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Structures of the O chains from lipopolysaccharides of *Campylobacter jejuni* serotypes O:23 and O:36

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ABSTRACT

Lipopolysaccharides of *C. jejuni* serotypes O:23 and O:36 have been shown to contain structurally variable O polysaccharide chains with repeating units of four closely-related types:
→ 3)-β-D-Glc*p*NAc-(1 → 3)-α-D-Gal*p*-(1 → 2)-6d-α-D-*alt*-Hep*p*-(1 →,
→ 3)-β-D-Glc*p*NAc-(1 → 3)-α-D-Gal*p*-(1 → 2)-6d-3-Me-α-D-*alt*-Hep*p*-(1 →,
→ 3)-β-D-Glc*p*NAc-(1 → 3)-α-D-Gal*p*-(1 → 2)-D-*glycero*-α-D-*alt*-Hep*p*-(1 →, and
→ 3)-β-D-Glc*p*NAc-(1 → 3)-α-D-Gal*p*-(1 → 2)-3-Me-D-*glycero*-α-D-*alt*-Hep*p*-(1 →.
Structural methods included ^{1}H- and ^{13}C-NMR spectroscopy, methylation linkage analysis, fast atom bombardment mass spectrometry of methylated glycans, and selective fragmentations by the Smith degradation and *N*-deacetylation–nitrous acid deamination.

INTRODUCTION

Campylobacter jejuni is now recognized as one of the most frequent causes of enteritis in humans[1]. The organism occurs in some 42 serovarieties. These are distinguishable in a widely used serotyping system[2] based on the detection of thermostable lipopolysaccharide (LPS) somatic (O) antigens by means of a passive hemagglutination assay. Previous studies of the composition of *C. jejuni* LPS[3,4], especially those of Naess and Hofstad[3], showed that, for several strains, mild acid hydrolysis liberated 3-deoxy-D-*manno*-octulosonic acid (Kdo)-terminated oligosaccharides with sugar residues commonly encountered in the core region of LPS, namely, D-glucose, D-galactose, L-*glycero*-D-*manno*-heptose, and sometimes 2-acetamido-2-deoxy-D-glucose and/or 2-acetamido-2-deoxy-D-galactose. Preston and Penner[5], using electrophoretic separations (SDS-PAGE), examined a large number of serotype reference strains, and confirmed that all serotypes elaborated LPSs of low M_r detectable by silver staining[6]. A minority of serotypes also

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synthesized LPSs of high M_r, which were not detectable by silver staining. However, detection by immunoblotting with specific homologous antisera revealed the characteristic ladder-like pattern of LPSs of high M_r with extended O chains. In collaboration with Professor J.L. Penner, we have undertaken a detailed investigation of a number of LPSs of high M_r and low M_r in order to determine the molecular basis for their serological differentiation. Partial structures have been established[7] for the core oligosaccharides from LPSs of low M_r from two of the most commonly occurring serotypes O:1 and O:4, and from serotypes O:23 and O:36 which also elaborate high-M_r LPSs, and between which immunological cross-sections have been observed[5]. In these studies, the LPS was isolated by phenol–water extraction of bacterial cells[8], and ultracentrifugation of the aqueous layer yielded material as a gel from which core oligosaccharides of similar composition[7] but with different structures were obtained on cleavage of the Kdo–lipid A linkage. Although complete structures for these core oligosaccharides have yet to be established, it is already clear from results obtained to date that variations in the outer-core structures are sufficient to account for serotypical differences. Oligosaccharides liberated from O:23 and O:36 LPSs were fractionated and the complete core oligosaccharides from the two serotypes were indistinguishable, but clearly different from those of serotypes O:1 and O:4. Other oligosaccharide fractions from the two serotypes showed structural differences, but all were biosynthetically incomplete, and their structures seemed unlikely to account for serotypical differences. Similarities in the core regions of LPS structure provide a possible basis for cross-reactions between the two serotypes, independently of similarities or differences in O-chain structures. In the cases of serotypes O:23 and O:36, a search for longer chain polysaccharide released from the gel revealed no material with a composition indicative of the regularity of structure normally found in O-chain repeating units, the presence of which had been implied[5] by SDS-PAGE. However, examination of the supernatant aqueous layer from which low-M_r LPS had been separated showed high-M_r LPS to be present in admixture with relatively large quantities of RNA. We have recently described[7] the liberation of O polysaccharide from this water-soluble LPS by acetic acid cleavage of the Kdo–lipid A linkage, followed by purification by gel-permeation chromatography (GPC), and we now report structural studies of the O polysaccharides from serotypes O:23 and O:36.

RESULTS AND DISCUSSION

Practical difficulties in growing *C. jejuni*, a microaerophilic organism, on blood agar plates limit the quantity of biomass, and hence of LPS and O polysaccharide, that can be readily acquired. When examination of the first preparations of O glycans from serotypes O:23 and O:36 revealed the presence of heptose constituents of unusual configuration, it was soon apparent that further quantities of material would be required for completion of the structural analyses, including

TABLE I

^{13}C-NMR data (δ in ppm) for O polysaccharides from the LPSs of *C. jejuni* serotypes O:23 and O:36

Residue/Structural unit	Polysaccharide sample				
	O:23A	O:23B	O:23C	O:36A	O:36B
GlcNAc N-$COCH_3$	174.8	174.6	174.8	174.6	174.6
N-$COCH_3$	22.5	22.4	22.4	22.5	22.4
C-2	55.8	55.8	55.8	55.8	55.8
C-1	102.3	102.3	102.3	102.3	102.2
Gal C-1	96.8	96.7	96.8	96.8	96.7
Hep / 3-MeHep OCH_3		59.0	59.0		
C-1	99.0	99.1	99.2, 99.1	98.9 [a]	98.9, 98.8
C-6 (CH_2)	33.9		33.8		33.9

[a] An additional signal at δ 100.0 cannot be accounted for.

assignments of absolute configurations for the heptoses. When further quantities of O polysaccharides were made available, the heptoses were found to be different, although the NMR data, especially those from ^{13}C spectra (Table I), pointed to glycans of otherwise closely similar structures. We therefore present compositional and methylation linkage analyses for three preparations of the O:23 glycan and two preparations of the O:36 glycan. More detailed investigations are reported for those glycan samples obtained in the relatively larger amounts necessary for complete structural analyses.

