HHS Public Access

Author manuscript

Org Biomol Chem. Author manuscript; available in PMC 2018 September 13.

Published in final edited form as:

Org Biomol Chem. 2017 September 13; 15(35): 7258–7262. doi:10.1039/c7ob01765k.

An Enzymatic Strategy to Asymmetrically Branched *N*-glycans

Angie D. Calderon[a], **Jun Zhou**[a], **Wanyi Guan**[a,b], **Zhigang Wu**[a], **Yuxi Guo**[a], **Jing Bai**[b], **Qing Li**[b], **Peng George Wang**[a], **Junqiang Fang**[c], and **Lei Li**[a]

[a]Department of Chemistry and Center for Diagnostics & Therapeutics, Georgia State University, Atlanta, GA 30303

[b]College of Life Science, Hebei Normal University, Shijiazhuang, Hebei 050024, China

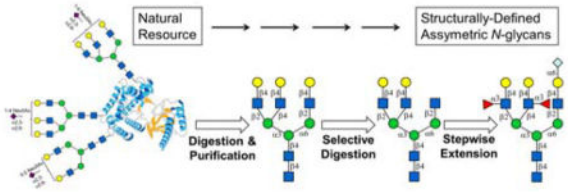
[c]National Glycoengineering Research Center, Shandong Provincial Key Laboratory of Glycochemistry and Glycobiology, Shandong University, Jinan, Shandong 250100, China

Abstract

An enzymatic strategy was developed to generate asymmetrically branched *N*-glycans from natural sources by using a panel of glycosidases and glycosyltransferases. Briefly, LacZ β-galactosidase was employed to selectively trim symmetrically branched *N*-glycans isolated from bovine fetuin. The yielding stuctures were then converted to asymetrically branched core structures by robust glycosyltransferase for further extension.

Graphical Abstract

Using a set of glycosidases and glycosyltransferases, an enzymatic strategy was developed to prepare asymmetric N-glycans excluding any chemical procedures.



Asparagine-linked glycosylation (*N*-glycosylation) is one of the most important post-translational modifications of eukaryotic proteins, and the *N*-glycan structures have been shown to modulate protein's structure and function in many ways. For example, *N*-glycans were found to play key roles in a variety of biological processes, including cell adhesion, immune responses, tumor metastasis, etc.[1] On the other hand, *N*-glycans can influence proteins folding, stability, and immunogenicity, among others.[1c, 2] Thus, elucidating the structures and functions of *N*-glycans is essential for understanding related biological and

Correspondence to: Junqiang Fang; Lei Li.

Electronic Supplementary Information (ESI) available: Experimental details for expression, purification and substrate specificity study of MGAT5, isolation and digestion of symmetrically branched N-glycans, as well as synthesis, purification and characterization of N-glycans. See DOI: 10.1039/x0xx00000x

pathological processes, and access of structurally defined *N*-glycans is prerequisite for this purpose.

The natural complexity in the biosynthesis of (*N*-)glycans has led to the generation of very diverse and complex glycan structures.[3] Take mouse zona pellucida glycoprotein 3 as an example, 58 distinct *N*-glycan structures were identified associated with a single glycosylation site (named *N*-glycan microheterogeneity).[4] Another representative example is that 13 high-mannose *N*-glycans structures were identified within 3 glycomers ($Man_5GlcNAc_2$, $Man_7GlcNAc_2$ and $Man_8GlcNAc_2$) released from bovine ribonuclease B.[5] Modern glycobiology studies have indicated that *N*-glycans with minor structural differences may exhibit distinct protein binding profiles,[6] and certain structures may play essential roles in various biological processes,[1b, 7] However, in-depth study of such roles have been largely limited by the lack of structurally defined *N*-glycans in adequate amount.

Current methods for the separation of *N*-glycan structures have been focusing on small quantities for analytical purposes.[8] And due to the diversity and complexity, as well as low abundance of most structures, it is extremely challenging to isolate adequate amounts of *N*-glycans with specific structures from natural sources. On the other hand, despite numerous efforts on chemical synthesis of *N*-glycans,[9] only a few groups had recently developed strategies for synthesizing libraries of structurally defined *N*-glycans, including Boons's general strategy for chemoenzymatic synthesis of asymmetric *N*-glycans,[6, 10] Wong's modular synthesis of HIV *N*-glycans,[11] and our core synthesis/enzymatic extension (CSEE) strategy to prepare *N*-glycan isomers.[12] Nevertheless, all strategies require expertise and proficient skills for chemical synthesis of key asymmetric *N*-glycan core or modular structures for further enzymatic diversification.

