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(54) **Title**: V3 IMMUNOGENS

(57) **Abstract**: The present invention relates, in general, to human immunodeficiency virus (HIV), and, in particular, to a vaccine for HIV-1, comprising synthetic V3 glycopeptides, and to methods of making and using same.

V3 IMMUNOGENS

[1] This application claims the benefit of U.S. Provisional Application Serial No. 61/811,992 filed April 15, 2013 the entire content of which application is hereby incorporated by reference.

[2] This invention was made with government support under Grant No. UM1 AI100645 awarded by the National Institutes of Health. The government has certain rights in the invention.

TECHNICAL FIELD

[3] The present invention relates, in general, to human immunodeficiency virus (HIV), and, in particular, to a vaccine for HIV-1 and to methods of making and using same.

BACKGROUND

[4] Development of an effective vaccine for prevention of HIV-1 infection is a global priority. While the RV144 trial using ALVAC prime, AIDSVAX B/E boost resulted in an estimated 31% vaccine efficacy, the protection induced was neither sufficiently robust for deployment, nor of sufficient durability for sustained vaccine efficacy.[8] In the RV144 trial, antibodies capable of neutralizing transmitted/founder viruses were not induced; rather the hypothesis is that antibodies targeted to the Env V2 region protected by effector mechanisms other than virus neutralization, such as antibody-dependent cellular cytotoxicity (ADCC). [9a, 10, 11]

[5] By contrast, antibodies capable of broadly neutralizing HIV-1 strains (BnAbs) have been isolated from HIV-1 chronically-infected subjects.[1] These antibodies are targeted to four general HIV-1 envelope targets: the gp41 membrane proximal external region (MPER), and on gp120, the CD4 binding site (CD4bs), the V1V2 glycan site, and several gp120 glycan sites.[2] However, only approximately 20% of infected subjects produce BnAbs, and then, only after years of infection.[12] When infused into rhesus macaques, BnAbs can protect against infection with

chimeric simian-human immunodeficiency viruses (SHIVs).[13] However, BnAbs have not been successfully induced by vaccine constructs thus far.

[6] A recently described set of epitopes to which potent BnAbs do bind are defined by the PGT121, 125 and 128 mAbs isolated from HIV-1 chronically infected subjects.[4,6] These antibodies are able to recognize a peptide-glycan epitope around the base of the gp120 V3 loop and includes N-linked glycans at amino acids 301 and 332.[6] These antibodies are the most potent BnAbs isolated to date, and are able to protect rhesus macaques from SHIV challenge at extremely low plasma levels.[14] Thus, induction of antibodies with specificities like these PGT antibodies is a key goal of HIV-1 vaccine development.

[7] Many reasons can be envisioned to account for the difficulties encountered in inducing such BnAbs. The heavily glycosylated envelope could well obstruct antibody access to BnAb sites.[2] The dominance of non-neutralizing epitope responses compared to the non-dominance of BnAb epitope responses might result in an inability of the B cell response to BnAb epitopes to either be induced or to be sustained.[1] To date, all BnAbs that have been isolated from chronically infected subjects exhibit unusual traits including high levels of somatic mutations, long heavy chain third complementarity determining regions (HCDR3s), and polyreactivity for non-HIV-1 antigens—all traits of antibodies that are potentially susceptible to control by host tolerance mechanisms.[1, 7]

[8] Thus, it seems likely from the unusual nature of BnAbs that unusual strategies will be required for their induction. Several discovery platforms have been proposed, including definition of BnAb epitope structures on the surface of native Env trimers,[2] identification of Env constructs that bind mature BnAbs and their ancestor antibodies avidly,[1b] and immunization with sequential Env constructs isolated during the course of HIV-1 infection.[15] However, none of these modes of operation take into account the heterogeneity of forms of recombinant Envs,[16]

and none prevent dominant, non-neutralizing antibody epitopes from inducing antibodies that out-compete BnAb-producing B cells targeted at sub-dominant Env BnAb epitopes. The ability to synthesize completely homogeneous glycopeptides that mimic the antigenic nature of native envelope proteins would facilitate the generation of constructs that can be configured to express primarily or only BnAb sub-dominant epitopes, thus limiting the likelihood of a dominant non-neutralizing response to be induced.

[9] New chemical methods have been developed to produce totally synthetic glycosylated erythropoietin.[17] These methods can be applied to the HIV/AIDS vaccine production. Attention has recently focused on the preparation, by chemical synthesis, of the V1V2 peptide-glycan epitope defined by BnAbs PG9 and CH01 lineages. In the context of these studies, a potential immunogen has been discovered that is capable of binding not only mature V1V2 BnAbs but also (in nM affinities) to their unmutated common ancestors (UCAs) (i.e., naïve B cell receptors (BCR)) of the V1V2 BnAb lineages. Previously, few Env constructs had been found that bind to the CH01 lineage UCA[18] and none had been found that bind to the PG9 UCA.[10]

[10] The present invention results, at least in part, from studies involving the design and chemical synthesis of immunogens that elicit neutralizing antibodies directed toward the V3 glycan epitope that is recognized by mAb PGT128.

SUMMARY OF THE INVENTION

[11] The present invention relates generally to HIV. More specifically, the invention relates to a vaccine for HIV-1 and to methods of making and using same.

[12] In certain aspects, the invention provides a synthetic peptide comprising sequence SEQ ID NO: 2, 4, 6, 7, 8 or 9, and wherein the peptide is cyclic via endogenous cysteins. In certain embodiments, the peptide is glycosylated at positions Asn301 and/or Asn 332. In certain

embodiments the peptide comprises a tag. In certain embodiments, the tag is biotin. In certain embodiments, there is a linker between the tag and the peptide, which linker could improve the peptide presentation when the peptide is immobilized via the linker. In certain aspects, the invention provides a synthetic peptide wherein the peptide is of SEQ ID NO: 2, 4, 6, 7, 8 or 9, and wherein the peptide is glycosylated at positions Asn301 and Asn 332. In certain aspects, the invention provides a synthetic glycopeptide of the structure in Figure 12 (peptide of SEQ ID NO: 8 glycosylated at positions Asn301 and Asn 332 with $Man_9GlcNAc_2$, wherein the peptide is cyclic via endogenous cysteins. In certain embodiments, the peptide is glycosylated with oilgomannose. In certain embodiments, the peptide has $Man_9GlcNAc_2$ glycans at positions Asn301 and Asn 332. In certain embodiments, the peptides are purified.

[13] In certain aspects, the invention provides a composition comprising a plurality of synthetic peptides of a given sequence as described herein, wherein the composition comprises purified homogenously glycosylated peptides. In certain embodiments, the glycosylation pattern is homogenous on all peptides of SEQ ID NO: 2, 4, 6, 7, 8 or 9 in the composition. In certain embodiments, the peptide is cyclic via endogenous cysteins. In certain embodiments, the compositions further comprise an adjuvant.

[14] In certain aspects, the invention provides a method of inducing antibodies against HIV-1 in a subject, the method comprising administering to the subject any one of the inventive peptides or compositions comprising the same in an amount sufficient to induce the anti-HIV-1 antibodies.

[15] In certain embodiments of the methods, the compositions used in the methods comprise anyone of the glycopeptides described herein. In certain embodiments of the methods, the compositions used in the methods comprise glycopeptide of the structure in Figure 12 (peptide of

SEQ ID NO: 8 glycosylated at positions Asn301 and Asn 332 with $Man_9GlcNAc_2$, wherein the peptide is cyclic via endogenous cysteins. In certain embodiments, the said subject is a human.

[16] In certain aspects, the invention provides a method of determining whether a subject has antibody responses to a V3 glycoprotein comprising obtaining a sample from a subject and determining binding of the sample to V3 glycopeptide and/or the glycan (e.g. free glycan or derivitazized glycan (biotinylated or amine conjugated) and the aglycone V3 peptide, wherein a sample (e.g. plasma, serum, blood, or any other suitable biological sample) which includes components (e.g antibodies) which bind preferentially to the V3 glycopeptide and/or the glycan (e.g. free glycan or derivitazized glycan (biotinylated or amine conjugated) but do not bind to the aglycone V3 peptide, is indicative of the subject having V3 glycopeptide antibodies. Various assays and methods are known in the art to determine if the antibodies induced by a V3 glycopeptide are neutralizing antibodies, and the breadth of neutralization. In certain embodiments, the individual is infected with HIV-1. In certain embodiments, the individual is immunized with a composition comprising an HIV-1 immunogen, including but not limited to any one of the synthetic V3 glycopeptides described herein. Thus in certain embodiments, the invention provides methods to determine whether a V3 glycopeptide is immunogenic in a vaccination regimen.

[17] In certain aspects, the invention provides a method for synthesis of a V3 glycopepetide for example according to the scheme in Example 5.

[18] An isolated antibody which binds to the peptide of claim 1 or the dimer of claim 5, wherein the antibody does not bind to the non-glycosylated peptide of SEQ ID NO: 1 (Aglycone V1V2 peptide of SEQ ID NO: 1).

[19] In certain aspects the invention provides a V3 glycopeptide immunogen as shown in Fig. 2, Figure 11. In certain aspects, the invention provides a method of inducing the production of broadly neutralizing antibodies in a subject comprising administering to the subject an amount of any one of the immunogenic peptides and/or compositions described here in an amount sufficient to effect said induction.

[20] Objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

[21] Figures 1A-1E. Initial evaluation of synthetic V1V2 glycopeptide constructs. (Fig. 1A) Structure of the four antigens, derived from the V1V2 region of gp120, bearing two N-linked $Man_5GlcNAc_2$, $Man_3GlcNAc_2$, or $GlcNAc_2$ oligosaccharides at N160 and N156, or zero glycans ("aglycone") (V1V2 sequence based on the AE.CM244 strain, displayed with HXB2 numbering). Surface plasmon reasonance (SPR) analysis of the interaction of V1V2 $Man_5GlcNAc_2$ (green), $Man_3GlcNAc_2$ (red), GlcNAc2 (blue), or aglycone (magenta) antigens with BnAbs PG9 (Fig. 1B), CH01 (Fig. 1C), or their respective umutated common ancestor (UCA) antibodies, PG9 UCA (Fig. 1D), and CH01-04 UCA (Fig. 1E). Concentration of antigens was 200 μg/ml for Figs. 1A and 1B, and 100 μg/ml for Figs. 1D and 1E, RU = response units

[22] Figures 2A -2C. Design gp120 V3 region BnAb epitope mimics. (Fig. 2A) Crystal structure of glycosylated gp120 outer domain containing a truncated V3 loop (mini-V3) (PDB ID 3TYG, with PGT128 Fab hidden). The proposed synthetic glycopeptides fragments are derived from the red-colored portion of the ribbon structure. For the glycans at N301 and N332, N-acetylglucosamine residues are colored blue, and mannose residues are colored green. (Figs. 2B and 2C) General structure of synthetic V3 glycopeptides, shown with clade B and C sequences

containing full-length and truncated V3 loops (HXB2 numbering). Mini-V3 constructs designed after Pejchal et al[6] by deleting residues 305-320 (highlighted yellow) and retaining P313. Sites of N-glycosylation are colored red, shown with $Man_3GlcNAc_2$. SEQ ID NOs:1-6 correspond to JRFL, CH505, CH040 peptides in Figure 2B in order of appearance. SEQ ID NO: 7 corresponds to the peptide in Figure 2C.

[23] Figure 3. General strategy for synthesizing gp120 V3 loop-based glycopeptides by either one- or two-fragment approaches. Reagents and conditions: (a) $Man_3GlcNAc_2$ -NH_2, PyAOP, DIEA, DMSO; (b) Cocktail R = 90:5:3:2 TFA/thioanisole/ethanedithiol/anisole; (c) Cocktail B = 88:5:5:2 TFA/phenol/water/trisopropysilane; (d) I_2, AcOH, H_2O; (e) Gnd·HCl, MPAA, TCEP, phosphate buffer, pH 7.2; (f) Gnd·HCL, TCEP, VA-044, t-BuSH, phosphate buffer, pH 7.2, 37°C. Acm = acetamidomethyl, Boc = tert-butoxycarbonyl, DIEA = N,N-diisopropylethylamine, DMSO = dimethylsulfoxide, Gnd = guanidine, MPAA = 4-mercaptophenylacetic acid, ψ = pseudoproline, PyAOP = (7-azabenzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate, R = alkyl or aryl, TFA = trifluoroacetic acid, TCEP = tris(2-carboxyethyl)phosphine, Trt = trityl, VA-044 = 2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride, X = any amino acid except proline.

[24] Figure 4. Synthesis of linear (non-looped) gp120 V3-based glycopeptides. Reagents and conditions (a) Gnd·HCl, TCEP, VA-044, t-BuSH, phosphate buffer, pH 7.2, 37°C.

[25] Figures 5A and 5B. V3 region glycoforms. (Fig. 5A) Consortium for Functional Glycomics (CFG) representations of high mannose and complex-type oligosaccharides. (Fig. 5B) Candidate glycopeptides based on the B.JRFL mini-V3 peptide backbone. Sites of N-glycosylation are colored red. Gal = galactose, GlcNac = N-acetylglucosamine, Man = mannose, Neu5Ac = N-acetylneuraminic acid.

[26] Figure 6. Synthesis of $Man_5GlcNAc_2$ heptasaccharide 27. Reagents and conditions (a) thioglycoside 22. NIS/TMSOT1, MS AW-300, CH_2Cl_2 0°C → r.t; (b) AcOH, H_2O), 63% (2 steps); (c) thioglycoside 24, NIS/TMSOTf, MS AW-300, CH_2Cl_2, 0°C → r.t., 64%; (d) NaOMe, MeOH, CH_2Cl_2; (e) $H_2NCH_2CH_2NH_2$, n-BuOH, PhMe, 90°C; (f) Ac_2O, Et_3N, MeOH quantitative (3 steps); (g) H_2, $Pd(OH)_2/C$, MeOH, H_2O, 75%; (f) sat. aq. NH_4HCO_3, 40°C, quantitative. Bn =benzyl, dfBz = 2,5-difluorobenzoyl, MS AW-300 = acid washed molecular sieves, NIS – N-iodosuccinimide, Phth = phthalimido, TMS = trimethylsilyl, Tol = tolyl.

[27] Figure 7. Outline for synthesis of $Man_3GlcNAc_2$ 30. Starting with trisaccharide core 21, $Man_9GlcNAc_2$ can be obtained as shown by first introducing trisaccharide donor 28 at the 3-OH, then unmasking the 6-OH for union with pentasaccharide donor 29, followed by global deprotection.

[28] Figure 8. Unified, maximally convergent strategy for synthesizing high mannose olosaccharides. Outline (omitting functional and protecting groups) illustrating how different combinations of branched and linear mannosyl donors can be used in stepwise fashion to elaborate the common $ManGlcNAc_2$ trisaccharide core and access higher-order oligomannose derivatives.

[29] Figure 9. Conjugation of glycopeptides to carrier protein via oxime ligation. Reagents and conditions: (a) 100 mM sodium phosphate buffer pH 6.5, 100 mM aniline.

[30] Figure 10. Potential application of oxime ligation to build up unimolecular multivalent HIV-1 vaccines.

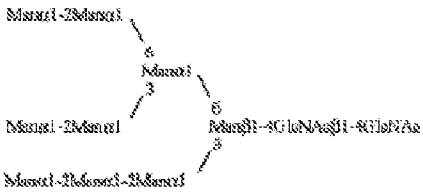
[31] Figure 11 shows one embodiment of a minimal V3 glycopeptide design (the linear peptide has SEQ ID NO: 8). Mini-V3 constructs was designed by deleting residues 305–320 and retaining P313. Sites of N-glycosylation are (N301/N332) colored red. Structure of synthetic V3

glycopeptides is based on the clade B JRFL mini-V3 peptide backbone. The mini-V3 constructs design is based on the structure of PGT 128 with a gp120 outer domain construct containing a truncated V3 loop (eODmV3) (Pejchal et al. 2011). The designed V3 immunogen is longer than the eODmV3 construct, having 30 aa of the V3 and with deletion of residues 305-320, while P313 is retained. The glycopeptide is cyclized through disulfide-bonding. The final V3 glycopeptide product has branched Man9 glycans (D1, D2, D3 arms) attached to proximal GlcNAc residues at positions N301 and N332.

[32] Figure 12 shows binding of V3 BnAb (e.g.PGT128) to the Man9 V3 glycopeptide depicted in the figure (the linear peptide has SEQ ID NO: 8). Binding of V3 Bnab to V3-Man9 glycopeptide. SPR binding curves show preferential binding of PGT128 and PGT 125 BnAbs and thus, indicating that the synthetic glycopeptide optimally presents the Man9 glycans on the minimal V3 backbone. No binding was observed to the V3 loop non-neutralizing mAb 19b (green) or the negative control anti-RSV mAb Synagis (grey). Both PGT128 and PGT125 are dependent on Man9 glycans on position N332 and N301 (Pejchal et al., 2011), with N332 being the primary glycan. Mabs were captured on anti-Fc IgG immobilized sensor surfaces and the V3-Man9 glycopeptide injected to monitor binding responses on a BIAcore 300 instrument and as previously described (Alam et al., 2013). Non-specific binding to the control Synagis surface and blank buffer signal were subtracted.

[33] Figure 13A-D show binding of PGT mAbs to synthetic $Man_9GlcNAc_2$ glycan. Binding of V3 Bnab to Man9 glycan. Both PGT128 and PGT125 bound to the Man9 glycan with affinities in μM (13A, 13B). 2G12 binding (13C) was only detected at the highest concentration of the glycan used, while there was no binding of the non-neutralizing V3 mAb 19b (magenta/top curve), or the V1V2 mAb PGT145 (green curve) (13D). Thus, the binding of

Man9GlcnAc2 glycan was detected only for V3-glycan dependent PGT BNAbs. These data indicate that synthetic Man9 glycans can be used to detect antibodies that interact with both D1 and D3 arm of the glycans and bind preferentially to higher order branched oligomannose glycans.



[34] Figure 14A and B show analytical LCMS from the first run through the synthesis. Figure 14 shows the Man_9V3 glycopeptide UV profile and Figure 14B shows Man_9V3 glycopeptide mass spectrometry profile. This is an analytical run so the quality will be improved. The product runs at a retention time of 2.6 min and the mass spectrum shows [M+4H]4+ (1784.7) and [M+5H]5+ (1427.7) peaks. The sample was run on a Waters Acquity UPLC instrument, C8 column, 10-60% acetonitrile/water over 5 min at a flow rate of 0.3 mL/min. The shoulder that is evident in the UV trace is from one of the glycopeptide fragments that was difficult to separate away from the final product.

DETAILED DESCRIPTION OF THE INVENTION

[35] New targets for HIV-1 vaccine development have been revealed by studies of recently identified broadly neutralizing antibodies (BnAbs).[1,2] Two classes of antibodies with potent neutralizing activities recognize glycan-dependent epitopes on the viral envelope spike (Env), specifically on the variable loop domains of gp120. The V1/V2-directed conformational BnAbs, typified by PG9 and PG16,[3] compose one group, characterized by a dependence on N160 glycosylation. The second group, which includes PGT121 and PGT128, binds the V3 region and requires an N-glycan at N332.[4] Crystallographic studies[5,6] indicate that these two classes of

BnAbs share a common mode of epitope recognition characterized by engagement of two N-glycans and a β-strand, and involving unusually long heavy chain third complementarity determining regions (HCDR3s). Although these interactions have been studied in great detail, the precise identity and arrangement of glycan residues that are necessary to mediate recognition remain uncertain. A more complete understanding of the involved glycan domains would facilitate structure-guided vaccine design efforts. Moreover, insights from models of B cell development suggest that the naïve ancestral B cells of potential BnAb lineages are rare due to host tolerance mechanisms that, for example, select against antibodies with long HCDR3s.[1,7] Therefore, successful induction of BnAb-like humoral responses will also likely require validation of strategies for overcoming the effects of host immunoregulation.

