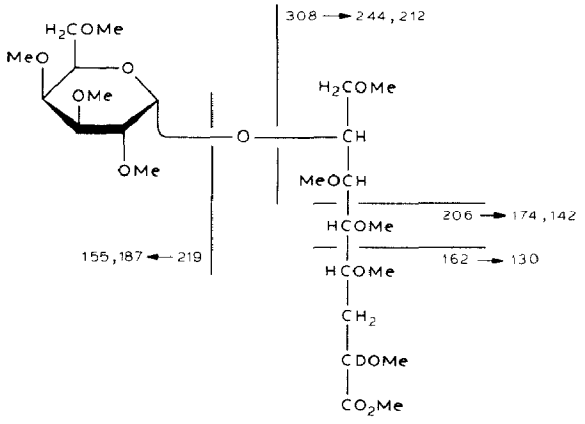
Compound	*Mol. wt.*[a]	*Retention time*[b]	m/z *(% of base peak)*[c]
2	543	2.45	45 (30.0), 59 (7.8), 71 (38.9), 75 (53.3), 85 (6.4), 88 (72.2), 89 (22.2), 101 (100), 111 (53.3), 115 (18.9), 130 (67.8), 133 (18.9), 145 (13.3), 155 (6.7), 162 (14.4), 174 (15.6) 187 (43.3), 206 (2.7), 212 (7.8), 219 (18.2), 244 (11.1), 308 (43.3)
4	531	2.34	45 (30.0), 59 (8.9), 71 (26.7), 75 (51.1), 88 (65.6), 92 (64.4), 101 (100), 111 (55.6), 115 (22.2), 118 (95.6), 129 (26.7), 133 (14.4), 145 (14.4), 150 (11.1), 155 (6.9), 187 (48.9), 200 (8.7), 219 (29.3), 296 (37.8)
7	383	1.00	45 (19.6), 71 (13.1), 72 (31.1), 75 (13.1), 87 (22.9), 88 (21.3), 101 (27.8), 102 (9.8), 120 (98.4), 129 (100.0), 142 (32.8), 145 (32.8), 146 (34.4), 161 (23.0), 205 (18.0), 222 (9.8)

[a]Determined by c.i.(ammonia)-m.s. on the basis of peaks at *m/z* for $(M + 1)^+$ and $(M + 18)^+$. [b]Relative to that of α-D-glucose penta-acetate (1.00), using a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded SE-54, a temperature programme of 140° for 3 min and then 3°/min → 220°, and H_2 as carrier gas (1.5 bar). [c]Determined by e.i.-m.s. (70 eV).

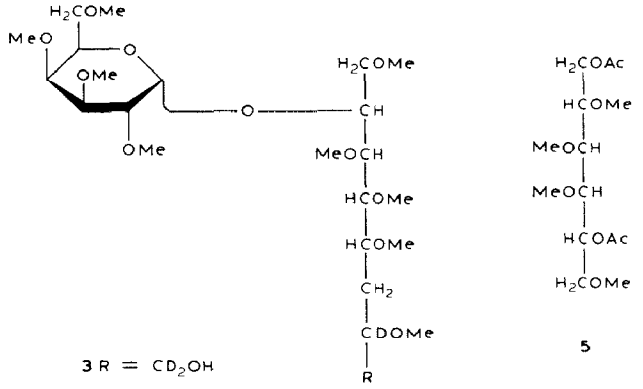
amounts of **5** and 7-*O*-acetyl-3-deoxy-1,2,4,5,6,8-hexa-*O*-methyl-D-*glycero*-D-*galacto/talo*-(1,1,2-$^2H_3$)octitol (**6**). C.i.(ammonia)-m.s. of **2** yielded a peak for $(M + 18)^+$ at *m/z* 561. The e.i. mass-spectral data are shown in Table I, and the fragmentation pattern is shown in the formula. C.i.(ammonia)- and e.i.-mass spectra (Table I) of **4** indicated that M^+ and the fragments containing C-1 of the alditol chain were 12 mass units smaller than for **1**, thereby confirming the presence and the position of the carboxyl group in **1** and the D-galactosyl→KDO structure.

