Conference paper

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Design, synthesis and evaluation of optimized saponin variants derived from the vaccine adjuvant QS-21

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Abstract: The saponin natural product QS-21 is one of the most potent investigational adjuvants, which are substances added to vaccines to enhance the immunogenicity of the antigen and potentiate the immune response. While QS-21 has been coadministered with vaccines against cancers and infectious diseases in many clinical trials, its inherent liabilities (scarcity, heterogeneity, instability, and dose-limiting toxicity) have limited its widespread clinical use. Furthermore, its molecular mechanisms of action are poorly understood. Structural modification of the natural product using chemical synthesis has become an important strategy to overcome these limitations. This review focuses mainly on research efforts in the group of the late Professor David Y. Gin on the development of optimized synthetic saponin adjuvants derived from QS-21. A number of QS-21 variants incorporating stable acyl chain amide linkages, truncated carbohydrate domains, and targeted modifications at the triterpene and central glycosyl ester linkage were designed, chemically synthesized, and immunologically evaluated. These studies delineated key minimal structural requirements for adjuvant activity, established correlations between saponin conformation and activity, and provided improved, synthetically accessible saponin adjuvants. Moreover, leveraging these structure–activity relationships, novel saponin probes with high potency and reduced toxicity were developed and used in biodistribution and fluorescence imaging studies, yielding early insights into their enigmatic mechanisms of action.

Keywords: adjuvant activity; biological activity; carbohydrates; chemical synthesis; ICS-28; medicinal chemistry; organic chemistry; structure-activity.

Introduction

The clinical success of anticancer and antiviral subunit vaccines based on well-defined antigens relies largely on an adjuvant, a substance that enhances their immunogenicity and, in turn, the immune response [1]. Unfortunately, only a handful of sufficiently potent adjuvants with low toxicity are available for human vaccination [2]. Among these, the promising saponin adjuvant QS-21, isolated from the cortex of the *Quillaja* Saponaria (QS) tree, has been employed in a number of vaccine clinical trials against cancers and infectious diseases. However, apart from two recent exceptions, wider clinical advancement has not been fully realized due to several drawbacks intrinsic to the natural product [3], including difficulty in isolation and purification to homogeneity, structural lability and toxic side effects. Moreover, the mechanisms of action of QS-21 have not been fully elucidated; partly due to the lack of tools to better investigate its immune-potentiating effects.

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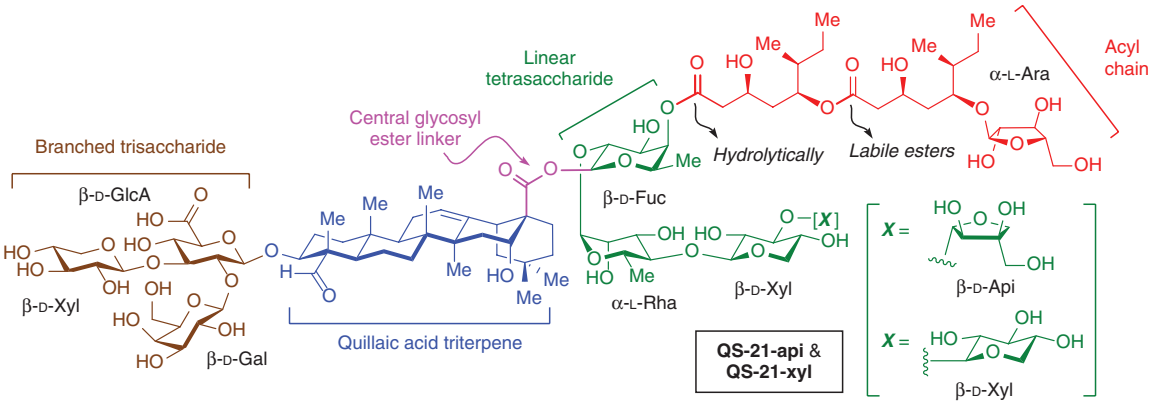


Fig. 1: Chemical structure of QS-21 saponin natural product (≈65 % QS-21-apiose; ≈35 % QS-21-xylose) with its four principal domains.

As a consequence, the development of new saponin adjuvants with improved properties that overcome the inherent issues of QS-21 is urgently needed [4].

Structurally, QS-21 is a mixture of isomers comprising four principal domains: a branched trisaccharide, a central triterpene core (quillaic acid), a linear tetrasaccharide terminating in either apiose (**QS-21-api**) or xylose (**QS-21-xyl**), and a glycosylated diester acyl chain (Fig. 1). Some strategies to address the aforementioned challenges have involved chemical derivatization of natural QS-21 in hopes to modulate its efficacy. However, the complexity and sensitivity of the saponin diverse functionalities limit the chemistry that can be performed on the parent compound, providing a narrow window for analog generation and structure/activity studies [5, 6]. One notable example of this derivatization strategy is the semisynthetic saponin adjuvant GPI-0100, which was prepared from QS-bark extracts via hydrolysis of the saponin acyl chain followed by amidation with dodecylamine at the branched trisaccharide glucuronic acid [6]. A more attractive approach to develop improved saponin variants is through structural modification of the QS-21 molecule with exquisite chemical control using synthetic organic chemistry. Advances on this front have capitalized on the original total synthesis by Gin and coworkers of both isomers of QS-21 (**QS-21-api** [7] and **QS-21-xyl** [8]) as well as on their subsequent semisynthetic strategy [9], by which the entire branched trisaccharide–triterpene half of the molecule can be obtained by chemical degradation of QS-extracts followed by selective protection. This semisynthetic technology enabled more dependable access to homogeneous QS-21 and more streamlined preparation of systematically designed QS-21 variants with increased stability, potent adjuvant activity and reduced toxicity. Moreover, it also opened the door to the development of novel saponin probes for investigation into their mechanisms of action [4]. Herein, I summarize these efforts on the design, synthesis, and evaluation of optimized QS saponin adjuvants [10], with particular emphasis on my research studies in the laboratory of Prof. David Y. Gin.

