Isomeric analysis of oligomannosidic N-glycans and their dolichol-linked precursors

Martin Pabst2,*, Josephine Grass2,*, Stefan Toegel4, Eva Liebminger3, Richard Strasser3, and Friedrich Altmann1,2

1Department of Chemistry; 2Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences, Muthgasse 18, 1190 Vienna, Austria; and 4Department of Orthopedics, Medical University Vienna, Vienna, Austria

Received on June 1, 2011; revised on August 30, 2011; accepted on September 6, 2011

Oligomannosidic (OM) N-glycans occur as a mixture of isomers, which at early stages of glycosidase trimming also comprise structures with one to three glucose residues. A complementary set of isomers is generated during the biosynthesis of the lipid-linked precursor. Here, we demonstrate the remarkable capacity of liquid chromatography (LC) with porous graphitic carbon and mass spectrometric detection for the determination of OM isomers. Protein-linked N-glycans were released enzymatically from samples with known isomer composition such as kidney bean proteins and ribonuclease B. Lipid-linked oligosaccharides were obtained by a direct mild acid hydrolysis of microsomes thus avoiding biphasic partitioning. A parallel analysis of pyridylaminated glycans by amide-silica and reversed-phase high-performance LC, the application of pyrophospho-dolichol, which is supposed to improve protein folding via lectin chaperoning (Doerrler and Lehrman 1999). OM N-glycans occur as mixtures of isomers. Interest in the exact isomeric structure of OM glycans arose from the observation that endoplasmic reticulum (ER) α1,2-mannosidases have differing substrate specificities and ER-resident mannose 6-phosphate receptor homology domain-containing lectins specifically associate with certain OM glycans (Gonzalez et al. 1999; Liebminger et al. 2009; Hosokawa et al. 2010a). The order of mannose removal affects the entry of unfolded non-native proteins into the calnexin/calreticulin cycle and influences the time frame for interaction in this glycoprotein folding cycle (Ruddock and Molinari 2006; Stigliano et al. 2011). Glycoproteins destined for the ER-associated degradation are substrate to ER-degradation enhancing α-mannosidase-like proteins (“EDEMs”; Olivari et al. 2006; Ruddock and Molinari 2006; Lederkremer 2009), which lead to the accumulation of certain isomers that flag misfolded proteins for degradation (Aebi et al. 2010). Thus, the particular type of an OM glycan present on a newly synthesized glycoprotein constitutes a signal of the protein folding state and influences a glycoprotein’s further fate (Hosokawa et al. 2010a). The analysis of OM N-glycans present in yeast αlg mutants has been key to deciphering the glycoprotein degradation signal and other critical steps of the ER quality-control process (Clerc et al. 2009; Stigliano et al. 2011). Moreover, for plants, it has also been shown that the presence of specific OM N-glycans affects root development and cell wall formation (Liebminger et al. 2009) highlighting the importance of approaches to analyze and assign these OM isoforms.

The predominance of oligomannose glycans on the surface of immunodeficiency viruses (Doores et al. 2010) and their involvement in host immune defense, i.e. recognition by one human anti-human immunodeficiency virus antibody (Scanlan et al. 2002), may be taken as reminder not to ignore the true structure of these oligomers.

Congenital disorders of glycosylation display a highly varying degree of severity and clinical manifestations (Freeze...
and Aebi 2005), where in certain cases the amount and structure of OM glycans may matter.

Determination of the isomeric structure of an OM glycan of a given size is mandatory for the deeper understanding of these processes and of course for the characterization of the substrate specificity of recombinant α-mannosidases. Nuclear magnetic resonance (NMR) spectroscopy certainly gives the most conclusive and definite answers, yet also requires rather large amounts of pure samples and is not the method of choice for enzymological or cell biological studies. About 20 years ago, a two-dimensional high-performance liquid chromatography (HPLC) strategy with fluorescence detection of pyridylaminated (PA) N-glycans was introduced for the separation and assignment of OM N-glycans (Oku et al. 1990; Tomiya et al. 1991; Trimble et al. 1991). This method relied on the collection of peaks by a normal-phase HPLC on amide-silica as the first dimension, drying the collected volumes and subjecting each peak to the reversed-phase HPLC as the second dimension or on off-line mass spectral (MS) detection of the glycan size (Suzuki et al. 2008). In a similar, early approach, Microsorb C18 column chromatography was employed to discriminate OM isomers (Moore and Spiro 1990). Regardless of the considerable effort associated with this two-dimensional HPLC, it was used in many biological studies (Gonzalez et al. 1999; Liebminger et al. 2009).

