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Structural and immunochemical identification of Le^a, Le^b, H type 1, and related glycolipids in small intestinal mucosa of a group O Le(a–b–) nonsecretor

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Total nonacid glycosphingolipids were isolated from small intestine mucosal scrapings of a red cell blood group O Le(a–b–) nonsecretor cadaver. Glycolipids were extracted and fractionated into five fractions based on chromatographic and immunostaining properties. These glycolipid fractions were then analysed by thin-layer chromatography for Lewis activity with antibodies reactive to the type 1 precursor (Le^c), H type 1 (Le^d), Le^a and Le^b epitopes. Fractions were structurally characterized by mass spectrometry (EI-MS and EI-MS/MS-TOF) and proton NMR spectroscopy. EI-MS/MS-TOF allowed for the identification of trace substances in fractions containing several other glycolipid species. Consistent with the red cell phenotype, large amounts of lactotetraosylceramide (Le^c-4) were detected. Inconsistent with the red cell phenotype, small quantities of Le^a-5, H-5-1 and Le^b-6 glycolipids were immunochemically and structurally identified in the small intestine of this individual. By EI-MS/MS-TOF several large glycolipids with 9 and 10 sugar residues were also identified. The extensive carbohydrate chain elongation seen in this individual with a Lewis negative nonsecretor phenotype supports the concept that Lewis and Secretor blood group fucosylation may be a mechanism to control type 1 glycoconjugate chain extension.

Keywords: ***FUT2***, ***FUT3***, **Lewis blood group system, nuclear magnetic resonance spectroscopy, saliva ABH nonsecretor, small intestine, tandem mass spectrometry**

Abbreviations: ***FUT1***, ***H*** **gene; *FUT2*, *Secretor* gene, (gene bank accession no. U17894); *FUT3*, *Lewis* gene or Fuc-TIII gene, (gene bank accession no. X53578); *FUT5*, Fuc-TV gene; $[Imm]^+$, immonium ion; Le^a-5, Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Le^b-6, Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Le^c-4, Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Le^d or H-5-1, Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; Le^x-5, Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glcβ1-1Cer; MAb, monoclonal antibody; MS, mass spectrometry; CID, collision-induced dissociation; EI, electron impact ionisation; MS/MS-TOF, tandem mass spectrometry using a time-of-flight mass spectrometer as the second mass spectrometer: *m/z*, mass-to-charge ratio; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; TLC, (high performance) thin layer chromatography**

Saccharide types are abbreviated to Hex for hexose, HexNAc for *N*-acetylhexosamine and dHex for deoxyhexose (fucose). Ceramide is abbreviated to Cer, and ceramide types are abbreviated to d for dihydroxy and t for trihydroxy base, n for non-hydroxy and h for hydroxy fatty acids

Introduction

The Lewis histo-blood group system comprises two major antigens, Le^a and Le^b, which are synthesized by exocrine epithelial cells, mostly of endodermal origin. Lewis antigens are shed into the exocrine secretions and plasma, and as plasma glycolipids are acquired by cells of the peripheral circulation (reviewed in [1]). The two fucosyltransferases of Lewis and Secretor, which are responsible for the synthesis of the Le^a and Le^b glycoconjugates, have been cloned, sequenced and expressed [2, 3]. The *Lewis* gene (*FUT3*) encodes an α(1,3/4)fucosyltransferase that can add fucose to either the type 1 precursor or H type 1, to form Le^a and Le^b antigens respectively [2]. The *Secretor* gene (*FUT2*) encodes

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an α(1,2)fucosyltransferase that can add a fucose onto the type 1 precursor to form H type 1, the precursor of Le^b [3]. There are several known inactivating or partially inactivating genetic mutations of both the Lewis [4–8] and Secretor [3, 9, 10] fucosyltransferases which are responsible for the different red cell and salivary blood group phenotypes. It is generally accepted that the Lewis genotype determines if an individual will express Lewis antigens, while the Secretor genotype determines in Lewis positive individuals what type and amount of Lewis antigen will be expressed (Le^a and/or Le^b) and whether soluble ABH antigens will be found in secretions. In individuals lacking the Lewis α(1,3/4) fucosyltransferase and/or the Secretor α(1,2) fucosyltransferase the fucosylated products of these transferases are not expected. At the serological level this is true, however in immunological studies expression of Lewis antigens in different tissues and body fluids of Lewis-negative individuals has been reported [11–17]. Although it is assumed that these antigens represent the appropriate Lewis structures, their unambiguous identification has not been structurally determined. The anti-Le^b reactive structures present in the plasma of a Le(a–b–) secretor individual have been structurally proven to be Le^b [18]. In this paper the identity of Lewis and related glycolipids in small intestinal mucosa of a phenotypically Lewis-negative nonsecretor individual are immunologically and structurally characterized.

Materials and methods

Sample

Human small intestine (jejunum and ileum) was obtained from a cadaver 12 h post mortem. The donor was a 21-year-old European male killed in a motor vehicle accident. The intestine was dissected longitudinally then gently washed free of particulate matter under running tap water. The intestinal lumen was scraped with a spoon as described [19] and the resulting epithelial cells and mucus obtained were stored frozen until the glycolipids could be extracted. The blood group, immunochemistry of the total mucosal glycolipids, and immunohistochemistry of jejunum of this individual (code 529) and others of related phenotypes have been reported previously [13].

Glycolipids

Total nonacid glycosphingolipids were prepared as previously described [20] with the addition of an extra solvent of chloroform:methanol:water (40:40:12 by vol) for the final elution of all silica columns. The total glycolipids of this sample showed poor solvent solubility and consequently were not fractionated by high pressure liquid chromatography. They were instead manually fractionated in an open 5-g silica (Silica gel S 31607, Riedel-de Haën AG, FRG) column. The column was eluted (flow rate 1.5 ml min^{-1}) with 200 ml of chloroform:methanol:water (60:35:8 by vol) and 100 fractions were collected. After testing each fraction by TLC and immunostaining (using the antibodies described below), these fractions were pooled into four fractions, with similar immunostaining and chromatographic properties. A fifth fraction was eluted with 100 ml of a chloroform:methanol:water 40:40:12 (by vol). From the approximately 35 mg of total glycolipids loaded onto the column only 19 mg were recovered, indicating a large loss of sample to the column. A range of solvents of increasing polarity, including pure water, could not further elute detectable glycolipids from the column.

Thin-layer chromatography and immunostaining

High performance silica TLC glass plates (Whatman Ltd, England) loaded with 10 μg of the total fraction and 1 μg of purified fractions were chromatographed in a solvent system of chloroform:methanol:water, 60:35:8 (by vol), dried and re-chromatographed in fresh solvent. Chemical detection was done with the anisaldehyde reagent [20], and immunostaining was performed by a modification of the method of Magnani [21, 22]. Monoclonal antibodies used in the immunostaining technique were; anti-Le^{ac} 071 (clone 318/2B3 or Le^a01) from BioCarb AB, Lund, Sweden; anti-Le^{ac} 069 (clone BRIC 87) from South West Regional Transfusion Centre, Southmead, Bristol, UK; anti-Le^a CO514 from Wistar Institute, Philadelphia, PA, USA; anti-Le^{ab} 073 (clone LM129/181) and anti-Le^{bH} 075 (clone LM137/276) from Glasgow and West of Scotland Blood Transfusion Service, Law Hospital, Carluke, UK. Monoclonal antibodies were all culture supernatants and were diluted between two and ten times in phosphate buffered saline with 2% bovine serum albumin. The immunological and serological properties of these reagents have been described in detail elsewhere [23, 24]. The nomenclature used for the blood group glycolipids is as previously described [24].

Mass spectrometry

Permethylated [25] and permethylated-reduced [26] glycolipid derivatives were prepared. Mass spectra were recorded on a hybrid tandem instrument with a magnetic sector as MS-1 and an orthogonally accelerated time-of-flight device as MS-2 (AutoSpec oa-TOF, VG Analytical, Manchester, UK) [27]. EI-MS was carried out in the positive mode at 40 eV electron energy, 250 μA trap current, source temperature of 150 °C, probe tip ramped from 50–400 °C at 25 °C min^{-1}, mass range 100–2900 mass units, and a scan time of 10 s per decade. Positive daughter ion spectra of the selected resolved monoisotopic ion peak produced by EI in the first MS was recorded in the TOF mass spectrometer at a lab frame collision energy of 800 eV, a push-out rate of 30 kHz in 2 s time intervals, and Ar as collision gas. The instrument was precalibrated in positive liquid secondary ion mass spectrometry using CsI prior to EI experiments. The mass range (0–4000 mass units) of the

TOF mass spectrometer was calibrated by collision induced dissociation of the 1954 m/z of the CsI cluster ion. In addition, all spectra were averaged over selected areas, background subtracted and peak detected using the Opus software facilities. Mass-to-charge values quoted in the text and in Figs. 2, 4, 5, 7 and 8 are experimental, while the values cited on the schematic drawings in these figures are calculated monoisotopic ^{12}C values.