[36] Described herein are both the design and chemical synthesis of immunogens that elicit neutralizing antibodies directed toward the V3 glycan epitope defined by the PGT128 BnAb class.[4,6] Minimal V3 region glycopeptides bearing two glycans of appropriate structure can mimic the antigenic nature of this epitope, and can provide an effective platform for immunogen development. This concept - based on the "two glycans and a strand" paradigm of recognition suggested by x-ray analysis[6] – has been successfully applied to the V1V2 region anti-glycan BnAb site (see Example 1). Given the likely rarity of naïve B cells relevant to BnAb ontogeny in the immune repertoire, preferred immunogens include those that exclude potentially interfering immunodominant epitopes. These immunogens can be evaluated not only based on their affinities for mature BnAbs, but also their germline precursors.

[37] In certain embodiment, the invention provides a composition comprising any one of the inventive peptides, wherein the composition comprises purified homogenously glycosylated peptides. In certain embodiments, about 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 99.9% of

the peptides in the composition are homogenously glycosylated peptides. In certain embodiments, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 99.9% of the peptides in the composition are homogenously glycosylated peptides. In certain embodiments, 70%-75%, 75.1%-80%, 80.1%-85%, 85.1%-90%, 90.1%-95%, 95.1%-99%, 96%-99%, 97%-99%, 98%-99% or 99.9% of the peptides in the composition are homogenously glycosylated peptides. In certain embodiment, the glycosylation pattern is homogenous on all V3 peptides in the composition. In certain embodiment, the glycosylation pattern is substantially identical on all V3 peptides in the composition.

[38] Various methods of determining the glycosylation pattern on a peptide are known in the art. In certain embodiments, glycosylation pattern on the peptides and % homogeneity can be determined by Liquid chromatography–mass spectrometry (LC-MS, or alternatively HPLC-MS).

[39] As indicated in the Examples that follow, V3 glycopeptides can be synthesized with well-defined glycans at N332 and N301 using clade B and clade C sequences (derived from Envs with known antigenicity toward V3 anti-glycan BnAbs). Variations of the peptide framework include full length vs. truncated V3 loops, as well as linear vs. constrained cyclic forms (via disulfide bond formation). Antigenicity testing provides the data needed to determine the peptide design motif that is optimal for binding to HIV-1 Env anti-glycan BnAbs. Using the best peptide "scaffold", derivatives can be synthesized bearing different glycans at N332 and N301 and the determination made as to the optimal carbohydrate design for anti-glycan BnAb binding. The constructs that exhibit the highest affinity for V3-directed anti-glycan BnAbs and their UCAs can be synthesized on larger scale and subjected to trials e.g., in non-human primates - immunogenicity can be evaluated for constructs both with and without conjugation to carrier protein.

[40] The present invention thus relates, at least in part, to immunogens that focus the immune response to the V3 glycan epitope on gp120 that lead to BnAbs and away from epitopes that lead to non-neutralizing antibodies. Central to the present design strategy is making the immunogen as minimal in size as possible so as not to introduce diverting, non-neutralizing epitopes. The sequences of preferred immunogens are described in Examples 2-4 below. Particularly preferred are the CH505 V3 glycopeptides shown Figs. 2B and 2C. Patient CH505 was followed from the time of acute HIV-1 infection to BnAb development (the sequence of the T/F virus is provided in Liao et al (Nature April 3, 2013 (doi:10.1038/nature12053) - CH505 Env sequences: Accession numbers KC247375-KC247667 and KC575845-KC576303; see also US Provisional Appln. 61/764,421, filed February 13, 2013).

[41] The immunogens can be formulated with appropriate carriers using standard techniques to yield compositions suitable for administration. The compositions can include an adjuvant, such as, for example, alum, poly IC, MF-59 or other squalene-based adjuvant, ASO1B or other liposomal based adjuvant suitable for protein immunization. Suitable vaccine strategies include, e.g., those described, for in the Examples that follow.

[42] Nucleic acid sequences (e.g., DNA sequences) encoding the immunogens can also be administered to a subject (e.g., a human) under conditions such that the immunogen is expressed *in vivo* and BNAbs are produced. The DNA can be present as naked DNA with a potent promoter such as the CMV promoter as used in the pCMVr plasmid (Churchyard et al, PLoS One 6:e21225 (2011)) or as an insert in a vector, such as a rAdenoviral (Barouch, et al. Nature Med. 16: 319-23 (2010), recombinant mycobacterial (i.e., BCG or M smegmatis) (Yu et al. Clinical Vaccine Immunol. 14: 886-093 (2007); ibid 13: 1204-11 (2006), or recombinant vaccinia type of vector (Santra S. Nature Med. 16: 324-8 (2010)).

[43] Immunogens of the invention, and nucleic acids (e.g., DNAs) encoding same, are suitable for use in generating an immune response (e.g., BNAbs) in a patient (e.g., a human patient) to HIV-1. The V3 N301, N332 peptide glycan can optimally be administered as a peptide-glycan formulated in a squalene based adjuvant such as MF59, or GLA-SE (Alving et al, Current Opinion in Immunology 24:310 (2012)). The mode of administration of the immunogen, or encoding sequence, can vary with the particular immunogen, the patient and the effect sought, similarly, the dose administered. Typically, the administration route is intramuscular or subcutaneous injection (intravenous and intraperitoneal can also be used). Additionally, the formulations can be administered via the intranasal route, or intrarectally or vaginally as a suppository-like vehicle. Optimum dosing regimens can be readily determined by one skilled in the art. The immunogens (and nucleic acids encoding same) are preferred for use prophylactically, however, their administration to infected individuals may reduce viral load.

[44] The present invention includes the specific minimal protein immunogens disclosed herein (e.g., those in Fig. 2B) and nucleic acids comprising nucleotide sequences encoding same. The proteins can be expressed, for example, in 293T cells, 293F cells or CHO cells (Liao et al, Virology 353:268-82 (2006)).

[45] Certain aspects of the invention can be described in greater detail in the non-limiting Examples that follows. (See also PCT/US2012/000570 and Prov Appln. 61/806,717.) (See chemical synthesis methods described in, for example, references 17, 24, 28-31, 31b, 32-36, 36b, and 37-49 below.)

EXAMPLE 1

[46] The successful preliminary V1V2 work is based, in part, on insights from the RV144 vaccine trial, where antibodies were induced that react with the gp70-V1V2 fusion protein of the

clade B case A2 Env.[9a,10] A molecular sieve analysis of viral sequences derived from vaccinated and placebo HIV-1-infected subjects demonstrated immune pressure at K169 in the V2 region, and antibodies directed to that region correlated with decreased transmission risk.[19] Monoclonal Abs have been isolated from RV144 vaccinees that bind to K169 from a number of trial subjects (prototype mAb CH58) and plasma antibodies to this region were the dominant responses induced.[10]

[47] Interestingly, V1V2 BnAbs isolated from rare HIV-1-chronically infected subjects also bind to K169 and surrounding amino acids, but also bind to high mannose glycans at N156 and N160.[3] The UCAs of V1V2 BnAbs CH01 and PG9 lineages have extremely long HCDR3s (of 24-30 aas) indicating their rarity in the germline repertoire, leading to rare sub-dominant antibody responses. It has been found that the RV144 vaccine component A244 gp120 expressed V1V2 region antigens that bound not only the mature PG9 and CH01 BnAbs, but also the CH01 UCA.[18] However, V1V2 BnAbs like CH01 and PG9 were not induced in RV144. Thus, the epitopes for CH58 ADCC-mediating V2 antibodies and V1V2 BnAbs were both expressed on the RV144 vaccine trial proteins, but the dominant response was CH58-like and not CH01/PG9-like.[10]

[48] A possible explanation for the dominance of the CH58 epitope is that the CH58-like UCAs are all antibodies with normal length HCDR3 regions whose expression is permitted during B cell ontogeny at a sufficiently high frequency for easy clonal B cell expansion. By contrast, the remarkably long HCDR3 regions associated with the CH01/PG9 UCAs leads to their elimination more frequently in the bone marrow by tolerance deletion, resulting in a much smaller pool of naïve B cells capable of responding to the V1V2 BnAb epitope. If this interpretation is correct, then an optimal immunogen for the V1V2 BnAb peptide-glycan

envelope region would be a completely homogeneous construct that selectively expresses the CH01/PG9 epitope but not the CH58 epitope, and, in addition, binds well to the CH01 and PG9 UCAs.

[49] Using chemical methods (see, for example, those referenced above), a set of homogeneous V1V2 glycopeptides bearing $Man_3GlcNAc_2$, $Man_5GlcNAc_2$, and $GlcNAc_2$ N-glycans were synthesized *de novo* (Fig. 1A). The V1V2 sequence was derived from the A244 gp120, based on data (referred to above) demonstrating its ability to bind to the PG9 and CH01 mature BnAbs as well as the CH01 UCA. The Man_5 glycan was targeted based on the crystal structure of PG9 Fab with a scaffolded V1V2 domain,[5] which showed interactions with high mannose glycans at N160 and N156 and the V1V2 C β-strand. Biacore analysis of the synthetic glycopeptides indicated that both the Man_3 and Man_5, constructs bind BnAbs PG9 and CH01 (Figs. 1B,C). These data support the concept that homogeneous glycopeptides with appropriate glycan motifs can emulate the antigenicity of full-length Envs toward V1V2 anti-glycan_BnAbs. Importantly, binding by the naked "aglycone" (Figs. 1B,C) or the solitary protein-free Man_3 and Man_5 oligosaccharides (data not shown) was not observed. Moreover, mixtures of "aglycone" and glycan also failed to show binding, demonstrating that the peptide and carbohydrate domains are both indispensible, and that covalent linkage between them is required for recognition.

[50] Remarkably, the Man_3-derivatized glycopeptide also displayed significant affinity for the UCAs of both PG9 and CH01 (Man_5 did as well, but to a lesser extent) (Figs. 1D,E). This is the first antigen known to bind to the PG9 UCA. Equally important is that the RV144 mAb CH58 and its UCA exhibited only weak reactivity toward the Man_3 construct, and had almost no affinity for the Man_5 derivative (data not shown). Thus, these two V1V2 peptide-glycans have selective reactivity toward the V1V2 BnAbs, and react poorly or not at all with the CH58 mature

and UCA antibodies. Therefore, these V1V2 peptide-glycan immunogens are prime candidates for selective induction of V1V2 BnAb lineages that are normally sub-dominant with infrequent UCAs.

EXAMPLE 2

[51] Described below is the synthesis of HIV-1 Env V3 glycopeptides and definition of the features of the peptide domain that support binding to V3 anti-glycan BnAbs.

[52] The overall design of the glycopeptide constructs is informed by the recently disclosed crystal structure of PGT128 Fab in complex with a glycosylated gp120 outer domain.[6] The bound gp120 fragment was a chimeric construct consisting of a truncated B.JRFL V3 domain ("mini-V3") grafted onto a B.HXB2 base sequence. The structure of the complex revealed that PGT128 engages two glycans (at N332 and N301) and the C-terminal V3 stem. The constructs therefore encompass these peptide and carbohydrate elements from the V3 region (colored red, blue, and green in Fig. 2A).

[53] Clade B and clade C sequences derived from Envs that are known to bind to V3 anti-glycan BnAbs (Fig. 2B) will be used. SPR analyses indicate that the B.JRFL and C.CH505 Envs are competent to bind PGTs 121, 125, and 128, whereas B.CH040 possesses affinity for PGTs 121, 125, and 130 (Haynes, B. F., unpublished data). A V1V2 pilot study demonstrated the feasibility of using partial Env sequences with appropriate glycosylation to emulate the binding characteristics of the full-length Envs.

[54] For the purposes of comparison, all of the constructs will be synthesized with $Man_3GlcNAc_2$ glycans at N332 and N301. The Man_3 sugar, unexpectedly, gave the best results in the V1V2 glycopeptide studies. Additionally, it has the virtue of being relatively accessible to chemical synthesis. Moreover, contacts between PGT128 and the interior mannose and N-

acetylglucosamine residues at N332 and N301 are evident in the x-ray structure.[6] If necessary, a more highly elaborated oligomannose glycan such as $Man_9GlcNAc_2$ could certainly be synthesized and used. Interactions with the outer mannose units may also be important (especially at N332).

[55] The initial set of constructs then will be based on the B.JRFL sequence, as the structure of this Env has been defined by cryo-electron microscopy at the ~11 Å level,[50] and since the JRFL "mini-V3" was used for the co-crystal structure with PGT128 discussed above.[6] B.JRFL will be used as a prototype to examine the effect of two types of structural modifications on antigenicity: (i) V3 loop truncation and (ii) V3 loop constraint. The former will be evaluated by comparing glycopeptides with full length and "mini-V3" segments. The "mini-V3" constructs follow the design of Pejchal et al,[6] as shown in Fig. 2B. To assess the effect of V3 loop constraint, the JRFL full length and mini-V3 constructs will each be made with and without the disulfide linkage between C296 and C331 (i.e., four constructs in all). As with the V1V2 glycopeptides synthesized previously, the N- and C- termini of all the V3 glycopeptides will be modified with acetyl and carboxamide moieties, respectively, to improve physiological stability, and also to avoid unnatural charges at the ends of the peptide.

[56] Based on studies of binding of these four glycopeptides with Env anti-glycan BnAbs, the optimal peptide scaffold will be selected, and sC.CH505 and B.CH040 versions having the same configuration (i.e., full length or mini-V3; cyclic or linear) will be synthesized. Both C.CH505 and B.CH040 are transmitted/founder Envs. In the case of CH505, over 200 mAbs have been isolated from this subject and over 400 single genome amplified viral sequences over time from transmission. It has been found that immune pressure is exerted at N332. The transmitted/founder Env B.CH040 was found to be the target of an early autologous neutralizing

antibody response that exerted selective pressure on the virus and may be easier to induce than BnAb reponses.[51] B.CH040-specific nAbs AbCH83 and AbCH84 were generated from day 132 B cell cultures, and were shown to target a conformational epitope at the base of V3. Binding of these neutralizing antibodies to B.CH040 Envs is blocked by PGTs 121, 125, and 130 (Haynes, B. F., unpublished data). Thus, a glycopeptide based on B.CH040 that is able to bind AbCH83 and AbCH84, as well as one or more of the broadly neutralizing PGTs, might be a good candidate for a priming vaccine, whereas immunogens based on either the B.JRFL or C.CH505 glycopeptides could form part of a boosting regimen.

[57] Evaluation of the constructs will be performed. The glycopeptides will be tested for binding to the PGT121, 125, 128, and 130 BnAbs, as well as their germline precursors, by SPR and ELISA. The constructs will also be tested for binding to mAbs, AbCH83 and AbCH84.[51] The immediate outcome of these studies will be the identification of an optimal peptide "scaffold" suitable for exploring glycan structure-activity relationships.. These investigations will also provide a logical starting point for the longer-term objective of delineating a minimal immunogen containing the relevant (likely sub-dominant) B cell determinants capable of driving the induction of PGT128-like BnAbs. Additionally, the results with the B.CH040 Env may also provide guidance for the development of rational prime/boost strategies for vaccination.

[58] Two general approaches to assemble the glycopeptides will be pursued (Fig. 3). The first would be a maximally convergent strategy where the N332 and N301 glycans are installed simultaneously—a "one-fragment approach." Using standard Fmoc SPPS techniques, a peptide with general structure **12** would be generated with acid-labile side chain protecting groups, except at positions 332 and 301, which would possess free carboxylic acid aspartate side chains. Pseudoproline protection would be implemented at the critical N-glycosylation consensus

sequence serine/threonine sites (at n+2, i.e., positions 303 and 305),[39] to ensure maximal efficiency in the double-aspartylation with the $Man_3GlcNAc_2$–NH_2 glycosyl amine. Subsequent acid-mediated side chain deprotection should afford glycopeptide **13**, with free side chain thiols at C296 and C331. Under oxidative conditions, disulfide bond formation ought to be facile,[52] providing ready access to constrained V3 loop constructs **14**.

[59] A "two-fragment approach" will also be pursued. By this logic, the doubly-glycosylated peptide is derived from the coupling of two mono-glycosylated peptides. In this route, advantage would be taken of the conserved proline residue at position 313 for uniting the two fragments using proline ligation methodology.[47] The requisite ligation partners would be derived from peptide **15**, bearing a C-terminal thioester, and peptide **16**, carrying an N-terminal mercaptoproline auxiliary. These fragments would each be independently glycosylated and subjected to acid-mediated deprotection, yielding mono-glycosylated peptides **17** and **18**. These two glycopeptides would then be joined under native chemical ligation conditions to afford doubly-glycosylated peptide **19**. The auxiliary thiol at P313 would then be removed via metal-free dethiylation,[43] followed by oxidative Acm cleavage and concomitant disulfide formation,[52] thereby converging on the cyclic glycopeptides **14** targeted by the "one-fragment approach."

[60] To access the desired linear non-disulfide bonded constructs, the plan is to apply mild free-radical desulfurization conditions to either **13** or **14** to furnish acyclic glycopeptides with general structure **20**, where removal of the sidechain thiols of C296 and C331 would effectively mutate those residues into alanines (Fig. 4). At this stage, making this chemical transformation is favored because reduced forms of **14** (i.e., **13**) will be susceptible to spontaneous oxidative cyclization over time with exposure to trace O_2. Constructs of type **20** could, in principle, be arrived at simply by incorporating alanines at positions 296 and 331 in the original SPPS.

However, from a strategic standpoint, it is generally more efficient to access structural diversity by branching from a late-stage intermediate, rather than an earlier one. Principles such as these form the basis of a chemical paradigm termed "diverted total synthesis."[53]

[61] From a chemical standpoint, there are no fundamental technical barriers that need to be addressed in the synthesis of these constructs. What remains to be seen is whether the choice of Man_3 glycans will be appropriate to support a level of binding sufficient to allow the projected peptide-level structural comparisons to be made. While binding to $Man_3GlcNAc_2$ was not observed for PGT128 on glycan arrays, such results do not necessarily correlate with what the reality may be when the glycan is presented in the context of a peptide backbone. Still, PGT128 paratope mutations affecting hydrogen bonds to terminal mannose residues of Man_9 at N332 markedly diminished neutralization activity and binding to gp120 and protein-free Man_9, suggesting that the outer mannose units may indeed be important, if not indispensible. If so, and the Man_3 glycopeptides fail to show binding, then derivatives will be synthesized bearing Man_9 at both glycosylation sites. It is possible that the approach of using partial Env fragments may not be appropriate for mimicking the PGT128 epitope. A limited V3 domain construct could be too flexible relative to full length Env, leading to poorer binding. Such concerns formed part of the rationale for testing the disulfide-constrained and truncated V3 loop variants. If these constructs fail, a potential alternative would be to move the cysteine residues closer together (to occupy non-natural locations), so as to constrain the loop further, an approach that has shown promise in the context of purely peptidic V3 loop immunogens.[55] A second option would be to extend the size of the peptide domain in order to obtain a more stably folded structure. Indeed, in the limiting case, the entire engineered "mini-V3" gp120 outer domain construct used by Pejchal et al[6] could be synthesized with glycans at N332 and N301, using a combination of convergent

aspartylation and NCL-based methods. While these technologies are sufficiently powerful to handle such an undertaking, the size of the target (190 aa) would probably not lend itself to a rapid prototyping of pure glycoforms. However, semi-synthetic methods (i.e., expressed protein ligation),[56] could well form the basis of a more practical approach where the glycopeptide fragment is generated by chemical synthesis, and the remaining peptide sequences are accessed by recombinant means.

EXAMPLE 3

[62] Described below is the synthesis of different V3 N332/N301 glycoforms and determination of the nature of the glycan residues that promote recognition by V3 anti-glycan BnAbs.

