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Synthesis and Preclinical Evaluation of QS-21 Variants Leading to Simplified Vaccine Adjuvants and Mechanistic Probes

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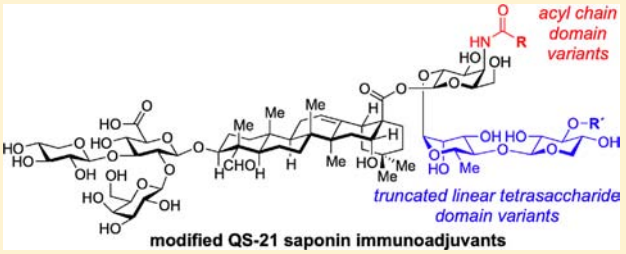
Supporting Information

ABSTRACT: QS-21 is a potent immunostimulatory saponin that is currently under clinical investigation as an adjuvant in various vaccines to treat infectious diseases, cancers, and cognitive disorders. Herein, we report the design, synthesis, and preclinical evaluation of simplified QS-21 congeners to define key structural features that are critical for adjuvant activity. Truncation of the linear tetrasaccharide domain revealed that a trisaccharide variant is equipotent to QS-21, while the corresponding disaccharide and monosaccharide congeners are more toxic and less potent, respectively. Modification of the acyl chain domain in the trisaccharide series revealed that a terminal carboxylic acid is well-tolerated while a terminal amine results in reduced adjuvant activity. Acylation of the terminal amine can, in some cases, restore adjuvant activity and enables the synthesis of fluorescently labeled QS-21 variants. Cellular studies with these probes revealed that, contrary to conventional wisdom, the most highly adjuvant active of these fluorescently labeled saponins does not simply associate with the plasma membrane, but rather is internalized by dendritic cells.



modified QS-21 saponin immunoadjuvants

INTRODUCTION

There is an ever increasing need to develop effective vaccines to combat acute and chronic infections and diseases, in both therapeutic and prophylactic settings. The traditional use of live, attenuated pathogens as immunogens in vaccines has proven successful in addressing a host of maladies, yet there are many pathogens and diseases for which this strategy is ineffective. As a consequence, many contemporary approaches to vaccine development employ recombinant or synthetic subunit vaccines, which offer improved safety and more precise targeting. However, they are often characterized by poor immunogenicity. As such, subunit antigens must be coadministered with an adjuvant to enhance the immune response.[1−8]

The discovery of novel adjuvants has emerged as a critical frontline effort in the development of modern vaccine formulations. The adjuvant component enables dose-sparing of rare, expensive, and otherwise impotent antigens. An adjuvant also extends the immunotherapeutic benefits to poor responders, such as elderly or immunocompromised patients. While numerous classes of adjuvants have been explored for vaccine therapies over the past several decades, Alum, a mixture of aluminum salts (hydroxide, phosphate, sulfate),[9−12] is still the most popular and is present in approximately half of all human vaccines in the U.S. First introduced for human use in the 1930s, Alum was the only approved adjuvant in man for more than 70 years. It was not until late 2009 that the FDA approved GlaxoSmithKline's AS04 adjuvant (a proprietary combination of Alum and monophosphoryl lipid A)[13] for the Cervarix vaccine to immunize against human papillomavirus (HPV). Thus, there remains a great need for novel adjuvants for use in vaccine therapy.

Currently, one of the most promising adjuvants undergoing clinical investigation is QS-21, a natural product extract obtained from the bark of the *Quillaja saponaria* Molina tree, found in the desert regions of Chile, Bolivia, and Southern Peru.[30] This semipurified extract consists of a mixture of triterpene saponins (Figure 1), whose principal constituents are characterized by complex bis(desmosides) **1** (QS-21-apiose) and **2** (QS-21-xylose).[31,32] These immunoactive constituents of QS-21 contain four distinct structural domains: the central triterpene (quillaic acid), a branched trisaccharide at the C3 position of the triterpene, a linear tetrasaccharide attached at C28 of the triterpene, and a branched acyl chain linked to the central fucose moiety of the linear tetrasaccharide.

The importance of QS-21 as an investigational adjuvant is evident in its use in more than 100 clinical trials involving >6000 total patients.[33] QS-21 has exhibited a remarkable ability to augment clinically significant responses to vaccine antigens targeting a wide landscape of diseases and degenerative disorders (e.g., cancer, Alzheimer's disease, malaria, HIV, hepatitis). Despite these promising indications, further

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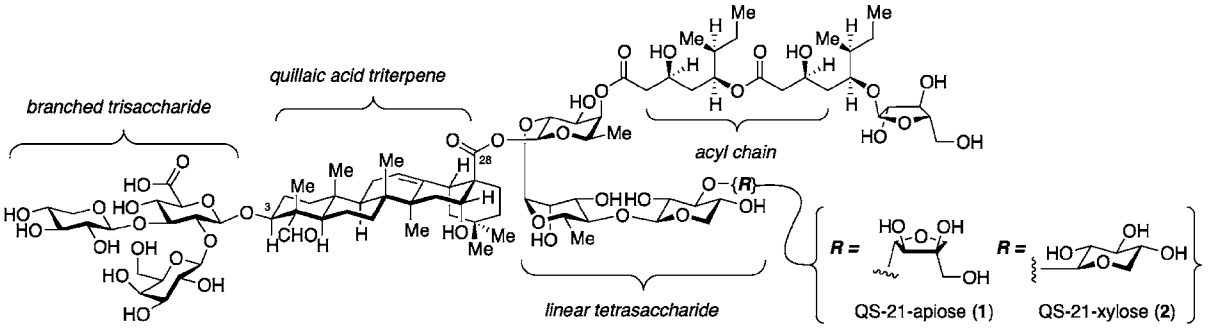

Figure 1. Structure of the saponin adjuvant QS-21 and four key structural domains.

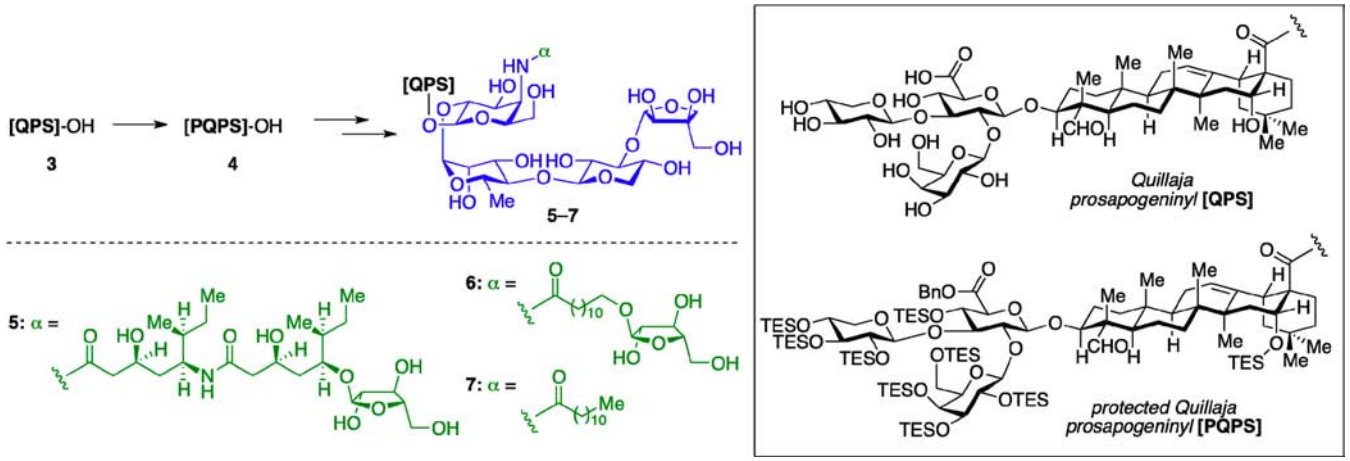

Figure 2. Semisynthetic approach to QS-21 analogues **5**–**7** with variations in the acyl chain domain (green).

