

The use of fast atom bombardment and laser desorption mass spectrometry in the analysis of complex carbohydrates

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ABSTRACT

Oligosaccharides occurring free in secretions or bound to lipid or protein, are known to modulate the biological response in many living systems. The structural characterization of these highly diverse oligosaccharides, that may be further complicated by the occurrence of non-carbohydrate substituents such as alkyl, acyl, sulfate, or phosphate groups, for example, represents the first step towards a rational approach that is able to relate structure to function. The structural delineation of carbohydrate residues at defined sites of attachment is especially important in recombinant glycoproteins because the type and extend of glycosylation affect their biological properties. In recent years the development of soft ionization procedures and the increase in mass range above 10,000 mass units at full acceleration, together with the development of highly sensitive detectors, has allowed the analysis of glycans containing more than 30 sugar units in the nano- and subnanomolar range.

INTRODUCTION

Mass spectrometry has been intensively used now for more than seventy years in the analysis of atoms, isotopes and small molecules (1). It is one of the basic tools in the fields of petrol-, lipid- or drug-chemistry. In the environmental and clinical disciplines MS techniques are used as definitive methods against which other assays have to be validated. Major obstacles in the application of MS techniques for sugar analysis were their thermal instability and the low volatility of carbohydrates even after derivatization. A major revolutionizing breakthrough was achieved with the development of "soft" or "cold" ionization methods that allowed the ionization and desorption of large polar compounds like intact proteins, glycoconjugates and nucleic acids without prior evaporation from liquid or solid matrices as documented in a series of recent reviews and the references therein (2-7). This progress would not have been possible without the pioneering work of the groups of D.Beckey, K.-A.Karlsson, N.K.Kochetkov and C.C.Sweeley (8-11) and the development of high mass instruments of high sensitivity operating in the nano- to femto-molar range like four-sector tandem mass spectrometers equipped with multichannel array detectors or time of flight instruments combined with plasma electrospray, or matrix-modulated laser desorption for molecules in excess of 100 000 daltons. As stated before (12), the analysis of complex glycan structures includes the determination of **a**) molecular mass and sugar components **b**) composition of the aglycon **c**) the anomeric configuration of sugar residues **d**) the sequence of sugar constituents and the pattern of branching **e**) presence of additional substituents, e.g. esters, acetals etc. **f**) the sites of glycosidic linkages **g**) the conformation of the sugar rings **h**) the preferred threedimensional structures.

Among these the parameters a), b), d), e) and partly also f) can be addressed by mass spectrometry. In this contribution we will summarize some of the results that we have obtained in the MS analysis of oligosaccharides from human milk (13-21) of phosphoinositol linked glycans from the variant surface glycoprotein (VSG) of *Trypanosoma b. brucei* (22-24) and finally of some high molecular weight glycosphingolipids with up to forty sugar residues, that are present in rabbit erythrocyte membranes (25-27).

GLYCANS FROM HUMAN MILK

It is known from the work of several groups of scientists that human milk is especially rich in complex carbohydrates (28-33). They may occur either as free oligosaccharides, as glycoproteins or as glycolipids. Interest has first been focussed on the oligosaccharide fraction because of its rôle in defense mechanisms against viral and bacterial infections and their relation to a number of tumor associated antigens (34-36). Until recently, the structures of more than 50 oligosaccharides have been described. From the work of the groups of R.Kuhn, J.Montreuil, V.Ginsburg and A.Kobata eight "core" structures are known (30-32). These "core" structures are further modified by the genetically controlled addition of fucose or sialic acid residues.

