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Chemoenzymatic Synthesis of HIV-1 V3 Glycopeptides Carrying Two *N*-Glycans and Effects of Glycosylation on the Peptide Domain

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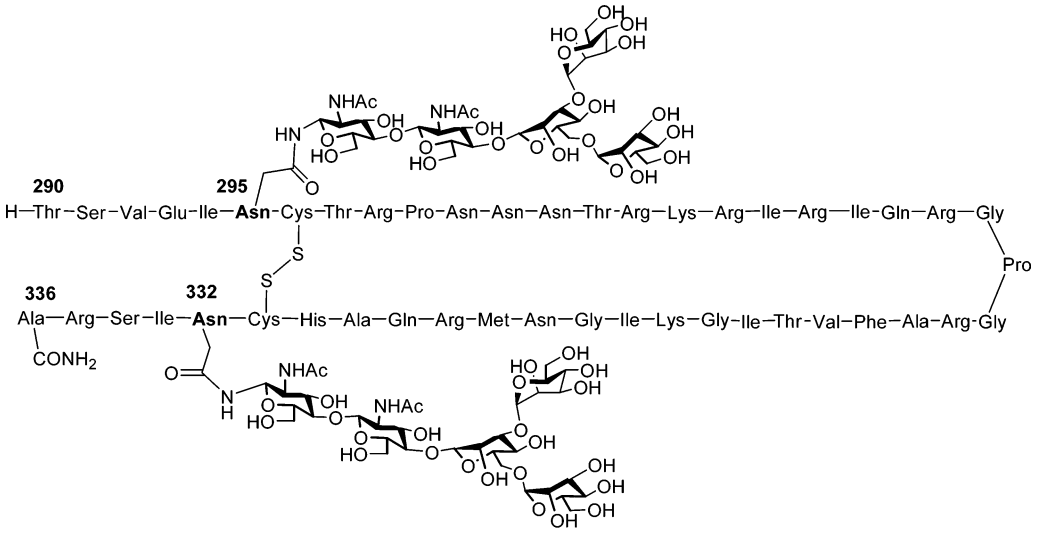

A highly efficient chemoenzymatic synthesis of HIV-1 V3 domain glycopeptides carrying two N-linked core tri- and pentasaccharides was achieved. The synthesis consisted of two key steps: a solid-phase synthesis of the cyclic, 47-mer V3 domain peptide containing two GlcNAc residues and a novel endoglycosidase-catalyzed transglycosylation that simultaneously added two *N*-glycan moieties to the peptide precursor from the oligosaccharide oxazoline donor substrates. The availability of the synthetic glycopeptides allowed the probing of the effects of glycosylation on the HIV-1 V3 domain. It was demonstrated that glycosylation influenced the global conformations of the V3 domain and provided protection of the V3 domain against protease digestion.

Introduction

Glycosylation is one of the most common posttranslational modifications of mammalian and viral proteins. The covalent attachment of carbohydrates has been shown to affect protein's structure and a wide range of biological functions.[1] However, a clear understanding of the effects of glycosylation is often hampered by glycoprotein's structural heterogeneity. Natural glycoproteins are usually produced as a mixture of glycoforms, from which structurally defined glycoforms are difficult to isolate. Various methods have thus been explored to synthesize homogeneous glycopeptides and glycoproteins for structural and functional studies.[2] The incorporation of new techniques, such as native chemical ligation,[3,4] novel solid-phase synthesis,[4,5] and enzymatic oligosaccharide transfer,[6,7] has significantly enhanced our ability to generate large natural glycopeptides and even glyco-

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proteins. Particularly, the chemoenzymatic method involving the endo-β-*N*-acetylglucosaminidase-catalyzed transglycosylation provides a highly convergent approach that allows the specific attachment of a large oligosaccharide to a preassembled GlcNAc-peptide in a single step, albeit in an inherent low transglycosylation yield.[7] Recently, we reported a significant improvement of the method by exploring oligosaccharide oxazolines as the donor substrates.[8] The use of the mimics of the presumed oxazolinium ion intermediate formed in a substrate-assistant mechanism not only expended the substrate availability but also led to a dramatic increase in the transglycosylation yield, leading to a highly efficient synthesis of large glycopeptides.

