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Cholic acid as template for multivalent peptide assembly

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Cholic acid, an amphiphilic steroid containing several selectively addressable functionalities, was exploited as a rigid template for multivalent peptide assembly. Thus, cholic acid-based templates suitable for chemoselective peptide ligation were synthesized, in which maleimide or bromoacetyl moieties were selectively introduced at the 3α, 7α, 12α-positions of cholic acid with varied length of linkers. Three peptides were chosen and tested for the chemoselective ligation. These include the HIV-1 peptide inhibitor DP178, the universal T-helper epitope derived from tetanus toxoid (830–844), and the minimum epitope sequence of the HIV-neutralizing antibody 2F5. It was found that the maleimide-functionalized templates are highly efficient for the ligation of all the peptides, while bromoacetyl templates led to low yield of ligation. Circular dichroism (CD) spectroscopic studies of the multivalent peptides (**10a** and **11a**) containing three strands of peptide DP178 indicate that the template-assembled peptides form three α-helix bundles with significantly enhanced α-helix contents than the single peptide. The results suggest that cholic acid is a valuable template for constructing α-helix bundles that may be useful as mimics of conformational epitopes for vaccine development.

Introduction

Template assembled multivalent peptides have been developed predominately for two applications: as novel artificial proteins to study the factors governing protein folding and stability,[1,2] and as chemically defined immunogens for vaccine development.[3,4] Enhanced inhibitory activities of peptide inhibitors were also attained through multivalent peptide assembly.[5–9] So far, several types of cyclic molecules have been exploited as templates for multivalent peptide assembly. These include cyclic peptide and derivatives,[10–13] porphyrin molecules,[14,15] calix[4]arene core,[7,16] cavitand macrocycles,[17,18] and monosaccharides.[19–25] New types of templates are demanded in order to control the topology of assembled peptides to achieve novel properties of the assembled peptides.

We have previously prepared monosaccharide-centered maleimide clusters and used them for the synthesis of multivalent HIV-1 gp41 peptides.[25] The long term goal of our template-assembled peptide project is to develop mimics of the trimeric gp41 fusion intermediates to serve as vaccines and inhibitors for blocking HIV-1 infection.[26–28] We report in this paper the use of cholic acid as a new template for multivalent peptide assembly. Cholic acid is a readily available, rigid, and facial amphiphilic molecule. The hydrophilic face contains 3 hydroxy groups at 3α, 7α, and 12α positions with defined spatial orientations and the other face consisting of the methyl groups and the backbone is hydrophobic. Because of its unique molecular property, cholic acid has been exploited for the design of artificial ion channel,[29,30] for drug targeting,[31,32] and as scaffold for the assembly of combinatorial libraries.[33–35] We demonstrate here the modification of cholic acid as a template for multivalent peptide assembly. We found that cholic acid-based maleimide clusters are highly efficient for chemoselective ligation of cysteine-containing peptides. Moreover, assembly of three strands of the potent HIV-1 inhibitor DP178, a 36-mer peptide,[36,37] on the cholic acid template led to the formation of a three-α-helix bundle structure, which may serve as a mimic of trimeric α-helical transition states of HIV-1 gp41 molecule during viral membrane fusion.

Results and discussion

Synthesis of functionalized templates

For chemoselective ligation to cysteine-containing peptides, the hydroxy groups of cholic acid (**1**) were selectively modified to introduce thiol-reactive functionality such as maleimide and bromoacetyl groups. The synthesis is summarized in Scheme 1. The preparation of the 3α,7α,12α-tri-O-allyl intermediate **4** was previously reported starting from methyl cholate.[38] We used a modified procedure for the synthesis. Instead of using methyl cholate as starting material, cholic acid was directly reduced with $LiAlH_4$ in THF to give the tetra-hydroxycholane **2** in 98% yield. Reaction of **2** with trityl chloride gave the trityl-protected compound **3**. Initial allylation of **3** with allyl bromide/NaH in THF according to the literature[38] was met with less success, leading to a mixture of mono-, di-, and tri-allylated derivatives even under reflux for 3 days with excess reagents. However, replacement of the allyl bromide with allyl iodide under the same conditions resulted in a quick and complete allylation of the three hydroxy groups, from which the tri-O-allyl derivative **4** was isolated in almost quantitative yield. Compound **4** was then subjected to photo-addition with cysteamine in the presence of initiator AIBN in MeOH under UV irradiation (254 nm).[39] The reaction was monitored by TLC and NMR. It was found that even after 6 days of reaction, only a small portion of the tri-allyl derivative **4** was converted into a mixture of the corresponding mono-, di-, and tri-amines with simultaneous de-O-tritylation of the starting material. It is likely that the strong UV absorbing trityl group might inhibit the radical reaction. To avoid this, we removed the trityl group to provide **5** before performing the photo-addition reaction. As a result, the photo-addition of cysteamine to the allyl groups of **5** proceeded rapidly and efficiently to give the tri-amine **6** in excellent yield (Scheme 1).

Starting from the intermediate tri-amine **6**, several templates suitable for chemselective peptide ligation were prepared. Thus, the three amino groups in **6** were directly converted into maleimide groups through reaction with methoxycarbonyl-maleimide to afford the trivalent maleimide cluster **7** in 78% yield. On the other hand, reaction of **6** with 6-maleimido-

