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Structure of the O56 antigen of *Escherichia coli*, a polysaccharide containing 7-substituted α-*N*-acetylneuraminic acid

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ABSTRACT

The O56 polysaccharide moiety of the O56 antigen (LPS) consists of D-glucose, D-galactose, 2-acetamido-2-deoxy-D-glucose, and *N*-acetylneuraminic acid in the molar ratios 1:1:1:1. Methylation analysis, periodate oxidation, mild acid hydrolysis, as well as ^{1}H and ^{13}C NMR spectroscopy showed that the O56 polysaccharide has the primary structure

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 7)\text{-}\alpha\text{-Neu5Ac-}(2\rightarrow$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p \end{array}$$

INTRODUCTION

N-Acetylneuraminic acid (Neu5Ac) is a widespread component of complex carbohydrates in eukaryotes, where it occurs mostly as a terminal unit of carbohydrate chains, although oligo-Neu5Ac substitution has also been detected in cell adhesion molecules[1,2]. Neu5Ac is also a constituent of a number of bacterial polysaccharides, either as a single terminal unit, as a homopolymer, or within a heteropolymer chain[3–5]. In all these cases, Neu5Ac is present as the α anomer; when in an internal position it is substituted at C-4, C-8, or C-9. Herein we report on the structure of an *E. coli* polysaccharide which contains α-Neu5Ac substituted at C-7.

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RESULTS AND DISCUSSION

Isolation and characterisation of the O56 (lipo)polysaccharide.—The bacteria, after growth in liquid culture, were extracted with aqueous 45% phenol and the aqueous phase was subjected to ultracentrifugation[6]. The sediment contained a LPS fraction (LPS I) which consisted of short-chain LPS and R-LPS. The supernatant solution contained an LPS (LPS II) which was isolated by fractional precipitation with cetyltrimethylammonium bromide[6,7]. LPS II, which had a long O-specific polysaccharide chain, was used for further structural studies.

The presence of only a little O-specific polysaccharide in LPS I and of only a little lipid A in LPS II was demonstrated by their SDS-PAGE patterns and by the fact that LPS I contained 13% of β-hydroxymyristic acid and LPS II contained 1.2% of this component which is characteristic of lipid A. LPS II consisted of D-glucose (Glc), D-galactose (Gal), 2-acetamido-2-deoxy-D-glucose (GlcNAc), and a thiobarbituric acid-reactive nonulosonic acid, which was identified as *N*-acetylneuraminic acid (Neu5Ac), in the molar ratios 1 : 1 : 1 : 1. Neu5Ac was identified by Neu5Ac aldolase[8], Determination of Neu5Ac with this reaction and with the thiobarbituric acid reaction[9] gave comparable values. Periodate oxidation destroyed the galactose residue and, after reduction, converted Neu5Ac into its C-8-analogue (see below).

Methylation analysis.—LPS II was methylated[10,11] with KH–MeI in Me_2SO. To analyse the substitution pattern of Glc, Gal, and GlcNAc, the purified methylation product was hydrolysed and the constituents were characterised as their partially methylated alditol acetates. To analyse the substitution pattern of Neu5Ac, methylated LPS-II was subjected to methanolysis[12] and the resulting methyl glycosides were acetylated. The results of the GLC–MS analysis are given in Table I and the mass spectrum of the Neu5Ac derivative is shown in Fig. 1. The results indicated that the O56 LPS II contained terminal Gal, 3-substituted GlcNAc, 2,3-disubstituted Glc, and 7-substituted Neu5Ac. The retention time of the Neu5Ac derivative

TABLE I

Methylation analysis[a] of non-sialic acid sugar components of the O56 polysaccharide (PS) and the oligosaccharide derived by Smith degradation (OS)