Exclusively, starting with sialylglycopeptide (SGP) isolated from egg yolk, we were able to generate 36 symmetric bi-antennary *N*-glycans.[10b] Herein, we introduce a novel enzymatic strategy to generate asymmetric *N*-glycans excluding any chemical synthesis. Briefly, two symmetric *N*-glycan structures, **N001** and **N301** (Fig. 1A) were firstly separated and purified from bovine fetuin in milligram scale, followed by selective degalactosylation and glycosylation to generate a panel of asymmetric *N*-glycan core structures (Fig. 1B), which can be further enzymatically extended to asymmetric bi-, tri- and tetra-antennary structures as described previously.[6, 12a]

Isolation of homogenous symmetric N-glycans N001 and N301 from bovine fetuin

Bovine fetuin is a serum glycoprotein that contributes to the attachment and spreading of cells, which thus has been widely used in cell culture. Bovine fetuin is also considered as a golden standard in glycomics and glycoanalysis, as glycan moieties (6 glycosylation sites) accounts for over one fourth of its molecular weight.[13] The 3 *N*-glycans represent 80% of total carbohydrates, and the 3 *O*-glycans represent the remaining 20%.[13] Among the *N*-glycans, totally 23 structures were previously identified, including around 80% tri-antennary ones (mass ratio) with one to four Neu5Ac attached (either α2,3-linked or α2,6-linked) to core structure **N301**, and 16% with zero to two Neu5Ac attached to **N001**.[13] No more

monosaccharide residues were found in fetuin other than Neu5Ac, Gal, GlcNAc and Man. While it is challenging to isolate homogenous forms of each glycans in preparative scale, it could be much practical to get mg to grams of core structures **N301** and **N001** in pure form after glycan trimming.

To test this, 400 mg of bovine fetuin was treated with PNGaseF (release *N*-glycans, monitored by SDS-PAGE) and neuraminidase from *Clostridium perfringens* (remove Neu5Ac, monitored by HPLC analysis of *N*-glycans) in one pot for two days (Supplementary Information III, Fig. S2). Two major *N*-glycan peaks (Fig. 2A) were found on the HPLC profile (running condition: Waters amide column: 130 Å, 5 μm, 4.6 mm × 250 mm; Solvent A : Water; Solvent B: Acetonitrile; Gradient elution B%: 65% to 50% within 25 min; Flow rate: 1 mL/min; monitor by UV_{210nm}) of the product, and MALDI-MS analysis showed that Peak 1(retention time = 15.0 min) and Peak 2 (retention time = 17.5 min) have m/z of 1663.5902 and 2028.7246 (Fig. 2B&C), corresponding to molecular weight of **N001** $[M + Na]^+$ and **N301** $[M + Na]^+$, respectively. Please note that, due to existence of interconverting α- and β-anomers of glycans with free reducing end in solution,[14] two adjacent bumps or a large shoulder can usually be observed on the glycan peak in HPLC analysis. Such a phenomenon was found and reported repeatedly in HPLC analysis of *N*-glycans.[10a, 12, 15] To purify the two glycans, fetuin protein were firstly precipitated by the addition of ice-cold acetone (3 volumes). After brief centrifugation, the supernatant was then concentrated and subjected to G-25 gel filtration (2.5 cm×100 cm) for rough separation. The *N*-glycan containing fractions (analyzed by MALDI-MS, Fig. S3) were then subject to HPLC purification as previously reported.[12a] Totally, 4.1 mg (63% yield, Supplemental Information III) of compound corresponding to peak 1 and 26 mg (65% yield) of compound corresponding to peak 2 were collected. Extensive NMR characterization (Supplemental Information IX) confirmed peak 1 as **N001** and peak 2 as **N301**. It is worth to note that, possible β1,3-linked Gal on β1,4-GlcNAc branch and/or core-fucose were observed on **N301**,[13, 16] however, we did not find any signals corresponding to them on NMR spectra of purified *N*-glycans, nor did we find impurity peaks on MS and HPLC profiles. One possible explanation is that only minor amount of such glycans exist in bovine fetuin, and HPLC purification steps eliminated these structures.

Selective degalactosylation of N001

Branch specificity of several β-galactosidases towards Galβ1,4-GlcNAcβ1,2-Manα1,6-(Galβ1,4-GlcNAcβ1,2-Manα1,3-)Manβ1,4-GlcNAc (**N001** without reducing end GlcNAc) were tested, with only *Escherichia coli* β-galactosidase (LacZ) exhibiting branch preferences (prefers α1,3-Man branch).[17] Interestingly, LacZ also had a branch preference towards Galβ1,4-GlcNAcβ1,6-(Galβ1,4-GlcNAcβ1,3-)Gal (prefers β1,6-GlcNAc branch) but not to Galβ1,4-GlcNAcβ1,6-(Galβ1,4-GlcNAcβ1,3-)Galβ1,4-GlcNAc.[17–18] For this reason, we first performed a substrate specificity assay of LacZ towards a pair of *N*-glycan isomers **N111** and **N211** (Fig. 1).[12a] The results (Supplemental Information VI) showed that LacZ digested **N111** much faster than **N211**, confirming LacZ has a preference toward the α1,3-Man branch.

