Published in final edited form as:
J Am Chem Soc. 2013 September 4; 135(35): . doi:10.1021/ja405990z.

Chemical synthesis of highly congested gp120 V1V2 N-glycopeptide antigens for potential HIV-1–directed vaccines

Baptiste Aussedat[a,†], **Yusuf Vohra**[a,†], **Peter K. Park**[a], **Alberto Fernández-Tejada**[a], **S. Munir Alam**[c], **S. Moses Dennison**[c], **Frederick H. Jaeger**[c], **Kara Anasti**[c], **Shelley Stewart**[c], **Julie H. Blinn**[c], **Hua-Xin Liao**[c,d], **Joseph G. Sodroski**[f,g,h,i], **Barton F. Haynes**[c,d,e], and **Samuel J. Danishefsky**[a,b,*]

[a]Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10065, United States

[b]Department of Chemistry, Columbia University, 3000 Broadway, New York, New York 10027, United States

[c]Duke Human Vaccine Institute, Duke University Medical Center, Durham, North Carolina 27710, United States

[d]Department of Medicine, Duke University Medical Center, Durham, North Carolina 27710, United States

[e]Department of Immunology, Duke University Medical Center, Durham, North Carolina 27710, United States

[f]Department of Cancer Immunology and AIDS, Dana-Farber Cancer Institute, Boston, Massachusetts, 02215, United States

[g]Department of Microbiology and Immunobiology, Harvard Medical School, Boston, Massachusetts, 02215, United States

[h]Department of Immunology and Infectious Diseases, Harvard School of Public Health, Boston, Massachusetts, 02115, United States

[i]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard, Cambridge, Massachusetts, 02139, United States

Abstract

Critical to the search for an effective HIV-1 vaccine is the development of immunogens capable of inducing broadly neutralizing antibodies (BnAbs). A key first step in this process is to design immunogens that can be recognized by known BnAbs. The monoclonal antibody PG9 is a BnAb that neutralizes diverse strains of HIV-1 by targeting a conserved carbohydrate-protein epitope in the variable 1 and 2 (V1V2) region of the viral envelope. Important for recognition are two closely spaced *N*-glycans at Asn^{160} and Asn^{156}. Glycopeptides containing this synthetically challenging bis-*N*-glycosylated motif were prepared by convergent assembly, and were shown to be antigenic for PG9. Synthetic glycopeptides such as these may be useful for the development of HIV-1 vaccines based on the envelope V1V2 BnAb epitope.

[*]Corresponding Author: s-danishefsky@ski.mskcc.org.

[†]***Author Contributions***

B.A. and Y.V. contributed equally.

The authors declare no competing financial interest.

Supporting Information. Detailed experimental procedures, including spectroscopic and analytical data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

INTRODUCTION

Few important structures in nature are more heavily glycosylated than is the envelope spike (Env) of human immunodeficiency virus type 1 (HIV-1).[1] A multitude of designed constructs that might simulate the unique architecture of Env have been considered and pursued in the context of potential HIV-1–directed vaccines. Yet, until recently, the only template for immunological recognition of this dense "glycan shield" has been the broadly neutralizing antibody (BnAb) 2G12.[2] Following its discovery, many laboratories,[3] including our own,[4] were able to generate mimics of the oligomannose cluster that constitutes its epitope, in the hope of eliciting 2G12-like antibodies. Unfortunately, these efforts were not successful. While many factors have been cited to explain the general difficulties surrounding BnAb induction,[5] the case of 2G12 is likely complicated further by the unusual domain-exchanged arrangement of its heavy chains, which is thought to be responsible for its unique mode of glycan recognition.[2d]

In 2009, two new and potent BnAbs, PG9 and PG16, were isolated from an HIV-1–infected donor from sub-Saharan Africa.[6] These monoclonal antibodies (mAbs) were found to neutralize 70–80% of circulating HIV-1 isolates. Initial epitope mapping suggested that PG9 and PG16 were targeting a new glycan-dependent Env epitope, entirely distinct from that of 2G12. A sensitivity to quarternary structure was also noted, as these BnAbs exhibited a preference for binding fully assembled trimeric viral spike over monomeric Env. Subsequently, a co-crystal structure of PG9 with gp120 variable regions 1 and 2 (V1V2) grafted onto a mini-protein scaffold revealed that the antibody engages high mannose glycans at Asn^{160} and Asn^{156} and an adjacent β-strand (Figure 1A).[7] In contrast to 2G12, which apparently does not interact with the gp120 peptide backbone, PG9 binds an epitope that contains *both carbohydrate and peptide components*, while possessing a normal heavy chain arrangement.

In light of our continuing involvement in the synthesis of glycoproteins and complex glycopolypeptide motifs,[8] we regarded these new structural observations with particular interest. It seemed not unlikely that successful design of vaccines based on the PG9 epitope would depend crucially on close simulation of the detailed surface glycopeptide architecture of Env. Given the importance of the glycan domains in forming this conserved epitope, it seemed that access to Env constructs that are well-defined and homogeneous with respect to glycosylation state would greatly facilitate immunogen development efforts. Past work has largely relied on recombinant Env preparations, supplied as mixtures of glycoforms.[9] This serious heterogeneity complicates efforts to draw precise correlations between glycan composition and immunoactivity. Absent a detailed understanding of the structural biology of the problem, informed vaccine design is, naturally, much more difficult. We were stirred by the prospect that *de novo* chemical synthesis could provide the complex, yet homogeneous probe substrates needed for rationally based advances in this urgent endeavor.

More specifically, we hypothesized that fully synthetic, homogeneous gp120 V1V2 polypeptide domains, bearing defined glycosyl patterns, might be able to function as minimal mimics of the PG9 epitope. If such uniform, synthetically-derived constructs were able to simulate the conformation of the pertinent native envelope glycoproteins, they would provide a logical starting point for immunogen design. Moreover, a minimal construct could, in theory, present the desired BnAb epitope without interference from other potentially more immunogenic Env determinants.[5]

Herein, we describe the chemical synthesis of gp120 V1V2 glycopeptides as *single glycoforms*[10] that were found to bind the BnAb PG9 with surprisingly high affinities. During the course of this work, we had to deal with and overcome the fundamental synthetic

challenge arising from the close spacing of large glycans along the peptide backbone. In engaging this challenge, we would be pressing against the limits of the prior art we had developed in the realm of glycopeptide ligations.[11]

RESULTS AND DISCUSSION

Design and Strategy

The structure of the gp120 V1V2 domain in the context of a bound PG9 mAb Fab consisted of four anti-parallel β-strands (A–D) that folded into what is known as a Greek key motif (Figure 1B).[7] Based on these x-ray crystallographic data, PG9 makes contacts with the C β-strand, and with the $Man_5GlcNAc_2$ glycans[12] (Figure 1C) at Asn^{160} and Asn^{156}, which reside on strand B. Since most of the structural features recognized by PG9 appear to be localized on the B and C strands,[13] we reasoned that an epitope mimic should, at the very least, encompass this region. Our initial prototype is shown in Figure 1D. The 35-amino acid peptide corresponds to positions 148–184 of gp120 (HXB2 numbering) derived from the A244 sequence,[14] an Env variant that is known to bind PG9 in monomeric form (i.e., without requiring trimerization).[15] With regard to the glycan structure, $Man_5GlcNAc_2$ was thought to be the best candidate on the basis of prior studies involving perturbations of glycan processing.[16,17] The primary target that emerged from this analysis was glycopeptide **1** with $Man_5GlcNAc_2$ units installed at the two glycosylation sites, Asn^{160} and Asn^{156}; we also planned to gain access to simpler glycoforms **2** and **3** bearing $Man_3GlcNAc_2$ and chitobiose ($GlcNAc_2$), respectively.[18] These could be used to probe the importance of the outer mannose residues for recognition.

