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The structure of the sialic acid-containing *Escherichia coli* O104 O-specific polysaccharide and its linkage to the core region in lipopolysaccharide *,**

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ABSTRACT

Mild acid hydrolysis of *Escherichia coli* O104 lipopolysaccharide released an O-specific polysaccharide, a tetrasaccharide repeating unit, the corresponding dimer, and a disaccharide fragment of the repeating unit. Complete and incomplete cores, and oligosaccharides comprising fragments of the repeating unit and the core region, were also obtained. On the basis of sugar and methylation analysis, FAB-mass spectrometry and NMR spectroscopy of the hydrolysis products, the repeating unit of the O-specific polysaccharide was shown to be the tetrasaccharide: → 4)-α-D-Gal*p*-(1 → 4)-α-Neu*p*5,7,9Ac_3-(2 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc (1 →. The linkage between the O-specific polysaccharide chain and the core region, which appeared to be of the R2 type, was established. These results indicate that *N*-acetylneuraminic acid, located in the O-specific polysaccharide, is an inherent lipopolysaccharide component.

INTRODUCTION

Sialic acids are important constituents of glycoconjugates in animal tissues[1]. The presence of sialic acid in lipopolysaccharides (LPS) of some enterobacterial strains of *Salmonella, Escherichia coli* (serotypes O24, O56, and O104), and *Citrobacter* was also reported[2,3]. The role of sialic acid in LPS is not known, although it is thought that in capsular polysaccharides of known structure, the sialic acid may contribute to the pathogenicity of bacteria by mimicking the host tissue compo-

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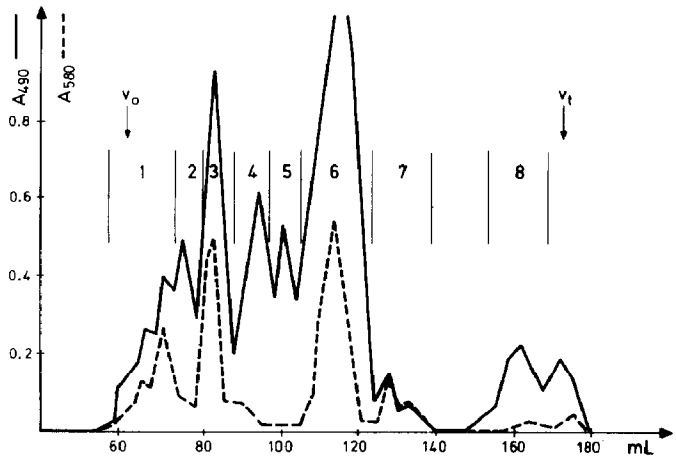

Fig. 1. Fractionation (Bio-Gel P-4 column, 1.6×100 cm, equilibrated with pyridine–acetic acid buffer, pH 5.6; flow rate, 2 mL/30 min) of the carbohydrate material isolated from *E. coli* strain O104 lipopolysaccharide, after acetic acid hydrolysis. The absorbance was measured at 490 nm for the phenol–sulfuric acid reaction and at 580 nm for the resorcinol reaction characteristic for sialic acid.

nent[4,5]. The structure of sialic acid containing lipopolysaccharides was not studied in detail until recently, when a branched octasaccharide O-specific repeating unit and its linkage to the core in LPS were described[6] for *Hafnia alvei* strain 2. The structure of the *E. coli* O104 polysaccharide and the type of linkage between the O-specific part and the core region are herein reported. The results substantiate the cross-reactivity previously described[3,5] between O104 lipopolysaccharide and *E. coli* K9 antigen, the structure of which has been established[7].

RESULTS AND DISCUSSION

The lipopolysaccharide of *E. coli* O104 acapsular strain was obtained in a 2.5% yield by phenol–water extraction of dried cells, followed by purification on a Sepharose 2B column[6,8]. It expressed a high-molecular-mass, ladder-like pattern typical for the smooth type of LPS[8] when analyzed by SDS-poly(acrylamide) gel electrophoresis (SDS-PAGE) and immunoblotting with homologous anti-*E. coli* O104 serum. Mild aqueous acetic acid hydrolysis released a lipid sediment, and a water-soluble carbohydrate portion which was separated into eight fractions by gel filtration on a Bio-Gel P-4 column (Fig. 1). The major oligosaccharide component, which contained sialic acid (Fraction 6), was subsequently divided into two subfractions by affinity chromatography on a Sepharose 4B–serotonin column[6]. Fraction 6a, which was not retained, was eluted with water, and Fraction 6b was desorbed with ammonium hydrogen carbonate buffer. The structures of the components of Fractions 1–7, were deduced by sugar analysis, methylation analysis, FAB-mass spectrometry, and NMR spectroscopy (see Scheme 1). Fraction 8, which contained 3-deoxyoctulosonic acid released from LPS, was not considered further.

