$^{0,2}A_n$ Cross-Ring Cleavage as a General Diagnostic Tool for Glycan Assignment in Glycoconjugate Mixtures

Sergey Y. Vakhrushev, Alina Zamfir and Jasna Peter-Katalinić
Biomedical Analysis Group, Institute for Medical Physics and Biophysics, University of Münster, Münster, Germany

In contrast to proteomics significantly less efficient analytical tools are presently available for high throughput glycomics using mass spectrometry. In this article, a strategy to use the $^{0,2}A_n$ ring cleavage ion at the reducing end of free glycans as a diagnostic ion for assignment of free glycans, in presence of glycopeptides containing similar glycosylation patterns, is presented for rapid distinction in complex mixtures by mass spectrometry. The MS to MS/MS automatic switching, already previously introduced for the on-line LC-MS and CE-MS analysis, is shown in this contribution to be highly functional to obtain diagnostic fragmentation patterns of free glycan precursors in rapid screening of highly complex glycoconjugate mixtures obtained from clinical samples, namely from the urine of patients suffering from congenital disorders of glycosylation. Congenital disorders of glycosylation (CDG) are inherited metabolic diseases based on defects in the glycosylation pathways of glycoconjugates. The urine of CDG patients was reported to contain O-glycans and glycosylated amino acids at concentrations two to three orders of magnitude higher in comparison with the healthy control, characterized by a high degree of heterogeneity concerning the type, number, and values of molecular ions. Using the $^{0,2}A_n$ ring cleavage ion approach by tandem MS, it was possible to sort out free glycans and get them assigned. (J Am Soc Mass Spectrom 2004, 15, 1863–1868) © 2004 American Society for Mass Spectrometry

Fragmentation of complex glycans is already under investigation over the last 20 years [1], but an assignment of a carbohydrate structure from a single experiment still represents a major task. A number of techniques has been applied for structural elucidation of components in carbohydrate mixtures, basically attempting to find diagnostic fragment ions responsible for single structures [2–13]. The possibility to obtain distinct fragmentation patterns under controlled collision energy and gas conditions along with the ability of automatic switching between MS and MS/MS mode by electrospray quadrupole time-of-flight Q-TOF instrument, represents a powerful option for high-throughput analysis of complex carbohydrate mixtures. This technique can be another option for such rapid investigation in combination with fully automated chip-ESI-QTOF-MS for high-performance glycoscreening and sequencing, developed by our group [14].

The electrospray ionization followed by formation of fragment ions by collision-induced dissociation allows sensitive mapping and sequencing of oligosaccharides for identification. In general, carbohydrates undergo two different types of fragmentation: those of glycosidic cleavages and those of cross-ring fragmentation. For sequencing, glycosidic cleavages along the chain B, C, Y, Z and those from the internal oligosaccharide chain B/Y, C/Y, are major tools for structural elucidation (nomenclature by Domon and Costelo [15]). According to the cross-ring fragmentation linkage and branching patterns can be established.

In this context, the formation of a D fragment ion was previously found to be diagnostic for the differentiation of isomeric triantennary N-glycans, whereas the $^{0,2}A$ together with the $^{2,4}A$ cross-ring fragment ions were diagnostic for detection of the core fucose [2]. The differentiation between two isomeric O-glycans from human mucins of Type 1 (Galβ1-3GlcNAc) and of Type 2 chains (Galβ1-4GlcNAc) was monitored by the presence of $^{0,2}A$ fragment ion MS/MS spectrum of Type 2 chain [3]. Such $^{0,2}A$ ions appeared to be highly diagnostic, allowing the precise assignment of the acetyl groups to the O-2 and/or O-3 of the acetylated galacturonic acid residues [4].

In this study, we propose the $^{0,2}A_n$ cross-ring cleavage ion at the reducing end as a diagnostic device for identification of free oligosaccharides in presence of glycopeptides in complex carbohydrate mixtures. The combination of this approach, along with the automatic MS to MS/MS switching and a computer assignment (to be described elsewhere) of fragment ions as a powerful tool for analytical glycobiology, will be pre-

Published online November 17, 2004

Address reprint requests to Dr. J. Peter-Katalinić, Biomedical Analysis Group, Institute for Medical Physics and Biophysics, University of Münster, D-48149 Münster, Germany. E-mail: jkp@uni-muenster.de

1044-0305/04/$30.00
doi:10.1016/j.jasms.2004.09.008

Received July 8, 2004
Revised September 10, 2004
Accepted September 10, 2004

sented in the application to the glycoscreening of urine of CDG patients.