Methylation product	Sugar residue	PS	OS
1,5-Di-OAc-2,3,4,6-tetra-OMe-hexitol	Gal(1 →	1	
1,2,3,5-Tetra-OAc-4,6-di-OMe-hexitol	→ 3)Glc(1 → 2 ↑	1	
1,3,5-Tri-OAc-4,6-di-OMe-2-deoxy-2-*N*-methylacetamidohexitol	→ 3)GlcNAc(1 →	1	
1,3,5-Tri-OAc-2,4,6-tri-OMe-hexitol	→ 3)Glc(1 →		1
1,5-Di-OAc-3,4,6-tri-OMe-2-deoxy-2-*N*-methylacetamidohexitol	GlcNAc(1 →		1

[a] Values are molar ratios.

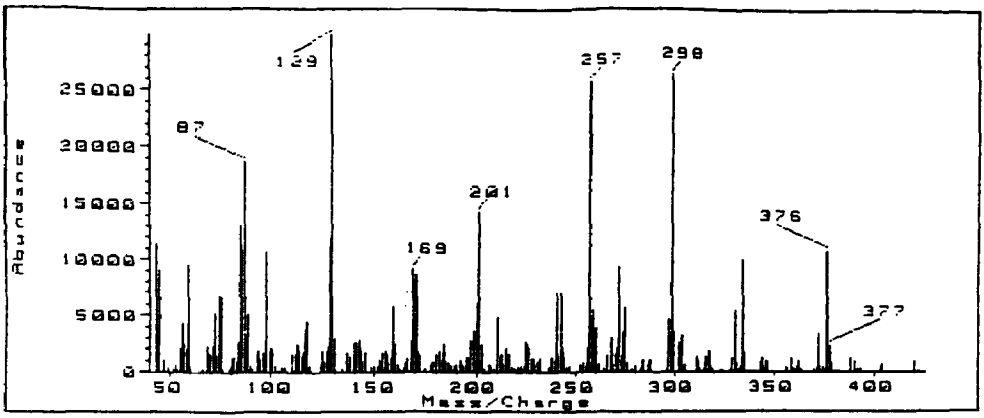

Fig. 1. Mass spectrum of the methyl ester of *N*-acetyl-7-*O*-acetyl-*N*-methyl-4,8,9-tri-*O*-methylneuraminic acid methyl glycoside, obtained from the O56 polysaccharide.

(relative to that of the derivative of terminal Neu5Ac), t_R 1.02, is in agreement with that (1.08) reported for synthetic *N*-acetyl-7-*O*-acetyl-*N*-methyl-4,8,9-tri-*O*-methylneuraminic acid methyl ester methyl glycoside[13]. Its fragmentation pattern is shown in Fig. 2. Apart from the presence of fragments B (*m/z* 376), E (*m/z* 201), F (*m/z* 89), and G (*m/z* 129), the absence of fragments C (*m/z* 346) and D (*m/z* 254) indicated that C-7 of the methylated Neu5Ac derivative was susbtituted with *O*-acetyl and not with *O*-methyl[13,14].

Isolation and methylation of a tetrasaccharide.—LPS II was hydrolysed (0.125 M H_2SO_4 80°C, 1 h) and the neutralised hydrolysate was fractionated by chromatography on Bio-Gel P-2. A tetrasaccharide was obtained which consisted of Gal, Glc, GlcNAc, and Neu5Ac in the same molar ratios (1:1:1:1) as for the native polysaccharide. The tetrasaccharide was methylated and the methylated product

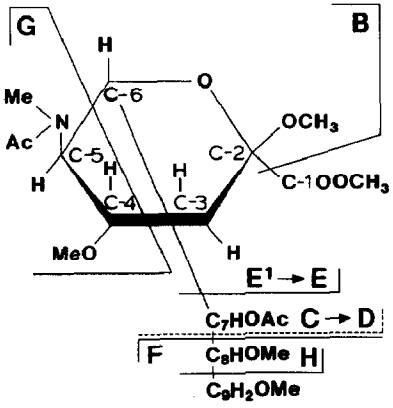

Fig. 2. Fragmentation pattern of the methyl ester of *N*-acetyl-7-*O*-acetyl-*N*-methyl-4,8,9-tri-*O*-methylneuraminic acid methyl glycoside.