Chemical composition of O chains and identities of the heptoses.—Compositional data for different O polysaccharide preparations from LPSs of *C. jejuni* serotypes O:23 and O:36 are given in Table II. For each glycan sample, three major constituents were identified by standard analyses as Gal, GlcNAc, and one or a mixture of two heptoses. Gal is also a constituent of core oligosaccharides of low-M_r LPSs from these serotypes[7], and presumably, but not previously proven, of core regions to which O chains may be attached. The formation also on hydrolysis of Glc, LDHep (L-*glycero*-D-*manno*-heptose), and GalNAc as minor components provided a qualitative indication that only a small proportion ($\sim$ 10%) of the Gal arose from core regions. Quantitative analyses of some heptoses were complicated by the formation on hydrolysis of relatively large proportions of 1,6- and 1,7-anhydroheptoses. Subsequent treatment of the hydrolysate with sodium borohydride (or deuteride) followed by acetylation therefore yielded both alditol acetates and anhydroheptose acetates. However, the formation of anhydrides provided a clue that the heptoses were sugars of unusual ring configuration.

Examination of the first sample of O glycan (O:23A) from serotype O:23 LPS showed that a 6-deoxyheptose was a major constituent. The formation of the corresponding alditol acetate from a mixture of methyl glycosides of 6-deoxy-D-*altro*-heptose (through the kindness of Professor P.J. Garegg, cf. ref. 9) showed this derivative to be indistinguishable (GLC–MS) from that from the LPS. In later experiments, the absolute configuration of the sugar was established as D through

TABLE II

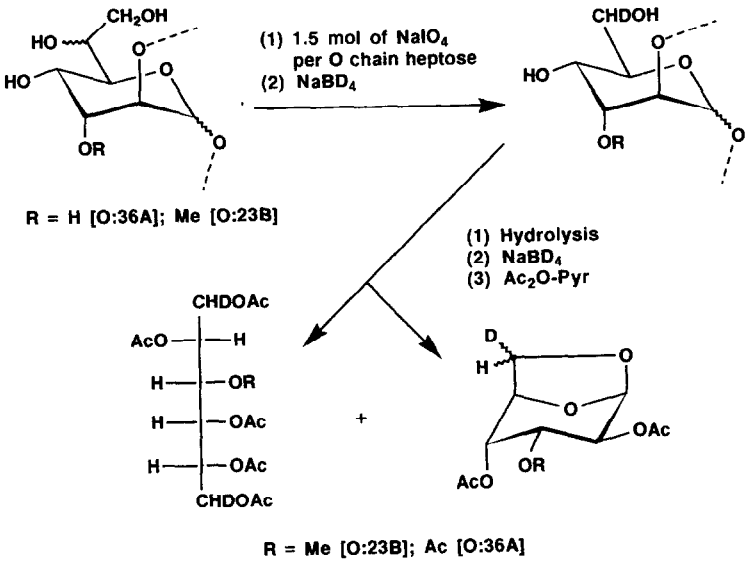
Sugar composition of O polysaccharides isolated from the LPSs of *C. jejuni* serotypes O:23 and O:36

Sugar [a]	Serotype and batch				
	O:23A	O:23B	O:23C	O:36A	O:36B
Gal	1.0	1.0	1.0	1.0	1.0
GlcNAc	0.9	0.9	0.9	1.5	1.5
6-dHep	0.8	0.1		0.1	0.8
3-Me-DD-Hep [b]		0.8	0.6		
DD-Hep [b]				1.2	0.5
3-Me-6-dHep			0.4		
Minor components					
Glc	0.1	0.02	0.1	0.2	tr [c]
GalNAc	0.05	tr	0.05	0.1	tr
LD-Hep	0.07	tr	0.05	0.1	tr

[a] Compositions are normalized with respect to galactose and are based on GLC of alditol acetates (and anhydroheptose acetates). 6-dHep = 6-deoxy-D-*altro*-heptose; DD-Hep = D-*glycero*-D-*altro*-heptose (elsewhere LD-Hep = L-*glycero*-D-*manno*-heptose from the core regions). [b] Ratios are inclusive of 1,6- and 1,7-anhydroheptoses. [c] Trace.

the formation of chiral glycosides and comparison with acetylated (*R*)- and (*S*)-2-butyl glycosides[10] formed from 6-deoxy-D-*altro*-heptose (6-dHep). The preparation of this sugar will be reported elsewhere in connection with the synthesis of oligosaccharides related to the O-chain repeating unit. Similar examination of the O glycan from the LPS of *C. jejuni* serotype O:36 showed that the first sample (O:36A) was closely related to that from serotype O:23. GLC–MS analysis of derivatives from the hydrolysate showed, in addition to alditol acetates from GlcNAc and Gal, a heptitol hepta-acetate and relatively large proportions of 1,6- and 1,7-anhydroheptose acetates, whose mass spectra were very similar to those reported for derivatives of D-*glycero*-D-*gulo*-heptose[11]. The ready formation of anhydrides suggested that the heptose (Hep) had an uncommon ring configuration, such as *altro*, *gulo*, or *ido*[12], the first-named being regarded as more likely in view of a possible biosynthetic relationship to the 6-dHep from the O:23A glycan.

In order to complete the structural studies, including configurational assignments for the Hep component of these glycans, further quantities of glycans were isolated, and those from both serotypes were found to differ from the previous samples in the nature of the heptose components. The second batch of O:23 LPS furnished O glycan (O:23B) in which the heptose component of the repeating trisaccharide unit was a 3-*O*-methylheptose, with only traces of 6-dHep being observed. The new sugar was characterized by GLC–MS of the derived alditol acetate which showed the relevant fragment ions (EIMS), together with two compounds whose molecular ions (from CIMS) indicated that they were *O*-acetyl-anhydroheptoses. In a search for larger quantities of LPS from the phenol–water extraction of cells from the same batch of serotype O:23, a second aqueous extraction of the phenol layer afforded a quantity of LPS from which O glycan



Scheme 1. Degradation by exocyclic cleavage of the O-chain heptose residues in high-M_r LPS from *Campylobacter jejuni* serotypes O:23 and O:36.

(O:23C) was liberated. Hydrolysis of this glycan gave inter alia another heptose in addition to 3-MeHep. GLC–MS of derivatives from the hydrolysate showed the new sugar to be a 6-deoxy-3-*O*-methylheptose. A further variation in the nature of heptose components was encountered when a second sample of O glycan (O:36B) from the LPS of serotype O:36 was isolated. Heptose components were both the aforementioned Hep and the 6-dHep first formed from the O:23A glycan.

