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Divergent Chemoenzymatic Synthesis of Asymmetrical-Core-Fucosylated and Core-Unmodified *N*-Glycans

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Abstract

A divergent chemoenzymatic approach for the preparation of core-fucosylated and core-unmodified asymmetrical *N*-glycans from a common precursor is described. An undecasaccharide was synthesized by sequential chemical glycosylations of an orthogonally protected core fucosylated hexasaccharide that is common to all mammalian core fucosylated *N*-glycans. Antennae-selective enzymatic extension of the undecasaccharide using a panel of glycosyl transferases afforded core fucosylated asymmetrical triantennary *N*-glycan isomers, which are potential biomarkers for breast cancer. A unique aspect of our approach is that a fucosidase (FucA1) has been identified that selectively can cleave a core-fucoside without affecting the fucoside of a sialyl Lewis^X epitope to give easy access to core-unmodified compounds.

Keywords

asymmetrical synthesis; bioorganic chemistry; chemoenzymatic synthesis; glycosylation; *N*-glycans

Of all post-translational modifications of proteins, complex *N*-linked glycans are the most prominent in terms of complexity and diversity.^[1] All eukaryotic *N*-linked glycans have a common pentasaccharide core that can be modified by various *N*-acetylglucosamine (βGal(1,4)GlcNAc, LacNAc) moieties, which in turn can be elaborated by glycosyltransferases to give branched structures. Most *N*-glycans have unique appendages at each antennae resulting in asymmetrical compounds with enormous structural diversity. The core pentasaccharide can be further modified with various monosaccharides such as bisecting *N*-acetylglucosamine and α(1,3)- and α(1,6)-fucosides.

In mammals, core fucose is found only α(1,6)-linked to the reducing GlcNAc moiety of the common core pentasaccharide, which is installed by the α(1,6)-fucosyltransferase FUT8.^[2]

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The biological importance of core fucosylation was demonstrated in FUT8 knockout mice, which exhibited growth retardation, lung emphysema and death within three days after birth.^[3] Changes in core fucosylation have been linked to breast^[4] and prostate cancer,^[5] chronic hepatitis and liver cirrhosis.^[6] Core fucosylation has a profound effect on protein function, and for example bi-antennary Fc-glycans of IgG antibodies that lack this moiety exhibit better binding of Fc γ receptors resulting in a 50–100 fold increase in antibody-directed cellular cytotoxicity (ADCC).^[7] The function of many other therapeutically important glycoproteins is regulated by core fucosylation and examples include α -fetoprotein (AFP),^[8] erythropoietin (EPO),^[9] human chorionic gonadotropin (hCG)^[10] and follicle-stimulation hormone (FSH).^[11] Core fucosylated complex *N*-glycans may serve as biomarkers for cancer.^[12] In general, glycomic analysis cannot provide exact structures of *N*-glycans because of their isobaric nature (i.e. different structures with identical molecular weights), which greatly complicates biomarker discovery.^[13] It is the expectation that well-defined glycan standards will make it possible to develop methodologies that can identify exact structures of glycans.^[14]

Although elegant chemical approaches for the preparation of core fucosylated *N*-glycans have been reported, these methods only allow the synthesis of one-compound-at-the-time or provided small numbers of related compounds that have symmetrical structures.^[15] It is also possible to install core fucose enzymatically by FUT8.^[16] This enzyme has, however, limited substrate specificity and cannot modify highly complex bi-, tri- and tetra-antennary *N*-glycans.

