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Design and synthesis of a template-assembled oligomannose cluster as an epitope mimic for human HIV-neutralizing antibody 2G12

Hengguang Li and Lai-Xi Wang*
Institute of Human Virology, University of Maryland Biotechnology Institute, University of Maryland, Baltimore, MD 21201, USA

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The synthesis and antibody-binding affinity of a novel template-assembled oligomannose cluster as an epitope mimic for human anti-HIV antibody 2G12 are described. Cholic acid was chosen as the scaffold and three high-mannose type oligosaccharide ($Man_9GlcNAc_2Asn$) moieties were selectively attached at the 3α, 7α, and 12α-positions of the scaffold through a series of regioselective transformations. Binding studies revealed that the synthetic oligosaccharide cluster is 46-fold more effective than the subunit $Man_9GlcNAc_2Asn$ in inhibiting 2G12-binding to immobilized gp120. The scaffold approach described in this paper provides an avenue to designing more effective epitope mimics for antibody 2G12 in the hope of developing a carbohydrate-based vaccine against HIV-1.

Introduction

Human immunodeficiency virus (HIV) is the cause of AIDS. Eliciting broadly neutralizing antibodies against HIV-1 has been the major goal of vaccine research in order to bring the expanding worldwide pandemic of HIV/AIDS under control. However, it has been a formidable task to create effective immunogens that are able to elicit neutralizing antibodies against a broad range of primary HIV-1 isolates.[1–3] So far, only a few human monoclonal antibodies (MAbs) have been identified that are broadly reactive to HIV-1 primary isolates. These include neutralizing antibodies 2F5 and 4E10 that target epitopes on the inner envelope glycoprotein gp41,[4–7] and neutralizing antibodies b12 and 2G12 that recognize epitopes on the outer envelope glycoprotein gp120.[8–12] The challenge now is how to duplicate the conserved epitopes and to incorporate them into effective vaccine design.

Human monoclonal antibody 2G12 is special among other anti-HIV antibodies in that it targets a unique carbohydrate antigen on HIV-1 gp120. Mutational and biochemical studies have shown that the epitope of 2G12 may involve high-mannose type oligosaccharides (oligomannoses) at the *N*-glycosylation sites N295, N332, N339, N386, and N392 of gp120, with the sugar chains at positions N295, N332, and N392 as being most critical for 2G12 binding.[12–14] As they are remodeled on the core structure of gp120, these oligosaccharides seem to form unique oligomannose clusters.[13,14] Most recently, Calarese *et al.*[15] solved the X-ray crystal structures of Fab 2G12 and its complexes with disaccharide Manα1,2Man and high-mannose oligosaccharide $Man_9GlcNAc_2$. The structural study revealed that 2G12 takes an unusual domain-swapped structure, in which the V_H domains of 2G12 exchange in its two Fab regions so that an extended multivalent binding surface is created to accommodate an oligomannose cluster. Calarese *et al.*[15] further proposed a model for the recognition between 2G12 and HIV-1 gp120 glycans (Fig. 1). The model suggested that 2G12 binds gp120 at the glycans of N332 and N392 in the primary combining sites, with a potential interaction with the glycan at position N339 at the V_H/V_H' interface (secondary binding site). Therefore, a novel oligosaccharide cluster composed of oligosaccharides at N332, N392 and N339 most likely constitutes the epitope for 2G12. As a first step toward a novel vaccine to raise 2G12-like broadly neutralizing antibodies against HIV-1, we describe in this paper the design and synthesis of a template-assembled oligomannose cluster to serve as a mimic for the proposed 2G12 epitope. Cholic acid was modified as a rigid scaffold and three oligomannose sugar chains were selectively attached at the 3α, 7α, and 12α positions of the scaffold. The binding of the synthetic epitope mimic to antibody 2G12 is also described.

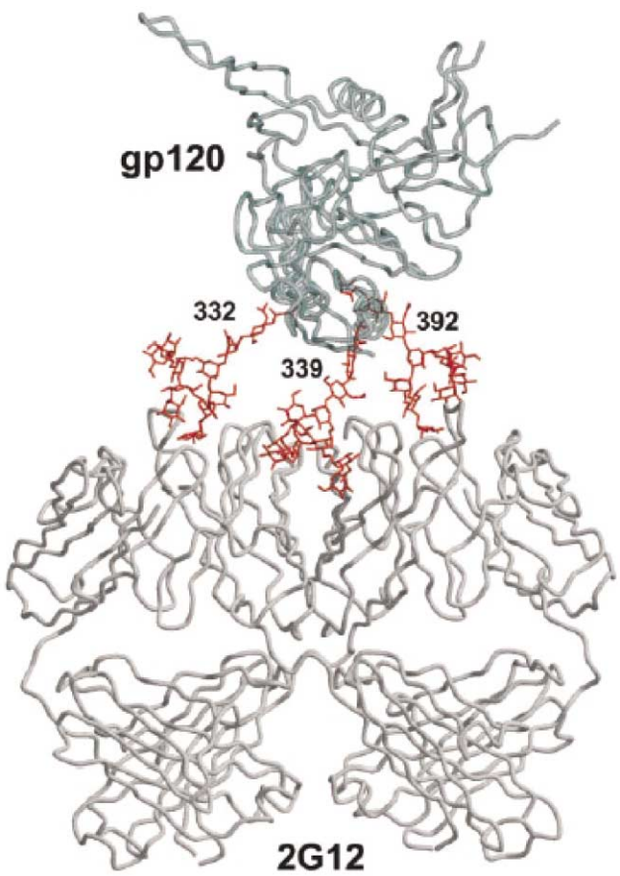

Fig. 1 *A model of 2G12-gp120 recognition*. Based on the model, three oligomannose (Man9GlcNAc2) moieties (shown in red) potentially mediate the binding of 2G12 to gp120 (two binding at the primary combining sites and one binding at the VH/VH′ interface) (Adapted from D. A. Calarese *et al.*, *Science*, 2003, **300**, 2065–71).

