Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 159–166

BIOORGANIC & MEDICINAL CHEMISTRY

Carbohydrate-Centered Maleimide Cluster as a New Type of Templates for Multivalent Peptide Assembling: Synthesis of Multivalent HIV-1 gp41 Peptides

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Received 6 March 2002; accepted 16 April 2002

Abstract—This paper describes a facile synthesis of carbohydrate-centered maleimide clusters and their application as a new type of templates for multivalent peptide assembling. Simultaneous introduction of multiple maleimide functionalities onto a carbohydrate core was achieved through the reaction of carbohydrate-based polyamines with methoxycarbonylmaleimide or with the *N*-hydroxylsuccinimide ester of 6-maleimidohexanoic acid. The clustered maleimides placed on the carbohydrate core allow rapid and highly chemoselective ligation with multiple copies of cysteine-containing peptides under virtually neutral conditions at room temperature. This mild and highly efficient ligation method is extremely valuable for synthesizing large and complex multivalent peptides that may not be easily obtained by conventional ligation methods. The usefulness of the maleimide clusters as a new type of templates for multivalent peptide synthesis was exemplified by the synthesis of two tetravalent gp41 peptides incorporating the sequence of the potent HIV inhibitor, T20. The synthetic multivalent gp41 peptides are useful as novel immunogens to raise specific antibodies for HIV studies. They are also useful probes for studying HIV membrane fusion mechanisms.
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Introduction

Template assembled multivalent peptides have found wide applications in recent years. For example, multiple antigenic peptides (MAPs), in which 4–8 copies of an antigenic peptide are attached to an oligo-lysine core, were synthesized and used as synthetic vaccines against HIV and other diseases.[1–3] Compared to the conventional vaccine preparations where an antigenic peptide is attached to a carrier protein such as keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA), the MAPs constructs are chemically unambiguous and immunologically focused, and often raise superior immune responses.[1,2,4–6] On the other hand, template assembled multivalent peptides have been exploited to design artificial proteins to study the folding and structure of proteins.[7,8] Enhanced inhibitory activities of peptide inhibitors were also attained through multivalent peptide assembly.[9–12] In addition to oligo-lysine core,[3] other types of templates that were developed for multivalent peptide construction include cyclic peptide and derivatives,[13–15] porphyrin molecules,[16,17] calix[4]-arene core,[10–12] and carbohydrates.[18–21] Although multivalent peptide may be assembled by a stepwise solid-phase peptide synthesis on an immobilized template, the solid-phase synthesis itself and the purification of the high molecular weight multivalent peptide product to real homogeneity, after cleavage and de-protection, present a clear challenge.[1] The recent development of techniques in chemoselective ligation of pre-assembled, unprotected peptide segments has significantly enhanced our ability to synthesize large, complex multivalent peptides and proteins.[3,7,8] Since polypeptides usually contain a batch of functional groups (e.g., carboxyl, amino, and hydroxyl groups, as well as aromatic side chains), the success of the ligation approach relies on a highly chemoselective reaction between a pair of mutually reactive functionalities that are placed on the unprotected peptide and the scaffold, respectively, which should not react crossly with any other functional groups in the polypeptides. Toward this end, two chemoselective reactions are most commonly used for multivalent peptide synthesis. One is the reaction of

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0968-0896/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(02)00339-5

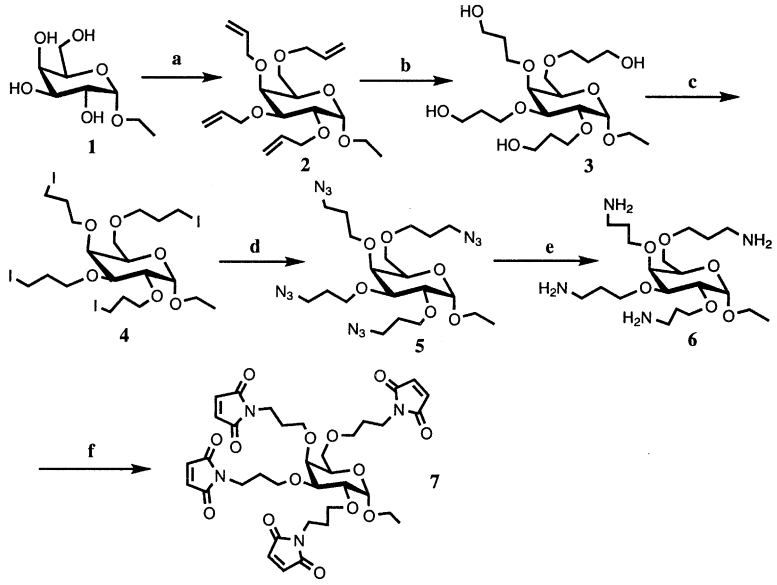
hydroxylamine or hydrazine nucleophiles with aldehydes/ketones, which results in the formation of oximes and hydrazones, respectively.[15,18,22,23] The reaction should be performed under acidic conditions (pH 4–5), and the oximes or hydrazones formed are chemically stable under acidic to neutral conditions. The other commonly used chemoselective reaction is the thioether formation between thiol groups (usually from cysteine residues) and bromoacetyl or chloroacetyl moieties through nucleophilic substitution.[24,25] Nevertheless, an efficient nucleophilic substitution between a thiol-chloroacetyl (or bromoacetyl) reactive pair can efficiently take place only under basic conditions (pH 8–9), and side reactions between free amino groups in the polypeptides and the haloacetyl groups may occur under the reaction conditions.[24]

