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Unusual transglycosylation activity of *Flavobacterium meningosepticum* endoglycosidases enables convergent chemoenzymatic synthesis of core fucosylated complex N-glycopeptides

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Abstract

Structurally well-defined, homogeneous glycopeptides and glycoproteins are indispensable tools for functional glycomics studies. By screening of various endo- β -N-acetylglucosaminidases using an appropriate synthetic donor and acceptor substrates, we have found that the *Flavobacterium meningosepticum* endo- β -N-acetylglucosaminidases glycosidases (GH family 18), including Endo-F2 and Endo-F3, were able to glycosylate α -1,6-fucosylated GlcNAc derivative to provide natural, core-fucosylated complex type N-glycopeptides. The Endo-F2 and Endo-F3 were efficient for transferring both sialylated and asialylated glycans and were highly specific for an α -1,6-fucosylated GlcNAc-peptide as acceptor for transglycosylation, as they showed only marginal activity on non-fucosylated GlcNAc-peptide. In contrast, we found that the commonly used endoglycosidases such as Endo-A and Endo-M, which belong to GH family 85, were unable to take α -1,6-fucosyl-GlcNAc derivative as acceptor for transglycosylation. The novel activity of Endo-F2 and Endo-F3 was successfully applied for a highly convergent chemoenzymatic synthesis of a full-size CD52 glycopeptide antigen carrying both terminal sialic acid and core fucose. This is the first report on endoglycosidases that are able to glycosylate α -1,6-fucosylated GlcNAc derivatives to form natural core-fucosylated glycopeptides.

Keywords

enzymatic transglycosylation; endoglycosidase; glycopeptides; chemoenzymatic synthesis; sugar oxazoline

Introduction

The oligosaccharide components of glycoproteins not only affect protein's structure and stability, but also directly participate in many important biological recognition processes such as cell adhesion, cell differentiation, tumor metastasis, host-pathogen interactions, and immune responses.^[1] Ample examples have shown that subtle changes in the glycan structures of glycoproteins can result in significant differences in functions.^[2] Core fucosylation, the attachment of a fucose α -1,6-linked to the innermost GlcNAc moiety of the asparagine (N)-linked glycans, is a natural modification frequently found in natural and

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recombinant glycoproteins. It has been demonstrated that core fucosylation affects N-glycan's conformations and regulates the interactions between N-glycan and various glycan-binding proteins.^[3] On the other hand, significant increases in core fucosylation have been associated with various cancers and are correlated to tumor progression, suggesting that core-fucosylated N-glycans and glycoproteins could serve as novel biomarkers for diagnosis.^[4, 5] However, functional glycomics studies are often hampered by the difficulties to obtain structurally well-defined complex oligosaccharides and glycoconjugates. Despite remarkable advance in chemical and chemoenzymatic synthesis of complex oligosaccharides and glycoconjugates in recent years,^[6] synthesis of sialylated and fucosylated complex glycopeptides and glycoproteins is still a challenging task, because of the labile nature of the α -sialyl/ α -fucosyl glycosidic linkages during multiple synthetic manipulations.^[7] It should be noted that a late-stage enzymatic introduction of the core fucose by α -1,6-fucosyltransferase (Fut8) does not seem to be feasible for full-size N-glycans because Fut-8 only accepts the truncated N-glycan core as substrate for α -1,6-fucosylation.^[5] In addition, a recent study on Fut8 showed an unusual substrate structural requirement for the Fut8-catalyzed α -1,6-fucosylation.^[8] Thus, a convergent synthetic approach that permits a native ligation between a pre-assembled oligosaccharide and an independently generated Fuc α 1,6GlcNAc-peptide/protein would be highly desirable.

We and others have recently explored the transglycosylation activity of glycosyl hydrolase (GH) family 85 endo- β -N-acetylglucosaminidases (ENGases) for glycopeptide and glycoprotein synthesis.^[9–17] It was shown that the Endo-A from *Arthrobacter protophormiae*^[18] and the Endo-M from *Mucor hiemalis*,^[19] could efficiently take synthetic sugar oxazolines as substrates for transglycosylation to form homogeneous glycopeptides/glycoproteins carrying truncated or modified N-glycans. In addition, glycosynthase mutants have been generated that are suitable for synthesizing glycoproteins carrying natural N-glycans without product hydrolysis.^[12–14, 16] Despite these advances, this convergent chemoenzymatic method has not been tested for synthesizing core-fucosylated complex N-glycopeptides and/or N-glycoproteins. In fact, so far there were no reports on any endoglycosidases capable of transferring oligosaccharides to a GlcNAc acceptor capped with an α -1,6-fucose. We report in this paper the first discovery of novel transglycosylation activities of a class of endo- β -N-acetylglucosaminidases that recognize core-fucosylated GlcNAc acceptors. We have found that the GH family 18 endoglycosidases from *Flavobacterium meningosepticum*,^[20, 21] particularly Endo-F2 and Endo-F3,^[22] can specifically glycosylate the 4-OH of the GlcNAc moiety in an α -1,6-fucosyl-GlcNAc-peptide acceptor to form a homogeneous, core-fucosylated complex glycopeptide, whereas the GH family 85 endoglycosidases Endo-A and Endo-M, which were previously reported to efficiently glycosylate non-fucosylated GlcNAc, were unable to take Fuc α 1,6GlcNAc derivative for transglycosylation. This is the first report on the transglycosylation activity of Endo-F2 and Endo-F3 and the first discovery of endoglycosidases capable of recognizing core-fucosylated GlcNAc as substrate. We also describe here a convergent chemoenzymatic synthesis of a full-size, sialylated and core-fucosylated CD52 glycopeptide antigen by applying the novel transglycosylation activity.

Results and Discussion

Chemical synthesis of Asn-linked disaccharide derivative Fmoc-Asn(Fuc α 1,6GlcNAc)-OH

For screening the transglycosylation activity of ENGases on fucosylated GlcNAc acceptor, we synthesized an Asn-linked disaccharide derivative, Fmoc-Asn(Fuc α 1,6GlcNAc)-OH (**9**), using the peracetylated GlcNAc-azide (**1**)^[23] as the starting material (Scheme 1). De-O-acetylation of **1**, followed by selective tritylation at the 6-OH and benzylation at the 3- and 4-OH gave compound **2**. The 6-O-trityl group was then selectively removed by mild acid hydrolysis to give the glycosyl acceptor **3**. Selective α -1,6-fucosylation of **3** with per-O-

trimethylsilyl α -fucopyranosyl iodide (**4**)^[24] as the glycosyl donor in the presence of 2,6-di-*tert*-butylpyridine, followed by *in situ* removal of the TMS protecting groups, gave the disaccharide derivative **5** in 61% yield. The newly formed glycosidic linkage was confirmed to be α -linkage as demonstrated by a relatively small coupling constant between H-1 and H-2 of the fucose (H-1, δ 4.82, d, $J_{1,2}$ = 3.68 Hz). After changing the O-protecting groups to O-acetyl groups, the resulting glycosyl azide **6** was reduced with Me₃P in THF and then coupled with Fmoc-Asp-OtBu to afford the Asn-linked derivative **7**. The *tert*-butyl group was selectively removed with a mild acid condition (20% TFA in CH₂Cl₂) without cleavage of the acid-labile α -1,6-fucosidic linkage to give the Asn-linked disaccharide **8** in 95% yield. Finally selective de-O-acetylation of **8** was achieved by treatment of **8** with K₂CO₃ in MeOH/THF to provide the N-Fmoc-protected Asn-disaccharide (**9**) in 85% yield (Scheme 1).

