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On the Emerging Role of Chemistry in the Fashioning of Biologics: Synthesis of a Bidomainal Fucosyl GM1-Based Vaccine for the Treatment of Small Cell Lung Cancer

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Received May 8, 2009

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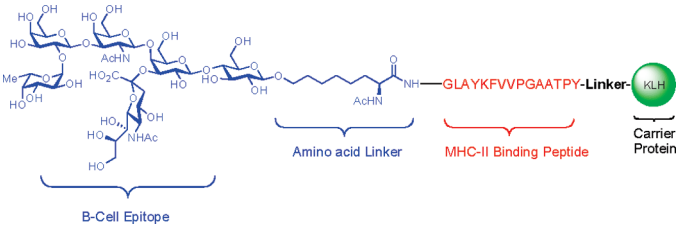
Published on June 25, 2009 on http://pubs.acs.org | doi: 10.1021/jo900918m



The synthesis of the novel small cell lung cancer (SCLC) fucosyl GM1-based vaccine construct, featuring insertion of the HLA-DR binding 15 amino acid sequence derived from *Plasmodium falciparum*, is described. The resultant glycopeptide has been synthesized in an efficient manner. Finally, successful conjugation of the glycopeptide to the keyhole limpet hemocyanin (KLH) carrier protein completed the preparation of the vaccine.

Introduction

Among the large number of emerging anticancer strategies, the prospect of mobilizing the immune system against the disease is especially attractive. One can imagine employing a vaccine-based therapeutic approach against a number of different primary tumors, as well as against metastatic cells, in an adjuvant mode.[1] Along these lines, we are pursuing the idea of targeting as immune system markers complex carbohydrate epitopes, which are overexpressed on cancer cell surfaces. Though this concept has occurred to others, our group has made a particularly strong commitment to accessing these structures by total synthesis.[2] A typical carbohydrate-based anticancer vaccine would consist of a complex carbohydrate hapten, overexpressed on the cancer cell, a carrier protein, and a linker attaching the carbohydrate to the protein (Figure 1). Beside being a potent immunogen, the carrier protein is known to provide the MHC-II binding peptides bound to the epitope, thus helping to present the carbohydrate to the T-cells for T-cell activation and initiation of the cellular response.[3,4] Consequently, one could imagine that the immunogenicity of a vaccine might well be enhanced by providing MHC-II binding peptides in the environs of the epitope, thereby serving to increase the number of epitopes presented to the CD4 + T cell. In a sense, this rationale is related to the idea of conjugating epitopes to carrier protein to create vaccines. However, this approach of placing an MHC-II binding sequence in a fixed relation to the antigen has been pursued mostly for vaccines unconjugated to carrier protein.[5] We hope to explore the possibility that introduction of an

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(2) For an overview of our synthetic carbohydrate-based vaccine program, see: (a) Danishefsky, S. J.; Allen, J. R. *Angew. Chem., Int. Ed.* **2000**, *39*, 836–863. (b) Keding, S. J.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 11937–11942.

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DOI: 10.1021/jo900918m Published on Web 06/25/2009
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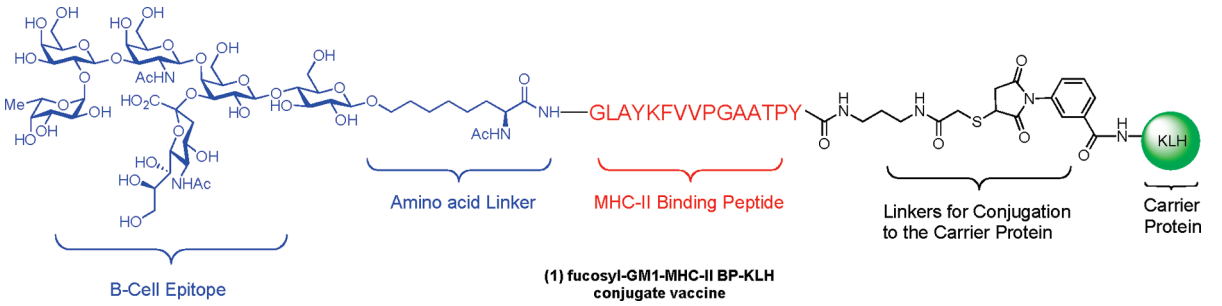
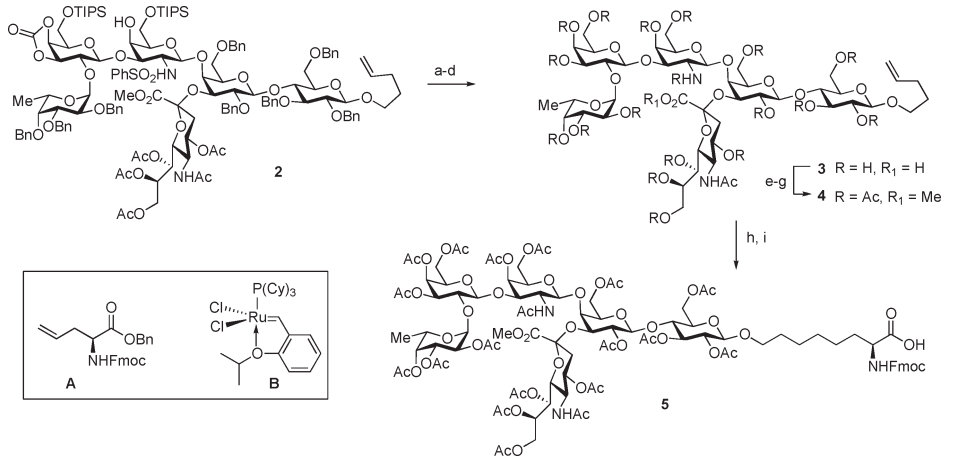


FIGURE 1. Next-generation bidomainal fucosyl GM1-based vaccine for the treatment of SCLC.

SCHEME 1[a]



[a]Key: (a) TBAF, AcOH, THF; (b) NaOMe, MeOH; (c) NaOH, THF; (d) Na, NH_3, THF; (e) Ac_2O, DMAP, Py; (f) DMAP, MeOH; (g) Ac_2O, DMAP, Py; 56% (7 steps); (h) **A**, **B**, CH_2Cl_2, rt; (i) H_2, Pt/C, MeOH−H_2O; 49%, two steps.