Herein, we report a chemoenzymatic strategy that makes it possible, for the first time, to prepare collections of highly complex asymmetrical multi-antennary *N*-glycans that are modified by core fucose. It builds on a chemoenzymatic approach that we recently reported^[17] in which complex oligosaccharides are chemically synthesized that can be extended in a branch selective manner by glycosyl transferases. Our new approach exploits hexasaccharide **5** which has an architecture that is common to all core fucosylated *N*-glycans in mammals (Figure 1).^[1] It is modified at positions where branching points can occur with the orthogonal protecting groups allyloxycarbonate (Alloc), levulinoyl (Lev), 2-naphthylmethyl (Nap) and fluorenylmethyloxycarbonate (Fmoc). These protecting groups can be removed in a sequential manner to give acceptors for chemical glycosylations with LacNAc and GlcNAc donors **6–8**. Removal of all protecting groups of the resulting products except the acetyl esters will give derivatives that in a branch selective manner can be elongated by glycosyltransferases resulting in core fucosylated asymmetrical glycans. A unique aspect of our approach is that late stage glyco-diversification can be accomplished by employing the fucosidase FucA1. This enzyme can selectively cleave a core-fucoside without affecting other fucosides to give entry into core-unmodified compounds. The methodology was employed for the chemical synthesis of undecasaccharide **9**, which enzymatically was extended to give core fucosylated compounds **1** and **2**. Treatment of the latter derivatives with FucA1 gave the corresponding derivatives **3** and **4**. Compounds **1** and **2**, which are trisialylated and core fucosylated triantennary *N*-glycans containing a sialyl Lewis^X epitope, are putative structures found in serum glycoproteins of breast cancer patients and may serve as biomarkers.^[18]

Orthogonally protected and core fucosylated hexasaccharide **5** was prepared from monosaccharide building blocks **10**, **11**, **12** and **13** and disaccharide **14** (Scheme 1).^[17,19] Coupling of glycosyl trifluoroacetimidate^[20] donor **14** with **10** using trifluoromethanesulfonic acid (TfOH) as the catalyst gave trisaccharide **15** as only the β -anomer due to neighboring group participation by the Troc (2,2,2-trichloroethoxycarbonyl) group. The Lev ester of **15** was easily removed with hydrazine acetate,^[21] and the resulting acceptor **16** was coupled with fucosyl donor **11** using trifluoromethanesulfonic anhydride (Tf₂O) as the activator in the presence of 2,4,6-tri-*tert*-butylpyrimidine (TTBP)^[19b] to provide the desired tetrasaccharide **17** in a yield of 90%. The stereochemistry of the newly formed α (1,6)-linked fucoside was confirmed by the coupling constant between the anomeric carbon and proton of the fucoside moiety (J_{C1-H1} =172.1 Hz). Subsequently, the Nap ether was removed by oxidation with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ)^[22] in a mixture of CH₂Cl₂ and phosphate buffer (pH 7.4), and the resulting alcohol **18** was coupled with glycosyl donor **12** using NIS/TfOH as the activator to afford α (1,3)-linked mannosyl pentasaccharide **19** in 82% yield. The benzylidene acetal of **19** was regioselectively opened using triethylsilane (Et₃SiH) and dichlorophenylborane (PhBCl₂)^[23] to provide glycosyl acceptor **20**, which was glycosylated with **13** using Tf₂O as the promoter in the presence of TTBP and 1-octene^[24] to give orthogonally protected core fucosylated hexasaccharide **5** in a yield of 72%.

With orthogonally protected hexasaccharide **5** in hand, attention was focused on the assembly of fucosylated triantennary undecasaccharide **9**, which was expected to be an appropriate precursor for branch-selective enzymatic modification (Scheme 2). Thus, the Fmoc group of **5** was selectively removed by the non-nucleophilic base triethylamine (Et₃N) without affecting the Lev and Alloc protecting groups to give glycosyl acceptor **21** in 88% yield.^[25] A TfOH-mediated glycosylation of trifluoroacetimidate^[20] donor **6** with **21** afforded octasaccharide **22**, which was treated with hydrazine acetate to remove the Lev ester to provide glycosyl acceptor **23**. Coupling of **23** with **7** using TfOH as the catalyst gave nonasaccharide **24** in 88% yield. Next, the Alloc protecting group of **24** was cleaved with Pd(PPh₃)₄^[26] to provide the corresponding hydroxyl **25**, which was coupled with **8** in the presence of catalytic TfOH to afford fully protected undecasaccharide **26** in 70% yield. Finally, partial deprotection of **26** to give the desired precursor undecasaccharide **9** was accomplished by removal of Troc groups using the aqueous sodium hydroxide in tetrahydrofuran (THF), followed by acetylation of the resulting amines with acetic anhydride and catalytic hydrogenolysis of the Nap and benzyl (Bn) ethers using Pd(OH)₂/C in presence of hydrogen. Detailed analysis of **9** by NMR confirmed the stereochemistry of all the glycosidic linkages. It also showed that the acetyl esters had remained intact.