commercial advancement of QS-21 is hampered by low isolation yields, inconsistent composition, and lack of purity. Moreover, the instability of QS-21, characterized by spontaneous hydrolysis of the acyl domain, limits its efficacy, enhances its local reactogenicity, and complicates formulation and storage protocols. Finally, these hurdles in advancing QS-21 are further exacerbated by the fact that the mechanism by which it potentiates the immune response is unknown. In the absence of insights on this front, there exists no rational path forward to design novel saponin adjuvants with improved vaccine efficacy.

Our previous synthetic studies on QS-21 have permitted access to each specific isomeric saponin within the mixture,[14−17] thereby obviating the problems associated with inconsistent purity and composition. While the initial synthetic route was rather lengthy, a significantly streamlined semisynthetic route was subsequently developed.[18] This involved isolation of the entire *Quillaja* prosapogenin half of the adjuvant ([**QPS**]−OH, **3**, Figure 2) from the commercial semipurified *Q. saponaria* extract Quil-A, followed by derivatization into a selectively protected form (protected *Quillaja* prosapogenin, [**PQPS**]−OH, **4**). Integration of this technology into the synthesis of novel saponins offers a convenient means by which to append distinct linear tetrasaccharide domain and acyl chain domain variants for evaluation.

Our initial work in this area involved the preparation and evaluation of three non-natural analogues comprising acyl chain domain variants **5**−**7** (Figure 2).[19] These syntheses involved modification of the fucose moiety within the linear tetrasaccharide domain of the saponin to reduce hydrolytic instability, allowing the attachment of three distinct lipophilic acyl chains. This initial study revealed that significant structural modification of the central fucosyl pyranose and of the acyl chain of QS-21 causes little or no impairment of adjuvant activity. Moreover, differing toxicity profiles were observed.

Although these initial findings highlighted the exciting prospect of designing improved saponin adjuvants, these three compounds offered a rather narrow insight into the structure−activity profile of these adjuvants. For example, while all three synthetic *Quillaja* saponins were competent immunostimulators, the complex acyl chain in **5** required an excessively long synthesis, the simplified acyl chain in **6** imparted unacceptable levels of toxicity, and the nonglycosylated acyl chain in **7** limited its aqueous solubility.

Herein, we report efforts that have defined specific substructures of the saponin adjuvant that are critical for adjuvant activity. Multiple variations of both the acyl chain and linear tetrasaccharide domains have been explored systematically, leading to the development of highly simplified structures that show excellent immunostimulatory properties with encouraging toxicity profiles. In addition, these efforts have led to the development of a synthetic *Quillaja* saponin scaffold that is amenable to attachment of fluorescent reporter groups while still retaining adjuvant activity. The development of this novel class of biochemical tools allows, for the first time, direct investigations of QS-21-derived molecular probes to unravel the cellular mechanisms of saponin immunostimulation.

RESULTS AND DISCUSSION

Synthesis and Evaluation of Initial Acyl Chain Domain Variants. The nonoptimal chemical and biological properties associated with the first three acyl chain domain variants **5**−**7**

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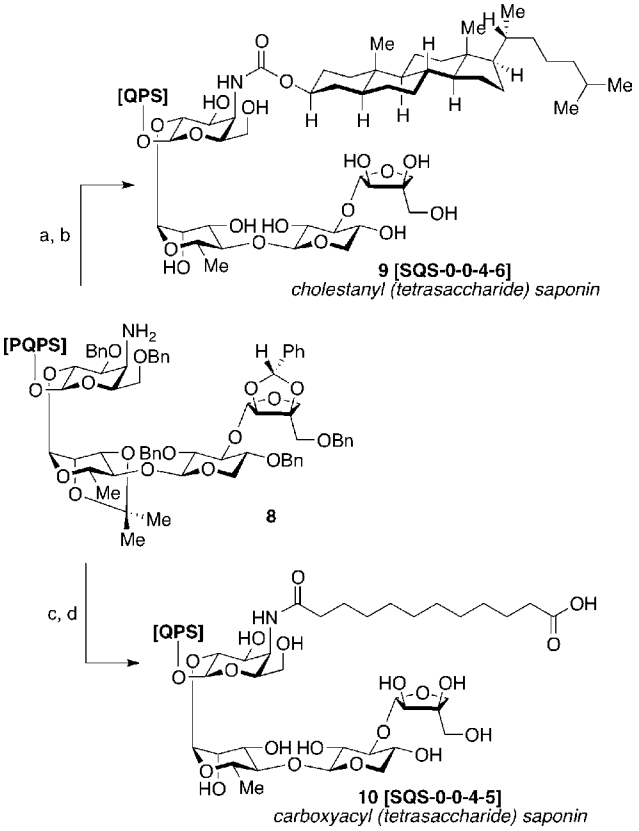
Scheme 1. Synthesis of Acyl Chain Domain Variants 9 and 10[a]



[a]Reagents and conditions: (a) 5α-cholestan-3β-ol (**11**), $COCl_2$, PhMe; add to **8**, 2,4,6-tri-*t*-butylpyridine, CH_2Cl_2, 21 °C, 58%; (b) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; TFA, H_2O, 0 °C, 58%; (c) $HO_2C(CH_2)_{10}CO_2Bn$ (**12**), EtOCOCl, Et_3N, THF, 0 °C; add **8**, 0 °C, 60%; (d) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; TFA/H_2O (3:1 v/v), 0 °C, RP-HPLC, 89%.

prompted us to investigate two additional variants (Scheme 1). The cholestanyl variant **9** was designed based on the known propensity of QS-21 to associate with cholesterol,[34] which has led to hypotheses that saponin binding to membrane-associated cholesterol may be a key enabling feature in its putative mechanism of action.[35] Indeed, the immunostimulatory complex ISCOMATRIX[20−22] comprises a proprietary mixture of QS-21, cholesterol, and various phospholipids, and is CSL Behring's favored adjuvant product in clinical trials with vaccines against influenza, hepatitis C virus (HCV), HPV, and cancer.[22] The carboxyacyl variant **10** was designed to overcome the water solubility issues associated with the simple dodecanoyl variant **7** through incorporation of a charged carboxylate moiety to increase polarity of the otherwise lipophilic acyl chain.

