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Note

Isolation and structural analysis of the tetrasaccharide 3-deoxy-5-*O*-[3-*O*-(3-*O*-α-D-glucopyranosyl-L-*glycero*-α-D-*manno*-heptopyranosyl)-L-*glycero*-α-D-*manno*-heptopyranosyl]-D-*manno*-octulosonic acid from the core region of the lipopolysaccharide of *Salmonella minnesota* strain R5 (rough mutant chemotype RcP$^-$) †

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The lipid A-proximal part of the core region of enterobacterial lipopolysaccharides (LPSs) contains[1] invariably the sugars 3-deoxy-D-*manno*-octulosonic acid (Kdo) and heptose present as L-*glycero*-D-*manno*- (L,D-Hep*p*) or D-*glycero*-D-*manno*-heptopyranose (D,D-Hep*p*). To a variable extent, other sugars were identified[1], e.g., in strains of *Escherichia coli* (D-Gal*p*, L-Rha*p*, D-Glc*p*N) or *Proteus mirabilis* (4-amino-4-deoxy-L-arabinose, D-Glc*p*, D-Gal*p*A). Typical for many enterobacterial and some other LPSs is the presence of the tetrasaccharide α-D-Glc*p*-(1 → 3)-Lα D-Hep*p*-(1 → 3)-Lα D-Hep*p*-(1 → 5)-Kdo which may be substituted at various positions. To facilitate the assignment of the signals obtained in the complex ^{13}C NMR spectra of larger core oligosaccharides, we have systematically characterised the core structures from deep rough mutants. In particular, we have characterised the Kdo- and Hep/Kdo-region of Re- and Rd-mutants, respectively. Thus, the disaccharides α-Kdo-(2 → 4)-Kdo[2] and Lα D-Hep*p*-(1 → 5)-Kdo[3,4], and the trisaccharides α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-Kdo[5,6] and Lα D-Hep*p*-(1 → 3)-Lα D-Hep*p*-(1 → 5)-Kdo[7] have been isolated and characterised by NMR and GLC–MS. Here, we report the isolation and structural analysis of the title tetrasaccharide **1** from the LPS of *Salmonella minnesota* R5, which also occurs in many other LPSs.

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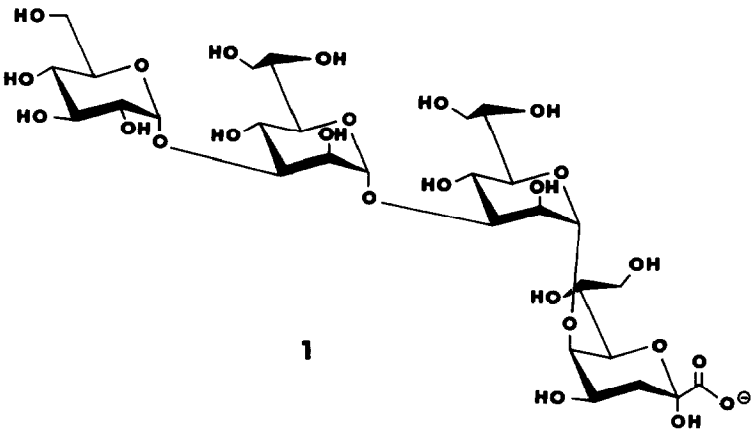

1

Hydrolysis of the LPS under conditions known to cleave ketosidic but not other glycosidic linkages released the core constituents of the LPS. Analysis by high-voltage paper electrophoresis (PE) revealed Kdo, small amounts of 3-deoxy-D-*manno*-octulopyranosonate 7-(2-aminoethyl phosphate)[8], and a product with M_{Kdo} 0.31. The latter was isolated and purified in two steps by gel-permeation chromatography, and shown to possess structure **1**.