The undecasaccharide **9** was employed for further extension by glycosyltransferases to prepare tri-sialylated and core fucosylated triantennary *N*-glycans **1** and **2** (Scheme 3). A challenging aspect of the preparation of these compounds was the installation of the sialyl Lewis^x epitope without affecting the acetyl esters of the LacNAc moiety at the C-2' arm and the terminal GlcNAc moiety at C-6. First, the LacNAc moiety of the C-2 arm was sialylated with α (2,3)-sialyltransferase (ST3Gal-IV), and cytidine-5'-monophospho-*N*-acetylneuraminic acid (CMP-Neu5Ac) in the presence of calf intestine alkaline phosphatase

(CIAP) to give **27**. The latter compound was fucosylated with $\alpha(1,3)$ -fucosyltransferase (FUT-V), guanosine 5'-diphospho- β -L-fucose (GDP-Fuc) and CIAP to afford **28** which has a sialyl Lewis^x motif at the bottom arm. Although ESI-MS analysis showed a loss of an acetyl ester, homogenous **29** was obtained after treatment of **28** with aqueous ammonia. Next, the GlcNAc moiety of **29** was converted into a LacNAc by $\beta(1,4)$ -galactosyltransferase (GalT-I), uridine 5'-diphosphogalactose (UDP-Gal) and CIAP to give **30**. The two LacNAc moieties of **30** were di-sialylated with ST3Gal-IV or $\alpha(2,6)$ -sialyltransferase (ST6Gal-I) in the presence of CMP-Neu5Ac and CIAP to give target compounds **1** and **2**, respectively. It is important to note that $\alpha(2,6)$ -sialylation of the upper C-6 antenna was slow, and after 24 h only one sialoside was attached. Addition of more enzyme and a prolonged incubation was required to drive the reaction to completion. To further diversify the *N*-glycans, compounds **1** and **2** were subjected to the fucosidase, FucA1, which selectively cleaved the core-fucoside without affecting the fucoside of sialyl Lewis^x epitope to give compounds **3** and **4**, respectively. The catalytic domain of the latter enzyme was expressed as soluble, secreted fusion proteins (amino acid residues 32–466) by transient transfection of HEK293 suspension cultures. The structural identity of the compounds was established by examining characteristic signals in ¹H NMR spectra (Figure 2). Compounds **1–4** exhibited a doublet (δ_{CH_3} = 1.16 ppm) that is typical for the fucoside of the sialyl Lewis^x moiety. On the other hand, compounds **1** and **2** had signals that were unique to the core fucoside (δ_{CH_3} = 1.22 ppm). After each enzymatic transformation, the product was purified by size exclusion chromatography using a Bio-Gel P2 gel filtration column and the resulting compounds were fully characterized by NMR and mass spectrometry of the permethylated derivatives. If any starting material was detected by mass spectrometry, the compound was resubjected to the enzyme and sugar nucleotide until a homogeneous product was obtained.

