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Immunological and Toxicological Assessment of Triterpenoid Saponins Bearing Lewis-X- and QS-21-Based Trisaccharides

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The search for safer and more effective vaccine adjuvants has intensified in recent years, with triterpenoid saponins like QS-21 and its analogues emerging as promising candidates. We report the synthesis of a novel QS-21 analogue featuring betulinic acid as aglycone, a lupane-type triterpenoid with low toxicity derived from white birch bark. Two convergent synthetic routes, involving different protecting groups and glycosyl donors (bromide and trichloroacetimidate), were optimized to construct the QS-21-based linear trisaccharide motif critical for adjuvant activity. This strategy also enabled efficient preparation of the structurally similar echinocystic acid analogue reported by Gin. The immunological and toxicological profiles of these chimeric saponins, along with Lewis-X-containing and rhamnose-modified derivatives, were evaluated in C57BL/6 wild-type and hDC-SIGN transgenic mice. While the synthetic saponins exhibited low toxicity in vitro and in vivo, replacing echinocystic acid with betulinic acid reduced immunogenicity when tested with ovalbumin as a model antigen compared to alhydrogel and QS-21. These findings provide a foundation for developing saponin-based adjuvants and demonstrate the utility of advanced glycosylation strategies for synthesizing complex unnatural triterpenoid saponins.

1. Introduction

In contrast to traditional vaccination approaches based on whole live or attenuated pathogens, subunit vaccines utilize purified antigens, such as peptides or saccharides, to elicit a protective immune response.[1] While subunit vaccines represent a promising strategy for preventing life-threatening diseases, their limited immunogenicity often necessitates co-administration with adjuvants to enhance their efficacy.[2] Adjuvants are substances that, when combined with an antigen, amplify the immune response triggered by the vaccine, thereby improving protection against pathogens.[3] Historically, aluminium-based mineral salts (alum) were the only clinically approved vaccine adjuvants.[4] However, the development of new-generation subunit vaccines has accelerated the search for novel adjuvants, including oil-in-water emulsions like MF59 and adjuvant systems such as AS01 and AS04.[5]

QS-21, a natural mixture of two bidesmosidic triterpenoid saponins, i.e., QS-21-Api and QS-21-Xyl in a 65:35 ratio (Figure 1A), is obtained from the bark of *Quillaja saponaria* tree, which is endemic to South America.[6] It has emerged as a highly promising vaccine adjuvant due to its ability to effectively enhance immune responses.[7] Notably, QS-21 stimulates both humoral and cell-mediated immunity by targeting antigen-presenting cells and T cells, although its precise mechanism of action remains incompletely understood.[8] Currently, QS-21 is used in combination with monophosphoryl lipid A as a key component of the adjuvant system AS01[9] and is also incorporated into the immunostimulatory complex Matrix M.[10] These formulations have been clinically approved for use in subunit vaccines against diseases such as malaria, shingles, and COVID-19, and have been evaluated in over 120 clinical trials for various antimicrobial and antitumor vaccines.[11]

Despite its promising immunostimulatory properties, the use of QS-21 as an adjuvant is limited by several significant challenges. These include inherent toxicity, limited natural availability, heterogeneity in *Q. saponaria* extracts, presence of structurally similar congeners, and instability of its acyl chain during storage, which can lead to undesirable toxic side effects.[12] To address issues of availability and homogeneity, Gin's group achieved the total syntheses of QS-21-Api[13] and QS-21-Xyl.[14] However, the lengthy synthetic routes (exceeding 70 steps) rendered these approaches economically unfeasible. Subsequently,

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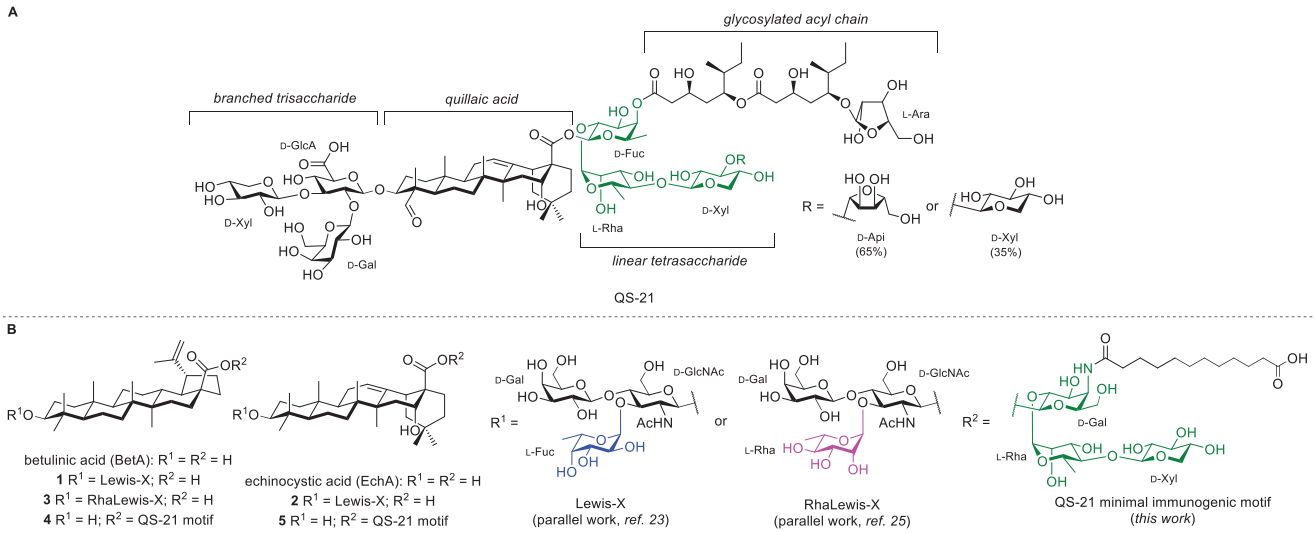


Figure 1. A) Structures of QS-21-Api and QS-21-Xyl; and B) Lewis-X- and RhaLewis-X-containing triterpenoid saponins 1–3 and QS-21-based triterpenoid saponins **4** and **5** evaluated for their toxicity and immunogenicity in this study.

the group explored structure-activity relationships of truncated synthetic variants, culminating in the development of a simplified analogue that retained adjuvant activity while reducing toxicity (compound **5**, Figure 1B).[15] Compared to QS-21, this synthetic variant lacks the branched trisaccharide at C3 and the aldehyde at C4 and features a linear trisaccharide at C28 linked to a minimalist non-glycosylated aliphatic chain containing a robust amide group, designed to enhance stability and reduce toxicity. Importantly, removing the terminal D-apiose or D-xylose, as well as replacing D-galactose with D-fucose in the linear trisaccharide of saponin **5**, did not impair its adjuvant profile.

