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Isolation and characterisation of disodium (4-amino-4-deoxy-β-L-arabinopyranosyl)-(1→8)-(D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy-D-*manno*-oct-2-ulopyranosid)onate from the lipopolysaccharide of *Burkholderia cepacia*

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Abstract

A trisaccharide was isolated from the core oligosaccharide in the lipopolysaccharide (LPS) of *Burkholderia cepacia* GIFU 645 (ATCC 25416, type strain) by methanolysis followed by HPLC and saponification. It was identified by MS, methylation analysis and ^{1}H and ^{13}C NMR spectroscopy as disodium (4-amino-4-deoxy-β-L-arabinopyranosyl)-(1→8)-(D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy-D-*manno*-oct-2-ulopyranosid)onate. In addition to the trisaccharide derivative, methanolysis gave dimethyl (D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy-D-*manno*-oct-2-ulopyranosid)onate in a relative proportion to the trisaccharide of 3:1, indicating a non-stoichiometric (~25%) substitution of the octulosonic acid by 4-amino-4-deoxyarabinose in the LPS. © 1998 Elsevier Science Ltd. All rights reserved

Keywords: *Burkholderia cepacia*; Lipopolysaccharide; 4-Amino-4-deoxy-L-arabinose (L-Ara4N); D-*glycero*-D-*talo*-Oct-2-ulosonic acid (Ko); 3-Deoxy-D-*manno*-oct-2-ulosonic acid (Kdo)

1. Introduction

Based on RNA homology, the genus *Burkholderia* was first proposed for seven species of bacteria, which were combined to group II of Pseudomonadaceae [1]. This group includes several important human and plant pathogens, such as *B. pseudomallei* and *B. cepacia*. The former is the causative agent of melioidosis which prevails in parts of Southeast Asia, Northern Australia, and Central and South America [2,3]. The latter has recently been recognised also as an important

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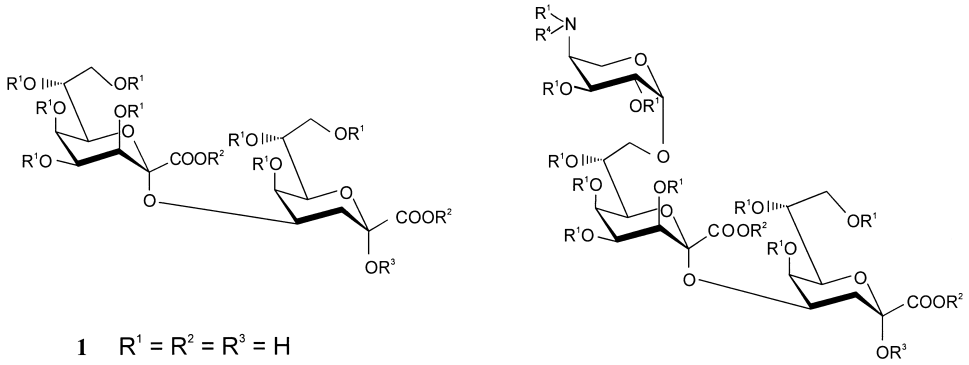

1 $R^1 = R^2 = R^3 = H$

1a $R^1 = R^2 = R^3 = Me$

1b $R^1 = H, R^2 = R^3 = Me$

1c $R^1 = H, R^2 = Na, R^3 = Me$

2 $R^1 = R^2 = R^3 = R^4 = H$

2a $R^1 = R^2 = R^3 = Me, R^4 = Ac$

2c $R^1 = R^4 = H, R^2 = Na, R^3 = Me$

Scheme 1.

opportunistic human pathogen. In particular, it has been reported that during pulmonary infection of patients suffering from cystic fibrosis, the occurrence of this bacterium is closely related to morbidity and mortality [4]. Despite increasing medical importance of these bacteria, their bacteriological and biochemical properties, virulence factors, and processes of pathogenesis are scarcely studied.

