# **Supporting Information**

# Linear synthesis and immunological properties of a fully synthetic vaccine containing a sialylated MUC1 glycopeptide

Pamela Thompson, Vani Lakshminarayanan, Nitin T. Supekar, Judy M. Bradley, Peter A. Cohen, Margreet A. Wolfert, Sandra J. Gendler\* and Geert-Jan Boons\*

Contents	
Figure S1	S2
Figure S2	S3
Synthesis	S4
Biology	S18
References	S20
NMR and MS data	S21



**Fig. S1** ELISA anti-MUC1 titers after immunizations with 1-3 or empty liposomes as indicated. ELISA plates were coated with BSA-MI-CTSAPDT(Neu5Ac- $\alpha$ 2,6- $\alpha$ GalNAc)RPAP conjugate for anti-MUC1(STn) antibody titers for 1, 3 and empty liposomes or BSA-MI-CTSAPDT( $\alpha$ GalNAc)RPAP conjugate for anti-MUC1(Tn) antibody titers for 2. Titers were determined by linear regression analysis, plotting dilution *vs.* absorbance. Titers were defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera. Each data point represents the titer for an individual mouse after 5 immunizations and the horizontal lines indicate the mean for the group of mice.



**Fig. S2** MUC1 epitope recognition of CD4<sup>+</sup> and CD8<sup>+</sup> T-cells. Mice were immunized with liposomes containing **1**, **2** or **3**. Lymph node derived T-cells expressing low levels of CD62L were obtained by cell sorting and cultured for 7 days in the presence of DCs pulsed with the corresponding immunizing construct. The resulting cells were analyzed by ICC for the presence of *A*) CD4<sup>+</sup>IFN $\gamma^+$  and *B*) CD8<sup>+</sup>IFN $\gamma^+$  T-cells. Data are shown as mean ± SEM (n=3).

#### Synthesis

General remarks. All reactions were carried out under nitrogen with anhydrous solvents, unless otherwise stated. CH<sub>2</sub>Cl<sub>2</sub> was distilled from CaH<sub>2</sub> prior to use in each reaction. Chemicals used were reagent grade as supplied except where noted. N-iodosuccinimide was used after recrystallization in 1,4-dioxane/CCl<sub>4</sub>. Column chromatography was performed on silica gel G60  $(60 - 200 \ \mu m \ 60 \ \text{Å})$ ; reactions were monitored by TLC on Silicagel 60 F<sub>254</sub>. The compounds were detected by examination under UV light and visualized by charring with cerium ammonium molybdate in 20% sulfuric acid. Solvents were removed under reduced pressure at  $\leq$  35 °C. 1H-NMR, gCOSY, and gHSQC spectra were recorded in CDCl<sub>3</sub> at 300 MHz or 500 MHz on a Varian Inova spectrometer with tetramethylsilane as an internal standard, unless otherwise stated. <sup>13</sup>C-NMR data reported from gHSQC spectra, unless otherwise stated. Reverse Phase HPLC was performed on an Agilent 1100 series system equipped with an autosampler, UV detector, and fraction collector. RP-HPLC was carried out using a Jupiter C4 analytical column (5 µm, 4.6 x 250 mm) at a flow rate of 1 mL/min. (A: 95% water, 5% acetonitrile, 0.1% TFA; B: 95% acetonitrile, 5% water, 0.1% TFA) High resolution mass spectra were obtained by using MALDI-ToF (Applied Biosystems 5800 Proteomics Analyzer or Bruker Microflex) with 2,5dihydroxybenzoic acid or  $\alpha$ -cyano-4-hydroxycinnamic acid as an internal standard matrix.

General methods for automated microwave-assisted solid-phase peptide synthesis (MW-SPPS). Peptides were synthesized by established protocols on a CEM Liberty Automated Microwave Peptide Synthesizer equipped with a UV detector using *N*- $\alpha$ -Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1-hydroxybenzotriazole (HOBt) as the activating reagents. Deprotection of the N- $\alpha$ -Fmoc was achieved using 20% 4-methyl piperidine in DMF.

General methods for manual MW-SPPS. Peptides were synthesized by established protocols on a CEM Discover SPS Microwave Peptide Synthesizer using *N*- $\alpha$ -Fmoc-protected amino acids and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/1hydroxybenzotriazole (HOBt) as the activating reagents. The couplings of the glycosylated amino acid *N*- $\alpha$ -Fmoc-Thr-(Ac<sub>3</sub>- $\alpha$ -D-GalNAc), *N*- $\alpha$ -fluorenylmethoxycarbonyl-*R*-(2,3bis(palmitoyloxy)-(2*R*-propyl)-(R)-cysteine, and palmitic acid were carried out using (2-(7-Aza1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HATU). Each manual coupling was monitored by standard Kaiser test. Deprotection of the N- $\alpha$ -Fmoc was achieved using 20% 4-methyl piperidine in DMF.



Scheme S1 Synthesis of galactosyl acceptor 5. (a) i) NaOMe, MeOH, ii) levulinic acid, 2-chloro-1-methylpyridinium iodide,  $Et_3N$ , dioxane, 65% over 2 steps; (b) 2,2-dimethoxypropane, PTSA, 75%; (c) i) HgCl<sub>2</sub>, CaCO<sub>3</sub>, ii) TDS-Cl, imidazole, 71% over 2 steps; (d) hydrazine acetate, EtOH/toluene, 99%.

**Phenyl(2-azido-6-***O***-levulinoyl)-1-seleno-α-D-galactopyranoside (S2).** Peracetylated galactose azide **S1** (13.6 g, 29.1 mmol) was dissolved in methanol (200 mL) and treated with 1M NaOMe/MeOH until pH = 9. The reaction was stirred at room temperature for 3 h. The reaction mixture was neutralized by the addition of acetic acid (1.5 mL). The mixture was concentrated *in vacuo* and dried on a high vacuum pump. The crude compound was dissolved in dioxane (370 mL) with CMPI (13.1 g, 58.1 mmol). Levulinic acid (4.04 g, 34.9 mmol) and triethylamine (24 mL, 174.3 mmol) were added. The reaction was stirred at room temperature overnight. The reaction mixture was diluted with EtOAc and washed with sat. aq. NaHCO<sub>3</sub> (200 mL x 3) and brine (200 mL). The organic layer was dried (MgSO<sub>4</sub>), filtered, and evaporated to dryness. The residue was purified by silica gel chromatography (0% → 55% EtOAc in hexanes) to afford compound **S2** in 65% yield. Analytical data for **S2**: R<sub>*f*</sub> = 0.43 (1:3 hexanes-EtOAc); <sup>1</sup>H NMR: δ 7.67 – 7.54 (3 H, m, aromatic), 7.39 – 7.22 (2 H, m, aromatic), 5.97 (1 H, d, *J* 5.3, H-1), 4.46 (2 H, q, *J* 6.7, H-5, H-6<sub>a</sub>), 4.10 (2 H, td, *J* 8.7, 3.6, H-6<sub>b</sub>, H-2), 4.02 (1 H, t, *J* 3.3, H-4), 3.83 (1 H, d, *J* 3.1, H-3), 3.02 (1 H, d, *J* 3.6, C4-O*H*), 2.83 – 2.67 (3 H, m, CO<sub>2</sub>C*H*<sub>2</sub>, C3-O*H*), 2.58 – 2.50 (2 H, m, C*H*<sub>2</sub>(C=O)), 2.23 – 2.14 (3 H, m, C*H*<sub>3</sub>) ppm. <sup>13</sup>C NMR: δ 134.87, 134.83, 134.36, 129.02,

128.85, 84.94 (C-1), 71.04, 80.86, 68.11, 62.47, 61.59, 37.96, 29.72, 27.68 ppm. HR-MALDI-ToF/MS: m/z for C<sub>17</sub>H<sub>21</sub>N<sub>3</sub>O<sub>6</sub>Se [M+Na]<sup>+</sup> calc 466.0493, found 466.0672.