To our experience, the fractionation and handling steps impair the measurement of quantitative ratios as the size fractionation by a normal-phase HPLC often does not work out as neat and clear cut as intended. One remedy to this problem may be to use gel filtration for size separation and to exploit the chromatographic selectivity, if small, of amide-silica for isomers (Hosokawa et al. 2010b). Porous graphitic carbon (PGC) displayed a promising selectivity for OM isomers of *Pichia pastoris* glycoproteins (Lipniunas et al. 1996), although the then used ultraviolet detection may have left a bit to desire regarding selectivity. The rise of MS detection opened new possibilities. MS detection after reversed-phase HPLC substituted the tedious size fractionation of PA-glycans (Nakagawa et al. 2007). The weak retention of PA-glycans by a reversed-phase surface may pose problems both for sample application to capillary or nano-format columns (unpublished observation) and for detection as only very low amounts of an organic modifier are used here. An alternative approach is the application of sophisticated MS strategies to unseparated samples (Zhao et al. 2008; Prien et al. 2009).

The analysis of LLO precursors of N-glycans is regarded a delicate task as it traditionally involves biphasic extractions of the dolichol-linked sugars and very often metabolic labeling to allow for detection (Lehman 2007). Non-radioactive methods based on fluorescent labeling of free oligosaccharides have recently been introduced (Gao and Lehman 2002; Grubenmann et al. 2004; O’Reilly et al. 2006; Hosokawa et al. 2010b). Analysis was confined to size sorting and isomer separation was obtained only to a limited extent as, for example, in a recent work on EDEM1 (Hosokawa et al. 2010a).

In this work, we have investigated the suitability of PGC in conjunction with MS detection for the separation and identification of the biosynthetically relevant isomers of OM N-glycans from glycoproteins as well as from dolichol-linked precursors.

**Results**

Here, we describe our observations on the separation of >30 OM glycans derived from either glycoproteins or lipid-linked precursors. The LC (liquid chromatography)-ESI (electrospray ionization)-MS system resembled essentially that used in previous papers (Pabst et al. 2007, 2010; Stadlmann et al. 2008). Much emphasis is laid on the reasoning for the assignment of a peak as a particular isomer. With retention times being thus highly important, deuterated (M\(^2\)-OM\(^2\))M\(^2\) = [8a] (the number in square brackets refers to the coordinates of the compound as depicted in Figure 1) and the complex-type, biantennary N-glycan A\(^4\)A\(^4\) (diantennary N-glycan with two β1,4-linked terminal galactoses; Figure 1) were added to all samples as internal standards in order to allow the correction of retention time shifts, which have to be taken into account to some extent on carbon columns (Pabst and Aebi 2008; Mélmer et al. 2010). Elution positions are given relative to (M\(^2\)-OM\(^2\))M\(^2\) = [9a].

Isomers are described with the “proglycan” code, which is explained in the following section and deciphered in Figure 1. For easier reading, these codes are accompanied by their accidental coordinates in Figure 1. The hurried reader may thus decide to skip the following section.

**Nomenclature for OM N-glycans**

The intention behind devising yet another nomenclature for OM glycans arises from the want for names understandable without a deciphering table, such as those used by the esteemed pioneers of glycan analysis (Oku et al. 1990; Tomiya et al. 1991). Obviously, we also wanted abbreviations complying with the “proglycan” system (www.proglycan.com). This abbreviation system is based on the notion that an N-glycan structure is essentially defined by its terminal residues.