Screening of transglycosylation activity using the fucosylated GlcNAc-Asn as the acceptor substrate and two synthetic sugar oxazolines as the donor substrates

With the synthetic disaccharide acceptor (**9**) in hand, we screened a series of ENGases for their ability to transfer an oligosaccharide to the Fuca1,6GlcNAc moiety. These include Endo-A,^[18] Endo-M,^[19] Endo-H from *Streptomyces plicatus*,^[25] Endo-D from *Streptococcus pneumoniae*,^[26] and Endo-F1, Endo-F2, and Endo-F3 from *Flavobacterium meningosepticum*.^[20–22] These enzymes all hydrolyze N-glycans at the *N,N'*-diacetylchitobiose core, but they have different substrate specificity. It is known that Endo-A, Endo-H, and Endo-F1 hydrolyze high-mannose type and/or hybrid type N-glycans;^[18, 20, 25] Endo-M can hydrolyze high-mannose type, hybrid type, and non-fucosylated bi-antennary complex-type N-glycans;^[19] Endo-D is specific for a truncated and core-fucosylated N-glycan;^[26] and Endo-F2 and Endo-F3 are able to cleave both core-fucosylated and non-fucosylated complex-type N-glycans,^[21, 22] but Endo-F3 prefers core-fucosylated N-glycans.^[27] Two synthetic sugar oxazolines, the Man3GlcNAc-oxazoline (**10**)^[10] and the sialylated N-glycan oxazoline (**12**),^[14] were used as donor substrates for screening the enzymes. If there was a transglycosylation activity, a corresponding core-fucosylated glycopeptide (**11** or **13**) would be produced, as shown in Scheme 2. Thus, a mixture of oxazoline (**10**) or (**12**) and acceptor **9** (donor/acceptor, 4:1, molar ratio) was incubated with an appropriate amount of the respective ENGase at 30 °C in a phosphate buffer (50 mM, pH 7.0) containing 10% DMSO. Inclusion of 10% DMSO significantly enhanced the solubility of the Fmoc derivative **9** in the buffer. The course of transglycosylation reaction was monitored by RP-HPLC analysis of reaction aliquots taken at intervals, and new peaks (products) were subject to ESI-MS analysis to confirm their identity. The screening results were summarized in Figure 1. It was revealed that Endo-A, Endo-M, Endo-D, and Endo-H did not show any transglycosylation activity towards the α -1,6-fucosylated GlcNAc derivative, although the first three enzymes were previously shown to be able to transglycosylate non-fucosylated GlcNAc-Asn acceptor. In contrast, the Endo-F3 demonstrated remarkable transglycosylation activity toward the α -1,6-fucosylated GlcNAc acceptor, with both the truncated glycan oxazoline (**10**) and the full-size complex type glycan oxazoline (**12**). The reaction led to the formation of the corresponding core-fucosylated N-glycan (**11**) and (**13**) respectively, in over 70% yield within 1 h. The yield was based on the conversion of the acceptor. It should be mentioned that only a single transglycosylation product was formed when oxazoline **10** or **12** were used, indicating the specificity for the enzymatic transglycosylation. A HPLC profile of the reaction between oxazoline **10** and acceptor **9** in the presence of Endo-F3 was shown in Figure S1 (supporting information). It was found that a gradual hydrolysis of the product by Endo-F3 occurred when the mixture was incubated with the enzyme for a prolonged time. This was expected, due to the inherent hydrolytic activity of Endo-F3. However, the Endo-F3 catalyzed transglycosylation from the sugar oxazoline was surprisingly fast, allowing a significant

accumulation of the transglycosylation product. These results suggest that the use of the highly active sugar oxazoline as donor substrate favours the transglycosylation. The Endo-F2 also showed transglycosylation activity on the 1,6-fucosylated GlcNAc acceptor, but the reaction was much slower than that catalyzed by Endo-F3. In comparison, the Endo-F1 only showed marginal transglycosylation activity when Man₃GlcNAc-oxazoline (**10**) was used as donor substrate, and did not show any activity when the sialylated glycan oxazoline (**12**) was used as donor substrate (Figure 1). These results clearly demonstrate the distinct substrate specificity of these endoglycosidases in transglycosylation. Products **11** and **13** from the transglycosylation by Endo-F2 and Endo-F3 were purified by RP-HPLC and first characterized by mass spectrometric analysis. The MALDI-TOF MS data of **11** (calculated, $M = 1392.50$; found (m/z), 1416.12 [$M + Na$]⁺) indicated that the product was an adduct of the Man₃GlcNAc-oxazoline and acceptor **9**; the ESI-MS of **11** revealed two major m/z species at 1393.58 and 1247.60, which corresponded to [$M + H$]⁺ and [$M - Fuc + H$]⁺, respectively. The presence of fragment [$M - Fuc + H$]⁺ suggested that the transferred oligosaccharide was attached to the GlcNAc moiety rather than the Fuc moiety in the acceptor.

Further characterization of product **11** was carried out by specific enzymatic transformations. Endo-D digestion of the isolated **11** gave tetrasaccharide Man₃GlcNAc that was identical to an authentic sample, and the Fmoc-Asn(Fuc α 1,6GlcNAc)-OH. Since Endo-D is known to specifically cleave the β -1,4-glycosidic linkage in the N,N'-diacetylchitobiose unit of a core-fucosylated Man₃GlcNAc₂ moiety [26], the observed result indicated that the transferred Man₃GlcNAc was attached specifically to the 4-OH of the GlcNAc moiety in acceptor **9** to form the desired β -1,4 glycosidic linkage. The sialylated product (**13**) was similarly characterized by MS analysis (See the experimental section in supporting information). To our knowledge, this is the first report showing a transglycosylation activity of Endo-F2 and Endo-F3 with a sugar acceptor.[28] The Endo-F2 and Endo-F3 also represent the first endoglycosidases capable of glycosylating core fucosylated GlcNAc acceptor to form a new core-fucosylated N-glycopeptide.

Endo-F2 and Endo-F3 do not accept non-fucosylated GlcNAc derivative as acceptor substrate for transglycosylation

The significant transglycosylation activity of Endo-F2 and Endo-F3 on Fmoc-Asn(Fuc α 1,6GlcNAc)-OH also prompted us to examine how these *Flavobacterium* enzymes act on non-fucosylated GlcNAc acceptor. We have previously shown that Endo-A and Endo-M are very efficient to transglycosylate non-fucosylated GlcNAc moiety.[9] Accordingly, we tested the transglycosylation of Endo-F1, Endo-F2, Endo-F3, and Endo-D on Fmoc-Asn(GlcNAc)-OH (**14**) using Man₃GlcNAc oxazoline (**10**) as the donor substrate (Scheme 3). The results were summarized in Figure 2. Surprisingly, Endo-F2 and Endo-F3 did not exhibit transglycosylation activity on the non-fucosylated GlcNAc acceptor under the screening conditions. On the other hand, the Endo-F1 enzyme, which showed only marginal activity on the fucosylated GlcNAc acceptor as described above, demonstrated significant transglycosylation activity with the non-fucosylated GlcNAc (**14**) to lead to a rapid formation of the corresponding product (**15**). The yield reached 45% within 30 min. However, a prolonged incubation did not result in further increase in yield. HPAEC-PAD analysis of the reaction mixture revealed that the moderate yield was mainly due to the simultaneous hydrolysis of the sugar oxazoline (**10**), which was completely gone within 30 min. Interestingly, the product Man₃GlcNAc₂-Asn(Fmoc) (**15**) was not hydrolyzed even for a prolonged incubation, indicating that the truncated N-glycan product was a poor substrate of Endo-F1.