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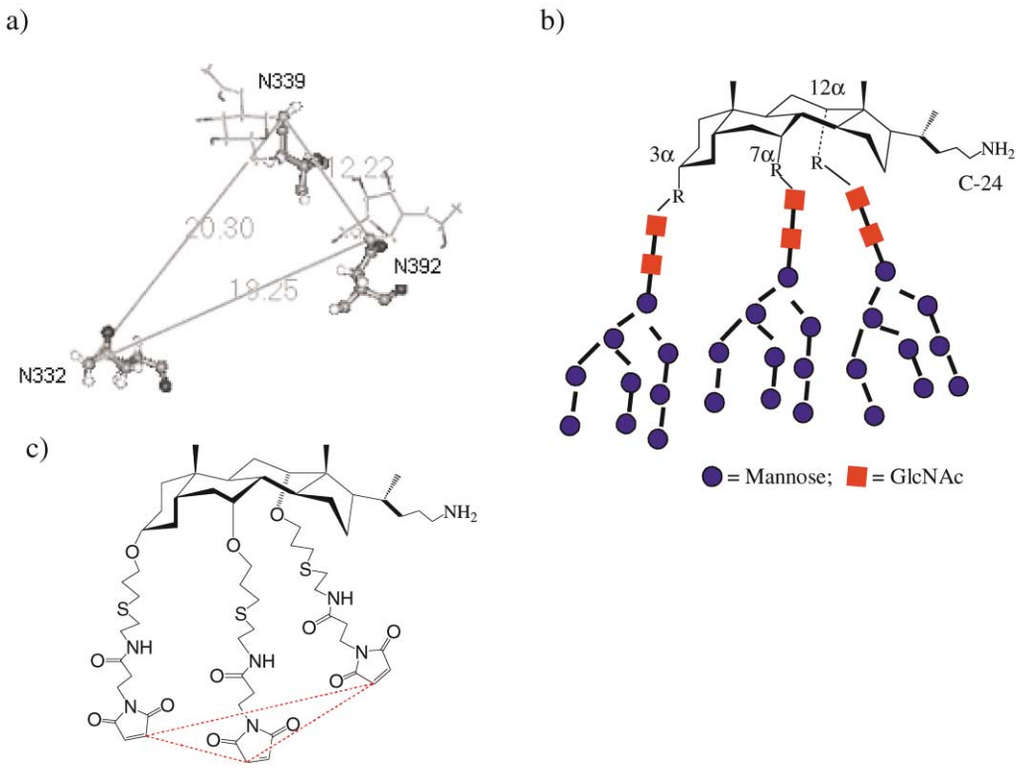


Fig. 2 *Structures of designed oligosaccharide cluster and template*: a) the geometry of the three *N*-glycosylation sites on the crystal structure of gp120 core; b) a general structure of the designed oligosaccharide cluster; c) structure of a maleimide cluster in which the three potential oligosaccharide attaching sites are between 12 and 20 Å in its low energy conformations.

