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Chemoenzymatic Synthesis of HIV-1 Glycopeptide Antigens

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Abstract

Glycosylation is one of the most common posttranslational modifications of proteins and can exert profound effects on the inherent properties and biological functions of a given protein. Structurally well-defined homogeneous glycopeptides are highly demanded for functional studies and biomedical applications. Various chemical and chemoenzymatic methods have been reported so far for synthesizing different N- and O-glycopeptides. Among them, the chemoenzymatic method based on an endoglycosidase-catalyzed ligation of free N-glycans and GlcNAc-tagged peptides is emerging as a highly efficient method for constructing large complex N-glycopeptides. This chemoenzymatic approach consists of two key steps. The first step is to prepare the GlcNAc peptide through automated solid-phase peptide synthesis (SPPS) by incorporating an Asn-linked GlcNAc moiety at a predetermined glycosylation site; and the second step is to transfer an N-glycan from the corresponding N-glycan oxazoline en bloc to the GlcNAc peptide by an endoglycosidase or its efficient glycosynthase mutant. In this chapter, we provide detailed procedures of this chemoenzymatic method by demonstrating the synthesis of two HIV-1 V3 glycopeptide antigens carrying a high-mannose-type and a complex-type N-glycan, respectively. The described procedures should be generally applicable for the synthesis of other biologically important N-glycopeptides.

Keywords

Solid-phase peptide synthesis (SPPS); N-glycopeptide; Chemoenzymatic synthesis; HIV glycopeptide antigens; Glycosynthase; Transglycosylation

1 Introduction

Glycosylation is one of the most prevalent posttranslational modifications of proteins. It is well documented that glycosylation can profoundly affect a protein's structure and functions, such as its folding, stability, intracellular trafficking, immunogenicity, and pharmacokinetics [1]. On the other hand, the sugar moiety attached can participate directly in a number of important biological recognition processes, including cell adhesion, cancer progression, host-pathogen interactions, and immune responses [2–5]. However, a detailed understanding of the structure-function relationships of glycoproteins is hampered by the structural heterogeneity caused by glycosylation. Natural and recombinant glycoproteins are

usually produced as mixtures of heterogeneous glycoforms that have the same protein backbone but differ in structures of the pendant glycans and/or the sites of glycosylation. It is usually extremely difficult to isolate a homogeneous glycoprotein from the mixtures of glycoforms through current separation technology. Thus, synthesis has become a practical means to obtain structural well-defined, homogeneous glycopeptides and glycoproteins.

Many elegant chemical methods have been reported for the synthesis of complex glycopeptides in recent years [6–8]. Notably, the development and expansion of the native chemical ligation concept for ligating large glycopeptides and polypeptides have now made it possible to construct complex glycopeptides and even sizable glycoproteins, as exemplified by the recent accomplishments of total chemical synthesis of fully glycosylated human erythropoietin (EPO) [9,10]. Nevertheless, total chemical glycoprotein synthesis is still a formidable task, which requires highly specialized skills that are not available in regular chemical and biochemical labs. In chemical N-glycopeptide or N-glycoprotein synthesis, the complex glycopeptides designed for native chemical ligation (NCL) are usually prepared by two approaches: one is the Lansbury coupling that involves the reaction of an oligosaccharide glycosylamine and a free aspartic acid in a *protected polypeptide* [11–13] and the other is the glycoamino acid building block strategy that incorporates a pre-assembled oligosaccharide-Asn building block in automated solid-phase peptide synthesis [7, 14, 15]. In both cases, the coupling yield is dependent heavily on the size and nature of the oligosaccharides involved. In addition, the final global deprotection using a strong acid cocktail may lead to partial decomposition of the acid-labile oligosaccharide moiety, resulting in low yield and the complication in purification. In general, native chemical ligation involving large glycopeptides is not trivial, often leading to low yield, particularly when the bulky glycans are near the ligation sites [9].