As part of our ongoing project on the development of multivalent peptides as HIV vaccines and viral membrane fusion inhibitors, we are interested in assembling novel multivalent gp41 peptides on a carbohydrate scaffold. The HIV-1 gp41 is an envelope glycoprotein that mediates the fusion of viral and cellular membranes, a critical step for HIV entry and infection. It was reported that synthetic gp41 C-peptides (peptides corresponding to the C-terminal ectodomain of gp41) such as T20 and C34, potently inhibit membrane fusion by both laboratory-adapted strains and primary isolates of HIV-1.[26–28] The critical roles of certain peptide domains of gp41 in mediating membrane fusion provide ideal targets for developing therapeutic and preventative agents against HIV/AIDS.[29] On the other hand, we chose carbohydrates as the scaffold for displaying the gp41 peptides because the rigid ring structure and the distinct configurations of multiple functionalities in carbohydrates make them unique platforms for topological accommodations of peptide chains. In fact, monosaccharides and oligosaccharides have been extensively exploited as scaffolds for the construction of dendritic glycoligands[30,31] and, more recently, for the synthesis of carbopeptides and carboproteins.[18–20] Although the reactive pairs of aminooxy-aldehyde and thiol-haloacetyl have been widely used for chemoselective ligation in multivalent peptide synthesis, we found that some of the gp41 peptides, such as the potent HIV fusion inhibitor T20 (a 36-mer peptide from the C-terminal ectodomain of HIV-1 gp41), are either insoluble under acidic conditions, thus preventing the application of the popular oxime chemistry, or are not stable under basic conditions and causing side reactions with haloacetyl derivatives, making the nucleophilic substitution not feasible for the ligation. Taking advantage of the well-established, highly efficient Michael-type addition of a thiol group to a maleimide moiety,[32,33] we reasoned that the reaction between a carbohydrate-centered maleimide cluster and a thiol-containing peptide should allow a highly chemoselective ligation under virtually neutral conditions, thus providing a new and efficient approach for multivalent peptide assembling. In this paper, we report a facile synthesis of carbohydrate-centered maleimide clusters. In addition, we report here the synthesis of two tetravalent gp41 peptides, each of which contains 4 strands of the 36-mer gp41 peptide, T20. By introducing a cysteine residue at the C-terminus of the gp41 peptide during solid phase synthesis, we found that a rapid and highly chemoselective ligation between the cysteine-containing gp41 peptide and a galactoside-based maleimide cluster can be achieved under neutral conditions, giving the target tetravalent peptides in high yield.

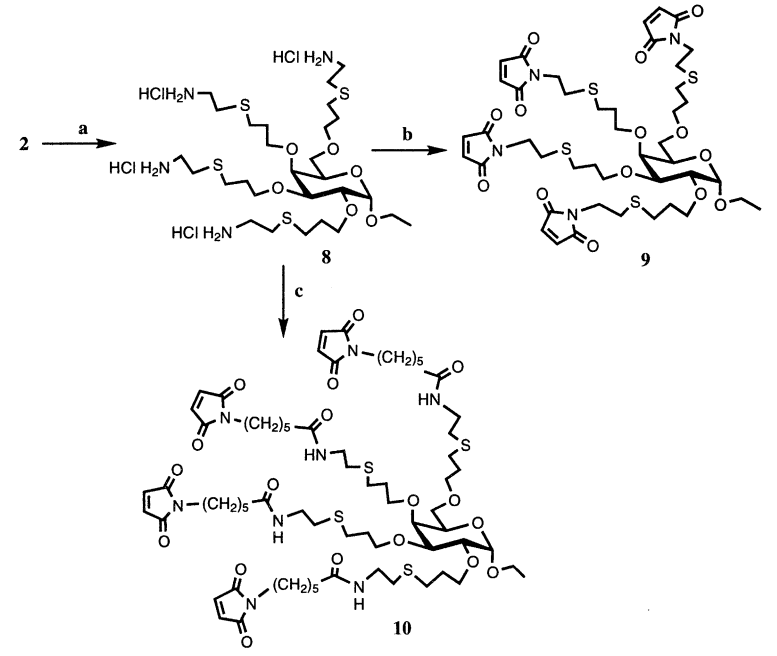
Results and Discussion

A galactoside-based, tetravalent maleimide cluster (**7**) was synthesized through a series of efficient chemical transformations (Scheme 1). First, compound (**1**) was allylated with allyl bromide to give the tetra-*O*-allyl galactoside (**2**) (94%). The tetra-allyl derivative was then subject to regioselective hydroboration with 9-borabicyclo-[3.3.1]nonane (9-BBN), and subsequent alkaline oxidation with H_2O_2 to give the tetra-*O*-(3-hydroxypropyl) galactoside (**3**) in 84% yield. To synthesize the tetra-amino derivative (**6**) that is required for introducing maleimide groups, the tetraol (**3**) was reacted with triphenylphosphine-iodine in DMF to give the tetraiodide (**4**) (70%), which was then converted into the azido-compound (**5**) by treatment with NaN_3 in DMF. Catalytic hydrogenation of **5** afforded the tetra-amino derivative (**6**) in a quantitative yield. Finally, Simultaneous introduction of 4 maleimide groups was achieved by treating the amine (**6**) with methoxycarbonylmaleimide in aqueous MeCN containing $NaHCO_3$ to give the tetravalent maleimide cluster (**7**) in 76% yield (Scheme 1).

