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Synthesis, conformation, and immunogenicity of monosaccharide-centered multivalent HIV-1 gp41 peptides containing the sequence of DP178

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Abstract—Several monosaccharide-centered multivalent HIV-1 gp41 peptides containing the sequence of DP178 were synthesized. Conformational studies showed that multivalent assembly enhanced the α-helical content of the peptide. Therefore, 2-, 3-, or 4-α-helix bundles of peptide DP178 could be obtained by assembling the peptide on a suitable bi-, tri-, or tetravalent template. Immunization studies indicated that while peptide DP178 alone was poorly immunogenic, the tetravalent peptide MVP-1 raised high titers of antibodies in mice that recognize not only peptide DP178 but also the native HIV-1 glycoprotein gp41, even in the absence of a carrier protein or adjuvant. The study suggests that carbohydrate-centered multivalent peptides provide not only a model for mimicking protein α-helix-bundle structure, but also an effective immunogen for raising high-titer antibodies against HIV-1 envelope glycoprotein gp41.
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1. Introduction

The worldwide epidemic of HIV/AIDS urges development of an effective HIV vaccine. It has been difficult to design immunogens that are able to raise broadly neutralizing antibodies against HIV-1 primary isolates.[1–3] An essential step in HIV-1 entry involves virus-cell membrane fusion mediated by HIV-1 envelope glycoprotein gp41. On the envelope of HIV-1, gp41 forms a trimeric complex associated with another envelope glycoprotein gp120. HIV-1 entry is initiated by the binding of envelope glycoprotein gp120 to host cell receptor CD4 and chemokine co-receptor CCR5 or CXCR4. The binding triggers conformational changes in gp41, leading to the exposure of gp41, insertion of the N-terminal fusion peptide into host membrane, and the subsequent formation of the fusogenic, six α-helix-bundle structure of the ectodomain of gp41, which provides the driving force for membrane fusion.[4] Several pieces of evidence suggest that the trimeric α-helical structure of the C-terminal region of gp41 is an ideal target for HIV-1 vaccine design. First, the C-terminal peptide region of gp41 is highly conserved among HIV-1 strains;[4,5] secondly, the epitopes of several broadly neutralizing anti-gp41 antibodies, including 2F5 and 4E10, were mapped to the C-ectodomain region of gp41,[6–10] suggesting that the region is accessible to antibody neutralization; and thirdly, synthetic peptides (C-peptides) derived from this region, such as DP178[11,12] and C34,[5,13] are potent inhibitors of HIV-1 infection. In particular, the sequence of the potent HIV-1 inhibitor DP178, also called T20 that was recently approved as a new drug for the treatment of AIDS, overlaps the epitope sequence NEQELLELDKWASLWN of neutralizing antibody 2F5.[6,7] However, attempts to use peptide DP178 and its conformation-constrained variants as immunogens failed to raise neutralizing antibodies reactive to HIV-1 strains.[14,15] The experimental data suggest that the actual epitopes may be more complex in nature than a single, linear peptide moiety.

Keywords: Multivalent peptides; HIV; gp41; Conformation; Carbohydrate; Immunogenicity; Antibody; Vaccine.
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Considering that in membrane fusion and viral entry, the functional unit of gp41 is an oligomeric (most likely a trimeric), parallel stranded, coiled coil complex rather than a monomeric gp41, and that the oligomeric gp41 complex undergoes conformational changes during viral membrane fusion,[4] we speculate that a conformation-restrained, multivalent (oligomeric) structure consisting of two or more gp41 C-peptide sequences may constitute real epitopes of the broadly neutralizing antibodies specific for the region. To capture or mimic the putative multivalent structure, we attempted to assemble the conserved gp41 C-peptides on a suitable scaffold to form novel multivalent peptides in the hope that the multivalent assembly will form α-helix bundles resembling the pre-fusogenic state of gp41. In this paper, we report the synthesis, conformations, and immunization studies of galactose-based, di-, tri-, and tetravalent HIV-1 gp41 peptides containing the sequence of DP178. We observed that the template-assembled peptides form α-helix bundles. Immunization studies showed that the synthetic multivalent peptides raise high titers of antibodies in mice that not only bind to the peptide immunogens used for the immunization, but also recognize HIV-1 envelope glycoprotein gp41.

2. Results and discussion

2.1. Synthesis of multivalent HIV-1 gp41 peptides containing DP178

We chose the sequence of the potent HIV-1 inhibitor DP178[11] for immunogen design for several reasons. First, this sequence is conserved and is involved in viral membrane fusion, an essential step for viral entry. Secondly, the sequence overlaps the extended linear epitope NEQELLELDKWASLWN (aa656-671 of gp160) of the broadly neutralizing antibody 2F5. Thirdly, a search of the database on T-helper epitopes in gp41 (http://hiv-web.lanl.gov) reveals that the DP178 sequence contains at least two T-helper epitopes, located at aa647-662 and aa650-661 of HIV-1 envelope gp160 (Fig. 1). With both B- and T-epitopes in the sequence, the resulting multivalent gp41 peptides containing DP178 are expected to be immunogenic even in the absence of a carrier protein or any other adjuvant.[16]

