Chemoenzymatic Synthesis of HIV-1 gp41 Glycopeptides: Effects of Glycosylation on the Anti-HIV Activity and α-Helix Bundle-Forming Ability of Peptide C34

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C34 is a 34-mer peptide derived from the C-terminal ectodomain of HIV-1 envelope glycoprotein, gp41. The C34 region in native gp41 carries a conserved N*-glycan at Asn637 and the sequence is directly involved in the virus–host membrane fusion, an essential step for HIV-1 infection. This paper describes the synthesis of glycoforms of C34 which carry a monosaccharide, a disaccharide, and a native oligosaccharide moiety. The synthesis of the glycopeptide which carries a native high-mannose type* N*-glycan was achieved by a chemoenzymatic approach by using an endoglycosidase-catalyzed oligosaccharide transfer as the key step. The effects of glycosylation on the inhibitory activity and the helix-bundle forming ability of C34 were investigated. It was found that glycosylation moderately decreases the anti-HIV activity of C34 and, in comparison with C34, glyco-C34 forms less compact six-helix bundles with the corresponding N-terminal peptide, N36. This study suggests that conserved glycosylation modulates the anti-HIV activity and conformations of the gp41 C-peptide, C34.*

Introduction

The human immunodeficiency virus type-1 (HIV-1), the causative agent of AIDS, is heavily glycosylated. The outer envelope glycoprotein, gp120, typically has 24 *N*-glycans,[1] and the transmembrane envelope glycoprotein, gp41, carries four conserved *N*-glycans.[2] It has been demonstrated that HIV-1 carbohydrates play a critical role in viral immune evasion,[3] and serve as ligands for dendritic-cell specific lectin DC-SIGN, during HIV-1 transmission.[4] However, the precise role of individual *N*-glycans, for example, the effects of individual glycosylation on the local conformation and immunogenicity of the HIV-1 envelope glycoproteins, are hitherto unclear. We believe that these problems can be adequately addressed by bioorganic synthesis and functional evaluation of related HIV-1 glycopeptides that represent partial structures of the envelope glycoproteins.

Peptide C34 is a 34-mer peptide derived from the C-terminal ectodomain region of gp41 (amino acids 628–661; Figure 1).[5–7] This sequence in the native gp41 carries an *N*-glycan at the conserved glycosylation site Asn637. The C34 sequence has a tendency to form six-helix bundles with the N-terminal peptide, N36, of gp41 which provides the driving force for virus–host membrane fusion.[5,6] Moreover, synthetic peptide C34 has been shown to be a potent inhibitor against HIV-1 infection.[5,8] Therefore, characterization of the effects of glycosylation in this region could provide important insights on the mechanism of HIV-1 membrane fusion and for HIV-1 inhibitor design. This paper describes the chemoenzymatic synthesis of the HIV-1 gp41 glycopeptides which corresponds to C34, and the evaluation of the effects of glycosylation on the anti-HIV activity and helix-bundle forming ability of peptide C34.

Results and Discussion

Chemoenzymatic synthesis of C34 glycopeptides

We sought to construct homogeneous glycoforms of C34 that contain a monosaccharide, a disaccharide, and a natural oligosaccharide moiety, respectively (Scheme 1). While glycopeptides that contain monosaccharides or a small oligosaccharide moiety can be prepared by conventional solid-phase peptide synthesis by using glycoamino acid building blocks,[9] the construction of large, biologically relevant glycopeptides is still a difficult task.[10] To construct the glycopeptide M9-C34 that carries a large, native high-mannose type *N*-glycan, we decided to take the chemoenzymatic approach by using the *Arthrobacter* endo-β-*N*-acetylglucosaminidase (Endo-A) as the key enzyme. Endo-A and another endoenzyme, Endo-M from *Mucor hiemalis*, are able to transfer an intact oligosaccharide moiety to a suitable *N*-acetylglucosamine (GlcNAc) containing peptide to form a new β-1,4-glycosidic linkage. Therefore, the novel transglycosylation activity was explored for chemoenzymatic synthesis and has emerged as a powerful method for the con-

