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Glycosynthases Enable a Highly Efficient Chemoenzymatic Synthesis of *N*-Glycoproteins Carrying Intact Natural *N*-Glycans

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Abstract: Homogeneous *N*-glycoproteins carrying defined natural *N*-glycans are essential for detailed structural and functional studies. The transglycosylation activity of the endo-β-*N*-acetylglucosaminidases from *Arthrobacter protophormiae* (Endo-A) and *Mucor hiemalis* (Endo-M) holds great potential for glycoprotein synthesis, but the wild-type enzymes are not practical for making glycoproteins carrying native *N*-glycans because of their predominant activity for product hydrolysis. In this article, we report studies of two endoglycosidase-based glycosynthases, EndoM-N175A and EndoA-N171A, and their usefulness in constructing homogeneous *N*-glycoproteins carrying natural *N*-glycans. The oligosaccharide oxazoline corresponding to the biantennary complex-type *N*-glycan was synthesized and tested with the two glycosynthases. The EndoM-N175A mutant was able to efficiently transfer the complex-type glycan oxazoline to a GlcNAc peptide and GlcNAc-containing ribonuclease to form the corresponding homogeneous glycopeptide/glycoprotein. The EndoA-N171A mutant did not recognize the complex-type *N*-glycan oxazoline but could efficiently use the high-mannose-type glycan oxazoline for transglycosylation. These mutants possess the transglycosylation activity but lack the hydrolytic activity toward the product. Kinetic studies revealed that the dramatically enhanced synthetic efficiency of the EndoA-N171A mutant was due to the significantly reduced hydrolytic activity toward both the $Man_9GlcNAc$ oxazoline and the product as well as to its enhanced activity for transglycosylation. Thus, the two mutants described here represent the first endoglycosidase-based glycosynthases enabling a highly efficient synthesis of homogeneous natural *N*-glycoproteins.

Introduction

Homogeneous glycopeptides and glycoproteins carrying defined glycan structures are indispensible tools for structural and functional investigations of glycoproteins.[1] Since natural glycoproteins are usually present as heterogeneous mixtures of glycoforms, from which pure glycoforms are difficult to isolate with current techniques, synthesis has become an essential source for obtaining homogeneous materials. A number of elegant synthetic methods have been developed, as discussed in recent reviews.[1−4] For example, mild chemoselective ligation methods, including native chemical ligation and auxiliary-assisted ligation, have been designed to overcome a series of technical difficulties in synthesizing homogeneous glycopeptides and glycoproteins.[5] Meanwhile, the chemoenzymatic approach, based on enzymatic elaboration of sugar chains on free monosaccharide-tagged polypeptides or proteins, represents another major focus of research in this field.[3,4] Both glycosyltransferases and endoglycosidases have been used to elaborate sugar chains for glycopeptide/glycoprotein synthesis. The chemoenzymatic synthesis using the transglycosylation activity of endo-β-*N*-acetylglucosaminidase (ENGase) constitutes a highly conver-

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gent approach, as this endoglycosidase is able to attach a large intact oligosaccharide moiety to a preassembled GlcNAc polypeptide or GlcNAc protein *in a single step and in a regio- and stereospecific manner* without the need for any protecting groups.[4,6] To date, two ENGases have frequently been used for the chemoenzymatic synthesis: the Endo-A from *Arthrobacter protophormiae*,[7−12] which is specific for high-mannose-type *N*-glycans, and the Endo-M from *Mucor hiemalis*,[13,14]which can act on both complex-type and high-mannose-type *N*-glycans. However, a major barrier that limits the use of this enzymatic method for synthetic purposes is its low efficiency caused by the inherently low transglycosylation yield with natural donor substrates (natural *N*-glycans or *N*-glycopeptides) as well as the rapid product hydrolysis. For example, in vitro remodeling of a glycoprotein using a natural complex-type *N*-glycopeptide as the donor substrate and wild-type Endo-M as the enzyme resulted only in a low yield (<5%) of the desired glycoprotein even when a large excess (over 100-fold) of the donor substrate was used.[15] To circumvent this problem, several research groups, including ours, have recently explored synthetic sugar oxazolines, which are highly activated species mimicking the presumed oxazolinium ion intermediates generated by a substrate-assisted mechanism of catalysis, as donor substrates for glycopeptide synthesis.[16−19] Extension of this approach to the synthesis of homogeneous glycoproteins carrying a defined oligosaccharide moiety was also achieved.[19] It was demonstrated that the activated oligosaccharide oxazoline corresponding to the $Man_3GlcNAc$ tetrasaccharide core and some of its structurally modified forms were able to serve as donor substrates for transglycosylation. Notably, the resulting glycopeptides carrying the modified *N*-glycans, regarded as the "ground state", became poor substrates for hydrolysis because of the slight structural modifications. Thus, a big difference in the reactivities of the highly activated oxazoline and the ground-state product enables an efficient synthesis of the corresponding glycopeptides or glycoproteins with truncated or modified *N*-glycans. Recently, we have successfully applied this chemoenzymatic approach to the glycoengineering of human IgG1-Fc and the synthesis of an array of homogeneous glycoforms of ribonuclease B.[20,21] However, if it were to be applied to glycoproteins carrying natural, full-size *N*-glycans, rapid product hydrolysis by the wild-type enzymes would be difficult to avoid, as the natural glycoforms, once formed, would become excellent substrates for the respective ENGases.

One solution to this problem is to use glycosidase mutagenesis to create novel glycosynthases that lack activity to hydrolyze the product but are able to take a highly activated species, such as glycosyl fluoride in the opposite anomeric configuration, as the donor substrate.[22] A general approach for creating glycosynthase from glycosidase is to mutate the essential nucleophilic residue to abolish its function for catalytic hydrolysis. The ENGases of the family 85 are a class of endoglycosidases that catalyze the reaction by a presumed substrate-assisted mechanism in which the 2-acetamido group in the substrate acts as the nucleophile. Thus, the common approach for generating glycosynthases by "knocking out" the nucleophilic residue could not apply to ENGases. To address this issue, we have recently performed site-directed mutagenesis in the putative catalytic region of Endo-M and identified a novel Endo-M mutant, N175A, that became the first glycosynthase derived from the family 85 endoglycosidases.[23] It was demonstrated that this mutant was able to use a full-size, high-mannose-type glycan oxazoline ($Man_9GlcNAc$ oxazoline) as a donor substrate for transglycosylation but lacked the activity to hydrolyze the resulting natural high-mannose-type *N*-glycopeptide product, implying a great potential for this type of mutant. However, a number of questions remain to be answered: Is this Endo-M mutant applicable for synthesizing glycopeptides carrying natural, full-size complex-type *N*-glycans without hydrolysis? Can this glycosynthase approach be efficiently extended to the synthesis of intact glycoproteins carrying native *N*-glycans? Does the corresponding Endo-A mutant behave similarly? Does the mutation change the substrate specificity (high-mannose type vs complex type)? What are the kinetic features of the mutant-