Hydrophilic interaction liquid chromatography (HILIC) is widely used for the analysis of native and derivatized glycans.^[27] We explored whether the isomeric *N*-glycan standards **1–4** can be separated by this type of chromatography. An XBridge[®] Amide column (5 μ m, 4.6 mm $\times$ 250 mm, Waters) was employed using an evaporative light scattering detector (ELSD) and a gradient of acetonitrile and ammonium formate (100 mM, pH 3.4). Gratifyingly, the isomers **1** (t_R = 21.26 min) and **2** (t_R = 18.33 min), and **3** (t_R = 20.94 min) and **4** (t_R = 17.89 min) could readily be separated (Figure 3). It is important to note that for each compound an adjacent and partially overlapping peak was observed, which is attributed to the presence of α - or β -anomers of the reducing GlcNAc moiety. Separation of the anomers is supported by LC-ESI-MS analysis which showed identical masses for the two peaks (t_R = 21.26 min, [*M*–3*H*]^{3–} = 1056.2554, t_R = 21.92 min, [*M*–3*H*]^{3–} = 1056.2545) and by rerunning the individual peaks giving identical chromatograms. Mutarotation, which is the equilibration of α - and β -anomers of reducing sugars, can be a relatively slow process especially in ammonium ion containing solvents, supporting partial separation of the two anomers.^[28]

In conclusion, we have demonstrated that from orthogonally protected hexasaccharide **5**, which resembles the common core of core-fucosylated *N*-glycans of all mammals, both core fucosylated and core unmodified glycans can be prepared. A key finding was that the fucosidase FucA1 can selectively cleave an $\alpha(1,6)$ -linked core-fucoside without affecting

other fucosides to give unmodified core-fucosylated glycans. This step can be performed at a late stage of synthesis, and thus core fucosylated and core unmodified glycans can easily be obtained from a single advanced compound, and does not require the time consuming preparation of two precursors (core fucosylated and core unmodified) for enzymatic extension. By using appropriate glycosyl transferases, the approach should allow the installation of a wide variety of terminal structures to provide entry into a wide array of glycans. The resulting asymmetrically branched *N*-glycans offer opportunities to investigate the specificities and biology of glycan binding proteins, to design probes for elucidating pathways of glycoconjugate biosynthesis, and to develop methodologies that can assign exact structures of glycans in complex biological mixtures. As a proof of principle, we have shown that separation methods can be established for isomeric glycans. Such capabilities will make it possible to identify exact structures of glycans in biological samples, which in turn will facilitate biomarker discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Orthogonally protected core fucosylated hexasaccharide **5**, glycosyl donors **6–8** and undecasaccharide **9**.

Figure 2.
 ^1H NMR comparison of glycans **1** vs. **3** and **2** vs. **4**.

Figure 3.
HPLC profiles of *N*-glycan isomers 1 vs. 2 and 3 vs. 4.

Scheme 1. Chemical synthesis of orthogonally protected core fucosylated hexasaccharide **5**.

Scheme 2. Chemical synthesis of the precursor undecasaccharide **9** for enzymatic extension.

Scheme 3.

Chemoenzymatic synthesis of core fucosylated and core-unmodified *N*-glycans **1–4**.

Reagents and conditions: a) ST3Gal-IV, CMP-Neu5Ac, CIAP, sodium cacodylate buffer (50 mM, pH 7.3), 37°C, overnight for **28** (86%), 48 h for **2** (83%). b) FUT-V, GDP-Fuc, CIAP, Mn^{2+} , Tris-HCl buffer (50 mM, pH 7.5), 37°C, overnight, 87%. c) 28–30% ammonium hydroxide/ H_2O 1:10 (v/v), rt, 2 h, 95 %. d) GalT-I, UDP-Gal, CIAP, Mn^{2+} , Tris-HCl buffer (50 mM, pH 7.5), 37°C, overnight, 91%. e) ST6Gal-I, CMP-Neu5Ac, CIAP, sodium cacodylate buffer (50 mM, pH 7.3), 37°C, 72 h, 81%. f) FucA1, 0.1M NaOAc buffer (pH 4.5), 37°C, 18 h, 85% for **3**, 82% for **4**.