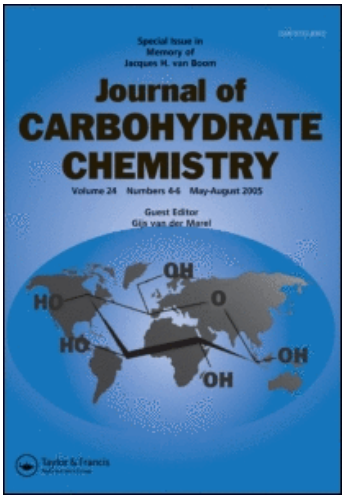

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The Application of Electrospray Mass Spectrometry for Structural Studies on a Tetrasaccharide Monopeptide from the Urine of a Patient with α-N-Acetylhexosaminidase Deficiency

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THE APPLICATION OF ELECTROSPRAY MASS SPECTROMETRY FOR STRUCTURAL STUDIES ON A TETRASACCHARIDE MONOPEPTIDE FROM THE URINE OF A PATIENT WITH α-N-ACETYLHEXOSAMINIDASE DEFICIENCY[1]

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ABSTRACT

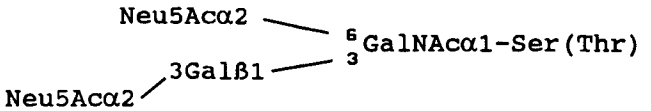
In this contribution the introduction of a new methodology to sequence the glycopeptides using electrospray mass spectrometry (ES-MS) as a direct and efficient approach combining structural data obtained by negative and by positive ion detection is proposed. The main characteristics of this procedure are: i) good sensitivity in the low and the high mass range ii) induction of fragmentation processes at higher orifice voltages iii) no necessity for derivatization.

ABBREVIATIONS

ES-MS:	electrospray mass spectrometry
FAB-MS:	fast atom bombardment mass spectrometry
LDI-MS:	laser desorption induced mass spectrometry
LSIMS:	liquid matrix secondary ion mass spectrometry
PD-MS:	plasma desorption mass spectrometry

INTRODUCTION

Sialyl oligosaccharides are excreted in a free form or as glycopeptides in human urine, and their structures and amounts are known to vary under different physiological and pathological conditions.[2] Lower amounts of disialyl T-antigen[3] as a tetrasaccharide monopeptide **1**, can be found in non-pathological human urine



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in addition to sialyl oligosaccharides, probably as catabolic products of glycophorine from erythrocyte membranes.[4] About 100 fold amounts of those, compared to the normal, were found in urine of two young brothers suffering from the α-*N*-acetylgalactosaminidase deficiency.[5,6]

The role of mass spectrometry in the analysis of glycoconjugates is well documented.[7] The established general strategy for MS analysis of *O*-linked glycans has been by well established procedures[8-11] including FAB-MS or LSIMS, single or tandem, after partial or full derivatization, following the release from the parent protein by ß-elimination or by *O*-glycanase. Fewer options are available for sequencing of naturally occuring glycopeptides, or those obtained by proteolytic digestion from parent glycoproteins. In FAB-MS,[12] LSIMS,[13] PD-MS[14] and LDI-MS[15] of *N*- and *O*-glycopeptides, the respective molecular ions have been easily identified, but virtually no sugar and/or peptide sequence fragments were observed. Additional structural data on sequence, branching and linkage sites were collected from series of MS experiments accompanied by chemical and/or enzymatic degradation reactions. The permethylation reaction is not well suited for the characterization of *O*-linked

glycopeptides, due to the lability of the GalNAc-serine (threonine) sugar-peptide bond under standard reaction conditions.

Recent development of electrospray mass spectrometry[16,17] gave rise to new options in analyses of glycoconjugates. This methodology has been in particular applied to analyse molecular distribution of proteins.[18,19] Here we report on the possibility of applying ES-MS for sequencing of the native low molecular weight *O*-glycopeptides.

RESULTS AND DISCUSSION

The molecular composition of the glycopeptides in terms of number and type of monosaccharides and amino acids involved can be calculated from the molecular $[M-H^+]^-$ ions observed in the negative ion FAB-MS, but only little direct evidence in terms of sequence ions is provided. The molecular ions of **1**, previously determined by negative ion FAB-MS,[6] were confirmed by negative ion ES-MS at Vc=50v as the monocharged molecular $[M-H^+]^-$ ions at m/z=1051 (**1**-Ser) and 1065 (**1**-Thr) (Fig. 1A). After increasing the cone voltage to Vc=60v the appearance of significant fragment ions was observed arising principally from the cleavages of glycosidic bonds and retention of charge on the glycosidic oxygen. The fragment $[M-NeuAc]^-$ at m/z= 760 and 774 of Y-type arose from the cleavage of only one bond, but abundant ions $[M-2NeuAc]^-$ at m/z=469 and 483 and $[NeuAcHexHexNAc]^-$ at m/z=673 after the cleavage of two glycosidic bonds (Fig. 1B). Significant shift of the molecular ion species from $[M-H^+]^-$ at lower cone voltage to the $[M-2H^++Na^+]^-$ and $[M-3H^++2Na^+]^-$ of high abundancy at higher cone voltage was also observed.

Structural data like sequence and linkage ions of the carbohydrate portion can generally be obtained from the positive ion FAB-MS after permethylation, but in such a case

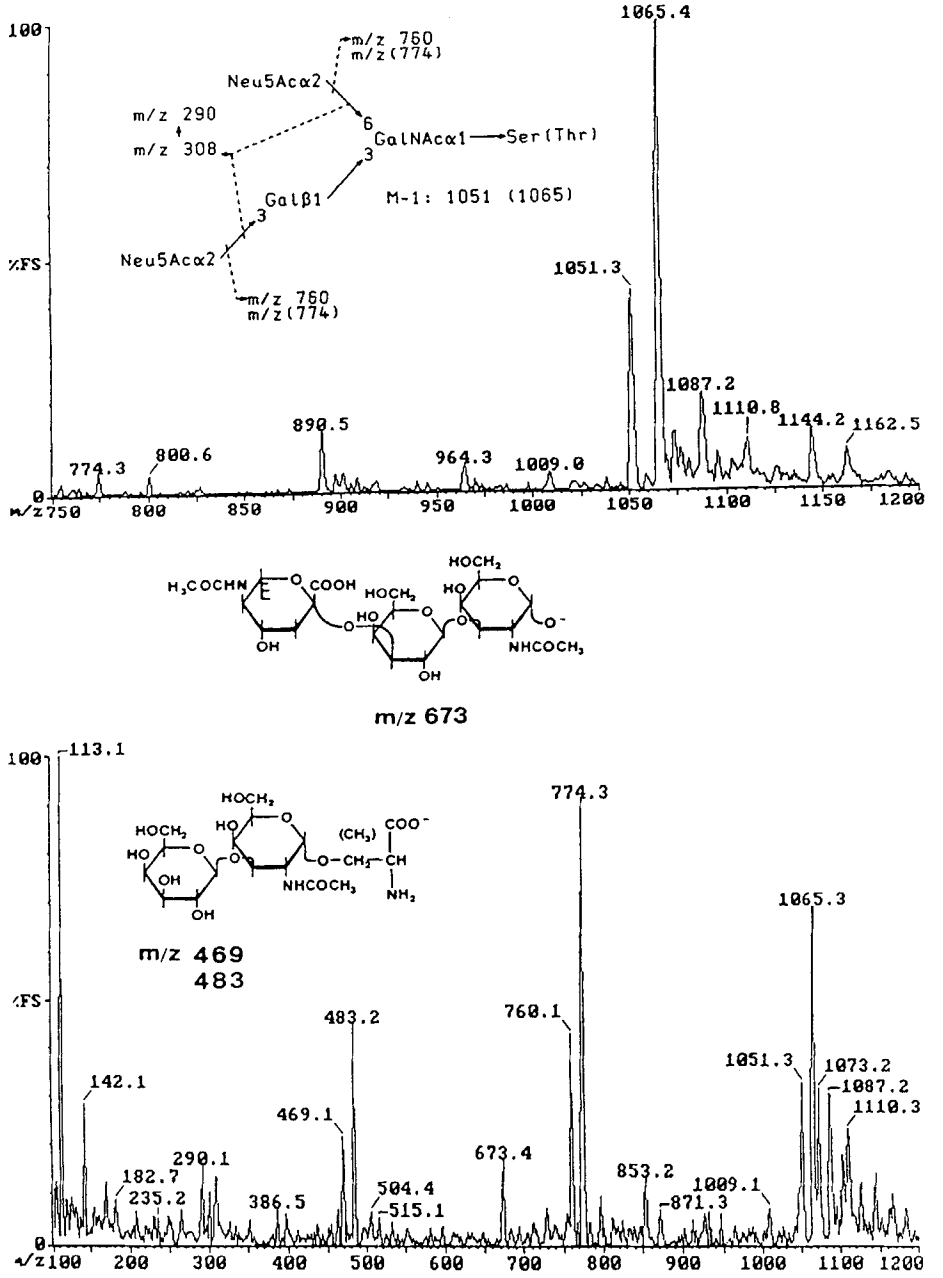
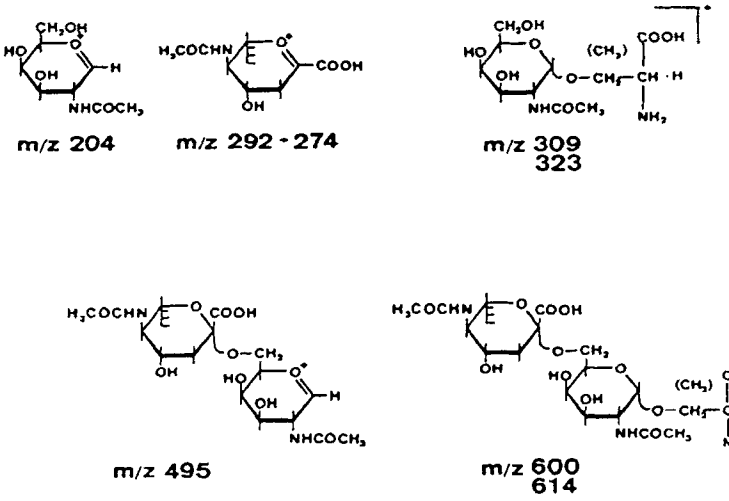


Fig. 1 Negative ion ES-MS of the native sample and respective fragmentation schema of the tetrasaccharide monopeptide 1. A.) The extended molecular ion area of a lower cone voltage scan (Vc=50v). B.) The full ES-MS of a higher cone voltage scan (Vc=60v).

the loss of the amino acid portion was observed.[6] The intact native glycopeptide can, however, be submitted to the positive ion high cone voltage ES-MS (Fig. 2). At a cone voltage of Vc=50v the singly charged molecular ions at m/z=1053 and 1067 ($[M+H]^+$) and m/z=1075 and 1089 ($[M+Na]^+$) respectively, were accompanied by abundant fragment ions. The B-type fragment ion at m/z=204 ($[HexNAc]^+$), resulted from the cleavages of three glycosidic linkages. As this ion at m/z=204 was observed so far in all samples containing an *O*-glycosidically linked GalNAc-Ser/Thr, it may be considered as a key ion for the identification of *O*-glycopeptides.[18] At the increased cone voltage of Vc=70v the sugar sequence from the non-reducing terminus was represented by the series of ions at m/z=784 and 798 of Z-type ($[M+Na-NeuAc]^+$), m/z=600 and 614 ($[M+H-NeuAc-Hex]^+$), and in particular the ions relevant for the characterization of the sugar-peptide link at m/z=309 and 323 ($[HexNAc-Ser/Thr]^+$). The monosaccharide fragments of B-type at m/z=204 ($[HexNAc]^+$), 292 and 274 ($[NeuAc]^+$) were accompanied by a disaccharide ion at m/z=495, characteristic for a 6-linked NeuAc to HexNAc (Fig. 2).

After substitution of the free glycopeptide *N*-terminus of **1** with an azobenzene-4-carbonyl group, the monocharged molecular $[M-H^+]^-$ ions were found at m/z=1259 and 1273 and the doubly charged species at m/z=630, 636 and 647 representing $[M-H^+]^{2-}$ and $[M-2H^++Na^+]^{2-}$ by negative ion ES-MS under cone voltage Vc=40v (Fig. 3A). The increase of Vc to more than 50v did not significantly induce the amount of fragmentation (spectrum not shown). In the positive ion ES-MS the molecular $[M+Na]^+$ ions appear at m/z=1283 and 1297. The Y-sequence ions are of only low abundance, at m/z=992 and 1006 ($[M+Na-NeuAc]^+$), beside the characteristic $HexNAc^+$ of high intensity at m/z=204. The ion at m/z=1151 could not be assigned (Fig.3B). The specificity of the linkage sites in **1** was confirmed also by methylation analysis.[20]



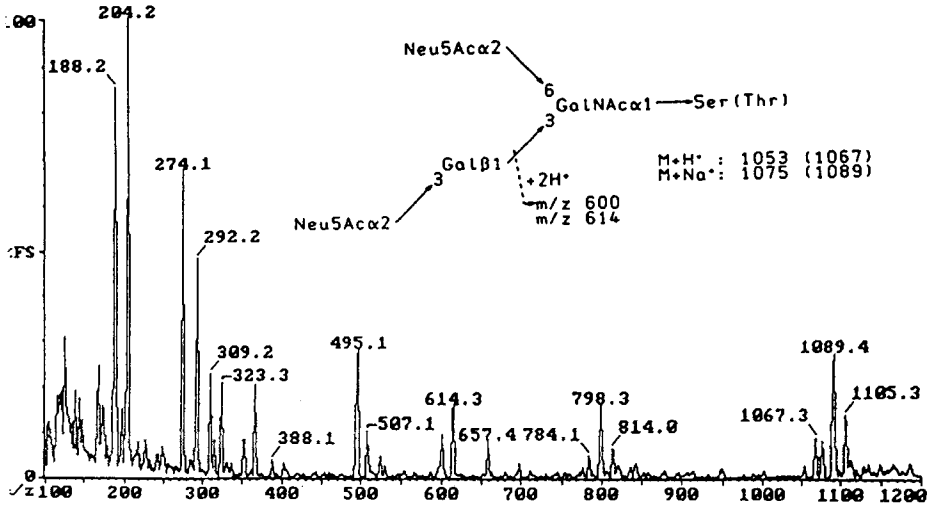


Fig. 2 Positive ion ES-MS of the native sample 1 and the structures of the fragment ions emerging during the higher cone voltage scan (Vc=50v).

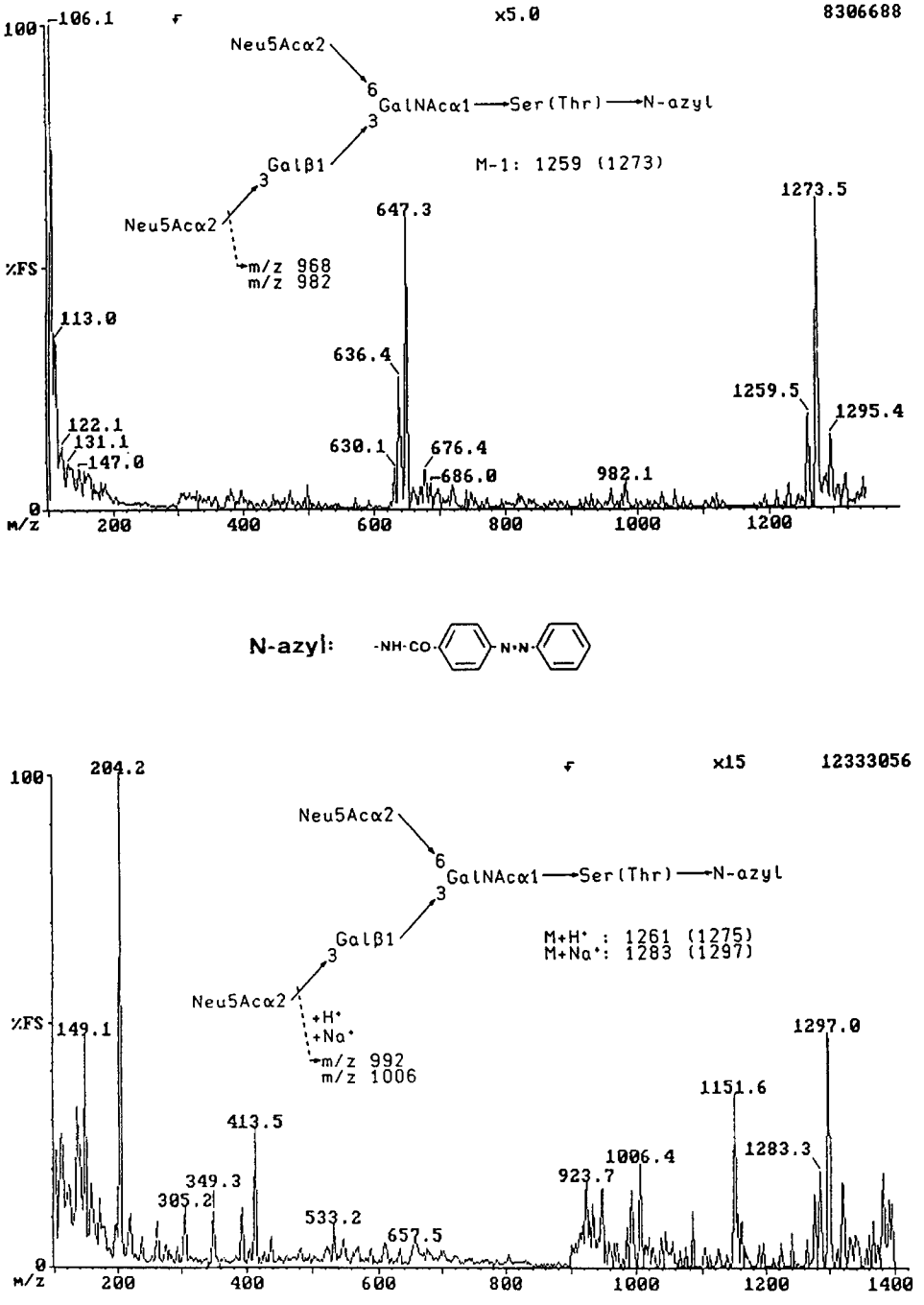


Fig. 3 Negative (A) and positive (B) ES-MS of the *N*-terminus derivatized **1** under lower cone voltage scan (Vc=40v and 50, respectively).

CONCLUSIONS

In this contribution, a novel approach for the sequencing of glycopeptides by positive and negative ES-MS is described. In general, molecular and fragment ions of non-derivatized glycopeptides obtained by ES-MS can contribute important data for the structural assignments of the carbohydrate and of the peptide portion, as well as the carbohydrate-peptide linkage in naturally occurring and recombinant glycoproteins or their proteolytic digests. The process of induced fragmentation by increasing the orifice voltages, giving rise to the higher relative abundance of diagnostic ions during the positive and negative ion detection, is not yet well understood.[21] The introduction of an aromatic chromophore, however, at the N-terminus of the glycopeptide portion apparently jeopardizes the process of fragmentation. The combined approach using positive and negative ion ES-MS can therefore be recommanded for acquisition of the native glycopeptide sequence data in a relatively short period of time using only several μg of material.

EXPERIMENTAL

Chromatography of the urine was performed essentially as described previously.[6] The yield of **1** was 34 mg/L urine. *N*-Azylation: To the glycopeptide (3-4 μmol), dissolved in 0.1 mL aq. $NaHCO_3$ (0.2 molar) in a Wheaton vial, a saturated solution of azobenzene-4-carbonyl chloride in acetone was added in two portions of 270 and 120 μL each, within a 2 h interval and the reaction mixture kept for an additional 2 h at room temperature. After centrifugation, the supernatant liquid was analysed by TLC using chloroform/methanol/0.2% aq. $CaCl_2$ (3:3:1; v/v/v) as the eluent. For derivative isolation, yellow coloured bands were scratched off of the plate and extracted with methanol. Positive and negative ion ES mass spectra of the native and the derivatized

glycopeptide were obtained on a VG BioTech BioQ mass spectrometer (Fisons VG Biotech, Altrincham, UK).[19] An electrostatic spray ion source operating at atmospheric pressure was attached to a quadrupole mass analyser with a mass range of 4000 Da. The electrospray potential was about 4kV. The voltage of the extracting cone (Vc) was set between 40 and 120 v. Glycopeptides were dissolved in acetonitrile/water (1:1; v/v) to give solutions of 0.2-2.0 μg/μL. 10μL of the solution were introduced into the ion source at a flow rate of ca. 3μL/min. Scanning was performed from m/z=100 to m/z=1800 in 10 sec using the mass calibration by horse heart myoglobin (16950.5 Da). The data system was operated as a multi-channel analyser and several scans summed to obtain the final spectrum.

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