Next, we monitored the digestion reaction of **N001** by LacZ to determine the best time to collect mono-agalactosylated **N001** (**N111**/**N211**) (Supplementary Information VII). Result showed that with appropriate amounts of LacZ, after 6 hours, all **N001** was digested and converted into mono-agalactosylated **N001** (85%) and di-agalactosylated **N001** (**N000**, 15%) (Fig. S6). Milligram scale LacZ digestion of **N001** were then performed and mono-agalactosylated **N001** (Fig. 3B, MALDI-MS m/z peak of 1501.5210) were purified by HPLC as previously reported.[12a] As our HPLC method cannot distinguish **N111** and **N211**, we applied an enzymatic strategy, using two human glycosyltransferases, α1,6-fucosyltransferase (FUT8) and mannosyl-α1,6-glycoprotein β1,6-*N*-acetyl-glucosaminyltransferase (MGAT5, also known as GnT-V). FUT8 strictly requires a free GlcNAc on the α1,3-Man branch of *N*-glycans for activity,[19] and MGAT5 requires a free GlcNAc on the α1,6-Man branch.[20] We also did detailed substrate specificity study of FUT8[12b] and MGAT5 (Supplementary Information V, Fig. S4) using a library of synthetic *N*-glycans, which double confirmed previous reports.[19–20] Thus, as illustrated in Fig. 3A, FUT8 can fucosylate **N211** but not **N111**, whereas MGAT5 can glycosylate **N111** but not **N211**. MALDI-MS analysis showed that all *N*-glycans were fucosylated (Fig. 3C, m/z peak corresponding to starting material disappeared, new m/z peak of 1647.5821 corresponding to **N6211** $[M + Na]^+$ appeared) in FUT8-catalyzed reaction, whereas no new peak was found corresponding to N4111 in MGAT5-catalyzed reaction (Fig. 3D). These results confirmed that the purified LacZ digestion product of **N001** is **N211** in pure form.

Selective degalactosylation of N301

Degalactosylation of **N301** was slower than that of **N001**. After 3 days of LacZ incubation, 4 peaks corresponding to *N*-glycans were observed on HPLC chromatograph (Fig. S7). We then performed degalactosylation of **N301** in milligram scale (20 mg), with each peak purified by semi-preparative HPLC. MALDI-MS analysis indicated that peak I (10%), II (73%), III (10%), and IV (7%) are corresponding to **N301**, mono-agalactosylated **N301** (**N391** or **N392** or **N393** or **mixture**, m/z peak of 1866.6707 as shown in Fig. 4B), di-agalactosylated **N301** (**N381** or **N382** or **N383** or mixture, m/z peak of 1704.6011 as shown in Fig. 4E) and **N300**, respectively. FUT8- and MGAT5-catalyzed reactions were performed to identify structures of agalactosylated **N301** as illustrated in Fig. 4A. In MGAT5-catalyzed reaction m/z peaks of 2069.7421 and 2086.5037 corresponding to **N4391** $[M + Na]^+$ and $[M + K]^+$ were observed while the peak corresponding to mono-agalactosylated **N301** disappeared (Fig. 4C), but in FUT8-catalyzed reaction, a product m/z peak corresponding to **N6393** was not observed (Fig. 4D). Such results indicated that purified mono-agalactosylated **N301** is **N391** in pure form. The structure was further confirmed by NMR characterization (Supplementary Information IX). Similarly, for di-agalactosylated **N301**, both MGAT5- and FUT8-catalyzed reactions yielded new m/z peaks on MALDI-MS profiles while the m/z peak of the starting glycan disappeared (Fig. 4F&G), suggesting that purified di-agalactosylated **N301** is pure **N382**.

These results indicated that LacZ prefers to digest the Gal residue on the α1,6-Man branch of tri-antennary *N*-glycan **N301**, and then the Gal residue on the β1,2-GlcNAcα1,3-Man branch, different from that towards bi-antennary *N*-glycan **N001**, which prefers to digest Gal

residue on the α1,3-Man branch. Nevertheless, LacZ was successfully used to generate asymmetric bi- and tri-antennary *N*-glycan core structures (**N211**, **N391** and **N382**) in mg scales.

