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Journal
Organic & Biomolecular Chemistry, 14(17)

ISSN
1477-0520

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Publication Date
2016-04-26

DOI
10.1039/c6ob00586a

Peer reviewed

Substrate Specificity of FUT8 and Chemoenzymatic Synthesis of Core-fucosylated Asymmetric *N*-glycans

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Abstract

Substrate specificity studies of human FUT8 using 77 structurally-defined *N*-glycans as acceptors showed a strict requirement towards the α1,3-mannose branch, but a great promiscuity towards the α1,6-mannose branch. Accordingly, a chemoenzymatic strategy was developed for efficient synthesis of core-fucosylated asymmetric *N*-glycans.

Graphical abstract
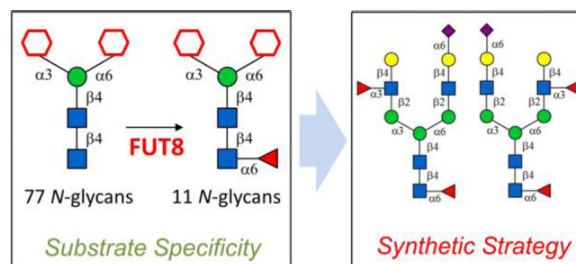
Asparagine-linked glycosylation (*N*-glycosylation) represents the most prevalent and structurally varied post-translational modification of eukaryotic proteins. Tremendous advances in glycobiology have demonstrated that asparagine-linked oligosaccharides (*N*-glycans) can modulate protein's structure and function in many ways. For example, they were found to play roles in a variety of biological processes, including cell adhesion, pathogen infections, immune responses and tumor metastasis.[1] On the other hand, *N*-glycans can influence proteins biosynthesis, folding, stability, antigenicity and immunogenicity.[1c, 2] *N*-Glycans found in nature possesses inherent complexity and diversity, due to variable connectivity of monosaccharide building blocks as well as additional modifications on the basic scaffolds. Core-fucosylation is a major *N*-glycan modification in eukaryotic glycomes.

In mammalian cells, core-fucosylation is exclusively found as α1,6-fucosylation of the innermost *N*-acetyl-glucosamine (GlcNAc) residue of *N*-glycans. Many glycoproteins are known to be core-fucosylated, and accumulating data indicate that this type of modification regulates the functions of certain glycoproteins. For example, depletion of *N*-glycan core-fucosylation in human IgG1 enhanced its antibody-dependent cytotoxicity (ADCC) activity to 50 – 100 folds.[3] It was shown that core-fucosylation regulates the functions of immunoglobulin by altering its physicochemical properties.[4] Another most recent example is that in humoral immune response, core-fucosylation of IgG B cell receptor was shown to mediate antigen recognition and cell signal transduction.[5] Alteration of core-fucosylation is frequently found to be closely related to human diseases including liver cancer, pancreatic cancer, lung cancer, breast cancer, etc.[6] A well-known example is α-fetoprotein, which was found highly core-fucosylated specifically in hepatocellular carcinoma, but not other liver diseases.[7] In addition, E-cadherin, a glycoprotein correlated with cancer metastasis, was found to be core-fucosylated in highly metastatic lung cancer cells but absent in low metastatic ones.[8] Compare to controls, increased core-fucosylated *N*-glycans and decreased non-fucosylated ones were also detected in the clinical ovarian cancer biomarker CA125.[9] Moreover, core-fucosylation is pivotal in development, and the loss of such *N*-glycan modification leads to severe phenotypes such as growth retardation, emphysema-like changes in lung, even death.[10] All these evidences strongly suggest that core-fucosylation plays vital roles in various biological processes and events.