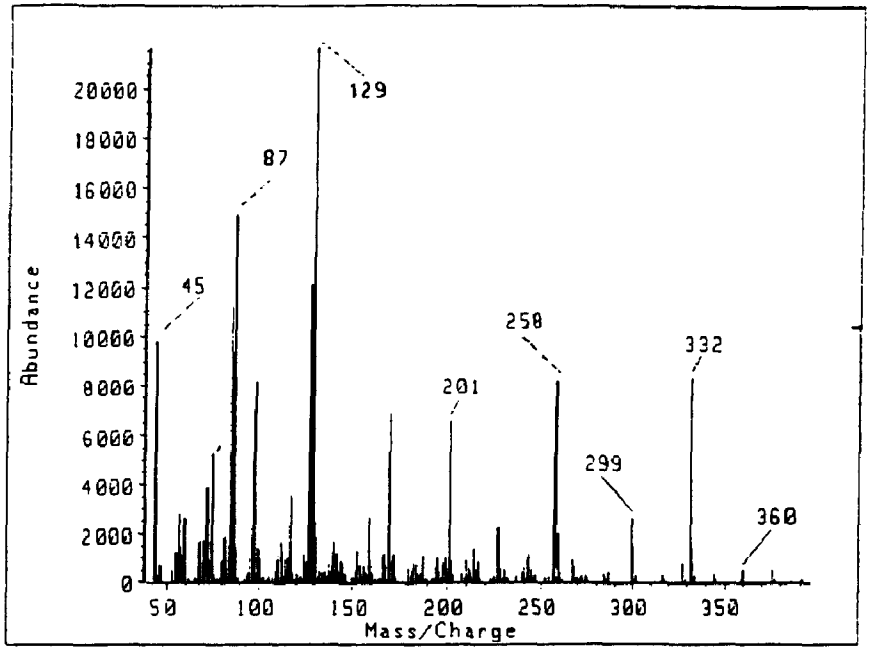

Fig. 3. Mass spectrum of the methyl ester of the *N*-acetyl-7-*O*-acetyl-*N*-methyl-4,8-di-*O*-methyl derivative of the C_8 analogue of neuraminic acid, obtained from the periodate-oxidised O56 polysaccharide.

was treated as described above. The result of the GLC–MS analysis indicated that the tetrasaccharide was branched with the sequence GlcNAc-(1 → 3)-[Gal-(1 → 2)]-Glc-(1 → 7)-Neu5Ac. It represents the chemical repeating unit of the O56 polysaccharide.

Smith degradation and methylation of the product.—LPS II was subjected to periodate oxidation and the product was reduced with sodium borohydride. Mild hydrolysis of the oxidised and reduced polysaccharide[4,15] (0.05 M CF_3CO_2H, 25°C, 24 h) yielded a trisaccharide which was methylated as described above. The results of the GLC–MS analysis (Table I) indicated that the product of the Smith degradation[15] consisted of terminal nonreducing GlcNAc, 3-substituted Glc, and the 7-substituted C_8-analogue of Neu5Ac. The mass spectrum obtained with the partially methylated and acetylated methyl ester methyl glycoside of the latter is shown in Fig. 3. These results confirm that, in the O56 polysaccharide, Neu5Ac is 7-substituted. They also show that galactose is linked to C-2 of the 3-linked main chain glucose.