MHC-II binding sequence could also improve the immunogenicity of vaccines incorporating standard carriers such as keyhole limpet hemocyanin (KLH).

To test the notion of upgrading the immunogenicity of a candidate carbohydrate-based vaccine in this way, we pursued the synthesis of the construct illustrated in Figure 1. Fucosyl GM1 is a carbohydrate epitope that is expressed on the surface of small-cell lung cancer (SCLC) cells.[6] This carbohydrate has been previously synthesized by our group as well as by others,[7] and it was selected based on the promising results demonstrated by its KLH conjugate in recent clinical trials.[8] A 15 amino acid peptide sequence derived from *Plasmodium falciparum* and illustrated in Figure 1 was chosen as the T-cell epitope. This sequence has been shown to be general for binding up to nine different genetic variants of human HLA-DR with binding capacity prevalently in the nanomolar range.[9]

The appendage of the fucosyl GM1 epitope to the peptide portion could be accomplished using the norleucine linker developed by our group.[10] The long aliphatic chain of this linker would be optimal in preventing potentially adverse interactions between the epitope and the peptide backbone. The amino acid functionality makes this linker a powerful handle for conjugation.

The synthesis of glycoprotein conjugates still presents a challenge for numerous reasons. One of the issues is compatibility and "collegiality" of the protecting groups required for peptidic and carbohydrate assemblies. Thus, a complex glycan is often unstable under the highly acidic conditions that are required to remove peptide protecting groups. Correspondingly, peptides may be unstable under the basic conditions that are required for the deprotection and retrieval of the oligosaccharide ensembles.[11] However, since the protecting groups of the selected peptide would be limited to *tert*-butylcarboxy for lysine and *tert*-butyl for tyrosines and threonine, there is, hopefully, no requirement

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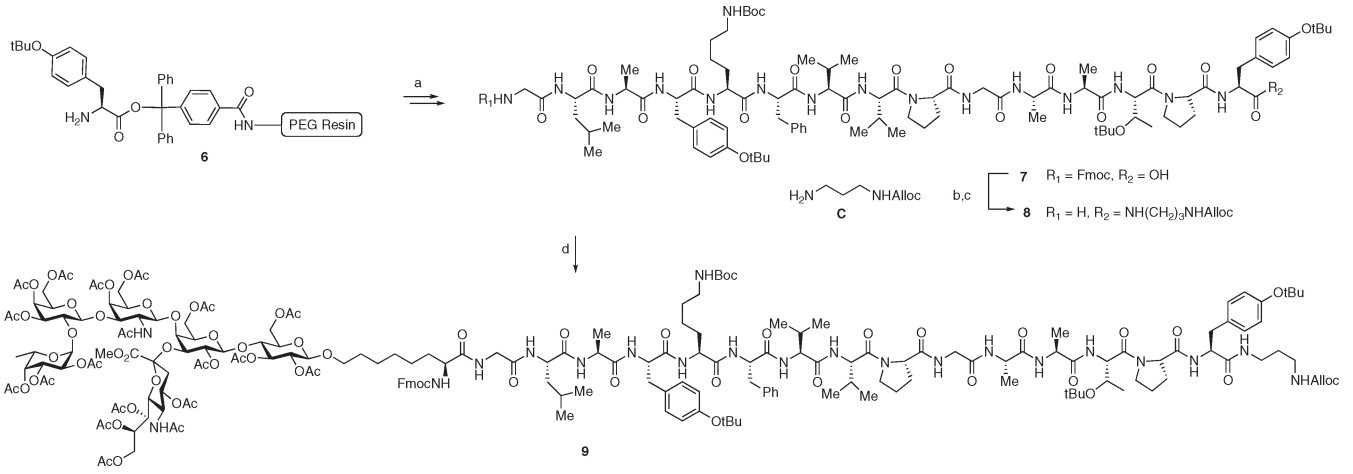
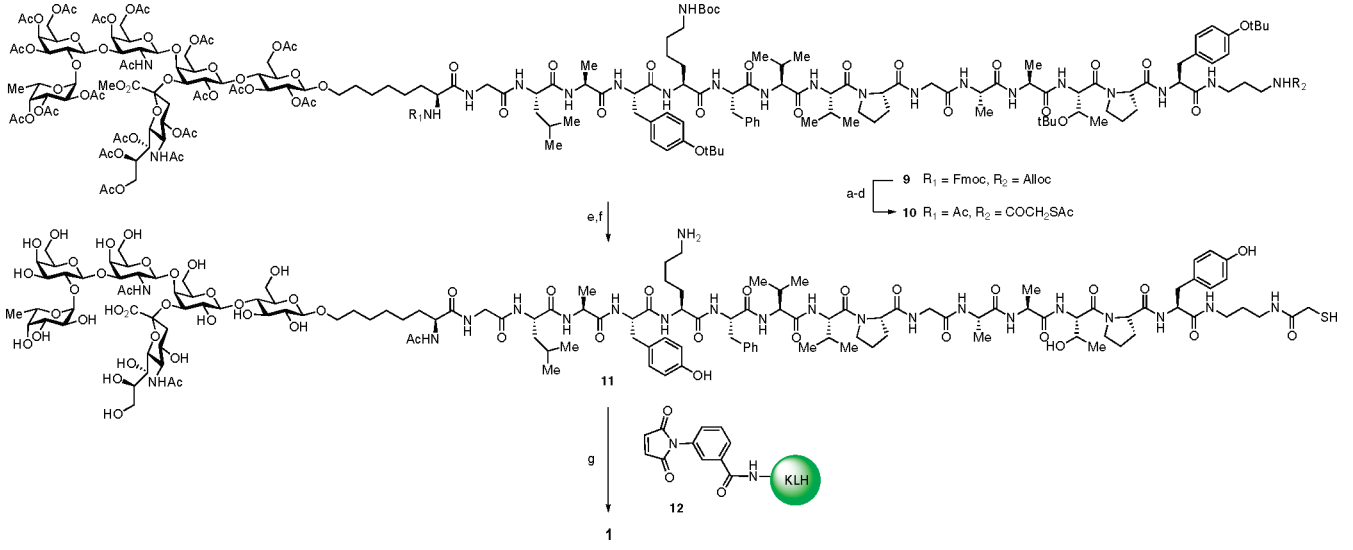
SCHEME 2[a]



[a]Key: (a) SPPS, then $AcOH/CF_3CH_2OH/CH_2Cl_2$ (1:1:8), 95%; (b) **C**, EDC, HOOBt, $CHCl_3/CF_3CH_2OH$ (3:1); (c) Piperidine, DMF; 71%, two steps; (d) **5**, EDCI, HOBt, DMF/CH_2Cl_2 (1:1), 81%.