On methylation analysis, **3** and **4** each yielded 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-galactitol (**5**), indicating the D-galactosyl unit to be a pyranoside. The other products were 7-*O*-acetyl-3-deoxy-1,2,4,5,6,8-hexa-*O*-methyl-D-*glycero*-D-*galacto/talo*-(1,1,2-$^2H_3$)octitol (**6**) and 1,7-di-*O*-acetyl-3-deoxy-2,4,5,6,8-penta-*O*-methyl-D-*glycero*-D-*galacto/talo*-(1,1,2-$^2H_3$)octitol (**7**), respectively. The e.i. mass spectrum of **6** has been reported[18], that of **7** is shown in Table I, and the fragmentation pattern is shown in the formula. Thus, **1** is D-galactopyranosyl-(1→7)-KDO.

The ^{13}C-n.m.r. data for **1** are shown in Table II. The resonance at 101.24 p.p.m. is assigned to C-1 of an α-D-galactopyranosyl unit (*cf.* 100.1 p.p.m. and



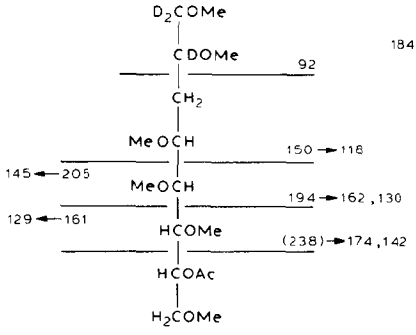
2 (mol. wt. 543)



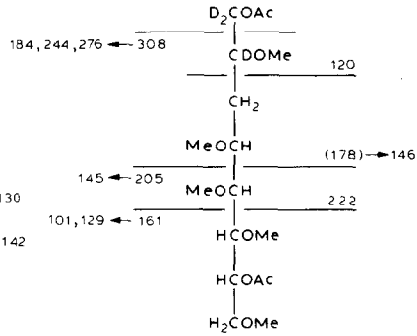
3 R = CD_2OH

4 R = CD_2OMe

5



6 (mol. wt. 355)



7 (mol. wt. 383)

104.5 p.p.m. for methyl α- and β-D-galactopyranoside[19], respectively), and the α configuration was confirmed[20] by the values $J_{C\text{-}1,H\text{-}1}$ 171.26 and $J_{1,2}$ 3.6 Hz. The ^{13}C-resonances of C-1/5 of the KDO moiety of **1** were similar to those of authentic KDO (Table II). The signal at 78.58 p.p.m. and the shifts of the resonances for C-6 and for C-8 indicated a substitution of KDO at C-7. Substitution at C-8 was excluded by comparing the data with those reported[10] for 8-substituted KDO. Thus, **1** is 3-deoxy-7-*O*-α-D-galactopyranosyl-D-*manno*-2-octulopyranosonate.

7-Substitution of KDO with a neutral sugar has not been detected hitherto in bacterial LPS, but occurs in the capsular polysaccharides of *E. coli* K6[21] and *Neisseria meningitidis*[22].

TABLE II

^{13}C-N.M.R. RESONANCES OF **1**, THE AMMONIUM SALT OF KDO, AND α-D-GALACTOPYRANOSE

Carbon atom	**1**	*KDO*	α-D-*Gal*p
1	177.38	177.59	
2	97.31	97.23	
3	34.36	34.45	
4	67.02	67.02	
5	67.30	67.41	
6	70.33	71.95	
7	78.58	70.03	
8	61.98	63.81	
1′	101.24		93.11
2′	69.36		69.17
3′	70.02		69.99
4′	70.09		70.13
5′	72.03		71.29
6′	61.98		62.01

EXPERIMENTAL

Bacteria and bacterial lipopolysaccharide (LPS). — *E. coli* strain EH100, a rough mutant derived[14] from *E. coli* 0100, was grown in a fermenter (14 L), killed with phenol (0.5%), and centrifuged. The sedimented bacteria were washed successively with ethanol, acetone (twice), and ether, and then dried. LPS was isolated (2–3%) from dry bacteria by the phenol–chloroform–light petroleum method[23].

