J|A|C|S
ARTICLES

Published on Web 01/20/2010

Design and Synthesis of Potent *Quillaja* Saponin Vaccine Adjuvants

Michelle M. Adams, Payal Damani, Nicholas R. Perl, Annie Won, Feng Hong, Philip O. Livingston,* Govind Ragupathi,* and David Y. Gin*

Molecular Pharmacology and Chemistry Program, and the Melanoma and Sarcoma Service, Department of Medicine, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, New York 10065

Received September 29, 2009; E-mail: gind@mskcc.org; ragupatg@mskcc.org; livingsp@mskcc.org

Abstract: The success of antitumor and antiviral vaccines often requires the use of an adjuvant, a substance that significantly enhances the immune response to a coadministered antigen. Only a handful of adjuvants have both sufficient potency and acceptable toxicity for clinical investigation. One promising adjuvant is QS-21, a saponin natural product that is the immunopotentiator of choice in many cancer and infectious disease vaccine clinical trials. However, the therapeutic promise of QS-21 adjuvant is curtailed by several factors, including its scarcity, difficulty in purification to homogeneity, dose-limiting toxicity, and chemical instability. Here, we report the design, synthesis, and evaluation of chemically stable synthetic saponins. These novel, amide-modified, non-natural substances exhibit immunopotentiating effects in vivo that rival or exceed that of QS-21 in evaluations with the GD3-KLH melanoma conjugate vaccine. The highly convergent synthetic preparation of these novel saponins establishes new avenues for discovering improved molecular adjuvants for specifically tailored vaccine therapies.

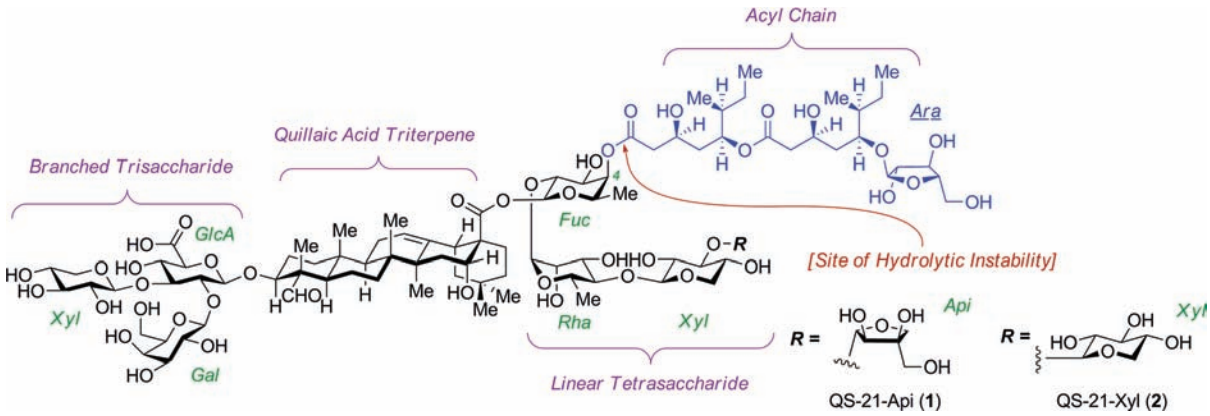
Introduction

The development of vaccines to combat cancer and vaccine-resistant infectious diseases has relied significantly on subunit antigen constructs. While defined molecular antigens offer advantages in terms of safety and precision in immune response targeting, they are typically less immunogenic. Often, these vaccine formulations require an adjuvant, a substance that potentiates immune response.[1,2] The critical roles of vaccine adjuvants lie in their ability to: (1) enable the use of otherwise impotent antigens; (2) extend the benefits of vaccination to poor responders (e.g., older or immune-compromised patients); and (3) effect dose-sparing of rare and expensive antigens in short supply (e.g., during an epidemic). Given that the majority of FDA-approved subunit vaccines rely on an adjuvant component, the challenge to discover novel immunopotentiators remains at the fore. QS-21 (Chart 1), a purified saponin fraction from the bark extracts of *Quillaja saponaria* (QS),[3,4] is a promising adjuvant in numerous prophylactic and therapeutic vaccines.[5] This saponin fraction comprises two principal isomers that share a triterpene, a branched trisaccharide, and a glycosylated pseudodimeric acyl chain.[6] The two isomeric forms differ in the constitution of the terminal sugar within the linear tetrasaccharide segment, wherein the major isomer, QS-21-Api (**1**, ~65% abundance), incorporates a β-D-apiose residue, and the minor isomer, QS-21-Xyl (**2**, ~35% abundance), terminates in a β-D-xylose substituent.[7]

Numerous clinical trials have been conducted with QS-21 adjuvant in vaccines against infectious diseases (malaria,[8,9] HIV,[10,11] hepatitis,[12] tuberculosis[13]) and cancer (melanoma,[14,15]