$[\rightarrow 3)$-β-D-Gal*p*-$(1\rightarrow 3)$-β-D-Gal*p*NAc-$(1\rightarrow 4)$-α-D-Gal*p*-$(1\rightarrow 4)$-α-Neu*p*5,7,9Ac_3-$(2\rightarrow]_n$

1 (Fraction 1)

β-D-Gal*p*-$(1\rightarrow 3)$-β-D-Gal*p*NAc-$(1\rightarrow 4)$-α-D-Gal*p*-$(1\rightarrow 4)$-α-Neu*p*5,7,9Ac_3-$(2\rightarrow 3)$-β-D-Gal*p*-$(1\rightarrow 3)$-β-D-Gal*p*NAc-$(1\rightarrow 4)$-α-D-Gal*p*-$(1\rightarrow 4)$-β-Neu*p*5,7,9Ac_3

2 (Fraction 3)

A B C D
β-D-Gal*p*-$(1\rightarrow 3)$-β-D-Gal*p*NAc-$(1\rightarrow 4)$-α-D-Gal*p*-$(1\rightarrow 4)$-β-Neu*p*5,7,9Ac_3

3 (Fraction 6a)

α-D-Gal*p*-$(1\rightarrow 4)$-β-Neu*p*5,7,9Ac_3

4 (Fraction 7)

β-D-Gal*p*-$(1\rightarrow 3)$-β-D-Gal*p*NAc-$(1\rightarrow 4)$-α-D-Glc*p*-$(1\rightarrow 2)$-α-D-Glc*p*-$(1\rightarrow 3)$-α-D-Glc*p*-$(1\rightarrow$

2 6
↑ ↑
1 1
α-D-Glc*p*NAc α-D-Gal*p*

5 (Fraction 4)

α-D-Glc*p*-$(1\rightarrow 2)$-α-D-Glc*p*-$(1\rightarrow 3)$-α-D-Glc*p*-$(1\rightarrow$

2 6
↑ ↑
1 1
α-D-Glc*p*NAc α-D-Gal*p*

6 (Fraction 5)

α-D-Glc*p*-$(1\rightarrow 2)$-α-D-Glc*p*-$(1\rightarrow 3)$-α-D-Glc*p*-$(1\rightarrow$
(30%) 6
↑
1
α-D-Gal*p*

7 (Fraction 6b)

Scheme 1. Proposed structures for the oligo- and poly-saccharides 1–7 present in Fractions 1–7 obtained by mild acetic acid hydrolysis of *E. coli* O104 LPS. The heptose-Kdo region in Fractions 4–6b, which was not studied in detail, is not shown.

Sugar analysis of Fractions 1–7, following hydrochloric acid hydrolysis and GLC of the resulting monosaccharide constituents in the form of their alditol acetate derivatives, and colorimetric determination with resorcinol for sialic acid, showed that the poly- and oligo-saccharides of Fractions 1, 3, and 6a had approximately the same composition with galactose, 2-acetamido-2-deoxygalactose, and *N*-acetylneuraminic acid as the sole constituents (Table I). Gal and GalNAc were subsequently shown to have the D configuration by oxidation with D-galactose oxidase[9]. Oligosaccharides in Fractions 3 and 6a may then originate from the

TABLE I

Sugar analysis (molar ratio) of the oligosaccharides **1–7** from Fractions 1–7 isolated from *E. coli* O104 lipopolysaccharide

Sugar component	Fraction							
	1 (**1**)	2	3 (**2**)	4 (**5**)	5 (**6**)	6a (**3**)	6b (**7**)	7 (**4**)
D-Gal	2.0	3.4	2.0	2.3	1.6	2.0	1.0	1.0
D-Glc		2.2	0.2	3.0	3.0		3.0	0.3
LD-Hep		1.3	0.2	2.2	2.5		1.8	0.3
D-GlcNAc	0.1	1.0	0.2	1.1	1.0			
D-GalNAc	2.7	2.0	0.9	0.9		0.8		
Neu5Ac	0.7	0.7	0.8	0.5		0.9		1.0

O-specific polysaccharide Fraction 1 and this was further corroborated by methylation analysis (Table II), which showed that oligosaccharide **2** of Fraction 3 was a dimer of oligosaccharide **3** of Fraction 6a, since it yielded the same methylated derivatives (double the amount), with, however, a O-3 substituted D-galactose unit in place of the terminal D-galactose unit in **3**. Methylation analysis for Fractions 1, 3, and 6a indicated furthermore that the O-specific polysaccharide **1** of Fraction 1 was composed of a tetrasaccharide repeating unit (**3**), and that this repeating unit contained O-3- and O-4-substituted D-galactose, O-3-substituted 2-acetamido-2-deoxy-D-galactose, and O-4-substituted *N*-acetylneuraminic acid. Substitution at O-4 for Neu5Ac was confirmed by EI-mass spectrometric identification[10] of methyl 4-*O*-acetyl-(5-*N*-acetyl-*N*-methyl)7,8,9-tri-*O*-methylneuraminate methyl glycoside after methanolysis and acetylation of permethylated Fraction 1. Minor Fraction 7, which contained only galactose and *N*-acetylneuraminic acid is probably a terminal fragment of repeating unit **3**. The presence of a heptose, besides other sugars, in

TABLE II

Methylation analysis (molar ratio) of the oligosaccharides **1–7** from Fractions 1–6 isolated from *E. coli* O104 lipopolysaccharide

