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Isolation and characterisation of 3-deoxy-D-*manno*-2-octulopyranosonate 7-(2-aminoethyl phosphate) from the inner core region of *Escherichia coli* K-12 and *Salmonella minnesota* lipopolysaccharides*†

Otto Holst‡, Eva Röhrscheidt-Andrzejewski, Helmut Brade,
Forschungsinstitut Borstel, Institut für Experimentelle Biologie und Medizin, Parkallee 22, D-2061 Borstel (F.R.G.)
and Daniel Charon
Centre d'Etudes Pharmaceutiques, Equippe No. 55 du C.N.R.S., F-92290 Châtenay-Malabry (France)

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ABSTRACT

The title compound (PE-Kdo) was isolated after hydrolysis of the lipopolysaccharides of *Escherichia coli* K-12 strain W3100 and *Salmonella minnesota* strains R4 and R7, and the location of the 2-aminoethyl phosphate group at position 7 was established by ^{13}C-n.m.r. spectroscopy. Derivatives of PE-Kdo were acetylated, silylated, and methylated in order to evaluate their usefulness for analysis by g.l.c.–m.s.

INTRODUCTION

Lipopolysaccharides (LPS), which are common constituents of the outer membrane of Gram-negative bacteria[1], comprise lipid A, the core region (proximal to lipid A), and the O-chain (O-antigen), which differ in their composition, structure, biosynthesis, and immunological and serological features. The core region contains an outer and an inner core, the latter being composed of L-*glycero*-D-*manno*-heptopyranose and 3-deoxy-D-*manno*-2-octulopyranosonate (Kdo). The inner core from LPS of *Salmonella minnesota* rough mutants has been investigated in detail[1] and involves, variously, one Kdo residue in the main chain 5-substituted by a heptopyranose and 4-substituted by Kdo (or partially by an α-(2→4)-linked Kdo-disaccharide). In addition, other substituents may be located at different hydroxyl groups of Kdo[2,3], of which a 3-deoxy-D-*manno*-2-octulopyranosonate (2-aminoethyl phosphate) (PE-Kdo) has been identified[2] in different enterobacterial LPS. However, only the product from *S. minnesota* was investigated[4] and it was concluded that the 2-aminoethyl phosphate residue was linked to C-7 of Kdo as in **1**.

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‡ Author for correspondence.

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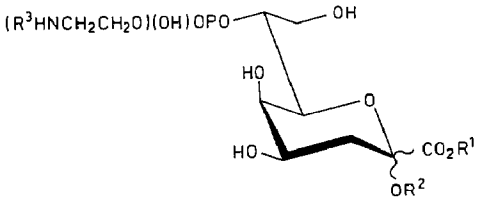
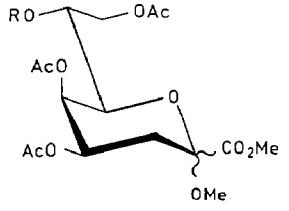
We now report that the PE-Kdo isolated from the LPS of *E. coli* K-12 and *S. minnesota* has the structure **1**.

RESULTS AND DISCUSSION

High-voltage paper electrophoresis (p.e.) of the products of hydrolysis of the LPS from *E. coli* K-12 W3100 revealed a component (M_{KDO} 0.73) which stained with thiobarbituric acid, ninhydrin, $AgNO_3$/NaOH, and molybdate, and which was shown to be PE-Kdo (**1**). Purification by ion-exchange and gel-permeation chromatography followed by preparative p.e. gave **1** in a yield of 0.3%. Chemical analysis[5,6] of **1** revealed Kdo, phosphate, and ethanolamine in the ratios ~1:1:1.

PE-Kdo (**1**) was isolated also (0.06%) from the LPS of *S. minnesota* R4 and R7 but, since it co-migrated with other compounds in p.e., the *N*-acetyl derivative **2** was prepared (M_{KDO} 2.06) which could be separated from the contaminants.