Generation of other core structures and asymmetric N-glycans

Several other asymmetric *N*-glycan core structures were generated (Fig. S8), including **N4391** that derived from **N391** via MGAT5-catalyzed reaction, as well as **N4382** and **N6382** that derived from **N382** via MGAT5 and FUT8-catalyzed reactions (Supplementary Information VIII and IV). These core structures are readily available for the synthesis of libraries of asymmetric complex *N*-glycans. For example, **N211** had been used as a core to generate a number of bi-antennary *N*-glycan isomers with or without core-fucosylation.[12] Taking the tri-antennary core **N391** as another example, stepwise enzymatic synthesis was performed to generate complex asymmetric *N*-glycans such as **N396** (a similar asymmetric tri-antennary N-glycan was prepared previously via chemoenzymatic approach). As shown in Fig. 5, firstly, in a 0.5 mL reaction system, 4 mg (4 mM) of **N391**was incubated with GDP-Fuc (10 mM), $MnCl_2$ (5 mM), and 0.1 mg of *Helicobacter pylori* α1,3-fucosyltransferase (Hp3FT).[12a] One microliter of the reaction mixture was sampled every 3 hours for analysis. MALDI-MS analysis showed a peak at m/z = 2158.7823, corresponding to **N394** $[M + Na]^+$ (Fig. 5B). After 20 hours, the reaction was freeze quenched at −80 °C for 30 min, and condensed for HPLC purification using a water/acetonitrile gradient elution, yielding 4.2 mg of **N394** (90% yield). The purified **N394** (3 mg) was then utilized for enzymatic syntheses of **N396** in two steps, catalyzed by bovine β1,4-galactosyltransferase (bGalT)[12a] and *Photobacterium damselae* α2, 6-sialyltransferase (Pd26ST),[21] respectively (Supplementary Information VIII for details). NMR analysis was performed to confirm the structure of the final product (2.5 mg, 93% yield for step 1, 85% yield for step 2) (Supplementary Information IX).

In summary, we have developed a pure enzymatic strategy to generate asymmetric complex *N*-glycans excluding chemical procedures. The strategy includes 1) glycosidase (PNGaseF and neuraminidase) assisted separation of bi- and tri-antennary *N*-glycans in mg scale; 2) β-galactosidase mediated selective degalactosylation and human FUT8- and MGAT5-catalyzed glycosylation to generate asymmetric *N*-glycan core structures. The yielded core structures can be readily utilized in the synthesis of asymmetrically branched bi-, tri- and tetra-antennary *N*-glycans. Large-scale preparation of asymmetrically branched *N*-glycans employing this strategy is undergoing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by National Institutes of Health (U01GM0116263 to P.G. Wang and L. Li), National Science Foundation of China (31200605 to W. Guan) and the Key Grant Project of Chinese Ministry of Education (313033). A. D. Calderon is grateful to Graduate Assistance in Areas of National Need (GAANN P200A150308). We are grateful to Dr. Donald Jarvis from the University of Wyoming for providing FUT8 and MGAT5 baculovirus

stock (Glycorepository project supported by NIH-NIGMS P41GM103390). We thank Dr. Xi Chen from UC Davis for providing α2,6-sialyltransferase.