The third variable (V3) domain of HIV-1 gp120 is the "principal neutralizing determinant (PND)" that represents an important target for HIV-1 vaccines.[9] There are three conserved *N*-glycans within or adjacent to the V3 loop, but the effects of the conserved *N*-glycans on the conformations and immunogenicity of the V3 domain are still poorly understood, mainly because of the large size and microheterogeneity of gp120 molecule.[10] The X-ray crystal structures of gp120 that were recently solved unfortunately left out the V3 domain, for the easiness of crystallization.[11] Some short, linear V3 glycopeptides carrying mono- or disaccharide moieties were previously synthesized for model NMR studies,[12] but a detailed understanding of the structural and immunological properties of the V3 domain requires the construction of homogeneous, larger V3 glycopeptides. We report in this paper the first chemoenzymatic synthesis of full-size, cyclic HIV-1 V3 domain glycopeptides by a novel enzymatic double glycosylation, which simultaneously adds two *N*-glycans to a preassembled polypeptide chain. We also describe here the effects of glycosylation on the global conformations and protease stability of the V3 domain peptides.

Results and Discussion

Synthesis. We sought to construct full-size V3 glycopeptides carrying *N*-glycans at the N295 and N332 glycosylation sites that are conserved in most HIV-1 strains.[10] It was reported that the high-mannose-type *N*-glycans at the N295 and N332 sites were an essential part of the epitope of the broadly neutralizing antibody 2G12.[13] Although the size (ranging from Man5 to Man9) of the high-mannose-type *N*-glycans at the two sites still awaits further characterization, we have chosen first to accomplish a full size V3 domain carrying two N-linked core pentasaccharides. A 47-mer glycopeptide sequence corresponding to the amino acid residues 290−336 of HXB2 gp120 was selected in order to include a number of T-helper epitopes and various B-cell epitopes for the consideration of an effective immunogen design (for epitope mapping of the V3 domain, see http://hiv-web.lanl.gov). The synthesis started with the preparation of the V3 peptide containing two GlcNAc moieties at N295 and N332 positions (Scheme 1). Fmoc-(Ac_3GlcNAc)-Asn-OH was used as the building block to replace the Asn residues at N295 and N332 during the automatic solid-phase peptide synthesis using the PAL-PEG-PS resin. The two Cys residues were temporarily protected as the Acm derivatives. After synthesis, the peptide was then retrieved from the resin with simultaneous side-chain deprotections by treatment with cocktail R (90:5:3:2, TFA−thioanisole−EDT−anisole). After de-*O*-acetylation with 5% aqueous hydrazine, the crude peptide was purified by RP-HPLC to give the linear GlcNAc-peptide (Acm-V3-G2) [ESI-MS calcd 5854.37, found 5853.88 (deconvoluted data)]. The total yield for the peptide synthesis is 16%. Removal of the Acm protecting groups with simultaneous cyclization was achieved by treatment with iodine in 10% acetic acid, giving the cyclic GlcNAc-peptide in 81% isolated yield (Scheme 1). The peptide was isolated by reverse-phase HPLC and characterized by ESI-MS [calcd 5710.19, found 5708.25]. Similarly, the corresponding nonglycosylated, cyclic V3 domain peptide was synthesized in the same manner in a 21% total yield [ESI-MS calcd 5304.13, found 5302.80].

We have previously reported that Man_3GlcNAc-oxazoline was a good substrate for Endo-A catalyzed transg-