1 R^1/R^3 = H

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

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

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

5 R = $PO(OMe)_2$

6 R = H

7 R = PO(OH)(OCH_2CH_2NHAc)

8 R = PO(OMe)(OCH_2CH_2NHAc)

TABLE I

^{13}C-N.m.r. chemical shifts (p.p.m.) for **1–4**, Kdo (ammonium salt), 2-aminoethyl phosphate (PE), and 2-acetamidoethyl phosphate (PE-NAc)

Carbon atom	*Kdo*	**1**	**2**	**3**	**4**	*PE*	*PE-NAc*
1	177.59	177.44	n.m.[a]	170.96	175.63		
2	97.23	97.50	n.m.[a]	100.18	101.29		
3	34.45	34.48	34.37	34.42	35.05		
4	67.02	66.78	66.62	66.06	66.36		
5	67.41	67.17	66.98	66.24	66.74		
6	71.95	70.58	70.62	70.88	70.67		
7	70.03	75.08	74.69	74.27	74.55		
8	63.81	61.86	61.78	61.51	61.91		
C-P		40.90	40.90	40.83	40.83	40.90	40.71
N-C		62.81	65.17	65.17	65.01	62.23	65.05

[a] n.m. = Not measurable.

TABLE II

$^2J_{P,C}$ (Hz) for **1**–**4**, 2-aminoethyl phosphate (PE), and 2-acetamidoethyl phosphate (PE-NAc)

Carbon atom	**1**	**2**	**3**	**4**	*PE*	*Pe-NAc*
1						
2						
3						
4						
5						
6	8.17	n.m.[a]	7.24	7.24		
7	5.52	n.m.[a]	5.43	5.43		
8						
C-P	8.05	n.m.[a]	7.24	7.24	7.88	7.44
N-C	5.03	n.m.[a]	5.43	5.43	4.53	5.16

[a] n.m. = Not measurable.

The ^{13}C-n.m.r. data for **1** isolated from *E. coli* K-12 and **2** from *S. minnesota*, the derivatives **3** and **4**, Kdo, 2-aminoethyl phosphate, and 2-acetamidoethyl phosphate are shown in Tables I and II. The resonances of C-1/5 of the Kdo moieties of **1** and **2** were similar to those of Kdo. The signal for C-7 of **1** was identified at 75.08 p.p.m. ($^2J_{P,C}$ 5.52 Hz, 5.05 p.p.m. downfield of the C-7 signal of Kdo), and that of **2** at 74.69 p.p.m. (shift of 4.66 p.p.m. downfield). These shifts are in the range (1.7–4.8 p.p.m.) expected for phosphorylated carbon atoms of carbohydrates[7]. Signals of neighbouring carbon atoms were shifted upfield: C-6 of **1** from 71.95 to 70.58 p.p.m. ($^2J_{P,C}$ 8.17 Hz), C-6 of **2** from 71.95 to 70.62 p.p.m., C-8 of **1** from 63.81 to 61.86 p.p.m., and C-8 of **2** from 63.81 to 61.78 p.p.m. These data identify **1** as 3-deoxy-D-*manno*-octulopyranosonate 7-(2-aminoethyl phosphate) and confirm the structure proposed[4] for PE-Kdo from *S. minnesota*.

Treatment of **3** with diazomethane and acetylation of the product gave only [methyl (methyl 4,5,8-tri-*O*-acetyl-3-deoxy-*a*-D-*manno*-octulopyranosid)onate] 7-(dimethyl phosphate) (**5**), *T* 2.64 (relative to that of *a*-D-glucose penta-acetate); c.i. (ammonia)-mass spectrum: *m/z* 501 $(M + 1)^+$ and 518 $(M + 18)^+$. Fig. 1 shows the e.i.-mass spectrum. Thus, the 2-acetamidoethyl phosphate linkage was cleaved during the treatment of **3** with methanolic diazomethane. Treatment of **3** with methanol (30 min, room temperature) had no effect (data not shown). Therefore, the method[8] developed for the methylation analysis of phosphodiesters of carbohydrates could not be used.

Likewise, silylation of **3** gave only [methyl (methyl 3-deoxy-4,5,8-tri-*O*-trimethylsilyl-*a*-D-*manno*-octulopyranosid)onate] 7-[bis(trimethylsilyl) phosphate] (**9**), *T* 3.22, *m/z* 707 $(M + 1)^+$ and 724 $(M + 18)^+$. The e.i.-mass spectrum is shown in Fig. 2. Hence, even under mild silylation conditions, the 2-acetamidoethyl phosphate linkage was cleaved.