NMR spectroscopy.—The ^{13}C NMR spectrum of the O56 polysaccharide exhibited four signals in the anomeric region, thus confirming the tetrasaccharide repeating unit. A gated decoupling experiment[16,17] showed one signal of an α-anomeric carbon atom (δ 96.6; $J_{C\text{-}1,H\text{-}1}$ 170 Hz) and two signals of β-anomeric carbon atoms (δ 103.3 and 102.2; $J_{C\text{-}1,H\text{-}1} \sim 163$ Hz); in an APT spectrum[18,19], the signal at δ 102.6 was negative and could thus be identified as due to C-2 of

Neu5Ac. In addition to the anomeric signals, the ^{13}C NMR spectrum also showed, inter alia, two signals of carbon atoms bearing nitrogen (δ 53.7 and 56.9), one signal due to a methylene group (δ 37.4), two signals from methyl groups (δ 23.2 and 23.6) and corresponding signals of carbonyl carbons of acetamido groups (δ 175.0 and δ 175.1), as well as a carboxyl signal at δ 173.9. In a gated decoupling experiment, the latter signal showed a large axial–axial coupling constant ($J_{C\text{-}1,H\text{-}3ax}$ 5.4 Hz) and a small axial–equatorial coupling constant ($J_{C\text{-}1,H\text{-}3eq}$ 1 Hz), indicative of α-Neu5Ac[20,21].

The ^1H NMR spectrum of the O56 polysaccharide contained three signals due to anomeric protons at δ 5.52 ($J_{H\text{-}1,H\text{-}2}$ 3.6 Hz), 4.69 ($J_{H\text{-}1,H\text{-}2}$ 7.6 Hz), and 4.73 ($J_{H\text{-}1,H\text{-}2}$ 8.2 Hz). A signal at δ 2.51 (broadened doublet with a visible coupling constant of ~ 8.0 Hz) was assigned to the equatorial H-3 (H-3*eq*) of Neu5Ac[22].

TABLE II

Assignments of the signals in ^1H and ^{13}C NMR spectra of the O56 polysaccharid

Residue	Proton	δ (ppm)	Visible coupling	J (Hz)		Carbon	δ (ppm)
→ 7)-α-Neu5Ac-(2 →						C-1	173.3
(A)						C-2	101.95
	H-3*ax*	2.00	m			C-3	37.45
	H-3*eq*	2.51	m				
	H-4,H-5	3.89	m			C-4	72.0
						C-5	53.7
	H-6	4.06	m			C-6	72.6
	H-7	3.73	b d	$J_{7,8}$	8.0	C-7	78.4
	H-8	3.77	d d d	$J_{8,9}$	3.0	C-8	69.15
	H-9	3.80	d d	$J_{9,9'}$	12.5	C-9	63.2
	H-9′	3.50	d d	$J_{8,9'}$	7.2		
→ 3)-β-D-Glc*p*NAc-(1 →	H-1	4.69	d	$J_{1,2}$	7.6	C-1	102.8
(B)	H-2	3.66	d d	$J_{2,3}$	9.0	C-2	56.9
	H-3	4.16	b t	$J_{3,4}$	9.0	C-3	78.2
	H-4,H-5	3.51	m			C-4	70.1
						C-5	76.3
	H-6	3.98	b d	$J_{6,6'}$	~ 12	C-6	61.9
	H-6′	3.79	b d				
→ 3)-β-D-Glc*p*-(1 →	H-1	4.73	d	$J_{1,2}$	8.2	C-1	101.6
(C) 2	H-2	3.65	d d	$J_{2,3}$	9.0	C-2	73.2
↑	H-3	4.07	t	$J_{3,4}$	9.0	C-3	80.2
	H-4	3.51	t	$J_{4,5}$	9.0	C-4	69.4
	H-5	3.57	m			C-5	75.8
	H-6	4.04	b d	$J_{6,6'}$	~ 12	C-6	61.9
	H-6′	3.81	b d				
α-D-Gal*p*-(1 →	H-1	5.52	d	$J_{1,2}$	3.6	C-1	96.8
(D)	H-2	3.82	d d	$J_{2,3}$	10.0	C-2	69.15
	H-3	3.92	d d	$J_{3,4}$	2.8	C-3	70.1
	H-4	4.00	b d	$J_{4,5}$	< 2	C-4	70.0
	H-5	4.12	b t	$\frac{1}{2}(J_{5,6} + J_{5,6'})$	5.0	C-5	71.0
	H-6,H-6′	3.80	d			C-6	61.9