(1) Jiang, Z. H.; Koganty, R. R. *Curr. Med. Chem.* **2003**, *10*, 1423–1439.
(2) Kwissa, M.; Kasturi, S. P.; Pulendran, B. *Exp. Rev. Vaccines* **2007**, *6*, 673–684.
(3) Dalsgaard, K. *Dansk Tids. Farm.* **1970**, *44*, 327–331.
(4) Kensil, C. R.; Patel, U.; Lennick, M.; Marciani, D. *J. Immunol.* **1991**, *146*, 431–437.
(5) Kensil, C. R. *Crit. Rev. Ther. Drug Carrier Syst.* **1996**, *13*, 1–55.
(6) Jacobsen, N. E.; Fairbrother, W. J.; Kensil, C. R.; Lim, A.; Wheeler, D. A.; Powell, M. F. *Carbohydr. Res.* **1996**, *280*, 1–14.
(7) Cleland, J. L.; Kensil, C. R.; Lim, A.; Jacobsen, N. E.; Basa, L.; Spellman, M.; Wheeler, D. A.; Wu, J. Y.; Powell, M. F. *J. Pharm. Sci.* **1996**, *85*, 22–28.
(8) Kester, K. E.; McKinney, D. A.; Tornieporth, N.; Ockenhouse, C. F.; Heppner, D. G.; Hall, T.; Wellde, B. T.; White, K.; Sun, P.; Schwenk, R.; Krzych, U.; Delchambre, M.; Voss, G.; Dubois, M. C.; Gasser, R. A.; Dowler, M. G.; O'Brien, M.; Wittes, J.; Wirtz, R.; Cohen, J.; Ballou, W. R.; Rts, S. M. V. E. G. *Vaccine* **2007**, *25*, 5359–5366.
(9) Abdulla, S.; Oberholzer, R.; Juma, O.; Kubhoja, S.; Machera, F.; Membi, C.; Omari, S.; Urassa, A.; Mshinda, H.; Jumanne, A.; Salim, N.; Shomari, M.; Aebi, T.; Schellenberg, D. M.; Carter, T.; Villafana, T.; Demoitie, M. A.; Dubois, M. C.; Leach, A.; Lievens, M.; Vekemans, J.; Cohen, J.; Ballou, W. R.; Tanner, M. *New Engl. J. Med.* **2008**, *359*, 2533–2544.
(10) Evans, T. G.; McElrath, M. J.; Matthews, T.; Montefiori, D.; Weinhold, K.; Wolff, M.; Keefer, M. C.; Kallas, E. G.; Corey, L.; Gorse, G. J.; Belshe, R.; Graham, B. S.; Spearman, P. W.; Schwartz, D.; Mulligan, M. J.; Goepfert, P.; Fast, P.; Berman, P.; Powell, M.; Francis, D.; Grp, N. A. V. E. *Vaccine* **2001**, *19*, 2080–2091.
(11) Kennedy, J. S.; Co, M.; Green, S.; Longtine, K.; Longtine, J.; O'Neill, M. A.; Adams, J. P.; Rothman, A. L.; Yu, Q.; Johnson-Leva, R.; Pal, R.; Wang, S. X.; Lu, S.; Markham, P. *Vaccine* **2008**, *26*, 4420–4424.
(12) Vandepapeliere, P.; Horsmans, Y.; Moris, P.; Van Mechelen, M.; Janssens, M.; Koutsoukos, M.; Van Belle, P.; Clement, F.; Hanon, E.; Wettendorff, M.; Garcona, N.; Leroux-Roels, G. *Vaccine* **2008**, *26*, 1375–1386.
(13) Garcon, N.; Chomez, P.; Van Mechelen, M. *Exp. Rev. Vaccines* **2007**, *6*, 723–739.
(14) Ragupathi, G.; Meyers, M.; Adluri, S.; Howard, L.; Musselli, C.; Livingston, P. O. *Int. J. Cancer* **2000**, *85*, 659–666.
(15) Ragupathi, G.; Livingston, P. O.; Hood, C.; Gathuru, J.; Krown, S. E.; Chapman, P. B.; Wolchok, J. D.; Williams, L. J.; Oldriel, R. C.; Hwu, W. J. *Clin. Cancer Res.* **2003**, *9*, 5214–5220.

Chart 1



breast,[16,17] small cell lung,[18] prostate[19]). Despite the promise of QS-21, however, there are several liabilities associated with its use as an adjuvant. First, high variability in molecular composition of saponins is seen even among QS trees of similar age and local environment.[20] Purification of the multicomponent QS-21 fraction entails elaborate low-yielding extraction and HPLC protocols.[21] Not surprisingly, this saponin adjuvant is currently not available commercially. Second, potency of QS-21 is proportional to dose, but the tolerated dose of QS-21 in cancer patients does not exceed 150 μg, above which significant local erythema and systemic flu-like symptoms arise. Finally, QS-21 was reported to degrade in a matter of days on storage in solutions of physiological pH at ambient temperature,[7] wherein the labile ester group within the acyl chain (Chart 1) of the molecule undergoes spontaneous hydrolysis to produce byproducts with severely attenuated adjuvant activity.[22,23] Importantly, the latter issue of chemical instability is a significant factor in precluding the advancement of QS-21 alone as an adjuvant for vaccines in developing countries, the epidemic strongholds of malaria, HIV, and tuberculosis. Efforts to address these impediments have largely focused on the incorporation of additives (copolymers, lipids, etc.)[13,24] to impart varying degrees of stability to QS-21. However, such complex formulations confer additional dimensions of heterogeneity to the vaccine and introduce challenges associated with maintaining formulation consistency for advancement to clinical evaluation.

An alternate approach to overcoming all of the problems associated with QS-21 is to improve this adjuvant through controlled structural modifications at the molecular level. The extent of these efforts has been quite limited, however, as the chemical sensitivity of the natural product presents an exceedingly narrow window for its functional group derivatization. This barrier can be overcome through chemical synthesis of the QS-adjuvants. The successful validation of this approach is exemplified herein by the design, synthesis, and evaluation of novel synthetic saponins based on the QS-21 parent architecture. These new hydrolytically stable molecular adjuvants exhibit in vivo immunostimulating potencies rivaling or exceeding that of naturally derived QS-21. Moreover, when compared to the natural product, these molecular entities can be obtained in homogeneous form with higher efficiency, and signal the potential for modulation of toxicity by further structural modification.

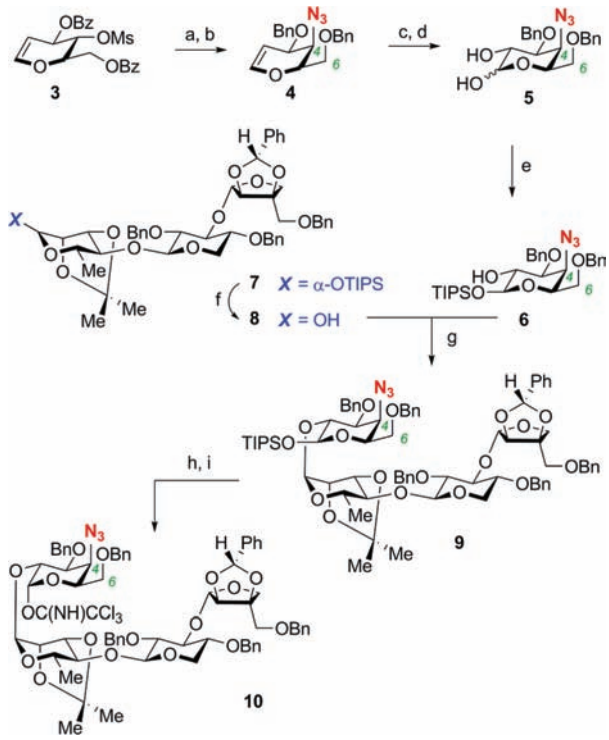
Results

Chemical Synthesis. A key observation from early stability studies on QS-21 revealed that the removal of the acyl chain of the natural product furnished a deacylated saponin byproduct that was acutely compromised in its ability to stimulate antibody and CTL responses against OVA antigen in mice.[22] The establishment of the unstable acyl chain as a critical component for the bioactivity of QS-21 prompts the investigations herein, which focus on the structural modification of this quadrant to arrive at novel saponin adjuvants. In this context, simple replacement of the unstable ester linkages within the acyl chain with more robust amide linkages would enhance the stability of the adjuvant with the intention of increasing potency and/or duration of therapeutic activity per dose.