Nuclear magnetic resonance spectroscopy

Native glycolipid fractions were deuterium exchanged in excess $CHCl_3$:CD_3OD (2:1 by vol) and then dissolved in approximately 0.5 ml d6-DMSO:D_2O (98:2 by vol) for proton NMR analysis at 60 °C. Spectra were recorded at 9.4 T (400 MHz) on a modified Varian XL 400 (Varian, Palo Alto, CA, USA), at 11.7 T (500 MHz) on a JEOL Alpha 500 (JEOL, Tokyo, Japan) or at 14.1 T (600 MHz) on a Varian Unity 600 (Varian, Palo Alto, CA, USA) with a digital resolution better than 0.5 Hz per point using 90° pulses, but no relaxation delay. Chemical shifts are given relative to tetra methyl silane (TMS), sometimes using the internal solvent peak. Processing was done off-line using the NMR1 program (New Methods Research Inc., Syracuse, NY, USA). Resolution enhancement was achieved using either a Lorentz-to-Gauss transformation, a squared shifted sinebell window or Maximum Entropy calculations as implemented in NMR1. Two-dimensional data were recorded using standard pulse sequences provided with the instrument.

Molecular biology

DNA was extracted from blood obtained at autopsy. Secretor genotyping was performed as described [3]. Lewis genotyping for the known point mutations at nucleotides 59, 202, 314, 508 and 1067 [4–8] were tested for as described [7]. To allow for further genetic investigation FUT3 PCR amplified fragments (nucleotides −20 to 1159) were cloned into the pCRII vector according to the manufacturer (Invitrogen, San Diego, CA). Several clones were isolated and Lewis genotyped as described [7]. Analysis of the purity of the DNA sample was performed using 11 different highly polymorphic markers from the Genethon [28] and the CHLC [29] genetic maps.

Results

Phenotype and genotype

The phenotype of this individual is Le(a–b–) nonsecretor as previously described [13]. The Secretor genotype was determined by PCR-RFLP and homozygous expression of the se^{G428A}, FUT2 nonsecretor confirmed the nonsecretor status [3]. The Lewis *FUT3* genotype was complex and inconclusive [7]. Initial typing of genomic DNA produced some unexpected weak reactions. By cloning four different Lewis alleles were identified. They were: allele 1 containing known mutations T59 → G, T202 → C, and T1067 → A [4, 5, 7]; allele 2, containing the 59 and 1067 mutations; alleles 3 and 4 each containing only the 59 or 1067 mutations respectively. The cloning results were consistent with the results originally detected on the genomic DNA but atypical of those expected for a Lewis genotype. The possibility that our DNA sample was contaminated with DNA from another person (*eg* from transfusion etc) was likely, and supported by additional weak bands seen with the polymorphic probes (not shown). The transfusion history of this patient prior to death could not be obtained.

Glycolipids

Thirty-five mg of the total glycolipid extract was manually fractionated on an open silica column and only 19 mg was recovered. Because of known solubility problems, it was assumed that the glycolipids irreversibly lost to the column represented mainly lactotetraosylceramide and the extended structures. The weights of the fractions recovered may not therefore be representative of the proportion of each fraction in the total sample, however the fractions recovered do appear to be representative of those seen in the total sample (Figure 1, plates I–VI). Glycolipids were separated by TLC and stained with the chemical reagent anisaldehyde (Figure 1, plate I) which gives a characteristic green colour for glycolipids [20]. The epithelial cell scraping of this individual was essentially free from stroma tissue, as supported by the presence of very little di- and triglycosylceramides which are major non-epithelial glycolipids [11, 19]. Mono, tetra and extended glycolipids (approx 9–10 sugars) were the dominating glycolipids.

Fraction 1

Representing mainly mono-, di- and triglycosylceramides, contained no Lewis blood group activity, and was not further analysed.

Fraction 2

Glycolipids migrated on the TLC plate predominantly as tetraglycosylceramides. These glycolipids were reactive only with the anti-Le^{ac} reagents in the 4 sugar region (Figure 1). EI-MS and NMR analyses showed that this fraction contained predominantly lactotetraosylceramide (Le^c-4) [30] and globotetraosylceramide (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-1 Cer or globoside) in a 2:1 ratio (results not shown). No neolactotetraosylceramide (Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-1Cer) was detected by NMR.

Fraction 3

Glycolipids migrated on the TLC plate as tetra- and pentaglycosylceramides (Figure 1). This fraction was reactive with the anti-Le^{ac} reagents in the 4- and 5-sugar regions, with the anti-Le^a reagent in the 5-sugar region, and the anti-Le^{ab} and anti-Le^{bH} reagents in the 5 and 6-sugar regions, suggesting

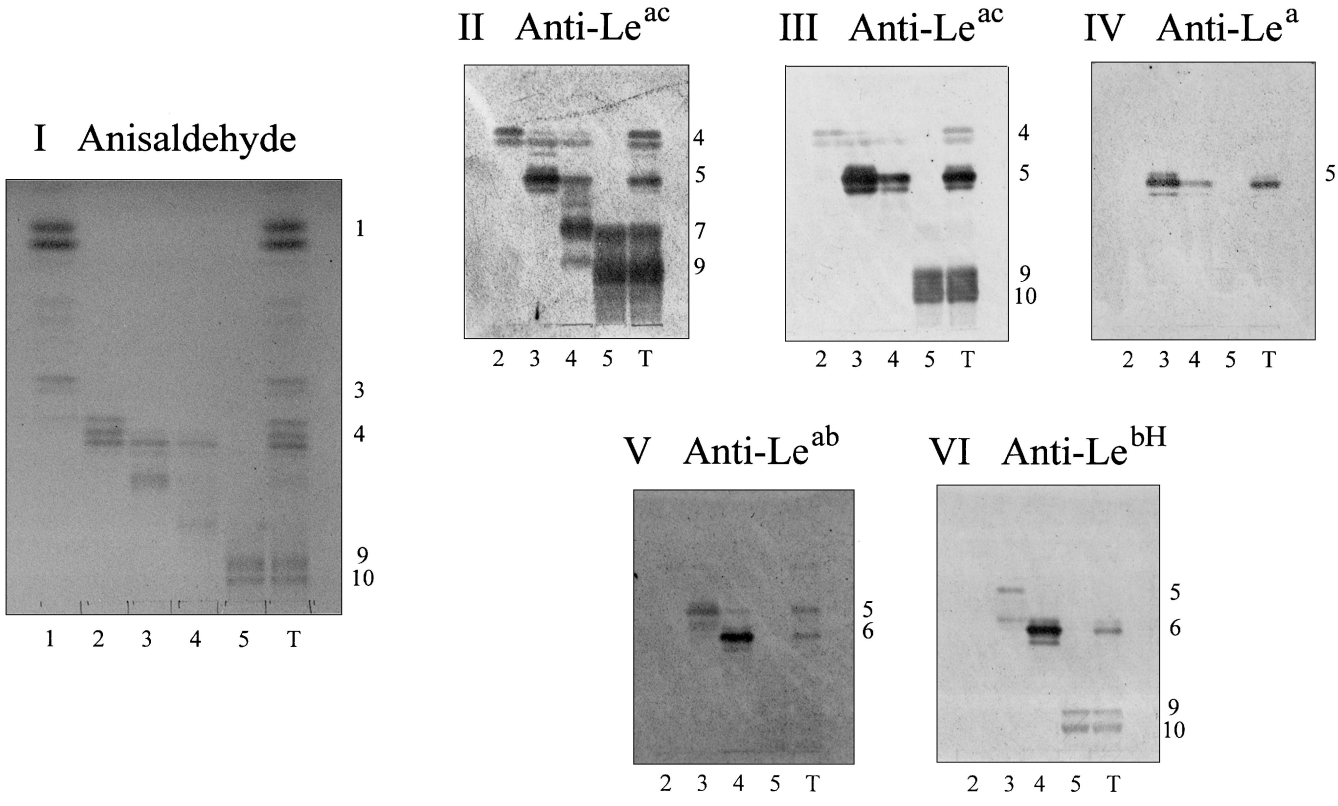

Figure 1. Thin-layer chromatographic analysis of nonacid glycolipid fractions isolated from small intestinal mucosa of a blood group O Le(a–b–) nonsecretor individual. Lanes labelled 2–5 indicate the manual silica column fractions of the prefractionation total sample (Lane T) as summarized in Table 1. The numbers on the right hand side of the chromatograms indicate the approximate number of sugar residues in each glycolipid band. It should be noted that the numbers indicating glycoconjugate sizes are only approximations, and fucosylation, branching and structure all affect TLC migration. Chemical staining for glycolipids with the reagent anisaldehyde is shown in plate I. The antibodies used for immunostaining were; plate II = 071 anti-Le^{ac}, plate III = 069 anti-Le^{ac}, plate IV = CO514 anti-Le^{a}, plate V = 073 anti-Le^{ab}, plate VI = 075 anti-Le^{bH}.

the presence of Le^{c}-4, Le^{a}-5, H-5-1 and traces of Le^{b}-6 glycolipids.