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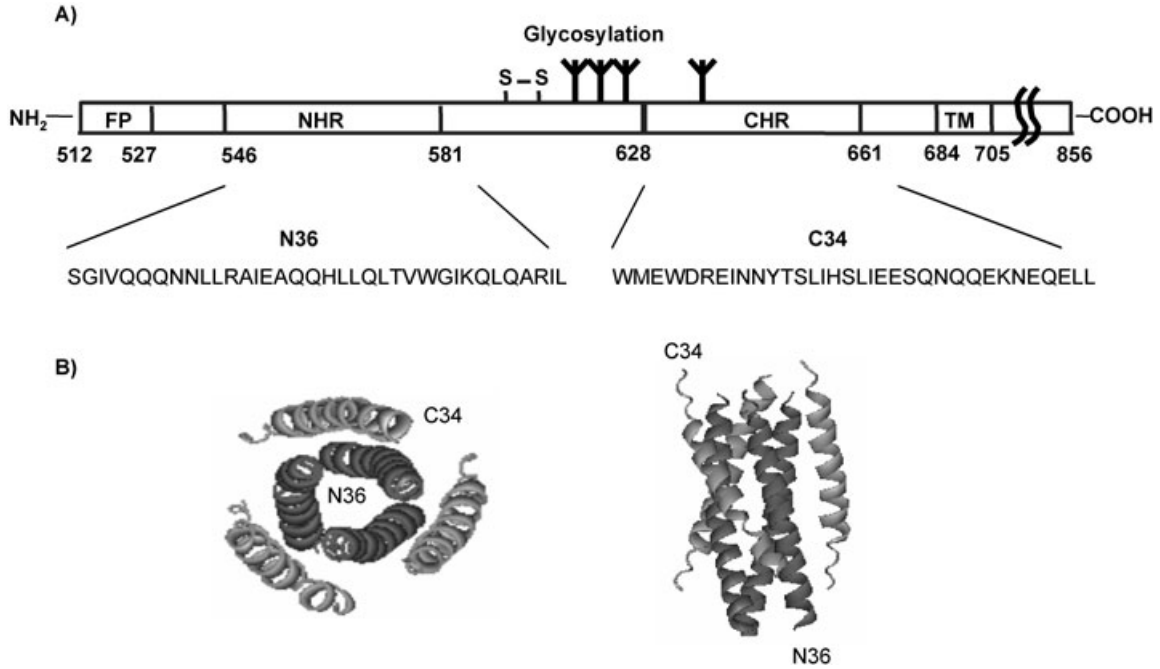
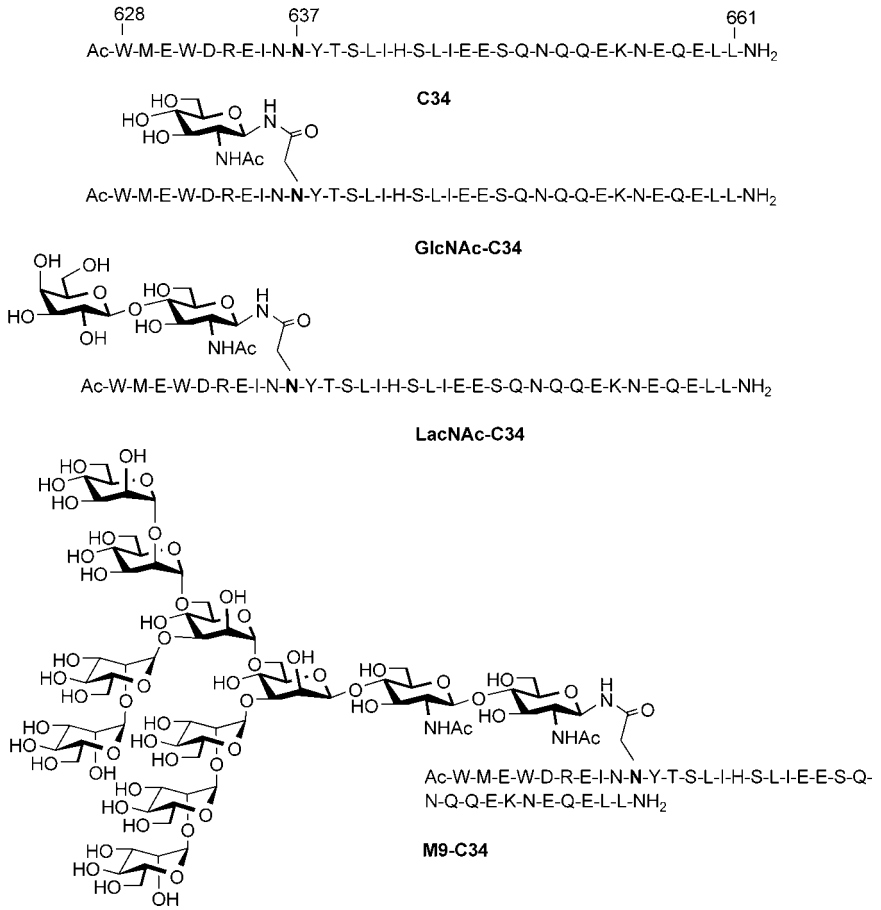