Lipopolysaccharide (LPS, endotoxin) is known as an important virulence factor of Gram-negative bacteria [5]. Several studies have been performed in order to determine the biological activities and the chemical structures of the O-chain polysaccharides in the LPSs of *B. pseudomallei* and *B. cepacia* [6–8]. Other parts of the LPSs, namely, the core oligosaccharide and lipid A, have also been chemically characterised [9–12]. However, the structure of the core region has not been determined. Preliminary results showed that these LPSs possess the following unusual chemical features: (i) the sugar that interlinks the core and lipid A, which is 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) in enterobacterial LPS, could not be detected in *B. cepacia* LPS by conventional colorimetric assay [9,10]; (ii) the linkage between the core and lipid A was resistant against mild acid hydrolysis [11,12]; and (iii) the LPSs included a hitherto unknown core constituent which was identified as (D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonic acid)-(2→4)-3-deoxy-D-*manno*-oct-2-ulosonic acid [α-K*o*p-(2→4)-Kdo (**1**)] [12].

Now we describe the identification of a new trisaccharide, (4-amino-4-deoxy-β-L-arabinopyranosyl)-(1→8)-(D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonic acid)-(2→4)-3-deoxy-D-*manno*-oct-2-ulosonic acid [β-L-Ara*p*4N-(1→8)-α-K*o*p-(2→4)-Kdo (**2**)], as a hitherto unknown but characteristic component of the inner core region of the LPS of *B. cepacia*.

2. Results and discussion

LPS was obtained from *B. cepacia* GIFU 645 (ATCC 25416) cells by the phenol–water extraction procedure followed by repeated ultracentrifugation in an overall yield of 2% (1.1 g, by wt.). In SDS-PAGE, the LPS showed one major band with high mobility indicative of rough (R)-type LPS. In addition, attenuate ladder bands were seen close to this major band, which were indicative of rough-smooth type LPS carrying one (S/R-type) or more (S-type) repeating units in the O-polysaccharide chain. Therefore, in the LPS of *B. cepacia* GIFU 645, the R-type dominated the S-type LPS. Chemical analyses further supported this finding. L-Rhamnose, D-glucose, L-*glycero*-D-*manno*-heptose, D-*glycero*-D-*manno*-heptose, 2-amino-2-deoxy-D-glucose, and phosphate were identified in the molar ratios of 0.9:1.0:3.3:0.5:0.5:0.7 as the major constituents. In addition, trace amounts of mannose, galactose, 2-amino-2-deoxygalactose, 2-amino-2-deoxymannose, and 2-amino-2,6-dideoxyglucose were detected, which, most likely, were minor components of the O-chain.

Kdo was not detectable in colorimetric assay under the conventional hydrolysis conditions, a finding which has also been described by other groups [9,10]. However, GLC-MS analysis following

strong methanolysis (2 M HCl/MeOH, 120 °C, 16 h) and permethylation revealed the expected permethylated Kdo derivatives [methyl (methyl 3-deoxy-4,5,7,8-tetra-*O*-methyl-α/β-D-*manno*-oct-2-ulopyranosid)onate]. Methanolysis under milder conditions (0.5 M HCl/MeOH, 85 °C, 45 min) followed by *N*-acetylation and permethylation resulted in methyl 4-deoxy-2,3-di-*O*-methyl-4-(*N*-methylacetamido)arabinopyranoside (derived from Ara*p*4N), dimethyl (3,4,5,7,8-penta-*O*-methyl-D-*glycero*-D-*talo*-oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy-5,7,8-tri-*O*-methyl-D-*manno*-oct-2-ulopyranosid)onate [from Ko*p*-(2→4)-Kdo (**1a**)] and a derivative of an unknown trisaccharide (**2a**) in the molar ratios of 3:3:1.

It is known that *B. cepacia* LPS carries Ara4N bound by a phosphodiester linkage to position 4 of the non-reducing glucosamine (GlcN II) of the lipid A backbone [10]. To eliminate this Ara4N residue, LPS was treated with 48% hydrofluoric acid (4 °C, 48 h). Mild methanolysis (0.5 M HCl/MeOH 85 °C, 45 min) of the resultant LPS-HF with subsequent *N*-acetylation, permethylation, and GLC-MS analysis revealed **1a** and **2a**, but no longer Ara4N. However, strong hydrolysis (4 M HCl, 65 °C, 16 h) of LPS-HF released Ara4N, indicating that, in addition to Ara4N attached to the lipid A backbone, a second, most likely, glycosidically linked Ara4N residue was present in the LPS of *B. cepacia*. The L configuration of this Ara4N residue was established by GLC-MS of permethylated (*S*)- and/or (*R*)-2-butyl glycosides.