Synthesis and evaluation of optimized QS saponin adjuvants

First-generation of stable amide-derived acyl chain variants

In the Gin group, the chemical instability of QS-21, which stems primarily from its hydrolytically labile acyl-fucose ester linkage, was first addressed by developing unnatural amide-derived acyl chain variants incorporating more robust amide groups instead of the unstable native esters [11]. This modification involved re-working of the linear tetrasaccharide to include a suitably protected “fucose surrogate” bearing an *N*-functionality at the C4-position, on which to anchor a variety of acyl chains through an amide linkage by chemoselective *N*-acylation. The first series of acyl chain analogs prepared by Gin and coworkers started with the most conservative variant SQS-0101 as a direct isosteric mimic of the natural acyl chain, except for the central ester-to-amide replacement (Fig. 2). More profound structural changes took the form of **SQS-**

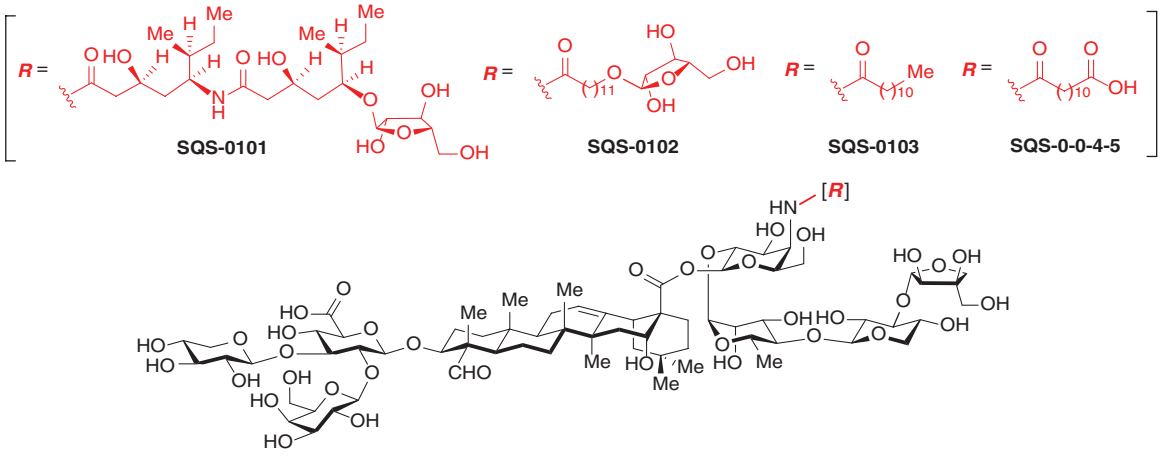


Fig. 2: Structure of first-generation, chemically stable, acyl chain amide variants developed by Gin and coworkers.

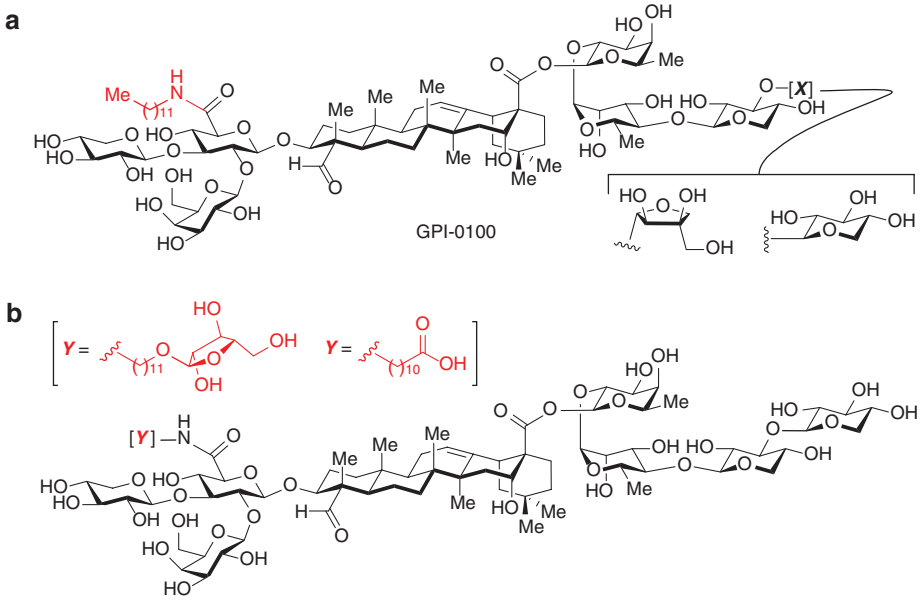


Fig. 3: (a) Structure of immunoactive isomeric constituents of GPI-0100 and (b) terminal-functionalized acyl chain analogs of GPI-0100 synthesized by Wang et al. [14].

0102, **SQS-0103**, and **SQS-0-0-4-5**, which incorporated simplified aliphatic acyl chains, either glycosylated (**SQS-0102**) or non-glycosylated (**SQS-0103** and **SQS-0-0-4-5**), the latter bearing a terminal carboxyl group to improve water solubility [12]. Immunological evaluation in mice of the stable saponin variants with the GD3–KLH melanoma conjugate vaccine demonstrated adjuvant activities comparable to QS-21, as assessed by measuring antibody responses by ELISA 1 week after booster injection (day 72). Moreover, while **SQS-0102** elicited considerably increased toxicity, as judged by mouse weight loss, all other QS saponins were significantly less toxic than QS-21, with only minor (**SQS-0101**) or no weight loss (**SQS-0103** and **SQS-0-0-4-5**) observed in these cases.

Drawing inspiration from the early semisynthesis of the heterogeneous GPI-0100 adjuvant by Marciani et al. [6], Wang and co-workers recently synthesized a number of saponin variants [13, 14], in which the hydrolytically labile native QS-21 acyl chain was replaced by simplified acyl chains incorporated at the branched trisaccharide glucuronic acid through an amide linkage (Fig. 3). By analogy to the Gin lab studies above, the introduction of polar groups (carboxylic acid and arabinose monosaccharide) at the terminus of the

otherwise lipophilic dodecylamide chain, increased the water-solubility and adjuvant activity of the variants to levels comparable with GPI-0100 [14].

Carbohydrate variants via systematic truncation of the QS linear tetrasaccharide

Based on the stable, simplified carboxylic-acid acyl chain variant **SQS-0-0-4-5**, which showed potent adjuvant activity and low toxicity, Gin and coworkers next investigated the minimal structural requirement of the linear tetrasaccharide for adjuvant activity [12]. Thus, we prepared additional simpler structures involving systematic truncation of this carbohydrate domain, providing trisaccharide variant **SQS-0-0-5-5**, disaccharide variant **SQS-0-0-6-5**, and monosaccharide variant **SQS-0-0-9-5** (Fig. 4). The synthesis of the linear

