Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Design of Fully Synthetic, Self-Adjuvanting Vaccine Incorporating the Tumor-Associated Carbohydrate Tn Antigen and Lipoamino Acid-Based Toll-like Receptor 2 Ligand

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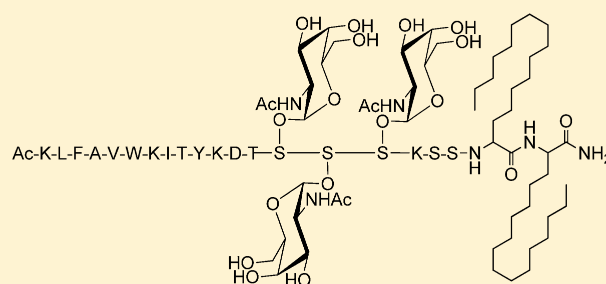
S *Supporting Information*

ABSTRACT: Overexpression of certain tumor-associated carbohydrate antigens (TACA) caused by malignant transformation offers promising targets to develop novel antitumor vaccines, provided the ability to break their inherent low immunogenicity and overcome the tolerance of the immune system. We designed, synthesized, and immunologically evaluated a number of fully synthetic new chimeric constructs incorporating a cluster of the most common TACA (known as Tn antigen) covalently attached to T-cell peptide epitopes derived from polio virus and ovalbumin and included a synthetic built-in adjuvant consisting of two 16-carbon lipoamino acids. Vaccine candidates were able to induce significantly strong antibody responses in mice without the need for any additional adjuvant, carrier protein, or special pharmaceutical preparation (e.g., liposomes). Vaccine constructs were assembled either in a linear or in a branched architecture, which demonstrated the intervening effects of the incorporation and arrangement of T-cell epitopes on antibody recognition.



INTRODUCTION

Malignant transformations resulting in defects in the cellular glycosylation machinery lead to aberrant changes of cell surface carbohydrates and overexpression of tumor-associated carbohydrate antigens (TACAs).[1] These TACAs represent attractive targets for the development of novel antitumor vaccines. For instance, *N*-acetylgalactosamine *O*-glycosides of serine or threonine (known as Tn antigen) are overexpressed in about 90% of human adenocarcinomas and more than 70% of lung, colon, and stomach carcinomas. The extent of its expression correlates with carcinoma differentiation and aggressiveness while it is essentially absent in normal cells.[2] However, the inherent immunogenicity of Tn antigens is too low to overcome the tolerance of the immune system.[1,3,4] Several conjugates of Tn antigen and immunogenic carrier proteins, such as keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA), have been used in clinical trials after promising results correlating with high antibody titers and cell-surface reactivity of the sera from immunized mice.[5,6] Alternatively, new strategies are focused on the covalent coupling of carbohydrate antigens to defined minimal CD4+ and CD8+ T-cell peptide epitopes and lipid moieties targeting the immunostimulating Toll-like receptor 2 (TLR2).[7,8] These multicomponent immunostimulating constructs improve vaccine specificity through their defined chemical composition, reproducible purity, and high antigen density.[10] However, from a synthetic point of view, covalent assembly of the vaccine components may present a challenge because it requires the use of manipulations that are compatible with different chemical classes (e.g., carbohydrates, lipids, and peptides).[7,11,12]

Lipoamino acids (LAAs) are synthetic compounds and combine the properties of both amino acids (amphoteric bifunctional moiety) and lipids (long alkyl chain). LAAs are easily accessible building blocks for the design and engineering of immunostimulating TLR2 ligands. To benefit from the immunostimulatory effects of TLR2 ligands, we developed lipopeptides incorporating two 16-carbon containing (C16) LAA residues that activate TLR2.[13−15] The use of LAAs as immunostimulants is advantageous because of their compatibility with standard solid-phase peptide synthesis (SPPS) protocols. This greatly improves synthesis and purification of their conjugates, unlike other TLR2 ligands previously used for anticancer vaccines, e.g., Pam_2Cys or Pam_3Cys.[16]

Herein, the design, synthesis, and immunological assessment of new self-adjuvanting cancer vaccine candidates is described. This system (5−7, Figure 1) consists of (1) a TLR2 targeting immunostimulant having two C16 LAAs and two serine residues at the C-terminus of the constructs, (2) a cluster of three Tn antigen B-cell epitopes, (3) a universal CD4+ T-cell helper epitope (PV,[17] KLFAVWKITYKDT) derived from polio virus, and (4) a CD8+ T-cell peptide epitope derived from

