An essential saccharide binding domain for the mAb 2C7 established for *Neisseria gonorrhoeae* LOS by ES-MS and MS^n

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A study of bacterial surface oligosaccharides were investigated among different strains of *Neisseria gonorrhoeae* to correlate structural features essential for binding to the MAb 2C7. This epitope is widely expressed and conserved in gonococcal isolates, characteristics essential to an effective candidate vaccine antigen. Sample lipoooligosaccharides (LOS), was prepared by a modification of the hot phenol-water method from which de-O-acetylated LOS and oligosaccharide (OS) components were analyzed by ES-MS-CID-MS and ES-MS^n in a triple quadrupole and an ion trap mass spectrometer, respectively. Previously documented natural heterogeneity was apparent from both LOS and OS preparations which was admixed with fragments induced by hydrazine and mild acid treatment. Natural heterogeneity was limited to phosphorylation and antenn extensions to the α-chain. Mild acid hydrolysis to release OS also hydrolyzed the β(1→6) glycosidic linkage of lipid A. OS structures were determined by collisional and resonance excitation combined with MS and multistep MS^n which provided sequence information from both neutral loss, and nonreducing terminal fragments. A comparison of OS structures, with earlier knowledge of MAb binding, enzyme treatment, and partial acid hydrolysis indicates a generic overlapping domain for 2C7 binding. Reoccurring structural features include a Hepz(1→3)Hepβ(1→5)KDO trisaccharide core branched on the nonreducing terminus (Hep-2) with an α(1→2) linked GlcNAc (γ-chain), and an α-linked lactose (β-chain) residue. From the central heptose (Hep-1), a β(1→4) linked lactose (α-chain), moeity is required although extensions to this residue appear unnecessary.

Key words: Gonorrhoeae/oligosaccharide/MAb 2C7/ES-MS^n

Introduction

In stark contrast to the downward trend in the incidence of gonococcal infection, the proportion of strains showing drug resistant have increased (Carbillo *et al.*, 1990; Harnett *et al.*, 1997; Mason *et al.*, 1998). Penicillin resistant strains were first reported in 1976 (Ashford *et al.*, 1976), and shifts to tetracycline therapy lead to a subsequent resistance, first reported in 1985 (Morse *et al.*, 1986). Since that time, drug resistant *Neisseria gonorrhoeae* isolates from various parts of the world have been described (Gascogne-Binzi *et al.*, 1992). In a recent study looking at all the current therapeutic alternatives between 1986 and 1993, a substantial increase in strains with decreased susceptibility to tetracycline and penicillin was noted (Nissinen *et al.*, 1997). Although some quinolones and extended-spectrum cephalosporins are currently proving adequate for a treatment regimen, species vulnerability will likely diminish. Antimicrobial resistance is a measure of endurance, and for *Neisseria gonorrhoeae*, this durability appears to be a matter of time.

Physiological adaptability and evasion of immune host response contributes to microbial persistence and many of these strategies can be manifested through and by the components of the outer surface lipoooligosaccharide (LOS). Foremost, and probably related, are the demonstrable antigenic variations (Burch *et al.*, 1997), and oligosaccharide (OS) heterogeneity (Gibson *et al.*, 1989, 1993; Yang and Gotschlich, 1996). Transparent to a significant part of these differences is reactivity with MAb 2C7 (Erwin *et al.*, 1996; Gulati *et al.*, 1997), where 94% of the culture-positive genital secretions were detected using the MAb 2C3 stick capture technique (Gulati *et al.*, 1997). A better understanding of the structural features necessary for 2C7 binding may provide an improved basis for gonococcal vaccine development. In this report, using several known and one unknown LOS, we describe structural characterization using simple chemical manipulations and the mass spectrometry (MS) techniques of electrospray (ES) ionization, collision induced dissociation (CID), and MS^n to build that data base.

Results

Composition and molecular weight profiling

To identify component residues and their number, LOS and OS preparations were analyzed for carbohydrate composition by GLC-MS, and profiled for molecular weight heterogeneity by mass spectrometry. For profiling, methyl derivatives were considered the method of choice to retain intact OS structure, enhance purification by organic solvent extraction, and increase ES detection sensitivity (Reinhold *et al.*, 1995, 1996). When followed by CID-MS/MS, the methyl derivatives also exhibit improved sequence and branching detail (Reinhold *et al.*, 1996). Data for the *N.gonorrhoeae* strains 1291c, 1291wt, 24–1, and 15253 is summarized in this report (Table I).