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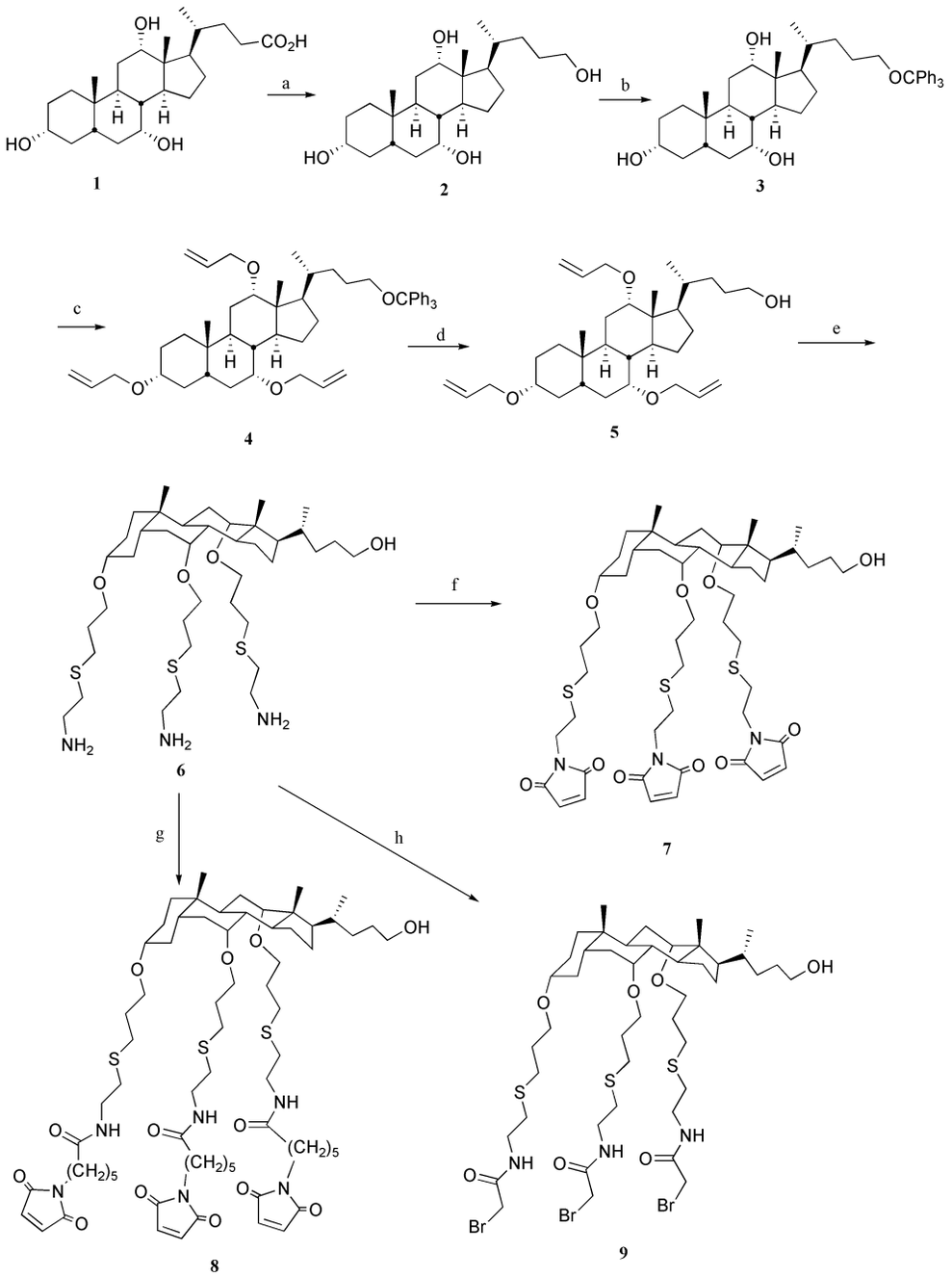
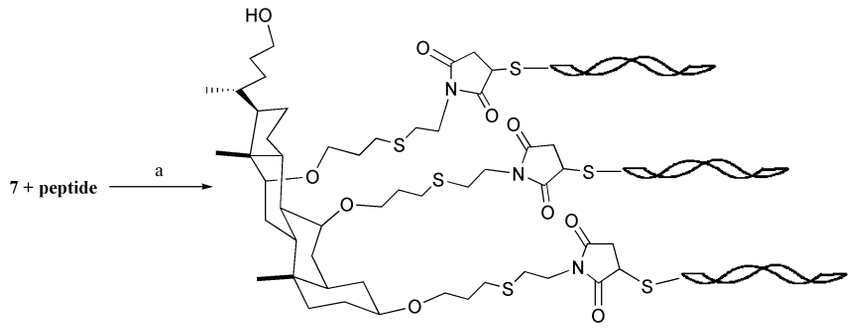


Scheme 1 Reagents and conditions: (a) $LiAlH_4$, THF, 0 °C to rt, 98%; (b) trityl chloride, Et_3N, DBU, DMF, r.t., 93%; (c) allyl iodide, NaH, THF, r.t. to 70 °C, 96%; (d) *p*-toluenesulfonic acid, DCM, MeOH, 100%; (e) 2-aminoethanethiol hydrochloride, ABIN, MeOH, UV (254 nm), r.t., 96%; (f) *N*-methoxycarbonylmaleimide, Et_3N, DMF, r.t., 78%; (g) 6-maleimidohexanoic acid *N*-hydroxylsuccinimide ester, DCM, r.t., 76%; (h) bromoacetic anhydride, DCM, r.t., 89%.

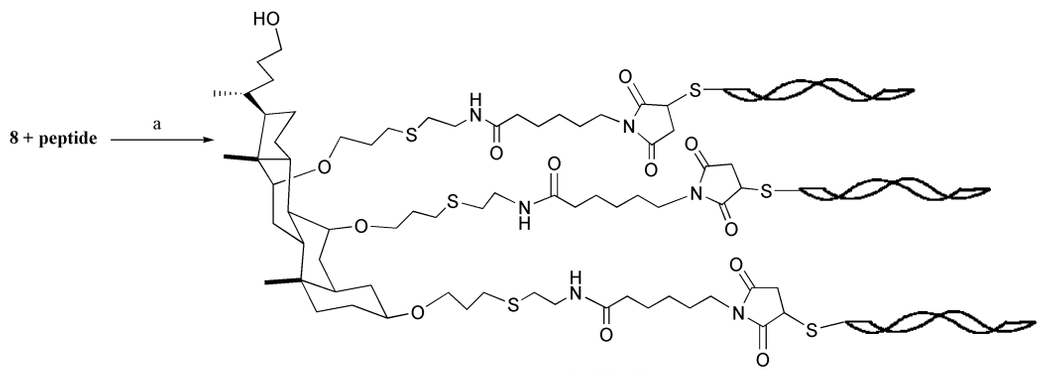
hexanoic acid *N*-hydroxylsuccinimide ester gave another trivalent maleimide cluster, compound **8**, with a longer spacer between the maleimide group and the cholic acid core. In addition, a bromoacetyl group was introduced at each amino group in **6** through *N*-bromoacetylation to give the bromoacetyl derivative **9**, which was prepared for the comparison of the efficiency in ligation (Scheme 1).