Received: June 12, 2012
Published: July 16, 2012

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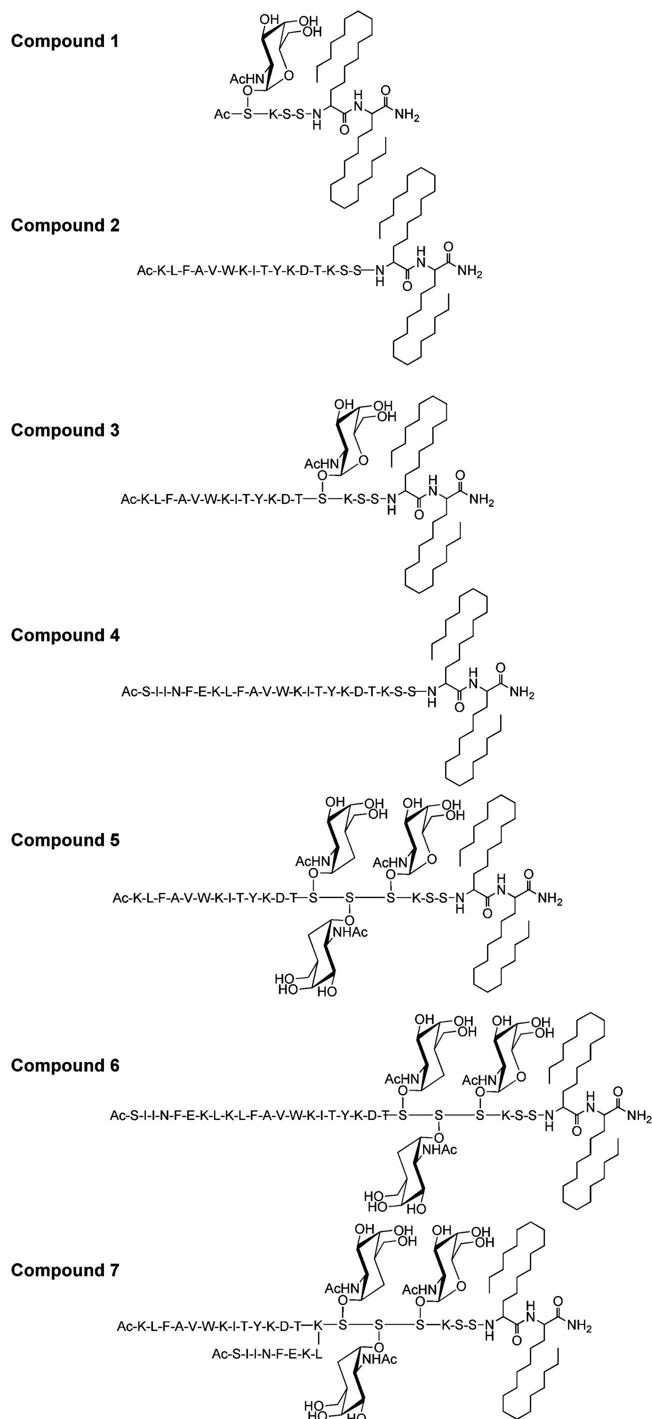

Figure 1. Structure of glycolipopeptide constructs.

ovalbumin ($OVA_{257-264}$;[18] SIINFEKL). The current study aimed to design a new Tn- and LAA-based anticancer vaccine delivery system capable of eliciting glycopeptide-specific antibody responses and to highlight the effect of molecular context on the elicited antibodies. To this end, the design of constructs (**5**–**7**, Figure 1) was varied to include both linear three- or four-component analogues (**5** and **6**, respectively) and a branched four-component analogue (**7**) which would provide information about the effect of incorporation or arrangement of T-cell epitopes on antibody recognition. Results demonstrated the immunogenicity and the intervening effects of vaccine components on the design of the novel LAA-based multi-component vaccine constructs.

■ RESULTS AND DISCUSSION

All compounds were synthesized on Rink amide MBHA resin following peptide Fmoc-chemistry using standard microwave-assisted SPPS protocols. Synthesis of linear glycolipopeptides (**5** and **6**, Figure 2) was performed by coupling two LAAs to the resin followed by coupling of two copies of Fmoc-Ser(tBu), one Fmoc-Lys(Boc) residue, and three copies of the glycosylated amino acid (Fmoc-Ser(*O*-*α*-D-GalNAc(OAc)3-OH). Coupling reactions were performed using standard HBTU/HOBt activation with 5 equiv of Fmoc-amino acids, 3.2 equiv of Dde-protected C16 LAA, and 1 equiv of the Tn-glycosylated amino acid. Coupling of the protected glycosylated amino acid was repeated three times to achieve adequate yield. A capping acetylation step was performed following the introduction of the first amino acid, and the glycosylated amino acid, to facilitate purification. Afterward, the peptide was elongated by either coupling the PV peptide sequence (KLFAVWKITYKDT) as in **5**, or coupling both PV and $OVA_{257-264}$ sequences (KLFAVWKITYKDTSIINFEKL) as in **6**.

The branched glycolipopeptide (**7**, Figure 3) was prepared in a manner similar to that for **5**, except that one copy of Fmoc-Lys(Dde)-OH was inserted in the backbone of the peptide after the introduction of the protected glycosylated amino acid residues to allow late branching after coupling of the PV amino acid sequence and subsequent N-terminal acetylation. Branching was performed by coupling of amino acid residues of OVA-derived CD8+ peptide epitope (SIINFEKL) on the side amino group of the lysine residue after Dde-deprotection using 2% hydrazine hydrate. After completion of synthesis, all resin-bound peptides were acetylated at the N-terminal and acetates of the glycoside residues were deprotected on the solid support using a solution of 6:1 v/v DMF/hydrazine hydrate for 6 h to afford compounds **5**–**7**. Purification was achieved using preparative reversed-phase HPLC C4 column (yields = 40–50%). All compounds were analyzed by mass spectrometry, and their purity was determined by analytical reversed- phase HPLC (Supporting Information).