The synthesis of maleimide clusters with variable length of spacers between the carbohydrate core and the maleimide was easily achievable by extending the spacers during the synthesis. The length of spacers between the carbohydrate core and the peptide chains is an important factor to determine the orientation and intra-molecular interaction of the peptide chains, which will eventually affect the properties of the resulting multivalent peptides.[3,7,34] For the purpose, two tetravalent maleimide clusters (compounds **9** and **10**) that have longer spacers between the maleimide and the carbohydrate core were synthesized (Scheme 2). Briefly, four amino functionalities were introduced into the tetra-*O*-allyl derivative (**2**) by photoaddition with cysteamine in MeOH.[35] Instead of using a large excess of cysteamine hydrochloride as previously reported,[35] we used only 3 molar equivalent per OH of cysteamine hydrochloride and monitored the reaction by measuring ^{1}H NMR. When the reaction is proceeding, the signals at δ 5.10–6.05 (for the allyl groups) decrease and the new signals at δ 2.68–2.90 (for SCH_2) increase. After disappearance of the allyl signals, the resulting product (**8**) was readily isolated in 80% yield by Sephadex G-15 gel filtration chromatography. We found that using less cysteamine hydrochloride did not affect the efficiency of the reaction but greatly facilitated the purification of the product by gel filtration. Treatment of **8** with methoxycarbonylmaleimide gave the tetravalent maleimide cluster (**9**) in 71% yield after chromatographic purification. On the other hand, coupling amine (**8**) with the *N*-hydroxylsuccinimide ester of 6-maleimidohexanoic acid afforded the tetravalent maleimide cluster (**10**) in 43% yield (Scheme 2).



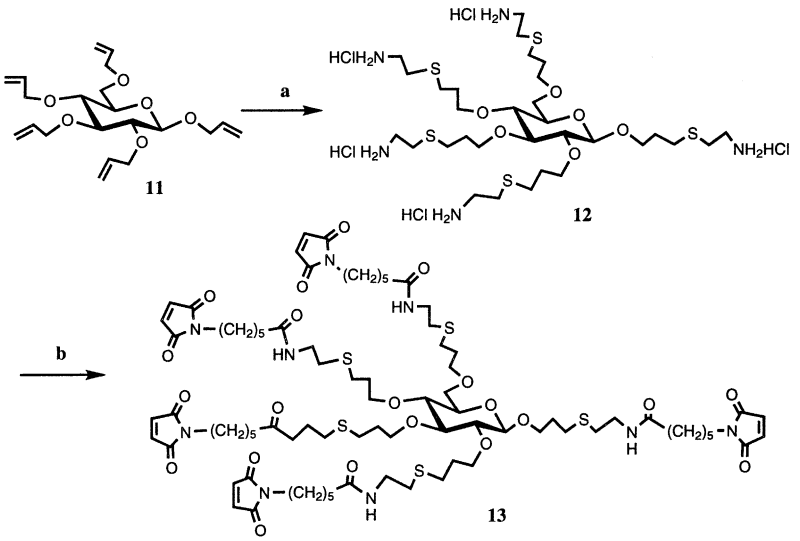
Scheme 1. (a) Allyl bromide, NaH/DMF, 0 °C to rt, 94%; (b) 9-BBN, THF, reflux, then 3 M aq NaOH, 30% H_2O_2, 0 °C to rt, 84%; (c) PPh_3/I_2, DMF, 80 °C, 70%; (d) NaN_3, DMF, rt, 78%; (e) Pd/C, H_2, MeOH, rt, 100%; (f) methoxycarbonylmaleimide, 1 M aq $NaHCO_3$, MeCN, rt, 76%.



Scheme 2. (a) Cysteamine hydrochloride, MeOH, hv, rt, 80%; (b) methoxycarbonylmaleimide, 1 M aq $NaHCO_3$, MeCN, rt, 71%; (c) 6-maleimidohexanoic acid *N*-hydroxylsuccinimidyl ester, 1 M aq $NaHCO_3$, THF, 0 °C to rt, 43%.

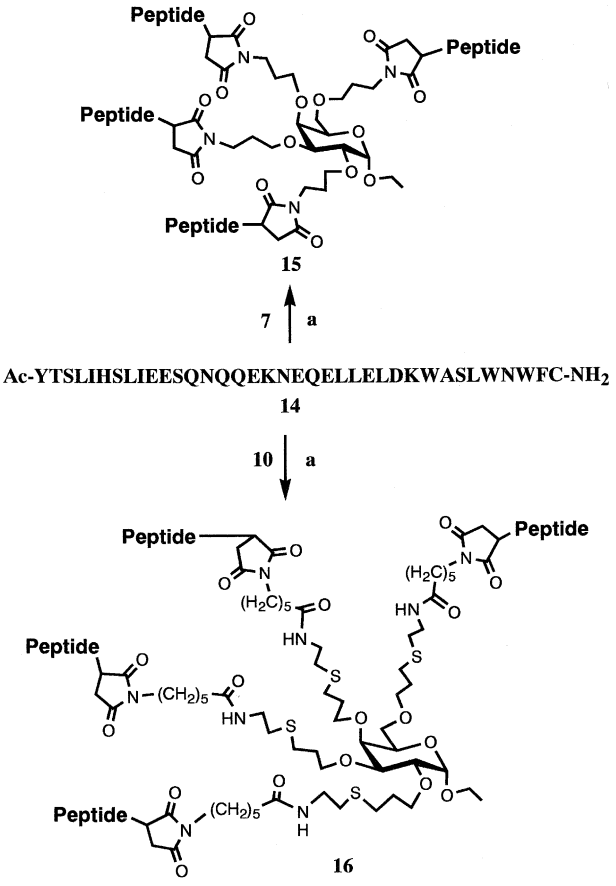
The established synthetic schemes are equally useful for the synthesis of maleimide clusters on different carbohydrate cores, which will allow the presentations of peptide chains in distinct orientations as well as in different valencies. As an example, a β-glucopyranoside-based, pentavalent maleimide cluster was readily synthesized (Scheme 3). Briefly, the penta-*O*-allyl β-glucoside (**11**), which was prepared according to the reported procedure,[36] was converted into the amino-compound (**12**) in 80% yield by photoaddition with cysteamine. Compound (**12**) was then reacted with the *N*-hydroxylsuccinimide ester of 6-maleimidohexanoic acid, giving the penta-valent maleimide cluster (**13**) in 39% yield (Scheme 3).