The EI mass spectrum of **2a** is shown in Fig. 1. It displayed fragment ions at *m/z* 291→259 (−MeOH) and 216→156 (−HOAc), indicating the presence of Kdo at the reducing end and Ara4NAc at the nonreducing end of **2a**. Two more fragment ions at *m/z* 522 and 597 were in agreement with Ara4NAc→Ko and Ko→Kdo moieties of the trisaccharide, respectively. CI-MS revealed a pseudomolecular ion $(M+NH_4)^+$ at *m/z* 847, which was in accord with the molecular mass (M_r 829) of the permethylated Ara4NAc→Ko→Kdo methyl ketoside (**2a**). These data suggested that the trisaccharide present in the LPS of *B. cepacia* is Ara4N→Ko→Kdo (**2**).

The primary products of mild methanolysis of LPS, **1b** and **2b**, were separated by reversed-phase HPLC and studied by ^1H and ^{13}C NMR spectroscopy. The data for **1b** confirmed its structure as dimethyl (D-glycero-α-D-*talo*-oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy-α-D-*manno*-oct-2-ulopyranosid)onate (Tables 1 and 2). However, the spectra of trisaccharide **2b** contained only two signals for methoxy groups rather than the expected three signals (one for the methyl ketoside and two for the methyl esters). Hence, esterification of one of the carboxyl groups in Ko or Kdo was incomplete. In the ^{13}C NMR spectrum a number of minor signals for anomeric carbons (δ 100–105) and carbonyl carbons (δ 170–176) indicated heterogeneity in Ko or/and Kdo residues. These results suggested that Ko or/and Kdo residues in **2b** formed multiple lactone or lactam bonds, as has been reported for Kdo attached to GlcN with a free amino group [14]. This finding was confirmed by MALDI-TOF MS (data not shown). Since the structure of **2b** could not be elucidated unequivocally, it is not shown in the Scheme. However, despite its heterogeneity it was found useful for further methylation analysis and NMR spectroscopic studies after saponification, yielding the disodium salt methyl ketoside **2c** which could be isolated as individual compound. Methylation analysis of **2c** revealed the presence of 4-substituted

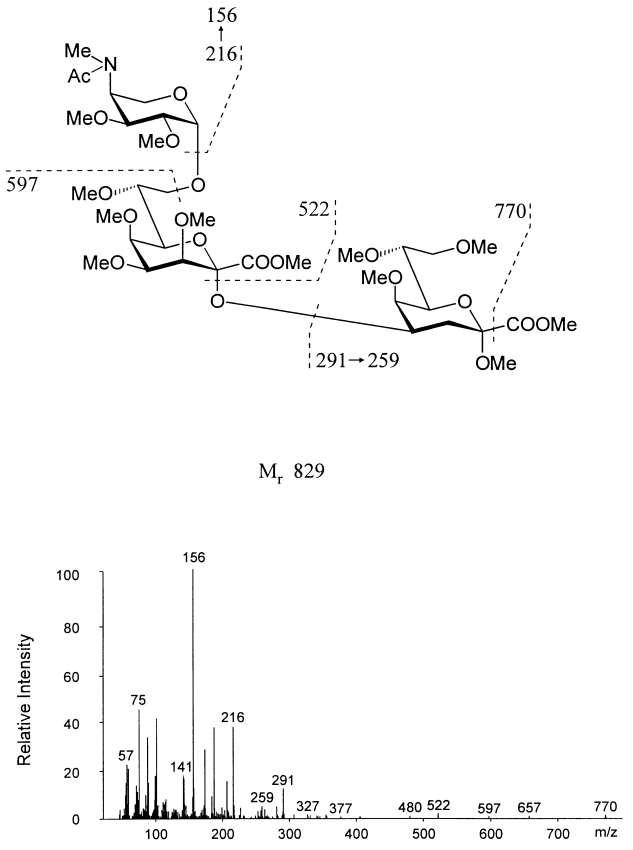

Fig. 1. Electron impact mass spectrum of **2a**, whose structure is shown in the inset.

