Toward the Development of Prophylactic and Therapeutic Human Papillomavirus Type-16 Lipopeptide Vaccines

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Four lipid−core peptide systems were synthesized using stepwise solid-phase peptide synthesis, incorporating a sequence from the human papillomavirus type-16 (HPV-16) E7 protein ($E7_{44-62}$), for the purpose of developing vaccines against HPV-16 associated cervical cancer. D-Mannose was conjugated to the vaccine in order to investigate whether the targeting of dendritic cell mannose receptors would improve vaccine efficacy. The ability of the vaccines to clear or reduce the size of HPV-16 associated tumors was assessed in C57BL/6 (H-2^b) mice using the TC-1 HPV-16 tumor model. Overall, significant reductions in the size of TC-1 tumors were observed in the mouse model, with the conjugation of mannose to these vaccines demonstrated to clear or reduce the size of TC-1 tumors to a greater extent than non-mannose-containing vaccines (37 out of 40 versus 21 out of 30 tumors cleared, respectively).

Introduction

Cervical cancer represents the second most common cancer worldwide, with 500 000 new cases diagnosed each year, resulting in 270 000 deaths.[1] Cervical cancer has been identified as near 100% attributable to human papillomavirus (HPV[a]) infection of the cervix, with HPV types 16 and 18 responsible for approximately 50% and 20% of cancers, respectively.[1] The ability to create prophylactic vaccines targeting these strains would therefore offer the capacity to prevent up to 70% of cervical cancers. Vaccines derived from the noninfectious protein coat of HPV-16 and -18, called virus-like particles (VLPs), have recently been approved for human use.[1] Because these vaccines seek to prevent HPV infection, and thus cervical cancer, they need to be administered prior to the commencement of sexual activity. A large proportion of the global population is already infected, and many women will continue to become infected with cervical cancer-associated HPV strains until global vaccine coverage is achieved.[2,3] The development of therapeutic vaccines, capable of eliminating HPV infected cells and clearing established HPV-associated tumors, would therefore be beneficial. Together, the application of prophylactic and therapeutic HPV vaccines offers the best approach to achieve HPV eradication.

HPV vaccines are designed to elicit $CD8^+$ cytotoxic T-lymphocytes (CTLs) that can eliminate HPV infected cells.[1] The continued expression of two HPV proteins, E6 and E7, is required to maintain HPV-associated tumor cell growth. Sequences from the E6 and E7 proteins, which contain epitopes capable of eliciting CTLs, are therefore commonly used for the development of vaccines against HPV infection.

The current study describes the development of four experimental vaccines (**1**−**4**, Table 1) against HPV-16 associated cervical cancer, synthesized using the lipid−core peptide (LCP) system[4] (Table 1). This vaccine delivery system incorporates (i) peptides (in this case, HPV peptides designed to elicit HPV-specific immune responses) attached to a multiple antigen peptide (MAP) system[5] and (ii) immunopotentiating lipids (lipoamino acids[6]) to stimulate strong immune responses. The vaccines contain four copies of a 19 amino acid long sequence (8Q: QAEPDRAHYNIVTFCCKCD;[7] $E7_{44-62}$) from the HPV-16 E7 protein. This sequence contains a CTL epitope, as well as a T-helper cell and B-cell epitope. The aim of this study was to determine whether conjugation of the 8Q peptide to an LCP system would elicit immune responses capable of protecting against HPV-16 associated tumor development in a mouse model and whether the attachment of D-mannose to these vaccines could increase vaccine efficacy, as improvements in vaccine efficacy have been reported in several systems[8,9] where antigens have been targeted to mannose receptors.

Results and Discussion

Chemistry. The lipid−core peptide systems **1**−**7** (Table 1) and control peptides **8**−**11** (Table 2) were synthesized using *tert*-butoxycarbonyl (Boc) manual stepwise solid-phase peptide synthesis (SPPS) on *p*-methylbenzhydrylamine (*p*MBHA) resin using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU)/*N*,*N*-diisopropylethylamine (DIPEA) in situ neutralization chemistry.[10] The synthesis of LCP systems **1**−**7** was achieved by coupling Boc-Gly-OH to the resin, followed by two cycles of the lipoamino acid 2-(*tert*-butoxycarbonylamino)-D,L-dodecanoic acid (Boc-C12-OH), then Boc-Gly-OH, Boc-C12-OH, Boc-Lys(Boc)-OH, and Boc-Lys-(Boc)-OH. Deprotection of the N-terminal Boc protecting groups

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[a] Abbreviations: ANOVA, analysis of variance; BOC, *tert*-butoxycarbonyl; Boc-C12-OH, 2-(*tert*-butoxycarbonylamino)-D,L-dodecanoic acid; C12, 2-amino-D,L-dodecanoic acid; CTLs, cytotoxic T-lymphocytes; DCM, dichloromethane; ddH_2O, deionized doubly distilled water; DMF, *N*,*N*-dimethylformamide; DIPEA, *N*,*N*-diisopropylethylamine; ESI-MS, electrospray ionization mass spectrometry; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate; HPLC, high-performance liquid chromatography; HPV, human papillomavirus; IFA, incomplete Freund's adjuvant; IPA, isopropyl alcohol; LCP, lipid−core peptide; *p*MBHA, *p*-methylbenzhydrylamine; MAP, multiple antigen peptide; OVA, ovalbumin; RP, reversed phase; RT, room temperature; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SPPS, solid-phase peptide synthesis; TCEP, tris(2-carboxyethyl)phosphine hydrochloride; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TLC, thin-layer chromatography; VLPs, virus-like particles.