The usefulness of the synthetic maleimide clusters was exemplified by the synthesis of the tetravalent gp41 peptides **15** and **16**, each of which contains four strands of sequence of the peptide inhibitor T20 (Scheme 4). T20 is a 36-mer peptide from the C-terminal ectodomain of HIV-1 gp41.[26,27] The peptide exhibits potent and broadly inhibitory activities against different strains of HIV through blocking viral membrane fusion, and is currently in clinical trials for the treatment of AIDS.[37] In order to ligate the peptide to the maleimide cluster, a cysteine residue was introduced at the C-terminus of the peptide during solid-phase peptide synthesis. The cysteine-containing peptide (**14**) was thus synthesized



Scheme 3. (a) Cysteamine hydrochloride, MeOH, hv, rt, 80%; (b) 6-maleimidohexanoic acid *N*-hydroxylsuccinimide ester, 1 M aq $NaHCO_3$, THF, 0 °C to rt, 39%.

using the Fmoc chemistry and purified by reverse-phase HPLC. While peptide (**14**) is insoluble in aqueous buffers below pH 6.5, it is readily soluble under neutral to slightly alkaline conditions (pH 7.0–7.5). To our surprise, the initial attempt to ligate the peptide (**14**) with the maleimide cluster (**7**) in a phosphate buffer (50 mM, pH 7.2) failed to give the desired tetravalent peptide. However, the reaction resulted in a polymer-like solid that, after lyophilization, can hardly dissolve again in aqueous buffers or aqueous organic solvents, or in pure organic solvents such as DMF and acetonitrile. Reverse phase HPLC analysis of the reaction mixture reveals a very broad peak following the peak of peptide (**14**). Mass spectrometric analysis of the solid failed to give any useful information. We assume that the solid material may result from aggregation of the peptide, which has a concentration of 1 mM in the reaction buffer. The clustered maleimide may serve as a cross-link reagent for the non-covalently aggregating peptides, promoting further and eventually irreversible aggregations of the peptides. It was previously revealed that, in aqueous buffer (pH 7.0), T20 is monomeric at the concentration below 10 μM, but exhibits complicated monomer/tetramer equilibrium and other aggregation patterns when the concentration is above 20 μM.[38] We also observed that, under native gel filtration conditions (PBS, pH 7.2) and at 0.5 mM, both T20 and the synthetic peptide (**14**) appear as a series of broad peaks that have molecular weights corresponding to the oligomeric and polymeric forms of the parent peptides (data not shown), indicating that indeed the peptides form aggregates at relatively high concentrations. We eventually found that the ligation of peptide (**14**) and the maleimide cluster (**7**) can proceed very smoothly in a 1:1 acetonitrile-phosphate buffer (pH 7.0) to give the desired tetravalent peptide (**15**) (Scheme 4). In the presence of high concentration of acetonitrile, the peptide could exist in monomeric form. The ligation is rapid and highly efficient. HPLC monitoring indicates that the reaction is actually finished within 30 min. The tetravalent peptide



Scheme 4. (a) 1:1 MeCN–phosphate buffer (pH 7.0), room temp, Yields: 82% for **15**; 84% for **16**.

(**15**) was purified in 82% yield by reverse-phase HPLC, and the purity and identity of the product was confirmed by analytic HPLC and ES–MS, respectively. We also prepared several short gp41 peptides that contain the broadly neutralizing antibody 2F5's epitope ELDKWA[39] and have a cysteine residue at either the

C- or *N*-termini, and used them for the ligation. We found that these peptides, which do not have solubility or aggregation problems, can be ligated to the clustered maleimides very rapidly in the range of pH 6.5–7.4 in an aqueous buffer, giving the desired multivalent peptides in virtually quantitative yields (data not shown).

Similarly, ligation of the peptide (**14**) to a different template, the tetravalent maleimide cluster (**10**), under the same reaction conditions as described for the synthesis of **15** gave the tetravalent gp41 peptide (**16**) in 84% yield after preparative HPLC (Scheme 4).

Conclusion

A facile synthesis of carbohydrate-centered maleimide clusters was described. The clustered maleimides placed on the carbohydrate core allow rapid and highly chemoselective ligation with multiple copies of cysteine-containing peptides under virtually neutral conditions. The mildness and high efficiency of the ligation reaction make the method extremely valuable for synthesizing large and complex multivalent peptides that may not be easily obtained by conventional ligation methods. Particularly, the use of carbohydrates as the core molecule allows the topological display of multiple peptide chains with defined spatial orientations, depending on the nature of the sugar molecules and the length of the spacers. The usefulness of the approach was exemplified by the synthesis of two tetravalent gp41 peptides incorporating the sequence of the potent HIV inhibitor, T20. The immunogenicity of the synthetic multivalent gp41 peptides are currently being evaluated and the results will be reported elsewhere. The synthetic multivalent gp41 peptides are also useful probes for studying the HIV membrane fusion mechanisms.

Experimental

General methods

Fmoc-protected amino acids were purchased from Novabiochem. HATU, DIPEA and Fmoc-PAL-PEG-PS were purchased from 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 NMR spectra were recorded on QE 300 with Me_4Si (δ 0) as the internal standard. The ES–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). Gel filtration was carried out on Sephadex G-15 (Pharmacia) using de-ionized water as the eluent. Photo-addition reactions were carried out in a quartz flask under N_2. Analytical HPLC was carried out with a Waters 626HPLC 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 25 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 600HPLC instrument on a Waters C18 column (Symmetry300, 19×300 mm) and/or on a Delta-Pak C18 column [Delta-Pak RCM 2×(2.5×10 cm)]. The column was eluted with a linear gradient of acetonitrile (10–60%) containing 0.1% TFA at a flow rate of 10 mL/min. The peptides were detected at 214/280 nm. Purified peptides were lyophilized and kept under nitrogen in a freezer (−20 °C).