Immunogenicity was evaluated in BALB/c ($H\text{-}2^d$) mice, which received a 30 μg dose each week for five weeks of tested compounds dissolved in sterile-filtered phosphate-buffered saline (PBS) either without additional adjuvant (**4**–**7**) or with complete Freund's adjuvant (CFA) (**5** and **6**). The negative control group was administered PBS only. Sera collected 9 days after the last immunization were tested by ELISA to assess antibody titers.[19]

Direct comparison of compounds **4**–**7** (Figure 4) resulted in three important observations. The first observation was that significant antibody response was induced without the need for any additional adjuvant, carrier protein, or special liposomal preparation, unlike previously reported Tn-containing glycolipopeptides.[8,20–22] The systemic IgG antibody response to immunization with compound **5** was significantly greater than that of the negative control groups (**5** vs PBS, $p < 0.05$ and **5** vs **4**, $p < 0.05$).

The second observation was that the current delivery system elicited glycopeptide-specific antibody responses. This was demonstrated by the differential binding of elicited antibodies to ELISA plates coated with compounds **2**, **3**, and **5**, which differ only in the number of Tn residues (0, 1, and 3,

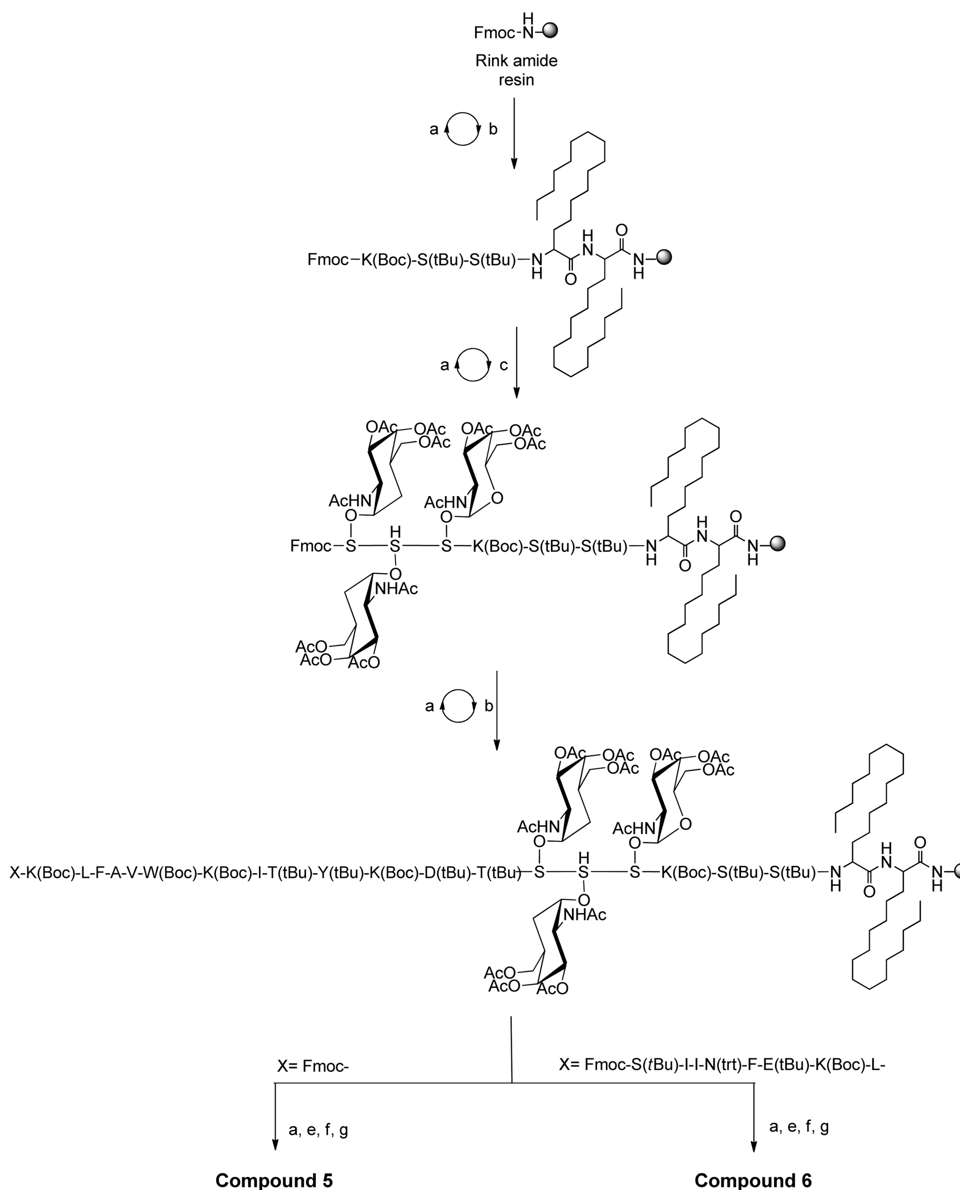

Figure 2. Synthesis of linear glycolipopeptides **5** and **6**. (a) Fmoc deprotection: 20% piperidine/DMF (1 × 2 min, 1 × 5 min); (b) coupling of Fmoc amino acids or Dde-LAA (3.2 equiv), HBTU, HOBt, DIPEA in DMF; (c) Coupling of Fmoc-Ser (*O*-*α*-D-GalNAc(OAc)$_3$)-OH (3 × 1.0 equiv), HBTU, HOBt, DIPEA in DMF; (d) Dde deprotection:2% hydrazine hydrate/DMF (1 × 5 min, 1 × 10 min; rt); (e) N-acetylation: Ac_2O/DIPEA/DMF (100 μL/100 μL/5 mL; 2× 10 min); (f) deacetylation: hydrazine hydrate/DMF (6:1 v/v), 6 h.; (g) cleavage from resin: TFA/TIS/H_2O (9.5:0.25:0.25).