Governing criteria in both of these acyl chain domain variants are simplicity and highly efficient synthesis. In this regard, 5α-cholestan-3β-ol (**11**) is commercially available, and dodecanedioic acid monobenzyl ester (**12**) is readily prepared in one step from its commercially available diacid precursor.[23] The preparation of these analogues began with the protected *Quillaja* triterpene bis(desmoside) **8** (Scheme 1), previously synthesized as a late stage intermediate in the preparation of the first-generation analogues **5**−**7**.[19] Its amino functionality was acylated with 5α-cholestan-3β-yl chloroformate, generated in

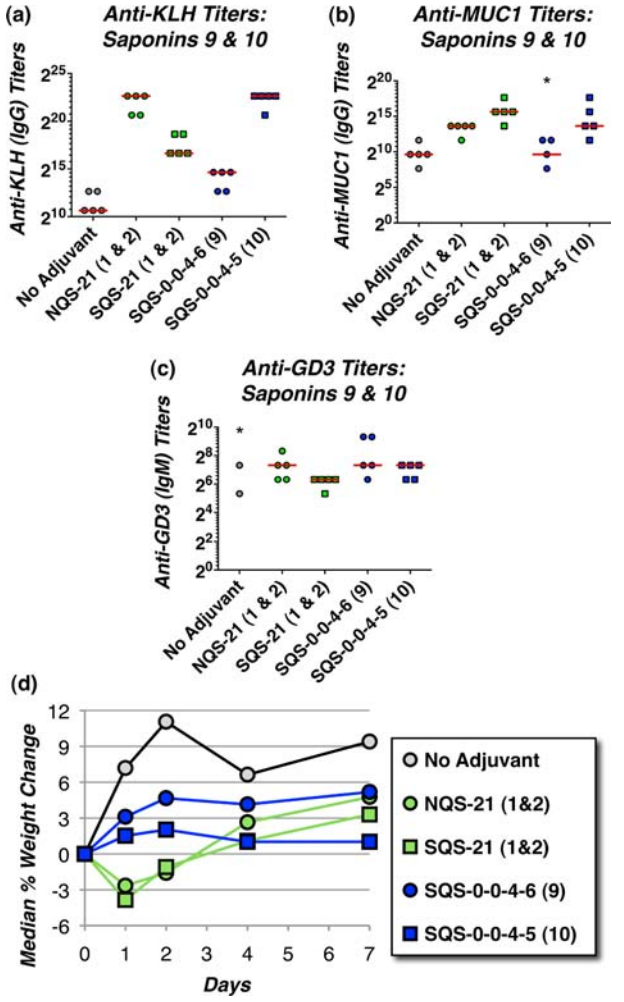


Figure 3. Preclinical evaluation of cholestanyl saponin **9** and carboxyacyl saponin **10** in a two-component vaccine containing both GD3−KLH and MUC1−KLH conjugates. Each adjuvant, plus a no-adjuvant negative control, was evaluated by vaccination of a group of five mice (C57BL/6J, female). Vaccinations were carried out by weekly subcutaneous injection of GD3−KLH (5 μg), MUC1−KLH (5.0 μg), and various saponin adjuvants (10 μg) for 3 weeks (days 0, 7, and 14), followed by a booster at day 65. Postboost serological data at day 72 are presented; *missing data points are from mice that did not exhibit an antibody response to the antigen in question. Median values of titers obtained are represented as a red horizontal bar. (a) Anti-KLH titers (IgG), (b) Anti-MUC1 titers (IgG), and (c) Anti-GD3 titers (IgM). (d) Toxicity assessment was done by tracking median weight loss over the course of a week after the first vaccine injection.

situ by treatment of 5α-cholestan-3β-ol with phosgene. The resulting cholestanyl adduct was then exposed to global deprotection conditions, via hydrogenolysis and acid hydrolysis, to afford the cholestanyl saponin **9** (SQS-0-0-4-6). In a similar manner, amine **8** was treated with the acyl chloride derivative of dodecanedioic acid monobenzyl ester to afford the corresponding fully protected saponin. Deprotection via hydrogenolysis and acid hydrolysis afforded the carboxyacyl saponin **10** (SQS-0-0-4-5).

Evaluation of these novel saponins for immunostimulatory activity involved vaccination of mice with a multiple antigen formulation that included both GD3−KLH and MUC1−KLH conjugates. These constructs represent clinically relevant oncology vaccine antigens (GD3: melanoma, sarcoma;

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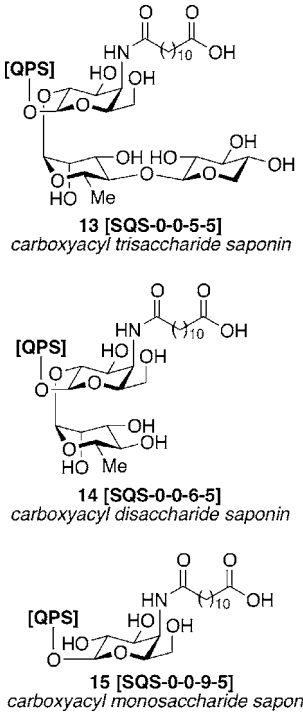
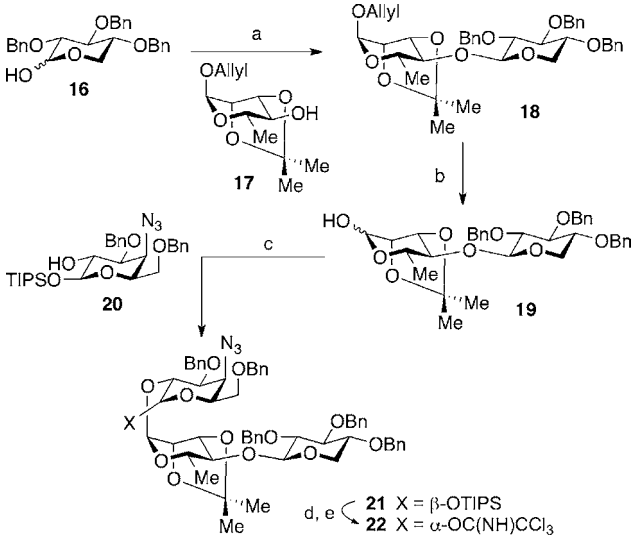


Figure 4. Truncated linear tetrasaccharide domain variants.

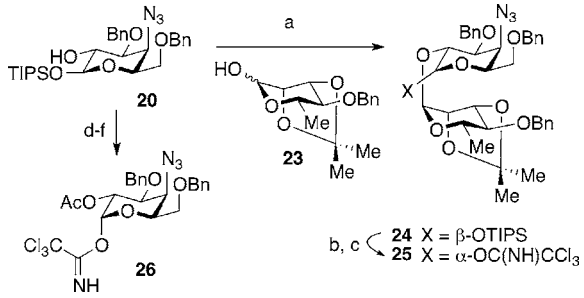
Scheme 2. Synthesis of Trisaccharide Donor 22[a]



[a]Reagents and conditions: (a) Ph_2SO, Tf_2O, TBP, CH_2Cl_2, −60 °C → 0 °C, 71%; (b) Et_2NH, $Pd(OAc)_2$, PPh_3, CH_2Cl_2, MeOH, 21 °C, >99% (9:1, α:β); (c) Ph_2SO, Tf_2O, TBP, CH_2Cl_2, −72 °C → 0 °C, 74%; (d) TBAF, AcOH, THF, 0 °C; (d) Cl_3CCN, DBU, CH_2Cl_2, 3 °C → 21 °C, 82% (2 steps).