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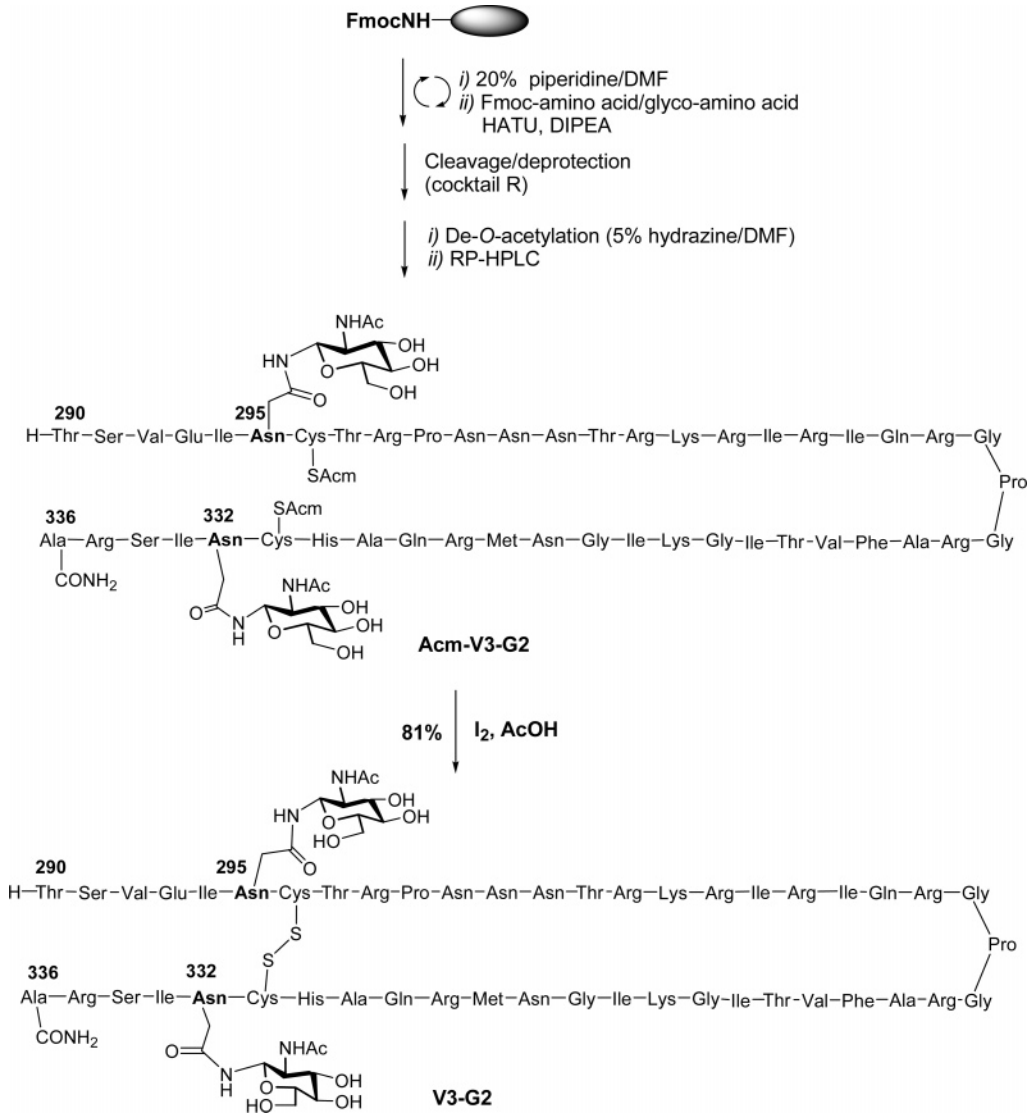
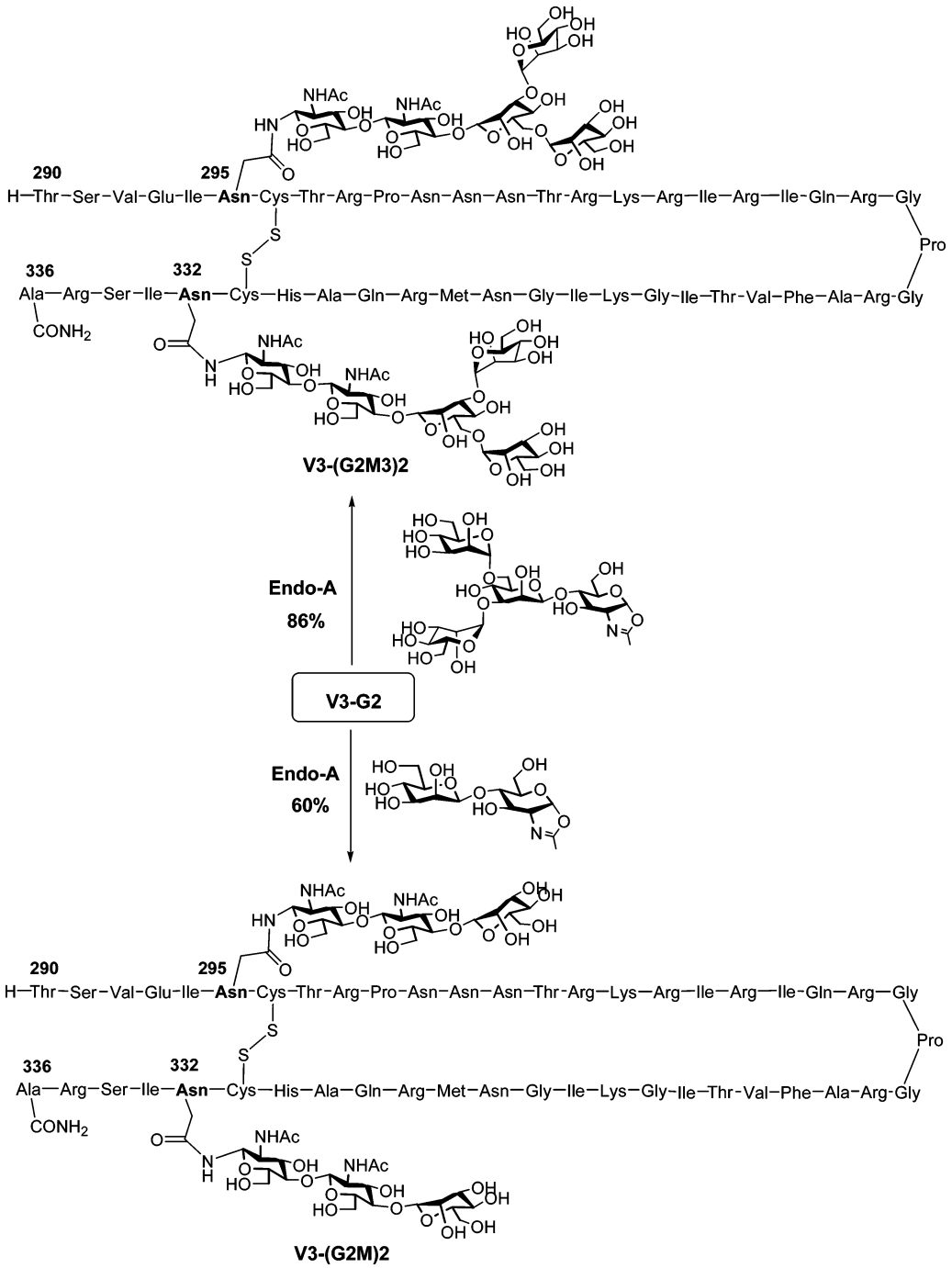
SCHEME 1. Solid-Phase Synthesis of Cyclic V3 Peptide Carrying Two GlcNAc Moieties