**Phenyl(2-azido-3,4-***O***-isopropylidene-6-***O***-levulinoyl)-1-seleno-α-D-galactopyranoside (S3). Compound S2 (1.7 g, 3.9 mmol) was dissolved in 2,2-dimethoxypropane (20 mL) and** *p***-toluenesulfonic acid (0.078 mmol) was added. The reaction mixture was stirred for 6 h and was then neutralized with triethylamine (0.5 mL). The mixture was concentrated** *in vacuo* **and purified by silica gel chromatography (0 → 35% EtOAc in hexanes) to afford compound S3 in 65% yield. Analytical data for S3: R<sub>f</sub> = 0.48 (2:1 hexanes-EtOAc); 1H NMR: δ (300 MHz, CDCl<sub>3</sub>) 7.60 (2 H, dd,** *J* **6.5, 3.0, aromatic), 7.37 – 7.18 (3 H, m, aromatic), 5.84 (1 H, d,** *J* **5.2, H-1), 4.60 (1 H, d,** *J* **2.5, H-5), 4.40 – 4.15 (3 H, m, H-6, H-4, H-3), 3.99 (1 H, dd,** *J* **6.7, 5.3, H-2), 2.70 (2 H, t,** *J* **6.6, CO<sub>2</sub>CH<sub>2</sub>), 2.52 (2 H, t,** *J* **6.6, CH<sub>2</sub>(C=O)), 2.17 (3 H, s, CH<sub>3</sub>), 1.52 (3 H, s, C(CH<sub>3</sub>)), 1.35 (3 H, s, C(CH<sub>3</sub>)) ppm. <sup>13</sup>C NMR: δ 134.48, 134.42, 134.36, 128.86, 128.70, 83.10 (C-1), 75.27, 72.69, 69.59, 64.09, 62.55, 38.17, 30.07, 28.21, 28.21, 26.28 ppm. HR-MALDI-ToF/MS:** *m/z* **for C<sub>20</sub>H<sub>25</sub>N<sub>3</sub>O<sub>6</sub>Se [M+Na]<sup>+</sup> calc 506.0806, found 506.1049.** 

#### Thexyldimethylsilyl(2-azido-3,4-O-isopropylidene-6-O-levulinoyl)-α-D-galactopyranoside

(S4). To a solution of compound S3 in 10:1 acetonitrile/water was added mercuric chloride and calcium carbonate. The reaction mixture was stirred at room temperature overnight and was then concentrated *in vacuo*. The residue was suspended in CH<sub>2</sub>Cl<sub>2</sub> and then filtered. The filtrate was then evaporated to dryness and purified by silica gel column chromatography. The purified hemiacetal was then dissolved in CH<sub>2</sub>Cl<sub>2</sub> and TDS-chloride and imidazole was added to the solution at 0 °C. The reaction was stirred at room temperature for 3 h. The reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub>and washed with sat. aq. NaHCO<sub>3</sub>. The organic layer was dried (MgSO<sub>4</sub>), evaporated to dryness, and purified by silica gel chromatography (0  $\rightarrow$  25% EtOAc in hexanes) to afford S4 in 71% yield. Analytical data for S4: R<sub>f</sub> = 0.60 (2:1 hexanes/EtOAc); <sup>1</sup>H NMR:  $\delta$  (300 MHz, CDCl<sub>3</sub>) 4.22 (1 H, d, J 8.2, H-1), 4.15 (2 H, dd, J 6.0, 4.9, H-6), 3.87 (1 H, dd, J 5.3, 2.2, H-4), 3.76 – 3.63 (2 H, m, H-5, H-3), 3.10 (1 H, t, J 8.1, H-2), 2.62 – 2.52 (2 H, m, CHH-CHH), 2.44 – 2.35 (2 H, m, CHH-CHH), 2.00 (3 H, s, CH<sub>3</sub>), 1.55 – 1.40 (1 H, m, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>, 1.35 (6 H, s, C(CH<sub>3</sub>)), 1.14 (3 H, s, C(CH<sub>3</sub>)), 0.70 (12 H, dd, J 4.3, 2.4, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.00 (6 H, d, J 2.4, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>) ppm. <sup>13</sup>C

NMR:  $\delta$  96.77 (C-1),77.87, 73.15, 71.38, 68.13, 64.29, 38.13, 30.34, 28.57, 28.57, 26.80, 20.30, -1.31 ppm. HR-MALDI-ToF/MS: *m*/*z* for C<sub>22</sub>H<sub>39</sub>N<sub>3</sub>O<sub>7</sub>Si [M+Na]<sup>+</sup> calc 508.2455, found 508.3337.

**Thexyldimethylsilyl(2-azido-3,4-***O***-isopropylidene)-α-D-galactopyranoside (5).** To a solution of **S4** (0.6 g, 1.27 mmol) in 2:1 ethanol/toluene (21 mL) was added hydrazine acetate (0.57 g, 6.35 mmol). The reaction stirred at room temperature for 7 h. The reaction mixture was diluted with ethyl acetate (100 mL) and was washed with sat. aq. NaHCO<sub>3</sub> and brine (100 mL). The organic layer was dried (MgSO<sub>4</sub>), evaporated to dryness, and purified by silica gel chromatography (0 → 25% EtOAc in hexanes) to afford **5** in 99% yield. Analytical data for **5**: R<sub>*f*</sub> = 0.57; <sup>1</sup>H NMR: δ (300 MHz, CDCl<sub>3</sub>) 4.24 (1 H, d, *J* 8.1, H-1), 3.88 (1 H, dd, *J* 5.4, 2.1, H-4), 3.73 (2 H, ddd, *J* 13.9, 10.3, 6.9, H-6<sub>a</sub>, H-5), 3.66 – 3.54 (2 H, m, H-3, H-6<sub>b</sub>), 3.10 (1 H, t, *J* 8.2, H-2), 1.72 (1 H, dd, *J* 9.3, 3.6, C6-OH), 1.48 (1 H, dt, *J* 13.7, 6.9, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 1.14 (3 H, s, C(CH<sub>3</sub>)), 0.69 (12 H, dd, *J* 3.8, 3.1, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), -0.00 (6 H, s, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>) ppm. <sup>13</sup>C NMR: δ 96.73 (C-1), 77.69, 74.01, 73.48, 67.97, 62.58, 28.43, 26.62, 20.05, -1.43 ppm. HR-MALDI-ToF/MS: *m/z* for C<sub>17</sub>H<sub>33</sub>N<sub>3</sub>O<sub>5</sub>Si [M+Na]<sup>+</sup> calc 410.2087, found 410.2278.



**Scheme S2** Synthesis of sialyl donor **4**. (a) i) Ac<sub>2</sub>O, pyridine, ii) Cs<sub>2</sub>CO<sub>3</sub>, AllylBr, DMF, 91% over 2 steps; (b) 1-adamantanethiol, BF<sub>3</sub>·OEt<sub>2</sub>, DCM, 84%; (c) Boc<sub>2</sub>O, DMAP, THF, 91%; (d) NaOMe, AllylOH, 67%; (e) i) TFA, ii) 4-NO<sub>2</sub>C<sub>6</sub>H<sub>4</sub>OCOCl, NaHCO<sub>3</sub>, H<sub>2</sub>O, MeCN, 61% over 2 steps; (f) i) Ac<sub>2</sub>O, pyridine, ii) AcCl, DIPEA, DCM, 80% over 2 steps.