Table 1
^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz)

Residue	Compound		
Proton	**1b**	**1c**[a]	**2c**
β-L-Ara*p*4N-(1→			
H-1			4.91 ($J_{1,2}$ 3.6)
H-2			3.59 ($J_{2,3}$10.0)
H-3			4.01 ($J_{3,4}$ 4.8)
H-4			3.54 ($J_{4,5ax}$ 2.3)
H-5ax			3.61 ($J_{5ax,5eq}$13.7)
H-5eq			4.01 ($J_{4,5eq}$ 2.0)
→8)-α-Ko*p*-(2→			
H-3	4.13 ($J_{3,4}$ 3.2)	3.90 ($J_{3,4}$ 3.1)	3.86 ($J_{3,4}$ 3.2, $^4J_{3,5}$ 1.4)
H-4	3.99 ($J_{4,5}$ 3.2)	3.81 ($J_{4,5}$ 3.1)	3.80 ($J_{4,5}$ 3.2)
H-5	4.14 ($J_{5,6}$~1.0)	3.94 ($J_{5,6}$ 1.3)	3.94 ($J_{5,6}$ 1.1)
H-6	3.69 ($J_{6,7}$ 9.4)	3.70 ($J_{6,7}$ 8.3)	3.48 ($J_{6,7}$ 7.9)
H-7	4.08 ($J_{7,8a}$ 6.5)	3.90 ($J_{7,8a}$ 6.7)	4.10 ($J_{7,8a}$ 8.5)
H-8a	3.75 ($J_{8a,8b}$ 11.9)	3.57 ($J_{8a,8b}$ 11.8)	3.64 ($J_{8a,8b}$10.6)
H-8b	3.97 ($J_{7,8b}$ 2.5)	3.81 ($J_{7,8b}$ 3.1)	3.82 ($J_{7,8b}$ 3.4)
→4)-α-Kdo*p*-(2→			
H-3ax	2.05 ($J_{3ax,4}$ 11.9, $J_{3ax,3eq}$ 13.0)	1.79 ($J_{3ax,4}$ 12.5, $J_{3ax,3eq}$ 11.6)	1.73 ($J_{3ax,4}$ 12.0, $J_{3ax,3eq}$12.8)
H-3eq	2.14 ($J_{3eq,4}$ 5.0)	1.90 ($J_{3eq,4}$ 5.7)	1.82 ($J_{3eq,4}$ 5.0)
H-4	4.18 ($J_{4,5}$ 2.9)	3.99 ($J_{4,5}$ 2.6)	3.89 ($J_{4,5}$ 2.9)
H-5	3.91 ($J_{5,6}$~1.0)	3.93 ($J_{5,6}$~0.8)	3.92 ($J_{5,6}$ 0.9)
H-6	3.58 ($J_{6,7}$ 9.0)	3.38 ($J_{6,7}$ 8.8)	3.34 ($J_{6,7}$ 8.7)
H-7	3.91 ($J_{7,8a}$ 6.1)	3.75 ($J_{7,8a}$ 7.0)	3.74 ($J_{7,8a}$ 7.1)
H-8a	3.65 ($J_{8a,8b}$ 11.9)	3.47 ($J_{8a,8b}$ 12.3)	3.45 ($J_{8a,8b}$ 12.4)
H-8b	3.89 ($J_{7,8b}$ 2.5)	3.74 ($J_{7,8b}$ 3.1)	3.74 ($J_{7,8b}$ 2.6)
OMe	3.21	3.00	2.95
COOMe	3.85, 3.88		

[a] Data from ref. [12].

Kdo*p*, 8-substituted Ko*p*, and terminal Ara*p*4N and, thus, confirmed the proposed structure.