FUT8 is the only enzyme responsible for core-fucosylation in mammals, which catalyzes the transfer of an L-fucose residue from GDP-β-L-fucose (GDP-Fuc) onto the innermost GlcNAc of *N*-glycan to form an α1,6-linkage (Fig. 1).[10–11] It has been reported that FUT8 is highly expressed in brain tissue (where core-fucosylated glycoproteins were found abundant), human blood platelets, cancer tissues, and other tissues during certain pathological processes. Previous substrate specificity study suggested that FUT8 requires both α1,3-mannose branch terminal GlcNAc residue and reducing end β-configuration of the acceptor.[12] In addition, galactosylation of the GlcNAc residue at β1,3-mannose branch and bisecting of *N*-glycans prevent FUT8 activity.[12a, 12b] Most recently, we and others showed that both human and *Caenorhabditis elegans* FUT8 can fucosylate asymmetric *N*-glycans (Fig. 1, **N211** and **N212**), indicating that they may have a much relaxed acceptor substrate preference towards the α1,6-mannose branch.[13] A comprehensive study is necessary to confirm such specificity, and structurally defined *N*-glycans that we synthesized previously[13b] represent perfect substrate candidates for test.

Given the significance, FUT8 and core-fucosylation have been extensively investigated in terms of glycomics and in vivo biological functions, while in depth molecular elucidation is largely limited by the unavailability of structurally well-defined *N*-glycans with core-fucosylation. In the past decade, a few chemical and enzymatic methodologies were developed for the preparation of such molecules. Unverzagt, used a modular chemical synthesis strategy where the α1,6-fucose was attached in the last step.[14] On the other hand, Huang applied a pre-activation-based one-pot chemical strategy to quickly assemble a dodecasaccharide *N*-glycan structure.[15] Enzymatically, Reichardt used *C. elegans* FUT8 to core-fucosylate chemically prepared simple *N*-glycan structures (Fig. 1, **N000** and **N010**),[16]

while Wang applied glycosynthases to conjugate chemically prepared glycan oxazolines and Fucα1,6-GlcNAc to form homogeneous core-fucosylated *N*-glycans and glycoproteins.[17] In all cases, only simple or symmetric core-fucosylated *N*-glycans were prepared, and an efficient strategy for rapid access of more complicated and asymmetric ones is yet to be developed. In this work, human FUT8 was overexpressed and purified, detailed acceptor substrate specificity was investigated towards 77 structurally defined *N*-glycans and derivatives. Accordingly, a chemoenzymatic strategy was developed for efficient synthesis of core-fucosylated asymmetric *N*-glycans.

Human FUT8 was chosen for substrate specificity study and synthetic purpose. Heterogeneous expression was achieved *via* a Titerless Infected-cell Preservation and Scale-up (TIPS) approach[18] (Supplementary Information II) using a baculorvirus system (see http://glycoenzymes.ccrc.uga.edu for system and construction details) provided by Dr. Jarvis from the University of Wyoming. After one-step Ni-Sepharose Excell (GE Healthcare) affinity purification, recombinant FUT8 with a purity of over 90% (Supplementary Figure S1) was obtained in a scale of 0.42 mg/L of cultures. Substrate specificity study was then performed in reactions with a total volume of 20 μL containing an acceptor *N*-glycan (0.3 mM), donor GDP-Fuc (1 mM), MES buffer (100 mM, pH 7.0) and 50 μg/mL of the recombinant human FUT8. After 4 h of incubation at 37 °C, the reactions were quenched by the addition of equal volumes of ice cold ethanol, followed by analysis using mass spectrometry (MS) and high performance liquid chromatography (HPLC) monitored by an evaporative light scattering detector (ELSD) (Supplementary Information III). Consistent with previous reports12 as shown in Figure 1, FUT8 could core-fucosylate *N*-glycans with an agalactosylated and unprotected GlcNAc residue on the α1,3-mannose branch (**N000, N010, N020, N030, N110**, and **N211–N215**), but not those either galactosylated or with protected GlcNAc residue on the branch. Among these N-glycans, FUT8 showed the highest activity towards biantennary complex *N*-glycan structures terminated with GlcNAc at the non-reducing end of both α1,3-and α1,6-mannose branches **N000** (91.3% conversion) and slightly decreased activities towards **N110** (63.4% conversion) and **N211–N213** (67.8% to 70.4% conversion), indicating that galactosylation with or without additional sialylation or chemical protection of GlcNAc on the α1,6-mannose branch can be well tolerated. The addition of an α1,3-linked fucose to the terminal *N*-acetyllactosamine (LacNAc) or sialyl LacNAc structures on **N211** and **N212** to form the Lewis x and sialyl Lewis x structures on the α1,6-mannose branch of **N214** (40.1%) and **N215** (34.8%), respectively, further decrease the efficiency for FUT8-catalyzed core fucosylation. Quite interestingly, FUT8 was found to be able to glycosylate **N04**, an *N*-glycan without a α1,3-mannose branch, even though with a neglectable percentage conversion (5.26%). The result was confirmed by milligram scale synthesis and product **N604** purification followed by mass spectrometry (MS) and proton nuclear magnetic resonance (^{1}H NMR) characterization (Supplementary Information VI). Overall, our substrate specificity study using a library of 77 structurally defined *N*-glycans confirmed that FUT8 has a quite strict requirement towards the α1,3-mannose branch, and suggested a very relaxed preference towards the α1,6-mannose branch.