Supporting evidence for the *altro* ring configuration of the Hep and 3-MeHep components of the O polysaccharides was obtained by subjecting the O:36A and O:23B glycans to (*a*) oxidation with limited quantities of periodate in order to cleave selectively the exocyclic diols and (*b*) reduction with sodium borodeuteride (Scheme 1). The products in the hydrolysates from the modified glycans were treated with sodium borodeuteride followed by acetylation. GLC–MS showed the products formed from the heptose residues to be 1,6-[$^2H_2$]-labelled hexitol acetates and 6-[^{2}H]-labelled 1,6-anhydrohexopyranose acetates which were indistinguishable, other than in isotopic content, from the corresponding derivatives formed from methyl α-D-altropyranoside and methyl 3-*O*-methyl-α-D-altropyranoside. In attempts to define the absolute configurations of sugars in the hydrolysates from the modified glycans by conversion into acetates of chiral 2-butyl glycosides, the results showed the Gal and GlcNAc to be D enantiomers, but 1,6-anhydrides were the only recognized products from the altrose components.

The relative configuration at C-6 of Hep was established by GLC comparison of the heptitol hepta-acetate with the corresponding derivative of D-*glycero*-D-*altro*-heptitol (kindly provided by Dr. M.B. Perry, National Research Council Canada, Ottawa), which compound is readily differentiated from the L-*glycero*-D-*altro*-

heptitol derivative[13]. In the light of the close structural relationship between the various heptoses, we may provisionally assign the heptose the DD rather than the LL configuration. Confirmation of the correctness of this conclusion will be sought in connection with syntheses directed to the preparation of oligosaccharides related to the O-chain repeating units.

The general conclusions based on compositional analysis of the different O-glycan samples were supported by the ^{13}C-NMR spectra (Table I). The spectra confirmed the presence of *N*-acetyl groups in each glycan, and of *O*-methyl and 6-deoxy methylene groups in those containing modified heptose residues. All glycans from serotypes O:23 and O:36 showed three superimposable anomeric carbon resonances, which were assigned individually later to β-GlcNAc (δ ~ 102.3), α-Gal (δ ~ 96.7), and α-Hep (δ ~ 99.0). For the O:23B glycan, anomeric proton resonances at δ 4.99 ($J_{1,2}$ 3.3 Hz), 4.91 (bs), and 4.71 ($J_{1,2}$ 6.8 Hz), and anomeric carbon resonances at δ_C 96.7 ($J_{C,H}$ 174 Hz), 99.1 ($J_{C,H}$ 169 Hz), and 102.3 ($J_{C,H}$ 165 Hz) were assigned[14] to α-Gal, α-3-MeHep, and β-GlcNAc residues, respectively. These observations supported the indications of trisaccharide repeating units from compositional analysis, and further suggested that the variable heptose residues in different chains were stereochemically related and existed in essentially identical environments.

Linkage analyses by methylation.—The results of methylation studies of the various O oligosaccharide samples derived from LPSs of serotypes O:23 and O:36 are shown in Table III. For each glycan sample, three main methylated sugar derivatives were formed from the O-chain regions. The 3-linked Gal*p* and 3-linked Glc*p*NAc residues were the same in all glycans. For the O:36B glycan, the heptose contribution was shared between those formed from Hep and 6-dHep. It is noteworthy that all the heptose residues were 2-linked and that the same derivative was formed from Hep and 3-MeHep, and correspondingly from 6-dHep and 3-Me-6-dHep. These observations emphasize further the close structural and stereochemical relationship between the various heptose components.

Among the minor components in the methylation analysis, small proportions of non-reducing Gal and GlcNAc end groups could represent termini of O chains. In four instances (O:23 A, B, and C, and O:36B), identification of sugar derivatives showed the residues to include terminal and 4-linked Glc, 3,4-branched Gal, 2-linked L-*glycero*-D-*manno*-heptose, and terminal and 4-linked GalNAc. Since GalNAc is known to be terminal in the complete oligosaccharide core, 4-linked GalNAc could represent the point of attachment of the O chain to the core. The 3,4-branched Gal residue is consistent with the attachment of a NeuAc residue by a (2 → 3) linkage to a 4-linked Gal, as in the complete oligosaccharide core. Although no direct evidence for terminal NeuAc residues was obtained from the linkage analysis, the FAB mass spectra showed characteristic ions at *m/z* 376 and 344 for terminal NeuAc residues.

The relative proportions of the three major sugar constituents for each glycan provided further evidence for trisaccharide repeating units, although some of the

TABLE III

Methylation analysis of O polysaccharides from the LPSs of *C. jejuni* serotypes O:23 and O:36

Methylated sugar [a,b] (and linkage type)	Serotype and batch				
	O:23A	O:23B	O:23C	O:36A	O:36B
2,4,6-Me_3Gal (-3 Gal 1-)	1.0	1.0	1.0	1.0	1.0
4,6-Me_2N–MeGlcNAc (-3 GlcNAc 1-)	0.6	0.7	0.7	1.1	0.6
3,4,7-$Me_3$6-dHep{(-2,6-dHep 1-) (-2,3-Me-6-dHep 1-)}	1.0	tr [c]	0.4	tr	1.4
3,4,6,7-Me_4Hep{(-2 Hep 1-) (-2,3-MeHep 1-)}		0.7	0.6	1.2	0.7

[a] Compositions are normalized with respect to 2,4,6-Me_3Gal based on GLC of partially methylated alditol acetates. [b] Most of the following methylated sugars were detected as minor components from each of the methylated glycans, in amounts ~ 0.1 with respect to 2,4,6-Me_3Gal, and their identities were confirmed by GLC–MS of partially methylated alditol acetates: 2,6-Me_2Gal (-3,4 Gal 1-), 3,4,6,7-Me_4LDHep (-2 LDHep 1-), 3,4,6-Me_3N–MeGlcNAc (GlcNAc 1-), 3,4,6-Me_3N–MeGalNAc (GalNAc 1-), 3,6-Me_3N–MeGalNAc (-4 GalNAc 1-). [c] Trace.