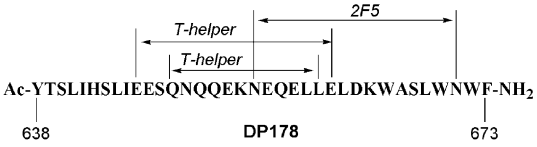
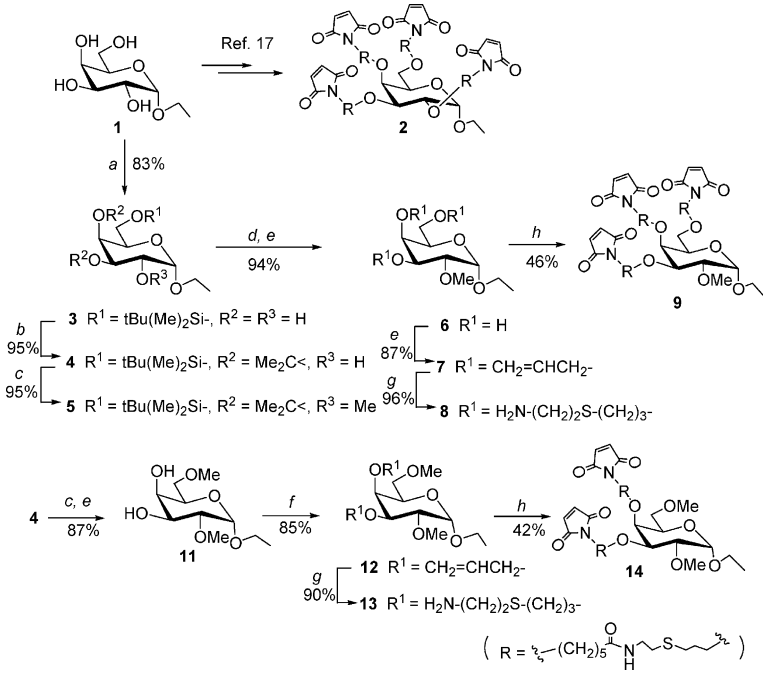


Figure 1. The sequence and epitope mapping of the HIV-1 inhibitor DP178.

We previously reported the synthesis of carbohydrate-centered maleimide clusters and their use as templates for multivalent peptide assembly.[17] The same approach was used for preparing galactose-based bi-, tri-, and tetravalent DP178 peptides. The tetravalent maleimide cluster (**2**) was readily synthesized starting from methyl α–D-galactopyranoside (**1**) according to the reported procedure.[17] To prepare galactose-based bi- and trivalent maleimide clusters, a series of regio-selective modifications were performed on methyl α–galactopyranoside (**1**). The synthesis was summarized in Scheme 1. In the trivalent maleimide cluster (**9**), malei-



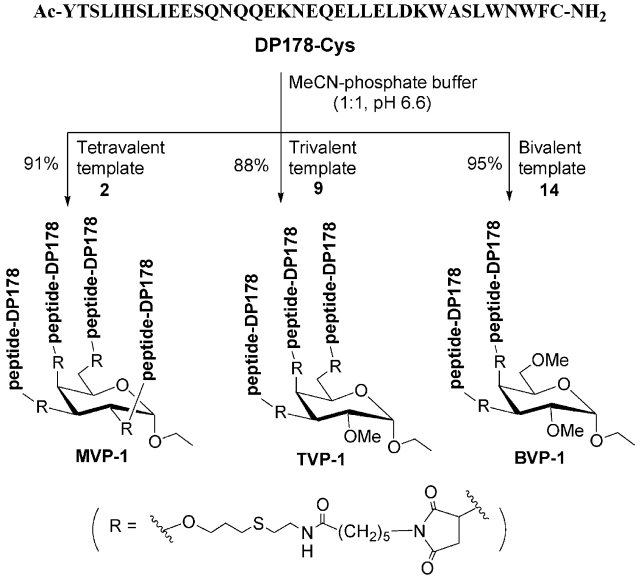
Scheme 1. Synthesis of maleimide clusters. Reactions and conditions: (a) *tert*-butyl(dimethyl)silyl chloride (1.2 mol equiv), pyridine, 0 °C–rt; (b) 2,2-dimethoxylpropane, *p*-TsOH (cat.), acetone, rt; (c) MeI, NaH, DMF, 0 °C–rt; (d) tetrabutylammonium fluoride, THF, rt; (e) 90% acetic acid, 90 °C; (f) allyl bromide, NaH, DMF, 0–25 °C; (g) cysteamine hydrochloride, MeOH, hν (254 nm), rt; (h) 6-maleimidohexanoic acid N-hydroxylsuccinimide ester, aq $NaHCO_3$, THF, 0–5 °C.

mide functionality was installed on the 3, 4, and 6-positions of the galactose-core. In the bivalent maleimide cluster (**14**), the maleimide functionality was selectively assembled on the 3 and 4-positions of the galactose-core, with the hydroxyl groups at 2- and 6-positions being protected by methyl groups (Scheme 1).

Chemoselective ligation between the respective maleimide cluster and the cysteine-containing DP178[17] gave the bi-, tri-, and tetravalent peptides (Scheme 2). The ligation was very efficient and normally completed within 30 min in a mixed solvent consisting of MeCN and phosphate buffer (pH 6.6) (1:1, v/v). The bi-, tri-, and tetravalent peptides, BVP-1, TVP-1, and MVP-1 were obtained in 95%, 88%, and 91% isolated yields, respectively, after HPLC purification. It should be pointed out that peptide DP178-Cys has a tendency to aggregate at 20 μM or higher concentration. For an efficient ligation, we found that the addition of MeCN was necessary to prevent the aggregation of the peptide in a phosphate buffer. The resulting multivalent peptides were readily purified by HPLC and characterized by ESI-MS.

2.2. Conformational studies

It has been demonstrated that template-assembled peptides may form α-helix bundles when a suitable template is applied.[18–20] We and others have recently reported that the 36-mer peptide DP178 formed three α-helix bundles when assembled on a cholic acid scaffold or on an oligolysine template.[21,22] On the other hand, Brask et al.[23] recently reported that assembly of a model peptide on several monosaccharide templates (Gal*p*, Glc*p*, and Alt*p*) resulted in four α-helix-bundle formation. Therefore, it is interesting to investigate how the DP178 peptides behave in the bi-, tri-, and tetravalent formats on the galactopyranoside scaffold. The circular dichroism (CD) spectra of the multivalent peptides



Scheme 2. Synthesis of the multivalent peptides.