Ethyl 2,3,4,6-tetra-*O*-allyl-α-D-galactopyranoside (2). A solution of ethyl α-D-galactopyranoside **1** (416.4 mg, 2.0 mmol) in anhydrous DMF (5 mL) was added dropwise to a stirred suspension of sodium hydride (60% dispersion in mineral oil, 960.0 mg, 24.0 mmol) in anhydrous DMF (15 mL). After 30 min, the reaction mixture was cooled to 0 °C, and allyl bromide (2.90 g, 24.0 mmol) was added dropwise. The resulting mixture was stirred for 1 h at 0 °C, and 3 h at room temperature. After the mixture was cooled in an ice-bath, excess sodium hydride was quenched by slow addition of methanol (5 mL). The volatiles were evaporated to dryness, and then the residue was mixed with ethyl acetate (150 mL) and washed with brine (3×30 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The oily residue was purified by flash chromatography (hexane/EtOAc 85:15) to afford compound **2** (690.0 mg, 94%) as a colorless oil; R_f 0.65 (hexane/EtOAc 7:3); ^{1}H NMR (300 MHz, $CDCl_3$/TMS) δ 6.05–5.84 (m, 4H, $OCH_2CH{=}CH_2$), 5.36–5.10 (m, 8H, $OCH_2CH{=}CH_2$), 4.94 (d, 1H, J = 3.7 Hz, H-1), 4.39 (dd, 1H, J = 12.6, 5.6 Hz, $OCHCH{=}CH_2$), 4.30–3.98 (m, 7H, $OCH_2CH{=}CH_2$), 3.93 (dd, 1H, J = 7.1, 6.4 Hz, H-5), 3.84 (s, 1H, br, H-4), 3.83 (dd, 1H, J = 9.8, 3.7 Hz, H-2), 3.80–3.60 (m, 2H, H-3, and $OCHCH_3$), 3.64–3.55 (m, 2H, H-6 and $OCHCH_3$), 3.52 (dd, 1H, J = 9.0, 6.1 Hz, H-6′), 1.24 (t, 3H, J = 7.1 Hz, OCH_2CH_3); ES–MS: 391.36 $(M+Na)^+$, 323.32 $(M-OEt)^+$, 207.14 $(M+2Na)^{2+}$.

Ethyl 2,3,4,6-tetra-*O*-(3-hydroxypropyl)-α-D-galactopyranoside (3). To a stirred solution of **2** (230.0 mg, 0.62 mmol) in dry THF (10 mL) was added dropwise a solution of 9-BBN in THF (0.5 M, 10.0 mL, 5.0 mmol) at room temperature. The reaction mixture was heated under reflux for 2 h, and excess of 9-BBN was then destroyed by dropwise addition of water at 0 °C. The hydroboration mixture was oxidized by treatment with 3 M aq NaOH (11.0 mL) and 30% H_2O_2 solution (11.0 mL) at 0 °C, followed by stirring overnight at room temperature. The mixture was saturated with K_2CO_3, and the phases were separated. The aqueous phase was extracted with THF (2×30 mL) and the combined organic phases were dried over Na_2SO_4, filtered, and concentrated. The oily residue was purified by column chromatography (ethyl acetate/Methanol 8:2) to yield tetraol **3** (231.0 mg, 84%) as a colorless oil; R_f 0.49 (EtOAc/MeOH 7:3); ^{1}H NMR (D_2O) δ 5.10 (d, 1H, J = 3.4 Hz, H-1), 4.02 (dd, 1H, J = 5.8, 4.8 Hz,

OC*H*HCH$_2$CH$_2$OH), 3.94 (s, 1H, br, H-4), 3.86–3.50 (m, 22H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$OH, OCH$_2$CH$_2$C*H*$_2$OH and OC*H*$_2$CH$_3$), 1.86–1.75 (m, 8H, OCH$_2$C*H*$_2$CH$_2$OH), 1.19 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$); ES–MS: 463.45 $(M+Na)^+$, 441.47 $(M+H)^+$, 395.43 $(M-OEt)^+$, 243.26 $(M+2Na)^{2+}$.

Ethyl 2,3,4,6-tetra-*O*-(3-azidopropyl)-α-D-galactopyranoside (5). To a stirred solution of Ph_3P (1.00 g, 3.81 mmol) in dry DMF (3 mL) was added iodine (0.97 g, 3.81 mmol). After 10 min, a solution of tetraol **3** (210.0 mg, 0.48 mmol) in DMF (2 mL) was added dropwise. The resulting mixture was stirred for 2 h at room temperature and another 2 h at 80 °C. Heating was then discontinued and the mixture was concentrated under reduced pressure to remove DMF. The residue was purified by flash chromatography (hexane/EtOAc 8:2) to give tetraiodide **4** (293.0 mg, 70%). The iodide 4 thus obtained was used immediately for the next step. Iodide **4**: R_f 0.65 (hexane/EtOAc 7:3); ^{1}H NMR ($CDCl_3$/TMS) δ 4.97 (d, 1H, $J=3.4$ Hz, H-1), 3.96–3.83 (m, 2H, H-4, and OC*H*HCH$_2$CH$_2$I), 3.80–3.47 (m, 14H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$I, and OC*H*$_2$CH$_3$), 3.36–3.24 (m, 8H, OCH$_2$CH$_2$C*H*$_2$I), 2.20–1.98 (m, 8H, OCH$_2$C*H*$_2$CH$_2$I), 1.23 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$).