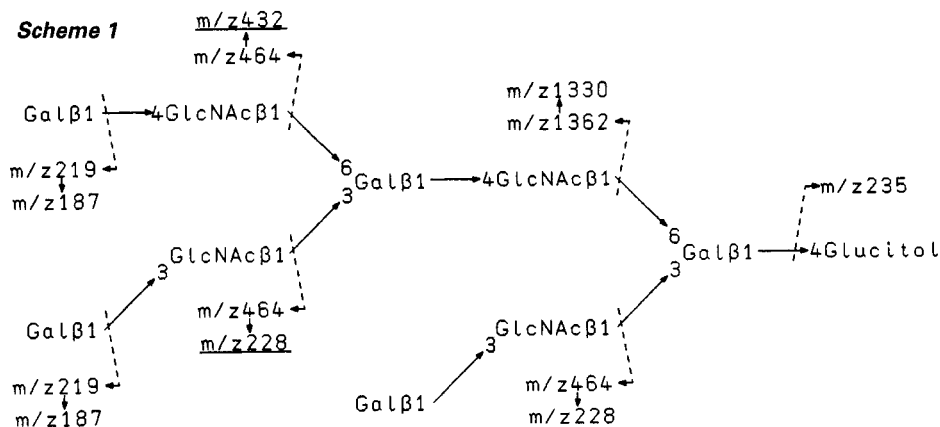
A first insight into the composition of milk oligosaccharides can be obtained by the determination of the molecular ions either using negative ion desorption of native compounds or positive ion desorption with peracetylated or permethylated compounds. From the studies mentioned above it is known, that in this group of oligosaccharides, glucose is always in terminal reducing position. Thus after reduction with borohydride or reductive amidation, each of the sugar components: fucose, sialic acid, galactose, glucosamine and glucitol or its amidated derivative contributes specific mass increments to the molecular weight. Therefore FAB MS can be also used favourably for monitoring fractionation procedures. The results of such an analysis of the neutral oligosaccharide fraction using gel permeation chromatography on Biogel P4 (32) and Fractogel TSK HW-40 are compiled in Table 1 (20).

Table 1: Molecular composition of fractions of neutral oligosaccharides from a donor of blood group O, Le^a, separated by chromatography on Biogel P4 (fraction 1-12) and Fractogel TSK HW-40 (fraction 13-18).

Fraction Nr.	Type of oligosaccharide	Molecular mass related ions		
		(M-1) ⁻ native	(M+Na) ⁺ reduced, peracetylated	(M+Na) ⁺ reduced, permethylated
1	Higher oligosaccharides ^a	> 2200		
2				
3				
4				
5				
6	Difucosyllacto- <i>N</i> -decaose	2093	3505	2637
7	Monofucosyllacto- <i>N</i> -decaose ^b	1947	3275	2463
8	Trifucosyllacto- <i>N</i> -octaose ^b	1874	3160	3623
9	Difucosyllacto- <i>N</i> -octaose ^b	1728	2930	2188
10	Monofucosyllacto- <i>N</i> -octaose ^b	1582	2700	2014
11	Tri- and Difucosyl- lacto- <i>N</i> -hexaoses	1509	2585	1913
		1363	2355	1739
12	Monofucosyllacto- <i>N</i> -hexaoses	1217	2125	1565
	Lacto- <i>N</i> -hexaoses	1071	1895	1391
13	Difucosyllacto- <i>N</i> -tetraose	998	1780	1290
14	Monofucosyllacto- <i>N</i> -tetraoses	852	1550	1116
15	Lacto- <i>N</i> -tetraoses	706	1320	942
16	11 ⁶ GalβLac ^c	503	1033	697
17	3 ³ Fuc-Lac	487	975	667
18	Lactose	341	745	493

a) Mixture of fucosylated lacto-*N*-deca- to tetradecaoses b) Incomplete separation
c) Reported by Yamashita and Kobata (37)

Analogous results were obtained with the acidic fraction. Among the high molecular weight fractions evidence was obtained for the occurrence of fucosylated or sialylated lacto-*N*-deca-, dodeca- and tetradecaoses. One of the decaose core structures could be purified to homogeneity after release of the fucose residues by mild acid hydrolysis. Its permethylated derivative was characterized both by FAB MS and ¹H-NMR. The major structurally important sequence ions of the reduced and permethylated compound are shown in the Scheme 1.