The common pentasaccharide core terminating with two mannoses is called MM in the “proglycan” system, whereby the first M stands for the 6-branch and the second for the 3-branch. Let us at first elongate this pentasaccharide with a GlcNAc residue on the lower arm (3-arm or C-branch). Then, this complex N-glycan is written as MGn, because the terminal residue is now a GlcNAc = Gn and no longer mannose. In GnM, the GlcNAc sits on the 6-arm. In the case of oligomannose glycans, we face a small problem. An α1,3-mannose on the 6-branch is again just depicted by an M. However, as it is no longer the invariable core mannose, we distinguish it by adding the linkage specifier “\(^3\)”, M\(^2\)M thus contains a 3-linked mannose on the upper arm. Adding another mannose in 6-linkage generates a branch and this is illustrated by brackets: (M\(^2\)-OM\(^2\))M. This is the common Man5 structure called M5.1 by the Takahashi group (Tomiya et al. 1991) or Man5A by the Hase group (Oku et al. 1990). The addition of an α1,2-mannose to the lower arm results in the designation (M\(^2\)-OM\(^2\))M. Now, it is time to mention that the number of mannoses in the molecule equals the number of figures plus 3.
Fig. 1. Structures of OM N-glycans. Structures that may occur in the course of biosynthesis and enzymatic degradation of OM glycans are listed. Glycans are labeled with their “proglycan” codes. The numbers in the upper left corner of each box are the retention time relative to that of true Man9 = (M2-6M2-3)M2-2 = [9a]. This number is set in brackets, where assignments are of tentative nature. Figures to the left of the table give the number of hexose residues, and the letters on the top of the grid are arbitrary. Structures obtained from chanterelles or Alg mutants are labeled with “Chan.” or “Alg + number,” respectively. The cartoons on top shall facilitate understanding of the structure drawings and of the “proglycan” code. Branch designation is as established elsewhere (Liebminger et al. 2009; Hosokawa et al. 2010a). Sugars symbols are: gray circles = mannose; black squares = N-acetylglucosamine; black circles = glucose; white circles = galactose. The “proglycan assistant” shows the order in which antennae are considered.
Larger OM glycans contain more than just single mannoses attached to the common core. If we elongate our Man6 isomer \((M^6G^3)M\) on the C-arm, we must find a code for the disaccharide Man6-2Man1-6. We may do this by writing \(M\rightarrow M^6\) or \(M^6\rightarrow M\) or \(M^6\rightarrow M^6\), which is a reminiscence of \(Na\) →, the term that encodes the disaccharide Neu5Acα2-6Galβ1-4 (linked to GlcNAcβ1-2Manα1-6) in complex-type glycans. The skeptic reader may briefly think about any other way to write—not draw—the Man7 isomers \((M^7G^3)M^2\), \((M^7G^3)M^3\) and \((M^7G^3)M^4\). The stringent logic of this system renders the codes machine readable as attested by the “proglycan translator” (www.proglycan.com).

At the very beginning of its existence, N-glycans consist of mannose and up to three glucose residues. With just one glucose, we denote the terminal residues on the 3-arm as G. Thus, adding a glucose to the Man8 glycan \((M^8G^3)M^2\) = [8a] results in \((M^8G^3)M^2\)G = [9b], which is the isomer recently described as G1M8B (Hosokawa et al. 2010a). For more glycans, we use the linear-code strings G-G or G-G-G. The well-known Glc3ManαGlcNAc2 precursor oligosaccharide is thus written as \((M^9G^3M^2)G\). Any processing step can be unambiguously described by the herein proposed system as illustrated in Figure 1 and Table I.

### Table I. Effect of particular sugar residues on retention time

<table>
<thead>
<tr>
<th>Substrate</th>
<th>RRT</th>
<th>Product</th>
<th>RRT</th>
<th>Coordinates</th>
<th>RRT</th>
<th>Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1,2-Glc</td>
<td>1.56</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.36</td>
<td>12a → 11a</td>
<td>0.2</td>
<td>5.6</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.57</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.29</td>
<td>11b → 10b</td>
<td>0.28</td>
<td>7.8</td>
</tr>
<tr>
<td>α2-6Gal</td>
<td>1.83</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.67</td>
<td>11c → 10c</td>
<td>0.16</td>
<td>4.4</td>
</tr>
<tr>
<td>α1,2-Mann</td>
<td>1.74</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.56</td>
<td>10d → 9d</td>
<td>0.18</td>
<td>5.0</td>
</tr>
<tr>
<td>left α1,2-Glc</td>
<td>1.36</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.14</td>
<td>11a → 10a</td>
<td>0.22</td>
<td>6.1</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.29</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.08</td>
<td>10b → 9b</td>
<td>0.21</td>
<td>5.8</td>
</tr>
<tr>
<td>left α1,3-Glc</td>
<td>1.67</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.38</td>
<td>10c → 9c</td>
<td>0.29</td>
<td>8.1</td>
</tr>
<tr>
<td>right α1,3-Glc</td>
<td>1.56</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.27</td>
<td>9d → 8d</td>
<td>0.29</td>
<td>8.1</td>
</tr>
<tr>
<td>C-branch</td>
<td>1.14</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.08</td>
<td>8d → 7d</td>
<td>0.11</td>
<td>3.1</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.27</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.05</td>
<td>8d → 7d</td>
<td>0.22</td>
<td>6.1</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.57</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.47</td>
<td>11b → 10d</td>
<td>-0.17</td>
<td>-4.7</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.29</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.56</td>
<td>10b → 9d</td>
<td>-0.27</td>
<td>-7.5</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.08</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.27</td>
<td>9b → 8d</td>
<td>-0.19</td>
<td>-5.3</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.00</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.09</td>
<td>8b → 7b</td>
<td>-0.09</td>
<td>-2.5</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>0.95</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.10</td>
<td>7c → 6a</td>
<td>-0.15</td>
<td>-4.2</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>1.19</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.05</td>
<td>6b → 5a</td>
<td>-0.08</td>
<td>-8.6</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>0.97</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.05</td>
<td>7b → 6a</td>
<td>-0.07</td>
<td>-19</td>
</tr>
<tr>
<td>α1,2-Man</td>
<td>0.96</td>
<td>((M^8G^3)G)-G-G</td>
<td>1.39</td>
<td>6b → 5a</td>
<td>-0.43</td>
<td>-119</td>
</tr>
</tbody>
</table>