Carbohydrate composition analysis by GLC-MS, using the methyl glycoside-trimethylsilyl derivatives, were consistent with previously published reports showing galactose, glucose, 2-acetamido-2-deoxy-glucose, heptose, and 2-keto-3-deoxy-octulosonic acid = KDO. Mass spectral ionization of native LOS samples has proven unsuccessful for a detailed understanding of structure, so acid release of the OS moiety, and extraction of this fraction after methylation was utilized in this study. Natural OS heterogeneity was retained within these methylated structures, a modification that enhances structural detail when probed by

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157
Table I. Structures and molecular masses of oligosaccharides from *N. gonorrhoeae* strains 1291wt/c, 15253, and 24-1

<table>
<thead>
<tr>
<th><em>N. gonorrhoeae</em> strain</th>
<th><em>M</em> after AcOH treatment [Da]</th>
<th><em>M</em> after AcOH and HF treatment [Da]</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1291wt/c</td>
<td>2130</td>
<td>1944</td>
<td>Hex−−HexNAc−−Hex−−Hex−−Hep−−KDO</td>
</tr>
<tr>
<td></td>
<td>2024</td>
<td>1944</td>
<td>HexNAc−−Hep−−PEA</td>
</tr>
<tr>
<td></td>
<td>1574</td>
<td>1495</td>
<td>Hex−−Hex−−Hep−−KDO</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>HexNAc−−Hep−−P</td>
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<td>Hex−−Hex−−Hep−−KDO</td>
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<td></td>
<td></td>
<td></td>
<td>HexNAc−−Hep−−P</td>
</tr>
<tr>
<td>15253</td>
<td>1903</td>
<td>1903</td>
<td>Hex−−Hex−−Hep−−KDO</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Hex−−Hex−−Hep−−HexNAc</td>
</tr>
<tr>
<td>24-1</td>
<td>1944</td>
<td>1944</td>
<td>Hex−−HexNAc−−Hex−−Hex−−Hep−−KDO</td>
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<tr>
<td></td>
<td>2189</td>
<td>2189</td>
<td>HexNAc−−HexNAc−−Hex−−Hex−−Hep−−KDO</td>
</tr>
<tr>
<td></td>
<td>2393</td>
<td>2393</td>
<td>Hex−−HexNAc−−Hex−−Hex−−Hep−−KDO</td>
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<td></td>
<td></td>
<td></td>
<td>HexNAc−−Hep</td>
</tr>
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</table>

Collision induced dissociation (CID). ES-MS spectra of these product ions showed only sodiated molecular ions (M+nNa)n+, and all samples were prepared in that way.

**Neisseria gonorrhoeae strain 1291wt/c**

To specifically access carbohydrate heterogeneity, LOS samples were O-deacylated (Gibson *et al*., 1993), dephosphorylated (phosphoethanolamine-, and H2PO3-), and methylated for molecular weight profiling. Strain 1291wt/c prepared in this manner provided a spectrum which supported major OS structural features of earlier work (John *et al*., 1991), and retained core and lipid A moieties of LOS (Figure 1). The highest molecular weight ion, *m/z* 3108.2, fits a carbohydrate composition that includes 3Hex, 2HexNAc, 2KDO, 2Hep, and the O-deacylated lipid A residue. Lower mass ions in the spectrum are a combination of those induced by acid lability and natural OS heterogeneity. Three sets of ions demonstrate the expected acid lability of the ketosidic linkage (*m/z* 2832.0, 2387.6, 1944.0, and 1938.1), but cleavage of the adjacent heptose (*m/z* 1667.9), and the internal β(1→6) glycosidic linkage of lipid A was surprising (*m/z* 2664.6, 2387.6, 1938.1). These fragments are easily differentiated from natural heterogeneity at the non-reducing terminus. From this consideration, and work described below, the ions observed in Figure 1 conform to the structures presented in Scheme 1. The two native OS structures detected were *m/z* 3108.2 and *m/z* 2658.9, differing by one lactosylamine moiety. An interesting feature of the spectrum is the 32 Da lower satellite peak associated with many fragments. This suggests methanol loss and may be comparable to the water loss ions observed earlier, considered to originate from KDO (Gibson *et al*., 1993). Support for this observation is questioned however, because it was not detected with the fragment *m/z* 1944.0, a structure composed of KDO. An alternative point of origin, consistent with these data, may reside in the β-methoxy group of N-myristyl glucosamine.