We term the general synthetic approach that our laboratory has applied to complex glycoprotein targets as convergent assembly.[11] In our usual *modus operandi*, *N*-linked sugars are installed via aspartylation of unprotected glycosyl amines, drawing from the precedent of Lansbury,[19] which we[20] and others[21] have extended in substantive ways. As we examined goal structures **1–3** in particular, we noted that the close spacing of the two glycans, especially with larger oligosaccharides, could present a difficult challenge for their incorporation. We anticipated that application of our methods in this demanding context would afford valuable teachings regarding the synthesis of the required clustered *N*-glycan motifs. As for the sugars themselves, $Man_3GlcNAc_2$ constitutes the common pentasaccharide core of all *N*-glycans; it has been synthesized previously by our laboratory and others.[22] By contrast, $Man_5GlcNAc_2$ seems to have received less attention as a synthetic target.[23,24] We start by describing our route to the desired glycans.

Synthesis of $Man_5GlcNAc_2$ and $Man_3GlcNAc_2$ glycans

Our studies commenced with experiments directed to the synthesis of the $Man_5GlcNAc_2$ heptasaccharide. The β-mannosyl linkage of the core trisaccharide **6** was constructed as we have in prior contexts[25] by uniting Crich donor **4**[26,27] with chitobiose acceptor **5**[25b] (Scheme 1). Fortunately, the minor quantities of undesired *a*-isomer formed (<10%) could be separated by careful chromatography to afford trisaccharide **6** as a single diastereomer in 86% yield. The PMB group was removed in 83% yield. Coupling of the resulting acceptor **7** with thioglycoside donor **8** was accomplished under NIS/TMSOTf activation conditions, yielding tetrasaccharide **9**. Cleavage of the benzylidene acetal with aqueous acetic acid afforded diol **10** in 63% overall yield from **7**.

Assembly of the heptasaccharide at first in protected form was accomplished convergently by selective mannosylation at C-6 of **10** with branched donor **15**. Synthesis of the requisite trisaccharide **15** was achieved by elaboration of mannosyl building block **11** (Scheme 2). Reductive ring opening was accomplished selectively with borane-THF complex in the

presence of copper triflate in 96% yield.[28] Cleavage of the PMB group afforded the 3,6-diol, **13**, which underwent double mannosylation with imidate donor **14** to furnish the bis-*a*-mannosylated trisaccharide **15** in 75% yield.

With the stage set for the key coupling, **15** was activated (NIS/TMSOTf) and joined with **10**, thus providing the fully elaborated protected heptasaccharide **16** in 64% yield (Scheme 3). A four-step sequence involving ester saponification, phthalimide cleavage, *N*-acetylation, and hydrogenolysis proceeded smoothly to give fully deprotected heptasaccharide **17** as a mixture of anomeric alcohols in 77% yield. This compound underwent apparently quantitative conversion to the *β*-anomeric amine **18** under Kochetkov amination conditions.[29]

The pentasaccharide, $Man_3GlcNAc_2$, was obtained from tetrasaccharide intermediate **10** by selectively coupling donor **8** to the C-6 hydroxyl group (Scheme 4). Although this reaction was complicated by a small amount of bis-glycosylation, the protected $Man_3GlcNAc_2$ unit was isolated in 94% yield. Subjection of this material to the 4-step global deprotection protocol described above resulted in a 74% overall yield of fully deprotected pentasaccharide **19** as a mixture of anomers. The *β*-anomeric amine **20** was subsequently generated by application of the Kochetkov conditions.

Convergent assembly of V1V2 glycopeptides

The most risky phase of the effort involved the assembly of the peptide domain of the targeted glycopeptide constructs, and their coupling to different oligosaccharides. Two basic strategies were considered. Our first thoughts envisioned installing both glycans simultaneously on the full-length peptide, bearing in mind our prior successes with two- and three-fold aspartylations on cyclic scaffolds.[4d] Pilot experiments using chitobiose as a model glycan, however, yielded only unmanageable mixtures of mono- and bis-glycosylated forms, presumably due to the steric demands imposed by the close proximity of aspartylation sites. Anticipating that driving the reaction to completion with larger oligosaccharides might require a substantial excess of precious glycosyl amine, we decided to pursue an alternative approach involving the ligation of two pre-built glycopeptide fragments. Here, the presence of Cys^{157} served to raise the possibility of native chemical ligation (NCL).[30]

We anticipated that this approach would not be without its own complications, given the close positioning of the glycans. Indeed, one of the coupling partners (Ile^{148}–Asn^{156}) must carry the sterically demanding oligosaccharide on its *C*-terminal thioester-bearing amino acid. Nevertheless, this scheme was successfully reduced to practice, as described below.

In the event, *N*-terminal fragment, peptide thioester **21**, was obtained by Fmoc solid phase peptide synthesis (SPPS) and post-resin *C*-terminal functionalization procedures[31] used by our laboratory in the context of other glycopeptide endeavors (Scheme 5).[32] Using our recently reported one-flask aspartylation/deprotection protocol,[20] the free carboxylic acid side chain at position 156 was joined to the $Man_5GlcNAc_2$ glycosyl amine **18**, followed by TFA treatment to provide glycopeptide thioester **22** in 44% yield after purification by reversed-phase HPLC. The formation of a side product of identical mass was observed in small quantities (5–10%), presumably due to base-induced epimerization of the thioester during the aspartylation. Fortunately, it could be easily separated during the purification.

For the *C*-terminal fragment, a similar one-flask sequence was used to convert protected peptide **23** to deprotected glycopeptide **24** in 22% yield. As has been previously observed, emplacement of a pseudoproline motif at Thr^{162} ($n + 2$ relative to Asp^{160}) was helpful in suppressing undesired aspartimide formation during the aspartylation.[20,21] The isolated yield for this fragment was eroded by factors that complicated the final purification of

glycopeptide **24**, including near overlap of the unglycosylated peptide, and the persistence of capped truncation products that had formed during the course of the SPPS (by an as yet undefined mechanism). Despite these obstacles, sufficient quantities of fragments of **22** and **24** could be synthesized and joined by NCL to afford the fully elaborated glycopeptide **1** bearing $Man_5GlcNAc_2$ units at Asn^{160} and Asn^{156} in 55% yield. The simpler glycoforms **2** and **3**, possessing two $Man_3GlcNAc_2$ and two chitobiose glycans, respectively, were prepared by an analogous route (see Supporting Information for details).

In all cases, the final ligation proved to be difficult. Indeed, three equivalents of thioester were required for the reaction to progress to completion.[33] Careful control of the reaction pH was needed to avoid apparent epimerization or excessive formation of succinimide (via cyclization of the asparagine side chain nitrogen onto the thioester). While certainly less than optimal, these ligations represent, to the best of our knowledge, the first examples of NCL with peptide thioesters carrying an *N*-glycan directly at the *C*-terminus. Furthermore, no other syntheses have been reported of linear glycopeptides bearing such closely spaced *N*-glycans, i.e., separated by three amino acids or less.[34] While the yields for the overall sequence are likely to benefit from optimization,[35] our concerns at first were focused more on the purity of the synthetic constructs rather than on maximizing material throughput. Fortunately, the synthesis, even in its present form, has produced sufficient quantities to initiate the biological studies now underway both *in vitro* and *in vivo* to chart a path forward to a clinically evaluable HIV-1 vaccine (*vide infra*).

Antigenicity studies

To assess the extent to which our synthetic V1V2 glycopeptides are able to recapitulate the mAb PG9 V1V2 BnAb epitope, we studied the binding of constructs **1**–**3** to PG9 by surface plasmon resonance (SPR) analysis (Figure 2). PG9 was captured by surface-immobilized anti-human Ig Fc, and the V1V2 glycopeptide constructs were injected as analytes on BIAcore 3000 instruments as described previously.[36] We found that the $Man_5GlcNAc_2$ V1V2 (**1**) and $Man_3GlcNAc_2$ V1V2 (**2**) glycopeptides both exhibited significant affinity for mAb PG9 (Figures 2A and 2B), with K_d's of 311 and 119 nM, respectively (obtained by using a global fit of multiple titrations to a 1:1 Langmuir model). By contrast, the chitobiose-bearing construct **3** did not bind mAb PG9 (Figure 2C), suggesting that the presence of *a*-linked mannose residues on the glycans is important for recognition. Furthermore, binding by the unglycosylated V1V2 peptide (i.e., "aglycone") (Figure 2D) or the solitary protein-free $Man_5GlcNAc_2$ and $Man_3GlcNAc_2$ oligosaccharides was not detected (Figure 2E and F). Mixtures of "aglycone" and glycan similarly failed to show measurable binding (not shown).

Taken together, these data demonstrate that PG9 recognition of our V1V2 constructs is critically dependent on both the peptide and carbohydrate domains. Covalent linkage between them is essential, since the apparent affinities for each individual component in isolation are very low. Indeed, NMR studies have shown that the K_d for binding of PG9 to $Man_5GlcNAc_2$–Asn alone is ~1–2 mM,[6] consistent with the general trend that individual protein-carbohydrate interactions tend to be weak. The overall high "avidity" observed may be attributed to the synergies afforded by multivalency, wherein the binding to PG9 is enhanced by multiple simultaneous interactions with the C β-strand, and the Asn^{160} and Asn^{156} glycans.[7] Conformational effects may also play a role, as glycosylation of the peptide backbone could have a favorable orienting influence on the involved peptide and/or sugar residues.[37] Evidence of such "cross-talk" between peptido and glyco domains has been observed by our laboratory in other settings.[38]

In light of these findings, it seems likely that proper evaluation of the optimal glycans and peptide sequences for mimicking the V1V2 BnAb epitope—and other similar glycopeptide

antigens—will require their presentation in their native *N*-linked context (or as some close isostere). Adopting this approach enabled us to make the unexpected discovery that the $Man_3GlcNAc_2$-based construct **2** binds PG9 just as well, and perhaps even a little better, than construct **1** (bearing $Man_5GlcNAc_2$).[39] Studies are underway to better understand the robust recognition of this non-canonical[40] glycan by PG9. In the meantime, we note that such fine structure preferences were not detected by previous approaches, such as glycan array analysis, that interrogated PG9 binding to isolated carbohydrates in the absence of a peptide backbone.[41]

More profound, perhaps, is the overall question of how the modestly sized glycopeptides **1** and **2** are able to simulate the antigenicity of native envelope glycoproteins so well. PG9 and other BnAbs that target the same V1V2 epitope (e.g., PG16 and CH01 to CH04[15]) are thought to be sensitive to quarternary structure, binding Env trimers better than monomeric Env.[6,13] Indeed, relatively few Env sequences are known to bind PG9 in monomeric form, so it is noteworthy that comparatively small (6–7 kDa), linear 35-mer Env glycopeptide fragments like **1** and **2** are able to bind PG9 with respectable affinities, with K_d's on the order of 10^{-7} M.[42] Such affinities compare favorably with the published K_d's of ~5×10^{-8} M for the full-length A244 Env monomer (from which **1** and **2** are derived),[15] and ~10^{-8} M for a recently reported trimeric Env construct.[13] We are actively investigating the nature of the binding interaction with PG9; preliminary results suggest that it cannot be fully explained by a simple "induced-fit" mode of association.

CONCLUSION

In summary, we have designed and chemically synthesized homogeneous gp120 V1V2 domain glycoforms that demonstrate robust antigenicity (K_d ~10^{-7}) for the HIV-1 gp120 V1V2 BnAb PG9. Key to these initial successes were significant achievements at the level of chemistry, which include the development of a scalable synthetic route to the $Man_5GlcNAc_2$ heptasaccharide **17**, and the execution of, arguably, some of the most ambitious glycopeptide ligations known to date. As a whole, this work represents a promising first step toward the development of experimental vaccine immunogens to be tested for the capacity to elicit gp120 V1V2 BnAb epitope-targeted antibodies. Further work is underway to characterize the antigenic properties of **1** and **2** in detail, and evaluate their immunogenicity in animal models. Similar and perhaps even more ambitious chemical synthesis strategies may be of use in preparing homogeneous glycopeptides for other HIV-1 gp120 BnAb epitopes.[43] This is an ongoing program whose results will be disclosed in due course.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by NIH grants for the Center for HIV/AIDS Vaccine Immunology, AI 067854 and the Duke Center for HIV/AIDS Vaccine Immunology-Immunogen Discovery, AI 100645. P.K.P. was supported by a postdoctoral fellowship (PF-11-014-01-CDD) from the American Cancer Society. A.F.-T. thanks the European Commission (Marie Curie International Outgoing Fellowship) for financial support. The authors thank Dr. George Sukenick, Ms. Hui Fang, and Ms. Sylvi Rusli of the NMR Analytical Core Facility at MSKCC for MS and NMR assistance. We also thank Dr. Ping Wang, Dr. Xiangyang Wu, Dr. Maciej Walczak, Dr. Gardner Creech, Dr. Steven Townsend, and Ms. Rebecca Wilson for advice and helpful discussions. We are grateful to Dr. Wayne Koff (International AIDS Vaccine Initiative, New York, NY) and Dr. Dennis Burton (The Scripps Research Institute, La Jolla, CA) for provision of mAb PG9.

References

1. Myers G, Lenroot R. AIDS Res Hum Retroviruses. 1992; 8:1459–1460. [PubMed: 1466982]
2. (a) Trkola A, Purtscher M, Muster T, Ballaun C, Buchacher A, Sullivan N, Srinivasan K, Sodroski J, Moore JP, Katinger H. J Virology. 1996; 70:1100–1108. [PubMed: 8551569] (b) Sanders RW, Venturi M, Schiffner L, Kalyanaraman R, Katinger H, Lloyd KO, Kwong PD, Moore JP. J Virol. 2002; 76:7293–7305. [PubMed: 12072528] (c) Scanlan CN, Pantophlet R, Wormald MR, Ollmann Saphire E, Stanfield R, Wilson IA, Katinger H, Dwek RA, Rudd PM, Burton DR. J Virology. 2002; 76:7306–7321. [PubMed: 12072529] (d) Calarese DA, Scanlan CN, Zwick MB, Deechongkit S, Mimura Y, Kunert R, Zhu P, Wormald MR, Stanfield RL, Roux KH, Kelly JW, Rudd PM, Dwek RA, Katinger H, Burton DR, Wilson IA. Science. 2003; 300:2065–2071. [PubMed: 12829775]
3. For recent reviews, see: Morelli L, Poletti L, Lay L. Eur J Org Chem. 2011; 2011:5723–5777.Wang L-X. Klyosov AA. Carbohydrate-Based Vaccines Against HIV/AIDS. Glycobiology and Drug Design. American Chemical SocietyWashington, DC2012:157–186.ACS Symposium Series 1102
4. (a) Mandal M, Dudkin VY, Geng X, Danishefsky SJ. Angew Chem Int Ed. 2004; 43:2557–2561.(b) Geng X, Dudkin VY, Mandal M, Danishefsky SJ. Angew Chem Int Ed. 2004; 43:2562–2565.(c) Dudkin VY, Orlova M, Geng X, Mandal M, Olson WC, Danishefsky SJ. J Am Chem Soc. 2004; 126:9560–9562. [PubMed: 15291558] (d) Krauss IJ, Joyce JG, Finnefrock AC, Song HC, Dudkin VY, Geng X, Warren JD, Chastain M, Shiver JW, Danishefsky SJ. J Am Chem Soc. 2007; 129:11042–11044. [PubMed: 17711286] (e) Joyce JG, Krauss IJ, Song HC, Opalka DW, Grimm KM, Nahas DD, Esser MT, Hrin R, Feng M, Dudkin VY, Chastain M, Shiver JW, Danishefsky SJ. Proc Natl Acad Sci U S A. 2008; 105:15684–15689. [PubMed: 18838688]
5. Haynes BF, Kelsoe G, Harrison SC, Kepler TB. Nat Biotechnol. 2012; 30:423–433. [PubMed: 22565972]
6. Walker LM, Phogat SK, Chan-Hui P-Y, Wagner D, Phung P, Goss JL, Wrin T, Simek MD, Fling S, Mitcham JL, Lehrman JK, Priddy FH, Olsen OA, Frey SM, Hammond PW, Protocol G, Kaminsky S, Zamb T, Moyle M, Koff WC, Poignard P, Burton DR. Principal Investigators. Science. 2009; 326:285–289. [PubMed: 19729618]
7. McLellan JS, Pancera M, Carrico C, Gorman J, Julien J-P, Khayat R, Louder R, Pejchal R, Sastry M, Dai K, O/'Dell S, Patel N, Shahzad-ul-Hussan S, Yang Y, Zhang B, Zhou T, Zhu J, Boyington JC, Chuang G-Y, Diwanji D, Georgiev I, Do Kwon Y, Lee D, Louder MK, Moquin S, Schmidt SD, Yang Z-Y, Bonsignori M, Crump JA, Kapiga SH, Sam NE, Haynes BF, Burton DR, Koff WC, Walker LM, Phogat S, Wyatt R, Orwenyo J, Wang L-X, Arthos J, Bewley CA, Mascola JR, Nabel GJ, Schief WR, Ward AB, Wilson IA, Kwong PD. Nature. 2011; 480:336–343. [PubMed: 22113616]
8. For recent reviews, see: Yuan Y, Chen J, Wan Q, Wilson RM, Danishefsky SJ. Biopolymers. 2010; 94:373–384. [PubMed: 20593459] Wilson RM, Dong S, Wang P, Danishefsky SJ. Angew Chem Int Ed. 2013; 52:7646–7665.
9. A recent analysis of the bivalent gp120 subunit vaccine AIDSVAX B/E found 16 isoforms of the clade B immunogen and 24 isoforms of the clade E immunogen: Yu B, Morales JF, O'Rourke SM, Tatsuno GP, Berman PW. PLoS ONE. 2012; 7:e43903. [PubMed: 22928048]
10. During the preparation of this manuscript, an abstract was published by Wang referring to work on homogeneous gp120 V1V2 glycopeptides: Wang L-X. Abstracts of Papers, 245th National Meeting of the American Chemical SocietyNew OrleansApr 7–11, 2013American Chemical SocietyWashington, DC2013:CARB–13.The Wang constructs were prepared by chemoenzymatic assembly using semisynthesis-derived glycans, as detailed in a subsequent publication, which was disclosed while this manuscript was undergoing review: Amin MN, McLellan JS, Huang W, Orwenyo J, Burton DR, Koff WC, Kwong PD, Wang LX. Nat Chem Biol. 2013; 9:521–526. [PubMed: 23831758]
11. (a) Miller JS, Dudkin VY, Lyon GJ, Muir TW, Danishefsky SJ. Angew Chem Int Ed. 2003; 42:431–434.(b) Warren JD, Miller JS, Keding SJ, Danishefsky SJ. J Am Chem Soc. 2004; 126:6576–6578. [PubMed: 15161285]
12. The scaffolded V1V2 construct was expressed in HEK 293S GnTI$^{-/-}$ cells, which are unable to process the $Man_5GlcNAc_2$ intermediate into hybrid and complex-type glycans. The entire

$Man_5GlcNAc_2$ oligosaccharide is evident in the electron density map for the Asn^{160} glycan, but only four mannose residues are visible for the Asn^{156} glycan.

13. In the context of the Env trimer, PG9 may engage a third glycan on Asn^{160} of a neighboring protomer: Julien JP, Lee JH, Cupo A, Murin CD, Derking R, Hoffenberg S, Caulfield MJ, King CR, Marozsan AJ, Klasse PJ, Sanders RW, Moore JP, Wilson IA, Ward AB. Proc Natl Acad Sci U S A. 2013; 110:4351–4356. [PubMed: 23426631]
14. An A244 strain recombinant gp120 glycoprotein (as a mixture of glycoforms) was used as part of the vaccine regimen in the recent, modestly efficacious RV144 HIV-1 vaccination clinical trial: Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S, Kaewkungwal J, Chiu J, Paris R, Premsri N, Namwat C, De Souza M, Adams E, Benenson M, Gurunathan S, Tartaglia J, McNeil JG, Francis DP, Stablein D, Birx DL, Chunsuttiwat S, Khamboonruang C, Thongcharoen P, Robb ML, Michael NL, Kunasol P, Kim JH. N Engl J Med. 2009; 361:2209–2220. [PubMed: 19843557]
15. Bonsignori M, Hwang KK, Chen X, Tsao CY, Morris L, Gray E, Marshall DJ, Crump JA, Kapiga SH, Sam NE, Sinangil F, Pancera M, Yongping Y, Zhang B, Zhu J, Kwong PD, O'Dell S, Mascola JR, Wu L, Nabel GJ, Phogat S, Seaman MS, Whitesides JF, Moody MA, Kelsoe G, Yang X, Sodroski J, Shaw GM, Montefiori DC, Kepler TB, Tomaras GD, Alam SM, Liao HX, Haynes BF. J Virology. 2011; 85:9998–10009. [PubMed: 21795340]
16. Doores KJ, Burton DR. J Virology. 2010; 84:10510–10521. [PubMed: 20686044]
17. A recent report suggests that there may be greater promiscuity with regard to recognition of the glycan at Asn^{156} by PG16 and PG9: Pancera M, Shahzad-ul-Hussan S, Doria-Rose NA, McLellan JS, Bailer RT, Dai K, Loesgen S, Louder MK, Staupe RP, Yang Y, Zhang B, Parks R, Eudailey J, Lloyd KE, Blinn J, Alam SM, Haynes BF, Amin MN, Wang L-X, Burton DR, Koff WC, Nabel GJ, Mascola JR, Bewley CA, Kwong PD. Nat Struct Mol Biol. 2013; 20:804–813. [PubMed: 23708607]
18. All of our V1V2 glycopeptide constructs are chemically different from the glycopeptides prepared by Amin *et al.* (ref. 10b). Although one of their constructs carried $Man_5GlcNAc_2$ at Asn^{160} and Asn^{156}, we used an Env sequence that was derived from a different HIV-1 strain (A244) than the two variants used in their study (CAP45 and ZM109). Moreover, in our constructs, the peptide domains are longer (35 amino acids versus 24), and we retained the native A244 sequence without mutating any residues or adding any affinity tags. Amin *et al.* mutated Lys^{155} and Phe^{176} to Cys, allowing for cyclization of their constructs via disulfide bond formation, and they incorporated a biotin tag at the *N*-terminus.
19. (a) Anisfeld ST, Lansbury PT. J Org Chem. 1990; 55:5560–5562.(b) Cohen-Anisfeld ST, Lansbury PT. J Am Chem Soc. 1993; 115:10531–10537.
20. Wang P, Aussedat B, Vohra Y, Danishefsky SJ. Angew Chem Int Ed. 2012; 51:11571–11575.
21. Ullmann V, Rädisch M, Boos I, Freund J, Pöhner C, Schwarzinger S, Unverzagt C. Angew Chem Int Ed. 2012; 51:11566–11570.
22. For previous reports detailing the synthesis of the reducing pentasaccharide, see: Paulsen H, Lebuhn R. Carbohydr Res. 1984; 130:85–101.Nakahara Y, Shibayama S, Nakahara Y, Ogawa T. Carbohydr Res. 1996; 280:67–84. [PubMed: 8581896] Wang ZG, Zhang X, Live D, Danishefsky SJ. Angew Chem Int Ed. 2000; 39:3652–3656.Totani K, Matsuo I, Ito Y. Bioorg Med Chem Lett. 2004; 14:2285–2289. [PubMed: 15081026] Hagihara S, Totani K, Matsuo I, Ito Y. J Med Chem. 2005; 48:3126–3129. [PubMed: 15857118] Wang ZG, Warren JD, Dudkin VY, Zhang X, Iserloh U, Visser M, Eckhardt M, Seeberger PH, Danishefsky SJ. Tetrahedron. 2006; 62:4954–4978.
23. Indeed, we were only able to locate a single report dealing with its preparation, not as the reducing sugar, but suitably functionalized for the construction of carbohydrate microarrays: Serna S, Etxebarria J, Ruiz N, Martin-Lomas M, Reichardt NC. Chem Eur J. 2010; 16:13163–13175. [PubMed: 20878803]
24. Interestingly, Cohen-Anisfeld and Lansbury did demonstrate their method using $Man_5GlcNAc_2$, but it was obtained from the urine of sheep with swainsonine-induced *a*-mannosidosis (see ref. 19b).
25. (a) Wang P, Zhu J, Yuan Y, Danishefsky SJ. J Am Chem Soc. 2009; 131:16669–16671. [PubMed: 19886622] (b) Walczak MA, Danishefsky SJ. J Am Chem Soc. 2012; 134:16430–16433. [PubMed: 22954207] (c) Walczak MA, Hayashida J, Danishefsky SJ. J Am Chem Soc. 2013; 135:4700–4703. [PubMed: 23461434]

