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Analysis of oligosaccharide epitopes of meningococcal lipopolysaccharides by fast-atom-bombardment mass spectrometry

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ABSTRACT

A mass-spectrometic approach is presented for the analysis of the structures of lipopolysaccharide-derived oligosaccharides, which are frequently difficult to define by classical methods since they contain chemically labile components. The method involves f.a.b.-m.s. of the oligosaccharides, their peracetylated and permethylated derivatives, their deuterioacetylated and methylated analogues, and the fragments obtained during graded methanolysis of the methylated analogues. Data obtained from two representative meningococcal LPS oligosaccharides define the sequence, patterns of branching, and the extent and location of the phosphorylethanolamine and *O*-acetyl substituents.

INTRODUCTION

The lipopolysaccharides (LPS) of *Neisseria meningitidis* have been implicated in the immune response to natural infection[1] and at least 11 serotypes (L1–L11) have been identified[2,3]. The epitopes of the LPS serotype reside in the carbohydrate moiety[4] and involve low-molecular-weight oligosaccharides of the R-type[5]. Because these oligosaccharides induce in animals antibodies that are bactericidal for meningococci, they are potential epitopes for use in a synthetic vaccine against meningococcal meningitis[4]. In previous work[6], using classical carbohydrate chemistry and n.m.r. techniques, it was shown that **1** is the core oligosaccharide associated with several LPS serotypes. The specificity of a serotype is generated either by the addition of glycose units or phosphorylethanolamine[7] and possibly *O*-acetyl groups to the core structure.

F.a.b.-m.s. is a powerful tool for the analysis of complex carbohydrates, including those that contain labile and/or polar functional groups[8]. The f.a.b.-mass spectra of underivatised oligosaccharides are a source of important structural information which can, in some instances, be enhanced by collision-induced dissociation[12]. However,

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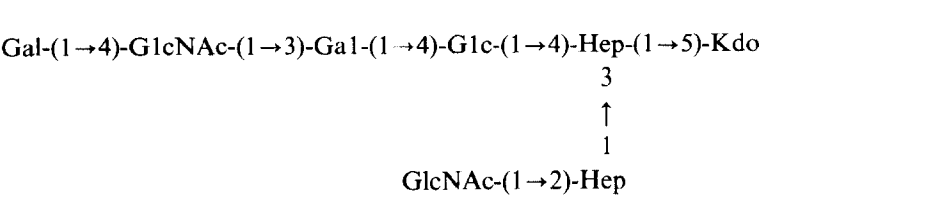

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underivatised oligosaccharides frequently afford fragment ions that are derived from at least two cleavage events, thereby making the assignment of sequence difficult or ambiguous. Therefore, unambiguous sequencing by f.a.b.-m.s. requires the prior preparation of such derivatives as the peracetyl or the permethyl. The use of appropriate deuterated derivatisation reagents allows the location of acylated residues[8] and information on sequence to be obtained which can be supplemented by f.a.b.-m.s. of methanolysates of permethylated derivatives[9,11].

We now report on the application of these strategies in the analysis of the structures of *Neisseria* LPS oligosaccharides. We show how chemical derivatisations and degradations can be combined with negative and positive f.a.b.-m.s. analyses to yield rapidly and sensitively complete sequences, branching patterns, and locations of substituents. We report on the stability of reducing terminal Kdo and of phosphorylethanolamine to acetylation and permethylation protocols, and discuss the products which are formed under these conditions, as well as describing the fragmentation behaviour of these derivatives in two different matrices. We describe for the first time the location of *O*-acetyl and phosphorylethanolamine substituents in the core oligosaccharides of *N. meningitidis* LPS.

EXPERIMENTAL

The prototype strains 981 (B, L5) and 891 (C, L4) were grown in a modified Franz medium and the LPS were extracted with phenol[5]. The oligosaccharides were obtained by heating the LPS in aqueous 1% acetic acid (2 h, 100°) and purified[5] by elution from a column of Bio-Gel P4 with 0.2M pyridinium acetate (pH 5.4). Only the largest of the oligosaccharides obtained from each serotype LPS was studied.

Deuterioacetylation was carried out under conditions of acid catalysis so that any endogenous acetyl groups present retained their original locations[8]. Briefly, the oligosaccharide (1–5 μg) was mixed with 2:1 trifluoroacetic anhydride–glacial acetic acid-d_4 (200 μl) for 10 min at room temperature, the reagents were then removed in a stream of nitrogen, and the water-soluble contaminants were removed on partition between chloroform and water.

Methylation was carried out using a modification of the method of Ciucanu and Kerek[10] in which the oligosaccharide (1–5 μg) was mixed with a slurry of sodium hydroxide in dimethyl sulphoxide (500 μl) and then methyl iodide (500 μl) was added. The mixture was shaken for 10 min at room temperature, the reaction was quenched

with cold water (1 mL), chloroform (1 mL) was added, and the solution was washed with water (4 × 1 mL).

Hakomori methylation and the isolation of products on C_{18} Sep-Pak cartridges was carried out as described[11].

Methanolyses of permethylated samples were carried out in Reacti Vials. Methanolic M HCl (10 μl) was added and each solution was kept at 40° then at 60°. Aliquots (1 μL) of the methanolysate were removed after 2, 20, and 40 min at 40°, and after 5, 30, 60, 120, and 240 min at 60°, and analysed directly by f.a.b.-m.s.. During the course of the experiment, additional methanolic HCl was added as necessary to prevent drying out.

F.a.b.-m.s. was carried out as described[13].

RESULTS AND DISCUSSION

Oligosaccharides from the LPS of R-type mutants of *Neisseria meningitidis* were obtained by mild acid hydrolysis under conditions which cleave the Kdo core. F.a.b.-m.s. was performed on ten samples, and detailed analyses are described of two representative samples (L5-F1 and L4-F1) which contain all the structural features found in the LPS core oligosaccharides. In order to facilitate the discussion, the complete structures of L5-F1 and L4-F1 are shown in **2** and **3**. Assignments of structure not afforded by f.a.b.-m.s., such as stereochemistry and linkage, which were carried out concurrently using n.m.r. and methylation analysis techniques, will be reported elsewhere.