In parallel to the development of chemical synthesis, there have also been tremendous progresses in the development of chemoenzymatic methods that combine chemical synthesis with key enzymatic transformations. In particular, the exploration of the transglycosylation activity of a class of *N*-acetyl- β glucosaminidases (endoglycosidases, ENGases) that enables the “native ligation” between unprotected glycans and GlcNAc-tagged peptides or proteins is emerging as a more appealing approach to constructing large complex N-glycopeptides and homogeneous N-glycoproteins [16–18]. A major progress in this pursuit is the discovery of a class of novel glycosynthases derived from endoglycosidases through site-directed mutations, which are devoid of hydrolysis activity but can use the highly activated glycan oxazolines (a potential transition state mimic) for transglycosylation to form a new glycosidic bond. The glycosynthase-catalyzed native ligation permits independent manipulations of the sugar and protein portions and provides a highly convergent approach to N-glycopeptide assembling. An array of glycosynthase mutants have been discovered that demonstrate distinct substrate specificity. These include the Endo-M N175A or N175Q mutants derived from *Mucor hiemalis* endoglycosidase (Endo-M, acting on both high-mannose and bi-antennary complex-type N-glycans) [19, 20]; the N171A mutant derived from the *Arthrobacter protophormiae* endoglycosidase (Endo-A, specific for high-mannose N-glycans) [21]; the D165A mutant derived from the *Flavobacterium meningosepticum* endoglycosidase (Endo-F3, capable of transferring bi- and tri-antennary N-glycans but highly selective for core-fucosylated GlcNAc acceptors) [22]; the D233A and D233Q

mutants of the endoglycosidase from *Streptococcus pyogenes* (Endo-S, specific for bi-antennary complex-type N-glycans and antibody Fc domain) [23]; and the D184M mutant of Endo-S2 from *Streptococcus pyogenes* of serotype M49, which is highly selective for antibody substrates and is able to transfer all major types of N-glycans [24]. These mutant enzymes, in combination with the use of respective N-glycan oxazolines as the donor substrates, have been successfully used for the synthesis of a series of biological important complex glycopeptides and glycoproteins [16, 18], such as the structurally well-defined mannose-6-phosphate (M6P)-containing glycoproteins [25, 26]. A particular application of this chemoenzymatic method is for the synthesis of a series of homogeneous HIV-1 V1V2 glycopeptide antigens and HIV-1 V3 glycopeptide antigens that would be otherwise difficult to obtain by other methods. Coupled with antibody binding studies, the synthetic HIV-1 glycopeptides enabled the characterization of the glycan specificity and fine neutralizing epitopes of a class of glycan-dependent broadly neutralizing antibodies against HIV-1 [27–30]. Moreover, the ability to reconstitute the glycopeptide neutralizing epitopes has facilitated the design, synthesis, and immunization studies of glycopeptide-based HIV vaccines in animals [31–33]. On the other hand, this chemoenzymatic method has been particularly useful for glycan remodeling of antibodies to provide homogeneous glycoforms for detailed structural and functional studies [23, 24, 34–41]. We have previously described a detailed protocol for the glycoengineering of antibodies using this chemoenzymatic glycan remodeling method [36].

For the synthesis of N-glycopeptides, this chemoenzymatic approach consists of two key steps. The first step is to incorporate an Asn-linked GlcNAc moiety at a predetermined glycosylation site through automated solid-phase peptide synthesis (SPPS) to give the GlcNAc-peptide precursor. It should be noted that in contrast to common O-glycosidic linkages labile for acidic conditions, the N-glycosidic linkage in the Fmoc-Asn (Ac₃GlcNAc)-OH is highly stable to acidic and basic treatment during SPPS. The second step is to transfer an N-glycan from the corresponding glycan oxazoline donor to the GlcNAc peptide to form the desired N-glycopeptide with native glycosidic linkage. In this chapter, we provide a detailed description of the chemoenzymatic method for N-glycopeptide synthesis. The procedures are demonstrated by the synthesis of two HIV-1 V3 glycopeptide antigens that are the minimal neutralizing epitopes of the broadly HIV-neutralizing antibodies, PGT128 and 10-1074 [29]. The described procedures should be generally applicable for the synthesis of other biologically important N-glycopeptides.

2 Materials

2.1 HPLC and Mass Spectrometric Analysis

1. Analytical HPLC (Waters e2695) with 2489UV/Vis detector.
2. Preparative HPLC (Waters 1525) with 2489UV/Vis detector and WFCIII fraction collector.
3. ESI-mass spectrometer (Waters SQ Detector 2).
4. Axima-CFR MALDI-TOF mass spectrometer (Shimadzu).
5. C18 analytical column (YMC-Triart C18, 4.6 × 250 mm, 5 μm).