However, acetylation of **3** gave two products: (*a*) methyl (methyl 4,5,8-tri-*O*-acetyl-3-deoxy-D-*manno*-octulopyranosid)onate (**6**), *T* 2.27, *m/z* 393 $(M + 1)^+$ and 410

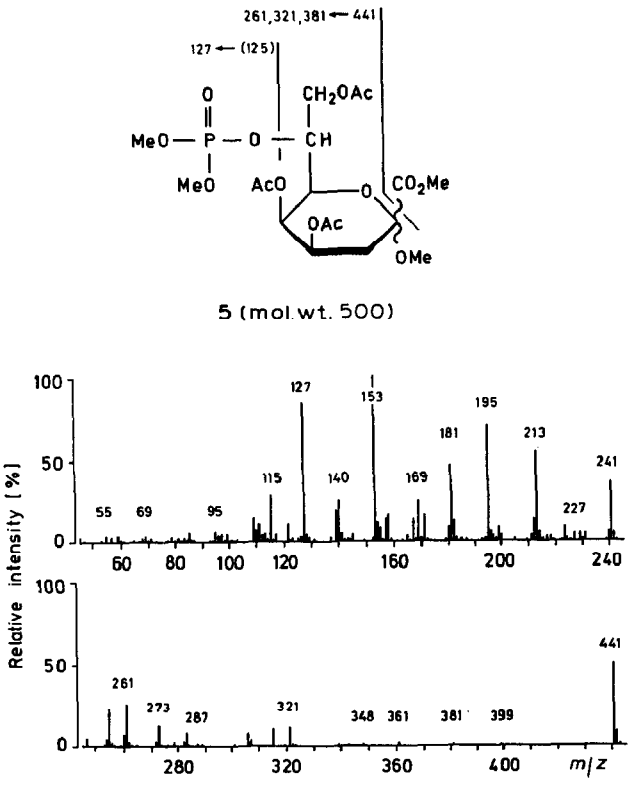
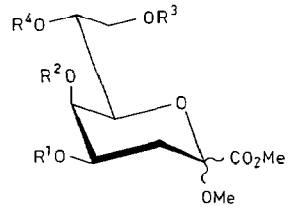


Fig. 1. Fragmentation pattern and e.i.-mass spectrum of **5**.

$(M + 18)^+$, the e.i.-mass spectrum of which is shown in Fig. 3; and (*b*) a small proportion of [methyl (methyl 4,5,8-tri-*O*-acetyl-3-deoxy-*α*-D-*manno*-octulopyranosid)onate] 7-(2-acetamidoethyl phosphate) (**7**), *m/z* 558 $(M + 1)^+$ and 575 $(M + 18)^+$, identified by direct-inlet probe (d.i.p.)-m.s.