respectively). The antibodies elicited by **5** did bind to ELISA plates coated with **5** but did not bind to ELISA plates coated with **2** or **3** (data not shown). The antibodies bound to ELISA plates coated with the compound **5** which contained three Tn residues. This observation was in accordance with previously reported results of multivalent Tn constructs.[9,23,24] It was also demonstrated that at least two adjacent Tn antigen residues were crucial for the binding of the anti-Tn monoclonal antibodies, whereas affinity was greater on a glycopeptide with three repeat Tn residues.[25]

The third observation was that sera of mice administered compounds **6** or **7** demonstrated statistically nonsignificant levels of IgG capable of binding to ELISA plates coated with compound **5**. However, mice administered compound **6** with CFA elicited significantly high levels of serum IgG titers capable of binding ELISA plates coated with compound **5**. These were similar to IgG titer levels elicited by compound **5** but lower than that in mice administered compound **5** mixed with CFA (**6** with **CFA** vs **5**, $p > 0.05$ and **6** with **CFA** vs **5** with **CFA,** $p < 0.05$). This observation supported the hypothesis that incorporation of CD8+ T-cell peptide derived from OVA at the N-terminus of compound **6** interfered with the ability of elicited antibodies to bind epitopes embedded in compound **5** and completely abolished binding when incorporated at the point of branching, as in compound **7**. Overall, the incorporation and the spatial arrangement of T-cell epitopes influenced antibody specificity of Tn-based multicomponent vaccines.

It was of interest to investigate the effect of incorporating Tn-glycosylated serine residues on TLR2 targeting LAA-based moieties. Compound **1** was tested as a model for TLR2 activity (Figure 5) using human embryonic kidney (HEK293) cells

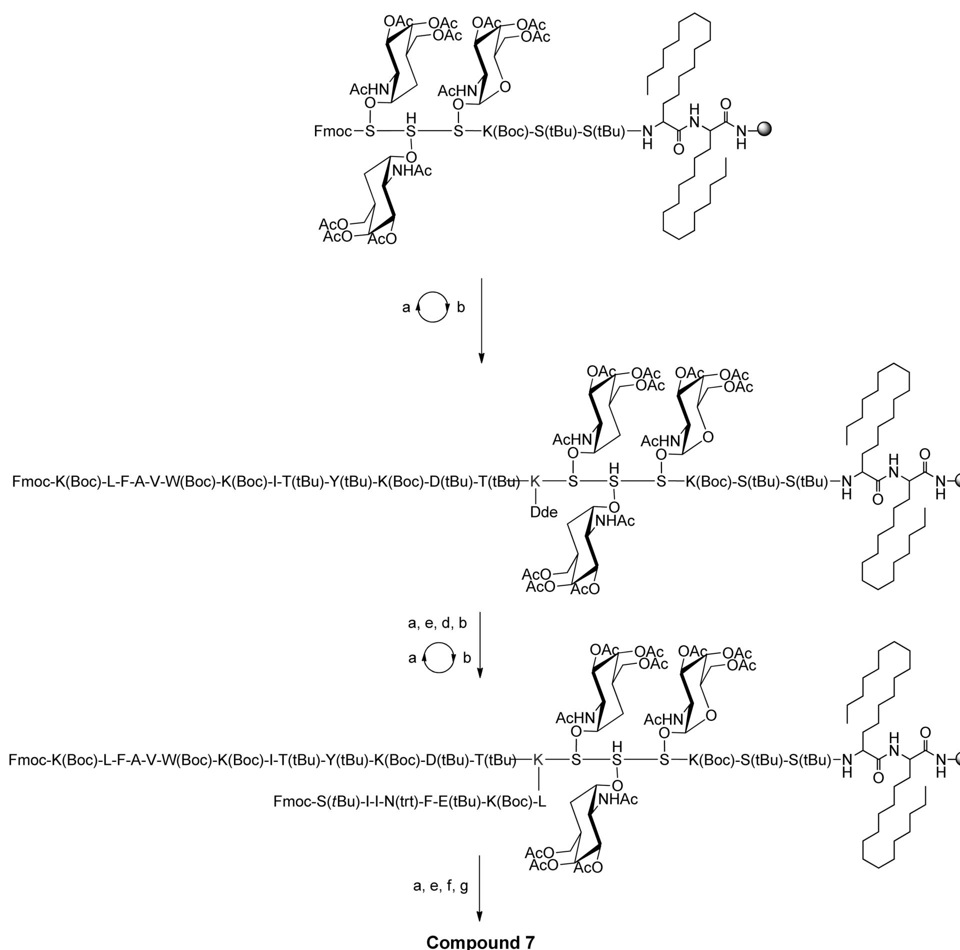

Figure 3. Synthesis of branched glycolipopeptide **7**. (a) Fmoc deprotection: 20% piperidine/DMF (1 × 2 min, 1 × 5 min); (b) coupling of Fmoc amino acids or Dde-LAA (3.2 equiv), HBTU, HOBt, DIPEA in DMF; (c) Coupling of Fmoc-Ser (*O*-*α*-D-GalNAc(OAc)$_3$)-OH (3 × 1.0 equiv), HBTU, HOBt, DIPEA in DMF; (d) Dde deprotection: 2% hydrazine hydrate/DMF (1 × 5 min, 1 × 10 min; rt); (e) N-acetylation: Ac_2O/DIPEA/DMF (100 *μ*L/100 *μ*L/5 mL; 2× 10 min); (f) deacetylation: hydrazine hydrate/DMF (6:1 v/v), 6 h.; (g) Cleavage from resin: TFA/TIS/H_2O (9.5:0.25:0.25).