6. C18 preparative column (Waters-SymmetryPrep C18, 19 × 300 mm, 7 μm).
7. Analytical HPLC mobile phase (Buffer A): 0.1% trifluoroacetic acid (v/v) in ddH₂O.
8. Analytical HPLC mobile phase (Buffer B): 0.1% trifluoroacetic acid (v/v) in acetonitrile.
9. Preparative HPLC mobile phase (Buffer A): 0.1% trifluoroacetic acid (v/v) in ddH₂O.
10. Preparative HPLC mobile phase (Buffer B): 0.1% trifluoroacetic acid (v/v) in acetonitrile.

2.2 Automated Solid-Phase Peptide Synthesis

1. Peptide synthesizer (Liberty Blue™).
2. Centrifuge (Allegra X-14R).
3. Lyophilizer (Virtis) with advanced Sentry 2.0 controller.
4. Standard *Fmoc*-amino acid derivatives: *Fmoc*-Ala-OH; *Fmoc*-Arg(Pbf)-OH; *Fmoc*-Asn(Trt)-OH; *Fmoc*-Asp(OtBu)-OH; *Fmoc*-Cys(Trt)-OH; *Fmoc*-Gln(Trt)-OH; *Fmoc*-Glu(OtBu)-OH; *Fmoc*-Gly-OH; *Fmoc*-His(Trt)-OH; *Fmoc*-Ile-OH; *Fmoc*-Lys(Boc)-OH; *Fmoc*-Pro-OH; *Fmoc*-Ser(tBu)-OH; *Fmoc*-Thr(tBu)-OH; *Fmoc*-Tyr(tBu)-OH.
5. *Fmoc* 6-Amino-hexanoic acid (*Fmoc*-ε-Acp-OH).
6. D-Biotin.
7. Building block *Fmoc*-Asn(Ac₃GlcNAc)-OH, prepared according to the previously reported method [28] (*see* Note 1).
8. Rink Amide AM resin (*see* Note 2).
9. Coupling reagent: *N,N'*-diisopropylcarbodiimide (DIC).
10. Hydroxybenzotriazole (HOBt).
11. *Fmoc*-deprotection reagent: 20% piperidine.
12. Cocktail R for global deprotection and releasing of peptide from the resin: trifluoroacetic acid (TFA) (90% v/v); thioanisole (5% v/v); 1,2-ethanedithiol (3% v/v); anisole (2% v/v) (*see* Note 3).

¹ *O*-Diethylisopropylsilyl (DEIPS) protected GlcNAc-Asn (*Fmoc*-Asn(DEIPS₃GlcNAc)-OH) could also be used as the building block, which is deprotected simultaneously in the resin cleavage step in cocktail R. The *O*-acetyl protecting groups in *Fmoc*-Asn(Ac₃GlcNAc)-OH after SPPS can be removed in basic condition in a later stage. If only one sugar building block required in the SPPS, *Fmoc*-Asn(Ac₃GlcNAc)-OH is preferred because of its easy preparation compared to *Fmoc*-Asn(DEIPS₃GlcNAc)-OH.

² The loading capacity of Rink Amide AM resin varies (usually 0.4–0.7 mmol/g). The amine equivalent in the Rink Amide AM resin should be verified by the common loading test before use.

³ Cocktail R should be freshly prepared before each usage. Use 30 mL Cocktail R for 1 g Resin. Cocktail R is highly corrosive and has an unpleasant smell. Operate the reaction carefully in a fume hood.

2.3 Chemoenzymatic Synthesis of Glycopeptides

1. Hydrazine hydrate (NH_2NH_2 50–60%).
2. $\text{Man}_9\text{GlcNAc}$ oxazoline, prepared according to the literature procedures [20] (see Note 4).
3. Sialylated complex-type (SCT) oxazoline, prepared according to the previously reported procedure [36] (see Note 5).
4. Recombinant enzyme EndoA-N171A, produced according to the procedures described previously [21] (see Note 6).
5. Recombinant enzyme EndoM-N175Q, produced according to the procedures described previously [19, 20] (see Note 7).
6. 1 M Phosphate buffer, pH 7.0.
7. Triethylamine (TEA).
8. 2-Chloro-1,3-dimethylimidazolinium chloride (DMC).
9. Sequencing-grade dimethylformamide (DMF).
10. Dichloromethane (DCM).
11. Ethyl ether (Et_2O).
12. Sephadex G-10 (GE Healthcare).
13. HisTrap FF column (1 mL, GE Healthcare).
14. PureCube glutathione cartridge (1 mL, Cube Biotech).