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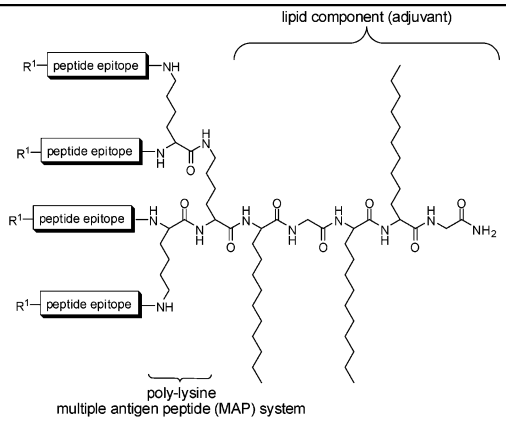
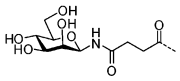
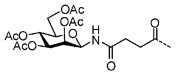
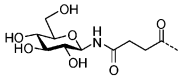
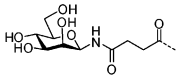
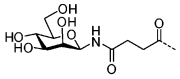
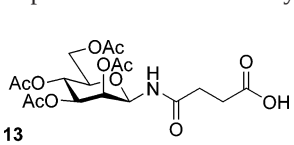
Table 1. HPV-16 8Q Peptide (**1**−**4**) and Control (**5**−**7**) LCP Systems



Compound	Peptide Antigen	R^1	Dose (μg)
1	8Q	H	30
2	8Q		40
3	8Q		40
4	8Q		40
5	OVA(Th+CTL)		40
6	8Q (random)		40
7	n/a	H	30

yielded LCP system **7** on resin. Peptide antigens (Table 1) were then synthesized onto the four N-terminal amines. For the synthesis of LCP systems **2**−**6**, the carbohydrates D-mannose (for LCP systems **2**, **3**, **5**, and **6**) and D-glucose (LCP system **4**) were conjugated to the amino terminus of each peptide antigen. These carbohydrates were coupled as per-O-acetylated analogues bearing an N-linked succinate linker (**13**, **14**; Figure 1) and were synthesized from 2,3,4,6-tetra-*O*-acetyl protected β-mannopyranosyl (**15**) and β-glucopyranosylazides by in situ reduction (Pd/C, $H_{2(g)}$) in the presence of succinic anhydride. Following



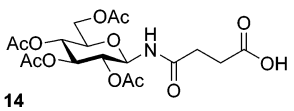
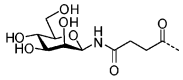
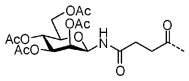
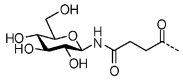


Figure 1. Acetylated mannose (**13**) and glucose (**14**) derivatives. Carbohydrates **13** and **14** were used for the synthesis of LCP systems **2**−**6**, as well as control peptides **9**−**11**.

Table 2. Control Peptides

Compound	Peptide	R^1	Dose (μg)
8	R^1-8Q-NH_2	H	30
9	R^1-8Q-NH_2		40
10	R^1-8Q-NH_2		40
11	R^1-8Q-NH_2		40
12	OVA/SIINFEKL	n/a	1/10

carbohydrate conjugation, a small sample of the resin-bound mannose 8Q LCP conjugate was kept in order to obtain **3**, and the carbohydrate *O*-acetyl protection for LCP systems **2** and **4**−**6** was removed by treating the resin with 1:11 hydrazine hydrate in DMF[11] for 4.5 h. The peptides were subsequently cleaved from the resins using anhydrous HF, purified by preparative reversed-phase high-performance liquid chromatography (RP-HPLC), and characterized by analytical RP-HPLC, electrospray ionization mass spectrometry (ESI-MS, where possible), and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The synthesis and characterization of control peptides **8**−**11** were performed analogous to the synthesis and characterization of LCP systems **1**−**7**.

Mannose was attached to LCP systems **2** and **3** in order to investigate whether targeting of dendritic cell mannose receptors could increase vaccine efficacy. Improvements in vaccine efficacy associated with targeting antigens to the mannose receptor have been reported in several systems.[8,9] As a control, D-glucose was attached to the 8Q sequence (in the case of LCP system **4**) because it displays weak mannose receptor binding in comparison to mannose[12] while maintaining the solubilizing effects of carbohydrate conjugation.

In addition to LCP systems **1**−**4**, controls **5**−**11** (Tables 1 and 2) were synthesized. The 8Q peptide with (**9**−**11**) or without (**8**) attached sugars was synthesized in order to demonstrate whether the 8Q peptide on its own, or with attached sugars, would stimulate appropriate vaccine-mediated immune responses to clear HPV-16 associated tumors despite not being conjugated to the LCP system. An LCP core, which contains the MAP system synthesized without attached peptides (**7**), and two mannose-conjugated LCP systems, one (**6**) containing a randomized version of the 8Q sequence [8Q (random), ADHIFKQPANTCDERYVCC] and another (**5**) containing a T-helper and CTL epitope from the ovalbumin (OVA) protein [OVA(Th + CTL), ISQAVHAAHAEINEAGRSIINFEKL], were synthesized to demonstrate whether the 8Q HPV peptide is necessary to protect against HPV-16 associated tumors.

Immunological Assessment. Assessment of each vaccine's capacity to protect against HPV-16 associated tumor development was performed following immunization of C57BL/6 (H-2^b) mice using the TC-1 tumor model.[13] Mice (n = 10/group) were immunized subcutaneously at the tail base with each