EI-MS of this methylated-reduced fraction produced peaks, that were interpreted as immonium ions, at *m/z* 1360, 1444 and 1472 (Figure 2). These peaks are consistent with monofucosylated pentaglycosylceramides composed of one deoxyhexose, one hexosamine and three hexoses with h16:0, h22:0 and h24:0 fatty acyl chains, respectively. Alternatively, these peaks could be assigned to pentaglycosylceramides composed of one hexosamine and four hexoses with n16:0, n22:0 and n24:0 fatty acyl chains, respectively. The presence of a large peak at *m/z* 624 corresponding with one deoxyhexose, one hexose and one hexosamine suggests the presence of a terminal trisaccharide containing deoxyhexose. Also present were peaks at *m/z* 1186, 1270 and 1298 which are consistent with the immonium ions of tetraglycosylceramides composed of one hexosamine and three hexoses with h16:0, h22:0 and h24:0 fatty acyl chains, respectively. The large peak at *m/z* 450 is consistent with oxonium ions from a terminal structure containing both a hexose and hexosamine residue. The CID spectrum of the *m/z* 1186 peak (not shown) produced expected fragments (experimental values) at *m/z* 450.2 corresponding with a hexose and hexosamine fragment, and at *m/z* 654.2 corresponding with a fragment with two hexoses and a hexosamine. A large peak was also seen at *m/z* 828.2 which is explained by a characteristic internal split of the hexose linked to the ceramide moiety [31].

The CID spectrum of the *m/z* 1359.2 peak is shown in Fig. 2. The presence of a peak at *m/z* 312.1 and absence of a peak at *m/z* 282 indicates the presence of an h16:0 fatty acid and the absence of an n16:0 fatty acid. The carbohydrate chain can thus be expected to be composed of one deoxyhexose, one hexosamine and three hexoses. The peak at *m/z* 624.2 indicates the presence of a terminal trisaccharide composed of one deoxyhexose, one hexose and one hexosamine, while the peak at *m/z* 828.1 indicates the same structure but with an additional hexose. It was not possible to distinguish to which terminal sugars of the disaccharide the deoxyhexose was linked. *M/z* 1169.2 is consistent with the whole immonium ion which has lost the deoxyhexose moiety and a proton, *m/z* 1032.2 is consistent with the complete terminal pentasaccharide, while *m/z* 1002.2 is explained by a characteristic internal split of the hexose linked

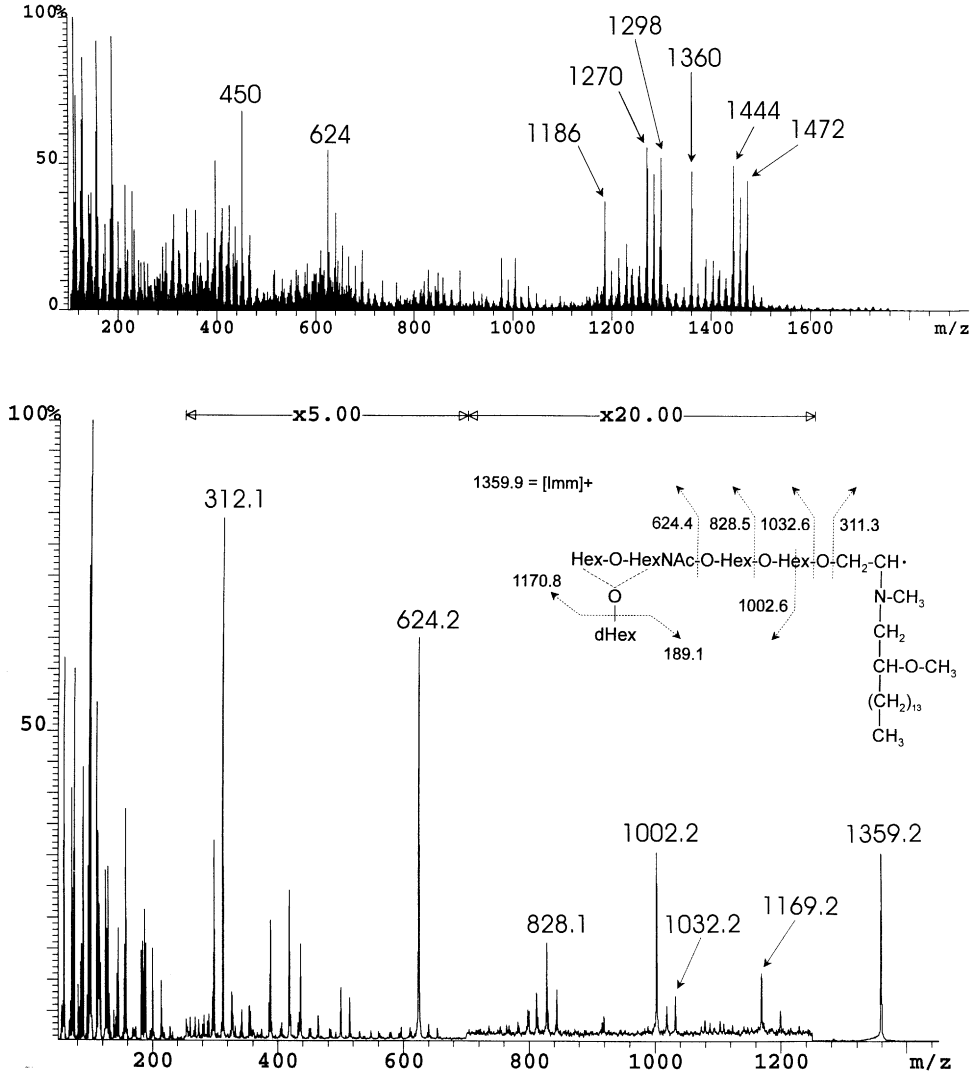

Figure 2. Mass spectrometry of the permethylated-reduced fraction 3. The upper spectrum shows the major peaks seen by EI-MS of this fraction. Peaks noted at *m/z* 450 and 624 correlate with saccharide oxonium ions, while the peaks at *m/z* 1186 and 1270 correlate with the immonium ions of an h16:0 and h22:0 tetraglycosylceramide. The peaks at *m/z* 1360, 1444 and 1472 correspond with h16:0, h22:0 and h24:0 immonium ions from a monofucosylated pentaglycoslyceramide. The lower spectrum is the EI-MS/MS-TOF collision-induced-dissociation of the *m/z* 1359.2 ion. The fragmentation pattern is indicative of a fucosylated pentaglycosylceramide with an h16:0 fatty acyl chain. In the inset schematic drawing the values cited are calculated monoisotopic ^{12}C mass values and the dHex may be linked in either of two positions, as indicated by the broken line.

to the lipid moiety [31]. Similar results were obtained for the EI-MS peaks seen at *m/z* 1444 and 1472, but with the expected h22:0 and h24:0 fatty acyl fragments. By NMR, major signals from Le^c-4 were seen in the anomeric region (Fig. 3). The β-doublet ($J_{1,2} = 8.2$ Hz) at 4.80 ppm, overlapping with two smaller signals (*vide infra*), together with a β-signal ($J_{1,2} = 6.5$ Hz) at 4.15 ppm are easily recognized as belonging to Le^c-4. The signals from the internal lactose unit of Le^c-4 are also present, at 4.28 ppm and 4.22/4.20 ppm. These resonances have contributions from all glycolipids present, since they all contain this internal lactose unit. The chemical shifts are in agreement with previously published data [32]. The remaining large signals in the anomeric region can be assigned to Le^x-5. An α-doublet ($J_{1,2} = 4.3$ Hz) from the fucose resonates at 4.88 ppm and the corresponding methyl doublet is present at 1.02 ppm. The fucose H5 quartet is also easily identified at 4.58 ppm. The internal β-GlcNAc H1 signal is seen at 4.75 ppm ($J_{1,2} = 7.5$ Hz) and the terminal galactose is assigned to a β-signal at 4.29 ppm. The relative ratio of the Le^c-4 to Le^x-5 was estimated as 1:1.

From the smaller signals in the anomeric region and the signals around 1 ppm, two more structures were identified. The H-5-1 carbohydrate moiety was identified from an α-signal ($J_{1,2} < 3$ Hz) at 5.00 ppm assigned to the terminal fucose, and two β-signals; the first at 4.63 ppm arising from

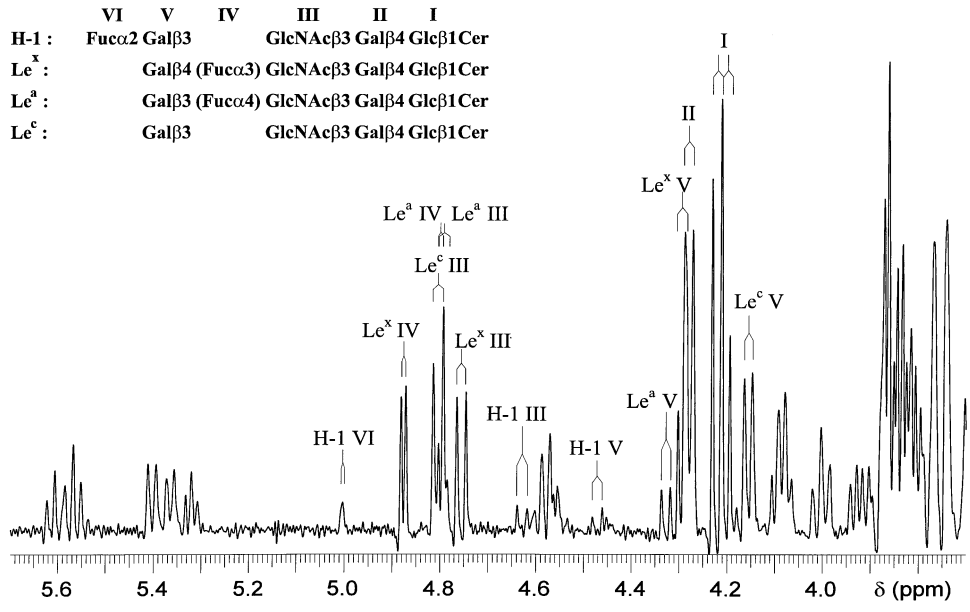

Figure 3. Anomeric region of a 400 MHz proton NMR spectrum of native glycolipids present in fraction 3. Signals identifying the Le^c-4, Le^a-5, Le^x-5, and H-5-1 (Le^d-5) structures are indicated.

the internal *N*-acetylglucosamine, and the second at 4.47 ppm belonging to the penultimate galactose. The presence of an Le^a-5 structure was indicated from two partially overlapping signals, one with an α-coupling and the other with a β-coupling, at 4.80 ppm and 4.79 ppm respectively. These signals were also overlapping with an Le^c-4 signal (*vide supra*). A third signal from the Le^a-5, a β-doublet ($J_{1,2} = 6.5$ Hz), was seen at 4.33 ppm. Both the H-5-1 and the Le^a-5 saccharides should have fucose methyl-signals in the 1.0–1.2 ppm region. At 1.07 and 1.06 ppm two such signals of suitable intensity were seen, but they could not be assigned specifically to either of the two fucoses. All signals were in good agreement with published values [18, 32, 33].