The CDGs were first discovered in 1980 [5], and in the following years a number of human disorders related to the N-glycosylation pathway were identified, according to deficient enzymes involved. At the present time, structural investigations on truncation of glycoforms are usually performed on transferrin by SDS-gel electrophoresis of patient's plasma. It was reported that O-glycans and glycosylated amino acids are largely accumulated in the urine of CDG patients [16]. Analysis of such samples represents a complex analytical task, due to the high degree of the sample heterogeneity concerning the type, number, and size of molecular species involved.

Experimental

Reagents and Samples

Methanol was obtained from Merck (Darmstadt, Germany) and used without further purification. Distilled and deionized water (Mili-Q Water Systems Millipore, Bedford, MA) was used for the preparation of sample solutions. The sample investigated in this work was a native mixture of glycans, together with O- and N-glycosylated sialylated peptides, denoted *$P_2$1Py2M3*, from the urine of patient A.L. suffering from symptoms assigned to the congenital disorder of glycosylation (CDG) [16].

For isolation of the components, the patients' urine was first filtered and submitted to the first gel filtration chromatography on Biogel P2 as described before [17]. The glycans were separated by the second gel filtration chromatography performed on Fractogel TSK HW 50 in 0.01 M pyridinium acetate at pH 5.4 as the eluting buffer. The fraction F1 was further separated by anion-exchange chromatography on MonoQ [17]. For ESI-MS analysis the sample was diluted in 1:1 vol/vol methanol:water at the concentration of approximately 5 pmol/μl calculated to an average molecular weight of 1200.

Method and Instrumentation

Mass spectrometry was performed on an orthogonal hybrid quadrupole time-of-flight mass spectrometer (QTOF Micromass, Manchester, U.K.) in the Micromass Z-spray geometry. A QTOF mass spectrometer is interfaced to a PC computer running the MassLynx 3.2 (Micromass, Manchester, U.K.) software to control the instrument, acquire and process MS data. Omega glass capillaries used in nanoESI- experiments were pulled using a vertical pipette puller (model 720, David Kopf Instruments, Tujunga, CA). The voltage was applied to the solution via a stainless steel wire inside the capillary. Nitrogen was used as a dissolvation gas and the source block temperature was kept at 80 °C. All mass spectra were acquired in the negative ion mode, which was shown to be advantageous for carbohydrate MS analysis [18]. The cone voltage values were in the 40–70 V range. Tandem MS was performed by CID at low energies using Ar as a collision gas. In order to obtain a maximum coverage of sequence ions the collision energy was adjusted during the experiment within the 35–45 eV range.

For sequencing of precursor ions, the automatic selection of the precursor ion in the MS to MS/MS mode switching was used, after setting a TIC threshold in counts/s or by preselecting ions via the "included masses only" option. In our case, 20–30 counts/s thresholds was used for switching from MS to MS/MS and 3000–5000 counts/s for switching back to the MS mode. Collision energy was in the 25–45 eV range. The acquisition time of the MS/MS was from 2 to 10 s.

Results and Discussion

Urine of a CDG patient was collected and fractions containing complex carbohydrates were isolated by gel permeation chromatography [16]. Single fractions were submitted to glycoscreening by negative ion nanoESI-QTOF MS showing a high degree of heterogeneity concerning the type of glycoconjugate and the degree of sialylation.

$ALP_2$1Py2M3 fraction as a representative sample has been investigated by negative ion nanoESI-QTOF MS glycoscreening as shown in Figure 1. Under the conditions used, a high number of singly charged molecular ions $[M-H]^-$ has been detected, reflecting a high level of heterogeneity concerning the type of oligosaccharides and glycopeptides related to their size and their degree of sialylation. The presence of both O- and N-glycans appearing either as free oligosaccharides or linked to one or more amino acids could be the reason for the high complexity of this sample.