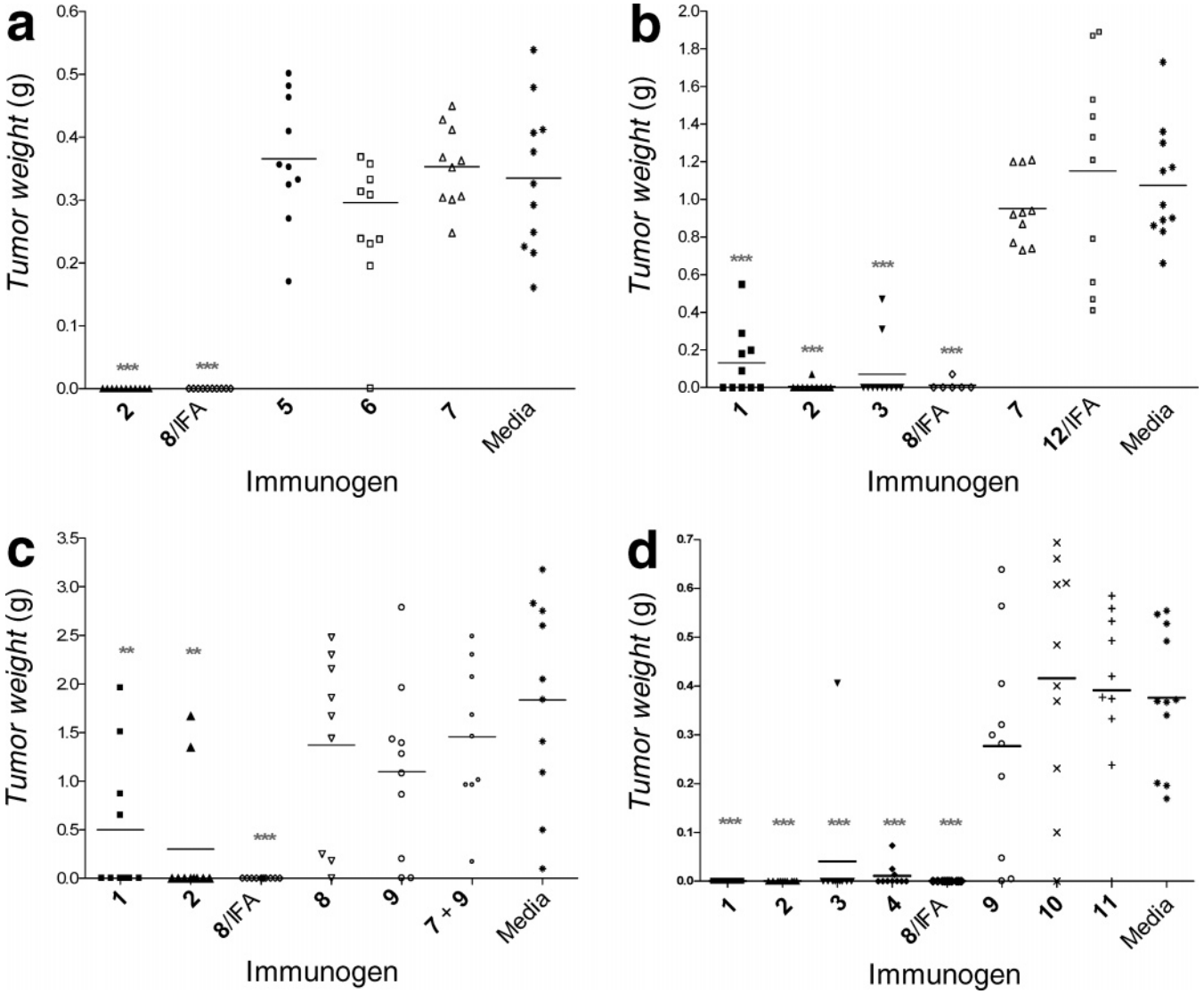


Figure 2. The mass of TC-1 tumors excised from mice 15 days after 1×10^6 TC-1 tumor cell challenge in the scruff of the neck (a, b) or lower back (c, d) following immunization with compounds **1**−**12** or RPMI 1640 media containing 0.02 mM β-mercaptoethanol. Statistically significant differences compared with media-immunized mice are represented as follows: (**) $P < 0.01$; (***) $P < 0.001$.

immunogen (doses in Tables 1 and 2) administered in RPMI 1640 media containing 0.02 mM β-mercaptoethanol. Alternatively, chicken egg OVA and the OVA SIINFEKL CTL epitope, as well as compound **8**, were administered as a 1:1 emulsion in incomplete Freund's adjuvant (IFA). Three weeks after immunization, the mice were challenged with TC-1 tumor cells[13] (1×10^6) either in the scruff of the neck or in the lower back. These cells express the HPV-16 E6 and E7 proteins, which are overexpressed in natural HPV-associated cancers. Fifteen days after TC-1 tumor cell challenge, the tumors were excised and weighed to assess the effect of immunization on tumor size (Figure 2). One-way analysis of variance (ANOVA) was used to compare the average TC-1 tumor size between groups, followed by the Bonferroni post hoc test. A *P*-value of less than or equal to 0.05 was considered to be significant.

Statistically significant tumor size reductions were observed in all groups vaccinated with LCP systems **1**−**3** when compared with mice that received RPMI 1640 media (Figure 2). Overall, mice vaccinated with the mannose containing LCP system **2** had the smallest resected tumors (or had no measurable tumors) (Figure 2), followed by mice vaccinated with compound **3** (Figure 2b,d). Mice vaccinated with the non-mannose conjugated compound **1** showed the largest tumor sizes of mice immunized with 8Q containing LCP systems (Figure 2b,c). Mice vaccinated with a mixture of the LCP core (**7**), which contains the MAP system synthesized without attached peptides, and mannose conjugated 8Q (**9**) developed tumors that did not differ in size when compared to the RPMI 1640 group (Figure 2c). These data suggested that LCP systems conjugated to 8Q were capable of preventing or reducing TC-1 tumor growth in immunized mice, with mannosylated analogues **2** and **3** appearing to offer superior anticancer activity and in many cases completely preventing the formation of detectable tumors (Figure 2).

To ensure that these results were not due to elicitation of non-8Q-specific immune responses, mice were immunized with alternative T-helper and CTL epitopes, the LCP core (**7**), or an LCP system incorporating a randomized version of the 8Q sequence (**6**). Alternative T-helper and CTL epitopes were administered as both OVA and the OVA CTL epitope SIINFEKL (**12**, Table 2) formulated in IFA or as an LCP system (**5**) incorporating the OVA SIINFEKL CTL epitope and a T-helper epitope from OVA. In each case (Figure 2a,b) the average resected tumor sizes were not significantly different compared to mice given RPMI 1640 media. This demonstrated that the 8Q peptide sequence was necessary for protection

against TC-1 tumor cell challenge and suggests that specific immune responses were responsible for this activity.

