DOI: 10.1002/chem.200501196

Glycopeptide Synthesis through *endo*-Glycosidase-Catalyzed Oligosaccharide Transfer of Sugar Oxazolines: Probing Substrate Structural Requirement

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Abstract: An array of sugar oxazolines was synthesized and tested as donor substrates for the *Arthrobacter* endo-β-N-acetylglucosaminidase (Endo-A)-catalyzed glycopeptide synthesis. The experiments revealed that the minimum structure of the donor substrate required for Endo-A catalyzed transglycosylation is a Manβ1→4-GlcNAc oxazoline moiety. Replacement of the β-D-Man moiety with β-D-Glc, β-D-Gal, and β-D-GlcNAc monosaccharides resulted in the loss of substrate activity for the disaccharide oxazoline. Despite this, the enzyme could tolerate modifications such as attachment of additional sugar residues or a functional group at the 3- and/or 6-positions of the β-D-Man moiety, thus allowing a successful transfer of selectively modified oligosaccharides to the peptide acceptor. On the other hand, the enzyme has a great flexibility for the acceptor portion and could take both small and large GlcNAc-peptides as the acceptor. The studies implicate a great potential of the endoglycosidase-catalyzed transglycosylation for constructing both natural and selectively modified glycopeptides.

Keywords: carbohydrates · enzymes · glycopeptides · glycosylation

Introduction

Glycoproteins are an important class of biomolecules that play crucial roles in many biological events such as cell adhesion, tumor metastasis, pathogen infection, and immune response.[1] However, a major problem in structural and functional studies of glycoproteins is their structural microheterogeneity. Natural and recombinant glycoproteins are typically produced as a mixture of glycoforms that differ only in the structure of the pendent oligosaccharides. The difficulty to obtain pure glycoforms from natural source has thus motivated chemists to take the challenge of assembling homogeneous glycopeptides (partial structures of glycoproteins) and even glycoproteins themselves by chemical/chemoenzymatic synthesis.[2] The introduction of new techniques, such as native chemical ligation and chemoselective ligation,[3] novel solid-phase synthesis,[4] and enzymatic oligosaccharide transfer[5–7] has significantly expanded our synthetic repertoire for constructing large homogeneous glycopeptides. We are particularly interested in the endo-β-N-acetylglucosaminidase (ENGase)-catalyzed oligosaccharide transfer for glycopeptide synthesis, because it allows the attachment of a large oligosaccharide to a pre-assembled, unprotected GlcNAc-peptide/protein in a single step in a regio- and stereospecific manner, thus providing a highly convergent approach.[6,7] The Endo-A from *Arthrobacter protophormiae*[8] and the Endo-M from *Mucor hiemalis*[9] are two ENGases that possess significant transglycosylation activity and have been widely used for synthesizing large glycopeptides.[6,7] However, ENGases are inherently a class of endoglycosidases that hydrolyze N-glycans by cleaving the β-1,4-glycosidic bond in the *N*,*N*′-diacetylchitobiose core. Like many glycosidase-catalyzed syntheses,[10] ENGase-catalyzed transglycosylation suffers from the inherent low transglycosylation yield and product hydrolysis. In addition, the ENGase-catalyzed synthesis has been limited thus far to the use of only natural N-glycans as the donor substrates, which themselves are difficult to obtain. To address these problems, we have recently communicated the use of synthetic oligosaccharide oxazolines, the presumed transition state analogues, as donor substrates for ENGase-catalyzed glycopeptide synthesis, which significantly enhanced the transglycosylation yield and also broadened the substrate availability by adopting synthetic substrates.[11] The method was based on the assumption that the ENGase-catalyzed reaction proceeds via a mechanism of the substrate-assisted cat-

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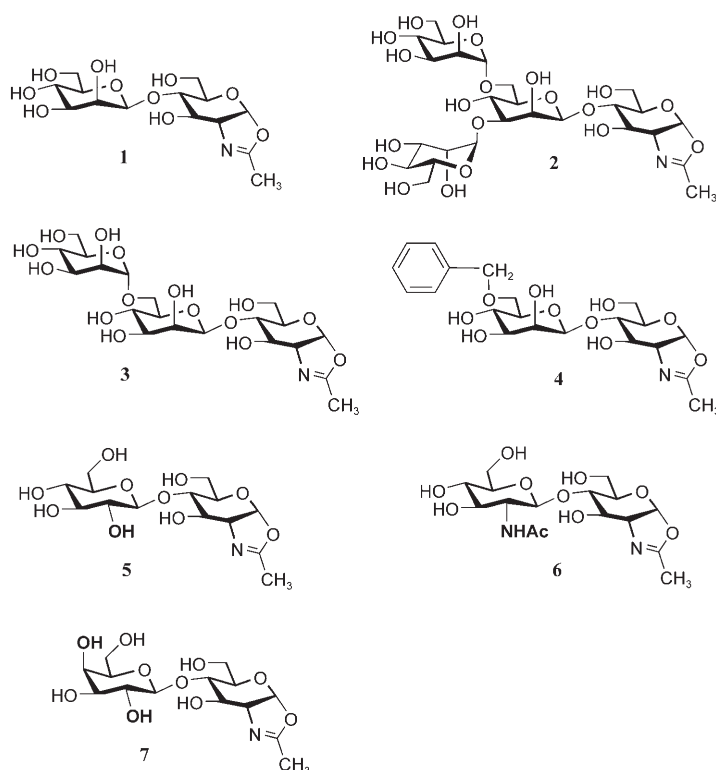
alysis involving an oxazolinium ion intermediate, as demonstrated for some chitinases[12] and N-acetyl-β-hexosaminidases.[13] Fujita and co-workers have first demonstrated that Endo-A and Endo-M did take a disaccharide oxazoline of Manβ1,4GlcNAc as a substrate for enzymatic transglycosylation.[14] There are also precedents using disaccharide oxazolines for chitinase- and hyaluronidase-catalyzed synthesis of oligo- and polysaccharides, respectively.[15–17] Our preliminary studies have shown that the di- and tetrasaccharide oxazolines corresponding to the N-glycan core structure could serve as donor substrates for a highly efficient chemoenzymatic synthesis of large N-glycopeptides.[11] To further explore the potential of this promising chemoenzymatic method, we describe in this paper the synthesis and evaluation of an array of oligosaccharide oxazolines (Scheme 1) as donor substrates for glycopeptide synthesis. The present study intends to probe the substrate requirement for the Endo-A catalyzed transglycosylation and to further explore the potential of the chemoenzymatic method for constructing both natural and modified N-glycopeptides.



Scheme 1. Structures of oligosaccharide oxazoline derivatives.

Results and Discussion

Synthesis of oligosaccharide oxazolines: The Manβ1→4GlcNAc moiety is a core disaccharide of N-glycans. This core disaccharide moiety was previously synthesized through stereocontrolled glycosylation of monosaccharides[11,14,18] Here we describe an alternative synthesis of the Manβ1→4GlcNAc core disaccharide and related derivatives using the readily available disaccharide 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1→4)-3,6-di-*O*-acetyl-D-glucal[19] as the starting material, which can be easily prepared from cellobiose on a large scale. The synthesis started with an azidonitration[20] of the cellobiose glycal to obtain the known 2-azido-2-deoxy-cellobiose derivative **8**.[19] Acetolysis of the isolated β-nitrate **8** with sodium acetate in acetic acid, followed by reduction of the azide to acetamido group with zinc dust in the presence of acetic anhydride, gave the 2-acetamido-2-deoxy-cellobiose derivative **9** in 65% yield. Treatment of compound **9** with TMSOTf[21] resulted in the formation of per-O-acetylated oxazoline derivative **10** (90%), which was de-O-acetylated with catalytic amount of MeONa in MeOH to give the oxazoline **5**. To synthesize the Manβ1→4GlcNAc core disaccharide and its derivatives, the configuration at C-2′ needs to be converted from the *gluco*-type to the *manno*-type. This was achieved by a series of selective transformations. First, a 4-pententyl group was introduced at the anomeric position of the disaccharide moiety, which could be directly transformed to an oxazoline moiety in the later stage by a one-step oxazoline formation reaction.[18] Thus treatment of **10** with 4-penten-1-ol under the catalysis of 10-camphorsulfonic acid (CSA) gave pentenyl 2-acetamido-β-glycoside **11** (90%). De-O-acetylation of **11**, followed by subsequent benzylidenation, gave compound **12** (53%). Selective benzoylation[22] of **12** with BzCN in MeCN at low temperature gave compound **14** in 34% isolated yield, which has a free hydroxyl group at the 2′-position. The inversion of the C-2′ configuration was achieved by following the previously described S_N2 inversion of the C-2 configuration for β-mannoside synthesis.[22,23] Thus, triflation of compound **14** with triflic anhydride/pyridine, followed by S_N2 substitution with benzoate, gave the protected Manβ1→4GlcNAc disaccharide derivative **15** in 65% yield in two steps. Compound **15** was converted to the O-acetylated derivative **16**, which was then modified to yield oxazoline derivative **17** by treatment with NIS/TESOTf.[18] Finally, de-O-acetylation of **17** gave disaccharide oxazoline **1** (Scheme 2).

The 6′-modified disaccharide oxazoline **4** was prepared from compound **15** in several steps (Scheme 3). Selective reductive ring-opening of the benzylidene group in **15** by treatment with Et_3SiH/TFA/trifluoroacetic anhydride/CH_2Cl_2[24] gave compound **18**, with a benzyl group attached to the 6′-O-position. The benzoyl groups were then replaced by acetyl groups to give compound **19**. Treatment of **19** with NIS/TESOTf resulted in the formation of oxazoline derivative **20** (78%), which was de-O-acetylated to give 6′-O-benzyl disaccharide oxazoline **4**.