Allyl(1-adamantanyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-2-thio-glycero-α-Dgalacto-non-2-ulopyranoside)oate (S7). 1-Adamantanethiol (1.05 g, 6.2 mmol) and compound S6 (2.92 g, 5.2 mmol) were dissolved in CH<sub>2</sub>Cl<sub>2</sub> (52 mL). BF<sub>3</sub>·OEt<sub>2</sub> (1.6 mL, 13 mmol) was added at room temperature and the reaction mixture was stirred for 19 h. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and was washed with sat. aq. NaHCO<sub>3</sub> (100 mL). The organic layer was dried with MgSO<sub>4</sub>, filtered, and evaporated to dryness. The residue was purified by silica gel chromatography (0  $\rightarrow$  30% acetone in toluene) to afford **S7** in 84% yield. Analytical data: R<sub>f</sub> = 0.38 (3:1 toluene/acetone); <sup>1</sup>H NMR:  $\delta$  (300 MHz, CDCl<sub>3</sub>) 6.05 – 5.87 (1 H, m, CH=CH<sub>2</sub>), 5.49 – 5.12 (5 H, m, CH=CH<sub>2</sub>, H-7, H-8, H-4), 4.90 (1 H, dd, *J* 12.3, 1.7, H-9<sub>a</sub>), 4.82 – 4.59 (2 H, m, CH<sub>2</sub>- CH=CH<sub>2</sub>), 4.55 (1 H, dd, *J* 10.5, 2.7, H-6), 4.39 – 4.24 (1 H, m, H-9<sub>b</sub>), 4.16 – 4.02 (1 H, m, H-5), 2.54 (1 H, dd, *J* 13.5, 4.7, H-3<sub>a</sub>), 2.18 – 1.57 (122 H, m, OAc x 4, NAc, H-3<sub>b</sub>, Ada) ppm. <sup>13</sup>C NMR  $\delta$ : 119.81, 74.01, 72.82, 69.48, 69.04, 66.72, 63.45, 49.83, 43.78, 39.92, 36.08, 29.80, 23.14, 20.96, 20.68, 20.67, 20.65 ppm. HR-MALDI-ToF/MS: *m*/*z* for C<sub>32</sub>H<sub>45</sub>NO<sub>12</sub>S [M+Na]<sup>+</sup> calc 690.2560, found 690.3072.

#### Allyl(1-adamantanyl-5-acetamido-5-N-tert-butoxycarbonyl-4,7,8,9-tetra-O-acetyl-3,5-

dideoxy-2-thio-glycero-α-D-galacto-non-2-ulopyranoside)oate (S8). To a solution of compound S7 (1.9 g, 2.8 mmol) in THF (70 mL) was added Boc<sub>2</sub>O (3.85 mL, 16.8 mmol) and DMAP (68 mg, 0.56 mmol). The reaction mixture was stirred at 60 °C for 24 h. The reaction mixture was quenched with methanol and evaporated to dryness. The residue was purified by silica gel chromatography (0 → 20% EtOAc in hexanes) to afford S8 in 91% yield. Analytical data:  $R_f = 0.28$  (3:1 hexanes/EtOAc); <sub>1</sub>H NMR: δ (300 MHz, CDCl<sub>3</sub>) 5.96 (1 H, ddd, *J* 11.8, 10.5, 5.3, C*H*=CH<sub>2</sub>), 5.64 (1 H, td, *J* 11.1, 4.7, H-4), 5.45 – 5.22 (4 H, m, CH=CH<sub>2</sub>, H-6, H-7), 5.15 (1 H, dd, *J* 9.4, 5.1, H-8), 4.87 (1 H, dd, *J* 11.4, 6.8, H-9<sub>a</sub>), 4.81 – 4.58 (3 H, m, H-5, CH<sub>2</sub>CH=CH<sub>2</sub>), 4.31 (1 H, dd, *J* 12.3, 8.9, H-9<sub>b</sub>), 2.63 (1 H, dd, *J* 13.5, 4.8, H-3<sub>a</sub>), 2.34 (3 H, s, N-COCH<sub>3</sub>), 2.20 – 1.79 (23 H, m, H-3<sub>b</sub>, OAc x 4, Ada), 1.74 – 1.57 (9 H, m, C(CH<sub>3</sub>)<sub>3</sub>), 1.48 (3 H, dd, *J* 18.2, 12.7, Ada) ppm. <sup>13</sup>C NMR δ: 131.31, 119.73, 74.10, 73.72, 73.10, 72.00, 69.33, 66.83, 66.36, 62.8, 53.23, 43.60, 41.72, 35.92, 29.68, 28.01, 26.94, 20.95, 20.81, 20.78, 20.58 ppm. HR-MALDI-ToF/MS: *m/z* for C<sub>37</sub>H<sub>53</sub>NO<sub>14</sub>S [M+Na]<sup>+</sup> calc 790.3084, found 790.3265.

Allyl(1-adamantanyl-5-*N-tert*-butoxycarbonyl-3,5-dideoxy-2-thio-glycero- $\alpha$ -D-galacto-non-2-ulopyranoside)oate (S9). Compound S8 (2.1 g, 2.69 mmol) was dissolved in allyl alcohol (30 mL) and 1M NaOMe was added until pH = 9. The reaction was stirred under vacuum for 4 h and was quenched neutralized with the addition of acetic acid (0.5 mL). The mixture was

concentrated *in vacuo* and the residue was purified by silica gel chromatography (0  $\rightarrow$  30% acetone in CHCl<sub>3</sub>) to afford **S9** in 80% yield. Analytical data:  $R_f = 0.38$  (1:1 toluene/acetone); HR-MALDI-ToF/MS: m/z for C<sub>27</sub>H<sub>43</sub>NO<sub>9</sub>S [M+Na]<sup>+</sup> calc 580.2556, found 580.3585.

#### Allyl(1-adamantanyl-5-N-4-O-carbonyl-3,5-dideoxy-2-thio-glycero-β-D-galacto-non-2-

ulopyranoside)oate (S10). Compound S9 (1.0 g, 1.79 mmol) was stirred in TFA (4 mL) for 1 h. The mixture was coevaporated with toluene and dried under high vacuum overnight. The residue was dissolved in acetonitrile (8 mL) and water (16 mL) and NaHCO<sub>3</sub> (0.75 g, 8.9 mmol) was added. The reaction mixture was cooled to 0 °C and 4-nitrophenylchloroformate (0.89 g, 4.45 mmol) was added. The reaction was stirred at 0 °C for 4 h. The mixture was washed with EtOAc (100 mL x 3) and the organic layer was washed with brine (50 mL x 2), dried (MgSO<sub>4</sub>), filtered, and concentrated *in vacuo*. The residue was purified by silica gel chromatography  $(0 \rightarrow 50\%)$ acetone in CHCl<sub>3</sub>) to afford S10 in 61% yield. Analytical data:  $R_f = 0.31$  (1:1 CHCl<sub>3</sub>/acetone), <sup>1</sup>H NMR: δ (300 MHz, CDCl<sub>3</sub>) 6.04 (1 H, s), 6.01 – 5.87 (1 H, m, CH=CH<sub>2</sub>), 5.37 (2 H, ddd, J 13.8, 11.5, 1.2, CH=CH<sub>2</sub>), 4.78 (1 H, dd, J 13.0, 5.9, CHHCH=CH<sub>2</sub>), 4.72 - 4.60 (2 H, m CHHCH=CH<sub>2</sub>,H-4), 4.47 (1 H, dd, J 9.8, 4.9, H-6), 3.97 – 3.74 (4 H, m, H-9, H-7, H-8), 3.52 (1 H, d, J 3.9, C8-OH), 3.43 (1 H, t, J 10.4, H-5), 3.32 (1 H, d, J 3.9, C7-OH), 2.74 (1 H, dd, J 12.8, 3.6, H-3<sub>a</sub>), 2.62 (1 H, d, J 6.6, C9-OH), 2.17 (1 H, t, J 12.7, H-3<sub>b</sub>), 1.94 (5 H, m, Ada), 1.64 (10 H, d, J 9.5, Ada). <sup>13</sup>C NMR: δ (75 MHz) 170.46 (CO<sub>2</sub>Allyl), 159.70 (N(C=O)O, 130.98, 120.21, 86.18, 77.65, 77.43, 77.22, 76.80, 75.68, 72.41, 70.79, 67.26, 63.98, 59.25, 51.32, 43.77, 39.32, 36.16, 30.05. HR-MALDI-ToF/MS: m/z for C<sub>23</sub>H<sub>33</sub>NO<sub>8</sub>S [M+Na]<sup>+</sup> calc 506.1825, found 506.2570.