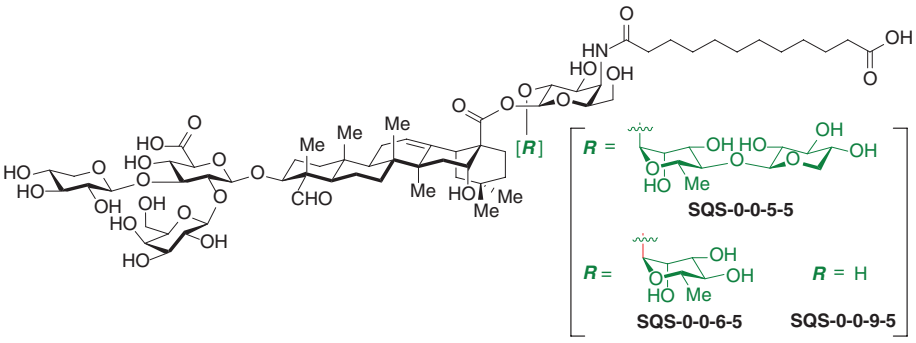
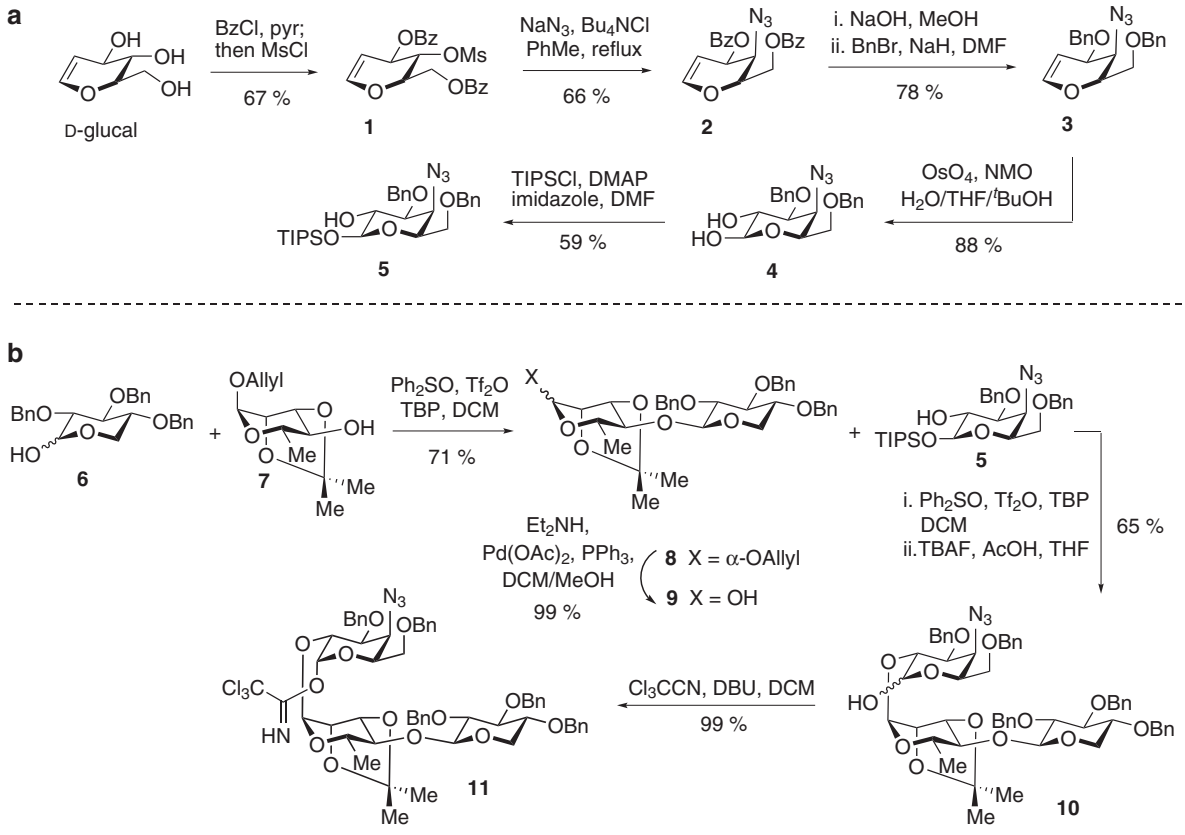
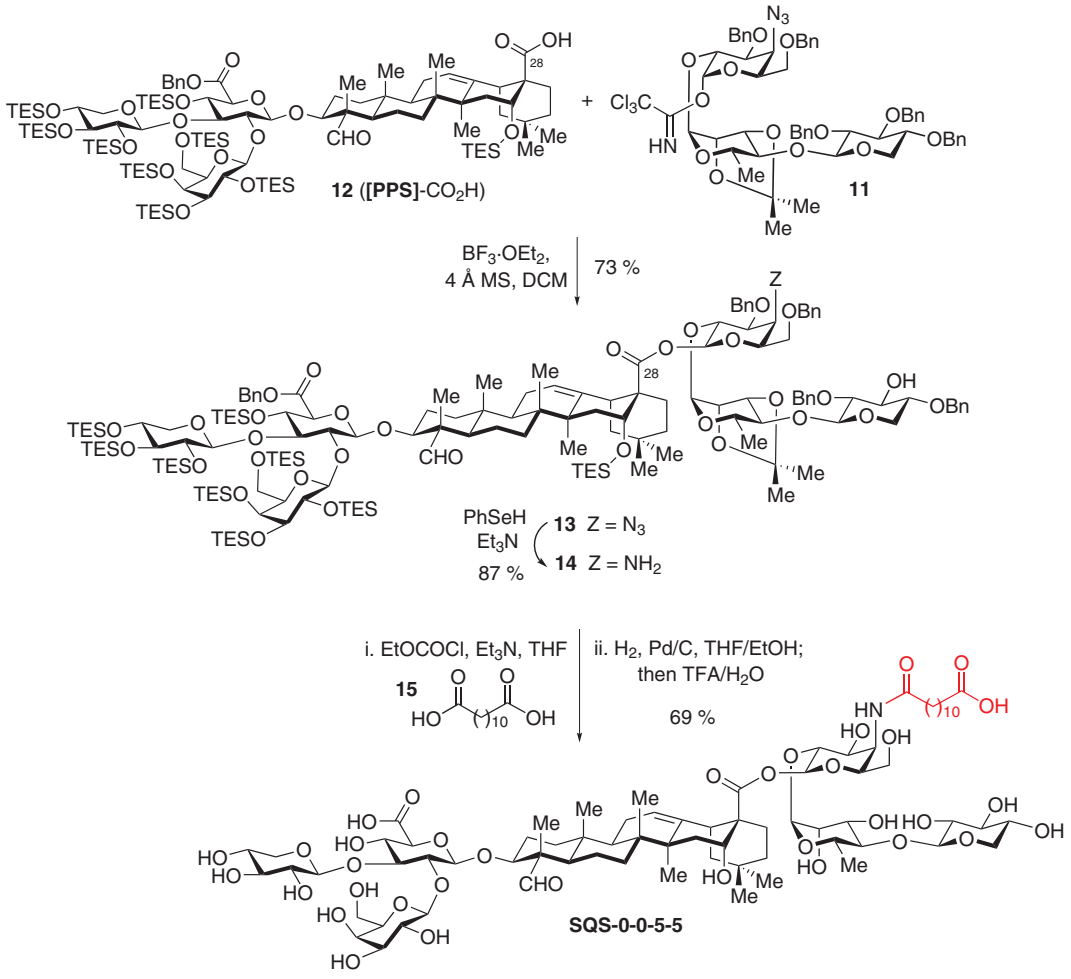


Fig. 4: Structures of carbohydrate-truncated variants with systematic truncation of the linear tetrasaccharide.



