SEQUENCING OF OLIGOSACCHARIDES BY MASS SPECTROMETRY APPLIED ON A 12-SUGAR GLYCOLIPID

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1. Introduction

Protein or lipid-bound oligosaccharides of the cell surface are at present of great research interest as they are targets for antibodies [1] and toxins [2] and are supposed to take part in intercellular recognition processes [3]. A problem has been the lack of chemical methods for the specific detection of separate saccharide sequences on a microscale. We will show that mass spectrometry is applicable for the microscale sequencing also of large saccharides. Although one would expect less success in this respect for saccharide [4] than for peptide [5], due to a higher mass, more functional groups, different kinds of linkage (position and configuration), branching of the chain and the heterocyclic structure of the saccharide monomers, a careful selection of derivatives and analysis conditions makes the characterization of even as large a molecule as a dodecasaccharide possible. This glycolipid represents the largest biomolecule structurally interpreted directly from mass spectrometry.

2. Materials and methods

The glycolipid used for analysis was isolated from epithelial cells of rat small intestine and showed strong blood group A activity (M. E. B., G. C. H., K.-A. K., H. L., in preparation). Two derivatives have been selected as the most suitable for electron impact mass spectrometry of glycosphingolipids, analysed pure [6–9] or in mixtures [10]. They are obtained by permethylation of the sample and by reduction with $LiAlH_4$ of the permethylated derivative. Spectra of the two derivatives supplement each other on important points. To obtain the heavier ions from this large glycolipid the in-beam technique was found essential, where the direct inlet probe is introduced in close proximity of the electron beam [11–13]. With the standard technique, ions above *m/e* 1000 from this glycolipid were not detectable although glycolipids with less sugars (up to 9) produced molecular ions [9]. Mass numbers were obtained by counting up by hand. Due to the large extent of hydrogens (mass 1.008) the exact masses in the higher region are higher by one unit than the nominal masses reproduced. The natural abundance of ^{13}C explains that the isotope peaks (nominal peaks plus one) dominate in the region above *m/e* 2000.

3. Results and discussion

The spectrum of the reduced derivative (fig.1) shows a series of rather intense peaks at *m/e* 2835–2977. These were derived from the complete saccharide chain and varying fatty acids (see top formula with proposed structure for one molecular species). With support from other ions (see below) one may conclude that the composition was 5 hexoses, 5 hexosamines and 2 fucoses and 2 series of fatty acids, one with non-hydroxy fatty acids with: 16 (*m/e* 2835), 20 (*m/e* 2891), 22 (*m/e* 2919), 23 (*m/e* 2933) and 24 (*m/e* 2947) carbon atoms; and the other with hydroxy fatty acids with: 16 (*m/e* 2865), 20 (*m/e* 2921), 22 (*m/e* 2949), 23 (*m/e* 2963) and 24 (*m/e* 2977) carbon atoms. The dominating long-chain base was phytosphingosine as shown by *m/e* 396 from the non-reduced derivative (fig.2). Therefore one of the major molecular species had the composition shown by the formulae.