[63] A systematic exploration will be undertaken of the effects of glycan structure at N332 and N301 on antigenicity toward known V3 glycan-directed BnAbs and their UCAs. As noted above, crystallographic studies[6] indicate that PGT128 uses a mode of recognition similar to the PG9-class[5] BnAbs where the epitope is formed by two glycans and a strand. In the electron density map, only the core pentasaccharide ($Man_5GlcNAc_2$) of the glycan at N301 is visible, whereas a Man_8 or Man_9 sugar is present at N332 (the terminal mannose of the D2 arm is not visible). Thus, the fine structures of the glycans that are necessary for recognition by V3 glycan-directed BnAbs have not been fully defined. Moreover, even less is known about the glycan preferences of the germline precursors of BnAbs. PGTs 125–128 and 130 showed binding to Man_8 and Man_9 oligosaccharides on glycan arrays,[4] but the involvement of other sugars is not definitively ruled out by negative results in such analyses. Experience with the V1V2 epitope indicates that failure of binding by isolated, protein-free carbohydrates is not conclusive, and argues for the importance of assaying these interactions with glycans presented in their native N-

linked contexts.

[64] Generally speaking, the N-glycans of HIV-1 virion-associated Env are thought to be mainly high mannose ($Man_{5-9}GlcNAc_2$) sugars—especially Man_5—with complex-type oligosaccharides composing the remaining minority fraction.[57] The structures of a few oligomannose and a representative complex-type glycan are shown in Fig. 5A. The initial focus will be on evaluating the oligomannose glycans for which synthetic routes have been developed, that is, Man_9, Man_5, and Man_3. The optimal peptide scaffold will be selected and glycopeptides synthesized that are doubly glycosylated with Man_5 and Man_9 (the Man_3 variant having been prepared as described above) (see Fig. 5B with B.JRFL mini-V3 sequence). Versions where the N332 glycan is kept constant as Man_9 and the N301 glycan is varied (Man_3, Man_5) will also be sybnthesized, since only $Man_5GlcNAc_2$ is visible at the latter site in the PGT128 Fab–gp120 outer domain co-crystal structure.

[65] The V3 glycopeptides will be evaluated as described above. Binding to PGTs 121, 125, 128, and 130 and their UCAs will be assessed by SPR and ELISA. Successful completion of these studies will help define the scope of oligomannose structures that can be recognized by these BnAbs in their native N-linked presentation. Using glycopeptides with homogeneous glycosylation will make it possible to elucidate the nature of these glycan-dependent epitopes with a level of specificity and control not provided by recombinant Env ligands with heterogeneous glycosylation. In particular, it is expected that the glycopeptides bearing different glycans at N332 and N301 will help to further characterize the properties of the secondary glycan-binding site of PGT128 (which recognizes the N301 glycan).

[66] The constructs for this study should be accessible following the general strategies outlined in Fig. 3. The glycopeptides possessing the same glycans at N332 and N301 can be

synthesized in the same manner as the constructs described above. The glycopeptides with discordant glycosylation at N332 and N301 will require a slightly modified route, but can, in principle, be reached by one- or two-fragment approaches. The one-fragment method would simply require that the carboxylic acid side chains of **12** at D332 and D301 be protected orthogonally, with sequential unmasking prior to the installation of each glycan. In the two-fragment mode, fragments **15** and **16** would each separately undergo aspartylation with different glycosylamines, giving rise to glycopeptides with two different sugars after ligation.

[67] Access to the requisite synthetic oligosaccharides is assured, because the chemistry for assembling the Man_9, Man_5, and Man_3 glycans has been validated (the synthesis of Man_5 is exemplified in Fig. 6). Recently, a more streamlined approach to the $ManGlcNAc_2$ core trisaccharide **21** was adopted,[58] which was used in preparing the Man_3 and Man_5 glycans, and is amenable to greater material throughput. The overall logic of assembly is geared toward maximal convergence, where the common intermediate **21** is sequentially elaborated with linear trimannoside **28** and branched pentamannoside **29** (Fig. 7). Global deprotection by a sequence similar to that shown in Fig. 6, followed by Kochetkov amination[59] would afford glycosylamine **30**. Hence, all of the glycans for this study can be accessed by a common strategy where **21** undergoes glycosylation at C-3 and C-6 with different oligomannosyl donors.

[68] Further exploration and optimization of glycan structure may be necessary after evaluation of the initial collection of targeted glycoforms, if constructs displaying sufficient affinities for mature BnAbs and/or their UCAs do not emerge as candidates for further development. The range of oligomannose structures to be probed can be broadened. A global strategy for accessing essentially any of the high mannose oligosaccharides can be mapped out (Fig. 8). Using the $ManGlcNAc_2$ trisaccharide core as a starting point, use can be made of a

limited number of linear and branched mannosyl donors in different combinations to achieve a "diverted total synthesis" of a full set of $Man_{3-9}GlcNAc_2$ glycans. In addition, hybrid or complex-type sugars can be incorporated. Constructs with complex-type N-glycans may be especially informative for the study of PGT121, which is sensitive to N332 for its neutralization activity,[4] binds complex-type, but not high mannose, oligosaccharides on glycan arrays, and yet still retains binding to high mannose-only forms of Env.[60] Hence, glycan recognition by PGT121 may be sufficiently promiscuous to accommodate either high mannose or complex-type sugars, which can be probed directly with homogeneous V3 constructs bearing each N-glycan form. Access to complex-type glycans can be obtained synthetically,[58, 61] or by isolation from natural sources—in particular, the biantennary complex-type glycan depicted in Fig. 5A (with α2,6-sialylation) can be obtained from egg yolk[62] and its use in glycopeptide/glycoprotein semi-synthesis has been demonstrated by others.[63]

EXAMPLE 4

[69] Described below are methods for conjugating synthetic V3 glycopeptides and generating immunogens for testing in animal models.

[70] An evaluation will be made of strategies for generating optimal humoral responses using the most promising constructs from above. It is contemplated that a minimal immunogen lacking interference from normally immunodominant epitopes and possessing sufficient affinity for the relevant UCA(s) will be able to initiate maturation of the desired sub-dominant B cell lineages that lead to BnAb induction. This concept will be tested by selecting two constructs that exhibit the best binding characteristics for V3-directed anti-glycan BnAbs and their UCAs and subjecting them to immunogenicity testing in rhesus macaques.

[71] Versions of the constructs that are conjugated to carrier protein will be produced for the

purposes of comparison. Carrier proteins used in currently licensed vaccines include tetanus toxoid (TT), diphtheria toxoid (DT), CRM_{197} (cross-reactive material of diphtheria $toxin_{197}$), *N. meningitidis* outer membrane protein (OMP), and *H. influenzae* protein D.[64] For the initial studies, CRM_{197}, a non-toxic mutant (G52→D) of diphtheria toxin, will be selected which, unlike TT and DT, does not require chemical detoxification with formaldehyde. Thus, it is a well-defined, homogeneous 63 kD protein with a complete set of free, surface-exposed lysine chains (39 total), devoid of cross-linking, which are available for conjugation with potential haptens.[65] Keyhole limpet hemocyanin (KLH) would be a potential alternative.

[72] A third option exists overall between these two limiting possibilities regarding the form of the immunogen (conjugated vs. unconjugated), which would be to join the V3 glycopeptides to a known T-helper peptide.

[73] A non-human primate (NHP) study is contemplated. A 100 μg dose can be used, based on favorable results in previous NHP studies with that dose of Env. If inferior immune responses are observed when dosing at 100 μg, then the amount of immunogen is not likely to be the problem. Thus, each IM immunization of 100 μg glycopeptide in 0.5 ml of adjuvant can be divided in two sites (~0.25 mL per site). The adjuvant can be a squalene-based emulsion of 17% oil-in-saline with TLR7 and TLR9 agonists (R848 and 2006 type B oligo-CpGs) that has been shown to be effective in supporting the induction of anti-Env antibodies (Haynes, B.F., unpublished data).

[74] The study groups for the first two V3 glycopeptide Env subunit immunogens can be:

Group 1: V3 glycopeptide #1, 100 μg IM × 5 (6 monkeys) with adjuvant

Group 2: V3 glycopeptide #2, 100 μg IM × 5 (6 monkeys) with adjuvant

Group 3: V3 glycopeptide #1 conjugated to CRM_{197}, 100 μg IM x 5 (6 monkeys)

with adjuvant

Group 4: V3 glycopeptide #2 conjugated to CRM_{197}, 100 μg IM x 5 (6 monkeys) with adjuvant

[75] Immunological evaluation can be performed as follows: each rhesus macaque can be heavily sampled systemically (blood/plasma) and mucosally (rectal/vaginal saliva fluids) before and two weeks after each immunization for profiling IgG and IgA vaccine-induced Env responses. Neutralization assays using both TZMbl and A3R5 assays can be performed. In addition, competitive inhibition assays can be performed for vaccine induction of antibodies that bind to various Env epitopes including measurement of antibodies that can block PGT128, block CD4 binding site antibodies CH31 and VRC01, and antibodies that can block the binding of PG9, CH01 and 2G12 that recognize other glycosylated BnAb Env regions. Samples at select day 14 post immunization time points can be obtained for both VH and VL 454 deep sequencing as well as memory B cell cultures and/or antigen-specific B cell sorting to profile the B cell repertoire and ontogeny of induced antibodies. Finally, if acceptable immunogenicity is obtained, with a degree of breath in tier 2 neutralization obtained, the animals can be challenged with SHIV SF162P3 IR low dose repeat challenge. If poor breadth is obtained, animals can be re-boosted with new immunogens directed by B cell lineage design, to awaken the desired lineages for induction of PGT-like V3 peptide-glycan BnAbs prior to challenge.

[76] Animals can be monitored for reactogenicity at the immunization sites, CBCs, and chemistries throughout the study.

[77] Evaluation of this first round of vaccine candidates can establish whether the V3 glycopeptides are immunogenic enough (when formulated with appropriate adjuvants) to be used as pure immunogens. This approach would be most desirable from the standpoint of minimizing

the likelihood of including potentially immunodominant epitopes that might overshadow a sub-dominant BnAb maturation pathway. Nevertheless, oligomerization of the antigen and/or the inclusion of more T-helper determinants may be necessary to achieve robust immune responses. Thus, parallel evaluation of glycopeptides conjugated to carrier protein can ensure that measurable humoral responses are obtained. Analysis of the quality of the humoral responses, namely, whether autologous and/or more broadly neutralizing activities are elicited, can reveal if the desired epitope conformation be can recognized by the immune system when presented in the context of a partial Env sequence.

[78] The methods described herein can be scaled up for production of candidate glycopeptides in sufficient quantities for *in vivo* immunogenicity studies. For the conjugated constructs, selecting an efficient method for linking to carrier protein will be important. Many potential bioconjugation chemistries could potentially be applied,[66] but chemoselective ligation strategies that rely on thiol functionalities may be unsuitable for disulfide-containing constructs, due to the potential for disulfide exchange and scrambling.[67] Therefore, use of an oxime ligation approach in contemplated (Fig. 9). The oxime linkage is physiologically stable, and its formation proceeds under neutral aqueous buffer conditions when accelerated by aniline.[68] The requisite heterobifunctional linkers are commercially available, and can be incorporated using standard protocols. Sulfo-N-succinimidyl-4-formylbenzamide can be used to modify the lysine side chains of CRM_{197} to present aldehydes (**31**), and incorporation of Boc-aminooxyacetic acid during SPPS can provide constructs of general structure **32** bearing oxyamine functionality at the N-terminus. Loading of glycopeptides **32** onto aldehyde-modified CRM_{197} **31** can be accomplished by oxime formation in aqueous buffer in the presence of aniline, to furnish conjugated constructs such as **33**. Degree of glycopeptide loading onto carrier protein (i.e., "*n*")

can be assessed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS).

[79] A potential further application of the oxime ligation chemistry would be in the oligomerization of multiple V3 domains, which could be advantageous if carrier protein loading becomes a necessity (to increase the ratio of antigen to carrier). The ligation could also be employed to join multiple different domains together, which would enable the production of fully synthetic unimolecular multivalent HIV-1 vaccines (Fig. 10 5).

[80] An alternative to using a carrier protein would be to join the antigen to a T-helper peptide, such as T1 (16 aa peptide from gp120 C4 region),[69, 70]. This option has some appeal, as a carrier protein could also carry immunodominant epitopes that might be detrimental for driving the kinds of B cell responses sought. It is possible that the V3 glycopeptide constructs may exhibit sufficient affinity for BnAbs and/or their UCAs through a kind of "induced-fit" mechanism, but still be sufficiently flexible that this desired conformation is not predominantly recognized by naïve B cell receptors. Remedies include strategies described above, (e.g., repositioning the disulfide constraint or extending the length of the peptide chain). It may also be feasible to constrain the V3 domain further by inserting it into a small protein that can serve as a scaffold (and access by semi-synthesis). If the neutralization activities observed are strain-specific, a strategy for addressing this would be to immunize sequentially, or as a mixture, constructs derived from different Envs. These constructs could also be linked together in a single molecular entity as discussed above (Fig. 10).

Example 5: Synthesis of V3 glycopeptides

[81] The overall design of these glycopeptide constructs is informed by the recently disclosed crystal structure of PGT128 Fab in complex with a glycosylated gp120 outer domain. See

Pejchal, R.; Doores, K. J.; Walker, L. M.; Khayat, R.; Huang, P.-S.; Wang, S.-K.; Stanfield, R. L.; Julien, J.-P.; Ramos, A.; Crispin, M.; Depetris, R.; Katpally, U.; Marozsan, A.; Cupo, A.; Maloveste, S.; Liu, Y.; McBride, R.; Ito, Y.; Sanders, R. W.; Ogohara, C.; Paulson, J. C.; Feizi, T.; Scanlan, C. N.; Wong, C.-H.; Moore, J. P.; Olson, W. C.; Ward, A. B.; Poignard, P.; Schief, W. R.; Burton, D. R.; Wilson, I. A. "A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield." *Science* **2011**, *334*, 1097–1103). The bound gp120 fragment was a chimeric construct consisting of a truncated B.JRFL V3 domain ("mini-V3") grafted onto a B.HXB2 base sequence. The structure of the complex revealed that PGT128 engages two glycans (at N332 and N301) and the C-terminal V3 stem. The planned constructs encompass these peptide and carbohydrate elements from the V3 region.

[82] One of the key goals of this study was to access the $Man_9GlcNAc_2$ glycan (**8**) by chemical synthesis. The logic of assembly is the same as the route we developed for the $Man_5GlcNAc_2$ oligosaccharide, See Aussedat, B.; Vohra, Y.; Park, P. K.; Fernández-Tejada, A.; Alam, S. M.; Dennison, S. M.; Jaeger, F. H.; Anasti, K.; Stewart, S.; Blinn, J. H.; et al. Chemical Synthesis of Highly Congested gp120 V1V2 N-Glycopeptide Antigens for Potential HIV-1-Directed Vaccines. *J. Am. Chem. Soc.* **2013**, *135*, 13113–13120.) which is geared toward maximal convergence, where the common intermediate **9**[Error! Bookmark not defined.] is sequentially elaborated with linear trimannoside **10** and branched pentamannoside **11** (Scheme 1). The pentasaccharide **11** was constructed from building block **12** by an iterative double-glycosylation approach (Scheme 2). Mannosyl bis-acceptor **12** was glycosylated at the C-3 and C-6 positions with imidate donor **13** in 80% yield. Cleavage of the Lev protecting groups on the resulting trimannoside **14** with hydrazine unveiled the bis-acceptor for the second double glycosylation event with donor **16**, which proceeded in 82% yield to furnish the desired pentameric

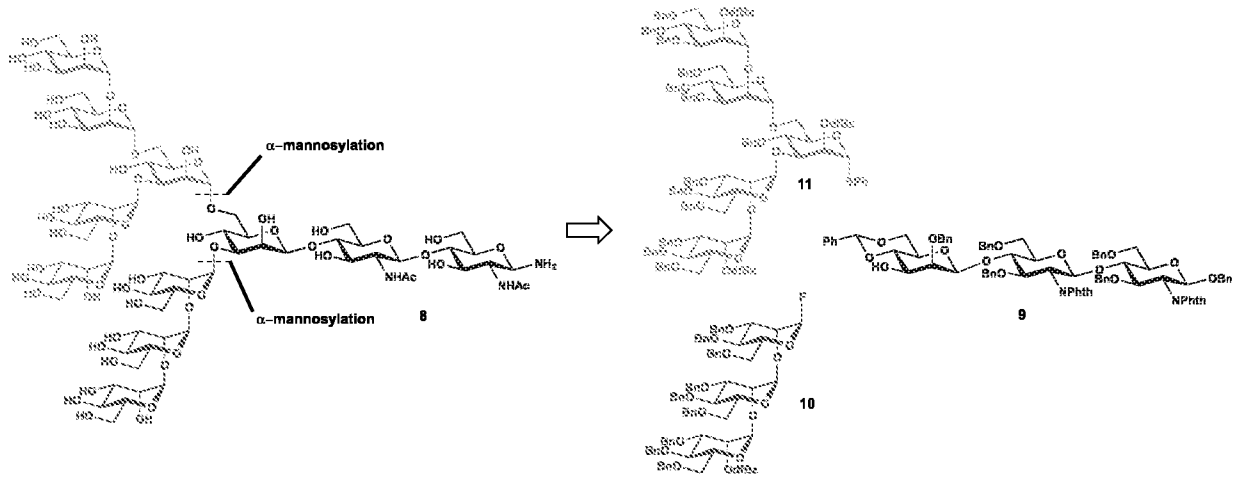
thioglycoside donor **11**.

[83] The linear trisaccharide **10** was obtained by stepwise elongation of mannosyl acceptor **17** with imidate **16** (Scheme 3). The resulting trimannosyl thioglycoside **20** was then hydrolyzed to the anomeric alcohol and converted to the fluoro donor **10** in 82% yield over the two steps.

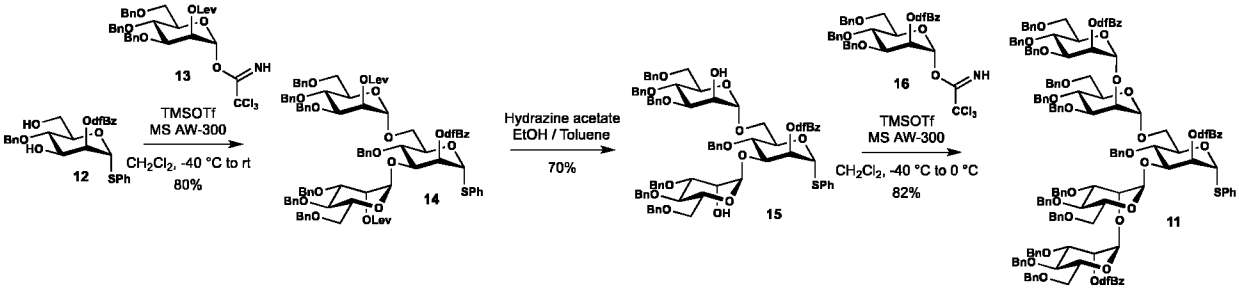
[84] The final assembly of the undecamer **23** was accomplished by first uniting fragments **9** and **10** using Cp_2HfCl_2/AgOTf as the promoter (Scheme 4). The benzylidene acetal was subsequently cleaved with aqueous acetic acid, and the resulting diol **22** was selectively glycosylated at the 6-position with pentasaccharide donor **11** to afford the protected undecasaccharide **23**. This substrate was subjected to a four-step deprotection sequence, then treated with aqueous sodium bicarbonate to generate glycosyl amine **8**.