The Endo-D is an interesting case. Our results indicated that Endo-D could use Man3GlcNAc-oxazoline (**10**) as donor substrate and GlcNAc-Asn derivative (**14**) as acceptor for transglycosylation, albeit at a low yield (Figure 2). This result is consistent with a recent report on Endo-D.^[17] However, when the α 1-6-fucosylated GlcNAc derivative (**9**) was used, no transglycosylation product was detected (Figure 1). Given the fact that Endo-D could efficiently hydrolyze α -1,6-fucosylated Man3GlcNAc2 core,^[26] it is conceivable to think that Endo-D should be able to accommodate α 1-6-fucosylated GlcNAc derivative (**9**) as an acceptor for transglycosylation. The failure to detect the transglycosylation product might be due to the quick Endo-D catalyzed hydrolysis of the product in situ before it was released from the catalytic site. Indeed, incubation of Endo-D and the purified core-fucosylated Man3GlcNAc2-Asn(Fmoc) (**11**) obtained from the Endo-F3 transglycosylation resulted in a very quick hydrolysis of **11** (see Experimental Section).

The presence of an α -1,6-linked fucose in the GlcNAc acceptor seems essential for recognition of the acceptor by Endo-F2 and Endo-F3 for an efficient transglycosylation. In contrast, attachment of a core fucose on the GlcNAc moiety completely blocked the accommodation of the acceptor by Endo-F1, Endo-A, and Endo-M for transglycosylation. It should be pointed out that the wild type Endo-F2 and Endo-F3 could still hydrolyze the transglycosylation product thus formed. One way to address this issue is to create glycosynthase mutants that is capable of working on the highly activated sugar oxazolines for transglycosylation but lacks product hydrolysis activity. We have previously shown that site-directed mutation at a specific Asn residue, the Asn175 in Endo-M and Asn171 in Endo-A, which promotes oxazoline formation during hydrolysis, could lead to glycosynthase mutants useful for synthesis.^[12–14, 16] The crystal structure of Endo-F3 was solved and the two conserved active residues at the catalytic site were identified as Asp126 and Glu128.^[29] In the substrate-assisted mechanism of the family 18 endoglycosidases,^[30] the Glu128 of Endo-F3 serves as a general acid to protonate the glycosidic bond; whereas the Asp126 was assigned a secondary role, assumingly having the same function as the Asn171 of Endo-A to promote the formation of the oxazolinium ion intermediate, and to stabilize it, by interactions with the 2-acetamido group in the GlcNAc moiety.^[29] Thus an interesting future experiment is to perform site-directed mutation at this aspartic acid residue to test whether efficient glycosynthase mutants can be generated.

Convergent chemoenzymatic synthesis of a sialylated and core-fucosylated complex-type glycoform of CD52 antigen

The remarkable transglycosylation activity of Endo-F2 and Endo-F3 with α -1,6-fucosylated GlcNAc acceptor, together with their flexibility in accepting different sugar oxazolines as donor substrates, opens a new avenue to a highly convergent chemoenzymatic synthesis of core-fucosylated complex type N-glycans, glycopeptides, and/or glycoproteins. As a test case, we chose to synthesize a sialylated and core-fucosylated complex-type glycoform of the CD52 glycopeptide antigen. CD52 is a GPI-anchored glycopeptide antigen found on human lymphocytes and sperm cells. It is a glycopeptide consisting of only 12 amino acid residues but carrying a large complex-type N-glycan at the Asn-3 residue.^[31] Several truncated CD52 glycoforms were made previously by chemical methods.^[32] We have previously used CD52 antigen as a model glycopeptide to test our chemoenzymatic method.^[15, 16, 33] However, a full-size complex-type CD52 antigen carrying both terminal sialic acid residues and core fucose moiety had not been made by either chemical or chemoenzymatic method. A convergent chemoenzymatic synthesis of the target CD52 antigen required the preparation of a CD52 polypeptide containing a Fuca1,6GlcNAc moiety at the Asn-3. To avoid a strong acidic condition for retrieving the polypeptide from the solid support in the Fmoc approach, we performed the solid-phase peptide synthesis on an acid-labile O-link TGT-resin, which can be cleaved by mild acid treatment (Scheme 4).

Fmoc-Asn(Ac₅Fuca1-6GlcNAc)-OH (**8**) was used as a building block to replace the residues at Asn-3 during the synthesis to introduce a Fuca1,6GlcNAc moiety. HATU (0.5 M in DMF) and DIPEA (1.0 M in DMF) (1:1, v/v) were used as the coupling reagent and piperidine (20% in DMF) was used as the deblocking reagent. 4-fold excess of Fmoc-protected building blocks were used for each coupling reaction cycle. The N-terminus amino group was protected with an acetyl group by treatment with Ac₂O/DIPEA. The disaccharide-containing polypeptide was released from the resin (**16**) by treatment with 20% TFA in CH₂Cl₂ at r.t. for 5 h, with simultaneous removal of the side-chain protecting groups (Trt and t-Bu). The Ac groups on the disaccharide were removed by treatment with 5% aqueous hydrazine at r.t. for 1 h. The crude product was then purified by preparative HPLC to give the Fuca1,6GlcNAc-CD52 (**17**) in an excellent overall yield (90%) (Scheme 4). The product was characterized by ESI-MS (calculated, M = 1598.64; found (*m/z*), 1599.16 [M + H]⁺) and a detailed NMR analysis (see supporting information).

With the Fuca1,6GlcNAc-CD52 (**17**) in hand, we next performed the transglycosylation with Endo-F3, using sialoglycan oxazoline (**12**) as donor substrate, which was efficiently synthesized from a free N-glycan by a single-step conversion in water.^[14] Thus, incubation of **12** and **17** (donor/acceptor, 2:1, molar ratio) with Endo-F3 in a phosphate buffer (pH 7.0) led to the formation of the CD52 glycoform (**18**) carrying a sialylated and core-fucosylated bi-antennary N-glycan. The reaction was monitored by HPLC. When the formation of **18** reached a plateau, the reaction was stopped and the product was isolated in 55% yield by HPLC. The efficiency of the transglycosylation was quite impressive, given the fact that only two-fold of the donor substrate was used. Using the Endo-F2 to replace Endo-F3 for the transglycosylation gave a 25% yield of **18** after a longer period (6 h) of incubation (data not shown). ESI-MS of **18** gave the expected *m/z* species (calculated for C₁₃₇H₂₂₁N₂₁O₉₀, M = 3600.34; found (*m/z*), 1201.66 [M + 3H]³⁺). Further characterization of the product was carried out by specific enzymatic transformations. Digestion of **18** with peptide-N4-(N-acetyl-β-D-glucosaminyl)asparagine amidase F (PNGase F), which specifically hydrolyzes the β-aspartylglucosylamine bond of N-glycopeptides and N-glycoproteins,^[34] yielded the expected sialoglycan **19** (ESI-MS: calculated for C₉₀H₁₄₈N₆O₆₆, M = 2368.84; found (*m/z*), 1185.82 [M + 2H]²⁺) and the expected aspartic acid-containing CD52 peptide (**20**) (ESI-MS: calculated for C₄₇H₇₄N₁₄O₂₆, M = 1250.49; found (*m/z*), 1251.56 [M + H]⁺, 626.38 [M + 2H]²⁺) (Figure S2, supporting information). On the other hand, incubation of purified **18** with large amount of Endo-F3 gave Fuca1,6GlcNAc-CD52 (**17**) and the corresponding N-glycan **21** (Figure S3, supporting information). These results suggest that the transferred glycan was attached to the GlcNAc in the peptide through the GlcNAc-β-1,4-GlcNAc linkage. Finally, a detailed ¹H-¹³C 2D HSQC NMR analysis of **18** permitted the assignment of all the sugar anomeric protons and anomeric carbons, as well as all the amino acid residues in the product (Figure S9, supporting information). These experiment data confirmed that the product obtained was the expected full-size, sialylated and core-fucosylated CD52 antigen.