9 $R^1/R^3 = Me_3Si$, $R^4 = PO(OSiMe_3)_2$

10 $R^2/R^4 = Me$, $R^1 = PO(OMe)_2$

11 $R^1 = R^3 = R^4 = Me$, $R^2 = PO(OMe)_2$

12 $R^1/R^3 = Me$, $R^4 = PO(OMe)_2$

13 $R^1 = R^2 = R^4 = Me$, $R^3 = PO(OMe)_2$

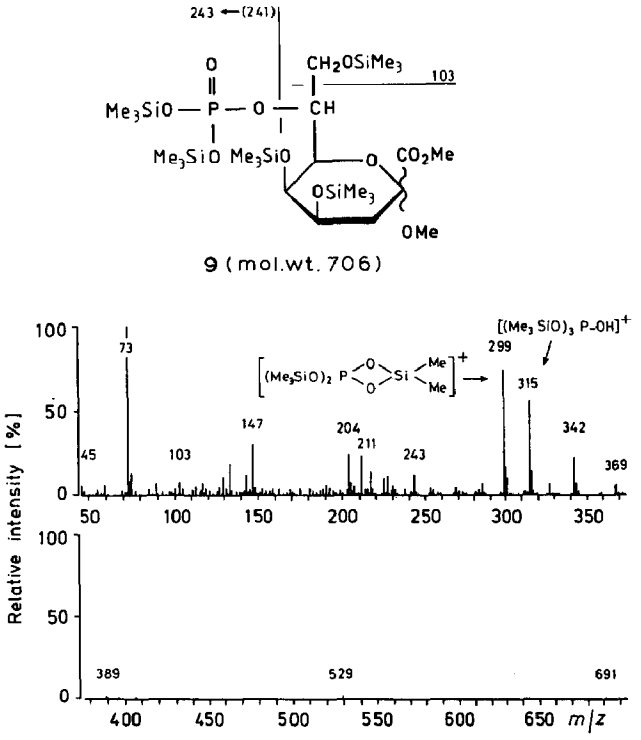


Fig. 2. Fragmentation pattern and e.i.-mass spectrum of **9**. Fragments indicated in the spectrum are according to ref. 15.

A mixture of **6** and **7** was treated with diazomethane, whereby the latter gave rise to **5** and a small proportion of [methyl (methyl 4,5,8-tri-*O*-acetyl-3-deoxy-α-D-*manno*-octulopyranosid)onate] 7-(2-acetamidoethyl methyl phosphate) (**8**), *m/z* 572 $(M + 1)^+$ and 589 $(M + 18)^+$. Therefore, mild acetylation does not yield **8** quantitatively. Furthermore, **7** and **8** were not detectable by g.l.c. and g.l.c.–m.s.

Compound **5** was deacetylated (methanolic 0.25M sodium methoxide, 15 min, room temperature) and methylated[9,10]. G.l.c. of the product revealed four peaks in the molar ratios ~0.2:0.3:1.0:0.6, which were shown by g.l.c.–m.s. to be [methyl (methyl 3-deoxy-1,5,7,8-tetra-*O*-methyl-α-D-*manno*-octulopyranosid)onate] 4-(dimethyl phosphate) (**10**, *T* 1.24), [methyl (methyl 3-deoxy-1,4,7,8-tetra-*O*-methyl-α-D-*manno*-octulopyranosid)onate] 5-(dimethyl phosphate) (**11**, *T* 0.93), [methyl (methyl 3-deoxy-1,4,5,8-tetra-*O*-methyl-α-D-*manno*-octulopyranosid)onate] 7-(dimethyl phosphate (**12**, *T* 1.02), and [methyl (methyl 3-deoxy-1,4,5,7-tetra-*O*-methyl-α-D-*manno*-octulopyranosid) onate] 8-(dimethyl phosphate) (**13**, *T* 1.18). Compounds **10**–**12** were identified by co-migration in g.l.c., and by g.l.c.–e.i.-m.s. with authentic derivatives of Kdo 4-, 5- (for both see ref. 11), and 7-phosphate, respectively. The e.i.-mass spectra of **12** and **13** are shown in Fig. 4. After carboxyl reduction and reductive cleavage[12] of the phosphates with $LiAlH_4$, hydrolysis in M trifluoroacetic acid (30 min, 100°), carbonyl reduction with