With the recent success in engineering yeast for QS-21 biosynthesis[16] and the ongoing development of novel subunit vaccines,[2] there remains a strong impetus to discover QS-21[17] and other saponin analogues[18] with improved efficacy, stability, and safety profiles.[19] Our research group has a long-standing interest in the development of triterpenoid saponins as medicinal agents.[20] Notably, we have demonstrated that lupane-type triterpenoid saponins, which feature betulinic acid (BetA) or betulin as aglycones, exhibit significantly reduced hemolytic activity compared to their oleanane-type counterparts bearing identical sugar units.[21] This reduced toxicity, combined with the readily availability of lupane-type triterpenoids from white birch bark, an industrial waste product of the forest industry,[22] make them highly attractive aglycones for the development of QS-21-like analogues.

In our first parallel work,[23] we report the synthesis of Lewis-X-containing triterpenoid saponins featuring BetA or echinocystic acid (EchA) as aglycones (**1** and **2**, respectively, Figure 1B), EchA being the aglycone of QS-21's minimalist variant (**5**, Figure 1B).[15a] The Lewis-X trisaccharide epitope functions as a specific ligand for dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a C-type lectin expressed on the surface of immature dendritic cells that plays a critical role in pathogen recognition.[24] Remarkably, we found that triterpenoid saponins **1** and **2** are among the most potent monovalent inhibitors of DC-SIGN-mediated HIV-1 infection transfer to CD4-positive cells, exhibiting IC_{50} values in the low micromolar range against multiple viral strains.[23] In contrast, triterpenoid saponins in which rhamnose replaces fucose (**3**, Figure 1B), as described in our second parallel work,[25] showed no anti-HIV-1 activity. This underscores the essential role of the fucose residue in mediating interactions with DC-SIGN.[26]

Given the critical role of DC-SIGN in antigen internalization and signaling,[24] DC-SIGN-specific ligands,[27] particularly those bearing fucose-based epitopes,[28] represent promising candidates for the design of dendritic cell-targeted vaccines and adjuvants capable of modulating immune responses. Previous studies have demonstrated that both unconjugated Lewis-X and Lewis-X covalently linked to antigens can enhance cell-mediated protective immunity in various vaccine contexts.[29] Additionally, it has been proposed that DC-SIGN may serve as a molecular target of QS-21, owing to the presence of a fucopyranose residue within the linear tetrasaccharide at C28.[30] Consequently, there is significant interest in evaluating the immunomodulatory potential of Lewis-X containing compounds, such as triterpenoid saponins **1** and **2**, for the development of innovative antiviral immunotherapies.

Within this framework, we present an efficient, convergent, and stereoselective synthesis of a novel QS-21 minimalist variant (**4**, Figure 1B), in which EchA is replaced by BetA. Additionally, we describe the synthesis of the previously reported QS-21 variant **5**[15a] using an original approach with a distinct protecting group strategy for the preparation of the linear trisaccharide. We evaluated QS-21 in liposomes, QS-21 analogues **4** and **5**, and Lewis-X- and RhaLewis-X-containing triterpenoid saponins **1**–**3** (reported in our parallel studies)[23,25] for in vivo toxicity and immunogenicity in both C57BL/6 wild-type and/or hDC-SIGN transgenic mice, using ovalbumin (OVA) as a model antigen. Our results demonstrate that, while the synthetic saponins exhibit low in vitro and in vivo toxicity, replacing EchA with BetA has a detrimental effect on their immunogenicity profiles.

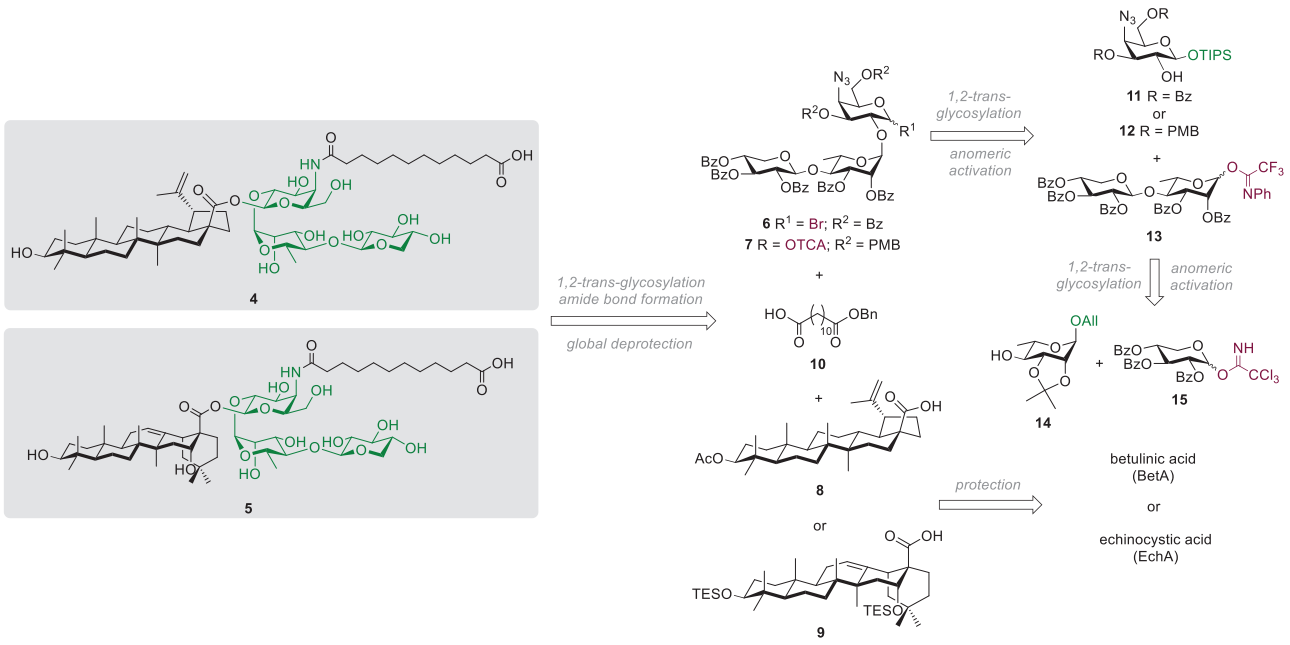
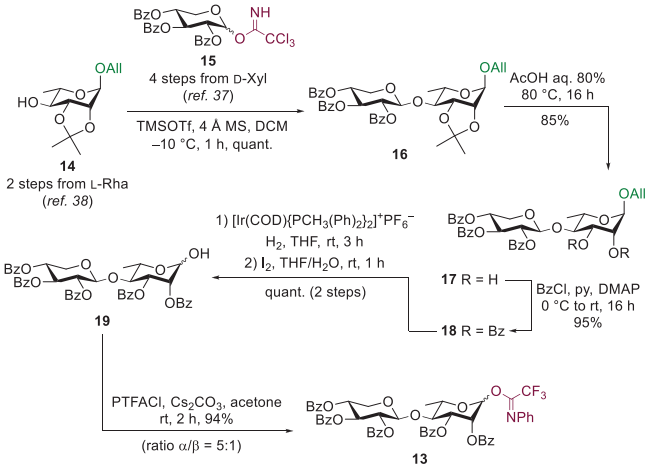