References

1. (a) Varki, A., Lowe, JB. Essentials of Glycobiology. Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Cold Spring Harbor (NY): 2009. (b) Zhao YY, Takahashi M, Gu JG, Miyoshi E, Matsumoto A, Kitazume S, Taniguchi N. Cancer Sci. 2008; 99:1304. [PubMed: 18492092] (c) Dwek RA. Chem Rev. 1996; 96:683. [PubMed: 11848770]
2. (a) Imperiali B, O'Connor SE. Curr Opin Chem Biol. 1999; 3:643. [PubMed: 10600722] (b) Helenius A, Aebi M. Science. 2001; 291:2364. [PubMed: 11269317] (c) Helenius A, Aebi M. Annu Rev Biochem. 2004; 73:1019. [PubMed: 15189166]
3. Krasnova L, Wong CH. Annu Rev Biochem. 2016; 85:599. [PubMed: 27145845]
4. Stanley, P., Schachter, H., Taniguchi, N. Essentials of Glycobiology. Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Cold Spring Harbor (NY): 2009.
5. Prien JM, Ashline DJ, Lapadula AJ, Zhang H, Reinhold VN. J Am Soc Mass Spectrom. 2009; 20:539. [PubMed: 19181540]
6. Wang Z, Chinoy ZS, Ambre SG, Peng W, McBride R, de Vries RP, Glushka J, Paulson JC, Boons GJ. Science. 2013; 341:379. [PubMed: 23888036]
7. (a) Takamatsu S, Shimomura M, Kamada Y, Maeda H, Sobajima T, Hikita H, Iijima M, Okamoto Y, Misaki R, Fujiyama K, Nagamori S, Kanai Y, Takehara T, Ueda K, Kuroda S, Miyoshi E. Glycobiology. 2016; 26:1180. [PubMed: 27329181] (b) Pang PC, Chiu PC, Lee CL, Chang LY, Panico M, Morris HR, Haslam SM, Khoo KH, Clark GF, Yeung WS, Dell A. Science. 2011; 333:1761. [PubMed: 21852454]
8. (a) Anumula KR. Anal Biochem. 2000; 283:17. [PubMed: 10929803] (b) Harvey DJ. Proteomics. 2001; 1:311. [PubMed: 11680878] (c) Hua S, An HJ, Ozcan S, Ro GS, Soares S, DeVere-White R, Lebrilla CB. Analyst. 2011; 136:3663. [PubMed: 21776491] (d) Mechref Y. Electrophoresis. 2011; 32:3467. [PubMed: 22180203] (e) Huang Y, Nie Y, Boyes B, Orlando R. J Biomol Tech. 2016; 27:98. [PubMed: 27582638] (f) Pu Y, Ridgeway ME, Glaskin RS, Park MA, Costello CE, Lin C. Ana Chem. 2016; 88:3440.
9. (a) Boltje TJ, Buskas T, Boons GJ. Nat Chem. 2009; 1:611. [PubMed: 20161474] (b) Unverzagt C, Kajihara Y. Chem Soc Rev. 2013; 42:4408. [PubMed: 23403448] (c) Maki Y, Okamoto R, Izumi M, Murase T, Kajihara Y. J Am Chem Soc. 2016; 138:3461. [PubMed: 26926155]
10. (a) Gagarinov IA, Li T, Torano JS, Caval T, Srivastava AD, Kruijtzer JA, Heck AJ, Boons GJ. J Am Chem Soc. 2017; 139:1011. [PubMed: 28002670] (b) Wu Z, Liu Y, Ma C, Li L, Bai J, Byrd-Leotis L, Lasanajak Y, Guo Y, Wen L, Zhu H, Song J, Li Y, Steinhauer DA, Smith DF, Zhao B, Chen X, Guan W, Wang PG. Org Biomol Chem. 2016; 14:11106. [PubMed: 27752690]
11. (a) Shivatare SS, Chang SH, Tsai TI, Tseng SY, Shivatare VS, Lin YS, Cheng YY, Ren CT, Lee CC, Pawar S, Tsai CS, Shih HW, Zeng YF, Liang CH, Kwong PD, Burton DR, Wu CY, Wong CH. Nat Chem. 2016; 8:338. [PubMed: 27001729] (b) Shivatare SS, Chang SH, Tsai TI, Ren CT, Chuang HY, Hsu L, Lin CW, Li ST, Wu CY, Wong CH. J Am Chem Soc. 2013; 135:15382. [PubMed: 24032650]
12. (a) Li L, Liu Y, Ma C, Qu J, Calderon AD, Wu B, Wei N, Wang X, Guo Y, Xiao Z, Song J, Sugiarto G, Li Y, Yu H, Chen X, Wang PG. Chem Sci. 2015; 6:5652. [PubMed: 26417422] (b) Calderon AD, Liu Y, Li X, Wang X, Chen X, Li L, Wang PG. Org Biomol Chem. 2016; 14:4027. [PubMed: 27080952]
13. Green ED, Adelt G, Baenziger JU, Wilson S, Van Halbeek H. J Biol Chem. 1988; 263:18253. [PubMed: 2461366]
14. Bertozzi, CR., Rabuka, D. Essentials of Glycobiology. Varki, A.Cummings, RD.Esko, JD.Freeze, HH.Stanley, P.Bertozzi, CR.Hart, GW., Etzler, ME., editors. Cold Spring Harbor (NY): 2009.
15. Li T, Huang M, Liu L, Wang S, Moremen KW, Boons GJ. Chem Eur J. 2016; 22:18742. [PubMed: 27798819]
16. Sun S, Shah P, Eshghi ST, Yang W, Trikannad N, Yang S, Chen L, Aiyetan P, Hoti N, Zhang Z, Chan DW, Zhang H. Nat Biotechnol. 2016; 34:84. [PubMed: 26571101]

17. van den Eijnden DH, Blanken WM, van Vliet A. Carbohydr Res. 1986; 151:329. [PubMed: 3094938]