SCHEME 3[a]



[a]Key: (a) piperidine, DMF; (b) Ac_2O, Py; (c) $Pd(PPh_3)_4$, $PhSiH_3$, CH_2Cl_2/DMF (1:1); (d) SAMA−OPbf, TEA, Py; (e) TFA/PhOH/H_2O/TIPS, (71% from **9**, five steps); (f) NaOH in MeOH/H_2O, pH = 10.5, 19%; (g) (i) TCEP gel; (ii) PBS buffer, pH = 7.2, 0.9 M NaCl, 0.1 M EDTA.

for prolonged treatment with acid in the deprotection phase. Thus, the glycoside linkages of peracetylated fucose and sialic acid moieties could be stable under the deprotection conditions. Of course, the cleavage of a methyl ester and 17 acetate groups could prove to be a challenging task that might well require careful selection of hydrolysis conditions.

Results and Discussion

The construction of the vaccine began from the known Fuc-GM1 hexasaccharide **2**, obtained through a previously disclosed sequence.[7a] After deprotection of the triisopropylsilyl groups with TBAF/acetic acid and subsequent cleavage of acetates, carbonate, and sialic acid methyl ester, the resultant product was debenzylated under Birch conditions.[12] The obtained acid **3** was exhaustively peracetylated to provide a corresponding lactone that was subsequently opened with methanol and DMAP. Acylation afforded **4** (56%, seven steps). Glycoside **4** was treated with Fmoc-L-allylglycine benzyl ester (**A**) and Hoveyda−Grubbs catalyst (**B**) under the previously developed conditions, and the resultant olefin cross-metathesis product was subjected to catalytic hydrogenation.[10a,10b,13] The side-chain olefinic linkage was reduced with concomitant selective removal of

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the benzyl protecting group in the presence of Fmoc-protected amine to afford the cassette **5** (49%, 2 steps) ready for coupling (Scheme 1).

The synthesis of peptide **7** was accomplished by standard Fmoc solid-phase peptide synthesis (SPPS), starting from the protected tyrosine, **6**, preloaded on NovaSyn TGT resin. Peptide **7** was obtained in 95% yield after cleavage from the resin, in more than 95% purity, as judged by LC/MS and ^{1}H NMR analysis. The elaboration of **7** to peptide **8** was executed by first conjugating **7** to the linker **C**[14] using the standard EDCI/HOBt protocol.[10c] The Fmoc protecting group was next removed by treatment with piperidine, providing fragment **8** in 71% yield (two steps). The attachment of **8** to carbohydrate epitope **5** proceeded in 81% yield, thereby providing glycopeptide **9** (see Scheme 2).

Compound **9** was treated sequentially with piperidine/DMF and Ac_2O/Py. The allylcarboxy protecting group was exchanged to the acetate-protected 2-sulfhydrylacetate linker by reduction with $Pd(PPh_3)_4/PhSiH_3$ followed by acylation with SAMA-OPbf.[10c] Finally, three *tert*-butyl and one *tert*-butylcarboxy groups were removed by treating **10** with TFA/PhOH/H_2O/TIPS providing the corresponding deprotected product in 71% yield (five steps) from **9** following purification by HPLC. This compound was treated with a degassed solution of NaOH in MeOH/H_2O (pH = 10.5), providing the desired deprotected product **11** in 19% yield following HPLC purification.[15] Minor amounts of a dehydration side product (ca. 5% yield after HPLC) were also isolated.

Next, the conjugation of construct **11** to maleimide-activated KLH **12** was examined (Scheme 3). Thus, **11** was pretreated with TCEP gel for 2 h and then treated with freshly prepared **12** at pH = 7.2. The efficiency of the coupling was estimated by a combination of Bradford protein assay[16] and neuraminic acid determination according to Svennerholm[17] to be 210 epitopes per molecule of KLH (MW = 8 MDa).

Conclusion

In summary, a new kind of fucosyl GM1 epitope-based vaccine has been efficiently prepared through the coupling of the fucosyl GM1 cassette with a promiscuous HLA-DR binding peptide. The resultant construct was further functionalized and deprotected to provide the glycopeptide, which was next conjugated to the carrier protein (KLH). The results of immunological evaluations of the vaccine will be forthcoming. This synthetic accomplishment is in keeping with an important theme in our laboratory, to the effect that there have emerged exciting opportunities for chemistry in the fashioning of structure types previously perceived as strictly "biologics". It goes without saying that chemistry provides vast opportunities (and challenges) in de novo design, thus offering far greater flexibility than is available though strictly biology-driven routes to biologics.

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Experimental Section

Synthesis of Acetylated Glycoside 4 (Steps a−c, Scheme 1). To a solution of the hexasaccharide **2**[7a] (335 mg, 0.131 mmol) in THF (6.0 mL) were added glacial AcOH (0.12 mL) and TBAF (1.0 M in THF, 1.31 mL). The reaction mixture was stirred at rt for 2 days, poured into ice−water (25 mL), and extracted with EtOAc. The organic extracts were dried over $MgSO_4$ and concentrated. The resulting triol was dissolved in anhydrous MeOH (6 mL), and sodium methoxide was added (25% solution in MeOH, 0.6 mL). The contents were stirred at rt for 3 days, and then water (6.0 mL) and THF (6.0 mL) were added. Stirring at rt for an additional 2 days was followed by neutralization with Dowex-H^+, filtration with MeOH washing, and concentration. The crude material was allowed to dry under high vacuum for 1 day.