lycosylation.[8] Thus, the transglycosylation reaction between the free GlcNAc-peptide as the acceptor (V3-G2) and the synthetic tetrasaccharide oxazoline[8] as the donor (donor/acceptor 3:1) under the catalysis of Endo-A (phosphate buffer, pH 6.5) was examined and monitored by RP-HPLC. It was found that the enzymatic transglycosylation proceeded very efficiently and completed within 2 h to give a single product, which was eluted slightly earlier than the starting GlcNAc-peptide under the analytical HPLC conditions. The product was easily purified by preparative HPLC and was characterized to be the desired glycopeptide V3-(G2M3)2 carrying two core pentasaccharide moieties (isolated yield 86%) (Scheme 2). Deconvolution of the ESI-MS data gave a molecular mass of 7089.68, which is in agreement with the calculated molecular mass, 7089.41, based on average isotope of the glycopeptide. Further structural characterization of the glycopeptide was performed by its Pronase digestion and isolation of the carbohydrate species. Thorough protease digestion yielded a single Asn-linked oligosaccharide that was identical to the authentic Asn-linked core pentasaccharide Manα1→6(Manα1→3)-Manβ1→4GlcNAcβ1→4GlcNAcβ1→Asn ($Man_3GlcNAc_2$-Asn) by ESI-MS and HPAEC analysis. The results indicated that the transferred oligosaccharide was attached to the GlcNAc moiety of the precursor GlcNAc-peptide in a GlcNAcβ1→4GlcNAc linkage. The results confirmed our previous observation that the Endo-A catalyzed transglycosylation proceeded in a regio- and stereospecific manner to form the β1→4-glycosidic bond as present in natural *N*-glycans.[8] Similarly, using the Manβ1→4GlcNAc disaccharide oxazoline as the donor substrate, another V3 glycopeptide, V3-(G2M)2 carrying two core trisaccharide moieties, was obtained in 60% yield [ESI-MS calcd 6440.85, found 6440.50]. It was found that, in contrast to the tetrasaccharide oxazoline donor, the enzymatic transglycosylation with the disaccharide oxazoline was much slower and a monotransglycosylation intermediate was observed during the reaction by HPLC monitoring. It was observed that in the presence of the GlcNAc-peptide acceptor, the transglycosylation of the di- and tetrasaccharide oxazolines was much faster than their enzymatic hydrolysis. In addition, no apparent enzymatic hydrolysis of the transglycosylation products was observed under the reaction conditions. The highly efficient enzymatic double glycosylation with the oli-