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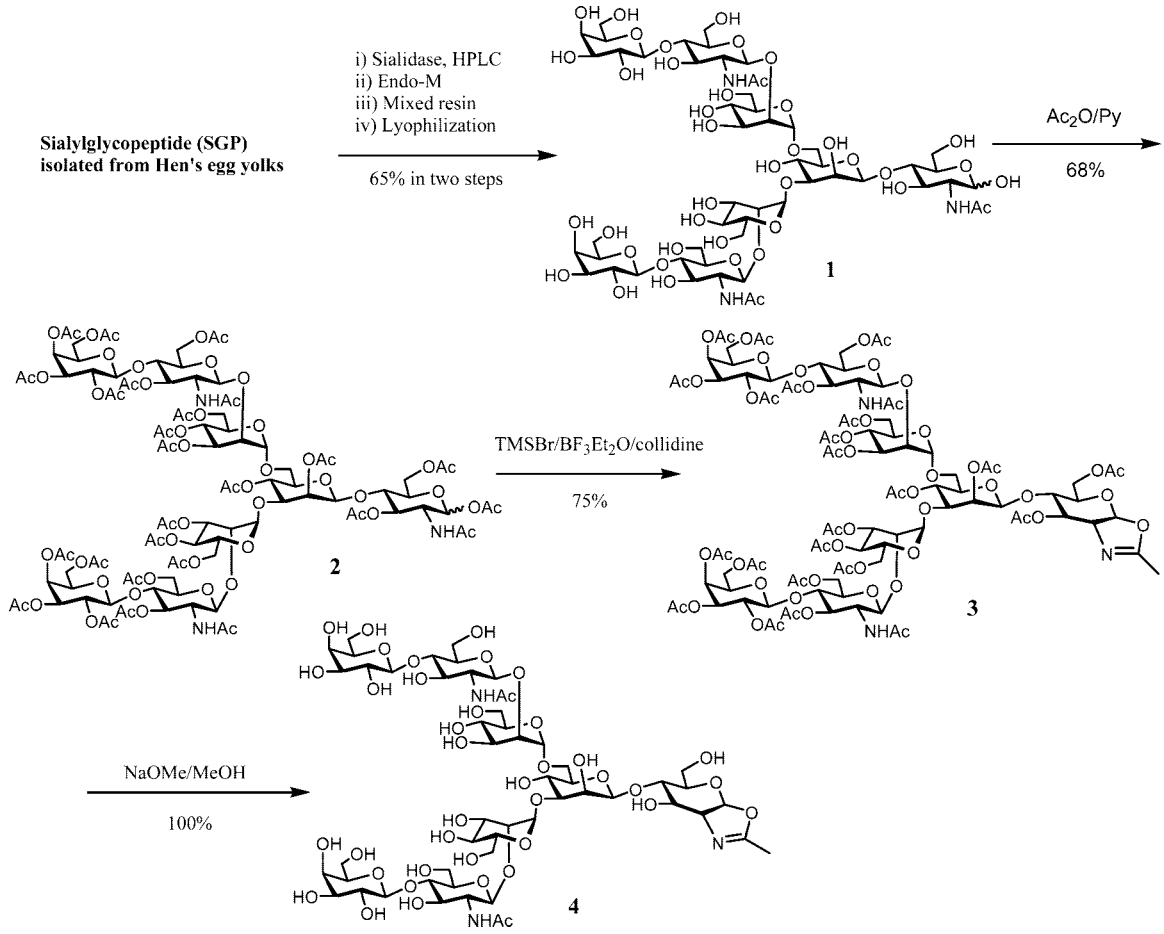
Scheme 1. Semisynthesis of the Oligosaccharide Oxazoline **4** Corresponding to the Complex-Type *N*-Glycan



catalyzed reactions? To answer these questions, we describe in this paper the synthesis of a key oligosaccharide oxazoline corresponding to the natural, biantennary complex-type *N*-glycan. The corresponding Endo-A mutant, EndoA-N171A, was generated. The substrate specificity and kinetics of the two mutant enzymes were evaluated. Our experiments have revealed that the EndoM-N175A mutant can take both the high-mannose-type and complex-type *N*-glycan oxazolines as substrates for transglycosylation without product hydrolysis but that the EndoA-N171A mutant recognizes only the high-mannose-type glycan oxazoline as the substrate for transglycosylation. Moreover, we have demonstrated that the glycosynthase approach can be efficiently applied to the synthesis of homogeneous *N*-glycoproteins carrying intact natural *N*-glycans.

Results and Discussion

Semisynthesis of the Oligosaccharide Oxazoline Corresponding to the Complex-Type *N*-Glycan. A semisynthesis of the complex-type *N*-glycan oxazoline (CT-oxazoline) was achieved using as the starting material the biantennary sialylglycopeptide (SGP), which was readily isolated from hen's egg yolk on a large scale according to previously reported procedures.[11,24] To simplify the synthesis, the sialic acid residues in SGP were removed by sialidase treatment, and this was followed by Endo-M-catalyzed hydrolysis to give the octasaccharide **1** in 65% yield in two steps. O-Acetylation of the free oligosaccharide **1** gave the peracetate **2**. Oxazoline ring formation was achieved by treatment of **2** with TMSBr and boron trifluoride in the presence of collidine, yielding the oxazoline derivative **3** in 75% yield. Finally, global de-O-acetylation with a catalytic amount of MeONa in MeOH afforded the complex-type *N*-glycan oxazoline **4** in quantitative yield (Scheme 1).

To evaluate whether the EndoM-N175A mutant can take oxazoline **4** as a donor substrate for transglycosylation, we used the GlcNAc-containing pentapeptide **5**[18] as the acceptor. The enzymatic reaction was performed in a phosphate buffer (pH 6.8) at 23 °C (3:1 donor/acceptor molar ratio). It was found that the enzymatic transglycosylation with oxazoline **4** proceeded smoothly with a steady formation of the glycopeptide product **6** (Scheme 2). After 4 h, the yield of the product reached 85% as monitored by reversed-phase HPLC (RP-HPLC). Incubation of EndoM-N175A with isolated **6** under the enzymatic reaction conditions indicated that the EndoM-N175A mutant did not hydrolyze the natural complex-type *N*-glycopeptide. These experimental data suggest that the EndoM-N175A mutant is a typical glycosynthase capable of using a highly activated species as the substrate for glycosylation but lacks the activity to hydrolyze the product. The identity of the transglycosylation product, glycopeptide **6**, was characterized by a combination of several analytic means. Deconvolution of the electrospray ionization mass spectrometry (ESI-MS) data for **6** revealed a molecular mass of 2197.32 Da, which is consistent with molecular mass of the expected adductive product (M_{calcd} = 2197.11 Da). Further characterization of the product was

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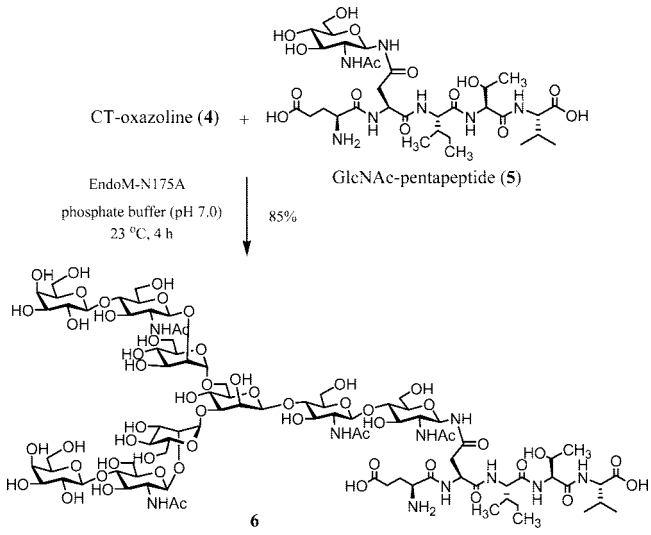
Scheme 2. Transglycosylation with the Complex-Type Glycan Oxazoline **4** by Glycosynthase EndoM-N175A



performed by Pronase digestion of **6** and subsequent analysis of the resulting Asn-linked oligosaccharide. Thus, treatment of glycopeptide **6** with Pronase gave an Asn-linked oligosaccharide that showed no difference from the authentic Asn-linked *N*-glycan (Galβ1→4GlcNAcβ1→2Manα1→6)[Galβ1→4GlcNAcβ1→2Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAcβ1-Asn prepared from the standard desialylated glycopeptide of hen's egg yolk[25] under ESI-MS and high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) analysis (Figure S4 in the Supporting Information). In addition, the ^{1}H NMR spectrum of the Asn-linked oligosaccharide obtained from Pronase digestion of glycopeptide **6** was essentially identical to that of the authentic Asn-linked glycan (Figure S5 in the Supporting Information). The anomeric proton H-1b (i.e., H-1′) of the second GlcNAc moiety appeared as a doublet at 4.58 ppm with a relatively large coupling constant ($J_{1',2'}$ = 7.5 Hz), clearly indicating that the newly formed glycosidic bond had the expected β stereochemistry. To further confirm the linkage type, we performed a detailed 2D NMR [^{1}H−^{13}C heteronuclear single-quantum correlation (HSQC) and ^{1}H−^{1}H nuclear Overhauser effect spectroscopy (NOESY)] analysis of the Asn-linked oligosaccharide obtained from glycopeptide **6** (Figure S6 in the Supporting Information). A clear NOE correlation between H-1b (the anomeric proton of the second GlcNAc) and H-4a (H-4 of the first GlcNAc) suggested that the newly generated glycosidic bond was of the β-1,4-linkage type. Taken together, these experimental data unambiguously confirmed that the mutant N175A-catalyzed transglycosylation proceeded in a regio- and stereospecific manner to form the desired *N*-glycopeptide, in which the newly generated glycosidic bond had the natural, β-1,4 glycosidic linkage.