To establish possible sites of phosphorylation (PEA-, H2PO3-) the 1291wt/c strain, was treated with mild acid only and the released lipid A removed by extraction. The hydrophilic OS residue was dried, methylated, and profiled by ES-MS (Figure 2). The mild acid conditions would be expected to cleave ketosidic and leave largely intact the glycosidic linkages and sites of phosphorylation. An ion composition of 3Hex, 2HexNAc, 2KDO, 2Hep, and the O-deacylated lipid A residue. Lower mass ions in the spectrum are a combination of those induced by acid lability and natural OS heterogeneity. Three sets of ions demonstrate the expected acid lability of the ketosidic linkage (*m/z* 2832.0, 2387.6, 1944.0, and 1938.1), but cleavage of the adjacent heptose (*m/z* 1667.9), and the internal β(1→6) glycosidic linkage of lipid A was surprising (*m/z* 2664.6, 2387.6, 1938.1). These fragments are easily differentiated from natural heterogeneity at the non-reducing terminus. From this consideration, and work described below, the ions observed in Figure 1 conform to the structures presented in Scheme 1. The two native OS structures detected were *m/z* 3108.2 and *m/z* 2658.9, differing by one lactosylamine moiety. An interesting feature of the spectrum is the 32 Da lower satellite peak associated with many fragments. This suggests methanol loss and may be comparable to the water loss ions observed earlier, considered to originate from KDO (Gibson *et al*., 1993). Support for this observation is questioned however, because it was not detected with the fragment *m/z* 1944.0, a structure composed of KDO. An alternative point of origin, consistent with these data, may reside in the β-methoxy group of N-myristyl glucosamine.

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The series of ions detected in Figure 1 provide some understanding of monomer array, however, the most effective technique for a determination of carbohydrate sequence remains parent ion selection, collisional activation and a mass scan of the products, MS-CID-MS. Application of these techniques to the phosphate analog, \( m/z \ 2024.0 \) (Figure 2), provided an oligomer sequence, branch point, and the monomer location of the phosphate residue (Figure 3). Confirming evidence for phosphorylation was indicated by the neutral loss of 94 and 112 Da (-MeOPO2, -MeOPHO3), providing the product ions, \( m/z \ 1929.6, 1912.7 \). Additional losses from the parent ion identifies two different nonreducing termini. The ion \( m/z \ 1765.5 \) indicates loss of a single nonreducing tHexNAc which was in sequence with an additional loss of a heptose phosphate residue, \( m/z \ 1436.5 \). This appears to be followed by an additional loss of 463 Da (tHex-HexNAc) yielding the fragment \( m/z \ 973.4 \). Each fragment losses was equal in mass to fully methylated residues which identifies their terminal location, and thus, the branching point. An indication of branching can again be ascertained from the fragment, \( m/z \ 565.3 \), which resides 14 Da lower than an expected sequence ion, \( m/z \ 580 \). The fragments (\( m/z \ 1765.5, 1436.5 \)) and the increment of 80 Da above a heptose interval (248 Da) localize a phosphate moiety on the penultimate residue, and the nonreducing terminal fragment \( m/z \ 610.2 \) supports that possibility. Thus, the overall topology of this OS was defined by a single MS-CID-MS spectrum. Other details of the structure (interresidue linkage, isomers, and anomers) have been incorporated into the drawn structures as described in previous reports (West and Clark et al., 1989; Erwin et al., 1996).

**Neisseria gonorrhoeae strain 15253**

In contrast to strain 1291, strain 15253 is recognized by the monoclonal antibody 2C7, a feature also shared by strain 24–1, discussed below. Mild acid release of the OS from strain 15253 followed by extractive removal of lipid A, provided a hydrophilic residue that was methylated and mass profiled (Figure 4). Two major ions were detected in the double and single charged state that differed in mass by a KDO residue, \( m/z \ 825.1^{2+}/1626.8, \) and \( m/z \ 965.3^{2+}/1902.8 \). As observed in Figure 2, partial loss of KDO occurs under the conditions used to release lipid A, and this spectrum suggests a comparable result. These results again support the acid lability of the Hep(1→4)KDO glycosidic
Scheme 1.
An essential saccharide binding domain for mAb 2C7

**Fig. 2.** ES-MS spectrum of *N. gonorrhoeae* LOS strain 1291wt/c following mild acid treatment, OS isolation, and methylation. Spectrum identifies two parent OS structures, PEA- (m/z 2130.9), and H$_2$PO$_3$-octomers (m/z 2024.0). Remaining ions attributed to acid treatment to release lipid A. Hydrolysis products, m/z 345.2 (KDO), m/z 518.4 (lactosamine), and m/z 722.5 (trisaccharide), observed at lower mass.