exact quantitation was less than convincing. Unambiguous evidence for the trisaccharide repeating units came from the FAB mass spectra, especially from series of glycosyloxonium ions. Fig. 1 shows the mass spectrum up to ~ 3,000 amu for the methylated O glycan from serotype O:23B, and Table IV summarizes the observed glycosyloxonium ions from each of the methylated O glycans. The mass spectra also contained many double-cleavage ions[15], e.g., those at m/z 698, 943, 1147, 1395, 1640, 2092, etc. in Fig. 1 and comparable ions for other O glycans, but these and some presently unexplained ions are omitted from Table IV. The most significant glycosyloxonium ions were at m/z 260 (terminal HexNAc), 464 (HexNAc, Hex), followed by those with successive increments of 218 (6-dHep) or 248 (Hep), 245 (HexNAc), and 204 amu (Hex). For O glycans containing both Hep and 6-dHep residues (or their methyl ethers), ions were observed for both chains containing homogeneous multiple repeating units with the same type of Hep residue. No conclusions could be drawn concerning the possible occurrence of chains with heterogeneous repeating units. In the light of observations elsewhere of preferential cleavage in methylated oligo- and poly-saccharides at HexNAc residues[15], it was tempting to postulate an initiating non-reducing Hex–HexNAc unit followed by repeating (Hep–Hex–HexNAc) sequences. However, the MS data for terminal HexNAc together with clearcut conclusions from degradative studies provided conclusive evidence for a (HexNAc–Hex) sequence within the repeating units. This apparently contradictory conclusion may be explained by a rapid secondary fragmentation occurring by β-elimination with loss of 3-*O*-glycosyl substituents from all of the preferentially generated GlcNAc cations, as evidenced by the high relative abundance of the unsaturated secondary ion at m/z 228 formed from each such cation. Evidence obtained thus far could be interpreted in terms of O-glycan structures in which trisaccharide repeating units of four related

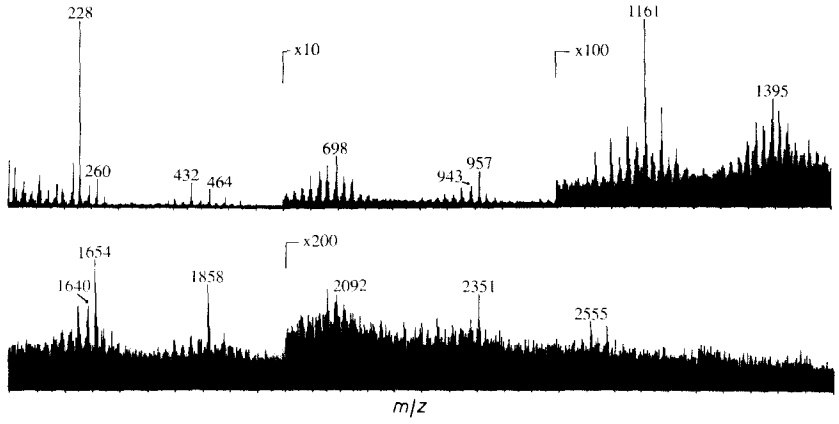


Fig. 1. Positive-ion FABMS spectrum of permethylated O glycan from *C. jejuni* serotype O:23B showing fragments up to ~ 3000 amu.

types may be present. Specific degradations, described below, followed by chemical and spectroscopic (NMR and MS) characterization of the resulting oligosaccharides led to formulation of structures **1** to **4** for the trisaccharide units.

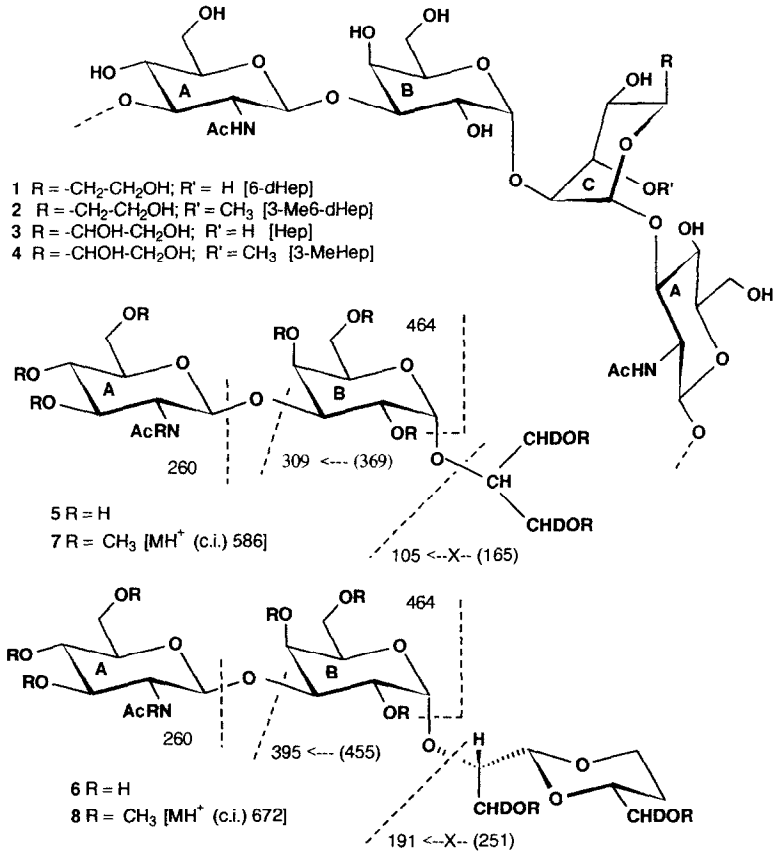
TABLE IV

FABMS data for methylated *C. jejuni* glycans

Fragment ions (m/z) from methylated glycans					Composition of glycosyloxonium ions
O:23A	O:23B	O:23C	O:36A	O:36B	
260	260	260	260	260	HexNAc
464	464	464	464	464	HexNAc Hex
682		682		682	HexNAc Hex 6-dHep
	712	712	712	712	HexNAc Hex Hep
927		927		927	$HexNAc_2$ Hex 6-dHep
	957	957	957	957	$HexNAc_2$ Hex Hep
1131		1131		1131	$HexNAc_2$ Hex_2 6-dHep
	1161	1161	1161	1161	$HexNAc_2$ Hex_2 Hep
1349		1349		1349	$HexNAc_2$ Hex_2 6-$dHep_2$
	1409	1409	1409		$HexNAc_2$ Hex_2 Hep_2
1594		1594		1594	$HexNAc_3$ Hex_2 6-$dHep_2$
	1654	1654		1654	$HexNAc_3$ Hex_2 Hep_2
1798		1798		1798	$HexNAc_3$ Hex_3 6-$dHep_2$
	1858	1858	1858		$HexNAc_3$ Hex_3 Hep_2
2016		2016			$HexNAc_3$ Hex_3 6-$dHep_3$
		2106			$HexNAc_3$ Hex_3 Hep_3
		2261			$HexNAc_4$ Hex_3 6-$dHep_3$
	2351	2351			$HexNAc_4$ Hex_3 Hep_3
2465		2465		2465	$HexNAc_4$ Hex_4 6-$dHep_3$
	2555	2555			$HexNAc_4$ Hex_4 Hep_3
3132					$HexNAc_5$ Hex_5 6-$dHep_4$