Figure 1. The schematic depiction of HIV-1 gp41. A) The glycosylation sites, the amino acid sequences of N36 and C34, and their locations in the gp41 ectodomain are shown. FP, fusion peptide; NHR, the N-terminal heptad repeat; CHR, the C-terminal heptad repeat; TM, the transmembrane domain. B) Top (left) and side views (right) of the crystal structure of the N36–C34 six-α-helix bundle (the fusion-active core structure of gp41).

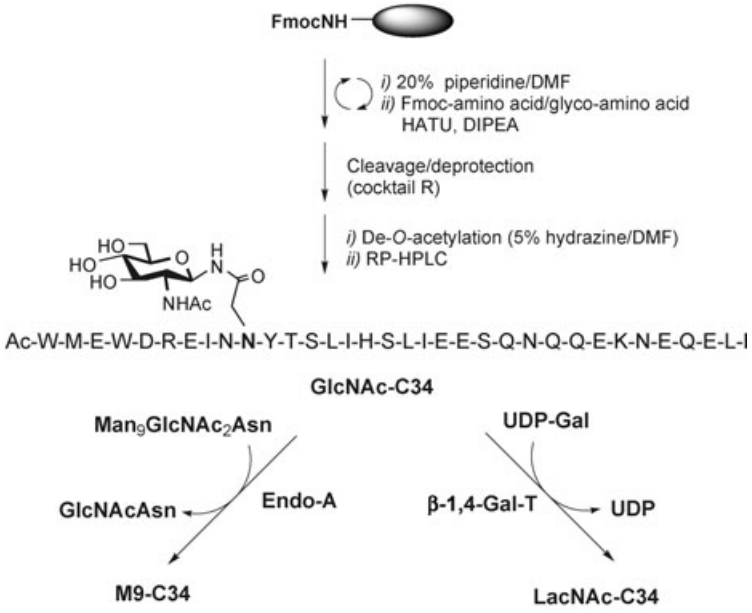


Scheme 1. The structures of C34 and glyco-C34.

struction of complex glycoconjugates that have been hitherto difficult to obtain by other methods.[11,12] Endo-A is specific for high-mannose type *N*-glycans, and we have used the enzyme previously to construct HIV-1 gp120 glycopeptides.[12,13]

The GlcNAc-C34 was synthesized on an automated solid-phase peptide synthesizer according to the previously reported procedure,[14] by using fluorenylmethoxycarbonyl (Fmoc) amino acids and/or Fmoc-Asn-(Ac_3GlcNAc)-OH as the building blocks. The synthetic crude GlcNAc-C34 was purified by reverse-phase HPLC and characterized by electron spray ionization-mass spectrometry (ESI-MS: calculated $M = 4492.84$; found $M = 4492.02 \pm 0.54$ (based on the deconvolution of the data)). Peptide C34 was synthesized and purified in the same way (ESI-MS: calculated $M = 4290.64$; found $M = 4289.16 \pm 0.64$ (based on the deconvolution of the data)). The oligosaccharide donor $Man_9GlcNAc_2Asn$ that was

used for transglycosylation was prepared from soybean flour following a modified procedure.[15] We observed that, as in the case of C34,[5,8,16] GlcNAc-C34 demonstrated a very low solubility in aqueous media. An initial attempt to perform the enzymatic transglycosylation in phosphate buffer failed to give any transglycosylation product due to the low concentration of the acceptor GlcNAc-C34 in the reaction media. Addition of acetone[17] improved neither the solubility of GlcNAc-C34 nor the transglycosylation. Eventually, we found that performing the enzymatic reaction in a phosphate buffer (pH 6.6) that contained 30% dimethyl sulfoxide (DMSO) led to the formation of the desired transglycosylation product M9-C34, which was isolated in 11% yield by RP-HPLC (Scheme 2). Therefore, the role of DMSO in the reaction media is two-fold: it enhances the solubility of GlcNAc-C34 and decreases the activity of water.[17]



Scheme 2. Chemoenzymatic synthesis of glyco-C34.