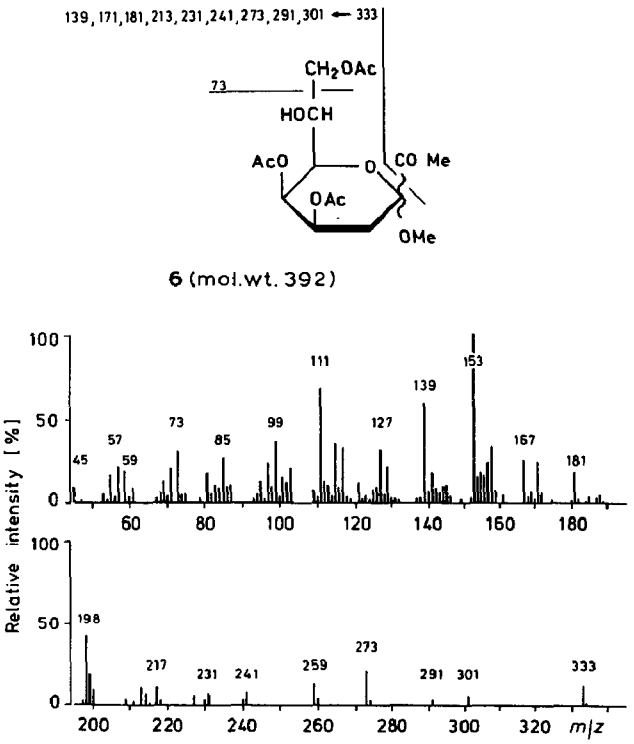


Fig. 3. Fragmentation pattern and e.i.-mass spectrum of **6**.

NaB^2H_4, and acetylation, 1,2,6,7-tetra-*O*-acetyl-3-deoxy-4,5,8-tri-*O*-methyl-[^{2}H]octitol (**14**, *T* 1.50 and 1.52 for the isomers) and 1,2,6,8-tetra-*O*-acetyl-3-deoxy-4,5,7-tri-*O*-methyl-[^{2}H]octitol (**15**, *T* 1.61 and 1.62) were identified, and for which the e.i.-mass spectra are shown in Fig. 5. Therefore, 7→8, and to a lesser extent 7→5→4, phosphate migration occurred during the acetylation and methylation of **3**.

After methylation of the LPS from *E. coli* K-12 strain W3100, methanolysis (M methanolic HCl, 16 h, 85°), and methylation, small amounts of **10–13** were identified by g.l.c.–m.s., and it is impossible to distinguish between PE-Kdo and Kdo phosphate as the source of these derivatives. Furthermore, g.l.c.–m.s. cannot prove the presence of Kdo phosphate in LPS. However, small amounts of Kdo phosphate (besides Kdo, PE-Kdo, and Rha-Kdo) were identified by p.e. after hydrolysis [0.1 M acetate buffer (pH 4.4), 1 h, 100°] of the LPS from *E. coli* K-12 strain W3100. Similar treatment of **1** and **2** gave no Kdo phosphate (p.e.). Thus, the LPS from *E. coli* K-12 strain W3100 contains both PE-Kdo and small amounts of phosphorylated Kdo.

Unless other methods are found for characterising PE-Kdo by g.l.c. and g.l.c.–m.s., n.m.r. spectroscopy is the method of choice for investigating the structure of PE-Kdo isolated from LPS.

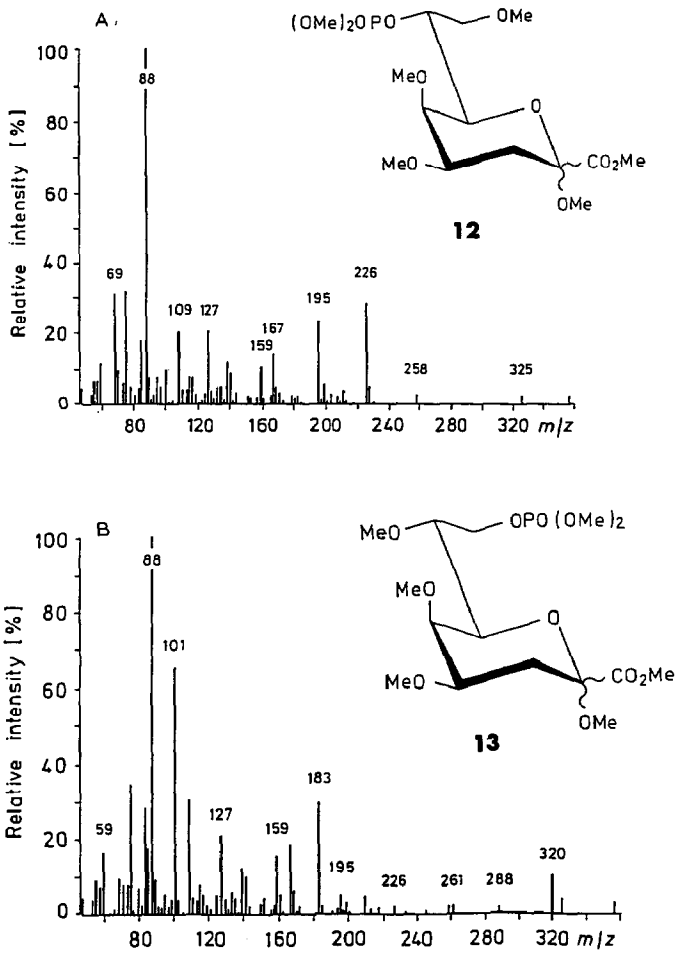


Fig. 4. E.i.-mass spectra of **12** and **13**.

