J|A|C|S
COMMUNICATIONS

Published on Web 08/21/2007

Fully Synthetic Carbohydrate HIV Antigens Designed on the Logic of the 2G12 Antibody

Isaac J. Krauss, Joseph G. Joyce, Adam C. Finnefrock, Hong C. Song, Vadim Y. Dudkin,[†] Xudong Geng,[‡] J. David Warren,[§] Michael Chastain, John W. Shiver, and Samuel J. Danishefsky*

Laboratory for Bioorganic Chemistry, Sloan-Kettering Institute for Cancer Research, 1275 York Avenue, New York, New York 10021, Department of Chemistry, Columbia University, Havemeyer Hall, 3000 Broadway, New York, New York 10027, and Vaccine and Biologics Research, Merck Research Laboratories, 770 Sumneytown Pike, West Point, Pennsylvania 19486

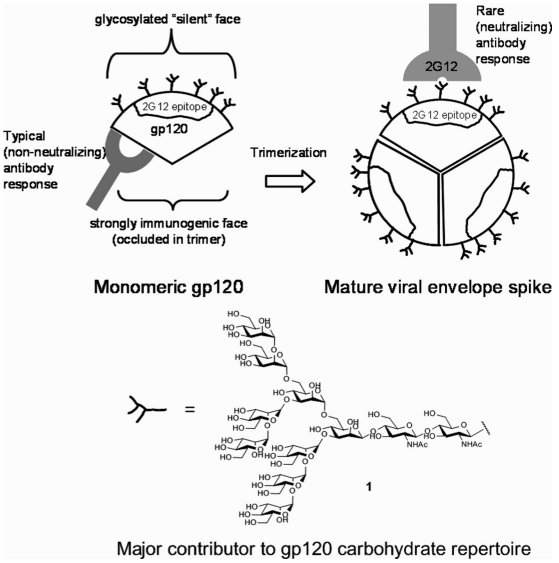
Received June 29, 2007; E-mail: s-danishefsky@ski.mskcc.org

Few individuals are fortunate enough to mount an effective immune response following HIV infection. This is despite the fact that the typical immunological reaction to the disease includes a massive antibody response against the envelope spike protein, gp120.[1] However, in the majority of cases (Scheme 1, left), the antibody repertoire is primarily targeted against regions of gp120 which are occluded in its mature trimeric form on the viral surface. The remaining exposed faces are heavily glycosylated, and few antibodies have been isolated which successfully bind to this "immunologically silent" region. 2G12 is an antibody which does bind to a portion of this "silent face" (Scheme 1, right) and is capable of neutralizing a broad range of HIV strains.[2] A successful vaccine approach would seek to elicit a focused immune response of 2G12-like antibodies by exposing the immune system to an effective structural mimic of the 2G12 epitope.

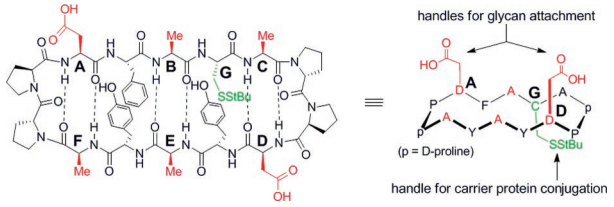
Alanine-scanning mutagenesis experiments[2b] as well as glycosidase digestion[3] have shown that the surface glycans of gp120 are necessary for 2G12 binding. Other evidence to the effect that the 2G12 epitope minimally consists of a carbohydrate motif, includes a cocrystal structure by Wilson. This shows that multiple copies of high-mannose glycan **1** bind to 2G12 as a cluster.[4,5] The crystal structure of 2G12 also shows that it possesses a unique domain-exchanged structure, which brings together the two Fab arms of the antibody to form an extended antigen recognition surface. Because this larger surface is capable of binding several copies of the carbohydrate (Scheme 1, right side), high binding affinity can be achieved.