The identity of M9-C34 was characterized by mass spectrometry (ESI-MS: calculated $M = 6155.29$; found $M = 6155.16 \pm 0.07$ (based on the deconvolution of the data)) Further structural characterization was performed by two specific transformations. First, treatment of M9-C34 with endo-β-*N*-acetylglucosaminidase from *Flavorbacter* (Endo-F1), which specifically cleaves *N*-glycans at the GlcNAcβ1–4GlcNAc core, gave two products: the $Man_9GlcNAc$ (detected by Dionex HPAEC) and GlcNAc-C34 (detected by HPLC). This indicates that the transferred oligosaccharide $Man_9GlcNAc$ was linked to the GlcNAc in the peptide through the expected β1,4-glycosidic linkage. Secondly, treatment of M9-C34 with pronase, which hydrolyzes all the peptide bonds of a given glycoprotein to release the Asn-linked *N*-glycan, led to the release of $Man_9GlcNAc_2Asn$ (detected by Dionex HPAEC analysis). This suggests that an intact *N*-glycan was attached to the Asn residue in the peptide. Taken together, the data unambiguously confirmed the structure of M9-C34. The chemoenzymatic approach, although giving a relatively low yield for the transglycosylation step, allowed quick access to the desired HIV-1 glycopeptide with high purity, which is otherwise difficult to obtain by other methods such as total chemical synthesis.[10] A glycopeptide carrying a disaccharide moiety, the *N*-acetyl lactosamine-C34 (LacNAc-C34), was synthesized by the enzymatic transfer of a galactose residue to the GlcNAc-C34 moiety from UDP-Gal under the catalysis of β-1,4-galactosyltransferase (GalT) (Scheme 2).[18] The product LacNAc-C34 was isolated in 78% yield by RP-HPLC (ESI-MS: calculated $M = 4654.98$; found $M = 4654.72 \pm 0.01$ (based on the deconvolution of the data)). It should be pointed out that in contrast to C34 and GlcNAc-C34 which have low solubility, LacNAc-C34 and M9-C34, are readily soluble in aqueous media. The purity and identity of the synthetic C34 and glyco-C34 were verified by analytical HPLC (Figure 2) and ESI-MS spectrometry (Figure 3), respectively.

Anti-HIV activity of C34 glycopeptides

The anti-HIV activities of the synthetic C34 glycopeptides were measured by using a cell-fusion assay.[19] The results are listed in Table 1. All the glycoforms of C34 demonstrated potent inhibitory activities against HIV-1 infection at *nanomolar* concentrations (Table 1). Attachment of a monosaccharide (GlcNAc) or a disaccharide moiety (LacNAc) to C34 had no significant effect on its anti-HIV activity. However, the glycopeptide carrying a large native *N*-glycan, M9-C34 ($IC_{50} = 7.7$ nM), demonstrated a moderate decrease in anti-HIV activity when compared with C34 ($IC_{50} = 1.1$ nM). Nevertheless, from perspective of anti-HIV drug development, the glycopeptides could be superior to C34 in two aspects. First, LacNAc-C34 and M9-C34 have a much better solubility under physiological conditions than C34, which overcomes a major drawback encountered for the clinical use of C34.[5,8,16] Secondly, because of the general protective effect of glycosylation, the glycopeptides could be more resistant to protease digestion in vivo than C34, although the overall in vivo efficiency of glyco-C34 and C34 is yet to be tested in animal models.

Table 1. Inhibitory activity of C34 and glyco-C34.[a]

Inhibitors	IC50 (nM)[b]		IC90 (nM)[c]	
	Mean	SD	Mean	SD
C34	1.10	0.07	2.16	0.11
GlcNAc-C34	1.32	0.05	2.46	0.10
LacNAc-C34	1.36	0.05	2.50	0.12
M9-C34	7.66	0.50	11.98	0.57

[a] Anti-HIV-1 activity was determined by a cell-fusion assay by using MT-2 and H9 cells chronically infected with HIV-1IIIB (H9/HIV-1IIIB) as target and effector cells, respectively. [b] IC50, concentration of the inhibitor that blocks HIV-1 fusion by 50% [c] IC90, concentration of the inhibitor that blocks HIV-1 fusion by 90%