SCHEME 2. Chemoenzymatic Synthesis of Cyclic V3 Glycopeptides Carrying Two *N*-Glyans



gosaccharide oxazolines came up with a surprise, as the simultaneous addition of two *N*-glycans to the sterically hindered GlcNAc residues in the cyclic V3 peptide seemed to be difficult at a glance. This is the first example of endoglycosidase-catalyzed double glycosylation for glycopeptide synthesis. Since many biomedically important glycoproteins such as erythropoietin[14] and HIV-1 gp120[10] bear multiple *N*-glycans, the highly convergent chemoenzymatic approach described here may open a new avenue toward the glycosylation engineering and total synthesis of large glycopeptide domains of this important class of biomolecules.

Effects of Glycosylation on the Global Conformations. The effects of glycosylation on the global conformations of the V3 domain were investigated by circular dichroism (CD) spectroscopy and the attenuated total reflection Fourier transformation infrared spectroscopy (ATR-FTIR). As shown in Figure 1A, a strong negative band at 198 nm for both the nonglycosylated peptide V3 and the glycopeptide V3-(G2M3)2 suggested that the V3 domain took largely an unordered structure in aqueous solution. A rough estimate of the possible secondary structures using the K2d software[15] revealed that the

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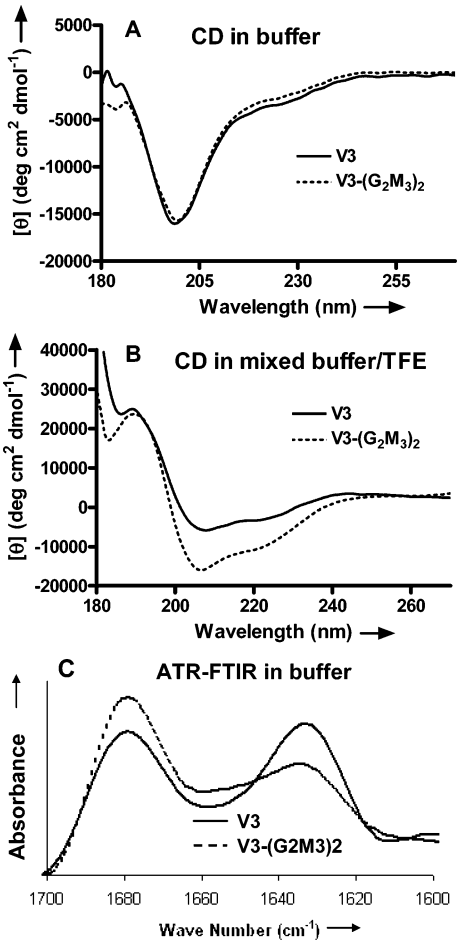

FIGURE 1. Effects of glycosylation on the conformations of V3 domain: (A) CD spectra in phosphate buffer (pH 6.5); (B) CD spectra in buffer containing 30% TFE; (C) the ATR-FTIR spectra in buffer. The CD spectra of the V3 peptide and V3 glycopeptide were measured at 20−40 μM in the media at 23 °C. The ATR-FTIR spectra were measured on a FTIR instrument equipped with a MTC detector and a BioATR cell.

glycopeptide carrying two *N*-glycans showed an enhanced β-turn or loop structure (39%) over the nonglycosylated peptide (24% β-turn or loop structure), suggesting that glycosylation has a tendency to enhance the β-turn structure. This was confirmed by the ATR-FTIR analysis, which is more sensitive to probe protein secondary structures in aqueous media.[16] In comparison, the glycopeptide clearly showed an enhanced band at 1682 cm^{-1} (an indication of enhanced β-turn structure) and a decreased band at 1633 cm^{-1} (an indication of an unordered or β-sheet structure) (Figure 1C). Previous CD and NMR studies on various cyclic nonglycosylated V3 peptides have demonstrated that although free V3 domain peptides took largely a random structure in water, a clear β-turn structure around the tip (GPGR) of the loop was apparent.[17] Our CD and ATR-FTIR studies suggest that glycosylation at the N295 and N332 positions has a tendency to promote β-turn or loop structure of the V3 domain in aqueous media.