Scheme 1: (a) Synthesis of fucose surrogate C4-deoxy-C4-azidogalactopyranoside **5**. (b) Synthesis of modified linear trisaccharide donor (**11**) devoid of the fourth sugar residue.



Scheme 2: Synthesis of the most elaborate of the carbohydrate-truncated analogs: linear trisaccharide variant **SQS-0-0-5-5** with a single-sugar truncation.

trisaccharide itself devoid of the fourth apiose sugar (as trichloroacetimidate donor, **11**) was accomplished in 16 steps (Scheme 1b) using a double dehydrative glycosylation procedure [15] starting from conveniently protected xylose and rhamnose monosaccharides and the previously prepared fucose surrogate, 4-azido-4-deoxygalactose **5**, synthesized in five steps from D-glucal (Scheme 1a). The protected prosapogenin **12** (**PPS**-CO_2H), obtained by semisynthesis from QS-extracts [9], was glycosylated with the trisaccharide imidate **11** ($BF_3 \cdot OEt_2$), and the corresponding intermediate **13** was subjected to phenylselenol reduction to arrive at amine **14** (Scheme 2). The last steps to **SQS-0-0-5-5** bearing a single sugar truncation involved acylation of the amine with dodecanedioic acid monobenzyl ester, followed by global deprotection via hydrogenolysis (H_2, Pd/C) and acid hydrolysis (TFA/H_2O) and subsequent HPLC purification. The disaccharide and monosaccharide variants (**SQS-0-0-6-5** and **SQS-0-0-9-5**) were prepared in a similar fashion from the corresponding protected carbohydrates.

Preclinical studies to evaluate the adjuvant activity of these saponins (20 μg) [12] involved mouse vaccination (groups of five mice) with the QS variant of interest and a four-component vaccine containing OVA (ovalbumin, a reliable immunogen that induces antibody responses), MUC1–KLH (MUC1: prostate, breast cancer peptide antigen, non-glycosylated tandem repeat domain), and GD3–KLH (GD3: melanoma, sarcoma glycolipid antigen, conjugated to the highly immunogenic protein KLH). Trisaccharide variant **SQS-0-0-5-5** elicited high antibody titers, similar to QS-21, whereas progressively weaker responses were observed with each carbohydrate truncation in disaccharide and monosaccharide variants **SQS-0-0-6-5** and **SQS-0-0-9-5**, respectively (Fig. 5). The toxicity of these saponins was assessed in terms of mouse weight loss. While

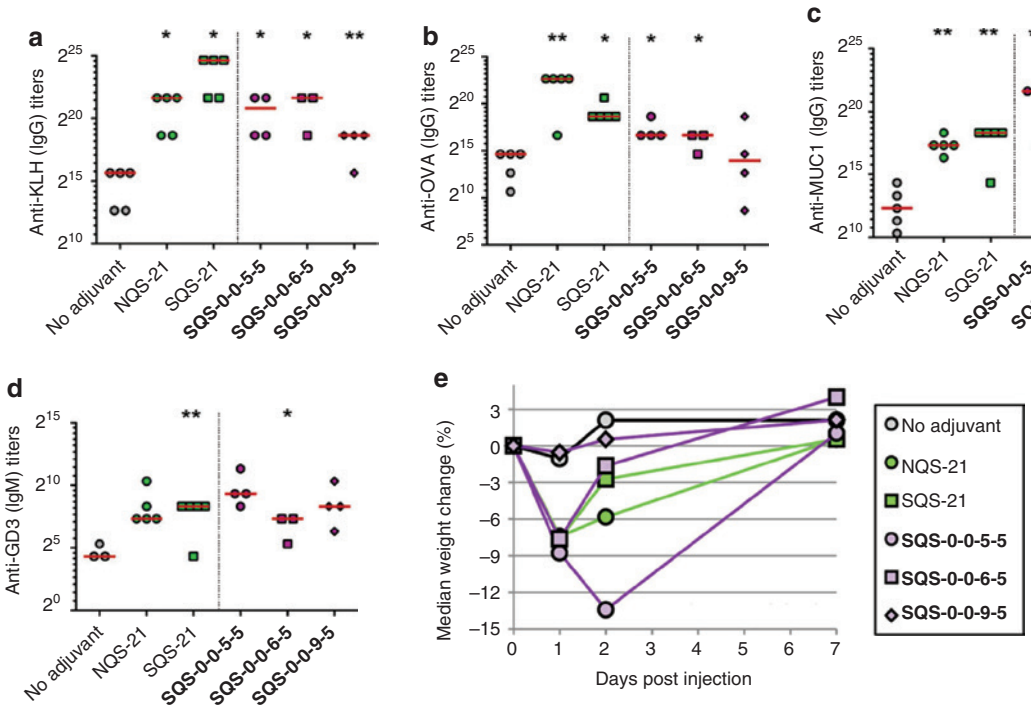


Fig. 5: Immunological evaluation of linear tetrasaccharide truncation variants **SQS-0-0-5-5**, **SQS-0-0-6-5**, and **SQS-0-0-9-5** (20 μg) with four-component vaccine of OVA (20 μg), MUC1–KLH (2.5 μg), GD3–KLH (10 μg). Adapted from Ref. [12]. Antibody titers after three biweekly vaccinations and booster against (a) KLH (IgG), (b) OVA (IgG), (c) MUC1 (IgG), and (d) GD3 (IgM). Horizontal bars represent median values of titers; statistical significance versus No Adjuvant control: *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. (e) Toxicity assessment based on median weight loss over 1 week after the first vaccination. NQS-21 = natural QS-21; SQS-21 = synthetic QS-21.

monosaccharide **SQS-0-0-9-5** was the least toxic (one dead mouse, and no weight loss observed), two mice died when administered with disaccharide variant **SQS-0-0-6-5** (–8 % weight change on day 1) and one mouse died with **SQS-0-0-5-5**, which also induced considerable weight loss (9 % on day 1, 13 % on day 2) (Fig. 5e). Although the toxicity issue was not completely resolved, trisaccharide variant **SQS-0-0-5-5** showed the best adjuvant activity within this group, comparable to QS-21, and was also more synthetically accessible (24 steps) than tetrasaccharide variant **SQS-0-0-4-5** (36 steps). This simplification opened the door to develop additional QS saponins with further structural variations, e.g. at the junction between the triterpene and linear oligosaccharide domains.