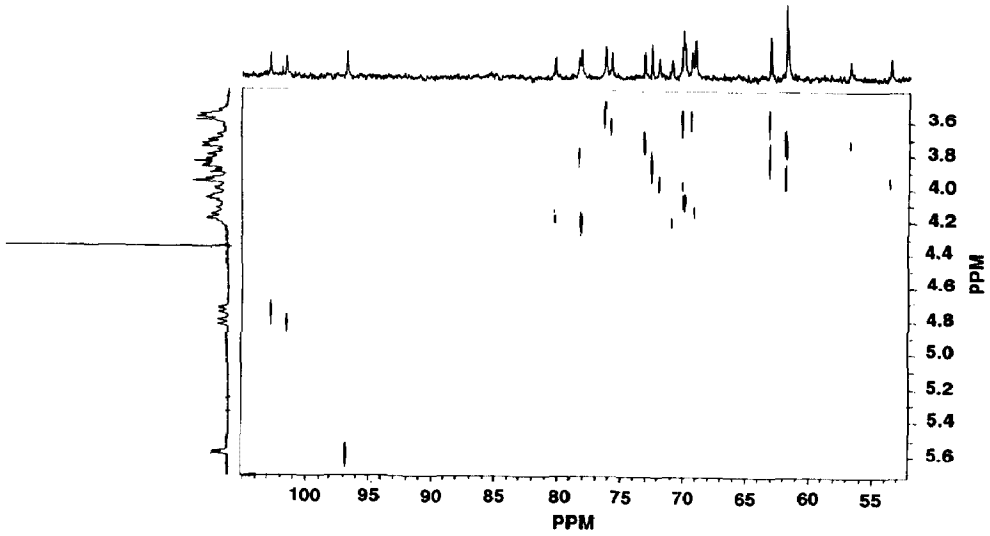

Fig. 4. 2D 300-MHz heteronuclear ^{13}C–^{1}H COSY spectrum.

The signals of the ^{1}H NMR spectrum were assigned (Table II using 1D HOHAHA[23] with excitation of the anomeric protons, 2D homonuclear COSY, and 1D NOE experiments in the differential mode[24]. Due to an overlap of the H-4 and H-5 signals of Neu5Ac, the coupling constants of its ring protons could not be determined. The signals of the ^{13}C NMR spectrum were assigned with 2D heteronuclear COSY (Fig. 4). The results are included in Table II.

The linkages and sequence of sugar residues in the repeating unit were established using a 500-MHz 2D ROESY[25] spectrum (Fig. 5) and 300-MHz 1D NOE experiments with pre-irradiation of anomeric as well as H-3*ax* and H-3*eq* protons of Neu5Ac (Fig. 6). Both methods revealed inter-residual contacts between H-1 of Glc*p*NAc and H-3 of Glc*p*, H-1 of Glc*p* with H-7 of NeuNAc, and H-1 of Gal*p* with H-2 of Glc*p*. Intra-residual proton interactions showed that Glc*p*NAc and Glc*p* were β and that Gal*p* was α. The α-anomeric configuration of Neu5Ac was evident from the gated decoupling NMR spectrum. These data, which are in full agreement with the results of the chemical studies, show that the O56 polysaccharide has the structure.