Allyl(1-adamantanyl-5-acetamido-7,8,9-tri-*O*-acetyl-5-*N*-4-*O*-carbonyl-3,5-dideoxy-2-thioglycero- $\beta$ -D-galacto-non-2-ulopyranoside)oate (4). Compound S10 (0.53 g, 1.1 mmol) was treated with 2:1 Py/Ac<sub>2</sub>O (30 mL) overnight. The mixture was quenched with allyl alcohol. The mixture was co-evaporated with toluene. The crude material was dissolved in CH<sub>2</sub>Cl<sub>2</sub> (10 mL) and DIPEA (1.9 mL, 10.9 mmol) was added. The reaction mixture was cooled to 0 °C and acetyl chloride (0.62 mL, 8.7 mmol) was added. The reaction was stirred at room temperature for 1 h. The reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> (50 mL) and was washed with sat. aq. NaHCO<sub>3</sub> (50 mL x 3). The organic layer was dried (MgSO<sub>4</sub>), filtered, evaporated to dryness, and purified by silica gel column chromatography (0 → 25% EtOAc in hexanes) to afford **4** in 80% yield. Analytical data for **4**:  $R_f = 0.28$  (2:1 hexanes/EtOAc); <sup>1</sup>H NMR:  $\delta$  (500 MHz, CDCl<sub>3</sub>) 6.05 – 5.90 (1 H, m, C*H*=CH<sub>2</sub>), 5.73 (1 H, t, *J* 2.9, H-7), 5.42 (1 H, dd, *J* 17.2, 1.3, CH=C*H*H), 5.33 (2 H, ddd, *J* 10.4, 4.5, 1.8, CH=CH*H*, H-8), 4.74 (5 H, dddd, *J* 22.3, 14.3, 8.9, 2.5, C*H*<sub>2</sub>CH=CH<sub>2</sub>, H-9<sub>a</sub>, H-4, H-6), 4.23 (1 H, dd, *J* 12.2, 7.9, H-9<sub>b</sub>), 3.69 (1 H, dd, *J* 11.3, 9.3, H-5), 2.83 (1 H, dd, *J* 12.8, 3.6, H-3<sub>a</sub>), 2.50 (3 H, s, NCOC*H*<sub>3</sub>), 2.20 (1 H, t, *J* 12.8, H-3<sub>b</sub>), 2.13 (6 H, d, OAc x 2), 2.08 – 1.97 (6 H, m, OAc, Ada), 1.92 (3 H, d, *J* 11.5, Ada), 1.73 – 1.62 (6 H, m, Ada), 1.56 (4 H, s, Ada) ppm. <sup>13</sup>C NMR:  $\delta$  131.33, 119.97, 75.69, 74.64, 73.44, 72.37, 66.91, 63.28, 60.48, 43.81, 38.84, 37.56, 29.85, 21.68, 21.11, 20.76 ppm. HR-MALDI-ToF/MS: *m/z* for C<sub>31</sub>H<sub>41</sub>NO<sub>12</sub>S [M+Na]<sup>+</sup> cale 674.2247, found 674.580.

#### Thexyldimethylsilyl[allyl(5-acetamido-7,8,9-tri-O-acetyl-5-N-4-O-carbonyl-3,5-dideoxy-

glycero- $\alpha$ -D-galacto-non-2-ulopyranoside)oate]-(2 $\rightarrow$ 6)-O-(2-azido-3,4-O-isopropylidene)- $\alpha$ -**D-galactopyranoside (6).** Sialyl donor **4** (87 mg, 0.13 mmol) and galactosyl acceptor **5** (78 mg, 0.20 mmol) were co-evaporated with toluene and dried overnight under high vacuum. The residue was dissolved in 1:1 acetonitrile/ CH<sub>2</sub>Cl<sub>2</sub> (4 mL) and molecular sieves were added. After 2 h, the mixture was cooled to -78 °C and NIS (45 mg, 0.20 mmol) and TfOH (4 µL, 0.04 mmol) were added. The reaction mixture was stirred at -78 °C in the dark for 30 min and was then diluted with CH<sub>2</sub>Cl<sub>2</sub> and washed with sat. aq. NaHCO<sub>3</sub>. The organic layer was dried (MgSO<sub>4</sub>), filtered, concentrated *in vacuo*, and and purified by silica gel column chromatography  $(0 \rightarrow 40\%)$ EtOAc in hexanes) to provide 6 in 86% yield. Analytical data for 6:  $R_f = 0.56$  (1:1) hexanes/EtOAc); <sup>1</sup>H NMR: δ (500 MHz, CDCl<sub>3</sub>) 5.74 (1 H, ddd, J 17.1, 6.0, 4.4, CH=CH<sub>2</sub>), 5.39 (1 H, dd, J 7.0, 1.6, H-7'), 5.24 – 5.16 (2 H, m, H-8', CH=CHH), 5.14 (1 H, dd, J 10.4, 1.0, CH=CHH), 4.51 (2 H, qd, J 12.8, 6.0, CH<sub>2</sub>CH=CH<sub>2</sub>), 4.36 (1 H, dd, J 9.4, 1.6, H-6'), 4.29 – 4.13 (1 H, m, H-9a'), 3.92 (1 H, dd, J 12.2, 6.9, H-9b'), 3.89 – 3.80 (2 H, m, H-4, H-4'), 3.77 (1 H, dd, J 10.3, 7.5, H-6<sub>a</sub>), 3.74 – 3.61 (2 H, m, H-5, H-3), 3.59 – 3.49 (2 H, m, H-5', H-6<sub>b</sub>), 3.08 (1 H, t, J 8.1, H-2), 2.70 (1 H, dd, J 12.2, 3.6, H-3<sub>a</sub>), 2.30 (3 H, d, J 6.4, NCOCH<sub>3</sub>), 1.99 – 1.77 (9 H, m, OAc x 3), 1.47 (1 H, dt, J 13.7, 6.8, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 1.36 (3 H, s, C(CH<sub>3</sub>)), 1.14 (3 H, d, J 8.4, C(CH<sub>3</sub>)), 0.75 – 0.66 (12 H, m SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), -0.01 (5 H, dd, J 11.2, 5.0, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>) ppm. <sup>13</sup>C NMR: δ 131.27, 120.71, 97.06 (C-1), 77.55, 95.16, 72.90, 72.41, 72.08, 70.24, 68.00, 67.49, 64.73, 63.43, 36.26, 34.11, 28.66, 26.52, 25.16, 21.69, 21.07, 20.97, 19.53, -2.52 ppm. HR-MALDI-ToF/MS: m/z for  $C_{38}H_{58}N_4O_{17}Si [M+Na]^+$  calc 893.3464, found 893.830.