MUC1: prostate, breast), conjugated to the immunological carrier KLH, and serve as useful models for comparing adjuvant performance. Monitoring antibody responses to both the antigen and the carrier protein provides a useful assessment of adjuvant performance in antigens of diverse immunogenicity, ranging from a poorly immunogenic glycolipid (GD3), to a moderately immunogenic nonglycosylated peptide (MUC1), and to a highly immunogenic protein (KLH). Groups of five mice (C57BL/6J, female, 6−8 weeks of age) were vaccinated with GD3−KLH and MUC1−KLH at a 5 μg antigen dose. The

Scheme 3. Synthesis of Disaccharide Donor 25 and Monosaccharide Donor 26[a]



[a]Reagents and conditions: (a) Ph_2SO, Tf_2O, TBP, CH_2Cl_2, −78 °C → 21 °C, 82%; (b) TBAF, AcOH, THF, 0 °C → 21 °C, 88%; (c) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 94%; (d) Ac_2O, py, 21 °C, 97%; (e) TBAF, AcOH, THF, 0 °C → 21 °C, 90%; (f) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 89%.

antigens were coadministered with the adjuvant of interest in a vaccination protocol involving three subcutaneous injections at days 0, 7, and 14, plus a booster at day 65. As the negative control, mice were vaccinated with the GD3−KLH and MUC1−KLH antigens without adjvant. As a positive control, vaccinations were performed with *naturally* derived QS-21 (NQS-21), obtained by fractionating a mixture of saponins from *Q. saponaria*,[24] and also with *synthetic* QS-21 as its reconstituted 65:35 mixture of apiose and xylose isomers (SQS-21). Antibodies against each antigen in mice sera were detected by ELISA (Figure 3a−c, see Supporting Information for experimental details). As a standard initial assessment of overall toxicity, the weight loss of the mice was monitored at 0, 24, 48, and 72 h after the first vaccination (Figure 3d).

The resulting antibody titers indicate that both acyl chain domain variants, cholestanyl saponin **9** (SQS-0-0-4-6) and carboxyacyl saponin **10** (SQS-0-0-4-5), induce anti-GD3 titers comparable to those of NQS-21 and SQS-21 (Figure 3c); however, stark differences were observed in comparisons of antibody levels generated against the MUC1 peptide and KLH protein antigens (Figure 3a,b). Cholestanyl saponin **9** was essentially inactive as an adjuvant in mice vaccinated with either of these antigens. In contrast, mice vaccinated in the presence of carboxyacyl saponin **10** exhibited anti-MUC1 and anti-KLH titers comparable to those of both positive controls (NQS-21, SQS-21). Interestingly, both synthetic saponins **9** and **10** exhibited noticeably lower toxicity, as judged by weight loss, in comparison to NQS-21 and SQS-21 (Figure 3d). On the basis of its superior immunostimulatory performance, carboxyacyl saponin **10** was selected as a lead for further structural modifications.

Synthesis and Evaluation of Truncated Linear Tetrasaccharide Domain Variants. Having identified in carboxyacyl saponin **10** an acyl chain domain that offers both ease of synthesis and potent immunostimulating effects, we next focused our efforts on modification of the linear tetrasaccharide domain of QS-21. Although the synthetic route for the preparation of the linear tetrasaccharide in **10** is highly streamlined, the synthesis of each of the selectively protected monosaccharide building blocks requires 3−7 steps, with preparation and installation of the terminal apiose sugar being particularly lengthy. Thus, we prepared systematically truncated versions of this linear tetrasaccharide in analogues

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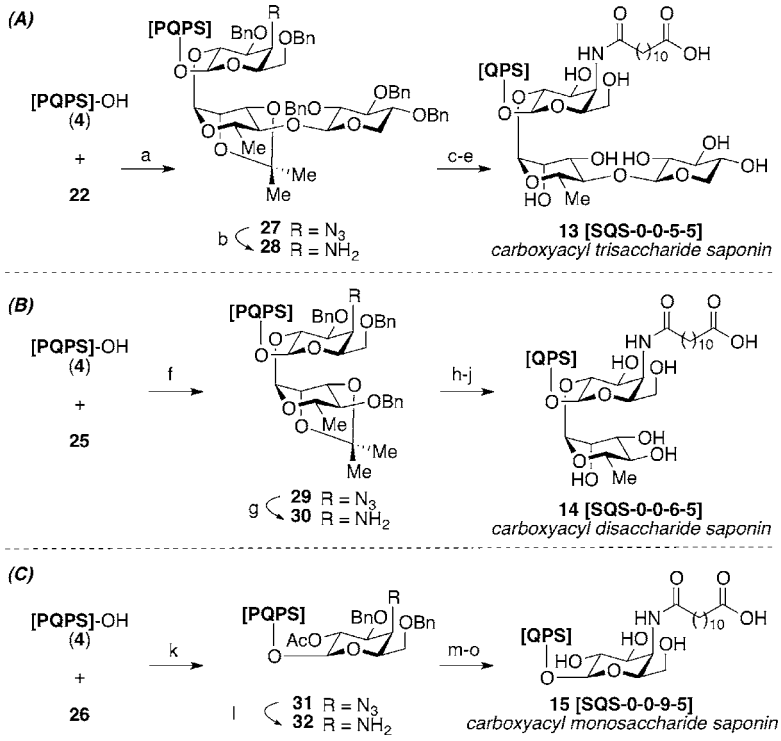
Scheme 4. Synthesis of Truncated Linear Tetrasaccharide Domain Variants 13−15[a]



[a]Reagents and conditions: (a) $BF_3{\cdot}OEt_2$, 4 Å M.S., CH_2Cl_2, −40 °C → 21 °C, 73%; (b) PhSeH, Et_3N, 40 °C, 87%; (c) $HO_2C(CH_2)_{10}CO_2Bn$ (**12**), EtOCOCl, Et_3N, THF, add to **28**, 0 °C, 75%; (d) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; (e) TFA, H_2O, 0 °C, RP-HPLC, 92% (2 steps); (f) $BF_3{\cdot}OEt_2$, 4 Å M.S., CH_2Cl_2, −78 °C → 21 °C, 41%; (g) PhSeH, Et_3N, 36 °C, 92%; (h) $HO_2C(CH_2)_{10}CO_2Bn$ (**12**), EtOCOCl, Et_3N, THF, add to **30**, 0 °C, 81%; (i) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; (j) TFA, H_2O, 0 °C, RP-HPLC, 70% (2 steps); (k) $BF_3{\cdot}OEt_2$, 4 Å M.S., CH_2Cl_2, −78 °C → 21 °C, 45%; (l) PhSeH, Et_3N, 36 °C, 90%; (m) $HO_2C(CH_2)_{10}CO_2Bn$ (**12**), EtOCOCl, Et_3N, THF, add to **32**, 0 °C, 72%; (n) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; (o) TFA, H_2O, 0 °C; NaOMe, MeOH, H_2O, 0 °C, RP-HPLC, 71% (3 steps).