BVP-1, TVP-1, and MVP-1 were measured at 23 °C in a phosphate buffer (pH 7.2). Alpha-helical content was calculated based on the mean residue ellipticity $[\theta]_{222}$ according to the proposed equation.[24] The α-helical contents for BVP-1, TVP-1, and MVP-1 were found to be 30%, 32%, and 39%, respectively, in the phosphate buffer. Under the same condition, the α-helical content of DP178 was measured to be 18%, which is consistent with the reported value.[25] The tetravalent peptide MVP-1 demonstrated the highest α-helical content among the synthetic multivalent peptides. A comparison of the CD spectra for DP178 and MVP-1 was shown in Figure 2. It was reported that DP178 turns to aggregate in buffer when the concentration is above 20 μM. We observed that the CD spectra of DP178, BVP-1, TVP-1, and MVP-1 were concentration independent in the range of 2–40 μM (data not shown). Thus, our data indicates that the template-assembled bi-, tri-, and tetravalent peptides all form α-helix bundles to some extent, with the tetravalent peptide MVP-1 possessing the highest content of α-helical structure.

2.3. Immunogenicity of the synthetic multivalent DP178 peptides

Since the tetravalent peptide MVP-1 forms an α-helix bundle with the highest α-helix content among the synthetic multivalent peptides, we predict that MVP-1 may partially mimic the pre-fusogenic state of the oligomeric gp41 during membrane fusion. Therefore, MVP-1 and DP178 were chosen to immunize mice in order to evaluate and compare their immunogenicity. Mice were primed with MVP-1 or DP178 (10 μg each), and boosted at days 14 (1° boost), 28 (2° boost), and 90 (3° boost) with the same amount of immunogen without any adjuvant. Serum was collected 10 days after each boost and the antibody responses were evaluated with enzyme-linked immunosorbent assays (ELISAs). As shown in Figure 3, the peptide DP178 alone was found to be poorly immunogenic. In contrast, the tetravalent format of DP178, MVP-1 was highly immunogenic even in the absence of any adjuvant. After the third boost, the anti-DP178 antibody titers reached $1{:}2\times10^6$ for the sera from mice immunized with MVP-1, while the

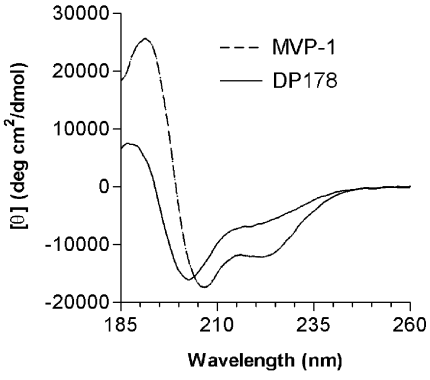


Figure 2. CD spectra of MVP-1 and DP178. The spectra were recorded with 10 μM of DP178 and 2.5 μM of MVP-1 in a phosphate buffer (50 mM, pH 7.2) at 23 °C.

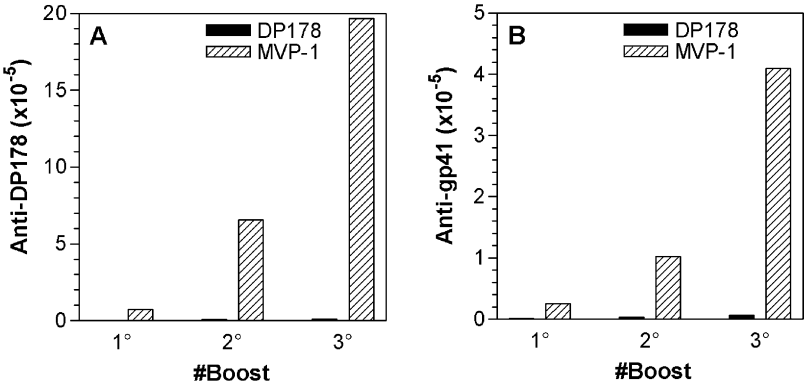


Figure 3. Antibody responses. Mice were primed with 10 μg of MVP-1 or DP178, and boosted at days 14 (1° boost), 28 (2° boost), and 90 (3° boost), respectively, with the same amount of immunogen without any adjuvant. Serum was collected 10 days after each boost and the antibody responses were evaluated with enzyme-linked immunosorbent assays (ELISAs). A, antibody response against immobilized DP178; B, antibody response against immobilized HIV-1 gp41.

anti-DP178 titer for the sera from mice immunized with DP178 was $1{:}9\times10^3$ after the third boost (Fig. 3A). Moreover, it was found that the antibodies raised from mice immunized with MVP-1 also recognized native HIV-1 envelope glycoprotein gp41, and the anti-gp41 antibody titer reached $1{:}4\times10^5$ after third boost (Fig. 3B). These results suggest that multivalent peptides containing both B- and T- epitopes are highly effective immunogens even in the absence of a carrier protein or any adjuvant. This raises the possibility that multivalent gp41 peptide immunogen may raise conformation-specific antibody targeting the prefusogenic state of gp41. The next step is to characterize these antibodies and to evaluate whether the antibodies can neutralize various HIV-1 isolates.

3. Conclusion

Several monosaccharide-centered multivalent HIV-1 gp41 peptides containing the sequence of DP178 were synthesized. It was found that multivalent assembly enhanced the α-helical content of the peptide, and led to the formation of 2-, 3-, or 4-α-helix bundles when a bi-, tri-, or tetravalent template was used. Immunization studies indicated that while peptide DP178 in the absence of a carrier protein or adjuvant was poorly immunogenic, the tetravalent peptide MVP-1 was able to raise high titers of antibodies in mice that recognize not only peptide DP178 but also the native HIV-1 glycoprotein gp41. The study suggests that the carbohydrate-centered multivalent peptides provide not only a model for mimicking protein α-helix-bundle structure, but also an effective immunogen that raises high-titer anti-gp41 antibodies, which may be exploited for HIV-1 vaccine development.