# Thexyldimethylsilyl[allyl(5-acetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero- $\alpha$ -D-galacto-non-2-ulopyranoside)oate]-(2 $\rightarrow$ 6)-*O*-(3,4-di-*O*-acetyl-2-azido)- $\alpha$ -D-

galactopyranoside (7). Sialyl disaccharide 6 (330 mg, 0.379 mmol) was dissolved in allyl alcohol (10 mL) and 1M NaOMe was added until pH = 9. The reaction was stirred under vacuum for 3 h and was neutralized by the addition of acetic acid. The mixture was concentrated in vacuo and the residue was dissolved in 70% aq. acetic acid (10 mL) and stirred at 70 °C for 2 h. The mixture was co-evaporated with toluene and dried overnight under high vacuum. The residue was then dissolved in 2:1 Py/Ac<sub>2</sub>O (6 mL) and stirred for 18 h. The mixture was guenched with allyl alcohol (3 mL) and was co-evaporated with toluene. The residue was purified by silica gel column chromatography ( $0 \rightarrow 100\%$  EtOAc in hexanes) to afford 7 in 65% yield. Analytical data for 7:  $R_f = 0.52$  (100% EtOAc); <sup>1</sup>H NMR:  $\delta_H$  (500 MHz, CDCl<sub>3</sub>) 5.74 – 5.60 (1 H, m, CH=CH<sub>2</sub>), 5.20 – 4.99 (4 H, m, CH=CH<sub>2</sub>, H-8', H-4), 4.85 (1 H, d, J 9.6, NH), 4.62 (1 H, dd, J 15.0, 7.0, H-4'), 4.55 (1 H, d, J 10.9, H-3), 4.49 (1 H, dd, J 12.7, 5.8, CHHCH=CH<sub>2</sub>), 4.40 (2 H, dd, J 15.1, 6.7, H-1, CHHCH=CH<sub>2</sub>), 4.05 (1 H, d, J 12.5, H-6<sub>a</sub>), 3.83 – 3.70 (3 H, m, H-6<sub>b</sub>, H-5, H-5'), 3.56 (2 H, d, J 10.8, H-9a', H-7'), 3.33 (1 H, t, J 9.2, H-2), 3.08 (1 H, t, J 10.3, H-9b'), 2.33 (1 H, d, J 12.7, H-3<sub>a</sub>'), 1.93 (6 H, d, J 4.0, Ac x 2), 1.83 (12 H, d, J 37.8, Ac x 4), 1.66 (4 H, d, J 6.4, Ac,  $H-3_{b}$ ), 1.55 – 1.33 (1 H, m SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.68 (12 H, s, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.01 (6 H, d, J 6.4, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>) ppm. <sup>13</sup>C NMR: δ 131.57, 120.53, 97.52 (C-1), 72.45, 72.91, 71.53, 69.00, 67.85, 67.16, 63.94, 63.94, 63.02, 49.90, 38.37, 34.36, 23.52, 21.42, 21.10, 21.03, 20.97, 20.54, 19.64 ppm. HR-MALDI-ToF/MS: m/z for C<sub>40</sub>H<sub>62</sub>N<sub>4</sub>O<sub>19</sub>Si [M+Na]<sup>+</sup> calc 953.3675, found 953.3832.

# Thexyldimethylsilyl[allyl(5-*N*-acetylacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero- $\alpha$ -D-galacto-non-2-ulopyranoside)oate]-(2 $\rightarrow$ 6)-*O*-3,4-di-*O*-acetyl-2-azido- $\alpha$ -D-

galactopyranoside (8). Disaccharide 7 (230 mg, 0.25 mmol) was dissolved in isopropenyl acetate (2.5 mL) and CSA (6 mg, 0.025 mmol) was added. The reaction mixture was heated at 65 °C overnight. The mixture was neutralized by the addition of triethylamine and was concentrated *in vacuo*. The residue was purified by column chromatography (0  $\rightarrow$  65% EtOAc in hexanes) to

afford **8** in 99% yield. Analytical data for **8**:  $R_f = 0.55$  (1:2 hexanes/EtOAc); <sup>1</sup>H NMR:  $\delta$  (300 MHz, CDCl<sub>3</sub>) 6.04 – 5.85 (1 H, m, CH=CH<sub>2</sub>), 5.47 (2 H, ddd, *J* 18.5, 9.2, 3.2, H-4', CH=C*H*H), 5.40 – 5.23 (3 H, m, CH=CH*H*, H-4, H-8'), 5.12 (1 H, dd, *J* 8.5, 1.7, H-7'), 4.92 (1 H, dd, *J* 10.1, 1.7, H-6'), 4.84 – 4.58 (4 H, m, H-3, CH<sub>2</sub>CH=CH<sub>2</sub>, H-1), 4.29 (1 H, dd, *J* 12.4, 2.7, H-9<sub>a</sub>'), 4.12 (2 H, dt, *J* 12.5, 6.8, H-5', H-9<sub>b</sub>'), 3.87 – 3.73 (2 H, m, H-5, H-6<sub>a</sub>), 3.60 – 3.38 (2 H, m, H-2, H-6<sub>b</sub>), 2.74 (1 H, dd, *J* 12.9, 5.1, H-3<sub>a</sub>'), 2.40 – 2.23 (6 H, m, NAc x 2), 2.22 – 1.93 (18 H, m, OAc x 6), 1.73 (2 H, ddd, *J* 20.5, 13.2, 9.1, H-3<sub>b</sub>', SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.92 (12 H, d, *J* 5.3, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.92 (12 H, d, *J* 5.3, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>), 0.22 (6 H, dd, *J* 7.6, 4.1, SiC(CH<sub>3</sub>)<sub>2</sub>C(CH<sub>3</sub>)<sub>2</sub>CH(CH<sub>3</sub>)<sub>2</sub>) ppm. <sup>13</sup>C NMR:  $\delta$  120.07, 97.24 (C-1), 71.88, 71.44, 69.99, 68.42, 67.33, 66.93, 66.91, 66.87, 63.69, 62.94, 62.16, 57.16, 38.94, 34.02, 28.09, 26.07, 21.22, 20.99, 20.92, 20.84, 20.18, 18.72, -1.68 ppm. HR-MALDI-ToF/MS: *m/z* for C<sub>42</sub>H<sub>64</sub>N<sub>4</sub>O<sub>20</sub>Si [M+Na]<sup>+</sup> calc 995.3781, found 995.2717.

# Allyl(5-*N*-acetylacetamido-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-glycero-α-D-galacto-non-2ulopyranoside)oate-(2→6)-*O*-(3,4-di-*O*-acetyl-2-azido-α-D-

galactopyranosyl)trichloroacetimidate (10). Compound 8 (80 mg, 0.0822 mmol) was dissolved in 3 mL THF and 70% HF/Py was added (0.86 mL, 32.886 mmol). The reaction mixture was stirred at room temperature for 5 h and was diluted with  $CH_2Cl_2$  and washed with sat. aq. NaHCO<sub>3</sub>. The organic layer was dried (MgSO<sub>4</sub>), filtered, and concentrated *in vacuo*. The residue was dissolved in  $CH_2Cl_2$  (1 mL) and treated with trichloroacetonitrile (82 µL, 0.822 mmol) and DBU (0.3 µL, 0.00246 mmol) for 2 h. The reaction mixture was evaporated to dryness and purified by silica gel column chromatography (0  $\rightarrow$  70% EtOAc in hexanes) to afford 10 in 76% yield. Analytical data for 10: Rf = 0.54 (1:2 hexanes/EtOAc).