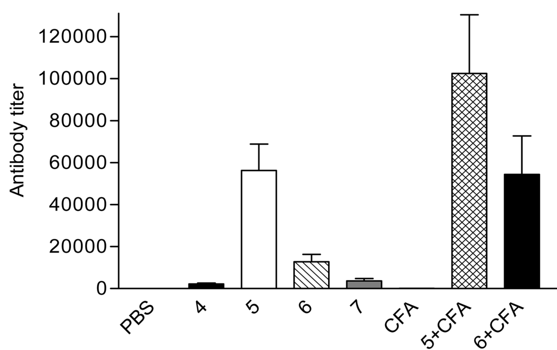

Figure 4. Reciprocal of IgG antibody titers at the final bleed (day 37). BALB/c (H-2^d) mice were administered five doses of tested compounds, and antibody titers were determined using ELISA plates precoated with compound **5**. Statistical analysis was performed using a one-way ANOVA followed by Tukey's multiple comparison test.

stably expressing TLR2 transfected with pNF-*κ*B-Luc Cis-Reporter plasmid. Negative control wells were treated with media while positive control wells were stimulated by 10 nM Pam_3Cys analogue. Compound **1** was able to induce significant TLR2 activation at a concentration range of 100−300 nM. These results indicated that the incorporation of Tn residue has no effect on TLR2 activity, while the C16 LAA acts as an immunostimulant for Tn-specific antibody response.

■ CONCLUSION

There has been a growing interest in the search for new vaccine delivery systems capable of stimulating TACA-specific effective immune responses. The current study represents the first report to describe a Tn-based vaccine delivery system incorporating LAA-based built-in adjuvant targeting TLR2. The combination of Tn antigens with LAA-based TLR2 ligands resulted in efficient, fully synthetic vaccine candidates, which can be easily synthesized through traditional SPPS. We have also demonstrated the effect of incorporation and arrangement of T-cell epitopes on antibody specificity of Tn-based multicomponent vaccines. The vaccine candidates presented in this study are applicable to humans because they do not require additional adjuvants or special pharmaceutical formulation (e.g., liposomes) and are advantageous over other existing vaccine candidates based on carrier proteins, as their the administration may include redundant carrier-associated epitopes and epitope suppression due to the presence of antibodies against the carrier.

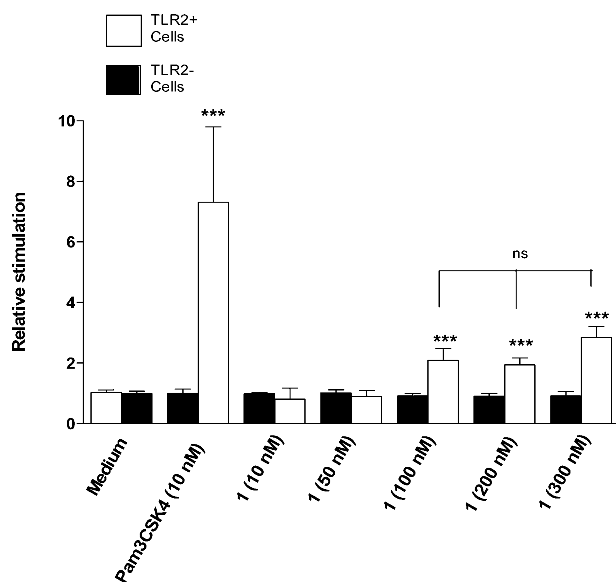

Figure 5. The ability of compound **1** to signal through TLR2 in a dose−response manner. HEK293 cells stably expressing TLR2 and transfected with an NF-κB-luciferase reporter gene were treated with tested compounds, and the several fold increase in NF-κB levels in treated cells were shown relative to that of the TLR2-expressing cells treated with media, which was set to a value of 1. Data are shown as means + SD of three-culture runs in two separate experiments. Variation between groups was analyzed using the two-tailed Student's *t* test (ns, $p > 0.05$; $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

■ EXPERIMENTAL SECTION

Materials and Methods. Protected L-amino acids and Rink amide resin were purchased from Novabiochem (Läufelfingen, Switzerland) or Reanal (Budapest, Hungary). Peptide synthesis grade trifluoroacetic acid (TFA), *N*,*N*-dimethylformamide (DMF), and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Auspep (Melbourne, Australia). HPLC grade acetonitrile (MeCN) was purchased from Honeywell-Burdick and Jackson (Morristown, NJ) or Labscan (Dublin, Ireland). All other reagents were purchased at the highest available purity from Sigma-Aldrich (Castle Hill, NSW, Australia) and were used without further purification. Dichloromethane (CH_2Cl_2) was distilled over calcium hydride. Other anhydrous-grade solvents were purchased from Sigma-Aldrich and were used directly. ESI-MS was performed with a Perkin-Elmer-Sciex API3000 instrument using Analyst 1.4 (Applied Biosystems/MDS Sciex, Toronto, Canada) software. Samples were introduced into MeCN−water mobile phase containing 0.1% (v/v) acetic acid.