In order to confirm that the lipid component of the LCP system was important as an adjuvant, the 8Q peptide (**8**) was assessed for its capacity to reduce or prevent TC-1 tumors when administered without additional adjuvant, either alone or conjugated to mannose and glucose derivatives (**9**−**11**). Mice vaccinated with compounds **8**−**11** did not exhibit significant tumor size reductions compared to mice given RPMI 1640 media. However, it was observed that when some of the monomeric peptides (**8**−**10**) were left in solution for a long period of time, the larger molecular weight species that formed as a result of intermolecular disulfide bond formation were able to stimulate immune responses capable of partially inhibiting TC-1 tumor growth (unpublished data, PMM & CO). As a positive control, the 8Q HPV peptide **8** was administered in IFA, a highly effective adjuvant that is not suitable for human use. All but 1 of the 40 mice vaccinated with control peptide **8** formulated in IFA were protected against TC-1 tumor cell challenge (Figure 2).

The 8Q LCP system **1** was observed to be poorly soluble in RPMI 1640 media and water, while 8Q LCP systems conjugated to carbohydrates (**3** and **4**) had greatly improved solubility. Because mannosylated 8Q LCP systems appear to be more effective at protecting against TC-1 tumor development compared with the non-mannosylated analogue **1**, an attempt was made to determine whether this may be due to mannose receptor binding. An experiment (Figure 2d) was performed in which mice were administered a glucose-containing 8Q LCP system (**4**) as well as mannose-containing 8Q LCP systems **2** and **3** and LCP system **1**. The results suggested that the glucose-containing LCP system **4** was not as effective at reducing the size of, or preventing, TC-1 tumors compared with LCP systems **1**−**3**. However, there were no statistically significant differences in tumor sizes between these groups.

Conclusion

Conjugation of mannose residues to 8Q containing LCP systems, both in per-O-acetylated and nonacetylated forms, resulted in smaller tumor sizes compared with non-carbohydrate conjugated and glucose conjugated analogues. Furthermore, incorporation of the 8Q peptide into the LCP system was observed to be essential for protection against TC-1 tumor cell challenge, since incorporation of a randomized 8Q sequence or alternative CTL and T-helper epitopes did not significantly affect tumor size when compared to media-immunized mice. The LCP systems incorporating the 8Q peptide (**1**−**4**) were also demonstrated to be self-adjuvanting and elicited vaccine-mediated immune responses capable of preventing TC-1 tumors, with the 8Q peptide established to be incapable of preventing TC-1 tumors when delivered on its own, either unconjugated or conjugated to mannose or glucose residues or physically mixed with the LCP core **7**. In conclusion, the current study has demonstrated the capacity of the LCP system to adjuvant the CTL epitope-containing 8Q HPV-16 E7 peptide in preventing HPV-16 associated tumors and its potential for the development of prophylactic and possibly therapeutic human lipopeptide HPV vaccines.

Experimental Section

Materials and Methods. Boc-L-amino acids and *p*MBHA resin were purchased from Renanal (Budapest, Hungary). Peptide synthesis grade *N*,*N*-dimethylformamide (DMF), dichloromethane (DCM), HBTU, and trifluoroacetic acid (TFA) were purchased from Auspep (Melbourne, VIC, Australia). Analytical grade acetic anhydride was purchased from Ajax Finechem (Seven Hills, NSW, Australia). D-(+)-Mannose was purchased from Lancaster Synthesis (Lancashire, England). HPLC grade MeCN was purchased from Labscan (Dublin, Ireland). HPLC grade isopropyl alcohol (IPA) and MeOH were purchased from Honeywell Burdick and Jackson (Morristown, NJ). Anhydrous HF was purchased from Matheson Tri-Gas (Irving, TX). N_2, H_2, and Ar gases (all ultrapure grade) were purchased from BOC gases (Brisbane, QLD, Australia). The chicken egg OVA SIINFEKL CTL epitope was purchased from Mimotopes (Victoria, Australia). All other reagents were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia) at the highest available purity. The racemic lipoamino acids 2-amino-D,L-dodecanoic acid (C12) and Boc-C12-OH were synthesized as previously described.[6]

Equipment. An all Kel-F apparatus (Toho Kasei Co., Tokyo, Japan) was used for HF cleavage. Deionized doubly distilled water (ddH_2O) was used throughout and was prepared by a Millipore (Billerica, MA) Simplicity 185 ultrapure water system. Flash chromatography was performed on silica gel 60 (230−400 mesh; Lomb scientific, Taren Point, NSW, Australia). Thin-layer chromatography (TLC) was performed on Merck (Darmstadt, Germany) aluminum backed silica gel 60 F_{254} plates. Detection was achieved using the following dips with heating for visualization: (a) 20% (w/v) ninhydrin in EtOH (for amines); (b) *p*-anisaldehyde dip (135 mL of EtOAc, 5 mL of concentrated H_2SO_4, 1.5 mL of glacial AcOH, 3.7 mL of *p*-anisaldehyde); (c) H_2SO_4 dip [20% (v/v) H_2SO_4 in EtOH]. Uncorrected melting points (°C) were obtained on a Buchi (Switzerland) melting point apparatus. Optical rotation was measured at room temperature (RT) in a 10 cm cell using a Perkin-Elmer (Wellesley, MA) 241MC polarimeter. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 500 MHz spectrometer (Bruker Biospin, Germany) at 298 K in deuterated chloroform ($CDCl_3$; Cambridge Isotope Laboratories, Andover, MA). Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane, with chemical shifts referenced to the residual $CHCl_3$ peak (δ 7.24 ppm). NMR spectra were processed using Topspin 1.3 (Bruker Biospin, Germany). Elemental analysis was performed on a Carlo Erba (Lakewood, NJ) elemental analyzer model 1106. SDS−PAGE was performed using precast 12% Bis-Tris Novex gels (Invitrogen), and Multimark colored standards (Invitrogen, 10 μL), in a Novex Xcell Surelock Mini-Cell (Invitrogen). The gels were subsequently stained using SimplyBlue SafeStain (Invitrogen, 20 mL) and imaged using a flat-bed scanner (Canon Canoscan N640P).