Results and discussion

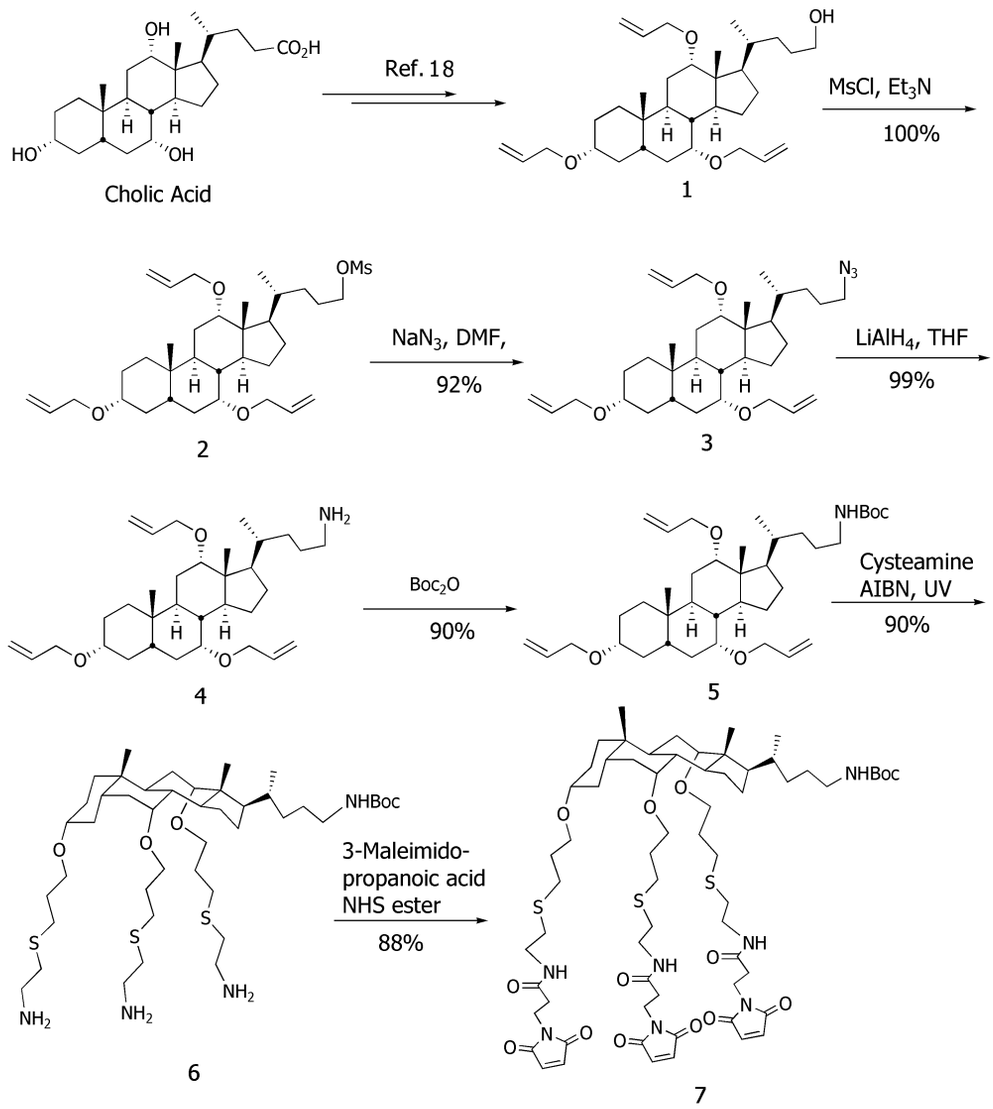
Design

Based on the crystal structure of the core gp120,[16,17] we have measured the distances between the β-amide nitrogen atoms of the asparagine at positions N332, N339, and N392 of gp120, to which the three high-mannose type oligosaccharides were attached. The distances between the asparagine (Asn) side chains of the pairs N332–N339, N332–N392, and N339–N392 were found to be 20.3 Å, 18.25 Å, and 12.2 Å (Fig. 2a). *We reasoned that if a suitable scaffold (template) could hold three high-mannose oligosaccharide (Man9GlcNAc$_2$Asn) chains with the oligosaccharide-attaching sites at the above measured distances, the resulting oligosaccharide cluster might well resemble the proposed epitope structure found on HIV-1 gp120.* Previously, we exploited cholic acid as a rigid template for multivalent peptide assembly.[18] The rigid structure and multiple functionality of cholic acid also makes it an attractive platform for multivalent oligosaccharide assembly. A general structure for the designed mimics is shown in Fig. 2b, where three oligomannose sugar chains are assembled at the 3α, 7α, and 12α positions of cholic acid through a suitable spacer R and another functional group X is installed at position C-24 for later coupling of the assembled oligosaccharide cluster to a carrier protein to make a vaccine. To have a preliminary estimate of a suitable length of the spacer between the template and the oligosaccharide chains, we have performed initial molecular modeling of some cholic acid-based maleimide clusters[18] that are suitable for coupling to SH-tagged oligosaccharides. The initial structures were generated on Chem3D, and the low energy conformations were searched using molecular mechanics (MM2) under default parameters together with a manual search. Fig. 2c showed the structure of a maleimide cluster, in which the distances between the three oligosaccharide attaching sites fall in the range of 12–20 Å in its low energy conformations. Therefore, we have chosen this maleimide cluster as the first template for constructing the oligosaccharide cluster.

Synthesis of the template-assembled oligosaccharide cluster

The 3α, 7α, 12α-tri-*O*-allyl intermediate **1** was synthesized starting from cholic acid according to our previously reported procedure.[18] For a later-stage coupling to a carrier protein, an amino group was introduced at the C24 position in compound **1** prior to manipulation of the allyl groups (Scheme 1). The primary hydroxyl group in **1** was first mesylated to give compound **2**. The methanesulfonyl group was then substituted with an azido group through treatment with NaN_3 in DMF, giving azide **3** in 92% yield. Reduction of the azido group with $LiAlH_4$ in THF afforded amine **4** in 99% yield without affecting the allyl groups. The amino group in **4** was subsequently protected with a Boc-group to give compound **5**. To introduce three maleimido groups on the template, the allyl derivative was subject to photo-addition with cysteamine hydrochloride in MeOH. However, it was found that the Boc-group was simultaneously removed under the photo-addition conditions, probably due to the acidity of the medium. When an aqueous $NaHCO_3$ solution was added to neutralize the acid, the photo-reaction proceeded rapidly and efficiently to give the desired product **6** in 90% yield without loss of the Boc-group. Finally, three maleimide groups were introduced simultaneously through treatment of compound **6** with *N*-(β-maleimidopropyloxy) succinimide ester to give the maleimide cluster **7** in 88% yield (Scheme 1).

For chemoselective ligation with the template, a thiol group was selectively introduced into Man$_9$GlcNAc$_2$Asn on the Asn portion of the oligosaccharide in two steps. First, Man$_9$GlcNAc$_2$Asn was *N*-acylated with *N*-succinimidyl *S*-acetylthioacetate (SATA)[19] to give the *N*-(*S*-acetylthioacetyl) derivative. The *S*-acetyl group was then selectively removed by hydroxylamine to give the SH-containing oligosaccharide **8**. Chemoselective ligation between the SH-tagged Man$_9$-oligosaccharide (**8**) and the maleimide cluster **7** was performed in a phosphate buffer (pH 6.6) containing MeCN. The ligation was monitored by HPLC. After completion of the ligation, the *N*-Boc group was selectively removed by treatment with



Scheme 1 Synthesis of the cholic acid template.