Chemoselective ligation of peptides to the cholic acid templates

To examine the chemoselective ligation, three antigenic peptides were chosen and tested. These include the potent HIV inhibitor DP178,[36,37] a T-helper epitope from tetanus toxoid (830–844),[40] and a minimum epitope sequence ELDKWA for HIV-neutralizing antibody 2F5.[41] For ligation, a cysteine residue was introduced at either the C- or N-terminus of the peptides during solid phase synthesis to give the Cys-containing peptides: P37C, T-helper, and P7C, respectively. In the case of the T-helper sequence, a tetra-peptide spacer GSSS was introduced at the N-terminus to increase the aqueous solubility of the otherwise hydrophobic T-helper epitope. It was observed that the ligation between the maleimide cluster **7** and the peptide P37C or the T-helper peptide proceeded very rapidly in a MeCN–phosphate buffer (1 : 1, v/v, pH 7.0) to give the trivalent peptides **10a** and **10b** in 82 and 89% yields, respectively (Scheme 2). Similarly, peptide ligation using the maleimide cluster **8** with a longer spacer afforded the trivalent peptides **11a** and **11b** in 83 and 91% yields, respectively (Scheme 2). Regardless of the length and complexity of the peptides and maleimide clusters used, all the ligation was complete within 3 hours under the above conditions. The desired multivalent peptides were purified by reverse phase HPLC and characterized by electron spray ionization mass spectrometry (ESI-MS). The typical ESI-MS and HPLC profiles of the purified multivalent peptides **10a** and



10a: peptide = **P-37C**, 82%
10b: peptide = **T-helper**, 89%



11a: peptide = **P-37C,** 83%
11b: peptide = **T-helper**, 91%

peptide =

P-37C: Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFC-NH_2
T-helper: CGSSSQYIKANSKFIGITEL-NH_2

Scheme 2 Reagents and conditions: (a) phosphate buffer (pH 6.6)/MeCN (1 : 1, v/v), r.t.

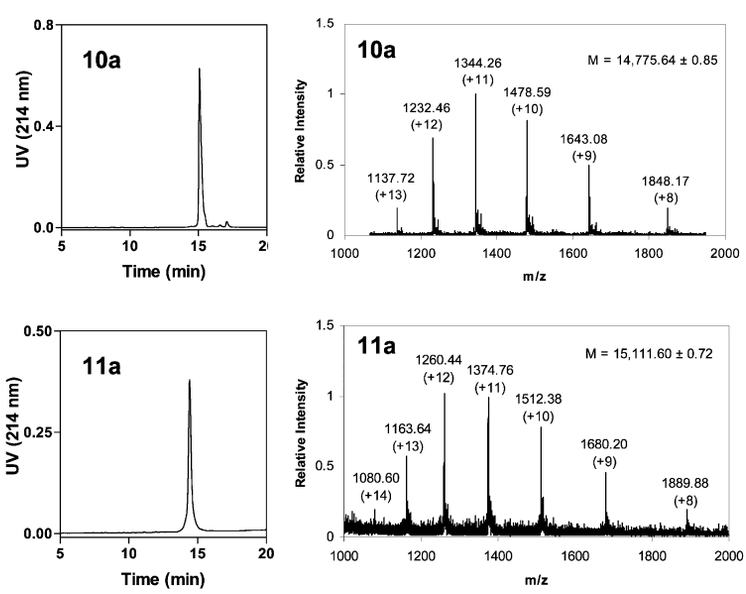
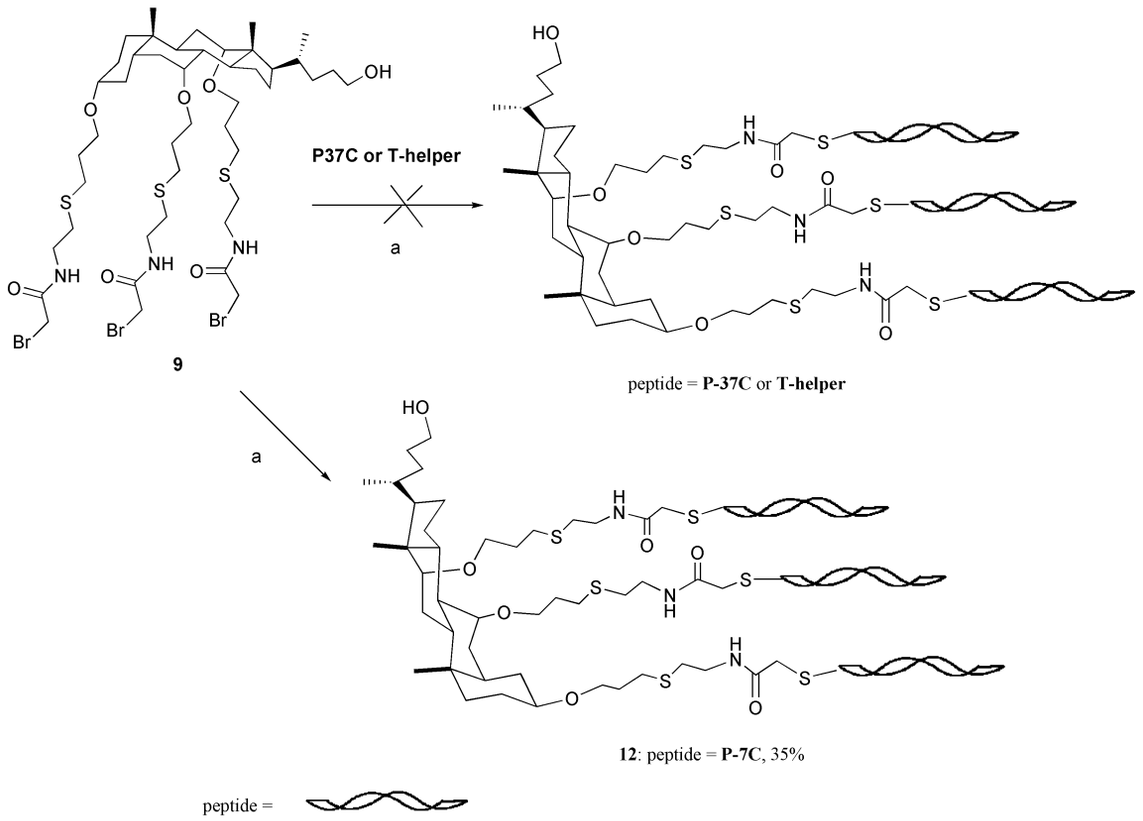