26. (a) Crich D, Sun S. J Org Chem. 1996; 61:4506–4507. [PubMed: 11667369] (b) Crich D, Sun S. J Org Chem. 1997; 62:1198–1199.(c) Crich D, Sun S. J Am Chem Soc. 1998; 120:435–436.(d) Crich D, Sun S. Tetrahedron. 1998; 54:8321–8348.
27. Crich D, Li H, Yao Q, Wink DJ, Sommer RD, Rheingold AL. J Am Chem Soc. 2001; 123:5826–5828. [PubMed: 11403627]
28. Shie C, Tzeng Z, Kulkarni SS, Uang B, Hsu C, Hung S. Angew Chem Int Ed. 2005; 44:1665–1668.
29. Likhosherstov LM, Novikova OS, Derevitskaja VA, Kochetkov NK. Carbohydr Res. 1986; 146:C1–C5.
30. Dawson PE, Muir TW, Clark-Lewis I, Kent SB. Science. 1994; 266:776–779. [PubMed: 7973629]
31. Kuroda H, Chen Y, Kimura T, Sakakibara S. Int J Pept Prot Res. 1992; 40:294–299.
32. Nagorny P, Fasching B, Li X, Chen G, Aussedat B, Danishefsky SJ. J Am Chem Soc. 2009; 131:5792–5799. [PubMed: 19341309]
33. One equivalent (combined) of MPAA-exchanged and unreacted ethyl thioester can be recovered after the reaction.
34. Closely spaced N-linked glycans on cyclic peptide scaffolds have been synthesized previously. For example, see: Sprengard U, Schudok M, Schmidt W, Kretzschmar G, Kunz H. Angew Chem Int Ed. 1996; 35:321–324.
35. Work is also in progress to evaluate alternative synthetic strategies that would yield a more “process-friendly” route.
36. Alam SM, McAdams M, Boren D, Rak M, Scearce RM, Gao F, Camacho ZT, Gewirth D, Kelsoe G, Chen P, Haynes BF. J Immunol. 2007; 178:4424–4435. [PubMed: 17372000]
37. The conformational consequences of N-glycosylation have been well studied. For reviews, see: Imperiali B, O’Connor SE. Curr Opin Chem Biol. 1999; 3:643–649. [PubMed: 10600722] Meyer B, Möller H. Wittmann V. Conformation of Glycopeptides and Glycoproteins. Glycopeptides and Glycoproteins: Synthesis, Structure, and Application. 267SpringerBerlin2007; :187–251.Topics in Current Chemistry
38. (a) Live DH, Kumar RA, Beebe X, Danishefsky SJ. Proc Natl Acad Sci U S A. 1996; 93:12759–12761. [PubMed: 8917491] (b) Live DH, Wang ZG, Iserloh U, Danishefsky SJ. Org Lett. 2001; 3:851–854. [PubMed: 11263898]
39. Similarly, using V1V2 glycopeptide isoforms bearing natively *N*-linked sugars, Amin *et al.* (ref. 10b) detected an astonishing preference of PG9 for a sialylated complex-type glycan at Asn^{156}.
40. $Man_3GlcNAc_2$, also known as “paucimannose,” is more characteristically associated with plant and invertebrate glycoproteins, although some have argued that it can be expressed in certain pathological states in mammals, such as inflammation or malignancy: Zipser B, Bello-DeOcampo D, Diestel S, Tai M-H, Schmitz B. J Carbohydr Chem. 2012; 31:504–518.
41. Others have remarked on the limitations of glycan array analysis in delineating BnAb anti-glycan specificities: “Protein-free glycan binding by anti-HIV antibodies is not always detectable; e.g., although PG9 recognizes a gp120-associated high-mannose glycan, no binding to protein-free glycans was detected in microarrays. Thus, although a positive result in a glycan microarray implies involvement of a particular glycan in an antibody epitope, a negative result does not rule out glycan recognition.” For the full discussion, see: Mouquet H, Scharf L, Euler Z, Liu Y, Eden C, Scheid JF, Halper-Stromberg A, Gnanapragasam PNP, Spencer DIR, Seaman MS, Schuitemaker H, Feizi T, Nussenzweig MC, Bjorkman PJ. Proc Natl Acad Sci U S A. 2012; 109:E3268–E3277. [PubMed: 23115339]
42. As a point of comparison, we note that Amin *et al.* (ref. 10b) prepared more than 25 V1V2 glycopeptides bearing various combinations of high mannose and complex-type glycans, and yet their highest affinity constructs bound PG9 Fab with K_d’s of $\sim 5 \times 10^{-6}$ M.
43. Walker LM, Huber M, Doores KJ, Falkowska E, Pejchal R, Julien J-P, Wang S-K, Ramos A, Chan-Hui P-Y, Moyle M, Mitcham JL, Hammond PW, Olsen OA, Phung P, Fling S, Wong C-H, Phogat S, Wrin T, Simek MD, Koff WC, Wilson IA, Burton DR, Poignard P. Principal Investigators PG. Nature. 2011; 477:466–470. [PubMed: 21849977]

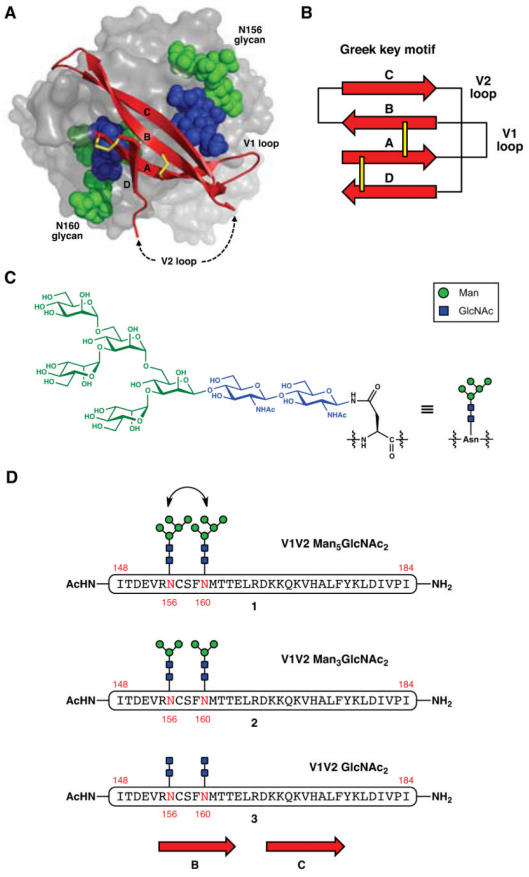


Figure 1.

Design of gp120 V1V2 domain broadly neutralizing epitope mimics. (A) Crystal structure of a scaffolded V1V2 domain from the CAP45 strain of HIV-1 (red ribbons) in complex with PG9 Fab (gray surface) (PDB ID 3U4E with scaffold hidden). The glycans at N160 and N156 are depicted with colored spheres representing atoms of the mannose (green) and *N*-acetylglucosamine (blue) residues. Disulfide bonds are shown as yellow sticks. Dashed arrows indicate where the disordered region of the V2 loop would be connected. Figure was created using PyMOL. (B) Schematic of the Greek key topology of the V1V2 domain. Strands are represented as arrows and disulfide bonds as yellow bars. (C) Chemical structure of $Man_5GlcNAc_2$–Asn. (D) Structures of candidate BnAb antigens, derived from residues 148–184 of the A244 strain gp120 (HXB2 numbering), encompassing the B and C β-strands (approximate location shown with red arrows) of the V1V2 domain, and bearing two *N*-linked $Man_5GlcNAc_2$, $Man_3GlcNAc_2$, or $GlcNAc_2$ oligosaccharides.