Figure 2. Retrosynthetic analysis of QS-21-based triterpenoid saponins **4** and **5**.

2. Results and Discussion

2.1. Synthetic Strategy

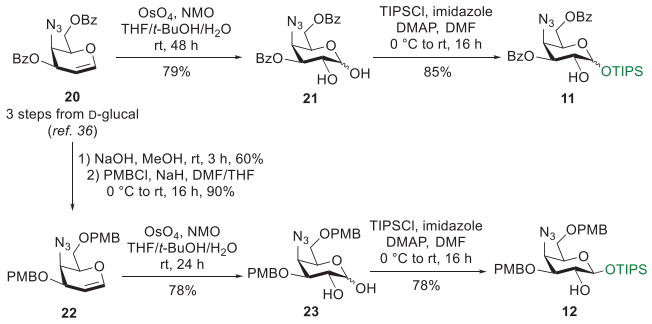
Inspired by Gin's works,[15a–c] we proposed a convergent synthetic pathway (Figure 2) involving the coupling of β-D-Xyl*p*-(1→4)-α-L-Rha*p*-(1→2)-4-azido-4-deoxy-D-Gal*p* donors **6** or **7** with triterpene acceptors **8**[31] (Scheme S2, Supporting Information) or **9**[15a] (Scheme S3, Supporting Information) to form the challenging acyl glycoside linkage at C28. This approach leverages both bromide[32] and Schmidt's trichloroacetimidate (TCA)[33] glycosylation chemistries. The aliphatic side chain at Gal-C4 would be constructed through azide reduction followed by amide formation with ester **10**.[34] To avoid reducing the double bond on the triterpenic scaffold under hydrogenolysis conditions, we opted not to use benzyl protecting groups. Additionally, strongly acidic conditions were avoided during glycosylation and deprotection steps to prevent Wagner-Meerwein rearrangement of the lupane scaffold, even in the presence of a hindered carboxylate at C28.[31,35] To address these limitations, we proposed using acetyl (Ac), benzoyl (Bz), *para*-methoxybenzyl (PMB), and triethylsilyl (TES) protecting groups, which can be removed under mild basic or acidic conditions. Trisaccharide donors **6** and **7** would be synthesized from a common β-D-Xyl*p*-(1→4)-L-Rha*p* disaccharide donor (**13**), which would be coupled to the free hydroxyl group at C2 of two distinct Gal acceptors (**11** and **12**). These two compounds would be prepared from commercially available D-glucal using an optimized procedure for a similar building block.[36] Disaccharide donor **13** would, in turn, be synthesized from known Xyl and Rha building blocks **15**[37] (Scheme S1, Supporting Information) and **14**,[38] respectively. The presence of a benzoyl group at Xyl-C2 and Rha-C2 would ensure the formation of 1,2-*trans*-glycosidic bonds through neighboring group participation.[39]



Scheme 1. Synthesis of disaccharide donor **13**.

2.2. Synthesis of Trisaccharide Donor 6 and First Attempts toward BetA Saponin 4

We began our synthesis with the preparation of the β-D-Xyl*p*-(1→4)-L-Rha*p* disaccharide (Scheme 1). First, allyl-protected 2,3-isopropylidene acceptor **14**,[38] synthesized in two steps from rhamnose, was glycosylated with perbenzoylated TCA donor **15**,[37] prepared in four steps from xylose, under TMSOTf-catalyzed conditions. This reaction afforded fully protected disaccharide **16** in quantitative yield with complete β-stereoselectivity. Next, the isopropylidene group was cleaved under acidic conditions and the resulting diol was benzoylated at positions C2 and C3 to yield fully benzoylated disaccharide **18** in 85 and 95% yields for the respective steps. Finally, the allyl group at the anomeric position was removed through iridium-catalyzed isomerization,[40] followed by iodine-promoted hydrolysis,[41] to



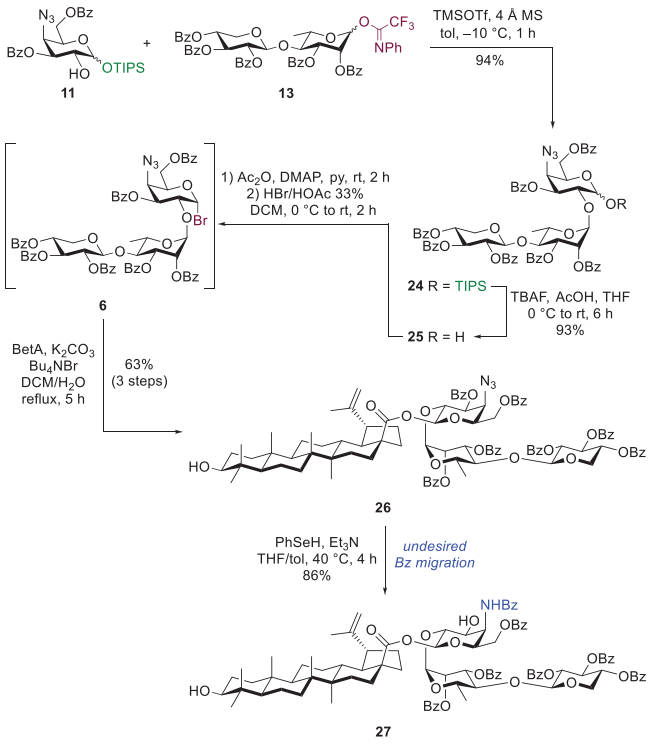
Scheme 2. Synthesis of galactose acceptors **11** and **12** from D-glucal.