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{NAc-}(1 \rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1 \rightarrow 7)\text{-}\alpha\text{-Neu5Ac-}(2 \rightarrow$$
$$\begin{array}{c} 2 \\ \uparrow \\ 1 \\ \alpha\text{-D-Gal}p \end{array}$$

Table III shows additional inter-residual proton interactions between H-1 of Gal and H-1 of GlcNAc as well as between H-3*ax* of Neu5Ac and H-3 of glucosamine. Further, some of the chemical shifts for the ring carbons of Neu5Ac were unusual. Thus, the C-3 signal was at δ 37.4 instead of δ 40, and the C-4 signal was at δ 72.0 instead of δ 67.8–69.8 (ref. 20). This may be due to spatial

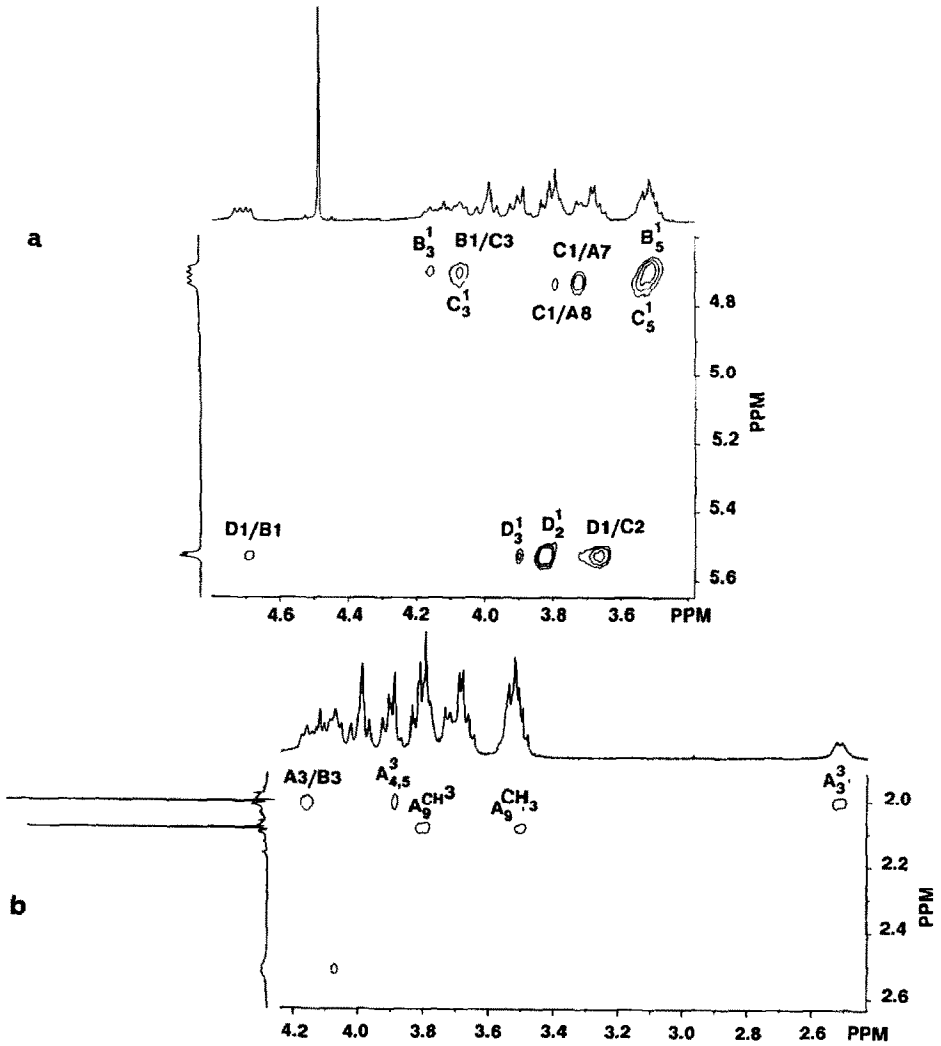

Fig. 5. 2D 500-MHz ROESY spectrum of the anomeric region (a) and the high-field region (b) of the O56 polysaccharidse. Numerals refer to the protons of the sugar residues specified by capital letters as in Table II. Symbols with a slash designate inter-residue interactions; e.g., B_3^1 corresponds to the interaction of H-1 with H-3 of residue B1, and B1/C3 to the interaction of H-1 of residue B with H-3 of residue C.

effects, such as, for example, the closeness of the acetamido group of GlcNAc and C-3 of Neu5Ac, and an apposition of the Gal residue to GlcNAc and Neu5Ac. Further studies of the origin of such effects are in progress.