To prepare trisaccharide oxazoline **3**, the 4′,6′-O-benzylidene group in **15** was selectively removed by treatment with 80% aqueous AcOH at 50°C. The resulting compound **21** was then selectively glycosylated at the 6′-OH position with 2,3,4,6-tetra-*O*-benzoyl-β-D-mannopyranosyl trichloroacetimidate under the catalysis of TMSOTf to give trisaccharide derivative **23** (76%). De-O-benzoylation of **23** with subsequent O-acetylation afforded the O-acetylated pentenyl gly-

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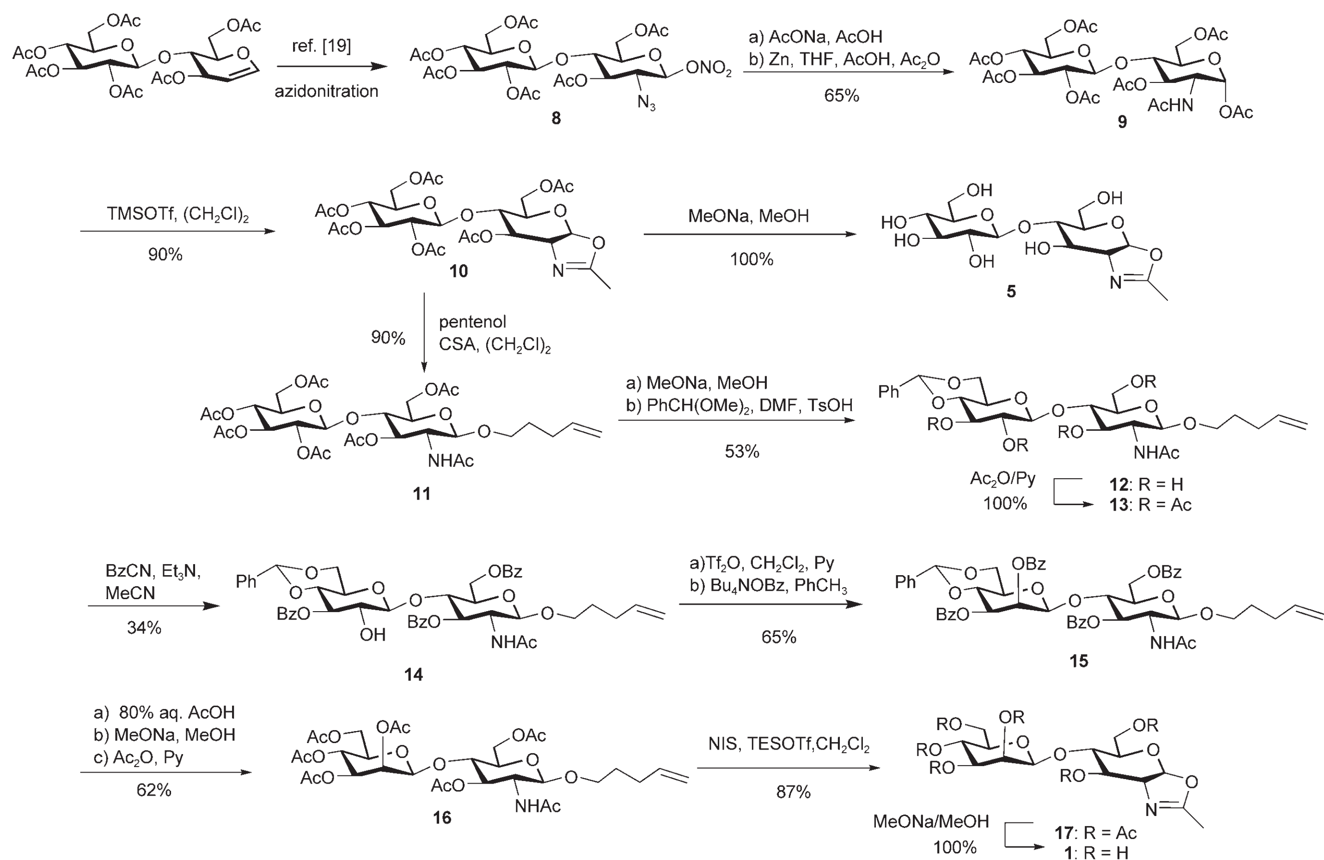
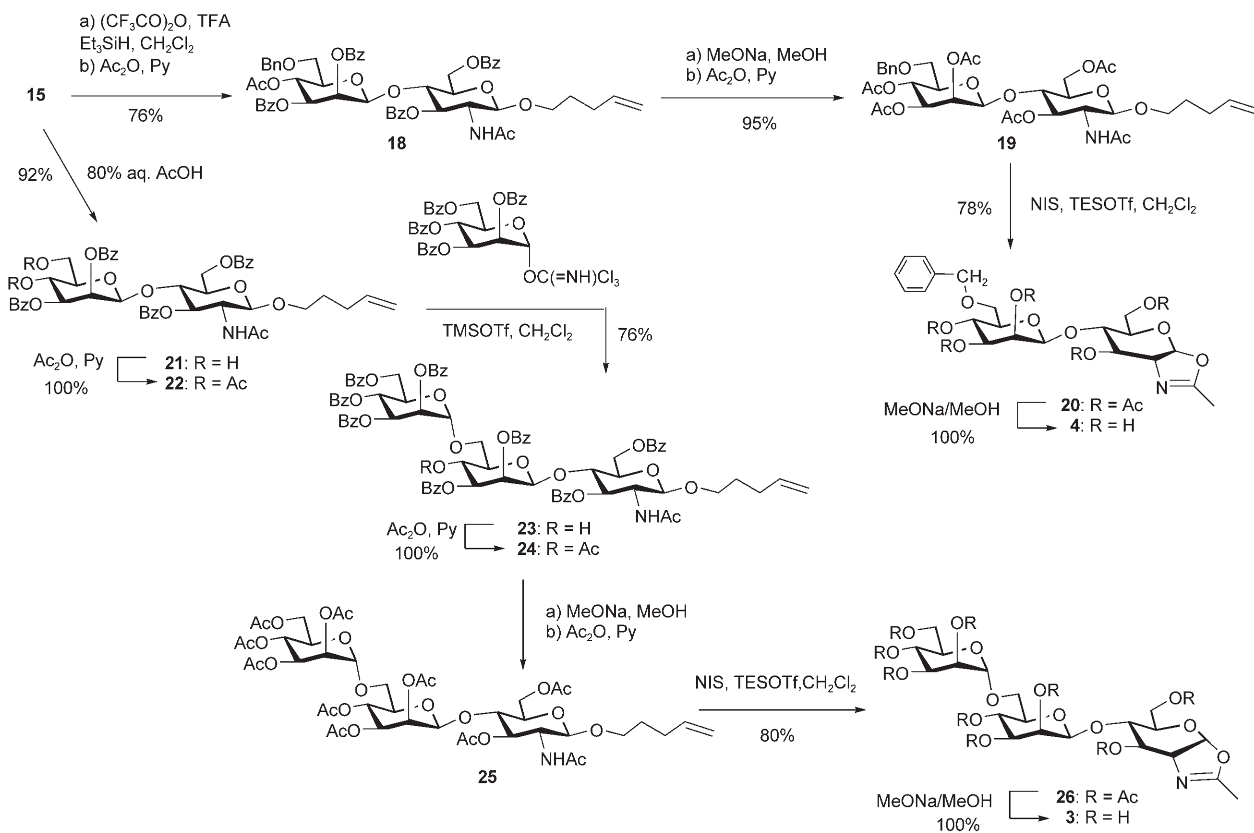


Scheme 2. Synthesis of disaccharide oxazolines.



Scheme 3. Synthesis of modified oxazoline derivatives.

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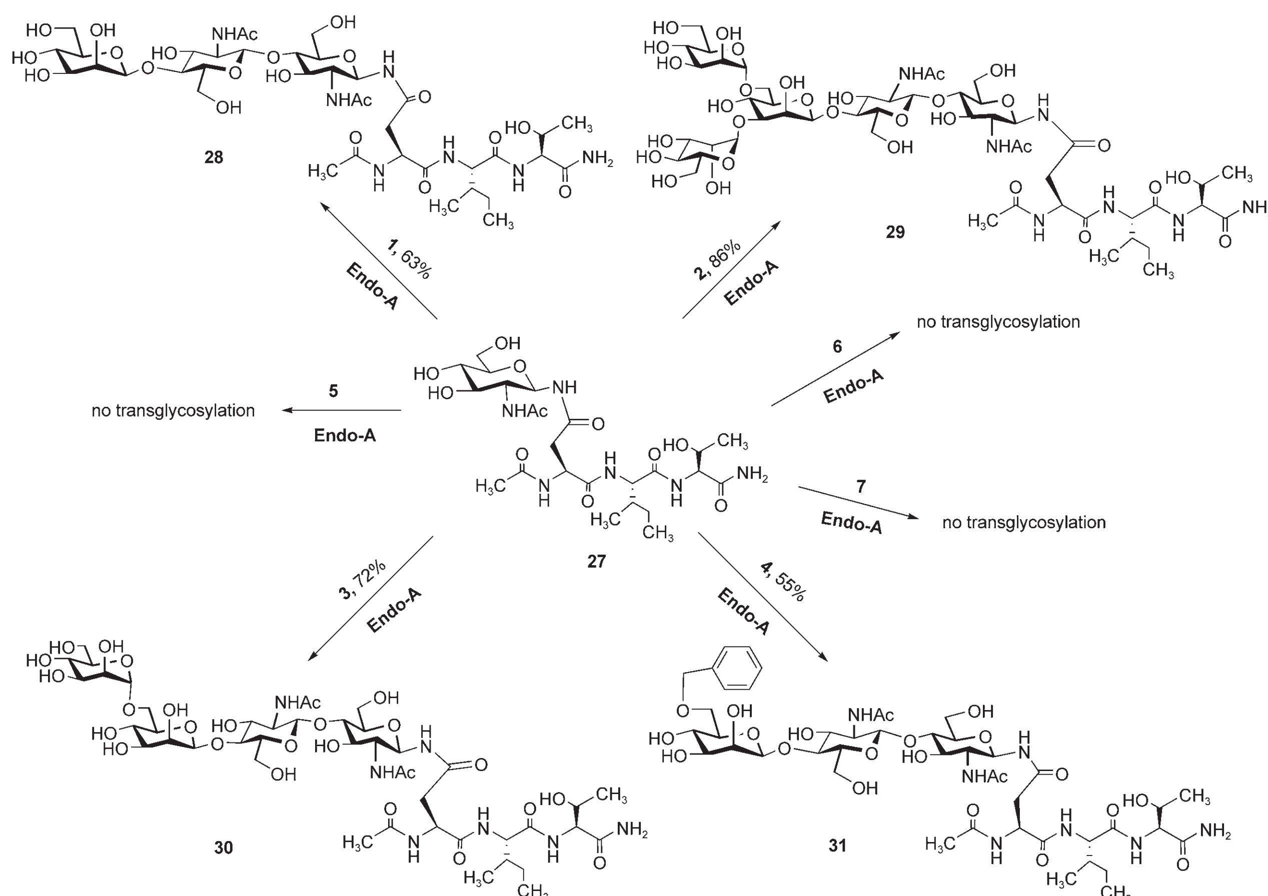
coside **25**. Finally, compound **25** was converted to the oxazoline derivative by treatment with NIS/TESOTf to give **26**, which was de-O-acetylated to provide trisaccharide oxazoline **3** (Scheme 3). On the other hand, the known chitobiose oxazoline **6**[15] and the LacNAc-oxazoline **7**[16] were prepared from O-acetylated *N,N'*-diacetylchitobiose and *N*-acetyllactosamine, respectively, following the reported procedure.[15,16]