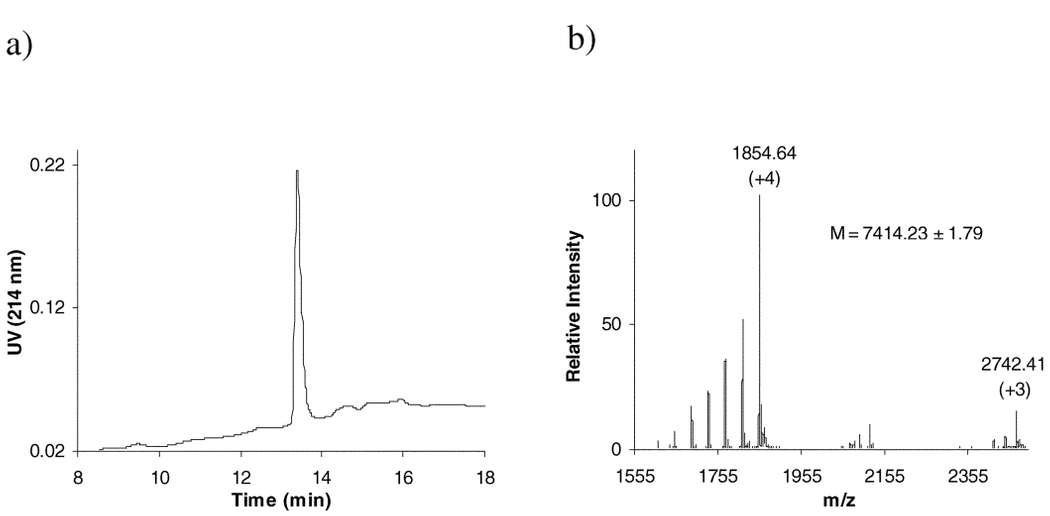
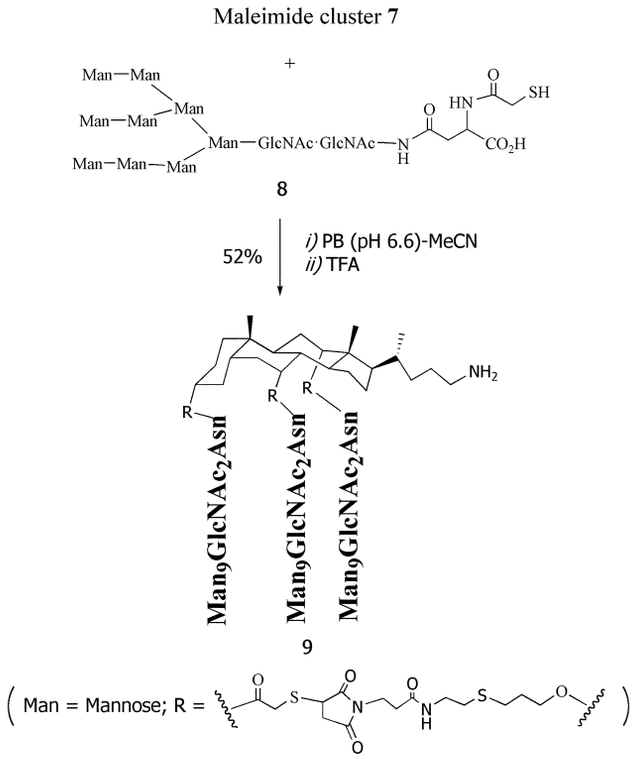


Fig. 3 *HPLC and ESI-MS profiles of synthetic mimic* **9**. a) HPLC; b) ESI-MS.

trifluoroacetic acid. Reverse-phase HPLC purification gave the final product **9** in 52% yield (Scheme 2). The identity of the synthetic mimic (**9**) was characterized by electron spray ionization-mass spectroscopy (ESI-MS) (Fig. 3). The ESI-MS spectrum revealed typical signals at 2472.41 $(M + 3H)^{3+}$ and 1854.64 $(M + 4H)^{4+}$, together with typical fragments at 1814.30 $(M - \text{Mannose} + 4H)^{4+}$, 1773.30 $(M - 2\text{Mannose} + 4H)^{4+}$, 1733.03 $(M - 3\text{Mannose} + 4H)^{4+}$, 1692.36 $(M - 4\text{Mannose} + 4H)^{4+}$ and 1652.02 $(M - 5\text{Mannose} + 4H)^{4+}$, which are in good agreement with the structure.



Scheme 2 Synthesis of the epitope mimic **9**.

Binding of the epitope mimic to 2G12

The binding affinity of the synthetic epitope mimic (**9**) to antibody 2G12 was examined by competitive inhibition of 2G12 binding to immobilized gp120. It was found that the synthetic mimic showed significantly enhanced affinity to antibody 2G12 compared to the monomeric subunit $Man_9GlcNAc_2Asn$ (Fig. 4). The IC_{50} (concentration for 50% inhibition) for epitope mimic **9** and $Man_9GlcNAc_2Asn$ was measured to be 21 μM and 960 μM, respectively. Therefore, compound **9** is about 46-fold more effective than $Man_9GlcNAc_2Asn$ in inhibition of 2G12 binding to immobilized gp120. The results suggest that the scaffold approach provides a reasonable means to generating mimics of the 2G12 epitope with increased affinity. However, it should be pointed out that gp120 could inhibit the binding of 2G12 to immobilized gp120 with an IC_{50} in nM range in our competitive assays (data not shown). Indeed, the spacers between the cholic acid template and the oligosaccharide moiety might be still too flexible. Therefore, a logical next step is to optimize the length of spacers, and the rigidity/configuration of the scaffold molecule in order to improve the affinity of the epitope mimics.

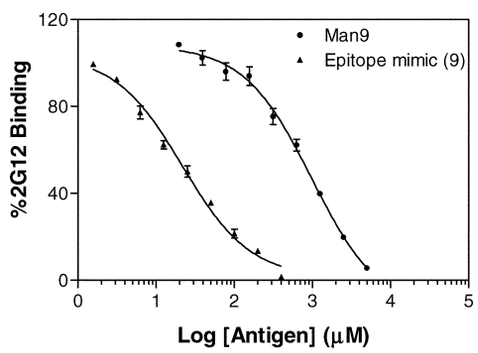


Fig. 4 *Inhibition of 2G12-binding to gp120*. 2G12-binding (%) was plotted against the log concentrations of the competing carbohydrate antigens. ●, Man9GlcNAc2Asn; ▲, synthetic mimic (**9**).