#### N-(9-Fluorenylmethyloxycarbonyl)-O-[Allyl(5-N-acetylacetamido-4,7,8,9-tetra-O-acetyl-

3,5-dideoxy-glycero-α-D-galacto-non-2-ulopyranoside)oate-(2→6)-O-(3,4-di-O-acetyl-2-

azido- $\alpha$ -D-galactopyranosyl)]-L-threonine *tert*-butyl ester (13). Acid-washed molecular sieves were added to a solution of sialyl disaccharide donor 10 (61 mg, 0.0623 mmol) and threonine acceptor 11 (75 mg, 0.188 mmol) in 0.5 mL Et<sub>2</sub>O. The mixture was stirred for 1 h and then cooled to 0 °C. TMSOTf (2.3 µL, 0.01251 mmol) was added and the reaction was complete in 15 min. The mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub> and was filtered into sat. aq. NaHCO<sub>3</sub> (20 mL). The organic layer was dried (MgSO<sub>4</sub>), filtered, concentrated *in vacuo*, and purified by silica gel column chromatography (0  $\rightarrow$  50% EtOAc in hexanes) to afford **13** in 78% yield. Analytical data for **13**:  $R_f = 0.32$  (1:1 hexanes/EtOAc); 1H NMR:  $\delta$  (500 MHz, acetone) 7.95 – 7.82 (8 H, m, aromatic), 7.73 (4 H, dd, *J* 14.6, 8.0, aromatic), 7.49 – 7.25 (4 H, m, aromatic), 6.16 – 5.89 (1 H, m, C*H*=CH<sub>2</sub>), 5.58 – 5.23 (6 H, m, H-4', CH=C*H*<sub>2</sub>, H-4, H-8', H-3), 5.23 – 5.08 (1 H, m, H-7'), 4.91 (2 H, m, H-6'), 4.89 – 4.77 (1 H, m, C*H*HCH=CH<sub>2</sub>), 4.77 – 4.57 (1 H, m, C*H*HCH=CH<sub>2</sub>), 4.52 – 4.21 (8 H, m, OC*H*CH<sub>3</sub> threonine, H-5, CHC*H*<sub>2</sub> Fmoc, H-9<sub>a</sub>', C*H*CO<sub>2</sub>Allyl, C*H*CH<sub>2</sub> Fmoc, H-5'), 4.09 (1 H, ddt, *J* 34.3, 13.4, 6.6, H-9<sub>b</sub>'), 3.99 – 3.87 (1 H, m, H-6<sub>a</sub>), 3.82 (3 H, dd, *J* 11.1, 3.3, H-2), 3.48 (1 H, dd, *J* 9.9, 5.4, H-6<sub>b</sub>), 2.92 – 2.69 (1 H, m, acetone-d<sup>6</sup>, H-3<sub>a</sub>'), 2.35 (6 H, d, *J* 15.7, NAc x 2), 2.20 – 1.89 (18 H, m, OAc x 3), 1.80 (1 H, t, *J* 12.0, H-3<sub>b</sub>'), 1.62 – 1.44 (9 H, m, C(CH<sub>3</sub>)<sub>3</sub>), 1.44 – 1.22 (3 H, m, CH<sub>3</sub> threonine) ppm. <sup>13</sup>C NMR:  $\delta$  127.94, 127.25, 125.57, 120.23, 118.80, 99.64 (C-1), 77.20, 70.27, 68.77, 68.69, 68.37, 67.75, 67.48, 66.95, 66.59, 66.57, 63.31, 62.13, 58.25, 56.90, 47.61, 38.77, 27.2, 26.87, 25.27, 20.70, 20.64, 20.32, 20.23, 18.40 ppm. HR-MALDI-ToF/MS: *m*/*z* for C<sub>57</sub>H<sub>71</sub>N<sub>5</sub>O<sub>24</sub> [M+Na]<sup>+</sup> calc 1232.4387, found 1232.5597.

N-(9-Fluorenylmethyloxycarbonyl)-O-[Allyl(5-N-acetylacetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-glycero- $\alpha$ -D-galacto-non-2-ulopyranoside)oate-(2 $\rightarrow$ 6)-O-(2-acetamido-3,4-di-Oacetyl-a-D-galactopyranosyl)]-L-threonine tert-butyl ester (14). Glycosylated amino acid 13 (180 mg, 0.149 mmol) was dissolved in 3:2:1 THF/Ac<sub>2</sub>O/HOAc (3 mL) and treated with Zn (126 mg, 1.93 mmol) and sat. aq. CuSO<sub>4</sub> (0.25 mL) for 45 min. The mixture was filtered over celite and co-evaporated with toluene. The residue was purified by silica gel column chromatography  $(0 \rightarrow 85\%$  EtOAc in hexanes) to afford 14 in 78% yield. Analytical data for 14:  $R_f = 0.33$  (1:3) hexanes/EtOAc) <sup>1</sup>H NMR: δ (500 MHz, acetone) 7.80 – 7.64 (2 H, m, aromatic), 7.64 – 7.47 (4 H, m, aromatic), 7.28 (2 H, dd, J 26.1, 20.5, aromatic), 5.99 – 5.71 (1 H, m, CH=CH<sub>2</sub>), 5.47 – 5.28 (1 H, m, H-4'), 5.25 – 5.08 (3 H, m, CH=CH<sub>2</sub>, H-4, H-8'), 5.08 – 4.96 (1 H, m, H-7'), 4.85 - 4.79 (3 H, m, H-6', H-3, H-1), 4.75 - 4.65 (1 H, m, CHHCH=CH<sub>2</sub>), 4.63 - 4.52 (1 H, m, CHHCH=CH<sub>2</sub>), 4.46 – 4.18 (6 H, m, CHCH<sub>2</sub> Fmoc, H-2, OCHCH<sub>3</sub> threonine, H-9<sub>a</sub>'), 4.15 – 3.87 (4 H, m, CHCH<sub>2</sub> Fmoc, CHCO<sub>2</sub>Allyl, H-5, H-9<sub>b</sub>'), 3.86 – 3.76 (1 H, m, H-6<sub>a</sub>), 3.49 – 3.31 (1 H, m, H-6<sub>b</sub>), 2.82 – 2.54 (1 H, m, H-3<sub>a</sub>'), 2.21 (6 H, d, J 16.6, NAc x 2), 2.09 – 1.74 (21 H, m, NAc, OAc x 6), 1.68 (9 H, dd, J 20.3, 8.4, H-3b'), 1.48 - 1.29 (9 H, m, C(CH<sub>3</sub>)<sub>3</sub>), 1.29 - 1.19 (3 H, m, CH<sub>3</sub> threonine) ppm. <sup>13</sup>C NMR: δ 128.03, 127.34, 125.46, 120.39, 119.14, 99.95 (C-1), 76.25,

70.47, 69.96, 68.82, 68.55, 67.98, 67.47, 66.57, 66.53, 66.39, 63.82, 61.75, 59.85, 56.87, 47.46, 47.04, 39.04, 29.51, 27.58, 27.30, 25.43, 22.77, 20.44, 20.42, 20.31, 20.16, 20.01, 19.33 ppm. HR-MALDI-ToF/MS: m/z for C<sub>57</sub>H<sub>73</sub>N<sub>3</sub>O<sub>24</sub> [M+Na]<sup>+</sup> calc 1248.4587, found 1248.5901.