4. Experimental

4.1. General methods

^{1}H NMR spectra were recorded on QE 300 with Me_4Si (δ0) as the internal standard. The ESI-MS spectra were measured on a Waters ZMD mass spectrometer. Analytical TLC was performed on glass plates coated with silica gel 60 F_{254} (E. Merck). Carbohydrates were detected by charring with 10% ethanolic sulfuric acid. Amines were detected by ninhydrin spraying. Flash chromatography was performed on silica gel 60 (200–400 mesh, EM Science). Photo-addition reactions were carried out in a quartz flask under N_2. Automatic solid-phase peptide synthesis was performed as described previously.[17] The final peptides were purified by reverse-phase HPLC. For the biotinylation of DP178, after removal of Fmoc at the last step in the solid-phase synthesis, the peptide on the resin was treated with biotin and HBTU in DMF. After coupling, the peptide was deprotected and cleaved from the resin, and finally purified by HPLC. Analytical HPLC was carried out with a Waters 626 HPLC instrument using a Waters Nova-Pak C18 column (3.9×150 mm) at 40 °C. The column was eluted with a linear gradient of acetonitrile (10–90%) containing 0.1% TFA in 20 min at a flow rate of 1 mL/min. Peptides were detected by UV absorbance at 214 and/or 280 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 water–MeCN containing 0.1% TFA. Peptides purified from HPLC were lyophilized and kept under nitrogen in a freezer (−20 °C). For the determination of concentrations of peptide DP178 and its multivalent derivatives, the UV absorbance was run on Beckman DU 640 spectrophotometer at 280 nm, and the concentration was calculated according to the equation proposed in the literature.[26] CD spectra were measured on JASCO-810 (CD-ORD) spectropolarimeter using quartz cuvette of 1 mm path length at 23 °C.

4.1.1. Ethyl 6-*O*-*t*-butyldimethylsilyl-α-D-galactopyranoside (3). Compound **1** (210 mg, 1.0 mmol) was dissolved in pyridine (5 mL), *tert*-butyldimethylsilyl chloride (182 mg, 1.2 mmol) was added at 0–5 °C. After stirring the resulting mixture at 0–5 °C for 30 min, and then at room temperature for 2 h, MeOH (1 mL) was added and

the solvent was removed under reduced pressure. Column chromatography (CH_2Cl_2/MeOH 95:5) of the residue gave compound **3** (270 mg, 83%). R_f 0.43 (CH_2Cl_2/MeOH 90:10); ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 4.92 (d, 1H, $J = 3.4$ Hz, H-1), 4.09 (br s, 1H, H-4), 3.94–3.74 (m, 6H, H-2, H-3, H-5, H-6, H-6$^+$, and OCH*H*CH$_3$), 3.62–3.49 (m, 1H, OC*H*HCH$_3$), 3.20 (br s, 1H, O*H*-4), 3.04 (d, 1H, $J = 4.9$ Hz, O*H*-3), 2.31 (dd, 1H, $J = 9.8$ Hz, O*H*-2), 1.24 (dd, 3H, $R = 7.1$, 6.8 Hz, OCH$_2$C*H*$_3$), 0.90 (s, 9H, (C*H*$_{33}$CSi)), 0.09 (s, 6H, C*H*$_3$SiC*H*$_3$); ES-MS: 667.47 (2M+Na)$^+$, 345.24 (M+Na)$^+$, 323.25 (M+H)$^+$.

4.1.2. Ethyl 6-*O*-*t*-butyldimethylsilyl-3,4-*O*-isopropylidene-α-D-galactopyranoside (4). Compound **3** (431 mg, 1.34 mmol) was dissolved in acetone (10 mL). 2,2-dimethoxypropane (278.5 mg, 2.68 mmol) and *p*TsOH (cat.) were added. The resulting mixture was stirred at room temperature for 3 h. The reaction mixture was then neutralized with aqueous $NaHCO_3$ and concentrated. The residue was taken up in EtOAc (50 mL) and washed with aqueous $NaHCO_3$, brine, and water. The organic layer was dried (Na_2SO_4) and concentrated. Column chromatography (CH_2Cl_2/MeOH 95:5) of the residue gave compound **4** (460 mg, 95%). R_f 0.67 (CH_2Cl_2/MeOH 93:7). ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 4.85 (d, 1H, $J = 3.2$ Hz, H-1), 4.28–4.16 (m, 2H, H-3, H-4), 4.03 (m, 1H, H-5), 3.92–3.75 (m, 4H, H-2, H-6, H-6$^+$, and OCH*H*CH$_3$), 3.62–3.49 (m, 1H, OC*H*HCH$_3$), 2.28 (dd, 1H, $J = 7.1$ Hz, O*H*-2), 1.50 (s, 3H, CC*H*$_3$), 1.34 (s, 3H, C*H*$_3$C), 1.24 (dd, 3H, $J = 7.1$, 6.6 Hz, OCH$_2$C*H*$_3$), 0.90 (s, 9H, (C*H*$_{33}$CSi)), 0.08 (s, 6H, C*H*$_3$SiC*H*$_3$); ES-MS: 385.26 (M+Na)$^+$, 363.26 (M+H)$^+$.