The ^{1}H and ^{13}C NMR data for **2c** are summarised in Tables 1 and 2. As expected, the spectra contained only one signal for a methoxy group (δ_H 2.95; δ_C 51.40). The coupling constant values $J_{3,4}$ 3.2, $J_{4,5}$ 3.2, and $J_{5,6}$ ~1 Hz, together with the diagnostic long-range coupling between H-3 and H-5 ($J_{3,5}$ 1.4) [13], allowed assignment of the *talo* configuration to the Ko*p* residue. The configuration of the Ara*p*4N residue was assigned on the basis of the chemical shift data and vicinal coupling constant values, which were relatively small for $J_{1,2}$ and $J_{2,3}$ and large for $J_{3,4}$. The β configuration of L-Ara*p*4N was determined by a relatively small $J_{1,2}$ coupling constant value of 3.6 Hz.

The ^{13}C NMR spectrum of **2c** (Table 2) contained characteristic signals for C-3 of Kdo (δ 34.29), one amino sugar signal for C-4 of Ara*p*4N (δ 52.84), three CH_2OH groups at δ 70.87, 63.81 and 58.91, three anomeric carbons at δ 102.74, 101.14 and 99.24, and two carbonyl groups at δ 175.73 and 174.14. The spectrum was assigned using an HMQC experiment and by comparison with data for the corresponding monomers: 4-amino-4-deoxy-β-L-arabinopyranose-1-phosphate

Table 2
^{13}C NMR chemical shifts (δ, ppm)

Residue Carbon	Compound		
	1b	**1c**[a]	**2c**
β-L-Ara*p*4N-(1→			
C-1			99.24
C-2			68.79
C-3			66.40
C-4			52.84
C-5			58.91
→8)-α-Ko*p*-(2→			
C-1	170.66	172.97	174.14
C-2	101.55	101.63	102.74
C-3	72.20	72.40	72.75
C-4	66.21	66.50	66.77
C-5	68.54	68.87	69.14
C-6	73.55	73.37	73.49
C-7	69.81	70.35	69.29
C-8	63.61	63.75	70.87
→4)-α-Kdo*p*-(2→			
C-1	170.80	174.05	175.73
C-2	99.98	100.50	101.14
C-3	33.34	33.78	34.29
C-4	69.88	70.21	70.73
C-5	64.46	64.95	65.41
C-6	72.20	72.21	72.16
C-7	69.48	69.84	70.12
C-8	63.54	63.67	63.81
OMe	51.93	51.64	51.40
COOMe	54.21, 54.28		

[a] Data from ref. [12].

from LPS of *Salmonella enterica* sv. Minnesota mutant R595 (U. Zähringer, unpublished), sodium salt of synthetic Ko [13] and Kdo methyl ketoside [15], as well as for **1c** [12]. The chemical shift data for the Ara*p*4N, Ko*p* and Kdo*p* residues were consistent with those for the reference compounds and demonstrated the α configuration of both Ko and Kdo residues. The signal for C-8 of the Ko residue in **2c** was significantly shifted downfield, as compared with its position in **1c**, that was evidently due to glycosylation at position 8.

In 2D NOESY and ROESY experiments, **2c** expressed interresidue NOEs between H-3eq and H-4 of Kdo and H-6 of Ko, which are characteristic for an α-K(d)o-(2→4)-linkage [16]. An interresidue NOE between H-1 of Ara*p*4N and H-8a,b of Ko demonstrated a β-L-Ara*p*4N-(1→8)-α-Ko fragment. Together these data demonstrated the sequence β-L-Ara*p*4N-(1→8)-α-Ko*p*-(2→4)-Kdo. The occurrence of an NOE between H-6 of Kdo and the methoxy group confirmed the α configuration of the Kdo residue.

Therefore, the data obtained showed that **2c** is a trisaccharide methyl ketoside which was derived from (4-amino-4-deoxy-β-L-arabinopyranosyl)-(1→8)-(D-*glycero*-α-D-*talo*-oct-2-ulopyranosylonic acid)-(2→4)-3-deoxy-D-*manno*-oct-2-ulopyranosylonic acid (**2**) present in the LPS of *B. cepacia*. Judging from the ratio of **1a** and **2a** (3:1) derived from LPS by mild methanolysis, and taking into account the stability of the glycosidic linkage of Ara*p*4N towards cleavage with acids ([20] and our data on methanolysis of LPS-HF, see above), it was concluded that substitution of Ko by Ara4N in the LPS is non-stoichiometric, the degree of substitution being estimated as ~25%.