We also examined the transglycosylation activities of wild-type Endo-M (WT-EndoM) and the Y217F mutant[23] with the newly synthesized oxazoline **4**. Both WT-EndoM and the Y217F mutant were able to use oxazoline **4** as the donor substrate for transglycosylation, and the initial transglycosylation rate for the Y217F mutant was much higher than those of WT-EndoM and the EndoM-N175A mutant (Figure 1). However, in contrast to

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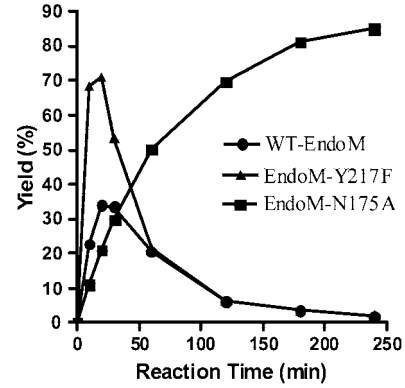


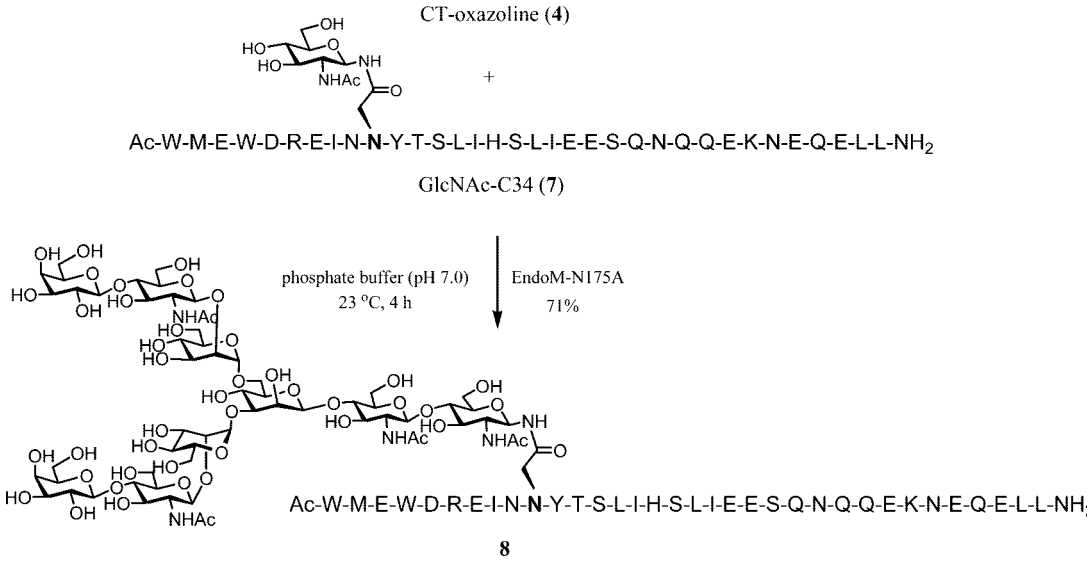
Figure 1. Transglycosylation with CT-oxazoline **4** by Endo-M and its mutants.

EndoM-N175A, which lacked the hydrolytic activity, both WT-EndoM and EndoM-Y217F gradually hydrolyzed the natural *N*-glycopeptide **6** thus produced, and after 2 h, essentially all of the glycopeptide product was hydrolyzed (Figure 1).

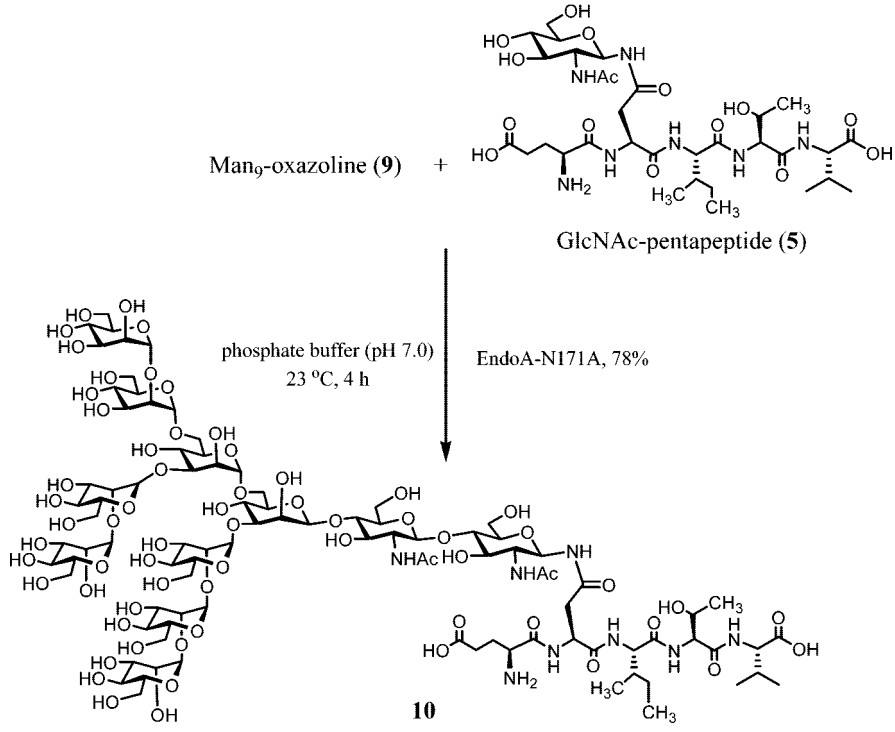
Synthesis of an HIV-1 gp41 C34 Glycopeptide Carrying a Natural Complex-Type *N*-Glycan. To demonstrate the usefulness of the glycosynthase for assembling large, natural complex-type *N*-glycopeptides, we chose as a target the HIV-1 gp41 C34 glycopeptide **7**, in which a complex-type *N*-glycan was attached at the conserved glycosylation site Asn-637 (using HIV-1 HXB2 numbering) (Scheme 3). We previously synthesized a high-mannose-type C34 glycopeptide using natural $Man_9GlcNAc_2Asn$ as the donor substrate and wild-type Endo-A as the enzyme, but that synthesis gave the product in only 12% yield under optimal transglycosylation conditions.[12] Incubation of the complex-type oxazoline **4** and presynthesized **7**[12] (5:1 donor/acceptor molar ratio) with EndoM-N175A for 4 h in a phosphate buffer (pH 7.0) containing 20% DMSO (used to increase the solubility of **7** in aqueous solution) gave the desired glycopeptide **8**, which was readily isolated by RP-HPLC in 71% yield (ESI-MS of **8**: calcd, M = 5912.62; found, 1479.62 $[M + 4H]^{4+}$, 1183.88 $[M + 5H]^{5+}$). These results indicate that the EndoM-N175A mutant is efficient for the synthesis of large complex-type *N*-glycopeptides.