Synthesis of Acetylated Glycoside 4 (Steps d−g, Scheme 1). To a blue solution of sodium (160 mg) in liquid NH_3 (50 mL) was added a solution of the white solid from above in THF (5.0 mL), and the resulting mixture was stirred at −78 °C for 2 h. The reaction was quenched by the addition of anhydrous MeOH (20 mL), warmed to rt, and concentrated with a stream of dry argon. The residue was diluted with MeOH (70 mL) and treated with Dowex 50wX8-400 until pH was nearly 5−6. The mixture was filtered and concentrated to provide a solid. This solid was dissolved in a mixture of pyridine (12.0 mL) and Ac_2O (6.0 mL) at rt. To the solution of tetrasaccharide was added DMAP (10 mg), and the mixture was stirred for an additional 2 days. The reaction mixture was cooled to 0 °C and treated with MeOH (24 mL). To this solution was added DMAP (15 mg), and the resultant mixture was stirred at rt for an additional 4 days. The reaction mixture was then concentrated and coevaporated with toluene (4 × 100 mL). The residue was dissolved in pyridine (5.0 mL) and Ac_2O (1.0 mL) at rt. The mixture was stirred for 1 day and then concentrated. The resultant oil was dissolved in MeOAc (10.0 mL) and MeI (0.2 mL). To the solution was added cesium carbonate (33 mg), and the mixture was stirred for 1 h and then diluted with methyl acetate (250 mL). The organic phase was washed with brine/$NH_4Cl_{(satd)}$ (1:1, 100 mL), $NaHCO_{3(satd)}$ (100 mL), and brine (100 mL) and dried over $MgSO_4$. Concentration followed by flash chromatography (silica, 5% methanol/dichloromethane) provided the acetylated glycoside **4** (142 mg, 56% from **2**): $[\alpha]^{24}_D = -40.4$ (*c* 1.00, $CHCl_3$); IR (film $CHCl_3$) 2969, 1746, 1689, 1530, 1371, 1231, 1131, 1058 cm^{-1}; ^{1}H NMR ($CDCl_3$, 600 MHz) δ 7.22 (d, J = 5.9 Hz, 1H), 5.76−5.69 (m, 1H), 5.61−5.58 (m, 1H), 5.43 (d, J = 3.5 Hz, 1H), 5.34−5.30 (m, 4H), 5.21−5.10 (m, 5H), 5.00 (d, J = 8.2 Hz, 1H), 4.97−4.89 (m, 4H), 4.82 (t, J = 8.8 Hz, 1H), 4.74 (td, J = 11.4, 3.6 Hz, 1H), 4.65 (d, J = 7.7 Hz, 1H), 4.52 (d, J = 7.8 Hz, 1H), 4.49−4.45 (m, 1H), 4.39−4.33 (m, 3H), 4.21−4.17 (m, 2H), 4.14−4.02 (m, 6H), 4.00−3.89 (m, 3H), 3.85−3.70 (m, 9H), 3.58−3.56 (m, 2H), 3.45−3.40 (m, 2H), 3.01 (dt, J = 12.7, 5.6 Hz, 1H), 2.81 (dd, J = 12.9, 4.1 Hz, 1H), 2.19 (s, 3H), 2.12 (s, 3H), 2.10 (s, 3H), 2.09 s, 3H), 2.03−1.97 (m, 33H), 1.95 (s, 3H), 1.94 (s, 3H), 1.93 (s, 3H), 1.80 (s, 3H), 1.70 (t, J = 12.8 Hz, 1H), 1.64−1.57 (m, 2H), 1.22−1.20 (m, 2H), 1.11 (d, J = 6.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 173.6, 171.0, 170.9, 170.9, 170.4, 170.4, 170.4, 170.3, 170.3, 170.3, 170.3, 170.3, 170.2, 170.2, 170.0, 169.7, 169.6, 169.5, 169.4, 169.2, 168.2, 137.7, 114.9, 102.0, 100.4, 98.7, 97.3, 94.4, 75.6, 73.7, 73.5, 73.3, 73.2, 72.8, 72.5, 72.0, 71.8, 71.7, 71.3, 70.9, 70.3, 70.2, 69.8, 69.4, 69.2, 69.1, 68.7, 68.1, 67.7, 67.4, 67.2, 67.0, 65.0, 63.3, 62.4, 62.4, 62.4, 60.6, 60.3, 55.5, 53.7, 52.5, 49.3, 37.1, 31.6, 29.7, 29.2, 28.5, 23.5, 23.0, 21.3, 20.9, 20.8, 20.7, 20.7, 20.7, 20.7, 20.6, 20.6, 20.5, 20.4, 20.4, 15.9, 14.1; ESI/MS exact mass calcd for $C_{83}H_{116}N_2O_{50}$ $[M + Na]^+$ 1963.7, $[M + Cl]^-$1975.6, found 1963.9, 1977.0.

Synthesis of Amino Acid 5 (Step h, Scheme 1). The first-generation Hoveyda−Grubbs catalyst (**B**, 9.6 mg, 0.016 mmol) was added to a solution of acetylated glycoside **4** (124 mg,

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(17) Svennerholm, L. *Biochim. Biophys. Acta* **1957**, *24*, 604–611.
(18) (a) Frechet, J. M. J.; Haque, K. E. *Tetrahedron Lett.* **1975**, *35*, 3055–3056. (b) Novabiochem catalog, 2006/2007.

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0.064 mmol) and allylglycine **A** (273 mg, 0.640 mmol) in CH_2Cl_2 (1 mL) at rt. The reaction mixture was stirred for 12 h and exposed to air for 3 h. The mixture was concentrated, and the resultant residue was purified by flash chromatography (100% ethyl acetate) to provide the coupled product.