This is the first report on the occurrence of a β-L-Ara*p*4N-(1→8)-α-Ko*p*-(2→4)-Kdo trisaccharide in a bacterial LPS. Previously, Ko has been found in LPS of several *Acinetobacter* strains as a constituent of the inner core region interlinking the core oligosaccharide and lipid A [17–19]. Furthermore, the linkage between the polysaccharide and lipid A moieties of the *Acinetobacter* LPS is resistant to mild acid hydrolysis. Based on this finding, we suggest that the presence of the Ko*p*→Kdo disaccharide in the inner core region is the reason for the known resistance of the LPS of *B. cepacia* towards acid hydrolysis. This accounts also for the finding that Kdo in this LPS could not be detected by the conventional thiobarbituric acid test [9,10]. Therefore, not only the Kdo residue that is attached to the lipid A backbone (Kdo I) [17–19], but also a Kdo residue in the side chain, e.g., Kdo II, can be replaced by Ko. The presence of a positively-charged substituent (L-Ara*p*4N) in the inner core region of *B. cepacia* LPS may influence the charge of the LPS molecule in a similar way as an Ara4N residue in the lipid A moiety. A similar structural element in the core oligosaccharide, namely, an L-Ara*p*4N-(1→8)-Kdo disaccharide, was found in the LPS of *Proteus mirabilis* rough mutant and wild-type strains [20].

3. Experimental

Extraction of LPS.—*B. cepacia* GIFU 645 (ATCC 25416, type strain), kindly provided by Dr. T. Ezaki (Gifu University School of Medicine, Gifu, Japan), was cultivated in a fermenter (30 L) with Nutrient broth No. 2 (Oxoid Ltd., Basingstoke, UK) at 35 °C for 24 h. After being killed by heating at 100 °C for 30 min, cells were harvested by centrifugation and washed twice each with water, ethanol and acetone. The LPS was extracted from diethyl ether-dried cells by the phenol–water procedure [21], and the aqueous phase material was degraded by enzymatic digestion (DNase, RNase, Trypsin and Proteinase K) and purified by repeated ultracentrifugation (100,000 *g*, 16 h, 3 times).

General methods.—GPC was performed on a column (3.0×100 cm) of TSK HW-40S (Merck) with 50 mM pyridinium acetate buffer (pH 5.0) or on a column (1.5×120 cm) of Sephadex G-10 with water. Elution was monitored with a Knauer differential refractometer. GLC was performed with a Hewlett–Packard Model 5890 chromatograph equipped with a capillary column of SPB-5 using the following temperature programs: program A, 3 min at 150 °C, then to 320 °C at 5 °C/min; program B, 3 min at 200 °C, then to 320 °C at 5 °C/min. GLC-MS in CI (with ammonia as reactant gas) and EI modes were performed on a Hewlett–Packard Model 5989 instrument equipped with a capillary column of HP-1 under the same condition as in GLC. SDS-PAGE was carried out in 18 and 20% polyacrylamide gel using a Mini-Protean II system (Bio-Rad), the gel was oxidised with periodate and stained [22]. MALDI-TOF MS was run on a Bruker Reflex™ II mass spectrometer in positive ion mode; 2,5-dihydroxybenzoic acid was used as a matrix.

NMR spectroscopy.—^{1}H NMR spectra were recorded at 360 MHz (Bruker AM-360) or

600 MHz (Bruker DRX-600 Avance) in D_2O at 300K using 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (δ_H 0) as internal standard. ^{13}C NMR spectra were recorded at 90.6 MHz (Bruker AM-360) with acetonitrile (δ_C 1.70) as internal standard. 2D ^1H,^1H COSY, NOESY, ROESY, and H-detected ^{13}C,^1H HMQC experiments were recorded using standard Bruker software (XWINNMR 1.3).