Methylated sugar [a]	Fraction						
	1 (**1**)	2	3 (**2**)	4 (**5**)	5 (**6**)	6a (**3**)	6b (**7**)
2,3,4,6-Me_4-Glc	[b]						0.8
2,3,4,6-Me_4-Gal	0.3	0.8	1.0	1.8	1.0	1.0	1.0
3,4,6-Me_3-Glc		0.3		1.0	1.7		0.3
2,3,6-Me_3-Gal	1.0	1.0	2.1	0.2		1.0	
2,4,6-Me_3-Gal	0.9	1.0	1.2	0.3			
2,3,4,6,7-Me_5-Hep	0.2	0.2		0.6	0.6		0.7
3,6-Me_2-Glc	0.2	0.3		1.0	0.3		
2,4-Me_2-Glc	0.2	0.6		1.3	1.0		1.3
3,4,6-Me_3-GlcNAc	tr [c]	0.4		1.0	0.6		
4,6-Me_2-GalNAc	0.8	1.7	1.7	1.1		0.6	

[a] As per-*O*-acetyl derivatives. [b] Component not detected. [c] Component present in trace amount.

the remaining Fractions 2, 4, 5, and 6b indicated that they were core-related oligosaccharides.

In order to establish the sequence of sugar residues in the repeating unit of the O-specific polysaccharide, tetrasaccharide **3** was subjected to FAB-mass spectrometry analysis. The underivatized tetrasaccharide gave, in the negative mode, a spectrum with a net pseudomolecular ion $[M - H]^-$ at m/z 919, together with two signals at m/z 877 and 835, for a partially deacetylated molecule, compatible with a global composition $(Hex)_2$, HexNAc, $Neu5Ac(OAc)_2$. In the positive mode, major ions at higher mass were found at m/z 943 $[M + Na]^+$ and 959 $[M + K]^+$, together with minor fragments at m/z 901 and 917 corresponding to partially deacetylated ions. The free acid form of the neuraminic acid containing oligosaccharide was also detected with ions at m/z 921, $[MH]^+$ and 879 (minus ketene). Two fragment ions at m/z 394 and 376, corresponding respectively to protonated $Neu5,7,9Ac_3$ and its dehydrated derivatives were also found in the latter spectrum, in agreement with the presence of a di-*O*-acetylNeu5Ac component.

Positive FAB-mass spectra of peracetylated tetrasaccharide **3** (Fig. 2, upper) afforded the sequence of sugar residues in the repeating unit. A terminal hexose was observed with an ion at m/z 331 corresponding to a B_1 fragment, according to the Domon and Costello nomenclature[11], and this was followed by an intense signal at m/z 618 (B_2, ref. 11) corresponding to Hex → HexNAc, a fragmentation that is expected in a sequence containing an *N*-acylhexosamine unit[12]. The next prominent fragment at m/z 946 (C_3, ref. 11) could be explained by loss of a 2,4-didehydroNeu5Ac component from the $[MNa]^+$ pseudomolecular ion, a fragmentation pattern that is supported by the presence of minor fragment ions at m/z 904, 886, and 826 resulting from the elimination of ketene and of one and two molecules of acetic acid, respectively, from the ion at m/z 946; a counterpart was observed in a fragment ion at m/z 400 (Y_1, ref. 11) which corresponds to charge retention on an 2,4-didehydroNeu5Ac species. Unequivocal assignment of B_1, B_2, C_3, and Y_1 ions was supported by a comparison of the FAB^+-mass spectrum of the corresponding per(deuterioacetylated) derivative of **3** (Fig. 2, lower), which showed the expected mass increments. Increase in mass by three units for fragment Y_1 was a further confirmation that Neu*p*5Ac is di-O-acetylated in the native polysaccharide at exocyclic O-7–9.

Mass-spectrometric analysis of octasaccharide **2**, a dimer of the tetrasaccharide repeating unit **3**, proved to be very helpful in confirming the sequence arrangement of the repeating unit in the O-specific polysaccharide. FAB^+- and FAB^--mass spectra of underivatized **2** showed respective pseudomolecular ions at m/z 1823 for $[MH]^+$ and m/z 1821 for $[M - H]^-$, in agreement with a mass of 1822 daltons, which confirms the dimeric character of the oligosaccharide fraction with respect to the repeating unit. Ions at m/z 2603 (B_8) and 1323 (B_4) in the FAB^+-mass spectrum of peracetylated **2** (Fig. 3, upper) are consistent with a sequence arrangement involving $Neup5Ac(OAc)_2 \rightarrow Galp$ as the anchoring site for the repeating unit, a point which is confirmed in the FAB^+-mass spectrum of per(de-