Fig. 1 HPLC and ESI-MS profiles of trivalent peptides **10a** and **11a**.

11a are shown in Fig. 1. Together with the HPLC data, the ESI-MS spectra showed the high purity and verified the correct primary structure of the synthetic artificial proteins.

Next, we examined the ligation using the bromoacetyl derivative **9**. Ligation between haloacetyl moiety and thiol functionality was used previously for constructing template-assembled multivalent peptides.[42,43] However, we found that neither the longer peptide P37C nor the medium-sized peptide T-helper gave the desired fully substituted product even when an excess amount (6-fold) of the cysteine-containing peptide was used in a mixed solvent (MeCN–borate buffer, pH 8.5). A mixture of mono-substituted and trace di-substituted ligation products were revealed by HPLC-MS analysis, together with the dimerization of the cysteine-containing peptide under the alkaline condition (data not shown). The reaction intermediates were not purified for further characterization. When a short peptide



P-37C: Ac-YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWFC-NH_2
T-helper: CGSSSQYIKANSKFIGITEL-NH_2
P7C: Ac-ELDKWAC-NH_2

Scheme 3 (a) Borate buffer (pH 8.5)/MeCN (1 : 1, v/v), r.t.

Ac-ELDKWAC-NH_2 (P3C) was used to react with the bromoacetyl template **9** under the above ligation condition, the desired trivalent peptide product **12** was formed together with several mono- and di-substituted by-products, from which **12** was isolated in 35% yield (Scheme 3). The results implicate a significant difference in the reactivity between the bromoacetyl and maleimide groups toward thiol functionality. The results again demonstrate that maleimide clusters are particularly useful for constructing very large and complex multivalent peptides through the highly efficient thiol–maleimide ligation.[25]

Conformational studies

One goal of our project is to create three-α-helix bundle structures to mimic the trimeric fusion intermediates of the HIV-1 gp41 molecule. It is known that template-assembled construction may induce α-helix bundle formation when a suitable template is applied.[1,2,23] The 36-mer peptide DP178 was derived from the C-terminal ectodomain of gp41, which is supposed to expose as a trimeric α-helical intermediate during viral membrane fusion.[27,28] Nevertheless, free peptide DP178 exists in solution in an almost random structure, with only 10–20% α-helical content.[44] Therefore, it will be interesting to investigate whether the cholic acid-assembled peptides adopt an enhanced α-helical structure, as exemplified by some other template-assembled peptides.[1,2,23] The circular dichroism (CD) spectra of the cholic acid-based trivalent peptides **10a** and **11a**, together with peptide DP178, were measured at 23 °C in a phosphate buffer (pH 7.2). As can be seen in Fig. 2, both the trivalent peptides **10a** and **11a** showed a significant degree of α-helicity, while the DP178 showed a typical random structure in the buffer. The content of α-helix was calculated to be 36, 41, and 18% for **10a**, **11a**, and DP178, respectively, based on their mean residue ellipticity $[\theta]_{222}$ and the proposed formula.[45] The trivalent peptide (**11a**) with a relatively long spacer between the peptide strand and the cholic acid core showed slightly higher α-helical content than the one with a relatively short spacer (**10a**). Since an accurate calculation of the α-helical content relies on the precise determination of peptide concentration, we carefully quantified the peptide concentration through UV absorbance at 280 nm because of the presence of several tryptophan residues in the peptide sequence. The α-helical content of DP178 in the buffer is in good agreement with the reported value.[44] It was also found that the CD spectra of **10a**, **11a**, and DP178 were concentration-independent in the range of 2–20 μM (data not shown). The results clearly suggest that three-α-helix-bundle

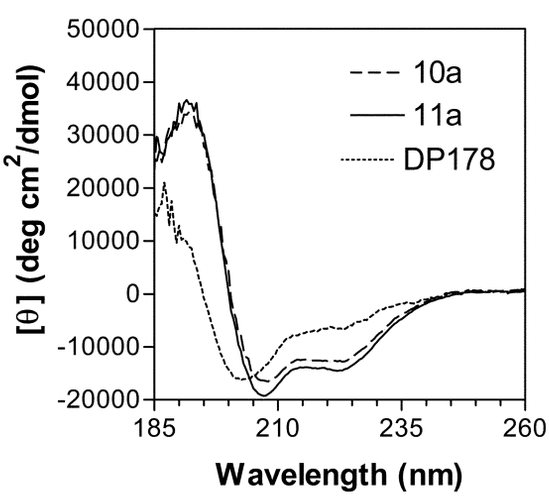


Fig. 2 CD spectra of peptide DP178 and the trivalent peptides **10a** and **11a**. The spectra were recorded at 5, 4, and 4 uM for peptides DP178, **10a**, and **11a**, respectively, in a phosphate buffer (50 mM, pH 7.2) at 23 °C.

structure was formed when three strands of the HIV peptides were installed on the cholic acid templates.