provide hemiacetal **19** in quantitative yield. Activation of the anomeric position of the latter derivative as an *N*-phenyl-2,2,2-trifluoroacetimidate (PTFA)[42] furnished disaccharide donor **13** in 94% yield.

Next, we synthesized 4-azido-4-deoxy Gal acceptors **11** and **12**. To achieve this, 4-azido-3,6-di-*O*-benzoyl-4-deoxy-D-galactal (**20**) was prepared from D-glucal using a reported three-step procedure (Scheme 2).[36] Osmium-catalyzed dihydroxylation[43] in the presence of *N*-methylmorpholine *N*-oxide (NMO) as a co-oxidant afforded the corresponding 2-hydroxy hemiacetal **21** in 79% yield. Regioselective protection of the anomeric position with a triisopropylsilyl (TIPS) group provided target acceptor **11** in 85% yield. In parallel, the benzoyl groups in derivative **20** were replaced with PMB groups through saponification followed by *para*-methoxybenzylation at positions C3 and C6, affording compound **22** in 54% yield over two steps. Target Gal acceptor **12** was then obtained by dihydroxylation of the double bond and regioselective silylation at the anomeric position under the aforementioned conditions, achieving 78% yield for each step.

With all the building blocks in hand, we proceeded to synthesize the target BetA saponin (Scheme 3). Glycosylation of dibenzoylated Gal acceptor **11** with PTFA-activated disaccharide donor **13**, promoted by TMSOTf in anhydrous toluene, afforded fully protected trisaccharide **24** in 94% yield with complete α-stereoselectivity. Next, the TIPS group was removed using TBAF buffered with acetic acid, furnishing hemiacetal **25** in 93% yield. The anomeric position was then acetylated and converted to bromide donor **6** by treatment with HBr 33% in acetic acid.[44] Glycosylation of the C28 position of free BetA was achieved using the resulting crude α-bromide under phase-transfer conditions[45] in the presence of K_2CO_3 and tetrabutylammonium bromide (Bu_4NBr) in a DCM/H_2O mixture, providing fully protected saponin **26** in a convenient 63% yield over three steps. The equatorial stereochemistry of the newly formed acyl glycosidic bond was confirmed by a ^{1}H NMR experiment, which revealed a $^3J_{H1,H2}$ coupling constant of 8.1 Hz.

Our next objective was the installation of the acyl side chain. To accomplish this, the azido group of protected saponin **26** was reduced using a freshly prepared solution of benzeneselenol (PhSeH)[15a] in the presence of triethylamine at 40 °C. However, the reduction of the azido group was accompanied by the migration of the neighboring benzoyl group at position C3 to the newly formed amine group at C4, as confirmed by 1D and 2D NMR analysis. Specifically, a broad singlet at 6.67 ppm in the ^{1}H

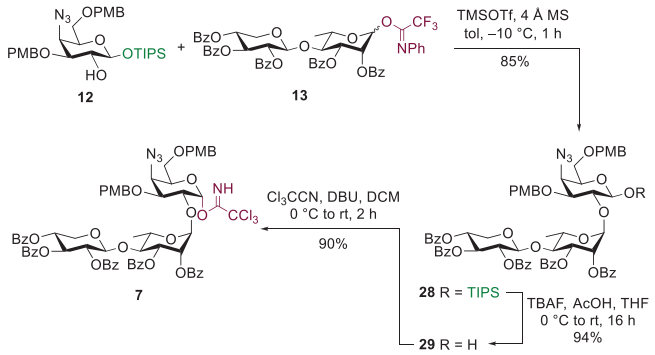


Scheme 3. Synthesis of trisaccharide bromide **6**, phase-transfer glycosylation with BetA, and unexpected benzoyl migration.

NMR spectrum indicated the formation of an undesired amide group in compound **27**. Additionally, a doublet at 3.54 ppm revealed the presence of a hydroxyl group, which exhibited a strong ^2J correlation with the proton at Gal-C3 in the COSY spectrum. Further evidence was provided by a COSY correlation between the NH group and the proton at position C4. Staudinger reaction (PPh_3 or PBu_3, THF/H_2O) was also applied to saponin **26**, but failed to provide any conversion of the azido group to the free amine. To circumvent this issue, we attempted to remove all benzoyl groups prior to the reduction step. Unfortunately, none of the reduction methods applied to the unprotected saponin proved successful in our hands (data not shown).

2.3. Synthesis of Trisaccharide Donor 7 and QS-21-Based Saponins 4 and 5

To prevent the migration of the neighbouring benzoyl group to the newly formed amino group, we focused on synthesizing a novel trisaccharide donor using a Gal acceptor bearing PMB groups instead of benzoyl groups at positions C3 and C6 (Scheme 4). Glycosylation of Gal acceptor **12** with disaccharide donor **13**, catalyzed by TMSOTf in anhydrous toluene, cleanly afforded protected trisaccharide **28** in 85% yield with complete stereoselectivity. The TIPS group at the anomeric position was then removed using TBAF/HOAc and the resulting hemiacetal was activated as a TCA donor by treatment with trichloroacetonitrile in the presence of DBU, providing trisaccharide donor **7** in 85% yield over two steps. Our initial approach involved coupling a trisaccharide bromide with BetA using our



Scheme 4. Synthesis of TCA trisaccharide donor **7**.

previously optimized three-step procedure. However, the acid labile PMB groups interfered with the bromination step, necessitating the development of an alternative strategy using a TCA donor instead of a bromine one.