Glycan pairs differing by one residue are listed as “substrate” and “product” of removal of this residue. Relative run times (RRTs) and approximate absolute retention time differences are given.

PGC-LC of reference samples

PGC-LC results of samples containing OM N-glycans hinted at the possibility to separate the isomers of Man7 and Man8. Therefore, we referred to samples, whose N-glycan structures had been thoroughly determined earlier. Such materials were the kidney bean glycoprotein II, whose N-glycans have been analyzed by NMR spectroscopy at the University of Utrecht.
Man7 isomers were seen on PGC-LC, whose ratio $(M6M3)M2-2 = [7a]$ and $(M2-6M3)M2 = [7b]$ (data not shown). The Man7 isomer $(M6M2-3)M2 = [7c]$, however, could not be detected without the acid hydrolysis step, which on the other hand was just strong enough to cleave the acid-labile sugar-phosphate linkage. Thus, this simple preparation of the Man7 pool. This is just one example of the difficulty inherently associated with the two-dimensional HPLC of PA-glycans. Ribonuclease B additionally contained classic Man5 = $(M6M^3)M = [5a]$ and, according to NMR data, also a smaller amount of $(M2-6M2-3)M^2 = [8c]$ (Fu et al. 1994). As realized later (see section on mannosidase digests), this isomer elutes very close to $(M2-6M3)M2-2 = [8a]$ and may at this ratio not show a separate peak. Indeed, this pair of Man8 isomers turned out as the only case of an unsatisfactory selectivity of the carbon column for OM N-glycans.

**Analysis of lipid-linked precursors from wild-type and ALG mutant plants**

Larger OM glycans may contain residual glucose residues, which leads to ambiguity in the isomer assignment. We decided for lipid-linked precursor structures as a source of glucose containing N-glycan structures. An additional motif came from the availability of Arabidopsis thaliana ALG mutants, which have specific defects in the synthesis pathway of the dolichol-linked glycan precursor (Samuelson et al. 2005).

Relying on the high sensitivity and specificity of MS detection, we developed a rather novel strategy for the analysis of lipid-linked precursors. The usual chloroform-methanol extraction step was substituted by a quick microsomal isolation procedure followed directly by mild-acid hydrolysis with 0.1 M trifluoroacetic acid instead of HCl as used in previous work (Lehrman 2007), as trifluoroacetic acid hydrolysis used to give higher yields of glycans. No free oligosaccharides could be detected without the acid hydrolysis step, which on the other hand was just strong enough to cleave the acid-labile sugar-phosphate linkage. Thus, this simple preparation appears to be highly specific for LLOs. The thus obtained oligosaccharides of, for example, plant leaves, porcine liver or yeast consisted of a series of glycans with the classical $(M2-6M2-3)G-G-G = [12a]$ as the major compound (Figure 3). The rather high elution time of this compound indicated that some of the glucose residues—in contrast to most mannose residues—had a profoundly strong positive effect on PGC retention. This inference was fully corroborated by analyses of the alg10 mutant, which cannot add the third glucose residue and hence accumulated $(M2-6M2-3)G-G = [11a]$ (data not shown).

Wild-type Nicotiana benthamiana (and similarly also A. thaliana, P. pastoris or the mouse liver) exhibited three peaks each for the Hex11, Hex10 and Hex9 glycans (Figure 3). The earliest eluting and largest peaks were assumed to consist of nine Man and 0–2 Glc residues, whereas the following peaks might represent Man8 isomers with 1–3 glucose. This was verified by digestion with branch-specific mannosidases (see next section).