*Isolation of dimethyl (*D-*glycero*-α-D-talo-*oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy*-α-D-*manno-oct-2-ulopyranosid)onate (**1b**), anhydro form(s) of methyl (4-amino-4-deoxy*-β-L-*arabinopyranosyl)-(1→8)-(*D-glycero-α-D-talo-*oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy*-α-D-*manno-oct-2-ulopyranosid)onate (**2b**),* and *disodium(4-amino-4-deoxy*-β-L-*arabinopyranosyl)-(1→8)-(*D-*glycero*-α-D-*talo-oct-2-ulopyranosylonate)-(2→4)-(methyl 3-deoxy*-α-D-*manno-oct-2-ulopyranosid)-onate* (**2c**).—*B. cepacia* LPS (300 mg) was subjected to methanolysis (60 mL 0.5 M HCl/MeOH, 85 °C, 3 h), and the methanolysate was evaporated to dryness. The residue was suspended in 50 mL water and extracted twice with 50 mL 2:3 diethyl ether–hexane to remove lipids. The water-soluble material was lyophilised and fractionated by GPC on TSK HW-40S to give six fractions. Each fraction was analysed by GLC-MS, and **1b** and **2b** were identified as their permethylated methyl glycosides in the third and second fractions. They were further purified by HPLC on a reversed-phase column (8×250 mm, Zorbax C_{18}, 5 μm, Bischoff, Leonberg) using isocratic elution with acetonitrile–water (5% for **1b** and 2% for **2b**) at 1 mL/min. Elution was monitored with a Knauer differential refractometer, and appropriate fractions were dried. **1b** (T = 17.3 min) and **2b** (T = 21.3 min) were obtained in yields of 6.2 and 3.1 mg, respectively.

2b (3.0 mg) was saponified with 0.1 M NaOH at 60 °C for 30 min and purified by GPC on Sephadex G-10 in water to give **2c** (1.9 mg).

Sugar analysis.—Neutral and acidic sugars, including Kdo, were analysed by GLC using temperature program A as peracetylated methyl glycosides derived by methanolysis (2 M HCl/MeOH, 120 °C, 16 h) or as alditol acetates obtained by hydrolysis (0.1 M HCl, 100 °C, 48 h), reduction ($NaBH_4$) and peracetylation (2:1 pyridine–Ac_2O, 65 °C, 1 h). Amino sugars, except for Ara4N, were estimated using an amino acid analyser (LKB alpha plus 4151) after acid hydrolysis (4 M HCl, 100 °C, 16 h). Kdo was also estimated by conventional colorimetric assay after hydrolysis with 0.1 M NaOAc buffer (pH 4.4) [23]. **1** and **2** were analysed by GLC using temperature program B as permethylated methyl glycosides (**1a** and **2a**) which were obtained by methanolysis of LPS (0.5 M HCl/MeOH, 85 °C, 45 min) followed by peracetylation, *O*-deacetylation (0.05 M NaOMe, 37 °C, 1 h), and methylation [24]. The absolute configurations of Ko and Kdo were determined by GLC (program A) of peracetylated (*S*)- or (*R*)-2-butyl esters and glycosides [25]. The absolute configuration of Ara4N was identified by GLC (program A) of permethylated (*S*)- or (*R*)-2-butyl glycoside derived from LPS-HF by hydrolysis (4 M HCl, 65 °C, 16 h) of LPS-HF using L-Ara4N from *S. enterica* sv. Minnesota mutant R595 LPS as reference [26].

Methylation analysis.—**2c** was treated with 0.1 M HCl/MeOH (60 °C, 30 min) and formation of methyl esters was completed by adding ethereal diazomethane. After *N*-acetylation [27] and purification by GPC on Sephadex G-10, the product was methylated [24], hydrolysed (2 M CF_3CO_2H, 100 °C, 1 h), and reduced ($NaBD_4$). After removal of boric acid by repeated (three times) evaporation with 0.5 M HCl in MeOH, the product was acetylated and analysed by GLC-MS using temperature program A.