Chemical analysis of **1** identified glucose, heptose, and Kdo in the molar ratios ~ 1:2:1. The absolute configuration of the glucose was established as D by GLC of the acetylated (*R*)-2-butyl glycoside. The retention time of the alditol acetate of the heptose was identical to that[9] of the same derivative of synthetic L-*glycero*-D-*manno*-heptopyranose, and different from that[9] of the alditol acetate of synthetic D-*glycero*-D-*manno*-heptopyranose. The retention time in GLC of the methylated octitol obtained from Kdo of the R5 core was identical to that of methylated 3-deoxy-D-*galacto/talo*-octitol obtained from synthetic Kdo, indicating its *manno* configuration.

Reduction ($NaBH_4$) and methylation of **1** afforded methylated methyl 3-deoxy-5-*O*-[3-*O*-(3-*O*-D-glucopyranosyl-L-*glycero*-D-*manno*-heptopyranosyl)-L-*glycero*-D-*manno*-heptopyranosyl]-D-*glycero*-D-*galacto/talo*-octonate ($T_{maltitol}$ 2.40), which was identified in GLC–MS by its EI-mass spectrum[10]. After carboxyl-reduction[4] followed by methylation analysis (hydrolysis in 8 M trifluoroacetic acid, reduction with NaB^2H_4), GLC–MS revealed 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-[1-^{2}H]glucitol, 1,3,5-tri-*O*-acetyl-2,4,6,7-tetra-*O*-methyl-[1-^{2}H]heptitol, and 1,5-di-*O*-acetyl-3-deoxy-2,4,6,7,8-penta-*O*-methyloctitol in the molar ratios ~ 1:2:1. In conclusion, **1** is 3-deoxy-5-*O*-[3-*O*-(3-*O*-D-glucopyranosyl-L-*glycero*-D-*manno*-heptopyranosyl)-L-*glycero*-D-*manno*-heptopyranosyl]-D-*manno*-octulosonic acid.

The signals obtained in ^{1}H (Table I) and ^{13}C NMR spectroscopy (Table II) were assigned by homonuclear ^{1}H,^{1}H and heteronuclear ^{1}H,^{13}C COSY NMR experi-

TABLE I

^{1}H NMR data (in ppm, 360 MHz, D_2O, $CH_3CN = 1.95$ ppm) and $J_{H,H}$ values of 1

Assignment	Chemical shift ($J_{H,H}$ in Hz)			
	→ 5)-Kdo	→ 3)-Hep*p*	→ 3)-Hep*p*	Glc*p*-(1 →
H-1		4.94	5.06	5.13
		(1.8)	(1.8)	(4.0)
H-2		4.02	4.11	3.43
		(2.2)	(3.1)	(10.0)
H-3*ax*	1.81	3.89	3.91	3.66
	(12.3; −12.7)	(10.5)	(9.5)	(9.1)
H-3*eq*	1.99			
	(4.0)			
H-4	3.97–4.04	3.86	3.94	3.27
		(9.0)	(9.4)	(9.6)
H-5	3.96–4.00	3.84–3.90	3.70–3.76	3.70–3.76
H-6a	3.75	3.86–3.92	3.90–3.94	3.71
	(9.0)			(2.7; −13.1)
H-6b				3.63–3.67
H-7a	3.78–3.82	3.56 [a]	3.58 [a]	
		(7.3)	(7.6)	
H-7b		3.52 [b]	3.54 [b]	
		(5.1; −11.9)	(5.6; −11.4)	
H-8a	3.67–3.72			
H-8b	3.58–3.62			

[a,b] Assignments exchangeable.