Conclusions

We have demonstrated that the GH family 18 *Flavobacterium meningosepticum* endoglycosidases Endo-F2 and Endo-F3 possess significant transglycosylation activities and are able to specifically glycosylate α-1,6-fucosylated GlcNAc-peptide by using sugar oxazoline as donor substrate. The novel activity of Endo-F2 and Endo-F3 was successfully applied for a convergent chemoenzymatic synthesis of a full-size CD52 glycopeptide antigen carrying both terminal sialic acid and core fucose. This is the first discovery of transglycosylation activity of Endo-F1, Endo-F2 and Endo-F3 that show distinct acceptor substrate specificity. This study also represents the first report on endoglycosidases capable of taking core-fucosylated GlcNAc-peptides for transglycosylation. In addition to the

usefulness for complex glycopeptide synthesis, the unusual transglycosylation activity discovered may be particularly useful for glycosylation remodelling of monoclonal antibodies that usually carry core fucosylated N-glycans at the Fc domain but can be specifically de-glycosylated by an endoenzyme to provide a core-fucosylated GlcNAc acceptor at the glycosylation site. Work along this line is currently undergoing in our laboratory and will be reported in due course.

Experimental Section

Materials

The Fmoc-protected amino acids and Fmoc-Ser(tBu)-O-TGT resin were purchased from Novabiochem Corporation (San Diego, CA). 2-(1-*H*-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was purchased from GenScript Corp (Piscataway, NJ). Diisopropylethylamine (DIPEA) was purchased from Applied Biosystems (Carlsbad, CA). Piperidine (20% in DMF) was purchased from American Bioanalytical (Natick, MA). *N,N*-Dimethylformamide sequencing grade was purchased from Fisher Biotech (Pittsburgh, PA). Acetonitrile HPLC grade was purchased from Fisher Scientific (Pittsburgh, PA). 3,4,6-Tri-*O*-acetyl-1 β -azido-GlcNAc (**1**) was synthesized following reference.^[23] 2,3,4-Tri-*O*-acetyl- α -L-fucosyl iodide (**4**) was synthesized by reported method.^[24] Man₃GlcNAc oxazoline (**10**) was synthesized as reported.^[10] The bi-antennary complex-type sialoglycan oxazoline (**12**) was prepared following the reported procedure.^[14] Fmoc-Asn(GlcNAc)-OH (**14**) was prepared by the reported procedure.^[35] Endo F1, Endo F2, and Endo F3 were purchased from CalBioChem (San Diego, CA). Endo H was purchased from New England Biolabs (Ipswich, MA). Endo D was purchased from United States Biological (Swampscott, MA). Endo-A was overproduced in *E. coli* and purified following the previously reported procedure,^[36] using the plasmid pGEX-2T/Endo-A that was kindly provided by Prof. Kaoru Takegawa. Endo-M was overproduced according to the previously reported method^[12]. PNGase F was purchased from New England Biolabs (Ipswich, MA). All other reagents were purchased from Sigma/Aldrich and used as received.

High-performance liquid chromatography (HPLC)

Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300™ C₁₈ column (5.0 μ m, 4.6 $\times$ 250 mm) or a XBridge™ BEH130 C₁₈ column (3.5 μ m, 4.6 $\times$ 250 mm) at 40 °C. The Symmetry300 column was eluted with a linear gradient of 0–90% MeCN containing 0.1% TFA within 30 min at a flow rate of 1 mL/min (*Method A*). The XBridge column was eluted with a linear gradient of 0–20% MeCN containing 0.1% TFA within 30 min at a flow rate of 0.5 mL/min (*Method B*). Preparative HPLC was performed on a Waters 600 HPLC instrument with a preparative Symmetry300™ C₁₈ column (7.0 μ m, 19 $\times$ 250 mm) or a XBridge™ Prep ShieldRP18 column (5.0 μ m, 10 $\times$ 250 mm). These columns were eluted with a suitable gradient of aqueous acetonitrile containing 0.1% TFA at a flow rate of 12 mL/min (for Symmetry300 column) or 4 mL/min (for XBridge column).

Nuclear magnetic resonance (NMR)

The ¹H NMR spectra were measured on JEOL ECX 400 MHz or Inova 500 MHz or Bruker DRX 500 MHz NMR spectrometers. All chemical shifts were assigned in ppm. The ¹³C NMR was measured at 125 MHz.

Mass spectrometry (MS)

The ESI-MS Spectra were measured on a Waters Micromass ZQ-4000 single quadrupole mass spectrometer. MALDI-TOF MS measurement was performed on an Autoflex II

MALDI-TOF mass spectrometer (Bruker Daltonics). The instrument was calibrated by using ProteoMass Peptide MALDI-MS calibration kit (MSCAL2, Sigma/Aldrich). The matrix used for glycans was 2,5-dihydroxybenzoic acid (DHB) and/or alpha-cyano-4-hydroxycinnamic acid (ACHA) (10 mg/mL in 50% acetonitrile containing 0.1% trifluoroacetic acid). The measuring conditions: 337 nm nitrogen laser with 100 μ J output; laser frequency 50.0 Hz; laser power 30–45%; linear mode; positive polarity; detection range 500–5000; pulsed ion extraction: 70ns; high voltage: on; realtime smooth: high; shots: 500–2000.

2-Acetamido-3,4-di-O-benzoyl-2-deoxy-6-O-trityl- β -D-glucopyranosyl azide (2)

3,4,6-Tri-*O*-acetyl-1 β -azido-GlcNAc (**1**) (610 mg, 1.64 mmol) was dissolved in anhydrous MeOH (15 mL) and NaOMe (wt. 25% in MeOH) (2.05 mL, 35.8 mmol) was added. The reaction mixture was stirred for 2 hr under argon. Then it was treated with Dowex 50WX8-100 ion-exchange resin H^+ form and filtered. The filtrate was concentrated to dryness under vacuum to give the crude β -glycosyl azide.

A mixture of the crude glycosyl azide, pyridine (10 mL) and trityl chloride (0.91 g, 3.25 mmol) was stirred at 60 $^{\circ}C$ overnight. Then it was cooled to rt and benzoyl chloride (0.47 mL, 4.06 mmol) was added. The solution was continued to stir at rt overnight. The residue was diluted with EtOAc and washed with 1N HCl, saturated $NaHCO_3$, water, and brine. The organic layer was dried over Na_2SO_4 and concentrated. The syrup was subject to a silica gel column chromatography (EtOAc/hexane, 1:2, v/v) to give product **2** as a white foam (810 mg, 70%, three steps). 1H NMR (400 MHz, $CDCl_3$) δ 7.91 (dd, J = 8.24, 0.92 Hz, 2H), 7.65 (dd, J = 8.24, 1.36 Hz, 2H), 7.32 – 7.49 (m, 11H), 7.08 – 7.16 (m, 10H), 5.84 (d, J = 8.72 Hz, 1H), 5.72 (dd, J = 10.08, 9.6 Hz, 1H), 5.53 (dd, J = 10.56, 10.52 Hz, 1H), 4.78 (d, J = 9.16 Hz, 1H), 4.30 (dd, J = 10.52, 9.16 Hz, 1H), 3.89 (m, 1H), 3.38 (dd, J = 10.56, 2.28 Hz, 1H), 3.16 (dd, J = 10.52, 4.12 Hz, 1H), 1.88 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.62, 167.17, 164.69, 143.52, 133.77, 133.24, 130.06, 129.66, 129.11, 128.65, 128.31, 127.85, 127.02, 88.59, 86.79, 73.29, 68.75, 62.06, 54.67, 23.35