[85] The V3 glycopeptides were assembled using two different approaches. For the constructs modified with the simpler chitobiose disaccharide, the glycans were installed by a double aspartylation on partially protected peptide **24**, bearing free carboxylic acid sidechains at positions 301 and 332 (Scheme 5). Deprotection of the glycopeptide was followed by treatment with iodine to form the cyclic disulfide **26**. The V3 glycopeptides bearing the more highly elaborated $Man_5GlcNAc_2$ and $Man_9GlcNAc_2$ glycans were put together using a "two-fragment" approach (as exemplified in Scheme 6). Peptide fragments **27** and **28** were individually glycosylated, then following TFA deprotection, united via native chemical ligation. The resulting ligated product was then oxidized with iodine to afford cyclized constructs, such as **31**.

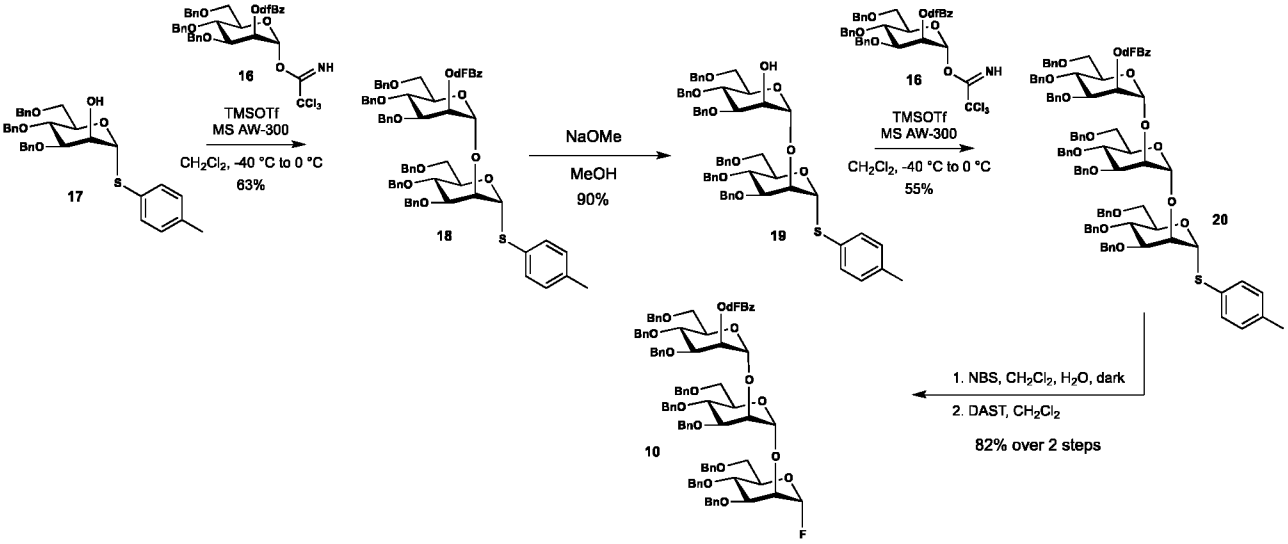
Schemes



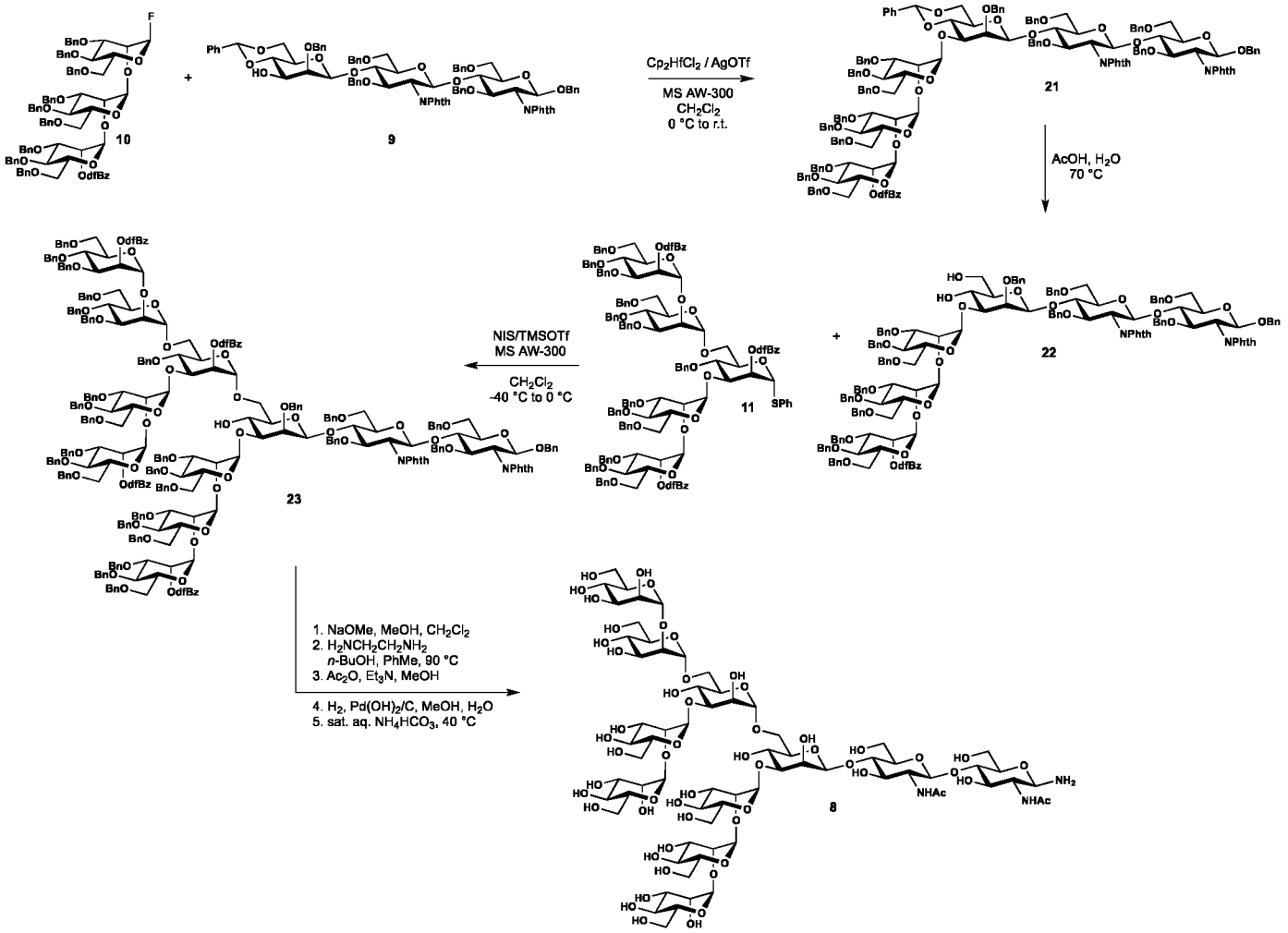
Scheme 1. Synthetic strategy to access $Man_9GlcNAc_2$ undecasaccharide **8**.



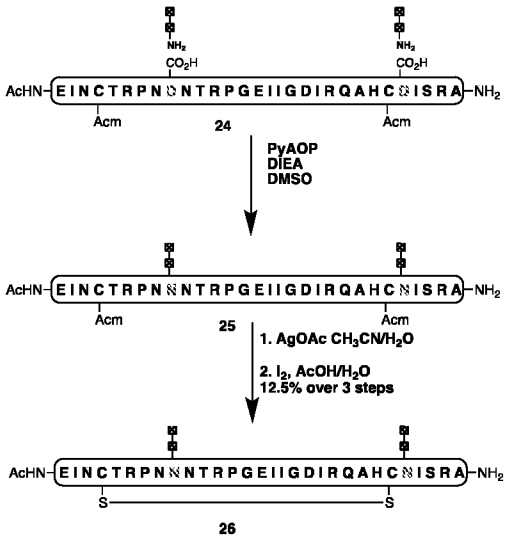
Scheme 2. Synthesis of branched pentamannoside **11**.



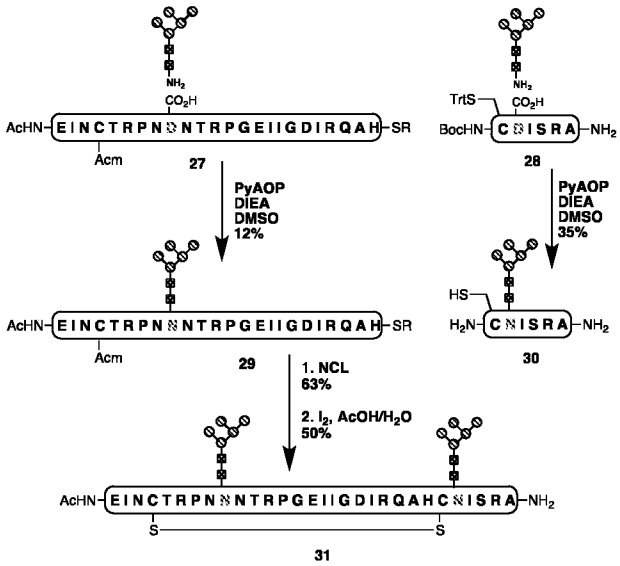
Scheme 3. Synthesis of linear trimannoside **10**.



Scheme 4. Synthesis of $Man_9GlcNAc_2$ undecasaccharide **8**.



Scheme 5. Synthesis of $GlcNAc_2$ V3 glycopeptide **26**.



Scheme 6. Synthesis of $Man_5GlcNAc_2$ V3 glycopeptide **31**.

[86] Figure 14A and 14B show an analytical LCMS from a run of the synthesis, showing glycosylated V3 peptide. This was an analytical synthesis and LCMS analysis (where small quantities were analyzed), so the spectrum could be improved.

[87] The product ran at a retention time of 2.6 min and the mass spectrum shows [M+4H]4+ (1784.7) and [M+5H]5+ (1427.7) peaks. The sample was run on a Waters Acquity UPLC instrument, C8 column, 10-60% acetonitrile/water over 5 min at a flow rate of 0.3 mL/min.

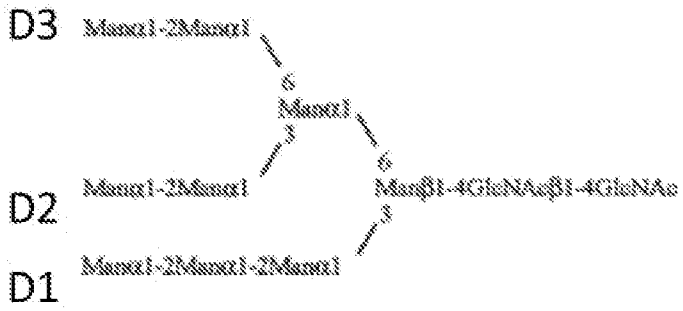
[88] The shoulder that is evident in the UV trace is from one of the glycopeptide fragments that was difficult to separate away from the final product. This will be corrected in a future synthesis. Since glycosylation is done chemically, the expectation is to achieve homogeneity in terms of glycosylation state.

Example 6

[89] Figures 11-13 depict various experimental data showing that synthetic glycopeptides, such as for example the peptide of Figure 1, can show differential binding to bnAbs vs non-neutralizing antibodies. From previous work it is know that V1V2 bnAbs PG9 and CH01 bound to disulfide-linked and mannose 5-derivatized glycopeptides, but did not show binding to aglycone V2 peptide. Both 2G12 mAb and 2G12 UCA bound well to Man5 V1V2 dimer, indicating recognition of the glycan cluster on a V2 backbone.

[90] Figure 12 shows that V3-Man9GlcNAc2 glycopeptide showed specific binding to PGT BnAbs (PGT 128, PGT 125).

[91] PGT 128 binds to the terminal mannose residues of both D1 and D3 arms of branched Man_8/Man_9 glycans (Pejchal et al., 2011). 2G12 makes central contacts with the terminal $Mana_{1,2}Man$, at the tip of D1 arm of oligomannose glycan (Calarese et al., 2003). Thus, we tested the binding of the glycan-dependent BNAbs for binding to synthetic Man_9 glycan itself.



[92] Figure 13 shows the Binding of PGT mAbs to synthetic Man_9-$GlcNAc_2$ glycan. In certain embodiments, the invention provides free sugars (Man_5 and/or Man_9 glycan). In other embodiments, the invention provides the Man9 and Man5 sugars with the biotin tag. In other embodiments, the invention provides the Man9 and Man5 sugars modified with glycosyl amine, for example to be used in the microarray platform.

[93] Some embodiments use the free glycans (Man5, Man9) and while other embodiments use the same glycans on a peptide backbone. The free glycans will allow us to do large screening of vaccine samples to be able to select those that are strongly positives for glycan binding and include all positives based on just glycan recognition. The peptide associated glycans will allow to further discriminate between those that bind to V1V2 vs V3. It is likely that some antibodies bind to oligomannose clusters and could potentially be missed on certain glycopeptide constructs. So the differential binding using a combination of glycan, glycopeptide and aglycone will allow to narrow down the samples for isolating B cells using a more specific glycopeptide hook. The reagents described herein can be used to identify antibodies which recognize glycans, for example Man_5 and/or Man_9 glycan, antibodies which recognize glycopeptides modified with these glycans, and antibodies which recognize the aglycone peptide. The aglycone can also include any suitable tag, for example but not limited to biotin, at either peptide end. Any other suitable linker, for example but not limited to PEG linker could be inserted between the tag and the peptide. In some embodiment, this additional linker could increase the chance of an antibody recognizing the glycopeptide and/or aglycone peptide, when the peptide is immobilized.

Example 7

[94] The $Man_9GlcNAc_2$ V3 ("Man_9 V3") glycopeptides of the invention, for example the peptide of Figure 11, will be used in various non-limiting examples of immunogenicity regimens. In one embodiment, a Man_9 V3 glycopeptide is used in repetitive immunizations intramuscularly (IM) alone with an adjuvant for example but not limited to as a squalene based adjuvant, for example MF59, or a Toll-like receptor 4 agonist, for example GLA/SE (see *Baldwin et al. J Immunol*; Prepublished online 30 January 2012). In another embodiment, a Man_9 V3glycopeptide will be used as a prime IM prior to IM boost with a V3 broad neutralizing

epitope. In another embodiment, a Man_9 V3glycopeptide will be used as an IM boost for a prime by AE.A244 gp120.

* * *

All documents and other information sources cited herein are hereby incorporated in their entirety by reference.

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WHAT IS CLAIMED IS:

1. A synthetic peptide comprising sequence SEQ ID NO: 2, 4, 6, 7, 8 or 9, wherein the peptide is glycosylated at positions Asn301 and Asn 332, and wherein the peptide is cyclic via endogenous cysteins.

2. The peptide of claim 1 wherein the peptide is of SEQ ID NO: 2, 4, 6, 7, 8 or 9, and wherein the peptide is glycosylated at positions Asn301 and Asn 332.

3. The peptide of claim 1, wherein the peptide is glycosylated with oilgomannose.

4. The peptide of claim 1, wherein the peptide has $Man_9GlcNAc_2$ glycans at positions Asn301 and Asn 332.

5. A synthetic glycopeptide of the structure in Figure 12 (peptide of SEQ ID NO: 8 glycosylated at positions Asn301 and Asn 332 with $Man_9GlcNAc_2$, wherein the peptide is cyclic via endogenous cysteins.

6. A composition comprising the synthetic peptide of any one of claims 1-5, wherein the composition comprises purified homogenously glycosylated peptides.

7. The composition of claim 6, wherein the glycosylation pattern is homogenous on all peptides of SEQ ID NO: 2, 4, 6, 7, 8 or 9 in the composition.

8. The composition of claim 6, wherein the peptide is cyclic via endogenous cysteins.

9. The composition of claim 6 further comprising an adjuvant.

10. A method of inducing antibodies against HIV-1 in a subject, the method comprising administering to the subject the composition of claim 6 in an amount sufficient to induce the anti-HIV-1 antibodies.

11. The method of claim 10, wherein the composition comprises glycopeptide of the structure in Figure 12 (peptide of SEQ ID NO: 8 glycosylated at positions Asn301 and Asn 332 with $Man_9GlcNAc_2$, wherein the peptide is cyclic via endogenous cysteins.

12. The method of claim 10 wherein said subject is a human.

13. A method of determining whether a V3 glycopeptide is immunogenic comprising obtaining a sample from a subject immunized with the composition of claim 6 and determining binding of the sample to V3 glycopeptide, the glycan and the aglycone V3 peptide, wherein a sample which includes antibodies which bind preferentially to the V3 glycopeptide and/or the glycan but do not bind to the aglycone V3 peptide, is indicative of a V3 glycopeptide which is an immunogen and induces antibodies to the V3 glycopeptide.

14. The method of claim 13, wherein the subject is immunized with the composition of claim 6.

15. A method for synthesizing the peptide of claim 1, according to the scheme in Example 5.

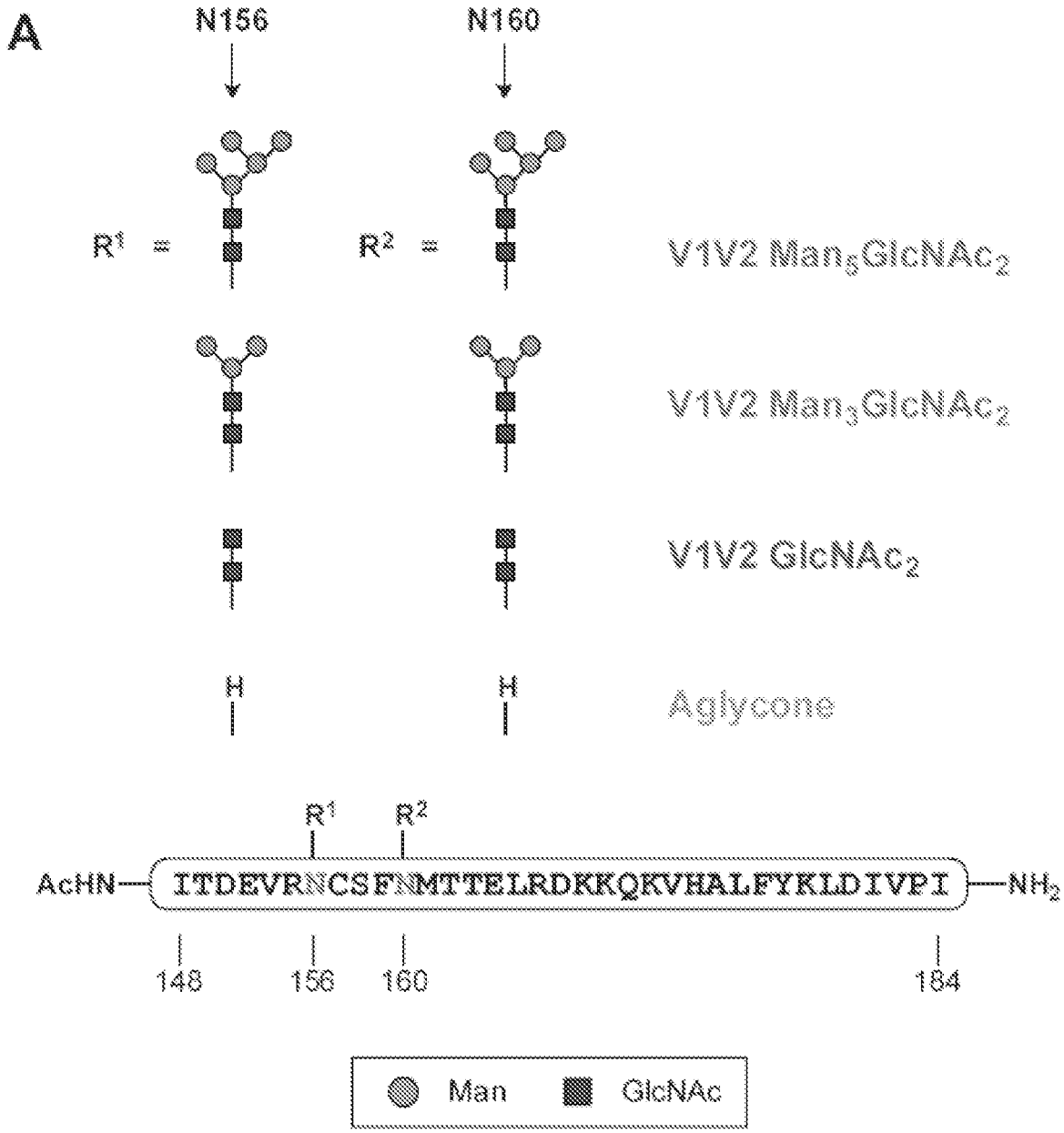

A
N156
N160
R1 =
R2 =
V1V2 Man5GlcNAc2
V1V2 Man3GlcNAc2
V1V2 GlcNAc2
H
H
Aglycone
R1
R2
AcHN
ITDEVRNCSFNMTTELRDKKQKVHALFYKLDIVPI
NH2
148
156
160
184
Man
GlcNAc