Conclusion

The synthesis and binding affinity of a template-assembled oligomannose cluster as an epitope mimic for anti-HIV antibody 2G12 are described. As an attempt to control the spatial orientation of an oligosaccharide cluster, cholic acid was chosen as the scaffold and three oligomannose sugar chains were selectively attached at the 3α, 7α, and 12α positions of cholic acid. Binding studies revealed that the synthetic oligosaccharide cluster is 46-fold more effective than the subunit $Man_9GlcNAc_2Asn$ in inhibiting 2G12-binding to immobilized gp120. Although the binding of the synthetic mimic to 2G12 is still in the μM range, while the binding of native gp120 to 2G12 is in the nM range, the template approach described in this paper is well suitable for designing more effective mimics for the epitope of antibody 2G12. Thus, the affinity of synthetic mimics can be improved through optimizing the length of spacers, and the rigidity and configuration of the scaffold molecule. The approach may eventually facilitate the development of a carbohydrate-based vaccine against HIV-1.

Experimental

General

^1H and ^{13}C NMR spectra were recorded on an Inova 500 with Me_4Si (δ 0) as the internal standard. The ESI-MS spectra were measured on a Waters ZMD mass spectrometer. TLC was performed on glass plates coated with silica gel 60 F254 (Merck) by visualizing the spots with UV light (254 nm) irradiation, 10% ethanolic sulfuric acid, ninhydrin spraying and/or iodine coloration. Flash column chromatography was performed on silica gel 60 (EM Science, 230–400 mesh). The photo-addition reaction was carried out in a quartz flask under N_2. Analytical HPLC was carried out with a Waters 626 HPLC instrument on a Waters Nova-Pak C18 column (3.9 × 150 mm) at 40 °C. The column was eluted with a linear gradient of MeCN–water containing 0.1% TFA at a flow rate of 1 mL min^{-1}. Compounds were detected at 214 nm. Preparative HPLC was performed with a Waters 600 HPLC instrument of a Waters C18 column (Symmetry 300, 19 × 300 mm). The column was eluted with a suitable gradient of MeCN–water containing 0.1% TFA at 12 mL min^{-1}.

3α,7α,12α-Triallyloxy-24-methanesulfonoxyl-5β-cholane (2)

To a solution of 3α,7α,12α-triallyloxy-24-hydroxyl-5β-cholane **1** (260 mg, 0.48 mmol) in ethyl acetate (10 mL) was added methanesulfonyl chloride (66.3 mg, 0.58 mmol) with stirring at 0 °C under N_2. Triethylamine (58 mg, 0.58 mmol) was added dropwise to the reaction mixture. The mixture was stirred at r.t. for 1 h, then diluted with ethyl acetate (20 mL). The mixture was washed with water (3 × 30 mL) and brine (10 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated using a rotary evaporator. The residue was subject to flash column chromatography using hexane–ethyl acetate (8 : 2, v/v) as the eluent to give compound **2** (276 mg, 100%) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$–TMS) δ 5.91 (m, 3H, C*H*=CH_2), 5.24 (m, 3H, CH_2=CHC), 5.10 (m, 3H, CH_2=CHC), 4.20 (t, 2H, J = 6.0 Hz, OCH_2CH=CH_2, (C-12)), 4.09 (dt, 2H, J = 15.0, 3.5 Hz, OCH_2CH=CH_2, (C-7)), 3.99 (dd, 2H, J = 5.5, 1.5 Hz, OCH_2CH=CH_2, (C-3)), 3.75 (dd, 1H, J = 7.3, 6.0 Hz, CH_2(24)), 3.71 (dd, 1H, J = 7.3, 6.0 Hz, CH_2(24)), 3.54 (s, 1H, C*H*(12)), 3.32 (d, 1H, J = 4.0 Hz, C*H* (7)), 3.13 (m, 1H, C*H* (3)), 3.00 (s, 3H, SO_2CH_3), 2.25 (q, 1H, J = 13.0 Hz, C*H* (11)), 2.16 (m, 2H, C*H* (8), C*H* (9)), 2.01 (q, 1H, J = 10.0 Hz, C*H* (11)), 1.85–0.95 (series of multiplest, 20H), 0.92 (d, 3H, J = 7.0 Hz, H_3C–C(20)), 0.89 (s, 3H, H_3C–C(10)), 0.67 (s, 3H, H_3C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$–TMS) 136.15, 135.84, 116.51, 115.80, 80.92, 79.29, 77.61, 77.35, 77.10, 75.28, 72.34, 70.98, 69.54, 69.44, 68.93, 46.53, 46.43, 42.80, 41.86, 39.89, 37.58,

35.41, 35.16, 31.70, 29.69, 28.72, 27.76, 27.63, 27.26, 26.05, 23.37, 23.06, 17.88, 12.79; ESI-MS calcd. for $C_{34}H_{56}O_6S$ (M): 592.38; Found: 593.46 $(M + H)^+$.