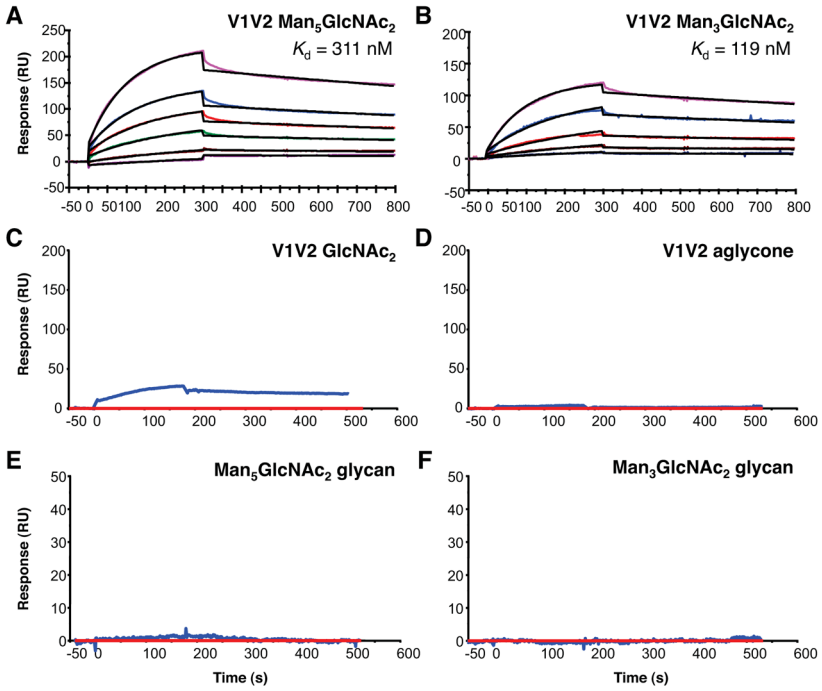
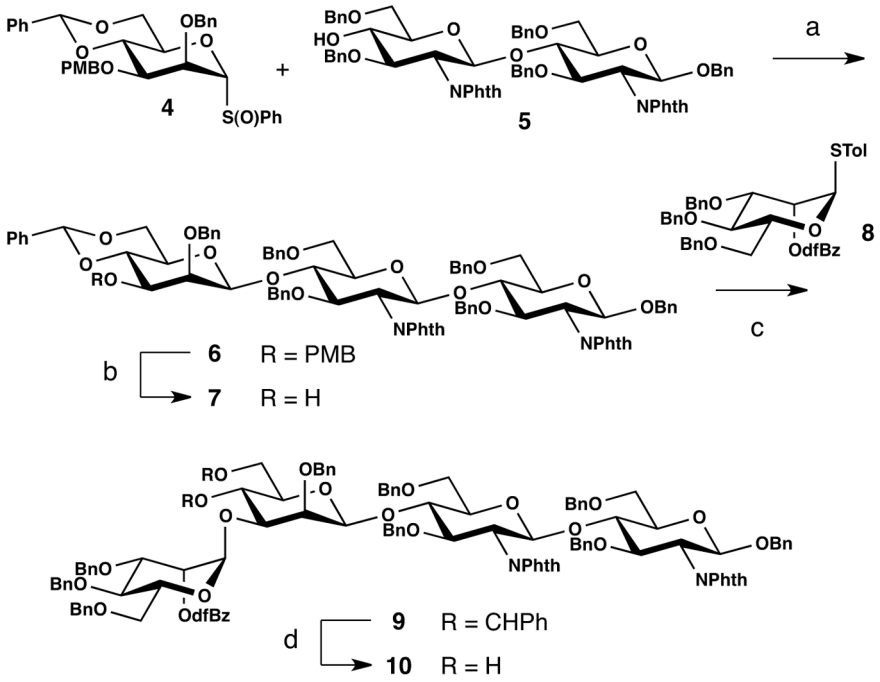


Figure 2.

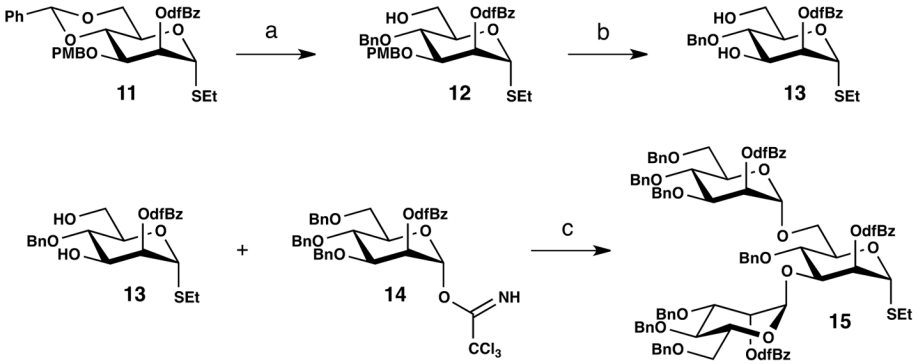
Binding of mAb PG9 to gp120 V1V2 glycopeptides. SPR sensorgrams showing binding of mAb PG9 to V1V2 glycopeptides derivatized with $Man_5GlcNAc_2$ (A) and $Man_3GlcNAc_2$ (B). V1V2 $Man_5GlcNAc_2$ binding curves are shown for glycopeptide concentrations at 5, 10, 20, 30 and 40 μg/mL and V1V2 $Man_3GlcNAc_2$ at 1, 2, 5, 10 and 20 μg/mL. Control SPR sensograms showing minimal to no binding of mAb PG9 to V1V2 $GlcNAc_2$ (C), V1V2 aglycone (D), $Man_5GlcNAc_2$ glycan alone (E), and $Man_3GlcNAc_2$ glycan alone (F). V1V2 $GlcNAc_2$ and aglycone peptides were injected at 200 μg/mL (C, D) and $Man_5GlcNAc_2$ and $Man_3GlcNAc_2$ glycans at 25 μg/mL (E, F) over PG9 captured on anti-human IgG (Fc-specific) surfaces. SPR data were derived following subtraction of non-specific signal on a control anti-RSV mAb (Synagis, red curve in C–F).



Scheme 1.

Synthesis of tetrasaccharide **10**.[a]

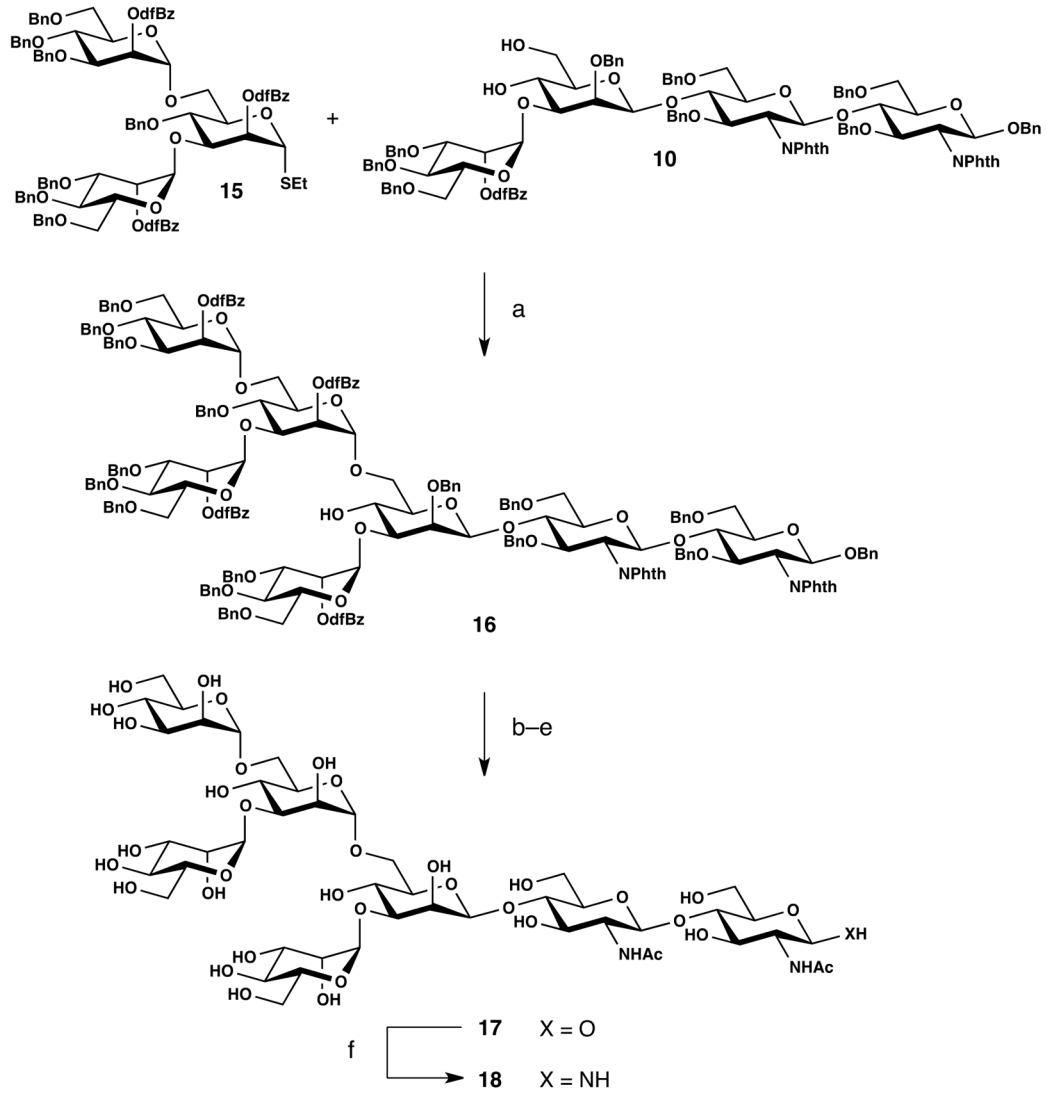
[a]Reagents and conditions: (a) Tf_2O, DTBP, MS AW-300, CH_2Cl_2, −78 °C, 86%; (b) DDQ, CH_2Cl_2, H_2O, 83%; (c) thioglycoside **8**, NIS/TMSOTf, MS AW-300, CH_2Cl_2, 0 °C→r.t., (d) AcOH, H_2O, 63% (2 steps).