The exotic isomer $M2-3M2-2 = [7e]$ is obtained from alg12 A. thaliana plants, which are unable to initiate the C-branch, which facilitates glucosylation of the A-branch (Figure 1).

**PGC-LC of mannosidase digests**

The branch-specific $A. thaliana \alpha_1,2$-mannosidase MNS1 disregards the B-arm (Figure 1), whereas MNS3 acts exclusively here (Liebminster et al. 2009). The treatment of Man9Glc3 with MNS1 or MNS3 therefore generated $(M6M^2-3)G-G-G$ and $(M6M2-3)G-G-G$, respectively, and treatment with both enzymes led to $(M6M^2)G-G-G = [10d]$. These experiments corroborated the above made interpretations of the elution pattern of LLOs (Figure 3). In fact, these degradations were performed with the LLO mixtures, which also contained Man9Glc2 and Man9Glc1. Therefore, we could also track the fate of $(M2-6M^2-3)G-G-G = [11a]$ and $(M2-6M^2-3)G-G = [10a]$.

A nice extra was that preexisting $(M2-6M^3)G-G-G = [11b]$ was degraded to Man7Glc3 = $(M6M^3)G-G-G = [10d]$ by MNS1 (Figure 3). Analogously, elution positions of $(M6M^3)G-G-G = [9d]$ and $(M6M^3)G = [8d]$ could be estimated. These assignments are strengthened by the observation that the retention times as a whole form a coherent mesh of data with often similar time increments for a particular sugar residue (Table 1), at least if retention time is also considered (Figure 3). Notably, other structural features can influence the increments as, for example, the presence of the C-arm $\alpha_1,2$-mannose increases the effects of $\alpha_1,3$-glucose residues. The observed retention time increments roughly matched those observed for non-glucosylated OM glycans (Table 1) and thus further confirmed the original assignments. The C-arm mannoside led to a decrease in retention time by several minutes irrespective of the presence of glucose. The B-arm mannoside in any case had a slightly increasing effect. For several residues, the overall retention time seems to influence the “unit contribution” (Figure 3).

When MNS1 was applied to the “regular” Man9 structure $(M2-6M^3)M2-2$, with both the A- and the C-branch mannosides being accessible, the major peak, however, eluted earlier than the substrate, also a bit earlier than $(M2-6M^3)M2-2 = [8a]$. We conclude that the A-arm mannoside had preferentially been removed to yield $(M2-6M2-3)M2-2 = [8c]$. Admittedly, the presence of a small amount of $(M2-6M^3)M2-2 = [8a]$ would have gone unnoticed because of the close elution of these Man8 isomers.
Fig. 2. Analysis of OM N-glycans. Reduced N-glycans were separated by PGC-LC-ESI-MS. (a) The sum spectrum of all N-glycan peaks, which occur as singly, or doubly charged ions without or with ammonium (indicated by an asterisk). (b) Selected ion chromatograms (SICs) were drawn from three different analyses. Panels designated with (R) show data of ribonuclease, (K) stands for kidney bean glycoproteins and (M) is for a Man9 sample digested with MNS1, which is an α1,2-mannosidase with a strong preference for the A- and C- arms as opposed to the B-arm. Retention times above peaks are given relative to that of “true” Man9 = (M^2-6M^2-3)M^2-2. In addition to the proglycan codes, shorter acronyms as used in earlier work (Tomiya et al. 1991) are given. SIC traces for M + H^+ or M + NH_4^+ ions were taken, whichever ion type was most abundant. The quantitative profile of the Man7 isomers in the kidney bean aided in correlating the peaks to literature data (30:60:10 in the order of elution shown here; Neeser et al. 1985).
The Man7 isomers gave four peaks of which \((M^6M^2-3)M^2 = [8c]\) dominated. Two smaller peaks—coinciding with already known isomers—were the products of the small activity of MNS1 toward the B-arm (Liebminger et al. 2009). The latest eluting peak, however, has not been observed elsewhere and was classified as \((M^2-6M^2-3)M = [7d]\). A similarly profound increase in retention by the inner \(\alpha_1,2\)-mannose of the A-arm was observed at the conversion of \((M^6M^3)M^2 = [6a]\) to \((M^6M^3)M = [5a]\) (Table I). MNS1 digestion led to the accumulation of a new Man6 isomer, which, according to previous work (Liebminger et al. 2009), was \((M^6M^2-3)M = [6c]\).