Conclusion

Cholic acid was exploited as a new type of template for multivalent peptide assembly. The results suggest that cholic acid-centered maleimide clusters are highly efficient for the construction of large and complex multivalent peptides. The cholic acid template allowed α-helix-bundle formation when a suitable peptide such as HIV-1 gp41 peptide DP178 was assembled. The resulting three-α-helix bundles of DP178 may mimic the conformational epitopes of gp41 that are exposed during viral membrane fusion, which should be useful for HIV-1 vaccine development.

Experimental

General

All Fmoc-protected amino acids used for peptide synthesis were purchased from Novabiochem. HATU, DIPEA and Fmoc-PAL-PEG-PS were purchased form Applied Biosystems. HPLC grade acetonitrile was purchased from Fisher Scientific. DMF was purchased from B & J Biosynthesis. All other chemicals were purchased from Aldrich/Sigma and used as received. ^{1}H and ^{13}C NMR spectra were recorded on QE 300 or Inova 500 with Me_4Si (δ 0) 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 F254 (E. 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 with silica gel 60 (EM Science, particle size 0.040–0.063 mm, 230–400 mesh). 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 acetonitrile (10–90%) containing 0.1% TFA in 20 min at a flow rate of 1 mL min^{-1}. Peptides were detected by UV absorbance at 214 and/or 280 nm. Preparative HPLC was performed with a Waters 600 HPLC instrument with 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.[46] CD spectra were measured on JASCO-810 (CD-ORD) spectropolarimeter using quartz cuvette of 1 mm path length at 23 °C.

3α,7α,12α,24-Tetrahydroxycholane (2). To a suspension of $LiAlH_4$ (3.85 g, 100 mmol) in dry THF (100 mL) at 0 °C was added dropwise a solution of cholic acid **1** (15.03 g, 35 mmol) in dry THF (200 mL) with vigorous stirring under nitrogen atmosphere. The reaction mixture was then stirred at room temperature overnight. Celite (3.00 g) was added to the sluggish mixture. The precipitate was removed *via* filtration and washed with hot methanol. The filtrate was concentrated, and the product was crystallized with acetone to compound **2** as a white solid (13.20 g, 98%): m.p. 238–239 °C (lit.,[38] m.p. 236–238 °C); ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 3.96 (bs, 1H, HO–C(12)), 3.82 (bs, 1H, HO–C(7)), 3.79 (d, 2H, J = 2.5 Hz, H_2–C(24)), 3.25 (b, 1H, HO–C(24)), 3.21 (m, 1H, H–C(12)), 3.15 (m, 1H, H–C(7)), 3.07 (m, 1H, H–C(3)), 2.15–1.10 (series of multiplet, 25H), 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)); ESI-MS: 395.58 $(M + H)^+$.

3α,7α,12α-Trihydroxy-24-trityloxy-5β-cholane (3). To a solution of compound **2** (6.23 g, 15.8 mmol) in DMF (100 mL) were added trityl chloride (6.60 g, 23.7 mmol), Et_3N (10 mL), and DBU (0.90 g, 5.9 mmol) in sequence with stirring at room temperature. After 24 h, water (400 mL) was added, and the mixture was stirred vigorously for 2 h. The precipitate was collected *via* filtration and washed with acetone. The crude product was re-crystallized in acetone to give **3** (9.35 g, 93%) as a white solid: m.p. 206–208 °C (lit.,[38] m.p. 187 °C); ^{1}H NMR (300 MHz, $CDCl_3$/TMS) δ 7.43 (d, 6H, J = 7.3 Hz, phenyl H-2, H-6), 7.29–7.18 (m, 9H, phenyl H-3, H-4, H-5), 3.96 (bs, 1H, H–C(12)), 3.82 (bs, 1H, H–C(7)), 3.41 (m, 1H, H–C(3)), 3.00 (m, 2H), 2.92 (s, 2H), 2.87 (s, 2H), 2.19 (m, 2H), 2.04 (s, 1H), 1.98–1.00 (series of multiplet, 16H), 0.96 (d, 3H, J = 6.1 Hz, H_3C–C(20)), 0.86 (s, 3H, H_3C–C(10)), 0.64 (s, 3H, H_3C–C(13)).

3α,7α,12α-Triallyloxy-24-trityloxy-5β-cholane (4). Sodium hydride (60% in mineral oil, 2.00 g, 29.2 mmol) was added in portions to a solution of compound **3** (3.12 g, 4.8 mmol) in dry THF (50 mL) under N_2, and stirred for 2 h at room temperature. Then allyl iodide (4.98 g, 30.0 mmol) was added dropwise and the mixture was heated at 70 °C with stirring for 4 h. After having cooled to room temperature, the reaction mixture was partitioned between ethyl acetate (50 mL) and water (30 mL). The organic layer was separated and washed with H_2O (2 × 20 mL) and brine, and dried over anhydrous Na_2SO_4, filtered, and concentrated under vacuum. The residue was subject to column chromatography on silica gel with hexane/ethyl acetate (90 : 10, v/v) as the eluent to give **4** (3.49 g, 96%) as a pale yellow syrup. ^{1}H NMR (300 MHz, $CDCl_3$/TMS) δ 7.44 (d, 6H, J = 7.1 Hz, benzyl H-2, H-6), 7.29–7.20 (m, 9H, benzyl H-3, H-4, H-5), 5.88 (m, 3H, *H*C=CH_2), 5.23 (m, 3H, H*H*C=CHC), 5.11 (m, 3H, *H*HC=CHC), 4.13 (m, 4H, O*H*$_2$CHC=CH_2, (C-7, C-12)), 3.99 (bs, 2H, O*H*$_2$CHC=CH_2, C-3), 3.88–3.50 (series of multiplet, 6H), 3.35 (t, 2H, J = 8.3 Hz, H_2–C(24)), 3.32 (bs, 1H, H–C(12)), 3.16 (m, 1H, H–C(7)), 3.03 (m, 1H, H–C(3)), 3.07 (m, 1H, H–C(3)), 2.30–1.02 (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)).