The f.a.b.-m.s. strategy most suitable for maximising the information on structure involved the following steps. (*1*) F.a.b.-m.s. of the underivatised material in the positive- and negative-ion modes indicates the composition in terms of hexoses, heptoses, Kdo, *etc.*, and the presence of substituents (*e.g.*, acetate, phosphate) or modifications (*e.g.*, lactone formation), if the quality and quantity of the sample are favourable. In practice, this step frequently yields data of poor quality due to the presence of salts that are difficult to remove entirely from biological samples. (*2*) F.a.b.-m.s. of the deuterioacetylated derivative in both the positive- and negative-ion modes affords a significant increase in sensitivity and a rapid and convenient means of cleaning-up samples contaminated with salts. The data allow determination of the extent of endogenous acetylation, identification of the acetyl-bearing residue(s), and yield information on sequence. (*3*) Positive-ion mode analysis of the permethylated derivative often produces a further improvement in sensitivity by reducing the mass of the derivative, and gives additional data on sequence. (*4*) Graded methanolysis[9] of the permethylated sample and monitoring by f.a.b.-m.s. of the products in a time course experiment determine the sequence and patterns of branching.

Structure of L5-F1. — (a) Underivatised material. F.a.b.-m.s. in the positive-ion mode produced a weak spectrum containing one major signal at *m/z* 1881 together with a minor signal 42 mass units lower. Strong matrix/salt cluster ions suggested that the poor data were a result of contamination with salts. Nevertheless, the ions observed indicated a composition of $Hex_5HexNAc_2Hep_2Kdo$ with and without one endogenous

1

R →4)-β-D-Glc*p*-(1→4)-β-D-Glc*p*-(1→4)-α-Hep-(1→5)-Kdo
3
↑
1
α-D-Glc*p*-(1→3)-α-Hep
2
↑
1
α-D-Glc*p*NAc
1
Ac(60%)

2 (L5-F1)

R →4)-β-D-Glc*p*-(1→4)-α-Hep-(1→5)-Kdo
3
↑
1
α-Hep-P-$EtNH_2$
2
↑
|
α-d-Glc*p*NAc
|
Ac(50%)

3 (L4-F1)

R = β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→

Hep = L-*glycero*-D-*manno*-heptopyranose

acetyl group. It is known[13] that the presence of even a single acetyl group in an oligosaccharide enhances the sensitivity in f.a.b.-m.s. and, therefore, it cannot be concluded that the mono-acetylated component preponderates. Normally the negative-ion mode is less sensitive to contamination by salts than the positive-ion mode[13], as was borne out by the acquisition of a considerably stronger negative-ion spectrum of L5-F1 than its positive-ion counterpart. The region containing the molecular ions in the negative-ion spectrum is reproduced in Fig. 1. $[M-H]^-$ ions are present at *m/z* 1837 and

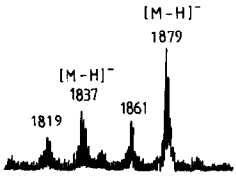


Fig. 1. Molecular ion region of negative-ion spectrum, acquired in G/TG, of native L5-F1.

1879, thus corroborating the data from the positive-ion spectrum. Additional ions at *m/z* 1819 and 1861 correspond to dehydrated forms of the non- and mono-acetylated species, which are observed commonly in the negative-ion spectra of carbohydrate-containing samples.

(b) Deuterioacetylated derivative. In order to determine the amount and location of the acetyl groups, the deuterioacetylated derivative was prepared, and spectra were obtained in the positive- and negative-ion modes in glycerol–1-thioglycerol (G–TG), the matrix most widely used in carbohydrate f.a.b.-m.s., and 3-nitrobenzyl alcohol (MNBA), a matrix introduced more recently[14]. In the resulting spectra, ion doublets separated in mass by 3 mass units indicate related species in which an endogenous acetyl group is represented by the ion of lower mass, whereas the fully deuterioacetylated component gives rise to the ion 3 mass units higher. The positive-ion spectrum obtained using the G–TG matrix is reproduced in Fig. 2a. The molecular ion clusters are resolved only partially in order to maximise sensitivity. Each cluster spans at least 7 mass units because of the isotopic spread. The mass at the centre of each cluster, which corresponds to the fully deuterioacetylated species, was assigned using the mass marker. The major molecular ion cluster at *m/z* 3279 corresponds to $[M + H]^+$ of fully deuterioacetylated $Hex_5Hep_2HexNAc_2Kdo$. Other major signals in the molecular ion region are derived from under-deuterioacetylated components, resulting from incomplete derivatisation, at *m/z* 3234 (45 mass units below *m/z* 3279) and *m/z* 3171 (45 mass units below *m/z* 3216), and an ion lacking a deuterioacetic acid moiety at *m/z* 3216. The signal at *m/z* 3324 (45 mass units above *m/z* 3279) is derived from a component carrying an additional deuterioacetyl group, most likely as the anhydride of the carboxyl group. A similar pattern of molecular ions was obtained using MNBA (Fig. 2b), except that sodium adduct ions were observed also. The results from this and related samples indicate that MNBA promotes the formation of sodium adduct ions and that peracylated samples slightly contaminated with salts, and which do not ionise well in G–TG, give excellent data in this matrix. In the negative-ion mode, using either matrix, molecular ion clusters were present at *m/z* 3277 (major signal; $[M - H]^-$ of fully deuterioacetylated species), *m/z* 3232 (minor signal, one deuterioacetyl group lacking), and *m/z* 3169 (major signal, loss of deuterioacetic acid from *m/z* 3232).