After EI-MS, MS/MS-TOF and NMR analyses this fraction was concluded to contain lactotetraosylceramide (Le^c-4), Le^x-5, Le^a-5, and H-5-1 glycolipids (estimated ratio 11:10:3:2 by NMR, respectively). Traces of Le^b-6 were immunochemically noted but could not be structurally identified due to low abundance.

Fraction 4

Glycolipids migrated on the TLC plates as tetra- to hepta-glycosylceramides (Figure 1). This fraction was reactive with the first anti-Le^{ac} reagent (plate II) in the 4–9 sugar regions, and with the second anti-Le^{ac} reagent (plate III) weakly in the 4- and strongly in the 5-sugar regions. Like the two anti-Le^{ac} reagents the anti-Le^a reagent detected reactivity in the 5-sugar region representative of Le^a-5. Reactivity with the anti-Le^{ab} reagent was seen in the 5- and 6-sugar regions and with the anti-Le^{bH} reagent in the 6-sugar region. These results suggest the presence of Le^c-4, Le^a-5, Le^b-6 and a 7-sugar glycolipid bearing Le^a and/or Le^c epitopes.

The EI-MS spectrum of this methylated-reduced fraction gave, as in fraction 3, peaks at *m/z* 1444 and 1472 characteristic of monofucosylated pentaglycosylceramides (Figure 4). Additional peaks, interpreted as immonium ions, were seen at *m/z* 1534, 1618 and 1646. These peaks are consistent with difucosylated hexaglycosylceramides composed of two deoxyhexoses, one hexosamine and three hexoses with h16:0, h22:0 and h24:0 fatty acyl chains, respectively. Another set of peaks, also interpreted as immonium ions, were seen at *m/z* 1879 and 1907. These peaks probably correspond with monofucosylated heptaglycosylceramides, composed of one deoxyhexose, two hexosamines and four hexoses with h22:0 and h24:0 fatty acyl chains, respectively (Figure 5). All of the above peaks are assigned to structures with hydroxylated ceramides, but could theoretically be explained by changing one of the deoxyhexoses for hexose and the fatty acid to a nonhydroxylated form. The CID spectrum of the peak at *m/z* 1645.9 is shown in Figure 4. The presence of a peak at *m/z* 424.2 and absence of a peak at *m/z* 394 indicates the presence of an h24:0 fatty acid and the absence of an n24:0 fatty acid. The carbohydrate chain can thus be expected to be composed of two deoxyhexoses, one hexosamine and three hexoses. The peak seen at *m/z* 798.4 is consistent with the terminal tetrasaccharide being composed of two deoxyhexoses, one hexose and a hexosamine, while the same structure but with an additional hexose produces a peak at *m/z* 1002.5. *M/z* 1176.6 is explained by the saccharide chain and a characteristic internal split of the

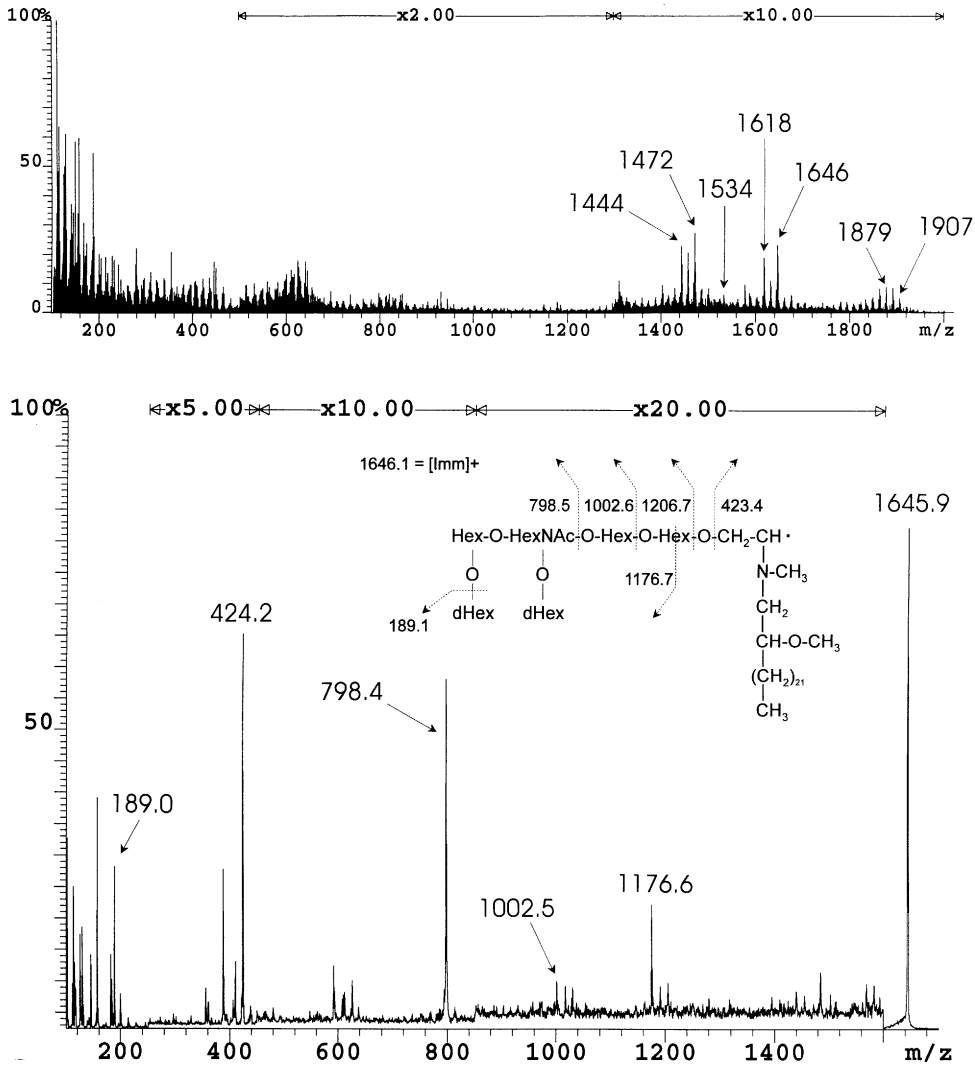

Figure 4. Mass spectrometry of the permethylated-reduced fraction 4. The upper spectrum shows the major peaks seen by EI-MS of this fraction. Peaks noted at *m/z* 1444 and 1472 correspond with h22:0 and h24:0 immonium ions from a monofucosylated pentaglycosylceramide (as in Figure 2). The peaks at *m/z* 1534, 1618 and 1646 correspond with h16:0, h22:0 and h24:0 immonium ions from a difucosylated pentaglycosylceramide. The peaks found at *m/z* 1879 and 1907 correspond with h22:0 and h24:0 immonium ions from monofucosylated heptaglycosylceramides (as in Figure 5). The lower spectrum is the EI-MS/MS-TOF collision-induced-dissociation of the *m/z* 1645.9 ion. The fragmentation pattern is indicative of a permethylated-reduced difucosylated hexaglycosylceramide with an h24:0 fatty acyl ceramide. In the inset schematic drawing the values cited are calculated monoisotopic ^{12}C mass values.

hexose linked to the lipid moiety [31]. Similar results were obtained for the EI-MS peak seen at *m/z* 1618, but with the expected h22:0 fatty acyl fragments.