As a result of studies performed on the permethylated milk oligosaccharides (4) and also on

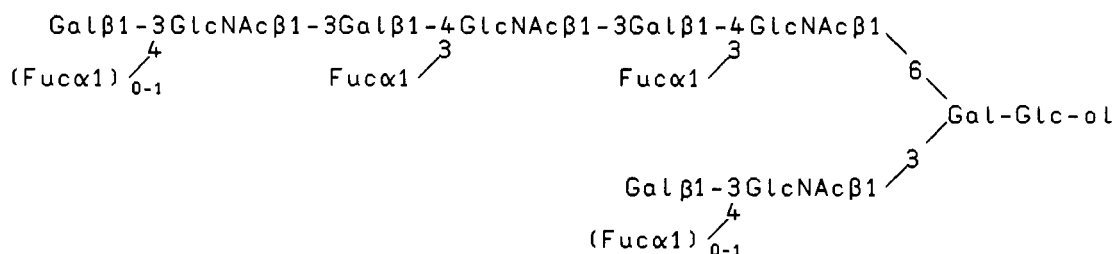
glycosphingolipids of related structure it became evident, that type 1 chains and their specific substitution patterns can be clearly discriminated from those of the type 2 chain due to the occurrence of structure specific daughter ions. The formation of these daughter ions is based on the highly preferred elimination of the substituents linked to position 3 of the GlcNAc residue. In table 2 an overview is given on the primary fragment ions and the key daughter ions that characterize the different type 1 or type 2 chains present in the neutral human milk oligosaccharides. A similar list of fragment- and daughter ions can be constructed for sialylated or sialylated and fucosylated oligosaccharides.

Table 2: Important primary mono- to tetrasaccharide fragments and their corresponding daughter ions from reduced and permethylated human milk oligosaccharides observed in a positive ion FAB mass spectrum.

Fragment	m/z	Fragment	m/z
dHex ⁺	189→157	Fuc-Hex-1-4-HexNAc ⁺	812→606
Hex ⁺	219→187→155	Fuc-1-3	
HexNAc ⁺	260→228	Fuc-1-4-HexNAc ⁺	812→402
NeuAc ⁺	376→344	Fuc-Hex-1-3	
Hex-ol ⁺	235	NeuAc-2-3-Hex-1-4-HexNAc ⁺	999→793
HexNAc-ol ⁺	276	Fuc-1-3	
Hex-1-3-HexNAc ⁺	464→228		
Hex-1-4-HexNAc ⁺	464→432	Fuc-1-4-HexNAc ⁺	999→402
Fuc-1-2-Hex-1-3-HexNAc ⁺	638→228	NeuAc-2-3-Hex-1-3	
Fuc-1-2-Hex-1-4-HexNAc ⁺	638→606		
Hex-1-4-HexNAc ⁺	638→432	NeuAc-2-6-HexNAc ⁺	999→589
Fuc-1-3		Fuc-Hex-1-3	
Fuc-1-4-HexNAc ⁺	638→402	Hex-Hex-1-4-HexNAc ⁺	668→636
Hex-1-3		Hex-HexNAc-Hex-1-3-HexNAc ⁺	913→228
NeuAc-Hex ⁺	580	Hex-HexNAc-Hex-1-4-HexNAc ⁺	913→881
NeuAc-Hex-1-3-HexNAc ⁺	825→228		
NeuAc-Hex-1-4-HexNAc ⁺	825→793		

As an example the FAB MS of a reduced and permethylated tridecasaccharide fraction isolated from the milk of a donor with blood group O, Lea, non secretor, is presented in Fig. 1. From the molecular ion M+Na⁺ at m/z 2811 it can be calculated that the oligosaccharide consists of three fucose residues, five galactoses, four N-acetyl-glucosamines and one glucitol. The molecule contains one terminal disaccharide unit Gal β1-3 GlcNAc as from the ions at m/z 464 and 228. As an indication for a branched structure, a second nonreducing terminal is represented by m/z 638. The simultaneous occurrence of two daughter ions at m/z 432 and 402 with a higher abundance of the latter indicates a terminal Gal β1- (Fuc α1)3/4 GlcNAc with a prevalence of the type 1 chain. Different from the structure shown for the above mentioned decaaccharide, one of the side chains of the core structure consists of an extended chain comprising up to three fucosylated lactosamine residues. This can clearly be deduced from sequence ions at m/z 1261 and 1884 with daughter ions at m/z 1055 and 1678 respectively. Evidence for structural heterogeneity in this fraction are ions at m/z 1087 and 1710 and a daughter ion at m/z 881 which show, that in the longer side chain one subterminally linked fucose residue may be missing.