Synthesis of Amino Acid 5 (Step i, Scheme 1). Pt/C (10% w/w, 15 mg) was added to a solution of the metathesis adduct from above in MeOH (3 mL) and H_2O (0.2 mL), and the hydrogen atmosphere was established. The reaction mixture was stirred for 4 days at rt, filtered through a short pad of silica gel, and concentrated. The residue was purified by flash chromatography (10% MeOH in CH_2Cl_2) to give the amino acid **5** (70 mg, 49% over two steps): $[\alpha]^{24}_D = -30.2$ (*c* 1.00, $CHCl_3$); IR (film $CHCl_3$) 3470, 2928, 2854, 1746, 1429, 1370, 1232, 1057 cm^{-1}; ^1H NMR ($CDCl_3$, 600 MHz) δ 8.16 (d, 1H, $J = 6.4$ Hz), 7.79 (d, 2H, $J = 7.4$ Hz), 7.67−7.65 (m, 2H), 7.39−7.37 (m, 2H), 7.30 (br.s, 2H), 5.62−5.60 (m, 1H), 5.48−5.47 (m, 2H), 5.39 (d, 1H, $J = 10$ Hz), 5.25−5.23 (m, 3H), 5.14−5.05 (m, 3H), 4.98−4.78 (m, 6H), 4.68 (d, 1H, $J = 7.5$ Hz), 4.64 (d, 1H, $J = 7.8$ Hz), 4.54−4.53 (m, 2H), 4.47 (d, 1H, $J = 11.2$ Hz), 4.40−4.32 (m, 3H), 4.29−4.10 (m, 7H), 4.07−3.76 (m, 15H), 3.72−3.65 (m, 3H), 3.57 (s, 1H), 3.48−3.44 (m, 1H), 3.25−3.17 (m, 1H), 2.86 (dd, 1H, $J = 3.9$ and 12.5 Hz), 2.27 (s, 3H), 2.16 (s, 3H), 2.14 (s, 3H), 2.12 (s, 6H), 2.11 (s, 3H), 2.08 (s, 3H), 2.06 (s, 6H), 2.03 (s, 6H), 2.02 (s, 6H), 2.00 (s, 3H), 1.98 (s, 6H), 1.95 (s, 6H), 1.82 (s, 3H), 1.61 (t, 2H, 12.5 Hz), 1.52 (br.s, 2H), 1.37−1.25 (m, 7H), 1.18 (d, 3H, $J = 6.2$ Hz); ^{13}C NMR ($CDCl_3$, 150 MHz) δ 180.2, 175.4, 173.6, 172.4, 172.4, 172.4, 172.3, 172.2, 172.2, 172.1, 171.8, 171.7, 171.7, 171.5, 171.4, 171.1, 169.7, 158.2, 145.5, 145.4, 142.6, 128.8, 128.2, 126.3, 126.3, 121.0, 102.6, 102.3, 101.7, 101.1, 98.7, 97.3, 77.6, 75.2, 75.0, 74.8, 74.4, 74.3, 74.2, 73.8, 73.3, 73.2, 73.0, 72.7, 72.4, 72.2, 71.9, 71.3, 71.0, 70.6, 69.7, 69.2, 68.9, 68.4, 67.7, 66.2, 65.0, 63.8, 63.6, 63.5, 62.6, 57.6, 55.8, 53.8, 50.0, 49.6, 48.6, 38.7, 34.1, 30.6, 30.2, 27.0, 26.8, 24.2, 22.8, 21.7, 21.7, 21.3, 21.0, 21.0, 20.9, 20.9, 20.8, 20.8, 20.8, 20.7, 20.6, 20.6, 20.6, 16.3; ESI/MS exact mass calcd for $C_{101}H_{133}N_3O_{54}$ $[M + Na]^+$ 2275.8, $[M + 2Na]^{2+}$ 1149.4, found 2275.5, 1149.3.

Synthesis of Peptide 7 (Step a, Scheme 2). NovaSyn TGT resin (purchased from NovaBiochem) was chlorinated, esterified with Fmoc-Tyr(tBu)-OH for 3 h, and then immediately Fmoc-deprotected according to the literature procedure.[18] A 0.21 g (ca. 0.05 mmol) portion of this resin was subjected to continuous flow automated peptide synthesis. For coupling steps, the resin was treated with a 4-fold excess of HATU and Fmoc amino acids in 1 M DIEA/DMF, and for deblocking, a solution of 2% piperidine/2% DBU in DMF was used. The amino acids used were, in order of synthesis: Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, Fmoc-Ala-OH, Fmoc-Ala-OH, Fmoc-Gly-OH, Fmoc-Pro-OH, Fmoc-Val-OH, Fmoc-Val-OH, Fmoc-Phe-OH, Fmoc-Lys(Boc)-OH, Fmoc-Tyr (tBu)-OH, Fmoc-Ala-OH, Fmoc-Leu-OH, Fmoc-Gly-OH. The resin was then transferred to a manual peptide synthesis vessel and treated with a cleavage solution of 5 mL of 1:1:8 trifluoroethanol/acetic acid/dichloromethane for 1.5 h. The beads were filtered, rinsed with another 10 mL of cleavage solution, filtered again, and then treated for another 1 h with 10 mL of the cleavage solution. This process was repeated for a total of three 2-h cleavage cycles, and the combined organic phase was concentrated in vacuo to afford 97 mg of peptide after cleavage (ca. 95% yield). This material was found to be >95% pure as judged by reversed-phase LC/ESI (C4 column) MS and ^1H NMR analysis: ^1H NMR (500 MHz, DMF-d_7)[19] δ 8.44 (t, $J = 5.5$ Hz, NH), 8.34 (br, NH), 8.27 (br, NH), 8.13 (t, $J = 8.6$ Hz, NH), 8.00 (m, NH), 7.94 (d, $J = 7.6$ Hz, 2H), 7.89 (d, $J = 6.5$ Hz, NH), 7.86 (d, $J = 7.1$ Hz, NH), 7.79 (br, NH), 7.73 (t, $J = 8.4$ Hz, 2H, NH), 7.62 (d, $J = 7.9$ Hz, NH), 7.60 (d, $J = 7.4$ Hz, NH), 7.55 (d, $J = 6.0$ Hz, NH), 7.45 (t, $J = 7.4$ Hz, 2H), 7.34 (t, $J = 7.6$ Hz, 2H), 7.31 (d, $J = 7.6$ Hz, 2H), 7.25 (m, 2H), 7.22−7.17 (m, 5H), 6.93 (t, $J = 8.3$ Hz, 2H), 6.88 (d, $J = 8.2$ Hz, 2H), 6.63 (m, NH), 5.12 (t, $J = 4.7$ Hz, NH), 4.71−4.67 (m, 2H), 4.66−4.59 (m, 1H), 4.54−4.47 (m, 3H), 4.46−4.41 (m, 2H), 4.41−4.37 (m, 2H), 4.30 (m, 2H), 4.29−4.23 (m, 2H), 4.00−3.85 (m, 4H), 3,77 (d, $J = 16.7$ Hz, 1H), 3.70 (m, 2H), 3.52 (br t, $J = 8.8$ Hz, 2H), 3.40 (q, $J = 8.5$ Hz, 1H), 3.23 (d, $J = 10.6$ Hz, 1H), 3.19−3.08 (m, 3H), 3.08−2.96 (m, 3H), 2.96 (s, 2H), 2.79 (s, 2H), 2.18−2.05 (m, 4H), 2.02−1.93 (m, 1H), 1.92−1.83 (m, 3H), 1.77−1.72 (m, 1H), 1.71−1.66 (m, 2H), 1.63 (m, 2H), 1.54 (m, 1H), 1.40 (s, 9H), 1.42−1.33 (m, 6H), 1.31 (s, 9H), 1.30 (s, 9H), 1.26 (s, 9H), 1.3−1.23 (m, 2H), 1.23 (s, 6H), 1.19 (s, 3H), 1.12 (d, $J = 6.2$ Hz, 6H), 1.05 (d, $J = 6.2$ Hz, 3H), 0.99 (d, $J = 6.8$ Hz, 3H), 0.97 (d, $J = 6.6$ Hz, 3H), 0.92 (d, $J = 6.2$ Hz, 6H), 0.90 (d, $J = 5.2$ Hz, 6H); LC/MS (ESI) $R_f = 16.6$ min (C4 column, 50−95% MeCN in H_2O, 30 min); exact mass calcd for $C_{108}H_{154}N_{16}O_{23}$ $[M + H]^+$ 2045.2, $[M + Na]^+$ 2067.1, $[M + 2H]^{2+}$, 1023.1, found 2044.7, 2066.6, 1022.9.