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References

[1] E. Yabuuchi, Y. Kosako, H. Oyaizu, I. Yano, H. Hotta, Y. Hashimoto, T. Ezaki, and M. Arakawa, *Microbiol. Immunol.*, 36 (1992) 1251–1275.
[2] K. Kanai and S. Dejsirilert, *Jpn. J. Med. Sci. Biol.*, 41 (1988) 123–157.
[3] A. Leelarasamee and S. Bovornkitti, *Rev. Infect. Dis.*, 11 (1989) 413–425.
[4] J.W. Nelson, S.L. Butler, D. Krieg, and J.R.W. Govan, *FEMS Immunol. Med. Microbiol.*, 8 (1994) 89–98.

[5] E.Th. Rietschel, L. Brade, B. Lindner, and U. Zähringer, *Biochemistry of Lipopolysaccharides*, in D.C. Morrison and J.L. Ryan (Eds.), *Bacterial Endotoxic Lipopolysaccharides*, Vol. I, *Molecular Biochemistry and Cellular Biology*, CRC Press, Boca Raton, FL, 1992, pp 3–41.
[6] Y.A. Knirel and N.K. Kochetkov, *Biochemistry (Moscow)*, 59 (1994) 1325–1383.
[7] M. Matsuura, K. Kawahara, T. Ezaki, and M. Nakano, *FEMS Microbiol. Lett.*, 137 (1996) 79–83.
[8] D. Shaw, I.R. Poxton, and J.R.W. Govan, *FEMS Immunol. Med. Microbiol.*, 11 (1995) 99–106.
[9] J.M. Manniello, H. Heymann, and F.W. Adair, *J. Gen. Microbiol.*, 112 (1979) 397–400.
[10] A.D. Cox and S.G. Wilkinson, *Mol. Microbiol.*, 5 (1991) 641–646.
[11] K. Kawahara, S. Dejsirilert, H. Danbara, and T. Ezaki, *FEMS Microbiol. Lett.*, 96 (1992) 129–134.
[12] U. Zähringer, H. Moll, P. Kosma, and K. Kawahara, *Abstracts of Papers*, 7th European Carbohydrate Symposium, Cracow, August 1993, B23.
[13] J. Gass, M. Strobl, A. Loibner, P. Kosma, and U. Zähringer, *Carbohydr. Res.*, 244 (1993) 69–84.
[14] H. Paulsen, Y. Hayauchi, and F.M. Unger, *Liebigs Ann. Chem.*, (1984) 1270–1287.
[15] K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
[16] K. Bock, J.U. Thomsen, P. Kosma, R. Christian, O. Holst, and H. Brade, *Carbohydr. Res.*, 229 (1992) 213–224.
[17] K. Kawahara, H. Brade, E.Th. Rietschel, and U. Zähringer, *Eur. J. Biochem.*, 163 (1987) 489–495.
[18] U. Zähringer, K. Kawahara, H. Brade, U. Sydel, E.Th. Rietschel, C. Krogmann, V. Sinnwell, H. Paulsen, and P. Kosma, *Abstracts of Papers*, 6th European Symposium on Carbohydrate Chemistry, Edinburgh, August 1991, A16.
[19] E.V. Vinogradov, K. Bock, B.O. Petersen, O. Holst, and H. Brade, *Eur. J. Biochem.*, 243 (1997) 122–127.
[20] Z. Sidorczyk, W. Kaca, H. Brade, E.Th. Rietschel, V. Sinnwell, and U. Zähringer, *Eur. J. Biochem.*, 168 (1987) 269–273.
[21] O. Westphal and K. Jann, *Methods Carbohydr. Chem.*, 5 (1965) 83–91.
[22] C.M. Tsai and C.E. Frasch, *Anal. Biochem.*, 119 (1982) 115–119.
[23] H. Brade, C. Galanos, and O. Lüderitz, *Eur. J. Biochem.*, 131 (1983) 195–200.
[24] I. Ciucanu and F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.
[25] G.J. Gerwig, J.P. Kamerling, and J.F.G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
[26] W.A. Volk, C. Galanos, and O. Lüderitz, *FEBS Lett.*, 8 (1970) 161–163.
[27] S. Hase and E.T. Rietschel, *Eur. J. Biochem.*, 63 (1976) 101–107.