linkage, concurrent with the ketosidic linkage. The most abundant ion, m/z 1902.8 (Figure 4), equals the molecular weight expected for a methylated OS sample (HexNAc, KDO, 2Hep, 4Hex), adducted with a single sodium ion. A small amount of natural heterogeneity was indicated by ions at lower mass. As an example, each of the two major fragments showed an ion in the profile with one less hexose residue, m/z 1698.7, 1422.4. These glycoforms may represent shortened OS structures from the α- or β-chain, or both from one chain. The ion abundance of these latter ions were too low to carry out collision studies.

Selection and collisional activation of the highest mass ion, m/z 964.2 2+, provided the MS$^2$ spectrum presented in Figure 5. A combination of nonreducing terminal, neutral loss fragments can be identified that define branching, sequence and the overall topology. The ability to distinguish reducing from nonreducing termini, and establish the presence of multiple branching (if not isomeric) is an advantage of methylation. In this sample two branches were indicated from the fragments m/z 1685.0 (-tHex), and 1645.0 (-tHexNAc). The former product was in sequence with a second hexose loss as indicated by the fragment m/z 1480.8 (-tHex-Hex). These losses could originate from either of two termini (α- or β-chain), or combine with losses from other antennae (double cleavage), as observed with the fragments, m/z 1221.6 (-tHex-tHex/-tHexNAc), m/z 1263.0 (-tHex/-tHex), and m/z 1426.9 (-tHex/-tHexNAc). The fragment m/z 987.6, with a mass composition equal to the β- and γ-chains (2Hex, HexNAc, Hep), suggests a single rupture between the heptose moieties. This remaining α-chain, tGal-Glc-Hep-(HO)KDO, appears to fragment further with loss of KDO to provide the trisaccharide, tGal-Glc-(HO)Hep, m/z 679.3 (Scheme 2). The fragment, m/z 463.1, tGal-Glc-OH, with retention of the glycosidic oxygen, is unusually high in abundance and may be explained by a facile E2-elimination from either branched heptose. A number of other fragments are seen to support the basic structure, and one of particular interest is the ion m/z 697.1, characterizing the β-chain. Many fragments can be assigned a structural representation from the determined mass and a known motif, however, this is not the case when the determined mass includes isomeric products. As an example, loss of lactose, m/z 1480.8 (Figure 5), could arise from either the α- or β-chain and trying to designate exact structure among isomeric possibilities is a common problem in multiply branched carbohydrate oligomers. Understanding these products...
Fig. 3. Collision spectrum (ES-CID-MS/MS) of selected $\text{H}_2\text{PO}_4$-octomer ($m/z$ 2024.0), from *N. gonorrhoeae* LOS strain 1291wt/c profile (Figure 2). Major fragments identified on structure (insert). Sample prepared as in Figure 2.

helps to define a fragmentation pathway that can aid in understanding precursor structure. Ion trap instrumentation, MS$^4$, is helpful in these situations.

MS$^4$ instrumentation is now commercially available and their brief history and general concepts of operation have been reviewed (Schwartz and Jardine, 1996; Jonscher and Yates, 1997; March, 1997). Studies applied to protein sequencing (Schwartz and Jardine, 1996), and carbohydrate samples have recently appeared (Asam and Glish, 1997; Reinhold and Sheeley, 1998; Sheeley and Reinhold, 1998). The latter application could have a major impact on carbohydrate biopolymers, where structural understanding is not complete in a single MS$^2$ experiment. Using this MS$^4$ technology we have defined the ion structure of $m/z$ 825.1$^+$ (Figure 4, strain 15253), and the isomeric lactose loss product from subsequent MS steps. The four separate scans, including ion selection, activation, and ejection have been arranged in a composite (Figure 6). The initial step (MS$^2$), yielded a fragment indicating loss of a terminal lactosyl residue, $m/z$ 1204.5. This product could arise by neutral loss from either the $\alpha$- or the $\beta$-chain, or be a mixture of both isomeric structures (Scheme 3). To identify which isomer, or product ratio, the ion was selected for MS$^3$ which produced the major fragment, $m/z$ 945.2, a composition consistent with loss of a tGlcNAc residue. Since this ion could also represent isomeric products (Scheme 3), the fragment was selected for MS$^4$. Two ions observed in the product spectrum suggest nonreducing termini ($m/z$ 711.3, $m/z$ 679.4), with the probable structures, tLac-(HO)HepOMe, and tLac-(HO)Hep, respectively. These products must arise from rupture of an identical heptose glycosidic linkage and their product abundance may be considered to closely approximate their isomeric ratios in the precursor, $m/z$ 945.2. Since the structure representing the $\beta$-chain, $m/z$ 679.4, was five times more abundant than the comparable ion representing the $\alpha$-chain, $m/z$ 711.3, these data establish a structure for the major components, $m/z$ 1205.5 and $m/z$ 945.2, and strongly suggest neutral loss of the $\alpha$-chain to be a highly favored process.