Figure 1A

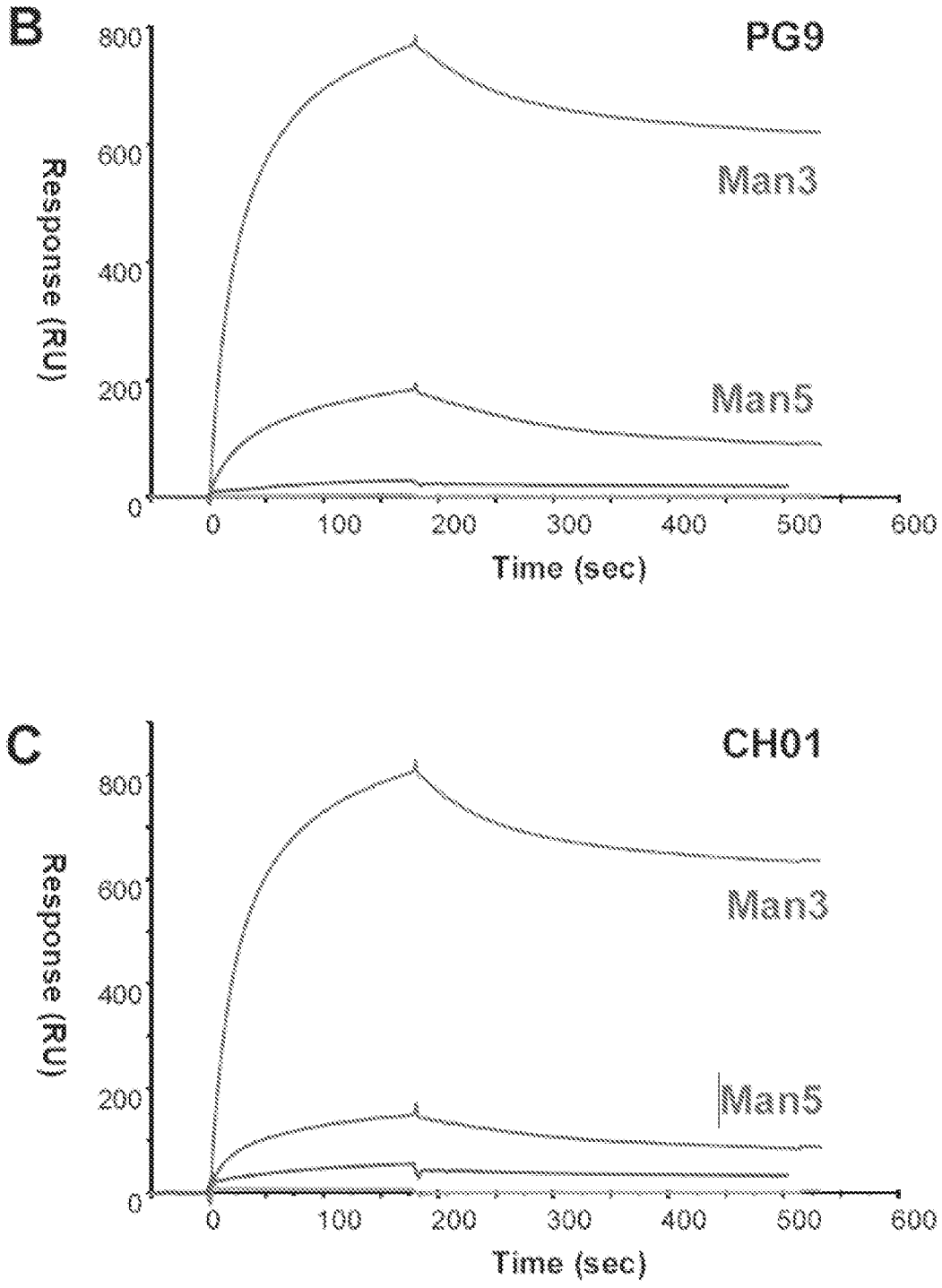


Figure 1B and 1C

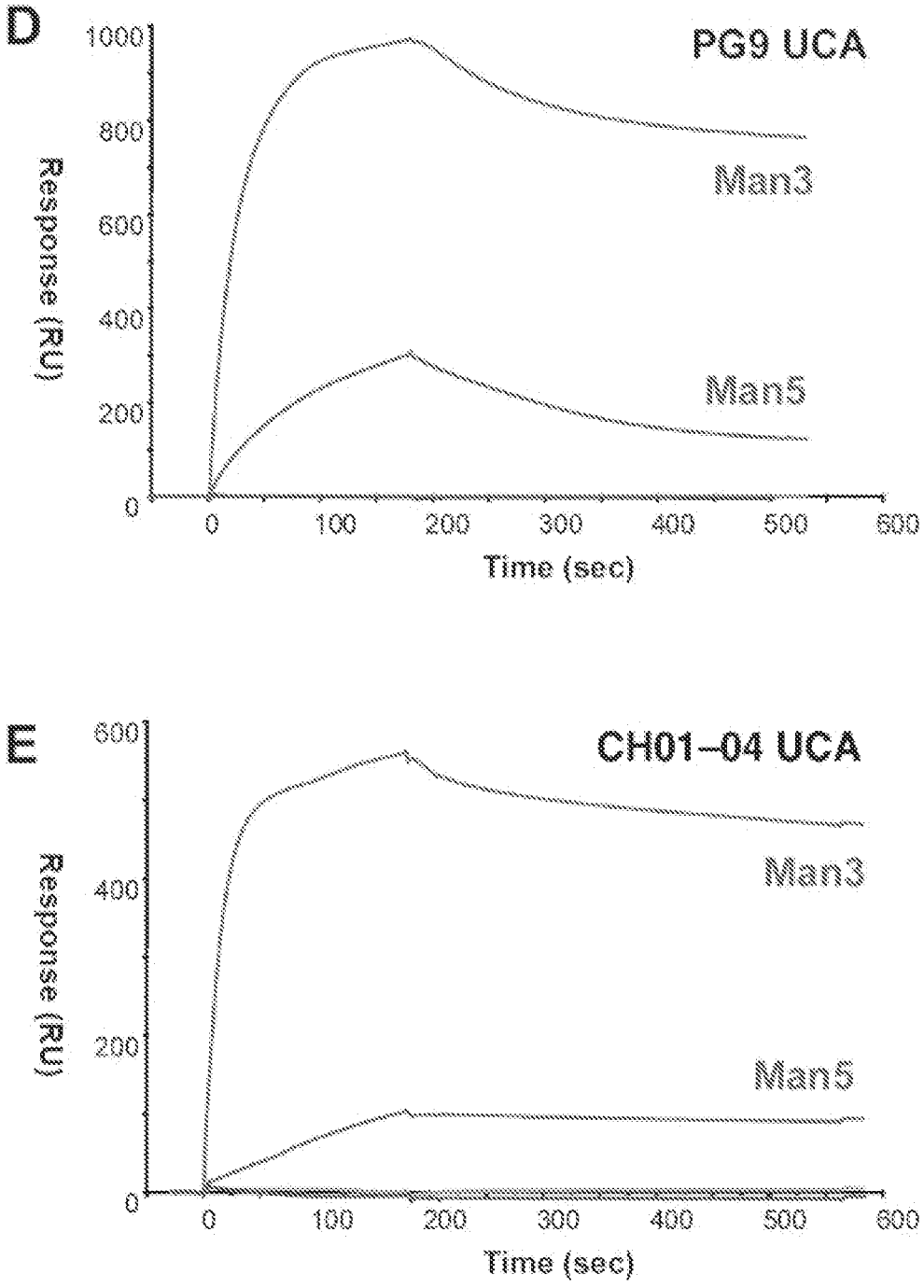


Figure 1D and 1E

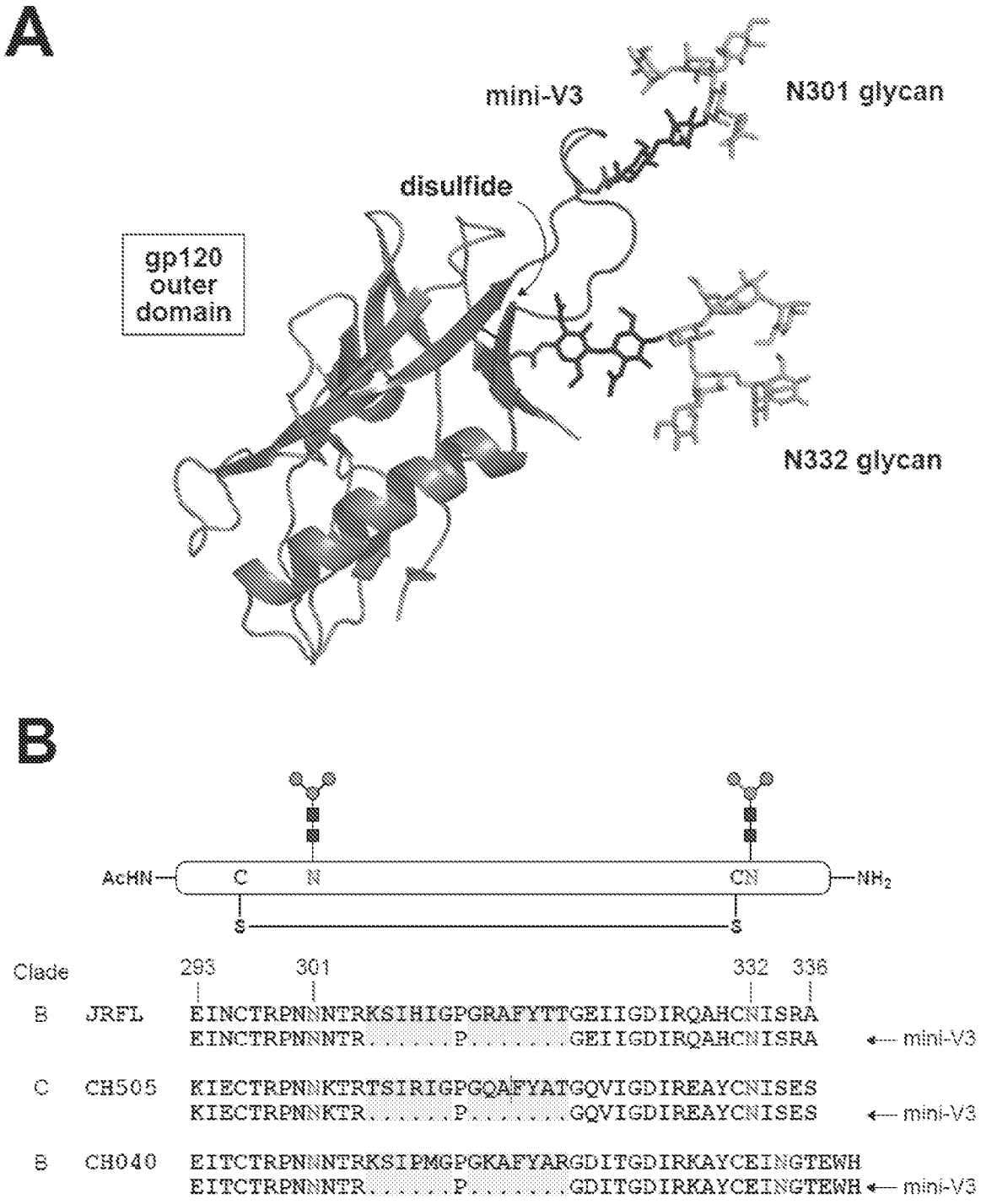


Figure 2A and 2B

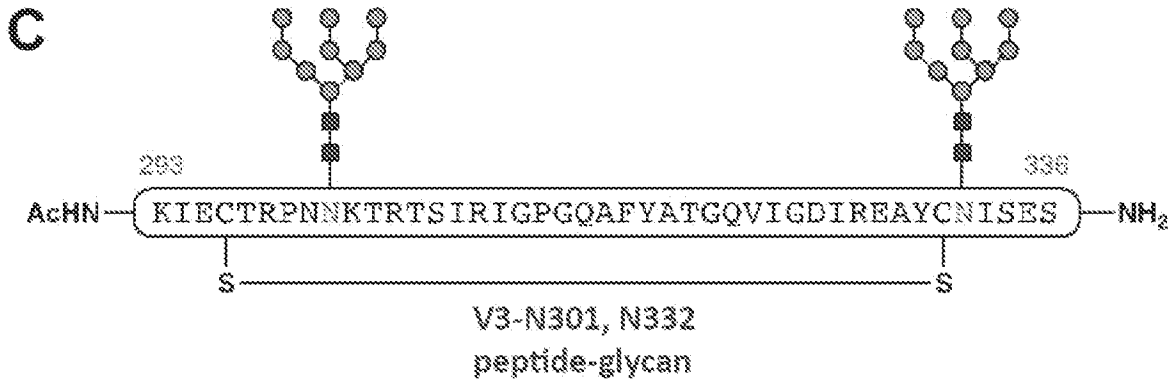

C
299
330
AcHN
KIECTRPNNKTRTSIRIGPGQAFYATGQVIGDIREAYCNISES
NH2
S
S
V3-N301, N332
peptide-glycan

Figure 2C

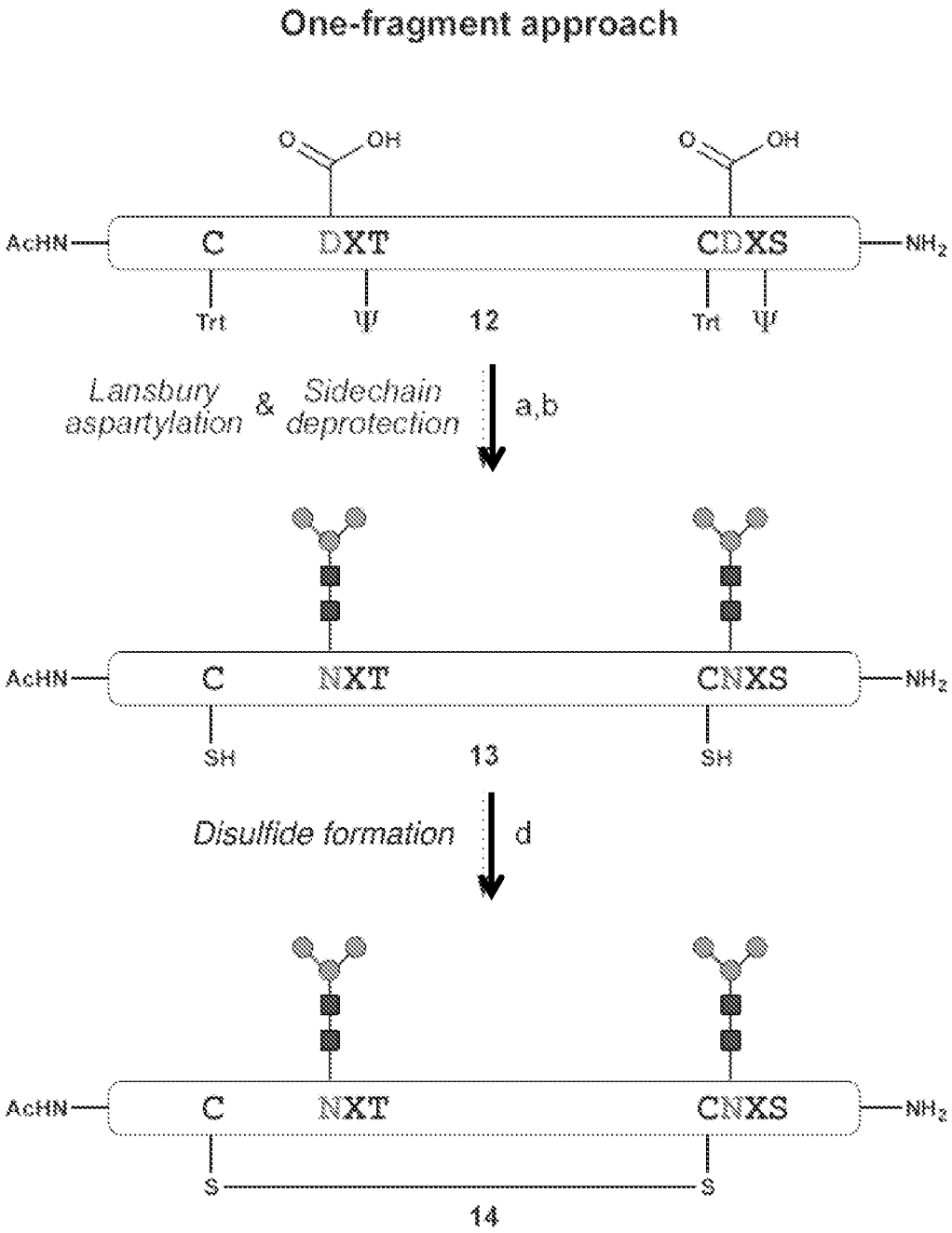


Figure 3A (One-fragment approach)

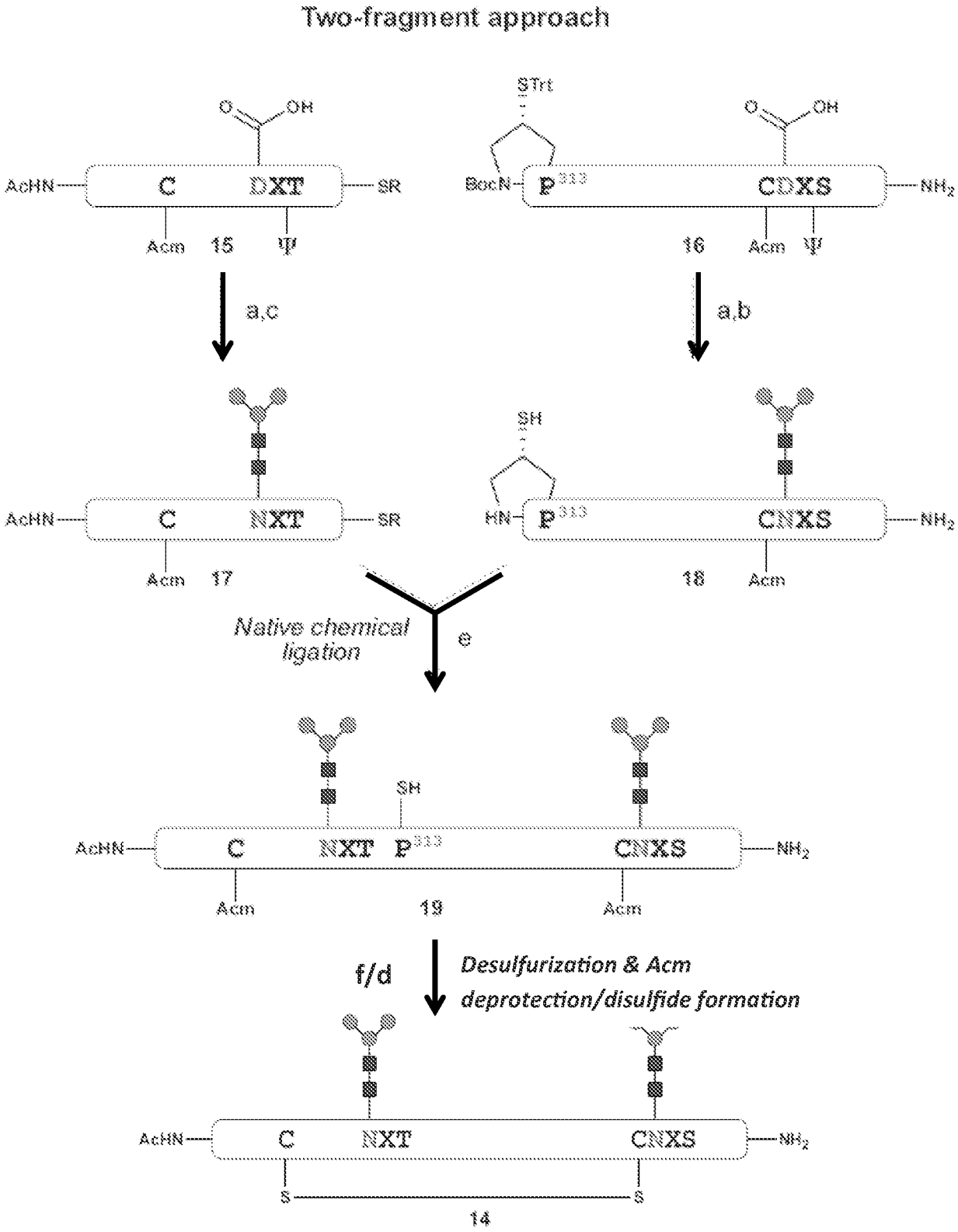


Figure 3B (Two-fragment approach)