Central glycosidic linkage variants with modified linker distance, stereochemistry and flexibility

Walkowicz et al. next explored modifications of length, stereochemistry, and flexibility in the central glycosyl ester linkage, and examined their effects on adjuvant activity and toxicity with the synthesis and evaluation of several linker variants [16]: β-ethanolamide **SQS-0-4-5-5**, β-carbamate **SQS-0-5-5-5**, and β-thioester **SQS-0-13-5-5**; α-ester **SQS-0-0-8-5**, α-amide **SQS-0-6-8-5**, and α-carbamate **SQS-0-5-8-5**; and β-ether **SQS-0-12-5-5**, and β-thioether **SQS-0-14-5-5**, respectively (Fig. 6). The syntheses of these variants proved challenging due to the sterically demanding glycosyl bond formation required to join the prosapogenin and oligosaccharide domains together in the context of the complex saponin scaffold. The corresponding glycosyl acceptors were synthesized by functionalization of the protected prosapogenin **12** (**PPS**-CO_2H) at its C28-carboxylic acid (Scheme 3) and then coupled to the trisaccharide glycosyl donors using two different strategies, with the glycosyl donor acting as an electrophile in a traditional glycosylation reaction (Scheme 4a), or as a nucleophile

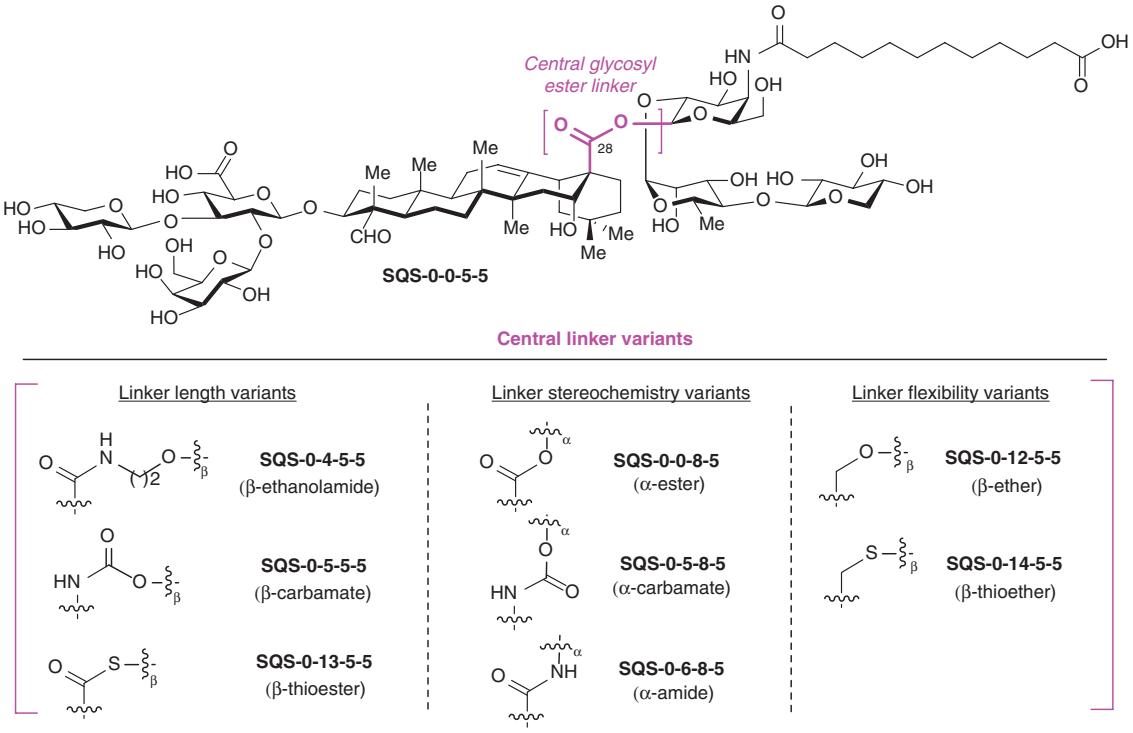
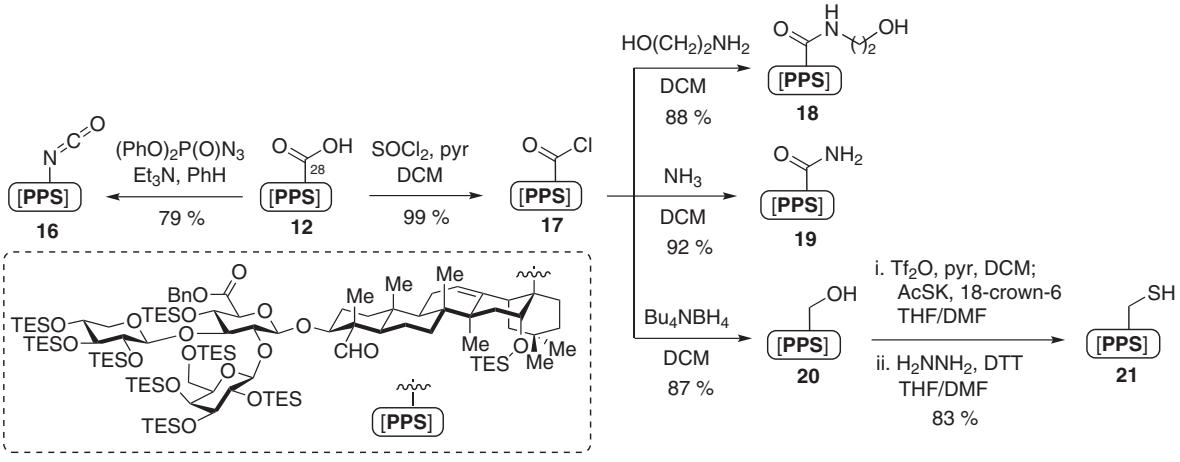