Access to these amide-stabilized structural variants would require a novel monosaccharide moiety to serve as a structural mimic to the acylated fucose sugar (Chart 1, *Fuc*-residue within **1** and **2**). Accordingly, the principal design criterion in this carbohydrate component is the replacement of the C4-oxygen functionality with an appropriate nitrogen group, as exemplified by C4-deoxy-C4-azidogalactopyranoside **6** (Scheme 1). The synthetic sequence to prepare the fucose surrogate **6** (Scheme 1) was initiated with 3,6-di-*O*-benzoyl-4-*O*-methanesulfonyl-D-glucal (**3**), derived in one step from D-glucal according to the procedure of Piancatelli.[25] This C4-mesylate **3** was a competent electrophile for S_N2 displacement with sodium azide

(16) Gilewski, T.; Ragupathi, G.; Bhuta, S.; Williams, L. J.; Musselli, C.; Zhang, X. F.; Bencsath, K. P.; Panageas, K. S.; Chin, J.; Hudis, C. A.; Norton, L.; Houghton, A. N.; Livingston, P. O.; Danishefsky, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 3270–3275.
(17) Musselli, C.; Ragupathi, G.; Gilewski, T.; Panageas, K. S.; Spinat, Y.; Livingston, P. O. *Int. J. Cancer* **2002**, *97*, 660–667.
(18) Krug, L. M.; Ragupathi, G.; Ng, K. K.; Hood, C.; Jennings, H. J.; Guo, Z. W.; Kris, M. G.; Miller, V.; Pizzo, B.; Tyson, L.; Baez, V.; Livingston, P. O. *Clin. Cancer Res.* **2004**, *10*, 916–923.
(19) Ragupathi, G.; Slovin, S. F.; Adluri, S.; Sames, D.; Kim, I. J.; Kim, H. M.; Spassova, M.; Bornmann, W. G.; Lloyd, K. O.; Scher, H. I.; Livingston, P. O.; Danishefsky, S. J. *Angew. Chem., Int. Ed.* **1999**, *38*, 563–566.
(20) Kamstrup, S.; San Martin, R.; Doberti, A.; Grande, H.; Dalsgaard, K. *Vaccine* **2000**, *18*, 2244–2249.
(21) Kensil, C. A. Saponin adjuvant compositions. U.S. Pat. 6,231,859, 2001.
(22) Kensil, C. R.; Soltysik, S.; Wheeler, D. A.; Wu, J. Y. Structure/function studies on QS-21, a unique immunological adjuvant from Quillaja saponaria In *Saponins Used in Traditional and Modern Medicine*; Waller, G. R., Yamasaki, K., Eds.; Plenum Press: New York, 1996; pp 165−172.
(23) Liu, G.; Anderson, C.; Scaltreto, H.; Barbon, J.; Kensil, C. R. *Vaccine* **2002**, *20*, 2808–2815.
(24) Drane, D.; Gittleson, C.; Boyle, J.; Moraskovsky, E. *Exp. Rev. Vaccines* **2007**, *6*, 761–772.
(25) Squarcia, A.; Vivolo, F.; Weinig, H. G.; Passacantilli, P.; Piancatelli, G. *Tetrahedron Lett.* **2002**, *43*, 4653–4655.

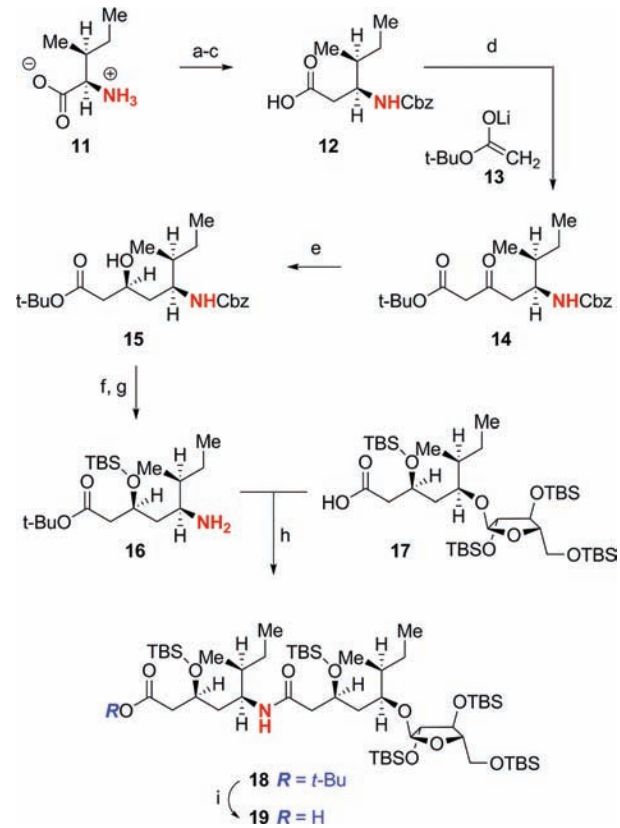
Scheme 1[a]



[a] Reagents and conditions: (a) NaN_3, Bu_4NCl, PhMe, 110 °C, 66%; (b) NaOH, MeOH, 23 °C; NaH, BnBr, DMF, 23 °C, 78%; (c) $PhI(OAc)_2$, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −50→−25 °C, 85%; (d) K_2CO_3, MeOH, H_2O, 23 °C, 84%; (e) TIPSCl, imidazole, DMAP, DMF, 23 °C, 59%; (f) TBAF, THF, 23 °C, 98%; (g) **8**, Tf_2O, Ph_2SO, TBP, CH_2Cl_2, −55 °C; add **6**, −78→23 °C, 67%; (h) TBAF, THF, 0 °C, 93%; (i) CCl_3CN, DBU, CH_2Cl_2, 0→23 °C, 95%.