2-Acetamido-3,4-di-O-benzoyl-2-deoxy- β -D-glucopyranosyl azide (3)

A solution of compound **2** (810 mg, 1.16 mmol) in 80% aqueous AcOH (40 mL) was stirred at 60 $^{\circ}C$ overnight. Then it was concentrated under vacuum. The residue was diluted with EtOAc and washed with water and brine. The organic layer was dried over Na_2SO_4 and concentrated. The resulted syrup was subject to silica gel column chromatography (EtOAc/hexane, 1:1, v/v) to give product **3** as a white foam (410 mg, 78%). 1H NMR (400 MHz, $CDCl_3$) δ 7.89 – 7.92 (m, 4H), 7.48 – 7.52 (m, 2H), 7.33 – 7.37 (m, 4H), 6.02 (d, J = 9.16 Hz, 1H), 5.78 (dd, J = 10.56, 9.16 Hz, 1H), 5.50 (dd, J = 10.08, 9.64 Hz, 1H), 4.97 (d, J = 9.16 Hz, 1H), 4.17 (dt, J = 10.52, 9.16 Hz, 1H), 3.84 – 3.89 (m, 2H), 3.73 (dd, J = 12.84, 4.6 Hz, 1H), 1.89 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.76, 166.80, 166.01, 133.86, 133.80, 129.94, 128.63, 128.54, 88.76, 77.43, 72.55, 69.08, 61.31, 54.61, 23.29.

2-Acetamido-3,4-di-O-benzoyl-2-deoxy-6-O-(α -L-fucopyranosyl)- β -D-glucopyranosyl azide (5)

To a stirring solution of tetra-*O*-trimethylsilyl- α -L-fucopyranose (1.23 g, 2.72 mmol) in anhydrous CH_2Cl_2 (8 mL) was added iodotrimethylsilane (0.37 mL, 2.72 mmol). The mixture was stirred at rt for 20 min to give the 2,3,4-Tri-*O*-acetyl- α -L-fucopyranosyl iodide (**4**) *in situ*. Then, a solution of compound **3** (0.62 g, 1.34 mmol) and 2,6-di-*tert*-butylpyridine (0.6 mL, 2.72 mmol) in anhydrous CH_2Cl_2 (7 mL) was added to the reaction solution and the resulting mixture was stirred at rt for 5 h. MeOH (5 mL) was added and the resulting mixture was stirred for 20 min to remove the TMS group. Then it was treated with Dowex 1X2-100 ion-exchange resin (OH^- form). And filtered. The filtrate was concentrated under

vacuum and the residue was subject to a silica gel column chromatography (CH₂Cl₂/MeOH, 15:1, v/v) to provide compound **5** as a white solid (0.50 g, 61%). ¹H NMR (400 MHz, CDCl₃) δ 7.86 – 7.89 (m, 4H), 7.48 (dt, *J* = 7.32, 1.36 Hz, 2H), 7.25 – 7.34 (m, 4H), 6.30 (d, *J* = 8.72 Hz, 1H), 5.68 – 5.75 (m, 2H), 4.93 (d, *J* = 9.64 Hz, 1H), 4.82 (d, *J* = 3.68 Hz, 1H), 4.22 (dd, *J* = 10.08, 9.16 Hz, 1H), 4.09 (dd, *J* = 13.28, 6.4 Hz, 1H), 3.94 – 4.04 (m, 2H), 3.78 – 3.84 (m, 2H), 3.58 (dd, *J* = 11.48, 3.68 Hz, 1H), 3.46 (broad s, 1H), 3.10 (d, *J* = 10.52 Hz, 1H), 2.90 (broad s, 1H), 1.87 (s, 3H), 1.26 (d, *J* = 6.44 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 170.96, 166.83, 165.86, 133.98, 133.84, 129.92, 128.65, 128.50, 128.46, 98.44, 88.44, 75.10, 72.64, 71.76, 71.51, 69.49, 68.58, 66.45, 65.05, 54.5, 23.24, 16.20.

2-Acetamido-3,4-di-O-acetyl-2-deoxy-6-O-(tri-O-acetyl-α-L-fucopyranosyl)-β-D-glucopyranosyl azide (6)

A solution of compound **5** (0.50 g, 0.83 mmol) and NaOMe in MeOH (0.5 M, 50 μL, 50 μmol) in anhydrous MeOH/THF (1/1, v/v, 20 mL) was stirred at rt under argon for 1 h. The reaction mixture was treated with Dowex 50WX8-100 ion-exchange resin (H⁺ form) and filtered. The filtrate was concentrated to dryness under vacuum. Pyridine (5 mL) and Ac₂O (5 mL) were added to the residue and the mixture was stirred at rt overnight. Then it was diluted with EtOAc and washed with saturated NaHCO₃, water, and brine. The organic layer was dried over Na₂SO₄ and concentrated under vacuum. The residue was subject to silica gel column chromatography using EtOAc as the eluent to give compound **6** as a white solid (0.46 g, 92%). ¹H NMR (400 MHz, CDCl₃) δ 5.72 (d, *J* = 8.68 Hz, 1H), 5.28 – 5.33 (m, 2H), 5.24 (dd, *J* = 10.52, 9.16 Hz, 1H), 5.03 – 5.10 (m, 3H), 4.75 (d, *J* = 9.64 Hz, 1H), 4.16 (dt, *J* = 6.88, 6.4 Hz, 1H), 3.86 (dt, *J* = 10.52, 9.2 Hz, 1H), 3.71 – 3.79 (m, 2H), 3.51 (dd, *J* = 11.48, 5.04 Hz, 1H), 2.14 (s, 3H), 2.08 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H), 1.97 (s, 3H), 1.96 (s, 3H), 1.12 (d, *J* = 6.4 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 171.07, 170.80, 170.70, 170.49, 170.12, 169.22, 96.76, 88.17, 75.07, 72.33, 71.11, 68.59, 68.04, 68.00, 66.36, 64.64, 54.33, 23.34, 20.82, 20.79, 20.72 (2C), 20.70, 15.96.