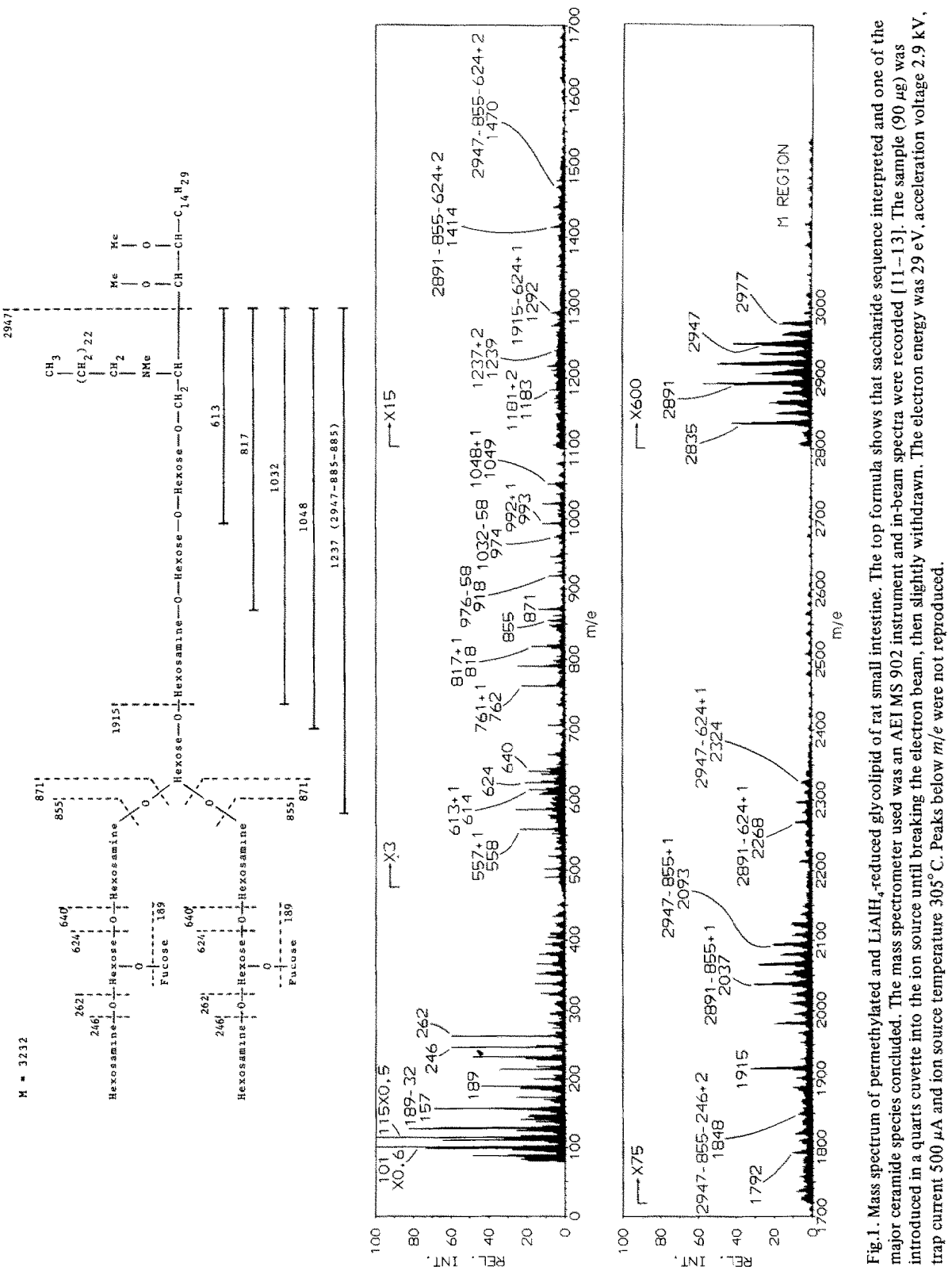

Fig.1. Mass spectrum of permethylated and $LiAlH_4$-reduced glycolipid of rat small intestine. The top formula shows that saccharide sequence interpreted and one of the major ceramide species concluded. The mass spectrometer used was an AEI MS 902 instrument and in-beam spectra were recorded [11–13]. The sample (90 μg) was introduced in a quarts cuvette into the ion source until breaking the electron beam, then slightly withdrawn. The electron energy was 29 eV, acceleration voltage 2.9 kV, trap current 500 μA and ion source temperature 305°C. Peaks below *m/e* were not reproduced.