4.1.3. Ethyl 6-*O*-*t*-butyldimethylsilyl-3,4-*O*-isopropylidene-2-*O*-methyl-α-D-galactopyranoside (5). A solution of compound **4** (284 mg, 0.78 mmol) in anhydrous DMF (5 mL) was added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 312 mg, 7.80 mmol) in anhydrous DMF (15 mL). After stirring the resulting suspension for 30 min, the reaction mixture was cooled to 0 °C, and then iodomethane (0.5 mL) was added. The resulting mixture was stirred at 0 °C for 1 h and at room temperature for 3 h. After the mixture was cooled in an ice bath, excess sodium hydride was consumed by slow addition of MeOH (5 mL). The mixture was concentrated to dryness under reduced pressure. The residue was then partitioned between EtOAc and water. The organic layer was washed with diluted HCl, brine, and water, dried over Na_2SO_4, and concentrated. The product was purified by column chromatography (hexane/EtOAc 85:15) to provide compound **5** (277 mg, 95%); R_f 0.45 (hexane/EtOAc 80:20). ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 4.94 (d, 1H, $J = 2.9$ Hz, H-1), 4.29–4.20 (m, 2H, H-3, H-4), 4.02 (m, 1H, H-5), 3.94–3.72 (m, 3H, H-6, H-6$^+$, and OCH*H*CH$_3$), 3.60–3.50 (m, 4H, OC*H*HCH$_3$, 2-OC*H*$_3$), 3.35 (dd, 1H, $J = 7.6$, 3.4 Hz, H-2), 1.53 (s, 3H, CC*H*$_3$), 1.34 (s, 3H, C*H*$_3$C), 1.26 (dd, 3H, $J = 7.1$, 6.6 Hz, OCH$_2$C*H*$_3$), 0.90 (s, 9H, (C*H*R$_{33}$CSi)), 0.08 (s, 6H, C*H*$_3$SiC*H*$_3$); ES-MS: 399.37 (M+Na)$^+$.

4.1.4. Ethyl 2-*O*-methyl-α-D-galactopyranoside (6). Compound **5** (221 mg, 0.59 mmol) was dissolved in THF (5 mL) and tetrabutylammonium fluoride in THF (1 M, 0.71 mL) was added. The mixture was stirred at room temperature for 1 h, and the solvent was then evaporated. Column chromatography (CH_2Cl_2/MeOH 95:5) of the residue gave ethyl 3,4-*O*-isopropylidene-2-*O*-methyl-α-D-galactopyranoside (146 mg, 95%): R_f 0.70 (CH_2Cl_2/MeOH 92:8). ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 5.00 (d, 1H, $J = 2.9$ Hz, H-1), 4.38–4.22 (m, 2H, H-3, H-4), 4.10–3.70 (m, 4H, H-5, H-6, H-6$^+$, and OCH*H*CH$_3$), 3.66–3.49 (m, 4H, OC*H*HCH$_3$, 2-OC*H*$_3$), 3.37 (dd, 1H, $J = 7.6$, 2.9 Hz, H-2), 2.29 (dd, 1H, $J = 10.7$, 3.8 Hz, O*H*-6), 1.54 (s, 3H, CC*H*$_3$), 1.36 (s, 3H, C*H*$_3$C), 1.25 (dd, 3H, $J = 7.1$, 6.9 Hz, OCH$_2$C*H*$_3$); ES-MS: 285.12 (M+Na)$^+$. Treatment of the product (92 mg, 0.35 mmol) with 90% acetic acid (3 mL) at 90 °C for 2 h gave compound **6** (77 mg, 99%) after column chromatography. R_f 0.54 (CH_2Cl_2/MeOH 80:20); ^{1}H NMR (300 MHz, D_2O): δ 5.21 (d, 1H, $J = 3.4$ Hz, H-1), 4.04–3.74 (m, 4H, H-3, H-4, H-5, OCH*H*CH$_3$), 3.74 (d, 2H, $J = 6.1$ Hz, H-6, and H-6$^+$), 3.68–3.48 (m, 2H, H-2, and OC*H*HCH$_3$), 3.47 (s, 3H, 2-OC*H*$_3$), 1.24 (dd, 3H, $J = 7.1$, 6.9 Hz, OCH$_2$C*H*$_3$); ES-MS: 245.16 (M+Na)$^+$.

4.1.5. Ethyl 3,4,6-tri-*O*-allyl-2-*O*-methyl-α-D-galactopyranoside (7). A solution of compound **6** (77 mg, 0.35 mmol) in anhydrous DMF (5 mL) was added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 416 mg, 10.4 mmol) in anhydrous DMF (15 mL). The reaction mixture was stirred at 0 °C for 30 min and allyl bromide (1 mL) was then added dropwise. The resulting mixture was stirred at 0 °C to room temperature for 5 h. The reaction was quenched by slow addition of MeOH (5 mL). The mixture was concentrated to dryness under reduced pressure. The residue was partitioned between EtOAc and diluted HCl. The organic layer was separated and washed with brine and water, dried over Na_2SO_4, and concentrated. Column chromatography (hexane/EtOAc 75:25) of the residue afforded compound **7** (100 mg, 87 %). R_f 0.42 (hexane/EtOAc 70:30); ^{1}H NMR (300 MHz, $CDCl_3$/TMS): 6.02–5.86 (m, 3H, OCH$_2$C*H*=CH$_2$), 5.36–5.10 (m, 6H, OCH$_2$CH=C*H*$_2$), 5.00 (d, 1H, $J = 3.2$ Hz, H-1), 4.39 (dd, 1H, $J = 12.3$, 5.4 Hz, OC*H*HCH = CH$_2$), 4.26–3.98 (m, 2H, OC*H*$_2$CH=CH$_2$), 3.91 (m, 1H, H-5), 3.83 (br s, 1H, H-4), 3.80–3.68 (m, 3H, H-3, H-6, and OC*H*HCH$_3$), 3.64–3.49 (m, 6H, H-2, H-6$^+$, OCH*H*CH$_3$, and 2-OC*H*$_3$), 1.24 (br t, 3H, $J = 6.9$ Hz, OCH$_2$C*H*$_3$); ES-MS: 365.28 (M+Na)$^+$.

4.1.6. Ethyl 3,4,6-tri-*O*-(6-amido-3-thiahexyl)-2-*O*-methyl-α-D-galactopyranoside trihydrochloride (8). To a solution of compound **7** (100 mg, 0.29 mmol) and AIBN (10 mg) in MeOH (15 mL) in a quartz flask was added cysteamine hydrochloride (198 mg, 1.74 mmol). After being degassed by bubbling N_2 into solution for 30 min, the solution was stirred and irradiated (UV, 254 nm) under N_2 atmosphere for 24 h. The solvent was evaporated and the residue was subject to gel filtration on a column of Sephadex G-15 using water as the eluent.