With the information about substrate specificity of FUT8 in hand, we were ready to design effective routes for synthesizing asymmetric *N*-glycans with core-fucosylation which have

been frequently found in the glycomes of mammalian cells, especially on disease related proteins.[19] For example, putative structures of m/z 2605 and 2780 found on human ovarian biomarker glycoprotein CA125 were determined by matrix-assisted laser desorption/ionization-mass spectrometry (MALDI-MS) and collision activated dissociation-tandem mass spectrometry (CAD-MS/MS).[19b] Nevertheless, a few possible isomers (Fig. 2) were not ruled out. To access such asymmetric *N*-glycans with core-fucosylation which can be used as glycan standards, a facile chemoenzymatic synthetic strategy was developed (Fig. 3 & 4).

Similar to our core synthesis/enzymatic extension strategy (CSEE) for the preparation of asymmetric *N*-glycans,[13b] we envisaged **N6111** and **N6211** as two key intermediates for the synthesis of core-fucosylated ones. As illustrated in Figure 3A, to obtain intermediate **N6111**, we chose chemically prepared *N*-glycan **N110**[13b] as a starting point, which was shown to be an well tolerated substrate of human FUT8 in the substrate specificity study described above. Briefly, in a 2.5 mL reaction system containing 100 mM MES buffer (pH 7.0), 7.2 mg (5 μmole, 2 mM) of **N110** was incubated at 37 °C with GDP-Fuc (4 mM), recombinant FUT8 (0.2 mg/mL), and 10 U/mL of thermosensitive alkaline phosphatase (FastAP, ThermoFisher) to drive the reaction forward by digesting byproduct GDP. Every other hour, 2 μL of the reaction mixture was withdrawn for analysis. MALDI-MS analysis showed a peak at m/z = 1611.5695, corresponding to **N6110** $[M + H]^+$. Meanwhile, on the HPLC-ELSD profile, a new peak (T_R = 6.99 min) was observed, of which the area increased while that of the peak corresponding to **N110** (T_R = 6.06 min) decreased. After **N110** was completely fucosylated (8 h incubation), the reaction was freeze-quenched at −80 °C for 30 min, and the mixture was concentrated to 300 mL for HPLC purification with a water/acetonitrile gradient elution (see Supplementary Information IV for details) to provide 6.9 mg of **N6110** (87% yield). The purified **N6110** was then galactosylated to afford **N6111a** by bovine β1,4-galactosyltransferase (B4GALT1) as described previously.[13b] In this case, only the GlcNAc residue on the α1,3-mannose branch was galactosylated, as the other one on the α1,3-mannose branch was protected by peracetylation. After HPLC purification, the GlcNAc residue on the α1,6-mannose branch of **N6111a** was de-acetylated with 3% of ammonium hydroxide to afford 6.5 mg (after HPLC purification) of key intermediate **N6111** (80% yield over 3 steps).