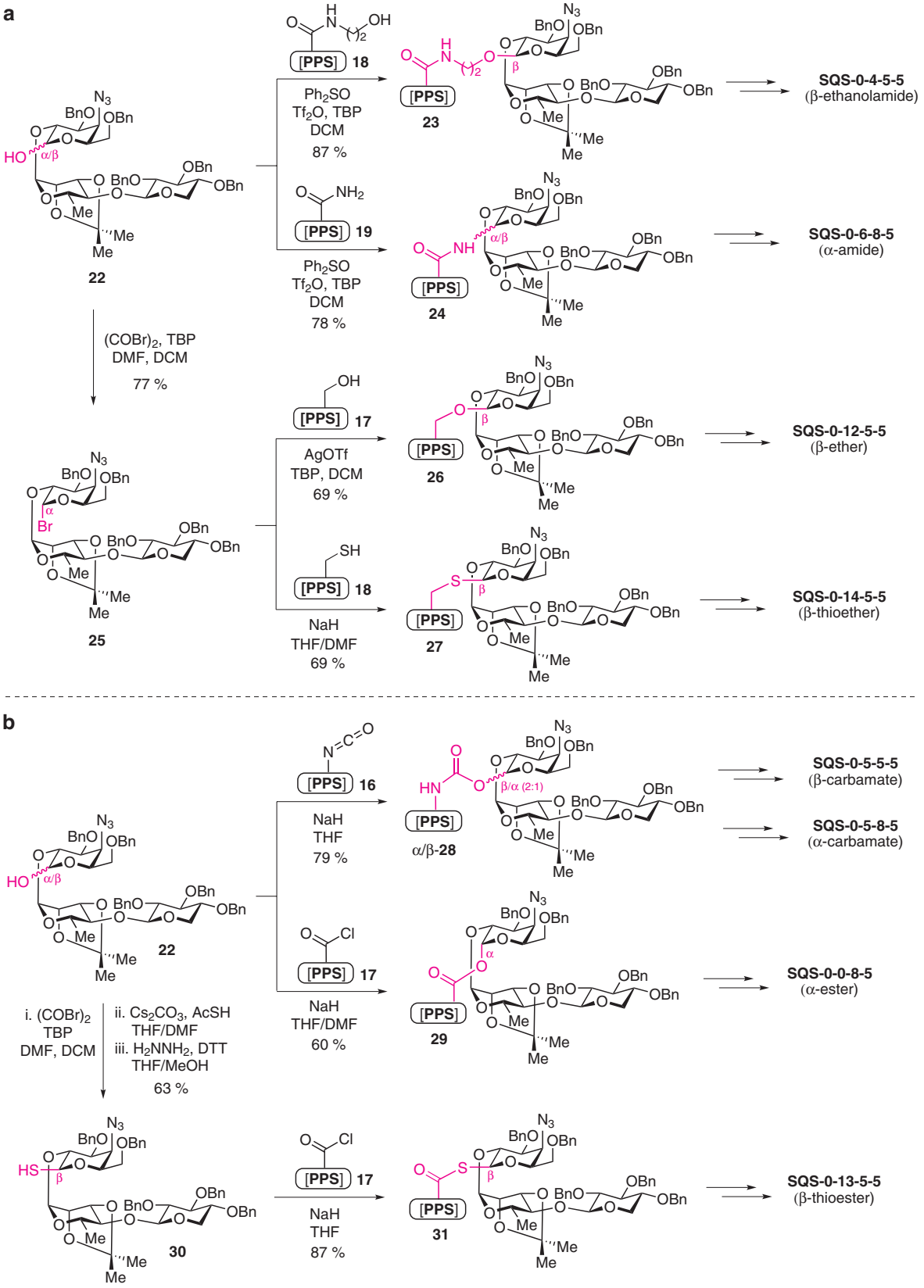
Fig. 6: Structures of central glycosidic linkage variants with modified linker length (left), stereochemistry (middle), and flexibility (right).

in a polarity-reversed coupling (Scheme 4b). The resulting prosapogenin–trisaccharide intermediates were then advanced to the fully deprotected central linker variants following the synthetic sequence described above, involving reductive acylation of the azide with the previous carboxylic acid acyl chain, and global deprotection (hydrogenolysis and acid hydrolysis).

Immunological evaluation in mice of these saponins variants with the four-antigen cocktail above revealed striking differences in and modulation of adjuvant activity, despite the seemingly minor structural perturbations. For instance, whereas longer linker length in β-ethanolamide **SQS-0-4-5-5** and β-carbamate **SQS-0-5-5-5** abrogated activity, a more modest increase in linker distance in β-thioester **SQS-0-13-5-5** resulted



Scheme 3: Synthesis of protected prosapogenin [**PPS**] glycosyl acceptors.



Scheme 4: Synthesis of QS saponin linker variants with glycosyl donor acting (a) as an electrophile in a traditional glycosylation or (b) as a nucleophile in a polarity-reversed coupling.

in potent adjuvant activity, with antibody responses comparable to QS-21 and the parent β-glycosyl ester **SQS-0-0-5-5** (Fig. 7). This novel variant **SQS-0-13-5-5** was essentially non-toxic at the 5 μg dose, but exhibited dose-limiting toxicity at the higher 20 μg dose. The stereoisomeric variants α-ester **SQS-0-0-8-5** and α-carbamate **SQS-0-5-8-5** were totally inactive; in contrast, the α-glycosyl amide **SQS-0-6-8-5** elicited high antibody titers but caused considerable mouse weight loss at both 5 and 20 μg doses. Finally, β-ether **SQS-0-12-5-5** and β-thioether **SQS-0-14-5-5**, with increased linker flexibility exhibited modest adjuvant activities, with only the 20 μg dose being moderately effective. In general, this marked decreased in activity observed with most linker variants indicate that the central glycosidic linkage is less tolerant to structural modifications than other domains of QS-21.

Remarkably, molecular dynamics simulations of these central linkage variants revealed distinct conformational features that correlated with the divergent adjuvant activities [16]. Thus, unlike the inactive analogs,