Synthesis of Compound 8 (Step b, Scheme 2). To compound **7** (30 mg, 0.0147 mmol) were added linker **C** (5.6 mg, 0.0352 mmol), HOOBt (5.7 mg, 0.0352 mmol) in 1:3 trifluoroethanol/$CHCl_3$, and EDC (6.2 mL, 0.0352 μmol). After 2 h, LC/MS indicated completion of the reaction. The mixture was concentrated under reduced pressure and purified via flash chromatography (silica, 2%→10% MeOH/ CH_2Cl_2), and the appropriate fractions were concentrated (R_f 0.5, 10% MeOH/ CH_2Cl_2) to afford 30 mg of product in 94% yield. This material was found to be >95% pure as judged by reversed-phase LC/ESI (C4 column): MS exact mass calcd for $C_{115}H_{166}N_{18}O_{24}$ $[M + H]^+$ 2185.2, $[M + Na]^+$ 2207.2, $[M + 2H]^{2+}$ 1093.1, found 2184.8, 2206.8, 1093.2.

Synthesis of Compound 8 (Step c, Scheme 2). The product from above (26.8 mg, 0.0123 mmol) was dissolved in 1.0 mL of DMF, and to this solution was added piperidine (0.25 mL). After 1 h, LC/MS analysis indicated the completion of the reaction. The mixture was concentrated under reduced pressure and purified via flash chromatography (silica, 10% → 12% MeOH/ CH_2Cl_2), and the appropriate fractions were concentrated (R_f 0.15, 10% MeOH/CH_2Cl_2) to afford 18 mg of product **8** in 75% yield. This material was found to be >95% pure as judged by reversed-phase LC/ESI analysis: $R_f = 15.7$ (C4 column, 40−85% MeCN in H_2O, 30 min); exact mass calcd for $C_{100}H_{156}N_{18}O_{22}$ $[M + H]^+$ 1963.2, $[M + Na]^+$ 1985.2, $[M + 2H]^{2+}$ 982.1, found 1962.9, 1984.9, 982.1.

Synthesis of Compound 9 (Step d, Scheme 2). Amine **8** (9.5 mg, 0.048 mmol) was combined with acid **5** (6.6 mg, 0.029 mmol), EDCI (1.8 mg, 0.093 mmol), and HOBt (1.3 mg, 0.0093 mmol), and this mixture was dissolved in 0.30 mL of 1:1 DMF/CH_2Cl_2. After 3 h of stirring under argon, the solvents were removed under high vacuum, the resultant oil was purified via flash chromatography (silica, 5%→10% MeOH/CH_2Cl_2), and the appropriate fractions were concentrated (R_f 0.5, 10% MeOH/CH_2Cl_2) to afford 10 mg of product **9** in 81% yield. This material was found to be >90% pure as judged by reversed-phase LC/ESI MS (C4 column) and ^1H NMR analysis: ^1H NMR (500 MHz, CD_3OD)[19] selected peaks δ 8.12 (d, 1H), 7.95 (m, 1H), 7.80 (d, $J = 9.1$ Hz, 2H), 7.74 (d, $J = 7.6$ Hz, 2H), 7.72 (m, 1H), 7.67 (d, $J = 8.2$ Hz, 2H), 7.66 (m, 1H), 7.62 (d, $J = 7.4$ Hz, 1H), 7.57 (d, $J = 7.5$ Hz, 1H), 7.56 (m, 1H), 7.45 (t, $J = 7.2$ Hz, 1H), 7.39 (t, $J = 7.7$ Hz, 1H), 7.33 (t, $J = 7.4$ Hz, 1H), 7.24 (dt, $J = 7.3$, 4.1 Hz, 1H), 7.18 (d, $J = 7.3$ Hz, 1H), 7.14 (t, $J = 7.6$ Hz, 1H), 7.08 (d, $J = 5.5$ Hz, 4H), 6.97 (m, NH), 6.93 (m, NH), 6.85 (d, $J = 7.9$ Hz, 2H), 6.80 (d, $J = 8.0$ Hz, 4H), 5.85 (ddd, $J = 16.2$, 10.5, 5.5 Hz, 1H), 5.55 (dt, $J = 9.5$, 4.1 Hz, 1H), 5.42 (dd, $J = 9.5$, 2.8 Hz, 2H), 5.32 (dd, $J = 9.6$, 2.1 Hz, 1H), 5.19 (m, 4H), 5.07 (m, 4H), 4.97 (m, 2H), 4.75 (m, 1H), 4.60 (d, $J = 7.5$ Hz, 3H), 4.49 (dt, $J = 16.3$, 8.1 Hz, 1H), 4.47−4.37 (m, 8H), 4.33−4.24 (m, 8H), 4.20−4.08 (m, 7H), 4.06 (dd, $J =$

(19) Due to the high degree of the NH exchange, the presence of the multiple peptide rotomers in the solution, as well as the high overlap, there is an ambiguity associated in the tabulation and interpretation of the ^1H NMR data. Refer to the attached ^1H NMR spectrum in the Supporting Information for additional details.