As there are three fucose residues in the molecule, the third fucose must necessarily reside in the shorter side chain. Assuming, this side chain to be of type 1 chain in analogy to other structures that have been completely analysed, the data are in agreement with the following structure:



This example is intended to show, that many of the carbohydrate structures that have also been characterized as specific cell surface antigens during embryogenesis, differentiation and oncogenesis do occur naturally in human milk either as free oligosaccharides or bound to protein (21).

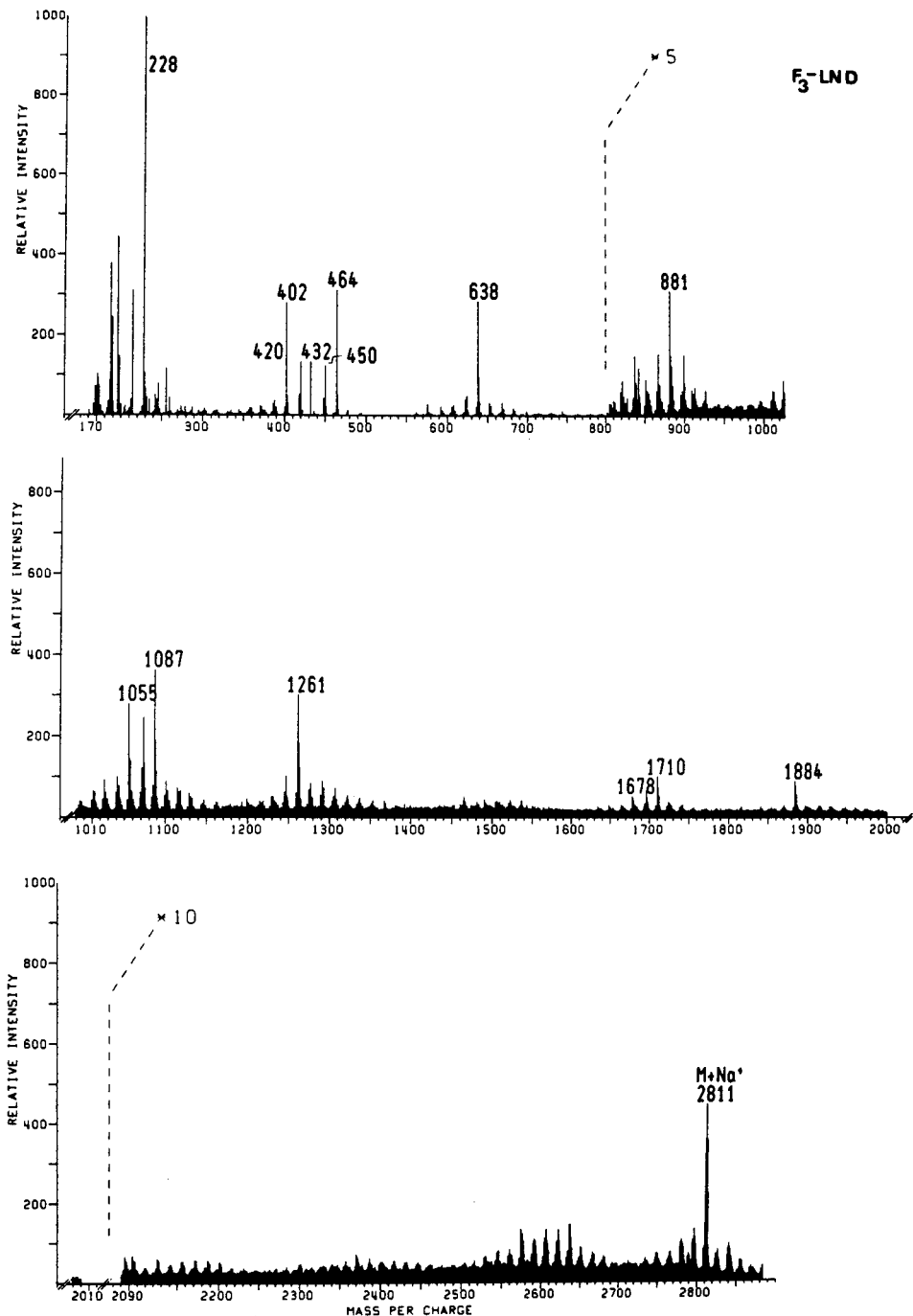


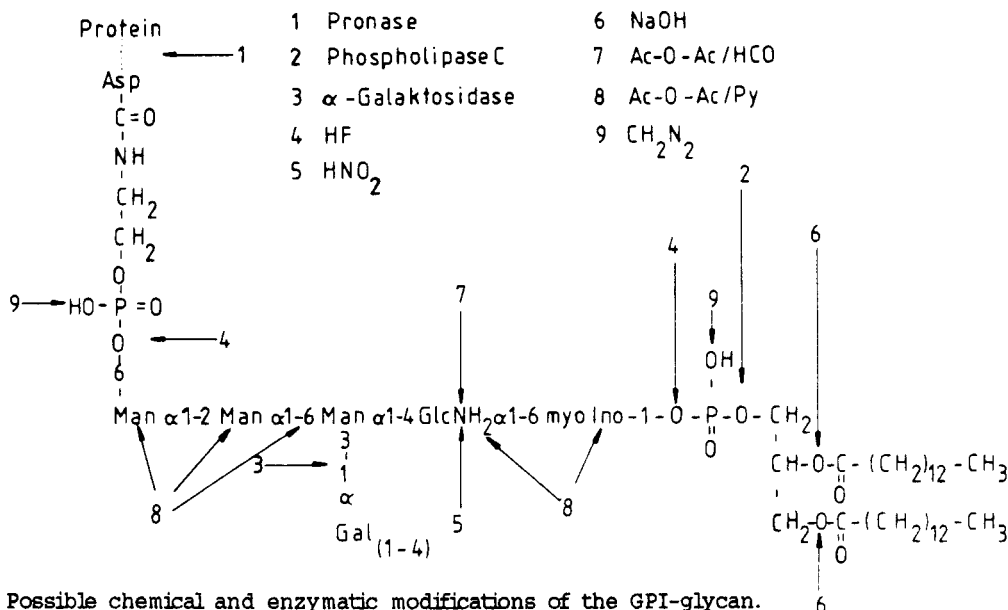
Fig. 1: Positive ion FAB mass spectrum of a reduced and permethylated trideca-saccharide isolated from the milk of a donor with blood group O, Lea.

FAB MS ANALYSIS OF GPI GLYCANS

In recent years a number of proteins have been described that are anchored via a phosphatidylinositol glycan [GPI] (38). One of the structures best studied is the GPI anchor of the variant surface glycoprotein of *Trypanosoma brucei* (39). The general structure of the GPI together with possible sites of chemical or enzymatic modifications are shown in the scheme 2.

Negative ion FAB-MS analysis of the intact GPI anchor comprising the C-terminal aspartic acid of VSG was performed with the peracetylated compound. Besides information on the type of acyl chains present in the diglyceride moiety it provided molecular ions M-1 at m/z 2928; 3216; 3504, that allow the calculation of the overall composition of the GPI glycan including the C-terminal aspartic acid of VSG. Further insight into the fine structure of the glycan is obtained from the FAB MS spectra of the peracetylated glycans that were released from

Scheme 2



Possible chemical and enzymatic modifications of the GPI-glycan.

membrane form VSG by treatment with HF. In Fig. 2 the mass spectrum of the peracetylated glycan fraction from the VSG of MITat 1.6 is shown. The molecular ions M+H⁺ at m/z 2448, 2736, 3024 and 3312 indicate, that the glycan structure may contain up to nine hexose units besides inositol and glucosamine. Sequence ions at m/z 619, 907, 1195 and 1483 give evidence that side chains with 2-5 hexose units are present. This clearly demonstrates, that the glycan structures of the GPI anchor of *T.b.brucei* may be more complex than those reported (40).