Fractions containing the product were pooled and lyophilized to give compound **8** (190 mg, 96%). ^{1}H NMR (D_2O): δ 5.19 (d, 1H, $J = 3.2$ Hz, H-1), 4.10–4.04 (m, 1H, SCH_2CH_2CHHO), 4.03–3.99 (m, 1H, H-4), 3.96 ~ 3.52 (m, 12H, H-2, H-3, H-5, H-6, H-6$^+$, SCH_2CH_2CHHO, $SCH_2CH_2CH_2O$, and OCH_2CH_3), 3.47 (s, 3H, 2-OCH_3), 3.32–3.24 (m, 6H, $ClHNH_2CH_2CH_2S$), 2.96–2.86 (m, 6H, $SCH_2CH_2NH_2HCl$), 2.76–2.68 (m, 6H, $OCH_2CH_2CH_2S$), 1.98–1.88 (m, 6H, OCH_2CH_2-CH_2S), 1.25 (br t, 3H, $J = 6.7$ Hz, OCH_2CH_3); ES-MS: 574.41 $(M+H-3HCl)^+$.

4.1.7. Ethyl 3,4,6-tri-*O*-(6-(6-maleimidohexanamido)-3-thiahexyl)-2-*O*-methyl-α-D-galactopyranoside (9). A solution of compound **8** (34 mg, 49.8 μmol) in 1 M aqueous solution of $NaHCO_3$ (1.0 mL) was added dropwise to a stirred solution of N-succimidyl 6-maleimidohexanoic acid ester (90 mg, 293 μmol) in THF (3 mL) at 0 °C. The resulting mixture was stirred at 0–5 °C for 1 h, then diluted with $CHCl_3$ (50 mL). The solution was washed with brine and water. The organic phase was dried over Na_2SO_4 and concentrated. The residue was subject to column chromatography (CH_2Cl_2/MeOH 95:5) to give compound **9** (26.01 mg, 46%). R_f 0.58 (CH_2Cl_2/MeOH 90:10); ^{1}H NMR ($CDCl_3$/TMS): δ 6.69 (s, 6H, $CH=CH$), 6.20–6.04 (m, 3H, $NHC(O)$), 4.97 (d, 1H, $J = 3.1$ Hz, H-1), 3.95–3.38 (m, 29H, H-2, H-3, H-4, H-5, H-6, H-6$^+$, NCH_2CH_2-$CH_2CH_2CH_2C(O)$, $(O)CNHCH_2CH_2S$, SCH_2CH_2-CH_2O, 2-OCH_3 and OCH_2CH_3), 2.72–2.58 (m, 12H, $OCH_2CH_2CH_2SCH_2CH_2NH$), 2.18 (t, 6H, $J = 7.3$ Hz, $NCH_2CH_2CH_2CH_2CH_2C(O)$), 1.94–1.80 (m, 6H, OCH_2-CH_2tlsbCH_2S), 1.74–1.54 (m, 12H, NCH_2CH_2-$CH_2CH_2CH_2CH_2C(O)$), 1.39–1.20 (m, 9H, NCH_2CH_2-$CH_2CH_2CH_2C(O)$, OCH_2CH_3); ESI-MS: 1153.63 $(M+H)^+$.

4.1.8. Ethyl 3,4-*O*-isopropylidene-α-D-galactopyranoside (10). To a solution of compound **4** (250 mg, 0.69 mmol) in THF (10 mL) was added tetrabutylammonium fluoride in THF (1 M, 1 mL). The solution was stirred at room temperature for 2 h and the solvent was evaporated. Column chromatography (CH_2Cl_2/MeOH 90:10) of the residue gave compound **10** (168 mg, 98%). R_f 0.53 (CH_2Cl_2/MeOH 90:10); ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 4.91 (d, 1H, $J = 3.0$ Hz, H-1), 4.24–4.20 (m, 2H, H-2, H-3), 4.12–4.44 (m, 1H, H-5), 3.99–3.74 (m, 4H, H-3, H-6, H-6$^+$, and $OCHHCH_3$), 3.66–3.49 (m, 1H, $OCHHCH_3$), 2.55–2.30 (br s, 2H, O*H*-2, and O*H*-6), 1.51 (s, 3H, CCH_3), 1.36 (s, 3H, CH_3C), 1.25 (br t, 3H, $J = 7.0$ Hz, OCH_2CH_3); ES-MS: 271.13 $(M+Na)^+$.

4.1.9. Ethyl 3,4-*O*-isopropylidene-2,6-di-*O*-methyl-α-D-galactopyranoside (11). A solution of compound **10** (248 mg, 1.0 mmol) in anhydrous DMF (5 mL) was added slowly to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 328 mg, 8.2 mmol) in anhydrous DMF (15 mL). After stirring the resulting suspension for 30 min, the reaction mixture was cooled to 0 °C, and iodomethane (0.7 mL) was added. The resulting mixture was stirred for at 0 °C to room temperature for 5 h. After the mixture was cooled with an ice bath, the reaction was quenched by slow addition of MeOH (5 mL). The mixture was concentrated to dryness under reduced pressure. The residue was partitioned between EtOAc and water. The organic layer was washed with diluted HCl, brine, and water, dried over Na_2SO_4, and concentrated. The residue was subject to column chromatography (hexane/EtOAc 70:30) to give compound **11** (243 mg, 88%). R_f 0.34 (hexane/EtOAc 70:30). ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 4.97 (d, 1H, $J = 3.0$ Hz, H-1), 4.26 (dd, 1H, $J = 7.9$, 5.4 Hz, H-3), 4.22–4.14 (m, 2H, H-4, H-5), 3.85–3.70 (m, 1H, $OCHHCH_3$), 3.66 (d, 2H, $J = 4.8$ Hz, H-6 and H-6$^+$), 3.64–3.50 (m, 4H, $OCHHCH_3$, 2-OCH_3), 3.43 (s, 3H, 6-OCH_3), 3.36 (dd, 1H, $J = 7.9$, 3.0 Hz, H-2), 1.54 (s, 3H, CCH_3), 1.34 (s, 3H, CH_3C), 1.25 (br t, 3H, $J = 7.0$ Hz, OCH_2CH_3); ES-MS: 299.17 $(M+Na)^+$.