3α,7α,12α-Triallyloxy-24-hydroxy-5β-cholane (5). *p*-Toluenesulfonic acid (45.0 mg, 0.23 mmol) was added to a solution of compound **4** (1.20 g, 1.6 mmol) in a mixed solvent of dichloromethane (40 mL) and EtOH (10 mL). The mixture was stirred at room temperature overnight, then diluted with dichloromethane (20 mL). The mixture was washed with 0.5 $NaHCO_3$ (15 mL), H_2O (2 × 15 mL) and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated under vacuum. The residue was subject to flash column chromatography on silica gel with hexane/ethyl acetate (70 : 30, v/v) as the eluent to give **5** (0.83 g, 100%) as a colorless syrup. ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 5.91 (m, 3H, *H*C=CH_2), 5.25 (m, 3H, H*H*C=CHC), 5.11 (m, 3H, *H*HC=CHC), 4.07 (d, 2H, J = 12.5 Hz, O*H*$_2$CHC=CH_2, (C-12)), 4.00 (d, 2H, J = 5.5 Hz, O*H*$_2$CHC=CH_2, (C-7)), 3.77 (dt, 1H, J = 7.5, 5.0 Hz, H*H*–C(24)), 3.71 (ddd, 1H, J = 6.0, 4.5, 1.5 Hz, *H*H–C(24)), 3.61 (b, 2H, O*H*$_2$CHC=CH_2, (C-3)), 3.54 (bs, 1H, H–C(12)), 3.32 (d, 1H, J = 2.5 Hz, H–C(7)), 3.13 (m, 1H, H–C(3)), 2.26 (dd, 1H, J = 13.0, 12.0 Hz, H–C(11)), 2.18 (ddd, 1H, J = 8.0, 8.0, 4.0 Hz, H–C(8)), 2.01 (dd, 1H, J = 10.0, 9.5 Hz, H–C(11)), 1.87–0.96 (series of multiplet, 24H), 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) 136.26, 116.45, 115.69, 95.00, 80.98, 79.34, 75.07, 68.58, 69.47, 68.93, 63.93, 63.90, 46.58, 46.55, 42.81, 42.25, 42.23, 40.03, 35.76, 35.62, 35.60, 35.24, 35.23, 32.02, 29.69, 29.11, 28.22, 27.86, 27.69, 23.44, 23.22, 18.03, 12.79; ESI-MS calcd. for $C_{33}H_{54}O_4$ (M): 514.40; Found: 515.57 $(M + H)^+$, 457.52 $(M - 58 + H)^+$, 399.47 $(M - 2 \times 58 + H)^+$, 341.47 $(M - 3 \times 58 + H)^+$.

3α,7α,12α-Tri-(6-amino-3-thiahexyoxyl)-24-hydroxy-5β-cholane (6). To a mixture of compound **5** (300 mg, 0.58 mmol) and 2-aminoethanethiol hydrochloride (397 mg, 3.50 mmol) in methanol (20 mL) in a Quartz flask was added AIBN (9.4 mg, 0.057 mmol). The solution was degassed by bubbling N_2, and irradiated by UV (254 nm) with stirring under N_2 for 20 h when ^{1}H NMR revealed that no residue allyl group existed. Dichloromethane (30 mL) was added and the mixture was washed with 0.5 M $NaHCO_3$ (2 × 10 mL), H_2O (2 × 5 mL) and brine. The organic layer was dried over anhydrous $NaSO_4$ and concentrated under vacuum. The residue was subject to flash column chromatography on silica gel with ethyl acetate/methanol (0 : 100 to 100 : 0, v/v) as the eluent to give **6** (415 mg, 96%) as a syrup. ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 3.74 (m, 1H, H–C(24)), 3.68 (m, 1H, H–C(24)), 3.61 (m, 2H, OCH_2CCH$_2$CH$_2$S), 3.56 (bs, 2H, OCH_2-CCH$_2$CH$_2$S), 3.53 (t, 2H, J = 7.0 Hz, OCH_2CCH$_2$CH$_2$S), 3.35 (m, 7H, CH$_2$CH_2NH$_2$, HO–C(24)), 3.22 (m, 1H, H–C(12)), 3.16 (m, 7H, NCH$_2$CH_2S, H–C(7)), 3.06 (m, 1H, H–C(3)), 2.86 (m, 6H, OCH$_2$CH$_2$CH_2S), 2.74 (m, 3H, OCH$_2$CHHCH$_2$S), 2.68 (t, 3H, J = 7.0 Hz, OCH$_2$CHHCH$_2$S), 2.20–1.05 (series of multiplet, 30H), 0.98 (d, 3H, J = 6.0 Hz, H$_3$C–C(20)), 0.94 (s, 3H, H$_3$C–C(10)), 0.72 (s, 3H, H$_3$C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$/TMS) 80.82, 79.95, 76.15, 66.19, 65.82, 62.47, 47.36, 46.46, 46.29, 42.86, 42.10, 40.64, 39.87, 38.91, 38.86, 35.72, 35.45, 35.12, 34.82, 32.11, 30.14, 29.93, 29.83, 28.93, 28.79, 28.61, 28.54, 28.47, 28.33, 28.00, 27.65, 27.59, 23.32, 22.72, 22.16, 17.52, 17.13, 14.47, 11.75; ESI-MS calcd. for $C_{39}H_{75}N_3O_4S_3$ (M): 745.48. Found: 746.58 $(M + H)^+$, 373.98 $(M + 2H)^{2+}$.