The perdeuterioacetylated derivative underwent extensive fragmentation in the positive-ion mode, and the resulting ions allowed a partial assignment of the sequence

and location of the endogenous acetyl group. There were significant differences in the number and abundance of the high-mass fragment ions produced in the two matrices. Characteristic A-type fragment ions[13,15] were formed in each matrix by cleavage at each of the first three residues in the backbone and at HexNAc and branched Hep in the side-chain (see Fig. 3). The terminal HexNAc gave a characteristic 3-mass-unit doublet, showing that the endogenous acetyl group was located on this residue. The relative intensities of the ions at m/z 336 and 339 indicate that ~60% of the molecules carried an

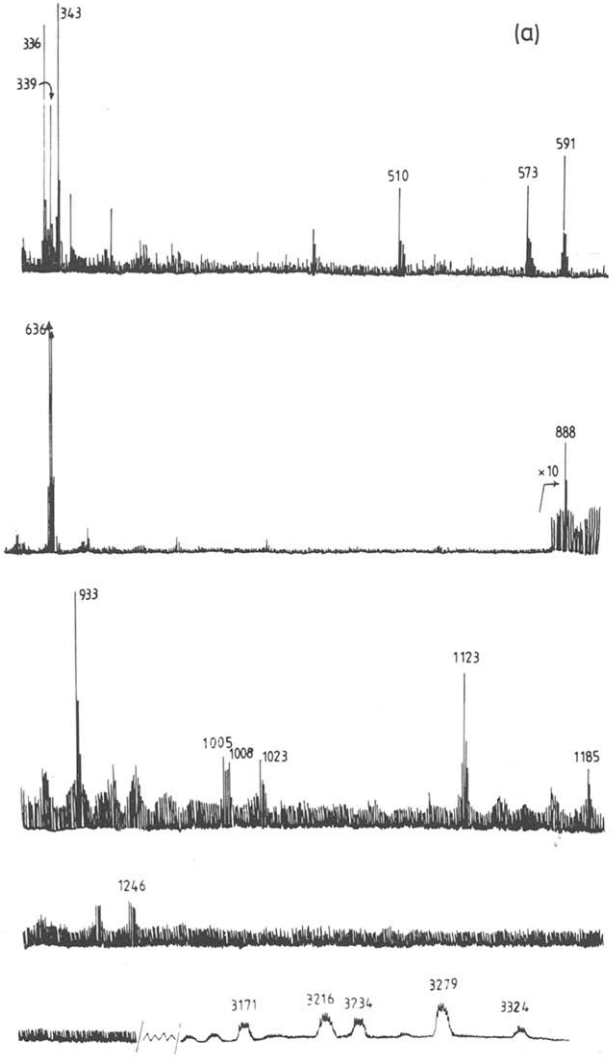
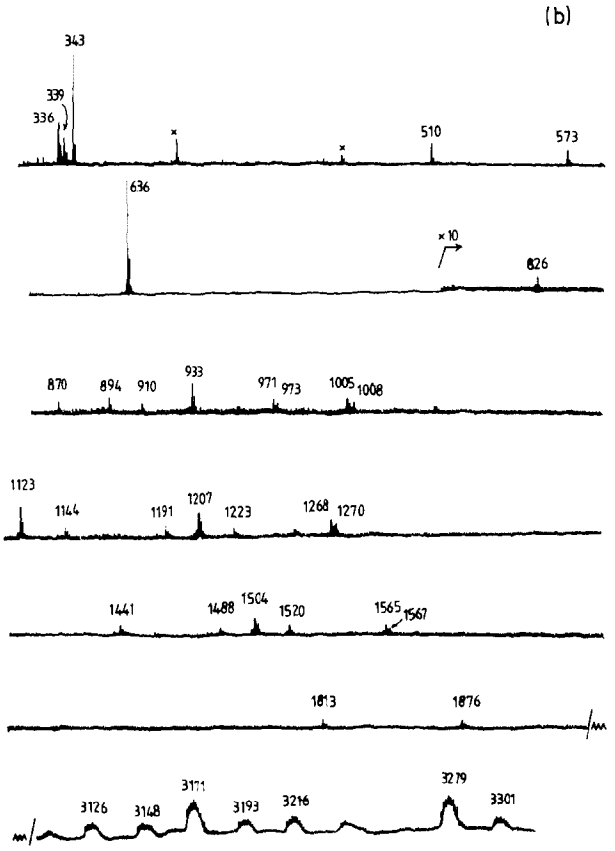


Fig. 2. (*a*) Positive-ion spectrum of deuterioacetylated L5-F1, acquired using G-TG; (*b*) molecular ion region of positive-ion spectrum of deuterioacetylated L5-F1, acquired using MNBA; (*c*) positive-ion spectrum of acetylated L5-F1, acquired using MNBA.


(b)
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636
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826
870
894
910
933
971
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1005
1008
1123
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1191
1207
1223
1268
1270
1441
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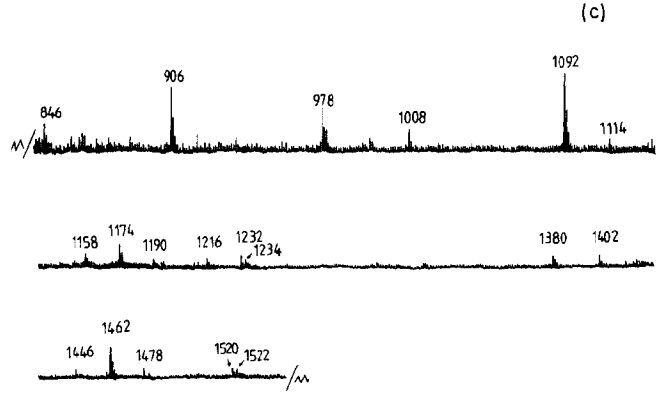
(c)
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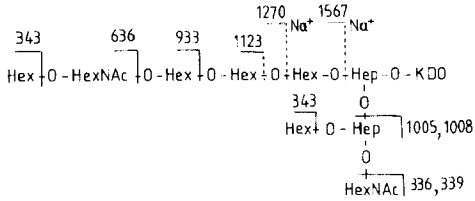
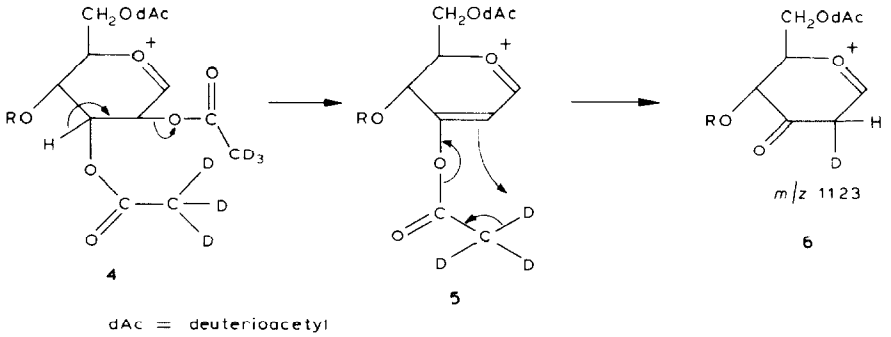