4.1.10. Ethyl 2,6-di-*O*-methyl-α-D-galactopyranoside (12). A solution of compound **11** (193 mg, 0.70 mmol) in 90% acetic acid was heated at 90 °C for 2 h. The solvent was evaporated and the residue was subject to column chromatography (CH_2Cl_2/MeOH 90:10) to give compound **12** (164 mg, 99%). R_f 0.41 (CH_2Cl_2/MeOH 90:10); ^{1}H NMR (300 MHz, $CDCl_3$/TMS): δ 5.10 (d, 1H, $J = 3.2$ Hz, H-1), 4.10–4.05 (m, 1H, H-4), 3.98–3.90 (m, 2H, H-3, H-5), 3.85–3.72 (m, 1H, $OCHHCH_3$), 3.68 (d, 2H, $J = 4.9$ Hz, H-6 and H-6$^+$), 3.67–3.50 (m, 2H, H-2, and $OCHHCH_3$), 3.49 (s, 3H, 2-OCH_3), 3.42 (s, 3H, 6-OCH_3), 1.26 (br t, 3H, $J = 7.0$ Hz, OCH_2CH_3); ES-MS: 259.28 $(M+Na)^+$.

4.1.11. Ethyl 3,4-di-*O*-allyl-2,6-di-*O*-methyl-α-D-galactopyranoside (13). A solution of compound **12** (142 mg, 0.60 mmol) in anhydrous DMF (8 mL) was added slowly to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 480 mg, 12 mmol) in anhydrous DMF (12 mL). The reaction mixture was stirred at 0 °C for 30 min and allyl bromide (1 mL) was then added dropwise. The resulting mixture was stirred at 0 °C to room temperature for 6 h. The reaction was quenched by slow addition of MeOH (5 mL). The mixture was concentrated to dryness under reduced pressure. The residue was partitioned between EtOAc and diluted HCl. The organic layer was separated and washed with brine and water, dried over Na_2SO_4, and concentrated. Column chromatography (hexane/EtOAc 70:30) of the residue afforded compound **13** (161 mg, 85 %). R_f 0.38 (hexane/EtOAc 70:30); ^{1}H NMR (300 MHz, $CDCl_3$/TMS): 6.02–5.86 (m, 2H, $OCH_2CH{=}CH_2$), 5.36–5.14 (m, 4H, OCH_2 $CH{=}CH_2$), 5.00 (d, 1H, $J = 3.5$ Hz, H-1), 4.39 (dd, 1H, $J = 12.2$, 5.2 Hz, $OCHHCH{=}CH_2$), 4.28–4.06 (m, 3H, $OCH_2CH{=}CH_2$), 3.89 (dd, 1H, $J = 6.4$, 5.9 Hz, H-5), 3.80 (s, 1H, br, H-4), 3.80–3.68 (m, 3H, H-3, H-6, and $OCHHCH_3$), 3.64–3.49 (m, 6H, H-2, H-6$^+$, $OCHHCH_3$, and 2-OCH_3), 3.38 (s, 3H, 6-OCH_3), 1.25 (br t, 3H, $J = 7.0$ Hz, OCH_2CH_3); ES-MS: 339.25 $(M+Na)^+$.

4.1.12. Ethyl 3,4-di-*O*-(6-maleimido-3-thiahexyl)-2,6-di-*O*-methyl-α-D-galactopyranoside dihydrochloride (14). To a solution of compound **13** (110 mg, 0.35 mmol) and AIBN (15 mg) in MeOH (15 mL) in a quartz flask was added cysteamine hydrochloride (227 mg, 2.0 mmol). After being degassed by bubbling N_2 into the solution for 30 min, the solution was stirred and irradiated (UV, 254 nm) under a N_2 atmosphere for 24 h. The solvent was evaporated and the residue was subject to gel filtration on a column of Sephadex G-15 using water as the eluent. Fractions containing the product were pooled and lyophilized to give compound **14** as its hydrochloride (171 mg, 90%). ^{1}H NMR (D_2O): δ 5.19 (d, 1H, $J = 3.2$ Hz, H-1), 4.10–4.04 (m, 1H, SCH_2CH_2CHHO), 4.03–3.98 (m, 1H, H-4), 3.96–3.52 (m, 10H, H-2, H-3, H-5, H-6, H-6$^+$, SCH_2CH_2CHHO, $SCH_2CH_2CH_2O$, and OCH_2CH_3), 3.47 (s, 3H, 2-OCH_3), 3.42 (s, 3H, 6-OCH_3), 3.32–3.24 (m, 4H, ClH-$NH_2CH_2CH_2S$), 2.96–2.86 (m, 4H, $SCH_2CH_2NH_2HCl$), 2.76–2.68 (m, 4H, $OCH_2CH_2CH_2S$), 1.98–1.88 (m, 4H, $OCH_2CH_2CH_2S$), 1.24 (br t, 3H, $J = 6.6$ Hz, OCH_2CH_3); ES-MS: 471.20 $(M+H-2HCl)^+$.