Smith degradation.—Smith degradation[16] was carried out on the O:36B glycan in which both Hep and 6-dHep residues would be expected to undergo endocyclic oxidation. GPC of the product from the oxidation–reduction–mild hydrolysis sequence furnished a fraction eluting in the di- to tri-saccharide range. Methylation linkage analysis showed the presence of non-reducing GlcNAc and 3-linked Gal residues. The ^{1}H-NMR spectrum showed anomeric proton signals at δ 4.75 ($J_{1,2}$ 8.3 Hz) and δ 5.12 and 5.15 ($J_{1,2}$ 3.8 Hz). The combined intensities of the latter signals pointed to the presence of two related compounds with terminal β-D-Glc*p*NAc and 3-linked α-D-Gal*p* residues bearing different residual aglyconic fragments arising from heptose residues. GLC–MS of the corresponding methylated derivatives in both CI and EI modes confirmed the presence of two such compounds. Proposed structures (**5** and **6**) are shown in Scheme 2 together with those of the methylated derivatives (**7** and **8**) and their more significant mass-spectral ions. Structure **5** is that of the expected Smith-degradation product in which the terminal glycerol unit could arise from 2-linked Hep or 6-dHep residues. Structure **6**, as a 1,3-dioxane, is postulated to arise specifically from the cleaved 6-dHep residue. An additional resonance in the ^{1}H-NMR spectrum at δ 5.08 (J 3.9 Hz), which may be assigned to an acetalic proton, supports the presence of a cyclic acetal unit at the reducing terminus. It is proposed that during the mild acid treatment step, following periodate oxidation and reduction, the acyclic glyceraldehyde moiety (from C-1,2,3) undergoes intramolecular transacetalation rather than hydrolysis.

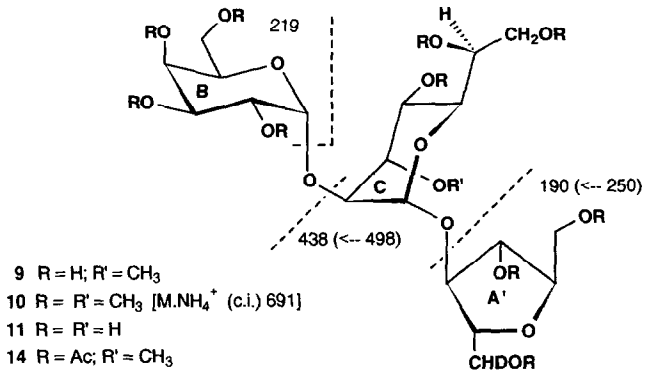
N-*Deacetylation and deamination*.—*N*-Deacetylation of the O:23B glycan with sodium hydroxide in dimethyl sulfoxide was followed sequentially by treatment of the product with nitrous acid and reduction with sodium borodeuteride. GPC afforded a fraction eluting in the trisaccharide range, the NMR data for which showed the presence of two anomeric centres, δ_H 5.03 (d, $J_{1,2}$ 3.8 Hz, α-Gal) and 4.97 (bs, Hep), and δ_C 99.1 (Hep) and 97.0 (Gal), and structure **9** is proposed. The fraction was methylated and linkage analysis showed derivatives of 2,3,4,6-Me_4Gal, 3,4,6,7-Me_4Hep, and 1,4,6-Me_3-anhydromannitol. GLC–MS in both EI and CI modes confirmed the presence of one major component whose structure (**10**), with important mass-spectral ions, is shown in Scheme 3. Brief comment may be made on the presence of three minor products from the deamination sequence, whose structures may be inferred solely on the basis of the mass-spectral data for their methylated derivatives. A second trisaccharide component showed mass-spectral ions pointing to the presence of a 6-deoxyheptose residue, and its retention time was identical to that observed for such a compound (**13**) obtained from the O:36B glycan. A disaccharide derivative of a hexosylheptitol is, presumably, a degradation product of the type observed on many occasions when 2,5-anhydromannose-terminated oligosaccharides (in this instance the main trisaccharide) are treated with sodium borohydride, and then undergo base-catalysed β-elimination before reduction of the reducing termini[17] is complete. The mass spectrum of another minor component showed it to be a hexosylanhydrohexitol. The possibility that this



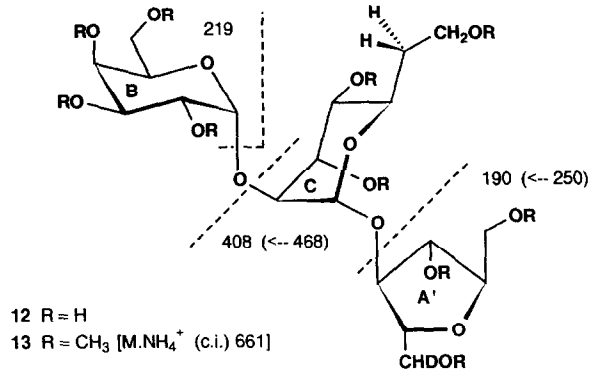
Scheme 2. Proposed structures are shown for compounds **5** and **6** formed during the Smith degradation of repeating units **1** and **3** of O-antigen chains in LPS from *Campylobacter jejuni* serotype O:36B. The salient features of the mass-spectral fragmentations are shown for the corresponding methylated derivatives **7** and **8**, with the convention that (369)309, or (369)309, indicates that an ion of the J_1 series is, or is not, observed.

product may have arisen from the point of attachment of the O chain to the core region is discussed later.