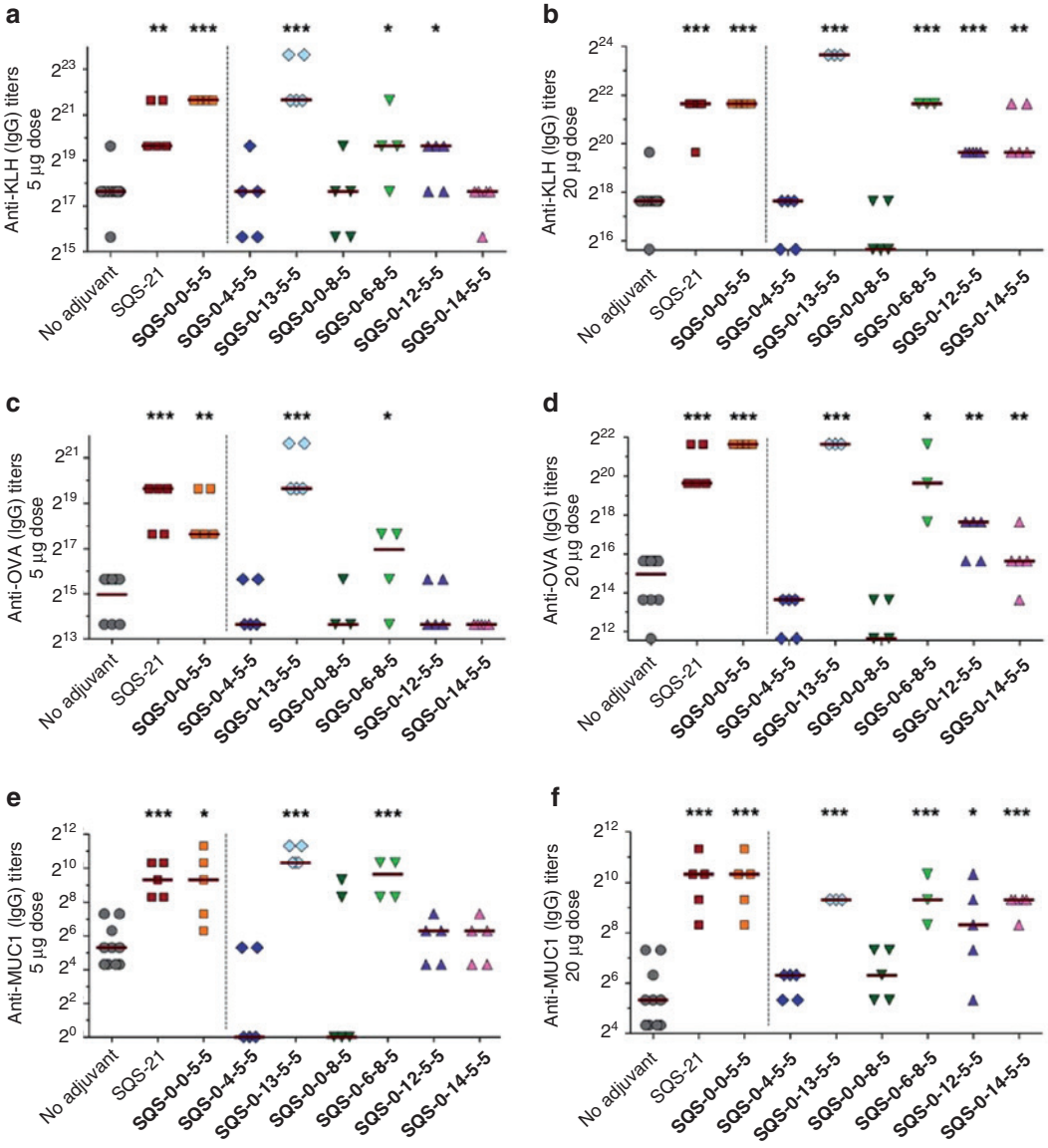


Fig. 7: Immunological evaluation of central linkage variants (β-ethanolamide **SQS-0-4-5-5**, β-thioester **SQS-0-13-5-5**, α-ester **SQS-0-0-8-5**, α-amide **SQS-0-6-8-5**, β-ether **SQS-0-12-5-5**, and β-thioether **SQS-0-14-5-5**) at 5 μg (left panels) and 20 μg (right panels) dose. Adapted from Ref. [16]. Antibody titers against (a, b) KLH (IgG), (c, d) OVA (IgG), (e, f) MUC1 (IgG).

e.g. α-ester **SQS-0-0-8-5**, which exhibited rather disorganized and less-ordered conformations around the central linker, the two most active variants, β-ester **SQS-0-5-5-5** and β-thioester **SQS-0-13-5-5**, as well as **QS-21-api**, all adopted comparatively rigid conformations, in which the linear oligosaccharide domain was extended away from the center of the triterpene and the acyl chain was folded back in parallel over the triterpene (Fig. 8). These conformational preferences and the extent of folding were quantitatively characterized by measuring the torsional angles around the central linkage, which together with the qualitative analysis above, provided a positive correlation between these specific conformational features and adjuvant activity that raises the possibility of a preferred, folded active conformation.

These results provide a molecular rationale for the striking variation in adjuvant activity despite the relatively subtle structural modifications, and yield important insights into the conformational preferences of QS saponins that correlate with activity. Thus, saponin conformation appears to be important for adjuvant activity, and may contribute to proper biodistribution, subcellular localization, and/or target binding.

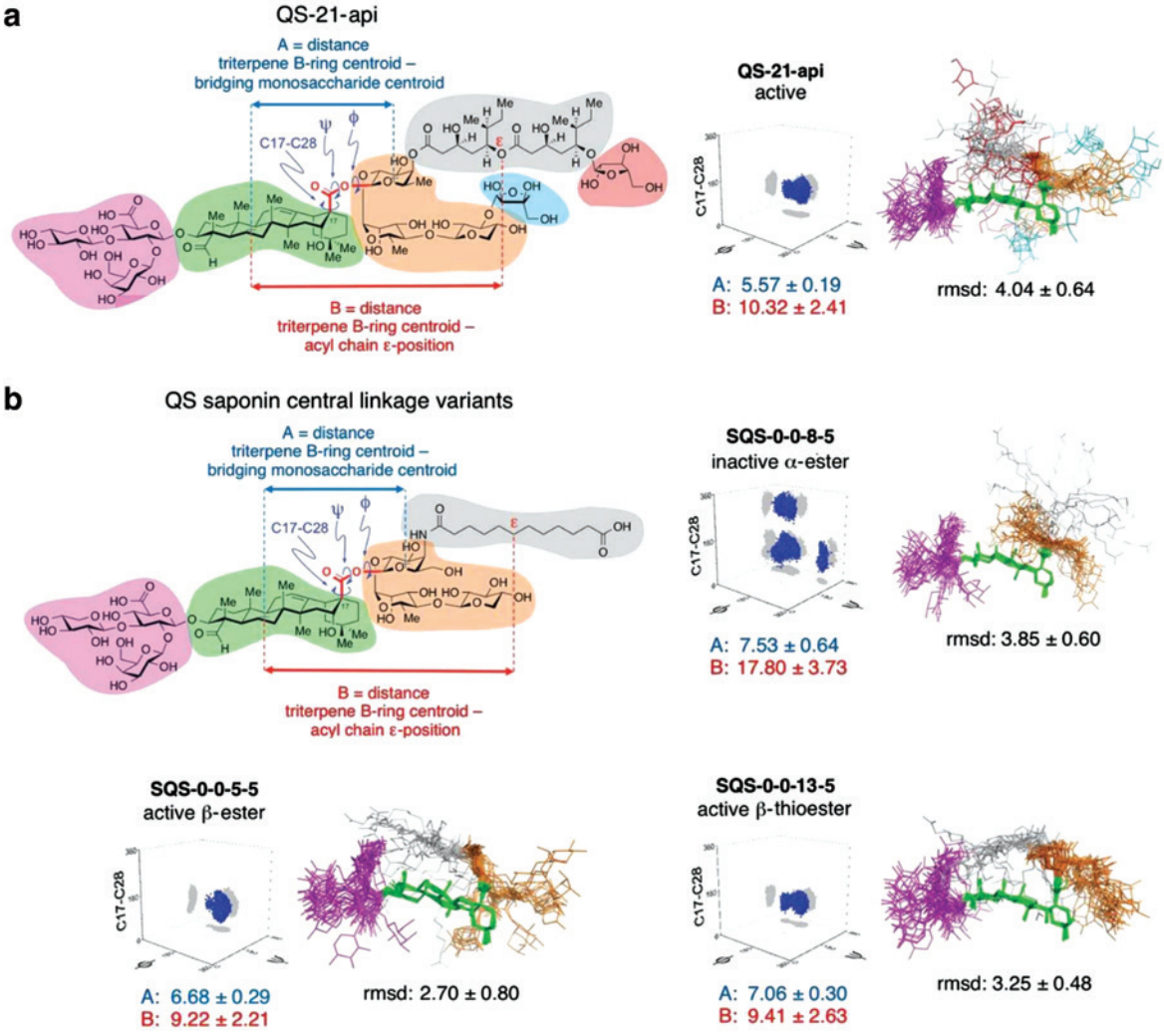
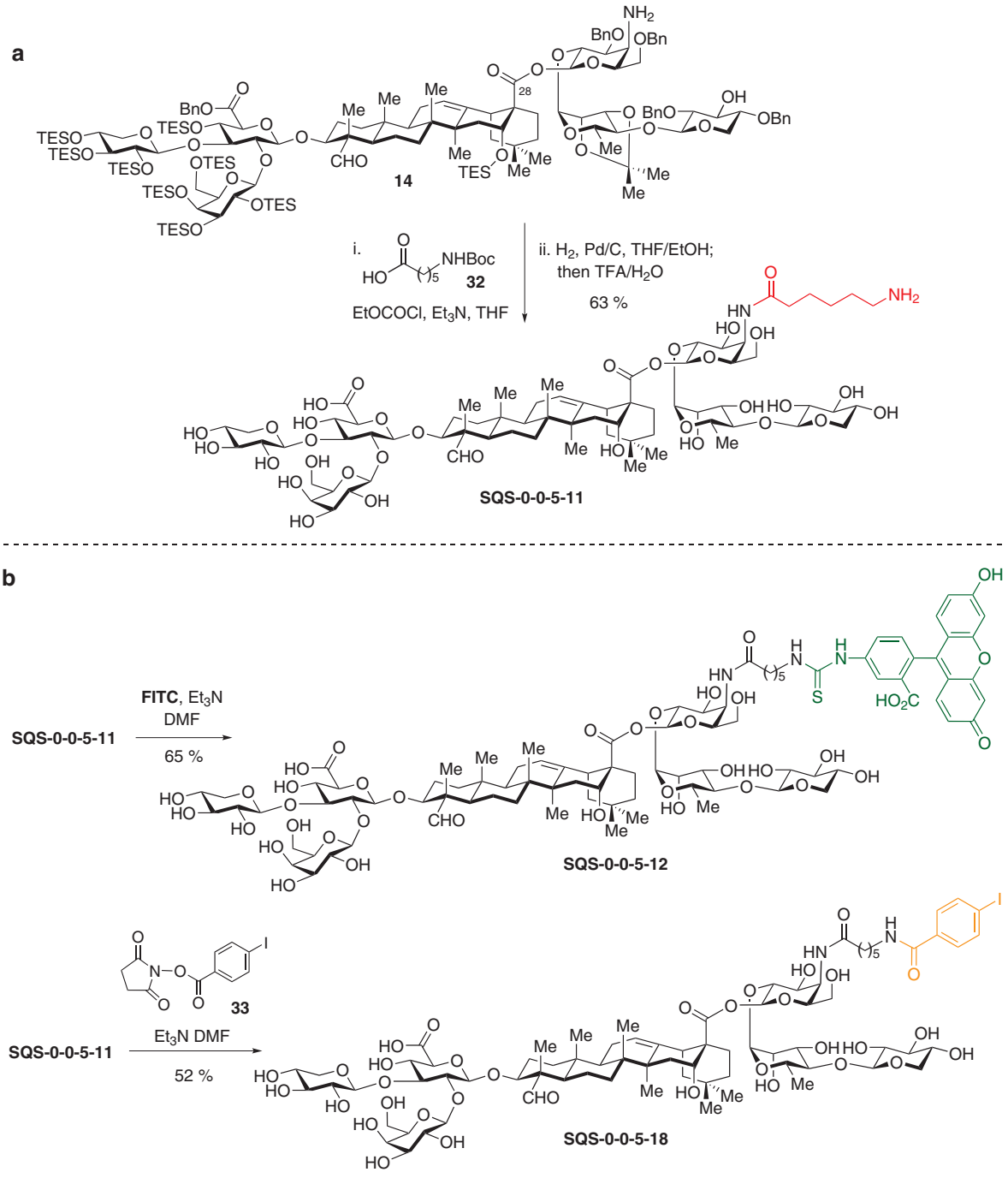