A digest of Man9 with jack bean \(\alpha\)-mannosidase depicted the degradation pathway down to the usual Man5 isomer \((M^6M^3)M\) as established by Tomiya et al. (1991). Upon longer incubation, an interesting set of smaller, “paucimannosidic” glycans was generated, which will be the topic of the following chapter.

**Paucimannosidic glycans**

Paucimannosidic glycans contain fewer mannose residues than OM glycans and they are the result of other than Golgi \(\alpha1,2\)-mannosidases. They may be formed by \(N\)-acetylglucosaminidase trimming of small complex-type sugars (Liebminger et al. 2011) or by the action of vacular \(\alpha\)-mannosidases akin to jack bean mannosidase. As to this definition, unphysiological Man5 isomers such as, for example, \((M^6M^2-3)U\) or \((M^2-6M^3)U\) may also be seen as paucimannosidic. However, both jack bean mannosidase and \(A.\ thaliana\) MNS1 generated just one and the same \((M^6M^3)M = [5a]\) isomer from Man9. The jack bean mannosidase digest contained two Man4 isomers with the major one representing \((M^6M^3)U = [4b]\) (Tomiya et al. 1991). The smaller peak will likely have been \(M^3M = [4a]\) based on the deliberation that this compound eluted far ahead of the Man5 substrate and that the removal of the pertinent \(\alpha1,3\)-linked mannose in the conversion of \(M^3U\) to \(MU = [3b\text{ to }2a]\) resulted in a similar upward shift (Table I). Two Man3 isomers were observed, of which none eluted at the time of the core pentasaccharide \(\text{MM} = [3a]\) (data not shown). According to the literature (Tomiya et al. 1991), the more abundant peak was considered as being \(M^3U = [3b]\). This was converted to \(MU = [2a]\) and finally to the trisaccharide \(UU\), where the \(\beta\)-mannose is unsubstituted in both the 3- and 6-positions.

A complementary set of small OM glycans was found in \(A.\ thaliana\) alg3 leaves, which lack the \(\alpha1,3\)-mannosyltransferase initiating the B-arm (Kajiura et al. 2010). The alg3 sample gave retention times of MM and larger dolichol-linked oligosaccharides as well as that of the dimannosyl compound UM, i.e. Man\(\alpha\)-3Man\(\beta\)-3GlcNAc\(\beta\)-4GlcNAc(itol).

**Glycan profiles of samples from various species**

Human chondrocytes were arbitrarily chosen as a representative of a mammalian specimen. The remarkable point here is that the isomeric analysis of oligomannose glycans comes together with the isomeric separation of all other types of \(N\)-glycans in the sample from neutral to tetra-sialylated...
structures. The structural assignment of biantennary N-glycans with differing linkages of β-galactoses and sialic acids has been the topic of a previous study (Pabst et al. 2007). The degree and linkage of sialic acid on biantennary N-glycans was influenced by proinflammatory cytokines (Pabst et al. 2010). The same chromatographic run, however, could also identify the OM structures (Figure 4). Aside of 10 OM structures, at least 65 complex-type glycans with up to four sialic acids were observed (Figure 4). These in part isobaric compounds represented typical biantennary and triantennary glycans (some of them contaminations from fetuin in the cell culture medium), hybrid-type structures and also glycans with masses indicative of “LacdiNAc” structures with GalNAc–GlcNAc units.

The comparison of mammalian samples (ribonuclease B, ovalbumin and a protein from Chinese hamster ovary cell culture obtained from Polymun Inc., Vienna) and plant samples (bean glycoproteins, A. thaliana or N. benthamiana leaves) revealed a very similar distribution of the same OM isomers. Chanterelle (mushroom) N-glycans, however, contained additional peaks of Man7, Man8 and Man9 isomers that were not found in plant and animal samples (Grass et al. 2011). Pichia pastoris N-glycans contained a Man9 major isomer with a similar but not identical retention time as (M2-6M3)G = [9b]. According to previous work (Trimble et al. 1991), this isomer presumably is the result of mannosidase trimming and addition of a yeast-typical α1,6-mannose and can be written as (M2-6M3)(M6M2-2) = [9e], yet a β-mannose containing structure cannot be excluded (Gomathinayagam, S., Mitchell, T., et al. 2011). Several new peaks of larger glycans were found (Table I). Although these unusual isomers of fungal OM glycans must remain unassigned, they witness the ability of the PGC-LC-ESI-MS approach to spot the structures different from the mammalian standard types.