Endo-A catalyzed transglycosylation of the synthetic oxazolines with GlcNAc-peptides: To test the transglycosylation potential of the synthetic oligosaccharide oxazolines, we synthesized a small GlcNAc-tripeptide acceptor, Asn(GlcNAc)-Ile-Thr, which represents the minimum consensus sequence for the N24 and N38 N-glycosylation sites in erythropoietin, an important therapeutic glycoprotein for the treatment of anemia.[25] Previously we reported that disaccharide oxazoline **1** and tetrasaccharide oxazoline **2** were good donor substrates for Endo-A.[11] As expected, the Endo-A catalyzed reaction of oxazolines **1** and **2** with the GlcNAc-peptide **27** (donor/acceptor 3:1) proceeded smoothly in a phosphate buffer (pH 6.5) to give the corresponding N-glycopeptides **28** and **29** in 63 and 86% yields, respectively. Trisaccharide oxazoline **3** was also an efficient donor substrate for the Endo-A catalyzed transglycosylation. Thus incubation of **3** and acceptor **27** (ratio 3:1) in the presence of Endo-A gave glycopeptide **30** carrying the N-linked tetrasaccharide moiety in 72% isolated yield. Interestingly, 6'-O-benzyl disaccharide oxazoline **4**, which has an aromatic substituent attached at the 6'-position, could still be recognized by Endo-A as a donor substrate. The Endo-A catalyzed reaction of **4** with acceptor **27** (3:1) afforded the modified glycopeptide **31** in 55% isolated yield (Scheme 4). Although the disaccharide oxazolines **1** and **4** were recognized by Endo-A as donor substrates for the transglycosylation, their reactions were found to proceed more slowly than those reactions of tetrasaccharide oxazoline **2** and trisaccharide oxazoline **3**. A quick comparison of the transglycosylation under the same conditions revealed that the relative transglycosylation rates with acceptor **27** were in the following order: tetrasaccharide oxazoline **2** > trisaccharide oxazoline **3** > disaccharide oxazoline **1** > Bn-substituted disaccharide oxazoline **4**. Although a quantitative comparison of the sugar oxazoline substrates will await detailed enzyme kinetic studies to obtain the kinetic data K_m and V_{max}, these results suggest that the attachment of additional β-mannosyl moieties at the 3- and 6-position of the β-mannose core, as present in the natural N-glycans, enhance the enzymatic recognition of the donor substrates. The results also suggested that Endo-A could tolerate modification at the 6'-position of the disac-



Scheme 4. ENGase-catalyzed transglycosylation with different oxazolines.

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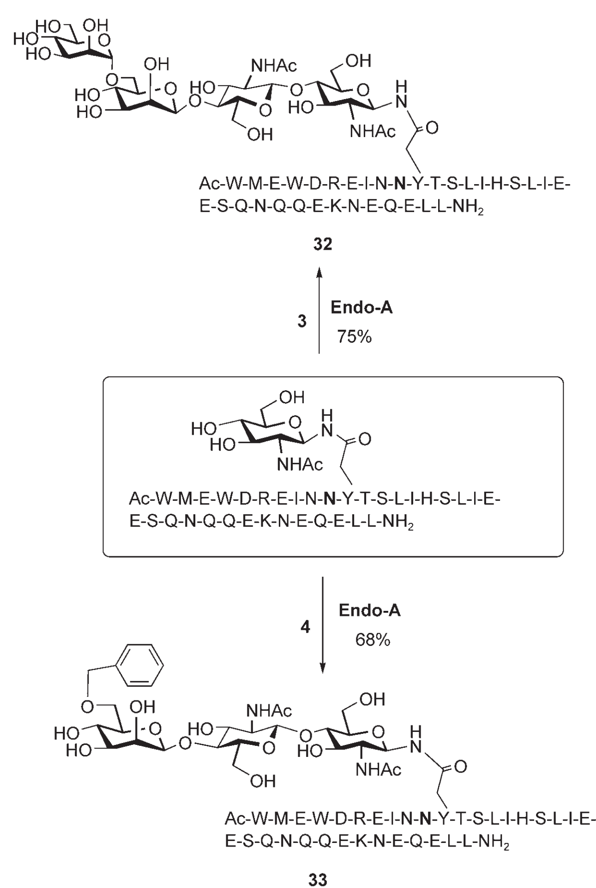
charide oxazoline, thus allowing the synthesis of modified glycopeptides. In all cases, the newly formed glycosidic bond in the resulting glycopeptides **28**, **29**, **30**, and **31** was determined by 2D NMR analysis (data not shown) to be a β-1,4-glycosidic linkage, confirming the previous conclusion that the Endo-A catalyzed transglycosylation using oligosaccharide oxazoline as the donor substrates proceeded in a regio- and stereospecific manner.[11] We observed that Endo-A could slowly hydrolyze the oxazolines **1**–**4** but, in the presence of a GlcNAc-peptide acceptor, the transglycosylation was found to be faster than the hydrolysis. The results suggest that the GlcNAc moiety is a better acceptor than water molecule in the Endo-A catalyzed reaction.

Next, the structurally related disaccharide oxazolines **5**–**7** were tested. Cellobiose-oxazoline **5**, which differs from Manβ-1,4GlcNAc-oxazoline **1** only by the configuration of the C′-2 hydroxyl group, was inactive in the enzymatic transglycosylation. Similarly, chitobiose-oxazoline **6** and LacNAc-oxazoline **7**, which were substrates of *Bacillus* chitinase,[15,16] could not be recognized by Endo-A. These results indicate that the structure of the core β-mannose moiety is required for Endo-A recognition. Replacement of the β-Man moiety in the disaccharide oxazoline substrate **1** with β-Glc, β-GlcNAc, and β-Gal moiety, respectively, resulted in total loss of its substrate activity. The experiments revealed that the Manβ1→4GlcNAc oxazoline moiety is the minimum structure that is recognized by the enzyme Endo-A for transglycosylation. Despite this, the enzyme could tolerate selective modification on the mannose moiety, for example, attachment of additional sugar moieties at the 3′, 6′-positions and even an aromatic group at the 6′-position.

In our preliminary communication,[11] we have shown that very large GlcNAc-peptides such as the 34-mer peptide GlcNAc-C34 could serve as an acceptor for Endo-A catalyzed transglycosylation of the di- and tetrasaccharide oxazolines **1** and **2**. In the present study, we found that the enzymatic transglycosylation of trisaccharide-oxazoline **3** and modified disaccharide oxazoline **4** to the large acceptor GlcNAc-C34 also proceeded successfully (Scheme 5). This led to the synthesis of the large glycopeptide **32** that contains the core N-linked tetrasaccharide (75% yield) and glycopeptide **33** which carries a modified trisaccharide (68%) when an excess oxazoline donor (donor/acceptor 5:1) was used. The relatively high-yield transglycosylation suggests that the Endo-A catalyzed transglycosylation of the sugar oxazolines was equally efficient for small and large acceptors. Finally, we also tested whether Endo-A could hydrolyze the resulting "ground-state" glycopeptides **28**–**33** carrying the N-linked core tri-, tetra-, and pentasaccharide, respectively. It was found that in the presence of large amount of Endo-A, only glycopeptide **29** that carries the core pentasaccharide was slowly hydrolyzed by Endo-A to release the tetrasaccharide Manα1→6(Manα1→3)Manβ1→4GlcNAc ($Man_3GlcNAc$) and the GlcNAc-peptide moiety. The other glycopeptides carrying the core tri- and tetrasaccharides **28**, **30**, **32**, as well as those carrying the modified trisaccharide **31** and **33**, were resistant to the enzymatic hydrolysis. Considering that the corresponding di-, tri-, and tetrasaccharide oxazolines **1**–**4** are active for the enzymatic transglycosylation, the results suggest that the sugar oxazolines as donor substrates are kinetically more favorable for the transglycosylation than the hydrolysis of the resulting ground-state" glycopeptides, allowing product accumulation.



Scheme 5. Synthesis of glycoforms of gp41 C-peptide C34.