Fig. 3. Fragmentation pattern observed for deuterioacetylated L5-F1.



endogenous acetyl group. As expected, the Hep cleavage ions at *m/z* 1005 and 1008 show the same ratio of intensities. A-type ions from the backbone are present as singlets at *m/z* 343, 636, and 933, and, therefore, this sample appears to have been acetylated exclusively on the side-chain HexNAc. The expected A-type cleavage at the fourth and fifth residues of the backbone did not take place; instead, the sample run in G–TG gave a major signal at *m/z* 1123, which is 107 mass units below that for the predicted A-type ion for cleavage at the fourth residue. A signal of similar intensity is present in the spectrum acquired in MNBA (Fig 2b). A clue to the nature of the *m/z* 1123 ion was provided by the spectrum (Fig. 2c) of acetylated L5-F1 acquired using the MNBA matrix. Each of the A-type ions shown in Fig. 3 has been shifted by the predicted amount, *i.e.*, 3 mass units, to lower mass for each acetyl group introduced. However, the unassigned signal at *m/z* 1123 appears to have shifted to *m/z* 1092, *i.e.*, by 31 mass units, which does not correspond to an integral number of acetyl groups. A shift of 31 mass units suggests loss of ketene with transfer of deuterium from the ketene-producing deuterioacetyl group to the sugar backbone. Concomitant loss of deuterioacetic acid will yield a fragment ion of the observed mass. A possible pathway for generation of *m/z* 1123 is the sequence **4→5→6**.

Loss of ketene cannot be differentiated from underacetylation, or apparent underacetylation if β-cleavages occur, unless deuterated reagents are used. The results from L5-F1 highlight the importance of using deuterated reagents, even when endogenous acetyl groups are not present, in order to corroborate the assignments of sequence. Failure to recognise the above unusual loss of ketene could lead to the incorrect assignment of a branched residue if β-cleavage is assumed to give rise to the "underacetylated" fragment ion.

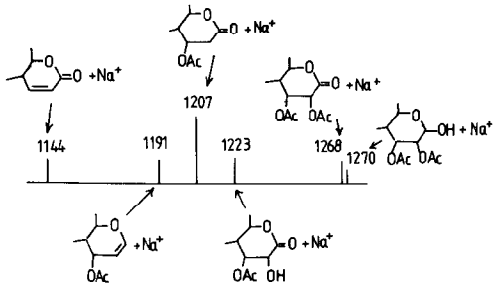


Fig. 4. Possible structures for signals in the spectra of acetylated and deuterioacetylated L5-F1, acquired using MNBA.

The spectra acquired using MNBA afford sets of fragment ions in the region *m/z* 1200–1900. These ions, which are not present in the spectrum obtained using G-TG (see Fig. 2), occur in characteristic clusters, each of which is separated from the next by the mass of a sugar residue. The pattern is exemplified by the signals in the region of *m/z* 1200 in Fig. 2b (see Fig. 4). The dominant signal at *m/z* 1207 is flanked by signals 16, 61, and 63 mass units higher and 16 and 63 mass units lower. In the peracetylated sample, the corresponding cluster is centred at *m/z* 1174 with accompanying signals at +16, +58, +60, −16, and −60. These ions can be rationalised as sodium adducts of sequence ions where the dominant ions retain the glycosidic oxygen. Possible structures for each member of the cluster are given in Fig 4. The presence of higher-mass sequence ions makes the MNBA matrix useful, especially when used alongside the G–TG matrix, with the caution that branched residues are not identified as readily in the latter since the non-branched hexoses and the branched heptose yield the same type of major fragment ion. The flanking 16-mass-unit signals are not present for the heptose cleavage position and it is possible that the absence of these satellites could be diagnostic of a branched residue. However, it is recommended that the methanolysis strategy described later be used to define branched residues since the ±16 satellites are weak at the unbranched positions and may not be observed when small quantities of material are examined.

(*c*) *Methylated derivative.* F.a.b.-m.s. of methylated L5-F1, prepared by the method of Ciucanu and Kerek (see Experimental), using a G–TG matrix, yielded the spectrum shown in Fig. 5a. Similar data were obtained for L5-F1 methylated by the Hakomori method. The MNBA matrix afforded similar data, except that sodium adducts were present also. The ion at *m/z* 2329 corresponds to the $[M + H]^+$ ion for $Hex_5HexNAc_2Hep_2Kdo$ in which all the hydroxyl, amide, and carboxylic acid groups are fully methylated. Minor ±14 satellite signals of the ion at *m/z* 2329 signal are attributed to over- and under-methylated components. It is likely that *C*-methylation of the Kdo residue results in the overmethylated species. Two additional major signals, at *m/z* 2283 and 2213, which are 46 and 116 mass units, respectively, less than the $[M + H]^+$ ion at *m/z* 2329 give the molecular ion region a characteristic appearance which has been observed in the spectra of all methylated samples containing reducing Kdo units.