The CID of the *m/z* 1878.9 is shown in Figure 5. The presence of a peak at 396.3 and absence of a peak at *m/z* 366 indicates the presence of an h22:0 fatty acid and the absence of an n22:0 fatty acid. The carbohydrate chain is thus expected to be composed of one deoxyhexose, two hexosamines and four hexoses. The peak at *m/z* 450.2 indicates a terminal disaccharide composed of hexose and hexosamine. The peak at *m/z* 624.2 indicates the presence of a terminal trisaccharide composed of one hexose, one hexosamine and one deoxyhexose. There is thus evidence of two terminal trisaccharide sequences. This could either be explained as a mixture of two different isomeric structures, or a branched structure. *M/z* 828.2 indicates a terminal tetrasaccharide composed of one deoxyhexose, one hexosamine and two hexoses indicative of the upper structure in the schematic diagram of Figure 5. This structure is consistent with the subterminally fucosylated structure (GL-3) described by Hanfland in the plasma of Le(a–b–) nonsecretors [34]. *M/z* 1059.4 indicates the addition of one hexosamine, *m/z* 1263.6 indicates the addition of a hexose and *m/z* 1437.3 is a typical complete saccharide fragment of

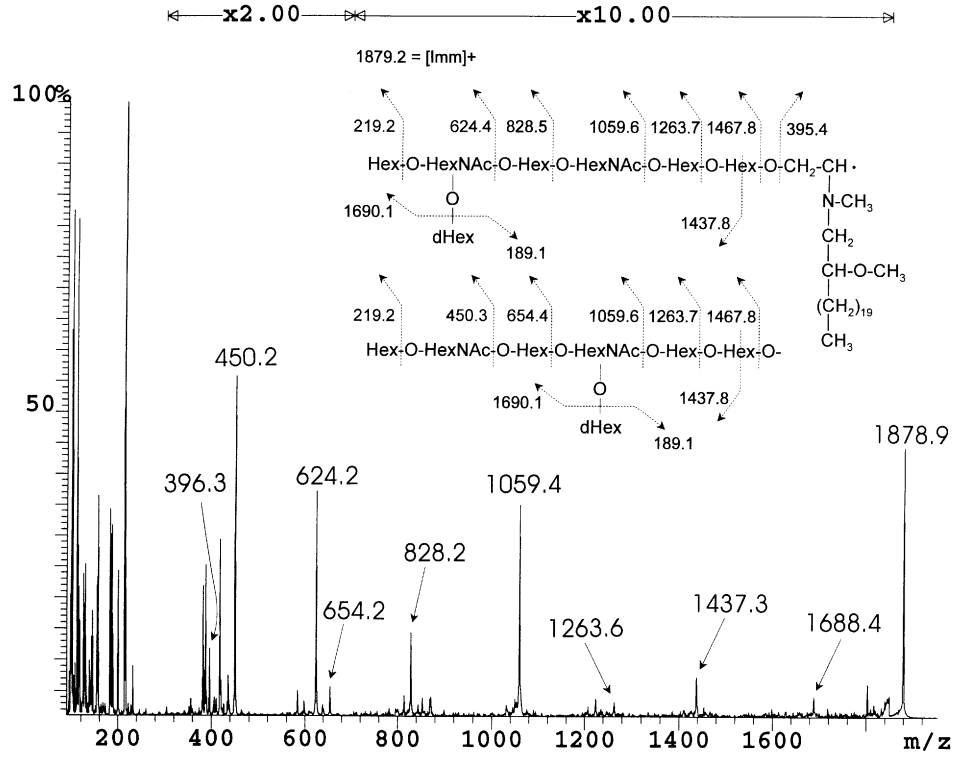

Figure 5. EI-MS/MS-TOF collision-induced-dissociation of the *m/z* 1878.9 ion present in fraction 4 (Figure 4). The fragmentation pattern is indicative of two permethylated-reduced monofucosylated heptaglycosylceramides with h22 : 0 fatty acyl ceramides. In the inset schematic drawing the values cited for the two different structures present are calculated monoisotopic ^{12}C mass values.

the proximal lipid-linked hexose [31]. *M/z* 1688.4 is most probably explained by the parent ion losing a deoxyhexose and a proton. The peaks at *m/z* 450.2 and 654.2 are indicative of the lower structure in the schematic diagram of Figure 5. The other peaks at *m/z* 1059.4, 1263.6, 1437.3 and 1688.4 can be explained by either of the schematic structures given in Figure 5. From the CID-TOF spectrum in Figure 5 it could be concluded that at least two isomeric heptaglycosylceramides were present, each with the deoxyhexose in a different position. The position of the deoxyhexose in the upper schematic diagram could not be concluded by mass spectrometry to be attached to the hexosamine. It could equally well be linked to the terminal hexose. Likewise in the structure shown in the lower schematic diagram there is ambiguity concerning the sequence of the terminal disaccharides. The possibility of a third monofucosylated heptaglycosylceramide, where the deoxyhexose is attached to the third sugar from the nonreducing end, cannot be excluded. Similar results identifying these two structures by EI-MS/MS-TOF were also obtained for the *m/z* 1907 ion (results not shown).

In the NMR spectrum (Figure 6) the dominating species is Le^c-4 with peaks at 4.80, 4.28, 4.22 and 4.15 ppm, all having a large (approx. 8 Hz) spin-spin coupling, identical to the peaks seen in the previous fraction. At approximately the 20% level of Le^c-4, signals from the Le^x structure are present. These signals appear at 4.88, 4.75, 4.29, 4.28, and 4.22 ppm. One can also identify the fucose methyl doublet at 1.02, but the H5 quartet is difficult to assign among the many peaks around 4.6 ppm. New, compared to the previous fraction, are α-signals at 4.92 and 4.78 ppm and β-signals at 4.65 and 4.54 ppm corresponding to a Le^b-6 structure. These signals are assigned to Fucα2, Fucα4, GlcNAcβ3 and Galβ3 respectively (see Figure 6). The lactosyl unit signals appear at their normal shifts of 4.28 and 4.22 ppm.

Just above the detection limit of the NMR-experiment many signals in the anomeric region are present. Spectra were recorded at three different temperatures (30, 50, and 60 °C) to assist with interpretation and a 'reporter' signal at 4.33 ppm belonging to H1 of the terminal Galβ3 of Le^a was concluded (as in the previous fraction). At 5.21 ppm an additional small α-signal can be found. Due to the low intensity of this signal in a relatively complex spectrum it could not be assigned to any specific structure, although internal α2 linked fucoses in a type 3 chain environment usually result in these large downfield shifted anomeric signals.

The heptaglycosylceramide structures identified by EI-MS/MS-TOF could not be conclusively identified by NMR.

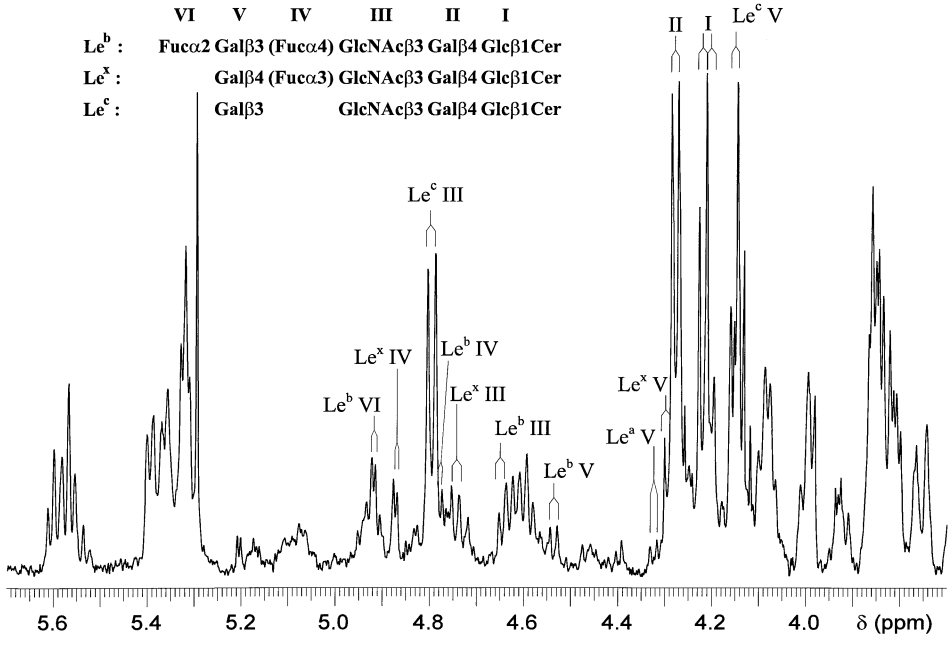

Figure 6. Anomeric region of a 500 MHz proton NMR spectrum of native glycolipids present in fraction 4. Signals identifying the Le^c-4, Le^x-5, and Le^b-6 structures are indicated.

An externally fucosylated seven sugar Le^a structure (Figure 5, upper schematic) will have the same reporter signals as a five sugar Le^a structure, and only the intensity of the lactosamine signals will change. Likewise a very internally fucosylated seven sugar structure (Figure 5, lower schematic) will generate signals at chemical shifts similar to the terminal Le^a epitope [35, 36]. Thus the only signal indicating an elongated internally fucosylated structure will be galactose, onto which elongation has taken place. Comparisons with the extended Le^x and Le^a structures would suggest such a signal between 4.35 and 4.40 ppm which could correspond to the small doublet seen at 4.40 ppm (Figure 6).

After EI-MS, MS/MS-TOF and NMR analyses this fraction was concluded to contain lactotetraosylceramide (Le^c-4), Le^x-5, Le^b-6, and Le^a-5 glycolipids (estimated ratio 22:5:3:1 by NMR, respectively). The heptaglycosylceramides, which were of low abundance, and identified by EI-MS/MS-TOF to be at least two different monofucosylated structures could not be concluded by NMR.