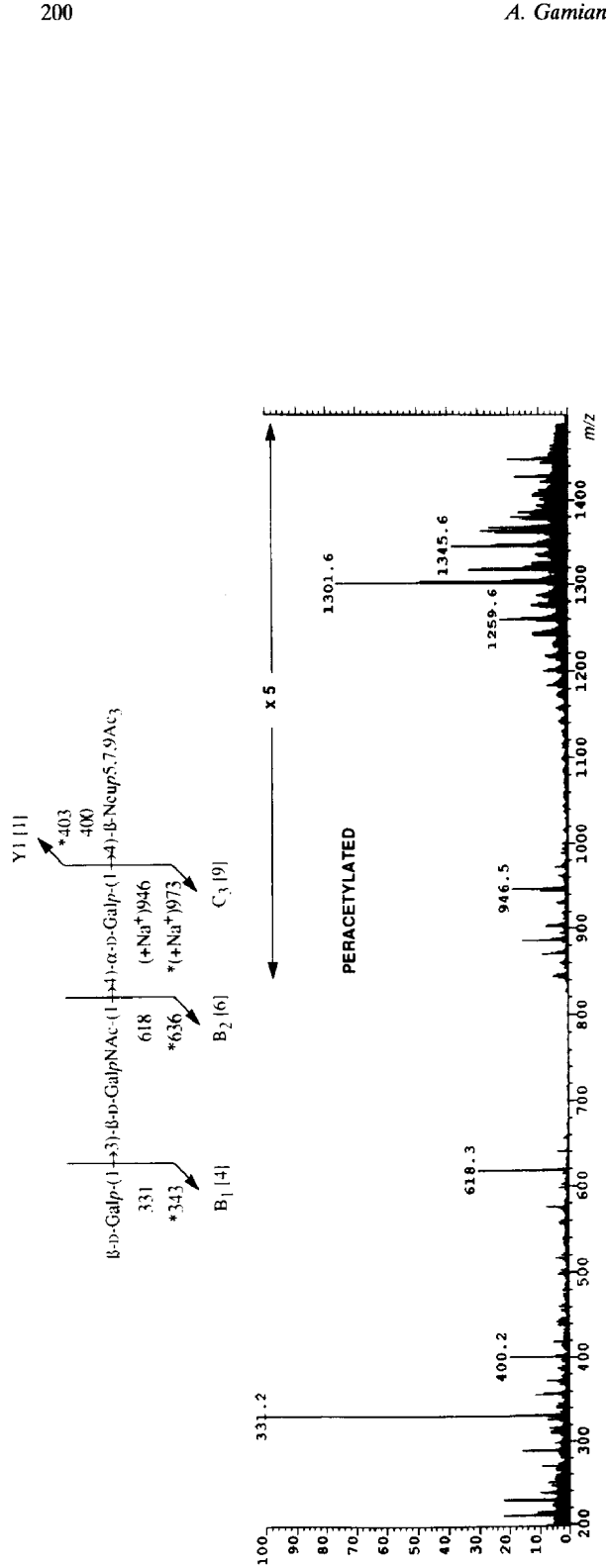

Fig. 2. Positive FAB-mass spectra and fragmentation pattern for peracetylated (upper) and *per(deuterioacetylated) (lower) tetrasaccharide **3**. The amount of deuterioacetylated groups in the fragment is in square brackets.