ments, and by comparison with data published for L*α*D-Hep*p*-(1 → 3)-L*α*D-Hep*p*-(1 → 5)-Kdo[7] (^{13}C), L*α*D-Hep*p*-(1 → 3)-L*α*D-Hep*p*[11,12] (^{13}C) and its allyl glycoside[13] (^{1}H), L*α*D-Hep*p*-(1 → 5)-Kdo[3] (^{13}C) and its *α*-allyl ketoside[13] (^{1}H), the *α*-allyl glycosides of L,D-Hep*p*[13] (^{1}H) and Kdo[6] (^{1}H,^{13}C), and *α*-D-Glc*p*[14] (^{1}H,^{13}C). Compared with the ^{13}C NMR data of allyl *α*-Kdo, *α*-D-Glc, and the terminal Hep of L*α*D-Hep*p*-(1 → 3)-L*α*D-Hep*p* and L*α*D-Hep*p*-(1 → 5)-Kdo, the glycosidic linkages were proved by downfield shifts of C-1′, C-1″, and C-1‴, respectively, and the substitution at O-5 of Kdo and O-3 of both L,D-Hep residues was indicated by downfield shifts for C-5, C-3′, and C-3″ of ~9, 7, and 8 ppm, respectively. A GATED ^{13}C NMR experiment[15] proved that all linkages were *α* (Table II).

TABLE II

^{13}C NMR data (in ppm, 90.6 MHz, D_2O, $CH_3CN = 1.70$ ppm) of 1

Residue	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8
→ 5)-Kdo	175.29	96.72	34.72	66.40	76.12	72.00	69.86	63.78 [a]
→ 3)-Hep*p*	102.17 [b]	70.90	78.01	66.67	72.85	69.63	63.78 [a,c]	
→ 3)-Hep*p*	102.64 [d]	70.70	79.71	66.15	72.63 [a]	69.34	64.10 [c]	
Glc*p*-(1 →	101.41 [e]	72.63	73.65	70.50	73.18	61.50		

[a] Non-resolved signal. [b] $J_{C\text{-}1',H\text{-}1'}$ 170.5 Hz. [c] Assignments exchangeable. [d] $J_{C\text{-}1'',H\text{-}1''}$ 172.6 Hz. [e] $J_{C\text{-}1''',H\text{-}1'''}$ 171.4 Hz.

In summary, our results indentified the structure of the isolated tetrasaccharide as **1**.

EXPERIMENTAL

General methods for quantitative analyses were as described[16]. Methylation of reduced **1** was performed according to Ciucanu and Kerek[17], and purification was done according to Waeghe et al.[18]. In methylation analysis of reduced and methylated **1**, the hydrolysis was in 8 M trifluoroacetic acid (4 h, 100°C).

The absolute configuration of Glc [obtained from **1** after hydrolysis (4 M trifluoroacetic acid, 4 h, 100°C)] was determined by GLC of the acetylated (*R*)-2-butyl glycoside[19,20] obtained after butanolysis (M HCl, 2 h, 85°C) and acetylation, compared with the same derivatives of authentic D- and L-Glc*p*, respectively.

GLC on a capillary column of SE 54 was performed as described[21]. Temperature programmes: 140°C for 3 min, then 3°C/min → 250°C, for partially methylated alditol acetates; 200°C for 3 min, then 5°C/min → 300°C, for reduced and methylated **1**. The retention time for reduced and methylated **1** was determined relative to that of maltitol nona-acetate (*T* 1.00). GLC of the acetylated (*R*)-2-butyl glycosides was carried out on a fused-silica capillary column with chemically bonded SPB-5 (30 m × 0.32 mm i.d., Supelco) at 0.1 MPa H_2 and 170°C. GLC–MS was performed as described[21].

The optical rotation of **1** was measured on a solution in water, using a Perkin–Elmer 141 polarimeter.

One-dimensional ^1H and ^{13}C and two-dimensional $^1H,^1H$ and $^1H,^{13}C$ COSY NMR spectra were recorded at 23°C for solutions of **1** in D_2O, using a Bruker AM 360 L spectrometer and Bruker standard software. CH_3CN was the internal standard (1.95 ppm in ^1H, 1.70 ppm in ^{13}C NMR spectroscopy).

Bacteria and bacterial LPS.—*S. minnesota* strain R5 (chemotype RcP^-) was grown in a fermenter (14 L), killed with phenol (0.5%), centrifuged, and washed with EtOH, acetone (twice), and ether. The LPS was extracted[22] from the dry bacteria in a yield of 2.9%.