Analytical RP-HPLC was performed using Shimadzu (Kyoto, Japan) instrumentation (Class Vp 6.12 software, SCL-10AVp controller, SIL-10A autoinjector, LC-10AT pump, LC-10AD pump, Waters 486 tunable absorbance detector). Analytical RP-HPLC was performed in gradient mode using a 1 mL/min flow rate, with detection at 214 nm. For all separations, except for peptide **10**, solvent A consisted of 0.1% (v/v) aqueous TFA and solvent B consisted of either 90% MeCN/0.1% TFA/H_2O (solvent B1), 90% IPA/0.1% TFA/H_2O (solvent B2), or 90% MeOH/0.1% TFA/H_2O (solvent B3). For peptide **10**, solvent A consisted of H_2O acidified to pH 6 (AcOH) and solvent B consisted of 90% MeCN/H_2O acidified to pH 6 (AcOH). Separation was achieved on a Vydac (Hesperia, CA) analytical C18 column (C18, 218TP54, 5 μm, 4.6 mm × 250 mm), a Vydac analytical C4 column (C4, 214TP54, 5 μm, 4.6 mm × 250 mm), or a short Vydac analytical C4 column (C4s, 214TP5405, 5 μm, 50 mm × 4.6 mm). All gradients were run over 30 min.

Preparative-scale RP-HPLC was performed on a Waters Delta 600 system (600E solvent delivery module; 490E multiwavelength UV detector; Fraction Collector III). Preparative RP-HPLC was performed in gradient mode using a 10 mL/min flow rate (unless otherwise specified) with detection at 230 nm. For all separations, except for peptides **3** and **10**, solvent A consisted of 0.1% (v/v) aqueous TFA and solvent B consisted of 90% MeCN/0.1% TFA/H_2O. For peptides **3** and **10** solvent A consisted of H_2O acidified to pH 6 with AcOH, and solvent B consisted of 90% (v/v) aqueous MeCN acidified to pH 6 with AcOH. The following columns were

utilized for separation: Vydac C4 preparative column (C4, 214TP1022, 10 μm, 250 mm × 22 mm), Vydac C18 preparative column (C18, 218TP1022, 10 μm, 250 mm × 22 mm), or a Vydac C4 semipreparative column (C4SP, 214TP1010, 10 μm, 250 mm × 10 mm). Prior to purification of the cysteine containing peptides, cysteine residues were reduced by treating each peptide with tris-(2-carboxyethyl)phosphine hydrochloride (TCEP, approximately 3 equiv per cysteine residue).

ESI-MS was performed on a Perkin-Elmer-Sciex API3000 triple quadrupole mass spectrometer fitted with an atmospheric pressure ESI source. The ion spray source was coupled to a binary Shimadzu HPLC system (SCL-10Avp system controller, 2 × LC-10ATvp pumps, DGU-12A degasser unit, Agilent 1100 series standard autosampler). Samples (1−10 μL) were injected via a manual injection port (Rheodyne, CA) into MeCN−H_2O mobile phases containing 0.1% (v/v) formic acid. ESI-MS data was acquired using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. All samples were run in positive ion mode.

Carbohydrate Synthesis. 2,3,4,6-Tetra-*O*-acetyl-*β*-D-mannopyranosylazide (15). Sodium azide (10.7 g; 170 mmol) was added to 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl bromide[14] (33.8 g, 82.2 mmol) dissolved in anhydrous DMF (140 mL) and left to stir at 70 °C under $Ar_{(g)}$. After 13 h the mixture was concentrated, dissolved in EtOAc (200 mL), and filtered to remove inorganic matter. The filtrate was washed with saturated $NaHCO_3$ solution (3 × 50 mL), 5% (v/v) HCl (3 × 50 mL), and saturated NaCl solution (50 mL), dried ($MgSO_4$), filtered, and concentrated to give a light-brown oil. The product was crystallized from EtOH (30 mL) to afford **15** (4.18 g, 13.6% yield) as white crystals. TLC R_f = 0.79 (EtOAc); mp 114−116 °C (literature value 124 °C[15]); $[\alpha]_D^{25}$ −76.4° (*c* 0.965, $CHCl_3$) (literature value −77°, $CHCl_3$[15]). ESI-MS: *m/z* $[M - N_3]^+$ 331.19 (calcd 331.3), $[M + Na^+]^+$ 396.10 (calcd 396.3). Anal. ($C_{14}H_{19}N_3O_9$) C, H, N. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.97, 2.03, 2.09, 2.19 (4 × 3H, s, CH_3), 3.74 (1H, m, H-5), 4.19 (1H, dd, *J* 12.5, 2.4 Hz, H-6a), 4.26 (1H, dd, *J* 12.3, 5.6 Hz, H-6b), 4.70 (1H, d, *J* 1.0 Hz, H-1), 5.02 (1H, dd, *J* 10, 3.2 Hz, H-3), 5.24 (1H, t, *J* 10 Hz, H-4), 5.43 (1H, d, *J* 2.2 Hz, H-2). ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.47, 20.60, 20.66, 20.66 (4 × CH_3), 62.29 (C-6), 65.39 (C-4), 69.24 (C-2), 70.95 (C-3), 74.67 (C-5), 85.12 (C-1), 169.49, 169.88, 169.88, 170.55 (4 × C═O).