In order to estimate the type of the glycoconjugate of the species present in this fraction, a computer algorithm for calculation of a glycan composition, in terms of Hex, HexNAc, dHex and NeuAc as monosaccharide building-block units was developed (to be described

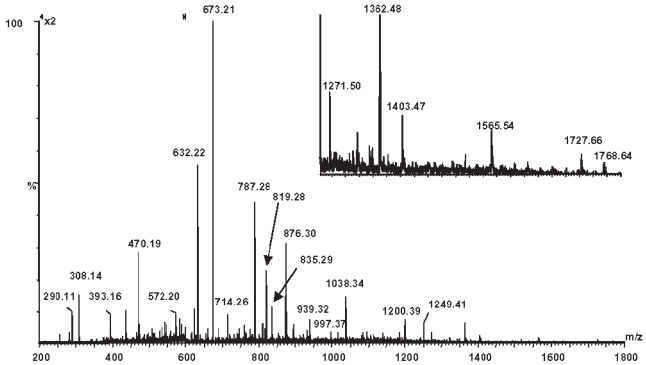

Figure 1. Negative ion nanoESI-QTOF MS of the fraction $ALP_2$1Py2M3 obtained from urine of the patient AL after gel permeation chromatography [16]. Capillary voltage −1100 V, cone voltage −50.

elsewhere). By using this program, major molecular ions from the MS in Figure 1 were submitted for calculation of the molecular composition. These data have been summarized in the Table 1. Although a majority of values for molecular ions could be directly associated with a carbohydrate composition, there were values which did not correspond to underivatized free glycan composition. Such molecular ions were at *m/z* 787.28, 939.32, 1249.41, and 1271.50, indicating that the sample might represent a mixture of free glycans and their conjugates. In order to test this prediction, a singly charged precursor ion at *m/z* = 876.30, assigned as a tetrasaccharide $NeuAcHexHexNAc_2$ by computer calculation, was submitted to the low energy CID MS/MS for identification (Figure 2). The MS/MS is dominated by the B ions at *m/z* 290.09, 452.15, 655.21, C ions at *m/z* 470.14, 673.24, and ring cleavage A ions at *m/z* 306.12, 424.16, 572.20, 583.25, 715.27, 775.28. The combination of B_2 and C_2 ions at *m/z* 452.15 and 470.14 indicate the attachment of NeuAc to Hex, where linkage position (α2-6) is determined by the presence of $^{0,4}A_2$ ion $-CO_2$ at *m/z* 306.12 [19, 20]. B_3 and C_3 at *m/z* 655.21 and 673.24 are indicative for attachment of a HexNAc to NeuAcHex unit to form a trisaccharide, which is itself linked to the reducing end HexNAc. Indicative for the free reducing end of the molecule was the loss of $\Delta m = 101$ u from the molecular $[M-H]^-$ precursor ion corresponding to the $^{0,2}A_4$ ion at *m/z* 775.28.

According to the fragmentation pattern depicted in Figure 3 the investigated structure should be assigned to a linear tetrasaccharide, where the $^{0,2}A_4$ sugar ring cleavage ion might represent a typical feature documenting a HexNAc moiety at the free reducing end. In order to obtain general evidence other components in the complex mixture were submitted to fragmentation process by low energy CID. The major objective was to fragment as many precursor ions as possible within the total nanoESI spraying time, delivering diagnostic $^{0,2}A_n$ ring cleavage ions.

Table 1. Computer assisted assignment of the major molecular ions detected in the $ALP_21Py2M3$ mixture by negative ion nanoESI-QTOF MS (Figure 1)