#### N-(9-Fluorenylmethyloxycarbonyl)-O-[Allyl(5-N-acetylacetamido-4,7,8,9-tetra-O-acetyl-

3,5-dideoxy-glycero- $\alpha$ -D-galacto-non-2-ulopyranoside)oate-(2 $\rightarrow$ 6)-O-(2-acetamido-3,4-di-Oacetyl- $\alpha$ -D-galactopyranosyl)]-L-threonine (15). Compound 14 (140 mg, 0.114 mmol) was treated with 1:1 TFA/ CH<sub>2</sub>Cl<sub>2</sub> (9 mL) for 3 h. The mixture was concentrated in vacuo and purified by silica gel column chromatography (0  $\rightarrow$  20% methanol in CH<sub>2</sub>Cl<sub>2</sub>) to afford 15 in 99% yield. Analytical data for 15:  $R_f = 0.8$  (20:1 CH<sub>2</sub>Cl<sub>2</sub>/MeOH); <sup>1</sup>H NMR:  $\delta$  (500 MHz, acetone) 7.88 (2 H, d, J 7.5, aromatic), 7.72 (2 H, t, J 7.2, aromatic), 7.43 (2 H, t, J 7.4, aromatic), 7.35 (2 H, td, J 7.4, 2.7, aromatic), 6.72 (1 H, d, J 8.6, NH), 6.08 - 5.94 (1 H, m, CH=CH<sub>2</sub>), 5.54 (1 H, td, J 10.7, 5.2, H-4'), 5.45 (1 H, d, J 17.3, CH=CHH), 5.40 - 5.25 (3 H, m, H-4, H-8', CH=CHH), 5.17 (1 H, d, J 7.8, H-7'), 5.05 (2 H, dd, J 8.2, 3.2, H-1, H-3), 4.99 (1 H, d, J 10.1, H-6'), 4.85 – 4.76 (1 H, m, CHHCH=CH<sub>2</sub>), 4.72 (1 H, dd, J 13.2, 5.7, CHHCH=CH<sub>2</sub>), 4.41 (4 H, p, J 10.4, H-2, CHCH<sub>2</sub> Fmoc, OCHCH<sub>3</sub> threonine), 4.35 – 4.20 (5 H, m, H-9a', CHCH<sub>2</sub> Fmoc, CHCO<sub>2</sub>Allvl, H-5', H-5), 4.13 (1 H, dd, J 12.4, 5.7, H-9<sub>h</sub>'), 3.94 (1 H, dd, J 10.0, 7.0, H-6<sub>a</sub>), 3.46 (1 H, dd, J 10.0, 5.6, H-6<sub>b</sub>), 3.16 - 2.69 (1 H, m, H-3<sub>a</sub>'), 2.34 (6 H, d, J 18.6, NAc x 2), 2.09 (6 H, dd, J 26.5, 11.1, OAc x 2), 2.00 – 1.85 (15 H, m, NAc, OAc x 4), 1.83 (1 H, dd, J 19.2, 7.6, H-3<sub>b</sub><sup>2</sup>), 1.34 (3 H, d, J 6.4, CH<sub>3</sub> threonine) ppm. <sup>13</sup>C NMR: δ 131.74, 127.93, 127.80, 127.33, 125.42, 118.87, 99.44 (C-1), 76.59, 70.26, 69.07, 68.96, 68.39, 67.92, 67.67, 66.81, 66.47, 66.28, 63.44, 61.90, 59.07, 57.15, 47.62, 47.47, 38.87, 29.49, 25.22, 25.33, 22.50, 20.55, 20.32, 20.10, 18.23 ppm. HR-MALDI-ToF/MS: m/z for C<sub>53</sub>H<sub>65</sub>N<sub>5</sub>O<sub>24</sub> [M+Na]<sup>+</sup> calc 1192.3961, found 1192.6213.



Scheme S3 Synthesis of glycopeptide 1.

Sialyl Tn-glycolipopeptide (1). Glycolipopeptide 1 was synthesized using MW-SPPS on Rink Amide AM LL Resin (0.1 mmol) using an automated CEM-Liberty instrument equipped with a UV-detector and a CEM-Discover SPS instrument. Side chain protection was as follows: N- $\alpha$ -Fmoc-O-tert-butyl-Asp-OH, N- $\alpha$ -Fmoc-N- $\epsilon$ -tert-Boc-L-lysine, N- $\alpha$ -Fmoc-O-tert-butyl-L-serine, N- $\alpha$ -Fmoc-O-tert-butyl-L-threonine, N- $\alpha$ -Fmoc-O-tert-butyl-L-tyrosine. Glycosylated amino acid 15 (157 mg, 0.13 mmol) was dissolved in DMF (2 mL) and HATU (51 mg, 0.13 mmol) and DIPEA (67  $\mu$ L, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps performed manually. The resin was then treated with Pd(PPh<sub>3</sub>)<sub>4</sub> (171 mg, 0.15 mmol) in CHCl<sub>3</sub>/HOAc/NMM (37:2:1, 5 mL) for 3 h. The resin was washed thoroughly with DCM (10 mL x 5) and was then treated with 60% hydrazine in methanol for 2 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in vacuo. The resin was swollen in DCM (5 mL) for 1 h. N-a-Fmoc-R-(2,3-bis (palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min, and was added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the N- $\alpha$ -Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation. Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in vacuo. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2.5%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in vacuo approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 °C, 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford 1 (34% based on resin loading capacity). MALDI-ToF/MS: m/z for  $C_{225}H_{379}N_{45}O_{60}S [M+2H]^+$  calc 4705.7710, found 4705.939.

**Tn-glycolipopeptide (2).** Glycolipopeptide **2** was prepared employing N<sup> $\alpha$ </sup>-Fmoc-Thr(AcO<sub>3</sub>- $\alpha$ -D-GalNAc) as a building block. Synthetic protocol was similar as that of glycopeptide **1** using MW-SPPS on Rink Amide AM LL Resin (0.1 mmol) using an automated CEM-Liberty instrument equipped with a UV-detector and a CEM-Discover SPS instrument. All amino acid couplings on the synthesizer were performed twice to avoid any possible side products due to incomplete amino acid couplings. Side chain protection was as follows: *N*- $\alpha$ -Fmoc-*O-tert*-butyl-Asp-OH, *N*- $\alpha$ -Fmoc-*N*- $\epsilon$ -*tert*-Boc-*L*-lysine, *N*- $\alpha$ -Fmoc-*O-tert*-butyl-*L*-serine, *N*- $\alpha$ -Fmoc-*O-tert*-