4-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-mannopyranosylamino)-4-oxobutanoic Acid (13). Succinic anhydride (0.5 g, 5.0 mmol) and **15** (1.0 g, 2.7 mmol) were dissolved in dry tetrahydrofuran (THF, 25 mL). This solution was treated with 10% Pd/C (100 mg), degassed, and left to stir at RT under $H_{2(g)}$ for 2 days. The solution was concentrated, taken up in EtOAc (150 mL), and filtered through Celite. The filtrate was washed with 5% (v/v) HCl (3 × 50 mL), dried ($MgSO_4$), and concentrated to give an orange oil. The oil was dissolved in a minimum volume of 9:1:0.1 EtOAc/hexane/AcOH, applied to a column (18 cm × 2.5 cm) of silica gel 60, and eluted with 9:1:0.1 EtOAc/hexane/AcOH. The fractions containing the pure product, as confirmed by TLC and ESI-MS, were combined, concentrated, dissolved in toluene (30 mL), and concentrated to give **13** (0.38 g, 32% yield) as a white foam. TLC R_f = 0.3 (9:1:0.1 EtOAc/hexane/AcOH); $[\alpha]_D^{25}$ −11.0° (*c* 0.807, $CHCl_3$). Anal. ($C_{18}H_{25}NO_{12}$) C, H, N. ESI-MS: *m/z* $[M - NHCO(CH_2)_2COOH^-]^+$ 331.21 (calcd 331.3), $[M + H^+]^+$ 448.32 (calcd 448.4), $[M + Na^+]^+$ 470.33 (calcd 470.4). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.95, 2.02, 2.06, 2.20 (4 × 3H, s, CH_3), 2.49, 2.66 (2 × 2H, m, $-CH_2-CH_2-$), 3.76 (1H, m, H-5), 4.07 (1H, dd, *J* 12.4, 2.1 Hz, H-6a), 4.27 (1H, dd, *J* 12.4, 5 Hz, H-6b), 5.10 (1H, dd, *J* 10, 3.3 Hz, H-3), 5.19 (1H, t, *J* 10 Hz, H-4), 5.32 (1H, t, *J* 3.1 Hz, H-2), 5.52 (1H, d, *J* 9.3 Hz, H-1), 6.66 (1H, d, *J* 9.3 Hz, −NH−). ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.82, 20.87, 20.95, 21.03 (4 × CH_3), 29.16 (CH_2), 30.95 (CH_2), 62.48 (C-6), 65.51 (C-4), 70.21 (C-2), 71.80 (C-3), 74.34 (C-5), 76.34 (C-1), 169.67, 169.93, 170.68, 170.84, 171.27, 176.14 (6 × C═O).

4-(2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucopyranosylamino)-4-oxobutanoic Acid (14). Succinic anhydride (1.09 g; 10.9 mmol) and 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucopyranosylazide[16] (3.7 g, 9.9 mmol) were dissolved in dry THF (40 mL). This solution was treated with a crystal of 4-di(methylamino)pyridine and 10% Pd/C (200 mg), degassed, and left to stir at RT under $H_{2(g)}$ for 24 h. The solution was then concentrated, taken up in EtOAc (150 mL), and filtered through Celite. The filtrate was washed with 5% (v/v) HCl (3 × 30 mL) and saturated NaCl solution (3 × 30 mL), dried ($MgSO_4$), and concentrated to give white crystals, which were triturated with hexane, filtered, and dried to give **14** (3.15 g, 71.3% yield) as white crystals. TLC R_f = 0.38 ($CHCl_3$/MeOH/AcOH, 90:8:2); mp 152−155 °C. Anal. ($C_{18}H_{25}NO_{12}$) C, H, N. ESI-MS: *m/z* $[M - NHCO(CH_2)_2COOH^-]^+$ 331.4 (calcd 331.3), $[M + H^+]^+$ 448.4 (calcd 448.4), $[M + Na^+]^+$ 470.3 (calcd 470.4). ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.99, 2.01, 2.03, 2.05 (4 × 3H, s, CH_3), 2.46 (2H, m, CH_2), 2.61 (1H, m, CH_a), 2.72 (1H, m, CH_b), 3.81 (1H, m, H-5), 4.06 (1H, dd, *J* 12.7, 2.0 Hz, H-6a), 4.27 (1H, dd, *J* 12.7, 4.0 Hz, H-6b), 4.91 (1H, t, *J* 9.5 Hz, H-2), 5.04 (1H, d, *J* 9.9 Hz, H-4), 5.23 (1H, d, *J* 9.0 Hz, H-1), 5.28 (1H, t, *J* 9.5 Hz, H-3), 6.57 (1H, d, *J* 9.5 Hz, NH). ^{13}C NMR (125 MHz, $CDCl_3$): δ 20.55, 20.55, 20.58, 20.70 (4 × CH_3), 28.59, 30.64 (2 × CH_2), 61.66 (C-6), 68.12 (C-4), 70.57 (C-2), 72.72 (C-3), 73.55 (C-5), 78.14 (C-1), 169.60, 169,92, 170.72, 171.25, 171.96, 176.36 (6 × C═O).

Peptide Synthesis. Peptides were synthesized by manual stepwise SPPS on *p*MBHA resin using HBTU/DIPEA in situ neutralization[10] and Boc chemistry. Prior to peptide synthesis, the resin was swollen in anhydrous DMF (10 mL/g resin) for 1 h. The HCl salt was then neutralized by 3 × 15 min treatments with 10% (v/v) DIPEA in anhydrous DMF (10 mL/g of resin) prior to peptide synthesis. Each amino acid coupling cycle consisted of Boc deprotection (2 × 1 min treatments with neat TFA), a 1 min DMF flow-wash, followed by 15−60 min couplings with 4 equiv of preactivated amino acid. Coupling yields were determined using the quantitative ninhydrin test.[17] Where necessary, couplings were repeated to give coupling yields greater than 99.7%. For coupling to a proline residue, the chloranil test[18,19] was utilized instead of the ninhydrin test. Amino acid activation was achieved by dissolving amino acids (4.4 equiv) in 0.5 M HBTU/DMF solution (4 equiv) to which DIPEA (12 equiv) was added. Amino acids were preactivated for 1 min prior to their addition to the resin. The same activation method was utilized for sugar derivatives **13** and **14** (with 5.74 equiv of DIPEA). Boc-amino acids with the following side chain protection were utilized for peptide synthesis: Arg(Tos), Asn(Xan), Asp(OcHx), Cys(*p*MeBzl), Gln(Xan), Glu(OcHx), His(DNP), Lys(2-Cl-Z), Ser(Bzl), Thr(Bzl), Tyr(2-Br-Z). The synthetic racemic lipoamino acid Boc-C12-OH[6] was utilized for LCP lipid core synthesis. After coupling glutamine residues, the resin was washed with DCM before and after Boc deprotection to prevent high-temperature catalyzed pyrrolidone carboxylic acid formation.[10] Acetylation was achieved by treating the resin with a mixture of acetic anhydride (0.5 mL), DIPEA (0.47 mL), and DMF (14 mL) for 5 min and repeating for 30 min. Following peptide synthesis, the following procedures were performed prior to peptide cleavage where necessary: (1) For peptides containing His(DNP) residues (**1**−**6**, **8**−**11**), the DNP group was cleaved by treating the resin with 20% (v/v) *β*-mercaptoethanol, 10% (v/v) DIPEA in DMF for 2 × 1 h treatments. (2) For peptides containing an N-terminal Boc-protecting group (**1**, **7**, **8**), the Boc-protecting group was removed, followed by a 1 min DMF flow-wash. (3) Peptides **2**, **4**−**6**, **9**, and **11** were de-O-acetylated by agitating the resin in 1:11 hydrazine hydrate in DMF[11] for 4.5 h. Following removal of these protecting groups, the resin was thoroughly washed with DMF, DCM, and MeOH and dried (vacuum desiccator) prior to cleavage using anhydrous HF.