It was previously reported that the C-terminal segment of the V3 peptide has a tendency to form α-helical structure in a mixed water/trifluoroethanol (TFE) solvent.[17] When the V3 peptides were transferred to a buffer containing 30% trifluoroenthanol (TFE), the CD spectra indeed revealed a significant amount of α-helical structures for both the nonglycosylated peptide V3 and the glycopeptide V3-(G2M3)2 (Figure 1B). Interestingly, in the mixed water/TFE media, the glycosylation in V3-(G2M3)2 could dramatically increase the amount of α-helical structure relative to that observed in the nonglycosylated V3 domain. Since conformational epitopes of the V3 domain have been implicated as important determinant for neutralizing antibodies against HIV-1,[18] the observed effects of glycosylation on the V3 global conformations suggest that glycosylation might be used as a tool to induce favorable conformational epitopes for an effective immunogen. A clear understanding of how the glycosylation affects the local conformations of various V3 domain segments will await detailed NMR studies.

Effects of Glycosylation on the Peptide Stability against Protease Digestion. The stability of the V3 glycopeptide against protease digestion was also studied. The protease furin was reported to be able to cleave the V3 domain of gp120 at the sequence 302−312.[19] Therefore, the glycopeptide V3-(G2M3)2 and nonglycosylated peptide V3 were treated with furin and the digestion was monitored by HPLC and ESI-MS. It was found that the glycopeptide was degraded much more slowly by furin than the "naked" V3 peptide (Figure 2A). The glycopeptide was also more resistant against digestion by pronase (actinase E) (Figure 2B). The results clearly indicated that the *N*-glycans attached at the N295 and N332 positions could provide protection against protease digestion. The results implicate that the V3 glycopeptides could be much more stable in vivo (against protease digestion) than the "naked" V3 peptides when used as immunogens. Taken together, the conformational and protease stability studies described in this paper provide a fine example of how glycosylation could affect the structural and biological properties of a polypeptide domain. The information will be also useful for the design of a glycopeptide-based HIV-1 vaccine.

Conclusions

A highly efficient synthesis of full-size HIV-1 V3 domain glycopeptides carrying two N-linked core pentassacharides was achieved by a novel enzymatic double glycosylation. The successful synthesis has allowed the probing of the effects of glycosylation on the global

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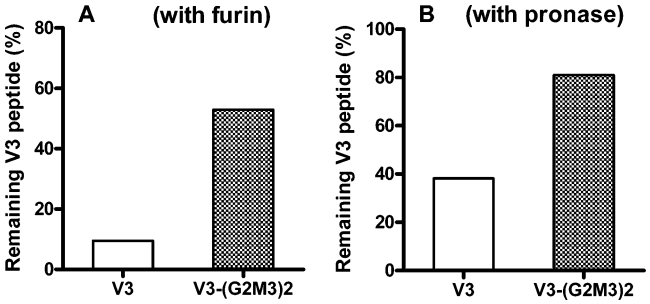

FIGURE 2. Stability of the peptide V3 and the glycopeptide V3-(G2M3)2 toward protease digestion: (A) digestion with furin; (B) digestion with pronase. Reaction conditions: a solution of each peptide (40 μM) in a phosphate buffer (pH 7.0, total 30 μL) was incubated with 10 units of furin (a unit was defined as the amount of furin that hydrolyzes 1 pmol of substrate in 1 min under the optimal condition) for 16 h at 30 °C, and the remaining starting peptide was quantified by HPLC and confirmed by ESI-MS. In the case of pronase, a solution of each peptide (40 μM) in a phosphate buffer (pH 7.4, total 100 μL) was incubated with 125 ng of pronase for 40 min at 30 °C, and the remaining starting peptide was quantified by HPLC and confirmed by ESI-MS.

conformations and protease stability of the HIV-1 V3 domain. The described chemoenzymatic approach will be particularly useful for constructing large glycopeptides bearing multiple *N*-glycans.