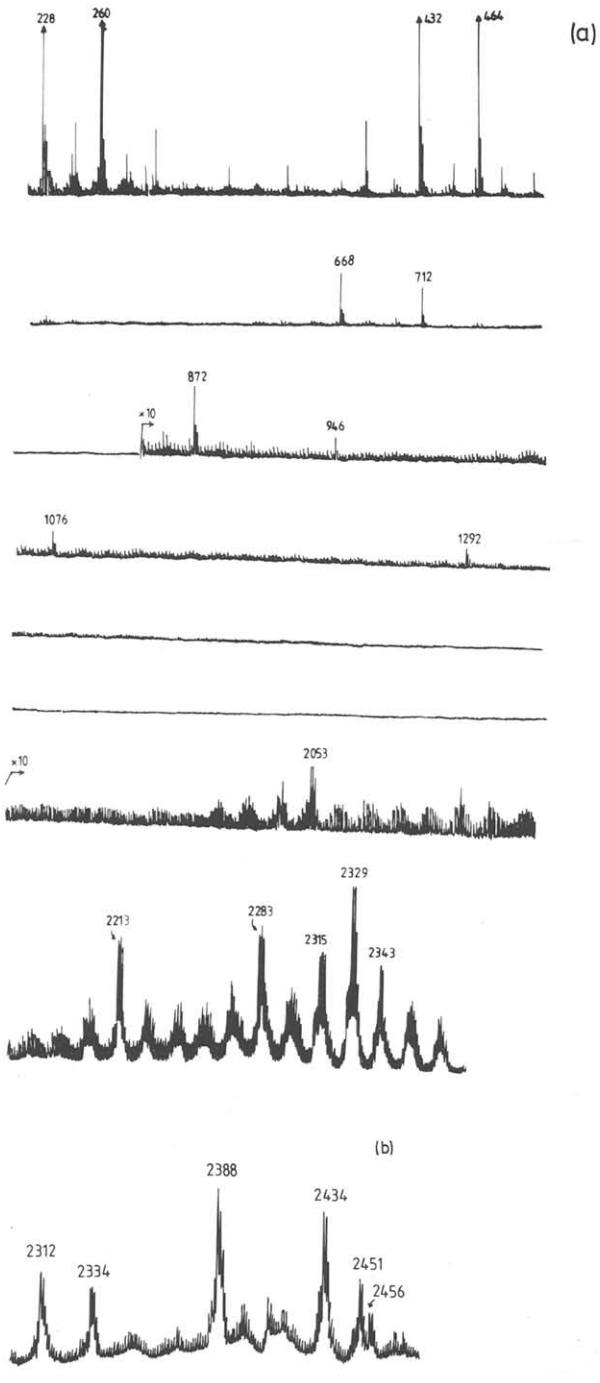
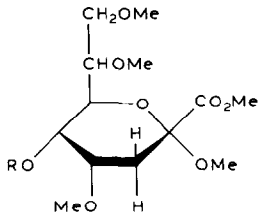
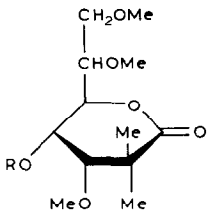


Fig. 5. Positive-ion spectra, obtained using G–TG, of (*a*) permethylated L5-F1, (*b*) deuteriomethylated L5-F1.



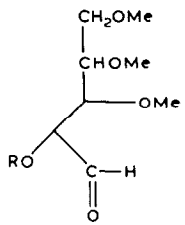
(methylated Kdo)

7



(7–46)

8



(7–116)

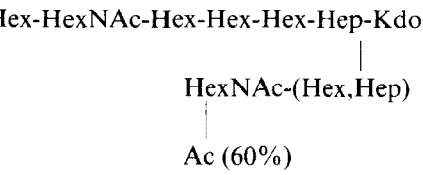
9

An obvious explanation for the ion at *m/z* 2283 is that it corresponds to the permethylated oligosaccharide that contains a lactonised Kdo residue, which differs in mass by 46 mass units from methyl-esterified Kdo. However, deuteriomethylation, carried out in order to confirm this assignment, gave an unexpected result. The molecular ion region of this derivative is reproduced in Fig. 5b. As predicted, the molecular ion for the fully methylated oligosaccharide has shifted by 105 mass units to *m/z* 2434, consistent with the introduction of 35 deuteriomethyl groups. Surprisingly, the ion at *m/z* 2283 has shifted by the same increment to *m/z* 2388, demonstrating that it cannot be the molecular ion of a lactone. The "−116" signal at *m/z* 2213 has shifted to *m/z* 2312 and therefore contains 2 fewer methyl groups than the intact molecule. These data indicate that the decrement of 46 mass units involves only atoms from the original carbohydrate skeleton, whereas the loss of 116 mass units involves two sites of methylation together with a contribution from the skeleton. This characteristic "−46" and "−116" pattern has been observed only for oligosaccharides that contain reducing Kdo units and its presence is a useful diagnostic marker. The same pattern of ions is produced by methylated L5-F1 obtained by both the Hakomori and the sodium hydroxide methods. It is proposed that the Kdo residue is partly degraded during methylation and two major products are formed. The structures **7–9** are compatible with the data, including the mass shifts on deuteriomethylation.

The minor signal at *m/z* 2053 corresponds to the $[M + H]^+$ ion of $Hex_5Hep_2HexNAc_2$, *i.e.*, without a Kdo residue. This species may have been present in the initial sample as a minor component, or could have been generated under the basic conditions used for the methylation. The latter explanation is supported by the data obtained from the deuterioacetylated derivative, formed under acid conditions, which gives similar sensitivity and no signals for molecular ions for components that lack Kdo.

A^+-type fragment ions are observed at *m/z* 260 ($HexNAc^+$), 464 ($HexHexNAc^+$) accompanied by 432 (464 − MeOH, therefore position 3 of the HexNAc residue carries OMe and not a sugar residue[13,16]), 668 ($HexHexNAcHex^+$), 712 (HexNAc-Hex,Hep^+), 872 ($HexHexNAcHexHex^+$), and 1076 ($HexHexNAcHexHexHex^+$). The signal at *m/z* 1292 is a double-cleavage ion formed by a combination of A-type cleavage at the heptose and loss of the side chain from the heptose via a β-elimination reaction. The signal at *m/z* 946 signal can be rationalised also as a double-cleavage ion that contains

the side chain and the backbone heptose, but lacks the remainder of the backbone residues. The assignments were confirmed by the appropriate mass shifts in the spectrum of the deuteriomethylated derivative. Combination of the data on the sequence ions from both the methylated and the deuterioacetylated derivatives with the composition determined in (*a*) allows assignment of the partial sequence:

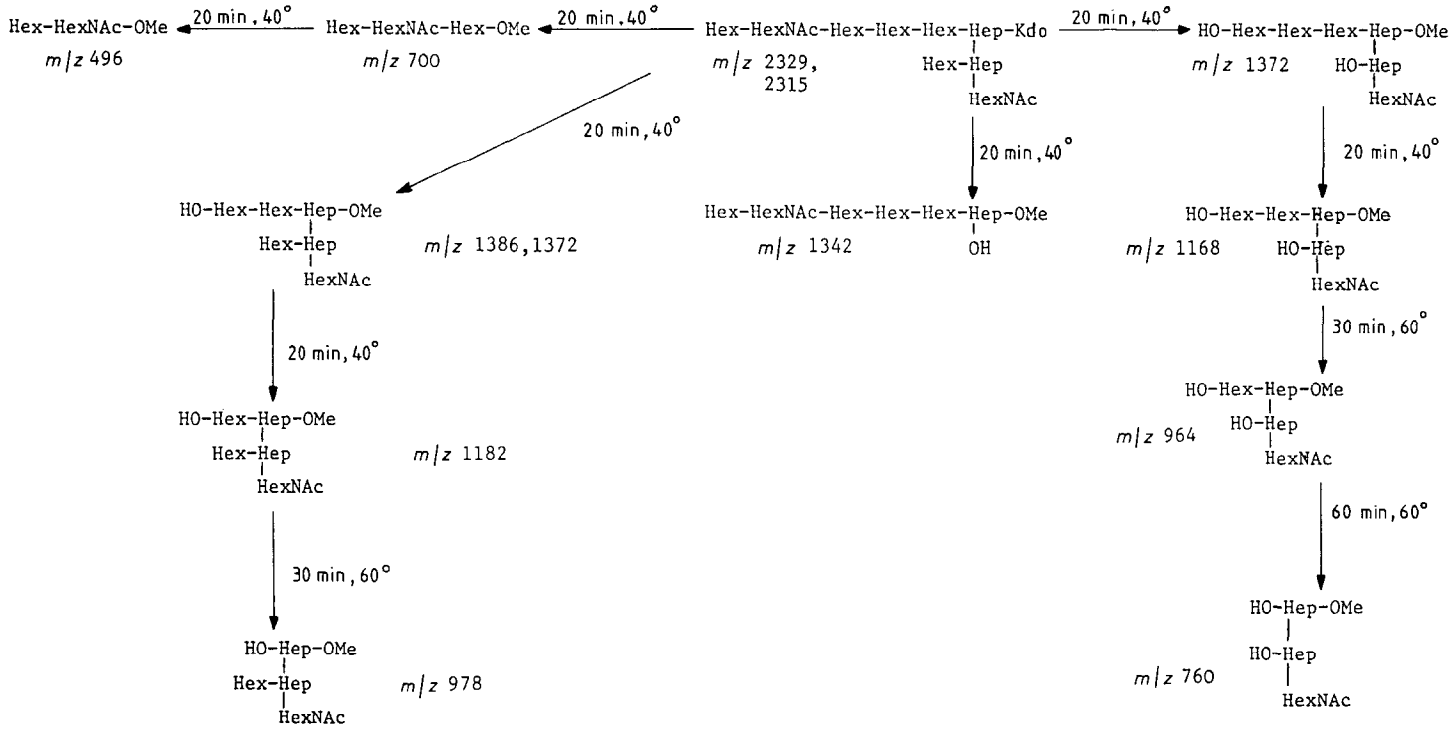


The observation of an A-type ion for Hex^+ in the spectra of the deuterioacetylated derivative and the absence of an equivalent signal for Hep^+ suggests that the second Hep is also branched. However, the absence of an ion, especially at the low-mass end of a f.a.b. mass spectrum, is not sufficient to determine the pattern of branching and so methanolysis of the remaining methylated sample is necessary.

(*d*) *Graded methanolysis.* During partial methanolysis of methylated compounds, random cleavage of glycosidic linkages occurs with the formation of methyl glycosides at newly generated reducing termini and the exposure of the hydroxyl group originally involved in the linkage. It is the mass difference between the OMe and OH groups which allows the patterns of branching to be determined, *e.g.*, loss of two residues to leave a single OH denotes a linear structure, and the generation of two OH groups indicates a branched structure. Monitoring the course of methanolysis by f.a.b.-m.s. allows data on the sequence to be obtained by following the generation of OH groups. The analysis is rapid, since aliquots of the degradation mixture are loaded directly onto the f.a.b. target. In fact, the methanolysis reagent, being mildly acidic, enhances the sensitivity of the analysis by displacing any salt ions and encouraging the generation of protonated species. Further, the ions which provide information on sequence are molecular ions rather than fragment ions, so that the problems of poor quality fragment-ion data, normally encountered at the sub-μg level, are circumvented. The data from the methanolysis studies are summarised in Scheme 1.

These data corroborate the partial sequences defined by the fragment ions in the spectra of the acetylated and methylated derivatives and, importantly, the key signal at *m/z* 1372, attributable to a molecule lacking two Hex and one HexNAc residues and carrying two free hydroxyl groups, shows that a non-reducing Hex residue is present on the side chain as shown in **2**.

Structure of L4-F1. — (a) *Underivatised material.* In the negative-ion mode (see Fig. 6), four molecular ions were observed with *m/z* 1636 ($Hex_3HexNAc_2Hep_2Kdo,P,EtNH_2$), 1618 (the lactonised form of 1636), and 1678 and 1660 for the same components bearing a single acetyl substituent. Two additional molecular ions



Scheme 1. Methanolysis data for methylated L5-F1.

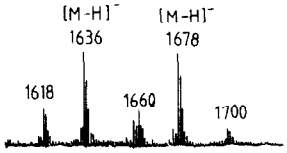


Fig. 6. Molecular ion region of negative-ion spectrum of native L4-F1, acquired in G–TG.

were observed at *m/z* 1658 and 1700, corresponding to the sodium salts of the free-acid forms at *m/z* 1636 and 1678. Sodium salts were not formed from the lactones, as expected. In the positive-ion mode, six molecular ions were observed, analogous to those in the negative-ion spectrum, namely, with *m/z* 1638 ($[M + H]^+$ for $Hex_3HexNAc_2Hep_2Kdo,P,EtNH_2$), 1620 (lactone), 1660 ($[M + Na]^+$ for the free-acid form), 1680 ($[M + H]^+$ for the free-acid form bearing one acetate), 1662 ($[M + H]^+$ for its lactone), and 1702 ($[M + Na]^+$ for the free-acid form bearing one acetate). Two additional $[M + H]^+$ ions were observed at *m/z* 1728 and 1770, which correspond to the thioesters formed by reaction of the matrix with the two lactones.