18. Renkonen O, Helin J, Vainio A, Niemela R, Penttila L, Hilden P. Biochem Cell Biol. 1990; 68:1032. [PubMed: 2121168]

19. (a) Longmore GD, Schachter H. Carbohydr Res. 1982; 100:365. [PubMed: 7083256] (b) Voynow JA, Kaiser RS, Scanlin TF, Glick MC. J Biol Chem. 1991; 266:21572. [PubMed: 1939186] (c) Kaminska J, Glick MC, Koscielak J. Glycoconj J. 1998; 15:783. [PubMed: 9870354] (d) Paschinger K, Staudacher E, Stemmer U, Fabini G, Wilson IB. Glycobiology. 2005; 15:463. [PubMed: 15604090]

20. (a) Kanie O, Crawley SC, Palcic MM, Hindsgaul O. Carbohydr Res. 1993; 243:139. [PubMed: 8324760] (b) Kanie O, Crawley SC, Palcic MM, Hindsgaul O. Bioorg Med Chem. 1994; 2:1231. [PubMed: 7757419]

21. Yu H, Chokhawala HA, Huang S, Chen X. Nat Protoc. 2006; 1:2485. [PubMed: 17406495]

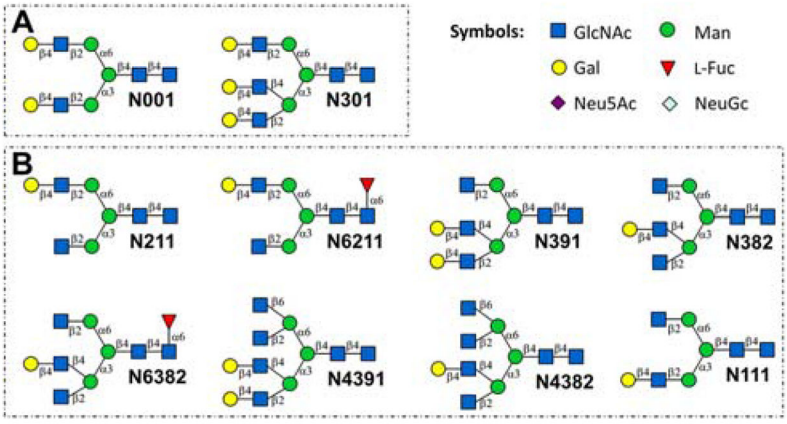


Figure 1.

Symmetric (A) and asymmetric (B) N-glycan core structures for the generation of *N*-glycan libraries. **N111** was not prepared in this study.

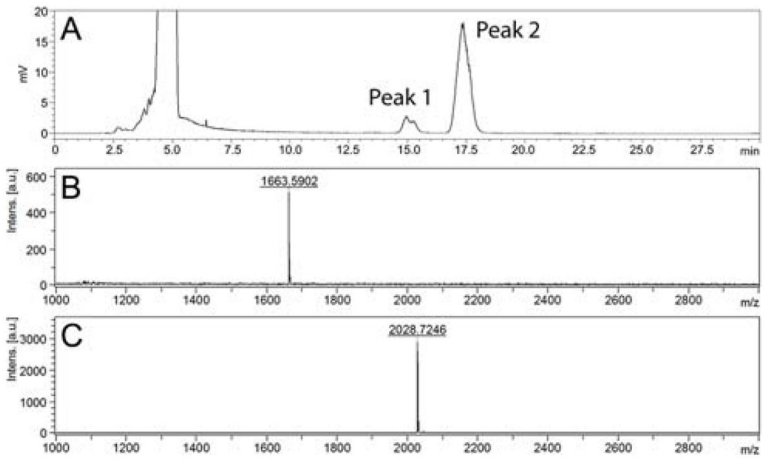


Figure 2.

HPLC analysis of PNGaseF and neuraminidase treated bovine fetuin (A), and MALDI-MS analysis of HPLC peak 1 (B) and peak 2 (C). The two adjacent bumps of peak 1 and peak 2 represent α- and β-anomer of the free reducing end glycans.