Specificity of the Corresponding Endo-A Mutant Glycosynthase for the High-Mannose-Type Oxazoline Substrate. Encouraged by the promising glycosynthase activity of EndoM-N175A, we next turned our attention to Endo-A, another endo-β-*N*-acetylglucosaminidase that possesses both transglycosylation and hydrolytic activity for high-mannose-type *N*-glycans.[7,8] On the basis of sequence alignment, we used site-directed mutagenesis to create the EndoA-N171A mutant, which can be regarded as the Endo-A equivalent of EndoM-N175A. A test of the EndoA-N171A with oxazoline **4** indicated that the EndoA-N171A mutant could not recognize the complex-type sugar oxazoline **4** as a substrate for transglycosylation. It was also found that the wild-type Endo-A could not take the complex-type glycan oxazoline either. Despite the fact that the sugar oxazoline **4** is a highly activated species and the fact that Endo-A could use $Man_3GlcNAc$ oxazolines and some structurally modified oxazolines with substituents at the 4- and 6-hydroxyl groups of the outer mannose residues of the $Man_3GlcNAc$ core,[18,19] no cross-reactivity was observed for Endo-A or its N171A mutant toward the complex-type oxazoline. These results imply that the attachment of β-1,2-linked GlcNAc residues on the outer mannose residues of the $Man_3GlcNAc$ core, as in the case of the complex-type *N*-glycan oxazoline **4**, render the substrate inactive to Endo-A enzymes. As wild-type Endo-A

Scheme 3. Synthesis of a Complex-Type Glycopeptide Derived from HIV-1 gp41



Scheme 4. Transglycosylation with a High-Mannose-Type Glycan Oxazoline by Glycosynthase EndoA-N171A



can quickly hydrolyze natural high-mannose-type *N*-glycans, we next tested how the EndoA-N171A would act on natural high-mannose-type *N*-glycans and their oxazolines. It was found that EndoA-N171A was unable to hydrolyze the high-mannose-type *N*-glycan $Man_9GlcNAc_2Asn$ but that it could use synthetic $Man_9GlcNAc$ oxazoline (**9**)[23] for transglycosylation to form the corresponding natural glycopeptide **10** with high efficiency (Scheme 4). Like EndoM-N175A, the EndoA-N171A mutant lacked the activity to hydrolyze the natural glycopeptide product thus formed. Therefore, EndoA-N171A represents another endoglycosidase-based glycosynthase that is valuable for the construction of large natural *N*-glycopeptides of the high-mannose type.

While EndoM-N175A can use both complex and high-mannose-type *N*-glycan oxazolines as substrates for transglycosylation, the EndoA-N171A mutant only uses the oxazoline corresponding to the high-mannose-type *N*-glycan, indicating a more strict substrate specificity for the Endo-A mutant. During the preparation of this manuscript, Fairbanks and co-workers[26] reported two other Endo-A mutants, E173H and E173Q, that demonstrated further decreased or aborted hydrolytic activities on the $Man_3GlcNAc_2$−peptide and related products thus formed, although their specific activities for transglycosylation with corresponding sugar oxazolines were also significantly reduced in comparison with that of wild-type Endo-A. We are aware that in this reported study, a relatively large amount of enzyme as well as a prolonged reaction time was employed in order to demonstrate the difference between the wild-type and mutant

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Table 1. Kinetic Parameters for the Hydrolysis of the $Man_9GlcNAc$ Oxazoline **9** by Wild-Type Endo-A and the EndoA-N171A Mutant

	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
WT-EndoA	130	0.43	290
EndoA- N171A	0.44	2.4	0.18

Table 2. Kinetic Parameters for the Transglycosylation Activities of EndoA-N171A and EndoM-N175A

	$Man_9GlcNAc$ oxazoline (**9**)			GlcNAc pentapeptide (**5**)		
	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})	k_{cat} (min^{-1})	K_m (mM)	k_{cat}/K_m (min^{-1} mM^{-1})
EndoA-N171A	3.4	2.7	1.3	2.1	5.0	0.40
EndoM-N175A	4.5	3.1	1.4	4.0	6.3	0.63

enzymes with respect to transglycosylation efficiency/product hydrolysis. Previously, it has been shown that Endo-A could use $Man_3GlcNAc$ oxazoline and its modified forms for transglycosylation to efficiently produce homogeneous *N*-glycopeptides and *N*-glycoproteins but that the enzymatic hydrolysis of the glycopeptide products that carry the $Man_3GlcNAc_2$-core pentasaccharide and its modified forms was only marginal *when a catalytic amount of wild-type Endo-A was used for the reactions*.[17−21] It remains to be demonstrated how the E173H and E173Q mutants act on sugar oxazolines corresponding to natural, full-size high-mannose-type *N*-glycans.

Kinetic Studies with the Glycosynthases. It is known that wild-type Endo-A can use natural high-mannose-type *N*-glycans such as $Man_9GlcNAc_2Asn$ as donor substrates for transglycosylation to form natural glycopeptides, despite the relatively low synthetic yield due to Endo-A-catalyzed hydrolysis of both the starting *N*-glycan and the product.[7,8,11,12] Therefore, we were interested in seeing how wild-type Endo-A would act on $Man_9GlcNAc$ oxazoline **9**. To our surprise, incubation of **9** and the GlcNAc pentapeptide **5** (an acceptor) in the presence of wild-type Endo-A gave no trace of the transglycosylation product, except for the hydrolysis of the **9**. This result implies that wild-type Endo-A might hydrolyze the $Man_9GlcNAc$ oxazoline much faster than it performs transglycosylation. To clarify this point, we performed kinetic studies on the hydrolysis and transglycosylation of $Man_9GlcNAc$ oxazoline **9** by wild-type Endo-A and its N171A mutant. Dionex HPAEC-PAD was used to monitor and quantify the formation of the hydrolysis product $Man_9GlcNAc$.[27] It was found that the K_m of the EndoA-N171A mutant (2.4 mM) is ~6-fold larger than that of wild-type Endo-A (K_m = 0.43 mM) for the substrate **9**. More significantly, the catalytic constant for the N171A mutant (k_{cat} = 0.44 min^{-1}) is 300-fold smaller than that of the wild type (k_{cat} = 130 min^{-1}). The specificity constant (k_{cat}/K_m) for N171A (0.18 min^{-1} mM^{-1}) for $Man_9GlcNAc$ oxazoline hydrolysis is thus ~1600-fold smaller than that for wild-type Endo-A (290 min^{-1} mM^{-1}) (Table 1). Taken together, these data indicate that the N-to-A mutation at position 171 dramatically diminishes the ability of the enzyme to hydrolyze the $Man_9GlcNAc$ oxazoline substrate, thus eliminating a key detrimental property of wild-type Endo-A in terms of its synthetic potential.

We next sought to determine the kinetic parameters for the mutant N171A-catalyzed transglycosylation with oxazoline **9** and the acceptor **5** (Table 2). Interestingly, while the K_m of **9** for the transglycosylation (2.7 mM) was found to be about the same as that for the hydrolysis reaction (2.4 mM), the k_{cat} for the oxazoline transglycosylation (3.4 min^{-1}) was found to be ~8-fold larger than that of the oxazoline hydrolysis (0.44 min^{-1}). These data suggest that while the affinity of N171A for the oxazoline substrate is independent of the presence or absence of the acceptor, the presence of a suitable acceptor (such as **5**) significantly promotes the reaction of the oxazoline to form the transglycosylation product. It is likely that the N171A mutation results in a diminished affinity of the enzyme for the nucleophilic water molecule at the catalytic site (as reflected by the diminished hydrolytic activity of the N171A mutant toward the oxazoline) and meanwhile leads to an enhanced affinity of the enzyme for the GlcNAc peptide acceptor for transglycosylation. Unfortunately, we could not accurately measure the kinetic parameters for the transglycosylation by wild-type Endo-A for comparison, because of the rapid hydrolysis of the donor substrate **9** as well as the presumed rapid in situ hydrolysis of the glycopeptide product once it has formed. The kinetic parameters of the corresponding Endo-M mutant, EndoM-N175A, for the transglycosylation between oxazoline **9** and acceptor **5** were also measured and are compared with those of the EndoA-N171A mutant in Table 2.

The two mutants were very similar with respect to both K_m (~3 mM for the oxazoline **9** and ~5 mM for the acceptor **5**) and k_{cat} (~4 min^{-1} for **9** and ~3 min^{-1} for **5**). These data suggest that the two mutants have comparable affinities for the high-mannose-type sugar oxazoline and the acceptor substrates. They also have similar catalytic efficiencies for the transglycosylation reactions (Table 2). In regard to the EndoM-N175A-catalyzed reaction between the complex-type glycan oxazoline **4** and the acceptor **5**, accurate kinetic parameters were not obtained because of the difficulty in reaching a saturated substrate concentration of the complex-type oxazoline substrate. The K_m for the oxazoline **4** was estimated to be larger than 10 mM. In comparison, the K_m of N175A for the high-mannose-type oxazoline **9** (3 mM) was much smaller than that for the complex-type oxazoline **4** (K_m > 10 mM). Therefore, the Endo-M mutant appears to have a higher affinity for the high-mannose-type glycan oxazoline than for the complex-type glycan oxazoline.