Experimental Section

Materials and Methods. The endo-β-*N*-acetylglucosaminidase from *Arthrobacter protophormiae* (Endo-A) was overproduced in *Escherichia coli* as a fusion protein linked to glutathione *S*-transferase (GST) and was purified according to the reported procedure.[20] The plasmid p*GEX-2T*/*Endo-A* used for the overexpression was kindly provided by Dr. K. Takegawa. The building block Fmoc-Asn(Ac_3GlcNAc)-OH used for the glycopeptide synthesis was prepared according to the reported method.[21] The ESI-MS spectra were measured on a single quadruple mass spectrometer.

Reverse-Phase HPLC (RP-HPLC). Analytical RP-HPLC was carried out on a C18 column (3.9 mm × 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 over 25 min. Peptides and glycopeptides were detected at double wavelengths (214 and 280 nm). Preparative RP-HPLC was performed on a preparative C18 column (19 mm × 300 mm). The column was eluted with a suitable gradient of MeCN containing 0.1% TFA at 12 mL/min.

High-Performance Anion Exchange Chromatography Coupled with Pulsed Electrochemical Detection (HPAEC-PED). For the analysis of Asn-linked oligosaccharide, the analytical anion-exchange chromatography was performed under the following conditions: column, CarboPac-PA1 (4 mm × 250 mm); eluent A, 0.1 M NaOH; eluent B, 1 M sodium acetate (NaOAc) in 0.1 M NaOH; gradient, 0−5 min 0% B, 5−25 min 0−15% B; flow rate, 1 mL/min.

Peptide Synthesis. Peptides were synthesized on an automatic solid-phase peptide synthesizer using fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids as building blocks, 2-(1-*H*-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as the coupling reagent and poly(ethylene glycol)-polystyrene resin with a peptide amide linker (PAL-PEG-PS resin) as the solid support. Synthesis was carried out on a 0.2 mmol scale and 4-fold Fmoc-amino acid building blocks were used for each coupling reaction. The peptides were released from the resin with simultaneous deprotection by treatment with cocktail R (TFA−thioanisole−EDT−anisole, 90:5:3:2) and precipitated by cold ether. The crude peptides were analyzed and purified by reverse-phase HPLC. In the case of GlcNAc-containing peptides, the crude peptides were treated with 5% hydrazine in water to remove *O*-acetyl groups before HPLC purification. The Acm group at cysteine 294 and cysteine 331 were removed by treatment of 0.1 M iodine in 10% acetic acid at 0.2 mg/mL concentration of peptide with simultaneous disulfide bond formation. The analytical data of the peptides and GlcNAc-peptides are shown below.

Cyclic V3 Peptide without GlcNAc (V3). HPLC (0−90% MeCN in 20 min): t_R = 9.43 min. ESI-MS: calcd 5304.13; found 663.73 $(M + 8H)^{8+}$, 758.47 $(M + 7H)^{7+}$, 884.69 $(M + 6H)^{6+}$, 1061.40 $(M + 5H)^{5+}$.

Acm-Protected, Linear V3 Peptide Containing Two GlcNAc (Acm-V3-G2). HPLC (0−90% MeCN in 20 min): t_R = 8.85 min. ESI-MS: calcd 5854.37; found 533.19 (M + 11H), 586.37 $(M + 10H)^{10+}$, 651.23 $(M + 9H)^{9+}$, 732.54 $(M + 8H)^{8+}$, 837.01 $(M + 7H)^{7+}$, 976.50 $(M + 6H)^{6+}$, 1171.35 $(M + 5H)^{5+}$.

Cyclic V3 Peptide Containing Two GlcNAc (V3-G2). HPLC (0−90% MeCN in 20 min): t_R = 9.32 min. ESI-MS: calcd 5710.19; found 714.54 $(M + 8H)^{8+}$, 816.47 $(M + 7H)^{7+}$, 952.37 $(M + 6H)^{6+}$, 1142.66 $(M + 5H)^{5+}$.