**Neisseria gonorrhoeae strain 24–1**

An additional *N. gonorrhoeae* clinical sample that tested positive to the 2C7 MAb was strain 24–1 (Densen *et al.*, 1987). Isolation of this OS and profiling by ES-MS showed three prominent ions, each matched by its corresponding single charged species, $m/z$ 983.5$^+$/1943.8 (59.5%), 1106.1$^+$/2189.9 (32.8%), and 1208.2$^+$/2393.4 (7.7%). The mass differences indicate structural heterogeneity incrementing from the most abundant species, $m/z$ 983.5$^+$/1943.8, by a HexNAc and a Hex residue. This predominant ion possessed a composition weight equal to 3Hex, 2Hep, 2HexNAc, and a single KDO, identical to that detected for strain
An essential saccharide binding domain for mAb 2C7

Fig. 4. ES-MS spectrum of *N. gonorrhoeae* strain 15253 LOS following mild acid treatment, and OS isolation and methylation. Two sets of major ions in the double and single charge state that differ by KDO residue. Smaller fragments represent each OS with one and two hexose less.

1291 wt/c. Importantly, this 24–1 species demonstrated 2C7 antibody binding, but only very weakly. There was no indication of phosphorylation, and HF treatment failed to alter the above results.

The most abundant OS structure was selected for study by collisionally activating its doubly charged ion, *m/z* 983.52+ (Figure 7). Two-thirds of the total ion current, *m/z* 486.1, and 752.32+/1480.7, can be accounted for by one facile bond rupture, and sodium addition to each of the two products. The rupture occurs at the nonreducing terminal α-chain lactosamine residue. This sodiated disaccharide accounts for a third of the ion current with the remaining two-thirds represented by its counterpart in the single and double charge state, *m/z* 1480.7, 752.32+. At much smaller abundance several fragments provide greater help in assigning the OS sequence and branching structure, but all are secondary products of the primary lactosamine cleavage ion, *m/z* 1480.7, e.g., -Hex, -HexNAc, -KDO, and -2Hex. The consecutive loss of two hexose residues, *m/z* 1276.7 and 1072.6, indicates a continued neutral loss from the α-chain to delineate a Hex-Hex-HexNAc-Hex-Hex-Hep sequence. In the same mass range a neutral loss of nonreducing terminal tHexNAc can be observed with the fragment *m/z* 1221.6. This fragment is probably in sequence with the ion, *m/z* 972.4, indicating a Hep moiety, -HexNAc-Hep, defining a γ-chain terminus and an absence of a β-chain. Again, within the same mass range the loss of the reducing-end KDO can be observed, *m/z* 1175.1, leaving a single core Hep unaccounted for by a single neutral loss. Its position in the sequence are clearly defined, however, by the fragments, *m/z* 1072.6, HexNAc-Hep-(HO)Hep-KDO. A 3-O-elimination of heptose-2 from the branched heptose-1 residue was suggested by the ion, *m/z* 548.2.

Understanding the most abundant OS structure in strain 24–1 provided a basis for considering other glycoforms within the sample, *m/z* 2189.9 and 2393.4. The ion at *m/z* 2189.9, an increment equal to an additional HexNAc residue, provided a CID spectrum very similar to that presented in Figure 7 but with a number of important differences (Figure 8). Instead of an abundant *m/z* 486 fragment, it was replaced by an ion 14 Da lower, *m/z* 472, indicating the lactosamine to be internal and not terminating. At lower abundance, the same pattern of secondary neutral losses were observed from the fragment *m/z* 1480.9. This linear sequence extends from the parent ion, *m/z* 2190.5 (*m/z* 1930.3, 1726.1, 1480.9, 1276.7, 1072.6, 330.7), and positions the branched residue (R), HexNAc-Hex-HexNAc-Hex-Hex-(R)Hep-KDO. A neutral loss of tHexNAc was also observed, *m/z* 1221.8, which was also in sequence with a Hep loss, *m/z* 973.6. The closely positioned fragment, *m/z* 976.22+, represents the doubly charged neutral trisaccharide loss product, *m/z* 1930.3.