3 Methods

3.1 HPLC Analysis and Purification of Peptides and Glycopeptides

1. Analytical RP-HPLC method: C18 column (YMC-Triart C18, 4.6×250 mm, 5 μm) at a flow rate of 0.5 mL/min using a linear gradient of 15–30% MeCN containing 0.1% TFA over 30 min followed by a 10 min isocratic 30% MeCN gradient.

⁴High-mannose-type N-glycan can be usually prepared by Endo-A-catalyzed hydrolysis of soybean agglutinin isolated from soybean flour. High-mannose oxazoline can be readily obtained with TEA and DMC in aqueous condition at 4 °C for 30 min. Sugar oxazoline is not stable in neutral or acidic conditions and should be dissolved in water containing trace amount of NaOH, lyophilized, and stored at –80 °C for long-term storage.

⁵Crude sialylated complex-type N-glycan (SCT) is usually obtained by Endo-S2-catalyzed hydrolysis of the sialoglycopeptide (SGP) isolated from egg yolk with subsequent purification with size-exclusion chromatography (G-10, GE Health). The SCT oxazoline can be readily obtained with TEA and DMC in aqueous condition at 4 °C for 30 min.

⁶The DNA sequence encoding Endo-A N171A mutant is cloned into a pGEX vector, and the enzyme is usually overexpressed in *E. coli* BL21 (DE3) as a fusion protein with a glutathione S-transferase (GST) tag. The recombinant enzyme can be purified with a PureCube glutathione cartridge (1 mL, Cube Biotech) to yield pure protein with a mass of 69 kDa. The protein could be kept at –80 °C for long-term storage.

⁷The DNA sequence encoding Endo-M N175Q mutant is cloned into a pET23b vector, and the enzyme carrying 6× His tag is overexpressed in *E. coli* BL21 (DE3) at 18 °C for 2 days (induced by 0.2 mM IPTG). The recombinant enzyme can be purified with a HisTrap FF column (1 mL, GE Healthcare) to yield pure protein with a mass of 85 kDa. The protein can be kept at –80 °C for long-term storage.

2. Preparative RP-HPLC method: C18 column (Waters-SymmetryPrep C18, 19 × 300 mm, 7 μm) at a flow rate of 10 mL/min using a linear gradient of 20–40% MeCN containing 0.1% TFA over 50 min.
3. Peptides and glycopeptides were detected at two wavelengths (214 and 280 nm).

3.2 Automated Solid-Phase Peptide Synthesis (SPPS) of the GlcNAc-Peptide Precursor

The methods described outline the automated solid-phase peptide synthesis (SPPS) of GlcNAc-peptide precursor of HIV-1 V3 glycopeptide.

1. The cyclic HIV-1 V3 GlcNAc peptide is synthesized by automated solid-phase peptide synthesis (SPPS) based on Fmoc chemistry on Rink Amide AM resin. The procedures are summarized in Fig. 1.
2. The automated synthesis is programmed according to the instructions from the manufacturer of the automated peptide synthesizer.
3. The synthesis is carried out on a 0.10 mmol scale.
4. Use 0.5 M DIC and 0.1 M HOBt as the coupling reagents.
5. Use 5 equiv of amino acid building blocks for each coupling reaction in the synthesis program.
6. Pre-dissolve each amino acid building block in DMF, and place it at the pre-determined site (channel) in the peptide synthesizer.
7. For the manual introduction the building block Fmoc-Asn (Ac₃GlcNAc)-OH: (a) dissolve 3 equiv of building block Fmoc-Asn(Ac₃GlcNAc)-OH and 5 equiv of HOBt in 3 mL DMF; (b) add 5 equiv of DIC and mix well with the resin for manual coupling; (c) place the resin and reactor back to continue the automated synthesis.
8. When all the coupling reactions are done, wash the resin with DMF (5 mL × 3 times) and DCM (5 mL × 3 times).
9. Dry the resin in vacuum.
10. Weigh the peptide-resin materials (total amount of GlcNAc peptide-containing resin after these procedures: 802 mg).
11. Suspend the obtained GlcNAc-peptide resin (802 mg) in 24 mL cocktail R in a 50 mL centrifuge tube (*see* Note 3).
12. Place the centrifuge tube on a shaker and gently shake at rt for 2 h.
13. Monitor the cleavage process by analytical RP-HPLC-MS (*see* Subheading 3.1, step 1).
14. Precipitate the crude GlcNAc peptide using an ice-cold ether: filter the reaction mixture onto 480 mL ice-cold ether (use 20 mL ether per mL of reaction mixture).
15. Centrifuge (3170 × *g* for 2 min).