N^ω-(2-Acetamido-3,4-di-O-acetyl-2-deoxy-6-O-(tri-O-acetyl-α-L-fucopyranosyl))-N^α-(9-fluorenylmethyloxycarbonyl)-L-asparagine *tert*-butyl ester (7)

To a solution of compound **6** (0.46 g, 0.76 mmol) in THF (10 mL) was slowly added trimethylphosphine in toluene (1.0 M, 1.15 mL, 1.15 mmol) and the mixture was stirred at rt for 2 h until TLC showed the completion of the reaction. The solvent was removed under vacuum and the residue was dissolved in DMF (2 mL). Then, N^α-(9-Fluorenylmethyloxycarbonyl)-L-aspartic acid α-*tert*-butyl ester (350 mg, 0.85 mmol), HATU (0.5 M in DMF, 4.5 mL, 2.25 mmol), and DIPEA (1.0 M in DMF, 3.0 mL, 3.0 mmol) were added. The resulting mixture was stirred at rt overnight under argon. The reaction solution was diluted with EtOAc and washed with water and brine. The organic layer was dried over Na₂SO₄ and concentrated to dryness under vacuum. The residue was subject to silica gel column chromatography using EtOAc as the eluent to give compound **7** as a white solid (570 mg, 77%, two steps). ¹H NMR (400 MHz, CDCl₃) δ 7.73 (d, *J* = 7.8 Hz, 2H), 7.63 (t, *J* = 7.32 Hz, 2H), 7.36 (t, *J* = 7.8 Hz, 2H), 7.26 – 7.31 (m, 2H), 7.15 (broad d, *J* = 8.24 Hz, 1H), 6.35 (d, *J* = 9.16 Hz, 1H), 5.93 (d, *J* = 7.76 Hz, 1H), 5.34 (dd, *J* = 11, 3.64 Hz, 1H), 5.26 (d, *J* = 2.72 Hz, 1H), 5.16 (dd, *J* = 10.96, 3.68 Hz, 1H), 4.98 – 5.06 (m, 4H), 4.96 (dd, *J* = 9.6, 8.24 Hz, 1H), 4.53 (m, 1H), 4.37 (dd, *J* = 10.52, 7.8 Hz, 1H), 4.32 (dd, *J* = 10.08, 7.36 Hz, 1H), 4.24 (t, *J* = 7.32 Hz, 1H), 4.06 (dt, *J* = 9.6, 8.24 Hz, 1H), 3.68 (m, 1H), 3.52 (dd, *J* = 11.92, 5.04 Hz, 1H), 2.87 (dd, *J* = 16.52, 4.6 Hz, 1H), 2.68 (dd, *J* = 16.48, 3.64 Hz, 1H), 2.12 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.96 (s, 9H), 1.93 (s, 3H), 1.42 (s, 9H), 1.07 (d, *J* = 6.44 Hz, 3H). ¹³C NMR (100 MHz, CDCl₃) δ 172.32, 172.11, 171.17, 170.93, 170.71, 170.25, 170.10, 169.32, 156.56, 144.10, 141.31, 127.69, 127.19, 127.14, 125.46, 125.42, 119.98, 96.84, 82.00, 75.25, 73.13, 71.15, 68.30, 68.02, 67.79, 67.25, 65.47, 64.66,

53.44, 50.94, 47.19, 38.69, 38.03, 27.99 (3C), 23.16, 20.89, 20.81, 20.79, 20.72, 20.56, 15.92.

N^w-(2-Acetamido-3,4-di-O-acetyl-2-deoxy-6-O-(tri-O-acetyl- α -L-fucopyranosyl)-N^α-(9-fluorenylmethyloxycarbonyl)-L-asparagine (8)

Compound **7** (100 mg, 0.1 mmol) was dissolved in a solution of TFA in CH₂Cl₂ (20%, 2 mL) and the mixture was stirred at room temperature for 1h. The residue was co-evaporated with toluene (3 mL) under vacuum and the resulted white solid was washed with Et₂O (5 mL) to give the pure compound **8** (90 mg, 95%). ¹H NMR (400 MHz, CDCl₃) δ 8.58 (d, *J* = 9.2 Hz, 1H, 1-NH of GlcNAc), 7.84 (m, 3H, 2-NHAc of GlcNAc, Ar-H), 7.68 (d, *J* = 7.6 Hz, 2H, Ar-H), 7.45 (d, *J* = 8.8 Hz, 1H, FmocNH), 7.38 (m, 2H, Ar-H), 7.28 (m, 2H, Ar-H), 5.19-5.15 (m, 3H, H1 of GlcNAc, H3 and H4 of Fuc), 5.03 (t, *J* = 9.6 Hz, 1H, H3 of GlcNAc), 4.91-4.80 (m, 3H, H1 and H2 of Fuc, H4 of GlcNAc), 4.34 (dd, *J* = 7.2, 13.2 Hz, α H of Asn), 4.26-4.15 (m, 4H, H5 of Fuc, CH₂ and H9 of Fmoc), 3.86 (q, *J* = 9.6 Hz, 1H, H2 of GlcNAc), 3.66 (m, 2H, H5 and H6a of GlcNAc), 3.45 (m, 1H, H6b of GlcNAc), 2.65-2.49 (m, 2H, β H of Asn), 2.06 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.93 (s, 3H, Ac), 1.90 (s, 3H, Ac), 1.86 (s, 3H, Ac), 1.68 (s, 3H, NHAc), 0.98 (d, *J* = 6.8 Hz, 1H, CH₃ of Fuc); ¹³C NMR (100 MHz, CDCl₃) δ 173.5, 170.8, 170.6, 170.3, 170.2, 170.1, 169.9, 169.7, 156.3, 144.3, 141.2, 128.1, 127.6, 125.8, 120.6, 96.5, 78.5, 74.2, 74.0, 71.1, 69.2, 67.9, 67.6, 66.4, 66.2, 64.5, 52.5, 50.5, 47.1, 37.4, 23.1, 21.0, 20.9, 20.8, 16.0. ESI-MS: calculated for C₄₃H₅₁N₃O₁₉, *M* = 913.31, found, 914.47 [M+H]⁺.

N^w-(2-Acetamido-6-O-(α -L-fucopyranosyl)-N^α-(9-fluorenylmethyloxycarbonyl)-L-asparagine (9)

A mixture of compound **8** (90 mg, 0.1 mmol) and K₂CO₃ (87 mg, 0.63 mmol) in MeOH/THF (3/1, v/v, 4 mL) was stirred at rt. The reaction was carefully monitored by ESI-MS until all the acetyl groups were selectively removed. Then a HCl solution (1N) was added to neutralize the solution to pH 2–3. The residue was subject to a preparative HPLC to give compound **9** as a white solid (60 mg, 85%). ¹H NMR (400 MHz, DMSO-*d*₆ + 1% D₂O) δ 8.20 (broad d, *J* = 9.16 Hz, 1H), 7.85 (d, *J* = 7.8 Hz, 2H), 7.67 (d, *J* = 5.92 Hz, 2H), 7.39 (t, *J* = 7.8 Hz, 2H), 7.30 (t, *J* = 7.32 Hz, 2H), 4.80 (dd, *J* = 9.2, 6.44 Hz, 1H), 4.59 (d, *J* = 3.2 Hz, 1H), 4.31 (dd, *J* = 7.8, 5.52 Hz, 1H), 4.15 – 4.25 (m, 3H), 3.83 (dd, *J* = 13.28, 6.4 Hz, 1H), 3.44 – 3.54 (m, 6H), 3.30 (dd, *J* = 9.64, 9.16 Hz, 2H), 3.09 (t, *J* = 9.16 Hz, 1H), 2.57 (dd, *J* = 16.04, 5.04 Hz, 1H), 2.44 (dd, *J* = 16.04, 7.32 Hz, 1H), 1.74 (s, 3H), 1.03 (d, *J* = 6.88 Hz, 3H). ¹³C NMR (100 MHz, DMSO-*d*₆ + 1% D₂O) δ 173.38, 171.71, 170.33, 156.56, 144.10, 144.05, 141.12, 128.33, 127.75, 125.70, 120.61, 100.03, 78.96, 77.33, 74.29, 71.95, 70.58, 69.91, 68.51, 67.95, 66.46, 66.38, 54.82, 50.5, 46.98, 37.19, 23.03, 16.81. ESI-MS: calculated for C₃₃H₄₁N₃O₁₄, *M* = 703.25, found, 702.31 [M-H][−].