(b) Deuterioacetylated derivative. The best data for this derivative were obtained using the MNBA matrix, and a typical positive-ion spectrum is reproduced in Fig. 7. Molecular ion clusters with the spread characteristic of ions due to the mono- and non-acetylated components are present in the range *m/z* 2600–2700. The calculated [M

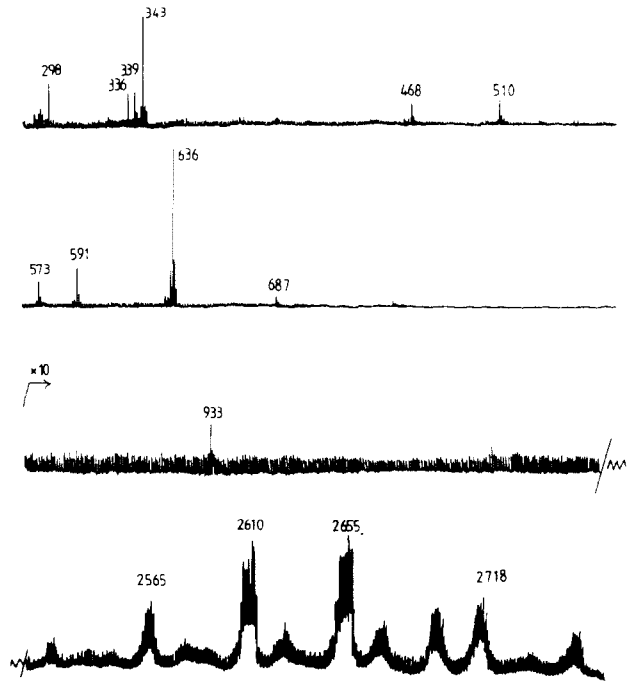


Fig. 7. Positive-ion spectrum of deuterioacetylated L4-F1, acquired using MNBA.

$+ H]^+$ ion for a deuterioacetylated molecule carrying one deuterioacetyl group on the phosphorylethanolamine moiety is *m/z* 2808. No signals were observed at this mass. Instead, the two most abundant $[M + H]^+$ ions were at *m/z* 2610 and 2655, consistent with their being A-type ions that have two and three degrees of underdeuterioacetylation, respectively. Similar results were obtained on related *Neisseria* oligosaccharides and it appears that samples containing the phosphorylethanolamine moiety are difficult to acetylate fully under acidic conditions. In a separate experiment (data not shown), when a deuterioacetylated sample was methylated, thereby replacing the *O*-deuterioacetyl groups, only a small proportion of the molecules retained a deuterioacetyl group, thus suggesting that the amino group of the phosphorylethanolamine moiety was not acetylated under acidic conditions. However, incomplete deuterioacetylation does not pose problems for interpretation or for sensitivity, and the spectra can be interpreted readily once underacetylation is recognised. The portion of the spectrum below *m/z* 1000 contains A^+-type fragment ions which yield information on the residues bearing the endogenous *O*-acetyl groups and also give some data on the sequence. The ions at *m/z* 336 and 339 have almost equal intensities and correspond to $HexNAc^+$ bearing a single endogenous acetate group and deuterioacetylated $HexNAc^+$, respectively, thus indicating that ~50% of the non-reducing HexNAc residues bear an endogenous acetyl group. The doublet (*m/z* 291/294), 45 mass units down from *m/z* 336/339, at *m/z* 291/294, represents one degree of underdeuterioacetylation. Additional information on the sequence is provided by the major ion at *m/z* 636 ($HexHexNAc^+$), and by those with *m/z* 343 (Hex^+) and 933 ($HexHexNAcHex^+$). The signal at *m/z* 298, 45 mass units down from *m/z* 343, represents a single degree of underdeuterioacetylation, whereas that at *m/z* 636 is accompanied by satellite ions at *m/z* 591 and 546 (1 and 2 degrees of underdeuterioacetylation) and 573 and 510 (loss of 1 and 2 deuterioacetic acid). The signal at *m/z* 687 is derived from a $HexHexNAc^+$ ion which carries a single trifluoroacetyl group in place of a deuterioacetyl group. This is due to the reagent being the mixed anhydride, CF_3COOAc.

F.a.b.-m.s. of the perdeuterioacetylated sample in the negative-ion mode yielded spectra containing molecular ion clusters for components having 2–5 degrees of underdeuterioacetylation. Because of the absence of sequence ions, negative-ion analysis of this derivative is not recommended as part of a sequencing strategy.

(3) Methylated derivative. Positive-ion f.a.b.-m.s. of the methylated derivative prepared by the Hakomori method (see Fig. 8) afforded data similar to those given by the product obtained by the NaOH method. A major $[M + H]^+$ ion was observed at *m/z* 2072 arising from the methylated species that contained N,N-dimethylethanolamine and a methylated phosphate ester. Over-methylated species were observed at *m/z* 2086 (intense ion), 2100 and 2114 (both much less intense than *m/z* 2072) for 1–3 degrees of over-methylation, respectively. By comparison with the behaviour of L5-F1, over-methylation is assumed to have occurred both at the nitrogen of the phosphorylethanolamine moiety and on the Kdo residue. A-type molecular ions occur 32 mass units below

