Bisecting Lewis X in Hybrid-Type N-Glycans of Human Brain Revealed by Deep Structural Glycomics

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ABSTRACT: The importance of protein glycosylation in the biomedical field requires methods that not only quantitate structures by their monosaccharide composition, but also resolve and identify the many isomers expressed by mammalian cells. The art of unambiguous identification of isomeric structures in complex mixtures, however, did not yet catch up with the fast pace of advance of high-throughput glycomics. Here, we present a strategy for deducing structures with the help of a deci-minute accurate retention time library for porous graphitic carbon chromatography with mass spectrometric detection. We implemented the concept for the fundamental N-glycan type consisting of five hexoses, four N-acetylhexosamines and one fucose residue. Nearly all of the 40 biosynthetized isomers occupied unique elution positions. This result demonstrates the unique isomer selectivity of porous graphitic carbon. With the help of a rather tightly spaced grid of isotope-labeled internal N-glycan, standard retention times were transposed to a standard chromatogram. Application of this approach to animal and human brain N-glycans immediately identified the majority of structures as being of the bisected type. Most notably, it exposed hybrid-type glycans with galactosylated and even Lewis X containing bisected 2-acetylglucosamine, which have not yet been discovered in a natural source. Thus, the time grid approach implemented herein facilitated discovery of the still missing pieces of the N-glycome in our most noble organ and suggests itself—in conjunction with collision induced dissociation—as a starting point for the overdue development of isomer-specific deep structural glycomics.
labeled internal standards. Chemical synthesis has recently been employed to synthesize a range of \[^{13}\text{C}\] -labeled N-glycans.\[^{26}\] An elution time matching that of a certain glycan does, however, not rule out the possibility of this glycan actually being another isomer unless an incidental co-elution can be ruled out by CID\[^{22,24}\] or by knowing the retention times of all possible isomers. In the absence of such knowledge, three recent comprehensive glycomic studies of brain tissues admittedly had to refrain from assigning explicit structures.\[^{22,27-29}\] Nevertheless, these and other studies revealed an unusually high amount of bisecting GlcNAc in brains of various species.\[^{22,27,29-32}\]

As of today, one can postulate that probably all human and mammalian glycosyltransferases and structural features are known and thus the glycome space, that is, the bona fide entirety of all isomeric N-glycan structures of a given mass level can be predicted.\[^{33}\] Establishing the relative retention times of all possible isomers, when separated by a shape-selective phase like PGC,\[^{21,34}\] provides a rational approach for structural assignment. At least, the many isomers not fulfilling the retention criterium can be ruled out right away. First steps in this direction have been attempted with a selection of the most likely occurring disialo N-glycans\[^{35}\] and with oligomannosidic N-glycans.\[^{36}\]

In the present work, we extend the range of synthetic reference structures with complex-type N-glycans containing fucose with its many attachment and interaction options, and we introduce a solution for overcoming retention time differences, which may derive from different columns, gradients, operators, or frequently observed “aging” (redox reactions) of PGC. Thirty-six isomers of glycans containing five hexoses, four N-acetylhexosamines and one fucose (HSN4F1), that may occur in mammals were biosynthetized. The PGC runs were conducted in the presence of eight synthetic (internal) standards with characteristic mass labels. With the help of this glycan retention Time Grid (glyco-TiGr), individual chromatographic runs—some with strongly deviating conditions—could be projected to a model chromatogram with deci-minute precision. Experimental retention times are thereby converted to “virtual minutes” (vi-min) that are used much in the sense of the well-tried “glucose units” known from hydrophilic interaction chromatography\[^{37,38}\] and capillary gel electrophoresis\[^{37,38}\] and the developed approach revealed yet undescribed structures with bisecting Lewis-X in the human brain N-glycome.

### EXPERIMENTAL SECTION

#### Materials

For the preparation of reference glycans from simple scaffolds, recombinant glycosyltransferases were expressed in the baculovirus insect cell system or purchased as detailed in the Supporting Information. Human brain samples were kindly provided by Dr. Lena Hirtler (Medical University, Vienna).