Fraction 5

Glycolipids migrated on the TLC plate as nona and larger (poly)glycosylceramides (Figure 1). This fraction was strongly reactive with the MAb 071 anti-Le^{ac} reagent (plate II) in the 7 and 9 sugar region with smearing of reactivity all the way from the origin. The MAb 069 anti-Le^{ac} reagent (plate III) was only reactive in the 9–10 sugar region. The wide banding pattern seen on plate III in the 9–10 sugar region suggests that more than one glycolipid structure is present. There was no reactivity with the anti-Le^a reagent (CO514) which has been previously shown by TLC not to react with the Le^c epitope on the Le^c-4 precursor [24]. This suggests reactivity seen with the Le^{ac} reagents may be due to the detection of Galβ1-3GlcNAc epitopes on extended structures. This fraction was unreactive with the anti-Le^{ab} reagent, but reacted in the 9–10 sugar region with the anti-Le^{bH} reagent. The lack of reactivity of the anti-Le^{ab} reagent suggests that the reactivity seen with the anti-Le^{bH} reagent is due to extended structures with H type 1 determinants.

EI-MS of this methylated-reduced fraction was complex because of the mixed nature and size of the glycolipids in this fraction (Figure 7). Peaks found at *m/z* 1879 and 1907 correlated with the heptaglycosylceramide described in fraction 4 (this was confirmed by CID). Peaks found at *m/z* 2231, 2259, 2315 and 2342 are consistent with the immonium ions of a branched monofucosylated nonasaccharide with one deoxyhexose, three hexosamines, five hexoses and h16:0, h18:0, h22:0 and h24:0 fatty acyl chains respectively (Figure 7). This structure is probably the nonaglycosylceramide originally described by Hanfland [34] to be in the plasma of Le(a–b–) nonsecretors. In the EI spectrum four other peaks are seen at *m/z* 2404, 2460, 2488 and 2517, which correspond with a difucosylated branched decasaccharide with h16:0, h20:0, h22:0 and h24:0 fatty acyl chains. This structure is based on the nonasaccharide but with an additional deoxyhexose. Alternative structures to the above could also exist but these were excluded with the assistance of EI-MS/MS-TOF. The peak seen at *m/z* 1280 was found by CID to be the terminal hexasaccharide of the

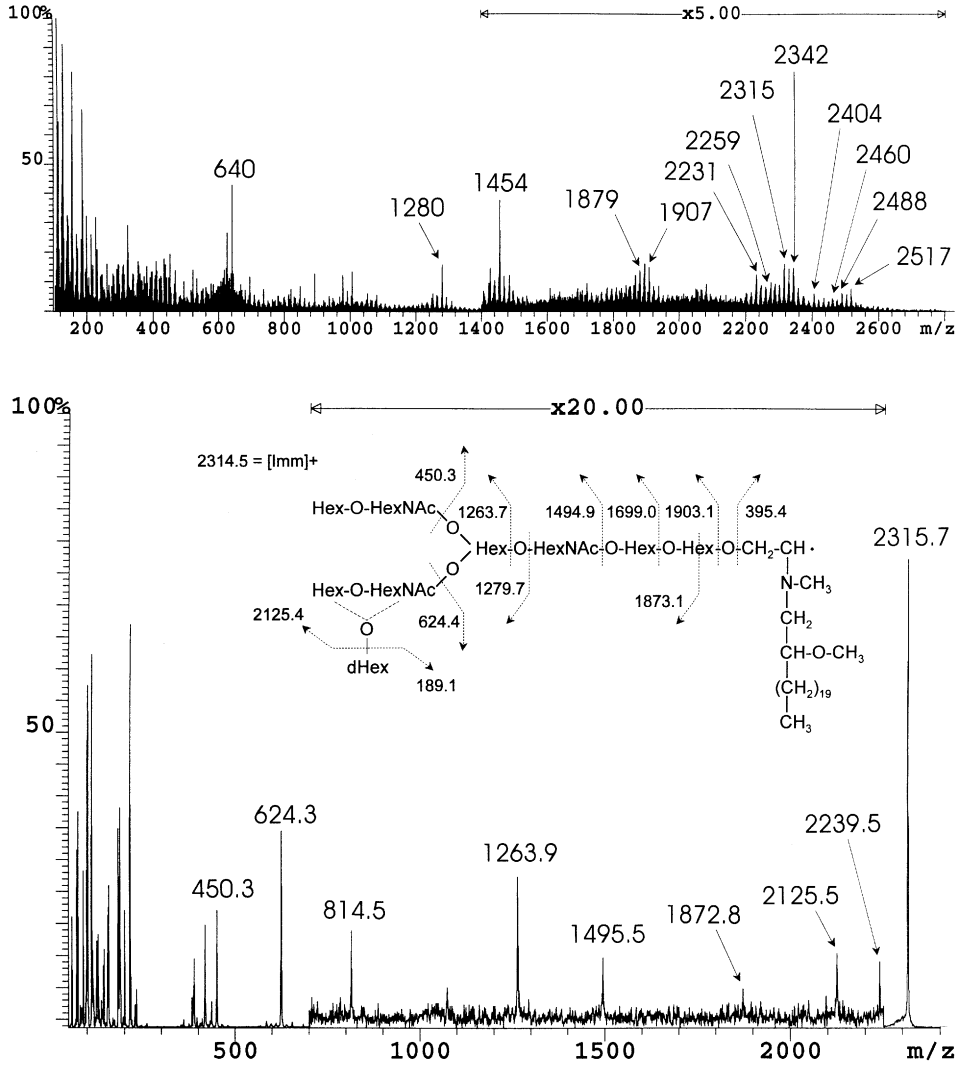

Figure 7. Mass spectrometry of the permethylated-reduced fraction 5. The upper spectrum shows the major peaks seen by EI-MS of this fraction. Peaks noted at *m/z* 1879 and 1907 correspond with h22:0 and h24:0 immonium ions from a monofucosylated pentaglycosylceramide (Figure 5). The peaks at *m/z* 2231, 2259, 2315 and 2342 correspond with h16:0, h18:0, h22:0 and h24:0 immonium ions from a monofucosylated branched nonaglycosylceramide. The peaks at *m/z* 2404, 2460, 2488 and 2517 correspond with h16:0, h20:0, h22:0 and h24:0 immonium ions from a difucosylated branched decaglycosylceramide (Figure 8). The lower spectrum is the EI-MS/MS-TOF collision-induced-dissociation of the *m/z* 2315.7 ion. The fragmentation pattern is indicative of a permethylated-reduced monofucosylated nonaglycosylceramide with an h22:0 fatty acyl ceramide. In the inset schematic drawing the values cited are calculated monoisotopic ^{12}C mass values and the dHex residue may be linked in either of two positions, as indicated by the broken line.

nonasaccharide, while the peak at *m/z* 1454 was found to be the terminal heptasaccharide of the decasaccharide (not shown). By identifying the major saccharide fragments of the two proposed structures present, the alternative possibilities of replacing a deoxyhexose for hexose and changing the fatty acid to a nonhydroxylated form, could be eliminated. Unlike the glycolipids with shorter carbohydrate chains, suggestive fragments derived from the fatty acyl chain were not present in the CID spectra of these extended structures.

The peak at *m/z* 2315.7, which is consistent with the immonium ions of a branched monofucosylated nonasaccharide with an h22:0 fatty acyl chain, was subjected to CID (Figure 7). Peaks corresponding to oxonium ions for two different terminal structures were seen, one containing hexose and hexosamine at *m/z* 450.3 and the other hexose, hexosamine and deoxyhexose at *m/z* 624.3. As stated previously these two different terminal structures could represent either different isomeric structures or a branch. It was concluded that they belonged to a branch, because fragmentation of the *m/z* 1280 ion resulted in both the *m/z* 450 and 624 peaks. The peak seen at *m/z* 1263.9 also corresponds with the hexasaccharide branch, and the peak at *m/z* 1495.5, the same branch but with an additional hexosamine.

The peak seen at *m/z* 814.5 probably represents fragmentation through the branch, with the fragment detected being derived from the branched saccharide which has lost hexose-hexosamine (*m/z* 1279.7 − 450.3 − 16 = 813.4 + 1). Absence of a similar peak from the non-fucosylated branch at *m/z* 639.3 suggests that this fragmentation may be linkage specific or affected by the deoxyhexose. It is generally accepted that this structure is type 1 on the non-fucosylated branch, and type 2 on the fucosylated branch [34]. Consistent with previous fragmentations, peaks were found at *m/z* 2125.5 consistent with the immonium ion which had lost deoxyhexose, and *m/z* 1872.8 consistent with the whole saccharide chain and a characteristic internal split of the lipid-linked hexose [31]. The peak seen at *m/z* 2239.5 was not assigned. Similar CID results identifying a branched monofucosylated nonasaccharide were obtained for peaks seen at *m/z* 2231, 2259 and 2342.