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Published on June 25, 2009 on http://pubs.acs.org | doi: 10.1021/jo900918m

11.2, 6.0 Hz, 2H), 4.05 (dt, J = 9.1, 4.0 Hz, 2H), 3.95 (m, 2H), 3.94−3.81 (m, 8H), 3.83 (s, 3H), 3.80 (m, 3H), 3.75 (m, 1H), 3.71 (m, 1H), 3.68 (m, 1H), 3.64 (m, 3H), 3,58 (m, 1H), 3.50 (s, 1H), 3,42 (m, 1H), 3.15 (dd, J = 13.3, 6.2 Hz, 2H), 3.12 (m, 1H), 3.11−3.03 (m, 4H), 2.97 (t, J = 6.2 Hz, 2H), 2.93−2.83 (m, 4H), 2.80 (dd, J = 12.7, 4.4 Hz, 1H), 2.21 (s, 3H), 2.20−2.13 (m, 2H), 2.11 (s, 3H), 2.09 (s, 3H), 2.06 (s, 9H), 2.01 (s, 3H), 2.00 (s, 3H), 1.99 (s, 3H), 1.97 (s, 6H), 1.96 (s, 6H), 1.94 (s, 3H), 1.92 (s, 3H), 1.91 (s, 3H), 1.89 (s, 3H), 1.89 (s, 3H), 2.13−1.77 (m, 13H), 1.75 (s, 3H), 1.58−1.48 (m, 12H), 1.36 (s, 3H), 1.36 (s, 3H), 1.34−1.24 (m, 8H), 1.23 (s, 18H), 1.21 (s, 9H), 1.15 (s, 6H), 1.15−1.09 (m, 6H), 1.05 (d, J = 6.2 Hz, 3H), 0.91 (d, J = 6.7 Hz, 3H), 0.89 (d, J = 8.6 Hz, 6H), 0.87 (d, J = 7.0 Hz, 6H), 0.85 (d, J = 6.6 Hz, 3H), 0.75 (m, 3H); R_f = 22.5 (C4 column, 50−95% MeCN in H_2O, 30 min); exact mass calcd for $C_{201}H_{287}N_{21}O_{75}$ $[M + 2H]^{2+}$ 2099.5, $[M + 2Na]^{2+}$ 2121.5, $[M + 3H]^{3+}$ 1400.0, found 2099.4, 2121.6, 1400.2.

Synthesis of Compound 10 (Step a, Scheme 3). Compound **9** (10.5 mg, 0.00250 mmol) was dissolved in 1.0 mL of DMF, and piperidine (0.25 mL) was added. After 1 h, LC/MS analysis indicated the completion of the reaction: R_f = 15.3 (C4 column, 50−95% MeCN in H_2O, 30 min); exact mass calcd for $C_{186}H_{277}N_{21}O_{71}$ $[M + 2H]^{2+}$ 1988.4, $[M + Na + H]^{2+}$ 1999.4, $[M+2Na]^{2+}$ 2010.4, $[M+3Na]^{3+}$ 1326.0, found 1988.4, 1999.37, 2010.45, 1326.1.

Synthesis of Compound 10 (Step b, Scheme 3). The mixture from above was concentrated under reduced pressure, redissolved in pyridine (1.0 mL), and treated with acetic anhydride (0.5 mL). After 4 h, the reaction mixture was concentrated and purified via flash chromatography (silica, 10% MeOH/CH_2Cl_2), and the appropriate fractions were concentrated (R_f 0.2, 10% MeOH/CH_2Cl_2) to afford 10 mg (quantitative yield) of product **9a**. This material was found to be >85% pure as judged by reversed-phase LC/ESI analysis: R_f = 17.8 (C4 column, 50−95% MeCN in H_2O, 30 min); exact mass calcd for $C_{188}H_{279}N_{21}O_{74}$ $[M + 2H]^{2+}$ 2009.5, $[M + 2Na]^{2+}$ 2031.4, $[M + 3H]^{3+}$ 1340.0; found 2009.4, 2032.0, 1340.0.

Synthesis of Compound 10 (Step c, Scheme 3). A solution of $Pd(PPh_3)_4$ (14 mg, 0.0125 mmol) and phenylsilane (46 μL, 0.373 mmol) in 3.5 mL of CH_2Cl_2 was prepared, and 0.35 mL of this solution was added to the solution of compound **9a** (10 mg, 0.00249 mmol) in DMF (0.35 mL). After 30 min, LC/MS analysis indicated the completion of the reaction. Pyridine (0.1 mL) was added, and the resultant mixture was concentrated under vacuum to provide crude product: R_f = 16.4 (C4 column, 50−95% MeCN in H_2O, 30 min); exact mass calcd for $C_{184}H_{275}N_{21}O_{72}$ $[M + 2H]^{2+}$ 1966.9, $[M + Na + H]^{2+}$ 1977.9, $[M + 2Na]^{2+}$ 1988.9, $[M + 3Na]^{3+}$ 1311.6, found 1967.5, 1978.6, 1989.7, 1312.6.

Synthesis of Compound 10 (Step d, Scheme 3). The residue was redissolved in pyridine (0.35 mL) and triethylamine (0.15 mL), and SAMAOPbf (11.2 mg, 0.0373 mmol) was added to this solution. The reaction mixture was stirred for 3 h, concentrated, and purified by flash chromatography (silica, 5% → 10% MeOH/CH_2Cl_2), and the appropriate fractions were concentrated (R_f 0.3, 10% MeOH/CH_2Cl_2) to afford product **10** contaminated with SAMAOPbf decomposition products (70% purity as judged by LC/MS): R_f = 16.5 (C4 column, 50−95% MeCN in H_2O, 30 min); exact mass calcd for $C_{188}H_{279}N_{21}O_{74}S$ $[M + 2Na]^{2+}$ 2026.4, $[M + 3Na]^{3+}$ 1351.3, found 2026.0, 1350.8. This product was advanced to the next step without further purification.