**Detection and quantification**

Most (complex-type) oligosaccharides preferentially form protonated ions during ESI-MS, but OM glycans tend to give ammonia adducts. This tendency grows with increasing size. Thus, two or even three peaks have to be taken into account for a quantitative assessment of results. To decide how to handle this problem, we analyzed a bean glycan sample both by LC-ESI-MS and by normal-phase HPLC of fluorescein-labeled glycans, which may be expected to yield true values for samples of low complexity and hence no peak overlapping.
Discussion

PGC-LC-ESI-MS is evolving into a most powerful method for the analysis of complex oligosaccharides (Schulz et al. 2007; Chu et al. 2009; Pabst and Altmann 2011). Nowadays, the reproducibility of capillary or nano HPLC is sufficient to fully exploit the amazing shape discrimination power of carbon columns for structural assignments. The use of internal standards and relative retention times is recommended in order to compensate for retention shifts, which are caused by as yet poorly understood red-ox reactions of the graphite phase and column clogging and which cannot be totally avoided (Tomkvist et al. 2004; Pabst and Altmann 2008; Melmer et al. 2010). With these simple precautions, isomers from the entire range of OM glycans including the glucosylated structures from lipid-linked precursors can be separated and discriminated from each other. For only a very few isomer pairs, low selectivity led to problems at a lopsided isomer ratio. The discrimination of Man8 isomers lacking either the A- or the B-arm mannose could benefit from increased separation power as provided by smaller diameter packings or longer columns. But even in its present form, this chromatographic approach appears as a useful alternative to non-separating multistage MS strategies using permethylated sugars (Zhao et al. 2008; Prien et al. 2009). But even for the MS\textsuperscript{3} analysis of permethylated sugars, the prior separation of isomers invalidates concerns about the artificial formation of isomers (Prien et al. 2010). Man5 isomers other than (M\textsuperscript{3}M\textsuperscript{3}) \textsuperscript{M} were not observed during this work, which was, however, not devised to detect minor components.

As regards the isomeric analysis of glucosylated precursors, we are not aware of a similarly comprehensive approach as the one reported here. The enrichment step by ultracentrifugation of vesicles (microsomes) rather than biphasic extractions is an optional detail, but—at least in our hands—it allowed the use of much smaller sample amounts (<0.5 g of leaves).

For reversed-phase and hydrophilic interaction chromatography (HILIC) separations of PA-labeled glycans, characteristic retention time increments were ascribed to each sugar residue for complex as well as for OM \textit{N}-glycans (Hase and Ikenaka 1990; Lee et al. 1990; Tomiya and Takahashi 1998; Yanagida et al. 1998). In graphitic carbon HPLC, a particular sugar residue also exhibits similar effects, but other features of the glycan structure apparently influence the exact shift (Table I). A striking example is the influence of the C-arm α,1,2-mannose on the retention effect of glucose residues. Several residues displayed a non-linear dependency of the glycan mass. The very strong retentive effect of glucose residues certainly helps to tell apart the removal/addition of glucose or mannose residues.

With the use of an MS detector, it would be obvious to substantiate the isomeric assignments by MSMS (tandem MS) fragmentation patterns. However, on-line collision-induced dissociation spectra of the underivatized glycans yielded essentially just B-, Y- and B/Y ions with just subtle and difficult to reproduce quantitative differences. Noteworthy, this held true for data acquired on the Q-TOF Ultima as well as the ion-trap MS (data not shown). Thus, for OM-glycans, at least for the larger ones, the gain achieved by MSMS experiments does not compensate the likely impairment of the chromatographic peak shape in the MS mode, which affects retention time and peak area measurement. The possibility of quantitation is an advantage of the herein presented chromatographic approach that is, as far as we know, not offered by isomer analysis via multistage MS of permethylated glycans (Zhao et al. 2008; Prien et al. 2009).

The isomer assignment of OM \textit{N}-glycans comes as a free byproduct of PGC-LC-ESI-MS analysis of \textit{N}-glycomes as shown with chondrocytes, where the same chromatographic run also delivered information about complex-type structures. With the increasing number of reference compounds available, an ever more complete assignment of the many peaks will be possible.