butyl-L-threonine,  $N-\alpha$ -Fmoc-O-tert-butyl-L-tyrosine. Glycosylated amino acid, N $^{\circ}$ -Fmoc-Thr(AcO<sub>3</sub>-α-D-GalNAc) (87 mg, 0.13 mmol) was dissolved in DMF (2 mL) and HATU (51 mg, 0.13 mmol) and DIPEA (67  $\mu$ L, 0.4 mmol) were premixed for 2 min and were added to the resin. The manual microwave-irradiated coupling reaction was monitored by Kaiser test and was complete after 10 min. The peptide was then elongated under MW-SPPS conditions described above until the final serine residue; the remaining steps performed manually. The resin was then treated with 60% hydrazine in methanol for 2 h. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2) and MeOH (5 mL x 2) and then dried in *vacuo*. The resin was swollen in DCM (5 mL) for 1 h. N-α-Fmoc-R-(2,3-bis (palmitoyloxy)-(2R-propyl)-(R)-cysteine (180 mg, 0.2 mmol) was dissolved in DMF (5 mL) and HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) were premixed for 2 min, and was added to the resin. The microwave-irradiated coupling reaction was monitored by the Kaiser test and was complete after 10 min. Upon completion of the coupling, the N- $\alpha$ -Fmoc group was cleaved using 20% 4-methyl piperidine in DMF (5 mL) under microwave irradiation. Palmitic acid (52 mg, 0.2 mmol) was coupled to the free amine as described above using HATU (76 mg, 0.2 mmol) and DIPEA (67 µL, 0.4 mmol) in DMF. The resin was washed thoroughly with DMF (5 mL x 2), DCM (5 mL x 2), and MeOH (5 mL x 2) and then dried in vacuo. The resin was swelled in DCM (5 mL) for 1 h, after which it was treated with reagent B (TFA 88%, water 5%, phenol 5%, and TIS 2.5%; 10 mL) for 2 h. The resin was filtered and washed with neat TFA (2 mL). The filtrate was concentrated in *vacuo* approximately 1/3 of its original volume. The peptide was precipitated using diethyl ether (0 °C, 30 mL) and recovered by centrifugation at 3,000 rpm for 15 min. The crude glycolipopeptide was purified by HPLC on a Jupiter analytical C-4 reversed phase column using a linear gradient of 0-100% solvent B in A over 40 min, and the appropriate fractions were lyophilized to afford 2 with 7% overall yield. MALDI-ToF/MS: m/z for C214H363N44O52S [M+H]<sup>+</sup> calc 4416.5740, found 4416.0864.

#### Biology

**Liposome preparation for immunizations.** The glycolipopeptide was incorporated into phospholipid-based small unilamellar vesicles (SUVs) by hydration of a thin film of the synthetic compounds, egg phosphatidylcholine, phosphatidylglycerol, and cholesterol in a HEPES buffer (10 mM, pH 7.4) containing NaCl (145 mM) followed by extrusion through a 0.1  $\mu$ m Nucleopore® polycarbonate membrane.

**Immunizations.** Eight to 12-week-old MUC1.Tg mice (C57BL/6; H-2b) that express human MUC1 at physiological level were immunized five-times at biweekly intervals at the base of the tail intradermally with liposomal preparations of three-component vaccine constructs (25  $\mu$ g containing 3  $\mu$ g of carbohydrate) and the respective control that lacks the tumor-associated MUC1 epitope. The mice were sacrificed one week after the last immunization. These animal studies have been approved by the Institutional Anima Care and Use Committee (IACUC) of the Mayo Clinic.

**Serologic assays.** Anti-MUC1 IgG, IgG1, IgG2a, IgG2b, IgG3 and IgM antibody titers were determined by enzyme-linked immunosorbent assay (ELISA) as described previously. Briefly, ELISA plates (Thermo Electron Corp.) were coated with a conjugate of the MUC1 glycopeptide conjugated to BSA through a maleimide linker (BSA-MI-CTSAPDT(Neu5Ac- $\alpha$ 2,6-GalNAc)RPAP). Serial dilutions of the sera were allowed to bind to immobilized MUC1. Detection was accomplished by the addition of phosphate-conjugated anti-mouse antibodies and *p*-nitrophenyl phosphate (Sigma). Titers were determined by linear regression analysis, with plotting of dilution versus absorbance. The antibody titer was defined as the highest dilution yielding an optical density of 0.1 or greater over that of normal control mouse sera.

**Cell culture.** Cell lines used in these studies include C57mg.MUC1 mammary gland tumor cells<sup>[1]</sup>, NK cells (KY-1 clone),<sup>[2]</sup> B16.MUC1 and B16.neo melanoma cells.<sup>[3]</sup> Cells were maintained in DMEM supplemented with FCS (5%), penicillin (100 U mL<sup>-1</sup>), streptomycin (0.1  $\mu$ g mL<sup>-1</sup>), L-glutamax (2 mM), and for B16.MUC1 cells G418 (150  $\mu$ g mL<sup>-1</sup>). All cells are derived originally from C57BL/6 mice.

**Determination of ADCC.** Tumor cells (C57mg.MUC1) were labeled with 100  $\mu$ Ci <sup>51</sup>Cr for 2 h at 37 °C, washed, and incubated with serum (1 in 25 dilutions) obtained from the vaccinated mice for 30 min at 37 °C. NK cells (KY-1 clone), which have high expression of CD16 receptor, were used as effectors. These cells were stimulated with IL-2 (200 units mL<sup>-1</sup>) for 24 h prior to assay. Effector cells were seeded with the antibody-labeled tumor cells in 96-well culture plates (Costar high binding plates) at an effector:target cell ratio of 50:1 for 4 h. Radioactive <sup>51</sup>Cr release was determined using the Topcount Microscintillation Counter (Packard Biosciences). Spontaneous and maximum release of <sup>51</sup>Cr was determined. The percentage of specific release was calculated according to the formula: (release-spontaneous release/maximal release-spontaneous release) x 100. Spontaneous release was below 14% of complete release.

**IFN**γ **ELISPOT assay.** At time of sacrifice, MAC sorted CD62L<sup>low</sup> T-cells from tumor-draining lymph nodes and CD8<sup>+</sup> T-cells from spleens were isolated from treated MUC1.Tg mice and used as responders in an IFNγ ELISPOT assay as described previously.<sup>[4]</sup> Spot numbers were determined using computer-assisted video image analysis by ZellNet Consulting, Inc. (Fort Lee, NJ). Splenocytes from C57BL/6 mice stimulated with Concavalin A were used as a positive control.

Intracellular cytokine (ICC) assay for IFN $\gamma$ . IFN $\gamma$  production by T-cells was assayed following stimulation with DCs pulsed with the immunizing construct for 7 days. Golgi plug was added during the last 6 hours, and harvested cells were stained with anti-mouse CD4, CD8 and IFN $\gamma$  per the BD intracellular staining protocol. Unpulsed DCs were used as control.

**Statistical analysis.** Multiple comparisons were performed using one-way ANOVA with Bonferroni's multiple comparison test. Differences were considered significant when P < 0.05.

#### References

- [1] P. Mukherjee, A. R. Ginardi, T. L. Tinder, C. J. Sterner, S. J. Gendler. *Clin. Cancer Res.* 2001, 7, 848s-855s.
- [2] E. L. Ho, J. W. Heusel, M. G. Brown, K. Matsumoto, A. A. Scalzo, W. M. Yokoyama. Proc. Natl. Acad. Sci. U. S. A. 1998, 95, 6320-6325.
- [3] K. G. Kohlgraf, A. J. Gawron, M. Higashi, M. L. VanLith, X. Shen, T. C. Caffrey, J. M. Anderson, M. A. Hollingsworth. *Cancer Immunol. Immunother.* 2004, 53, 1068-1084.
- [4] P. Mukherjee, L. B. Pathangey, J. B. Bradley, T. L. Tinder, G. D. Basu, E. T. Akporiaye, S. J. Gendler. *Vaccine* 2007, 25, 1607-1618.

## <sup>1</sup>H NMR Compound S2:



F2 (ppm)



3.5 F2 (ppm) S22

1.0

2.0

2.5

3.0

0

5.5

5.0

4.5

4.0

#### <sup>1</sup>H NMR Compound S4:

















### <sup>1</sup>H NMR Compound S9:



### <sup>1</sup>H NMR Compound S10:



#### <sup>1</sup>H NMR Compound 4:

STANDARD 1H OBSERVE - profile



## <sup>1</sup>H NMR for Compound 6:



F2 (ppm)





#### <sup>1</sup>H NMR Compound 13:











ΟН



# HR-MALDI-ToF Compound 2:



## HR-MALDI-ToF intermediate for Compound 2:



