SUPPLEMENTARY INFORMATION

Synthetic Multivalent Gp120 V3 Glycopeptides Display Enhanced Recognition by Glycan-Dependent HIV-1 Broadly Neutralizing Antibodies

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Figure S1. a-c). SPR sensorgrams of the binding between PGT128 IgG and synthetic glycopeptide 5 (a), bivalent glycopeptide 7 (b) and trivalent glycopeptide 9 (c). The PGT128 IgG was run from 1000 nM with 1:2 serial dilutions. Data were fit with a 1:1 Langmuir binding model (fitting was shown in black line). $K_a$ was given in $M^{-1}$; $K_d$ in $S^{-1}$ and $K_D$ in $M$. 
Figure S2. SPR sensorgrams of the binding between 10-1074 IgG and synthetic glycopeptide 5 (a), bivalent antigen 7 (b) and trivalent antigen 9 (c). d-f). The 10-1074 IgG were run from 500 nM with 1:2 serial dilutions. Data were fit with a 1:1 Langmuir binding model (fitting was shown in black line). $K_a$ was given in $M^{-1}S^{-1}$; $K_d$ in $S^{-1}$ and $K_D$ in $M$. 
Figure S3. SPR sensorgrams of the binding between PGT128 and 10-1074 IgG and synthetic trivalent JR-FL V3 peptide 10 and trivalent high-mannose glycan 11. Trivalent peptide 10 was immobilized on CM5 chip until 200 RU, and trivalent high-mannose glycan 11 was immobilized on the chip until 25 RU, with about the same amount of glycan as the high-mannose glycopeptide (5, 7 and 9). 10-1074 did not bind to the trivalent peptide 10 and high-mannose glycan 11. PGT128 did not bind to trivalent peptide 10 and but showed weak binding to the trivalent high-mannose glycan 11. However, the binding affinity of PGT128 to the high-mannose glycan 11 was apparently weaker than the trivalent glycopeptide 9 carrying high-mannose glycan.
Figure S4. a-c). SPR sensorgrams of binding between PGT128 and 10-1074 Fabs and mono-, bi- and trivalent glycopeptides derived from CN54 strain (12, 13 and 14, a-c, respectively). Fabs were run from 1000 nM with 1:2 serial dilutions. Data were fit with a 1:1 Langmuir binding model (fitting was shown in black). $K_a$ was given in M$^{-1}$S$^{-1}$; $K_d$ in S$^{-1}$ and $K_D$ in M.
Figure S5. ELISA binding of 10-1074 Fab to JR-FL Env trimer and gp120 monomer.
Scheme S2. Synthesis of trivalent high-mannose glycan 11.
Scheme S3. Synthesis of CN54 V3 monovalent glycopeptide 12.
Scheme S5. Synthesis of CN54 V3 trivalent glycopeptide 14.
Experimental Section

General Procedures

Solid phase peptide synthesis (SPPS) was performed with microwave irradiation on an automatic peptide synthesizer (Liberty, CEM). Analytical reverse-phase HPLC was carried out on a Waters 626 HPLC system equipped with a dual absorbance UV detector (λ = 214 or 280 nm) using a C18 column (YMC-Triart C18, 4.6 X 250 mm, 3.5 µm) at a flow rate of 1 mL/min. The column was eluted using a linear gradient of 15-45% MeCN containing 0.1% TFA over 30 min. ESI-MS spectra were measured on a Micromass ZQ-4000 single-quadrupole mass spectrometer. Preparative reverse-phase HPLC was carried out on a Waters 600 HPLC system equipped with a dual absorbance UV detector using a C18 column (Waters XBridge, Prep Shield RP 10 X 250 mm, 5µm) at a flow rate of 4 mL/min. The column was eluted using a linear gradient of 15-50% MeCN containing 0.1% TFA over 35 min.
**Surface Plasmon Resonance (SPR) Measurement**

SPR binding analysis was evaluated on a BIAcore T200 system (GE Healthcare) at 25 °C. Biotinylated glycopeptides were immobilized on neutravidin-coated CM5 sensor chips in HBS-P buffer (10 mM HEPES, 150 mM NaCl, P20 surfactant 0.05% v/v, pH 7.4) until 200 response units (RU) was achieved, with almost identical amount of mono- bi- and trivalent glycopeptides on the chip. PGT128 and 10-1074 (IgG and Fab) were injected individually over four cells at two-fold increasing concentration in HBS-P buffer with a flow rate of 40 μL/min for 180 s. HBS-P buffer with a flow rate of 40 μL/min was injected for 210 s to allow for dissociation. Regeneration was performed by injection of 3M MgCl₂ with a flow rate of 50 μL/min for 3 min followed by injection of HBS-P buffer with a flow rate of 50 μL/min for 5 min. Data processing was carried out using the BIAcore T200 evaluation software to subtract appropriate blank references and to fit the sensorgrams globally using a 1:1 Langmuir binding model to obtain the apparent kinetic parameters.
ELISA Analysis

IgG ELISA:
The 96-well ELISA microtiter plates were first coated with 10 \( \mu \text{g/mL} \) streptavidin in PBS (100 \( \mu \text{L/well} \)) and incubated at 4 °C overnight. The plates were washed with PBS/0.05% Tween-20 and blocked with 2% sodium caseinate (w/v) in PBS at room temperature for 1 h. After washing three times, 2 \( \mu \text{g/mL} \) of the respective biotinylated glycopeptides (5, 7 and 9) in 1% casein PBS was added (100 \( \mu \text{L/well} \)). Plates were incubated at 37 °C for 1 h. Then plates were washed three times and titrated against 1:2 serial dilutions of the PGT128 and 10-1074 antibodies in 1% sodium caseinate, starting from 20 \( \mu \text{g/mL} \) (67 nM) (100 \( \mu \text{L/well} \)). Then the plates were incubated at 37 °C for 1 h. After washing three times, a solution (100\( \mu \text{L} \)) of 1:3000 diluted horseradish peroxidase (HRP)-conjugated goat anti-human IgG (H + L) antibody in 1% PBS was added to the plates. The plates were incubated for 1 h at 37 °C. After washing three times, a solution of 3, 3’, 5, 5’-tetramethylbenzidine (TMB) was added. Color was allowed to develop for 5 min, and then quenched by adding a solution of 1 M H\textsubscript{3}PO\textsubscript{4} to each well. The readout was measured at a wavelength of 450 nm.

Fab ELISA:
The Fab ELISA was performed using a modified procedure from the IgG. The PGT128 and 10-1074 Fab were added in a 1:2 serial dilutions in 1% sodium caseinate, starting from 10 \( \mu \text{g/mL} \) (200 nM) (100 \( \mu \text{L/well} \)). A horseradish peroxidase (HRP)-conjugated goat anti-human IgG (Fab specific) (Sigma) antibody diluted 1:3000 in 1% PBS was used as the secondary antibody. The other steps were the same as the IgG ELISA.
Compounds Synthesis

Peptide 1 was obtained from SPPS. Synthesis was based on Fmoc chemistry using PAL-PEG-PS resin (0.18 mmol/g) on a 0.1 mmol scale. Couplings were performed using 6 equiv. of Fmoc-protected amino acids, 6 equiv. of TBTU and 12 equiv. of DIPEA in DMF. The couplings were carried out at 45 °C for 20 min. The glycosyl amino acid Fmoc-(Ac$_3$GlcNAc)-Asn-OH was introduced at the desired glycosylation sites and Fmoc deprotection was carried out with 20% piperidine in DMF containing 0.1 M HOBt. Upon completion of the V3 sequence, Fmoc-ε-Acp-OH was extended at the N-terminus as spacer and then the 4-pentynoic acid was coupled at the N-terminus to install the alkyne group. The resin was washed with DMF (3X) and DCM (3X) then cleavage was carried out using cocktail R (TFA/Thioanisole/Ethanediethiol/Anisole = 90/5/3/2) treatment for 2 h. The resin was then filtered and the solution was added to cold diethyl ether for precipitation. The crude peptide was dissolved in glacial acetic acid and then lyophilized. The crude peptides were purified on preparative RP-HPLC to afford the peptide 1 (195 mg, 46% yield over all steps).

ESI-MS: Calcd., M = 4185.62; found (m/z): 698.52 [M + 6 H]$^6+$, 838.02 [M + 5 H]$^5+$, 1047.27 [M + 4 H]$^4+$, 1396.03 [M + 3 H]$^3+$. RP-HPLC retention time, $t_R = 21.1$ min.

HPLC and ESI-MS profiles of peptide 1:
Peptide 2 was obtained from SPPS using similar procedure for Peptide 1. Upon completion of the V3 sequence, Fmoc-ε-Acp-OH was extended at the N-terminus as spacer and then the biotin tag was coupled. Then cleavage was carried out using cocktail R (TFA/Thioanisole/Ethanediethiol/Anisole = 90/5/3/2) treatment for 2 h. The resin was then filtered and the solution added to cold diethyl ether for precipitation. The crude peptide was dissolved in glacial acetic acid and then lyophilized. The crude peptide was cyclized in a 20% aqueous DMSO solution. De-O-acetylation of the acetylated GlcNAc moiety was performed in a 5% aqueous hydrazine solution. The crude peptides were purified on preparative RP-HPLC to afford the Peptide 2 (182 mg, 42% yield over all steps).


HPLC and ESI-MS profiles of peptide 2:
Glycopeptide 4 was prepared from chemoenzymatic method. Glycopeptide 1 (1.9 mg, 0.46 µmol) was incubated at 30 °C together with Man9-oxazoline 3 (4.6 mg, 2.76 µmol) and Endo-AN171A (80 µg) in phosphate buffer (100 mM, pH 7, 200 µl). The reaction was monitored by analytical RP-HPLC. Upon completion of the reaction, the reaction was quenched using 0.1% aq. TFA. The product was purified by RP-HPLC to give glycopeptide 4 as a white powder (2.33 mg, 87%).

ESI-MS: Calcd., M = 5848.08; found (m/z): 975.61 [M + 6 H]^6+, 1170.53 [M + 5 H]^5+, 1462.91 [M + 4 H]^4+. RP-HPLC retention time, t_R = 21.0 min.

HPLC and ESI-MS profiles of glycopeptide 4:
Glycopeptide 2 (2 mg, 0.46 µmol) was incubated at 30 °C together with Man9-oxazoline 3 (4.6 mg, 2.76 µmol) and Endo-AN171A (80 µg) in phosphate buffer (100 mM, pH 7, 200 µl). The reaction was monitored by analytical RP-HPLC. Upon completion of the reaction, the reaction was quenched using 0.1% aq. TFA. The product was purified by RP-HPLC to give glycopeptide 5 as a white powder (2.46 mg, 89%).

ESI-MS: Calcd., M = 5994.34; found (m/z): 889.12 [M + 7 H]^7+, 999.95 [M + 6 H]^6+, 1199.95 [M + 5 H]^5+. RP-HPLC retention time, t_R = 20.1 min.

HPLC and ESI-MS profiles of glycopeptide 5:
Peptide scaffold 6 was obtained from SPPS using Fmoc chemistry started from the PAL-PEG-PS resin (0.18 mmol/g) on a 0.05 mmol scale. Fmoc-Lys(N$_3$)-OH, Fmoc-ε-Acp-OH and biotin acid were coupled in 6 equiv.. Upon completion of synthesis, the resin was washed with DMF (3X) and DCM (3X) then cleavage was carried out using cocktail R (TFA/Thioanisole/Ethanedithiol/Anisole = 90/5/3/2) treatment for 2 h. The resin was then filtered and the solution was added to cold diethyl ether for precipitation. The crude peptide was dissolved in glacial acetic acid and then lyophilized and purified on preparative RP-HPLC to afford the Peptide 6 (29 mg, 65% yield).

ESI-MS: Calcd, M = 891.14; found (m/z): 891.51 [M + H]$^+$, 913.50 [M + Na]$^+$. RP-HPLC retention time, $t_R = 26.7$ min.

HPLC and ESI-MS profiles of peptide 6:
Peptide 6 (0.23 mg, 0.25 μmol) and glycopeptide 4 (3.1 mg, 0.525 μmol) were dissolved in 200 μL DMF. Copper (I) acetate (6 μg, 0.05μmol) were added. The mixture was incubated at 45 °C for 20 h. The reaction mixture was diluted in 1 mL water and then lyophilized. The obtained mixture were dissolved in water and purified on preparative RP-HPLC to afford the bivalent glycopeptide antigen 7 (2.1 mg, 68% yield).


HPLC and ESI-MS profiles of bivalent glycopeptide antigen 7:
Peptide scaffold 8 was obtained from SPPS using similar procedure to peptide 6. The crude peptides were purified on preparative RP-HPLC to afford the peptide 8 (31 mg, 53% yield).

ESI-MS: Calcd., M = 1158.47; found (m/z): 579.86 [M + 2H]^{2+}, 1158.71 [M + 1H]^{+}, 1180.69 [M + Na]^{+}. RP-HPLC retention time, t_{R} = 31.2 min.

HPLC and ESI-MS profiles of peptide 8:
Peptide 8 (0.29 mg, 0.25 μmol) and glycopeptide 4 (4.6 mg, 0.788 μmol) were dissolved in 250 μL DMF. Copper (I) acetate (9 μg, 0.075 μmol) were added. The mixture was incubated at 45 °C for 20 h. The reaction mixture were diluted in 1 mL water and then lyophilized. The obtained mixture were dissolved in water and purified on preparative RP-HPLC to afford the trivalent glycopeptide antigen 9 (2.0 mg, 43% yield).


HPLC and ESI-MS profiles of trivalent glycopeptide antigen 9:
Peptide 8 (0.29 mg, 0.25 μmol) and JR-FL peptide (3.1 mg, 0.788 μmol) were dissolved in 250 μL DMF. Copper (I) acetate (9 μg, 0.075μmol) were added. The mixture was incubated at 45 °C for 20 h. The reaction mixture were diluted in 1 mL water and then lyophilized. The obtained mixture were dissolved in water and purified on preparative RP-HPLC to afford the trivalent peptide antigen 10 (2.1 mg, 63% yield).


HPLC and ESI-MS profiles of trivalent peptide 10:
Peptide 8 (0.29 mg, 0.25 μmol) and Alkyne high-mannose glycan (1.8 mg, 0.788 μmol) were dissolved in 150 μL DMF. Copper (I) acetate (9 μg, 0.075 μmol) were added. The mixture was incubated at 45 °C for 20 h. The reaction mixture were diluted in 1 mL water and then lyophilized. The obtained mixture were dissolved in water and purified on preparative RP-HPLC to afford the trivalent high-mannose antigen 11 (1.1 mg, 61% yield).

ESI-MS: Calcd., M = 7390; found (m/z): 2464.87 [M + 3H]^3+, 1484.48 [M + 4H]^4+, 1479.24 [M + 5H]^5+. RP-HPLC retention time, t<sub>R</sub> = 24.6 min.

HPLC and ESI-MS profiles of trivalent glycan 11:
CN54 V3 glycopeptide 12 was prepared using the same procedures for glycopeptide 5.

ESI-MS: Calcd., M = 5921.58; found (m/z): 1185.53 [M + 5H]^{5+}, 1482.15 [M + 4H]^{4+}. RP-HPLC retention time, t_R = 21.9 min.

HPLC and ESI-MS profiles of monovalent CN54 V3 glycopeptide 12:
Bivalent CN54 V3 glycopeptide 13 was prepared using the same procedures for glycopeptide 7.

ESI-MS: Calcd., M = 12449.1; found (m/z): 1245.78 [M + 10H]\(^{10+}\), 138414 [M + 9H]\(^{9+}\), 1556.90 [M + 8H]\(^{8+}\), 1779.37 [M + 7H]\(^{7+}\), 2075.74 [M + 6H]\(^{6+}\). RP-HPLC retention time, \(t_R = 22.2\) min.

HPLC and ESI-MS profiles of trivalent peptide 13:
Trivalent CN54 V3 glycopeptide 14 was prepared using the same procedures for glycopeptide 9.


HPLC and ESI-MS profiles of trivalent peptide 14:
Full References