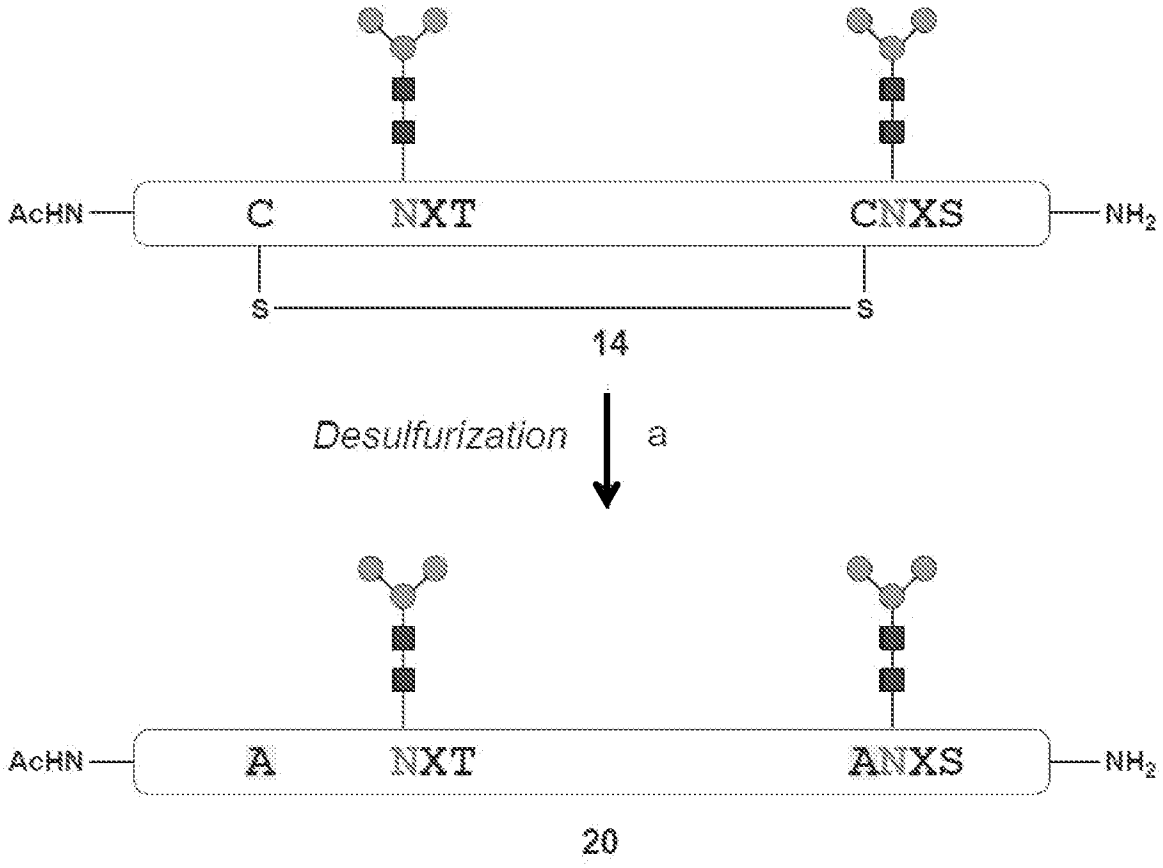

AcHN
C
NXT
CNXS
NH2
S
S
14
Desulfurization
a
AcHN
A
NXT
ANXS
NH2
20

Figure 4

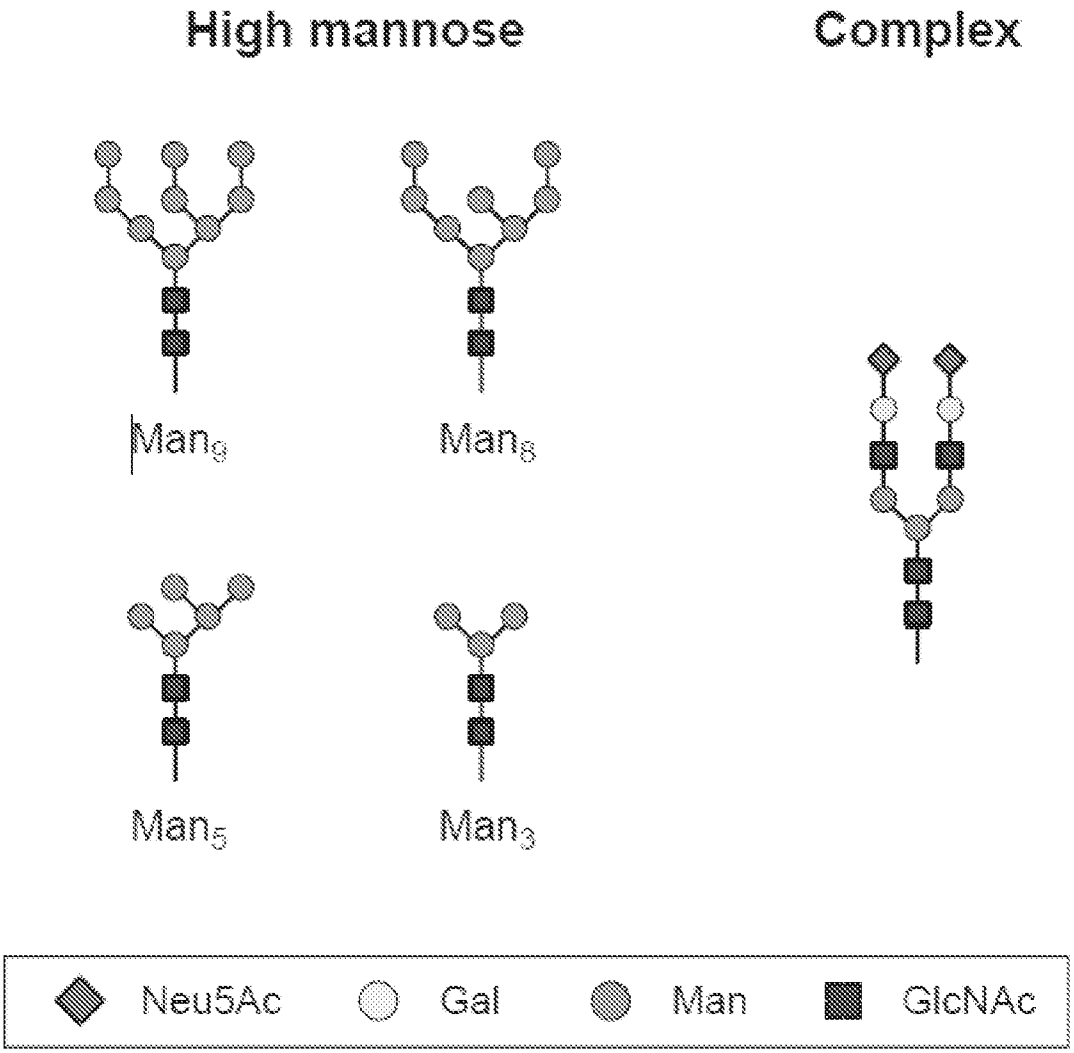
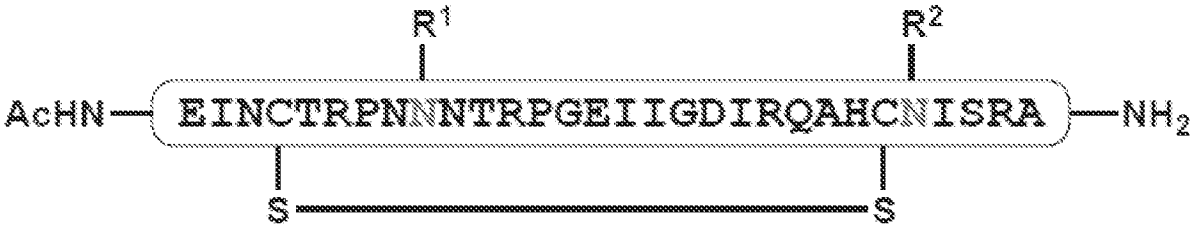

High mannose
Complex
Man9
Man8
Man5
Man3
Neu5Ac
Gal
Man
GlcNAc

Figure 5A



	N301		N332
R^1 =	Man_3	R^2 =	Man_3
	Man_5		Man_5
	Man_9		Man_9
	Man_3		Man_9
	Man_5		Man_9

Figure 5B

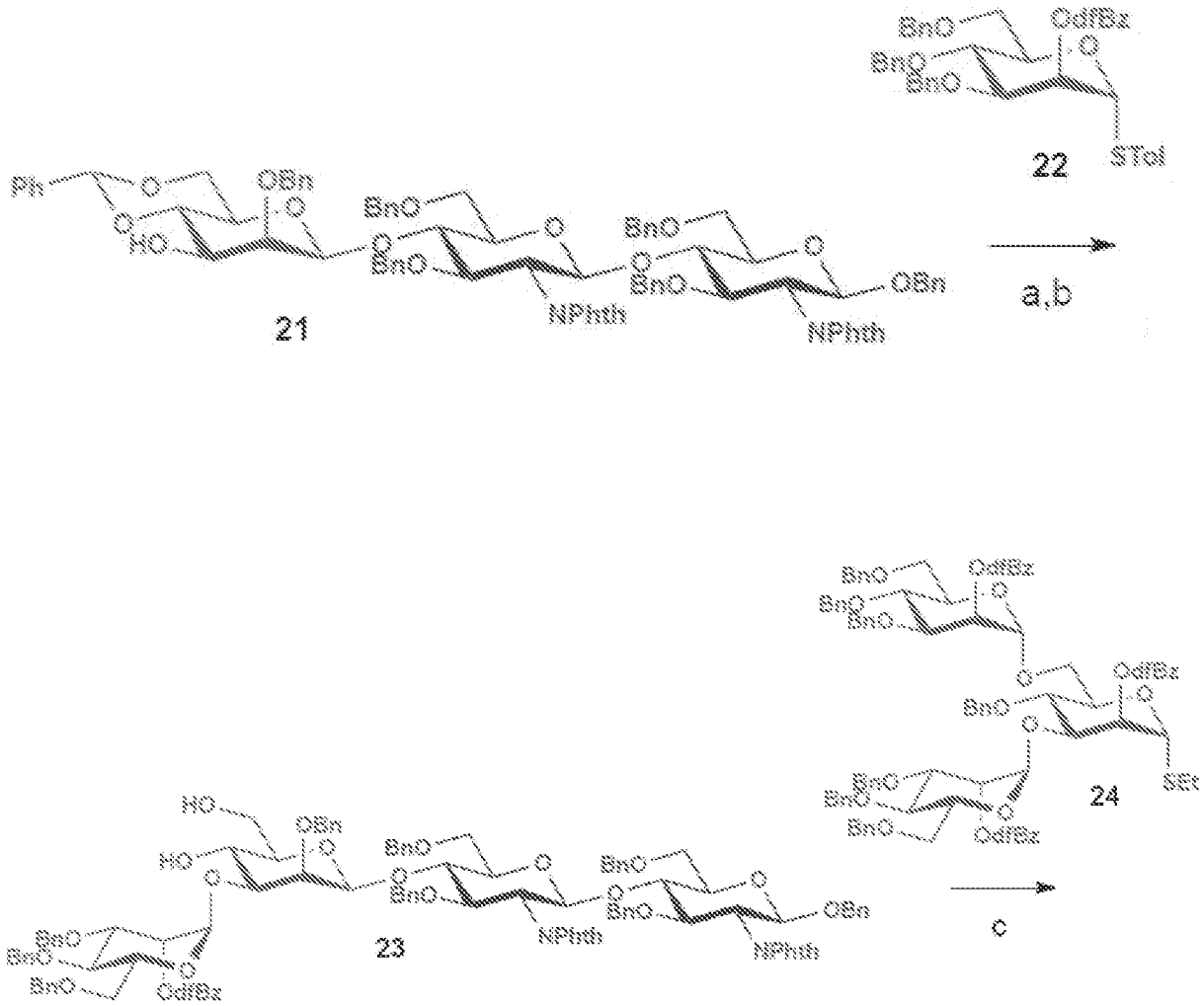


Figure 6

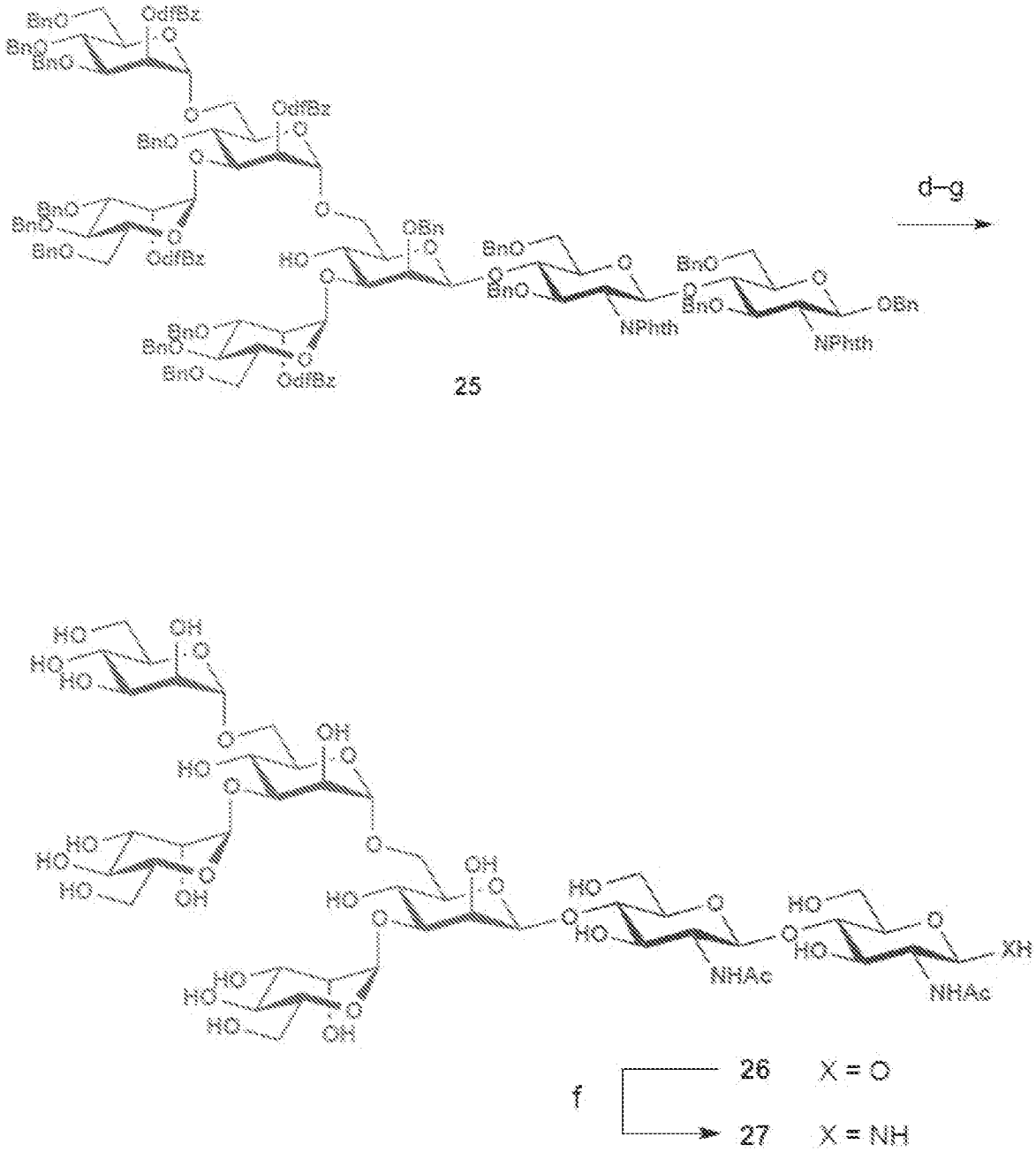


Figure 6 (Cont.)

