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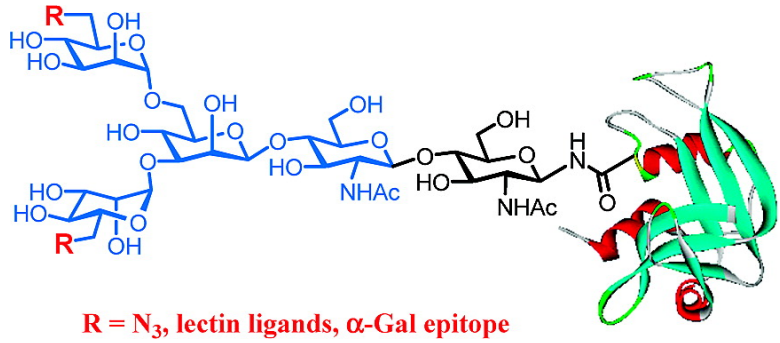

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Expeditious Chemoenzymatic Synthesis of Homogeneous N-Glycoproteins Carrying Defined Oligosaccharide Ligands

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Abstract: An efficient chemoenzymatic method for the construction of homogeneous *N*-glycoproteins was described that explores the transglycosylation activity of the endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) with synthetic sugar oxazolines as the donor substrates. First, an array of large oligosaccharide oxazolines were synthesized and evaluated as substrates for the Endo-A-catalyzed transglycosylation by use of ribonuclease B as a model system. The experimental results showed that Endo-A could tolerate modifications at the outer mannose residues of the $Man_3GlcNAc$-oxazoline core, thus allowing introduction of large oligosaccharide ligands into a protein and meanwhile preserving the natural, core *N*-pentasaccharide ($Man_3GlcNAc_2$) structure in the resulting glycoprotein upon transglycosylation. In addition to ligands for galectins and mannose-binding lectins, azido functionality could be readily introduced at the N-pentasaccharide ($Man_3GlcNAc_2$) core by use of azido-containing $Man_3GlcNAc$ oxazoline as the donor substrate. The introduction of azido functionality permits further site-specific modifications of the resulting glycoproteins, as demonstrated by the successful attachment of two copies of αGal epitopes to ribonuclease B. This study reveals a broad substrate specificity of Endo-A for transglycosylation, and the chemoenzymatic method described here points to a new avenue for quick access to various homogeneous N-glycoproteins for structure−activity relationship studies and for biomedical applications.

Introduction

Protein glycosylation is a ubiquitous posttranslational modification capable of transforming a protein's properties in different ways. It is known that glycosylation can profoundly impact a protein's folding, stability, and intracellular trafficking.[1] On the other hand, the covalently linked oligosaccharides of glycoproteins can serve as specific ligands for lectins and receptors on cell surface, thus directly participating in many important cellular communication processes, such as cell adhesion, cell differentiation, pathogen−host interaction, development, and immune responses.[2] However, a detailed understanding of the functions of glycoproteins is often hindered by the structural microheterogeneity caused by the diverse patterns of glycosylation. In most cases, natural and recombinant glycoproteins are produced as mixtures of glycoforms that have the same polypeptide backbone but differ in the pendant sugar chains. As pure glycoforms are extremely difficult to isolate from natural and recombinant sources with current techniques, synthetic natural and unnatural homogeneous glycopeptides and glycoproteins are urgently needed for both structural/functional studies and biomedical applications. The past decade has witnessed tremendous progress in this field, and many chemical, enzymatic, and bioengineering methods have been explored in order to overcome a series of technical obstacles in the road toward the ultimate assembly of homogeneous glycoproteins carrying defined oligosaccharides.[3] In particular, the exploration of various chemical and enzymatic ligation methods, including native chemical ligation, auxiliary-assisted ligation, and expressed protein ligation, as well as the use of glycoenzymes for sugar chain elongation in glycoprotein synthesis, have significantly expanded our synthetic repertoire.[4−7]

The chemoenzymatic method based on the transglycosylation activity of endo-β-*N*-acetylglucosaminidase (ENGases) has attracted much attention in this field in recent years.[7] ENGases are a class of hydrolytic enzymes that remove N-glycans from glycoproteins by hydrolyzing the β-1,4-glycosidic bond in the *N*,*N*′-diacetylchitobiose core. But a few ENGases, including the

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Endo-M from *Mucor hiemali*[8] and Endo-A from *Arthrobactor protophormiae*,[9−11] possess transglycosylation activity, that is, the ability to transfer the released oligosaccharide moiety to an acceptor such as an *N*-acetylglucosamine (GlcNAc)-containing peptide to form a new homogeneous glycopeptide. A big advantage of the endoglycosidase-based method is its high convergence, as the enzyme is able to attach a large intact oligosaccharide to a GlcNAc-polypeptide *in a single step and in a regio- and stereospecific manner* to form a homogeneous glycopeptide or glycoprotein in natural glycosidic linkages, without the need for any protecting groups.[7] To address the relatively low transglycosylation yield, the limitation of the use of only natural N-glycans as donor substrate, and the problem of product hydrolysis, we and other groups have recently explored synthetic sugar oxazolines as donor substrates for transglycosylation, with a focus on its application for glycopeptide synthesis.[6,7,12,13] The highly activated synthetic sugar oxazoline can be regarded as a mimic of the presumed oxazolinium ion intermediate generated by a substrate-assisted catalytic mechanism. It was demonstrated that oligosaccharide oxazolines corresponding to the N-glycan core $Man_3GlcNAc$, that is, Manα1,3(Manα1,6)Manβ1,4GlcNAc and some of its truncated and selectively modified forms, were able to serve as donor substrates for transglycosylation. Interestingly, the resulting glycopeptides that carry truncated or modified N-glycans were poor substrates for hydrolysis because of the structural modifications, resulting in accumulation of the glycopeptide product. Moreover, we have also reported a preliminary study showing that this approach could be extended to glycoprotein synthesis and glycosylation engineering without loss of the transglycosylation efficiency.[6] In addition to the truncated or modified N-glycans, full-size natural N-glycan such as $Man_9GlcNAc_2$ might also be introduced into a GlcNAc-protein by a novel glycosynthase, EndoM-N175A mutant, that we have recently reported.[14] EndoM-N175A can take the high-mannose sugar oxazoline as substrate for transglycosylation but lacks the ability to hydrolyze the resulting natural N-glycopeptide because of the mutation. These studies implicate a great potential of the endoglycosidase-based method for synthesizing both modified and natural N-glycoproteins. We describe in this paper the expansion of this chemoenzymatic approach to the synthesis of an array of homogeneous N-glycoproteins carrying defined oligosaccharide ligands potentially useful for biological recognition. This chemoenzymatic approach involves two key steps, as depicted in Figure 1. The first step is to prepare a GlcNAc-protein, which can be achieved by selective deglycosylation of a natural or recombinant glycoprotein by an endoenzyme such as Endo-H to remove the heterogeneous N-glycan, leaving only the innermost GlcNAc residue attached to the Asn at the glycosylation site. Alternatively, a GlcNAc-protein can be synthesized by modern chemical protein synthesis techniques,

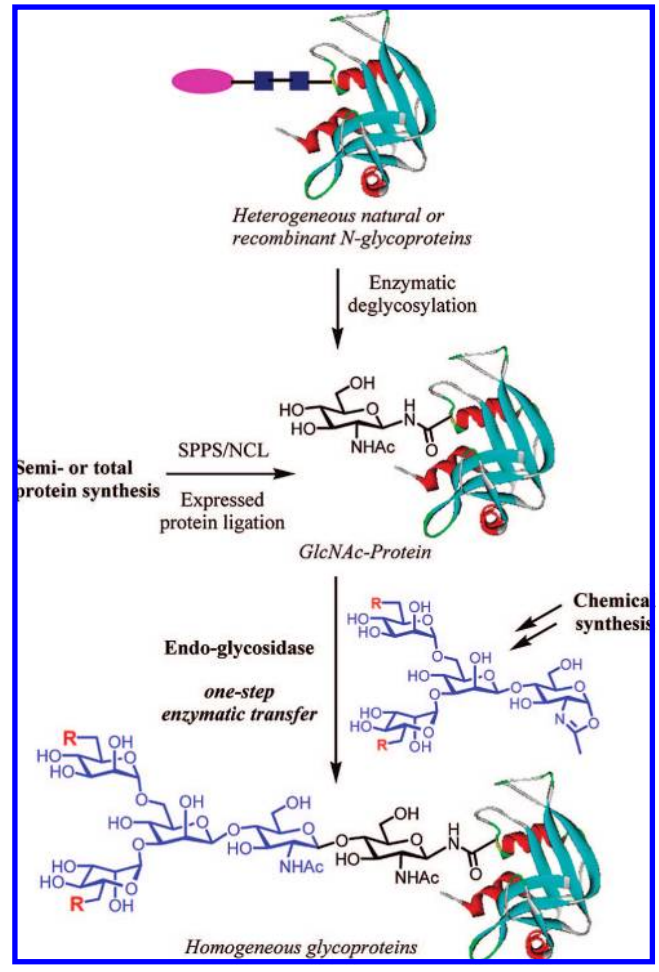

Figure 1. Chemoenzymatic strategy for N-glycoprotein synthesis

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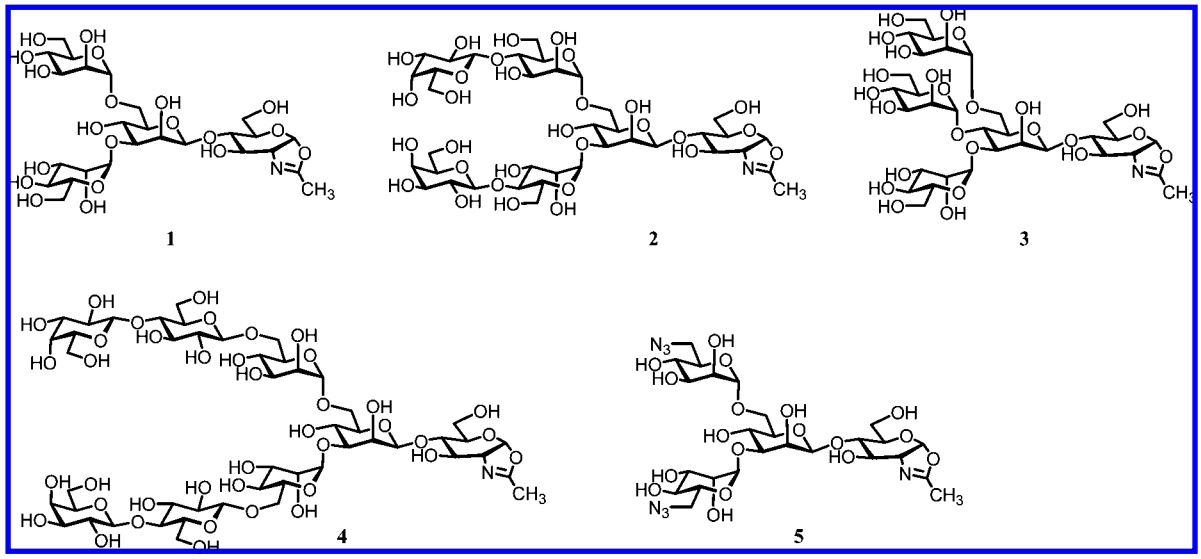

Figure 2. Synthetic oligosaccharide oxazolines.

in which the stable Asn-linked GlcNAc can be incorporated at any desired site(s) during the synthesis. The second step is the regio- and stereospecific enzymatic ligation of a preassembled oligosaccharide moiety (in the form of the activated oxazoline) to the GlcNAc-protein by the endoglycosidase-catalyzed transglycosylation. We have previously shown that Endo-A could tolerate modifications on the outer mannose residues of the $Man_3GlcNAc$ oxazoline.[6,13] Moreover, unnatural modification on the outer residues of the N-glycan core also retarded the enzymatic hydrolysis of the glycoprotein product, thus permitting accumulation of the transglycosylation product.[6,13] Therefore, to introduce specific oligosaccharide ligands into a protein, we have decided to glue the ligands at the outer residues of the $Man_3GlcNAc$ oxazoline core, so that a native core N-pentasaccharide ($Man_3GlcNAc_2$) at the glycosylation site would be generated after transglycosylation. The preservation of a natural core N-glycan structure may be important for maintaining the native conformations of the glycoproteins upon ligand introduction, as the N-glycan core, especially the innermost chitobiose (GlcNAcβ1,4GlcNAc) moiety, plays a primary role in impacting a protein's conformations.[15]

Results and Discussion

Design and Synthesis of Novel Oligosaccharide Oxazolines as Donor Substrates. We have previously synthesized several oligosaccharide oxazolines corresponding to the N-glycan core, such as oxazolines **1** and **2** (Figure 2), and evaluated them as substrates of Endo-A for transglycosylation.[6,13] We have found that Endo-A could tolerate certain modifications on the outer mannose residues of $Man_3GlcNAc$-oxazoline and even the Manβ1,4GlcNAc-oxazoline core. These results imply a broad substrate specificity for the oxazoline substrates and suggest that the outer residues might tolerate various modifications, a property ideal for introducing various carbohydrate ligands. To further examine the substrate specificity of Endo-A for oxazolines modified with functional groups and/or large carbohydrate ligands, we have designed three novel oligosaccharide oxazolines **3**−**5** (Figure 2), which will allow the introduction of specific carbohydrate ligands into proteins if proven as substrates for ENGases. For oxazoline **3**, an additional mannose residue was added at the bisecting location of the N-glycan core $Man_3GlcNAc$. If this oxazoline is a substrate for Endo-A, then a novel oligomannose ligand can be introduced into a protein, which may be useful for targeting the protein to specific cells, for example, macrophages and dendritic cells that express mannose receptors or DC-SIGN.[16] Oligosaccharide oxazoline **4** carries two lactose moieties β-1,6-linked to the α-mannoside residues in the $Man_3GlcNAc$ core. Lactose moiety is known to be recognized by various lectins such as PNA and galectins[17] and can serve as a substrate for various glycosyltransferases like α1,3-galactosyltransferase and sialyltransferases. The incorporation of two lactose moieties on the core N-glycan is likely to enhance the avidity of the ligand to various lectins.[18] In oligosaccharide oxazoline **5**, two azido groups were introduced at the 6-positions of the terminal mannose residues in the $Man_3GlcNAc$ core. Azido group is one of the most fascinating functional groups in bioconjugate chemistry because of its small size and its chemoselective reactivity. Thus, once it is introduced into a protein, a variety of functional groups could be site-specifically and chemo-selectively attached through orthogonal Staudinger ligation or the Huisgen 1,3-dipolar cycloaddition.[19]