MALDI-TOF mass spectra were measured on a Voyager-DE PRO biospectrometry workstation by PerSeptive Biosystem. A solution of 10 mg/mL α-cyano-4-hydroxycinnamic acid containing 0.1% TFA was used for generation of probe−matrix mixture. Analytical RP-HPLC was performed using the Agilent 1100 series system equipped with an autosampler, UV-detector, and fraction-collector with a 1 mL/min flow rate and detection at 214 nm. Separation was achieved on either a Vydac analytical C4 column (214TP54; 5 μm, 4.6 mm × 250 mm) or a Vydac analytical C18 column (218TP54; 5 μm, 4.6 mm × 250 mm). Analysis was run in gradient mode using 0.1% TFA/H_2O as solvent A and either 90% MeCN/0.1% TFA/H_2O (solvent B1) or 90% MeOH/0.1% TFA/H_2O (solvent B2). Two methods were run: method A, 20−100% solvent B1 over 45 min, method B, 50−100% solvent B2 over 30 min. Preparative RP-HPLC was performed on a Waters Delta 600 system using 5 mL/min flow rate and detection at 230 nm. Separations were performed in gradient mode with 0.1% TFA/H_2O as solvent A and 90% MeCN/0.1% TFA/H_2O as solvent B on Vydac preparative C4 column (214TP1022; 10 μm, 22 mm × 250 mm).

General Procedure for Glycolipopeptide Synthesis. All compounds (**1**−**7**) were assembled on Rink amide MBHA resin (0.7 mmol/g) by microwave-assisted solid-phase peptide synthesis (SPPS) at 40 w and 75 °C. SPPS was carried out by using a SPS mode CEM Discovery reactor (CME Corporation, Matthews, NC). Fmoc-amino acids with the following side-chain protection were used throughout the SPPS: Fmoc-Ser(t-Bu), Fmoc-Lys (Boc), Fmoc-Lys (Dde), Fmoc-Asp, Fmoc-Tyr(t-Bu), Fmoc-Thr(t-Bu), Fmoc-Ile, Fmoc-Trp(Boc), Fmoc-Val, Fmoc-Ala hydrate, Fmoc-Phe, Fmoc-Leu, Fmoc-Glu(t-Bu), Fmoc-Asn(trt). Normal coupling was performed in DMF with 5 equiv of the Fmoc-amino acids, 3.2 equiv of lipoamino acid (Dde-C16), and 1 equiv of the Fmoc-amino acid glycoside (Tn antigen). Coupling activation was achieved using HBTU (0.30 mmol)/DIPEA (0.45 mmol) for 3.1 mmol of amino acid and 1 mmol resin scale. Coupling time was (1 cycle × 15 min, double coupling) for Fmoc-amino acids, (2 cycles × 15 min, double coupling) for Dde-protected lipoamino acid and (1 cycle × 30 min, triple coupling) for Fmoc-amino acid glycoside (double coupling helps to ensure complete coupling). Deprotection of Fmoc-protecting group was performed by treatment with 20% piperidine/DMF (1 × 2 min) and then (1 × 5 min) utilizing the microwave radiation whereas deprotection of the Dde-protecting group was achieved using 2% hydrazine hydrate/DMF (1 × 5 min) and then (1 × 10 min) at rt. A capping process with acetic anhydride/DIPEA/DMF 1:1:3 (2 × 5 min), after the introduction of the first amino acid and after the introduction of the glycosylated amino acid, was recommended to facilitate purification. Compounds were acetylated after removal of the protecting group of the last amino acid in the peptide synthesis by treatment of the resin with 100 μL of Ac_2O, 100 μL DIPEA in 5 mL of DMF (2 × 10 min). After completion of synthesis, the acetates of the glycoside residue were deprotected while on the solid support using a solution of 6:1 v/v DMF/hydrazine hydrate for 6 h and then the resin was washed with DMF and DCM several times and dried prior to cleavage. Cleavage of the peptide from the resin was achieved by stirring the resin with a cleavage cocktail composed of TFA (4.25 mL), triisopropylsilane (0.25 mL), and water (0.25 mL) for 2.5 h. The resin was washed with TFA (4 × 4 mL), the combined solutions were concentrated in vacuo, and the crude product was precipitated with ice-cold diethyl ether and filtered. The crude precipitate was then dissolved in acetonitrile/water/1% TFA and lyophilized prior to purification. Crude fractions were analyzed by ESI-MS and analytical RP-HPLC and where appropriate combined to give pure product (>95%).