With **N6111** in hand, core-fucosylated asymmetric *N*-glycans **N6122, N6123** and **N6244** were prepared in a step-wise manner similar to that of the non-core-fucosylated ones.[13b] Besides B4GALT1, 3 bacterial glycosyltransferases (GTs) were employed in the synthesis, including a sialidase activity reduced mutant (E271F/R313Y) of *Pasteurella multocida* α2,3-sialyltransferase 1 (PmST1m)[20] to catalyze the formation of α2,3-sialosides, a *Photobacterium damselae* α2,6-sialyltransferase (Pd2,6ST)[21] to catalyze the formation of α2,6-sialosides, and a C-terminal 66 amino acids truncated version of *Helicobacter pylori* α1,3-fucosyltransferase (Hpα1,3FT)[13b] to fucosylate branched GlcNAc residues (Fig. 3A). All glycans were purified by HPLC after each glycosylation step and characterized by MALDI-MS (Fig. 3B). N-Glycan purity was confirmed by HPLC-ELSD (Supplementary Information V). It is worth to mention that none of the 4 GTs showed altered activity towards core-fucosylated *N*-glycan substrates compare to that for non-core-fucosylated ones,

indicating that the α1,6-core fucose has negligible effect on acceptor glycan interaction with the GTs. More core-fucosylated asymmetric *N*-glycans can thus be readily synthesized from **N6111** following previously reported routes.[13b]

In contrast to **N110** with a per-acetylated GlcNAc at the α1,6-mannose branch which is a suitable acceptor for FUT8, **N210** with a per-acetylated GlcNAc at the α1,3-mannose branch is not a suitable substrate for FUT8. Therefore, a key intermediate **N6211** was needed for further enzymatic extension. It was synthesized from **N210** by β1,4-galactosylation of the non-protected GlcNAc on the α1,6-mannose branch for the formation of **N211** followed by deprotection of the GlcNAc on the α1,3-mannose branch and FUT8-catalyzed core-fucosylation of the asymmetric *N*-glycan (Fig. 4). Starting from **N6211**, desired *N*-glycans **N6222, N6223** and **N6144** were then enzymatically synthesized in a step-wise manner as described above (Fig. 4). The purified products were characterized by MALDI-MS, ^{1}H NMR (Supplementary Information VII), and HPLC-ELSD (Supplementary Information V).