A mixture of iodide **4** (260.0 mg, 0.30 mmol) and NaN_3 (1.24 g, 19.07 mmol) in dry DMF (10 mL) was stirred overnight at room temperature. The mixture was evaporated at reduced pressure to dryness, and the residue was partitioned in CH_2Cl_2 (100 mL) and water. The organic layer was washed with brine (3×20 mL), dried (Na_2SO_4), filtered, and concentrated. The oily residue was purified by flash chromatography (hexane/EtOAc 8:2) to provide tetraazide **5** (124.0 mg, 78%); R_f 0.41 (hexane/EtOAc 7:3); ^{1}H NMR ($CDCl_3$/TMS) δ 4.95 (d, 1H, $J=3.4$ Hz, H-1), 3.96–3.85 (m, 2H, H-4, and OC*H*HCH$_2$CH$_2$N$_3$), 3.80–3.47 (m, 14H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$N$_3$, and OC*H*$_2$CH$_3$), 3.47–3.35 (m, 8H, OCH$_2$CH$_2$C*H*$_2$N$_3$), 1.95–1.76 (m, 8H, OCH$_2$C*H*$_2$CH$_2$N$_3$), 1.25 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$); ES–MS: 563.39 $(M+Na)^+$, 467.42 $(M-OEt-N_2)^+$.

Ethyl 2,3,4,6-tetra-*O*-(3-aminopropyl)-α-D-galactopyranoside (6). Azide **5** (61.0 mg, 0.11 mmol) was hydrogenated with Pd/C (10%, 10 mg) in methanol (5 mL) overnight at room temperature. The mixture was filtered through a bed of Celite, and the filtrate was then concentrated to give tetraamine **6** (51.5 mg, 100%); ^{1}H NMR (D_2O) δ 5.10 (d, 1H, $J=3.4$ Hz, H-1), 4.06–3.98 (m, 1H, OC*H*HCH$_2$CH$_2$NH$_2$), 3.96–3.92 (m, 1H, H-4), 3.90∼3.48 (m, 14H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$NH$_2$, and OC*H*$_2$CH$_3$), 2.89–2.70 (m, 8H, OCH$_2$CH$_2$C*H*$_2$NH$_2$), 1.90–1.65 (m, 8H, OCH$_2$C*H*$_2$-CH$_2$NH$_2$), 1.18 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$); ES−MS: 437.48 $(M+H)^+$, 219.16 $(M+2H)^{2+}$.

Ethyl 2,3,4,6-tetra-*O*-(3-maleimidopropyl)-α-D-galactopyranoside (7). A solution of amine **6** (17.8 mg, 0.04 mmol) in 1 M aqueous solution of $NaHCO_3$ (1 mL) was treated with methoxycarbonylmaleimide (37.94 mg, 0.24 mmol) at 0 °C. After 5 min, the mixture was diluted with water (1 mL) and acetonitrile (2 mL), and then stirred at room temperature for 4 h. After adding CH_2Cl_2 (50 mL), the organic layer was separated and washed with brine (3×10 mL). The organic layer was then dried (Na_2SO_4), filtered, and concentrated. The oily residue was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to give maleimide **7** (23.2 mg, 76%); R_f 0.42 (CH_2Cl_2/MeOH 95:5); ^{1}H NMR ($CDCl_3$/TMS) δ 6.70 (s, 2H, C*H*=C*H*), 6.69 (s, 4H, C*H*=C*H*), 6.68 (s, 2H, C*H*=C*H*), 4.98 (d, 1H, $J=3.4$ Hz, H-1), 3.95–3.82 (m, 2H, H-4, and OC*H*HCH$_2$CH$_2$N), 3.80∼3.35 (m, 22H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$N, OCH$_2$CH$_2$C*H*$_2$N, and OC*H*$_2$CH$_3$), 1.96–1.78 (m, 8H, OCH$_2$C*H*$_2$CH$_2$N), 1.25 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$); ES–MS: 779.28 $(M+Na)^+$, 401.29 $(M+2Na)^{2+}$.

Ethyl 2,3,4,6-tetra-*O*-(6-amino-3-thia-hexyl)-α-D-galactopyranoside tetrahydrochloride (8). To a solution of the tetra-*O*-allyl derivative **2** (404.6 mg, 1.10 mmol) and AIBN (30.0 mg) in methanol (15 mL) in a Quartz flask was added cysteamine hydrochloride (1.50 g, 13.20 mmol). After being degassed by bubbling N_2 into solution for 30 min, the resulting mixture was stirred and irradiated (UV, 254 nm) under N_2. The reaction was monitored by measuring the ^{1}H NMR of a small portion of the reaction mixture, which was dried and deuterium-exchanged with D_2O before recording the NMR. During the progress of the reaction, the signals at δ 5.10–6.05 (for the allyl groups) decreased and the new signals at δ 2.68–2.90 (for SCH_2) increased. After 24 h, NMR indicated the disappearance of the allyl signals. MeOH was then evaporated and the residue was purified by gel filtration on a Sephadex G-15 column using water as the eluent. Fractions containing the product were pooled and lyophilized to give amine **8** (723.4 mg, 80%) as a colorless glass-like solid; ^{1}H NMR (D_2O) δ 5.08 (d, 1H, $J=3.4$ Hz, H-1), 4.06–3.98 (m, 1H, OC*H*HCH$_2$CH$_2$S), 3.96–3.92 (m, 1H, H-4), 3.90∼3.48 (m, 14H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$S, and OC*H*$_2$CH$_3$), 3.26∼3.14 (m, 8H, SCH$_2$C*H*$_2$NH$_2$HCl), 2.90–2.82 (m, 8H, SC*H*$_2$CH$_2$NH$_2$-HCl), 2.72–2.68 (m, 8H, OCH$_2$CH$_2$C*H*$_2$S), 1.96–1.78 (m, 8H, OCH$_2$C*H*$_2$CH$_2$S), 1.20 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$); ES–MS: 677.1 $(M+H\text{-}4HCl)^+$, 339.1 $(M+2H\text{-}4HCl)^{2+}$.