Compound 1. Two LAA residues were coupled to Fmoc-deprotected Rink amide resin followed by coupling of the Fmoc-Ser(*t*Bu), Fmoc-Ser(*t*Bu), and Fmoc-Lys(Boc) following methods mentioned in the general procedures. Coupling of the glycosyl amino acid (Fmoc-Ser(*O*-α-D-GalNAc(OAc)$_3$-OH) was achieved using activated solution of 3 × 1 equiv of the protected Tn antigen as described in the general procedures followed by Fmoc deprotection and N-terminal acetylation. Deacetylation of the carbohydrate residue was performed on resin using 6:1 v/v DMF/hydrazine hydrate for 6 h. The resin was cleaved as previously mentioned to afford compound **1**. Scale: 0.1 mmol. HPLC: t_R = 19.6 min (method A, C4 column), t_R = 18.7 min (method B, C4 column); ESI-MS: *m*/*z* 1159.1 $[M + H]^+$ (calculated: 1159.51), MALDI-TOF (*m*/*z*) 1157.84 $[M]^+$, 1179.87 $[M + Na]^+$, 1195.785 $[M + K]^+$.

Compound 2. The Fmoc-Rink amide resin was treated in a fashion similar to that for compound **1**, except the coupling of glycosylated amino acid residues (Tn) which is replaced by coupling of the 13 amino acid residues of PV peptide sequence (KLFAVWKITYKDT) followed by N-terminal acetylation and cleavage from resin to afford compounds **2**. Scale: 0.025 mmol. HPLC: t_R = 18.3 min (method A, C4 column), t_R = 17.8 min (method B, C4 column); ESI-MS: *m*/*z* 1233.1 ($[M + 2H]^{2+}$, calcd 1232.56), 822.6 $[M + 3H]^{3+}$, 616.4 $[M + 4H]^{4+}$, MALDI-TOF (*m*/*z*) 2464.98 $[M + H]^+$, 2484.9 $[M + Na]^+$, 2501.02 $[M + K]^+$.

Compound 3. The Fmoc-Rink amide resin was treated in a fashion similar to that for compound **1** where the peptide was further elongated by coupling PV peptide sequence (KLFAVWKITYKDT)

after the glycosylated amino acid, followed by N-terminal acetylation, carbohydrate deacetylation and cleavage from resin to afford compounds **3**. Scale: 0.05 mmol. HPLC: t_R = 18.2 min (method A, C4 column), t_R = 17.8 min (method B, C4 column); ESI-MS: *m/z* 1378.0 ($[M + 2H]^{2+}$, calcd 1377.7), 918.9 $[M + 3H]^{3+}$, 689.6 $[M + 4H]^{4+}$); MALDI-TOF (*m/z*) 2754.34 $[M + H]^+$, 2776.4 $[M + Na]^+$, 2795.40 $[M + K]^+$.

Compound 4. The Fmoc-Rink amide resin was treated in a fashion similar to that for **2**, except that the peptide was further elongated after the PV sequence by conjugation of eight amino acids of the following sequence: SIINFEKL to afford compound **4**. Scale: 0.025 mmol. HPLC: t_R = 18.1 min (method A, C4 column), t_R = 17.5, 17.7, 17.9 min (method B, C4 column); ESI-MS: *m/z* 1704.7 ($[M + 2H]^{2+}$, calcd 1705.12), 1137.1 $[M + 3H]^{3+}$, 853.0 $[M + 4H]^{4+}$, 688.6 $[M + 5H]^{5+}$, MALDI-TOF (*m/z*) 3408.35 $[M]^+$, 3431.24 $[M + Na]^+$, 3447.25 $[M + K]^+$.

Compound 5. The Fmoc-Rink amide resin was treated in a fashion similar to that for compound **3**, except the number of glycosylated amino acid residues (three copies of Tn). Scale: 0.05 mmol. HPLC: t_R = 17.4 min (method A, C4 column), t_R = 17.6 min (method B, C4 column); ESI-MS: *m/z* 1668.1 ($[M + 2H]^{2+}$, calcd 1667.97), 1112.4 ($[M + 3H]^{3+}$), 834.5 $[M + 4H]^{4+}$, 667.7 $[M + 5H]^{5+}$; MALDI-TOF (*m/z*) 3332..33 $[M + H]^+$, 3354.39 $[M + Na]^+$, 3371.3927 $[M + K]^+$.

Compound 6. The Fmoc-Rink amide resin was treated in a fashion similar to that for **5**, except that the peptide was further elongated after the PV sequence by conjugation of eight amino acids of the following sequence: SIINFEKL to afford compound **6**. Scale: 0.05 mmol. HPLC: t_R = 17.8 min (method A, C4 column), t_R = 17.5 min (method B, C4 column); ESI-MS: *m/z* 1427.3 ($[M + 3H]^{3+}$, calcd 1427.35), 1070.9 $[M + 4H]^{4+}$, 857.0 $[M + 5H]^{5+}$, 714.14 $[M + 6H]^{6+}$; MALDI-TOF (*m/z*) 4280.82 $[M + H]^+$, 4303.1869 $[M + Na]^+$, 4320.86 $[M + K]^+$.

Compound 7. The Fmoc-Rink amide resin was treated in a fashion similar to that for **5**, except that after the introduction of the protected Tn antigen (Fmoc-Ser(O-α-D-GalNAc(OAc)$_3$)-OH), Fmoc-Lys(Dde) was introduced into the backbone of the peptide to allow branching after coupling of the PV amino acid sequence and N-terminal acetylation. Dde-deprotection was performed using 2% hydrazine hydrate/DMF and was followed by coupling of amino acid residues of CD8+ peptide epitope (SIINFEKL). The peptide was N-terminally acetylated followed by O-deacetylation of carbohydrate residues as previously mentioned to afford compound **7** after cleavage from the resin. Scale: 0.05 mmol. HPLC: t_R = 17.8 min (method A, C4 column), t_R = 17.5 min (method B, C4 column); ESI-MS: *m/z* 1484.7 ($[M + 3H]^{3+}$, calcd 1484.09), 1113.6 $[M + 4H]^{4+}$, 891.1 $[M + 5H]^{5+}$ 890.854, 742.4 $[M + 6H]^{6+}$; MALDI-TOF (*m/z*) 4450.7 [M + H] +, 4473.87 [M + Na]+, 4491.1 [M + K]+.