16. Discard the supernatant.
17. Resuspend the solid with ether, and repeat the process for two more times to obtain the crude GlcNAc peptide (5) (*see* Note 8).
18. Dissolve all the crude GlcNAc peptide (5) in 200 mL ddH₂O.
19. Add 4 mL hydrazine hydrate (*see* Note 9).
20. Gently shake the reaction mixture at rt for 3 h.
21. Monitor the process by analytical RP-HPLC-MS (*see* Note 10). HPLC condition: same as described in Subheading 3.1, **step 1**.
22. Lyophilize the reaction mixture.
23. Purify the crude product (6) by preparative RP-HPLC (*see* Subheading 3.1, **step 2**).
24. Analyze the HPLC fractions by analytical RP-HPLC (Fig. 2a) and MALDI (Fig. 2b).
25. Lyophilize the fractions containing pure GlcNAc peptide.
26. Weigh the amount of the pure GlcNAc peptide (182 mg, 42% yield), which appears as a white powder.

3.3 Chemoenzymatic Synthesis of the HIV-1 V3 Glycopeptide

The methods described outline enzymatic transglycosylation to form homogeneous large glycopeptides. The detailed procedures were demonstrated by the chemoenzymatic synthesis of two HIV-1 V3 glycopeptide antigens carrying a high-mannose-type N-glycan (Man₉GlcNAc₂) and a sialylated complex-type N-glycan (SCT), respectively.

3.3.1 Chemoenzymatic Synthesis of the HIV-1 V3 Glycopeptide Carrying a High-Mannose-Type N-Glycan Using Glycosynthase Endo-A N171A

1. The general procedures of the chemoenzymatic synthesis of HIV-1 V3 glycopeptides are depicted in Fig. 3.
2. The cloning, overexpression, and purification of Endo-A N171A enzyme follow the procedures described previously [21] (*see* Note 6).
3. Add 20 µL of Endo-A N171A (final concentration 0.4 µg/µL) to a solution of GlcNAc peptide (2 mg) and Man₉GlcNAcoxazoline (4.6 mg, 6 equiv) in 200 µL phosphate buffer (100 mM, pH 7).

⁸The crude GlcNAc peptide (5) can be directly used without purification in the next cyclization step.

⁹The high dilution condition is critical for intramolecular cyclization without intermolecular disulfide bond formation. A final concentration of GlcNAc peptide at 2 mg/mL is applied. The cyclization of the GlcNAc peptide could also be conducted with 20% aqueous DMSO solution. The deacetylation could then be performed in a 5% aqueous hydrazine solution following the reported procedure [29]. However, the protocol described in this chapter is much more straightforward, which combines the de-O-acetylation and cyclization in a single step.

¹⁰Sample should be spun down before injection into LC-ESI-MS system. Adjust the injection amount to avoid potential contamination to column and instrument.

4. Incubate the mixture at 30 °C for 2 h.
5. Monitor the transglycosylation process by LC-ESI-MS (*see* Note 10). HPLC condition: same as described in Subheading 3.1, step 1.
6. Quench the reaction with 0.1% TFA and centrifuge at $26,452 \times g$.
7. Purify the transglycosylation product using preparative RP-HPLC (*see* Subheading 3.1, **step 2**).
8. Analyze the HPLC fractions by analytical RP-HPLC (Fig. 4a) and MALDI-TOF MS (Fig. 4b).
9. Combine and lyophilize the fractions containing the pure glycopeptide.
10. Weigh the amount. The high-mannose-type glycopeptide (9) obtained (2.4 mg, 87%) appears as a white powder (*see* Note 11).