Evidence for the proposed sequence was obtained from both spectra. The reduced derivative gave peaks corresponding to a successively increasing part of the saccharide chain from the non-reducing end, at *m/e* 157, 189, 246, 262, 624, 640, 855, 871 and 1915. Before reduction (fig.2) the amino sugars are 14 units heavier, thus an analogous series of peaks is found at *m/e* 157, 189, 228, 260, 606, 638, 851, 883, 1955, 2168 and 2200. The absence of peaks in these 2 series for oligosaccharides with 5–8 sugar residues is an argument against a linear structure and favours the branching point indicated. Support of this may be taken from re-arrangement ions of the reduced derivative explained below the formula of fig.1. These contain the fatty acid and an increasing part of the saccharide. Addition of one hydrogen, probably at the cleaved glycosidic bond, explains the following triplets of peaks due to 20, 22 and 24 carbon non-hydroxy fatty acids:

For one sugar at *m/e* 558, 586 and 614, for two sugars at *m/e* 762, 790 and 818;

For 3 sugars at *m/e* 993, 1021 and 1049;

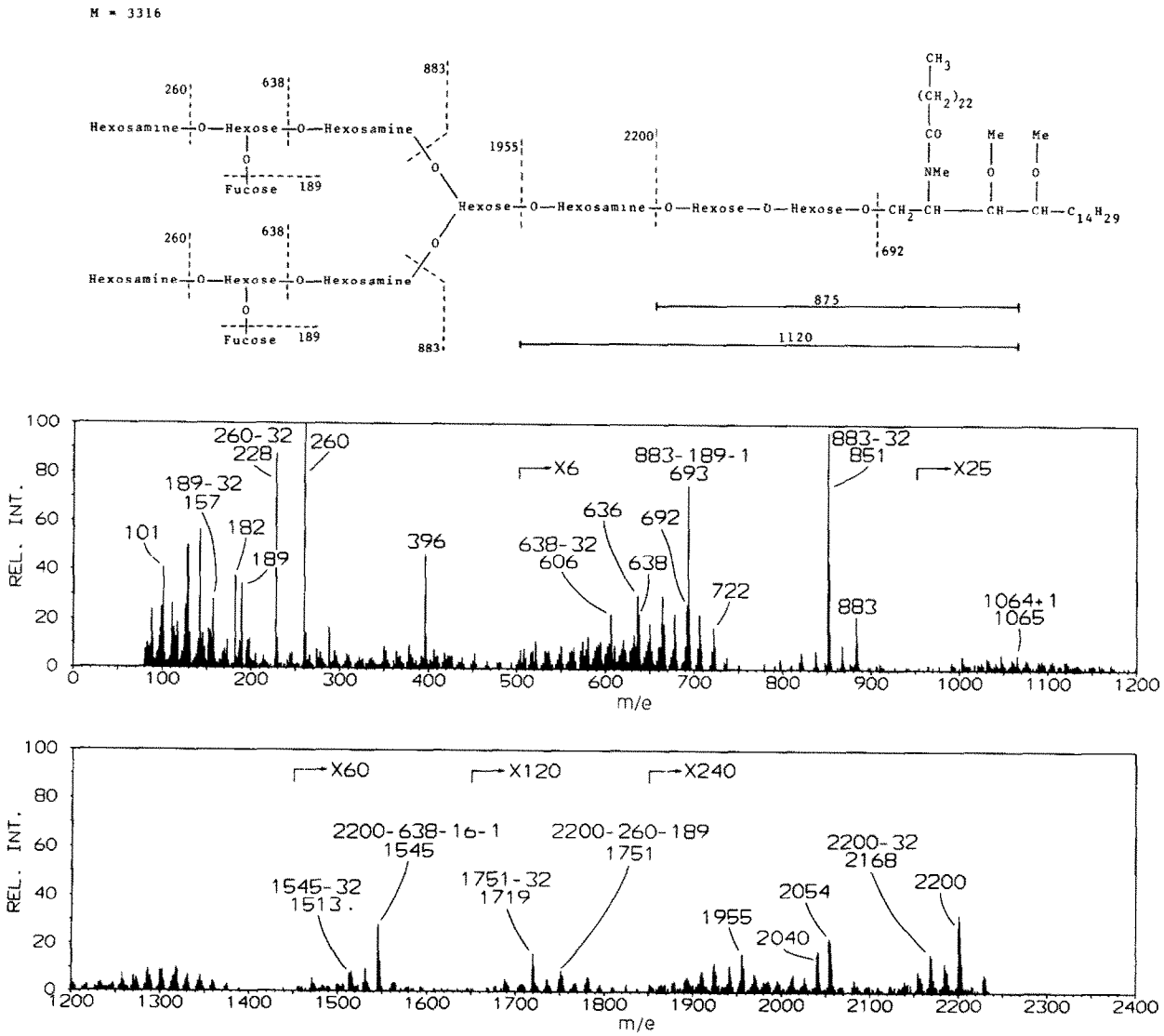

Fig.2. Mass spectrum of permethylated glycolipid of rat small intestine. The amount of substance used was 80 μg, the electron energy was 26 eV, acceleration voltage 4 kV, trap current 500 μA and ion source temperature 280°C. For further data, see legend of fig.1.

For 4 sugars at *m/e* 1182, 1211 and 1239. Note that the last-mentioned triplet added 2 mass units due to cleavage of 2 glycosidic bonds at the branch. If only one of the 2 branches is split, it results in the peaks at *m/e* 2037, 2065 and 2093. Loss of one complete branch and one or 3 sugars of the other branch explains the triplet at *m/e* 1792, 1820 and 1848, or 1414, 1442 and 1470.

For the non-reduced derivative (fig.2) the terminal decasaccharide may loose 2 (*m/e* 1719 and 1751) or 3 sugars (*m/e* 1513 and 1545). Loss of 190 units from the terminal tetrasaccharide (*m/e* 693) and not of 206 (189 + 1 + 16) units is further evidence for fucose linked to hexose and not to hexosamine [10].