In conclusion, the available of previously synthesized 77 structurally-defined *N*-glycans allowed detailed substrate specificity study of a recombinant human FUT8. Results showed a quite strict requirement of FUT8 towards the α1,3-mannose branch but a great promiscuity towards the α1,6-mannose branch. Interestingly, human FUT8 can also core-fucosylated an *N*-glycan without the α1,3-mannose branch, which was not observed before. In addition, a facile chemoenzymatic strategy was developed and employed for the efficient synthesis of a panel of core-fucosylated asymmetric *N*-glycans. These well-defined structures are valuable standards and probes for glycomics analysis and elucidating functions of glycan-binding protein.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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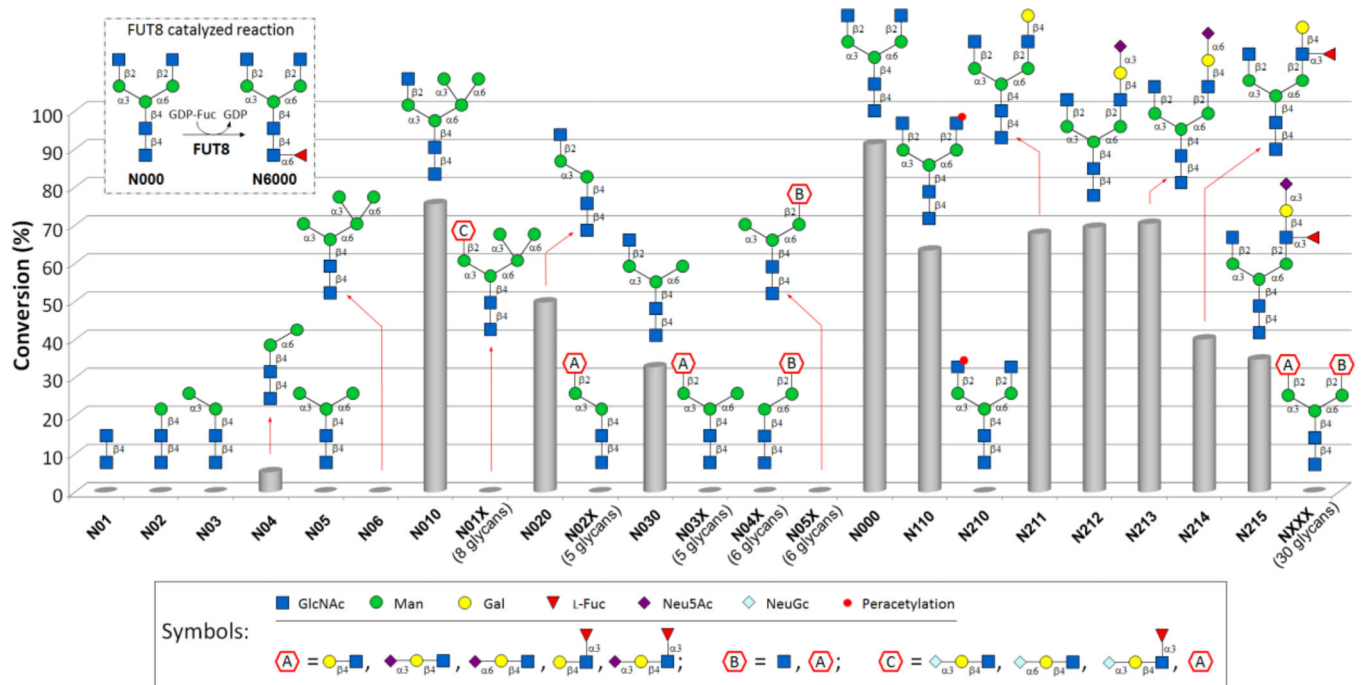21. Yu H, Huang S, Chokhawala H, Sun M, Zheng H, Chen X. Angew Chem Int Ed Engl. 2006; 45:3938. [PubMed: 16721893]