3.3.2 Chemoenzymatic Synthesis of the HIV-1 V3 Glycopeptide Carrying a Sialylated Complex-Type N-Glycan Using Glycosynthase Endo-M N175Q

1. The cloning, overexpression, and purification of Endo-M N175Q enzyme follow the procedures described previously [19,20] (*see* Note 7).
2. Add 20 μL of Endo-M N175Q (final concentration 0.4 $\mu\text{g}/\mu\text{L}$) to a solution of the GlcNAc peptide (2 mg) and the sialylated complex-type oxazoline (5.5 mg, 6 equiv) in 200 μL phosphate buffer (100 mM, pH 7).
3. Incubate the mixture at 30 °C for 2 h.
4. Monitor the transglycosylation process by LC-ESI-MS (*see* Note 10). HPLC condition: same as described in Subheading 3.1, **step 1**.
5. Quench the reaction with 0.1% TFA.
6. Centrifuge the reaction mixture at $26452 \times g$.
7. Purify the transglycosylation product using preparative RP-HPLC (*see* Subheading 3.1, **step 2**).
8. Analyze the HPLC fractions by analytical RP-HPLC (Fig. 4c) and MALDI-TOF MS (Fig. 4d).
9. Combine and lyophilize the fractions containing the pure glycopeptide.
10. Weigh the amount. The glycopeptide (10) obtained (2.5 mg, 86%) appears as a white powder (*see* Note 12).

¹¹The addition of a high-mannose N-glycan to the GlcNAc peptide accounts for a mass of 5994.28 Da. It is confirmed by the MALDI-TOF MS analysis, showing a signal $[\text{M}+\text{H}]^+$ at 5995.37 m/z (Fig. 4b).

¹²The addition of a SCT N-glycan to the GlcNAc-V3 peptide accounts for a mass of 6331.52 Da. It is confirmed by the MALDI-TOF MS analysis, showing a signal $[\text{M}+\text{H}]^+$ at 6332.71 m/z (Fig. 4d).