Chemoenzymatic transglycosylation assay of various endoglycosidases using Man₃GlcNAc oxazoline (10**) as donor substrate and Fmoc-Asn(Fuc α 1,6GlcNAc)-OH (**9**) as acceptor substrate**

A mixture of acceptor **9** (25 μ g, 36 nmol) and Man₃GlcNAc oxazoline (**10**) (100 μ g, 145 nmol) in a phosphate buffer (50 mM, pH 7.0, 12 μ L) was incubated at 30 °C with individual Endo- β -N-acetylglucosaminidase (4 μ g). The reaction was monitored by analytic HPLC by taking reaction aliquots at 10 min, 20 min, 30 min, 1 h, 2 h, 4h and 20 h. The yields were calculated based on the peak area of product and starting material acceptor. seven ENGases, including Endo-F1, Endo-F2, Endo-F3, Endo-A, Endo-M, Endo-D, and Endo-H, were measured for their transglycosylation activity in this assay. The time-course was shown in Figure 1. Panel A. The product (**11**) was purified by RP-HPLC and confirmed by MS analysis. Fmoc-Asn(Man₃GlcNAc₂Fuc)-OH (**11**): t_R = 16.8 min (analytical HPLC, method

A). MALDI-TOF MS: Calculated for $C_{59}H_{84}N_4O_{34}$, $M = 1392.50$; found, 1416.12 $[M + Na]^+$; ESI-MS: found 1393.58 $[M + H]^+$, 1247.60 $[M - Fuc + H]^+$, 1085.43 $[M - Fuc - Man + H]^+$, 923.45 $[M - Fuc - 2Man + H]^+$, 761.39 $[M - Fuc - 3Man + H]^+$.

Endo-D digestion of compound 11

A solution of **11** (50 μ g) in a phosphate buffer (50 mM, pH 7.0, 20 μ L) was incubated with Endo-D (1 μ g) at 30 °C for 2 h. The digestion was monitored by RP-HPLC combined with ESI-MS analysis. Endo-D digestion resulted in complete hydrolysis of **11** and the formation of Fmoc-Asn(Fuc α 1,6GlcNAc)-OH which was confirmed by ESI-MS analysis. This digestion data validated the β 1,4-linkage of the *N,N'*-diacetylchitobiose unit in compound **11**.

Chemoenzymatic transglycosylation assay of various Endoglycosidases using complex-type sialoglycan oxazoline (**12**) as donor substrate and Fmoc-Asn(Fuc α 1,6GlcNAc)-OH (**9**) as acceptor substrate

A mixture of acceptor **9** (25 μ g, 36 nmol) and sialoglycan oxazoline **12** (290 μ g, 145 nmol) in phosphate buffer (50 mM, pH 7.0, 12 μ L) was incubated at 30 °C with individual endo- β -N-acetylglucosaminidase (4 μ g). The reaction was monitored by analytic HPLC by taking reaction aliquots at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 20 h. The yields were calculated based on the peak area of product and starting material acceptor. Seven ENGases, including Endo-F1, Endo-F2, Endo-F3, Endo-A, Endo-M, Endo-D, and Endo-H, were measured for their transglycosylation activity in this assay. The time-course was shown in Figure 1. Panel B. The product (**13**) was purified and confirmed by MS analysis. Fmoc-Asn(Sia $_2$ Gal $_2$ GlcNAc $_2$ Man $_3$ GlcNAc $_2$ Fuc)-OH (**13**): $t_R = 16.3$ min (analytical HPLC, method A). MALDI-TOF MS: Calculated for $C_{107}H_{161}N_7O_{69}$, $M = 2704.95$; found, 2728.48 $[M + Na]^+$; ESI-MS: found 1353.98 $[M + 2H]^{2+}$, 1208.19 $[M - Sia + 2H]^{2+}$.

Endo-F3 digestion of compound 13

A solution of **13** (10 μ g) in a phosphate buffer (50 mM, pH 7.0, 20 μ L) was incubated with Endo-F3 (2 μ g) at 30 °C for 20 h. The digestion was monitored by RP-HPLC combined with ESI-MS analysis. The Endo-F3 digestion led to complete hydrolysis of **13** and the formation of the expected complex type N-glycan and the Fmoc-Asn(Fuc α 1,6GlcNAc)-OH as confirmed by MS analysis.

Chemoenzymatic transglycosylation assay of various endoglycosidases using Man $_3$ GlcNAc oxazoline (**10**) as donor substrate and Fmoc-Asn(GlcNAc)-OH (**14**) as acceptor substrate

A mixture of acceptor **14** (20 μ g, 36 nmol) and Man $_3$ GlcNAc oxazoline (100 μ g, 145 nmol) in phosphate buffer (50 mM, pH 7.0, 12 μ L) was incubated at 30 °C with individual Endo- β -N-acetylglucosaminidase (4 μ g). The reaction was monitored by analytic HPLC by taking reaction aliquots at 10 min, 20 min, 30 min, 1 h, 2 h, 4 h and 20 h. The yields were calculated based on the peak area of product and starting material acceptor. The time-course was shown in Figure 2. The product was purified by RP-HPLC to give Fmoc-Asn(Man $_3$ GlcNAc $_2$)-OH (**15**): $t_R = 17.0$ min (analytical HPLC, method A). MALDI-TOF MS: Calculated for $C_{53}H_{74}N_4O_{30}$, $M = 1246.44$; found, 1270.25 $[M + Na]^+$; ESI-MS: found 1247.68 $[M + H]^+$.

Endo-M digestion of compound 15

A solution of **15** (10 μ g) in a phosphate buffer (50 mM, pH 7.0, 20 μ L) was incubated with Endo-M (1 μ g) at 30 °C for 2 h. The digestion was monitored by RP-HPLC combined with

ESI-MS analysis. The Endo-M digestion of compound **15** gave the glycan Man₃GlcNAc and the Fmoc-Asn(GlcNAc)-OH as confirmed by MS analysis.

Solid-phase synthesis of the CD52 peptide bearing Fuc α 1-6GlcNAc (**17**)