4.1.13. Ethyl 3,4-di-*O*-(6-(6-maleimidohexanamido)-3-thiahexyl)-2,6-di-*O*-methyl-α-D-galactopyranoside (15). A solution of compound **14** hydrochloride (60 mg, 0.11 mmol, 49 μmol) in 1 M aqueous solution of $NaHCO_3$ (1.0 mL) was added dropwise to a stirred solution of N-succimidyl 6-maleimidohexanoic acid ester (101 mg, 0.33 mmol) in THF (3 mL) at 0 °C. The resulting mixture was stirred at 0–5 °C for 1 h, then diluted with $CHCl_3$ (40 mL). The solution was washed with brine and water. The organic layer was dried over Na_2SO_4 and concentrated. The residue was subject to column chromatography (CH_2Cl_2/MeOH 90:10) to give compound **15** (39.5 mg, 42%).Yield: 42%; R_f 0.49 (CH_2Cl_2/MeOH 90:10); ^{1}H NMR ($CDCl_3$/TMS): δ 6.69 (s, 4H, C*H*=C*H*), 6.05 (br s, 2H, N*H*C(O)), 4.98 (d, 1H, $J = 3.1$ Hz, H-1), 3.95–3.41 (m, 23H, H-2, H-3, H-4, H-5, H-6, H-6$^+$, $NCH_2CH_2CH_2CH_2CH_2C(O)$, (O)CNH-$CH_2CH_2S$, $SCH_2CH_2CH_2O$, 2-OCH_3, and OCH_2CH_3), 3.38 (s, 3H, 6-OCH_3), 2.72–2.60 (m, 8H, OCH_2CH_2-$CH_2SCH_2CH_2NH$), 2.18 (t, 4H, $J = 7.3$ Hz, $NCH_2CH_2CH_2CH_2CH_2C(O)$), 1.94–1.80 (m, 4H, $OCH_2CH_2CH_2S$), 1.74–1.54 (m, 8H, $NCH_2CH_2CH_2$-$CH_2CH_2C(O)$), 1.39–1.19 (m, 7H, NCH_2CH_2-$CH_2CH_2CH_2C(O)$, OCH_2CH_3); ESI-MS: 857.46 $(M+H)^+$.

4.2. Synthesis of multivalent gp41 peptides—General procedures

Peptide DP178-Cys was dissolved in a mixed solvent of phosphate buffer (50 mM, pH 6.6) and acetonitrile (1:1, v/v) to make a 5 mg/mL solution. A solution (2 mg/mL) of the synthetic maleimide cluster (**2** or **9** or **15**) in DMF was added to the peptide solution. Generally, 1.5-molar equivalent of the peptide was used to react with 1-molar equivalent maleimide functionality. The resulting mixture was gently shaken at room temperature for 2 h. The solution was lyophilized and the product was purified by reverse-phase HPLC.

Tetravalent peptide MVP-1: yield, 91%; ESI-MS: 1653.72 $(M+12H)^{12+}$, 1526.60 $(M+13H)^{13+}$, 1417.63 $(M+14H)^{14+}$, 1323.20 $(M+15H)^{15+}$.

Trivalent peptide TVP-1: yield 95%; ESI-MS: 2135.70 $(M+7H)^{7+}$, 1868.98 $(M+8H)^{8+}$, 1661.43 $(M+9H)^{9+}$, 1495.23 $(M+10H)^{10+}$, 1359.47 $(M+11H)^{11+}$, 1246.31 $(M+12H)^{12+}$, 1150.49 $(M+13H)^{13+}$.

Bivalent peptide BVP-1: yield 88%; ESI-MS: 2011.08 $(M+5H)^{5+}$, 1675.98 $(M+6H)^{6+}$, 1436.80 $(M+7H)^{7+}$, 1257.36 $(M+8H)^{8+}$, 1117.81 $(M+9H)^{9+}$, 1006.04 $(M+10H)^{10+}$, 914.76 $(M+11H)^{11+}$.

4.3. Circular dichroism (CD)

Multivalent gp41 peptides (MVP-1, TVP-1, and BVP-1) and peptide DP178 were dissolved in a phosphate buffer (50 mM, pH 7.2) at concentrations ranging from 4–40 μM. The actual concentration of the peptides was measured by UV absorption and calculated according to the proposed formulation based on the absorbance of Trp residues at 280 nm.[26] The CD spectra were measured at 23 °C and an average of three scans was taken. Alpha-helical content was calculated based on the mean residue ellipticity $[\theta]_{222}$ and the proposed equation.[24]

4.4. Immunization of mice

Groups of five female BALB/c mice (8–10 weeks old from Jackson Laboratory) were immunized intraperitoneally with 10 μg each of the synthetic peptide immunogen. The mice were boosted with the same quantity (10 μg) of individual immunogen at days 14 (first boost), 28 (second boost), and 90 (third boost). The immunogens were formulated in a phosphate-buffered saline (PBS) (10 μg/200 μL for each injection) without any adjuvant. Blood was drawn from the retro-orbital plexus 10 days after each boost. Sera from the same group were pooled and used for detecting epitope-specific antibodies by enzyme-linked immunosorbent assay (ELISA).

4.5. Enzyme-linked immunosorbent assay (ELISA)

To detect DP178-specific antibody, microtiter ELISA plates were coated with 8 μg/mL Neutravidin (streptavidin) in PBS and incubated at 4 °C overnight. After washings with PBS/0.5% Tween-20, nonspecific binding was blocked with 5% (w/v) BSA in PBS at room temperature for 1 h. Plates were then washed and 2 μg/mL of biotinylated DP178 in 1% BSA was added. Plates were incubated at 37 °C for 1 h. The plates were again washed and mouse serum was added in a 1:2 or 1:3 serial dilution in 1% BSA/PBS. The plates were incubated at 37 °C for 2 h and washed. To the plates was added a solution of 1:2000 diluted horseradish peroxidase (HRP)-conjugated

goat anti-mouse IgG. After incubation for 1 h at 37 °C, the plates were washed again and a solution of $3,3^{+},5,5^{+}$-tetramethyl benzidine (TMB) was added. Color was allowed to develop for 5 min, and the reaction was then quenched by adding a solution of 0.5 M H_2SO_4 to each well. The optical density was then measured at 450 nm. To detect gp41-specific antibody in the serum, the plates were coated with 1 μg/mL gp41 in PBS at 4 °C overnight. After washing, blocking, and washing, the plates were incubated with a series dilution of the mouse serum. The antibody titers were then measured as described above. Antibody titers were calculated as reciprocals of the highest dilutions, which gave optical density values at least twofold above the negative control.

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