Some indication on position of binding for Gal → GlcNAc may also be given by mass spectrometry [9,10,14,15]. The peak at *m/e* 182 in the spectrum of the non-reduced derivative (fig.2) is evidence for the presence of Gal 1 → 4 GlcNAc [9,10]. The relative intensity of this peak and the presence of the triplet at *m/e* 918, 946 and 974 of fig.1, which is evidence for Gal 1 → 3 GlcNAc of the core tetrasaccharide [14,15], suggest that one of the branches may contain Gal 1 → 4 GlcNAc.

Ceramide ions of the non-reduced derivative (fig.2) were due to phytosphingosine combined with non-hydroxy 20, 22 and 24 carbon acid (at *m/e* 636, 664 and 692, respectively) and with hydroxy 20, 22 and 24 carbon acid (at *m/e* 666, 694 and 722, respectively).

Mass spectrometry therefore provides conclusive evidence for the branched sequence of a dodecaglycosylceramide. The carbohydrate sequence and binding positions obtained are not unexpected since this general structure has been deduced from degradative studies of soluble ovarial cyst glycoproteins [16]. A similar sequence has also been proposed for some complex glycolipids of human erythrocytes [17]. However, in a strict sense the present glycolipid is the largest cell surface saccharide identified conclusively. A strong blood group A activity (M. E. B., G. C. H., K.-A. K., H. L., in preparation) is explained by the terminal trisaccharide of the two branches which is most probably the A determinant GalNAc α1 → 3 Gal[2 ← 1α Fuc] β1 → [1].

Even if the complete sequence of a complex glycolipid would not be safely interpreted in all cases by using this technique, the saccharide plus fatty acid ions of the reduced derivative (fig.1) provide us with the number of sugars present. This is not possible with conventional degradation methods which are unable to give reliable data when $\gtrsim$5 sugars are present [18–20]. Of particular interest is our recent application of the technique on mixtures of glycolipid antigens [10,21]. By a temperature-programmed distillation in the ion source and a continuous recording of spectra, a specific information from separate sequences may now be obtained from mixtures with 1–12 sugars. This would be of obvious help for functional studies of cell surface saccharides [1–3]. The present method and derivatives have also been applied on glycopeptides [22].

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