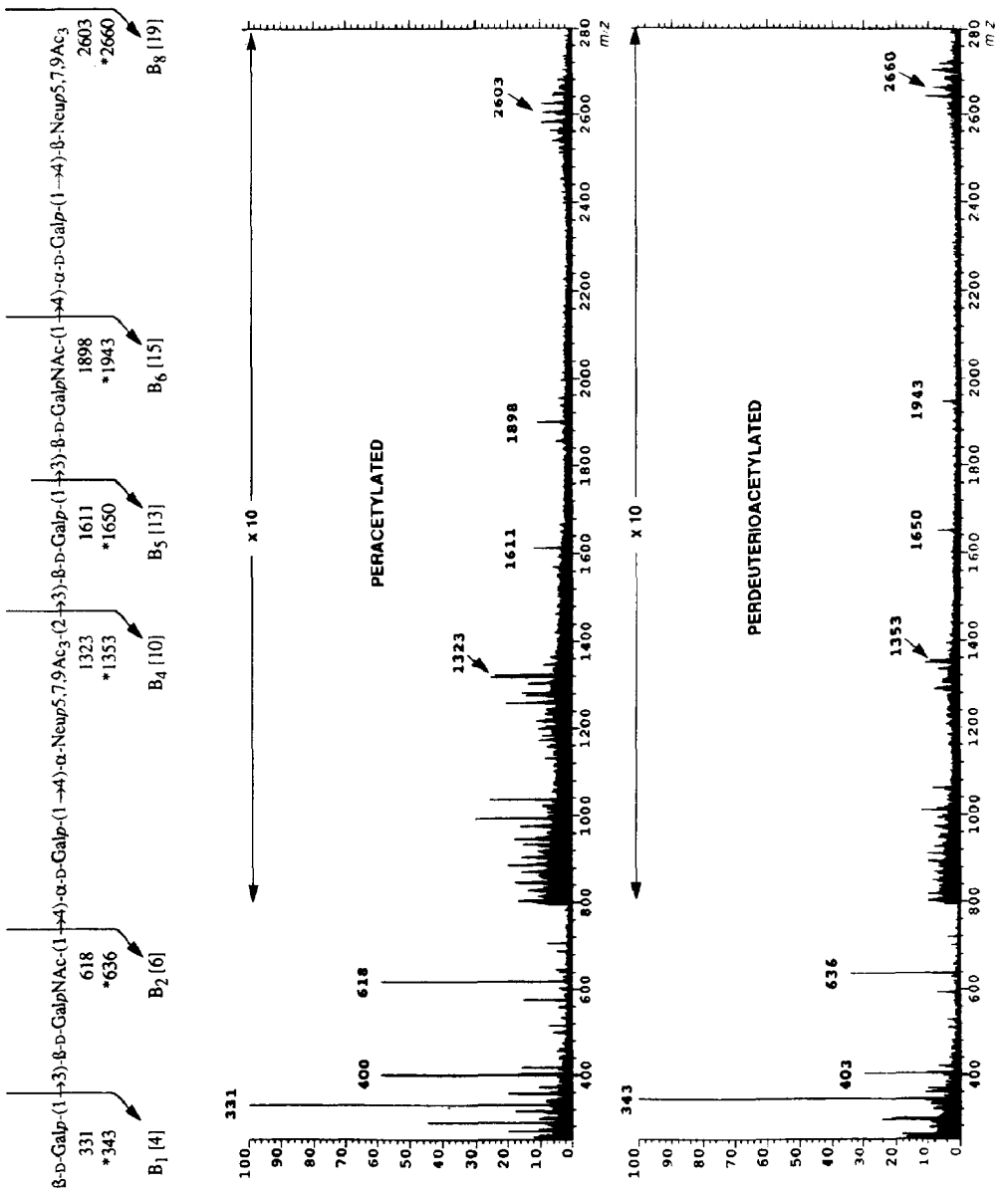

Fig. 3. Positive FAB-mass spectra and fragmentation pattern for peracetylated (upper) and (*) per(deuterioacetylated) (lower) octasaccharide **2**.

uterioacetylated) **2** (Fig. 3, lower), with related ions at m/z 2660 and 1323. Di-*O*-acetylation of Neu*p*5Ac in the native polysaccharide, as well as the number of unsubstituted hydroxyl groups in the monosaccharide constituents, was again clearly seen from comparison of sequence-ions between the acetylated and deuterioacetylated molecules.

Assignment of the anomeric configuration of sugar units in 1–7 and confirmation of the position of *O*-acetyl substituents in the Neu5Ac component were obtained from ^{1}H and ^{13}C NMR analysis. The 400-MHz, ^{1}H NMR spectrum of the O-specific polysaccharide **1** showed two doublet signals for β (D) anomeric protons at δ 4.51 and 4.75 ($^3J_{1,2} \sim 8.5$ Hz), and one signal at δ 5.05 ($^3J_{1,2} < 4$ Hz) for an α (D)-linked residue, as well as signals for two acetamido (δ 1.97 and 2.04) and two acetoxy (δ 2.14) groups. Two one-proton multiplets were also present at δ 1.82 (quasi triplet) and 2.92 (unresolved), thus indicating[6,7] the presence of an α-linked neuraminic acid residue in the *E. coli* O104 polysaccharide. In addition, one-doublet proton signal (δ 5.13) was assigned to H-7 ($^3J_{7,8}$ 8.9 Hz) of *N*-acetyl-7,9-di-*O*-acetylneuraminic acid (Neu5,7,9Ac$_3$), in agreement with Kamerling et al. data[13], and this assignment was corroborated by the chemical displacement at δ 1.97 found for the acetamido group of this unit. As expected from the reported ease of *O*-acetyl group migration in the latter component[13], the region for *O*-acetyl group signals in the spectrum of **1** was found to be rather complicated. An enhanced resolution was obtained with the proton spectrum of the repeating unit **3** (Fig. 4A). Indeed, the expanded region for *O*-acetyl group resonance for **3** showed (Fig. 4B and Table III) nine acetyl signals in the range δ 1.93–2.15 indicating[13] the presence of both 7,9- and 8,9-di-*O*-acetyl derivatives of *N*-acetylneuraminic acid. Furthermore, two characteristic, low-field shifted signals at δ 5.19 for H-7 of Neu5,7,9Ac$_3$ and at δ 5.11 for H-8 of Neu5,8,9Ac$_3$ (Fig. 4A), with an integral ratio of 3:2, led us to assume that originally two *O*-acetyl groups were located at positions O-7 and O-9 of the neuraminic acid and that partial migration of the acetyl group from O-7 to O-8 occurred without significant *O*-deacylation. An enhanced resolution for the δ 5.19 and 5.11 signals for **3** was obtained with the 500-MHz ^{1}H-NMR spectrum of **4**, the low-field portion of which is shown in Fig. 4C.

Disaccharide **4** exhibited, in its ^{1}H NMR spectrum, an *N*,*O*-acetyl region similar to that of tetrasaccharide **3**, except that the *N*-acetyl signal at δ 2.028, 2.031 for 2-acetamido-2-deoxy-D-galactose was lacking. The molar ratio of *O*-acetyl to *N*-acetyl signals was 1.92:1, confirming the di-*O*-acetylation of neuraminic acid. A signal at δ 5.02 for the D-galactose residue with $^3J_{1,2}$ 3.88 (Fig. 4C) indicated furthermore that the anomeric configuration of the linkage with neuraminic acid was α (D). Therefore, the second D-galactose residue in tetrasaccharide **3**, and consequently in **1**, has the β-anomeric configuration. This point was corroborated by the comparative ^{13}C NMR spectra of **3** and **4** (Table IV), which allowed the assignment of the C-1 resonances for both residues together with the neuraminic acid signals. From these data, the absence of a signal in the region δ 81–90