3α,7α,12α-Triallyloxy-24-azido-5β-cholane (3)

Compound **2** (142 mg, 0.24 mmol), sodium azide (156 mg, 2.4 mmol), and tetrabutylammonium bromide (TBAB) (5 mg, 16 μmol) were mixed in DMF (15 mL) and water (5 mL). The mixture was heated to 70 °C for 3 h with stirring. Ethyl acetate (20 mL) was added to the reaction mixture, and the mixture was washed with water (3 × 100 mL) and brine (20 mL). The organic layer was dried over Na_2SO_4, and concentrated using a rotary evaporator. The residue was subject to flash column chromatography using hexane as the eluent to give product **3** (118 mg, 92%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$–TMS) δ 5.92 (m, 3H, C*H*=CH_2), 5.25 (m, 3H, CH_2=CHC), 5.11 (m, 3H, CH_2=CHC), 4.09 (dm, 2H, J = 12.5 Hz, OCH_2-CH=CH_2, (C-7)), 4.00 (dd, 2H, J = 4.5, 1.0 Hz, OCH_2CH=CH_2, (C-3)), 3.76 (ddd, 1H, J = 6.0, 4.5, 1.0 Hz, CH_2 (24)), 3.70 (ddd, 1H, J = 6.0, 5.0, 1.0 Hz, CH_2 (24)), 3.53 (s, 1H, H–C(12)), 3.32 (d, 1H, J = 3.0 Hz, H–C(7)), 3.23 (m, 2H, OCH_2CH=CH_2, (C-12)), 3.14 (m, 1H, H–C(3)), 2.25 (q, 1H, J = 12.5 Hz, H–C(11)), 2.17 (m, 2H, H–C(8), H–C(9)), 2.01 (q, 1H, J = 10.0 Hz, H–C(11)), 1.85–0.95 (series of multiplets, 20H), 0.92 (d, 3H, J = 6.5 Hz, H_3C–C(20)), 0.89 (s, 3H, H_3C–C(10)), 0.66 (s, 3H, H_3C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$–TMS) 136.33, 123.07, 115.61, 100.10, 84.02, 80.95, 79.16, 77.52, 77.26, 77.01, 76.93, 75.20, 75.15, 69.57, 69.25, 52.27, 46.57, 42.73, 42.19, 40.08, 35.24, 35.82, 29.10, 28.01, 27.80, 27.63, 25.79, 23.21, 20.68, 17.99, 13.79, 12.71, 3.25; ESI-MS calcd. for $C_{33}H_{53}N_3O_3$ (M): 539.41; Found: 540.56 $(M + H)^+$, 500.51 $(M - N_3 + H)^+$.

3α,7α,12α-Triallyloxy-24-amino-5β-cholane (4)

To a solution of azide **3** (120 mg, 0.22 mmol) in anhydrous THF (20 mL) was added $LiAlH_4$ (16 mg, 0.33 mmol) in portions under N_2. The suspension was stirred at r.t. for 20 h. EtOH (5 mL, 95%) was added to quench the reaction. The mixture was then filtered through a pad of Celite. The filtrate was concentrated with a rotary evaporator, and the residue was dried over P_2O_5 in vacuum to provide amine **4** (110mg, 99%) as a colorless syrup, which was used for the next step immediately without further purification. ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 5.92 (m, 3H, C*H*=CH_2), 5.25 (m, 3H, C*H*=CHC), 5.10 (m, 3H, C*H*2=CHC), 4.07 (m, 2H, OCH_2CH=CH_2, (C-12)), 4.00 (d, 2H, J = 4.5 Hz, OCH_2CH=CH_2, (C-7)), 3.75 (dd, 2H, J = 9.0, 5.5 Hz, OCH_2CH=CH_2, (C-3)), 3.72 (d, 1H, J = 4.5 Hz, –CH2 (24)), 3.69 (m, 1H, –CH2 (24)), 3.67 (s, 2H, H_2N), 3.54 (s, 1H, H–C(12)), 3.49 (s, 2H, OCH_2CH=CH_2, (C-12)), 3.32 (s, 1H, H–C(7)), 3.14 (m, 1H, H–C(3)), 2.35–2.15 (m, 3H, H–C(11), H–C(8), H–C(9)), 2.00 (m, 1H, H–C(11)), 1.83–0.96 (series of multiplet, 18H), 0.92 (d, 3H, J = 6.0 Hz, H_3C–C(20)), 0.89 (s, 3H, H_3C–C(10)), 0.66 (s, 3H, H_3C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$–TMS) 136.31, 123.07, 115.61, 100.10, 84.02, 80.95, 79.16, 77.52, 77.26, 77.01, 76.93, 75.20, 75.15, 69.57, 69.25, 52.27, 46.57, 42.73, 42.19, 40.08, 35.24, 35.82, 29.10, 28.01, 27.80, 27.63, 25.79, 23.21, 20.68, 17.99, 13.79, 12.71, 3.25; ESI-MS calcd. for $C_{33}H_{55}NO_3$ (M): 513.42; Found: 514.76 $(M + H)^+$.

3α,7α,12α-Triallyloxy-24-*tert*-butyloxylcarboamido-5β-cholane (5)