Immunological Assessment of Vaccine Candidates. Female BALB/c (H-2^d) mice (4−6 week old, Animal Resource Centre, Perth, Western Australia, Australia) were used for immunization. Mice (n = 5/group) received a primary sc dose of 30 μg of compound (**4**−**7**) dissolved in a total volume of 50 μL of PBS followed by four further similar boosts at weekly intervals. The negative control group was administered 50 μL of PBS or CFA while the positive control group received 30 μg of compound (**5** or **6**) dissolved in a 50 μL solution of PBS and complete Freund's adjuvant (CFA) (1:1). All controls were boosted with 50 μL of PBS. Blood was collected from the tail artery of each mouse one day prior to each dose and nine days after the last booster immunization. The blood was left to clot at 37 °C for 1 h and then centrifuged for 10 min at 3000 rpm to remove clots. Sera were then stored at −20 °C. Determination of serum IgG antibodies against compounds **1**−**4** was performed using a previously described ELISA.[26] Briefly, serial 2-fold dilutions of samples were produced in 0.5% skim milk/PBS−Tween 20 buffer, starting at a concentration of 1:100. Optical density was read at 450 nm in a microplate reader following the addition of peroxidase-conjugated goat antimouse IgG (sera). The antibody titer was defined as the lowest dilution with an optical density more than three standard deviations greater than the mean absorbance of control wells containing normal mouse sera. Statistical analysis of antibody titers between groups was performed using a two-way ANOVA followed by the Tukey's multiple comparison test. GraphPad Prism 4 software was used for statistical analysis, with $p < 0.05$ taken as statistically significant.

Toll-like Receptor 2 Assay. Human embryonic kidney (HEK293) cells stably expressing TLR2 (a gift from Dr. Ashley Mansell, Centre for Functional Genomics and Human Disease, Monash Institute of Reproductive and Development, Clayton, Victoria, Australia) were used in TLR2 stimulation experiment. Each experiment was repeated to validate results. HEK293 cells were cultured and maintained (six-well plates, 1 × 106/well) in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in 5% CO_2. Culture media was composed of DMEM containing heat-inactivated serum supreme (10%, BioWhittaker, Walkersville, MD), gentamycin sulfate (500 μg/mL, Genticin, G418 sulfate), L-glutamine (2 mM), and sodium pyruvate (1 mM). HEK293 cells were transiently transfected with pNF-κB-Luc cis-reporter plasmid (1 μg per 1 × 10^6 cells, Stratagene, La Jolla, CA) and pRL-TK containing a *Renilla luciferase* gene (100 ng/well) as an internal control. Transfection was performed using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) at a reagent-to-DNA ratio of 3:1 (vol/wt) according to the manufacturer's instructions. Plasmid DNA (6 μg) was mixed with Lipofectamine (150 μL) and OPTI-MEMI (134.4 μL) and incubated for 10 min at room temperature followed by dilution with OPTI-MEMI medium (2.55 mL). After the cell culture media was changed to OPTI-MEMI medium (Invitrogen, Carlsbad, CA), transfection mixture (500 μL/well) was added to cells and were incubated at 37 °C in 5% CO_2. Culture media was changed to DMEM after 24 h, and cells were transferred to 24-well plate (1.5 × 105 cells/well). Each triplicate of wells were treated for 4 h with test compound (**1**) using different concentrations (10, 50, 100, 200, and 300 nM). Negative control triplicate wells were treated with media while positive control wells were stimulated by 10 nM Pam_3Cys standard. Cells were then lysed using reporter lysis buffer (Promega Corporation, Madison, WI), and luciferase activity was measured using the Dual-Glo luciferase assay system (Promega). Cell lysates were centrifuged (13 000 rpm, 30 s), and supernatant (10 μL) was mixed with Luciferein (50 μL) and measured using an illuminometer (Turner Designs, Sunnyvale, CA). The firefly luciferase activity was normalized to that of *Renilla luciferase* to correct for transfection efficiency and cell viability. Relative stimulation was calculated relative to media control wells. Data were presented as means ± SD of the indicated number of experiments. Differences between groups were determined using the two-tailed Student's t test and were considered statistically significant if the P value was <0.05.

■ ASSOCIATED CONTENT

Supporting Information

HPLC chromatograms and MS spectra of compounds **1**−**7**. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

Boc, *tert*-butoxycarbonyl; CFA, complete Freund's adjuvant; Dde, 1-(4,4-dimethyl-2,6-dioxacyclohexylidene)ethyl; Fmoc, fluorenylmethyloxycarbonyl; HEK293, Human embryonic kidney; LAA, lipoamino acid; PBS, phosphate buffered saline; *p*MBHA, *p*-methylbenzhydrylamine; SPPS, solid-phase peptide synthesis; TACA, tumor-associated carbohydrate antigen; TLR2, toll-like receptor 2

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