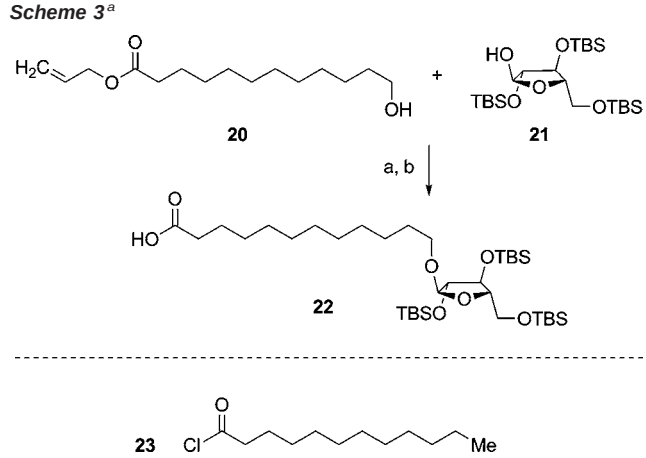
to provide the corresponding azide, whose ester groups were subsequently exchanged for benzyl ethers by Zemplén saponification (NaOMe) and alkylation with benzyl bromide to furnish azido-galactal **4** (51% from **3**). Stereoselective 1,2-bis(acyloxylation)[26] of glycal **4** ($PhI(OAc)_2$, $BF_3{\cdot}OEt_2$) was accomplished in 85% yield, allowing for subsequent acetate methanolysis (**5**, 84%). Selective protection of the hemiacetal with TIPSCl provided the C4-azidogalactoside **6** (59%), which serves as the fucose surrogate incorporating the C4-nitrogen functionality. It is worth noting that the fucose mimic **6** bears an "extra" C6-benzyloxy group as compared to the natural fucose moiety, which incorporates only a C6-methyl group. This artifact of the synthesis not only allows for a relatively short synthetic sequence to **6**, but also imparts an additional functional handle with which to explore future novel compositions of matter. Glycosylation of the azido-sugar **6** with the trisaccharide hemiacetal **8**, derived from anomeric desilyation of the previously prepared trisaccharide **7**,[27] proceeded under the dehydrative coupling protocol[28] (Ph_2SO, Tf_2O) to afford the selectively protected tetrasaccharide **9** (70%). Subsequent removal of the tri-*iso*-propylsilyl acetal within **9** was accomplished with TBAF to provide the corresponding hemiacetal (93%), which was then converted to the anomeric α-trichloroacetimidate (**10**, 95%). This constitutes a successful route to a fully elaborated tetrasaccharide fragment that incorporates the required galacto-C4-azido group on which to anchor a variety of amide acyl chains.

Scheme 2[a]



[a] Reagents and conditions: (a) NaOH, BnOCOCl, H_2O, 0→23 °C, >99%; (b) EtOCOCl, Et_3N, THF; CH_2N_2, 0→23 °C, 78%; (c) CF_3CO_2Ag, Et_3N, THF, −50→23 °C, 79%; (d) CDI, THF, 23 °C; **13**, THF, −78 °C, 60%; (e) H_2, $RuCl_2{\cdot}$(*S*)-BINAP, MeOH, 23 °C, 89%; (f) TBSOTf, 2,6-lutidine, CH_2Cl_2, −78 °C, 94%; (g) Pd/C, H_2, MeOH, 23 °C, 97%; (h) **17**, EtOCOCl, Et_3N, THF, 0 °C; add **16**, 23 °C, 80%; (i) TMSOTf, 2,6-lutidine, CH_2Cl_2, 0→23 °C, 76%.

Scheme 3[a]



[a] Reagents and conditions: (a) **21**, Tf_2O, Ph_2SO, TBP, CH_2Cl_2, −45 °C; add **20**, −78→0 °C, 91% (1.1:1, α:*β*, SiO_2 separation); (b) $BaOH{\cdot}8H_2O$, MeOH, 23 °C, 81%.

Initial efforts toward the preparation of acyl chain variants of QS-21 focused on **19** (Scheme 2), **22**, and **23** (Scheme 3) as promising acylation agents to access novel hydrolytically stable saponin adjuvants. The most complex amide acyl chain of these

(26) Shi, L.; Kim, Y. J.; Gin, D. Y. *J. Am. Chem. Soc.* **2001**, *123*, 6939–6940.
(27) Deng, K.; Adams, M. M.; Gin, D. Y. *J. Am. Chem. Soc.* **2008**, *130*, 5860–5861.
(28) Garcia, B. A.; Poole, J. L.; Gin, D. Y. *J. Am. Chem. Soc.* **1997**, *119*, 7597–7598.

is the glycosylated pseudodimeric amide **19** (Scheme 2), incorporating the most conservative structural variations of the acyl chain. This substrate serves as an isosteric mimic of the natural acyl substituent, differing only in the central ester-to-amide replacement, which should serve to further enhance stability of this stereochemically elaborate substructure. In addition, two simplified lipophilic amide acyl chains (Scheme 3), in the form of either a glycosylated (e.g., **22**) or a nonglycosylated (e.g., **23**) linear aliphatic moiety, were also pursued. Synthetic QS saponins bearing the latter two hydrophobic chains would not only be prepared by considerably shorter synthetic sequences as compared to that of **19**, but also provide a clear indication of whether the elaborate stereochemical array within the native acyl chain is required for adjuvant activity.

The synthesis of the isosteric amide acyl chain **19** (Scheme 2) began with D-alloisoleucine (**11**), obtained from L-isoleucine by the three-step epimerization-resolution protocol of Sakai.[29] Following amino protection of **11** as its benzyl carbamate (BnOCOCl, >99%), the carboxylic acid was subjected to Arndt−Eistert homologation via its derivatization to the corresponding α-diazoketone and Wolff rearrangement to provide the β-amino acid **12** (62%). Subsequent activation of the carboxylic acid in **12** with carbonyldiimidazole allowed for Claisen condensation with the Li-enolate of *t*-butyl acetate (**13**) to provide β-keto ester **14** (60%), a suitable substrate for Noyori catalytic asymmetric hydrogenation.[30] This proceeded with $RuCl_2$•(*S*)-BINAP and H_2, providing the β-hydroxy ester **15** (89%) with complete catalyst-controlled diastereoselectivity. Hydroxyl group silylation (TBSOTf, 94%) was followed by hydrogenolytic unmasking of carbamate **15** to afford amine **16** in 97% yield. Importantly, the deliberate selection of the *t*-butyl ester protective group in this sequence precluded unproductive lactamization of amine **16**, allowing for its acylation (80%) with the glycosylated acyl chain fragment **17**, previously prepared in the synthesis of QS-21.[31−33] This transformation provided, after acid-catalyzed *t*-butyl ester removal (76%), the selectively protected fully intact acyl chain **19**, the direct O→N amide mimic of the QS-21 acyl chain.