13−**15** (SQS-0-0-5-5, SQS-0-0-6-5, and SQS-0-0-9-5) (Figure 4).

We first sought to assemble the selectively protected linear trisaccharide **22** (Scheme 2), in which the terminal apiose sugar is omitted. As shown, commencing with allyl 2,3-di-*O*-isopropylidinerhamnopyranoside (**17**), the C4-hydroxyl group was glycosylated with tri-*O*-benzylxylopyranose (**16**) under dehydrative coupling conditions with Ph_2SO and Tf_2O.[36,37] The condensation proceeded with β-anomeric selectivity to afford the disaccharide **18** in 71% yield. Selective removal of the allyl acetal in **18** proceeded in quantitative yield to provide disaccharide **19**, which served as a competent glycosyl donor in a second dehydrative glycosylation with the previously prepared 4-deoxy-4-azidogalactopyranoside acceptor **20**,[19] to furnish trisaccharide **21** with complete α-selectivity. Subsequent conversion of the silyl acetal in **21** to a latent leaving group was performed in a two-step procedure, involving desilylation followed by treatment with CCl_3CN in the presence of DBU to give the α-trichloroacetimidate **22**.

The two other truncated carbohydrate fragments, **25** and **26**, were prepared in an analogous fashion from similar substrates (Scheme 3). Thus, the C2-hydroxyl group of the 4-deoxy-4-azidogalactopyranoside acceptor **20** underwent dehydrative glycosylation with 4-*O*-benzyl-2,3-di-*O*-isopropylidenerhamnopyranoside (**23**) to afford disaccharide **24**, allowing for subsequent conversion of its anomeric functionality from the TIPS acetal to the trichloroacetimidate **25**. Finally, preparation of monosaccharide **26** was accomplished through straightforward functional group modifications of 4-deoxy-4-azidogalactopyranoside **20**, involving C2-*O*-acetylation, carefully monitored desilylation to avoid unwanted acyl transfer, and trichloroacetimidate formation.

The protected carbohydrate donors **22**, **25**, and **26** were then advanced to saponin analogues **13**, **14**, and **15**, respectively, having truncated linear tetrasaccharide domains (Scheme 4). These late stage assembly steps are exemplified by the synthesis of the saponin **13** (Scheme 4a), in which the previously prepared selectively protected *Quillaja* prosapogenin **4** ([**PQPS**]−OH)[18] is glycosylated at its C28-carboxylic acid by the trisaccharide **22** under Schmidt coupling conditions to afford the glycosyl ester **27** with complete β-anomeric selectivity. Reduction of the azide **27** to its corresponding amine **28** was accomplished with PhSeH under basic conditions. On the basis of the promising activity of the carboxyacyl saponin **10** above (Figure 3), the amino group of **28** was also acylated with dodecanedioic acid monobenzyl ester **12** to install the acyl chain domain. Subsequent global deprotection via hydrogenolysis and acid hydrolysis provided the carboxyacyl trisaccharide **13** as the single-sugar truncation analogue of the immunoactive carboxyacyl tetrasaccharide saponin **10**. Following similar synthetic protocols, the progressively carbohydrate-truncated saponins carboxyacyl disaccharide **14** (Scheme 4b) and carboxyacyl monosaccharide **15** (Scheme 4c) were prepared from their corresponding protected saccharides **25** and **26**, respectively.

Evaluation of these three truncated saponin analogues for immunoadjuvant activity involved vaccination of mice with a three-component vaccine that contained the GD3−KLH and

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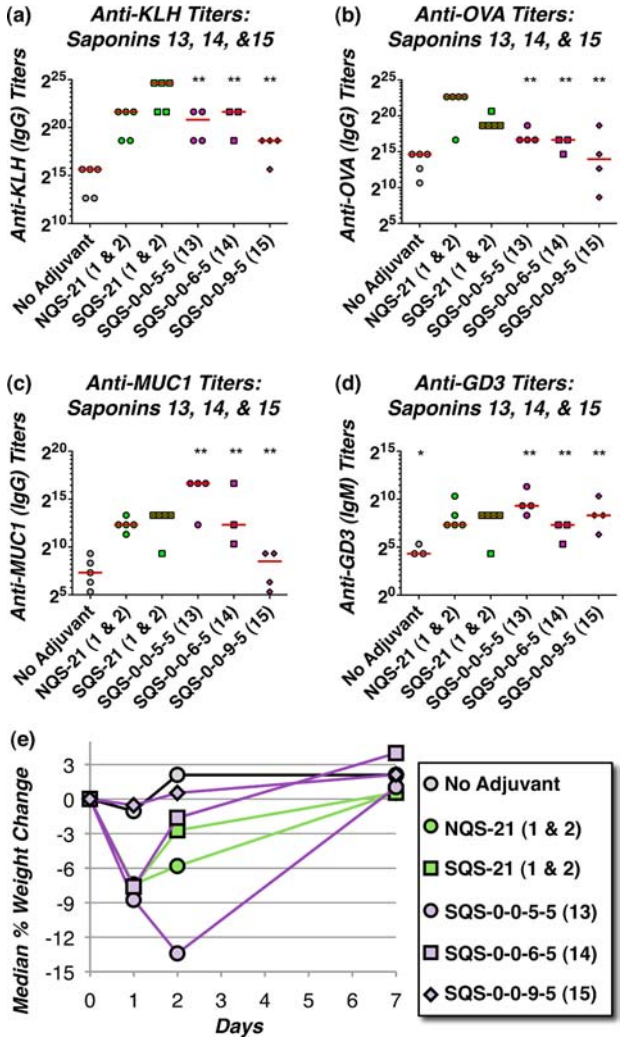
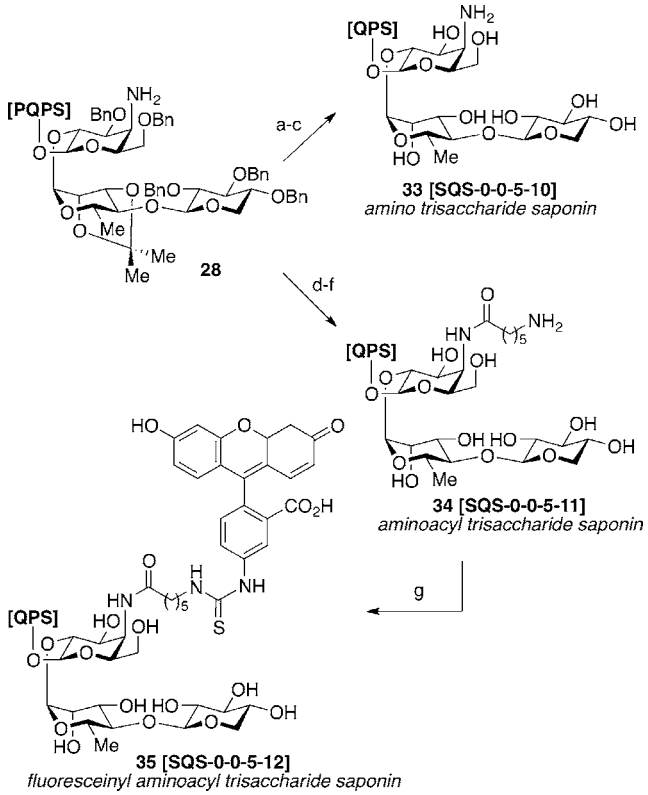


Figure 5. Preclinical evaluation of carboxyacyl trisaccharide saponin **13**, carboxyacyl disaccharide **14**, and carboxyacyl monosaccharide **15** with GD3–KLH, MUC1–KLH, and OVA three-component vaccine. Each adjuvant, plus a no-adjuvant negative control, was evaluated by vaccination of a group of five mice (C57BL/6J, female). Vaccinations were carried out by weekly subcutaneous injection of GD3–KLH (5 μg), MUC1–KLH (2.5 μg), OVA (20 μg), and various saponin adjuvants (20 μg) for 3 weeks (days 0, 7, and 14), followed by a booster at day 65. Postboost serological data at day 72 are presented; *missing data points are from mice that did not exhibit an antibody response to the antigen in question; **missing data points are from dead mice. Median values of titers obtained are represented as a red horizontal bar. (a) Anti-KLH titers (IgG), (b) anti-OVA titers (IgG), (c) anti-MUC1 titers (IgG), and (d) anti-GD3 titers (IgM). (e) Toxicity assessment was done by tracking median weight loss over the course of a week after the first vaccine injection.

MUC1–KLH conjugates as well as ovalbumin (OVA), the main protein in egg white, which is known to be a reliable immunogen that induces both antibody and T-cell responses in immunized mice. The resulting antibody responses to GD3 were similar in the mice vaccinated with carboxyacyl trisaccharide **13**, carboxyacyl disaccharide **14**, carboxyacyl monosaccharide **15**, and the positive controls, NQS-21 and SQS-21 (Figure 5d). However, antibody titers against KLH, OVA, and MUC1 peptide decreased with each subsequent saccharide truncation (Figure 5a–c). Although trisaccharide **13** induces greater weight loss (Figure 5d), two of five mice died after vaccination with disaccharide **14**, while only one mouse died when treated with trisaccharide **13** or monosaccharide **15**. In addition, while monosaccharide **15** was the least toxic among the saponins tested, as assessed by weight loss, it was also the least efficacious. Thus, taking mortality, toxicity, and efficacy into consideration, the carboxyacyl trisaccharide saponin **13** was deemed superior to the disaccharide **14** and the monosaccharide **15**. Overall, trisaccharide **13** also exhibits comparable efficacy to SQS-21, but is more readily accessible (26 vs 76 synthetic steps for QS-21-apiose (**1**)).

Scheme 5. Synthesis of Acyl Chain Domain Variants 33–35[a]



[a]Reagents and conditions: (a) Boc_2O, CH_2Cl_2, 21 °C; (b) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; (c) TFA, H_2O, 0 °C, RP-HPLC, 59% (3 steps); (d) $HO_2C(CH_2)_5NHBoc$ (**36**), EtOCOCl, Et_3N, THF, add to **28**, 0 °C; (e) H_2 (50 psi), Pd/C (Degussa), THF, EtOH, 21 °C; (f) TFA, H_2O, 0 °C, RP-HPLC, 63% (3 steps); (g) FITC (**37**), Et_3N, DMF, 21 °C, RP-HPLC, 75%.

Synthesis and Evaluation of Functionalized Acyl Chain Domain Variants. The above results reveal the importance of incorporating at least the trisaccharide moiety of the linear tetrasaccharide domain to maintain optimal adjuvant activity. They also present an attractive path forward for accessing additional simplified adjuvants, given that the significant efforts required for the synthesis and installation of the selectively protected apiose sugar can be circumvented. Thus, the simplified amino trisaccharide intermediate **28** was subsequently used as the workhorse scaffold for the investigation of an additional property of the acyl chain domain, ionic charge. Initial studies with the 12-carboxydodecanoyl chain in both carboxyacyl tetrasaccharide **10** and carboxyacyl trisaccharide **13** had signaled that adjuvant activity can be maintained with the introduction of a carboxylate anion (at physiological pH).

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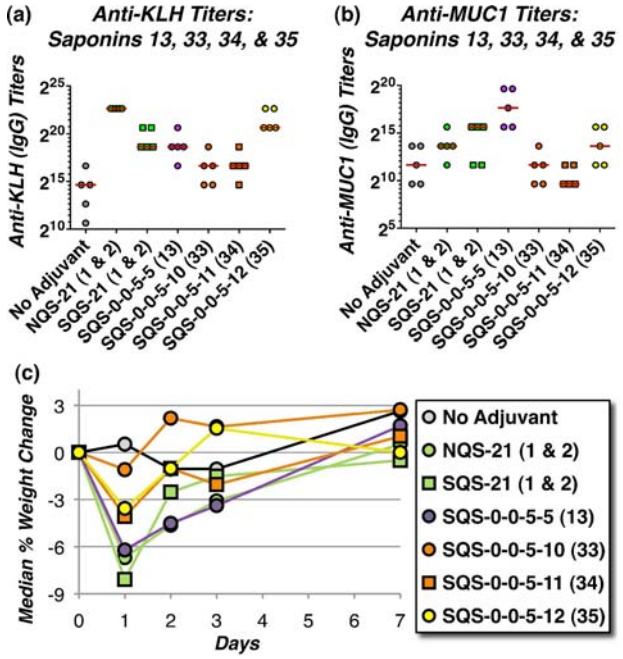


Figure 6. Preclinical evaluation of carboxyacyl trisaccharide saponin **13**, amino trisaccharide saponin **33**, aminoacyl trisaccharide saponin **34**, and fluoresceinyl aminoacyl trisaccharide saponin **35** with MUC1–KLH vaccine. Each adjuvant, plus a no-adjuvant negative control, was evaluated by vaccination of a group of five mice (C57BL/6J, female). Vaccinations were carried out by weekly subcutaneous injection of MUC1–KLH (2.5 μg) and various saponin adjuvants (10 μg) for 3 weeks (days 0, 7, and 14), followed by a booster at day 65. Postboost serological data at day 72 are presented. Median values of titers obtained are represented as a red horizontal bar. (a) Anti-KLH titers (IgG) and (b) anti-MUC1 titers (IgG). (c) Toxicity assessment was done by tracking median weight loss over the course of a week after the first vaccine injection.