$[M-H]^-$ *m/z*	Theoretical *m/z*	Proposed composition
470.19	470.15	NeuAcHex*
632.22	632.20	$NeuAcHex_2$
673.21	673.23	NeuAcHexHexNAc*
714.26	714.26	$NeuAcHexNAc_2$*
787.28	–	n.a.*
819.28	819.29	NeuAcdHexHexHexNAc*
835.29	835.28	$NeuAcHex_2HexNAc$*
876.30	876.31	$NeuAcHexHexNAc_2$*
939.32	939.33	$dHexHex_3HexNAc(Ser)$*
997.37	997.34	$NeuAcHex_3HexNAc$
1038.34	1038.36	$NeuAcHex_2HexNAc_2$*
1200.39	1200.42	$NeuAcHex_3HexNAc_2$*
1249.41	–	n.a.*
1271.50	–	n.a.
1362.48	1362.47	$NeuAcHex_4HexNAc_2$*
1403.47	1403.50	$NeuAcHex_3HexNAc_3$*
1565.54	1565.55	$NeuAcHex_4HexNAc_3$*
1727.66	1727.60	$NeuAcHex_5HexNAc_3$
1768.64	1768.63	$NeuAcHex_4HexNAc_4$

Components marked by asterisk (*) have been submitted to MS/MS analysis. Abbreviations: Hex: hexose, HexNAc: N-acetylhexosamine, dHex: deoxyhexose, NeuAc: N-acetylneuraminic acid, n.a.: not assigned.

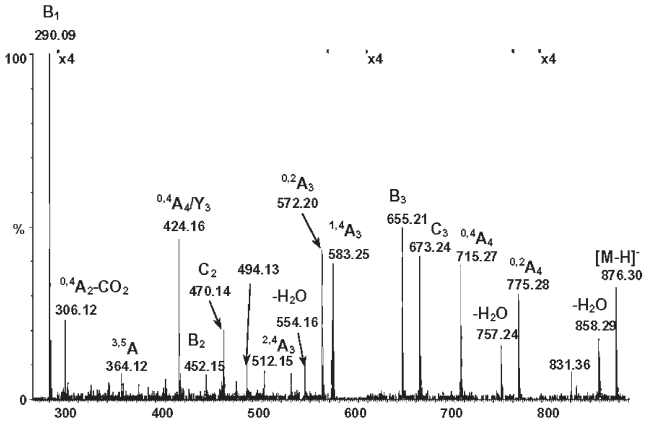

Figure 2. Negative ion nanoESI-QTOF MS/MS of the precursor ion at *m/z* 876.30 assigned to $NeuAcHexHexNAc_2$. Capillary voltage −1100 V, cone voltage −50 V, collision energy −45 eV.

MS to MS/MS automatic switching, previously introduced for on-line LC-MS and CE-MS analysis [21], was considered for further explorations of fragmentation patterns under short acquisition time. MassLynx 3.2 software (Micromass, Manchester, UK), as tested for an off-line work, required some adjustments. It could be observed that the acquisition of MS/MS in the time frame from 2 to 10 s already provides a highly informative fragmentation pattern, decreasing the total time frame for screening of one sample to 2 minutes. Considering the short ion accumulation time in the MS/MS, this method could therefore be optimized for screening complex biological samples, like urine fractions in our case. Following the concept of the $^{0,2}A_n$ sugar ring cleavage ion for identification of free glycans in mixtures with glycopeptides, the MS to MS/MS automatic switching method has been applied. Exploring experimental conditions and requirements for formation of $^{0,2}A_n$ ring cleavage ion, a series of measurements has been done. Starting from precursor ions at *m/z* 673.21, 876.30, 1038.34, 1200.39, 1362.48, and 1403.47 MS/MS analysis has been performed under different cone voltage and collision energy conditions to obtain respective fragment ions at *m/z* 572.17, 775.28, 937.29, 1099.37, 1261.43, and 1203.41 assigned as diagnostic $^{0,2}A_n$ ions.

We could conclude that no universal cone voltage and collision energy parameters can be set to obtain diagnostic $^{0,2}A_n$ ions for all molecular species present in the mixture. For molecular ions predicted as disaccharides the optimum cone voltage was in the 40–50 V range and the collision energy in the 25–35 eV range, whereas for ions assigned as oligosaccharides containing 4–6 building block monosaccharides optimal values were 50–70 V and 40–45 eV, respectively. The necessity

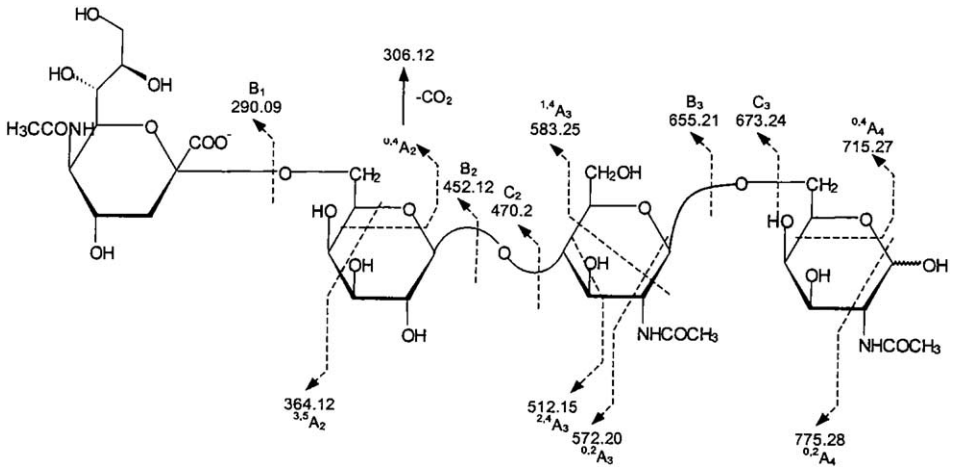

Figure 3. Molecular structure and fragmentation scheme of NeuAcGalGlcNAcGalNAc, precursor ion at *m/z* 876.30, derived from MS/MS data (Figure 2).

of higher energy for ionization and higher collisional energy for sequencing larger molecules poses a prerequisite to run a series of at least two experiments with distinct ionization and fragmentation conditions to obtain a full set of data about the presence and identity of all ionic species generated from the precursor ion, which can be realized conveniently by automatic MS to MS/MS switching.

The fragmentation of the precursor ions presented in Figure 1 were recorded under optimized conditions for each component as shown in Figures 4, and 5. In Table 2 the overview of diagnostic $^{0,2}A_n$ fragment ions applied for assignment of free glycans is presented.

The resulting MS/MS are presented in two distinct groups, according to the diagnostic loss of $\Delta m = 101$ u assigned to the HexNAc $^{0,2}A_n$ cross-ring cleavage ion. In the group shown in Figure 4 the glycans could be assigned according to this principle as free glycan species, whereas in the second series, depicted in Figure 5, no losses of $\Delta m = 101$ u have been observed.