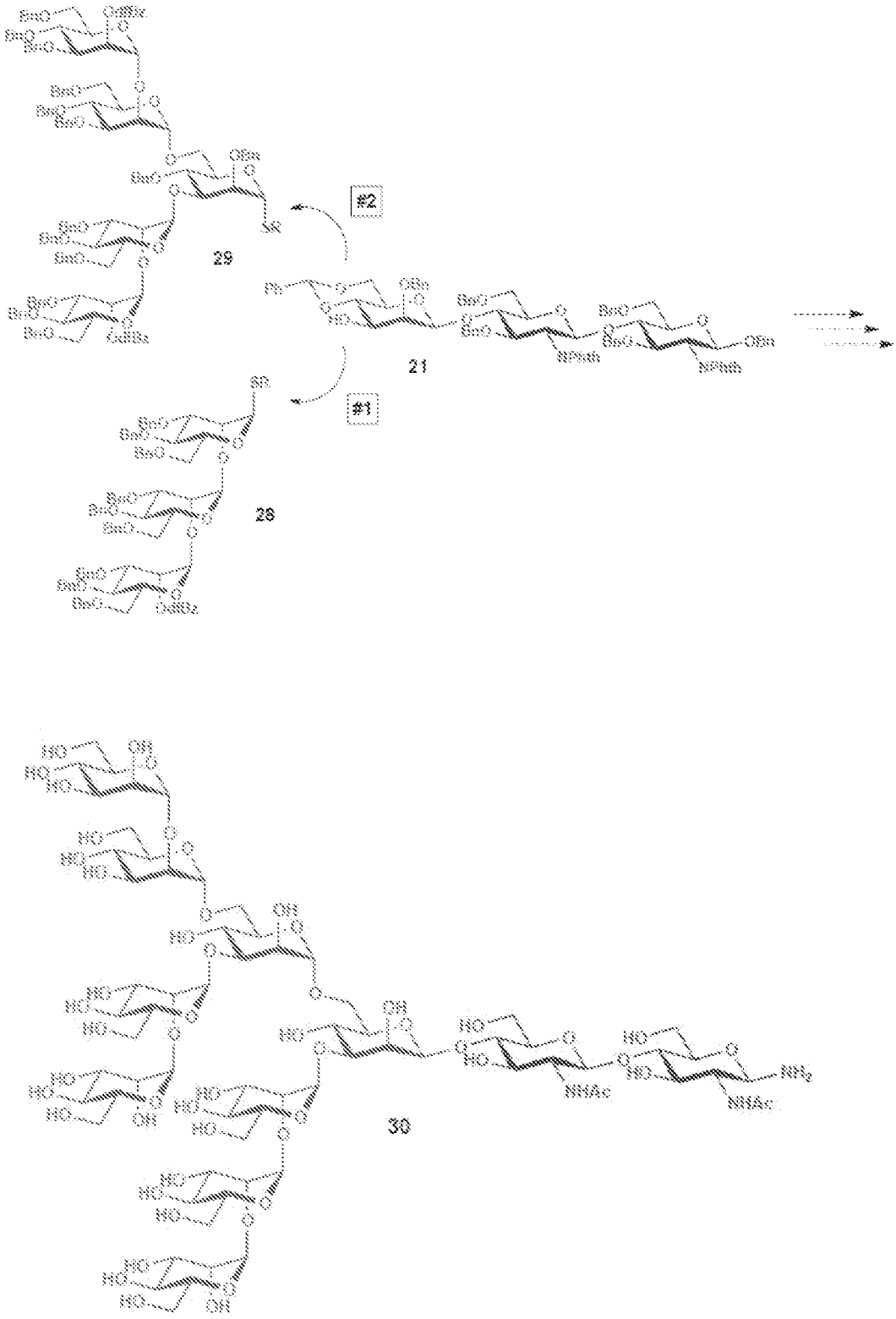


Figure 7

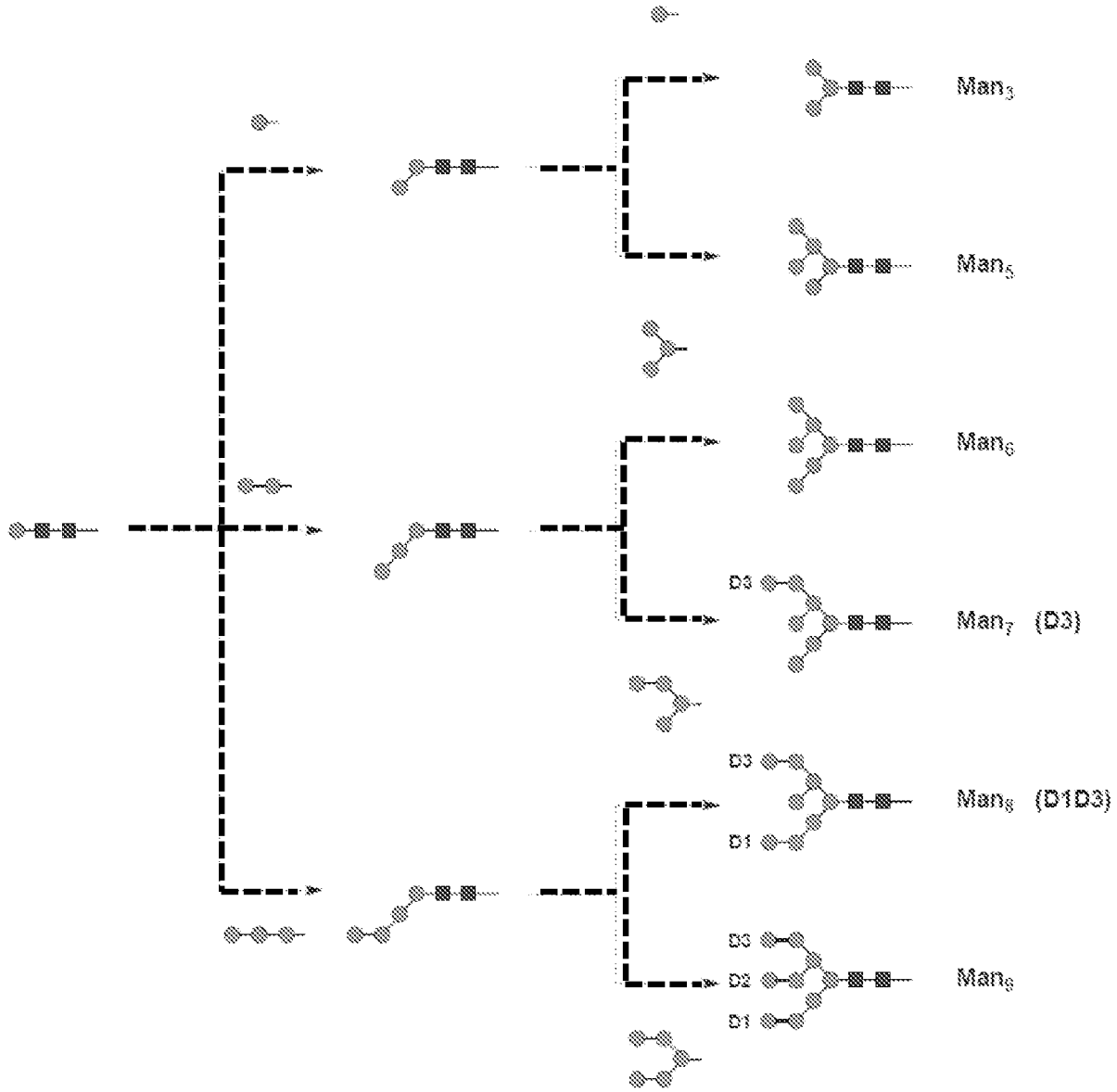


Figure 8

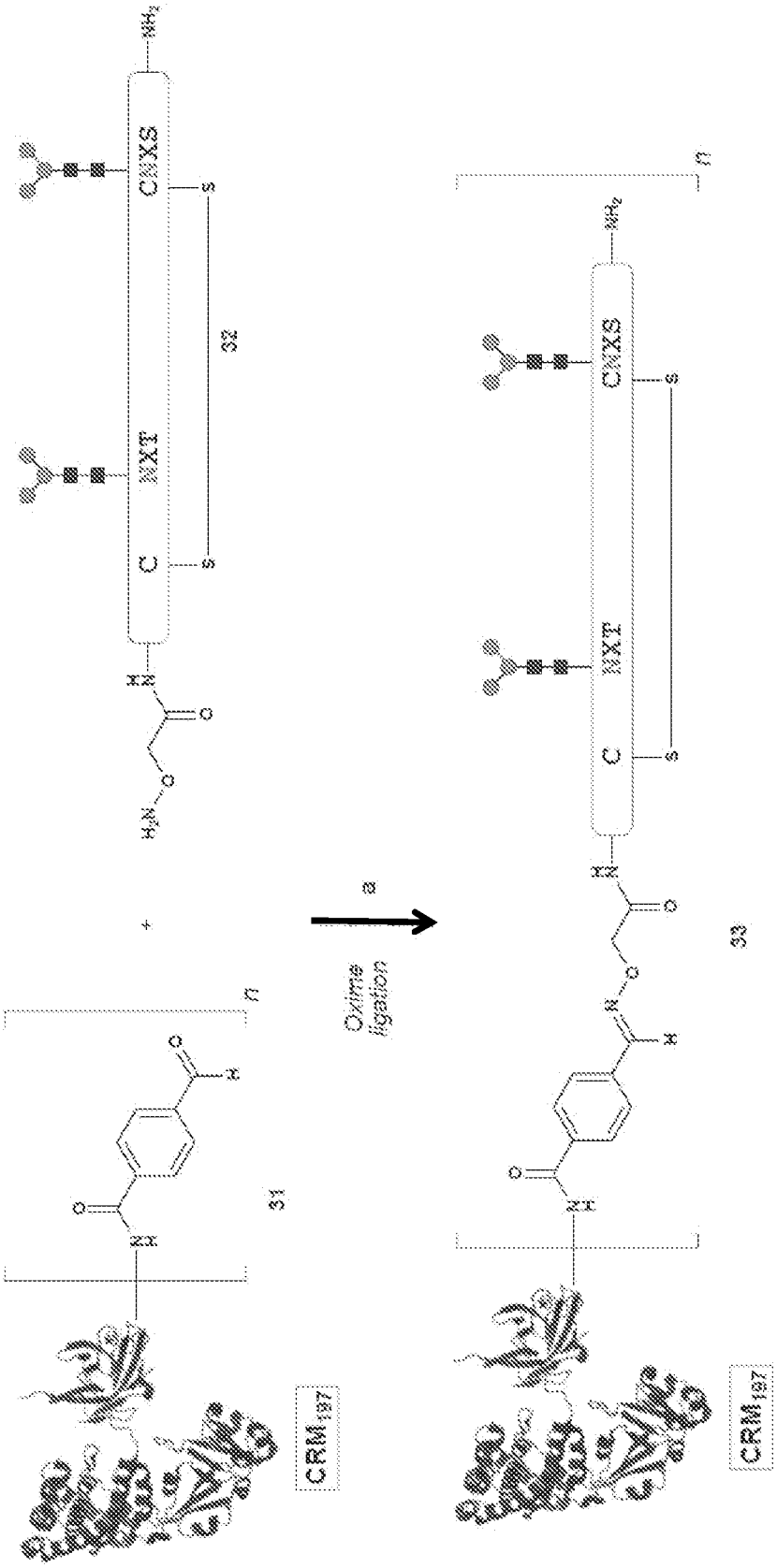
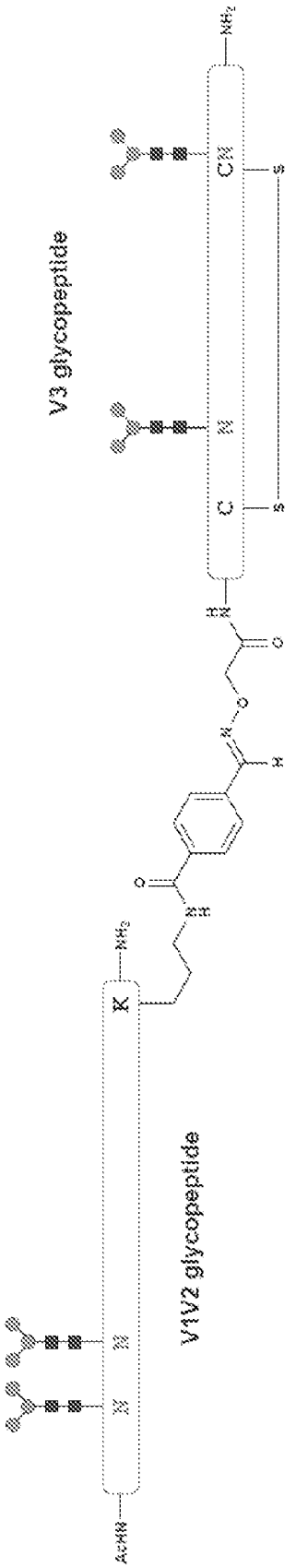


Figure 9



Potential application of oxime ligation to build up unimolecular multivalent HIV-1 vaccines.

Figure 10

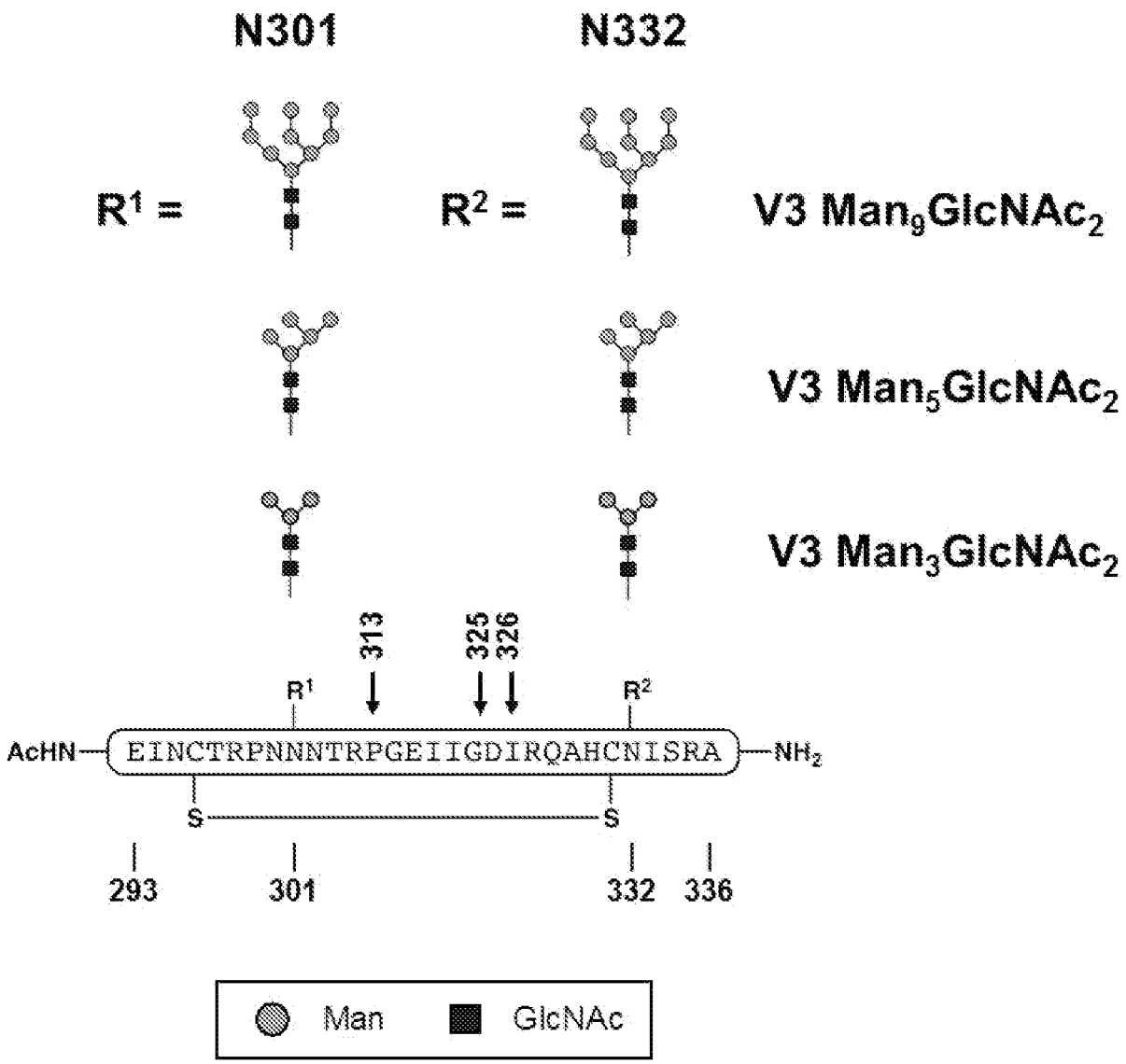
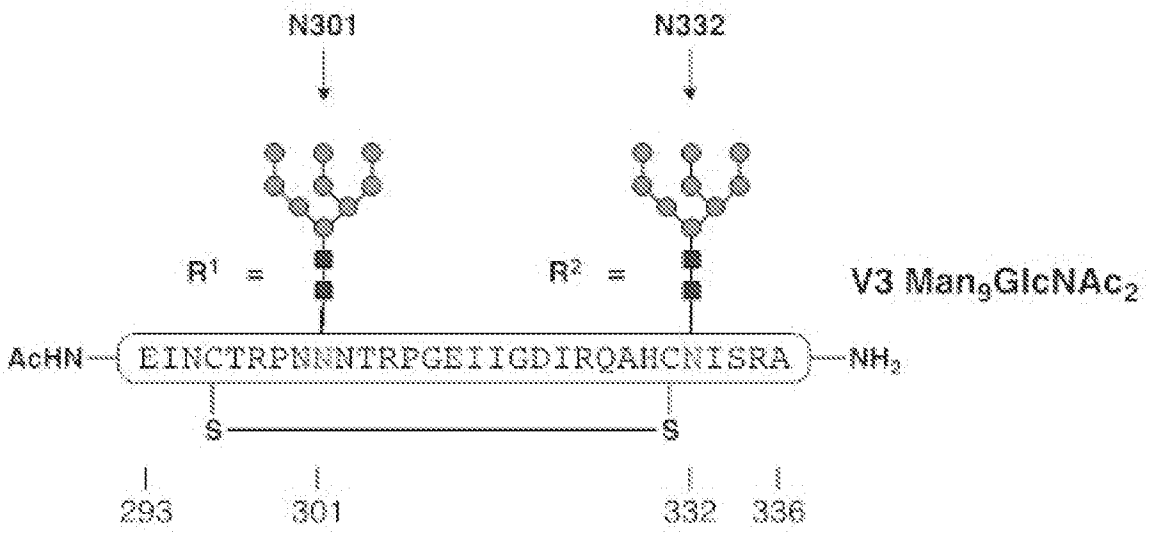

N301
N332
R1 =
R2 =
V3 Man9GlcNAc2
V3 Man5GlcNAc2
V3 Man3GlcNAc2
313
325
326
R1
R2
AcHN
EINCTRPNNNTRPGEIIGDIRQAHCNISRA
NH2
S
S
293
301
332
336
Man
GlcNAc

Figure 11


N301
N332
R1 =
R2 =
V3 Man9GlcNAc2
AcHN
EINCTRPNNNTRPGEIIGDIRQAHCNISRA
NH2
S
S
293
301
332
336

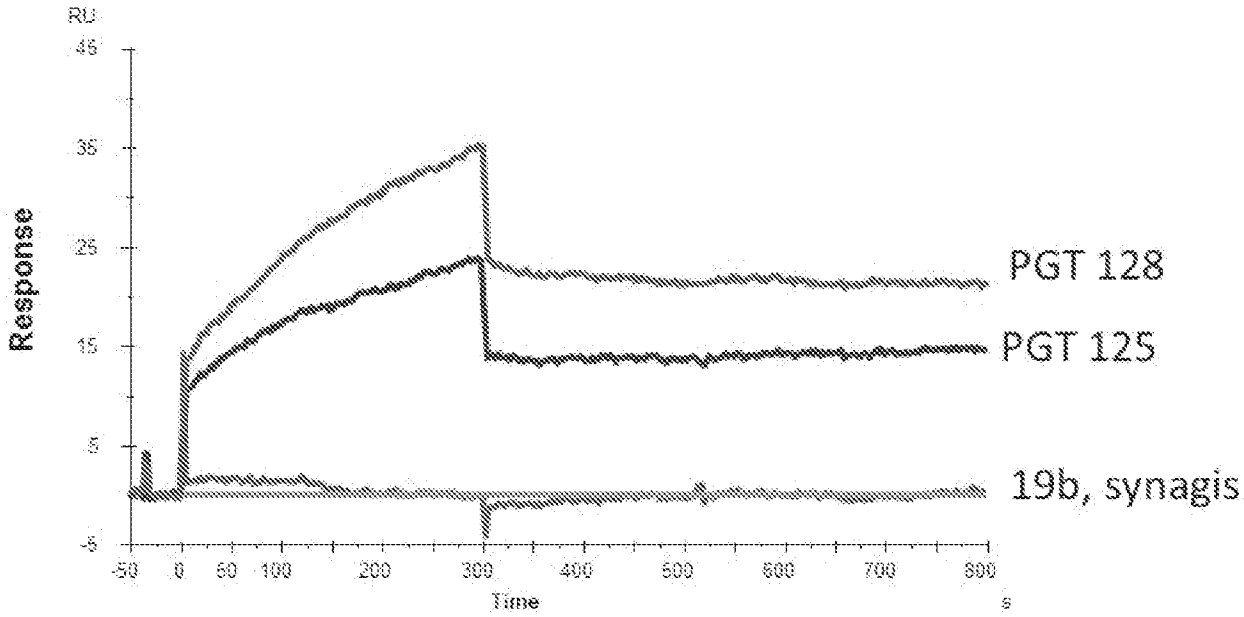

RU
45
35
25
15
5
-5
Response
-50
0
50
100
200
300
400
500
600
700
800
Time
s
PGT 128
PGT 125
19b, synagis