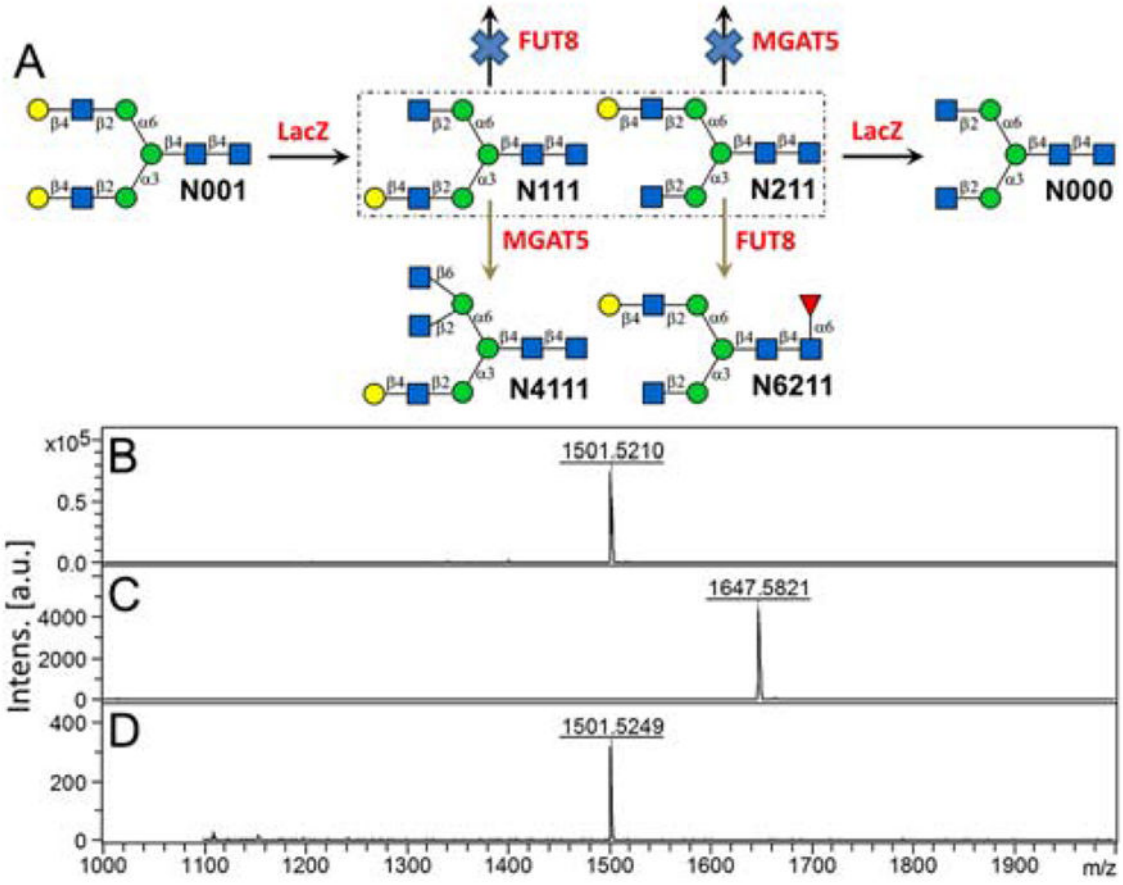


Figure 3.

Rationale of enzymatic *N*-glycan structure identification of monoagalactosylated **N001** (A); and MALDI-MS analysis of mono-agalactosylated **N001** (B), mono-agalactosylated **N001** + FUT8 (C), mono-agalactosylated **N001** + MGAT5 (D). LacZ, *E. coli* β-galactosidase; FUT8, human α1,6-fucosyltransferase; MGAT5, human mannosyl-α1,6-glycoprotein β1,6-*N*-acetyl-gucosaminyltransferase.

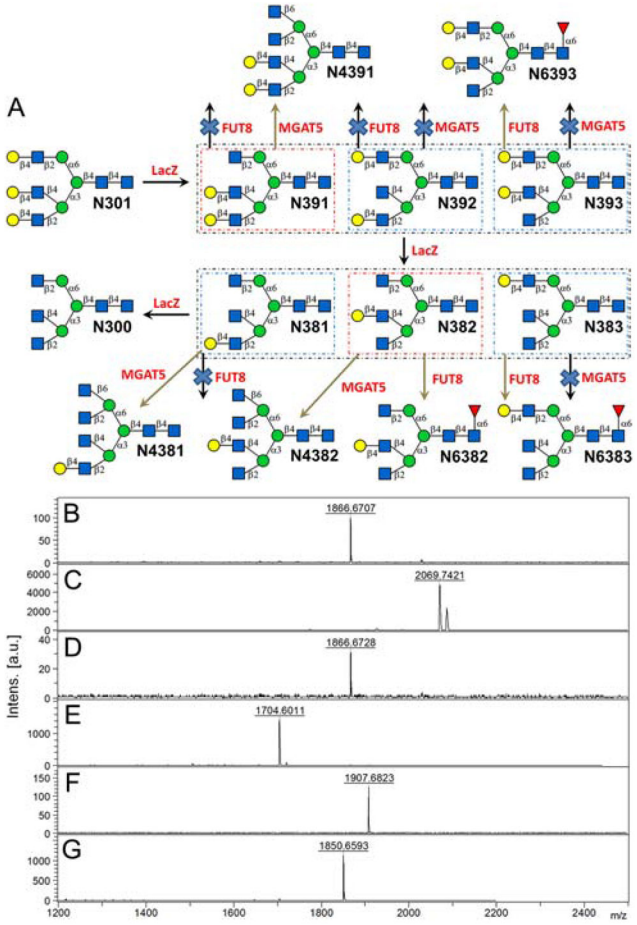


Figure 4.