Figure 1.
FUT8-catalyzed reaction and substrate specificity of human FUT8 using 77 *N*-glycans as potential acceptors (see Supplementary Figure S2 for the full list of *N*-glycans, Supplementary Table S1 for conversion percentage towards each glycan). The average conversion percentage of three replicates for each acceptor was shown.
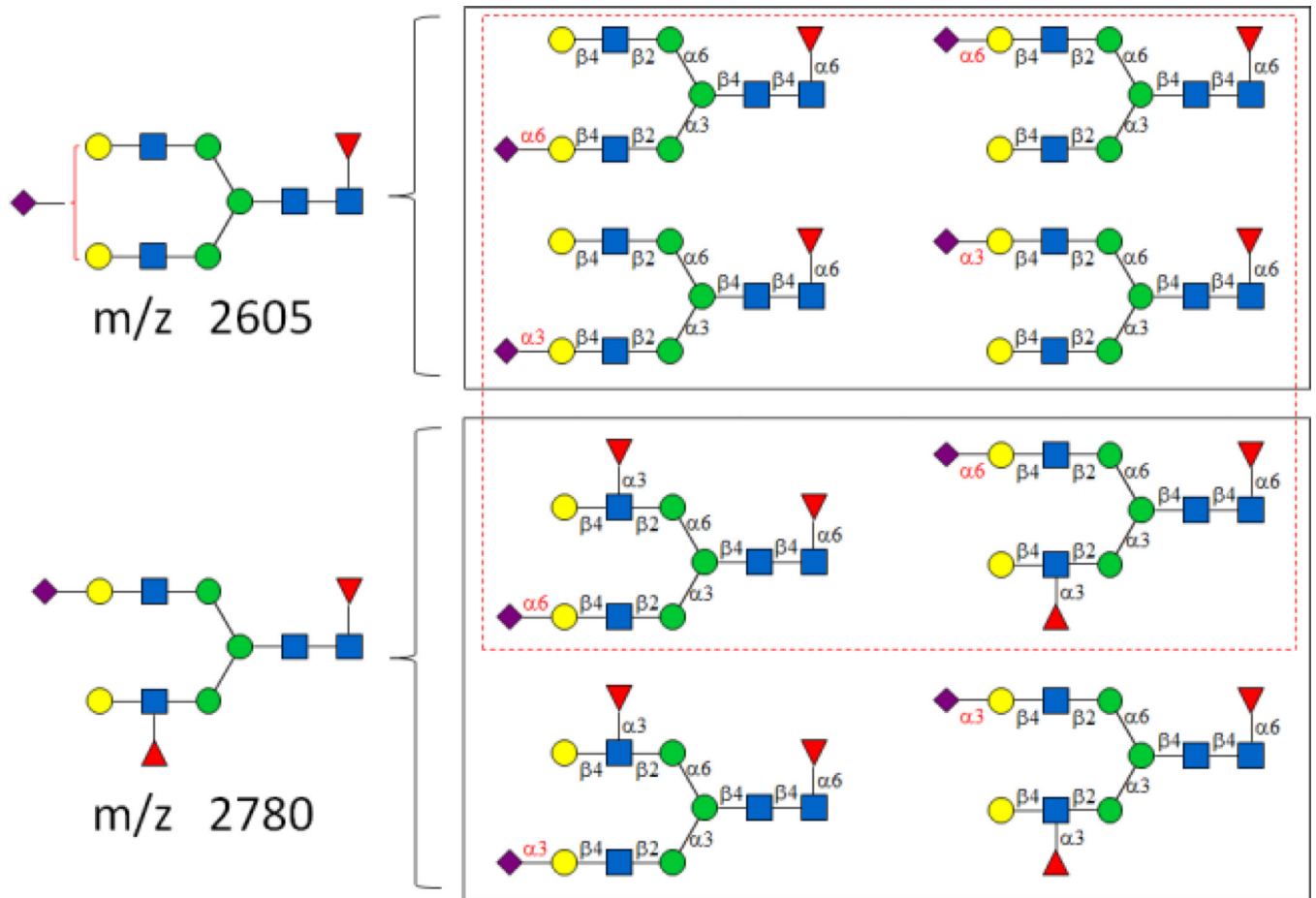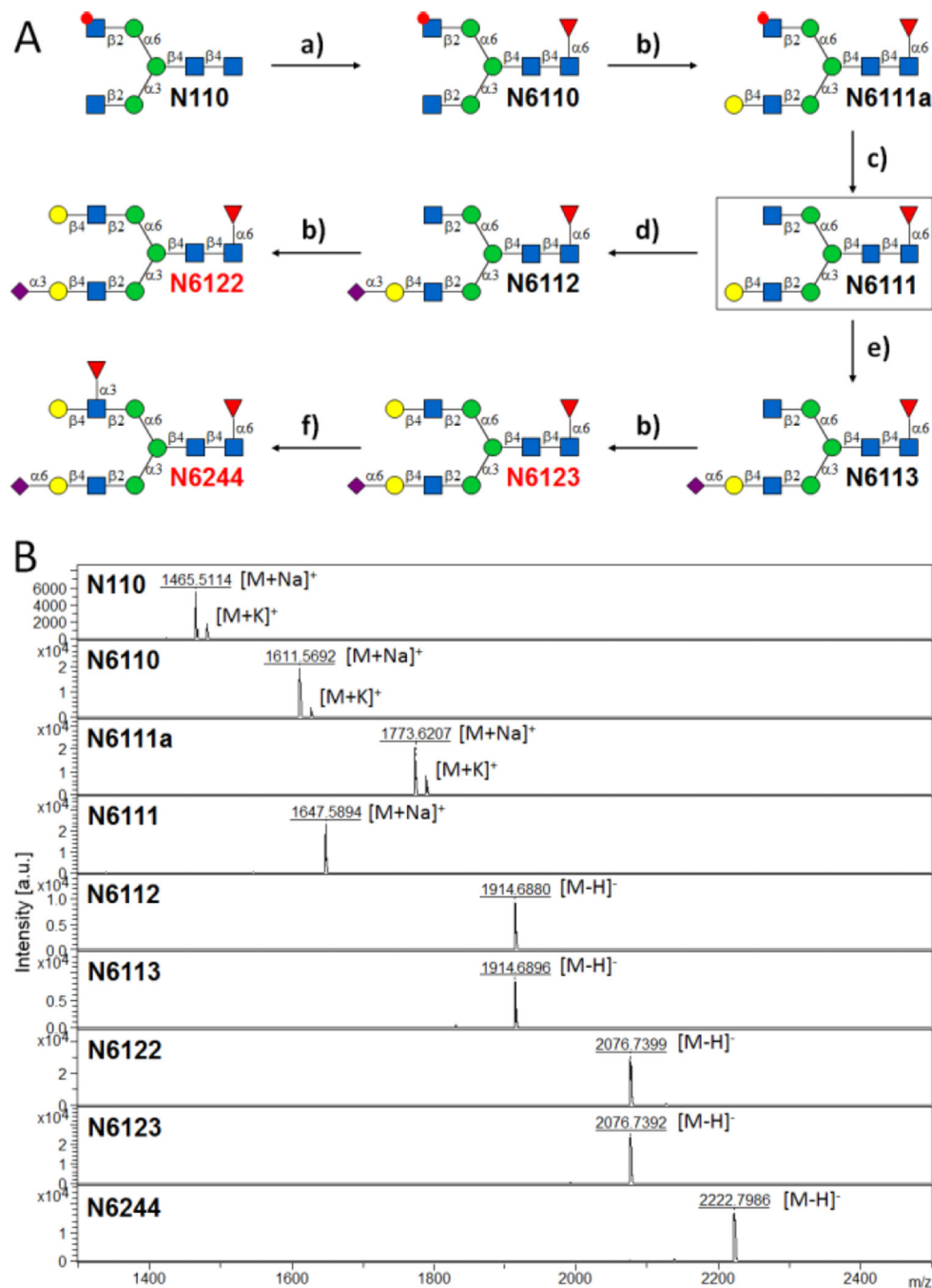
Figure 2.
Two *N*-glycans (m/z) identified from CA125 and possible *N*-glycans (black squared). Red
dash squared N-glycan structures were synthesized in this study.