Ethyl 2,3,4,6-tetra-*O*-(6-maleimido-3-thia-hexyl)-α-D-galactopyranoside (9). A solution of amine **8** (23.9 mg, 29.0 μmol) dissolved in 1 M aqueous solution of $NaHCO_3$ (1 mL) was treated with methoxycarbonylmaleimide (27.03 mg, 17.4 μmol) at 0 °C. After 5 min, the mixture was diluted with water (1 mL) and acetonitrile (2 mL), and then stirred at room temperature for 1 h. The mixture was diluted with CH_2Cl_2 (50 mL) and washed with brine (3×10 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated. The oily residue was purified by flash chromatography (CH_2Cl_2/MeOH 96:4) to give maleimide **9** (20.3 mg, 71%); R_f 0.45 (CH_2Cl_2/MeOH 96:4); ^{1}H NMR ($CDCl_3$/TMS) δ 6.72 (s, 8H, C*H*=C*H*), 4.94 (d, 1H, $J=3.4$ Hz, H-1), 3.95–3.82 (m, 2H, H-4, and OC*H*HCH$_2$CH$_2$N), 3.80∼3.45 (m, 22H, H-2, H-3, H-5, H-6, H-6′, OC*H*$_2$CH$_2$CH$_2$S, SCH$_2$C*H*$_2$N, and OC*H*$_2$CH$_3$), 2.90–2.60 (m, 16H, OCH$_2$CH$_2$C*H*$_2$SC*H*2CH$_2$N), 1.96–1.82 (m, 8H, OCH$_2$C*H*$_2$CH$_2$S), 1.23 (t, 3H, $J=7.1$ Hz, OCH$_2$C*H*$_3$); ES–MS: 1019.49 $(M+Na)^+$, 951(M-OEt)$^+$.

Ethyl 2,3,4,6-tetra-*O*-[(6-(6-maleimidohexanamido)-3-thia-hexyl)]-α-D-galactopyranoside (10). A solution of amine **8** (138.6 mg, 0.17 mmol) in 1 M aqueous solution of $NaHCO_3$ (1.5 mL) was added dropwise to a stirred solution of 6-maleimidohexanoic acid *N*-hydroxylsuccinimide ester (272.5 mg, 0.88 mmol) in THF (3 mL), which was cooled with a bath of ice-water. The resulting mixture was stirred for 1 h at 0–5 °C. The mixture was then diluted with $CHCl_3$ (70 mL) and washed with brine (3×10 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated. The oily residue was purified by flash chromatography (CH_2Cl_2/MeOH 95:5) to give maleimide **10** (103.6 mg, 43%); R_f 0.62 (CH_2Cl_2/MeOH 9:1); ^1H NMR ($CDCl_3$/TMS) δ 6.68 (s, 8H, C*H*=C*H*), 6.26–6.20 (m, 2H, N*H*), 6.12 (t, 1H, $J=6.8$ Hz, N*H*), 6.04 (t, 1H, $J=6.8$ Hz, N*H*), 4.92 (d, 1H, $J=3.2$ Hz, H-1), 3.95–3.86 (m, 2H, H-4, and OC*H*HCH_2CH_2N), 3.80∼3.40 (m, 30H, H-2, H-3, H-5, H-6, H-6′, OCH_2CH_2CH_2S, SCH_2CH_2N, NCH_2(CH_2)$_4$ and OCH_2CH_3), 2.75–2.60 (m, 16H, OCH_2CH_2CH_2-SCH_2CH_2N), 2.18 (t, 8H, $J=7.3$ Hz, N(CH_2)$_4$CH_2), 1.96–1.82 (m, 8H, OCH_2CH_2CH_2S), 1.74–1.54 (m, 16H, NCH_2CH_2CH_2CH_2CH_2), 1.37–1.24 (m, 8H, N(CH_2)$_2$CH_2(CH_2)$_2$), 1.22 (t, 3H, $J=7.1$ Hz, OCH_2CH_3); ES–MS: 1450.01 $(M+H)^+$, 725.56 $(M+2H)^{2+}$.

(6-Amino-3-thia-hexyl) 2,3,4,6-tetra-*O*-(6-amino-3-thia-hexyl)-β-D-glucopyranoside pentahydrochloride (12). To a solution of penta-*O*-allyl derivative **11**[36] (628.5 mg, 1.65 mmol) and AIBN (50.0 mg) in methanol (15 mL) in a Quartz flask was added cysteamine hydrochloride (2.80 g, 24.67 mmol). After being degassed by bubbling N_2 into the solution for 30 min, the resulting mixture was stirred and irradiated (UV, 254 nm) under N_2. The reaction was monitored by ^1H NMR, After 24 h when NMR showed the disappearance of the allyl signals, the solvent removed by evaporation and the residue was subjected 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 amine **12** (1.25 g, 80%) as a colorless glass-like solid; ^1H NMR (D_2O) δ 4.43 (d, 1H, $J=7.8$ Hz, H-1), 4.04–3.40 (m, 13H, H-5, H-6, H-6′, OCH_2CH_2CH_2S), 3.40 (dd, 1H, $J=8.5$, 9.1 Hz, H-4), 3.36 (dd, 1H, $J=9.1$, 9.3 Hz, H-3), 3.26–3.16 (m, 10H, SCH_2CH_2NH_2HCl), 3.11 (dd, 1H, $J=7.8$, 9.3 Hz, H-2), 2.90–2.82 (m, 10H, SCH_2-CH_2NH_2HCl), 2.72–2.64 (m, 10H, OCH_2CH_2CH_2S), 1.96–1.84 (m, 10H, OCH_2CH_2CH_2S); ES–MS: 766.1 $(M+H-5HCl)^+$, 383.7 $(M+2H-5HCl)^{2+}$.