EndoA-N171A is More Thermally Stable Than EndoM-N175A. Originally, Endo-A was a bacterial enzyme isolated from *A. protophormiae*[7,8] and Endo-M a fungus enzyme isolated from *M. hiemalis*.[13] For the present study, the Endo-A and Endo-M mutants were successfully overproduced in *Escherichia coli*. As Endo-M appeared to be much more sensitive than Endo-A during our transglycosylation reactions, we examined and compared the thermal stabilities of the two mutants. Thus, the two mutant enzymes were incubated in a phosphate buffer (50 mM, pH 6.8) at different temperatures (30−50 °C) for 10 min, and then their transglycosylation activities were measured using **9** as the donor and **5** as the acceptor substrate. As shown in Figure 2, preincubation of the EndoM-N175A at 45 °C for 10 min resulted in complete loss of its transglycosylation activity, while the EndoA-N171A mutant retained its full activity after the same treatment. Therefore, the EndoA-171A mutant was much more thermally stable than the EndoM-N175A mutant.

Glycosynthase-Catalyzed Synthesis of Homogeneous Glycoproteins Carrying Full-Size Natural *N*-Glycans. We have demonstrated that the two endoglycosidase mutants EndoM-N175A and EndoA-N171A were able to take the *N*-glycan oxazolines as donor substrates for transglycosylation to form the corresponding natural *N*-glycopeptides without hydrolysis of the products. These results prompted us to examine the

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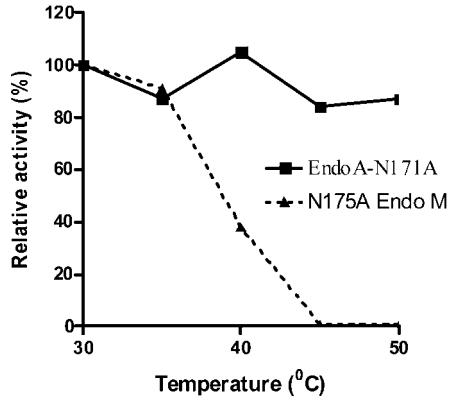

Figure 2. Thermal stabilities of EndoA-N171A and EndoM-N175A.

efficiencies of the two glycosynthases for the synthesis of full-size homogeneous glycoproteins carrying natural *N*-glycans. Again, we chose bovine ribonuclease B, a natural glycoprotein consisting of 124 amino acid residues, as a model system to test the two glycosynthases. Natural bovine ribonuclease B (RNase B) carries a heterogeneous high-mannose-type *N*-glycan ($Man_{5-9}GlcNAc_2$) at Asn-34. It was previously used by us and others for showcasing new chemoenzymatic methods for glycoprotein synthesis and glycosylation remodeling.[7,15,19,21,28] Thus, the heterogeneous *N*-glycan in the natural RNase B was removed by Endo-H treatment, leaving only the innermost *N*-acetylglucosamine (GlcNAc) at the Asn-34 site to give the homogeneous GlcNAc−RNase B, which was used as the acceptor substrate for the enzymatic transglycosylation. It was found that incubation of the complex-type glycan oxazoline **4** and GlcNAc−RNase B (10:1 donor/acceptor molar ratio) with the mutant EndoM-N175A in a phosphate buffer (pH 7.0) at 23 °C resulted in the formation of the transglycosylation product **11**. After 8 h, ~80% of the GlcNAc−RNase B was converted into **11**, which was eluted slightly earlier than GlcNAc−RNase B in RP-HPLC. The product could easily be isolated by HPLC and was characterized as the RNase B glycoform carrying a complex-type *N*-glycan. Deconvolution of the ESI-MS data for **11** gave a molecular mass of 15 308 Da, which is in good agreement with the calculated molecular mass (15305 Da) of the expected RNase B glycoform carrying the complex-type *N*-glycan. For further characterization, the attached *N*-glycan in glycoprotein **11** was released by PNGase F treatment. MALDI-TOF MS of the released *N*-glycan gave a single species $[M + Na]^+$ at *m/z* 1664.53, which is consistent with the biantennary complex-type *N*-glycan (Galβ1→4GlcNAcβ1→2Manα1→6)-[Galβ1→4GlcNAcβ1→2Manα1,3]Manβ1→4GlcNAcβ1→4GlcNAc (M_{calcd} = 1641.49 Da). It was found that the transglycosylation with GlcNAc−RNase B could be driven to completion to form the glycoprotein product when additional CT-oxazoline **4** was added (Scheme 5). It was also observed that a relatively large amount of enzyme was required for an efficient transglycosylation, particularly when the substrate concentration was low. Therefore, future research should be directed to the improvement of the specific activity of the mutants for transglycosylation. This might be achieved by mutagenesis and screening of a mutant library based on the EndoM-N175A mutant.

For the synthesis of the RNase B glycoform carrying a full-size high-mannose-type *N*-glycan, GlcNAc−RNase B was incubated with the $Man_9GlcNAc$ oxazoline **9** (8:1 donor/acceptor molar ratio) in a phosphate buffer in the presence of the mutant EndoA-N171A at 23 °C. Under these conditions, the transglycosylation was found to take place smoothly to form the desired glycoprotein product **12**, which was isolated by HPLC in 82% yield (Scheme 5). Again, the obtained glycoprotein product was confirmed to be the desired $Man_9GlcNAc_2$−RNase B **12** by ESI-MS analysis of the glycoprotein and MALDI-TOF MS analysis of the *N*-glycan released by PNGase F treatment. The deconvoluted ESI-MS data for **12** (M_{found} = 15 549 Da) matches well with the calculated molecular mass of glycoprotein **12** (M_{calcd} = 15 547 Da). The ESI-MS profiles of the synthetic glycoproteins **11** and **12** are shown in Figure 3.

These experimental data clearly indicate that the two endoglycosynthase mutants EndoM-N175A and EndoA-N171A, when coupled with the use of an appropriate *N*-glycan oxazoline as the donor substrate, enable an efficient synthesis of homogeneous *N*-glycoproteins carrying either a natural complex-type or a natural high-mannose-type *N*-glycan without product hydrolysis. It should be pointed out that the application of the glycosynthases for glycoprotein synthesis would rely on access to a GlcNAc protein as the precursor. Fortunately, a given GlcNAc protein could be potentially prepared by several approaches, including (a) overproduction of the protein in a high-yield yeast or insect expression system coupled with Endo-H deglycosylation; (b) total protein synthesis via native chemical ligation or expressed protein ligation with incorporation of a GlcNAc moiety at a predetermined site during the synthesis;[29] and (c) overproduction of a GlcNAc-containing protein in *E. coli* through the novel nonsense codon suppression technology.[30]

Conclusion

The properties and synthetic potential of two novel endoglycosidase-based glycosynthases, EndoM-N175A and EndoA-N171A, have been described. The two glycosynthases were able to take an appropriate highly activated glycan oxazoline as the donor substrate for transglycosylation to form homogeneous natural *N*-glycoproteins without hydrolysis of the product. Thus, the creation of these glycosynthases provides a beautiful solution to the problem of product hydrolysis associated with the wild-type enzymes. EndoM-N175A was found to be capable of transferring complex-type *N*-glycan oxazolines to form homogeneous, complex-type *N*-glycoproteins, while the EndoA-N171A mutant is a more convenient glycosynthase for the synthesis of high-mannose-type *N*-glycoproteins. Therefore, the novel glycosynthases described here provides a timely and potentially powerful tool for the synthesis of various homogeneous *N*-glycoproteins carrying defined natural *N*-glycans, which are essential for detailed structural and functional studies.

Experimental Section

Materials. The GlcNAc pentapeptide **1** derived from erythropoietin (amino acid sequence 37−41) was synthesized as described in the literature.[18] GlcNAc−C34 (**7**), a 34-mer peptide derived from HIV-1 gp41, was prepared by automatic solid-phase peptide synthesis using

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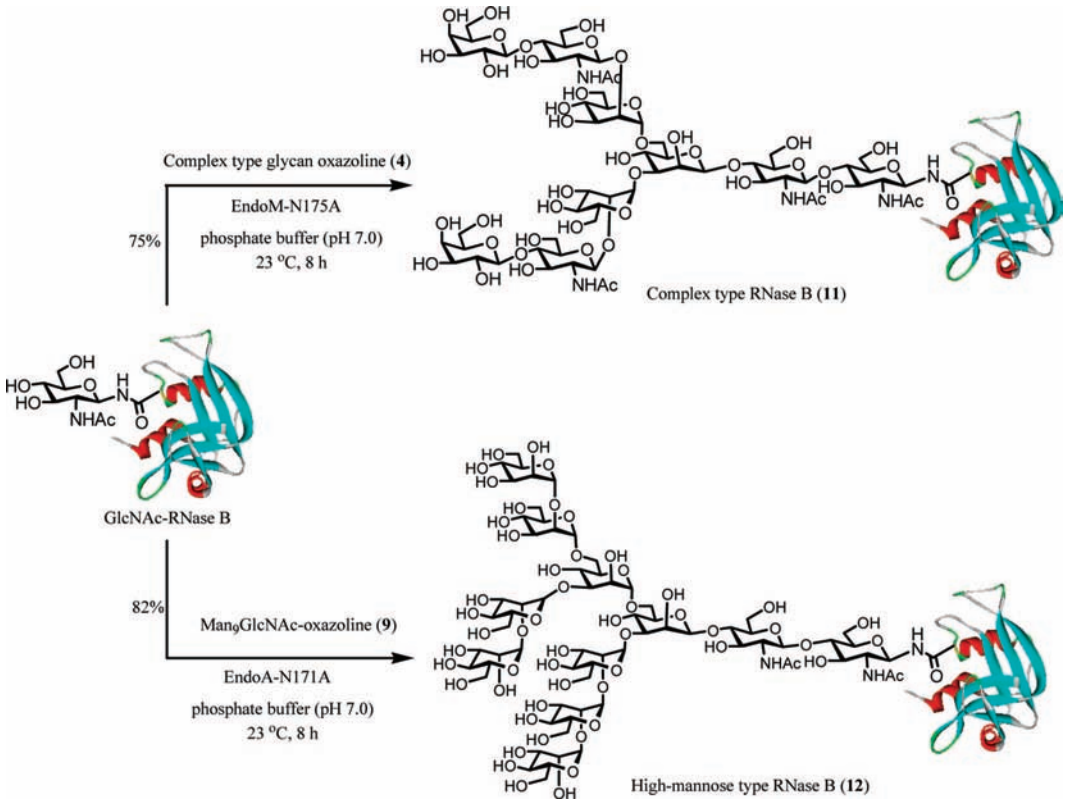
(29) (a) Dawson, P. E.; Kent, S. B. *Annu. Rev. Biochem.* **2000**, *69*, 923–960. (b) Schwarzer, D.; Cole, P. A. *Curr. Opin. Chem. Biol.* **2005**, *9*, 561–569.

(30) (a) Wang, L.; Schultz, P. G. *Angew. Chem., Int. Ed.* **2005**, *44*, 34–66. (b) Zhang, Z.; Gildersleeve, J.; Yang, Y. Y.; Xu, R.; Loo, J. A.; Uryu, S.; Wong, C. H.; Schultz, P. G. *Science* **2004**, *303*, 371–373. (c) Fahmi, N. E.; Dedkova, L.; Wang, B.; Golovine, S.; Hecht, S. M. *J. Am. Chem. Soc.* **2007**, *129*, 3586–3597.

Scheme 5. Chemoenzymatic Synthesis of Homogeneous Glycoforms of RNase B



the Fmoc approach, according to our previously reported method.[12] $Man_9GlcNAc$ oxazoline **9** was synthesized from $Man_9GlcNAc_2Asn$ according to our previously reported method.[23] The biantennary complex-type SGP Lys-Val-Ala-Asn[(NeuAc-Gal-GlcNAc-Man)$_2$Man-GlcNAc$_2$)]-Lys-Thr was prepared from hen's egg yolks following the reported procedure.[11,24] The recombinant wild-type Endo-A was overproduced in *E. coli* and purified by affinity chromatography according to the literature.[10] The pGEX-2T/Endo-A plasmid used for the expression was kindly provided by Prof. Takegawa. The recombinant wild-type Endo-M and the two Endo-M mutants EndoM-N175A and EndoM-Y217F were overproduced according to our previously reported method.[23] Neuraminidase (*Vibrio cholerae*) and peptide *N*-glycosidase F (PNGase F) were purchased from New England Biolabs. All of the other reagents were purchased from Sigma-Aldrich and used as received.

Methods. Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300 C18 column (5.0 μm, 4.6 $\times$ 250 mm) at 40 °C. The column was eluted with a linear gradient of MeCN at a flow rate of 1 mL/min using one of the following four gradient methods, depending on the properties of the compounds to be separated: (A) 0−20% MeCN containing 0.1% trifluoroacetic acid (TFA) for 20 min; (B) 0−30% MeCN containing 0.1% TFA for 18 min; (C) 0−90% MeCN containing 0.1% TFA for 30 min; (D) 23−29% MeCN containing 0.1% TFA for 30 min. Preparative HPLC was performed on a Waters 600 HPLC instrument with a preparative C18 column (Symmetry300, 19 $\times$ 300 mm). The column was eluted with a suitable gradient of water/acetonitrile containing 0.1% TFA. NMR spectra were measured with JEOL ECX 400 MHz and/or Inova 500 MHz NMR spectrometers. The chemical shifts were assigned in parts per million. ESI-MS spectra were measured on a Micromass ZQ-4000 single-quadruple mass spectrometer. MALDI-TOF MS measurements were performed on an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics). The instrument was calibrated using a Proteo-Mass Peptide MALDI-MS calibration kit (MSCAL2, Sigma-Aldrich). The matrix used for glycans was 2,5-dihydroxybenzoic acid (DHB) (10 mg/mL in 50% acetonitrile containing 0.1% TFA). The parameters for measurement were the following: 337 nm

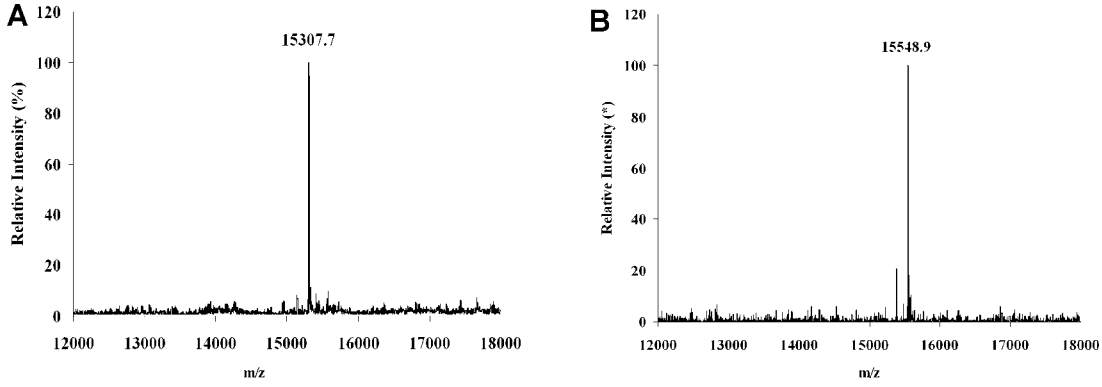


Figure 3. Deconvoluted ESI-MS profiles of the synthetic glycoforms of RNase B: (A) RNase B carrying the biantennary complex-type *N*-glycan; (B) RNase B carrying the $Man_9GlcNAc_2$ glycan.

nitrogen laser with 100 μJ output; laser frequency, 50.0 Hz; laser power, 35%; linear mode; positive polarity; detection range, 1000−3000; pulsed ion extraction, 70 ns; high voltage, on; realtime smoothing, high; shots, 500−2000.