The highest molecular weight glycoform in the 24–1 OS (Figure 6) was incremented by an additional hexose residue, *m/z* 2393.4. Collisional activation of the doubly charged ion, *m/z* 1208.22+, again showed a spectrum with similarities observed in Figures 7 and 8. This single hexose extension, combined with additional characteristic losses, positioned the residue as a...
Scheme 2.
An essential saccharide binding domain for mAb 2C7

Fig. 5. Collision spectrum (ES-CID-MS/MS) of OS octamer (m/z 964.2^2+), selected from *N. gonorrhoeae* LOS strain 15253 profile (Figure 4). Major fragments identified on structure (inset). Complete list presented in Scheme 2.

Discussion

The cell surface components responsible for many aspects of gonococcal disease are termed lipooligosaccharide (LOS). Previous studies (Gulati *et al*., 1996) have described a conserved, widely expressed epitope within LOS molecules that could be identified by MAb 2C7 (Mandrell *et al*., 1988; Gibson *et al*., 1988; Gulati *et al*., 1997). The MAb bound 13 of 20 separate gonococcal LOS preparations in a solid-phase RIA (Mandrell *et al*., 1988), and recognized 94% of culture-positive genital secretions using a stick capture technique (Gulati *et al*., 1997).

Correlation of antibody reactivity with a conserved OS structure among different isolates could provide component features necessary for vaccine development. The object of this study was to outline and compare domains of several LOS structures that contribute to 2C7 reactivity.

In this report two *N. gonorrhoeae* LOS strains were characterized that showed positive banding in a Western blot assay for the 2C7 antibody (24–1, 15253); also characterized were two that do not (1291c and the revertant wild type; Gulati *et al*., 1996). The LOS domains responsible for 2C7 binding have been previously related to the oligosaccharide nonreducing termini and are not influenced by neisserial lipid A or core KDO residues (Mandrell *et al*., 1988; Erwin *et al*., 1996; Gulati *et al*., 1997).

SDS-PAGE analysis of the LOS isolates have indicated characteristic heterogeneity and these identical fractions were analyzed in this study. To characterize oligosaccharide heterogeneity the samples were O-deacylated (Gibson *et al*., 1993), and dephosphorylated with the methylated products analyzed by ES-MS (Figure 1). The acid treatment (mild acid, HF), caused degradation of the LOS sample, most of which can related to acid lability of the ketosidic linkage. The fragments and their relationships, however, allow a provisional assignment of structure from the product ion masses (Scheme 1). Previous studies have also shown heterogeneity following mild acid treatment (John *et al*., 1991; Yamasaki *et al*., 1991; Erwin *et al*., 1996), and an additional fragility was also noted in the β(1→6) chitobiose linkage of lipid A (Scheme 1, m/z 2214.7, 1938.1, 2387.6, 2664.8). From these recognized points of chemical lability, a determination of oligosaccharide heterogeneity can be realized, m/z 3108.2 and 2658.9, which represent extensions of α-chain by a lactosylamine residue (Figure 1, inset).

This finding is consistent with the two spots observed with SDS-PAGE analysis (Gulati *et al*., 1997).

Molecular heterogeneity was also demonstrated when isolate 1291wt/c was profiled without dephosphorylation. ES-MS of the acid released and methylated OS showed two high mass ions suggesting phosphate (m/z 2024.0) and phosphoethanolamine (m/z 2130.9) mass increments (Figure 2) to a core OS, m/z 1944.0 (Figure 1). Interestingly, no OS structures were detected without terminal extension to the α-chain providing the sequence, tHex-HexNAc-Hex-HexNAc-Hex-Hex-(R)Hep-KDO.
either phosphate or phosphoethanolamine adducts, and each showed the corresponding lactosamine heterogeneity observed in Figure 1. Thus, four entities appear to represent the heterogeneity in isolate 1291wt/c, although only two bands were apparent on the SDS-PAGE analysis (Erwin et al., 1996; Gulati et al., 1997). Collisional activation of the OS, and a mass scan of the products provided sequence information from both neutral loss, and nonreducing terminal ions. Sequence ions along the \( \alpha \)-chain indicate a hepse-1 branch point with nonreducing and reducing fragments. Neutral loss sequence ions from the \( \gamma \)-chain terminus defined the heptose-2 position, the point of phosphorylation, and an absence of a \( \beta \)-chain. Polar residues retain charge, and when positioned at or near a chain terminus, a prominent neutral loss ion sequence is common. This structure demonstrates that principle (Figure 3, inset).