HF Cleavage. HF cleavage (10 mL of HF/g of resin) was performed at −5 °C for 2 h. *p*-Cresol (5% (v/v)) was utilized as a scavenger, with 5% (v/v) *p*-thiocresol also added where peptides contained *p*MeBzl protected Cys residues (**1**, **2**, **4**, **6**, **8**, **9**, **11**). For peptides containing *O*-acetyl protected sugars (**3**, **10**), 10% (v/v) *p*-cresol was used as a scavenger, with no *p*-thiocresol added.

Following HF cleavage, the HF was removed under reduced pressure. The peptides were then precipitated in ice-cold ether, filtered, and washed with ice-cold ether. The peptides, except for **3** and **10**, were then dissolved in 40% MeCN/0.1% TFA/H_2O, frozen using MeOH−dry ice, and lyophilized to give the crude peptides. Alternatively, peptides **3** and **10** were dissolved in 50% (v/v) aqueous MeCN, frozen using MeOH−dry ice, and lyophilized to give the crude peptides.

Synthesis of LCP Systems 1−7. Synthesis of LCP systems **1−7** was performed as previously described.[4,20] Briefly, Boc-Gly-OH was coupled to *p*MBHA resin, followed by two cycles of Boc-C12-OH, then Boc-Gly-OH, Boc-C12-OH, Boc-Lys(Boc)-OH, and Boc-Lys(Boc)-OH. Following deprotection of the N-terminal Boc protecting groups, a portion of the resin was washed, dried, and cleaved to yield **7**. For peptides **1−6**, four copies of the peptide epitope of interest were synthesized on the exposed amines. The N-terminal Boc protecting groups were then removed. For peptide **1**, the resin was then washed, dried and cleaved. For peptides **2−6**, the modified carbohydrate of interest (**13** or **14**) was coupled to the N-terminal amines. Peptides **2**, and **4−6** were then de-O-acetylated. The resins were then washed, dried, and cleaved to yield the crude peptides, which were semipurified by preparative RP-HPLC.

Analytical Data for LCP System 1. Purified yield: 51.5%. HPLC: t_R = 21.292 min (0−100% solvent B1, C4 column), t_R = 18.858 min (0−100% solvent B2, C4 column). SDS−PAGE: MW 11 kDa (expected 9.9 kDa). ESI-MS: *m*/*z* $[M + 7H^+]^{7+}$ 1412.4 (calcd 1413.7), $[M + 9H^+]^{9+}$ 1100.4 (calcd 1099.8), $[M + 12H^+]^{12+}$ 824.6 (calcd 825.1). MW 9889.45 g/mol.

Analytical Data for LCP System 2. Purified yield: 45.2%. HPLC: t_R = 21.833 min (0−100% solvent B1, C4 column), t_R = 18.192 min (0−100% solvent B2, C4 column). SDS−PAGE: MW 12 kDa (expected 10.9 kDa). ESI-MS: *m*/*z* $[M + 8H^+]^{8+}$ 1367.3 (calcd 1367.8), $[M + 10H^+]^{10+}$ 1094.5 (calcd 1094.4), $[M + 11H^+]^{11+}$ 994.9 (calcd 995.0), $[M + 12H^+]^{12+}$ 913.2 (calcd 912.2), $[M + 14H^+]^{14+}$ 781.3 (calcd 782.0), $[M + 15H^+]^{15+}$ 729.8 (calcd 729.9), $[M + 16H^+]^{16+}$ 683.4 (calcd 684.4), $[M + 17H^+]^{17+}$ 645.3 (calcd 644.2), $[M + 18H^+]^{18+}$ 608.3 (calcd 608.5). MW 10 934.36 g/mol.

Analytical Data for LCP System 3. Purified yield: 45.4%. HPLC: t_R = 21.133 min (0−100% solvent B1, C4 column), t_R = 19.033 min (0−100% solvent B2, C4 column). SDS−PAGE: MW 12 kDa (expected 11.6 kDa); MW 11606.95 g/mol. Treating 5 mg of **3** with 0.6 M aqueous LiOH (degassed with $He_{(g)}$) for 2 h at RT (to cleave *O*-acetyl protection) followed by acidification and reduction with TCEP (approximately 3 eq/cys) yielded an ESI-MS spectrum identical to that for LCP system **2**.

Analytical Data for LCP System 4. Purified yield: 10.6%. HPLC: t_R = 20.467 min (0−100% solvent B1, C4 column), t_R = 17.342 min (0−100% solvent B2, C4 column). SDS−PAGE: MW 12 kDa (expected 10.9 kDa). ESI-MS: *m*/*z* $[M + 10H^+]^{10+}$ 1095.1 (calcd 1094.4), $[M + 11H^+]^{11+}$ 994.3 (calcd 995.0), $[M + 14H^+]^{14+}$ 782.4 (calcd 782.0), $[M + 15H^+]^{15+}$ 729.3 (calcd 729.9), $[M + 16H^+]^{16+}$ 684.1 (calcd 684.4). MW 10 934.36 g/mol.

Analytical Data for LCP System 5. Purified yield: 69.0%. HPLC: t_R = 21.925 min (0−100% solvent B1, C4 column), t_R = 20.100 min (0−100% solvent B2, C4 column). SDS−PAGE: MW 12 kDa (expected 12.9 kDa). ESI-MS: *m*/*z* $[M + 9H^+]^{9+}$ 1441.3 (calcd 1440.6), $[M + 10H^+]^{10+}$ 1295.7 (calcd 1296.7), $[M + 11H^+]^{11+}$ 1177.3 (calcd 1178.9), $[M + 12H^+]^{12+}$ 1079.7 (calcd 1080.7), $[M + 15H^+]^{15+}$ 865.4 (calcd 865.4). MW 12 956.54 g/mol.