Our group has been involved for some years in the synthesis of carbohydrate-based vaccines,[6] as well as complex glycans such as **1**.[7] These experiences placed us in a favorable position to apply the above information about the 2G12-gp120 interaction in a vaccine approach. Although we had previously synthesized structures[6] containing **1** attached to the proximal portion of a gp120 amino acid sequence (5 amino acids), the peptide alone had exhibited no affinity for 2G12. Indeed, little is known currently about the extent to which 2G12 directly interacts with the peptide portion of gp120.[8] It is possible that the primary importance of the peptide component lies in its structural role of presenting the glycans in a particular orientation. Assuming this to be the case, an unnatural peptide might provide us with much greater design flexibility than one based on the natural sequence. In choosing an unnatural peptide, we desired that it be modular and tunable, allowing for variation

Scheme 1. Typical vs. Potentially Neutralizing Antibodies against HIV



Scheme 2. Cyclic Peptide Scaffold



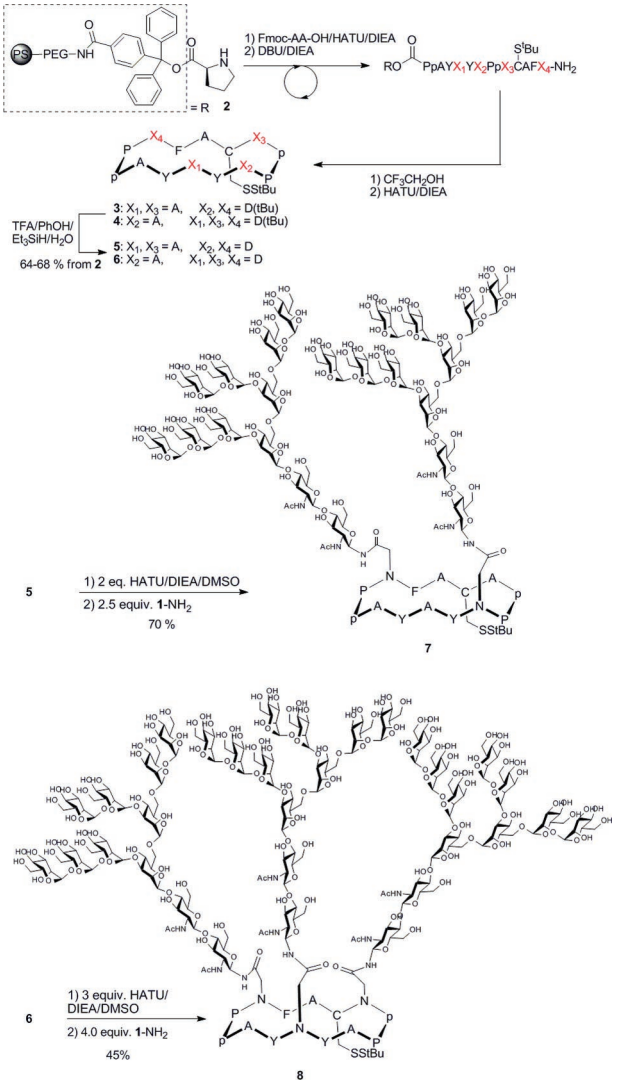
in the number of glycan attachments, as well as the distances between glycans. Additionally, such a scaffold would contain a suitable chemical handle for conjugation to a carrier protein (necessary for immune adjuvation during vaccination).