The synthesis of oxazoline **3** is summarized in Scheme 1. Disaccharide **8** was prepared via direct β-mannosylation by the method of Crich and Dudkin.[20] The 2-azido-2-deoxy derivative **6**[21] was chosen as the acceptor since it was demonstrated to be a better glycosyl acceptor in the direct β-mannosylation reaction than the corresponding 2-phthalimido or 2-*N*-acetyl derivative.[20]

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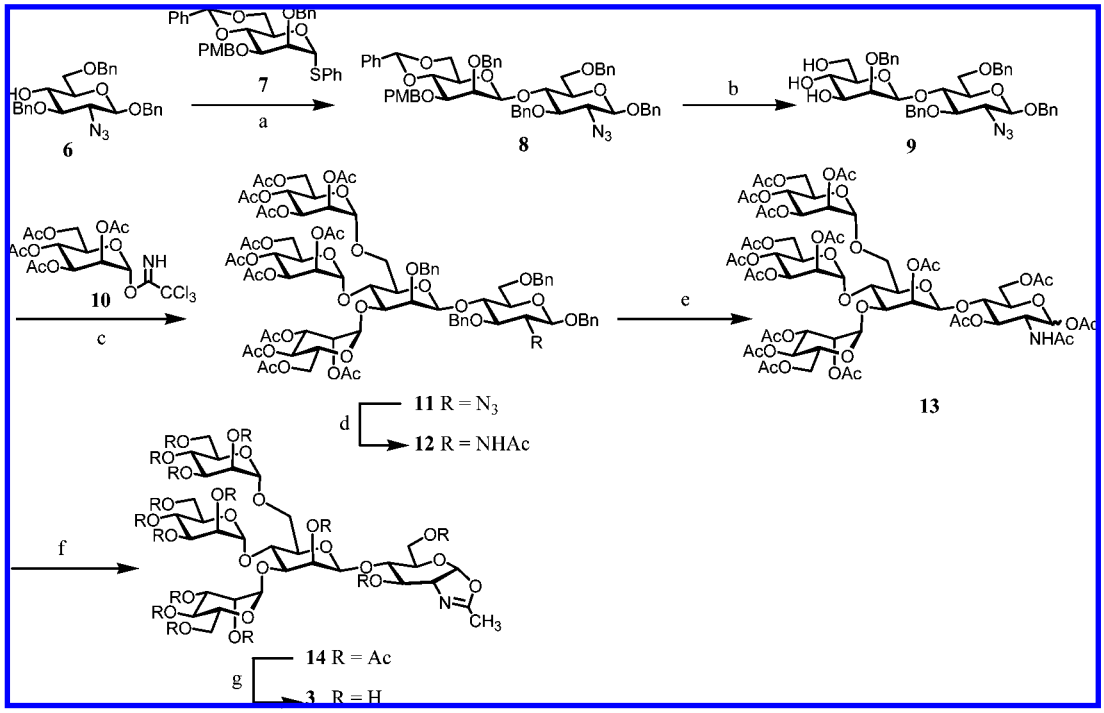
Scheme 1. Synthesis of Oligosaccharide Oxazoline **3**[a]



[a] Reagents and conditions: (a) BSP, TTBP, Tf_2O, CH_2Cl_2, 67%. (b) TFA, CH_2Cl_2, 85%. (c) TMSOTf, CH_2Cl_2, 79%. (d) AcSH, 86%. (e) (i) $Pd(OH)_2$-C, H_2, MeOH; (ii) Ac_2O, pyridine, 90% (2 steps). (f) TMSBr, $BF_3 \cdot OEt_2$, 2,4,6-collidine, CH_2Cl_2, 67%. (g) MeONa, MeOH, quant.

Thus, reaction of **6** and glycosyl donor **7**[22] by use of a mixture of 1-benzenesulfinyl piperidine (BSP)/2,4,6-tri-*tert*-butylpyrimidine (TTBP)/trifluoromethanesulfonic (triflic) anhydride (Tf_2O) as the promoter gave the disaccharide **8** in 67% yield. The 4,6-*O*-benzylidene group and the 3-*O*-*p*-methoxybenzyl (PMB) group of **8** were removed by treatment with TFA to give the triol derivative **9**. Glycosylation of **9** with an excess (8 mol equiv) of mannosyl trichloroacetimidate (**10**)[23] under the catalysis of trimethylsilyl triflate (TMSOTf) gave the pentasaccharide derivative **11** in 79% yield. The newly attached mannosyl residues were confirmed to be in the desired α-O-glycosidic linkages by NMR analysis. Next, the 2-azide group in **11** was converted to an acetamido group by treatment with thioacetic acid to give **12**. The O-benzyl groups were then removed by hydrogenation, and the resulting hydroxyl groups were acetylated to provide the fully acetylated derivative **13**. Oxazoline ring formation was achieved by treatment with trimethylsilyl bromide (TMSBr), $BF_3 \cdot OEt_2$, and 2,4,6-collidine to provide the oxazoline derivative **14** in 67% yield. Finally, de-O-acetylation with a catalytic amount of MeONa in MeOH afforded the pentasaccharide oxazoline **3** in quantitative yield (Scheme 1).

The synthesis of sugar oxazoline **4** is summarized in Scheme 2. Selective removal of the PMB group in disaccharide **8** with 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ), followed by regioselective opening of the 4,6-*O*-benzylidene with $Cu(OTf)_2$ and BH_3 in tetrahydrofuran (THF),[24] gave the diol **16** in 58% yield in two steps. Double glycosylation of diol **16** with 6-*O*-acetyl-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate **17**[25] provided the tetrasaccharide **18** in excellent yield. Selective removal of the two O-acetyl groups in **18** by mild acidic hydrolysis (acetic chloride in MeOH and dichloromethane) afforded the diol **19**. This compound can serve as a key intermediate for introducing various functional groups at the 6-positions of the α-mannoside residues. To introduce two lactose moieties at the N-glycan core, compound **19** was glycosylated with the lactose glycosyl donor **20**,[26] giving the octasaccharide derivative **21**. Compound **21** was then changed to the peracetylated derivative **23** through several steps of protecting group manipulations. Oxazoline formation was carried out by treatment of **23** with TMSBr and $BF_3 \cdot Et_2O$ to give the octasaccharide derivative **24** (35%). The relatively low yield of **24** was mainly due to the slow reaction, as the starting material **23** was partially recovered after reaction for 3 days. De-O-acetylation of **24** with MeONa/MeOH gave the octasaccharide oxazoline **4** in quantitative yield.

The synthesis of azido-containing oxazoline **5** was carried out with the tetrasaccharide derivative **18** as the starting material (Scheme 3). The 2-azido group of **18** was converted to an acetamido group by treatment with thioacetic acid to give **25**. The 6-*O*-acetyl group was then selectively removed by mild acidic hydrolysis without affecting the benzoyl groups to give the diol **26**. Tosylation of **26**, followed by azide substitution, provided compound **27** in 84% yield, with two azido moieties attached at the 6-positions of the terminal α-mannosyl residues. Removal of the benzoyl groups gave compound **28**. Since there was no efficient method to selectively deprotect the benzyl groups without affecting the azido functionality in oligosaccharide synthesis, we decided to apply a two-step conversion to restore the azido functionality. First, the benzyl groups in **28** were removed by catalytic hydrogenation. Under this condition,

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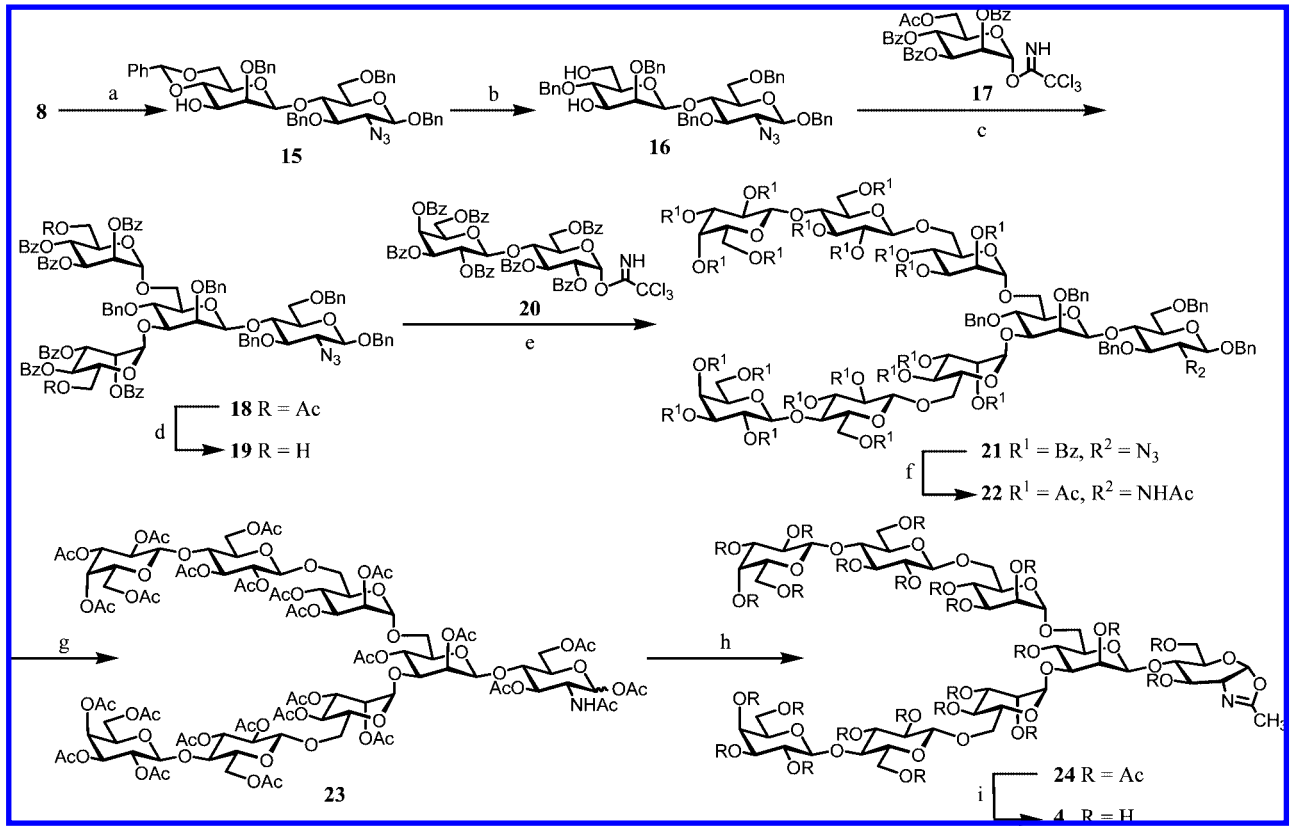
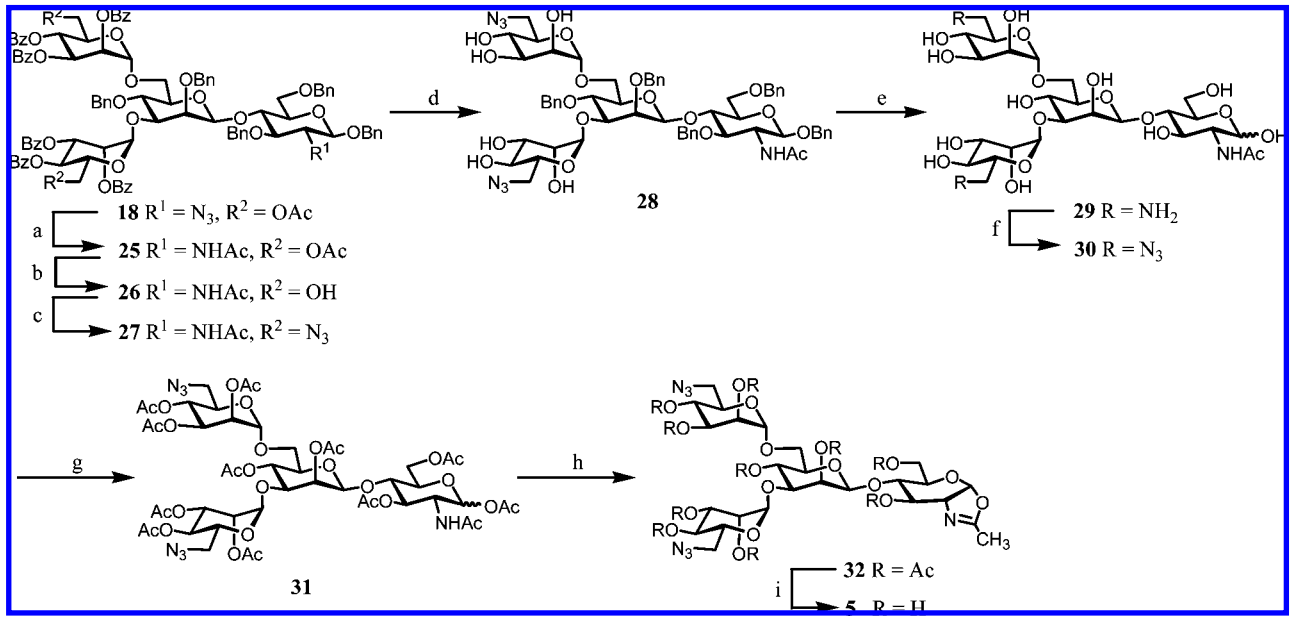
Scheme 2. Synthesis of Sugar Oxazoline **4**[a]



[a] Reagents and conditions: (a) DDQ, CH_2Cl_2, H_2O, 80%. (b) $Cu(OTf)_2$, BH_3·THF, THF, 73%. (c) TMSOTf, CH_2Cl_2, 96%. (d) AcCl, CH_2Cl_2, MeOH, 90%. (e) TMSOTf, CH_2Cl_2, 65%. (f) (i) MeONa, CH_2Cl_2, MeOH; (ii) Ac_2O, pyridine; (iii) AcSH, pyridine, $CHCl_3$, 79% (3 steps). (g) (i) $Pd(OH)_2$-C, H_2, MeOH; (ii) Ac_2O, pyridine, 77% (2 steps). (h) TMSBr, BF_3·OEt_2, 2,4,6-collidine, CH_2ClCH_2Cl, 35%. (i) MeONa, MeOH, quant.

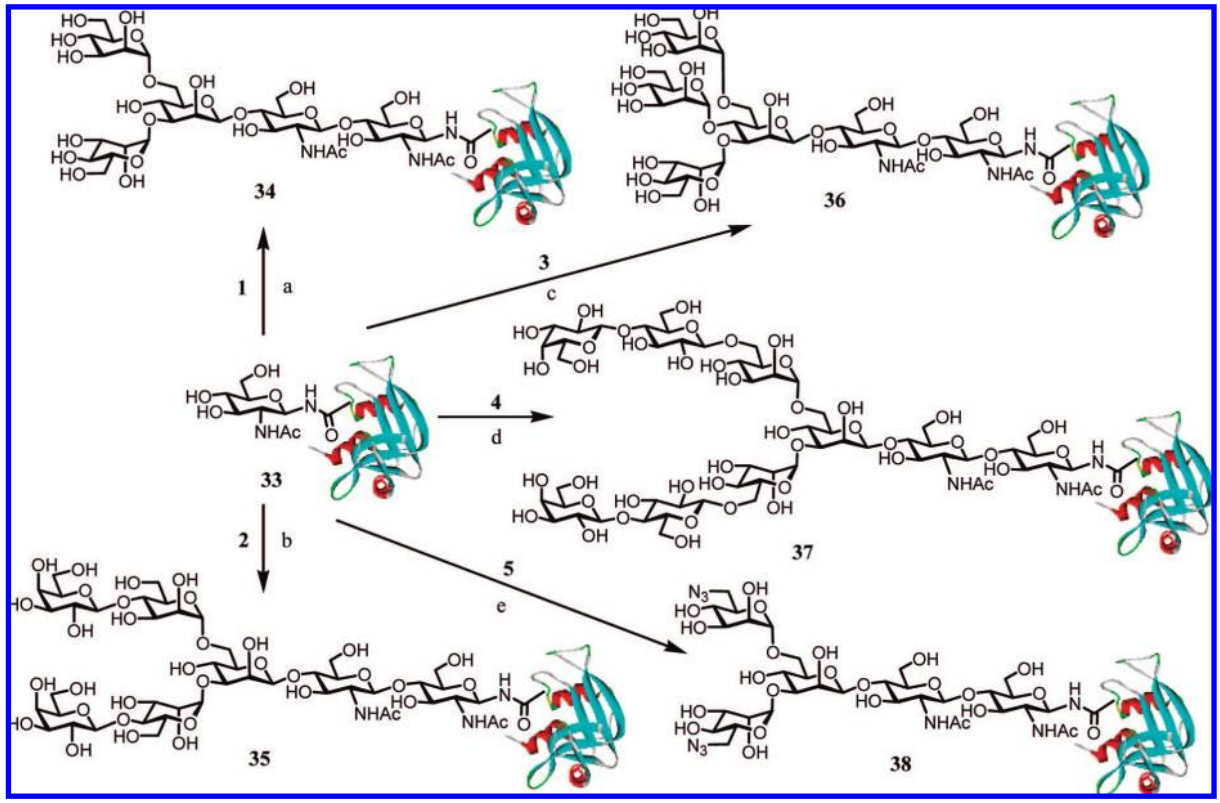
Scheme 3. Synthesis of Sugar Oxazoline **5**[a]



[a] Reagents and conditions: (a) AcSH, pyridine, $CHCl_3$, 85%. (b) AcCl, CH_2Cl_2, MeOH, 72%. (c) (i) TsCl, pyridine; (ii) NaN_3, DMF, 84% (2 steps). (d) MeONa, MeOH, 85%; (e) $Pd(OH)_2$-C, H_2, MeOH; (f) TfN_3, K_2CO_3, $CuSO_4$, CH_2Cl_2, MeOH, H_2O. (g) Ac_2O, pyridine, 61% (3 steps). (h) TMSBr, BF_3·OEt_2, 2,4,6-collidine, CH_2Cl_2, 52%. (i) MeONa, MeOH, quant.

the azido groups were simultaneously reduced to amino groups to give compound **29**. Then the amino groups in **29** were changed back to azido functionality by the copper-catalyzed diazo transfer reaction[27] to form the azido derivative **30**. The reaction was monitored by electrospray ionization mass spectrometry (ESI-MS) and the conversion proceeded efficiently to give the diazido compound, which was isolated as the peracetylated derivative **31** in 61% yield in three steps. Treatment of

Scheme 4. Enzymatic Transglycosylation[a]



[a] Reagents and conditions: (a) Endo-A, phosphate buffer (50 mM, pH 6.5), 82%. (b) Endo-A, phosphate buffer (50 mM, pH 6.5), 96%. (c) Endo-A, phosphate buffer (50 mM, pH 6.5), 71%. (d) Endo-A, phosphate buffer (50 mM, pH 6.5), 38%. (e) Endo-A, phosphate buffer (50 mM, pH 6.5), 89%.

31 with TMSBr, $BF_3 \cdot OEt_2$, and 2,4,6-collidine gave the oxazoline derivative **32** in 52% yield. Finally, de-*O*-acetylation with MeONa/MeOH afforded the azido-containing sugar oxazoline **5** in quantitative yield (Scheme 3).

Enzymatic Transglycosylation To Form Homogeneous Glycoproteins. To examine the efficiency of the ENGase-catalyzed transglycosylation for glycoprotein synthesis with preassembled oligosaccharide oxazolines as the donor substrates, we have chosen bovine ribonuclease B (RB) as a model system that has been used by us and others for testing new synthetic strategy.[5,6,9] Bovine RB is a small natural glycoprotein that consists of 124 amino acids. It carries heterogeneous high-mannose-type oligosaccharides (Man_{5-9}) attached at the single glycosylation site, Asn-34. As demonstrated in our previous work,[6] the heterogeneous *N*-glycans on native RB were removed by treatment with Endo-H to provide the homogeneous GlcNAc-protein **33**, in which only the innermost GlcNAc residue of the N-glycan was left at the Asn-34 site. This GlcNAc-containing protein (GlcNAc-RB) was used as the acceptor to examine the transglycosylation with synthetic oligosaccharide oxazolines. We have previously demonstrated that oxazoline **1**, corresponding to the *N*-glycan $Man_3GlcNAc$ core, and oxazoline **2**, which has two galactose residues attached at the 4-positions of the outer mannose residues of the core, were excellent substrates for Endo-A-catalyzed transglycosylation with GlcNAc-RB to form the glycoproteins **34** and **35**.[6] Thus the Endo-A-catalyzed transglycosylations with oxazolines **3**−**5** were examined in a similar way in phosphate buffer (50 mM, pH 6.5) at 30 °C. It was found that sugar oxazoline **3**, which carries a bisecting mannose at the 4-position of the core β-mannose residue, could serve as a donor substrate for Endo-A to react with GlcNAc-RB (**33**) to give a new, homogeneous glycoprotein **36**, in which a novel oligomannose glycan was introduced (Scheme 4). This newly formed glycoprotein **36** was eluted slightly earlier than GlcNAc-RB under appropriate reverse-phase HPLC (RP-HPLC) conditions (see Experimental Section) and was purified by RP-HPLC in 71% yield. The ESI mass spectrum of the glycoprotein **36** (Figure 3A) clearly indicates its homogeneity. Deconvolution of the ESI mass spectrum of **36** gave a molecular mass of 14 740 Da, which is in good agreement with the calculated mass (14 745 Da) of glycoprotein **36**.

The oxazoline **4** was also able to serve as a donor substrate of Endo-A for transglycosylation, but the enzymatic reaction proceeded in a much slower path than the enzymatic reactions with oxazolines **1**, **2**, and **3**. After 3 days of reaction, the newly formed glycoprotein **37** was isolated by RP-HPLC in 38% yield, and the unreacted starting material GlcNAc-RB (**33**) was recovered. Deconvolution of the ESI mass spectrum of **37** (Figure 3B) gave a molecular mass of 15 229 Da, which agreed well with the calculated mass (15 231 Da) of glycoprotein **37**. The relatively low reactivity of octasaccharide oxazoline **4** in comparison with core tetrasaccharide oxazoline **1** and hexasaccharide oxazoline **2** might be attributed to the unnatural modification on the 6-positions of the α-mannosyl residues and the relatively large size of the lactose moieties. Consistent with this assumption, the sugar oxazoline **5**, which has two small azido groups attached at the 6-position of the outer mannose residues, was found to be an excellent substrate for Endo-A. Thus, transglycosylation of GlcNAc-RB (**33**) with sugar oxazoline **5** under the catalysis of Endo-A proceeded efficiently

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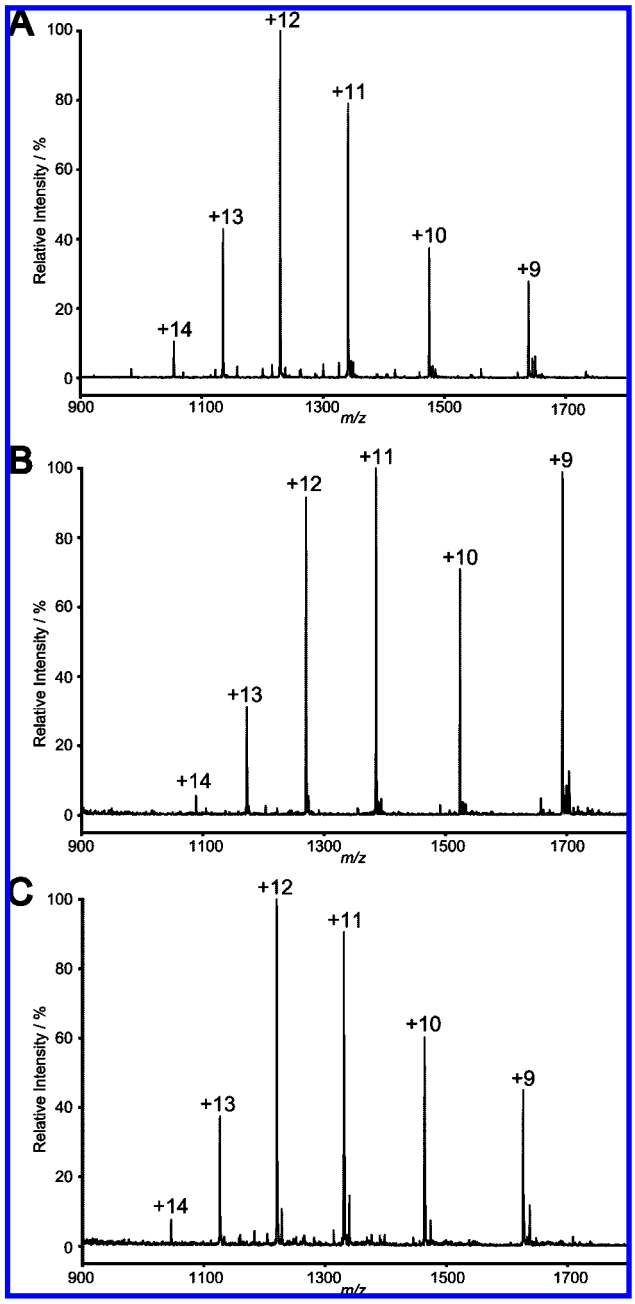
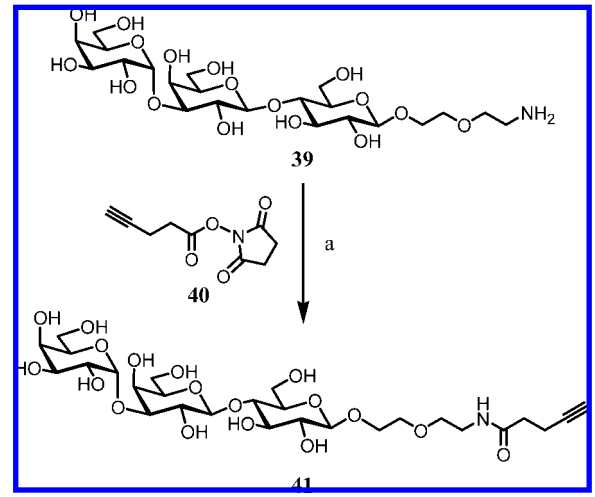


Figure 3. ESI mass spectra of synthetic glycoproteins: (A) glycoprotein **36**, (B) glycoprotein **37**, and (C) glycoprotein **38**. Peaks are labeled according to the corresponding charge states.

to give the glycoprotein **38** in 89% yield (Scheme 4). Again, deconvolution of the ESI mass spectrum of **38** (Figure 3C) gave a molecular mass of 14 630 Da, indicating the attachment of the azidooligosaccharide moiety to the GlcNAc-RB to form glycoprotein **38** (calculated molecular mass 14 619 Da).

Ligand Introduction via Huisgen 1,3-Dipolar Cycloaddition. The efficient chemoenzymatic introduction into proteins of a core *N*-pentasaccharide carrying azido functionality opens an avenue to further site-specific functionalization of the protein, as the azido group can serve as a unique tag for introducing various functional groups through highly chemoselective bioorthogonal reactions.[19] These include the Staudinger ligation, in which the azide reacts with a functionalized phosphine to form

Scheme 5. Synthesis of Alkyne-Functionalized αGal Epitope[a]



[a] Reagents and conditions: (a) $NaHCO_3$, MeOH, MeCN, H_2O, 79%.

a stable amide bond,[28] and the azide-alkyne [3 + 2] cycloaddition to form a stable triazole linkage under catalysis.[29] To demonstrate usefulness of the combination of the chemoenzymatic transglycosylation and the "Click chemistry", we selected αGal epitope as a ligand to introduce into RB via the transglycosylation product glycoprotein **38**. For the purpose, an alkyne-functionalized αGal trisaccharide epitope **41** was prepared with the amino-containing trisaccharide derivative **39** as the starting material (Schemes 5 and 6), which we have previously synthesized for HIV-1 immunotargeting purposes.[30] To incorporate αGal epitopes into glycoprotein **38** that contains two azido groups, we examined several catalytic methods for the 1,3-dipolar cycloaddition. It was found that the cycloaddition reaction between glycoprotein **38** and the alkyne-oligosaccharide **41** proceeded most efficiently under the catalysis of $CuSO_4$/L-ascorbic acid in the presence of bathophenanthroline disulfonic acid (to serve as a ligand for the catalyst).[31] Thus, when an excess of **41** was used, an esentially quantitative conversion of the azidoprotein **38** to the new glycoprotein **42** was fulfilled after 12 h at room temperature, as monitored by HPLC and ESI-MS. After the reaction, the resulting glycoprotein **42** was easily isolated in 87% yield by RP-HPLC, in which two copies of the αGal epitope were introduced through the 1,3-dipolar cycloaddition. The purity and identity of the new glycoprotein **42** were revealed by SDS−PAGE and ESI-MS analysis (Figure 4). The SDS gel of **42** showed a single band at 18 kDa, which is about 2 kDa larger than the glycoprotein **38**, implicating the introduction of two αGal epitopes (Figure 4A). Deconvolution of its ESI-MS (Figure 4B) gave a molecular mass of 15 971 Da, which matched well with the calculated mass (M = 15 976 Da) of glycoprotein **42**. It should be pointed out that addition of the bathophenanthroline ligand in the reaction mixture is

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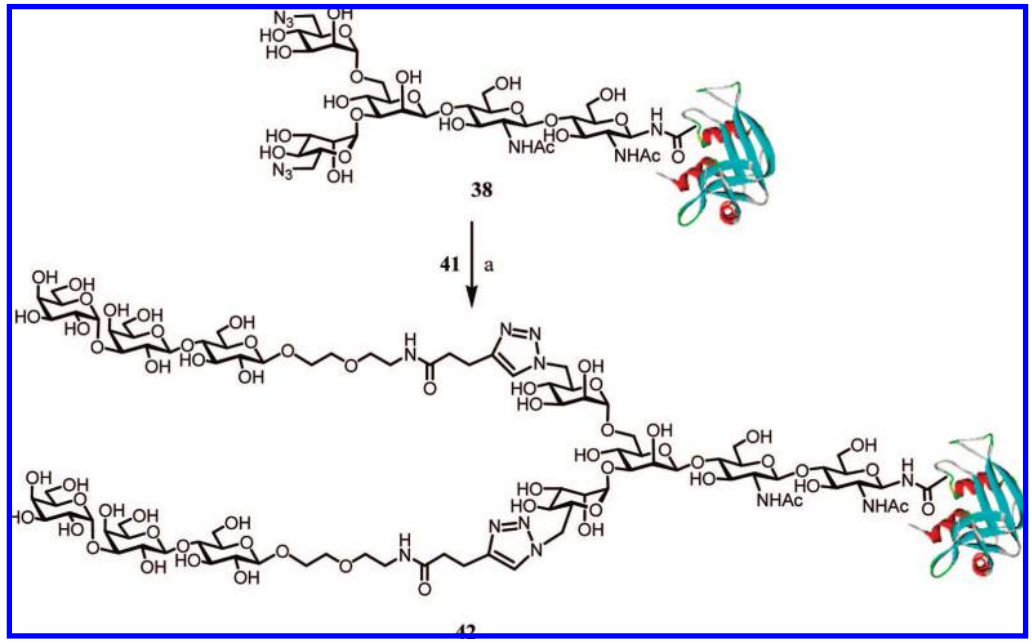
Scheme 6. Catalytic 1,3-Dipolar Cycloaddition To Introduce αGal Epitope[a]



[a] Reagents and conditions: (a) Tris buffer (0.1 M, pH 8.0), $CuSO_4$, L-ascorbic acid, bathophenanthrolinedisulfonic acid, 87%.

essential for the success of cycloaddition, as an inefficient reaction was observed in the absence of bathophenanthroline disulfonic acid, giving ca. 28% of a monosubstituted glycoprotein and less than 5% of the double-substituted glycoprotein **42** after 24 h of reaction (confirmed by ESI-MS analysis). On the other hand, we have also observed that the use of $CuSO_4$/copper wire-catalyzed reaction[32] resulted in precipitation of the protein. There are precedents that certain catalytic conditions for the Huisgen 1,3-dipolar azide-alkyne cycloaddition would lead to precipitation of protein substrates.[33] Thus, choice of appropriate cycloaddition conditions is critical for the success of ligation, particularly when it is applied to protein-based reactants. The recognition of the αGal epitopes (an array of oligosaccharides with terminal Galα1,3Gal moieties) on cells of most animals such as pigs by natural human anti-αGal antibodies is mainly responsible for the hyperacute rejection in xenotransplantation.[34] A successful introduction of αGal epitopes into proteins under physiological pH and temperature in a site-specific manner may find interesting applications such as for potential immunotargeting.[30]

Binding of the Synthetic Glycoproteins with Respective Lectins. The introduction of specific oligosaccharide ligands into a protein or glycoprotein would enable various useful applications on the basis of ligand−receptor interactions. Thus, we have performed a preliminary study on the binding of the synthetic ligand-containing glycoproteins with respective lectin and antibody, using surface plasmon resonance (SPR) technology. It was found that the oligomannose-containing glycoproteins **34** and **36** could be efficiently recognized by ConA, a lectin that binds terminal α-mannosyl residues.[35] Interestingly, glycoprotein **36** exhibited a slightly better affinity for ConA than glycoprotein **34**, implicating a positive contribution of the additional bisecting mannose residue. But the enhancement was not significant. In contrast, the ribonuclease A that shares the same sequence as RB but lacks the N-glycan did not bind to ConA at all (Figure 5A). For the terminal galactose-containing glycoproteins, it was observed that the glycoproteins **35** and **37**, which carried the Galβ1,4Man and Galβ1,4Glc (lactose) moieties, respectively, could efficiently bind to PNA, a lectin that recognizes terminal β-linked galactose moieties.[35] The two glycoproteins showed very similar affinity to lectin PNA. However, the αGal-containing glycoprotein **42**, as well as the ribonuclease A that does not contain glycan, did not bind to PNA (Figure 5B). These results confirm the importance of the anomeric configuration of carbohydrate ligands in lectin recognition. To detect the binding of αGal-glycoprotein **42** to human anti-αGal antibodies, the whole human IgG that contains about 1−2% anti-αGal antibodies were immobilized on chips and the antibody chips were used for probing the synthetic glycoproteins. As expected, the αGal-containing glycoprotein **42** could be readily recognized by the immobilized whole human IgG that contains a small fraction of anti-αGal antibodies, whereas the lactose-containing glycoprotein **37** and ribonuclease A did not react to the immobilized human IgG (Figure 5C).

Conclusion

An array of large oligosaccharide oxazolines were synthesized and evaluated as substrates for the Endo-A-catalyzed transglycosylation for the synthesis of homogeneous N-glycoproteins carrying defined oligosaccharide ligands. The results suggest that Endo-A could tolerate modifications at the outer mannose residues of the $Man_3GlcNAc$-oxazoline core. Thus a broad substrate specificity of the enzyme was revealed, which allows introduction of large oligosaccharide ligands into a protein and

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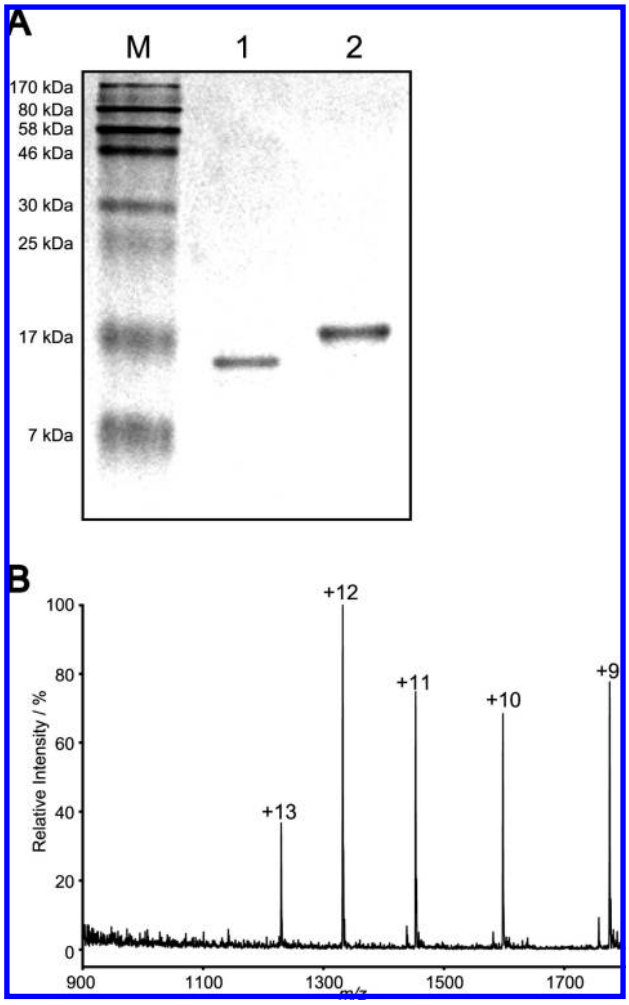

Figure 4. SDS−PAGE and ESI-MS analysis of the αGal-incorporated glycoprotein product (**42**) generated by "Click" chemistry. (A) Coomassie blue-stained SDS−polyacrylamide gel (lane M is protein marker with sizes on the left; lane 1 is the starting material, glycoprotein **38**; and lane 2 is the purified product, glycoprotein **42**). (B) ESI mass spectrum of glycoprotein **42**. Charge states are labeled.

meanwhile preserves the natural N-pentasaccharide ($Man_3GlcNAc_2$) core upon transglycosylation. In particular, the incorporation of the azido functionality at the N-pentasaccharide ($Man_3GlcNAc_2$) core permits further structural modifications of the resulting glycoproteins, as demonstrated by the successful ligation of two copies of αGal epitopes to ribonuclease B. This novel chemoenzymatic approach opens a new avenue to various homogeneous N-glycoproteins that will be useful for basic research and biomedical applications.

Experimental Section

Materials. Endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was overproduced in *Escherichia coli* following the reported procedure.[10] ConA, PNA, and IgG from normal human serum were purchased from Sigma (St. Louis, MO).

General Procedures. Thin-layer chromatography (TLC) was performed by use of silica gel on aluminum plates (Sigma−Aldrich). Flash column chromatography was performed on silica gel 60 (230−400 mesh). SDS−PAGE was performed with an 18% (w/v) gel. NMR spectra were recorded on a JEOL ECX 400 MHz spectrometer. The chemical shifts were assigned in parts per million (ppm). ESI-MS spectra were measured on a Micromass ZQ-4000 single-quadrupole mass spectrometer. Analytical RP-HPLC was performed on a Waters 626 HPLC instrument with a Symmetry300 C18 column (5.0 μm, 4.6 × 250 mm) at 40 °C. The column was eluted with a linear gradient of 23−29% MeCN containing 0.1% TFA for 30 min at a flow rate of 1.0 mL/min. Preparative HPLC was performed with a Waters 600 HPLC instrument of a Waters C18 column (Symmetry 300, 19 × 300 mm). The column was eluted with a suitable gradient of MeCN-H_2O containing 0.1% TFA at a flow rate of 12 mL/min.

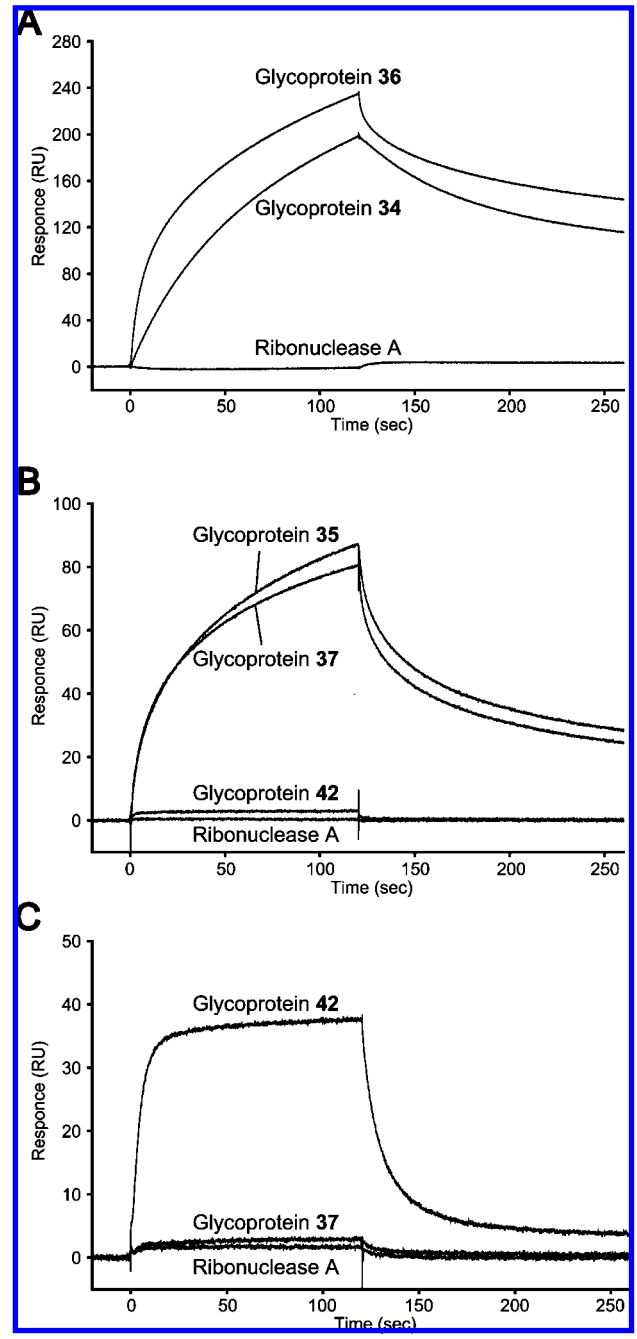

Figure 5. SPR sensorgrams of the binding between respective synthetic glycoproteins and immobilized lectin or human serum antibody. (A) Binding to immobilized ConA; (B) binding to immobilized PNA; (C) binding to immobilized whole IgG-type antibodies from human serum. For comparison, respective glycoprotein or ribonuclease A was injected onto the sensor chip surface at a concentration of 1.0 μM.

Benzyl 2-*O*-Benzyl-4,6-*O*-benzylidene-3-*O*-*p*-methoxybenzyl-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (8). A mixture of phenyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-*p*-methoxybenzyl-1-thio-α-D-mannopyranoside **7**[22]

(450 mg, 0.79 mmol), BSP (182 mg, 0.87 mmol), TTBP (329 mg, 1.58 mmol), and activated 3 Å molecular sieves (3.46 g) in CH_2Cl_2 (16 mL) was stirred for 30 min at −60 °C under an argon atmosphere. Then Tf_2O (159 μL, 0.95 mmol) was added at this temperature. After 5 min, a solution of benzyl 2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside **6**[21] (250 mg, 0.53 mmol) in CH_2Cl_2 (2.6 mL) was added and the mixture was stirred at −60 °C for 2 h. The mixture was filtered through a Celite pad. The filtrate was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated. The residue was subjected to silica gel column chromatography (hexanes/EtOAc, 8:1) to afford **8** (329 mg, 67%) as a colorless syrup. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.49−6.83 (m, 29H), 5.51 (s, 1H), 5.05 (d, 1H, J = 10.6 Hz), 4.92 (d, 1H, J = 11.9 Hz), 4.84 (d, 1H, J = 11.9 Hz), 4.77 (d, 1H, J = 11.9 Hz), 4.69 (d, 1H, J = 11.9 Hz), 4.67 (d, 1H, J = 11.9 Hz), 4.62 (d, 2H, J = 11.4 Hz), 4.52 (d, 1H, J = 11.9 Hz), 4.49 (s, 1H), 4.40 (d, 1H, J = 11.9 Hz), 4.28 (d, 1H, J = 8.2 Hz), 4.10−4.03 (m, 2H), 3.96 (t, 1H, J = 9.4 Hz), 3.78 (s, 3H), 3.69 (br d, 1H, J = 2.7 Hz), 3.63 (dd, 1H, J = 11.3, 2.1 Hz), 3.56−3.45 (m, 3H), 3.40 (dd, 1H, J = 9.9, 3.1 Hz), 3.32 (t, 1H, J = 9.2 Hz), 3.26 (m, 1H), 3.08 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.10, 138.48, 138.42. 137.60, 137.57, 136.76, 130.50, 129.01, 128.80, 128.49, 128.43, 128.26, 128.11, 128.07, 127.97, 127.94, 127.89, 127.76, 127.49, 126.04, 113.67, 101.42, 101.28, 100.38, 81.47, 78.61, 77.90, 77.31, 77.00, 75.11, 75.04, 74.72, 73.54, 72.29, 70.79, 68.40, 68.29, 67.32, 65.68, 55.21.

Benzyl 2-*O*-Benzyl-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (9). To a stirred solution of compound **8** (80 mg, 85 μmol) in CH_2Cl_2 (4 mL) was added TFA (0.75 mL) at −20 °C. The mixture was stirred for 30 min at this temperature and then for 1 h at 0 °C. MeOH (2 mL) and CH_2Cl_2 (20 mL) were added to the reaction mixture, and the mixture was sequentially washed with saturated $NaHCO_3$ and brine and then dried over $MgSO_4$. After removal of the solvent, the residue was purified by chromatography on a silica gel column (hexanes/EtOAc, 2:3) to yield **9** (53 mg, 85%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.41−7.24 (m, 20H), 5.02 (d, 1H, J = 10.5 Hz), 4.94 (d, 1H, J = 11.5 Hz), 4.94 (d, 1H, J = 11.9 Hz), 4.71 (d, 1H, J = 11.9 Hz), 4.71−4.65 (m, 2H), 4.54 (d, 1H, J = 11.5 Hz), 4.50−4.47 (m, 2H), 4.31 (d, 1H, J = 8.3 Hz), 3.95 (t, 1H, J = 9.4 Hz), 3.73 (dd, 1H, J = 11.2, 2.0 Hz), 3.67 (dd, 1H, J = 11.0, 3.6 Hz), 3.62 (m, 1H), 3.57 (br d, 1H, J = 3.7 Hz), 3.52−3.48 (m, 2H), 3.39−3.30 (m, 3H), 3.19 (br s, 1H), 3.01 (m, 1H), 2.45 (br s, 1H), 2.30 (br s, 1H), 1.80 (br s, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.21, 137.98, 137.39, 136.70, 128.55, 128.42, 128.27, 128.06, 128.02, 127.91, 127.82, 127.65, 127.41, 100.96, 100.31, 81.35, 77.95, 77.08, 75.56, 75.25, 75.03, 74.79, 73.84, 73.65, 70.76, 68.96, 68.12, 65.72, 62.16.

Benzyl 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)]-{[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)]}-2-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (11). A solution of compound **9** (37 mg, 50 μmol) and 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl tricholoroacetimidate **10**[23] (199 mg, 0.40 mmol) in CH_2Cl_2 (4 mL) containing activated 4 Å molecular sieves (240 mg) was stirred under an atmosphere of argon at room temperature for 1 h. After the mixture was cooled to −40 °C, a solution of TMSOTf in CH_2Cl_2 (1 M, 81 μL, 81 μmol) was added and the resulting mixture was stirred at room temperature for 24 h. The mixture was filtered through a Celite pad, poured into saturated $NaHCO_3$, and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated and the residue was subjected to flash silica gel column chromatography (hexanes/EtOAc, 1:1) to provide **11** (69 mg, 79%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.44−7.18 (m, 20 H), 5.32−5.19 (m, 8H), 5.14−5.10 (m, 3H), 4.96 (s, 1H), 4.94−4.90 (m, 2H), 4.88 (d, 1H, J = 10.5 Hz), 4.78 (d, 1H, J = 11.9 Hz), 4.70−4.64 (m, 2H), 4.51 (d, 1H, J = 12.3 Hz), 4.46 (d, 1H, J = 11.9 Hz), 4.46 (s, 1H), 4.34 (d, 1H, J = 8.3 Hz), 4.30 (m, 1H), 4.19−4.13 (m, 2H), 4.08 (dd, 1H, J = 11.9, 1.8 Hz), 4.00−3.86 (m, 5H), 3.84 (d, 1H, J = 2.8 Hz), 3.79−3.69 (m, 5H), 3.55 (t, 1H, J = 9.0 Hz), 3.44−3.29 (m, 4H), 3.18 (m, 1H), 2.16, 2.12, 2.10, 2.10, 2.06, 2.04, 2.02, 2.01, 2.00, 1.98, 1.94, 1.89 (12s, 36 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.71, 170.49, 170.28, 169.97, 169.83, 169.71, 169.69, 169.62, 169.56, 169.49, 138.18, 138.03, 137.62, 136.72, 128.76, 128.38, 128.32, 128.24, 128.05, 127.99, 127.91, 127.53, 127.33, 126.47, 100.44, 99.94, 99.36, 97.62, 83.19, 80.98, 78.02, 77.20, 76.03, 75.78, 74.78, 74.69, 73.83, 73.71, 70.71, 69.65, 69.58, 69.28, 69.17, 68.95, 68.74, 68.50, 68.42, 68.34, 68.27, 66.08, 65.95, 65.65, 65.43, 62.53, 62.48, 61.80, 20.84, 20.78, 20.73, 20.65, 20.61, 20.56.

Benzyl 2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)]-{[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)]}-2-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (12). Compound **11** (59 mg, 35 μmol) was treated with thioacetic acid (2 mL) for 24 h at room temperature. The reaction mixture was concentrated under reduced pressure and the residue was purified by silica gel column chromatography (hexanes/EtOAc, 2:3) to give **12** (52 mg, 86%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.43−7.18 (m, 20H), 6.06 (d, 1H, J = 7.8 Hz), 5.35−5.17 (m, 9H), 5.13 (d, 1H, J = 12.3 Hz), 5.09 (d, 1H, J = 1.8 Hz), 4.99 (d, 1H, J = 7.8 Hz), 4.90−4.84 (m, 3H), 4.77 (d, 1H, J = 12.4 Hz), 4.59−4.52 (m, 4H), 4.43 (d, 1H, J = 12.4 Hz), 4.32 (dd, 1H, J = 12.3, 5.5 Hz), 4.27 (dd, 1H, J = 12.9, 3.7 Hz), 4.09−3.96 (m, 6H), 3.92−3.57 (m, 10H), 3.48 (m, 1H), 3.31 (m, 1H), 3.21 (m, 1H), 2.16, 2.12, 2.09, 2.08, 2.05, 2.03, 2.02, 1.99, 1.97, 1.94, 1.90, 1.83 (12s, 39H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.93, 170.38, 170.31, 170.27, 169.92, 169.80, 169.77, 169.70, 169.62, 169.55, 169.46, 138.56, 138.12, 137.84, 137.55, 128.73, 128.35, 128.28, 128.20, 128.07, 127.70, 127.60, 127.50, 127.25, 126.49, 99.86, 99.62, 99.43, 99.28, 97.00, 82.75, 77.87, 77.63, 77.20, 76.83, 75.99, 74.74, 74.31, 73.72, 73.68, 73.55, 70.71, 69.54, 69.52, 69.43, 69.39, 69.36, 68.97, 68.69, 68.45, 68.33, 67.57, 66.08, 66.04, 65.38, 62.56, 62.47, 62.18, 56.49, 23.34, 20.82, 20.77, 20.76, 20.71, 20.66, 20.63, 20.59, 20.56.

2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)]-{[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)]}-2-*O*-acetyl-β-D-mannopyranosyl-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-D-glucopyranose (13). To a solution of **12** (52 mg, 30 μmol) in MeOH (3 mL) was added 20% palladium(II) hydroxide on activated carbon (30 mg). The mixture was vigorously stirred at room temperature under a hydrogen atmosphere overnight. The mixture was filtered through a Celite pad and the filtrate was concentrated in vacuo. The residue was dissolved in pyridine (4 mL), and Ac_2O (4 mL) was added. The mixture was stirred at room temperature overnight and then was concentrated. The residue was then dissolved in CH_2Cl_2, and the solution was washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and concentrated. Silica gel column chromatography (EtOAc) of the residue afforded **13** (41 mg, 90%, α/β = 79/21) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS, selected signals) δ 6.06 (d, 0.79H, J = 3.2 Hz), 5.65 (d, 0.21H, J = 8.3 Hz), 2.22−1.93 (m, 51H); ^{13}C NMR (100 MHz, $CDCl_3$, selected signals) δ 171.07, 170.65, 170.62, 170.40, 170.24, 170.07, 170.03, 169.86, 169.77, 169.70, 169.62, 169.57, 169.48, 169.22, 169.05, 98.33, 97.17, 97.03, 96.30, 92.23, 90.64, 22.84, 22.66, 20.88, 20.83, 20.68, 20.61, 20.50, 20.44, 20.32. ESI-MS: calcd for $C_{64}H_{88}NO_{42}$ $[M + H]^+$, 1542.48; found, 1542.58.

2-Methyl-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)]-{[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→6)]}-2-*O*-acetyl-β-D-mannopyranosyl-(1→4)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline (14). To a solution of **13** (214 mg, 0.14 mmol) in CH_2Cl_2 (21 mL) mL) containing activated 4 Å molecular sieves (1.7 g) were added 2,4,6-collidine (365 μL, 2.8 mmol), TMSBr

(357 μL, 2.8 mmol), and $BF_3 \cdot OEt_2$ (347 μL, 2.8 mmol). The reaction mixture was stirred at room temperature for 24 h. The mixture was diluted with CH_2Cl_2, filtered through a Celite pad, washed sequentially with saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. The residue was chromatographed (hexanes/EtOAc, 1:1 to 1:4) to give **14** (137 mg, 67%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.91 (d, 1H, J = 7.4 Hz), 5.55 (d, 1H, J = 2.3 Hz), 5.36−5.16 (m, 10H), 5.10 (d, 1H, J = 1.0 Hz), 5.02 (s, 1H), 4.98 (d, 1H, J = 0.9 Hz), 4.79 (s, 1H), 4.37−3.89 (m, 16H), 3.67 (m, 1H), 3.60 (d, 1H, J = 8.7 Hz), 3.41 (m, 1H), 2.20, 2.15, 2.12, 2.12, 2.11, 2.11, 2.10, 2.09, 2.09, 2.05, 2.04 (11s, 33H), 2.03 (d, 3H), 2.01, 1.98, 1.96, 1.96 (4s, 12H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.56, 170.47, 170.34, 169.75, 169.70, 169.67, 169.64, 169.58, 169.52, 169.40, 165.86, 99.55, 99.05, 98.93, 98.11, 78.33, 77.20, 76.45, 74.71, 74.00, 70.18, 69.52, 69.49, 69.39, 69.32, 69.18, 69.05, 68.94, 68.49, 68.36, 67.81, 67.18, 65.98, 65.81, 65.58, 64.43, 63.39, 62.36, 62.15, 62.01, 20.87, 20.80, 20.70, 20.62, 20.58, 20.54, 13.49. ESI-MS: calcd for $C_{62}H_{84}NO_{40}$ $[M + H]^+$, 1482.46; found, 1482.37.

2-Methyl-[α-D-mannopyranosyl-(1→3)-[α-D-mannopyranosyl-(1→4)]-{[α-D-mannopyranosyl-(1→6)]}-β-D-mannopyranosyl-(1→4)-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline (3). To a solution of **14** (98 mg, 66 μmol) in MeOH (6 mL) was added MeONa in MeOH (0.5 M, 13.2 μL, 6.6 μmol). After being stirred at room temperature overnight, the reaction mixture was concentrated to dryness. The residue was dissolved in water and lyophilized to give the oxazoline **3** (56 mg, quantitative). ^{1}H NMR (400 MHz, D_2O) δ 5.96 (d, 1H, J = 7.4 Hz), 5.00 (d, 1H, J = 1.4 Hz), 4.94 (s, 1H), 4.88 (d, 1H, J = 1.9 Hz), 4.60 (s, 1H), 4.23 (dd, 1H, J = 3.0, 1.6 Hz), 4.08−4.05 (m, 2H), 3.91−3.48 (m, 26H), 3.27 (m, 1H), 1.94 (d, 3H, J = 1.8 Hz); ^{13}C NMR (100 MHz, D_2O) δ 168.67, 102.92, 101.94, 101.02, 100.07, 99.97, 82.39, 77.92, 73.91, 73.75, 73.55, 72.86, 70.97, 70.54, 70.40, 70.20, 69.97, 69.17, 66.84, 66.50, 66.34, 65.25, 61.77, 61.09, 60.99, 60.94, 13.01. ESI-MS: calcd for $C_{32}H_{54}NO_{25}$ $[M + H]^+$, 852.30; found, 852.40.

Benzyl 2-*O*-Benzyl-4,6-*O*-benzylidene-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (15). To a solution of **8** (2.08 g, 2.22 mmol) in a mixture of CH_2Cl_2/H_2O (17.5/1, 74 mL) was added DDQ (1.16 g, 5.11 mmol) at 0 °C. After 30 min, the reaction mixture was warmed to room temperature and further stirred for 1 h. The reaction mixture was diluted with CH_2Cl_2, washed with saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. Concentration and purification by column chromatography on silica gel (hexanes/EtOAc, 10:1 to 3:1) provided **15** (1.46 g, 80%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.47−7.25 (m, 25H), 5.44 (s, 1H), 5.05 (d, 1H, J = 10.1 Hz), 4.94 (d, 1H, J = 11.9 Hz), 4.92 (d, 1H, J = 11.4 Hz), 4.73 (d, 1H, J = 12.3 Hz), 4.69 (d, 1H, J = 12.4 Hz), 4.66−4.61 (m, 2H), 4.58 (s, 1H), 4.47 (d, 1H, J = 11.9 Hz), 4.30 (d, 1H, J = 7.8 Hz), 4.07 (dd, 1H, J = 10.3, 4.8 Hz), 4.01 (t, 1H, J = 9.2 Hz), 3.73−3.64 (m, 4H), 3.56−3.44 (m, 3H), 3.35−3.30 (m, 2H), 3.08 (m, 1H), 2.32 (d, 1H, J = 8.7 Hz); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.36, 138.00, 137.46, 137.16, 136.71, 129.08, 128.59, 128.43, 128.21, 128.15, 128.12, 127.98, 127.94, 127.92, 127.86, 127.55, 126.25, 101.92, 101.57, 100.34, 81.50, 79.02, 78.84, 77.49, 75.77, 75.14, 74.75, 73.68, 70.87, 70.79, 68.35, 68.15, 66.88, 65.67.

Benzyl 2,4-Di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (16). A 1 M solution of $BH_3 \cdot$THF in THF (6 mL, 6 mmol) was added to compound **15** (980 mg, 1.20 mmol) at room temperature. The mixture was stirred for 5 min, and copper(II) trifluoromethanesulfonate (43 mg, 0.119 mmol) was added to the solution. After being stiredg for 5 h, the mixture was cooled down to 0 °C, and the reaction was quenched by sequential additions of Et_3N (167 μL, 1.2 mmol) and MeOH (2.2 mL). The resulting mixture was concentrated under reduced pressure followed by coevaporation with MeOH. The residue was purified by flash column chromatography (hexanes/EtOAc, 3:1) on silica gel to give **16** (718 mg, 73%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.42−7.25 (m, 25H), 5.05 (d, 1H, J = 10.5 Hz), 4.95−4.92 (m, 2H), 4.81 (d, 1H, J = 11.0 Hz), 4.71−4.66 (m, 3H), 4.59 (d, 1H, J = 11.4 Hz), 4.57 (d, 1H, J = 11.0 Hz), 4.49 (d, 1H, J = 12.4 Hz), 4.50 (s, 1H), 4.31 (d, 1H, J = 8.2 Hz), 3.94 (t, 1H, J = 9.4 Hz), 3.71 (dd, 1H, J = 11.0, 1.9 Hz), 3.66 (dd, 1H, J = 11.0, 3.2 Hz), 3.65−3.57 (m, 2H), 3.52−3.42 (m, 3H), 3.38−3.30 (m, 3H), 3.05 (m, 1 H), 2.28 (m, 1 H), 1.59 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.35, 138.20, 138.05, 137.40, 136.73, 128.55, 128.50, 128.43, 128.38, 128.33, 128.05, 127.94, 127.89, 127.77, 127.65, 127.19, 100.97, 100.32, 81.37, 78.32, 77.17, 76.40, 75.25, 74.92, 74.82, 74.64, 74.07, 73.67, 70.77, 68.12, 65.80, 62.02.

Benzyl 6-*O*-Acetyl-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[6-*O*-acetyl-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (18). A solution of compound **16** (76 mg, 93 μmol) and 2,3,4-tri-*O*-acetyl-6-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate **17**[25] (315 mg, 464 μmol) in CH_2Cl_2 (3.9 mL) containing activated 4 Å molecular sieves (469 mg) was stirred under an atmosphere of argon at room temperature for 15 min. After the mixture was cooled to −40 °C, a solution of TMSOTf in CH_2Cl_2 (1 M, 46 μL, 46 μmol) was added and the resulting mixture was stirred at room temperature overnight. The mixture was filtered through a Celite pad. The filtrate was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated and the residue was subjected to flash silica gel column chromatography (hexanes/EtOAc, 5:1 to 3:1) to provide **18** (165 mg, 96%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.11−7.13 (m, 55H), 5.98−5.87 (m, 3H), 5.84−5.79 (m, 2H), 5.54 (d, 1H, J = 1.9 Hz), 5.37 (s, 1H), 5.20 (d, 1H, J = 12.3 Hz), 5.85−5.06 (m, 2H), 4.99 (d, 1H, J = 10.5 Hz), 4.92 (d, 1H, J = 11.9 Hz), 4.77 (d, 1H, J = 12.4 Hz), 4.73−4.64 (m, 3H), 4.60 (s, 1H), 4.52 (d, 1H, J = 11.9 Hz), 4.35 (d, 1H, J = 8.2 Hz), 4.29−4.23 (m, 2H), 4.18−4.11 (m, 3H), 4.06−4.00 (m, 2H), 3.94 (m, 1H), 3.85−3.61 (m, 6H), 3.44 (m, 1H), 3.40−3.26 (m, 3H), 2.06 (s, 3H), 2.00 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.69, 170.36, 165.45, 165.40, 165.33, 165.09, 164.97, 138.52, 138.47, 137.72, 137.45, 136.93, 133.58, 133.44, 133.38, 133.23, 133.19, 132.90, 129.82, 129.80, 129.76, 129.70, 129.44, 129.22, 129.14, 129.02, 128.77, 128.57, 128.54, 128.51, 128.46, 128.34, 128.29, 128.25, 128.12, 127.99, 127.93, 127.83, 127.46, 127.35, 126.98, 100.78, 100.34, 99.84, 97.89, 83.24, 81.02, 78.33, 76.14, 75.67, 75.49, 74.95, 74.65, 74.39, 74.30, 73.69, 70.85, 70.35, 70.01, 69.94, 69.67, 69.17, 68.94, 68.60, 68.25, 66.75, 66.41, 65.36, 62.78, 62.27, 20.67, 20.58.

Benzyl 2,3,4-Tri-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (19). To a solution of **18** (124 mg, 67 μmol) in 1:2 (v/v) CH_2Cl_2-MeOH (12 mL) was added acetyl chloride (0.4 mL). After being stirred overnight, the mixture was concentrated under reduced pressure, and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc, 3:1 to 1:1) to give **19** (107 mg, 90%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.08−7.12 (m, 55H), 6.04 (dd, 1H, J = 10.1, 3.2 Hz), 5.89 (dd, 1H, J = 10.1, 3.2 Hz), 5.82 (dd, 1H, J = 3.5, 1.7 Hz), 5.79−5.73 (m, 2H), 5.58 (dd, 1H, J = 3.2, 1.8 Hz), 5.39 (d, 1H, J = 1.4 Hz), 5.15 (d, 1H, J = 1.8 Hz), 5.13 (d, 1H, J = 12.4 Hz), 5.07−5.01 (m, 2H), 4.95 (d, 1H, J = 11.9 Hz), 4.77 (d, 1H, J = 11.0 Hz), 4.72 (d, 1H, J = 9.6 Hz), 4.69−4.65 (m, 2H), 4.62 (s, 1H), 4.51 (d, 1H, J = 12.4 Hz), 4.33 (d, 1H, J = 8.2 Hz), 4.28 (t, 1H, J = 9.4 Hz), 3.97 (m, 1H), 3.90−3.86 (m, 2H), 3.82 (t, 1H, J = 9.4 Hz), 3.73−3.54 (m, 8H), 3.48−3.27 (m, 5H), 2.85 (m, 1H), 2.40 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 166.39, 166.25, 165.36, 165.33, 165.20, 138.48, 138.44, 137.58, 137.49, 136.99, 133.65, 133.54, 133.33, 133.18, 132.87, 129.88, 129.83, 129.67, 129.34, 129.22, 129.15, 129.06, 128.93, 128.59, 128.55, 128.49, 128.44, 128.39, 128.36, 128.28, 128.24, 128.17, 128.07, 128.03, 127.95, 127.81, 127.75, 127.71, 127.42, 127.34, 127.21, 100.74,

100.43, 99.86, 97.78, 82.62, 81.23, 78.06, 76.12, 75.67, 75.44, 74.80, 74.70, 74.38, 74.33, 73.79, 71.56, 71.23, 70.78, 70.53, 70.35, 69.80, 69.40, 68.11, 67.98, 67.26, 67.12, 65.32, 61.33, 61.04.

Benzyl 2,3,4,6-Tetra-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-*β*-D-mannopyranosyl-(1→4)-2-azido-3,6-di-*O*-benzyl-2-deoxy-*β*-D-glucopyranoside (21). A mixture of compound **19** (24 mg, 14 μmol) and 2,3,4,6-tetra-*O*-benzoyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl trichloroacetimidate **20**[26] (83 mg, 68 μmol) in CH_2Cl_2 (1.1 mL) containing activated 4 Å molecular sieves (132 mg) was stirred under an atmosphere of argon at −40 °C for 30 min. A solution of TMSOTf in CH_2Cl_2 (1 M, 6.8 μL, 6.8 μmol) was added and the resulting mixture was stirred at room temperature overnight. The mixture was filtered through a Celite pad. The filtrate was poured into saturated $NaHCO_3$ and extracted with CH_2Cl_2. The organic layer was washed with brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated and the residue was subjected to flash silica gel column chromatography (hexanes/EtOAc, 3:1 to 1:1) to provide **21** (34 mg, 65%) as a white amorphous. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.11−6.85 (m, 125H), 5.98−5.51 (m, 14H), 5.33−5.29 (m, 2H), 5.05−4.35 (m, 23H), 4.20−3.34 (m, 26H), 3.16 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.74, 165.67, 165.59, 165.47, 165.33, 165.27, 165.24, 165.16, 165.09, 164.99, 164.96, 164.68, 138.84, 138.81, 138.31, 137.81, 137.10, 133.44, 133.30, 133.22, 133.17, 133.00, 132.92, 132.84, 132.70, 129.91, 129.84, 129.75, 129.60, 129.53, 129.40, 129.34, 129.28, 129.21, 129.17, 129.12, 128.86, 128.82, 128.74, 128.68, 128.65, 128.49, 128.36, 128.32, 128.20, 128.16, 128.12, 128.10, 128.00, 127.95, 127.79, 127.71, 127.49, 127.38, 127.10, 127.04, 126.88, 102.09, 101.44, 100.93, 100.84, 100.31, 98.92, 97.47, 81.19, 80.53, 78.46, 77.20, 76.18, 75.85, 75.01, 74.93, 74.79, 74.44, 74.05, 73.28, 72.91, 72.80, 72.72, 71.80, 71.61, 71.54, 71.26, 70.93, 70.72, 70.13, 70.04, 69.76, 69.54, 69.34, 68.91, 68.32, 67.43, 67.17, 67.05, 66.96, 66.86, 65.61, 62.67, 62.36, 60.99, 60.37.

Benzyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-*β*-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-*β*-D-glucopyranoside (22). Compound **21** (148 mg, 38 μmol) was dissolved in 1:1 (v/v) CH_2Cl_2-MeOH (8 mL), and a solution of 0.5 M MeONa in MeOH (0.4 mL, 0.2 mmol) was added. After being stirred at room temperature overnight, the solution was neutralized with Dowex 50W (H^+), filtered, and concentrated. The residue was dissolved in dry pyridine (5 mL) and treated with Ac_2O (5 mL) at room temperature overnight. The mixture was evaporated to dryness under reduced pressure, and the residue was diluted with CH_2Cl_2, washed sequentially with 1 M HCl, saturated $NaHCO_3$ and brine, dried over $MgSO_4$, filtered, and concentrated. To the residue were added $CHCl_3$ (1 mL), pyridine (1 mL), and thioacetic acid (2 mL), and the mixture was stirred at room temperature for 48 h. Evaporation of the solvent under reduced pressure was followed by silica gel column chromatography (hexanes/EtOAc, 2:5 to 1:3) to yield **22** (80 mg, 79%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 7.43−7.18 (m, 25H), 6.11 (d, 1H, J = 7.8 Hz), 5.35−4.33 (m, 38H), 4.13−3.41 (m, 26H), 3.35 (m, 1H), 3.25 (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.25, 170.20, 170.15, 170.06, 169.96, 169.73, 169.65, 169.57, 169.55, 169.46, 168.92, 138.75, 138.30, 138.21, 137.64, 137.49, 128.33, 128.31, 128.17, 127.99, 127.94, 127.69, 127.61, 127.52, 127.46, 127.23, 101.21, 100.92, 100.62, 100.26, 99.64, 98.49, 97.12, 79.61, 78.44, 77.11, 76.65, 76.14, 76.06, 75.49, 75.37, 75.06, 74.96, 74.59, 73.19, 72.73, 72.60, 72.25, 72.16, 71.24, 71.05, 70.90, 70.85, 70.53, 70.49, 70.15, 69.73, 69.52, 69.46, 69.15, 69.00, 68.97, 68.73, 68.57, 67.30, 66.87, 66.49, 66.38, 65.83, 65.38, 61.79, 60.67, 60.51, 52.70, 23.04, 20.72, 20.54, 20.42.

2,3,4,6-Tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-acetyl-*β*-D-mannopyranosyl-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-D-glucopyranose (23). To a solution of **22** (37 mg, 14 μmol) in 25:30:1 (v/v/v) CH_2Cl_2−MeOH−AcOH (1.2 mL) was added 20% palladium(II) hydroxide on activated carbon (37 mg). The reaction mixture was vigorously stirred at room temperature under hydrogen atmosphere for 24 h. The mixture was filtered through Celite and the filtrate was concentrated in vacuo. Pyridine (5 mL) and Ac_2O (5 mL) were added, and the mixture was stirred at room temperature overnight. The mixture was concentrated, diluted with CH_2Cl_2, and washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and concentrated. Silica gel column chromatography (CH_2Cl_2/MeOH, 40:1) of the residue afforded **23** (26 mg, 77%, α/β = 90/10) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS, selected signals) δ 6.04 (d, 0.90H, J = 3.6 Hz), 5.75 (d, 0.10H, J = 9.2 Hz), 5.61 (d, 0.90H, J = 9.2 Hz), 5.56 (d, 0.10H, J = 8.7 Hz), 2.18−1.91 (m, 78H); ^{13}C NMR (100 MHz, $CDCl_3$, for the major α-anomer) δ 171.17, 170.60, 170.12, 170.09, 170.00, 169.93, 169.82, 169.64, 169.49, 169.39, 169.29, 168.91, 168.79, 168.72, 101.18, 100.84, 100.73, 100.55, 97.05, 96.59, 96.26, 90.55, 75.95, 75.71, 72.58, 72.35, 72.28, 71.73, 71.07, 71.01, 70.79, 70.73, 70.39, 70.21, 69.73, 69.49, 69.34, 69.20, 68.91, 67.71, 67.29, 66.55, 66.39, 65.84, 65.37, 61.74, 61.52, 60.56, 60.37, 50.18, 22.70, 20.56, 20.39, 20.28. ESI-MS: calcd for $C_{100}H_{136}NO_{66}$ $[M + H]^+$, 2406.73; found, 2307.06.

2-Methyl-[2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl-(1→3)-[2,3,4,6-tetra-*O*-acetyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-acetyl-*β*-D-glucopyranosyl-(1→6)-2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-*β*-D-mannopyranosyl-(1→4)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline (24). In a manner similar to the preparation of the oxazoline derivative **14**, compound **23** (10 mg, 4.3 μmol) was converted to **24** (3.5 mg, 35%). ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.92 (d, 1H, J = 6.8 Hz), 5.55 (t, 1H, J = 3.2 Hz), 5.36−4.88 (m, 20H), 4.83 (d, 1H, J = 1.4 Hz), 4.52−4.44 (m, 5H), 4.38 (d, 1H, J = 7.7 Hz), 4.26−3.43 (m, 26H), 3.37 (m, 1H), 2.22−1.94 (m, 75H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.80, 170.53, 170.31, 170.11, 170.00, 169.91, 169.68, 169.63, 169.57, 169.50, 169.41, 169.04, 166.03, 101.00, 100.97, 100.90, 100.82, 99.67, 98.93, 97.81, 97.21, 76.17, 75.97, 74.19, 72.68, 72.57, 72.26, 71.29, 71.24, 70.96, 70.56, 70.18, 70.10, 69.93, 69.59, 69.24, 69.11, 69.03, 68.57, 68.45, 67.91, 67.73, 66.99, 66.54, 65.99, 64.83, 63.71, 61.84, 60.73, 60.60, 20.74, 20.57, 20.45, 13.80. ESI-MS: calcd for $C_{98}H_{132}NO_{64}$ $[M + H]^+$, 2346.71; found, 2346.65.

2-Methyl-[*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-α-D-mannopyranosyl-(1→3)-[*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-α-D-mannopyranosyl-(1→6)]-*β*-D-mannopyranosyl-(1→4)-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline (4). In the same manner as described for the conversion of **14** to **3**, de-O-acetylation of compound **24** (3.5 mg, 1.5 μmol) with a catalytic amount of MeONa in MeOH gave compound **4** (2.0 mg) in quantitative yield. ^{1}H NMR (400 MHz, D_2O) δ 5.98 (d, 1H, J = 7.3 Hz), 4.97 (s, 1H), 4.83 (s, 1H), 4.63 (s, 1H), 4.43 (d, 1H, J = 7.7 Hz), 4.42 (d, 1H, J = 7.8 Hz), 4.33 (d, 1H, J = 7.8 Hz), 4.32 (d, 1H, J = 7.3 Hz), 4.28 (m, 1H), 4.09−3.18 (m, 47H), 1.97 (s, 3H); ^{13}C NMR (100 MHz, D_2O) δ 168.62, 102.98, 102.67, 102.56, 102.54, 101.34, 99.92, 99.80, 81.12, 78.50, 78.42, 77.79, 75.39, 74.82, 74.79, 74.29, 74.23, 73.05, 72.86, 72.55, 72.27, 71.69, 70.99, 70.42, 70.24, 70.00, 69.85, 69.07, 68.78, 68.58, 66.51, 66.43, 65.83, 65.69, 65.12, 62.51, 61.89, 61.06, 60.98, 60.11, 13.10. ESI-MS: calcd for $C_{50}H_{84}NO_{40}$ $[M + H]^+$, 1338.46; found, 1338.85.

Benzyl 6-*O*-Acetyl-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[6-*O*-acetyl-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (25). To a solution of **18** (200 mg, 0.108 mmol) in $CHCl_3$ (2 mL) were consecutively added pyridine (2 mL) and thioacetic acid (2 mL) at room temperature. After being stirred for 48 h, the mixture was concentrated and purified by flash column chromatography on silica gel to afford **25** (172 mg, 85%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.13−7.13 (m, 55H), 5.99−5.90 (m, 3H), 5.82−5.79 (m, 2H), 5.61 (t, 1H, J = 2.3 Hz), 5.36 (d, 1H, J = 0.9 Hz), 5.28−5.25 (m, 2H), 5.22 (d, 1H, J = 12.8 Hz), 5.07 (d, 1H, J = 11.0 Hz), 5.02 (d, 1H, J = 7.8 Hz), 4.87 (d, 1H, J = 11.5 Hz), 4.82 (d, 1H, J = 11.9 Hz), 4.76 (d, 1H, J = 12.4 Hz), 4.70 (d, 1H, J = 11.0 Hz), 4.66 (s, 1H), 4.65 (d, 1H, J = 11.9 Hz), 4.55 (d, 1H, J = 10.9 Hz), 4.51 (d, 1H, J = 11.9 Hz), 4.46 (d, 1H, J = 11.9 Hz), 4.27−4.08 (m, 8H), 3.94 (d, 1H, J = 3.2 Hz), 3.85−3.72 (m, 4H), 3.71−3.66 (m, 2H), 3.47−3.38 (m, 2H), 3.35 (m, 1H), 2.09 (s, 3H), 1.99 (s, 3H), 1.58 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.75, 170.31, 170.04, 165.55, 165.44, 165.39, 165.32, 165.10, 164.77, 138.80, 138.54, 137.95, 137.73, 137.44, 133.55, 133.42, 133.37, 133.29, 133.15, 133.07, 129.81, 129.75, 129.68, 129.61, 129.20, 129.00, 128.96, 128.80, 128.75, 128.55, 128.50, 128.46, 128.39, 128.34, 128.30, 128.26, 128.19, 128.12, 128.08, 127.95, 127.78, 127.60, 127.50, 127.41, 127.31, 126.95, 100.28, 99.82, 99.32, 97.33, 83.11, 78.30, 77.20, 77.15, 75.50, 75.38, 74.82, 74.52, 74.26, 73.56, 73.21, 70.98, 70.34, 70.15, 70.10, 69.69, 69.19, 69.11, 68.80, 68.26, 66.70, 66.49, 62.82, 62.40, 56.62, 23.28, 20.67, 20.56.

Benzyl 2,3,4-Tri-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (26). The title compound was prepared from **25** (85 mg, 46 μmol) in a manner similar to that described for **19**. Flash silica gel column chromatography (hexanes/EtOAc, 1:1 to 2:3) of the crude product afforded **26** (59 mg, 72%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.13−7.08 (m, 55H), 6.05 (dd, 1H, J = 10.1, 3.2 Hz), 5.92 (t, 1H, J = 10.1 Hz), 5.85−5.81 (m, 3H), 5.61 (t, 1H, J = 2.6 Hz), 5.41 (d, 1H, J = 1.4 Hz), 5.37 (d, 1H, J = 1.4 Hz), 5.14 (d, 1H, J = 12.4 Hz), 5.04 (d, 1H, J = 11.0 Hz), 5.03 (d, 1H, J = 7.3 Hz), 4.90 (d, 1H, J = 11.9 Hz), 4.88 (d, 1H, J = 11.0 Hz), 4.77 (d, 1H, J = 12.4 Hz), 4.69−4.63 (m, 3H), 4.49 (d, 1H, J = 10.9 Hz), 4.48 (d, 1H, J = 12.4 Hz), 4.47 (d, 1H, J = 11.9 Hz), 4.31 (t, 1H, J = 8.5 Hz), 4.21 (t, 1H, J = 8.7 Hz), 4.07 (m, 1H), 3.97 (m, 1H), 3.88−3.70 (m, 7H), 3.65−3.55 (m, 4H), 3.49 (m, 1H), 3.37 (m, 1H), 3.30−3.21 (m, 2H), 2.38 (dd, 1H, J = 7.8, 6.0 Hz), 1.45 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.16, 166.35, 165.76, 165.54, 165.39, 165.19, 164.84, 138.74, 138.35, 137.78, 137.55, 137.37, 133.63, 133.51, 133.17, 132.96, 129.84, 129.70, 129.64, 129.11, 129.02, 128.94, 128.56, 128.50, 128.42, 128.33, 128.26, 128.20, 127.98, 127.94, 127.80, 127.57, 127.45, 127.27, 100.44, 99.77, 99.08, 97.56, 82.29, 78.06, 76.67, 76.60, 75.96, 75.50, 74.83, 74.44, 74.25, 73.61, 73.34, 71.53, 71.47, 70.99, 70.49, 70.36, 70.23, 69.42, 68.74, 68.40, 66.99, 61.23, 60.92, 56.73, 23.18.

Benzyl 6-Azido-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→3)-[6-azido-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (27). To a solution of **26** (304 mg, 0.171 mmol) in pyridine (2 mL) was added TsCl (195 mg, 1.02 mmol). The mixture was stirred overnight at room temperature. The reaction mixture was then concentrated, diluted with CH_2Cl_2, and washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$. After removal of solvent, the tosylated crude product was dissolved in DMF (5 mL), and NaN_3 (222 mg, 3.41 mmol) was added. The reaction solution was stirred at 80 °C overnight. After removal of DMF, the residue was poured into brine and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and concentrated. Silica gel column chromatography (hexanes/EtOAc, 2:1 to 1:1) of the residue afforded **27** (261 mg, 84%) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 8.09−7.16 (m, 55H), 5.95 (dd, 1H, J = 10.3, 3.5 Hz), 5.85−5.76 (m, 4H), 5.64 (s, 1H), 5.44 (d, 1H, J = 7.8 Hz), 5.40 (s, 1H), 5.21−5.18 (m, 2H), 5.05 (d, 1H, J = 11.0 Hz), 5.00 (d, 1H, J = 7.8 Hz), 4.92−4.86 (m, 2H), 4.87 (d, 1H, J = 11.9 Hz), 4.76 (s, 1H), 4.71 (d, 1H, J = 11.0 Hz), 4.64−4.59 (m, 2H), 4.53 (d, 1H, J = 12.3 Hz), 4.49 (d, 1H, J = 11.9 Hz), 4.19−4.10 (m, 2H), 4.02 (d, 1H, J = 2.8 Hz), 3.91−3.73 (m, 6H), 3.59−3.35 (m, 5H), 3.29−3.24 (m, 2H), 1.61−1.57 (m, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.00, 165.49, 165.47, 165.26, 165.10, 164.95, 138.88, 138.51, 138.05, 137.81, 137.45, 133.56, 133.51, 133.37, 133.14, 133.04, 129.82, 129.76, 129.63, 129.60, 129.32, 129.06, 128.94, 128.92, 128.81, 128.71, 128.54, 128.44, 128.24, 128.13, 128.11, 128.04, 127.99, 127.87, 127.77, 127.64, 127.56, 127.42, 127.26, 100.47, 99.70, 99.36, 97.43, 82.14, 78.06, 77.14, 76.69, 75.42, 75.04, 74.91, 74.37, 73.37, 73.00, 70.90, 70.79, 70.27, 70.02, 69.97, 69.93, 69.42, 69.20, 67.98, 67.56, 67.50, 55.96, 51.19, 50.88, 23.28.

Benzyl 6-Azido-α-D-mannopyranosyl-(1→3)-[6-Azido-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-benzyl-β-D-mannopyranosyl-(1→4)-2-acetamido-3,6-di-*O*-benzyl-2-deoxy-β-D-glucopyranoside (28). Compound **27** (262 mg, 0.143 μmol) was dissolved in 1:4 (v/v) CH_2Cl_2−MeOH (20 mL), and a solution of 0.5 M MeONa in MeOH (0.8 mL, 0.4 mmol) was added. After being stirred at room temperature for 2 h, the solution was neutralized with Dowex 50W (H^+), filtered, and concentrated. The residue was subjected to flash silica gel column chromatography (CH_2Cl_2/MeOH, 10:1) to give **28** (146 mg, 85%) as a white amorphous solid. ^{1}H NMR (400 MHz, CD_3OD, selected signals) δ 7.42−7.14 (m, 25H), 1.80 (s, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 173.40, 140.56, 140.29, 139.49, 139.44, 139.17, 129.77, 129.62, 129.45, 129.41, 129.33, 129.27, 129.22, 129.16, 129.09, 129.04, 128.95, 128.83, 128.75, 128.54, 128.42, 104.16, 101.83, 101.38, 82.54, 81.62, 80.23, 78.08, 76.69, 76.13, 76.10, 76.02, 75.97, 75.46, 74.86, 74.48, 73.54, 72.58, 72.48, 72.32, 71.99, 71.88, 69.90, 69.58, 69.47, 67.51, 56.45, 53.36, 52.79, 23.21. ESI-MS: calcd for $C_{61}H_{74}N_7O_{19}$ $[M + H]^+$, 1208.50; found, 1208.90.

2,3,4-Tri-*O*-acetyl-6-azido-α-D-mannopyranosyl-(1→3)-[2,3,4-tri-*O*-acetyl-6-azido-α-D-mannopyranosyl-(1→6)]-2,4-di-*O*-acetyl-β-D-mannopyranosyl-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-D-glucopyranose (31). The title compound was prepared via a three-step procedure:

(1) To a solution of **28** (100 mg, 83 μmol) in MeOH (5 mL) was added 20% palladium(II) hydroxide on activated carbon (100 mg). The reaction mixture was vigorously stirred at room temperature under hydrogen atmosphere for 7 h. After addition of H_2O (5 mL), the reaction mixture was stirred overnight at room temperature under hydrogen atmosphere. The mixture was filtered through a Celite pad and the filtrate was concentrated in vacuo to give 6-amino-α-D-mannopyranosyl-(1→3)-[6-amino-α-D-mannopyranosyl-(1→6)]-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose **29**: ESI-MS: calcd for $C_{26}H_{48}N_3O_{19}$ $[M + H]^+$, 706.29; found, 706.40. Compound **29** was used in the next step without further purification.

(2) Compound **29** was dissolved in H_2O (4 mL) and treated with K_2CO_3 (91 mg, 0.66 mmol) and $CuSO_4$ (6.0 mg, 37 μmol). To the solution was added MeOH (9.4 mL) and a freshly prepared solution of trifluoromethanesulfonyl azide (0.53 mmol)[36] in CH_2Cl_2 (4 mL). After being stirred at room temperature for 24 h, the reaction mixture was concentrated to afford crude 6-azido-α-D-mannopyranosyl-(1→3)-[6-amino-α-D-mannopyranosyl-(1→6)]-β-D-mannopyranosyl-(1→4)-2-acetamido-2-deoxy-D-glucopyranose **30**: ESI-MS: calcd for $C_{26}H_{43}KN_7O_{19}$ $[M + K]^+$ 796.23; found, 796.36. Compound **30** was used directly in the next step.

(3) Compound **30** was acetylated with acetic anhydride (10 mL) in pyridine (10 mL) at room temperature for overnight. The mixture

(36) Alper, P. B.; Hung, S. C.; Wong, C.-H. *Tetrahedron Lett.* **1996**, *37*, 6029–6032.

was concentrated, diluted with CH_2Cl_2, and washed sequentially with 1 M HCl, saturated $NaHCO_3$, and brine. The organic layer was dried over $MgSO_4$ and concentrated. Silica gel column chromatography (CH_2Cl_2/MeOH, 60:1) of the residue afforded **31** (61 mg, 61% from **28**, α/β = 38/62) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$, TMS, selected signals) δ 6.07 (d, 0.38H, J = 3.6 Hz), 5.96 (d, 0.62H, J = 9.6 Hz), 5.73 (d, 0.38H, J = 9.1 Hz), 5.61 (d, 0.62H, J = 8.7 Hz), 2.22−1.92 (m, 36H); ^{13}C NMR (100 MHz, $CDCl_3$, selected signals) δ 171.09, 170.65, 170.39, 170.36, 170.09, 170.03, 169.99, 169.88, 169.78, 169.61, 169.49, 169.34, 168.91, 168.85, 98.13, 97.65, 97.25, 96.63, 96.55, 96.19, 92.37, 90.40, 22.73, 22.64, 20.71, 20.58, 20.53, 20.48, 20.36. ESI-MS: calcd for $C_{48}H_{66}N_7O_{30}$ $[M + H]^+$, 1220.39; found, 1220.47.

2-Methyl-[2,3,4-tri-*O*-acetyl-6-azido-α-D-mannopyranosyl-(1→3)-[2,3,4-tri-*O*-acetyl-6-azido-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-glucopyrano]-[2,1-*d*]-2-oxazoline (32). Compound **32** (26 mg, 52%) was obtained from **31** (53 mg, 43 μmol) following the procedure described for **14**. ^{1}H NMR (400 MHz, $CDCl_3$, TMS) δ 5.92 (d, 1H, J = 6.8 Hz), 5.59 (m, 1H), 5.44 (d, 1H, J = 3.2 Hz), 5.30−5.16 (m, 6H), 5.01−4.99 (m, 2H), 4.83 (s, 1H), 4.81 (s, 1H), 4.27−4.06 (m, 5H), 3.93−3.91 (m, 2H), 3.69−3.62 (m, 3H), 3.45−3.26 (m, 5H), 2.24 (s, 3H), 2.16−2.15 (m, 6H), 2.14, 2.11, 2.09, 2.06 (4s, 12H), 2.05−2.04 (m, 3H), 2.02, 1.99, 1.97 (3s, 9H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.72, 170.55, 169.99, 169.87, 169.79, 169.59, 169.56, 169.51, 169.38, 165.96, 99.23, 98.52, 97.33, 76.44, 76.01, 72.66, 70.36, 69.86, 69.78, 69.70, 69.08, 68.69, 67.89, 67.77, 67.38, 66.87, 66.57, 64.57, 63.30, 50.76, 50.60, 20.86, 20.69, 20.53, 13.62. ESI-MS: calcd for $C_{46}H_{62}N_7O_{28}$ $[M + H]^+$, 1160.36; found 1160.41.

2-Methyl-[6-azido-α-D-mannopyranosyl-(1→3)-[6-azido-α-D-mannopyranosyl-(1→6)]-*β*-D-mannopyranosyl-(1→4)-1,2-dideoxy-α-D-glucopyrano]-[2,1-*d*]-2-oxazoline (5). Compound **32** (27 mg, 23 μmol) was de-O-acetylated in a manner similar to the preparation of compound **3** to give the title compound (**5**) (17 mg, quantitative). ^{1}H NMR (400 MHz, D_2O) δ 5.97 (d, 1H, J = 7.3 Hz), 4.96 (d, 1H, J = 1.4 Hz), 4.83 (d, 1H, J = 1.4 Hz), 4.64 (s, 1H), 4.27 (dd, 1H, J = 3.0, 1.6 Hz), 4.08 (m, 1H), 4.04 (d, 1H, J = 3.2 Hz), 3.96 (dd, 1H, J = 3.4, 1.6 Hz), 3.91−3.87 (m, 2H), 3.82−3.39 (m, 17H), 3.28 (m, 1H), 1.95 (d, 3H, J = 1.4 Hz); ^{13}C NMR (100 MHz, D_2O) δ 168.68, 102.72, 101.44, 99.92, 99.68, 81.05, 77.86, 74.36, 72.20, 71.52, 71.01, 70.46, 70.37, 70.13, 69.97, 69.81, 69.05, 67.70, 67.53, 65.56, 65.11, 61.83, 51.37, 51.16, 13.01. ESI-MS: calcd for $C_{26}H_{42}N_7O_{18}$ $[M + H]^+$, 740.26; found, 740.46.

Glycoprotein 36. A mixture of pentasaccharide oxazoline **3** (0.36 mg, 0.42 μmol) and GlcNAc-RB **33**[6] (1.95 mg, 0.14 μmol) in a phosphate buffer (50 mM, pH 6.5, 75 μL) was incubated at 30 °C with Endo-A (20 milliunits). The reaction was monitored by analytical HPLC. After 7 h, additional oxazoline **3** (0.36 mg, 0.42 μmol) and Endo-A (20 milliunits) were added to the solution, and the mixture was incubated at 30 °C. This procedure was repeated twice to push the reaction to completion. The product was then purified by preparative HPLC (23−29% MeCN containing 0.1% TFA in 30 min) to afford the glycoprotein **36** (0.099 μmol, 1.46 mg, 71%). The isolated glycoprotein product was quantified by UV absorbance at 280 nm, with a standard solution (at an accurate molar concentration) of GlcNAc-RB as the reference. ESI-MS of **36**: calcd, M = 14 745; found, 1638.71 $[M + 9H]^{9+}$, 1475.01 $[M + 10H]^{10+}$, 1341.05 $[M + 11H]^{11+}$, 1229.32 $[M + 12H]^{12+}$, 1134.91 $[M + 13H]^{13+}$, and 1053.91 $[M + 14H]^{14+}$.

Glycoprotein 37. Incubation of oxazoline **4** (1.15 mg, 0.86 μmol), GlcNAc-RB **33** (1.0 mg, 0.072 μmol), and Endo-A (20 milliunits) was performed in the same way as described for the preparation of **36** to give glycoprotein **37** (0.028 μmol, 0.42 mg, 38%). ESI-MS of **37**: calcd, M = 15 231; found, 1693.04 $[M + 9H]^{9+}$, 1523.86 $[M + 10H]^{10+}$, 1385.46 $[M + 11H]^{11+}$, 1270.13 $[M + 12H]^{12+}$, 1172.57 $[M + 13H]^{13+}$, and 1088.84 $[M + 14H]^{14+}$.

Glycoprotein 38. Glycoprotein **38** was prepared from **5** (0.64 mg, 0.86 μmol) and GlcNAc-RB **33** (1.0 mg, 0.072 μmol), as described in the preparation of **36**, to give glycoprotein **38** (0.0643 μmol, 0.94 mg, 89%). ESI-MS of **38**: calcd, M = 14 619; found, 1626.58 $[M + 9H]^{9+}$, 1464.00 $[M + 10H]^{10+}$, 1331.10 $[M + 11H]^{11+}$, 1220.23 $[M + 12H]^{12+}$, 1126.47 $[M + 13H]^{13+}$, and 1046.13 $[M + 14H]^{14+}$.

2-[2-(4-Pentynamido)ethoxy]ethyl α-D-galactopyranosyl-(1→3)-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside (41). To a solution of 2-(2-aminoethoxy)-ethyl α-D-galactopyranosyl-(1→3)-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranoside **39**[30] (5.0 mg, 8.5 μmol) in aqueous $NaHCO_3$ (0.3 M, 250 μL) and MeOH (50 μL) was added a solution of *N*-succinimido 4-pentynonate **40**[37] (2.5 mg, 13 μmol) in MeCN (250 μL). The residue was purified by preparative HPLC (0−10% MeCN in 30 min) to give **41** (4.5 mg, 6.7 μmol, 79%). ^{1}H NMR (400 MHz, D_2O) δ 5.03 (d, 1H, J = 3.5 Hz), 4.41−4.39 (m, 2H), 4.09−4.06 (m, 2H), 3.96−3.82 (m, 4H), 3.76−3.46 (m, 16H), 3.32−3.29 (m, 2H), 3.23 (t, 1H, J = 8.3 Hz), 2.41−2.32 (m, 5H); ESI-MS: calcd for $C_{27}H_{46}NO_{18}$ $[M + H]^+$, 672.27; found, 672.45.

Glycoprotein 42. Compound **41** (37.6 μL, 20 mM in H_2O), $CuSO_4$ (9.4 μL, 50 mM in H_2O), bathophenanthrolinedisulfonic acid (18.8 μL, 20 mM in H_2O), and L-ascorbic acid (9.4 μL, 50 mM in H_2O) were added to a solution of glycoprotein **38** (0.55 mg, 0.038 μmol) in Tris buffer (0.17 M, pH 8.0, 113 μL). The reaction mixture was incubated at room temperature overnight. The solution was subjected to preparative HPLC purification (23−29% MeCN containing 0.1% TFA in 30 min) to afford the glycoprotein **42** (0.033 μmol, 0.52 mg, 87%). ESI-MS: calcd MS = 15 976; found, 1775.43 $[M + 9H]^{9+}$, 1598.08 $[M + 10H]^{10+}$, 1453.03 $[M + 11H]^{11+}$, 1331.98 $[M + 12H]^{12+}$, and 1229.71 $[M + 13H]^{13+}$.

Surface Plasmon Resonance Measurements. SPR measurements were performed with a BIACore T100 instrument (GE Healthcare). ConA, PNA, and IgG from human serum were immobilized on CM5 sensor chips in an acetate buffer (10 mM, pH 5.0) by use of the amine coupling kit provided by the manufacturer. Approximately 4000 resonance units (RU) of protein was immobilized. Analyses were performed at 25 °C with a flow rate of 30 mL/min in either HBS-P+ buffer (10 mM HEPES, 150 mM NaCl, and 0.05% surfactant P20, pH 7.4), for PNA and IgG, or HBS-P+ buffer containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, for ConA. Injection times for glycoproteins were 2 min followed by 10 min of dissociation. Regeneration was performed by either a 1-min pulse of 100 mM glycine (pH 1.7) stripping buffer, for ConA and IgG, or a 1-min pulse of 0.3 M lactose, for PNA.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of key synthetic compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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