Figure 8 shows the CID results for the peak at *m/z* 2516.7, which is consistent with the immonium ions of a branched difucosylated decasaccharide with an h24:0 fatty acyl chain. Peaks corresponding to oxonium ions of only one terminal structure, consisting of hexose, hexosamine and deoxyhexose, were seen at *m/z* 624.3. This was supported by the absence of peaks at *m/z* 450 and 799 which would correspond to oxonium ions for a terminal structure containing hexose and hexosamine, and the other with hexose, hexosamine and two deoxyhexoses respectively. Further evidence that this structure was predominantly monofucosylated on both branches, came from CID of the terminal heptasaccharide branch peak at *m/z* 1454 which also did not reveal any evidence for difucosylation of a single branch (not shown). It was not possible to distinguish whether the deoxyhexose was linked to either the hexose or the hexosamine. The peak at *m/z* 1436.6 is consistent with the heptasaccharide branch, and the peak at *m/z* 1669.5 to the same branch but with an additional hexosamine. As with the nonaglycosylceramide, the peak seen at *m/z* 814.5 probably represents a similar fragmentation through the branch, with the fragment detected being derived from the branched saccharide which has lost a deoxyhexose-hexose-hexosamine arm (*m/z* 1437.8 − 624.4 = 813.4 + 1). The parent ion also produced peaks at *m/z* 2326.4 consistent with a loss of deoxyhexose and a proton, and *m/z* 2047.4 consistent with the whole saccharide chain and a characteristic internal split of the hexose linked to the lipid moiety [31]. Similar CID results, identifying the difucosylated branched decaglycosylceramide were obtained for the peaks at *m/z* 2404, 2460 and 2488.

The NMR spectrum of this sample was very complex and further purification is required before full interpretation can be made. Analysis of a 600 MHz proton NMR spectrum of this mixed fraction (not shown), did however indicate some substructures. The major determinant is Le^x, as indicated by a large α-signal at 4.88 ppm. Other Le^x signals are heavily

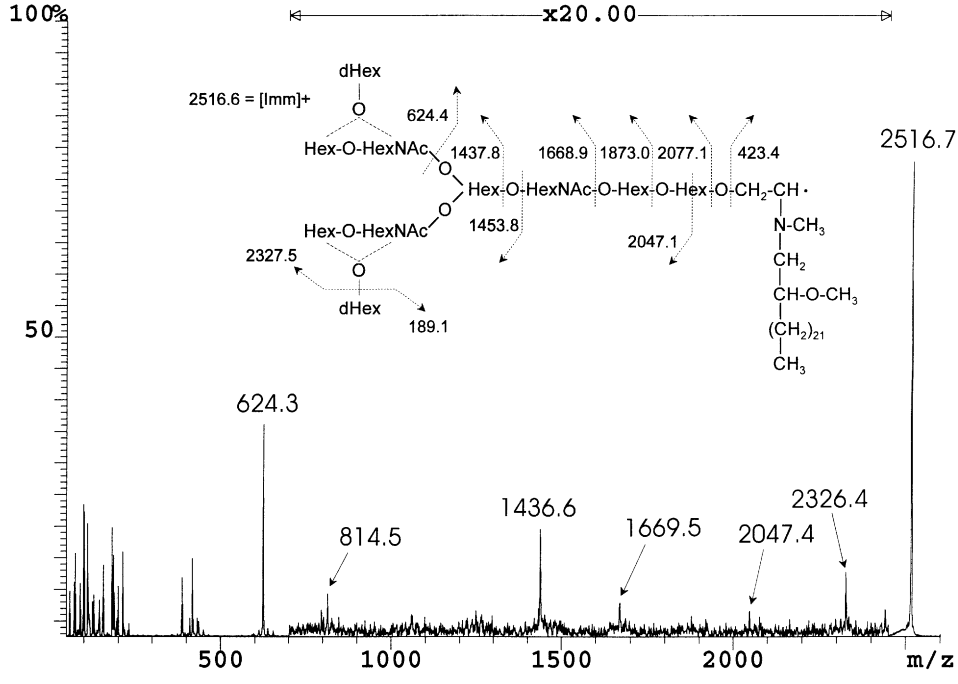

Figure 8. EI-MS/MS-TOF collision-induced-dissociation of the *m/z* 2516.7 ion present in fraction 5. The fragmentation pattern is indicative of a permethylated-reduced difucosylated decaglycosylceramide with an h22:0 fatty acyl ceramide. The absence of a peak at *m/z* 450 indicates the dHex residues are present on both branches and not both on a single branch. In the inset schematic drawing the values cited are calculated monoisotopic ^{12}C mass values and the dHex residues may be linked in either of two positions, as indicated by the broken line.

overlapped. Another large α-signal is present at 4.85 ppm, and it is also identified as a Fucα3 signal. It's slight up-field shift could indicate it residing on a six-linked branch. Branching is deduced from two β signals at 4.49 and 4.54 ppm. Neither of these are GalNAc as the chemical shifts of the C-2 protons are below 3.6 ppm [37]. Their positions do not correspond with the shifts for six-linked branches seen in B-i series glycolipids [38], but taking into account the fucosylation induced down-field shift of GlcNAc anomeric proton signals, these signals are indicative of branched structures which are fucosylated at least on the six-linked GlcNAc. A smaller part of this fraction is also made up of a non-GlcNAc-fucosylated six-linked branch (GlcNAcβ6 H1 at 4.42 ppm). From the 2D-COSY spectrum it is also evident that a Fucα4 moiety (4.78 ppm) is present. A normal Le^a would also have a GlcNAcβ signal at 4.77 ppm, but this is not seen. The result here is consistent with the Le^a trisaccharide being six-linked to the branching Gal. Of further interest is the presence of small Fucα2 anomeric signals at 5.03 ppm and 4.99 ppm, which can be assigned to H type 2 and H type 1 respectively. The small size of these signals makes it impossible to find either the H1-H2 cross-peaks in the COSY-spectrum, or the other anomeric signals in the 1D spectrum.

After EI-MS, MS/MS-TOF and limited NMR analyses this fraction was concluded to contain several large glycolipids including the heptaglycoslyceramides seen in fraction 4. NMR data suggests that the major structures present in this fraction are branched and fucosylated. MS/MS-TOF data concluded the presence of the nonaglycosylceramide structure originally described in the plasma of Le(a–b–) nonsecretor individuals [34]. In addition, a branched difucosylated decaglycosylceramide was clearly identified. On the basis of immunochemistry, the reported structure of the nonaglycosylceramide [34], and an NMR reporter signal for H type 1, it is probable that some of the decaglycosylceramides have H type 1 epitopes. A small part of this fraction also contains the H type 2 determinant. It was additionally evident from the NMR analyses that a Le^a epitope is present on a branched structure. This is not seen with the anti-Le^a reagent CO514 (Figure 1, plate IV), but is suggested by the wide banding pattern seen with the anti-Le^{ac} reagent (plate III). The difference in reactivity may be due to a shielding of the epitope by the opposite branch to the CO514 reagent. This fraction is very complex, contains several variants of a similar motif, and further fractionation is required to accurately resolve all the structures present.

Discussion

Glycolipid structures of the gastrointestinal epithelium are mainly based on type 1 chains and fucosylated under the control of the *Secretor* and *Lewis* genes. The expression of type 1 ABH and Lewis glycolipids in the small intestine is in concordance with the red cell phenotype of the donor [11, 13].

In Lewis negative nonsecretor individuals no functional Lewis or Secretor fucosyltransferases are inherited, and it is generally accepted that these individuals do not express Lewis or salivary ABH antigens. The Lewis negative phenotype is however not an all-or-nothing state, as exemplified by the traces of Lewis activity which can be immunohistochemically demonstrated in some tissues, [e.g. 11–16].

The amount of Le^a and/or Le^b antigens made by Lewis negative individuals is however relatively small, e.g. in the glycolipids isolated from the plasma of a Le(a–b–) secretor it has been estimated that the amount of Lewis antigens made was about 5% of that made by a Lewis positive individual (or about 0.5% of the total glycolipids) [18]. These traces of Lewis antigens are serologically undetectable on the red cell, but they can be immunochemically

Table 1. Nonacid glycolipid fractions isolated from small intestinal mucosa of a Le(a–b–) nonsecretor individual. Fraction 1 containing mono-, di- and triglycosylceramides was not tested (nt). Immunoreactivity and identities of Lewis related glycolipids found in fractions 2–5 and the unfractionated sample (lane T), based on thin-layer chromatography, immunostaining, EI-MS, EI-MS/MS-TOF and NMR. Different ceramide species found are not shown.

Fraction	mg	Immunoreactivity					Lewis related glycolipids structurally identified				
		-Le^{ac} MAb 071	-Le^{ac} MAb 069	-Le^a MAb CO514	-Le^{ab} MAb 073	-Le^{bH} MAb 075	Le^c-4	Le^x-5	Le^a-5	H-5-1	Le^b-6
1	10.3	nt	nt	nt	nt	nt					
2	4.0	+	+	–	–	–	✓				
3	0.4	+	+	+	+	+	✓	✓	✓	✓	
4	0.3	+	+	+	+	+	✓	✓	✓		✓
5	4.0	+	+	–	–	+					
T	19.0										

demonstrated in plasma and red cell glycolipid extracts [24, 39]. Likewise, Lewis antibody neutralizing substances can be serologically detected in Le(a–b–) saliva [17]. The difficulty in defining the presence of Lewis antigens in a biological sample is that all Lewis reactive antibodies cross-react to some extent with type 1 and/or type 2 Lewis-related structures which may also be present. As a consequence, reactivity with a Lewis reagent cannot be assumed to reflect the presence of a particular Lewis antigen in a sample. Structural identification is thus required to confirm the presence of unexpected Lewis antigens.