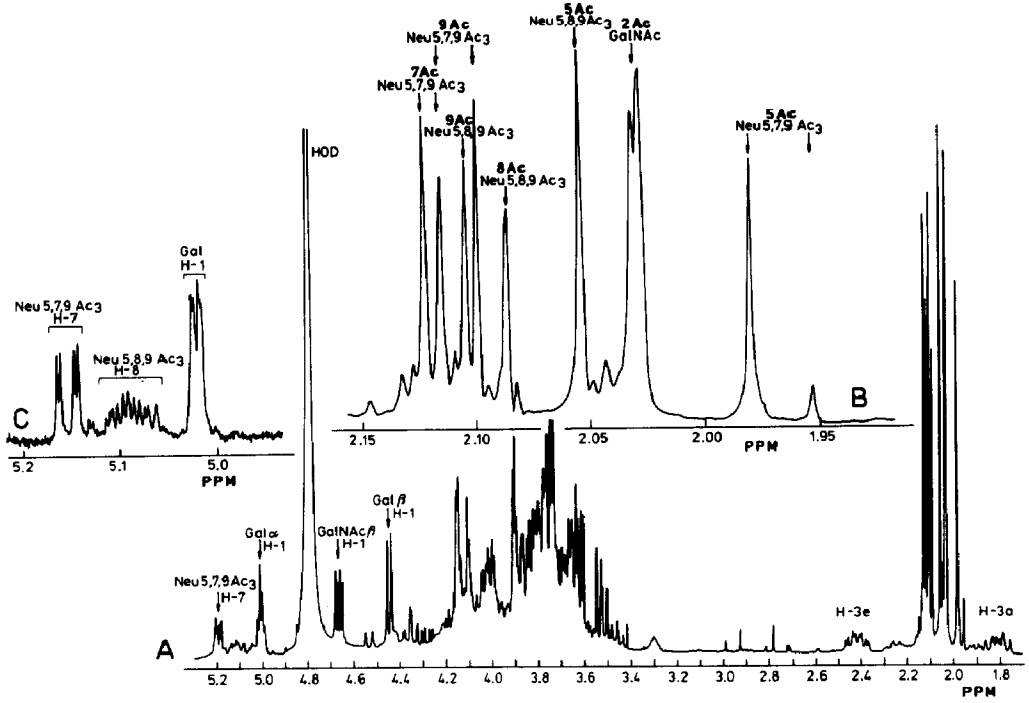

Fig. 4. 400-MHz, ^{1}H NMR spectrum of tetrasaccharide repeating unit **3** (A), with expanded portion of the high-field part of the spectrum (B) showing *N,O*-acetyl signals. For comparison, the low field part of the 500-MHz, ^{1}H NMR spectrum of the disaccharide component **4** is shown in (C).

TABLE III

^{1}H NMR chemical shift (δ)[a] values of *N,O*-acetyl signals in spectra of compounds **3** and **4**

Tentative assignment			Compound		Lit.[13]
For	In		**3** [b]	**4** [c]	
5Ac	Neu5,7,9Ac$_3$	α	1.953	1.952	1.948
		β	1.980	1.980	1.980
7Ac	Neu5,7,9Ac$_3$	α	2.115	2.109	2.119
		β	2.123	2.124	2.134
9Ac	Neu-5,7,9Ac$_3$	α	2.115	2.113	2.122
		β	2.098	2.098	2.106
5Ac	β-Neu5,8,9Ac$_3$		2.054	2.055	2.059
8Ac	β-Neu5,8,9Ac$_3$		2.086	2.087	2.090
9Ac	β-Neu5,8,9Ac$_3$		2.104	2.103	2.107
2Ac	GalNac		2.028, 2.031 [d]	[e]	[e]

[a] For solutions in ^2H_2O at 295 K and internal acetone (δ 2.23) as a standard. [b] Recorded at 400 MHz [c] Recorded at 500 MHz. [d] Poorly resolved. [e] Not present or not determined.

TABLE IV

Partial ^{13}C and ^{1}H NMR data for oligosaccharides **3** and **4**

Group or residue [a]	H or C atom	^{1}H NMR [c] (δ) [c]		^{3}J (Hz)		^{13}C NMR (δ) [c]
Compound **3**						
A β-D-Gal*p*-(1 →	1	4.45 (4.45)	$J_{1,2}$	8.3	(7.7)	105.61 (105.56)
B → 3)-β-D-Gal*p*NAc(1 →	1	4.67 (4.68)	$J_{1,2}$	8.9	(8.6)	103.34 (103.32)
	2					52.29 (52.30)
	3					80.44 (80.45)
C → 4)-α-D-Gal*p*-(1 →	1	5.01 (5.03)	$J_{1,2}$	3.95	(3.95)	95.93 (95.94)
	4					77.36 (77.36)
D → 4)-Neu5,7,9Ac$_3$	3*a*	1.82 (1.83)	$J_{3a,4}$	11	(10.7)	
	3*e*	2.43 (2.48)	$J_{3e,4}$	2.3	(4.34)	36.57 (36.37)
	6	4.35 (4.241)	$J_{5,6}$	11	(10.5)	50.46 (50.39)
	7	5.19 (5.137)	$J_{6,7}$	1.8	(2.0)	
			$J_{7,8}$	8.9	(8.8)	
→ 4)-Neu5,8,9Ac$_3$	8	5.11 (5.109)	$J_{7,8}$	8.4	(8.4)	
			$J_{8,9a}$	2.7	(2.6)	
			$J_{8,9b}$	5.2	(5.5)	
	9	4.54 (4.522)	$J_{8,9a}$	2.3	(2.6)	
			$J_{9a,9b}$	1.3		
→ 4)-Neu 5Ac	5	4.02 (3.899)				
	6	4.07 (3.979)				
Compound **4**						
α-D-Gal	1	5.02	$J_{1,2}$	3.88		97.26
Neu5Ac	3					38.62
						38.42
4)-Neu5,7,9Ac$_3$	5					52.38
						52.33
	7	5.15	$J_{6,7}$	1.9		
			$J_{7,8}$	9.2		
→ 4)-Neu5,8,9Ac$_3$	8	5.09				