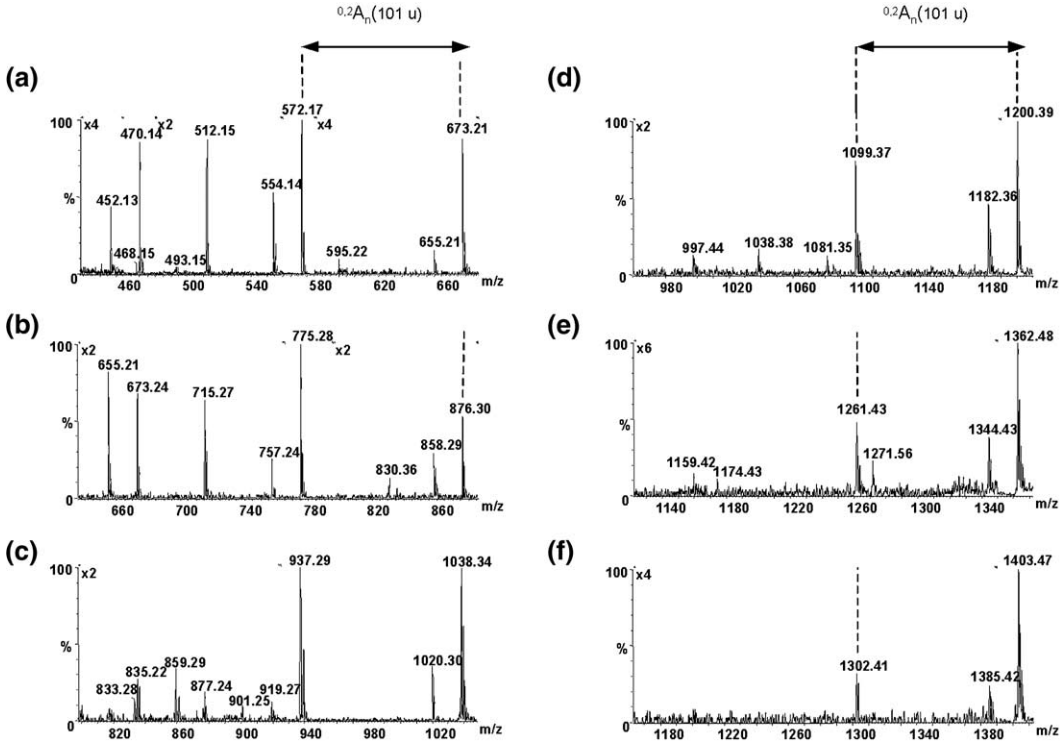

Figure 4. $^{0,2}A_n$ fragment ions, obtained by MS/MS of the relevant precursor ions marked by asterisk in the Table 1. Capillary voltage 1100 V, cone voltage 65 V (**c**), 60 V (**f**), 50 V (**a**), (**b**), (**d**), (**e**), collision energy 45 eV. The ion at *m/z* $[M-H]^-$ - 101 in (**a**)–(**f**) was indicating the presence of N-acetylhexosamine unit at the reducing end.

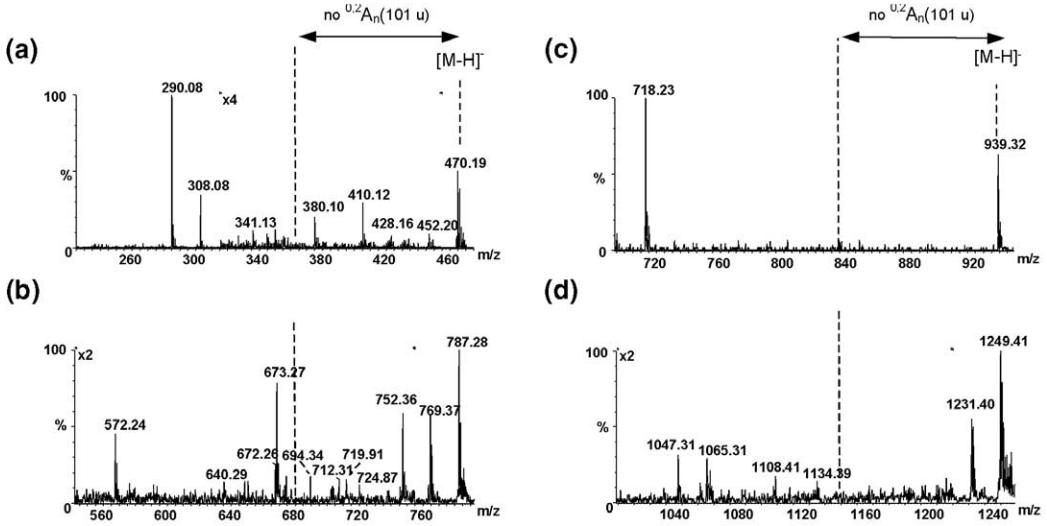

Figure 5. $^{0,2}A_n$ fragment ions, obtained by MS/MS of the relevant precursor ions marked by asterisk in the Table 1. Capillary voltage 1100 V, cone voltage 50 V (**a**), (**c**), (**d**), 40 V (**b**), collision energy 45 eV (**c**), (**d**), 35eV (**b**), 25eV (**a**). The absence of ion at *m/z* $[M-H]^-$ - 101 in (**a**)–(**d**) was indicating the absent of N-acetylhexosamine unit at the reducing end.

According to the MS/MS of precursor ions assigned as free glycans (Figure 4), the diagnostic $^{0,2}A_n$ ions were at *m/z* 572.17 for 673.21 (4A), 775.28 for 876.30 (4B), 937.29 for 1038.34 (4C), 1099.37 for 1200.39 (4D), 1261.43 for 1362.48 (4E), and 1302.41 for 1403.47 (4F). On the other hand $\Delta m=101$ u losses were not observed from the precursor species depicted in Figure 5, at *m/z* 470.19 (5A), 787.28(5B), 939.32 (5C), and 1249.41 (5D). The loss of $\Delta m=101$ u was also not generated from the precursor ion at *m/z* 470.19 (NeuAcHex) but of $\Delta m=60$ u indicating a $^{0,2}A_2$ Hex ring cleavage (Figure 5a). The ions at *m/z* 787.28, 939.32, and 1249.41 might represent glycoconjugates with the blocked reducing terminus, since they could not be assigned either by the computer calculation or by the loss of $\Delta m=101$ or $\Delta m=60$ u as free glycans.

Conclusions

In this study, the use of the $^{0,2}A_n$ ion represented by a loss of 101 u from the molecular precursor ion for assignment is presented. Species such as an N- or O-glycan, containing a HexNAc unit at the reducing end, GlcNAc or GalNAc, can be identified by such rapid screen, but for a full assignment other sequence and ring cleavage ions must be considered.

For an efficient analysis of heterogeneous samples, a crucial aspect is the assignment and identification of possibly all components present, in particular by high throughput techniques. Such techniques were not introduced for the complex carbohydrate analysis previously.

The appearance of the $^{0,2}A_n$ ring cleavage ion in the MS/MS suggests its application as a diagnostic tool to distinguish between free glycans from glycoconjugates and can be considered together with B and Y cleavage ions for structural identification.

Acknowledgments

The authors thank Deutsche Forschungsgemeinschaft (Bonn-Bad Godesberg, Germany) for financial support.

Table 2. The overview of diagnostic $^{0,2}A_n$ fragment ions applied for assignment of free oligosaccharides in the $ALP_2$1Py2M3 mixture

$[M-H]^-$ *m/z*	Proposed composition	$^{0,2}A_n$ fragment ion *m/z*	Terminal HexNAc
470.19	**NeuAcHex**	**410.12**	**No**
673.21	**NeuAcHexHexNAc**	**572.17**	**Yes**
714.26	$NeuAcHexNAc_2$	613.25	Yes
819.28	NeuAcdHexHexHexNAc	718.23	Yes
835.29	$NeuAcHex_2HexNAc$	734.28	Yes
876.30	**$NeuAcHexHexNAc_2$**	**775.28**	**Yes**
1038.34	**$NeuAcHex_2HexNAc_2$**	**937.29**	**Yes**
1200.39	**$NeuAcHex_3HexNAc_2$**	**1099.37**	**Yes**
1362.48	**$NeuAcHex_4HexNAc_2$**	**1261.43**	**Yes**
1403.47	**$NeuAcHex_3HexNAc_3$**	**1302.41**	**Yes**
1565.54	$NeuAcHex_4HexNAc_3$	1464.53	Yes

The MS/MS of precursor ions in bold are presented in Figures.

References

1. Domon, B.; Müller, D. R.; Richter, W. J. Determination of interglycosidic linkages in disaccharides by high performance tandem mass spectrometry. *Int. J. Mass Spectrom. Ion Processes* **1990,** *100,* 301–311.
2. Šagi, D.; Peter-Katalinić, J.; Conradt, H. S.; Nimtz, M. Sequencing of tri- and tetra-antennary N-glycans containing sialic acid by negative mode ESI QTOF tandem MS. *J. Am. Soc. Mass Spectrom.* **2002,** *13,* 1138–1148.
3. Robbe, C.; Capon, C.; Coddeville, B.; Michalski, J. C. Diagnostic ions for the rapid analysis by nano-electrospray ionization quadrupole time-of-flight mass spectrometry of O-glycans from human mucins. *Rapid Commun. Mass Spectrom.* **2004,** *18,* 412–420.
4. Quemener, B.; Cabrera Pino, J. C.; Ralet, M. C.; Bonnin, E.; Thibault, J. F. Assignment of acetyl groups to O-2 and/or O-3 of pectic oligogalacturonides using negative electrospray ionization ion trap mass spectrometry. *J. Mass Spectrom.* **2003,** *38,* 641–648.
5. Jaeken, J. Familial psychomotor retardation with markedly fluctuating serum prolactin, FSH, and GH levels, partial TBG deficiency, increased serum arylsulfatase A, and increased CSF protein: A new syndrome? *Pediat. Res.* **1980,** *16,* 179.
6. Chai, W.; Piskarev, V.; Lawson, A. M. Branching pattern and sequence analysis of underivatized oligosaccharides by combined MS/MS of singly and doubly charged molecular ions in negative-ion electrospray mass spectrometry. *J. Am. Soc. Mass Spectrom.* **2002,** *13,* 670–679.
7. Chai, W.; Piskarev, V.; Lawson, A. M. Negative-ion electrospray mass spectrometry of neutral underivatized oligosaccharides. *Anal. Chem.* **2001,** *73,* 651–657.
8. Harvey, D. J. Collision-induced fragmentation of underivatized N-linked carbohydrates ionized by electrospray. *J. Mass Spectrom.* **2000,** *35,* 1178–1190.
9. Harvey, D. J. Electrospray mass spectrometry and fragmentation of N-linked carbohydrates derivatized at the reducing terminus. *J. Am. Soc. Mass Spectrom.* **2000,** *11,* 900–915.
10. Pfenninger, A.; Karas, M.; Finke, B.; Stahl, B. Structural analysis of underivatized neutral human milk oligosaccharides in the negative ion mode by nano-electrospray MS(n). Part 1: Methodology. *J. Am. Soc. Mass Spectrom.* **2002,** *13,* 1331–1340.
11. Harvey, D. J. Ionization and collision-induced fragmentation of N-linked and related carbohydrates using divalent cations. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 926–937.
12. Que, A. H.; Novotny, M. V. Structural characterization of neutral oligosaccharide mixtures through a combination of capillary electrochromatography and ion trap tandem mass spectrometry. *Anal. Bioanal. Chem.* **2003,** *375,* 599–608.
13. Butler, M.; Quelhas, D.; Critchley, A. J.; Carchon, H.; Hebestreit, H. F.; Hibbert, R. G.; Vilarinho, L.; Teles, E.; Matthijs, G.; Schollen, E.; Argibay, P.; Harvey, D. J.; Dwek, R. A.; Jaeken, J.; Rudd, P. M. Detailed glycan analysis of serum glycoproteins of patients with congenital disorders of glycosylation indicates the specific defective glycan processing step and provides an insight into pathogenesis. *Glycobiology* **2003,** *13,* 601–22.
14. Zamfir, A.; Vakhrushev, S.; Sterling, A.; Niebel, H.-J.; Allen, M.; Peter-Katalinić J. Fully automated Chip-Based Mass Spectrometry for complex carbohydrate system analysis. *Anal. Chem.* **2004,** *76,* 2046–2054.
15. Domon, B.; Costello, C. E. A systematic nomenclature for carbohydrate fragmentation in FABMS/MS of glycoconjugates. *Glycoconj. J.* **1988,** *5,* 397–409.
16. Nahrings, C. Diploma Thesis, University of Bonn, 1994.
17. Williger, K. Ph.D. Thesis, University of Bonn, 1993.
18. Peter-Katalinić, J. Analysis of glycoconjugates by fast atom bombardment mass spectrometry and related MS techniques. *Mass Spectrom. Rev.* **1994,** *13,* 77–90.
19. Meisen, I.; Peter-Katalinić, J.; Müting, J. Discrimination of neolacto-series gangliosides with α2-3- and α2-6-linked N-acetylneuraminic acid by nanoelectrospray ionization low-energy collision-induced dissociation tandem quadrupole TOF MS. *Anal Chem.* **2003,** *75,* 5719–5725.
20. Wheeler, S. F.; Harvey, D. J. Negative ion mass spectrometry of sialylated carbohydrates: discrimination of N-acetylneuraminic acid linkages by MALDI-TOF and ESI-TOF mass spectrometry. *Anal. Chem.* **2000,** *72,* 5027–5039.
21. Zamfir, A.; Peter-Katalinić, J. Glycoscreening by on-line sheathless capillary electrophoresis/electrospray ionization-quadrupole time of flight-tandem mass spectrometry. *Electrophoresis* **2001,** *22,* 2448–2457.