EXPERIMENTAL

General methods were as described[3]. Kdo was determined by the thiobarbituric acid assay[5] and phosphate was measured according to Lowry *et al.*[6]. 2-Aminoethyl phosphate was quantified with an LKB Biochrom 4151 PLUS analyser. D.i.p.-m.s. was performed for 3 min at 60°, then 30° . min^{-1}→350°. The syntheses of compounds **3** and **4** and of Kdo 7-phosphate were performed by D. Charon and will be published elsewhere.

Bacteria and bacterial lipopolysaccharides (LPS). — *S. minnesota* [chemotypes Rd_2 (strain R4) and Rd_1 (strain R7)] and *E. coli* K-12 (strain W3100) were grown in a fermenter (14 L), killed with phenol (0.5%), centrifuged, and washed with ethanol, acetone (twice), and ether. LPS from *E. coli* K-12 strain W3100 and *S. minnesota* strains R4 and R7 were obtained after extraction[13] of dry bacteria with phenol–chloroform–light petroleum in yields of 3.5, 8.3, and 8.5%, respectively.

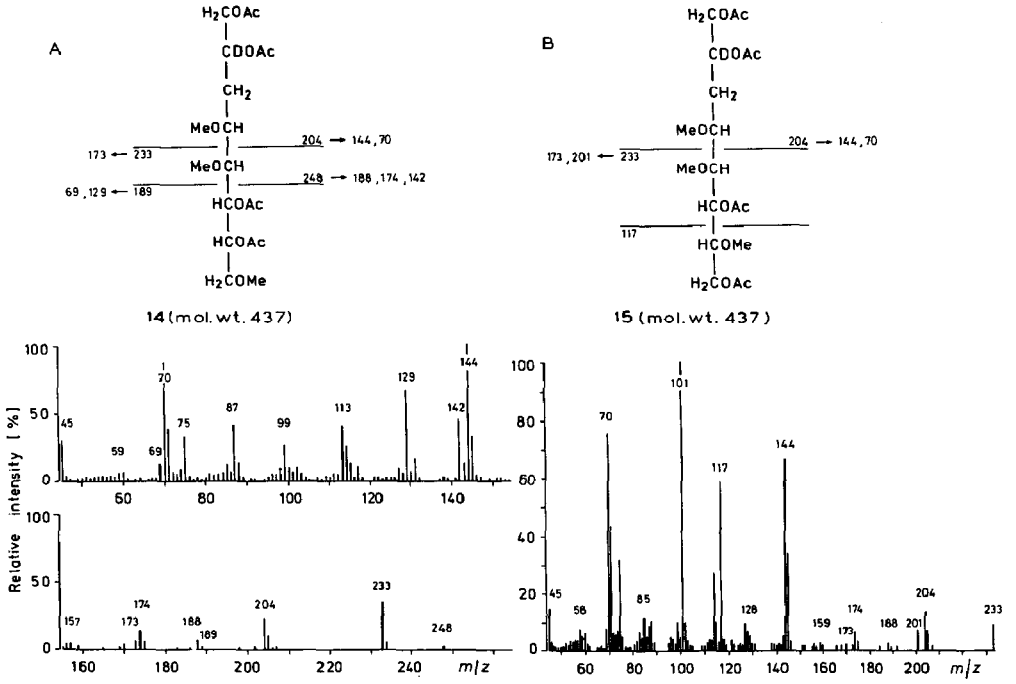


Fig. 5. Fragmentation patterns and e.i.–mass spectra of **14** (A) and **15** (B).