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References

1. Helenius A, Aebi M (2001) Intracellular functions of N-linked glycans. *Science* 291:2364–2369 [PubMed: 11269317]
2. Jefferis R (2009) Glycosylation as a strategy to improve antibody-based therapeutics. *Nat Rev Drug Discov* 8:226–234 [PubMed: 19247305]
3. Hart GW, Copeland RJ (2010) Glycomics hits the big time. *Cell* 143:672–676 [PubMed: 21111227]
4. Moremen KW, Tiemeyer M, Nairn AV (2012) Vertebrate protein glycosylation: diversity, synthesis and function. *Nat Rev Mol Cell Biol* 13:448–462 [PubMed: 22722607]
5. Varki A (2017) Biological roles of glycans. *Glycobiology* 27:3–49 [PubMed: 27558841]
6. Payne RJ, Wong CH (2010) Advances in chemical ligation strategies for the synthesis of glycopeptides and glycoproteins. *Chem Commun (Camb)* 46:21–43 [PubMed: 20024291]
7. Unverzagt C, Kajihara Y (2013) Chemical assembly of N-glycoproteins: a refined toolbox to address a ubiquitous posttranslational modification. *Chem Soc Rev* 42:4408–4420 [PubMed: 23403448]
8. Wilson RM, Dong S, Wang P et al. (2013) The winding pathway to erythropoietin along the chemistry-biology frontier: a success at last. *Angew Chem Int Ed* 52:7646–7665
9. Wang P, Dong S, Shieh JH et al. (2013) Erythropoietin derived by chemical synthesis. *Science* 342:1357–1360 [PubMed: 24337294]
10. Murakami M, Kiuchi T, Nishihara M et al. (2016) Chemical synthesis of erythropoietin glycoforms for insights into the relationship between glycosylation pattern and bioactivity. *Sci Adv* 2:e1500678 [PubMed: 26824070]
11. Aussedat B, Fasching B, Johnston E et al. (2012) Total synthesis of the alpha-subunit of human glycoprotein hormones: toward fully synthetic homogeneous human follicle-stimulating hormone. *J Am Chem Soc* 134:3532–3541 [PubMed: 22280541]
12. Nagorny P, Sane N, Fasching B et al. (2012) Probing the frontiers of glycoprotein synthesis: the fully elaborated beta-subunit of the human follicle-stimulating hormone. *Angew Chem Int Ed* 51:975–979
13. Aussedat B, Vohra Y, Park PK et al. (2013) Chemical synthesis of highly congested gp120 V1V2 N-glycopeptide antigens for potential HIV-1-directed vaccines. *J Am Chem Soc* 135:13113–13120 [PubMed: 23915436]
14. Yamamoto N, Tanabe Y, Okamoto R et al. (2008) Chemical synthesis of a glycoprotein having an intact human complex-type sialyloligosaccharide under the Boc and Fmoc synthetic strategies. *J Am Chem Soc* 130:501–510 [PubMed: 18085777]
15. Sakamoto I, Tezuka K, Fukae K et al. (2012) Chemical synthesis of homogeneous human glycosyl-interferon-beta that exhibits potent antitumor activity in vivo. *J Am Chem Soc* 134:5428–5431 [PubMed: 22404596]
16. Wang LX, Amin MN (2014) Chemical and chemoenzymatic synthesis of glycoproteins for deciphering functions. *Chem Biol* 21:51–66 [PubMed: 24439206]
17. Fairbanks AJ (2017) The ENGases: versatile biocatalysts for the production of homogeneous N-linked glycopeptides and glycoproteins. *Chem Soc Rev* 46:5128–5146 [PubMed: 28681051]
18. Li C, Wang LX (2018) Chemoenzymatic methods for the synthesis of glycoproteins. *Chem Rev* 118:8359–8413 [PubMed: 30141327]
19. Umekawa M, Li C, Higashiyama T et al. (2010) Efficient glycosynthase mutant derived from *Mucor hiemalis* endo-beta-N-acetylglucosaminidase capable of transferring oligosaccharide from both sugar oxazoline and natural N-glycan. *J Biol Chem* 285:511–521 [PubMed: 19880511]

20. Umekawa M, Huang W, Li B et al. (2008) Mutants of *Mucor hiemalis* endo-beta-N-acetylglucosaminidase show enhanced transglycosylation and glycosynthase-like activities. *J Biol Chem* 283:4469–4479 [PubMed: 18096701]
21. Huang W, Li C, Li B et al. (2009) Glycosynthases enable a highly efficient chemoenzymatic synthesis of N-glycoproteins carrying intact natural N-glycans. *J Am Chem Soc* 131:2214–2223 [PubMed: 19199609]
22. Giddens JP, Lomino JV, Amin MN et al. (2016) Endo-F3 glycosynthase mutants enable chemoenzymatic synthesis of core-fucosylated triantennary complex type glycopeptides and glycoproteins. *J Biol Chem* 291:9356–9370 [PubMed: 26966183]
23. Huang W, Giddens J, Fan SQ et al. (2012) Chemoenzymatic glycoengineering of intact IgG antibodies for gain of functions. *J Am Chem Soc* 134:12308–12318 [PubMed: 22747414]
24. Li T, Tong X, Yang Q et al. (2016) Glycosynthase mutants of endoglycosidase S2 show potent transglycosylation activity and remarkably relaxed substrate specificity for antibody glycosylation remodeling. *J Biol Chem* 291:16508–16518 [PubMed: 27288408]
25. Priyanka P, Parsons TB, Miller A et al. (2016) Chemoenzymatic synthesis of a phosphorylated glycoprotein. *Angew Chem Int Ed* 55:5058–5061
26. Yamaguchi T, Amin MN, Toonstra C et al. (2016) Chemoenzymatic synthesis and receptor binding of mannose-6-phosphate (M6P)-containing glycoprotein ligands reveal unusual structural requirements for M6P receptor recognition. *J Am Chem Soc* 138:12472–12485 [PubMed: 27500601]
27. Amin MN, McLellan JS, Huang W et al. (2013) Synthetic glycopeptides reveal the glycan specificity of HIV-neutralizing antibodies. *Nat Chem Biol* 9:521–526 [PubMed: 23831758]
28. Toonstra C, Amin MN, Wang LX (2016) Site-selective chemoenzymatic glycosylation of an HIV-1 polypeptide antigen with two distinct N-glycans via an orthogonal protecting group strategy. *J Org Chem* 81:6176–6185 [PubMed: 27380452]
29. Orwenyo J, Cai H, Giddens J et al. (2017) Systematic synthesis and binding study of HIV V3 glycopeptides reveal the fine epitopes of several broadly neutralizing antibodies. *ACS Chem Biol* 12:1566–1575 [PubMed: 28414420]
30. Cai H, Orwenyo J, Guenaga J et al. (2017) Synthetic multivalent V3 glycopeptides display enhanced recognition by glycan-dependent HIV-1 broadly neutralizing antibodies. *Chem Commun (Camb)* 53:5453–5456 [PubMed: 28466900]
31. Cai H, Orwenyo J, Giddens JP et al. (2017) Synthetic three-component HIV-1 V3 glycopeptide immunogens induce glycan-dependent antibody responses. *Cell Chem Biol* 24:1513–1522.e4 [PubMed: 29107699]
32. Cai H, Zhang R, Orwenyo J et al. (2018) Multivalent antigen presentation enhances the immunogenicity of a synthetic three-component HIV-1 V3 glycopeptide vaccine. *ACS Cent Sci* 4:582–589 [PubMed: 29806004]
33. Cai H, Zhang RS, Orwenyo J et al. (2018) Synthetic HIV V3 glycopeptide immunogen carrying a N334 N-glycan induces glycan-dependent antibodies with promiscuous site recognition. *J Med Chem* 61:10116–10125 [PubMed: 30384610]
34. Lin CW, Tsai MH, Li ST et al. (2015) A common glycan structure on immunoglobulin G for enhancement of effector functions. *Proc Natl Acad Sci U S A* 112:10611–10616 [PubMed: 26253764]
35. Kuroguchi M, Mori M, Osumi K et al. (2015) Glycoengineered monoclonal antibodies with homogeneous glycan (M3, G0, G2, and A2) using a chemoenzymatic approach have different affinities for FcγRIIIa and variable antibody-dependent cellular cytotoxicity activities. *PLoS One* 10:e0132848 [PubMed: 26200113]
36. Giddens JP, Wang LX (2015) Chemoenzymatic glyco-engineering of monoclonal antibodies. *Methods Mol Biol* 1321:375–387 [PubMed: 26082235]
37. Quast I, Keller CW, Maurer MA et al. (2015) Sialylation of IgG Fc domain impairs complement-dependent cytotoxicity. *J Clin Invest* 125:4160–4170 [PubMed: 26436649]
38. Parsons TB, Struwe WB, Gault J et al. (2016) Optimal synthetic glycosylation of a therapeutic antibody. *Angew Chem Int Ed* 55:2361–2367

39. Liu R, Giddens J, McClung CM et al. (2016) Evaluation of a glycoengineered monoclonal antibody via LC-MS analysis in combination with multiple enzymatic digestion. *MAbs* 8:340–346 [PubMed: 26514686]
40. Li T, Dilillo D, Bournazos S et al. (2017) Modulating IgG effector functions by Fc glycan engineering. *Proc Natl Acad Sci U S A* 114:3485–3490 [PubMed: 28289219]
41. Giddens JP, Lomino JV, Dilillo DJ et al. (2018) Site-selective chemoenzymatic glycoengineering of Fab and Fc glycans of a therapeutic antibody. *Proc Natl Acad Sci U S A* 115:12023–12027 [PubMed: 30397147]

Fig. 1.
Synthesis of the HIV-1 V3 GlcNAc peptide (6) through automated solid-phase peptide synthesis

Fig. 2. HPLC and MALDI-TOF MS profiles of the synthetic GlcNAc peptide (6). (a) The HPLC analysis of **6**; (b) the MALDI-TOF MS analysis of **6**

Fig. 4. HPLC and MALDI-TOF MS profiles of the synthetic glycopeptides carrying a high-mannose N-glycan (**9**) and a complex-type N-glycan (**10**). (**a**) The HPLC analysis of **9**; (**b**) the MALDI-TOF MS analysis of **9**; (**c**) the HPLC analysis of **10**; (**d**) the MALDI-TOF MS analysis of **10**