The O:36B glycan with repeating units **1** and **3**, in which 6-dHep and Hep components were present in the approximate proportions of 2:1, was subjected to the *N*-deacetylation–nitrous acid deamination sequence, and furnished, as major products, a mixture of two trisaccharides (**11** and **12**) in the corresponding proportions. The ^{1}H-NMR spectrum showed signals for only two anomeric protons at δ 5.03 (d, $J_{1,2}$ 3.7 Hz) and 4.97 (bs) in the mixture of compounds, but the ^{13}C-NMR spectrum showed two sets of anomeric resonances, at δ_C 98.5 (6-dHep) and 96.6 (Gal) for the more abundant and δ_C 98.9 (Hep) and 97.0 (Gal) for the less abundant components. Linkage analysis confirmed the formation of the same sugars as those from methylated trisaccharide **10**, but now originating from the



9 R = H; R' = CH_3
10 R = R' = CH_3 [M.NH_4^+ (c.i.) 691]
11 R = R' = H
14 R = Ac; R' = CH_3



12 R = H
13 R = CH_3 [M.NH_4^+ (c.i.) 661]

Scheme 3. Proposed structures are shown for (*a*) compound **9** formed during the *N*-deacetylation–deamination degradation of repeating units **4** of O-antigen chains in the LPS from *Campylobacter jejuni* serotype O:23B; and (*b*) compounds **11** and **12** formed similarly from O-antigen chains from *C. jejuni* serotype O:36B. The salient features of the mass-spectral fragmentations are shown for the corresponding methylated derivatives **10** and **13**.

2-linked Hep residues of **11**, together with those formed from the methylated derivative **13** of deoxytrisaccharide **12**. The mixture of derivatives obtained from methylation of the degradation products from the O:36B glycan was examined as above by GLC–MS in both CI and EI modes. The less abundant of the two main components was identical to that from the O:23B glycan and the MS of the more abundant component was fully in accord with that of the corresponding trisaccharide **13** containing the 6-dHep residue. Three minor components were present, hexosylheptitol and hexosyl-6-deoxyheptitol derivatives as degradation products from the two trisaccharides, and a third component identical to that of the hexosyl-anhydromannitol derivative observed from the O:23B glycan.

Anomeric configuration of heptose residues.—^{13}C-NMR data for the O glycans and ^{13}C- and ^{1}H-NMR data for the mixture of trisaccharides **11** and **12** suggest that the Hep and 6-dHep residues have α-D-glycosidic configurations. However, the chemical shift data for α- and β-altropyranosides[18] are not sufficiently distinc-

tive to be predictive of anomeric configurations in the absence of independent evidence. A further possible complication concerns the ring conformation of altropyranosides, given their propensity to form 1,6-anhydroaltropyranoses on hydrolysis. For high-field ^{1}H-NMR examination, trisaccharide **9**, formed on *N*-deacetylation–deamination of the O:23B glycan, the simplest derivative isolated with a single type of Hep residue, was converted into the *O*-acetyl derivative **14** to aid proton assignments. The signals for anomeric protons (α-Gal at δ 5.23, $J_{1,2}$ 3.5 Hz, α-Hep at δ 4.97, bs) and methylene carbons were assigned in an inverse ^{1}H–^{13}C heteronuclear multiple-quantum correlation (HMQC) experiment. Other proton signals were assigned from two-dimensional phase-sensitive double-quantum filtered (DQF-COSY) and total correlation spectroscopy (TOCSY) experiments (Table V). Coupling constants for the assigned proton resonances were clearly indicative of a $^4C_1$ conformation for the 3-*O*-methyl-*altro*-heptose. In a rotating frame nuclear Overhauser enhancement spectroscopy (ROESY) experiment, the only strong NOE intra-residue interactions were those of H-1 with H-2, and H-2 and H-3 with OMe; none were detected between H-1 and H-5 or OMe-3. A 2D-NOE (NOESY) spectrum of methyl 4,6-*O*-benzylidene-3-*O*-methyl-α-D-altropyranoside[19] showed similar intra-residue interactions in validation of the anomeric configuration assignment for the 3-*O*-methyl-D-*glycero*-α-D-*altro*-heptopyranose residues in the O:23B glycan. With the close similarities between the O glycans, it is reasonable to assign the α-*altro* configuration to the heptose variants in other trisaccharide repeating units.

Structural conclusions.—The O polysaccharides of *C. jejuni* serotypes O:23 and O:36 were chosen for detailed study to determine whether similarities in LPS structure could account for their serological cross-reactions. Our results show that the O-glycan chains from the LPSs of *C. jejuni* serotypes O:23 and O:36 contain repeating trisaccharide units with closely related structures, –HexNAc–Hex–Hep–, in which the only variable feature is the modification of otherwise similar 2-linked heptopyranose residues with the same unusual *altro* configuration. Only one of the four heptose variants, whose absolute configuration has been established unam-

TABLE V

First-order ^{1}H-chemical shifts (δ) and vicinal coupling constants (Hz) for **14** at 500.1 MHz [a]

Position	Residue		
	3-Me-Hep	Gal	Anhydromannitol
1	4.98 (s)	5.23 (3.5)	4.26
2	3.88 (4.0)	5.16 (10.8)	4.20 (4.0)
3	3.75 (3.7)	5.33 (3.2)	4.15
4	5.09 (10.0)	5.53 (2.8)	5.41
5	4.12	4.63 (5.8)	4.29
6	5.36	4.12	4.43
7	4.42 (7.0, 12.0)		
OCH_3	3.42 (s)		

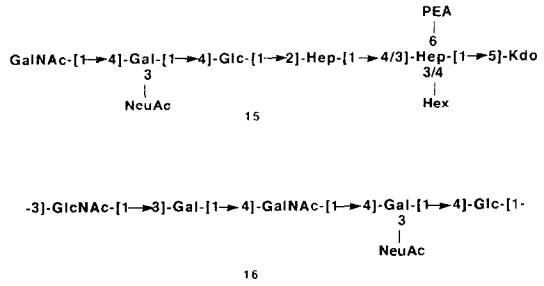
[a] Coupling constants are not quoted for unresolved multiplets.

biguously as 6-deoxy-D-*altro*-heptose, has been encountered previously as a polysaccharide constituent. This sugar occurs in capsular polysaccharides from *Eubacterium saburreum* where its 2-linked residues are present in the α-furanosyl form[9]. As far as we are aware, the other heptoses have not been reported previously. The absolute configurations of these heptoses of the same relative *altro* ring configuration remain to be confirmed. In the light of chemical and spectroscopic evidence for similarities in O-glycan structure, and of the occurrence of the sugars in different batches of LPS from immunologically cross-reacting serovariants, it is highly probable that the other heptoses may be assigned correctly as 6-deoxy-3-*O*-methyl-D-*altro*-heptose, D-*glycero*-D-*altro*-heptose, and 3-*O*-methyl-D-*glycero*-D-*altro*-heptose. The FABMS data in conjunction with those for linkage analysis of the methylated glycans provide convincing evidence for regular trisaccharide repeating units for those samples in which one heptose variant is dominant. For those samples in which heptose and 6-deoxyheptose derivatives are present in comparable proportions, present mass-spectral data neither support nor exclude the possibility that two heptose variants might occur in the same chain.