To a solution of amine **4** (240 mg, 0.47 mmol) in THF (20 mL) and $NaHCO_3$ (0.1 M, 5 mL) was added di-*tert*-butyl dicarbonate (153 mg, 0.70 mmol). The mixture was stirred at r.t. for 30 min, and then diluted with ethyl acetate (30 mL). The solution was washed with water (3 × 10 mL), saturated $NaHCO_3$ and brine (10 mL). The organic layer was dried over Na_2SO_4 and concentrated. The residue was subject to flash column chromatography using hexane–ethyl acetate (9 : 1, v/v) as the eluent to give compound **5** (280 mg, 96%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$–TMS) δ 5.92 (m, 3H, C*H*=CH_2), 5.24 (m, 3H, H*H*C=CHC), 5.10 (m, 3H, CH_2=CHC), 4.54 (br s, 1H, HNCO), 4.07 (m, 2H, OCH_2CH=CH_2, (C-12)), 4.00 (dd, 2H, J = 4.5, 1.5 Hz, OCH_2CH=CH_2, (C-7)), 3.78 (m, 1H, –CH_2 (24)), 3.70 (m, 1H, –CH_2 (24)), 3.53 (s, 1H, H–C(12)), 3.32 (d, 1H, J = 2.0 Hz, H–C(7)), 3.13 (m, 2H, OCH_2CH=CH_2, (C-3)), 3.04 (m, 1H, H–C(3)), 2.25 (q, 1H, J = 12.5 Hz, H–C(11)), 2.17 (m, 2H, H–C(8), H–C(9)), 1.99 (q, 1H, J = 10.0 Hz, H–C(11)), 1.44 (s, 9H, H_3C(Boc)), 1.85–0.93 (series of multiplet, 20H), 0.92 (d, 3H, J = 6.5 Hz, H_3C–C(20)), 0.89 (s, 3H, H_3C–C(10)), 0.65 (s, 3H, H_3C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$–TMS) 136.29, 136.12, 116.51, 115.58, 115.50, 115.46, 115.80, 80.95, 79.50, 79.22, 77.35, 77.10, 75.23, 74.92, 69.47, 68.88, 46.53, 42.69, 42.17, 40.05, 39.95, 35.61, 35.22, 33.14, 29.09, 28.72, 28.61, 28.26, 28.10, 27.73, 26.78, 23.38, 23.36, 23.20, 17.99, 12.85, 12.70, 6.01; ESI-MS calcd. for $C_{38}H_{63}NO_5$ (M): 592.38; Found: 593.46 $(M + H)^+$.

3α,7α,12α-Tri-(6-amino-3-thiahexyoxyl)-24-*tert*-butyloxylcarboamido-5β-cholane (6)

To a mixture of compound **5** (250 mg, 0.40 mmol) and 2-aminoethanethiol hydrochloride (360 mg, 3.20 mmol) in a mixed solvent of MeOH (20 mL) and $NaHCO_3$ (0.5 M, 10 mL) in a quartz flask was added AIBN (6.6 mg, 0.04 mmol). The solution was degassed by bubbling N_2, and irradiated by UV (254 nm) with stirring under N_2. After a 20 h reaction, ^{1}H NMR revealed that no residual allyl groups existed. The reaction mixture was diluted with dichloromethane (50 mL) and washed with brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was subject to gel filtration on a column of Sephadex LH-20 using MeOH as the eluent to give **6** (304 mg, 90%) as a glass-like solid. ^{1}H NMR (500 MHz, $CDCl_3$–TMS) δ 5.03 (br s, 1H, HNBoc), 3.89 (s, 6H, H_2N), 3.65 (m, 1H, H–C(24)), 3.59 (m, 1H, H–C(24)), 3.53 (m, 2H, OCH_2CCH$_2$CH$_2$S), 3.47 (s, 1H, H–C(12)), 3.40 (s, 2H, OCH_2-CH$_2$CH$_2$S), 3.25 (s, 1H, OCH_2CH$_2$CH$_2$S), 3.23 (m, 2H, OCH_2-CH$_2$CH$_2$S), 3.15 (m, 1H, H–C(7)), 3.08 (m, 1H, H–C(3)), 3.05 (m, 2H, CH$_2$CH_2NH$_2$), 2.96 (m, 4H, CH$_2$CH_2NH$_2$), 2.71 (m, 6H, NCH$_2$CH_2S), 2.66 (m, 2H, OCH$_2$CH$_2$CH_2S), 2.62 (m, 4H, OCH$_2$CH$_2$CH_2S), 1.84 (m, 6H, OCH$_2$CH_2CH$_2$S), 1.44 (s, 9H, H_3C-(Boc)), 2.20–1.00 (series of multiplets, 26H), 0.90 (d, 3H, J = 6.4 Hz, H_3C–C(20)), 0.88 (s, 3H, H_3C–C(10)), 0.64 (s, 3H, H_3C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$–TMS) 132.08, 80.12, 79.31, 78.48, 75.54, 67.80, 66.32, 65.89, 65.70, 49.54, 45.97, 45.87, 42.21, 41.56, 40.73, 40.13, 40.04, 39.33, 38.38, 34.99, 34.95, 34.60, 34.14, 33.94, 33.53, 32.67, 30.14, 30.06, 30.02, 29.91, 28.70, 28.63, 28.58, 28.41, 28.15, 27.45, 27.28, 26.08, 23.42, 23.05, 22.64, 22.58, 22.46, 17.63, 13.73, 12.10, 10.64; ESI-MS calcd. for $C_{44}H_{84}N_4O_5S_3$ (M): 844.56. Found: 845.73 $(M + H)^+$, 728.67 $(M - CH_2S(CH_2)_2NH_2 + H)^+$, 423.49 $(M + 2H)^{2+}$.

3α,7α,12α-Tri-[6-(β-maleimidopropylamido-3-thiahexyoxyl)]-24-amino-5β-cholane (7)

To a solution of free amine **6** (33.3 mg, 39 μmol) and *N*–(β-maleimidopropyloxy) succinimide ester (BMPS) (32.1 mg, 0.14 mmol) in THF (5 mL) was added 0.1 M $NaHCO_3$ (0.5 mL). The mixture was stirred at room temperature for 2 h, and then diluted with dichloromethane (20 mL). The organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was removed under vacuum. The residue was subject to flash column chromatography on silica gel with ethyl acetate–methanol (90 : 10, v/v) as the eluent to give **7** (30 mg, 88%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$–TMS) δ 6.71 (s, 6H, C*H*=CH), 4.70 (br s, 1H, HNBoc), 3.84 (m, 3H, HNCOC), 3.63 (m, 1H, H–C(24)), 3.55 (m, 1H, H–C(24)), 3.51