Fortunately, synthetic access to the two remaining acyl chain fragments **22** and **23** (Scheme 3) was significantly less effort-intensive given the absence of stereochemical complexity within these substructures. For example, the glycosylated acyl chain **22** could be readily obtained in a three-step sequence[34] involving dehydrative glycosylation of allyl 12-hydroxydodecanoate (**20**) with 2,3,5-tri-*O*-TBS-arabinofuranose (**21**)[31,32] to provide the intermediate glycoside (91%) as a separable mixture of anomers (1.1:1, α:β). The α-anomer was then advanced via $Ba(OH)_2$-mediated saponification of the allyl ester to provide the acyl chain **22** (81%). Finally, the nonglycosylated aliphatic acyl chain variant was the easiest to procure given that lauryl chloride (**23**) is commercially available.

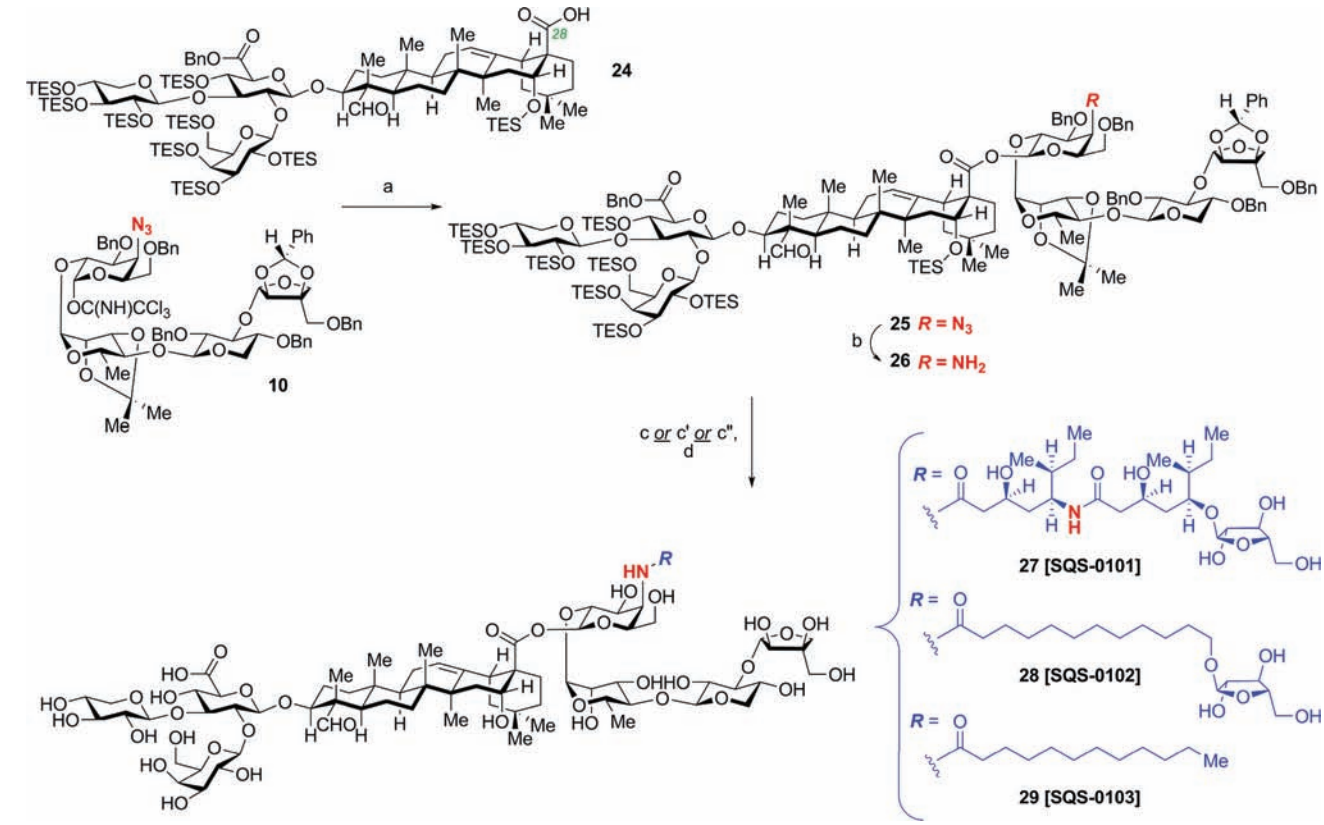
The late-stage convergent assembly of the amide acyl chain variants of QS-21 (Scheme 4) capitalized on the recent disclosure of our semisynthetic approach to access homogeneous samples of QS-saponin adjuvants.[27] Although the acyl chain and linear tetrasaccharide quadrants of the QS saponins are synthesized de novo, the remaining half of the natural product, comprising the trisaccharide−triterpene conjugate, could be isolated by controlled chemical degradation of QS-extracts and selective protection to yield the protected prosapogenin **24** (Scheme 4) in only three steps.[27] Thus, Schmidt glycosylation[35] of the C28 carboxylic acid in **24** with the tetrasaccharide glycosyl trichloroacetimidate donor **10** provided the glycosyl ester **25** (82%) with complete anomeric selectivity. The lone azide group in **25** was responsive to reduction with benzeneselenol to reveal the corresponding amine **26** (91%), onto which various acyl chains could be appended. In the cases of *N*-acylation with the glycosylated acyl chain carboxylic acid derivatives **19** and **22**, couplings were accomplished by initial carboxylate activation with ethyl chloroformate. For introduction of the aliphatic lauryl chain, direct acylation with lauryl chloride (**23**) could be accomplished. Subsequent sequential hydrogenolysis and acid hydrolysis effected global deprotection of the resulting advanced intermediates to provide the amide acyl chain SQS-saponin variants **27** (SQS-0101), **28** (SQS-0102), and **29** (SQS-0103) in 79%, 69%, and 68% yields, respectively, from the amine precursor **26**.

Evaluation of Immune Response Augmentation. Currently, there exists no rapid in vitro biological screen for assessing the potential efficacy of saponin vaccine adjuvants, given that the mechanism by which saponins augment immune response is unknown. As a result, evaluation of these novel saponins as immunostimulants proceeded directly to preclinical studies involving mouse vaccination with the melanoma antigen GD3 ganglioside conjugated to the KLH carrier protein (GD3-KLH, Chart 2). This is a clinically relevant vaccine model that has proven useful for comparing the immunopotentiating ability of various adjuvants.[36,37] Monitoring antibody responses to both the carbohydrate antigen and the protein carrier provides a useful assessment of adjuvant performance to antigens of different immunogenicity, ranging from a poorly immunogenic glycolipid (GD3) to a highly immunogenic protein (KLH). Moreover, previous experience with this vaccination protocol has consistently correlated antibody titers against KLH to that of T-cell response to this antigen.[37]

Groups of five mice (C57BL/6J, female, 6−8 weeks of age) were vaccinated with GD3-KLH at a 10 μg dose (Figure 1). The antigen was coadministered with the adjuvant of interest in a vaccination protocol involving three subcutaneous injections at 1-week intervals (days 0, 7, and 14) plus a booster at day 65. As the negative control, mice were vaccinated with the GD3-KLH antigen only. As a positive control, vaccinations were performed with naturally derived QS-21 (NQS-21), obtained by fractionating a mixture of saponins from *Quillaja saponaria*.[38] The adjuvant dose employed in these comparative studies was 10 μg, a quantity of QS-21 known to induce measurable antibody responses with acceptable toxicity effects

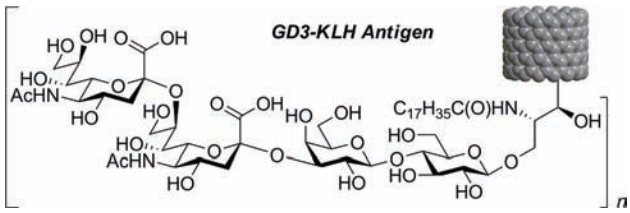
(29) Noda, H.; Sakai, K.; Murakami, H. *Tetrahedron: Asymmetry* **2002**, *13*, 2649–2652.
(30) Noyori, R.; Ohkuma, T.; Kitamura, M.; Takaya, H.; Sayo, N.; Kumobayashi, H.; Akutagawa, S. *J. Am. Chem. Soc.* **1987**, *109*, 5856–5858.
(31) Wang, P.; Kim, Y. J.; Navarro-Villalobos, M.; Rohde, B. D.; Gin, D. Y. *J. Am. Chem. Soc.* **2005**, *127*, 3256–3257.
(32) Kim, Y. J.; Wang, P.; Navarro-Villalobos, M.; Rohde, B. D.; Derryberry, J.; Gin, D. Y. *J. Am. Chem. Soc.* **2006**, *128*, 11906–11915.
(33) Deng, K.; Adams, M. M.; Damani, P.; Livingston, P. O.; Ragupathi, G.; Gin, D. Y. *Angew. Chem., Int. Ed.* **2008**, *47*, 6395–6398.
(34) Lee, R. E.; Mikusova, K.; Brennan, P. J.; Besra, G. S. *J. Am. Chem. Soc.* **1995**, *117*, 11829–11832.
(35) Schmidt, R. R.; Kinzy, W. *Adv. Carbohydr. Chem. Biochem.* **1994**, *50*, 21–123.
(36) Kim, S. K.; Ragupathi, G.; Musselli, C.; Choi, S. J.; Park, Y. S.; Livingston, P. O. *Vaccine* **2000**, *18*, 597–603.
(37) Kim, S. K.; Ragupathi, G.; Cappello, S.; Kagan, E.; Livingston, P. O. *Vaccine* **2001**, *19*, 530–537.
(38) Kensil, C. R. *Methods Mol. Med.* **2000**, *42*, 259–271 (Vaccine Adjuvants).

Scheme 4[a]



[a] Reagents and conditions: (a) $BF_3 \cdot OEt_2$, 4 Å ms, CH_2Cl_2, −78→23 °C, 82%; (b) PhSeH, Et_3N, 30 °C, 91%; (c) **19**, EtOCOCl, Et_3N, THF, 0 °C; add **26**, 10 °C, 93%; (c′) **22**, EtOCOCl, Et_3N, THF, 0 °C; add **26**, 12 °C, 89%; (c″) **23**, TBP, CH_2Cl_2, 23 °C, 98%; (d) Pd/C, H_2, THF, EtOH, 23 °C; TFA, H_2O, 23 °C, RP-HPLC, 85% (for **27**), 78% (for **28**), 69% (for **29**).

Chart 2



in mice. Antibody titers against GD3 (IgM and IgG) and KLH (IgG) were determined by ELISA. Remarkably, all of the synthetic SQS-adjuvants (**27**−**29**) are at least as active as NQS-21 in immunopotentiating ability, as illustrated by the IgM and IgG response to GD3 (Figure 1A and B) measured 1 week following the booster (day 72). In each case, antibody titers were significantly higher than the group with GD3-KLH alone. In addition, the IgG antibody response against KLH was also strikingly elevated (Figure 1C) with all of the novel SQS-saponin adjuvants. Antibody subtyping of the anti-GD3 IgG isotype (Figure 1D) revealed a significant bias toward the mouse IgG2b subtype with all of our synthetic saponin adjuvants (SQS-0101, -0102, and -0103), a result similar to that of naturally derived NQS-21. Production of other mouse anti-GD3 IgG subtypes, including IgG1, IgG2a, and IgG3, was low or negligible as indicated by class-specific ELISA employing the standard 0.1 absorbance level threshold for positive reactivity.

The activity of these anti-GD3 antibodies was further investigated for their ability to effect tumor cell binding and lysis. These properties were first assessed by flow cytometry, monitoring the cell surface reactivity of a tumor cell-line expressing GD3 antigen (Figure 1E). Sera drawn 7 days after the booster were evaluated by FACS using the SK-Mel-28 (GD3 positive) cell line. The individual FACS results are presented at 10 μg SQS-adjuvant dose for direct comparison. Prevaccination sera from mice showed less than 10% positive cells, while sera obtained after vaccination with all three synthetic adjuvants showed significant positive reactivity with SK-Mel-28. The median percent positive cell reactivities were 15% for the no-adjuvant control, 87% for NQS-21 (**1** and **2**), 78% for SQS-0101 (**27**), 83% for SQS-0102 (**28**), and 64% for SQS-0103 (**29**). These cell-surface reactivity results further reinforce the comparable adjuvant activity of synthetic SQS-0101, -0102, and -0103 relative to that of NQS-21. In addition, cell surface reactivity against SK-Mel-28 was further characterized by complement-dependent cytotoxicity (CDC) assays (Figure 1F) using rabbit complement and sera from the mice vaccinated with GD3-KLH (10 μg) plus SQS-101 (**27**), SQS-102 (**28**), or SQS-103 (**29**), each at 20 μg doses. Less than 6% median reactivity was detected in prevaccination sera and in mice vaccinated with GD3-KLH alone, both serving as negative controls. However, sera obtained from mice immunized with GD3-KLH plus SQS-101, SQS-102, and SQS-103 all showed significantly enhanced cytotoxicity against SK-Mel-28 over the negative controls.

As a standard initial overall assessment of toxicity, the weight loss of the mice was monitored at 0, 1, 2, 3, and 7 days after the first vaccination (Figure 2). For the negative control involving vaccinations with GD3-KLH only (no adjuvant), no appreciable weight loss was observed. For the positive control, the presence of NQS-21 (10 μg) elicited notable and expected median weight loss of 9% after the initial injection. The group

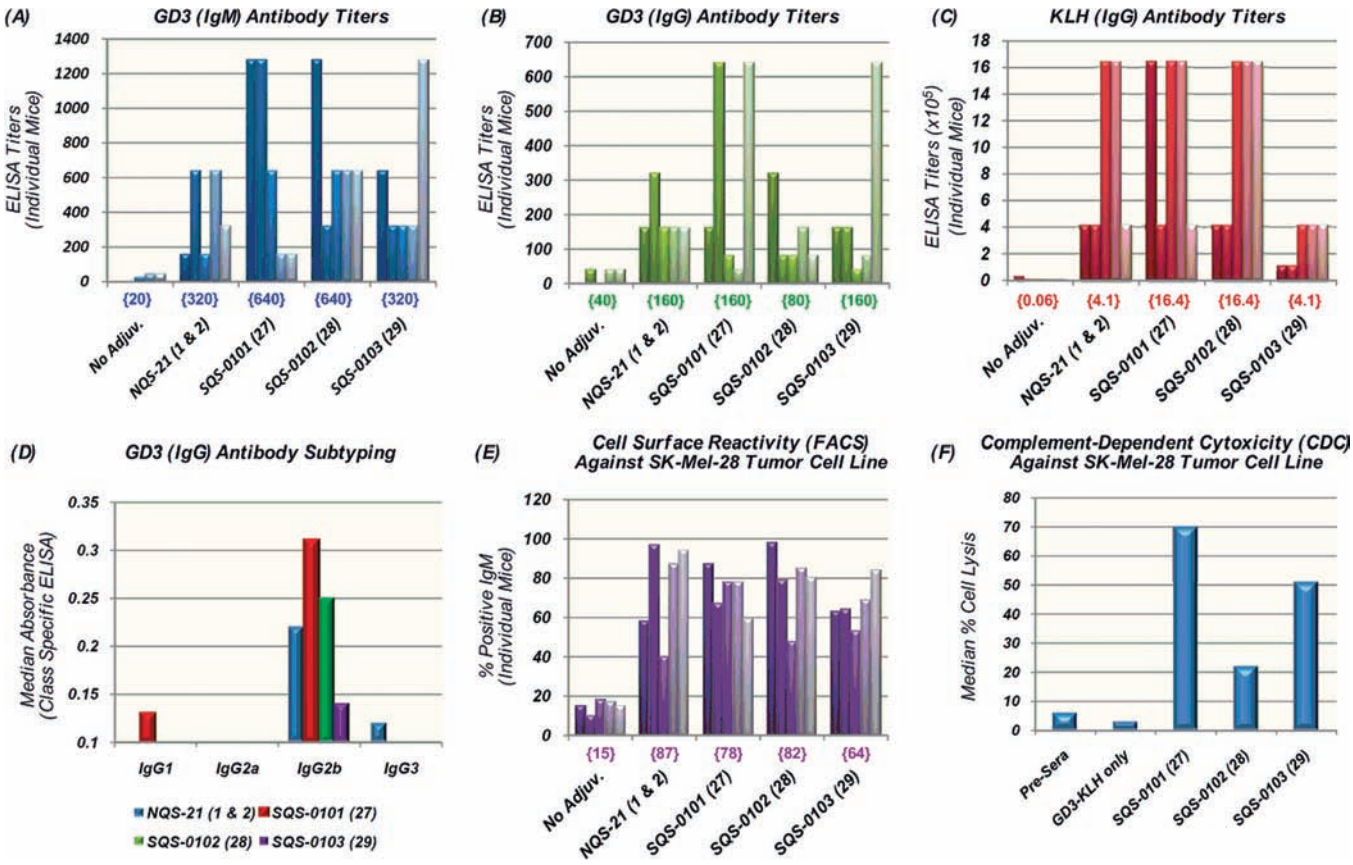


Figure 1. Preclinical immunological evaluation of SQS-adjuvants with GD3-KLH conjugate vaccine. Each adjuvant, plus a no-adjuvant negative control, was evaluated by vaccination of a group of five mice (C57BL/6J, female). Vaccinations took the form of weekly subcutaneous injection of antigen (10 μg) and various saponin adjuvants (10 μg for ELISA and FACS results; 20 μg for CDC results) for 3 weeks (days 0, 7, and 14), followed by a booster at day 65. Postboost serological data at day 72 are presented. (A) Anti-GD3 titers (IgM) after vaccination; median values in curly brackets. (B) Anti-GD3 titers (IgG) after vaccination; median values in curly brackets. (C) Anti-KLH titers (IgG) after vaccination; median values in curly brackets. (D) Anti-GD3 IgG subtyping as assessed by class-specific indirect ELISA; median absorbance levels >0.1 are considered positive. (E) Cell surface reactivity of anti-GD3 against SK-Mel-28 tumor cell line expressing GD3 antigen following vaccination; median values in curly brackets. (F) Complement-dependent cytotoxicity (CDC) activity of anti-GD3 against SK-Mel-28 tumor cell line expressing GD3 antigen.