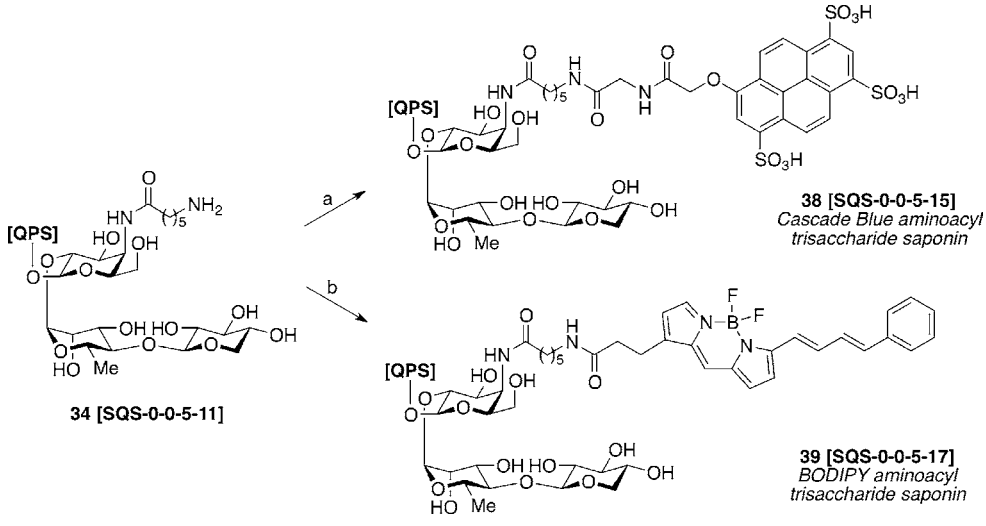
To explore whether a cationic moiety would impart the same beneficial properties, two amine-containing saponins **33** and **34** were prepared from selectively protected precursor **28** (Scheme 5). Acylation of the amino group in **28** with $(Boc)_2O$ provided the corresponding carbamate. The fully protected intermediate was then subjected to the standard hydrogenolysis/hydrolysis deprotection protocol to provide amino trisaccharide **33** (SQS-0-0-5-10), which lacks the lipophilic acyl chain domain entirely. Preparation of a second amine-containing variant involved acylation of the amino group in **28** with 6-((*t*-butoxycarbonyl)-amino)hexanoic acid (**36**) followed by hydrogenolysis/hydrolysis deprotection, providing the extended lipophilic aminoacyl trisaccharide **34** (SQS-0-0-5-11).

We recognized that the amine-containing saponin **34** also opened the door to late-stage, chemoselective elaboration with other moieties, such as fluorescent labels that might be useful in studying saponin subcellular localization. Along these lines, treatment of aminoacyl trisaccharide **34** with fluorescein isothiocyanate (FITC) (**37**) afforded the fluoresceinyl aminoacyl trisaccharide **35** (SQS-0-0-5-12).

Immunological evaluation of this new group of saponins was accomplished through vaccination of mice with the MUC1–KLH conjugate. Antibody responses to KLH and MUC1 in mice vaccinated with carboxyacyl trisaccharide saponin **13** (SQS-0-0-5-5) were again comparable to both NQS-21 and SQS-21 (Figure 6a,b). In contrast, mice vaccinated with either amino trisaccharide **33** (SQS-0-0-5-10) or aminoacyl trisaccharide **34** (SQS-0-0-5-11) generated antibody titers that were similar to no-adjuvant treatment negative control. This suggests that the positive charge imparted by the ammonium group in the acyl chain domain attenuates saponin adjuvant activity. The toxicity (weight loss) of the amine-containing saponins **33** and **34** was also decreased compared to NQS-21, SQS-21, and carboxyacyl saponin **13** (Figure 6c).

Notably, upon masking the free amine of **34** by acylation with FITC, efficacy was restored in the resulting fluoresceinyl aminoacyl trisaccharide **35** (SQS-0-0-5-12) to levels comparable to both positive controls NQS-21 and SQS-21 (Figure 6a,b). Importantly, in addition to this potent adjuvant activity, **35** exhibited lower toxicity (weight loss) compared to NQS-21, SQS-21, and carboxyacyl saponin **13** (Figure 6c). To the best of our knowledge, while other fluorescently labeled saponins have

Scheme 6. Synthesis of Fluorescently Labeled Variants 38 and 39[a]



[a]Reagents and conditions: (a) 3-(diethoxyphosphoryloxy)-1,2,3-benzotriazin-4(3*H*)-one, Et_3N, DMF, triethylammonium 2-(2-(3,6,8-trisulfonatopyren-1-yloxy)acetamido)acetate (**40**), 21 °C, RP-HPLC, 36%; (b) BODIPY 581/591 C_3 succinimidyl ester (**41**), Et_3N, DMF, 21 °C, RP-HPLC, 28%.

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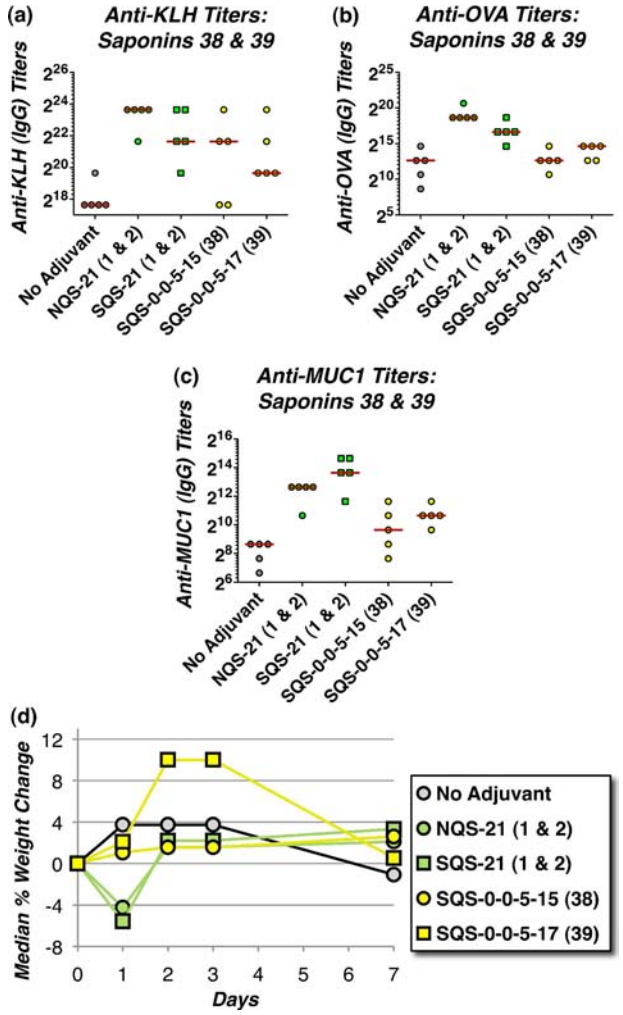

Figure 7. Preclinical evaluation of Cascade Blue-labeled saponin **38** and BODIPY-labeled saponin **39** with OVA and MUC1–KLH two-component vaccine. Each adjuvant, plus a no-adjuvant negative control, was evaluated by vaccination of a group of five mice (C57BL/6J, female). Vaccinations took the form of weekly subcutaneous injection of OVA (20 μg), MUC1-KLH (2.5 μg), and various saponin adjuvants (20 μg) for 3 weeks (days 0, 7, and 14), followed by a booster at day 65. Postboost serological data at day 72 are presented. Median values of titers obtained are represented as a red horizontal bar. (a) Anti-KLH titers (IgG), (b) anti-MUC1 titers (IgG), and (c) anti-GD3 titers (IgM). (d) Toxicity assessment was done by tracking median weight loss over the course of a week after the first vaccine injection.