(m, 2H, OCH_2CH$_2$CH$_2$S), 3.46(s, 1H, H–C(12)), 3.41 (s, 6H, CH_2NHCOC), 3.23 (s, 1H, OCH2CH$_2$CH$_2$S), 3.21 (m, 1H, OCH2CH$_2$CH$_2$S), 3.14 (m, 2H, OCH_2CH$_2$CH$_2$S), 3.07 (m, 1H, H–C(7)), 3.02 (m, 1H, H–C(3)), 2.63 (m, 9H, NCH$_2$CH_2S, CH_2-maleimide), 2.55 (m, 9H, OCH$_2$CH$_2$CH_2S, CH_2-maleimide), 2.04 (s, 6H, CH_2CH$_2$-maleimide), 1.79 (m, 6H, OCH$_2$CH_2-CH$_2$S), 1.44 (s, 9H, H$_3$C-(Boc)), 2.20–1.00 (series of multiplets, 26H), 0.90 (d, 3H, J = 6.4 Hz, H$_3$C–C(20)), 0.88 (s, 3H, H$_3$C–C(10)), 0.64 (s, 3H, H$_3$C–C(13)); ^{13}C NMR (500 MHz, CDCl$_3$–TMS) 134.19, 80.20, 79.68, 78.91, 75.80, 66.63, 66.26, 65.79, 60.35, 52.33, 46.22, 46.14, 42.51, 41.83, 41.09, 39.61, 38.52, 38.43, 35.38, 35.24, 35.14, 34.90, 34.59, 34.55, 34.33, 34.31, 33.63, 32.93, 31.83, 31.51, 30.27, 30.24, 30.06, 29.05, 29.01, 28.52, 28.42, 28.32, 28.06, 27.76, 27.60, 27.53, 26.26, 23.33, 22.83, 22.68, 21.02, 17.96, 14.16, 12.36, 10.64; ESI-MS calcd. for $C_{65}H_{99}N_7O_{14}S_3$ (M): 1297.64. Found: 1298.76 $(M + H)^+$, 1030.73 $(M - (CH_2)_2S(CH_2)_2NHCO(CH_2)_2$-maleimide $+ H)^+$, 650.02 $(M + 2H)^{2+}$.

Synthesis of the epitope mimic (9)

$Man_9GlcNAc_2Asn$ was prepared from soybean agglutinin according to the reported procedure.[20] To introduce a SH-group into the oligosaccharide for ligation purposes, $Man_9GlcNAc_2Asn$ was reacted with *N*-succinimidyl *S*-acetylthioacetate[19] in a phosphate buffer (pH 7.4) containing 20% MeCN to give the *N*-(*S*-acetylthioacetyl) $Man_9GlcNAc_2Asn$ derivative, which was subsequently treated with hydroxyl amine to remove the *S*-acetyl group, giving the desired SH-tagged oligosaccharide **8** after HPLC purification. ESI-MS of **8**: 2072.56 $(M + H)^+$, 1036.71 $(M + 2H)^{2+}$, 955.68 $(M - Man + 2H)^{2+}$, 874.71 $(M - 2Man + 2H)^{2+}$, 793.66 $(M - 3Man + 2H)^{2+}$, 712.56 $(M - 4Man + 2H)^{2+}$, 631.51 $(M - 5Man + 2H)^{2+}$, 550.66 $(M - 6Man + 2H)^{2+}$.

To a solution of the SH-tagged oligosaccharide **8** (8.7 mg, 4.2 μmol) in a degassed sodium phosphate buffer (pH 6.6) (1 mL) was added a solution of the maleimide derivative **7** (1.1 mg, 0.8 μmol) in MeCN (1 mL). The mixture was shaken gently at room temperature. The progress of the reaction was monitored by HPLC. After completion of the reaction (1 h), TFA (4 mL) was added and the mixture was kept at 4 °C overnight to remove the *N*-Boc group. The desired oligosaccharide cluster **9** (3.1 mg, 52%), with a free amino group at the C-24 position, was obtained by HPLC purification: t_R 13.4 min (10–90% MeCN in 20 min). ESI-MS: calcd. M = 7414.21; Found, 2472.41 $(M + 3H)^{3+}$, 1854.64 $(M + 4H)^{4+}$, 1814.30 $(M - Man + 4H)^{4+}$, 1773.30 $(M - 2Man + 4H)^{4+}$, 1733.03 $(M - 3Man + 4H)^{4+}$, 1692.36 $(M - 4Man + 4H)^{4+}$, 1652.02 $(M - 5Man + 4H)^{4+}$.

Competitive enzyme-linked immunosorbent assays (ELISAs)

Microtiter plates were coated with human cell line 293-expressed HIV-1_{IIIB} gp120 (100 ng ml^{-1}) overnight at 4 °C. After washing, non-specific binding was blocked with 5% BSA in PBS for 1 h at room temperature. The plates were then washed three times with 0.1% Tween-20/PBS. Serial dilutions (1 : 2) of various carbohydrate antigens were mixed with an equal volume of mAb 2G12 (fixed final concentration of 5 ng ml^{-1}) and added to the plates. The plates were incubated for 1 h at 37 °C and washed with washing buffer. To the plates was added a 100 μl solution of 1 : 3000 diluted horseradish peroxidase-conjugated goat anti-human IgG in 0.5% BSA/PBS. After incubation for 1 h at 37 °C, the plates were washed again and a 100 μl solution of 3,3′,5,5′-tetramethyl benzidine (TMB) was added. Color was allowed to develop for 5 min, and the reaction was quenched through adding a 100 μl solution of 0.5 M H_2SO_4 to each well. The optical density was read at 450 nm.

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