EXPERIMENTAL

Bacteria and cultivation.—E. coli strain Su 3684-41 (O56:K^-:H^-, Freiburg collection number 2405) was grown at 37°C to the late logarithmic phase in a fermenter in 10-L batches of standard I broth (Merck).

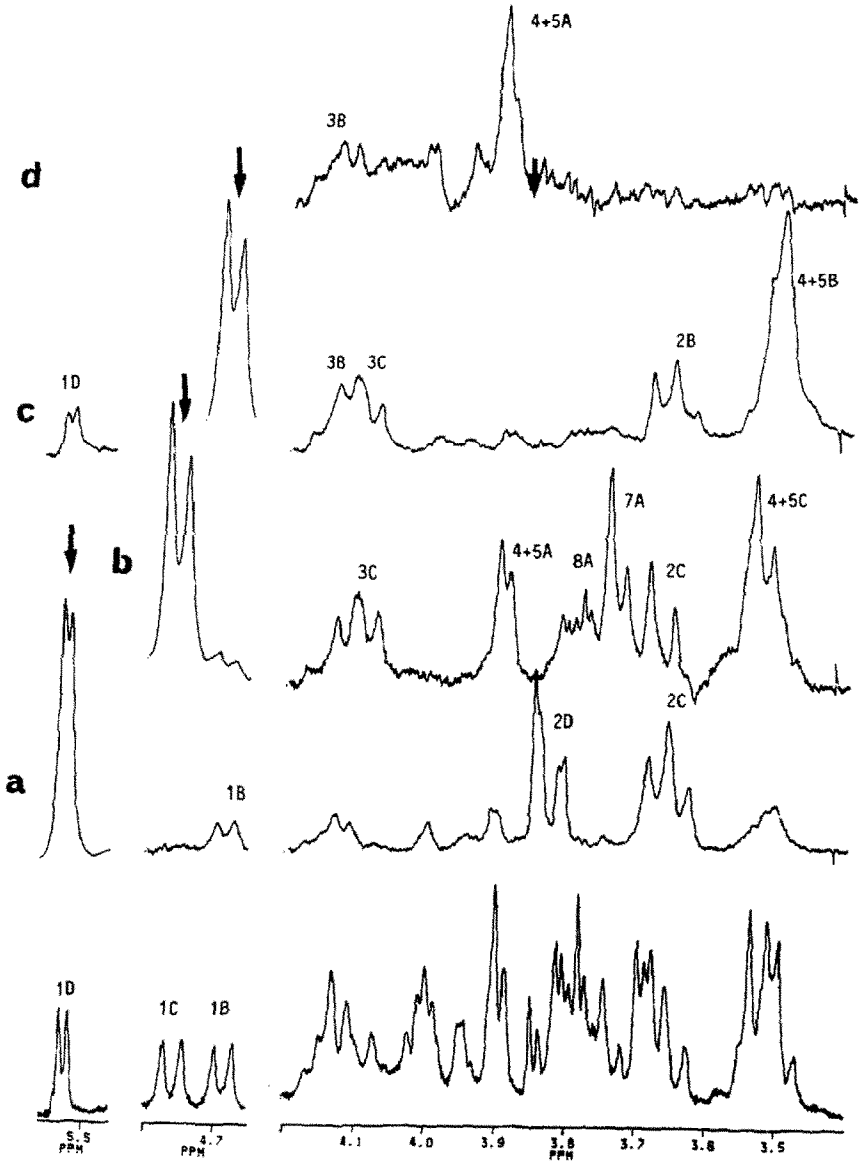

Fig. 6. 1D NOE experiments with pre-irradiation of the anomeric protons (a–c) and the H-3*ax* of Neu5Ac (d) of the O56 polysaccharide. Arrows indicate pre-irradiated protons. The signal of H-3*ax* is not shown. The designation is as in Fig. 5.