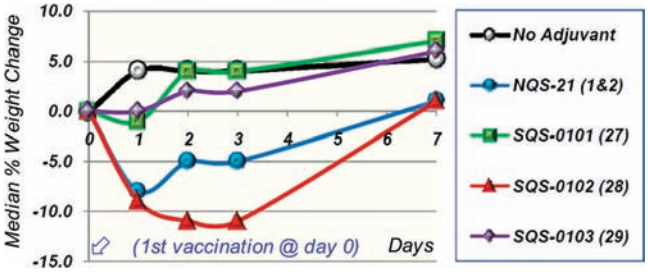


Figure 2. Toxicity assessment via median weight loss. Initial toxicity assessment was performed by tracking median weight loss of each group of mice following the first vaccination with GD3-KLH (10 μg) antigen with various synthetic saponin adjuvants (10 μg).

vaccinated with SQS-0102 had an 11% median weight loss, while the maximum weight loss of the remaining groups incorporating SQS-0101 and SQS-0103 was less than 5%. Overall, weight loss with 10 μg SQS-0102 was slightly, but not significantly, more than with NQS-21 ($p > 0.15$), while weight loss with SQS-0101 ($p = 0.05$) and SQS-0103 ($p < 0.025$) was significantly less than that with NQS-21.

Discussion

QS-21 remains the immunopotentiator of choice in many cancer and infectious disease vaccine trials despite its liabilities, which include scarcity, difficulty in purification to homogeneity, dose-limiting toxicity, and chemical instability. The challenges associated with QS-21 have prompted the generation of alternate structures inspired by QS saponins for the discovery of adjuvants with greater stability and more favorable therapeutic profiles. With the current semisynthetic approach to complex QS saponins,[27] the preparation of novel adjuvants with unprecedented control over molecular structure is now possible. Using this strategy, the first generation of SQS-amide analogues (**27**−**29**) has been successfully prepared. Each of these structures exhibits at least comparable adjuvant activity to that of natural NQS-21, as evidenced by initial ELISA, FACS, and CDC data (Figure 1). For the strongly immunogenic KLH protein carrier, expected high antibody titers were evident. Like that of NQS-21, the anti-KLH titers for all of the SQS-analogues were at least 20-fold higher than the negative control group comprising GD3-KLH antigen in the absence of a saponin adjuvant. For the weakly immunogenic GD3 antigen, initial IgM production followed by class switching to IgG was observed with all three synthetic saponins at levels comparable to that of NQS-21. Upon subtyping these antibodies, the IgG2b subclass was shown to predominate. The mouse IgG2b and IgG2a subclasses are known to induce potent immunotherapeutic effector functions,[39] including complement-dependent cytotoxicity (CDC) and antibody-

(39) Nimmerjahn, F.; Ravetch, J. V. *Science* **2005**, *310*, 1510–1512.

dependent cellular cytotoxicity (ADCC). Importantly, the cytotoxic activity of these anti-GD3 antibodies was unambiguously verified to effect CDC on the SK-Mel-28 (GD3 positive) tumor cell line.

On the basis of these initial immunological data, our first few hydrolytically stable structural variants (**27**−**29**) clearly show that the elaborate stereochemical array within the native QS-21 acyl chain is not essential to maintain adjuvant activity comparable to the natural saponin. This finding, in combination with the integration of semisynthetic prosapogenin **24**[27] into the synthesis, shortens the synthetic route to potent saponin adjuvants like **28** and **29** by more than 30 steps when compared to the original chemical synthesis of QS-21.[31–33] Finally, differences in toxicity profiles between the amide variants and NQS-21, based on tracking the weight loss in mice during vaccination, are also notable. Whereas toxicity of SQS-0102 (**28**) is at least as pronounced as that of NQS-21 (**1** and **2**), SQS-0101 (**27**) and SQS-0103 (**29**) exhibit lower overall toxicity effects. Indeed, the specific reasons behind this structure-dependent toxicity remain to be defined. Future investigations on this front would entail adjuvant dose-escalation studies, followed by detailed biochemical toxicity evaluations of those mice exhibiting >10% weight loss upon vaccination. Nevertheless, the weight loss differential observed even at the 10 μg adjuvant dose (Figure 2) is a strong indication that toxicity of the saponin could be independently modulated through specific structural perturbations while maintaining potent adjuvant activity. These initial results speak to the likelihood of the future discovery of even simpler molecular variants that possess potent immunostimulatory activities and attenuated toxicity.

It is worth noting that the mechanism by which QS-21 (or our SQS-analogues) potentiates immune response is unknown. Hypotheses have been put forth that the saponin, through lectin-mediated cell membrane interactions, may facilitate uptake of the antigen into antigen-presenting cells (APCs), leading to specific cytokine profiles that enhance T- and/or B-cell responses.[5,40] The lone aldehyde group within the QS triterpene has also been suggested to react with putative T-cell surface receptors via Schiff base formation, providing T-cells with a costimulatory signal for T-cell activation.[40] Within the limited available data concerning the mechanism of action of QS-21, there is strong evidence to suggest that a depot effect is not operative[5] and that the saponin is not a ligand for Toll-like receptors 2 and 4 (TLR-2, TLR-4),[41] proteins that activate innate immune responses by recognizing pathogen-associated molecular patterns. While these findings are highly significant, there remain an infinite number of mechanistic possibilities to consider. Despite the striking efficacy of QS-21 and related saponins as immunological adjuvants, there is little guidance (beyond broad immunological intuition) with which to marshal exploratory efforts on their mechanisms of action. This paucity of data has plagued the saponin adjuvant field, whereby seminal mechanistic information in this arena is essentially nonexistent, despite the decades-long use of saponin adjuvants in clinical trials. Thus, this demonstration of high adjuvant potency with simpler, hydrolytically stable SQS-adjuvants (**27**−**29**) not only signals the potential for discovery of novel immunotherapeutics, but also should enable the controlled installation of chemical, optical, and radio-isotopic reporters as valuable molecular probes on the adjuvant molecule to address long-standing mechanistic questions concerning saponin immunopotentiation.

Acknowledgment. This work was supported by grants from the NIH (R01 GM058833, PO1 CA052477, P50 AT002779), the DOD Prostate Cancer Research Program #W81XWH-05-1-0085, and Mr. William H. and Mrs. Alice Goodwin and the Commonwealth Foundation for Cancer Research and The Experimental Therapeutics Center of Memorial Sloan-Kettering Cancer Center.

Supporting Information Available: Experimental details for synthetic procedures and analytical data for isolable synthetic intermediates. This material is available free of charge via the Internet at http://pubs.acs.org.

JA9082842

(40) Marciani, D. J. *Drug Discovery Today* **2003**, *8*, 934–943.

(41) Pink, J. R.; Kieny, M.-P. *Vaccine* **2004**, *22*, 2097–102.