Isolate 15253 is recognized by MAb 2C7 and shows a single band on Western blotting and SDS-PAGE (Gibson et al., 1993; Gulati et al., 1997). ES-MS analysis of the released and methylated OS supported that observation. The two major ions (differing by a KDO residue), illustrate the expected reducing-end raggedness as a consequence of acid treatment. Minor fragment ions were detected, however, one and two hexose residues lower in mass (Figure 4). Selection and collisional activation of the highest mass ion, \( m/z \) 965.3\(^{2+}\), provided a spectrum with considerable structural detail (Figure 5). Neutral loss sequence ions from the \( \gamma \)-chain indicate a \( \beta \)-chain structure which was documented with additional fragments that indicate it to be a disaccharide. Further analysis by ion trap MS was investigated to confirm the isomeric disaccharide structure in each \( \alpha \)- and \( \beta \)-chain. These results clearly showed the enhanced CID lability of the \( \alpha \)-chain lactosamine when contrasted with an identical residue linked to the \( \beta \)-chain (Scheme 3, Figure 6).

Comparative structural analysis and binding activity of these gonococcal strains have focused on the presence or absence of heptose phosphorylation and monomer increments to the \( \alpha \)-chain.

Structural understanding of the three major components detected in isotype 241 OS were readily related to HexNAc and Hex-HexNAc extensions of the \( \alpha \)-chain. Neutral loss sequence ions indicated a penultimate heptose residue and an absence of the \( \beta \)-chain. More difficult to correlate with these structures was the weak single band observed for 2C7 binding in a Western blot vs. the three major bands detected by SDS-PAGE (Gulati et al., 1996, 1997). Since it has been shown that saccharide extensions of heptose-1 (\( \alpha \)-chain) do not influence 2C7 binding, this would suggest none of three major peaks bind to 2C7, and the minor band observed in the Western blot would be an unrelated structure. Such an unrelated peak was not observed in the MS spectrum and may have been below the detection limits or, equally possible, was suppressed by components in higher concentration. This interpretation would indicate \( \beta \)-chain extension is a prerequisite for binding comparable to the structural data obtained for isolate 15253.

In summary, by comparing these OS structures, with earlier knowledge of MAb binding, enzyme treatment, and partial acid hydrolysis, a generic overlapping domain for 2C7 binding becomes evident. Reoccurring structural features include an Hep\( \alpha(1\rightarrow3)\)Hep\( \beta(1\rightarrow5)\)KDO trisaccharide core branched on the nonreducing terminus (Hep-2) with an \( \alpha(1\rightarrow2) \) linked GlcNAc (\( \gamma \)-chain), and an \( \alpha \)-linked lactose (\( \beta \)-chain) residue. From the central heptose (Hep-1), a \( \beta(1\rightarrow4) \) linked lactose (\( \alpha \)-chain) moiety is required although extensions to this residue appears unnecessary (Scheme 4). Structural conformation may only be resolved with synthesis (Figure 9).
Materials and methods

Composition

Total carbohydrate compositions were carried out following 2 N TFA hydrolysis for 1 h and analyzed as trimethylsilyl derivatives by GLC-MS (Reinhold, 1972).

Gonococcal strains

Gonococci (nonpiliated and transparent), were grown on solid media supplemented with 1% Isovitalex equivalent (McQuillen et al., 1994), for 12–14 h in candle extinction jars at 37°C (James and Swanson, 1978). LOSs were prepared from whole organisms using a modification of the hot phenol-water method (Westphal et al., 1952). Oligosaccharides were prepared from the LOSs by hydrolysis in 1% acetic acid for 2 h at 100°C followed by centrifugation (200 x g, 5 min; Schmidt et al., 1969; Griffiss et al., 1987). The supernatant was washed three times with CHCl₃ (1:1, vol:vol), and the cloudy phase of the final wash was lyophilized (Schmidt et al., 1969; Griffiss et al., 1987). Gonococci were also isolated from patients attending the Sexually Transmitted Diseases Clinic (Boston Medical Center). All isolates were confirmed by routine biochemical testing. The strain 1291c was one of several mutants that survive the biological influence of pyocin (bacteriocins produced by Pseudomonas aeruginosa), and selects
for mutants with truncated LOS structures (John et al., 1991). In this study, however, the strain was cultured without pyocin which appeared to induce a partial back mutation to the wild type, 1291wt/c.