In analogy to the modular systems designed by Dumy[9] and Robinson,[10] we settled on a cyclic peptide scaffold (Scheme 2).[11] In this system, containing D-Pro-L-Pro sequences[12] to promote β-turns at both ends of the macrocycle, positions **A**−**F** (red) should present side chains above the plane of the macrocycle as drawn. Placement of aspartate residues in any of these positions enables attachment of glycan **1** by Lansbury aspartylation[13] at up to six positions and with variable spacing. Position **G** (green, with side chain projecting from the opposite face of the scaffold) contains a cysteine residue, suitable for linkage to a carrier protein or biological marker (vide infra). In similar 14-residue cyclic peptide systems, Robinson has demonstrated the preservation of β-sheet character

[†] Current address: Merck & Co., Department of Medicinal Chemistry, West Point, PA 19486.
[‡] Current address: Novartis Institute for Biomedical Research, Inc., Cambridge, MA 02139.
[§] Current address: Weill Medical College of Cornell University, New York, NY 10021.

10.1021/ja074804r CCC: $37.00

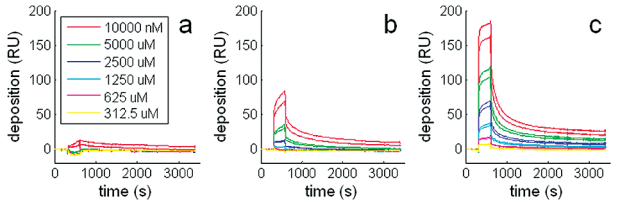
Scheme 3. Synthesis of 2G12-epitope Mimics



in small libraries of cyclic peptides containing differential substitution at these positions.[9b]

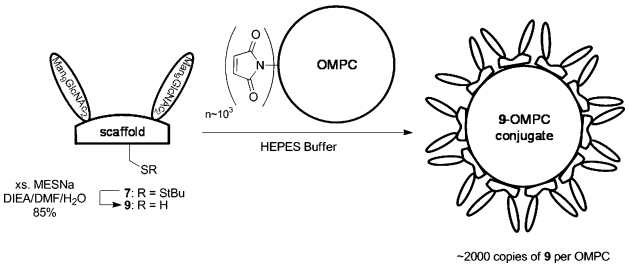
We thus sought to couple multiple copies of **1** to the scaffold in a maximally convergent manner (see Scheme 3). Cyclic peptides **3** and **4** (containing 2 and 3 aspartate residues, respectively) were prepared in parallel by automated solid-phase synthesis from prolinated trityl resin **2**, followed by cleavage from the resin and an extremely facile macrocyclization.[14] Following *tert*-butyl deprotection of aspartate esters to give **5** and **6**, we were in a position to attach the oligosaccharides via Lansbury aspartylation. Few cases of multifold Lansbury aspartylation have been reported to date.[15] Whereas the procedure for monofold aspartylation normally employs excess peptide to consume the valuable glycosyl amine fully, in the case where the peptide contains multiple aspartate residues, this strategy would clearly result in monocoupled side-products. It was thus necessary to optimize a procedure to give a high yield with respect to peptide. Thus, after conversion of fully deprotected **1**-OH[6] to glycosyl amine **1**-NH_2 by Kotchetkov amination,[16] the peptide was activated with HATU under carefully optimized conditions with minimal activation time. **1**-NH_2 was then quickly added, resulting in a high yield of the double aspartylation product **7** (with the cysteine thiol still masked as mixed disulfide; vide infra). Alternatively, trivalent antigen **8** could be constructed

Chart 1. Binding of Antigens to 2G12[a]



[a] Real-time SPR response units (RU) during the injection of monovalent (a), divalent (b), and trivalent (c) antigens over a 2G12-coupled surface as described in the text. Response curve for peptide alone (**5**) was similar to that in panel a but is not displayed. Each antigen concentration was run in duplicate; 2G12 activity decreases slightly during the $MgCl_2$ regeneration phase. The kinetics cannot be described by a simple Langmuir isotherm.