Typical Procedure for the Endo-A Catalyzed Double Transglycosylation. A mixture of the oligosaccharide oxazoline (30 μmol, 3 molar equiv to each GlcNAc in the acceptor) and the acceptor $(GlcNAc)_2$-peptide (5 μmol) in a phosphate buffer (50 mM, pH 6.0, 1 mL) was incubated at 23 °C with the enzyme Endo-A (100 milliunits). The reaction was monitored by analytical HPLC on a C18 column (3.9 mm × 150 mm) at 40 °C with a linear gradient (0−90% MeCN containing 0.1% TFA in 25 min, flow rate 1 mL/min). In the case of the tetrasaccharide oxazoline, the double transglycosylation was complete within 2 h, yielding a single transglycosylation product that was eluted earlier than the peptide acceptor. However, in the case of the disaccharide oxazoline, the reaction was much slower and about 70% double transglycosylation was achieved after 6 h. The enzymatic reaction was stopped by heating in a boiling water bath for 3 min. The product was purified by preparative HPLC on a preparative column (19 mm × 300 mm). The fractions containing the desired glycopeptide product were pooled and lyophilized. The purity and identity of the glycopeptides were analyzed by anyltical HPLC and ESI-MS.

Cyclic V3 Domain Glycopeptide Carrying Two Core Asn-Linked Pentasaccharides V3-(G2M3)2. HPLC (0−90% MeCN in 20 min): t_R = 8.95 min. ESI-MS: calcd 7089.41; found 887.21 $(M + 8H)^{8+}$, 1013.87 $(M + 7H)^{7+}$, 1182.67 $(M + 6H)^{6+}$, 1419.25 $(M + 5H)^{5+}$. Yield: 86%. Further structural characterization of the glycopeptide was performed by pronase digestion and product analysis. Briefly, glycopeptide V3-(G2M3)2 was digested with pronase in a phosphate buffer (pH 8.2) at 37 °C for 6 h. The reaction mixture was lyophilized and the residue was subjected to Sephadex G-10 gel filtration. The carbohydrate positive fractions (detected by anthrone assay) were pooled and subjected to ESI-MS and HPAEC-PED analysis. The isolated oligosaccharide was characterized to be $Man_3GlcNAc_2Asn$ [ESI-MS calcd for $Man_3GlcNAc_2Asn$ 1024.37; found 1025.55 $(M + H)^+$]. Under the HPAEC condition (described above), the product showed a single peak at 14.13 min, which is identical to the standard $Man_3GlcNAc_2Asn$, the core Asn-linked pentasaccharide. These results confirm that the transglycosylation added the tetrasaccharide to the Asn-linked GlcNAc residue in the peptide to form the core Asn-linked pentasaccharide structure in the resulting glycopeptide.

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The cyclic V3 domain glycopeptide carrying two core Asn-linked trisaccharides V3-(G2M)2: HPLC (0−90% MeCN in 20 min): $t_R = 9.18$ min. ESI-MS: calcd 6440.85; found 806.12 $(M + 8H)^{8+}$, 920.15 $(M + 7H)^{7+}$, 1075.23 $(M + 6H)^{6+}$, 1289.93 $(M + 5H)^{5+}$. Yield: 60%.

Circular Dichroism (CD) Spectroscopy. CD spectra were recorded at 23 °C in a sodium phosphate buffer (5 mM, pH 7.0) in a 0.1 cm cuvette, scanning at 20 nm/min with a bandwidth of 1 nm and data pacing of 0.5 nm. Each spectrum represents the average of three individual scans after subtracting the background spectra. The samples were prepared at concentrations of 20−40 μM.

Attenuated Total Reflection Fourier Transformation Infrared (ATR-FTIR) Spectroscopy. The ATR-FTIR spectra were measured with a Tensor 27 FTIR instrument equipped with an MCT detector cooled with liquid nitrogen. Each sample (10 μL in 5 mM sodium phosphate buffer, pH 7.0) was loaded into the BioATR II cell, and 1024 scans at 2 cm^{-1} resolution were collected for each sample under constant purging with nitrogen. Spectra were corrected for water vapor, and background spectra of the same buffer were subtracted. The bands were resolved by Fourier self-deconvolution in the Opus 4.2 software package using a Lorentzian line shape and parameters equivalent to 20 cm^{-1} bandwidth and noise reduction factor of 0.1.

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Supporting Information Available: HPLC profiles and ESI-MS spectra of the synthetic HIV-1 V3 domain glycopeptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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