Conclusion

An array of oligosaccharide oxazolines was synthesized and evaluated as donor substrates for the Endo-A catalyzed glycopeptide synthesis. It was revealed that the minimum substrate structure required for the Endo-A catalyzed transglycosylation is a Manβ1→4GlcNAc oxazoline moiety. Despite this, the enzyme can tolerate modifications at the 3′- and/or 6′-positions of the disaccharide oxazoline, allowing the transfer of both larger and selectively modified oligosaccharide moieties to the peptide acceptor. On the other hand, the enzyme has a great flexibility for the acceptor portion and could efficiently take both small and large GlcNAc-peptide as the acceptor substrate. Since this high-yield enzymatic ligation allows independent manipulations of the donor (sugar oxazoline) and the acceptor (GlcNAc-peptide/protein) portions by well-established oligosaccharide and pep-

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tide chemistry, it provides a highly convergent approach for constructing both natural and modified glycopeptides. Studies directed to the exploration of this chemoenzymatic approach for constructing large complex glycopeptides and glycoproteins are underway.[26]

Experimental Section

General procedures: TLC was performed on aluminum plates coated with silica gel 60 with detection by charring with 10% (v/v) sulfuric acid in methanol or by UV detection. Flash column chromatography was performed on silica gel 60 (EM Science, 230–400 mesh). ^{1}H and ^{13}C NMR, and 2D NMR spectra were recorded on Inova 500 NMR in $CDCl_3$, D_2O, or CD_3OD, as specified. Chemical shifts are expressed in ppm downfield using external Me_4Si (0 ppm) as the reference. The ESI-MS spectra were measured on a micromass ZQ-400 single quadruple mass spectrometer. Analytic HPLC was carried out with a Waters 626 HPLC instrument on a Waters NovaPak C18 column (3.9×150 mm) at 40°C. The column was eluted with a linear gradient of 0–90% MeCN containing 0.1% TFA at a flow rate of 1 mL min^{-1} over 25 min. Peptide and glycopeptides were detected at two wavelengths (214 and 280 nm). Preparative HPLC was performed with Waters 600 HPLC instrument on a Waters C18 Column (symmetry 300, 19×300 mm). The column was eluted with a suitable gradient of MeCN containing 0.1% TFA at 12 mL min^{-1}.

***O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-2-acetamido-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose (9):** A suspension of **8** (15 g, 22.5 mmol)[19] and anhydrous NaOAc (20 g) in AcOH (250 mL) was heated to 100°C for 1 h. The reaction was diluted with EtOAc and washed with $NaHCO_3$ and brine, dried by Na_2SO_4, and filtered. The filtrate was concentrated in vacuo to give a white solid. The solid was dissolved in THF (100 mL) and AcOH (50 mL). To the solution were sequentially added Ac_2O (10 mL) and zinc dust (30 g) in portions. The mixture was stirred at RT for 1 h and filtered through a Celite pad. The filtrate was concentrated in vacuo and the residue was subject to flash column chromatography (hexanes/EtOAc 1:2) to give **9** (9.94 g, 65%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 6.09 (d, J=3.5 Hz, 1H, H-1), 5.72 (d, J=8.5 Hz, 1H, NH), 5.21 (dd, J=8.8, 10.5 Hz, 1H, H-3), 5.15 (t, J= 9.0 Hz, 1H, H-3′), 5.08 (t, J=9.0 Hz, 1H, H-4′), 4.93 (t, J=9.0 Hz, 1H, H-2′), 4.55 (d, J=8.4 Hz, 1H, H-1′), 4.44–4.35 (m, 3H), 4.12–4.04 (m, 2H), 3.88–3.82 (m, 2H), 3.70–3.66 (m, 1H), 2.19, 2.12, 2.09, 2.06, 2.04, 2.01, 1.99, 1.95 (s each, 3H each, 8 CH_3CO); ESI-MS: m/z: calcd for $C_{28}H_{39}NO_{18}$: 677.22; found: 678.34 [M+H]$^+$.

***O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (10):** TMSOTf (3.2 mL, 1.1 equiv) was added under argon atmosphere to a solution of **9** (10.8 g, 16 mmol) in dry $ClCH_2CH_2Cl$. The mixture was stirred at 50°C overnight. After cooling to RT, the mixture was neutralized by Et_3N and concentrated. The residue was subjected to a flash column chromatography (hexanes/EtOAc 1:2) to afford **10** (8.85 g, 90%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.90 (d, J=7.3 Hz, 1H, H-1), 5.64 (d, J=1.8 Hz, 1H, H-3), 5.16 (t, J=9.2 Hz, 1H, H-3′), 5.11 (t, J=9.8 Hz, 1H, H-4′), 5.00 (t, J=8.4 Hz, 1H, H-2′), 4.70 (d, J=8.4 Hz, 1H, H-1′), 4.37–4.09 (m, 5H), 3.79–3.76 (m, 1H, H-5), 3.63–3.62 (m, 1H), 3.48–3.45 (m, 1H, H-5′), 2.10, 2.09, 2.08, 2.08, 2.03, 2.00, 1.98 (s each, 3H each, 7 CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 169.8, 169.7, 169.4, 168.5, 165.9, 101.2, 98.1, 77.2, 72.1, 71.1, 70.4, 69.2, 67.1, 66.6, 63.9, 62.6, 60.9, 13.0; ESI-MS: m/z: calcd for $C_{26}H_{35}NO_{16}$: 617.2, found: 618.43 [M+H]$^+$.

***O*-(β-D-Glucopyranosyl)-(1→4)-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (5):** A solution of **10** (12 mg, 19 μmol) in MeOH containing NaOMe (2 μmol) was stirred at RT for 2 h. Then the mixture was concentrated in vacuo. The residue was dissolved in water and lyophilized to give oxazoline **5** (6 mg, quantitative) as a white solid. ^{1}H NMR (CD_3OD, 500 MHz): δ = 6.03 (d, J=7.0 Hz, 1H, H-1), 4.66 (d, J=7.5 Hz, 1H, H-1′), 4.32 (s, 1H, H-2), 4.13 (dd, J=7.5, 8.4 Hz, 1H, H-2′), 3.91–3.88 (m, 4H), 3.72–3.50 (m, 12H), 3.55–3.32 (m, 3H), 1.85 (s, 3H, CH_3); ^{13}C NMR(CD_3OD, 125 MHz): δ = 168.6, 101.2, 99.9, 77.4, 76.4, 72.8, 70.9, 70.4, 69.3, 66.8, 65.2, 61.7, 61.1, 13.0; ESI-MS: m/z: calcd for $C_{14}H_{23}NO_{10}$: 365.13; found: 366.87 [M+H]$^+$.

4-Pentenyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside (11): 4-Penten-1-ol (10 mL, 53 mmol) and CSA (300 mg) was added to a solution of **10** (8.85 g, 14.3 mmol) in anhydrous $ClCH_2CH_2Cl$ (80 mL). The mixture was stirred under an argon atmosphere at 90°C for 2 h. After cooling to RT, the mixture was neutralized by Et_3N and concentrated. The residue was subjected to flash column chromatography (hexanes/EtOAc 1:3) to afford **11** (9.1 g, 90%) as an amorphous solid. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 5.83–5.75 (m, 2H, -CH=, NH), 5.18 (t, J=9.0 Hz, 1H), 5.12–4.95 (m, 5H), 4.57–4.54 (m, 2H), 4.45 (d, J=8.0 Hz, 1H, H-1′), 4.39 (dd, J=12.0, 4.5 Hz, 1H), 4.16–4.02 (m, 4H), 3.87–3.69 (m, 6H), 3.64–3.61 (m, 1H), 3.50–3.45 (m, 3H), 2.14, 2.11, 2.08, 2.06, 2.04, 2.01, 1.98 (s each, 3H each, 7 CH_3CO), 1.75–1.64 (m, 2H, -$OCH_2CH_2CH_2$-); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 169.9, 169.6, 169.5, 169.3, 169.2, 168.5, 168.4, 137.0, 114.0, 100.1, 99.8, 75.3, 71.9, 71.7, 71.4, 71.0, 70.7, 67.9, 66.9, 61.3, 60.7, 52.5, 29.01, 19.9, 19.8, 19.7, 19.6; ESI-MS: m/z: calcd for $C_{31}H_{45}NO_{17}$: 703.27, found: 704.07 [M+H]$^+$.

4-Pentenyl *O*-(4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside (12) and 4-pentenyl *O*-(2,3-di-*O*-acetyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside (13): A solution of the compound **11** (9 g, 13 mmol) in MeOH (100 mL) containing NaOMe (1.3 mmol) was stirred at RT for 2 h. Then the mixture was neutralized by Dowex W50-X8 (H^+ form), filtered, and the filtrate was concentrated. The residue was dissolved in DMF (50 mL) and dimethyl acetal benzaldehyde (6.3 mL, 3.4 equiv) and *p*-toluenesulfonic acid (1.0 g). The mixture was stirred at 50°C for 10 h. After cooling to RT, the mixture was neutralized by Et_3N and concentrated. The residue was subjected to flash column chromatography (EtOAc/MeOH 10:1) to give **12** (3.65 g, 53%). ESI-MS: m/z: calcd for $C_{26}H_{37}NO_{11}$: 539.24; found: 540.35 [M+H]$^+$.

The compound was further characterized by transformation to its acetylated derivative **13**. To a solution of **12** (50 mg) in pyridine (2 mL) was added Ac_2O (0.2 mL). The mixture was stirred at RT for 5 h. The mixture was then poured into cold $NaHCO_3$ solution and stirred for 2 h at RT. The mixture was extracted with CH_2Cl_2 and the organic layer washed with $NaHCO_3$, HCl (1 N) and brine, dried by Na_2SO_4, and filtered. The filtrate was concentrated in vacuo and the residue was subjected to flash column chromatography (hexanes/EtOAc 2:1) to give **13** (72 mg, quantitative) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.51–7.32 (m, 5H, C_6H_5), 5.84–5.78 (m, 2H, -CH=, NH), 5.53 (s, 1H, PhCH=), 5.32 (t, J=9.5 Hz, 1H), 5.12 (t, J=9.2 Hz, 1H), 5.06–4.95 (m, 3H), 4.66 (d, J= 7.5 Hz, 1H, H-1), 4.55–4.39 (m, 3H), 4.16–4.04 (m, 2H), 3.88–3.71 (m, 5H), 3.64–3.63 (m, 2H), 3.55–3.46 (m, 3H), 2.16, 2.10, 2.08, 2.06, 2.00 (s each, 3H each, 5 CH_3CO), 1.75–1.66 (m, 2H, -$OCH_2CH_2CH_2$-); ESI-MS: m/z: calcd for $C_{34}H_{45}NO_{15}$: 707.28; found: 708.58 [M+H]$^+$.