Scheme 4. Conjugation of Antigen to Immunogenic Carrier

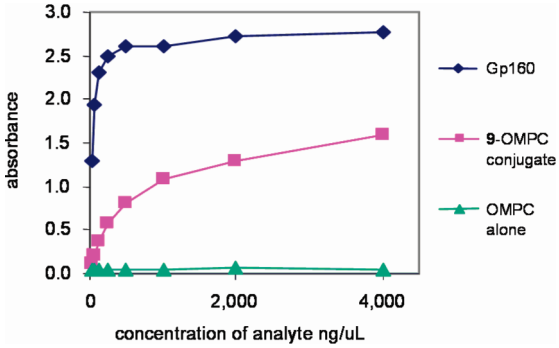


in a parallel fashion by triple Lansbury aspartylation using peptide **6**. These reactions represent the most complex examples of this type of coupling performed to date.

With a panel of antigens in hand, their relative binding affinities for 2G12 were studied by surface plasmon resonance. Each antigen was tested on a freshly prepared 2G12 surface. Two flow cells of a CM5 chip (Biacore) were activated with an EDC/NHS mixture; 40 nM 2G12 in sodium acetate pH 5.5 was injected over one; both were then blocked with 1.0 M ethanolamine. The antigens were injected over both surfaces at 50 μL/minute, and signals from control channel were subtracted from the 2G12 channel. Injection of non- and monoglycosylated peptides (**5** and **7mono**[17]) onto the 2G12-coated surface showed no measurable response (Chart 1a). The lack of response with monovalent glycopeptide highlights the importance of multivalency to the 2G12-glycopeptide interaction.[18] However, divalent and trivalent glycopeptides **7** and **8** showed strong binding to the 2G12 surface, suggesting homology to the natural epitope on gp120. Interestingly, the association and dissociation do not follow ideal Langmuir curves; we hypothesize that a more complex interaction involving conformational shifts may occur at the interface.

Having confirmed the ability of our synthetic glycopeptides to serve as 2G12 epitope mimics, we took steps to covalently attach selected structures to a carrier protein for immune adjuvation (Scheme 4). The ^{t}Bu-disulfide protecting group of **7** was unmasked by disulfide exchange with excess MESNa in high yield to give the dimerization-prone free sulfhydryl structure **9**. This product was covalently coupled to the purified outer membrane protein complex (OMPC) derived from *Neisseria meningitidis*. OMPC is a macromolecular lipoprotein complex which serves as a highly effective immunostimulatory carrier for poorly immunogenic peptide and carbohydrate antigens.[19] OMPC was maleimidated on a portion of its surface-accessible lysine residues using sulfosuccinimidyl-4-[*N*-maleimidomethyl]-cyclohexane-1-carboxylate, and the activated carrier was reacted with **9** at near-neutral pH to give OMPC conjugate **10**. The number of copies of glycopeptide monomer incorporated in the conjugate was determined to ~2000 based on quantitative amino acid analysis.

Chart 2. Binding of OMPC conjugate to 2G12



The ability of the **9**-OMPC conjugate to bind 2G12 was qualitatively assessed by an ELISA sandwich assay. Plates were coated with gp160,[20] 9-OMPC conjugate, or OMPC alone and then incubated with 2G12. HRP conjugated antihuman IgG was used as secondary antibody. Although the negative control (OMPC alone) showed no binding to 2G12, the **9**-OMPC conjugate showed a clear response, although it was not as robust as that of the positive control, gp160 (Chart 2).

In conclusion, we have designed and synthesized multivalent glycopeptide constructs which do not contain any relation to a native gp120 peptide sequence, yet still mimic gp120's binding to 2G12. At the same time, these constructs contain suitable functional handles for attachment to a carrier protein, facilitating their use in vaccines. Conjugation to the carrier protein has been readily accomplished, and animal vaccination studies are in progress.

Acknowledgment. Support for this work was provided by the National Institutes of Health (Grant CA103823). I.J.K. is grateful for an NIH postdoctoral fellowship (AI063976). U.S. Army breast cancer research program postdoctoral fellowship support is gratefully acknowledged by V.Y.D. (BC020513) and X.G. (BC022120). J.D.W. is grateful for an NIH postdoctoral fellowship (CA62948).