With the second-generation trisaccharide donor in hand, we proceeded to perform the glycosylation at the C28 position of the triterpene acceptors (Scheme 5). The challenging coupling reaction was carried out using an excess of 3-*O*-acetyl betulinic acid (**8**)[31] or 3,16-di-*O*-triethylsilyl echinocystic acid (**9**)[15a] as glycosyl acceptors, promoted by $BF_3{\cdot}Et_2O$ in anhydrous toluene.[15a] Under these conditions, fully protected saponins **30** and **31** were obtained in 77% yields. The β-configuration of the glycosidic bond was confirmed by ^{1}H NMR spectroscopy, which revealed $^3J_{H1,H2}$ coupling constants of 8.3 Hz for saponin **30** and 7.7 Hz for saponin **31**, corresponding to the anomeric proton of the Gal residue.

With fully protected saponins in hand, we turned our attention to introducing the side chain. To this end, saponin derivatives **30** and **31** were subjected to azido group reduction using PhSeH. Gratifyingly, no migration was observed, as evidenced by the absence of amide or hydroxy signals in the NMR spectra. After purification, this procedure provided the corresponding amino-containing BetA and EchA saponins in 86 and 54% (87% brsm) yields, respectively. The side chain was then installed by coupling the corresponding amino-containing saponins with dodecanedioic acid benzyl ester **10**[34] in the presence of *N*,*N*′-dicyclohexylcarbodiimide (DCC) and DMAP, furnishing protected derivatives **32** and **33** in 83 and 91% yields, respectively.

We then proceeded to carry out the final deprotection sequence to obtain our target compounds. First, BetA saponin derivative **32** was treated with ceric ammonium nitrate (CAN) in acetonitrile/H_2O to remove the PMB groups. Next, the ester groups were cleaved by reaction with NaOH in a THF/MeOH/H_2O mixture, furnishing target BetA saponin **4** in 69% yield over two steps. Notably, the acyl glycosidic bond at C28 remained intact during the saponification step, consistent with our previous observations for the synthesis of BetA saponins.[45]

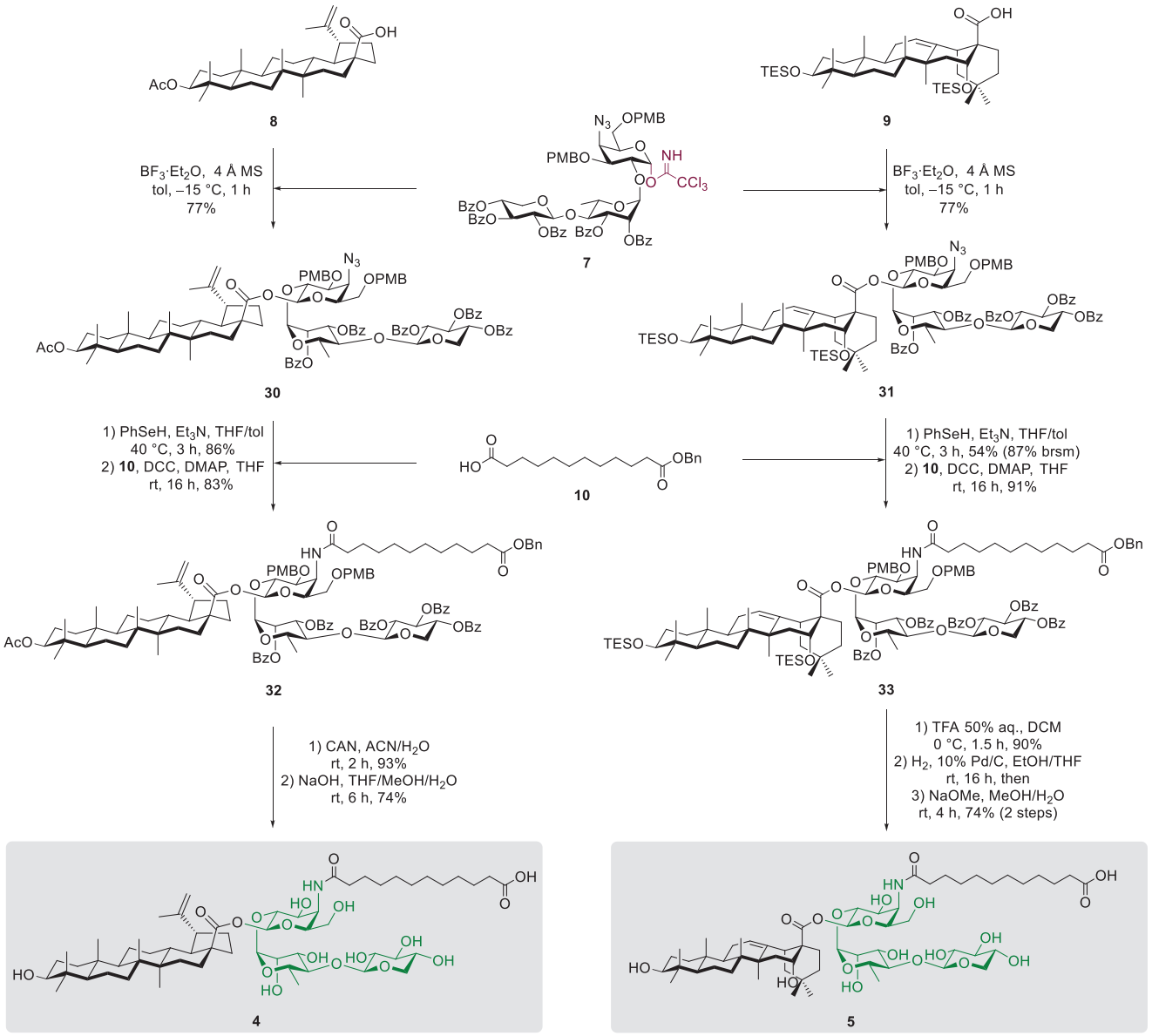
For the final deprotection to access known EchA saponin **5**,[15a] a slightly different approach was employed. First, the PMB and TES groups of derivative **33** were removed using TFA in DCM at 0 °C, affording the corresponding tetraol in 90% yield. To prevent undesired cleavage of the more labile ester bond, as observed for EchA in our parallel work,[23] we applied a deprotection sequence developed by Gin and co-workers.[15a] The terminal benzyl ester group on the side chain was firstly cleaved by hydrogenolysis using 10% Pd/C as the catalyst, followed by removal of the remaining ester protecting groups via Zemplén deacetylation (NaOMe in MeOH/H_2O). Under these conditions, target EchA saponin **5** was obtained in 74% over two deprotection steps.