**Glycan Preparations.** N-Glycans from immunoglobulins, bovine fibrin, white beans, and brain were prepared using PNGase F as described.\[^{39,40}\] Glycans were reduced with either NaBH\(_4\) or NaBD\(_4\) and eventually fractionated by PGC-LC monitored by MALDI-TOF MS.\[^{39}\] These glycans were used as scaffolds for the glycosyltransferases and glycosidases mentioned above for the preparation of the structures depicted in Figure 1. Some modifications were performed with UDP-\[^{13}\text{C}\] -galactose (Figure 2). Details of the preparation procedures are provided in the Supporting Information.

**Liquid Chromatography–Electrospray Ionization–Mass Spectrometry.** Analytical PGC-LC-ESI-CID-MS/MS was performed with a capillary column (Hypercarb, 100 mm × 0.32 mm, 5 μm particle size; Thermo Scientific, Waltham, MA, USA) eluted with 10 mM ammonium bicarbonate as the aqueous solvent.\[^{37,41}\] Details of the preparation procedures are provided in the Supporting Information.

### RESULTS

Thirty-six isomers of the fundamental composition HSN4F1 that may naturally occur in mammals plus four isomers eventually cropping up in glyco-engineered plants were biosynthetized from scaffold glycans by the use of recombinant glycosyltransferases (Figure S1). The choice of this type of composition was guided by reports on the role of fucosylation for synapsin expression and on the prevalence of other isomeric structures than the standard biantennary core fucosylated oligosaccharides, such as found on serum proteins like IgG, in brain glycoproteins.\[^{22,30,43,44}\]

The many isomeric structures (Figure 1) need to be named, and we hope to the reader inclined to accept the system applied herein, which allows to fully define structures with short text strings, naming the terminal residues as explained in ref 36 and in the Supporting Information.

**Preparation of Standards I—Individual Structures.** The biosynthesis of biantennary glycans either with core...
fucose, Lewis X (LeX), or Lewis A (LeA) determinants or with the blood group H (bgH) α1,2-fucose started from isolated GnGnF6, A4GnF6, GnA4F6, and A4A4F6 peaks from human IgG. The structures are depicted in Figure 1 with consecutive reference numbers. One route entailed immediate β1,3-galactosylation, yielding the core-fucose-series (structures 1–4). De-fucosylation of these structures in Man5Gn generated a series of reference glycans on the one hand and internal standards on the other hand. Deuterium introduction at the reducing end, galactosylation with one or two residues of 13C-galactose, or use of 13C-N-acetylated compounds allowed for mass increases of 1, 6, and 8 Da and in combination a panel of well over 10 different increments. Careful choice of the preparation scheme equipped many of the standards with individual mass labels, thus allowing unambiguous identification of otherwise isobaric compounds in one chromatographic analysis. A scheme for biosynthesis of a set of isomers with inherently different mass increments and the resulting extract ion chromatograms (EICs) are depicted for core fucosylated and LeX isomers (Figure 2) and for LeA and bgH isomers (Figures S3 and S4). The key point was the use of a mixture of A4A4, A4A3, A3A4, and A3A3—derived from human IgG as described above—with 13C-galactose as the 3-linked terminal hexose, thereby introducing three mass levels for four (or eight in the case of the bgH series) isomers. These were combined with NaBH4 reduction (Lewis A series), NaBD3 reduction (both Lewis X and Core series as they elute far apart), and 13C-acetyl groups (bgH series). Within these mixtures, isobaric core fucose, Lewis X, or Lewis A structures could be assigned via the individual standards (see above), the marked difference in abundance of 3- and 6-arm galactose in human IgG, and the pronounced fragmentation of the 3-arm one in positive mode CID (Figures S5 and S6). In the case of the bgH-series, the bias of the α1,2-FucT for type I chains, together with single standards, likewise allowed for unambiguous assignment of all peaks (Figure S4).

Preparation of hybrid-type glycans starting from Man5Gn led to Man5GnF6-bi (structure 30). This compound was treated with βGalT and β4GalT, and 13C-galactose was expected to generate one compound each with Gal on the 3-arm. This naive assumption, however, had to be revised as β4GalT generated—in very different ratios—two products. Comparison of positive mode CID spectra revealed a similar architecture of two products (Figure 3), whereas the late eluting β4GalT product showed a characteristic, preferential loss of GlcNAc (Figure 3). Negative ion CID substantiated the view that in this third peak, galactose was bound to the bisecting GlcNAc (structure 27; M3GnF6A-bi) (Figure S7). For conversions with FucT-2 (resulting in structure 24; M3GnF2-A-bi) and FucT-4 (resulting in structure 31; M3GnAF-bi), therefore, these three possibilities had to be considered (Figure S8). FucT-3 did not act on the galactosylated hybrid-type glycan, which is in line with a recent report on the suppressive effect of bisecting GlcNAc. The Le X elaboration of the 3-arm (structure 26) likewise did not work out.

A final pettiness was the definition of the structure of the 6-arm in the case of Man4 glycans. The anachronistic uncertainty about the structure of Man4 glycans was probed with α1,6-specific glycosidase (Figure S9). In agreement with mechanistic studies, the α1,6-linked mannose is preferentially cleaved, which leads to a pronounced forward shift on PGC.
The removal of the core fucose (peaks show the positive mode CID spectra of peaks 541c with peaks warping in 2D-electrophoresis. Experimental data are projected except.

Therefore, the 6-arm of all Man4 structures is de... product of internal re-arrangement. This was observed for all Man4 isomers in this study, and tentative selection of possible isomers. The red arrow denotes a product of internal re-arrangement.

This was observed for all Man4 isomers in this study, and therefore, the 6-arm of all Man4 structures is defined as “M4n-36.

Preparation of Standards III—The Time Grid Concept. To make a glycan retention times library useful for PGC-LC, individual runs must undergo a process akin to image warping in 2D-electrophoresis. Experimental data are projected to a model chromatogram. The “glucose unit” method, popular for fluorescence-labeled glycans,8,9 was recently adopted for PGC-LC.47 However, in our hands, ionization efficiency and peak shape of the isomaltose oligosaccharides of eight and more glucose units failed to meet the required quality. Besides, the likelihood of differing sorption isotherms for compounds without and with amide functions makes these simple sugars doubtful for a stationary phase as delicate as PGC. We therefore decided for a set of eight isotope-labeled N-glycans with elution times covering the entire time range of the isomers in focus (Figure 2). This multipoint retention Time Grid (glyco-TiGr) allowed us to correct for elution time shifts, so that real elution times obtained in a particular run could be converted to virtual times (virtual minutes = vi-min) in an arbitrarily defined model chromatogram. Interpolation of sample retention times between “glyco-TiGr” mix “sign posts”—facilitated by a dedicated Excel sheet (Supporting Information)—generates a list of normalized retention times that can be looked up in the retention time library (Table 1). Notably, while applied to the composition HSN4F1 in this study, the very same “TiGr mix” can also be used to express elution of glycans with other masses in a manner essentially independent of the individual column and gradient conditions as demonstrated by the accidental collection of glycans with compositions other than HSN4F1 (Table S1). The criteria for choosing the TiGr mix structures were their distribution over the elution range of the compounds of interest and their accessibility. More TiGr standards will eventually be added to cover the elution regions of larger and sialylated N-glycans.

Admittedly, not all of the 40 isomers are satisfactorily separated. However, some notoriously difficult and usually ignored questions can be answered at first sight without consultation of negative mode MS/MS, which helps to define type2 and location of outer arm fucosylation3,49 and provide a sufficient quality of the fragment spectrum. Linkage isomers are, however, easily distinguished by retention time, as seen at the example of IgA N-glycans, which contain some β1,3-galactose (Figure S10, Table S2).

Application to Brain N-Glycans. Using the four series of biantennary standards (core, LeX, LeA, and bgH), we set out

Table 1. Retention Times of HSN4F1 Isomers in the Virtual Model Chromatograma

| # | proglycan code | ret. time (vi-min) | # | proglycan code | ret. time (vi-min) | # | proglycan code | ret. time (vi-min) |
|---|---|---|---|---|---|---|---|---|
| 31 | M3Gn(AF)-bi | 11.2 | 41 | A'4P | 24.0 | 12 | (FA)A3 | 34.5 |
| 21 | M3A'P-bi | 16.6 | 6 | (AF)A4 | 25.4 | 16 | P3-A3 | 35.6 |
| 30 | Man5GnF2bi | 17.5 | 36 | Gn(A'-+P') | 26.2 | 15 | A'-p3 | 36.2 |
| 22 | M3F2 | 18.5 | 8 | (AF)A3 | 26.5 | 1 | A'SPb | 36.2 |
| 25 | M3A'P-bi | 18.8 | 12 | (AF') | (27.9) | 18 | P3-A3 | 36.8 |
| 38 | A'4b | 19.6 | 7 | A'(AF) | 27.9 | 17 | A'b-p3 | 37.2 |
| 39 | A'4b | 19.8 | 9 | A'(FA) | 27.9 | 19 | A'b-pb | 37.6 |
| 24 | M3Gn4-4-bi | 21.0 | 11 | A'(FA) | 31.2 | 19 | A'b-4 | 37.8 |
| 23 | M32-2-bi | 21.2 | 14 | F3-A4 | 31.6 | 33 | A'-GnF6 | 38.3 |
| 29 | Man5(AnF) | 21.8 | 28 | Man5AnPp | 32.4 | 3 | A'SPb | 39.6 |
| 27 | M3Gn4A-bi | 22.7 | 12 | (AF') | (32.5) | 4 | A'SPb | 40.4 |
| 37 | (A'4-P')Gn | 23.5 | 10 | (FA)A4 | 32.5 | 34 | GnA-p3 | 41.1 |
| 5 | A'(AF) | 23.9 | 20 | F3-A4 | 32.9 | 35 | GnA-GnPb | 42.5 |
| 40 | A'4P | 24.0 | 13 | A'b-p4 | 34.1 | 36 | GnA-p3 | 44.1 |

aStructure can be deciphered by reference to Figure 1, by use of the proglycan084 applet or reading the explanation in the Supporting Information. To allow for facile referencing to Figure 1, the consecutive numbers (#) are added. Two anisobaric TiGr-reference glycans are marked by “( )”. Bold print denotes structures not described so far to the best of the authors knowledge. Retention times are given as virtual minute (vi-min) of the reference chromatogram.
to compare their retention times with that found in the mouse brain. None of the 20 isomers coeluted with the three main peaks (Figure 4). The early elution time rather pointed at hybrid-type glycans, and in fact, peak 541b coeluted with the reference.

Man5GnF6bi (structure 30) is a compound previously found in the brain. Galactosylated derivatives of this reference compound coeluted with brain peaks 541a and 541c and the galactose was bona fide imagined as linked to the 3-arm GlcNAc.

This could have been the end of the story, if we would not have been sensitized by the occurrence of two products in the galactose incorporation experiment with βGalT4 (Figure 3). In fact, brain peak 541c perfectly coeluted with product 3 and was insensitive to galactosidase, as previously observed for bisecting LacNac. Likewise, the structure exhibited the informative preferential loss of one terminal GlcNAc in positive mode CID (Figure S11) and the matching E- and F-type ions in CID of negative ions (Figure 4). Thus, peak 3 was identified as bearing a bisecting LacNac (structure 27; M5GnF6A-bi). The isomer with a substituted 3-arm was not observed.

Making use of the very distinct elution positions of glycans with galactose on the 3-arm versus on bisecting GlcNAc (Figure 3a), we probed brain peak 541a with a fucosidase able to act on the Lewis terminus. This converted peak 541a to a compound coeluting with glycan 541b (Figures 3a and 4c). Peak 1 furthermore gave a positive mode MS spectrum with a preferential loss of one GlcNAc as typical for a nonsubstituted 3-arm GlcNAc (Figure 4d). Thus, peak 1 harbored a glycan termed M3Gn(AF)-bi, in which the bisecting GlcNAc was fully elaborated to a Lewis X trisaccharide (structure 31; M3Gn(AF)-bi), whereas the corresponding isomer M3(AF)bi could not be found.