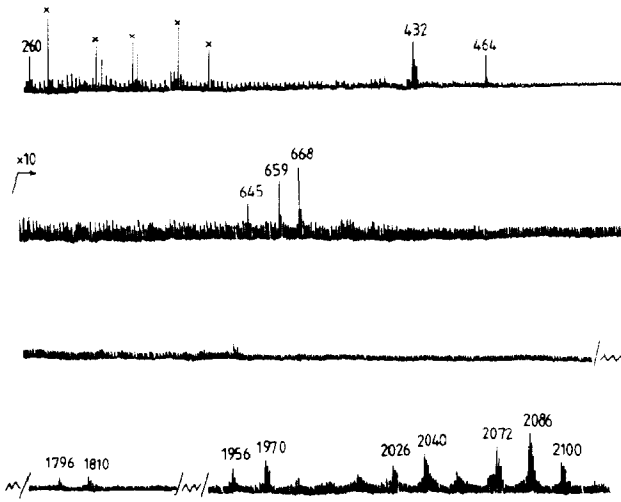


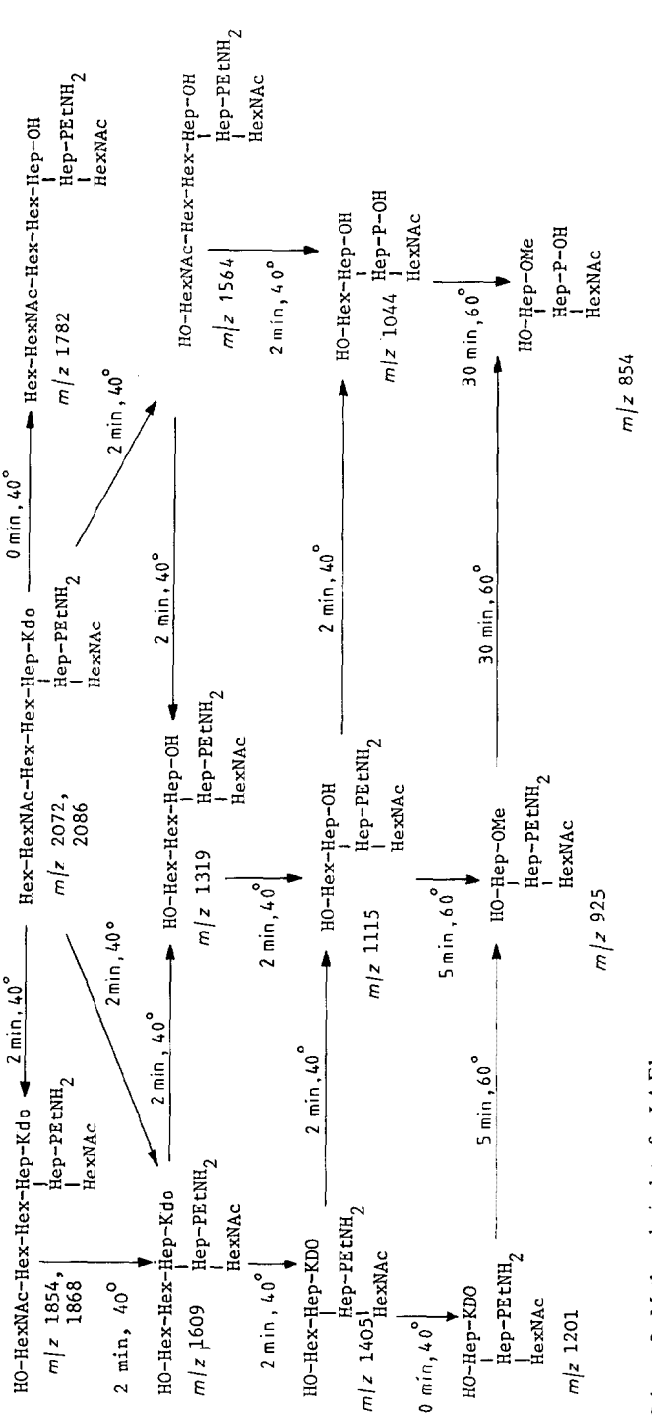
Fig. 8. Positive-ion spectrum of permethylated L4-F1, acquired using G–TG.

each $[M + H]^+$ ion. Additional intense signals at *m/z* 2012 and 2026, 46 mass units from the molecular ions, and at *m/z* 1956 and 1970, 116 mass units from the molecular ions, are rationalised as described above for L5-F1. The minor ion at *m/z* 1810 corresponds to the $[M + H]^+$ ion for the species from which the Kdo was cleaved during the base-catalysed methylation. Its absence form the spectrum of the acetylated derivative supports this assignment, as discussed above. A^+-type fragment ions were observed at *m/z* 668 ($Hex_2HexNAc^+$), 659 (HexNAc,Hep,P,$EtNH_2^+$), 464 (Hex,$HexNAc^+$), 432 (*β*-elimination of MeOH from 464, which indicates that the position 3 of the HexNAc is occupied by OMe), 260 ($HexNAc^+$), and 219 (Hex^+). These data indicate that the molecule is branched, since there are at least two sets of ions for non-reducing termini, namely, the $HexNAc^+$, HexNAc,Hep,P,$EtNH_2^+$ set, and the Hex^+, Hex–$HexNAc^+$, Hex–HexNAc–Hex^+ series. These two sets of ions account for 5 of the 8 residues, as well as the $PEtNH_2$ group, but do not define the branched residue (which must be the remaining Hep or Hex, since Kdo must be at the reducing terminus in order to generate the "−46" and "−116" ions).

Combination of the data for both derivatives allows assignments of the partial structure.

Hex-HexNAc-Hex-(Hex,Hep)-Kdo
|
HexNAc-Hep-P-$EtNH_2$
|
Ac (50%)

(d) Methanolysis. The data obtained on methanolysis of L4-F1 and shown in Scheme 2 remove any remaining ambiguities. The key signal at *m/z* 1201 corresponds to



Schema 2. Methanolysis data for I.4-F1.

a fragment with a single OH group and a composition of $Hep_2HexNAc,P,EtNH_2,Kdo$, which allows the second Hep residue to be assigned to the branch point.

The above f.a.b.-m.s. strategies for analysis of the structure of oligosaccharides containing acetyl and phosphorylethanolamine substituents and reducing terminal Kdo residues, isolated from the core of the LPS from *Neisseria meningitidis*, allowed the sequence and the extent and location of *O*-acetyl and phosphorylethanolamine groups to be established for the first time. The fast, sensitive, and convenient protocol involves f.a.b.-m.s. of methylated and acetylated derivatives and their deuterium-containing analogues, and graded methanolysis of methylated derivatives.

The f.a.b.-m.s. data show that, after methylation, the reducing terminal Kdo units afford three major components, for which we have proposed tentative structures (**4**–**6**). It is possible that the formation of such structures during methylation analysis of oligosaccharides containing reducing Kdo units could account for the reported[17] multiple peaks in g.l.c. for the Kdo residue. Although it is possible to prevent such degradation by reduction of the oligosaccharide prior to methylation[18], such a step is not necessary for f.a.b.-m.s. studies. On the contrary, the characteristic molecular ion fingerprint afforded by the three major products allows facile identification of oligosaccharides containing reducing Kdo units, and this possibility is particularly useful when mixtures of compounds are examined.

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