Supporting Information Available: Complete ref 4 and ref 19b; experimental procedures and spectra, including polarimetric data, for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Burton, D. R. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10018−10023.
(2) (a) Trkola, A.; Purtscher, M.; Muster, T.; Ballaun, C.; Buchacher, A.; Sullivan, N.; Srinivasan, K.; Sodroski, J.; Moore, J. P.; Katinger, H. *J. Virol.* **1996**, *70*, 1100−1108. (b) Scanlan C. N.; Pantophlet, R.; Wormald, M. R.; Saphire, E. O.; Stanfield, R.; Wilson, I. A.; Katinger, H.; Dwek, R. A.; Rudd, P. M.; Burton D. R. *J. Virol.* **2002**, *76*, 7306−7321.
(3) Sanders, R. W.; Venturi, M.; Schiffner, L.; Kalyanaraman, R.; Katinger, H.; Lloyd, K. O.; Kwong, P. D.; Moore, J. P. *J. Virol.* **2002**, *76*, 7293−7305.
(4) Calarese, D. A.; et. al. *Science* **2003**, *300*, 2065−2071.
(5) Wong, Wilson, and others have elegantly demonstrated that the D1 (linear trimannose) arm of **1** is the most important region of the oligosaccharide for interaction with 2G12: Calarese, D. A.; Lee, H.-K.; Huang, C.-Y.; Best, M. D.; Astronomo, R. D.; Stanfield, R. L.; Katinger, H.; Burton, D. R.; Wong, C.-H.; Wilson, I. A. *Proc. Natl. Acad. Sci., U.S.A.* **2005**, *102*, 13372−13377. Future antigen designs guided by this study may employ simplified oligosaccharides.
(6) Vaccines, based on cancer antigens: (a) Danishefsky, S. J.; Allen, J. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 836−863. (b) Keding, S. J.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11937−11942. (c) Ragupathi, G.; Koide, F.; Livingston, P. O.; Cho, Y. S.; Endo, A.; Wan., Q.; Spassova, M. K.; Keding, S. J.; Allen, J.; Ouerfelli, O.; Wilson, R. M.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2006**, *128*, 2715−2725. Vaccines against bacterial pathogens: (d) Svenson, S. B.; Lindberg, A. A. *FEMS Microbiol. Lett.* **1977**, *1*, 145−148. (e) Pawlowski, A.; Källenius, G.; Svenson, S. B. *Vaccine* **2000**, *18*, 1873−1885. Vaccines against fungal pathogens: (f) Torosantucci, A.; Bromuro, C.; Chiani, P.; De Bernardis, F.; Berti, F.; Galli, C.; Norelli, F.; Bellucci, C.; Polonelli, L; Costantino, P.; Rappuoli, R.; Cassone, A. *J. Exp. Med.* **2005**, *202*, 597−606.
(7) Geng, X.; Dudkin, V. Y.; Mandal, M.; Danishefsky, S. J. *Angew. Chem., Int. Ed. Engl.* **2004**, 2562−2565. Dudkin, V. Y.; Orlova, M.; Geng, X.; Mandal, M.; Olson, W. C.; Danishefsky, S. J. *J. Am. Chem. Soc.* **2004**, *126*, 9560−9562.
(8) Discontinuous, amino acid residues of gp120 important for 2G12 binding have been identified: Tumanova, O. Yu.; Kuvshinov, V. N.; Il'ichev, A. A.; Nekrasov, B. G.; Ivanisenko, V. A.; Kozlov, A. P.; Sandakhchiev, L. S. *Mol. Biol.* **2002**, *36*, 517−521.
(9) (a) Dumy, P.; Eggleston, I. M.; Cervigni, S.; Sila, U.; Sun, X.; Mutter, M. *Tetrahedron Lett.* **1995**, *36*, 1225−1258. (b) Dumy, P.; Renaudet, O. *Org. Lett.* **2003**, *5*, 243−246. (c) Singh, Y.; Dolphin, G. T.; Razkin, J.; Dumy, P. *ChemBioChem* **2006**, *7*, 1298−1314.