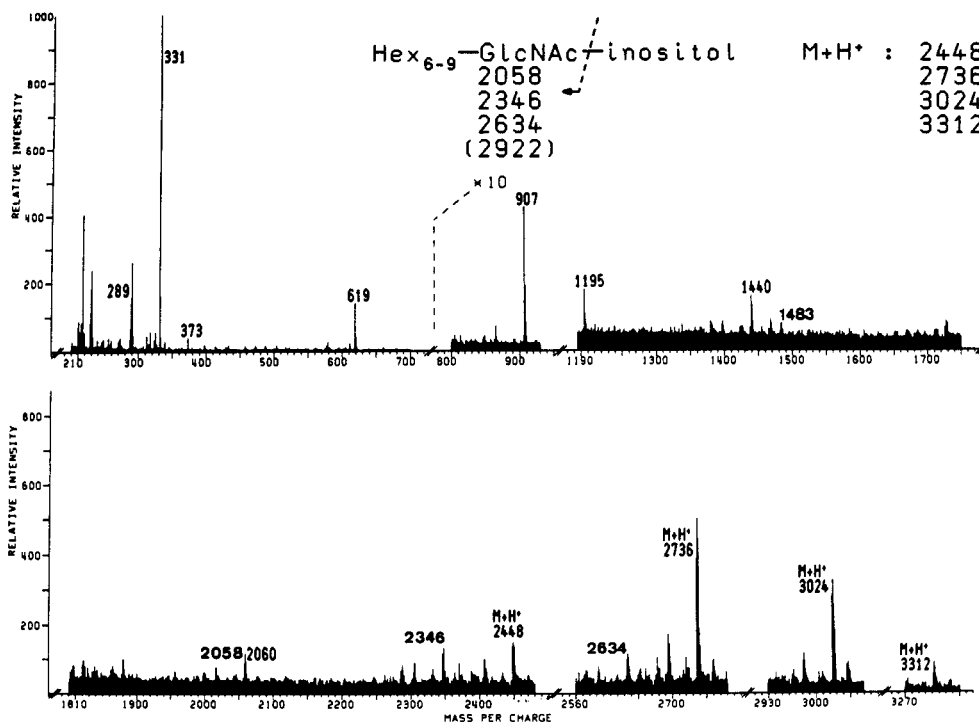


Fig. 2: Positive ion FAB mass spectrum of the peracetylated glycan fraction released from VSG of *Trypanosoma b.brucei* by treatment with HF.

The glycan mixture can be further analysed after selective N-acetylation with a mixture of normal and deuterated acetic anhydride and extensive treatment with α-galactosidase from jack bean.

The analysis of the products of enzymatic degradation after peracetylation shows molecular species M+H⁺ at m/z 1584, 1872 and 2160, indicating the presence of three to five hexose residues that resisted α-galactosidase treatment besides the inositol and the glucosamine