Figure 3.
Chemoenzymatic synthesis of core-fucosylated asymmetric *N*-glycans from **N110** (**A**), and MALDI-MS analysis of purified glycans (**B**). Reaction conditions: a) FUT8, GDP-Fuc, FastAP; b) β4GALT1, UDP-Gal, FastAP, Mn^{2+}; c) 30% ammonium hydroxide : H_2O (1 : 10), 6 h; d) PmST1m, CMP-Neu5Ac; e) Pd2,6ST, CMP-Neu5Ac; f) Hpα1,3FT, GDP-Fuc, FastAP, Mn^{2+}. FUT8, human α1,6-fucosyltransferase; B4GALT1, bovine β1,4-galactosyltransferase; PmST1m, *Pasteurella multocida* α2,3-sialyltransferase 1 mutant E271F/R313Y; Pd2,6ST, *Photobacterium damselae* α 2,6-sialyltransferase; Hpα1,3FT, c-

terminal 66 amino acids truncated *Helicobacter pylori* α1,3-fucosyltransferase; FastAP, thermo-sensitive alkaline phosphatase.
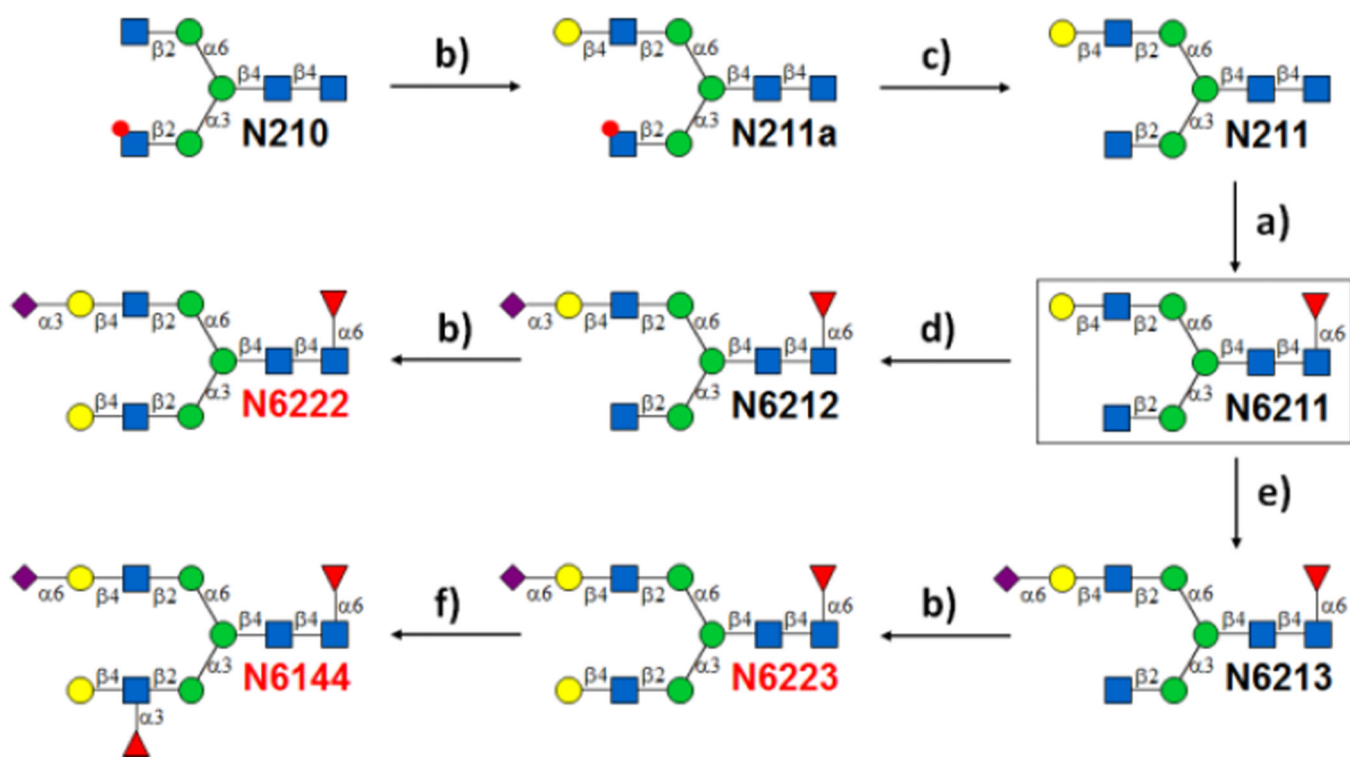
Figure 4.
Enzymatic synthesis of core-fucosylated *N*-glycans starting with **N210**. See Figure 3A for detailed description of each step.