**Dephosphorylation**

Dephosphorylation conditions were carried out by treating the LOS and OS samples with aqueous hydrogen fluoride (48%) for 24 h at 10°C. Residues were dried under vacuum and utilized directly for methylation.

**Methylation**

Methylation was achieved by dissolving samples in a NaOH/DMSO suspension, prepared by vortex mixing DMSO and powdered sodium hydroxide (Ciucanu and Kerek, 1984). After 1 h at room temperature, 50 µg of methyl iodide were added and the suspensions set for 1 h at room temperature with occasional vortexing (Reinhold et al., 1996). The methylated product was extracted by adding 1 ml of chloroform, and the suspensions were back-washed four times with 2–3 ml of 30% acetic acid. Methylated samples were dried following chloroform extraction, and redissolved to a concentration of 10 µM in a 1 mM solution of sodium acetate in 70:30 methanol/water just prior to analysis. The chloroform layer was taken to dryness and stored at -20°C.

**Electrospray ionization mass spectrometry**

Mass spectral measurements were performed on either a triple quadrupole or an ion trap mass spectrometer equipped with electrospray ionization. For both instruments samples were dissolved in methanol: water solutions (6:4, v/v) containing 0.25 mM NaOH and analyzed by syringe pump flow injection directly into the electrospray chamber through a stainless steel hypodermic needle, at a flow rate of 0.85 µl/min for the triple quadrupole instrument. The voltage difference between the needle tip and the source electrode was -3.5 kV.

The ion trap mass spectrometer (ITMS) used in this study was a Finnigan-MAT LCQ (Finnigan Corporation, San Jose, CA).

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Fig. 7. Collision spectrum (ES-CID-MS/MS) of OS octamer (m/z 983.52+), selected from *N.gonorrhoeae* LOS strain 24–1 profile spectrum (data not shown). Major fragments identified on structure (insert). Primary loss of nonreducing terminal LacNAc from which sequence ions derived.
An essential saccharide binding domain for mAb 2C7

Fig. 8. Collision spectrum (ES-CID-MS/MS) of OS nonamer (m/z 1106.1^{2+}), selected from *N. gonorrhoeae* LOS strain 24–1 profile spectrum (data not shown). Major fragments identified on structure (inset). Spectrum process by an entropy based algorithm (Reinhold and Reinhold, 1992).

Scheme 4.

also coupled with an ESI source through a vacuum chamber that allows for differential pumping between the ion source and the analyzer region. Samples were prepared the same but flow injected at a rate of 1.5 µl/min. Ions are injected axially into the ion trap by a gate lens and a trapping field was established with a 100–1100 kHz radio frequency applied to the ring electrode.

Acknowledgments

We acknowledge the fellowship provided to W.M. by the Fonds zur Foerderung der Wissenschaftlichen Forschung (FWF, Grant #JO1389CHE), Austria. Financial support: NIH (AI-01061 D.P.M.) (AI-33087 P.A.R.). The mass spectral studies were carried out, in part, at the Boston University Medical Center Mass Spectrometry Resource (GM54045 V.R.). Ion trap studies were kindly provided by Professor Paul Younos. The triple quadrupole was purchased with SIG RR10493, awarded to C.E. Costello.

Abbreviations

Hex, hexose; HexNAc, 2-acetamido-2-deoxy-hexose; Gal, galactose; Glc, glucose; LacNAc, lactose amine Gal-GlcNAc; ES, electrospray; CID, collision induced dissociation; MS, mass spectrometry; IT-MS, ion trap-mass spectrometry; Hep, heptose; KDO, 2-keto-3-deoxy-octulosonic acid; PEA, phosphatidylethanolamine; (HO)Hex, notation designating exposed hydroxyl as a consequence of linkage rupture by collisional activation; tHex, notation designating nonreducing terminal fragment.
Fig. 9. Collision spectrum (ES-CID-MS/MS) of OS decamer (m/z 1208.2+), selected from *N. gonorrhoeae* LOS strain 24–1 profile spectrum (data not shown). Major fragments identified on structure (inset). Spectrum process by a entropy based algorithm (Reinhold and Reinhold, 1992).

References


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