Analytical Data for LCP System 6. Purified yield: 58.4%. HPLC: t_R = 19.842 min (0−100% solvent B1, C4 column), t_R = 18.208 (0−100% solvent B2, C4 column). SDS−PAGE: MW 11 kDa (expected 10.9 kDa). ESI-MS: *m*/*z* $[M + 10H^+]^{10+}$ 1094.5 (calcd 1094.4), $[M + 11H^+]^{11+}$ 994.9 (calcd 995.0), $[M + 12H^+]^{12+}$ 913.0 (calcd 912.2), $[M + 14H^+]^{14+}$ 781.3 (calcd 782.0), $[M + 15H^+]^{15+}$ 729.7 (calcd 729.9), $[M + 16H^+]^{16+}$ 683.7 (calcd 684.4), $[M + 17H^+]^{17+}$ 645.3 (calcd 644.2). MW 10r t934.36 g/mol.

Analytical Data for LCP System 7. Yield: 6.9%. HPLC: t_R = 22.658 and 23.033 min (0−100% solvent B1, C4 column), t_R = 20.925 and 21.208 min (0−100% solvent B2, C4 column), t_R = 27.933 min (20−100% solvent B3, C4 column). HPLC purity, ≥95%. ESI-MS: *m*/*z* $[M + H]^+$ 1109.1 (calcd 1108.6), $[M + 2H]^{2+}$ 555.4 (calcd 554.8). MW 1107.60 g/mol.

Analytical Data for Control Peptide 8. Yield: 6.5%. HPLC: t_R = 16.125 min (0−100% solvent B1, C18 column), t_R = 21.792 min (10−50% solvent B1, C18 column), t_R = 14.258 min (0−100% solvent B2, C18 column), t_R = 24.225 min (0−100% solvent B3, C18 column). HPLC purity = 95.3%. ESI-MS: *m*/*z* $[M + 2H^+]^{2+}$ 1107.8 (calcd 1107.2), $[M + 3H^+]^{3+}$ 739.0 (calcd 738.5), $[M + 4H^+]^{4+}$ 554.8 (calcd 554.1), $[M + 6H^+]^{6+}$ 371.5 (calcd 369.7). MW 2212.49 g/mol.

Analytical Data for Control Peptide 9. Yield: 7.5%. HPLC: t_R = 16.025 min (0−100% solvent B1, C18 column), t_R = 21.567 min (10−50% solvent B1, C18 column), t_R = 14.008 min (0−100% solvent B2, C18 column), t_R = 23.708 min (0−100% solvent B3, C18 column). HPLC purity = 98.4%. ESI-MS: *m*/*z* $[M + 2H^+]^{2+}$ 1238.2 (calcd 1237.9), $[M + 3H^+]^{3+}$ 825.6 (calcd 825.6). MW 2473.72 g/mol.

Analytical Data for Control Peptide 10. Yield: 18.6%. HPLC: t_R = 15.833 (−2Ac) and 15.492 (−1Ac) min (0−100% solvent B, C4 column), t_R = 11.183 (−2Ac) and 11.458 (−1Ac) min (0−100% solvent B, C4s column), t_R = 9.042 (−2Ac) and 9.317 (−1Ac) min (0−100% solvent B2, C4s column). HPLC purity = N/A. ESI-MS: *m*/*z* $[M - Ac + 2H]^{2+}$ 1301.1 (calcd 1300.0), $[M - 2Ac + 2H]^{2+}$ 1280.6 (calcd 1279.0), $[M - Ac + 3H]^{3+}$ 868.4 (calcd 867.0), $[M - 2Ac + 3H]^{3+}$ 854.2 (calcd 853.0). MW (−Ac) 2598.08 g/mol. MW (−2Ac) 2556.07 g/mol.

Analytical Data for Control Peptide 11. Yield: 12.9%. HPLC: t_R = 15.842 min (0−100% solvent B1, C18 column), t_R = 18.458 min (10−60% solvent B1, C18 column), t_R = 14.508 min (0−100% solvent B2, C18 column), 23.950 min (0−100% solvent B3, C18 column). HPLC purity, ≥95%. ESI-MS: *m*/*z* $[M + H^+]^+$ 2474.7 (calcd 2474.7), $[M + 2H^+]^{2+}$ 1238.5 (calcd 1237.9), $[M + 3H^+]^{3+}$ 826.6 (calcd 825.6). MW 2473.72 g/mol.

Immunological Assessment of LCP Systems (1−7) and Control Peptides (8−12). All protocols were approved by the Bancroft Centre Research Animal Ethics Committee (approval number P415). C57BL/6 (H-2^b) mice (10/group) were immunized at the tail base with compounds **1−7** and **9−11** in RPMI 1640 media, with **8** in RPMI 1640 media or emulsified in incomplete Freund's adjuvant (IFA), and with OVA/SIINFEKL (**12**) in IFA. For priming, 30 μg of immunogen [40 μg for carbohydrate containing immunogens (**2−6**, **9−11**); 1μg/10μg for the OVA/SIINFEKL group (**12**)] was administered in either IFA (1:1) or RPMI media (50 μL total volume). β-Mercaptoethanol (0.02 mM) was included in all samples to prevent cysteine oxidation prior to immunization. Three weeks after immunization, the mice were challenged with TC-1 cells[13] (1×10^6) injected into the scruff of the neck (parts a and b of Figure 2) or lower back (parts c and d of Figure 2). Fifteen days after the tumor cell challenge, tumors were excised and weighed to investigate tumor growth.

Statistical Analysis. Comparison of the average resected TC-1 tumor masses between groups was performed using a one-way ANOVA followed by the Bonferroni post hoc test. GraphPad Prism 4 was used for statistical analysis, with $P < 0.05$ taken as statistically significant.

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Supporting Information Available: Elemental analysis results, SPPS details, preparative RP-HPLC data, and SDS−PAGE details. This material is available free of charge via the Internet at http://pubs.acs.org.

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