4-Pentenyl *O*-(3-*O*-benzoyl-4,6-*O*-benzylidene-β-D-glucopyranosyl)-(1→4)-3,6-di-*O*-benzoyl-2-acetamido-2-deoxy-β-D-glucopyranoside (14): BzCN (2.6 mL, 3.3 equiv) in MeCN (25 mL) was added at −30°C in portions over 3 h to a solution of **12** (3.3 g, 61 mmol) in MeCN (50 mL) containing Et_3N (3 mL). The reaction was monitored by TLC (hexanes/EtOAc 2:1). After stirring at −30°C for 5 h, the reaction was quenched by adding MeOH. The mixture was diluted with EtOAc, washed with 0.1 M HCl, $NaHCO_3$ and brine, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo and the residue was subject to flash silica gel column chromatography (hexanes/EtOAc 2:1) to give **14** (1.77 g, 34%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.20–7.26 (m, 20H, C_6H_5), 6.33 (d, J=8.5 Hz, 1H, NH), 5.75–5.69 (m, 1H, -CH=), 5.64 (dd, J=9.5, 2.0 Hz, 1H, H-3), 5.28 (t, J=9.2 Hz, 1H, H-3′), 5.14 (s, 1H, PhCH=), 5.01–4.87 (m, 3H), 4.80 (d, J=8.5 Hz, 1H, H-1), 4.63–4.60 (m, 2H), 4.20–4.15 (m, 1H), 4.05–4.03 (m, 2H), 3.95–3.94 (d, J=4.5 Hz, 1H), 3.86–3.81 (m, 2H), 3.69–3.65 (m, 1H), 3.55–3.51 (m, 1H), 3.44 (t, J= 9.0 Hz, 1H, H-4′), 3.35 (dd, J=10.5, 4.8 Hz, 1H), 3.21–3.16 (m, 1H), 2.87 (t, J=10.5 Hz, 1H), 2.06–2.03 (m, 2H, -$OCH_2CH_2CH_2$-), 1.83 (s, 3H, CH_3CO), 1.68–1.59 (m, 2H, -$OCH_2CH_2CH_2$-); ESI-MS: m/z: calcd for $C_{47}H_{49}NO_{14}$: 851.32; found: 852.42 [M+H]$^+$.

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4-Pentenyl *O*-(2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-mannopyranosyl)-(1→4)-3,6-di-*O*-benzoyl-2-acetamido-2-deoxy-β-D-glucopyranoside (15): Tf_2O (690 μL, 2 equiv) at 0 °C for 1 h was added to a solution of **14** (1.7 g, 19 mmol) in CH_2Cl_2 (50 mL) containing pyridine (1 mL). The reaction was monitored by TLC. After the completion of the reaction, the mixture was diluted with CH_2Cl_2, washed successively with cold HCl (0.1 N), cold saturated $NaHCO_3$ and H_2O, dried over Na_2SO_4, and filtered. The filtrate was concentrated and co-evaporated with toluene. The residue was then dissolved in toluene (20 mL) and Bu_4NOBz (3 g, 76 mmol) was added. The mixture was stirred under reflux for 2 h when TLC indicated the completion of the reaction. After evaporation, the mixture was diluted with CH_2Cl_2, washed successively with saturated $NaHCO_3$ and H_2O, dried over Na_2SO_4, and filtered. The filtrate was concentrated in vacuo and the residue was subject to flash silica gel column chromatography (hexanes/EtOAc 1:3) to afford **15** (1.25 g, 65 %) as a white foam. ^1H NMR (500 MHz, $CDCl_3$): δ = 8.11–7.26 (m, 25 H, 5 C_6H_5), 5.87 (d, J = 3.5 Hz, 1 H, H-2′), 5.77–5.69 (m, 2 H), 5.61 (d, J = 9.5 Hz, 1 H, NH), 5.45–5.38 (m, 3 H), 5.33 (t, J = 9.2 Hz, 1 H, H-3), 4.97–4.90 (m, 3 H), 4.69 (dd, J = 12.0, 3.5 Hz, 1 H, H-6a), 4.60 (dd, J = 12.0, 3.8 Hz, 1 H, H-6b), 4.53–4.48 (m, 2 H), 4.19–4.14 (m, 2 H), 3.99 (t, J = 10.0 Hz, 1 H, H-4′), 3.79–3.68 (m, 3 H), 3.48–3.26 (m, 3 H), 2.08–2.02 (m, 2 H, -O$CH_2CH_2CH_2$-), 1.83 (s, 3 H, CH_3CO), 1.68–1.59 (m, 2 H, -OCH_2 CH_2 CH_2-); ^{13}C NMR($CDCl_3$, 125 MHz): δ = 170.1, 166.4, 166.3, 165.7, 165.5, 136.0, 133.4, 133.3, 133.1, 130.0, 129.9, 129.8, 128.6, 128.4, 128.2, 126.1, 114.9, 101.7, 101.2, 99.2, 77.4, 77.1, 76.8, 72.8, 70.4, 68.8, 68.0, 67.5, 62.0, 53.7, 30.0, 28.6, 23.3; ESI-MS: m/z: calcd for: $C_{54}H_{53}NO_{15}$: 955.34; found: 956.37 $[M+H]^+$.

4-Pentenyl *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-3,6-di-*O*-acetyl-2-acetamido-2-deoxy-β-D-glucopyranoside (16): A solution of **15** (300 mg, 0.31 mmol) in 80 % HOAc (10 mL) was stirred at 50 °C for 4 h. The mixture was concentrated in vacuo, and the residue was dissolved in MeOH (20 mL) containing NaOMe (0.03 mmol). The solution was stirred at RT for 5 h and then neutralized with Dowex W50-X8 (H^+ form). The solution was filtered, and the filtrate was concentrated to dryness. The residue was then treated with pyridine (5 mL) and Ac_2O (1 mL) at RT for 10 h. The reaction mixture was then poured into cold $NaHCO_3$ solution and stirred for 2 h. The reaction mixture was extracted with CH_2Cl_2 and the organic layer was washed with aq. $NaHCO_3$, HCl (1 N) and brine, dried by Na_2SO_4, and filtered. The filtrate was concentrated in vacuo and the residue was subject to flash silica gel column chromatography (hexanes/EtOAc 1:3) to give **16** (140 mg, 62 %). ^1H NMR (500 MHz, $CDCl_3$: δ = 5.83–5.75 (m, 1 H, -CH=), 5.41 (d, J = 9.0 Hz, 1 H, NH), 5.39 (d, J = 3.5 Hz, 1 H, H-2′), 5.19 (t, J = 9.0 Hz, 1 H, H-4′), 5.10 (t, J = 9.0 Hz, 1 H, H-3), 5.03–4.94 (m, 3 H), 4.69 (s, 1 H, H-1′), 4.46 (d, J = 8.0 Hz, 1 H, H-1), 4.40–4.30 (m, 2 H), 4.20 (dd, J = 12, 4.5 Hz, 1 H), 4.13–3.96 (m, 2 H), 3.93–3.81 (m, 3 H), 3.64–3.44 (m, 4 H), 2.14, 2.11, 2.09, 2.07, 2.04, 2.01, 1.98 (s each, 3 H each, 7 CH_3CO), 1.75–1.64 (m, 2 H, -O$CH_2CH_2CH_2$-); ESI-MS: m/z: calcd for $C_{31}H_{45}NO_{17}$: 703.27; found: 704.47 $[M+H]^+$.

***O*-(2,3,4,6-Tetra-*O*-acetyl-β-D-mannopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (17):** NIS (15 mg, 1.2 equiv) and TESOTf (25 μL) were added to a solution of **16** (75 mg, 106 μmol) in anhydrous CH_2Cl_2 (20 mL). The mixture was stirred at RT for 0.5 h and then diluted with CH_2Cl_2, and washed with $NaHCO_3$ and brine. The organic layer was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 1:2) to give **17** (57.3 mg, 87 %) as a white solid. ^1H NMR ($CDCl_3$, 500 MHz): δ = 5.90 (d, J = 7.0 Hz, 1 H, H-1), 5.54 (t, J = 2.5 Hz, 1 H, H-3), 5.41 (d, J = 3.0 Hz, 1 H, H-2′), 5.25 (t, J = 10 Hz, 1 H, H-4′), 5.05 (dd, J = 9.5, 3.0 Hz, 1 H, H-3′), 4.81 (s, 1 H, H-1′), 4.27 (dd, J = 12.5, 5.0 Hz, 1 H, H-6′), 4.19–4.09 (m, 4 H), 3.70–3.68 (m, 2 H), 3.48–3.46 (m, 1 H), 2.20, 2.11, 2.09, 2.09, 2.04, 2.04, (s each, 3 H each, 6 CH_3CO). 1.99 (s, 3 H, CH_3); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 170.7, 170.6, 170.5, 170.0, 169.6, 169.3, 166.3, 99.3, 99.0, 76.1, 72.5, 70.8, 70.4, 68.4, 67.8, 66.0, 64.8, 63.6, 62.5, 60.4, 20.1, 20.9, 20.8, 20.7, 20.6, 20.5, 14.2; ESI-MS: m/z: calcd for $C_{26}H_{35}NO_{16}$: 617.2; found: 618.37 $[M+H]^+$.

***O*-(β-D-Mannopyranosyl)-(1→4)-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (1)**: A solution of **17** (57 mg, 100 μmol) in MeOH (2 mL) containing NaOMe (10 μmol) was stirred at RT for 2 h. Then the mixture was concentrated. The residue was dissolved in water and lyophilized to give the disaccharide oxazoline **1** (34 mg, quantitative) as a white solid. ^1H NMR (D_2O, 500 MHz): δ = 6.03 (d, J = 7.5 Hz, 1 H, H-1), 4.66 (s, 1 H, H-1′), 4.32 (s, 1 H, H-2′), 4.13 (s, 1 H, H-2), 3.91–3.88 (m, 4 H), 3.72–3.50 (m, 4 H), 3.55–3.32 (m, 2 H), 1.85 (s, 3 H, CH_3-); ^{13}C NMR (D_2O, 125 MHz): δ = 168.6, 101.2, 99.9, 77.4, 76.4, 72.8, 70.9, 70.4, 69.3, 66.8, 65.2, 61.7, 61.1, 13.0 ESI-MS: m/z: calcd for $C_{14}H_{23}NO_{10}$: 365.13; found: 366.57 $[M+H]^+$.

4-Pentenyl *O*-(4-*O*-acetyl-2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-mannopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (18): Trifluoroacetic anhydride (120 mL, 0.88 mmol) and Et_3SiH (240 μL, 0.44 mmol) were added at 0 °C to a solution of **15** (140 mg, 0.15 mmol) in dry CH_2Cl_2 (5 mL). After the reaction mixture was stirred at 0 °C for 5 min, TFA (112 μL, 0.44 mmol) was added dropwise over 2 min. The reaction was stirred from 0 °C to RT overnight. The mixture was diluted with EtOAc (50 mL), and the solution was washed with $NaHCO_3$ and brine, dried by Na_2SO_4, and filtered. The filtrate was dissolved in pyridine (5 mL) containing Ac_2O (0.5 mL). The mixture was stirred overnight at RT and then diluted with CH_2Cl_2, washed with $NaHCO_3$ and brine. The organic layer was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 2:1) to give **18** (107 mg, 76 %) as a white foam. ^1H NMR (500 MHz, $CDCl_3$): δ = 8.08–7.26 (m, 25 H, 5 C_6H_5), 5.81 (d, J = 2.5 Hz, 1 H, H-2′), 5.77–5.71 (m, 1 H, -CH=), 5.56 (d, J = 8.0 Hz, 1 H, NH), 5.47 (t, J = 10.0 Hz, 1 H, H-4′), 5.34 (t, J = 9.0 Hz, 1 H, H-3), 5.28 (dd, J = 7.0, 3.0 Hz, 1 H, H-3′), 4.97–4.90 (m, 3 H), 4.72 (dd, J = 11.5, 2.0 Hz, 1 H, H-6a), 4.59 (dd, J = 11.5, 4.0 Hz, 1 H, H-6b), 4.48 (m, 2 H), 4.37 (d, J = 12.0 Hz, 1 H, PhCH_2-), 4.29–4.17 (m, 2 H), 3.86–3.81 (m, 2 H), 3.49–3.28 (m, 4 H), 2.10–2.06 (m, 2 H, -OCH_2-CH_2-CH_2-), 1.80, 1.76 (s each, 3 H each, 2 CH_3CO), 1.72–1.64 (m, 2 H, -OCH_2-CH_2-CH_2-); ESI-MS: m/z: calcd for $C_{56}H_{57}NO_{16}$: 999.37; found: 1000.16 $[M+H]^+$.

4-Pentenyl *O*-(2,3,4-*O*-triacetyl-6-*O*-benzyl-β-D-mannopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-acetyl-2-deoxy-β-D-glucopyranoside (19): A solution of the **18** (100 mg, 0.1 mmol) in MeOH (10 mL) containing NaOMe (0.1 equiv) was stirred at RT for 2 h. Then the mixture was neutralized by Dowex W50-X8 (H^+ form), filtered and concentrated. The foregoing compound was dissolved in pyridine (5 mL) containing Ac_2O (0.5 mL). The mixture was stirred at RT overnight and then diluted with CH_2Cl_2, and washed with $NaHCO_3$ and brine. The organic layer was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 1:2) to give **19** (71 mg, 95 %). ^1H NMR (500 MHz, $CDCl_3$): δ = 7.32–7.26 (m, 5 H, C_6H_5), 5.78–5.76 (m, 1 H, -CH=), 5.41 (d, J = 9.0 Hz, 1 H, NH), 5.37 (d, J = 2.3 Hz, 1 H, H-2′), 5.22 (t, J = 9. 5 Hz, 1 H, H-4), 5.14–4.94 (m, 5 H), 4.66 (s, 1 H, H-1′), 4.55–4.35 (m, 4 H), 4.19 (dd, J = 12.0, 4.0 Hz, 1 H, H-6), 3.89–3.77 (m, 3 H), 3.60–3.54 (m, 4 H), 3.45–3.41 (m, 2 H), 2.12, 2.09, 2.04, 2.01, 1.97, 1.94, 1.93 (s each, 3 H each, 6 CH_3CO), 1.68–1.58 (m, 2 H, -O$CH_2CH_2CH_2$-); ^{13}C NMR($CDCl_3$, 125 MHz): δ = 169.6, 169.5, 169.1, 168.8, 137.0, 136.6, 127.6, 127.1, 126.9, 114.0, 100.3, 96.5, 72.5, 71.5, 71.3, 68.2, 68.0, 61.5, 52.9, 29.0, 27.6, 22.4, 19.9, 19.8, 19.7; ESI-MS: m/z: calcd for $C_{36}H_{49}NO_{16}$: 751.77; found: 752.09 $[M+H]^+$.

***O*-(2,3,4-Tri-*O*-acetyl-6-*O*-benzyl-β-D-mannopyranosyl)-(1→4)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (20):** NIS (12 mg, 1.3 equiv) and TESOTf (13 μL) were added to a solution of **19** (38 mg, 51 μmol) in anhydrous CH_2Cl_2 (5 mL), and the mixture was stirred at RT for 0.5 h until TLC indicated the completion of the reaction. The mixture was diluted with CH_2Cl_2 and washed with $NaHCO_3$ and brine. The mixture was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc/Et_3N 1:2:0.01) to give **20** (26 mg, 78 %) as a white solid. ^1H NMR ($CDCl_3$, 500 MHz): δ = 7.33–7.32 (m, 5 H, C_6H_5), 5.88 (d, J = 7.0 Hz, 1 H, H-1), 5.54 (dd, J = 4.5, 2.5 Hz, 1 H, H-3), 5.39 (d, J = 3.5 Hz, 1 H, H-2′), 5.35 (t, J = 10 Hz, 1 H, H-4′), 5.03 (dd, J = 10.0, 3.0 Hz, 1 H, H-3′), 4.80 (s, 1 H, H-1′), 4.52 (d, J = 12.0 Hz, 1 H, PhCH_2-), 4.19 (d, J = 12.0 Hz, 1 H, PhCH_2-), 4.19–4.09 (m, 4 H), 3.70–3.68 (m, 2 H), 3.73–3.54 (m, 4 H), 2.28, 2.10, 2.01, 1.98, 1.90 (s each, 3 H each, 5 CH_3CO-), 1.89 (s, 3 H, CH_3); ^{13}C NMR($CDCl_3$, 125 MHz): δ = 170.7, 170.1, 169.8, 169.3, 166.2, 138.0, 128.4, 127.9, 127.7, 99.4, 98.8, 73.9, 73.5, 70.9, 70.7,

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69.7, 68.5, 63.6, 21.1, 20.9, 20.8, 20.7, 13.9, 11.4; ESI-MS: *m/z*: calcd for $C_{31}H_{39}NO_{15}$: 665.23; found: 666.16 $[M+H]^+$.

***O*-(6-Benzyl-β-D-mannopyranosyl)-(1→4)-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (4):** A solution of compound **20** (22 mg, 36 μmol) in MeOH containing NaOMe (5 μmol) was stirred at RT for 2 h. Then the mixture was concentrated. The residue was dissolved in water and lyophilized to give the oxazoline **4** (15 mg, quantitative) as a white solid. ^{1}H NMR (CD_3OD, 500 MHz): δ = 7.39–7.32 (m, 5H, C_6H_5), 5.99 (d, *J* = 7.2 Hz, 1H, H-1), 4.64–4.59 (m, 3H, H-1′, 2 PhCH_2-), 4.23 (t, *J* = 2.8 Hz, 1H, H-2′), 4.06–4.04 (m, 1H), 3.85–3.29 (m, 11H), 2.13 (s, 3H, CH_3-); ^{13}C NMR (CD_3OD, 125 MHz): δ = 168.7, 137.2, 128.8, 128.6, 128.4, 101.3, 99.9, 77.6, 75.1, 73.3, 72.8, 70.9, 70.4, 69.4, 69.3, 13.0; ESI-MS: *m/z*: calcd for $C_{21}H_{29}NO_{10}$: 455.18; found: 456.16 $[M+H]^+$.

4-Pentenyl *O*-(2,3-di-*O*-benzoyl-β-D-mannopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (21) and 4-pentenyl *O*-(4,6-di-*O*-acetyl-2,3-di-*O*-benzoyl-β-D-mannopyranosyl)-(1→4)-2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (22): A solution of compound **15** (110 mg, 0.21 mmol) in 80% HOAc (10 mL) was stirred at 50 °C for 4 h. Concentration of the reaction mixture followed by purification on a silica gel column (CH_2Cl_2/MeOH 30:1) gave compound **21** (95 mg, 92%). ESI-MS: *m/z*: calcd for $C_{47}H_{49}NO_{15}$: 867.31; found: 868.28 $[M+H]^+$.

The identity of compound **21** was further characterized by conversion to the 4′,6′-di-*O*-acetyl derivative **22**. To a solution of **21** in pyridine (5 mL) was added Ac_2O (0.5 mL). The mixture was stirred at RT for 4 h, and then diluted with CH_2Cl_2. The solution was washed with $NaHCO_3$ and brine, and the organic layer was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 1:1) to give **22** (96 mg, 100%). ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.11–7.26 (m, 20H, 5C_6H_5), 5.82 (d, *J* = 3.0 Hz, 1H, H-2′), 5.72–5.69 (m, 1H, -C*H*=), 5.38 (dd, *J* = 9.0 Hz, 1H, H-3), 5.27 (dd, *J* = 9.0, 3.5 Hz, 1H, H-3′), 5.01–4.94 (m, 3H, H-1′, =CH_2-), 4.60 (m, 2H), 4.51 (d, *J* = 8.0 Hz, 1H, H-1), 4.21–4.16 (m, 2H), 4.06–3.96 (m, 2H), 3.85–3.82 (m, 1H), 3.74–3.69 (m, 3H), 3.48–3.38 (m, 2H), 2.14–2.10 (m, 2H, -O$CH_2$$CH_2$$CH_2$-), 2.01, 1.85, 1.79 (3s, 9H, 3CH_3CO), 1.68–1.58 (m, 2H, -O$CH_2$$CH_2$$CH_2$-); ESI-MS: *m/z*: calcd for $C_{51}H_{53}NO_{17}$: 951.33; found: 952.26 $[M+H]^+$.

4-Pentenyl *O*-[(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→6)-(2,3-di-*O*-benzoyl-β-D-mannopyranosyl)]-(1→4)-2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (23) and 4-pentenyl *O*-[(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-(1→6)-(4-*O*-acetyl-2,3-di-*O*-benzoyl-β-D-mannopyranosyl)]-(1→4)-2-acetamido-3,6-di-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (24): A suspension of compound **22** (70 mg, 75 μmol) and 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl trichloroacetimidate (60 mg, 81 μmol) in dry CH_2Cl_2 (4 mL) containing activated 4 Å molecular sieves (100 mg) was stirred under an atmosphere of argon at RT for 30 min. After cooling to −20 °C, a solution of TMSOTf in CH_2Cl_2 (0.1 M, 40 μL, 4 μmol) was added and the resulting mixture was stirred from −20 °C to RT for 3 h. The reaction was quenched with Et_3N and diluted with CH_2Cl_2 (10 mL). The solution was washed with brine and the organic layer was dried over Na_2SO_4 and filtered. The filtrate was concentrated in vacuo and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 2:1) to give compound **23** (82 mg, 76%) as a white foam. ESI-MS: *m/z*: calcd for $C_{81}H_{75}NO_{24}$: 1445.47; found: 1446.52 $[M+H]^+$.

Compound **23** was further characterized by its conversion to the 4′-*O*-acetyl derivative **24**. Compound **23** (40 mg, 27 μmol) was dissolved in pyridine (2 mL) containing Ac_2O (0.5 mL). The mixture was stirred at RT overnight. The mixture was diluted with CH_2Cl_2 and washed with $NaHCO_3$, brine, dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 2:1) to give **24** (42 mg, quantitative) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.09–7.32 (m, 40H, 8C_6H_5), 6.11 (d, *J* = 10.0 Hz, 1H, H-4″), 5.92–5.87 (m, 3H, N*H*, H-4′, H-3″), 5.78–5.76 (m, 1H), 5.72–5.67 (m, 1H, =C*H*-), 5.65–5.60 (m, 2H), 5.37 (dd, *J* = 10.0, 3.5 Hz, 1H, H-3′), 5.05 (s, 1H, H-1″), 4.96–4.89 (m, 3H), 4.82 (d, *J* = 8.5 Hz, 1H), 4.70–4.60 (m, 4H), 4.49–4.42 (m, 2H), 4.28–4.26 (m, 2H), 4.19 (t, *J* = 9.5 Hz, 1H, H-4), 3.71–3.62 (m, 3H), 3.46–3.30 (m, 3H), 2.10–2.06 (m, 2H, -CH_2 CH_2O), 1.95, 1.77 (s each, 3H each, 2CH_3CO), 1.68–1.58 (m, 2H, -O$CH_2$$CH_2$$CH_2$-); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 170.61, 169.7, 166.3, 166.2, 115.28, 99.0, 98.1, 96.9, 77.4, 77.1, 76.8, 72.7, 72.4, 71.5, 70.0, 68.8, 67.8, 66.9, 63.0, 62.9, 52.44, 30.27, 29.8, 28.3, 23.1, 20.8; ESI-MS: *m/z*: calcd for $C_{83}H_{77}NO_{25}$: 1487.48; found: 1488.61 $[M+H]^+$.

4-Pentenyl *O*-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-triacetyl-β-D-mannopyranosyl)]-(1→4)-2-acetamido-3,6-di-O-acetyl-2-deoxy-β-D-glucopyranoside (25): A solution of **24** (150 mg, 101 μmol) in MeOH (10 mL) containing NaOMe (10 μmol) was stirred at RT for 2 h. Then the mixture was neutralized by Dowex W50-X8 (H^+ form), filtered and concentrated. The foregoing compound was dissolved in pyridine (5 mL) and Ac_2O (1 mL). The mixture was stirred at RT overnight. The mixture was diluted with CH_2Cl_2 and washed with $NaHCO_3$, brine, and water. The organic layer was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc 1:2) to give **25** (95 mg, 95%). ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.87–5.62 (m, 2H, NH, -C*H*=), 5.43 (d, *J* = 2.8 Hz, 1H, H-2″), 5.28–5.10 (m, 5H), 5.02–4.94 (m, 3H), 4.77 (s, 1H, H-1″), 4.68 (s, 1H, H-1′), 4.67 (d, *J* = 8.8 Hz, 1H, H-1), 4.43–4.10 (m, 6H), 3.81–3.80 (m, 2H), 3.55–3.28 (m, 3H), 2.11, 2.10, 2.08, 2.06, 2.00, 1.99, 1.97, 1.93, 1.92 (9s, 30H, 9CH_3); ESI-MS: *m/z*: calcd for $C_{43}H_{61}NO_{25}$: 991.35; found: 992.15 $[M+H]^+$.

***O*-[(2,3,4,6-Tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→6)-(2,3,4-tri-*O*-acetyl-β-D-mannopyranosyl)]-(1→4)-3,6-di-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (26):** NIS (13 mg, 1.3 equiv) and TESOTf (14 μL) were added to a solution of **25** (50 mg, 50 μmol) in dry CH_2Cl_2 (5 mL). The mixture was stirred at RT for 0.5 h when TLC indicated the completion of the reaction. The mixture was diluted with CH_2Cl_2 and washed with $NaHCO_3$ and brine. The organic layer was dried by Na_2SO_4 and filtered. The filtrate was concentrated and the residue was purified by flash chromatography on silica gel using (EtOAc/hexanes 2:1 containing 1% Et_3N) to give **26** (37 mg, 80%) as a white solid. ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.91 (d, *J* = 7.5 Hz, 1H, H-1), 5.61–5.60 (s, 1H, H-3), 5.40 (d, *J* = 3.5 Hz, 1H, H-2′), 5.31–5.27 (m, 3H), 5.20 (t, *J* = 10.0 Hz, 1H, H-4′), 5.03 (dd, *J* = 3.0, 10.0 Hz, 1H, H-3″), 4.85 (s, 1H, H-1′), 4.84 (s, 1H, H-1″), 4.34 (dd, *J* = 4.0, 12.0 Hz, 1H, H-6), 4.18–4.08 (m, 5H), 3.92 (dd, *J* = 4.4, 11.0 Hz, 1H), 3.76–3.74 (m, 1H), 3.67–3.59 (m, 2H), 3.45–3.43 (m, 1H), 2.18, 2.15, 2.12, 2.11, 2.10, 2.07, 2.04, 2.01, 1.99, (s each, 3H each, 10CH_3CO), 1.96 (s, 3H, CH_3); ESI-MS: *m/z*: calcd for $C_{43}H_{61}NO_{25}$: 905.28; found: 906.53 $[M+H]^+$.

***O*-[(α-D-Mannopyranosyl)-(1→6)-(β-D-mannopyranosyl)]-(1→4)-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline (3)**: A solution of **26** (22 mg, 24 μmol) in MeOH containing NaOMe (3 μmol) was stirred at RT for 2 h. Then the mixture was concentrated. The residue was dissolved in water and lyophilized to give **3** (13 mg, quantitative) as a white solid. ^{1}H NMR (CD_3OD, 500 MHz): δ = 6.00 (d, *J* = 7.2 Hz, 1H, H-1), 4.79 (s, 1H, H-1′), 4.62 (s, 1H, H-2′), 4.59 (s, 2H, H-1″, H-2″), 4.20 (s, 1H, H-2), 4.04–4.02 (m, 1H), 3.9–3.26 (m, 14H), 2.01 (s, 3H, CH_3-); ^{13}C NMR (CD_3OD, 125 MHz): δ = 168.6, 100.0, 99.7, 99.6, 77.8, 74.5, 73.0, 72.7, 70.4, 70.1, 69.9, 69.4, 66.8, 66.3, 65.1, 61.7, 60.8, 13.0; ESI-MS: *m/z*: calcd for $C_{20}H_{33}NO_{15}$: 527.19; found: 528.29 $[M+H]^+$.

Acceptor GlcNAc-peptide 27: GlcNAc-tripeptide **27** was synthesized on a Pioneer automatic peptide synthesizer (Applied Biosystems) using Fmoc chemistry on a PAL-PEG-PS resin (on 0.2 mmol scale), following the previously described procedure.[7] After the solid-phase synthesis, the peptide was retrieved from the resin with 95% TFA, followed by the treatment with 5% hydrazine to remove the O-acetyl groups. The resulting crude GlcNAc-peptide was purified by preparative RP-HPLC to provide the GlcNAc-tripeptide **27** (104 mg, 88%). ^{1}H NMR (D_2O, 500 MHz): δ = 4.98 (d, *J* = 9.8 Hz, 1H, NH-Ac), 4.65 (d, *J* = 7.0 Hz, 1H, H-1), 4.25 (d, *J* = 4.4 Hz, 1H, Thr-H_α), 4.17 (d, *J* = 7.5 Hz, 1H, Leu-H_α), 4.16 (dd, *J* = 4.5, 6.5 Hz, 1H, Asn-H_α), 3.81 (dd, *J* = 12.4, 1.8 Hz, 1H, H-3), 3.74 (t, *J* = 10.0 Hz, 1H, H-2), 3.68 (dd, *J* = 12.3, 4.8 Hz, 1H, H-6), 3.53 (t, *J* = 8.4 Hz, 1H, H-4), 3.45–3.38 (m, 3H, Leu-H_β, H-5, H-6′), 2.77 (dd, *J* = 5.8, 16.0 Hz, 1H, Asn-H_β), 2.65 (dd, *J* = 16.0, 7.8 Hz, 1H, Asn-H_β), 1.95 (s, 3H, $NHCOCH_3$), 1.94 (s, 3H, $NHCOCH_3$), 1.87–1.90 (m, 1H, Leu-H_β), 1.45–1.48 (m, 2H, Leu-CH_2), 1.14 (d, *J* = 6.6 Hz, 3H, Thr-CH_3), 0.85

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(d, $J=7$ Hz, 3H, Leu-CH$_3$), 0.80 (t, $J=7$ Hz, 3H, Leu-CH$_3$); ESI-MS: m/z: calcd for $C_{24}H_{42}N_6O_{11}$: 590.29; found: 591.29 $[M+H]^+$.

General procedure for the ENGase-catalyzed transglycosylation: A mixture of the respective oligosaccharide oxazoline (10–30 μmol) and the GlcNAc-tripeptide **27** (3 molar equivalents of the donor) or the GlcNAc-C34[7] (5 molar equivalents of the donor) in a phosphate buffer (50 mM, pH 6.5, 0.5–1 mL) was incubated at 25 °C with the enzyme Endo-A (100 mU). The oxazoline derivative was added in portions during the reaction. The reaction was monitored by analytical HPLC by taking aliquots at intervals. It was observed that the transglycosylation reaction with the disaccharide oxazoline derivatives **1** and **4** proceeded slowly and took more than 48 h for reaching the plateau, while the transglycosylation with the tetrasaccharide oxazoline **2** proceeded quickly and would be complete within 2 h. The reaction was stopped by heating in a boiling water bath for 3 min when the peak of the transglycosylation product reached the maximum. The product was purified by preparative HPLC on a Waters preparative column (Symmetry 300, 19x300 mm) to afford the respective glycopeptide, which was characterized by NMR and ESI-MS).

Glycopeptide 28: yield: 3.0 mg, 63 % (based on the reaction with 5 μmol of **27**); ^{1}H NMR (D$_2$O, 500 MHz): δ = 4.97 (d, $J=10.0$ Hz, 1H, NH-Ac), 4.66 (d, $J=7.0$ Hz, 1H, H-1), 4.65 (s, 1H, H-1″), 4.53 (d, $J=7.5$ Hz, 1H, H-1′), 4.24 (d, $J=4.4$ Hz, 1H, Thr-H$_\alpha$), 4.20 (d, $J=7.5$ Hz, 1H, Leu-H$_\alpha$), 4.15 (dd, $J=6.2$, 4.4 Hz, 1H, Asn-H$_\alpha$), 4.00 (d, $J=3.2$ Hz, 1H, H-2″), 3.86–3.32 (m, 18H), 2.77 (dd, $J=5.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 2.65 (dd, $J=7.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 1.99 (s, 3H, NHCOCH$_3$), 1.95 (s, 3H, NHCOCH$_3$), 1.93 (s, 3H, NHCOCH$_3$), 1.92–1.82 (m, 1H, Leu-H$_\beta$), 1.42–1.34 (m, 2H, Leu-CH$_2$), 1.14 (d, $J=6.6$ Hz, 3H, Thr-CH$_3$), 0.85 (d, $J=7.0$ Hz, 3H, Leu-CH$_3$), 0.80 (t, $J=7$ Hz, 3H, Leu-CH$_3$); ESI-MS: m/z: calcd for $C_{38}H_{65}N_7O_{21}$: 955.42; found: 956.39 $[M+H]^+$.

Glycopeptide 29: yield: 5.5 mg, 86 % (based on the reaction with 5 μmol of **27**); ^{1}H NMR (D$_2$O, 500 MHz): δ = 5.02 (s, 1H, H-1⁗), 4.97 (d, $J=10.0$ Hz, 1H, NH-Ac), 4.84 (d, $J=1.40$ Hz, 1H, H-1‴), 4.65 (d, $J=7.1$ Hz, 1H, H-1), 4.55 (d, $J=7.8$ Hz, 1H, H-1″), 4.53 (d, $J=7.5$ Hz, 1H, H-1′), 4.24 (d, $J=4.4$ Hz, 1H, Thr-H$_\alpha$), 4.18 (d, $J=7.5$ Hz, 1H, Leu-H$_\alpha$), 4.15 (dd, $J=4.4$, 6.2 Hz, 1H, Asn-H$_\alpha$), 3.99 (dd, $J=2.0$, 3.5 Hz, 1H,H-2″), 3.90 (dd, $J=1.5$, 3.5 Hz, 1H, H-2⁗), 3.86–3.46 (m, 29H), 2.77 (dd, $J=5.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 2.65 (dd, $J=7.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 1.99 (s, 3H, NHCOCH$_3$), 1.95 (s, 3H, NHCOCH$_3$), 1.93 (s, 3H, NHCOCH$_3$), 1.92–1.82 (m, 1H, Leu-H$_\beta$), 1.42–1.34 (m, 2H, Leu-CH$_2$), 1.14 (d, $J=6.6$ Hz, 3H, Thr-CH$_3$), 0.85 (d, $J=7$ Hz, 3H, Leu-CH$_3$), 0.80 (t, $J=7$ Hz, 3H, Leu-CH$_3$); ESI-MS: m/z: calcd for $C_{50}H_{89}N_7O_{31}$: 1279.53; found: 1280.62 $[M+H]^+$.

Glycopeptide 30: yield: 4 mg, 72 % (based on the reaction with 5 μmol of **27**); ^{1}H NMR (D$_2$O, 500 MHz): δ = 4.97 (d, $J=8.7$ Hz, 1H, NH-Ac), 4.84 (s, 1H, H-1‴), 4.72 (d, $J=7.5$ Hz, 1H, H-1), 4.65 (s, 1H, H-1″), 4.53 (d, $J=7.5$ Hz, 1H, H-1′), 4.24 (d, $J=4.5$ Hz, 1H, Thr-H$_\alpha$), 4.20 (d, $J=7.5$ Hz, 1H, Leu-H$_\alpha$), 4.15 (dd, $J=4.4$, 6.2 Hz, 1H, Asn-H$_\alpha$), 4.00 (d, $J=3.2$ Hz, 1H, H-2″), 3.88–3.32 (m, 23H), 2.75 (dd, $J=5.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 2.61 (dd, $J=7.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 2.00 (s, 3H, NHCOCH$_3$), 1.94 (s, 3H, NHCOCH$_3$), 1.93 (s, 3H, NHCOCH$_3$), 1.92–1.82 (m, 1H, Leu-H$_\beta$), 1.42–1.34 (m, 2H, Leu-CH$_2$), 1.14 (d, $J=6.6$ Hz, 3H, Thr-CH$_3$), 0.85 (d, $J=7$ Hz, 3H, Leu-CH$_3$), 0.80 (t, $J=7$ Hz, 3H, Leu-CH$_3$); ESI-MS: m/z: calcd for $C_{44}H_{75}N_7O_{26}$: 1117.48; found: 1118.60 $[M+H]^+$.

Glycopeptide 31: yield: 2.87 mg, 55 % (based on the reaction with 5 μmol of **27**); ^{1}H NMR (D$_2$O, 500 MHz): δ = 7.38–7.32 (m, 5H, C_6H_5), 4.97 (d, $J=8.5$ Hz, 1H, NH-Ac), 4.66 (d, $J=7.2$ Hz, 1H, H-1), 4.53 (d, $J=7.5$ Hz, 1H, H-1′), 4.24 (d, $J=4.4$ Hz, 1H, Thr-H$_\alpha$), 4.19 (d, $J=7.5$ Hz, 1H, Leu-H$_\alpha$), 4.15 (dd, $J=4.4$, 6.2 Hz, 1H, Asn-H$_\alpha$), 3.98 (d, $J=3.8$ Hz, 1H, H-2″), 3.86–3.40 (m, 21H), 2.77 (dd, $J=5.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 2.65 (dd, $J=7.8$, 16.0 Hz, 1H, Asn-H$_\beta$), 1.99 (s, 3H, NHCOCH$_3$), 1.95 (s, 3H, NHCOCH$_3$), 1.93 (s, 3H, NHCOCH$_3$), 1.90–1.82 (m, 1H, Leu-H$_\beta$), 1.42–1.34 (m, 2H, Leu-CH$_2$), 1.14 (d, $J=6.6$ Hz, 3H, Thr-CH$_3$), 0.85 (d, $J=7$ Hz, 3H, Leu-CH$_3$), 0.80 (t, $J=7$ Hz, 3H, Leu-CH$_3$); ESI-MS: m/z: calcd for $C_{45}H_{71}N_7O_{21}$: 1045.47, found: 1046.40 $[M+H]^+$.

Glycopeptide 32: Yield: 5.6 mg, 75 % (based on the reaction with 1.5 μmol of GlcNAc-C34); ESI-MS: m/z: calcd for: 5018.22; found: 1674.04 $[M+3H]^{3+}$, 1255.64 $[M+4H]^{4+}$, 1004.78 $[M+5H]^{5+}$.

Glycopeptide 33: Yield: 5.0 mg, 68 % (based on the reaction with 1.5 μmol of GlcNAc-C34); ESI-MS: m/z: calcd for: 4946.44; found: 1649.68 $[M+3H]^{3+}$, 1237.50 $[M+4H]^{4+}$, 990.20 $[M+5H]^{5+}$.

Pronase digestion of glycopeptides 32 and 33: Glycopeptide **32** (0.5 mg) was digested with pronase (30 μg, Sigma) in a phosphate buffer (pH 8.2, 100 μL) at 37 °C for 4 h. The reaction mixture was subjected to Sephadex G-10 gel filtration and the carbohydrate positive fractions (detected by anthrone assay) were pooled and subjected to ESI-MS analysis. ESI-MS: m/z: calcd for Man$_2$GlcNAc$_2$Asn: 862.78; found: 863.62 $[M+H]^+$. Similarly the Bn-tagged glycopeptide **33** (0.3 mg) was digested with pronase as described above, and the digestion product was isolated and characterized. ESI-MS: m/z: calcd for Bn-Manβ1→4GlcNAcβ1→4GlcNAcβ1→Asn: 790.31; found: 791.40 $[M+H]^+$.

Acknowledgements

We thank Professor K. Takegawa for kindly providing the pGEX-2T/Endo-A plasmid that was used to overexpress Endo-A. The work was supported by the National Institutes of Health (AI054354 and AI051235).

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Received: September 28, 2005
Published online: February 7, 2006

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