Synthesis of Compound 11 (Step e, Scheme 3). Phenol (60 mg), triisopropylsilane (0.15 mL), and water (0.2 mL) were added to trifluoroacetic acid (3.0 mL). The resultant solution (1.0 mL) was added to a vial with compound **10** from above (ca. 10 mg, 0.00249 mmol). The reaction mixture was stirred for 40 min before being diluted with dichloromethane (3 mL), concentrated, and purified by reversed-phase HPLC: R_f = 18.7 (C4 column, 35−75% MeCN in H_2O, 30 min) to afford pure product (6.7 mg, 71% yield from **9**). The product was >95% pure as judged by LC/MS: exact mass calcd for $C_{171}H_{247}N_{21}O_{72}S$ $[M + 2H]^{2+}$ 1891.3, $[M + 3H]^{3+}$ 1261.2, found 1891.4, 1261.5.

Synthesis of Compound 11 (Step f, Scheme 3). The product from above was redissolved in degassed 1:1 MeOH/water (10 mL), and degassed 0.03 M NaOH (0.75 mL) was added. The reaction mixture was stirred for 40 h before being neutralized with MAC-3 Dowex resin to pH = 5, filtered, and purified by HPLC: R_f = 14.5 (C18 column, 10−85% MeCN in H_2O, 30 min) to afford **11** (1.0 mg, 19% yield from **11a**, 13% from **9**). The product was found to be >95% pure as judged by LC/MS and ^{1}H NMR: ^{1}H NMR (500 MHz, D_2O)[19] δ 7.46 (d, J = 9.7 Hz, 1H), 7.38 (d, J = 7.4 Hz, 1H), 7.37 (d, J = 7.6 Hz, 2H), 7.32 (t, J = 7.4 Hz, 2H), 7.26 (d, J = 7.0 Hz, 2H), 7.07 (d, J = 8.5 Hz, 2H), 6.87 (d, J = 8.6 Hz, 2H), 6.81 (d, J = 7.4 Hz, 2H), 5.28 (s, 1H), 5.26 (d, J = 3.9 Hz, 1H), 4.70 (dd, J = 5.2, 1.5 Hz, 2H), 4.66 (d, J = 6.9 Hz, 2H), 4.62 (d, J = 8.0 Hz, 1H), 4.60 (d, J = 5.5 Hz, 2H), 4.47 (t, J = 7.5 Hz, 2H), 4.16 (t, J = 6.1 Hz, 2H), 4.10 (at, J = 5.5 Hz, 4H), 4.07 (d, J = 8.5 Hz, 2H), 3,48 (m, 1H), 3.42 (dd, J = 10.0, 2.0 Hz, 1H), 3.28 (t, J = 8.9 Hz, 1H), 3.20 (dt, J = 13.1, 6.4 Hz, 1H), 3.12 (dd, J = 13.5, 6.8 Hz, 1H), 2.58 (dd, J = 12.1, 4.4 Hz, 1H), 2.31 (m, 1H), 2.24 (s, 3H), 2.05 (s, 3H), 2.04 (s, 3H), 2.01 (s, 3H), 1.87 (t, J = 13.2 Hz, 1H), 1.81 (dd, J = 6.5 Hz, 1H), 1.42 (d, J = 8.5 Hz, 3H), 1.26 (d, J = 6.1 Hz, 3H), 1.24 (d, J = 6.6 Hz, 3H), 1.01 (d, J = 6.8 Hz, 3H), 0.99 (d, J = 6.8 Hz, 3H), 0.94 (d, J = 6.3 Hz, 3H), 0.91 (d, J = 6.7 Hz, 3H), 0.89 (d, J = 6.3 Hz, 3H), 0.87 (d, J = 6.7 Hz, 3H); exact mass calcd for sodium salt $C_{134}H_{208}N_{21}NaO_{54}S$ $[M+2H]^{2+}$ 1516.7, $[M+3H]^{3+}$ 1011.5, $[M + CF_3CO_2^-]^{2-}$ 1572.2, found 1517.7, 1012.3, 1572.9.

Preparation of Conjugate 1 (Step g, Scheme 3). A solution of sulfo-SMCC (10 mg/mL, 0.10 mL) in 0.1 M sodium phosphate, 0.9 M NaCl (pH = 7.2), was added to the reconstituted with water solution of KLH (Aldrich, H7017, 10 mg/mL, 1.0 mL). The resultant solution was stirred for 1 h and then purified over a G-25 Sephadex column using 0.1 M sodium phosphate, 0.9 M NaCl, 0.1 M EDTA, pH = 7.2 for elution. The fractions containing KLH were collected and combined, giving a total volume of 3.0 mL. Compound **11** (2 mg, 0.665 μmol) in 0.2 mL of the pH = 7.2 buffer was treated with TCEP gel for 2 h, filtered, combined with the solution of KLH (0.6 mL), and reacted under argon for 2 h. The resultant solution was purified by repetitive centrifugation over a molecular filter (30 kDa cut off) resulting in ca. 1 mL of the final solution of the vaccine construct. The degree of the epitope incorporation was estimated to be 210 epitopes per molecule of KLH using Bradford protein assay with KLH as a standard and Svennerholm sialic acid assay to determine the carbohydrate concentration.[16,17]

Acknowledgment. This work was supported by the National Institutes of Health (Grant No. CA28824 to S.J.D.). P.N. thanks the National Institutes of Health for a Ruth L. Kirschstein postdoctoral fellowship (CA125934-02), W.H.K. is grateful for a Korea Research Foundation Grant funded by the Korean government (KRF-2007-357-c00060), and D.L. is thankful for support provided by the Terri Brodeur Breast Cancer Foundation. We thank Yu Rao, Yu Yuan, and Jennifer Stockdill for fruitful discussions during the preparation of this manuscript.

Supporting Information Available: ^{1}H and ^{13}C NMR spectra for compounds **4** and **5**, LC/MS data for compounds **7**, **8a**, **8**, **9**, **10b**, **10**, **11a**, **11**, and selected intermediates, and ^{1}H NMR spectra for compounds **7**, **8a**, **9**, **10b**, **11a**, and **11**. This material is available free of charge via the Internet at http://pubs.acs.org.