Figure 12

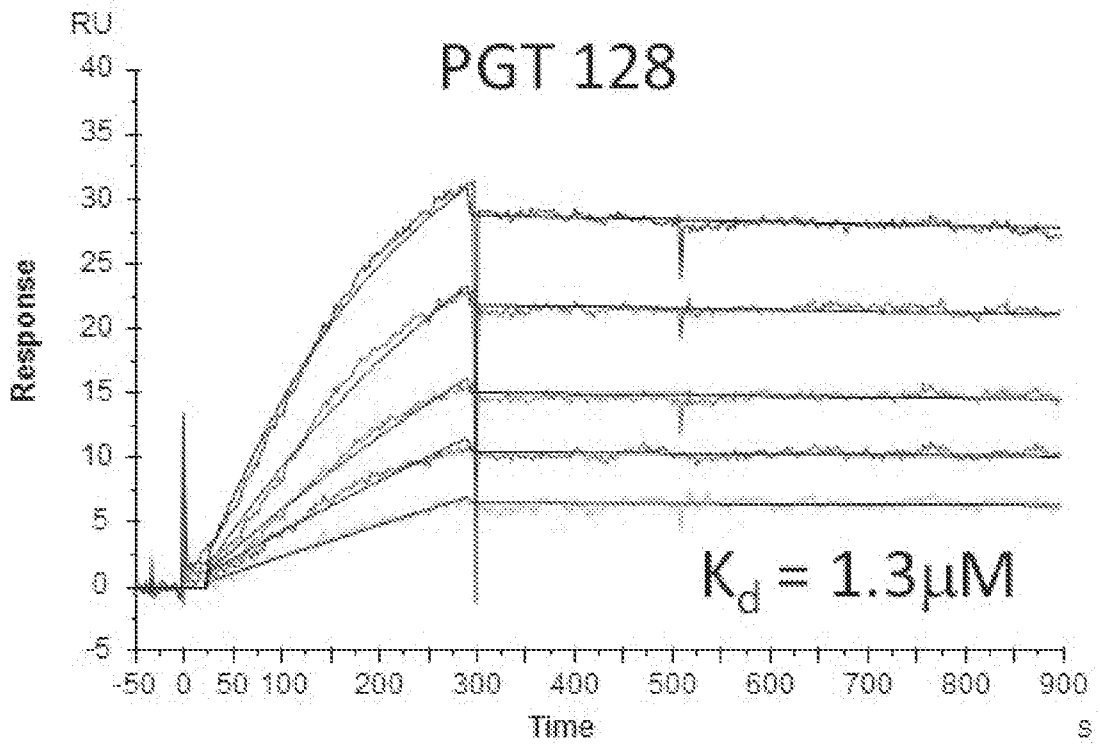


Figure 13A

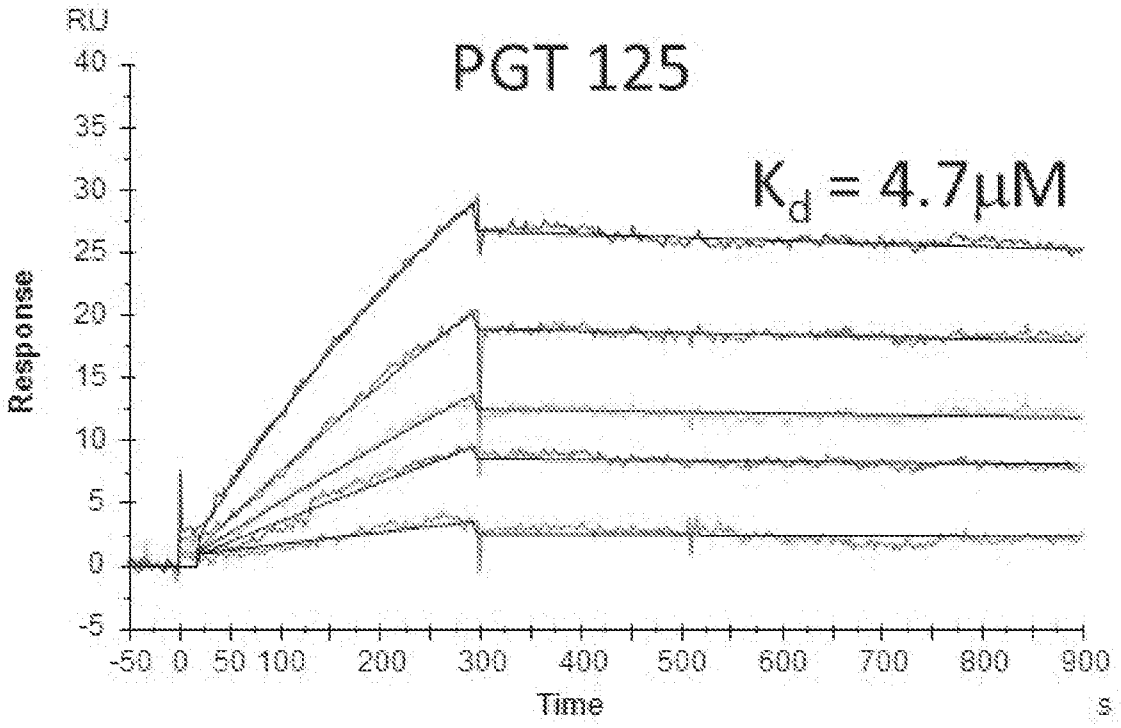


Figure 13B

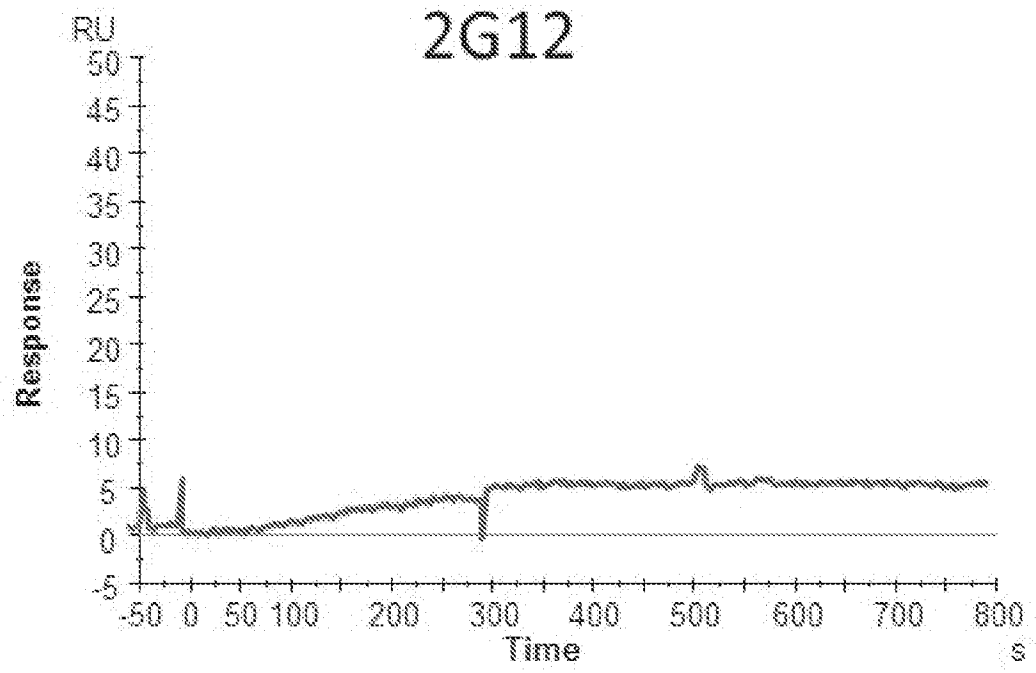


Figure 13C

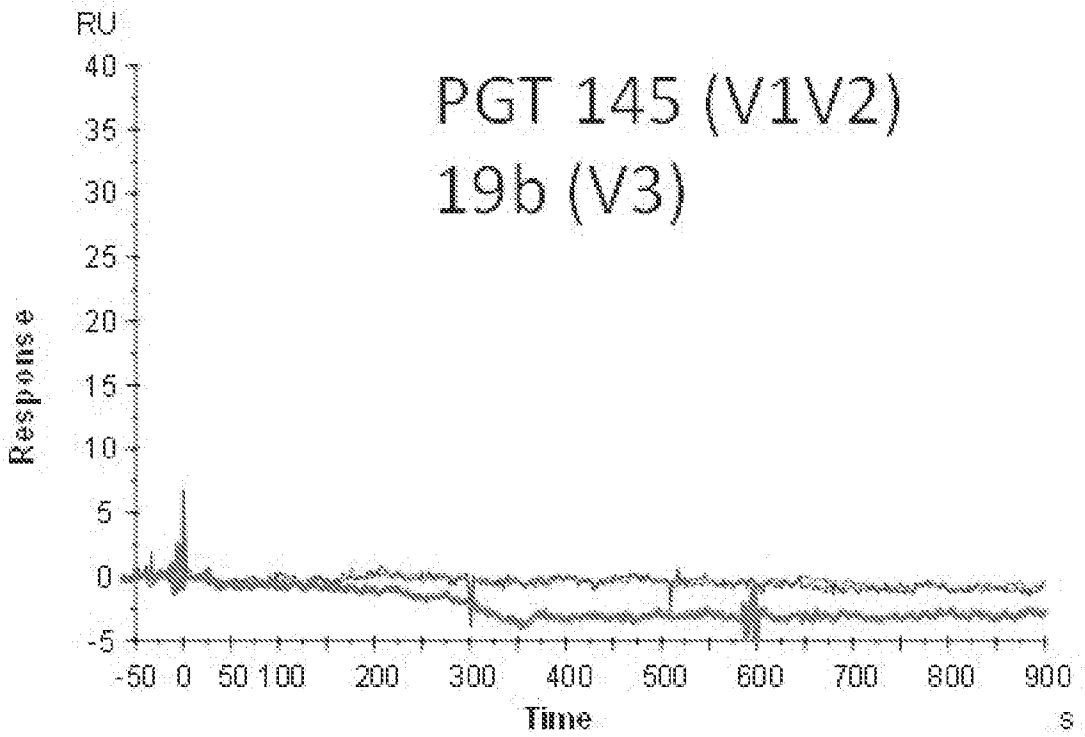


Figure 13D

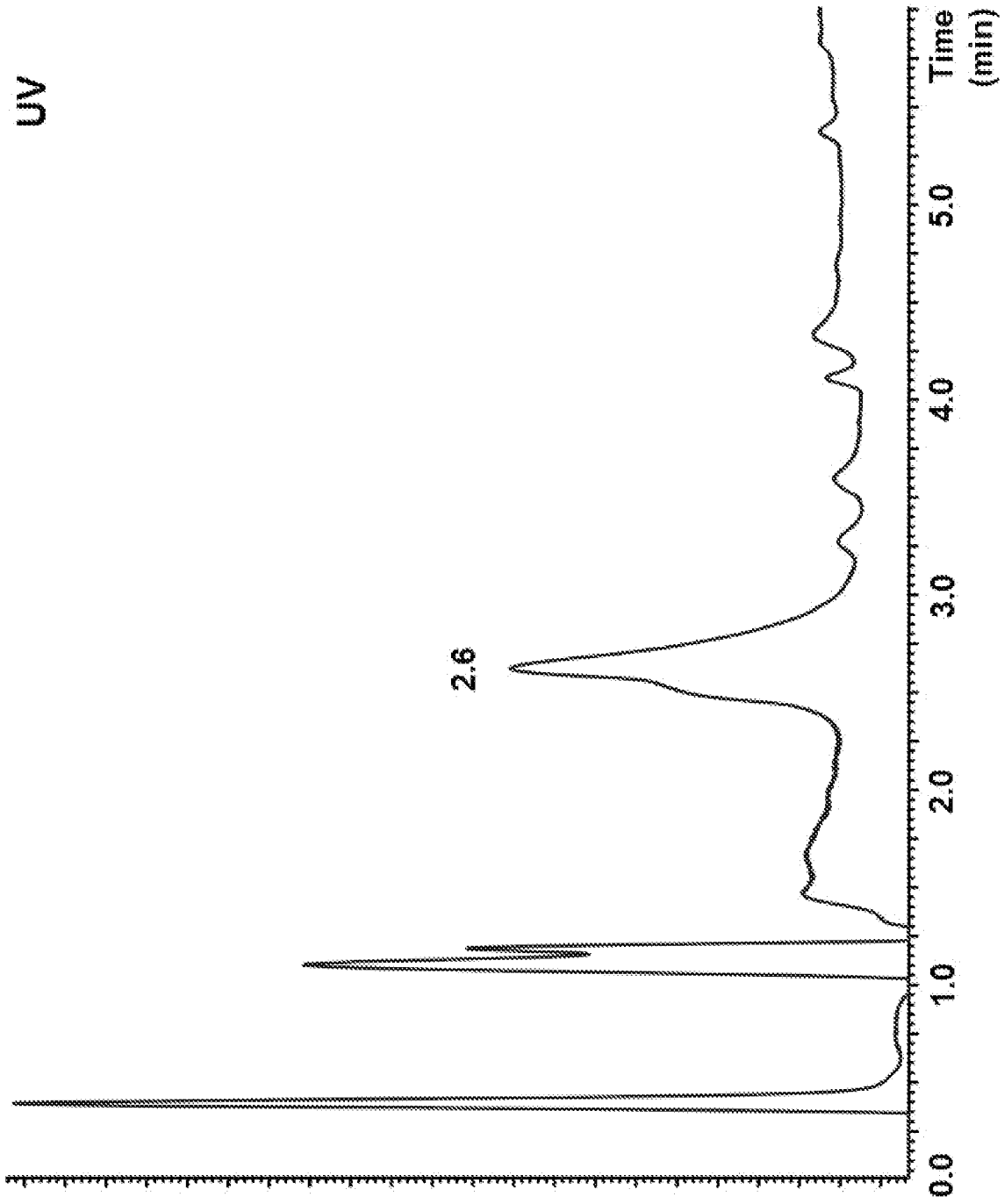


Figure 14A

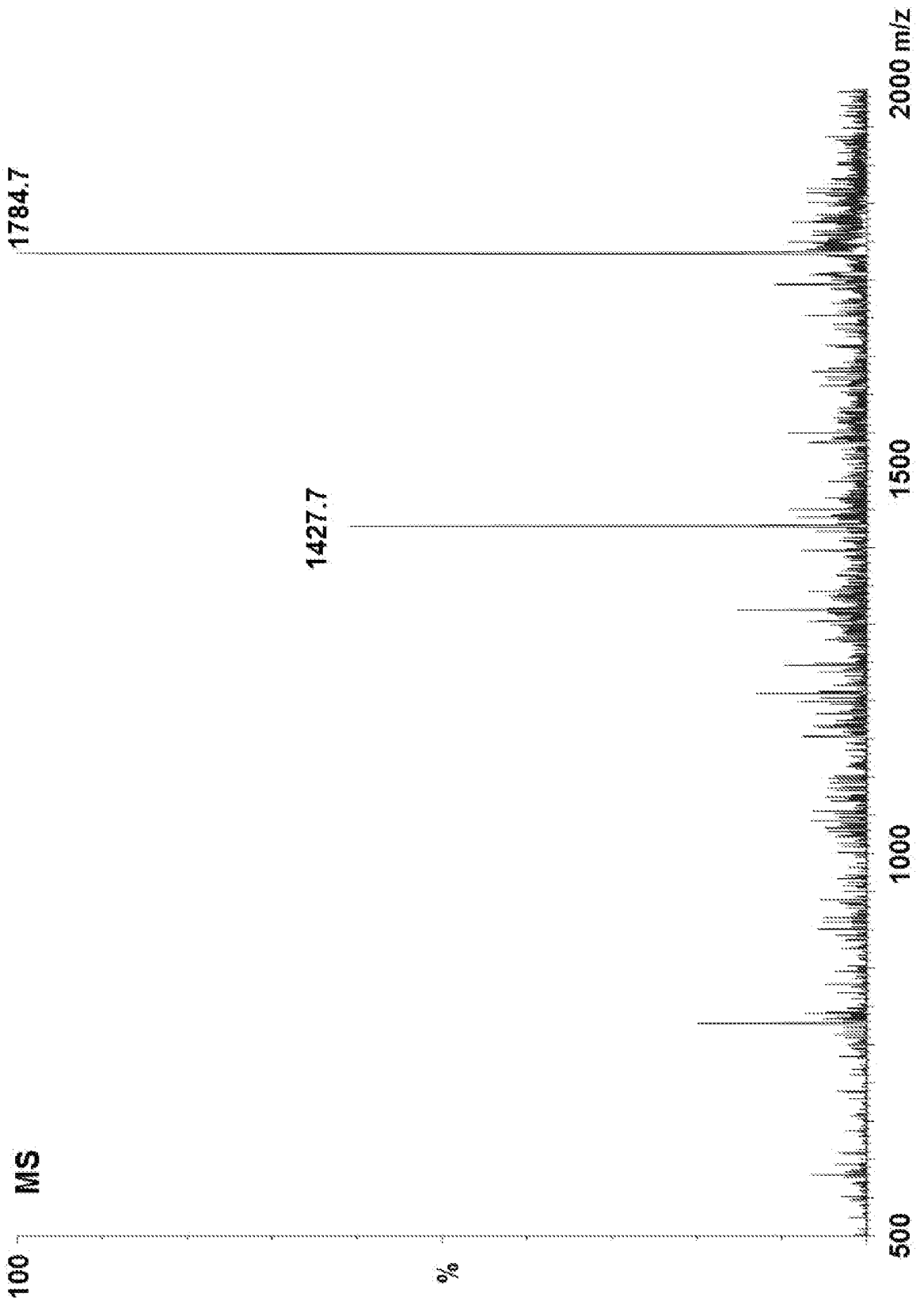


Figure 14B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/034189

A. CLASSIFICATION OF SUBJECT MATTER

C07K 19/00(2006.01)i, C07K 14/00(2006.01)i, G01N 33/53(2006.01)i, A61P 31/18(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K 19/00; C07K 14/00; G01N 33/53; A61P 31/18

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & Keywords: broad neutralizing antibody, Man9GlcNAc2 glycan, Asn301, Asn332

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOUQUET et al., 'Complex-type N-glycan recognition by potent broadly neutralizing HIV antibodies' Proceedings of the National Academy of Sciences, Vol.109, No.47, pp.E3268-E3277 (2012) See abstract and pages E3271 and E3275.	1-9,13-14
A		15
X	GENG et al., 'In pursuit of carbohydrate-based HIV vaccines, Part 2: The total synthesis of high-mannose-type gp120 fragments-Evaluation of strategies directed to maximal convergence' Angewandte Chemie, Vol.116, No.19, pp.2616-2619 (2004) See abstract and schemes 1-5.	15
A	NCBI, PDB accession no. 3TYG_A (10 October 2012) See the whole document.	1-9,13-15
A	PEJCHAL et al., 'A potent and broad neutralizing antibody recognizes and penetrates the HIV glycan shield' Science, Vol.334, No.6059, pp.1097-1103 (2011) See abstract and figures 3 and 4.	1-9,13-15

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents,such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 August 2014 (28.08.2014)	**02 September 2014 (02.09.2014)**

Name and mailing address of the ISA/KR
International Application Division
Korean Intellectual Property Office
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/034189

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 10-12
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 10-12 pertain to methods for treatment of the human body by therapy and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2014/034189

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DOORES et al., 'Envelope glycans of immunodeficiency virions are almost entirely oligomannose antigens' Proceedings of the National Academy of Sciences, Vol.107, No.31, pp.13800-13805 (2010) See abstract and figure 4.	1-9,13-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/US2014/034189

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
None			