A rather large, single peak of composition HSN4F2 (15.0 min) was converted to a peak akin to 541a upon core-defucosylation and finally to M5Gn-A-bi (peak 540b) upon complete defucosylation and thus was assigned the structure shown in Figure 4. The porcine sample additionally contained diantennary N-glycans that very likely originated from contaminating blood.

**DISCUSSION**

The selectivity achieved for the 40 isobaric N-glycans containing one fucose, five hexoses, and four N-acetylhexosamine residues almost overshoot our expectations. In order to secure this treasure, normalization of retention times was realized by a set of synthetic stable isotope-labeled N-glycans spanning the entire oligosaccharide elution range. Obviously, these glyco-TiGr standards can also be applied to N-glycans with compositions other than HSN4F1 (Table 1). Likewise, the approach can be easily extended to larger, later eluting...
glycans. Population of further mass levels with a near to comprehensive collection of possible isomers would obviously result in an unparalleled isomer assignment power. Searched against a properly well-sorted virtual retention time library, the vi-min value of a peak at least excludes most of the possible isomers. Consultation of CID spectra then merely would help to confirm an assignment or choose between the very few remaining options. Vice versa, ambiguity of CID data can be met by information from chromatography. Notably, establishing libraries for just a few mass levels will allow us to infer the structures of glycans of other masses, as demonstrated here for the H5N4F2 peak. The hitherto, often covertly eluded distinction between different types of outer arm fucosylation or of β1,3 and β1,4 galactose (Supporting Information S2) becomes an easy task with the glyco-TiGr approach. Even the highly important but all too often neglected definition of the exact type of outer arm fucosylation becomes possible by just a one-shot analysis without the need for exo-glycosidase digestions and re-analysis.

The focus of the current work on the H5N4F1 composition level revealed the potential of the approach introduced herein and surfaced several hitherto undescribed structures for multimeric human IgA but foremost for brain glycoproteins.

Three prominent H5N4F1 N-glycan isomers were found in brains of mouse, pig, and humans. Most remarkably, one exhibited a bisecting LacNAc unit, that is, galactosylated bisecting GlcNAc or even a bisecting LeX unit, β4Galt, but not β3Galt5, was able to generate this exotic feature. Bisecting LacNAc has been found as a minor component in IgG,49,50 and surfaced several hitherto undescribed structures for multimeric IgA but foremost for brain glycoproteins.

The concept laid out herein could be the starting point for a full exploitation of the outstanding shape selectivity of porous graphitic carbon and thus for a methodology that truly appreciates the amazing and probably functionally significant isomeric diversity of N-glycans. Likewise, this study spotlights the brain N-glycome as a structurally unexplored territory, with broad implications for future deep structural glycomic studies on brain disorders such as Alzheimer’s disease.

DATA AVAILABILITY

The data supporting the results of this study is available within the article and its Supporting Information files. Access to MS raw data files will be provided upon request.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.1c03793.

Supporting methods, Tables with vi-min values of standards and IgA glycans, Figures exemplifying pathways for biosynthesis, examples of elution patterns of standards, positive and negative mode MS/MS spectra of standards, an example of hybrid-type isomers, determination of the Man4Gn structure, analysis of multimeric IgA glycans, MALDI-TOF MS of brain N-glycans, and explanation of the proglycan code (PDF) Excel sheet for “vi-min” calculations (XLSX)

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Author Contributions

J.H. C.G., M.P., and F.A. conceived the study. J.H., A.T. J.U., J.F., L.N., and D.S. performed enzymatic expressions and glycan preparations. J.H., C.G., D.M., L.N., and M.P. conducted the...
analytical experiments. J.H. and F.A. evaluated the data and composed the manuscript. All authors reviewed the manuscript.

**Notes**
The authors declare no competing financial interest.

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