(10) (a) Jiang, L.; Moehle, K.; Dhanapal, B.; Obrecht, D.; Robinson, J. A. *Helv. Chim. Acta* **2000**, *83*, 3097−3112. (b) Favre, M.; Moehle, K.; Jiang, J.; Pfeiffer, B.; Robinson, J. A. *J. Am. Chem. Soc.* **1999**, *121*, 2679−2685. For a review, see (b) Robinson, J. A. *Synlett*, **2000**, *4*, 429−441.
(11) For an example of an approach utilizing conformationally flexible linkers connected to pyranose and steroid scaffolds, see: (a) Wang, L. X.; Ni, J.; Singh, S.; Li, H. *Chem. Biol.* **2004**, *11*, 127−134. (b) Li, H.; Wang, L. X. *Org. Biomol. Chem.* **2004**, *2*, 483−488. (c) Ni, J.; Song, H.; Wang, Y.; Stamatos, N. M.; Wang, L. X. *Bioconjugate Chem.* **2006**, *17*, 493−500. After submission of this manuscript we noted a very recent approach utilizing cyclic peptide scaffolds and a simplified fragment of oligosaccharide **1**: (d) Wang, J.; Li, H.; Zou, G.; Wang, L. X. *Org. Biomol. Chem.* **2007**, *5*, 1529−1540.
(12) Bean, J. W.; Kopple, K. D.; Peishoff, C. E. *J. Am. Chem. Soc.* **1992**, *114*, 5328−5334.
(13) Cohen-Anisfeld, S. T.; Lansbury, P. T. *J. Am. Chem. Soc.* **1993**, *115*, 10531−10537.
(14) The macrocyclization was essentially instantaneous at room temperature, indicating that cross-strand hydrogen bonding may preorganize the acyclic peptide for cyclization. Moreover, downfield ^{1}HNMR chemical shifts of selected N**H** resonances, slow D_2O exchange times, and the temperature profile of these chemical shifts provided preliminary evidence of the desired β-sheet character of the peptide scaffold.
(15) Sprengard, U.; Schudok, M.; Schmidt, W.; Kretzschmar, G.; Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 321−324.
(16) Likhosherstov, L. M; Novikova, O. S.; Derevitskaja, V. A.; Kochetkov, N. K. *Carbohydr. Res.* **1986**, *146*, C1−C5.
(17) Obtained as a minor side-product of the double aspartylation (**5** ≥ **7**).
(18) In ref 7b, monovalent glycopeptides showed no measurable binding to 2G12. However, dimerization through a cysteine sulfhydryl function resulted in a divalent compound which showed measurable binding to 2G12 but could not be conjugated to a carrier protein.
(19) (a) McGaughey, G. B.; Citron, M.; Danzeisen, R. C.; Freidinger, R. M.; Garsky, V. M.; Hurni, W. M.; Joyce, J. G.; Liang, X.; Miller, M.; Shiver, J.; Bogusky, M. J. *Biochemistry* **2003**, *42*, 3214−3223. (b) Joyce, J. G.; et. al. *Carbohydrate Res.* **2003**, *338*, 903−922. (c) Joyce, J.; Cook, J.; Chabot, D.; Hepler, R.; Shoop, W.; Xu, Q.; Stambaugh, T.; Aste-Amezaga, M.; Wang, S.; Indrawati, L.; Bruner, M.; Friedlander, A.; Keller, P.; Caulfield, M. *J. Biol. Chem.* **2006**, *281*, 4831−4843.
(20) gp160 is the biosynthetic precursor of gp120 and gp41. It is later cleaved to gp120 (the extracellular domain) and gp41 (the transmembrane domain), each of which trimerize and reassemble to form the mature viral envelope spike (see ref 1).

JA074804R