Rationale of enzymatic *N*-glycan structure identification of mono- and di-agalactosylated **N301** (A); and MALDI-MS analysis of mono-agalactosylated **N301** (B), mono-agalactosylated **N001** + MGAT5 (C), mono-agalactosylated **N001** + FUT8 (D), di-agalactosylated **N301** (E), di-agalactosylated **N001** + MGAT5 (F), di-agalactosylated **N001** + FUT8 (G). LacZ, *E. coli* β-galactosidase; FUT8, human α1,6-fucosyltransferase; MGAT5, human mannosyl-α1,6-glycoprotein β1,6-*N*-acetyl-gucosaminyltransferase.

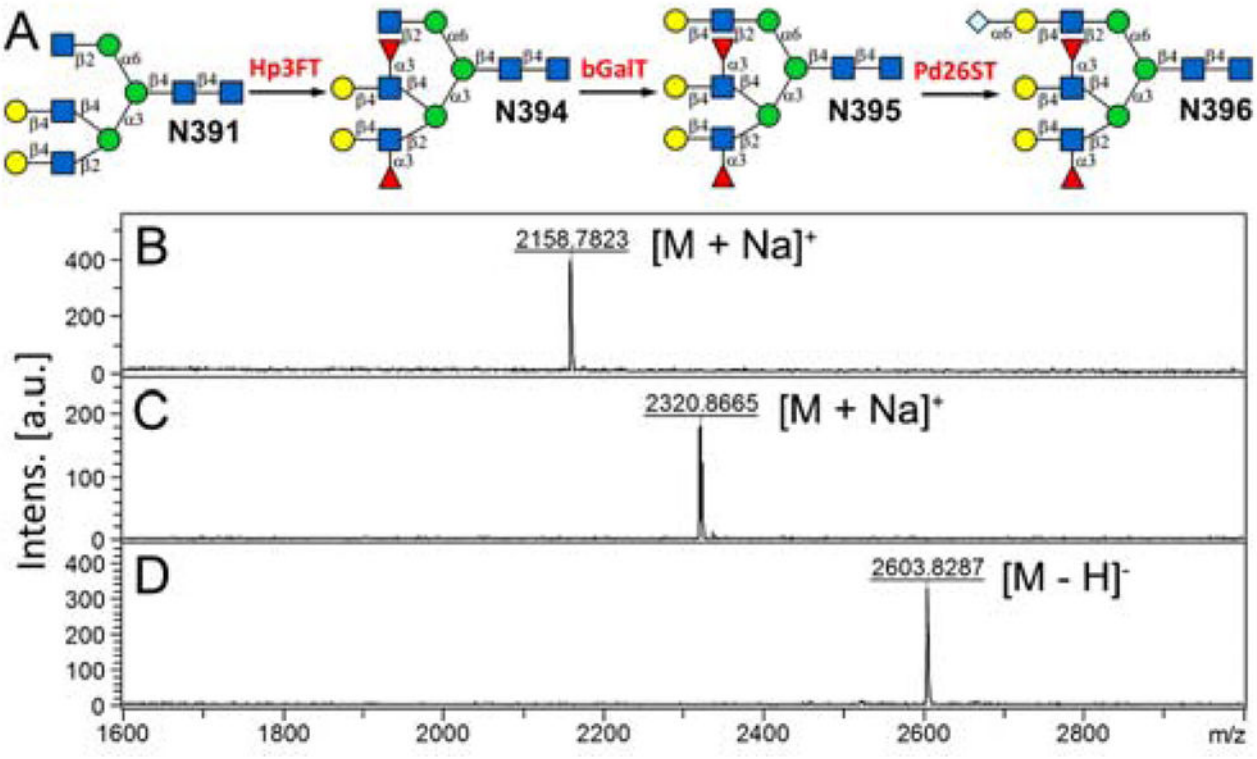


Figure 5.

Enzymatic extension of **N391** to generate asymmetric tri-antennary glycans (A) and MALDI-MS analysis of **N394** (B), **N395** (C) and **N396** (D). Hp3FT, *Helicobacter pylori* α1,3-fucosyltransferase; bGalT, bovine β1,3-galactosyltransferase; Pd26ST, *Photobacterium damslae* a2,6-sialyltransferase.