The CD52 peptide was synthesized manually by the Fmoc-chemistry using Fmoc-protected amino acid derivatives. An O-link TGT resin (Novabiochem Corp) was used as the solid support, in which the first amino acid (Ser) was attached through the acid-labile ester linkage. To introduce a Fuc α 1-6GlcNAc residue at the N-glycosylation site, Fmoc-Asn(Ac₅Fuc α 1-6GlcNAc)-OH (**8**) was used as building blocks to replace the residues at Asn-3 in the solid-phase peptide synthesis. HATU (0.5 M in DMF) and DIPEA (1.0 M in DMF) (1:1, v/v) were used as the coupling activator and piperidine (20% in DMF) was used as the deblocking reagent. Synthesis was carried out on a 10 μ mol scale and 4-fold excess of Fmoc-protected building blocks were used for each coupling reaction cycle. The N-terminus amino group was protected with acetyl group by treatment with Ac₂O/DIPEA. The resulted resin (**16**) was cleaved by treatment with TFA/CH₂Cl₂ (1/4, v/v) at r.t. for 5 h to release the crude peptide and simultaneously remove the protecting groups of Trt and t-Bu. The Ac groups on the disaccharide were removed by treatment with 5% aqueous hydrazine at rt for 1h. The residue was subject to preparative HPLC purification to give the glycopeptide Fuc α 1-6GlcNAc-CD52 (**17**) as a white powder (14.4 mg, overall yield 90%). Analytical HPLC of **17** (*Method B*): t_R = 23.8 min; ¹H NMR (D₂O, 400 MHz): δ 4.91 (d, J = 9.6 Hz, 1H, H1 of GlcNAc), 4.76 (d, J = 3.6 Hz, 1H, H1 of Fuc), 4.71-4.58 (m, 3H, α H of Ser, α H of Asp, α H of Asn), 4.39-4.25 (m, 7H, α H of Ser x 3, α H of Pro, α H of Gln x 2, α H of Thr), 4.19-4.10 (m, 3H, α H of Thr, β H of Thr x 2), 3.99 (q, J = 6.8 Hz, 1H, H5 of Fuc), 3.86-3.60 (m, 18H), 3.52-3.38 (m, 3H), 2.82-2.64 (m, 4H, β H of Asn x 2, β H of Asp x 2), 2.27-2.15 (m, 5H, Gln-H, Pro-H), 2.08-1.82 (m, 13H, Ac, Gln-H, Pro-H), 1.08 (m, 9H, Thr-CH₃ x 2, Fuc-CH₃); ¹H-¹³C HSQC NMR (¹³C 125 MHz, ¹H 500 MHz, D₂O) δ 4.91/78.4 (GlcNAc-1), 4.76/99.2 (Fuc-1), 4.69/53.1 (Ser- α), 4.68/49.8 (Asp α), 4.60/49.6 (Asn- α), 4.39/56.1 (Ser- α), 4.35/61.8 (Pro- α), 4.31/56.3 (Ser- α), 4.30/56.0 (Gln- α), 4.28/53.3 (Gln- α), 4.26/58.9 (Ser- α), 4.25/59.1 (Thr- α), 4.18/53.5 (Thr- α), 4.16/67.8 (Thr- β), 4.13/67.9 (Thr- β), 3.99/67.5 (Fuc-5), 3.85/68.3 (GlcNAc-6), 3.64/68.3 (GlcNAc-6), 2.80/35.8 (Asn- β), 2.74/36.4 (Asp- β), 2.25/31.4 (Gln- β), 2.21/30.7 (Pro), 2.02/22.1 (Ac), 1.95/22.8 (Ac), 1.89/30.6 (Pro), 1.83/27.2 (Ac), 1.10/16.2 (Thr-CH₃), 1.08/19.7 (Fuc-CH₃), 1.06/16.2 (Thr-CH₃); MALDI-TOF MS: Calculated for C₆₁H₉₈N₁₆O₃₄, M = 1598.64; found, 1622.32 [M + Na]⁺; ESI-MS: 1599.16 [M + H]⁺, 800.27 [M + 2H]²⁺.

Chemoenzymatic synthesis of sialylated and core-fucosylated complex-type glycoform of CD52 (**18**)

A mixture of CD52 peptide **17** (2 mg, 1.25 μ mol) and sialoglycan oxazoline **12** (5 mg, 2.5 μ mol) in a phosphate buffer (50 mM, pH 7.0, 250 μ L) was incubated at 30 °C with Endo-F3 (80 μ g) for 1h. The reaction was quenched with 1% TFA (10 μ L) and the residue was subject to RP-HPLC to give Sia₂Gal₂GlcNAc₂Man₃(Fuc)GlcNAc₂-CD52 (**18**) as a white powder (2.5 mg, 55%). t_R = 21.5 min (analytical HPLC, method B). ¹H NMR (D₂O, 400 MHz): δ 5.00 (s, 1H, H1 of Man⁴), 4.89 (d, J = 9.6 Hz, 1H, H1 of GlcNAc¹), 4.81 (s, 1H, H1 of Man^{4'}), 4.75 (s, 1H, H1 of Fuc), 4.72-4.58 (m, 4H, α H of Ser, H1 of Man³, α H of Asp, α H of Asn), 4.56 (d, J = 7.6 Hz, H1 of GlcNAc²), 4.48 (d, J = 7.2 Hz, H1 of GlcNAc⁵, H1 of GlcNAc^{5'}), 4.41-4.24 (m, 9H, α H of Ser x 3, α H of Pro, α H of Gln x 2, H1 of Gal⁶, H1 of Gal^{6'}, α H of Thr), 4.18-4.14 (m, 3H, α H of Thr, β H of Thr x 2), 4.12 (m, 1H), 4.07 (m, 1H), 3.98 (m, 2H), 3.89-3.35 (m, 82H), 2.87-2.61 (m, 4H, β H of Asn x 2, β H of Asp x 2), 2.54 (m, 2H, H_{3ax} of Sia x 2), 2.27-2.18 (m, 5H, Gln-H, Pro-H), 2.07-1.83 (m, 29H, Ac, Gln-H, Pro-H), 1.60 (t, J = 12.0 Hz, 2H, H_{3eq} of Sia x 2), 1.07 (m, 9H, Thr-CH₃ x 2, Fuc-CH₃); ¹H-¹³C HSQC NMR (¹³C 125 MHz, ¹H 500 MHz, D₂O) δ 5.00/99.6 (Man⁴-1), 4.89/78.7 (GlcNAc¹-1), 4.81/97.5 (Man^{4'}-1), 4.75/99.8 (Fuc-1), 4.68/54.4 (Ser- α), 4.66/51.3

(Asp- α), 4.65/101.3 (Man³-1), 4.59/51.5 (Asn- α), 4.56/101.7 (GlcNAc²-1), 4.48/100.1 (GlcNAc⁵-1, GlcNAc^{5'}-1), 4.38/56.3 (Ser- α), 4.35/61.5 (Pro- α), 4.31/56.8 (Ser- α), 4.31/56.3 (Gln- α), 4.30/104.4 (Gal⁶-1, Gal^{6'}-1), 4.27/53.7 (Gln- α), 4.26/59.8 (Ser- α), 4.24/60.1 (Thr- α), 4.18/54.2 (Thr- α), 4.15/67.8 (Thr- β), 4.13/67.8 (Thr- β), 3.98/67.5 (Fuc-5), 2.83/35.8 (Asn- β), 2.74/36.2 (Asp- β), 2.54/40.3 (Sia-3_{ax}), 2.25/31.3 (Gln- β), 2.02/22.3 (Ac), 1.95/22.4 (Ac), 1.87/26.8 (Ac), 1.60/40.3 (Sia-3_{eq}), 1.08/15.9 (Thr-CH₃), 1.07/19.7 (Fuc-CH₃), 1.05/15.9 (Thr-CH₃); ESI-MS: Calculated for C₁₃₇H₂₂₁N₂₁O₉₀, M = 3600.34; found 1201.66 [M + 3H]³⁺

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. The time courses of endoglycosidases-catalyzed transglycosylation on Fmoc-Asn(Fuca1,6GlcNAc)-OH (**9**) monitored by HPLC. Panel A: Man3GlcNAc oxazoline (**10**) as the donor substrate; Panel B: sialoglycan oxazoline (**12**) as the donor substrate.

Figure 2.
HPLC-monitored time courses of Endoglycosidases-catalyzed transglycosylation on Fmoc-Asn(GlcNAc)-OH using Man3GlcNAc oxazoline as the donor substrate.

Scheme 1.
Synthesis of Fmoc-Asn(Fuca 1,6GlcNAc)-OH (**9**)

Scheme 2.

Screening transglycosylation activity using the Fmoc-Asn(Fuca1,6GlcNAc)-OH (**9**) as the acceptor and synthetic sugar oxazolines (**10** and **12**) as the donor substrates

Scheme 3.

Screening transglycosylation activity using non-fucosylated GlcNAc-Asn-Fmoc (**14**) as the acceptor and Man₃GlcNAc oxazolines (**10**) as the donor substrate

Scheme 4.
Chemoenzymatic synthesis of sialylated and core-fucosylated CD52 antigen