3α,7α,12α-Tri-(6-maleimido-3-thiahexyoxyl)-24-hydroxy-5β-cholane (7). To a solution of free amine **6** (10 mg, 13 μmol) in DMF (10 mL) were added *N*-methoxycarbonylmaleimide (20 mg, 0.13 mmol) and Et_3N (1.0 mL) dropwise with stirring. After 6 h, the reaction mixture was diluted with ethyl acetate (20 mL) and washed with H_2O (2 × 20 mL) and brine. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered, and concentrated. The residue was subject to column chromatography on silica gel with ethyl acetate/hexane (5 : 95, v/v) as the eluent to provide **7** (10 mg, 78%) as a pale-yellow oil. ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 6.73 (s, 6H, OCHC=CH), 3.79 (d, 2H, J = 2.5 Hz, H$_2$–C(24)), 3.74 (m, 6H, CH$_2$CH_2-NCO), 3.68–3.57 (m, 6H, OCH_2CCH$_2$CH$_2$S), 3.57–3.42 (m, 6H, SCH_2CH$_2$N), 3.22 (b, 1H, HO–C(24)), 3.20 (m, 1H, H–C(12)), 3.17 (m, 1H, H–C(7)), 3.05 (m, 1H, H–C(3)), 2.72 (m, 6H, OCH$_2$CH$_2$CH_2S,), 2.69–2.60 (m, 6H, OCH$_2$CH_2CH$_2$S), 2.15–0.98 (series of multiplet, 24H), 0.92 (d, 3H, J = 6.5 Hz, H$_3$C–C(20)), 0.88 (s, 3H, H$_3$C–C(10)), 0.65 (s, 3H, H$_3$C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$/TMS) 187.05, 176.13, 170.73, 170.72, 170.75, 166.02, 160.98, 157.37, 156.08, 155.85, 134.12, 134.27, 134.30, 134.36, 135.06, 136.6, 104.75, 102.52, 99.57, 94.82, 94.80, 93.96, 93.91, 91.26, 90.05, 85.27, 60.01, 57.73, 50.85, 49.91, 48.23, 37.06, 37.02, 37.10, 36.13, 36.01, 36.00, 34.78, 34.66, 34.63, 34.29, 29.14; 29.10, 29.00, 25.67, 25.31, 24.67, 19.22, 18.55, 18.08, 11.86. ESI-MS calcd. for $C_{51}H_{75}N_3O_{10}S_3$ (M): 985.45. Found: 986.84 $(M + H)^+$, 771.73 $(M - 215 + H)^+$, 556.59. $(M - 2 \times 215 + H)^+$.

3α,7α,12α-Tri-[6-(6-maleimidohexanamido-3-thiahexyoxyl)]-24-hydroxy-5β-cholane (8). To a solution of free amine **6** (7.2 mg, 9.6 μmol) in dichloromethane (10 mL) was added 6-maleimidohexanoic acid *N*-hydroxylsuccinimide ester (30 mg, 96 μmol). The mixture was stirred at room temperature for 3 h and the solvent was evaporated under vacuum. The residue was subject to column chromatography on silica gel with ethyl acetate/methanol (95 : 5, v/v) as the eluent to afford **8** (9.6 mg, 76%) as a colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 6.72 (s, 6H, HC=CH), 6.61 (t, 1H, J = 6.5 Hz, HN), 6.48 (t, 1H, J = 6.5 Hz, HN), 6.19 (t, 1H, J = 6.5 Hz, HN), 3.59 (td, 2H, J = 13.0, 6.5 Hz, H$_2$–C(24)), 3.52 (t, 6H, J = 7.0 Hz, OCH_2CCH$_2$CH$_2$S), 3.47 (bs, 6H, CH$_2$CH_2NCO) 3.43 (m, 6H, NCH_2CH$_2$S), 3.25 (b, 1H, HO–C(24)), 3.21 (m, 1H, H–C(12)), 3.15 (m, 1H, H–C(7)), 3.07 (m, 1H, H–C(3)), 2.66–2.59 (m, 12H, OCH$_2$CH$_2$CH_2S, SCH_2-CH$_2$N), 2.18 (m, 6H, NCOCH_2CH$_2$), 2.15–1.06 (series of multiplet, 48H), 0.92 (d, 3H, J = 6.5 Hz, H$_3$C–C(20)), 0.89 (s, 3H, H$_3$C–C(10)), 0.65 (s, 3H, H$_3$C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$/TMS) 182.11, 178.58, 175.72, 174.17, 174.22, 165.45, 165.50, 164.76, 164.58, 163.88, 163.59, 138.62, 138.61, 137.85, 136.67, 136.20, 135.96, 86.51, 75.43, 72.47, 67.51, 67.29, 66.91, 63.38, 49.95, 48.27, 45.13, 45.91, 46.11, 43.35, 41.56, 40.88, 41.09, 41.82, 39.45, 37.76, 36.58, 35.33, 34.91, 34.96, 34.54, 34.57, 34.58, 34.30, 34.37, 34.89, 33.52, 33.62, 32.28, 31.17, 30.18, 29.18, 29.23, 29.56, 28.85, 28.63, 28.83, 28.12, 27.91, 27.03, 26.41, 26.68, 26.76, 25.83, 25.67, 25.31, 24.67, 17.89, 11.76; ESI-MS calcd. for $C_{69}H_{108}N_6O_{13}S_3$ (M): 1325.70; found: 1348.09 $(M + Na)^+$, 1326.17 $(M + H)^+$, 663.82 $(M + 2H)^{2+}$.

3α,7α,12α-Tri-[6-(2-bromoacetylamido-3-thiahexyoxyl)]-24-hydroxy-5β-cholane (9). To a solution of free amine **6** (12 mg, 16 μmol) in dichloromethane (10 mL) was added bromoacetic anhydride (17 mg, 64 μmol). The mixture was stirred at room temperature for 3 h when TLC showed that no more free amine remained in the reaction mixture. The solvent was removed under vacuum. The residue was subject to column chromatography on silica gel with ethyl acetate/hexane (60 : 40, v/v) as the eluent to give **9** (16 mg, 89%) as a pale white oil. ^{1}H NMR (500 MHz, $CDCl_3$/TMS) δ 4.04 (m, 1H, H–C(24)), 4.00 (m, 1H, H–C(24)), 3.93 (m, 6H, OCH_2CCH$_2$CH$_2$S), 3.89 (m, 6H, CH$_2$CH_2NH), 3.61 (m, 6H, OCH$_2$CH$_2$CH_2S), 3.32 (m, 1H, H–C(12)), 3.25 (b, 1H, HO–C(24)), 3.14 (m, 7H, NCH$_2$CH_2S, H–C(7)), 3.08 (m, 1H, H–C(3)), 2.71 (m, 3H, OCH$_2$CHHCH$_2$S), 2.63 (m, 3H, OCH$_2$CHHCH$_2$S), 2.20–1.05 (series of multiplet, 28H), 1.82 (bs, 6H, OCCH$_2$Br), 0.92 (d, 3H, J = 6.0 Hz, H$_3$C–C(20)), 0.89 (s, 3H, H$_3$C–C(10)), 0.66 (s, 3H, H$_3$C–C(13)); ^{13}C NMR (500 MHz, $CDCl_3$/TMS) 168.53, 168.51, 168.51, 80.82, 79.95, 76.15, 66.19, 65.82, 65.81, 62.47, 46.52, 46.29, 42.86, 42.10, 40.64, 39.87, 38.91, 38.86, 35.72, 35.45, 35.12, 34.82, 32.11, 30.14, 29.93, 29.83, 28.93, 28.79, 28.61, 28.54, 28.47, 28.33, 28.00, 27.65, 27.59, 27.34, 25.60, 23.32, 22.72, 22.16, 17.52, 17.13, 14.47, 13.55, 11.75; ESI-MS calcd. for $C_{45}H_{78}Br_3N_3O_7S_3$ (M): 1109.25. Found: 1110.52 $(M + H)^+$, 555.58 $(M + 2H)^{2+}$.