This paper describes an investigation of the Lewis reactive nonacid glycolipids in small intestinal mucosa of a phenotypically Le(a–b–) nonsecretor individual. The secretor negative status of this sample was confirmed by homozygous expression of the se^{G428A}, nonsecretor mutation [3]. The Lewis genotype was complex and inconclusive, with four different Lewis alleles being identified by cloning. Three of the four *FUT3* alleles identified are known Lewis negative alleles, while the fourth expressed the T59 → G loss-of-enzyme-anchoring mutation alone [4, 5, 7]. The possibility that the DNA of this individual was contaminated or had acquired foreign DNA during medical treatment from a transfusion was likely (medical records were not available). This was supported by the presence of additional bands when the DNA was tested for polymorphic markers. It was not established which alleles belonged to this individual, however the DNA of this individual is currently undergoing further genetic analysis. Despite the inconclusive Lewis genotype, the red cell phenotype and glycolipid profiles were those expected of a Lewis negative nonsecretor [24].

Previously it has immunohistologically been shown for this Le(a–b–) nonsecretor individual that although Le^a and Le^b were absent from the brush border, anti-Le^b reactivity was detected in the area of Golgi apparatus in the deep areas of the Leiberkühn's crypts [13]. No immunohistological evidence for Le^a or H type 1 was found. It should however be noted that immunohistology of paraffin embedded sections probably detects only glycoproteins, with glycolipids being lost during deparaffination. On the basis of TLC immunochemistry of the glycolipids isolated from the mucosal scrapings of this individual, reactivity with antiserum suggested the presence of trace amounts of Le^a-5 and Le^b-6 [13].

The final structural identification of the manually fractionated glycolipids in this individual rely on a combination of techniques including separation by thin-layer chromatography, immunostaining with highly characterized antibodies, EI-MS, EI-MS/MS-TOF, together with diagnostic proton NMR analyses. As expected and consistent with the accepted biosynthetic pathway for the formation of Lewis antigens, this individual expressed mainly Le^c-4 (lactotetraosylceramide) and extended glycolipids [24]. Inconsistent with the red cell phenotype, small amounts of Le^a-5, H-5-1, and Le^b-6 were also structurally identified. Le^a-5 was identified on the basis of immunoreactivity with three different anti-Le^a reagents, MS fragments indicative of a fucosylated pentaglycosylceramide and NMR signals diagnostic of the Le^a structure. H-5-1 was identified on the basis of immunoreactivity with an anti-Le^{bH} reagent known to detect the H type 1 epitope, MS fragments indicative of a fucosylated pentaglycosylceramide (although the peaks could also belong to other related structures) and NMR signals diagnostic for an H type 1 structure. Le^b-6 was identified on the basis of immunoreactivity with two different anti-Le^b reagents, MS fragments indicative of a difucosylated hexaglycosylceramide and NMR signals diagnostic of the Le^b structure. Although each structure identified was present in small quantities in a mixture and often not unambiguously identified by any one procedure, the data together are sufficient to confidently assign the structures.

Because of the loss of sample to the fractionation column it is difficult to be certain of the actual amount of Le^a-5, Le^b-6, and H-5-1 glycolipid antigen present in this sample, however it is reasonable to estimate the combined amount of these 'inappropriate' antigens as being less than 1% of the total sample. This is similar to the relative amount (0.5%) present in the plasma of a Le(a–b–) secretor [18].

The glycolipids present in group O Le(a–b–) nonsecretors have previously been studied in pooled plasma. In this previous study glycolipids were isolated, fractionated and subjected to structural analysis in an effort to define the Le^c antigen [34]. Another analysis of the pentaglycosylceramides from the same preparation identified H type 2 and Le^x [40]. Neither of these investigations report detecting Le^a-5, H type 1, or Le^b-6 glycolipids. The discrepancy between these reports and the results here possibly represents the different biological tissues being studied. More probably, the differences seen represent the different strategies taken. Here, we deliberately pooled fractions on the basis of TLC mobility and immunoreactivity so that fractions containing trace amounts of Lewis antigens could be resolved. Alternative strategies, pooling not on the basis of immunoreactivity, would probably pool the trace amounts of Lewis active glycolipids in with other more predominant glycolipids, thereby masking them.

In addition to the Lewis-related structures described above, two different monofucosylated heptaglycosylceramides were determined by MS/MS-TOF. The first, which is probably subterminally fucosylated, may represent the structure GL-3 described by Hanfland [34]. The second, where the innermost hexosamine appears to be fucosylated, has not been previously described in the plasma of Le(a–b–) nonsecretors. This structure, to the best of our knowledge, has not been previously described in a biological sample, although type 1 and type 2 forms of it have been prepared by enzymatic modification of larger structures [35, 41]. The structure described here is possibly responsible for the immunostaining seen with the first anti-Le^{ac} reagent via its

terminal Le^c epitope. This is supported by the observation that the internally fucosylated type 1 Le^a structure (III^4FucLc_6) prepared by Stroud *et al.* [35] did not react with anti-Le^a, but reacted instead with antibodies directed to the unsubstituted type 1 precursor. Unfortunately our glycolipid was present only in very trace amounts in a mixed fraction, and without NMR results complete structural determination, including monosaccharide identification, chain type and linkage was not possible.

Also present in this sample were extended branched structures. Although the fraction was too mixed for unambiguous NMR structural determination, basic structures were determined by MS/MS-TOF. The monofucosylated branched nonasaccharide originally described by Hanfland [34] with a variety of hydroxylated ceramides was concluded to be present. In addition, this structure, but with a second fucose on the opposite branch (and also with a range of hydroxylated ceramides), was also found. NMR signals for H type 1 and H type 2 together with immunochemistry for H type 1 suggest that some of the decaglycosylceramides have H epitopes. These structures have not previously been reported in Lewis negative individuals but a similar structure, having H on both branches, is known in the rat small intestine [42]. The fucosylated nona and decaglycosylceramides were the only extended structures which could be found by MS in this fraction.

In this group O individual, on the basis of weight and anisaldehyde staining, the amount of extended structures is estimated at about 20% of the total. Unfortunately, because of the loss of a large amount of material to the column, this value is only approximate, and probably underestimated. The amount is however considerably more than that isolated from the small intestinal glycolipids from individuals of the Le(a−b+) and Le(a+b+) phenotypes [13]. The results here therefore further support the concept that Secretor and Lewis fucosylation may be an important control mechanism of saccharide extension and branching. It is also of interest to note that there was only a small amount of glycolipid between the 4 and 9 sugar regions (as visualized by anisaldehyde), and essentially no Lewis reactive epitopes were present in this area. The immunochemical and structural results suggest that branching is favoured and the branched precursor is a suitable precursor for fucosylation (by non-Lewis/Secretor fucosyltransferases) into the 9–10 sugar glycolipids detected. All extended structures found were fucosylated, which suggests that fucosylation is a signal for the termination of saccharide chain elongation. The only major non-fucosylated blood group related structure found in this individual was lactotetraosylceramide. It is probable that a large amount of lactotetraosylceramide exists unmodified because the branching enzyme in an individual of this phenotype is unable to cope with the large amount of this precursor, and consequently it passes through the Golgi before it can be modified. In most individuals lactotetraosylceramide is converted into Lewis or ABH related structures, with very little being extended or branched [24].

It is also important to consider whether the extended structures identified may reflect to some extent the efficiency of the methodology used to isolate extended structures. Alternative methodology, such as that used to isolate polyglycosylceramides [43], may find other extended structures.

The three glycolipids Le^a-5, H type 1, and Le^b-6 identified are not expected in this Lewis-negative nonsecretor individual. The formation of H type 1 antigens in this individual, who genetically does not have a functional Secretor transferase, must therefore involve fucosyltransferases other than Secretor. The formation of small amounts of H-5-1 and extended H is probably a result of H transferase activity which has been shown to fucosylate the type 1 precursor, but with a much lower efficiency than that of the Secretor transferase [44]. It seems clear that because of 'redundancy' and 'degeneracy' in the specificity of glycosyltransferases [45], trace amounts of almost any glycoconjugate could occur in individuals lacking particular transferases. The 'inappropriate' expression of Lewis antigens is not so clear because of the inconclusive Lewis genotype of this individual. It is possible that the allele with only the T59 → G loss-of-enzyme-anchoring mutation belongs to this individual (although this mutation alone is not known in Europeans), and is making the trace Lewis antigens detected [4]. It is not known whether this occurs, or whether some other transferase is responsible for the Lewis antigens formed i.e. FUT5 [46]. It should be noted that small amounts of Lewis antigens are seen also in the plasma of other Lewis negative nonsecretors [24].

In this paper we also show the usefulness of a novel tandem mass spectrometry technique, that is EI-MS/MS-TOF, to resolve the structures of complex methylated-reduced glycolipid derivatives in biological mixtures. The structures of trace and/or ambiguous (e.g. normal and hydroxy fatty acids vs hexose and deoxyhexose) ion peaks seen in MS-1 were able to be easily identified by CID. Furthermore, CID produces a number of sequence oxonium ions, which in most cases will define sugar sequence, branching positions and even isomeric forms. It is expected that new information like binding positions will eventually emerge from these spectra once reference substances have been analysed.

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