[6-(6-Maleimidohexanamido)-3-thia-hexyl] 2,3,4,6-tetra-*O*-[6-(6-maleimidohexanamido)-3-thia-hexyl)-β-D-glucopyranoside (13). A solution of amine **12** (109.8 mg, 0.12 mmol) in 1 M aqueous solution of $NaHCO_3$ (1.2 mL) was added dropwise to a stirred solution of 6-maleimidohexanoic acid *N*-hydroxylsuccinimide ester (214.2 mg, 0.70 mmol) in THF (3 mL) that was cooled with a bath of ice-water. The resulting mixture was stirred for 1 h at 0–5 °C, and then diluted with $CHCl_3$ (70 mL). The organic layer was separated and washed with brine (3×10 mL), dried (Na_2SO_4), filtered, and concentrated. The oily residue was subjected to column chromatography (CH_2Cl_2/MeOH 95:5) to give pentamaleimide **13** (76.7 mg, 39%); R_f 0.35 (CH_2Cl_2/MeOH 95:5); ^1H NMR ($CDCl_3$/TMS) δ 6.69 (s, 10H, C*H*=C*H*), 6.28–6.15 (m, 5H, N*H*), 4.18 (d, 1H, $J=7.8$ Hz, H-1), 3.95–3.50 (m, 13H, H-5, H-6, H-6′, OCH_2CH_2CH_2S), 3.50 (t, 10H, $J=7.2$ Hz, NCH_2(CH_2)$_4$), 3.50–3.38 (m, 10H, SCH_2CH_2N), 3.24–3.16 (m, 2H, H-3, H-4), 3.04–2.96 (m, 1H, H-2), 2.70–2.58 (m, 20H, OCH_2CH_2CH_2SCH_2CH_2N), 2.18 (t, 10H, $J=7.3$ Hz, N(CH_2)$_4$CH_2), 1.92–1.78 (m, 10H, OCH_2CH_2CH_2S), 1.74–1.54 (m, 20H, NCH_2CH_2CH_2-CH_2CH_2), 1.38–1.24 (m, 10H, N(CH_2)$_2$CH_2(CH_2)$_2$); ES–MS: 1733.16 $(M+H)^+$, 866.84 $(M+2H)^{2+}$.

Peptide (14). The peptide **14** was synthesized on a Pioneer Peptide Synthesizer (Applied Biosystems, Foster City, CA, USA) using Fmoc-chemistry on Fmoc-PAL-PEG-PS resin. 4-fold excess of N^{α}-Fmoc-protected amino acids were used for each coupling and HATU/DIPEA were used as the coupling reagents. The N-terminus of the peptide was capped with an acetyl group. Cleavage of the peptide from the resin with simultaneous deprotection was performed with TFA/thioanisole/EDT/anisole (90:5:3:2) (cocktail R), and the crude peptide was precipitated with cold ether. Purification of the peptide was carried out by preparative HPLC as described in the general methods. The purified **14** appeared as a single peak on analytic HPLC. Under the analytic conditions (General Methods), the peptide was eluted at 15.84 min. ES–MS of **14**: 1532.84 $(M+3H)^{3+}$, 1149.80 $(M+4H)^{4+}$, 920.12 $(M+5H)^{5+}$.

Tetravalent peptide (15). To a solution of peptide **14** (20.0 mg, 4.35 μmol) in phosphate buffer (50 mM, pH 7.0, 3.0 mL) and acetonitrile (3.0 mL) was added dropwise a solution of maleimide **7** (0.52 mg, 0.69 μmol) in DMF (104 μL). After shaken at room temperature for 2 h, the reaction mixture was lyophilized. The residue was purified by RP-HPLC as described in general methods, giving the tetravalent peptide **15** (10.7 mg, 82%) as a white powder. Analytical HPLC showed that the purified **15** showed up at 16.47 min as a single peak. ES–MS of **15**: 1914.75 $(M+10H)^{10+}$, 1741.00 $(M+11H)^{11+}$, 1596.01 $(M+12H)^{12+}$, 1473.36 $(M+13H)^{13+}$, 1368.29 $(M+14H)^{14+}$, 1276.76 $(M+15H)^{15+}$.

Tetravalent peptide (16). To a solution of peptide **14** (20.0 mg, 4.35 μmol) in phosphate buffer (50 mM, pH 7.0, 3.0 mL) and acetonitrile (3.0 mL) was added a solution of maleimide **10** (1.05 mg, 0.72 μmol) in DMF (210 μL). After 2 h at room temperature, the reaction mixture was lyophilized and the residue was purified by preparative HPLC as described in General Methods to afford the tetravalent peptide **16** (12 mg, 84%). Analytical HPLC showed a single peak for **16** with a retention time of 16.85 min. ES–MS of **16**: 1653.72 $(M+12H)^{12+}$, 1526.60 $(M+13H)^{13+}$, 1417.63 $(M+14H)^{14+}$, 1323.20 $(M+15H)^{15+}$.

Acknowledgements

The work was financially supported by the Institute of Human Virology, University of Maryland Biotechnology Institute. We thank Prof. George Lewis for stimulating discussions.

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