Creation and Expression of the EndoA-N171A Mutant. Mutation of Asn171 to Ala was performed using a Quik-Change mutagenesis kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. The oligonucleotide primers are listed here, with the mutated site underlined: forward, 5′-GAC GGC TGG TTT ATT GCC CAA GAA ACA GAA GGG-3′; reverse, 5′-CCC TTC TGT TTC TTG GGC AAT AAA CCA GCC GTC-3′. Mutations were verified by DNA sequencing. The recombinant EndoA-N171A was overexpressed and finally purified by affinity chromatography following our previously described procedures.[23]

Synthesis of β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[β-D-Galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose (1). SGP (70 mg, 24.4 μmol) was incubated with neuraminidase (*V. cholerae*) (5 units) in a phosphate buffer (200 μL, 50 mM, pH 6.0) at 30 °C. The desialylation reaction was monitored by analytic HPLC (method A, t_R = 10.5 min). The reaction was complete after 12 h. The product was purified by preparative HPLC to give the asialo-SGP (55 mg, 98%). ^{1}H NMR (D_2O, 400 MHz): δ 5.01 (s, 1H, H-1 of Man^4), 4.92 (d, 1H, J = 8.0 Hz, H-1 of GlcNAc^1), 4.81 (s, 1H, H-1 of Man$^{4'}$), 4.68 (s, 1H, H-1 of Man^3), 4.50−4.44 (m, 3H, H-1 of GlcNAc^2, GlcNAc^5 and GlcNAc$^{5'}$), 4.35 (m, 2H, H-1 of Gal^6 and Gal$^{6'}$), 1.96 (s, 3H, Ac), 1.92 (s, 3H, Ac), 1.91 (s, 3H, Ac), 1.88 (s, 3H, Ac), 1.24 (d, 3H, J = 7.6 Hz, Thr−CH_3), 1.09 (d, 3H, J = 7.6 Hz, Ala−CH_3), 0.82 (d, 6H, J = 7.6 Hz, Val−CH_3). ESI-MS: calcd for $C_{90}H_{155}N_{13}O_{54}$, M = 2283.24 Da; found, m/z 1141.16 $[M + 2H]^{2+}$, 761.82 $[M + 3H]^{3+}$.

The asialo-SGP thus obtained (55 mg) was incubated with Endo-M (50 milliunits) in a phosphate buffer (50 mM, pH 6.5, 300 μL) at 30 °C for 16 h. The residue was treated with DOWEX 50WX2−400 and DOWEX 1×2-100 ion-exchange resins to completely remove cation and anion species, and the solution was filtered and concentrated. The residue containing the neutral oligosaccharide was dissolved in water and loaded onto a column (1.5 × 85 cm) of Sephadex G-25. The material was eluted with 0.1 M acetic acid. The fractions containing the oligosaccharide were combined and lyophilized to give **1** (23 mg, 66%). ^{1}H NMR (D_2O, 400 MHz): δ 5.10 (d, 1H, J = 3.6 Hz, H-1 of GlcNAc^1), 5.01 (s, 1H, H-1 of Man^3), 4.82 (s, 1H, H-1 of Man$^{3'}$), 4.65 (s, 1H, H-1 of Man^2), 4.46 (d, 2H, J = 8.0 Hz, H-1 of GlcNAc^4 and GlcNAc$^{4'}$), 4.34 (d, 2H, J = 8.0 Hz, H-1 of Gal^5 and Gal$^{5'}$), 4.15 (m, 1H), 4.08 (m, 1H), 4.01 (m, 1H), 1.95 (s, 3H, Ac), 1.94 (s, 3H, Ac), 1.93 (s, 3H, Ac). ESI-MS: calcd for $C_{54}H_{91}N_3O_{41}$, M = 1437.51 Da; found, m/z 1438.49 $[M + H]^+$, 719.93 $[M + 2H]^{2+}$.

Synthesis of 2,3,4,6-Tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-acetyl-β-D-mannopyranosyl-(1→4)-1,3,6-tri-*O*-acetyl-2-acetamido-2-deoxy-D-glucopyranose (2). Compound **1** (23 mg, 16 μmol) was dissolved in pyridine (2 mL) and acetic anhydride (2 mL), and the solution was stirred at room temperature (rt) for 20 h. The reaction mixture was concentrated to dryness under vacuum, and the residue was subjected to column chromatography on silica gel using 20:1 CH_2Cl_2/MeOH as the eluent, affording the fully acetylated compound **2** (26 mg, 68%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.42 (m, 1H, H-1 of GlcNAc^1), 5.03 (s, 1H, H-1 of Man^3), 4.82 (s, 1H, H-1 of Man^3), 4.64 (s, 1H, H-1 of Man^2), 4.51 (m, 2H, H-1 of GlcNAc^4 and GlcNAc$^{4'}$), 4.36 (m, 2H, H-1 of Gal^5 and Gal$^{5'}$), 2.19−1.95 (m, 78H, 26 × Ac). ESI-MS: calcd for $C_{100}H_{137}N_3O_{64}$, M = 2403.75 Da; found, m/z 2404.85 $[M + H]^+$, 1203.31 $[M + 2H]^{2+}$.

Synthesis of 2-Methyl-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranosyl-(1→2)-3,4,6-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-acetyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyranose]-[2,1-*d*]-oxazoline (3). Compound **2** (26 mg, 10.8 μmol) was dissolved in anhydrous 1,2-dichloroethane (2 mL), and then TMSBr (20 μL, 0.17 mmol), $BF_3 \cdot Et_2O$ (20 μL, 0.17 mmol), and 2,4,6-collidine (23 μL, 0.17 mmol) were added sequentially under an argon atmosphere. The mixture was stirred at rt for 12 h and then diluted with chloroform (20 mL) and washed with saturated sodium bicarbonate solution and brine. The organic layer was dried over anhydrous sodium sulfate and filtered. The filtrate was concentrated, and the residue was purified by column chromatography on silica gel using 20:1 CH_2Cl_2/MeOH as the eluent to give a crude product as a yellow solid. The crude product was further purified by gel filtration (Sephadex LH20, eluted with MeOH) to afford compound **3** as a pale-yellow foam (19 mg, 75%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.99 (d, 1H, J = 7.0 Hz, H-1 of oxazoline), 5.16 (s, 1H, H-1 of Man^3), 4.94 (s, 1H, H-1 of Man^3), 4.72 (s, 1H, H-1 of Man^2), 4.52 (m, 2H, H-1 of GlcNAc^4 and GlcNAc$^{4'}$), 4.34 (m, 2H, H-1 of Gal^5 and Gal$^{5'}$), 2.19−1.85 (m, 75H, 25 × Ac). ESI-MS: calcd for $C_{98}H_{133}N_3O_{62}$, M = 2343.73 Da; found, m/z 2344.96 $[M + H]^+$, 1173.42 $[M + 2H]^{2+}$.

Synthesis of 2-Methyl-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→3)-[β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→2)-α-D-mannopyranosyl-(1→6)]-β-D-mannopyranosyl-(1→4)-1,2-dideoxy-α-D-glucopyranose]-[2,1-*d*]-oxazoline (4). Compound **3** (19 mg, 8.1 μmol) was dissolved in anhydrous methanol (1 mL) and treated with MeONa (0.5 M in MeOH, 2 μL, 1 μmol), and the de-*O*-acetylation was monitored by ESI-MS. After 22 h, the solvent was removed by evaporation, and the residue was subjected to gel filtration (Sephadex G-10, eluted by water containing 0.03% Et_3N). The fractions containing the product were combined and lyophilized to give the oxazoline **4** as a pale-yellow solid (11 mg, quantitative yield). ^{1}H NMR (D_2O, 500 MHz): δ 6.05 (d, 1H, J = 7.0 Hz, H-1 of oxazoline), 5.12 (s, 1H, H-1 of Man^3), 5.07 (s, 1H, H-1 of Man^3), 4.89 (s, 1H, H-1 of Man^2), 4.57 (m, 2H, H-1 of GlcNAc^4 and GlcNAc$^{4'}$), 4.42 (m, 2H, H-1 of Gal^5 and Gal$^{5'}$), 2.02 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.84 (s, 3H, CH_3 of oxazoline). ESI-MS: calcd for $C_{54}H_{89}N_3O_{40}$, M = 1419.50 Da; found, m/z 1420.36 $[M + H]^+$.