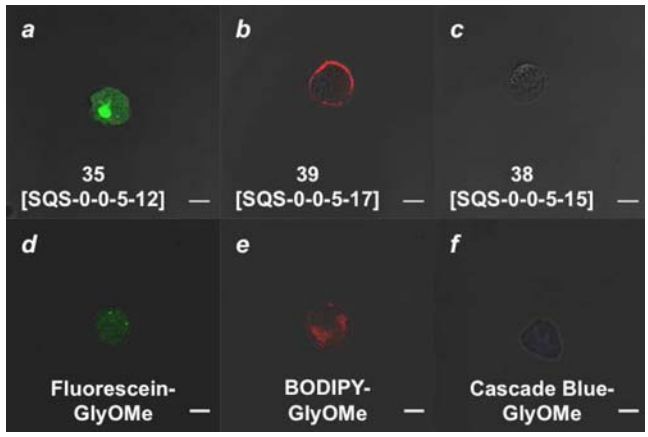

Figure 8. Subcellular localization of fluorescently labeled saponins **35**, **38**, and **39** in immature dendritic cells. Immature dendritic cells were treated with (a) 1 μM fluoresceinlabeled saponin **35** (SQS-0-0-5-12), (b) 1 μM BODIPY-labeled saponin **39** (SQS-0-0-5-17), or (c) 1 μM Cascade Blue-labeled saponin **38** (SQS-0-0-5-15) for 1 h then visualized by confocal microscopy. As controls, immature dendritic cells were treated with glycine methyl ester labeled with each corresponding fluorophore (d–f).

been reported,[25] the fluorescein-labeled saponin **35** represents the first example of a fluorescent saponin with confirmed adjuvant activity.

Synthesis and Evaluation of Fluorescently Labeled Saponin Probes. Building upon the promising adjuvant activity and toxicity profile of the fluorescein-labeled saponin **35** above, we next investigated two additional fluorescently labeled saponins (Scheme 6) to provide probes with alternative excitation and emission wavelengths and increased stability at acidic pH that may be encountered in the intracellular environment. Chemoselective acylation of the aminoacyl trisaccharide scaffold **34** with Cascade Blue derivative **40**[26] afforded the corresponding Cascade Blue-labeled saponin **38** (SQS-0-0-5-15). Analogous acylation with BODIPY 581/591 C_3 succinimidyl ester (**41**) afforded BODIPY-labeled saponin **39** (SQS-0-0-5-17).

Preclinical evaluation of these fluorescently labeled saponins revealed that, although median antibody titers to KLH from mice treated with Cascade Blue-labeled saponin **38** were comparable to SQS-21, antibody responses to both OVA and MUC1 were attenuated (Figure 7a–c). Since Cascade Blue imparts polyanionic character to the saponin, the attenuation in antibody response to OVA and MUC1 suggests that saponin adjuvant activity is highly sensitive to charge. In mice treated with BODIPY-labeled saponin **39**, antibody titers generated to KLH, OVA, and MUC1 were all lower compared to NQS-21 and SQS-21. Although introducing either of these two fluorophores attenuates adjuvant activity, both fluorescently labeled saponins were also less toxic compared to the positive controls (Figure 7d).

Subcellular Localization of Fluorescently Labeled Saponins. Currently, there are only indirect means to investigate the mechanism of action of immunopotentiating saponins. Theories for the saponin mode of action include perturbation of cell membrane components to increase immune cell maturation as well as increased delivery of antigens into cells.[38,39] Although there is limited knowledge on which cell types are involved in saponin-mediated immunostimulation, we elected to investigate dendritic cells, as there is mounting evidence of their importance in adjuvant activity across a panel of various adjuvants, including MF59,[27] α-galactosylceramide,[28] and MPL-A.[29]

Immature dendritic cells were treated with the fluorescently labeled saponins **35**, **38**, and **39**, and imaged via confocal microscopy. Interestingly, the immunopotentiating fluorescein-labeled saponin **35** (SQS-0-0-5-12) (Figure 8a) localized to a discrete structure within the cell, distinguishing itself from the other two probes **38** (SQS-0-0-15) and **39** (SQS-0-0-5-17), which are poor immunopotentiators. BODIPY-labeled saponin **39** (Figure 8b) localized to the plasma membrane, while Cascade Blue-labeled saponin **38** (Figure 8c) did not associate with dendritic cells at all. These findings suggest that, in order for a saponin to be an efficacious adjuvant, it is necessary for it

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to be internalized into dendritic cells. This mechanism is shared by Alum, wherein internalization results in activation of the NALP-3 inflammasome.[11] The low efficacy observed in Cascade Blue-labeled saponin **38** may result from its inability to engage the cell membrane due to the repulsion between its three sulfonate anions with the phospholipid anions that adorn the plasma membrane. The importance of the saponin scaffold in the trafficking of these fluorescently labeled saponins within dendritic cells was established by comparison to the trafficking of the corresponding fluorescently labeled glycine methyl esters (Figure 8d–f; see Supporting Information for synthesis and characterization).

To determine whether the fluorescein-labeled saponin **35** remains intact intracellularly, peripheral blood mononuclear cells were treated with **35** and the cellular lysate was analyzed by RP-HPLC. A major peak (t_r = 18.1 min) corresponding to **35** was observed (Supporting Information Figure S1), supporting the supposition that the immunoactive fluorescein-labeled saponin **35** survives as an intact molecule within the cells.

CONCLUSION

The synthesis and preclinical evaluation of the tailored QS-21 derived saponins reported herein have defined key structural features that are critical for adjuvant activity and have enabled the development of the first generation of chemical probes to study saponin mechanisms of action. Through systematic truncation of the linear tetrasaccharide domain, it was discovered that an optimal balance of synthetic accessibility, toxicity, and efficacy is achieved with the corresponding trisaccharide variant **13** (SQS-0-0-5-5) (Figure 4). As a result, synthetic challenges associated with the 40-step synthesis of the initially identified carboxyacyl tetrasaccharide **10** (SQS-0-0-4-5) have been circumvented with the identification of this significantly simplified analogue **13**, which is accessed in only 26 steps. This will allow future investigations of other domains of QS-21, such as the branched trisaccharide and triterpene core, to be carried out more efficiently.

The aminoacyl trisaccharide saponin **34** has provided an efficient avenue for late-stage, chemoselective functionalization with a series of fluorophores. This led to the identification of the first adjuvant-active, fluorescently labeled saponin **35** (SQS-0-0-5-12), which is equipotent to and also less toxic than NQS-21 and SQS-21 (Figure 6). As an example of the utility of these molecules, the panel of fluorescent saponins was tracked with confocal microscopy (Figure 8), leading to the intriguing finding that only the most efficacious saponin adjuvant was internalized into dendritic cells. Further detailed investigations into the trafficking of fluorescently labeled saponin **35** and other saponin adjuvants in various cell types are warranted to assess the role of dendritic cell internalization in saponin mechanisms of action. While we have described herein the ability to decorate aminoacyl trisaccharide saponin **34** with fluorophores, a similar strategy can also be envisioned to attach radiolabels for in vivo biodistribution studies and affinity tags to identify molecular targets of saponin adjuvants in the future. Thus, the development of this new class of saponin adjuvant probes affords unique opportunities to dissect the mechanisms of action of QS-21 and other saponin adjuvants.

ASSOCIATED CONTENT

Supporting Information

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

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Notes

The authors declare the following competing financial interest(s): J. R. G., P. O. L., G. R., and D. Y. G. are founders of and have financial interests in Adjuvance Technologies, Inc.
[¶]Deceased March 22, 2011.

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