[a] See structure **3** for A, B, C, and D (in D, Ac-7 or -8). [b] Comparative value found in ref. 7 for the tetrasaccharide repeating sequence β-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → 4)-α-D-Gal*p*-(1 → 4)-β-Neu*p*5Ac in *E. coli* serotype K9 polysaccharide, and in ref. 13 for acetylated sialic acid residues, in brackets. [c] Chemical shifts relative to that of acetone (δ 2.23 and 31.07 downfield from the signal for Na 4,4-dimethyl-4-silapentane-1-sulfonate, for ^{1}H and ^{13}C, respectively).

confirmed that all monosaccharide components were in the pyranose form. The presence of disaccharide **4** among the products of mild acid hydrolysis of LPS, and the concomitant absence of free Neu5Ac, was a further indication that disaccharide **4** terminates the repeating unit in intact LPS, a conclusion that was confirmed by the absence of methyl tetra-*O*-methylneuraminate methyl glycoside after methanolysis and acetylation of permethylated polysaccharide **1**.

The type of linkage between the O-specific polysaccharide and the core oligosaccharide was established by the comparative analysis of Fractions 4, 5 and 6b, which were additionally purified on Ultrapherogel-SEC 2000 gel exclusion LC. From the sugar (Table I) and the methylation analysis (Table II), Fraction 5

Fig. 5. Immunoblotting of SDS-PAGE resolved lipopolysaccharides from *Salmonella toucra* 048 (1), *E. coli* 024 (2), *E. coli* 56R (3), and *E. coli* O104 (4) strains, in reaction with anti-56R serum.

appeared to contain a complete core oligosaccharide corresponding to *E. coli* R2 type[14,15]. Fraction 6b contained an incomplete core oligosaccharide, since the terminal 2-acetamido-2-deoxy-D-glucopyranose component was missing. The oligosaccharide (**5**) of Fraction 4, on the other hand, contained an additional disaccharide fragment linked to a complete R2 core oligosaccharide at O-4 of the subterminal D-glucopyranose unit, since it yielded the same methylated derivative as compared to that of oligosaccharide **6** from Fraction 5 with, however, additional stoichiometric amounts of 2-acetamido-2-deoxy-D-galactopyranose and D-galactopyranose methyl ethers corresponding to the β-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → sequence. Also, the presence of 3,6-di-*O*-methyl-D-glucose in place of a 3,4,6-tri-methyl ether from **6**, indicated that the site of attachment of the O-specific chain to the core is at position O-4 of its subterminal D-glucose unit. In *E. coli* 0100 LPS, similar complete and incomplete core oligosaccharides and mode of core substitution were also observed[14]. The *E. coli* R2 type core is also present in *E. coli* O56 strain[16]. Interestingly, a cross reactivity was observed in immunoblotting experiments (Fig. 5), between O104 and O56 R LPS in the presence of anti-*E. coli* O56 core serum, a result that corroborates the conclusion that *E. coli* O104 core is of the R2 type.

These results are consistent with a tetrasaccharide repeating unit, → 4)-α-D-Gal*p*-(1 → 4)-α-Neu*p*5,7,9Ac$_3$-(2 → 3)-β-D-Gal*p*-(1 → 3)-β-D-Gal*p*NAc-(1 → , for the *E. coli* O104 O-specific polysaccharide, a sequence which would subsequently be attached, through a β-D-Gal*p*NAc residue, to the subterminal α-D-Glc*p* residue of *E. coli* R2 type core. Structural analysis of the remaining Fraction 2 (Tables I and II) suggested that it is a hexasaccharide fragment linked to the core oligosaccharide, since it comprises one terminal Gal, one O-4- and one O-3-linked Gal units together with two O-3-linked GalNAc and one Neu5Ac units, besides core components.

A close similarity between the repetitive sequence of the neuraminic acid-containing *E. coli* serotype K9 capsular polysaccharide[7] and O104 polysaccharide was noticed and is evident from ^{1}H NMR data (Table III) since the only difference lies in the *O*-acetyl substitution of the Neu5Ac constituent. This suggests that the repeating unit in both antigens could have a closely related structure. It would, then, be of interest to compare the biosynthesis of both polysaccharides. Such a study could furthermore be useful in the understanding of the role of sialic acid in LPS with reference to host-bacteria interaction.