*Isolation and purification of 3-deoxy-5-*O-[*3*-O-(*3*-O-α-D-*glucopyranosyl*-L-glycero-α-D-manno-*heptopyranosyl*)-L-glycero-α-D-manno-*heptopyranosyl*]-D-manno-*octulosonic acid* (**1**).—The LPS (3 g) was hydrolysed (1 h, 100°C) in 100 mM sodium acetate buffer (pH 4.4, 300 mL), and the hydrolysate was dialysed against water (3 × 500 mL) at 4°C. The combined diffusates were concentrated and then separated on a column (100 × 1.5 cm) of Bio-Gel P2 (Bio-Rad) in water. The first of two fractions was rechromatographed on a column (3.5 × 40 cm) of TSK HW40 (S) (Merck) in water, from which four fractions were obtained and analysed in PE. Only one spot (M_{Kdo} 0.31) was present in the second fraction, yielding **1** {50 mg, 1.6% of LPS, $[\alpha]_D^{22}$ + 128.4° (*c* 0.7)} after lyophilisation.

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REFERENCES

1 O. Holst and H. Brade, in D.C. Morrison and J.L. Ryan (Eds.), *Bacterial Endotoxic Lipopolysaccharides*, Vol. 1, CRC Press, Boca Raton, FL, 1992, pp 135–170.
2 H. Brade, U. Zähringer, E.T. Rietschel, R. Christian, G. Schulz, and F.M. Unger, *Carbohydr. Res.*, 134 (1984) 157–166.
3 R. Christian, G. Schulz, and F.M. Unger, *Tetrahedron Lett.*, 26 (1985) 3951–3954.
4 A. Tacken, E.T. Rietschel, and H. Brade, *Carbohydr. Res.*, 149 (1986) 279–291.
5 H. Brade, L. Brade, and F.E. Nano, *Proc. Natl. Acad. Sci. USA*, 84 (1987) 2508–2512.
6 K. Bock, J.U. Thomsen, P. Kosma, R. Christian, O. Holst, and H. Brade, *Carbohydr. Res.*, 229 (1992) 213–224.
7 O. Holst and H. Brade, *Carbohydr. Res.*, 219 (1991) 247–251.
8 O. Holst, E. Röhrscheidt-Andrzejewski, H. Brade, and D. Charon, *Carbohydr. Res.*, 204 (1990) 93–102.
9 O. Holst, H. Brade, K. Dziewiszek, and A. Zamojski, *Carbohydr. Res.*, 204 (1990) 1–9.
10 H. Brade, H. Moll, and E.T. Rietschel, *Biomed. Mass Spectrom.*, 12 (1985) 602–609.
11 K. Dziewiszek, A. Banaszek, and A. Zamojski, *Tetrahedron Lett.*, 28 (1987) 1569–1572.
12 H. Paulsen and A.C. Heitmann, *Liebigs Ann. Chem.*, (1988) 1061–1071.
13 H. Paulsen, A. Wulff, and M. Brenken, *Liebigs Ann. Chem.* (1991) 1127–1145.
14 K. Bock and H. Thøgersen, *Annu. Rep. NMR Spectrosc.*, 13 (1982) 1–57.
15 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
16 W. Kaca, J. De Jongh-Leuvenink, U. Zähringer, E.T. Rietschel, H. Brade, J. Verhoef, and V. Sinnwell, *Carbohydr. Res.*, 179 (1988) 289–299.
17 I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
18 T.J. Waeghe, A.G. Darvill, M. McNeill, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.
19 G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
20 S. Kondo, U. Zähringer, E.T. Rietschel, and K. Hisatsune, *Carbohydr. Res.*, 188 (1989) 97–104.
21 O. Holst, E. Röhrscheidt-Andrzejewski, H.-P. Cordes, and H. Brade, *Carbohydr. Res.*, 188 (1989) 212–218.
22 C. Galanos, O. Lüderitz, and O. Westphal, *Eur. J. Biochem.*, 9 (1969) 245–249.