2.4. Toxicological and Immunological Evaluation of Saponins

Prior to conducting the in vivo toxicological and immunological studies in mice, synthetic saponins **1**–**5** were solubilized in Dulbecco's phosphate buffered saline (DPBS) containing 5.0% DMSO (see SI for details). To rule out any potential interference from bacterial lipopolysaccharide (LPS, endotoxin) contamination in the synthetic samples, the LPS levels were quantified using an endotoxin quantification test based on the Limulus amebocyte lysate (LAL) assay. The results confirmed that the endotoxin levels in the saponin solutions were below 5.0 EU·kg^{-1} of mouse body weight per administered dose (Figure S1, Supporting Information), thus meeting the recommended safety limit for subcutaneous injection in mice.[46]

As an in vitro marker of toxicity, the hemolytic activity of the synthetic saponins, along with QS-21 formulated with or without liposomes, was measured using sheep erythrocytes[21] at concentrations reflecting the doses administered in the in vivo experiments (Figure 3A; Table S1, Supporting Information). Notably, our results revealed that QS-21-based BetA saponin **4** exhibited no significant hemolytic activity at a concentration of 0.080 mg·mL^{-1}, in contrast to the structurally similar EchA saponin **5** and QS-21. Furthermore, no significant hemolytic activity was observed for the Lewis-X-containing saponins **1** and **2**, as well as the rhamnose analogue **3**. Our findings also demonstrated that formulating QS-21 into liposomes significantly reduced its hemolytic activity toward sheep erythrocytes. For this reason, only the liposomal formulation of QS-21 was used in the in vivo studies.

To evaluate the toxicological impact of immunization with synthetic saponins **1**–**5** on the overall health of mice, weight variations and clinical symptoms (Table S3, Supporting Information) were monitored at various time points following each administration. Notably, as shown in Figure 3C,D, no significant weight loss, adverse clinical signs (e.g., changes in activity level, fur condition, posture, breathing, or eye appearance), or inflammation at the injection site were observed after the administration of saponins **1**–**5**. In contrast, and consistent with in vitro hemolytic assay results (Figure 3A), QS-21 formulated in liposomes induced significant weight loss and clinical signs of toxicity, such as reduced activity and visible discomfort, after each immunization. These findings highlight the potential toxicity associated with QS-21. Collectively, these toxicological data demonstrate the safety of synthetic saponins **1**–**5** at the tested doses, underscoring their potential for further preclinical development. This is particularly promising for Lewis-X-containing saponins **1** and **2**, which have been shown to exhibit anti-HIV-1 activity in our parallel work.[23]



Scheme 5. Successful synthetic routes for the preparation of QS-21-based triterpenoid saponins **4** and **5**.

As no suitable in vitro assays currently exist to determine adjuvant activity, the synthetic saponins **1**–**5** were directly evaluated in vivo for their immunological (Figure 4) profiles. In this in vivo study, groups of C57BL/6 wild-type mice and hDC-SIGN transgenic mice (only for saponins **1**–**3**), both male and female aged 8–14 weeks, were immunized with the model antigen OVA (40 µg/dose) either alone (negative control) or in combination with adjuvants. Alhydrogel 2%, QS-21 formulated in liposomes, and the previously reported synthetic EchA saponin **5**,[15a] which contains QS-21′s minimal immunogenic motifs required for adjuvant activity, were used as positive controls. The mice were immunized every two weeks (days 0, 14, and 28) with synthetic saponins **1**–**5** (40 µg/dose, administered via subcutaneous injection) or QS-21 in liposomes (18.5 µg/dose, administered via intramuscular injection) (see Table S2, Supporting Information, for details of vaccine formulations). Blood samples were collected 35 days after the first immunization. The production of OVA-specific immunoglobulin antibodies (IgG1) was assessed using serially diluted mouse sera by titration on OVA-coated enzyme-linked immunosorbent assay (ELISA) plates at days 0, 14, 28, and 35 (Figures S7 and S8, Supporting Information). Specific antibody detection was performed using IgG1 conjugated to horseradish peroxidase, with absorbance measured at 490 nm. A significant serum IgG response was detected in all groups injected with OVA by day 35 after the initial immunization, as shown in Figure 4A–D. Additionally, spleens were harvested on day 35 to analyze the cellular response elicited by the tested adjuvants using flow cytometry. Briefly, splenocytes were isolated and processed using two distinct methods. One portion was directly stained for viability and surface markers to analyze T cell proportions (Table S4, Supporting Information). The other was first stimulated with SIINFEKL peptide or PMA/ionomycin,