Material and methods

\textbf{N-Glycan preparations}

\textit{N}-Glycans of kidney beans, chanterelles and \textit{P. pastoris} proteins were prepared by pepsin digestion, glycopeptide extraction and peptide:\textit{N}-glycosidase A (proglycan, Vienna) treatment as described (Grass et al. 2011). Pure Man9 for the mannosidase digestion experiments was extracted in the same way from 0.5 g leaves of \textit{A. thaliana mns1 mns2 mns3} triple
knockdown mutant plants (Liebminger et al. 2009). Chondrocyte N-glycans were obtained as described (Pabst et al. 2010). In the case of the pure proteins ribonuclease B (Sigma-Aldrich), 5 µg was digested overnight at 37°C with 0.2 U of peptide:N-glycosidase F (Roche, Vienna, Austria) in 50 mM ammonium acetate of pH 8.4. For deamination, the sample was incubated at pH of ~5 for 15 min. The glycans were reduced in 1% sodium borohydride (typically 500 µL) at room temperature for 4 h. Salt was removed using a 10 mg HyperSep Hypercarb solid-phase extraction cartridge (Thermo Fisher Scientific, Vienna) (Packer et al. 1998). Samples were vacuum-dried and taken up in small volumes of water before analysis.

**Preparation of dolichol-linked glycans**

LLOs were extracted from leaves (0.5 g) of *N. benthamiana* and *A. thaliana* wild-type ecotype Col-0; alg3-, alg10 and alg12 [European Arabidopsis Stock Centre (http://arabidopsis.info/home.html) and Versailles T-DNA insertion line collection (http://www-ijpb.versailles.inra.fr/en/cla/cra_accueil.htm)] as well as from porcine liver by hydrolysis of the microsomal pellet. In brief, tissue (typically 1 g) was homogenized at 4°C in 5 mL of Tris–HCl buffer, pH 7.3, containing 0.5 mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid, 250 mM sucrose and 0.5 mM phenylmethylsulfonyl fluoride. The suspension was filtered through Miracloth (Merek, Darmstadt, Germany) and centrifuged at 3000 × g for 20 min. The supernatant was ultracentrifuged at 100,000 × g for 45 min. The microsomal pellet was resuspended in 1 mL of 0.1 M trifluoroacetic acid and incubated at 80°C for 1 h. After centrifugation at 16,000 × g for 3 min, the supernatant was made slightly alkaline with sodium hydroxide. Glycans were purified on a carbon solid-phase extraction cartridge (see above), then reduced and desalted again.

**Glycan analysis**

Reduced oligosaccharides were analyzed by PGC-LC-ESI-MS on a Hypercarb column (0.32 × 150 mM, Thermo Fisher Scientific) coupled to an Ultimate 3000 (Dionex, Vienna, Austria) capillary HPLC and a Q-TOF Ultima MS (Waters) as described previously (Pabst et al. 2007, 2010; Stadlmann et al. 2008). MSMS experiments were also conducted on a Thermo LCQ ion trap.

Retention time variations were corrected with the help of two internal standards, i.e. boroduteride-reduced (M2-6M3)Thermo LCQ ion trap. et al. 2008). MSMS experiments were also conducted on a Hypercarb column (0.32 × 150 mM, Thermo Fisher Scientific, Vienna) (Packer et al. 1998). Samples were vacuum-dried and taken up in small volumes of water before analysis.

**Funding**

This research was funded by the Austrian Science Fund (FWF): P22274 and P20817.

**Acknowledgements**

This work was in part supported by the “Österreichische Forschungsförderungsgesellschaft (FFG)” within the Laura-Bassi Center “Production of Glycan Optimized Biopharmaceuticals in Plants”. We thank Karin Polacek for wonderful plant glycan preparations, Akhlaq Farid for the screening of *A. thaliana* Alg mutants, Christopher Hasenhindl for proteins of *P. pastoris* and Thomas Dalik for invaluable technical assistance.

**Conflict of interest**

None declared.

**Abbreviations**

\[A\] = diantennary N-glycan with two \( \beta1,4 \)-linked terminal galactoses; EDEM, endoplasmic reticulum-degradation enhancing \( \alpha \)-mannosidase-like proteins; ER, endoplasmic reticulum; ESI, electrospray ionization; Glc, glucose; GlcNAc, \( N \)-acetylglucosamine; HILIC, hydrophilic interaction chromatography; HPLC, high-performance liquid chromatography; LC, liquid chromatography; LLO, lipid-linked oligosaccharide; Man, mannoside; (M\(^2-6M\(^3\))\(^2\)) \( \rightarrow \) and similar terms, glycans structures see Figure 1; MS, mass spectrometry; MSMS, tandem MS; NMR, nuclear magnetic resonance; OM, oligomannoside; PA, pyridylamino; PGC, porous graphitic carbon.

**References**


**c** α 1,2-