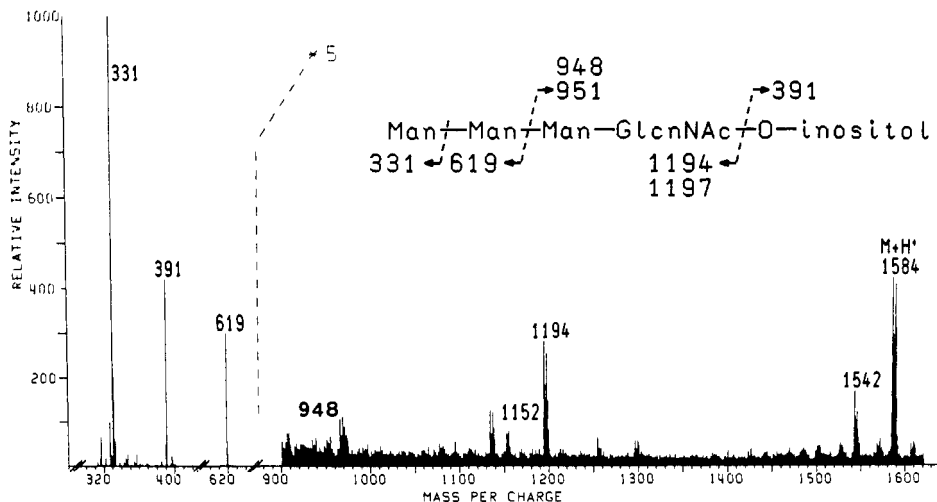


Fig. 3: FAB mass spectrum of the peracetylated pentasaccharide obtained after α -galactosidase treatment of the HF cleavage product. The amino group of the glucosamine was mass-labelled by partial deuterio acetylation.

residues. In Fig. 3 the mass spectrum of the peracetylated pentasaccharide is shown, where the amino group of the glucosamine was N-acetylated with a 1:1 mixture of normal and deuterated acetic anhydride.

The sequence data are in complete agreement with a linear structure as published by Ferguson (39). Especially the occurrence of ions at m/z 619 and 948/951 preclude the existence of a branched mannotriose structure. These data are at variance with a structural proposal published previously by our group (41). This proposal was based mainly on the occurrence of a trihexosyl ion at m/z 907 in several preparations of a heptasaccharide Hex5-GlcNAc-inositol that appeared homogenous by criteria of HPLC and HPTLC. We have to conclude that these preparations contained a mixture of at least two cochromatographing components with one component carrying an unbranched Hex3-chain.

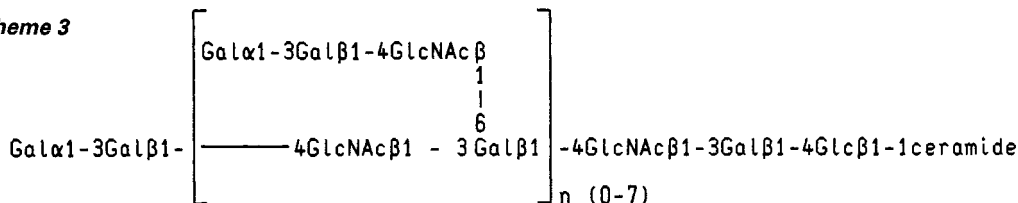
LASER DESORPTION MASS SPECTROMETRY OF NATIVE AND DERIVATIZED HIGH-MASS GLYCOSPHINGOLIPIDS

The use of lasers for desorption processes in mass spectrometry was first reported in 1968 (42). The molecular ions $[M + \text{cation}]^+$ of peptides, di- to tetrasaccharides, cardiac glycosides and nucleotides with molecular weight up to 1200 Daltons have been obtained by laser desorption on a magnetic sector instrument (43). The construction of the laser microprobe mass analyser (LAMMA), combined with a time-of-flight mass spectrometer by Hillenkamp *et al.* (44), granted the option to analyse high molecular weight biological samples.

The discovery, that the UV-laser induced desorption/ionization (UV-LDI) in combination with an improved matrix technique can be used for the analysis of high polymers (over 100,000 Dalton) in combination with an improved matrix technique changed dramatically the mass detection limits by mass spectrometry (45). In order to obtain a high ionization yield of biomolecules via photochemical proton transfer reactions, the use of small organic compounds e.g. nicotinic acid as a matrix is required. Single charged molecular ions are the most abundant species observed at a sensitivity range of about 1 pmol.

The structural properties of glycosphingolipids (GSLs) from rabbit erythrocytes, shown to possess blood group B-like activity have been studied by fast atom bombardment mass spectrometry (FAB-MS) (27). This class of GSLs exhibits a highly regular structure with molecular species differing each by five sugar units.

Scheme 3



General Structure of the neutral glycosphingolipids from rabbit erythrocytes

The FAB mass spectra of permethylated high molecular weight GSL in the range above 6000 Da are characterized by the appearance of multiple ion clusters of similar intensity separated by 14 a.m.u. that are possibly produced by interaction with matrix derived ions and that are

spread over an area of 200 to 300 mass units. Thus in the presence of several molecular species differing e.g. in the composition of the ceramide residue, an unequivocal assignment of molecular ions may be difficult. This is exemplified with the molecular ion region of the FAB spectrum of a GSL carrying 25 sugar residues with the molecular composition of $C_{288}H_{514}N_{10}O_{128}Na$ (Fig.4) (12).