EXPERIMENTAL

Bacterial strain and isolation, purification, and serological characterization of LPS. — *Escherichia coli* serotype O104: K$^-$: H-12 strain H519 (PCM 270) and *E. coli* O24 strain E41a were kindly provided by Dr. F. Ørskov, Statens Seruminstitut, Copenhagen. *Salmonella toucra* O48 strain was from the National Salmonella Centre, Gdansk, and *E. coli* R56 (PCM 225) was a spontaneous mutant from a collection of the Institute of Immunology (Wroclaw). Bacteria were cultivated in liquid medium with aeration at 37°. After 24 h, cells were harvested and freeze-dried. Lipopolysaccharide was isolated by phenol–water extraction[17] and purified on a Sepharose 2B column as described[6,8]. The LPS of *E. coli* O104 (200 mg) was hydrolyzed with 1% acetic acid (20 mL) for 1 h at 100° and, after removal of the precipitated lipid A by centrifugation, the supernatant was freeze-dried and then fractionated on a column (1.6 × 100 cm) of Bio-Gel P-4. Gel-filtration chromatography was performed in 0.05 M aqueous pyridinium acetate buffer (pH 5.6). The column eluate was monitored with a Knauer differential refractometer type 188.00. When needed, this purification was followed by LC on a gel-permeation column Ultrapherogel-SEC 2000 (Beckmann, 7.5 mm × 30 cm) fitted to a Perkin–Elmer pump model 250 working in the isocratic mode with the same solvent. SDS-PAGE and immunoblotting experiments were carried out as previously described[8,18]. Electrophoresis was done in a 15% acrylamide slab gel and 5% acrylamide stacking gel. The LPS suspensions (1 mg/mL) were boiled in the sample buffer for 10 min and a constant current of 30 mA was applied to 2-μL samples. The resulting gel was then subjected to electrophoretic transfer to nitrocellulose which was kept with rabbit anti-*E. coli* R 56 serum diluted to 1:200, and then with goat anti-rabbit IgG–horseradish peroxidase conjugate prior to staining.

NMR spectroscopy. — For NMR measurements, the samples were repeatedly treated with ^2H_2O, with intermediate lyophilization, and then dissolved in ^2H_2O (0.3 mL), containing a trace of acetone which was used as internal reference. ^{1}H NMR spectra were recorded at 400 MHz with AM-400 or at 500 MHz with AM-500 Bruker instruments. ^{13}C NMR spectra were recorded at 100 or 125 MHz with the same equipment. Spectra were measured at 300 or 295 K.

FAB-mass spectrometry. — FABMS was carried out on a Fisons-VG type ZAB-SEQ, double-focusing mass spectrometer working at 8 kV accelerating volt-

age. The LSIMS ion source was equipped with a Cs ion gun, giving a beam of $2\mu A/35$ keV for positive and negative mode. Thioglycerol or a 1:1 (v/v) thioglycerol–glycerol mixture were used as the liquid matrix in the positive mode, and triethanolamine in the negative mode. Samples, dissolved in MeOH, were generally acidified with 5% acetic acid. Peracetylation and per(deuterioacetylation) of oligosaccharides were done as described[19].

Analysis of component sugars. — Dried (P_2O_5 under reduced pressure) oligo- and poly-saccharide samples (~ 1 mg) were treated with aq M HCl (0.6 mL) for 4 h at 100°. After reduction with $NaBH_4$ (20 mg, 16 h, 4°), and acetylation (1:1, v/v, Ac_2O–pyridine), hexosamines and neutral sugars were analysed by GLC using a Varian 2000 gas chromatograph fitted with a flame ionization detector and a glass OV-225 (2 m × 3 mm) column, with N_2 as the carrier gas. Separation was performed at 180° for hexitol acetates, or 220° for hexosamine derivatives. 3-Deoxyoctulosonic acid (Kdo) was estimated with periodate–thiobarbituric acid reagent[20], *N*-acetylneuraminic acid (Neu5Ac) with resorcinol reagent[21] and *O*-acetyl groups by the method of Hestrin[22].

Methylation analysis. — Samples of oligo- and poly-saccharides (1 mg) were methylated according to Hakomori[23], and the products were purified with Sep Pak C18 cartridges. A portion of the methylated product was hydrolyzed with 2 M trifluoroacetic acid for 2 h at 120°, and then reduced with $NaBD_4$ and acetylated for GLC–MS analysis. Another portion (~ 0.5 mg) of the methylated sample was depolymerized by methanolysis (M HCl in MeOH, 0.7 mL, 4 h, 80°) followed by concentration to dryness with a N_2 stream, and acetylation with acetic anhydride–pyridine at 100° for 35 min. GLC analysis was carried out on the above Varian equipment (0V-225, 2 m × 3 mm column) with a temperature programming of 1°/min from 150 to 200°. For GLC–MS analysis, a Hewlett–Packard 5971 A system equipped with an HP-1 glass capillary column (0.2 mm × 12 m), a temperature programming of 8°/min from 150 to 270°, and 70 eV ionization potential was used.

Determination of the absolute configuration of the monosaccharides. — The O-specific polysaccharide (0.5 mg) was hydrolyzed with M HCl for 4 h at 100° to release neutral sugar. Another portion (0.5 mg) was treated with 4 M HCl for 5 h at 100° to release hexosamines. Both samples showed positive reactions upon treatment with D-galactose oxidase[9], indicating the D-configuration for the galactose and galactosamine units in the polysaccharide.

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