^{13}C-N.m.r. spectroscopy. — Spectra (90.56 MHz) were obtained on solutions of **1**–**4**, 2-aminoethyl phosphate, and 2-acetamidoethyl phosphate in D_2O at 23° with a Bruker AM360L instrument (spectral width, 172 kHz; 32k of memory; internal acetonitrile, 1.70 p.p.m.).

*Isolation and purification of 3-deoxy-*D-manno-*2-octulopyranosonate 7-(2-aminoethyl phosphate)* (**1**) *and 3-deoxy-*D-manno-*2-octulopyranosolate 7-(2-acetamidoethyl phosphate)* (**2**). — LPS from *E. coli* K-12 strain W3100 (1.5 g) and *S. minnesota* strains R4 and R7 (2.7 g in total) were hydrolysed (1 h at 100°) in 100mM sodium acetate buffer (pH 4.4) and dialysed against water (3 × 500 mL) at 4°. This procedure was repeated with the retentate. The combined dialysates were desalted with Amberlite IRA-120 (H^+) resin, neutralised with pyridine, and concentrated to dryness. A solution of the residue in water was eluted from a column (3.0 × 1.5 cm) of polyethyleneimine cellulose (Sigma) with water and 50mM pyridinium acetate (pH 5.5). Fractions were concentrated and analysed for **1** (M_{Kdo} 0.73) by p.e. The appropriate fractions were combined and concentrated to dryness, and a solution of the residue in water was eluted from a column (100 × 1 cm) of Bio-Gel P2 (Bio-Rad) with 50mM pyridinium acetate (pH 5.2). Fractions were monitored by t.l.c. on Silica Gel 60 (Merck) by detection with orcinol–sulfuric acid. Positive fractions were combined, analysed by p.e., then purified by preparative p.e. to give **1** (4.5 mg, 0.3% of LPS). The fraction containing PE-Kdo from *S. minnesota* had to be *N*-acetylated prior to preparative p.e., to give **2** (1.5 mg, 0.06% of LPS).

*Derivatives of [methyl (methyl 3-deoxy-α-*D*-manno-octulopyranosid)onate] 7-(2-acetamidoethyl phosphate)* (**3**). — To a solution of **3** (0.2 mg) in methanol (0.2 mL) was added ethereal diazomethane (1 mL). The solution was stirred for 30 min at room temperature, then concentrated, and the residue was acetylated (18 h, room temperature), using 4-dimethylaminopyridine as catalyst. G.l.c. [SE-54 fused-silica capillary column, 25 m × 0.32 mm i.d. (Weeke, Mühlheim), for 5 min, 150°, then 5° . min^{-1}→300°], g.l.c.-m.s., and d.i.p.–m.s. revealed [methyl (methyl 4,5,8-tri-*O*-acetyl-3-deoxy-α-D-*manno*-octulopyranosid)onate] 7-(dimethyl phosphate) (**5**), which was saponified with methanolic 0.25M sodium methoxide, then methylated by a modified[10] Hakomori[9] procedure. After purification on a SEP-PAK C_{18}-cartridge[14], the product was investigated by g.l.c. [OV 210 capillary column, 25 m × 0.35 mm i.d. (Weeke, Mühlheim); 0.8 bar H_2; 3 min, 150°, then 5° . min^{-1}→250°] and g.l.c.–m.s. Compound **3** was also methylated after treatment with diazomethane, and the product was analysed by g.l.c. (SE-54, 5 min, 160°, then 5° . min^{-1}→300°) and g.l.c.-m.s.

Compound **3** was acetylated, then treated with diazomethane, and the product at each stage was analysed by g.l.c. (SE-54, 5 min, 160°, then 5°.min^{-1}→300°), g.l.c.–m.s., and d.i.p.-m.s.

Compound **3** (0.5 mg) was treated with chlorotrimethylsilane–hexamethyldisilazane–pyridine (1:1:5, 0.7 mL) for 30 min at room temperature, and the product was analysed by g.l.c. (SE-54, 5 min, 150°, then 5°.min^{-1}→300°) and g.l.c.–m.s.

ACKNOWLEDGMENTS

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