With the technique of UV-LDI also native high molecular weight GSL normally not amenable to FAB MS can be successfully analysed as shown in Fig. 6.

By contrast, in the positive ion UV-LDI mass spectra molecular ions are usually of high intensity whereas the formation of fragment ions is low. Thus also mixtures of high molecular weight samples are amenable to this technique of analysis.

Normally resolution is lower than that of modern high mass sector instruments and the formation of ions containing more than one cation has to be taken into account. In Fig. 5 the UV-LDI mass spectrum of a mixture of permethylated GSL with 20, 25, 30, 35 and 40 sugar residues is presented.



Fig. 4: Molecular ion region of a permethylated GSL with 25 sugar residues obtained from a thioglycerol matrix by FAB MS using a V.G. ZAB HF instrument. Mass determination of the center of the most intense ion cluster was performed by interpolation of $C_{s_n}J_{x-1}^+$ ions.

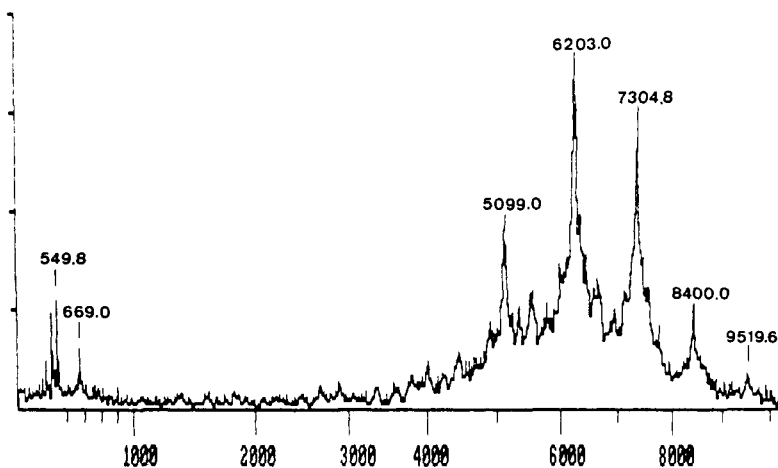


Fig. 5: Positive ion UV-LDI mass spectrum of a mixture of permethylated GSL from rabbit erythrocytes (27) with up to forty sugar residues.

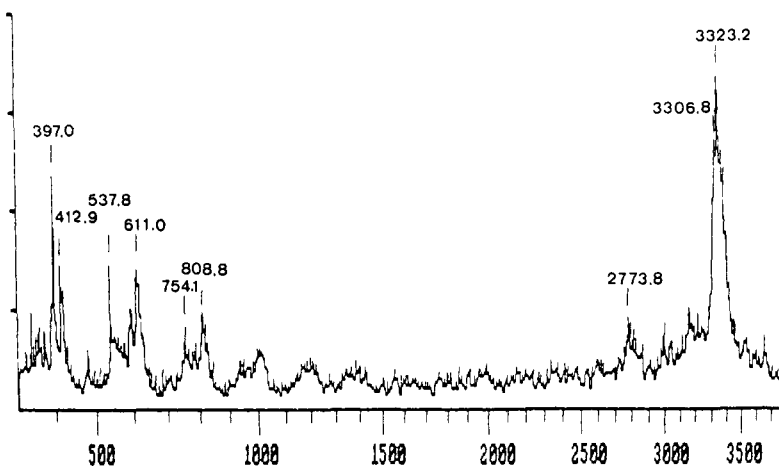


Fig.6: Positive ion UV-LDI mass spectrum of a native ceramide pentadeca-saccharide isolated from rabbit erythrocyte membranes with a molecular ion at m/z 3307.

It can be envisaged, that with the improvement of the resolving power of time of flight mass spectrometers more complex glycans derived from polysaccharides, glycopeptides produced by partial proteolysis or intact glycoproteins can be analysed by this method.

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