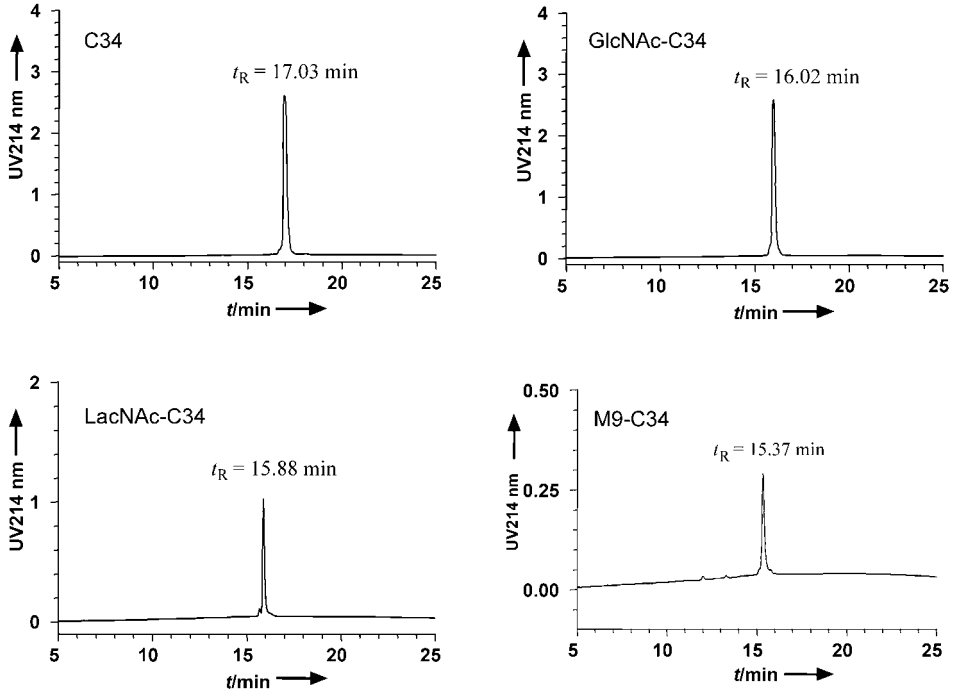


Figure 2. HPLC profiles of C34 and glyco-C34. The analytical HPLC was performed with a Waters 626 HPLC instrument on a Waters Nova-Pak C18 column (3.9×150 mm) at 40 °C. The column was eluted with a linear gradient of 0–90% MeCN (0.1% TFA), at a flow rate of 1 mL min^{-1} over 25 minutes. Peptides and glycopeptides were detected at double wavelengths (214 and 280 nm).

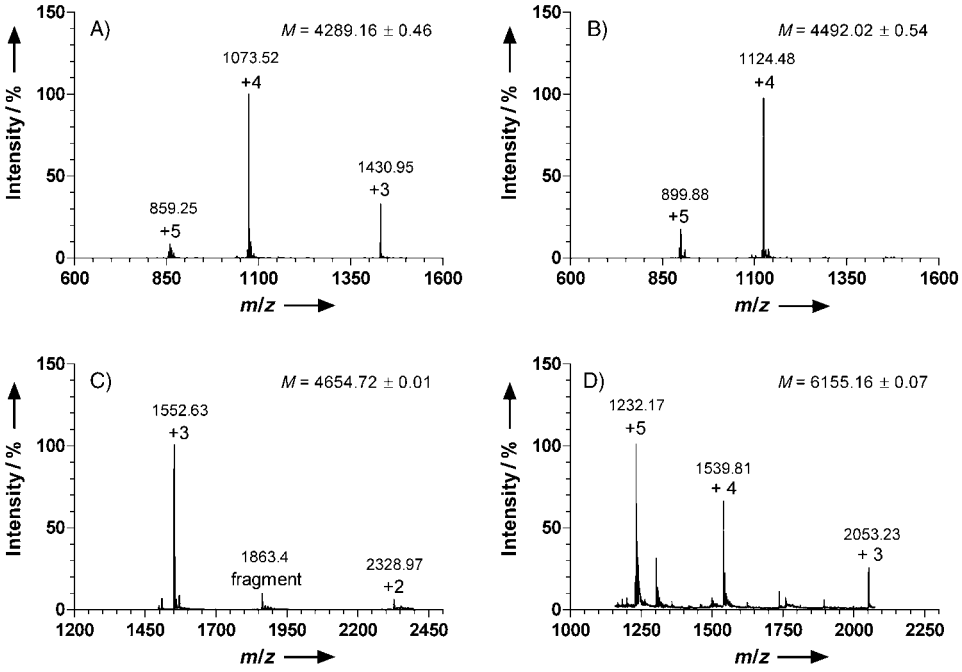


Figure 3. The ESI-MS profiles of synthetic C34 and glyco-C34. The mass spectra were measured on a micromass ZQ-4000 single quadruple mass spectrometer. A, C34; B, GlcNAc-C34; C, LacNAc-C34; D, M9-C34.

Effects of glycosylation on the helix-bundle forming ability of peptide C34

It is known that C34 has an almost featureless and random structure in aqueous media.[8,20] We investigated the conformations of the synthetic glyco-C34 in the presence or absence of the N-peptide, N36, by circular dichroism (CD) analyses. In the absence of N36, all the C34 glycopeptides demonstrated almost the same CD spectra as that of C34. This suggests that C34 glycosylation did not change its solution conformations. However, it was found that in the presence of N36, the formation of N–C-peptide α-helical bundles were induced. CD spectra of equimolar mixtures of N36 and the respective C-peptide showed typical α-helical conformations between the peptide complexes (Figure 4). The stability of the helical bundles was assessed by monitoring the changes in the ellipticity as a function of temperatures. It was observed that the apparent melting temperature (T_m) for N36–glyco-C34 were generally lower than that of N36–C34 (Table 2). This indicates that glycosylation of C34 partially disrupted the six-helix bundle formation. The T_m results are consistent with the observed α-helical contents calculated for the complexes. The higher the α-helical content, the more stable is the complex (Table 2). It should be pointed out that natural HIV-1 gp41 bears an *N*-glycan at the C34 sequence. Therefore, the N36–glyco-C34 complex (particularly M9-C34) would resemble the actual fusion-active structure of gp41 more closely than the N36–C34 complex which lacks the natural *N*-glycan. It was previously reported that immunization in mice with a model polypeptide N36(L6)C34, which folds into a stable six-helix bundle, raised antibodies specific for the bundles, but the antibodies were unable to neutralize HIV-1.[21] One explanation was that once a stable fusogenic structure of gp41 was formed, the structure, that is the stable six-helix bundle of gp41, would become inaccessible to antibody neutralization. Alternatively, the antibodies raised by N36–C34 might primarily recognize the peptide backbone un-

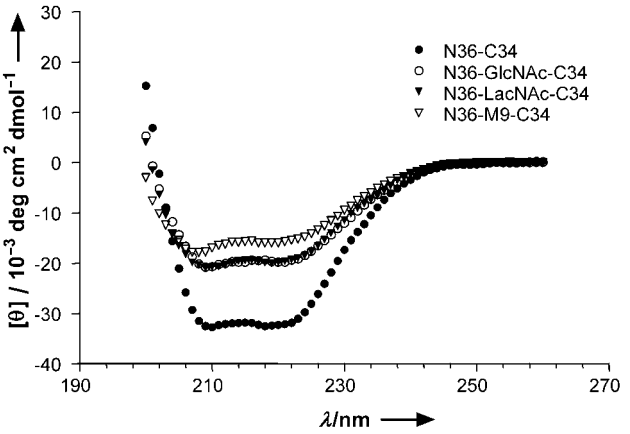


Figure 4. The CD spectra of the N36–C34 and N36–glyco-C34 complexes. Equimolar amounts (10 μM each) of the N- and C-peptide was incubated at 37 °C for 30 min in PBS. The CD spectra of each mixture were then measured at 4 °C on a Jasco spectropolarimeter.

Table 2. The estimated α-helical content and T_m of the N- and C-peptide complexes.

	$[\theta]_{222}$ [° cm^2 dmol^{-1}]	T_m [°C]	α-helix [%][a]
N36	−10466	/	32
C34	−17	/	0
N36/C34	−31777	62	96
N36/GlcNAc-C34	−19392	54	58
N36/LacNAc-C34	−19231	56	58
N36M$^-$9-C34	−15386	51	47

[a] α-helical content was calculated with the equation: $\alpha\text{-helix}\% = [\theta]_{222}/(-33000)\times 100\%$.

derneath the natural *N*-glycan, which is actually inaccessible to antibody neutralization in HIV-1 gp41 due to the shielding effect of the *N*-glycan. Therefore, a plausible target in vaccine design would be the transition state (prefusogenic) structure of gp41 that might be exposed to the immune system during membrane fusion processes. The less compact complex formed by N36–M9-C34 (with ca. 50% α-helical content) might capture at least part of the transition state structure of gp41 that is exposed during membrane fusion. Moreover, the *N*-glycan itself might serve as a target for immune recognition, as demonstrated by the carbohydrate epitope for neutralizing antibody 2G12.[22] Therefore, it would be interesting to test whether glyco-C34 or its complex with N36 will serve as new types of immunogens for future HIV-1 vaccine development.[23]

Conclusion

We have successfully synthesized several glycoforms of the gp41 peptide, C34, by a chemoenzymatic approach. The availability of the glycopeptides allowed the assessment of glycosylation effects on the structure and function of C34. It was found that glycosylation affects the anti-HIV activity of C34 and its ability to form six-helix bundles with the corresponding N-peptide, N36. The less compact complex between N36–glyco-C34 might arrest certain conformation-dependent neutralizing epitopes valuable for HIV-1 vaccine design.

Experimental Section

Materials and methods: Bovine milk galactosyltransferase (GalT), UDP-Gal, and α-lactalbumin were purchased from Sigma/Aldrich Chemical Company (St. Louis, MO). Endo-F1 was purchased from PROZYME (San Leandro, CA). Endo-A was a gift kindly provided by Prof. K. Takegawa. All Fmoc-protected amino acids used for peptide synthesis were purchased from Novabiochem (La Jolla, CA). The building block Fmoc-Asn(Ac_3GlcNAc)-OH used for glycopeptide synthesis was synthesized according to reported procedure.[24] 2-(1-H-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), *N*,*N*-diisopropylethylamine (DIPEA), and Fmoc-PAL-PEG-PS were purchased from Applied Biosystems. HPLC grade acetonitrile was purchased from Fisher Scientific (Pittsburgh, PA). Dimethyl formamide (DMF) was purchased from B & J Biosynthesis (Morristown, NJ). All other chemicals and biochemicals were purchased from Pierce or Sigma/Aldrich and used as received. The ESI-MS spectra were measured on a micromass ZQ-4000 single quadruple mass spectrometer.

Reverse-phase HPLC: Analytical HPLC was carried out with a Waters 626 HPLC instrument on a Waters Nova-Pak C18 column (3.9×150 mm) at 40 °C.[25] The column was eluted with a linear gradient of 0–90% MeCN (0.1% TFA) at a flow rate of 1 mL min^{-1} over 25 min. Peptides and glycopeptides were detected at double wavelengths (214 and 280 nm). Preparative HPLC was performed with a Waters 600 HPLC instrument by using a Waters C18 column (Symmetry 300, 19×300 mm). The column was eluted with a suitable gradient of MeCN (0.1% TFA) at 12 mL min^{-1}.

Dionex high-performance anion exchange chromatography coupled with pulsed electrochemical detection (HPAEC-PED): The analytical anion-exchange chromatography was performed on a Dionex DX600 chromatography system (Dionex Corporation, CA) equipped with an electrochemical detector (ED50, Dionex Corporation, CA). The following conditions were used: column, CarboPac-PA1 (4×250 mm); two eluent system: eluent A (0.1 M NaOH); eluent B (1 M NaOAc in 0.1 M NaOH); gradient: 0–5 min, 0% eluent B; 5–25 min, 0–15% eluent B; flow rate, 1 mL min^{-1}. Under these conditions, $Man_9GlcNAc_2Asn$ and $Man_9GlcNAc$ were separated and appeared at 17.1 and 16.8 min, respectively.

Peptide synthesis: Peptides were synthesized on a Pioneer automatic peptide synthesizer (Applied Biosystems) by using Fmoc-protected amino acids as building blocks, HATU as the coupling reagent, and polyethylene glycol-polystyrene resin with a peptide amide linker (PAL-PEG-PS resin) as the solid support. 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 with cold ether. The crude peptides were purified by preparative HPLC. In the case of GlcNAc-C34, the crude peptides were treated with 5% hydrazine in water to remove O-acetyl groups before HPLC purification. The purity and identity of the peptides were analyzed by HPLC and ESI-MS. ESI-MS of C34: calculated $M = 4290.64$; found, 1430.95 $[M+3H]^{3+}$, 1073.52 $[M+4H]^{4+}$, 859.25 $[M+5H]^{5+}$. ESI-MS of GlcNAc-C34: calculated $M = 4492.84$; found, 1124.48 $[M+4H]^{4+}$, 899.88 $[M+5H]^{5+}$.

Endo-A catalyzed transglycosylation, M9-C34 synthesis: A mixture of $Man_9GlcNAc_2Asn$ (5 mg) and GlcNAc-C34 (7 mg) in ammonium acetate buffer (300 µL, 50 mM, 30% DMSO, pH 6.0) was incu-

bated at 37 °C with recombinant Endo-A (50 milliunits). The reaction was monitored by analytical HPLC on a Waters Nova-Pak C18 column (3.9×150 mm) at 40 °C with a linear gradient (0–90% MeCN containing 0.1% TFA in 25 min, flow rate 1 mL min^{-1}). When the transglycosylation product, M9-C34, which appears earlier than the GlcNAc-C34, reached the maximal, the enzymatic reaction was stopped by heating in a boiling water bath for 3 min. The product was purified by preparative HPLC on a Waters preparative column (Symmetry 300, 19x300 mm) to give M9-C34 (1.7 mg, 11% based on the oligosaccharide donor used). Excess GlcNAc-C34 was recovered. ESI-MS of M9-C34: calculated $M=6155.29$; found, 2053.23 $[M+3H]^{3+}$, 1539.81 $[M+4H]^{4+}$, 1232.17 $[M+5H]^{5+}$.

GalT-catalyzed synthesis of LacNAc-C34: A mixture of GlcNAc-C34 (2 mg), UDP-Gal (1 mg), a-lactalbumin (100 μg), $MnCl_2$ (20 mM), and bovine milk GalT (0.3 unit) in 2 mL of HEPES buffer (50 mM, pH 7.5) was incubated for 20 h at 37 °C. The mixture was lyophilized and the product was purified by RP-HPLC to give LacNAc-C34 (1.6 mg, 78%). ESI-MS of LacNAc-C34: calculated $M=4654.98$; found, 2328.97 $[M+2H]^{2+}$, 1552.63 $[M+3H]^{3+}$.

Inhibition of HIV-1 infection by synthetic peptides and glycopeptides: Anti-HIV-1 activities of the peptides and glycopeptides were determined by a cell-fusion assay by using MT-2 and HIV-1_{IIIB} chronically infected H9 (H9/HIV-1_{IIIB}) cells as target and effector cells, respectively.

Briefly, HIV-1_{IIIB}-infected H9 cells were labeled with a fluorescent reagent, 2′,7′-bis-(2-carboxyethyl)-5-and-6-carboxyfluorescein acetoxyethyl ester (BCECF-AM, Molecular Probes, Inc., OR) and incubated with MT-2 cells (ratio = 1:10) in a 96-well plate at 37 °C 5% CO_2 humidified atmosphere for 2 h in the presence of the peptides or glycopeptides at different concentrations. The fused and not fused Calcein-labeled HIV-1-infected cells were counted under an inverted fluorescence microscope (Zeiss, Germany) with an eyepiece micrometer disc. Four fields per well were counted. The percentage of inhibition of cell fusion was calculated based on the experimental results.

Circular dichroism spectroscopy: N36 was incubated with an equimolar amount of C34 or glyco-C34 in phosphate buffered saline (PBS, 50 mM sodium phosphate, 150 mM NaCl, pH 7.2) at 37 °C for 30 min. The final concentration of N- and C-peptides was 10 μM each. The CD spectra of the mixtures were then measured at 4 °C on a Jasco spectropolarimeter (Model J-715, Jasco Inc., Japan). Measurement conditions were: 5.0 nm bandwidth, 0.1 nm resolution, 0.1 cm path length, 4.0 sec response time, and a 50 nm min^{-1} scanning speed). The spectra were corrected by the subtraction of a blank corresponding to the solvent. Thermal denaturation was monitored at 222 nm by applying a thermal gradient of 5 °C min^{-1}. The melting curve was smoothed and the midpoint of the thermal unfolding transition (T_m) values was calculated by using the Jasco software.

Acknowledgements

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Keywords: chemoenzymatic synthesis · glycopeptide · glycosylation · inhibitors · vaccines

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