General. — The ammonium salt of KDO was a gift of Dr. P. Kosma (Vienna) and β-D-galactose dehydrogenase (EC 1.1.1.48) from *Pseudomonas fluorescens* was obtained from Sigma. Galactose was identified as its alditol hexa-acetate by g.l.c., after hydrolysis (48 h, 100°) in 0.1M HCl, and KDO was determined by the thiobarbiturate assay[24]. Reduction with sodium borohydride or sodium borodeuteride was performed conventionally, as was acetylation with pyridine–acetic anhydride (1:1, 30 min, 100°). Optical rotations were measured with a Perkin–Elmer 141 polarimeter. High-voltage p.e. was carried out at 40 V/cm in pyridine–acetic acid–formic acid–water[25] (1:10:1.5:90; pH 2.8), with detection by alkaline silver nitrate for reducing sugars[26], ninhydrin (0.2% in acetone) for free amino groups, a molybdate reagent for phosphorus[27], and the thiobarbiturate reagent[28] for KDO.

G.l.c. was performed with a Varian 3700 gas chromatograph equipped with a flame-ionisation detector and a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded SE-54 (0.2 μm) (Weeke, Mühlheim); the carrier gas was H_2 (1.5 bar). Temperature programme: 150° for 5 min, 5°/min→300°. G.l.c.–m.s. was carried out on a Hewlett–Packard instrument (Model 5985) equipped with an SE-54 column and an HP-1000 data system. E.i.-mass spectra were recorded at 70 eV and c.i.-mass spectra were obtained with ammonia as reactant gas. The ion-source temperature was 200°.

The absolute configuration of the galactose released (4M trifluoroacetic acid, 4 h, 100°) from **1** was determined by (*a*) g.l.c. of the acetylated (−)-2-butyl glycosides[29,30] obtained after butanolysis (M HCl, 2 h, 85°), using a fused-silica capillary column (25 m × 0.32 mm i.d.) with chemically bonded CP SIL 5 at 170° and H_2 (0.4 bar) as the carrier gas; and (*b*) by reaction with β-D-galactose dehydrogenase, using tris(hydroxymethyl)methylamine–HCl buffer (0.1M, pH 8.8) and a three-fold excess of NAD^+.

N.m.r. spectra of **1** [^1H, 360 MHz, 23°, internal CH_3CN (1.95 p.p.m.); ^{13}C, 90.56 MHz, 23°, internal CH_3CN (1.70 p.p.m.)] were recorded for a solution of **1** with a Bruker AM360L spectrometer.

Isolation and purification of **1**. — LPS (520 mg) was hydrolysed (1 h, 100°) in 100mM sodium acetate buffer (pH 4.4, 50 mL) and the hydrolysate was dialysed against water (3 × 500 mL) at 4°. The diffusates were combined, desalted with Amberlite IR-120 (H^+) resin, neutralised with pyridine, and concentrated to dryness. A solution of the residue in water was eluted from a column (2.6 × 1.5 cm) of polyethyleneimine-cellulose (Sigma) with 50mM pyridine acetate (pH 5.5). Fractions were analysed for **1** (M_{KDO} 0.63) by p.e. The appropriate fractions were

combined and concentrated, and a solution of the residue in water was eluted from a column (100 × 1 cm) of Bio-Gel P2 (Bio-Rad) with 50mM pyridine acetate buffer (pH 5.2). Fractions were analysed by t.l.c. on Silica Gel 60 (Merck), using acetonitrile–water (85/15) and detection with orcinol–sulfuric acid. Appropriate fractions were combined and analysed by p.e., and homogeneous amorphous **1** (9.5 mg, 1.8% of LPS) was obtained, after preparative p.e., by lyophilisation. Compound **1** had $[\alpha]_D$ +90° (*c* 1.2, water); for the n.m.r. studies, it was purified further by gel-permeation chromatography, using distilled water as the irrigant (yield, 7.0 mg; 1.35% of LPS).

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