Isolation of the polysaccharide.—The bacteria were extracted with aqueous 45% phenol and the dialysed aqueous phase was subjected to ultracentrifugation[6]. From the supernatant solution, the O56 polysaccharide was obtained by fractional centrifugation with cetyltrimethylammonium bromide and the complex precipitated was converted into the sodium salt as described[6].

TABLE III

NOe data [a] for the O56 polysaccharide

NOE observed on		A, H-3*ax*	Pre-irradiated proton		C, H-1	D, H-1
residue	proton		A, H-3*eq*	B, H-1		
→ 7)-α-Neu5Ac-(2 →	H-3*ax*		+			
(A)	H-3*eq*	+				
	H-4 + H-5	+	+		+	
	H-7				+	
	H-8				+	
→ 3)-β-D-Glc*p*NAc-(1 →	H-1					
(B)	H-2			+		+
	H-3	+		+		
	H-4 + H-5			+		
→ 3)-β-D-Glc*p*-(1 →	H-2				+	+
(C) 2 ↑	H-3				+	
	H-4 + H-5				+	
α-D-Gal*p*-(1 →	H-1			+		+
(D)	H-2					

[a] The test was performed using standard Bruker software NOEMULT.

Methylation.—A modification[11] of the Hakomori procedure[10] was used and the methylated product was purified with a Sep Pak C_{18} cartridges[26]. A sample of the methylated material was hydrolysed with 90% formic acid and subsequently with 0.125 M H_2SO_4, neutralised with $Ba(OH)_2$, and reduced with $NaBD_4$. Another sample was subjected to methanolysis[12] with 0.5 M HCl in MeOH (24 h, 85°C) and, after addition of *tert*-butyl alcohol, evaporated to dryness under a stream of nitrogen[27]. Both samples were peracetylated and subjected to GLC–MS.

Periodate oxidation and Smith degradation.—These procedures have been described[15,28]. The trisaccharide obtained by Smith degradation was chromatographed on a column (2 × 100 cm) of Bio-Gel P-2 with water as eluant.

Analytical methods.—Glucose, galactose, and glucosamine were determined as their alditol acetates by GLC as well as with D-glucose oxidase, D-galactose oxidase, and the Elson–Morgan reaction, respectively. For the identification of Neu5Ac, the polysaccharide was hydrolysed, and the liberated neuraminic acid was re-*N*-acetylated with acetic anhydride—$NaHCO_3$ and reacted with Neu5Ac aldolase–lactic acid dehydrogenase–NADH[8]. GLC–MS was carried out with a Hewlett–Packard 5988A instrument, using a DB 5 capillary column (0.2 mm × 30 m) with He as carrier gas and a temperature program of 50 → 180°C at 70°C/min and then 180 → 250°C at 5°C/min. EI-mass spectra were obtained with an ionising energy of 70 eV and were compared with those of model alditol acetates[29] or methyl glycosides[12]. NMR spectra were recorded in D_2O with a Bruker WM 300 spectrometer at 300 MHz (^1H NMR) or at 75 MHz (^{13}C NMR) with acetone as internal standard (δ 2.225 for ^1H spectra; δ 31.45 for ^{13}C spectra). Standard Bruker software was used for 2D COSY, COSYRTC, two-step COSYRTC2, and

heteronuclear $^{13}C-^{1}H$ COSY (HXCORR). 1D NOE experiments were performed in the differential mode using the Bruker NOEMULT program. 1D HOHAHA spectra were obtained with variable mixing times during accumulation[23]. The DANTE pulse sequence[30] was used for selective excitation. For ROESY spectra, the Rance pulse sequence[31] was applied.

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