Peptides

All peptides were synthesized using the standard Fmoc-based solid phase peptide synthesis on a Fmoc-PAL-PEG-PS resin.[25] The P37C, Ac-YTSLIHSLIEESQNQQEKNEQELLELDKW-ASLWNWFC-NH$_2$, was reported previously.[25] The characteristic data for the T-helper epitope and the minimum epitope sequence of the HIV-neutralizing antibody 2F5 are shown below.

T-helper

CGSSSQYIKANSKFIGITEL-NH$_2$, t_R 8.6 min (under the analytical HPLC conditions described in the general methods); ESI-MS: Calcd. M = 2145.48; Found: 1073.90 $(M + 2H)^{2+}$, 716.42 $(M + 3H)^{3+}$.

P-7C

Ac-ELDKWAC-NH$_2$, t_R 7.9 min (under the analytical HPLC conditions described in the general methods). ESI-MS: Calcd. M = 905.86; Found, 905.53 $(M + H)^+$, 453.42 $(M + 2H)^{2+}$.

Ligation of cysteine-containing peptide to the cholic acid-based maleimide clusters

The peptides (1.5 mol. equivalent per maleimide functionality) were dissolved in degassed phosphate buffer (pH 6.6) containing 50% acetonitrile to a final concentration of *ca*. 2 μmol mL^{-1}. The mixture was gently shaken under N_2 at room

temperature. The processing of ligation was monitored with analytic HPLC. The reaction mixture was purified with RP-HPLC. The peptides were lyophilized and their identity was characterized by ESI-MS.

Trivalent peptide (10a)

82% yield; t_R 15.1 min (under the analytical HPLC conditions described in the general methods); ESI-MS: Calcd. M = 14770.88; Found, 1847.83 $(M + 8H)^{8+}$, 1642.74 $(M + 9H)^{9+}$, 1478.59 $(M + 10H)^{10+}$, 1344.26 $(M + 11H)^{11+}$, 1232.32 $(M + 12H)^{12+}$, 1137.72 $(M + 13H)^{13+}$, 1056.34 $(M + 14H)^{14+}$.

Trivalent peptide (10b)

20% yield; t_R 12.0 min (under the analytical HPLC conditions described in the general methods); ESI-MS: Calcd. M = 7421.90; Found, 1485.67 $(M + 5H)^{5+}$, 1238.18 $(M + 6H)^{6+}$, 1061.51 $(M + 7H)^{7+}$, 928.94 $(M + 8H)^{8+}$, 825.98 $(M + 9H)^{9+}$.

Trivalent peptide (11a)

83% yield; Retention time (t_R) 14.4 min (under the analytical HPLC conditions described in the general methods); ESI-MS: Calcd. M = 15110.13; Found, 1889.74 $(M + 8H)^{8+}$, 1680.20 $(M+ 9H)^{9+}$, 1512.32 $(M + 10H)^{10+}$, 1374.92 $(M + 11H)^{11+}$, 1260.44 $(M +12H)^{12+}$, 1163.64 $(M +13H)^{13+}$, 1080.46 $(M+14H)^{14+}$.

Trivalent peptide (11b)

91% yield; t_R 12.4 min (under the analytical HPLC conditions described in the general methods); ESI-MS: Calcd. M = 7761.16; Found, 1553.33 $(M + 5H)^{5+}$, 1294.71 $(M + 6H)^{6+}$, 1109.88 $(M + 7H)^{7+}$, 971.31 $(M + 8H)^{8+}$, 863.56 $(M + 9H)^{9+}$.

Trivalent peptide (2)

The peptide **P-7C** (9.7 mg, 11 μmol) was dissolved in degassed sodium borate buffer (pH 8.5) containing 50% acetonitrile (3 mL). The bromoacetyl derivative **9** (2.6 mg, 2.4 μmol) in acetonitrile was added with gentle shaking. The processing of ligation was monitored with analytic HPLC. The desired trivalent peptide **12** was purified with RP-HPLC and lyophilized to afford a white solid (3 mg, 35%). t_R 14.5 min (under the analytical HPLC conditions described in the general methods); ESI-MS: Calcd. M = 3582.65; Found, 1792.26 $(M + 2H)^{2+}$, 1195.14 $(M + 3H)^{3+}$, 896.74 $(M + 4H)^{4+}$, 717.68 $(M +5H)^{5+}$.

Circular dichroism (CD)

The samples of trivalent peptide **10a**, trivalent peptide **11a**, peptides DP178, and the control sample 3α,7α,12α-tri-(6-amino-3-thiahexyoxyl)-24-hydroxy-5β-cholane **6** were prepared in a concentration range from 8 to 18 μM in 50 mM phosphate buffer (pH 7.2). The accurate concentration of the peptides was measured by UV-absorption and calculated based on the proposed formulation.[46] The measurement was run at 23 °C. Blank was subtracted from the CD spectra, which were the average of three-time scans.

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