Our results to date fail to provide a clear answer as to whether the two serotypes, O:23 and O:36, are really different or whether differences in LPS structure merely reflect variations that are independent of supposed serotype. In unpublished results, a number of individual colonies of each serotype were cultivated, but samples of the isolated LPSs showed no specific correlation of heptose variant with particular colonies.

Finally, it is relevant to comment on the biosynthetic implications of our observations. On the assumption that all heptoses have the D-*altro* ring configuration, it is reasonable to speculate that D-*glycero*-D-*altro*-heptose is the parent sugar of the group. Nothing is known of the origin of this sugar, but it may be noted that it is a C-3 epimer of D-*glycero*-D-*manno*-heptose which itself undergoes epimerization at C-6 to give L-*glycero*-D-*manno*-heptose, a constituent of the core region of the majority of LPSs from Gram-negative bacteria, including those from *C. jejuni* now under investigation.

O-Methyl sugars are quite common constituents of polysaccharides but, in the absence of evidence for glycosyl esters of nucleotides bearing *O*-methyl-glycosyl residues, it is most likely that the modification is a post-polymerization event. In contrast, deoxygenation in the biosynthesis of the 6-deoxyhexoses, L-rhamnose and L-fucose, is a well understood process that involves a series of sugar nucleotide transformations[20]. Residues of these sugars occur in polysaccharides in quite different locations from those of the glycose units of the precursor sugar nucleotides. Furthermore, the functional changes occurring in the biosyntheses of these 6-deoxyhexoses are accompanied by configurational changes at several chiral centres. Unless the configurationally related heptoses are synthesized by unrelated pathways, 6-deoxy-D-*altro*-heptose is likely to be formed from D-*glycero*-D-*altro*-heptose in a series of reactions mechanistically similar to those in L-rhamnose and L-fucose biosynthesis but, in contrast, without change of configuration within the



Scheme 4. The proposed structure **16** is shown for connection of the inner disaccharide segment of the O chains to the terminal GalNAc residue of core oligosaccharide **15** of LPSs from *Campylobacter jejuni* serotypes O:23 and O:36.

pyranose ring. In the absence of direct experimental evidence, the apparent facility with which the heptose residues are varied might argue for both *O*-methylation and deoxygenation occurring within an assembled glycan chain rather than at the glycosyl nucleotide level. If these modifications were to take place in the glycosyl donors before glycan assembly occurs, biosynthesis would presumably require separate glycosyl transferases for the different heptose residues, and these residues when incorporated might, in their turn, exhibit selectivity and require different glycosyl transferases also in their roles as glycosyl acceptors.

We comment finally on the minor features revealed in the methylation linkage analyses and in the deamination reactions. Evidence has been presented elsewhere for the same core region in LPSs of low M_r from LPSs of serotypes O:23 and O:36 as shown[7] in structure **15**. Methylation linkage analyses for the O glycans from LPSs of serotypes O:23A, B, and C, and O:36B pointed to the presence, as minor components, of several of the characteristic components from the core region (but not all were detected in the case of O:36A). GalNAc residues as end groups are consistent with the presence of residual core oligosaccharides from LPSs of low M_r in the O-glycan preparations. On the other hand, 4-linked GalNAc residues could represent the point of connection of O chains to the terminal unit of the core region. An observation in support of such a proposal comes from the formation of an incompletely defined hexosyl-anhydrohexitol as a minor product from the *N*-deacetylation–deamination sequence of reactions. The sequence of residues implied here is inconsistent with this disaccharide fragment arising from repetitive trisaccharide units in the O chain. We therefore propose the partial structure **16** for the connection of the O antigen chain to the core region as shown in Scheme 4.

EXPERIMENTAL

Materials.—*Campylobacter jejuni* serotypes O:23 and O:36 were grown in the laboratory of Professor J.L. Penner[5]. Lipopolysaccharides were isolated by phenol–water[8] extraction and those of high M_r containing extended O chains were

located in the non-sedimenting, aqueous supernatant liquid together with large quantities of RNA. O-Specific polysaccharides were obtained after mild acid hydrolysis of the solid residue from lyophilization of this aqueous solution (1% CH_3CO_2H, 100°, 1h) and purified by GPC on a Bio-Gel P-6 column as described elsewhere [7].

Analytical procedures.—GLC was carried out on a Hewlett–Packard model 5890A chromatography. Separations were carried out using capillary columns with the following programs: *A*, DB-23 (15 m × 0.5 mm or 30 m × 0.25 mm) isothermally at 220°; *B*, DB-23 (30 m × 0.35 mm) isothermally at 190°; *C*, DB-17 (15 m × 0.25 mm) isothermally at 190°; *D*, DB-23 (30 m × 0.25 mm), 200° (10 min), 200 → 230° at 2°/min, 230° (10 min); *E*, DB-5 (15 m × 0.25 mm), 180 → 280° at 5°/min (20 min). GLC–mass spectrometry (GLC–MS) was conducted on a VG Micromass 16F instrument with electron impact ionization at 70 eV, equipped with a Pye Unicam chromatograph series 204, and using capillary columns as above.

Fast atom bombardment mass spectra (FABMS) were acquired using a VG-Analytical ZAB-SE instrument equipped with an Ion Tech Saddle field gun. Permethylated samples (1–2 μL) in MeOH were loaded onto the target with a matrix (1–2 μL) of thioglycerol or 3:1 thioglycerol–glycerol. The samples were bombarded with Xe atoms (1.2 mA anode current, 8 keV anode potential), the spectra were recorded with a VG 11-250 data system under the multi-channel analyzing mode, and 2–3 scans were accumulated. Resolution was set at 1500 to 2000 (10% valley definition) and CsI was used as calibrant. For high-mass analyses, resolution was decreased to about 600 to increase the sensitivity. All ions are reported as the nominal mass of the ^{12}C-containing component. The GLC–MS (EI and CI) of the products from Smith degradation and *N*-deacetylation–deamination experiments were also performed using the VG-Analytical ZAB-SE spectrometer. The EI conditions were 70 eV; trap current, 100 μA; source temperature, 200°; and scanning from *m/z* 800 to 60. The CI conditions were 100 eV; emission current, 1 mA; source temperature, 180°; and ammonia as reagent gas. GLC conditions were those cited above.