EndoM-N175A-Catalyzed Transglycosylation with Complex-Type Glycan Oxazoline 4: Synthesis of Glu-Asn[(Gal-GlcNAc-Man)$_2$Man-GlcNAc$_2$]-Ile-Thr-Val (6). CT-oxazoline **4** (1 mg, 0.7 μmol) and GlcNAc pentapeptide **5** (110 μg, 0.14 μmol) in phosphate buffer (50 mM, pH 7.0, 35 μL) was incubated with EndoM-N175A (7 μg) at 23 °C for 4 h. The glycopeptide **6** was obtained via preparative HPLC purification (260 μg, 85%). Analytic HPLC (method B): t_R = 14.2 min. ESI-MS: calcd for $C_{86}H_{145}N_{11}O_{54}$, M = 2197.11 Da; found, m/z 1099.78 $[M + 2H]^{2+}$, 916.75 [M − GalGlcNAc + 2H]$^{2+}$, 733.46 $[M + 3H]^{3+}$. In a comparative study, the same reaction was carried out with wild-type Endo-M and mutant Y217F, and aliquots were taken at intervals for HPLC analysis (method B) to monitor the product formation (the results were shown in Figure 1).

EndoM-175A-Catalyzed Transfer of Complex *N*-Glycan to GlcNAc−C34: Synthesis of (Gal-GlcNAc-Man)$_2$Man-GlcNAc$_2$−C34 (8). A solution of **4** (1 mg, 0.7 μmol) and **7** (630 μg, 0.14 μmol) in a phosphate buffer (50 mM, pH 7.0, 50 μL) containing 20% DMSO was incubated with EndoM-N175A (10 μg) at 23 °C for 4 h. The glycopeptide **8** was obtained via preparative HPLC purification (590 μg, 71%). Analytic HPLC (method C): t_R = 19.4

min. ESI-MS: calcd for $C_{248}H_{388}N_{55}O_{109}S$, $M = 5912.62$ Da; found, *m/z* 1479.62 $[M + 4H]^{4+}$, 1388.22 $[M - GalGlcNAc + 4H]^{4+}$, 1183.88 $[M + 5H]^{5+}$, 1110.81 $[M - GalGlcNAc + 5H]^{5+}$.

EndoA-N171A-Catalyzed Transglycosylation with $Man_9GlcNAcOxazoline$ 9: Synthesis of Glu-Asn($Man_9GlcNAc_2$)-Ile-Thr-Val (10). A solution of **9** (1 mg, 0.6 μmol) and **5** (100 μg, 0.128 μmol) in a phosphate buffer (50 mM, pH 7.0, 30 μL) was incubated with EndoA-N171A (6 μg) at 23 °C for 4 h. The transglycosylation product was then purified by preparative HPLC to give glycopeptide **10** (250 μg, 78%), Analytic HPLC (method B): $t_R = 13.8$ min. ESI-MS: calcd for $C_{94}H_{159}N_9O_{54}$, $M = 2437.94$ Da; found, *m/z* 1220.78 $[M + 2H]^{2+}$, 1139.66 $[M - Man + 2H]^{2+}$.

EndoM-N175A-Catalyzed Transfer of a Complex-Type *N*-Glycan to GlcNAc−RNase B: Synthesis of RNase B Glycoform 11 Carrying a Complex-Type *N*-Glycan. A solution of **4** (500 μg, 0.35 μmol) and GlcNAc−RNase B (500 μg, 0.036 μmol) in a phosphate buffer (50 mM, pH 7.0, 5 μL) was incubated with EndoM-N175A (200 μg) at 23 °C for 8 h. The transglycosylation product was isolated by preparative HPLC to afford the glycoprotein product **11** (415 μg, 75%). Analytic HPLC (method D): $t_R = 21.3$ min. ESI-MS: calcd, $M = 15\,305$ Da; found, 15 307.7 Da (data deconvolution). Further structural characterization of the glycoprotein was performed by treatment of the glycoprotein with PNGase F. The *N*-glycan released from the glycoprotein was confirmed to be the complex-type *N*-glycan (Galβ1→4GlcNAcβ1→2Manα1→6)[Galβ1→4GlcNAcβ1→2Manα1→3]Manβ1→4GlcNAcβ1→4GlcNAc. MALDI-TOF MS of the released *N*-glycan: calcd for $C_{62}H_{104}N_4O_{46}$, $M = 1641.49$ Da; found, *m/z* 1664.53 $[M + Na]^+$.

EndoA-N171A-Catalyzed Transfer of a High-Mannose-Type Glycan to GlcNAc−RNase B: Synthesis of $Man_9GlcNAc_2$−RNase B (12). A solution of **9** (500 μg, 0.30 μmol) and GlcNAc−RNase B[19] (500 μg, 0.036 μmol) in a phosphate buffer (50 mM, pH 7.0, 10 μL) was incubated with EndoA-N171A (200 μg) at 23 °C for 8 h. The reaction was monitored by analytic HPLC, and the glycoprotein product was isolated by preparative HPLC to give $Man_9GlcNAc_2$−RNase B (**12**) as a white foam after lyophilization (460 μg, 82%). Analytic HPLC (method D): $t_R = 21.2$ min. ESI-MS: calcd, $M = 15\,547$ Da; found, 15 548.9 Da (data deconvolution). Further structural characterization of the glycoprotein product was performed by treatment of the glycoprotein with PNGase F. The glycan released from the glycoprotein was confirmed to be $Man_9GlcNAc_2$. MALDI-TOF of the released *N*-glycan: calcd for $C_{70}H_{118}N_2O_{56}$, $M = 1882.64$ Da; found, *m/z* 1905.99 $[M + Na]^+$, 1744.32 $[M - mannose + Na]^+$.

Kinetic Studies. For the measurement of the kinetic parameters of $Man_9GlcNAc$ oxazoline hydrolysis by Endo-A and its mutant, solutions of **9** at various concentrations (0.3125, 0.625, 1.25, 2.5, 5.0, and 10 mM) in a Tris-HCl buffer (200 mM, pH 8.0, total volume 5 μL) were incubated with 10 ng of EndoA or 3 μg of N171A at 30 °C. Each reaction was terminated after 30 min by the addition of an equal volume of 100% THF in aqueous sodium hydroxide (0.1 M). The aliquots were then analyzed by HPAEC-PAD, and the hydrolysis product $Man_9GlcNAc$ was quantified according to our previously described method.[27] For the measurement of the kinetic parameters for the transglycosylations by EndoA-N171A and EndoM-N175A with oxazoline **9** and GlcNAc pentapeptide **5**, the enzymatic reactions were performed at 30 °C for 30 min in a Tris-HCl buffer (200 mM, pH 8.0, total volume, 5 μL). To determine the K_m value for **9**, solutions of the oxazoline at various concentrations (0.3125, 0.625, 1.25, 2.5, 5.0, and 10 mM) were incubated with 6 μg of EndoA-N171A or EndoM-N175A and a fixed concentration (20 mM) of **5** in the buffer. To determine the apparent K_m value for **5**, the oxazoline concentration was fixed at 10 mM and the concentrations of **5** were varied as follows: 0.3125, 0.625, 1.25, 2.5, 5.0, 10, and 20 mM. The reaction was monitored by analytic HPLC. The parameters K_m and V_{max} were obtained by fitting the experimental data to the Michaelis−Menten kinetics model using GraphPad Prism.

Thermal Stability of EndoA and EndoM Mutants. To determine the thermal stabilities of the mutants, 5 μg of EndoA-N171A or EndoM-N175A was incubated at various temperatures (30, 35, 40, 45, and 50 °C) for 10 min. Then the enzyme was cooled on ice for 5 min, after which its activity was assayed as follows: the treated enzyme was incubated with the oxazoline **9** (10 mM) and the GlcNAc pentapeptide **5** (20 mM) in a Tris-HCl buffer (200 mM, pH 8.0, total volume 5 μL) at 30 °C for 1 h; the reaction was then terminated by addition of 10% TFA, and the transglycosylation product **10** was quantified by HPLC analysis.

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Supporting Information Available: ^{1}H NMR spectra of the asialoglycopeptide from hen's egg yolk, the complex-type oligosaccharide **1**, and the complex-type glycan oxazoline **4**; HPAEC-PED analysis, 1H NMR spectrum, and $^1H-^{13}C$ HSQC and $^1H-^1H$ NOESY 2D NMR spectra of the Asn-linked *N*-glycan obtained from Pronase digestion of glycopeptide **6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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