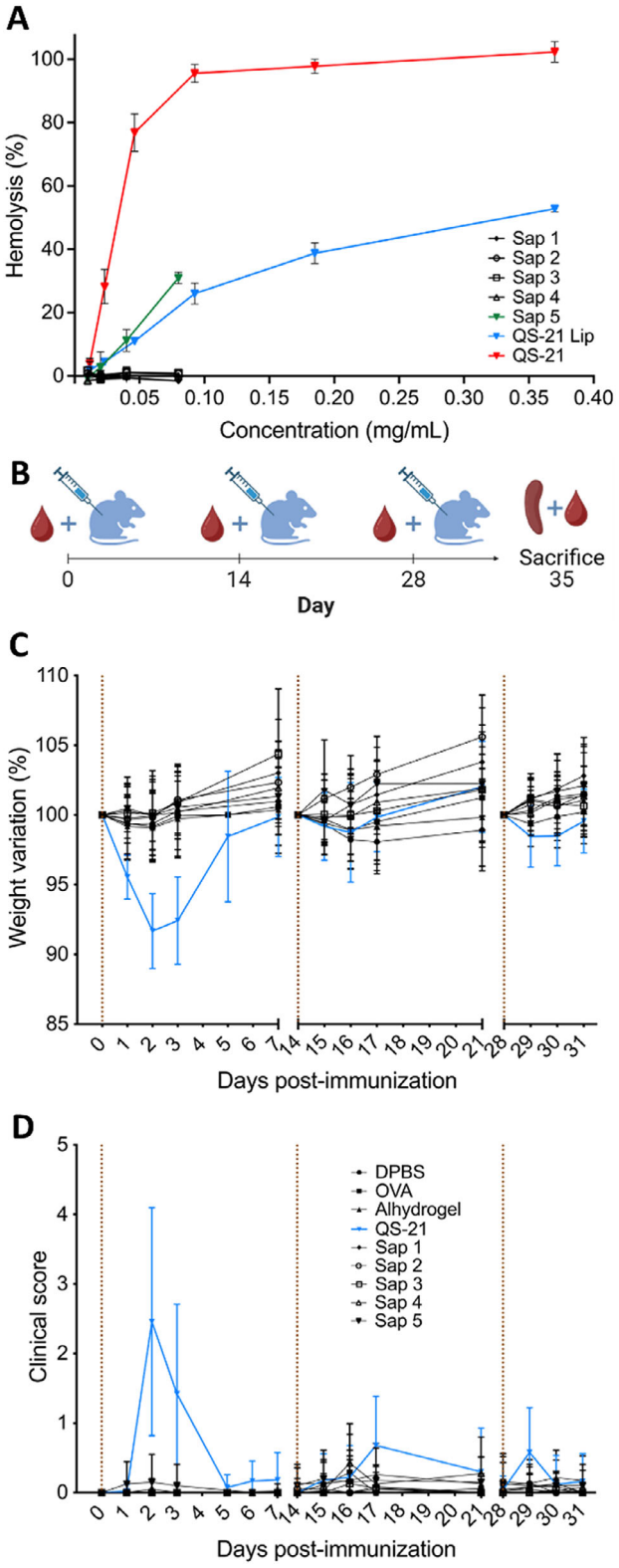


Figure 3. Evaluation of synthetic saponins **1**–**5** safety and their minimal effects on mouse health. A) Hemolytic activity of QS-21 and synthetic saponins **1**–**5** on sheep erythrocytes. Data are presented as the mean ± SD from at least two independent experiments. B) Immunization and blood sampling timeline (created with BioRender.com). C) Mouse body weight was measured at different time points following each immunization. D) Clinical signs observed in mice were compiled at the same time points. Data are presented as the mean ± SD from at least two independent experiments (n = 18–26).

then stained as before, followed by intracellular staining to assess CD4 + and CD8 + T cells (Figures S2–S4, Supporting Information).

As shown in Figure 4A–D, the IgG1 antibody responses in mice immunized with synthetic triterpenoid saponins **1**–**5**, bearing either a Lewis-X-containing trisaccharide (**1** and **2**), a rhamnose-modified Lewis-X trisaccharide (**3**), or QS-21′s minimal immunogenic motifs (**4** and **5**), were unfortunately lower than those observed in mice immunized with QS-21. Overall, the levels of IgG1 antibodies for saponins **1**–**5** were not significantly higher than those in the control group injected with OVA alone. Furthermore, no significant differences in antibody responses were observed when comparing wild-type mice to hDC-SIGN transgenic mice immunized with saponins **1**–**3**. Analysis of splenic T cell activation at day 35 revealed that only the clinically used QS-21 adjuvant could induce a robust CD8 + T cell activation compared to the control groups (Figure 4E–H; Figures S2–S4, Supporting Information). Notably, these preliminary in vivo data indicate that replacing EchA with BetA as the aglycone reduces immunogenicity when tested with OVA as a model antigen, particularly in comparison to established adjuvants like alhydrogel and QS-21. Additionally, the presence of the Lewis-X antigen at the C3 position of EchA does not appear sufficient to elicit robust IgG production in hDC-SIGN transgenic mice. These findings align with prior studies suggesting that the absence of the C16 hydroxyl group[15a] and/or conformational constraints[17c] of triterpenoid saponins may impair their adjuvant activity. Specifically, saponin **4**, which incorporates BetA as its aglycone, demonstrate markedly reduced adjuvant activity, likely due to these structural limitations. It is also possible that higher injection doses of the synthetic saponins could have yield better immunological results. However, the mice were administered the highest concentrations of the synthetic triterpenoid saponins within their solubility limits in aqueous buffer (40 µg/500 µL/dose).

3. Conclusion

This study describes the convergent synthesis of BetA saponin **4**, featuring QS-21′s minimal trisaccharide motif at the C28 position. Two distinct glycosylation strategies and protecting group approaches were employed to construct the monodesmosidic lupane-type scaffold. In the first synthetic route, an in situ-formed bromide trisaccharide donor was utilized in a phase-transfer glycosylation, affording the target compound in good overall yield (63% over three steps). However, during the reduction of the azido functionality, an unexpected benzoyl migration occurred, shifting from the C3 hydroxyl to the C4 amine of the 4-deoxygalactose residue. To address this issue, a second synthetic route was developed, employing $BF_3{\cdot}OEt_2$-mediated Schmidt glycosylation and incorporating PMB instead of benzoyl groups. This approach successfully yielded both the target QS-21-based BetA saponin **4** and the previously reported, structurally similar EchA saponin **5** following global deprotection.

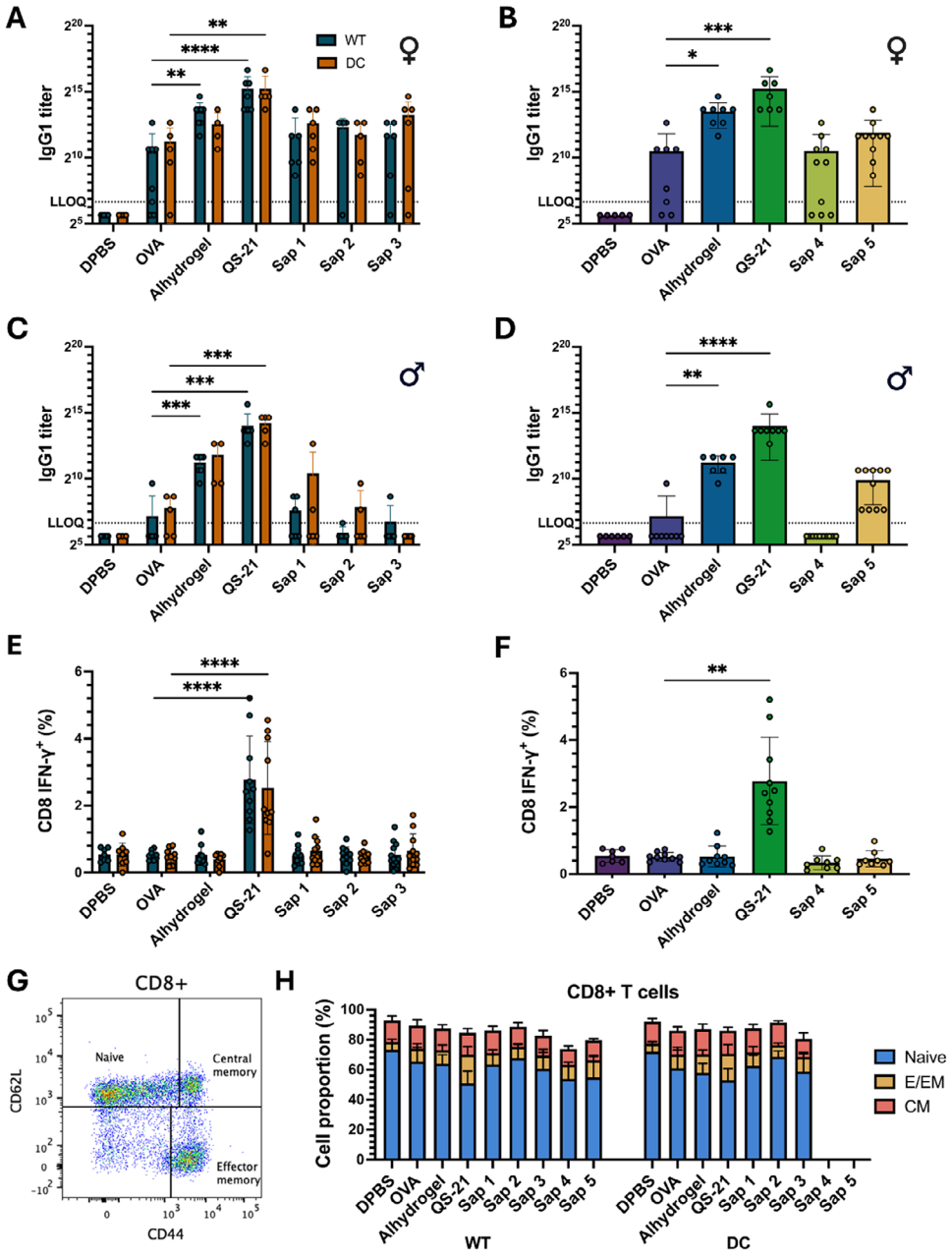


Figure 4. Effects of immunization with synthetic saponins **1**–**5** and QS-21 on the humoral and cellular immune responses. A–D) IgG1 titers in serum samples were measured in (A, B) female and (C, D) male mice 35 days after the first immunization. Data are presented as the mean ± SD from at least two independent experiments (n = 5–10). E,F) The proportion of IFN-γ+ CD8 + T cells after direct ex vivo stimulation with SIINFEKL peptide, and G,H) unstimulated CD8 + T cells memory populations based on CD44 and CD62L expression, were also measured. Mice were immunized via subcutaneous or intramuscular injection (see Figure 3B for timeline) with OVA, either alone or with adjuvants (alhydrogel, QS-21 in liposomes or synthetic saponins **1**–**5**). See Table S2 (Supporting Information) for details regarding the quantities, injection volumes, and administration routes. Data are presented as the mean ± SD (n = 7–12, including both male and female mice), and gating strategies are detailed in Figures S5 and S6 (Supporting Information). A,C,E) Statistical analysis was performed using ARTool,[47] followed by ART-C post hoc pairwise comparisons[48] using R software.[49] B,D,F) Statistical analysis was performed by comparing each group to the OVA group using the Kruskal–Wallis test, followed by post-hoc Dunn's tests using GraphPad Prism 10 software. Significance levels are indicated as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$. CM: central memory T cells. E/EM: effector T cells/memory T cells. LLOQ: lower limit of quantitation. WT: C57BL/6 wild-type mice. DC: hDC-SIGN transgenic mice.

The immunological and toxicological properties of these QS-21-based saponins, along with Lewis-X-containing saponins **1**–**3**, were evaluated in vivo in both wild-type and hDC-SIGN transgenic mice. While the synthetic saponins exhibited low toxicity, replacing EchA with BetA significantly reduced immunogenicity when tested with OVA as a model antigen, particularly in comparison to benchmark adjuvants such as alhydrogel and QS-21. These findings underscore the critical importance of preserving the oleanane-type scaffold with a hydroxyl group at the C16 position for optimal adjuvant activity.

Collectively, these results, together with those reported in our parallel studies, provide a foundation for the development

of DC-SIGN-targeted "antiviral adjuvants". For instance, an EchA bidesmosidic saponin bearing both the Lewis-X trisaccharide and QS-21's minimal immunogenic motifs could serve as a promising candidate for this purpose. Efforts toward the synthesis and biological evaluation of this bidesmosidic EchA saponin are currently underway in our research group.

Supporting Information

Synthetic procedures, experimental data, additional schemes, figures, tables, and NMR spectra (Figures S9–S117, Supporting Information) for all new compounds. This material is available free of charge via the internet. The authors have cited additional references within the Supporting Information.[50]

Author Contributions

The manuscript was written with contributions from all authors. All authors have approved the final version of the manuscript. Conceptualization (O.J.G.M. and C.G.), formal analysis (O.J.G.M., N.V., Y.A.-B., T.C., and C.G.), funding acquisition (A.P., A.L., and C.G), investigation (O.J.G.M., Y.A.-B., B.S., N.V., T.C., M.H., and G.L.), project administration (A.L. and C.G.), supervision (A.P., A.L., and C.G.), writing – original draft preparation (O.J.G.M., Y.A.-B., and C.G.), writing – review and editing (O.J.G.M., Y.A.-B., B.S., N.V., T.C., A.P., A.L., and C.G.).

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Conflict of Interests

The authors declare no competing financial interests.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: immunogenicity · Lewis-x trisaccharide · QS-21 analogues · toxicity · triterpenoid saponins

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