The ^1H- and ^{13}C-NMR spectra were recorded with a Bruker AM 300 spectrometer. The oligosaccharide samples were exchanged (×3) with D_2O (99.8%), lyophilized, and dissolved in D_2O (99.9%). The ^1H-NMR spectra were recorded using a 5-mm probe at 25° and/or 40°, and chemical shifts were measured relative to internal acetone (δ 2.17). The ^{13}C-NMR spectra were broad-band decoupled and recorded at 25° using a 5-mm probe, and chemical shifts were measured relative to internal acetone (δ 30.4). NMR spectra at high field were recorded with a Bruker AMX 500 spectrometer for a solution of **14** in $CDCl_3$ and measured relative to Me_4Si. All experiments were performed with standard Bruker software.

Glycose analyses were performed on alditol and anhydroglycose acetate derivatives[21]. *O*-Polysaccharide samples (100–200 μg) in a 1-mL "Reactivial" were hydrolysed with 2 M trifluoroacetic acid (0.5 mL) at 100° for 6 h. Excess of acid was evaporated in a stream of air and the residue was treated with sodium

borodeuteride for 2–3 h. Excess of hydride was destroyed by the addition of acetic acid, the solution was concentrated to dryness, and MeOH containing 5% of acetic acid was evaporated repeatedly from the residue. The alditol mixture (with anhydroglycoses if present) with residual sodium acetate was acetylated with acetic anhydride (0.5 mL) at 100° for 1 h. Ethanol was added to the solution which was concentrated to dryness for analysis by GLC or GLC–MS using program *A*. Methylation analyses were performed on 0.3–1.0 mg samples, using (*a*) the Hakomori method[22] with the methylated product recovered by reverse-phase chromatography on a C-18 cartridge, or (*b*) the method of Ciucanu and Kerek[23]. Samples of the methylated glycan were used for positive-ion FABMS and, after conversion into partially methylated alditol acetates by hydrolysis, reduction, and acetylation as described above, for linkage analysis by GLC and GLC–MS using programs *B* and *C*.

Where enantiomerically defined sugars were available as standards, absolute configurations were determined by conversion of glycan hydrolysates into chiral glycosides[10] by heating in (*R*)- or (*S*)-2-butanol containing 10% of CF_3CO_2H for 6 h at 105°, followed by acetylation and GLC analysis using program *D*.

Chemical modification and degradations.—Controlled periodate oxidation of samples (1 mg) of O glycan from O:23B and O:36A was carried out by treatment with 1.5 equiv of sodium metaperiodate per trisaccharide unit in 0.1 M sodium acetate buffer (0.9 mL, pH 4) at 4° for 16 h. The extent of reaction was monitored[24] by change in absorbance at 222 nm. Excess of periodate was destroyed by the addition of ethanediol and the solution was desalted by elution with water though a column (1.2 × 110 cm) of Bio-Gel P6. Oxidized glycan was reduced with sodium borodeuteride, excess of hydride was destroyed, and borate was removed as described previously, and a solution of the modified glycan in water was desalted by passage through a column of Bio-Gel P-6. The hydrolysate (2 M CF_3CO_2H at 100° for 6 h) was divided into two portions for conversion into (*a*) alditol and anhydroglycose acetates, and (*b*) chiral butyl glycoside acetates for GLC and GLC–MS analysis.

Smith degradation of the O:36B polysaccharide was carried out under the conditions reported by Pritchard et al.[25]. Glycan (10 mg) was treated with 40 mM sodium metaperiodate in 0.1 M sodium acetate buffer (2 mL, pH 4) at 4° for 72 h with reaction monitored by change in absorbance at 222 nm. The product was purified and then treated with sodium borodeuteride and isolated as described above. The reduced glycan was hydrolysed in 1 M CF_3CO_2H (4 mL) for 1 h at 45°. After concentration to remove acid, the hydrolysate was treated again with sodium borodeuteride. The reaction mixture was worked-up in the usual way and GPC on a column of Bio-Gel P-6 gave two fractions detected by the phenol–H_2SO_4 assay[26]. The fraction (2.7 mg) eluting in the mono- to tri-saccharide range was examined by NMR spectroscopy and the remaining material was converted into permethylated derivatives for direct GLC–MS examination in the EI and CI modes using program *E*, and for linkage analysis of the cleavage products as partially methylated alditol acetates.

N-Deacetylation was carried out as described by Kenne and Lindberg[27], and the product was then deaminated. Dimethyl sulfoxide (4.5 mL) was added to a solution of O:23B glycan (19 mg) in water (0.9 mL) containing NaOH (440 mg) and benzenethiol (100 mg), and the mixture was stirred for 21 h at 115°. The solution was concentrated under high vacuum to remove Me_2SO, a solution of the residue in water (5 mL) was concentrated by ultrafiltration using a membrane of 1000-Da cut-off, and the retentate was lyophilized to yield product (10 mg). *N*-Deacetylation was shown by the almost complete absence of the *N*-acetyl resonance at δ 2.1 in the ^{1}H-NMR spectrum. Aqueous 33% acetic acid (1.6 mL) and aq 5% sodium nitrite (1.6 mL) were added successively to a solution of *N*-deacetylated glycan in water (1.6 mL), and the mixture was stirred for 1 h. The solution was deionized with Amberlite IR-120 (H^+) and IR-45 (acetate) resins, concentrated, and treated with sodium borodeuteride. Normal work-up followed by GPC on Bio-Gel P-6 furnished a trisaccharide product (2.5 mg) which was divided into portions for (*a*) ^{1}H- and ^{13}C-NMR spectroscopy; (*b*) conversion into a permethylated derivative for direct GLC–MS examination in the EI and CI modes using program *E*, and linkage analysis of the cleavage products as partially methylated alditol acetates; and (*c*) conversion into an acetylated derivative (**14**) for high-field NMR examination at 500 MHz. *N*-Deacetylation of O:36B glycan (14 mg) in a similar manner afforded *N*-deacetylated glycan (5 mg) which was deaminated and reduced to give a mixture of trisaccharides (1.8 mg) for chemical and spectroscopic examination.

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