Scheme 2.

Synthesis of trisaccharide donor **15**.[a]

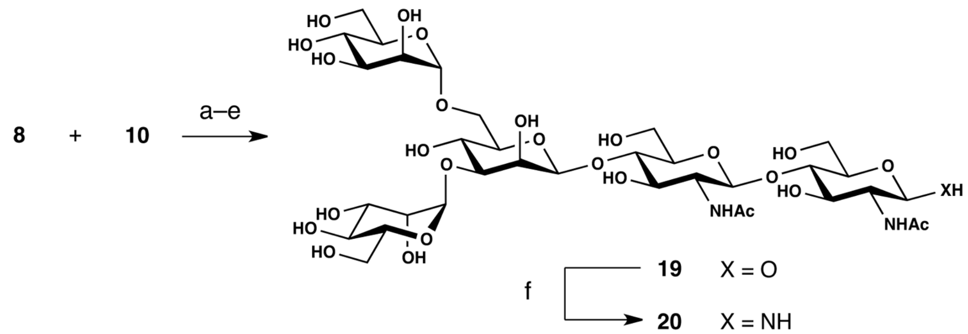
[a]Reagents and conditions: (a) $Cu(OTf)_2$, BH_3·THF, THF, 0 °C, 96%; (b) DDQ, CH_2Cl_2, H_2O, 89%; (c) TMSOTf, MS AW-300, CH_2Cl_2, 0 °C, 75%.



Scheme 3.

Synthesis of heptasaccharide **18**.[a]

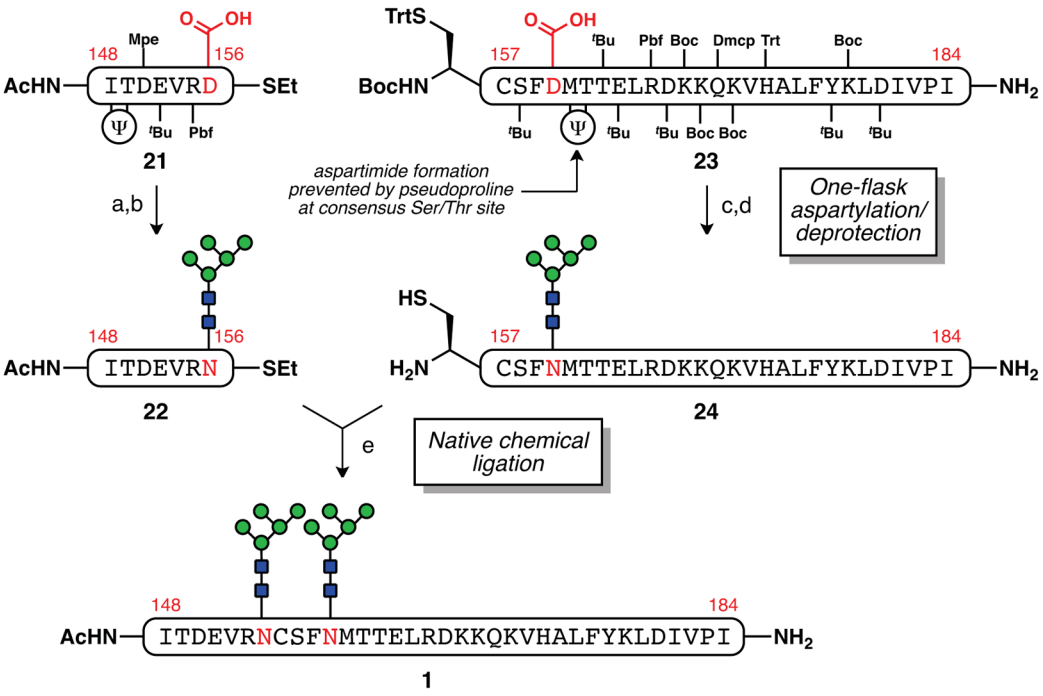
[a]Reagents and conditions: (a) NIS/TMSOTf, MS AW-300, CH_2Cl_2, 0 °C→r.t., 64%; (b) NaOMe, MeOH, CH_2Cl_2; (c) $H_2NCH_2CH_2NH_2$, *n*-BuOH, PhMe, 90 °C; (d) Ac_2O, Et_3N, MeOH; (e) H_2, $Pd(OH)_2$/C, MeOH, H_2O, 77% (4 steps); (f) sat. aq. NH_4HCO_3, 40 °C, quantitative.



Scheme 4.

Synthesis of pentasaccharide **20**.[a]

[a]Reagents and conditions: (a) NIS/TMSOTf, MS AW-300, CH_2Cl_2, 0 °C→r.t., 94%; (b) NaOMe, MeOH, CH_2Cl_2; (c) $H_2NCH_2CH_2NH_2$, *n*-BuOH, PhMe, 90 °C; (d) Ac_2O, Et_3N, MeOH, 95%; (e) H_2, $Pd(OH)_2/C$, MeOH, H_2O, 74% (4 steps); (f) sat. aq. NH_4HCO_3, 40 °C, quantitative.



Scheme 5.

Synthesis of V1V2 glycopeptide **1**.[a]

[a]Reagents and conditions: (a) $Man_5GlcNAc_2$–NH_2 (**18**), PyAOP, DIEA, DMSO; (b) Cocktail B = 88:5:5:2 TFA/phenol/water/triisopropylsilane, 44% (2 steps); (c) $Man_5GlcNAc_2$–NH_2 (**18**), PyAOP, DIEA, DMSO; (d) Cocktail R = 90:5:3:2 TFA/thioanisole/ethanedithiol/anisole, 22% (2 steps); (e) 6 M Gnd·HCl, 200 mM Na_2HPO_4, 200 mM MPAA, 20 mM TCEP·HCl, pH 7.2, 55%.