Fig. 8: Conformational ensembles and torsional angle distributions of the central glycosidic linkage derived from unrestrained molecular dynamics simulations of (a) **QS-21-api** and (b) characteristic saponin variants: inactive α-glycosyl ester **SQS-0-0-8-5**, and active β-glycosyl ester **SQS-0-0-5-5** and β-glycosyl thioester **SQS-0-0-13-5**. Adapted from Ref. [4]. The above trends also hold for the rest of the linker variants.

Development of acyl chain variants bearing fluorescent and iodinated probes for mechanistic studies

The previous trisaccharide amine precursor **14** was used to investigate the effect of the ionic charge on adjuvant activity with the synthesis of aminoacyl trisaccharide variant, **SQS-0-0-5-11** (Scheme 5a), which incorporates a 6-aminohexanoic acyl chain with a positively charged terminal amine. In mice vaccinated with



Scheme 5: (a) Synthesis of amine-terminated acyl chain variant **SQS-0-0-5-11**. (b) Synthesis of functionalized acyl chain variants bearing fluorescent (**SQS-0-0-5-12**) and iodinated (**SQS-0-0-5-18**) tags.

the MUC1–KLH conjugate antigen, **SQS-0-0-5-11** elicited low antibody responses (Fig. 9), revealing that the positive charge in this variant attenuates adjuvant activity, as opposed to the effect observed with negatively charged dodecanedioic trisaccharide analog **SQS-0-0-5-5**, which retained potent adjuvant activity. None-

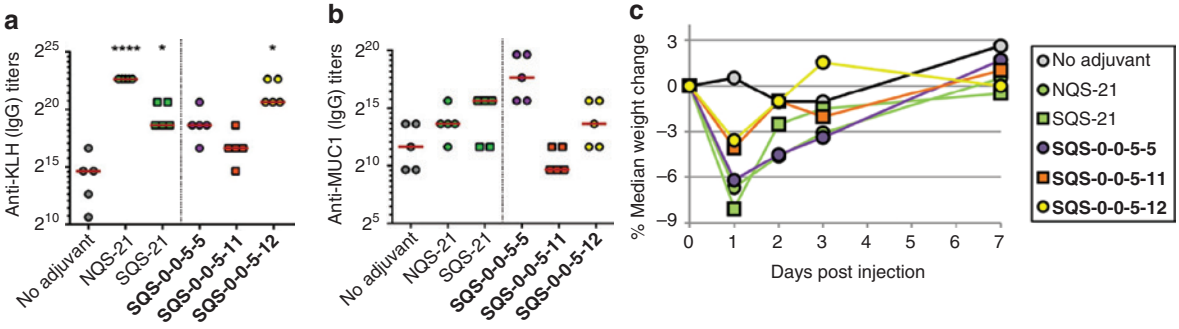


Fig. 9: Immunological evaluation of functionalized acyl chain variants **SQS-0-0-5-11** and fluorescent **SQS-0-0-5-12** (10 μg saponin) with MUC1–KLH (2.5 μg) vaccine. Adapted from Ref. [12]. Antibody titers against (a) KLH (IgG), (b) MUC1 (IgG). (c) Toxicity assessment based on median weight loss over 1 week after the first vaccine injection.

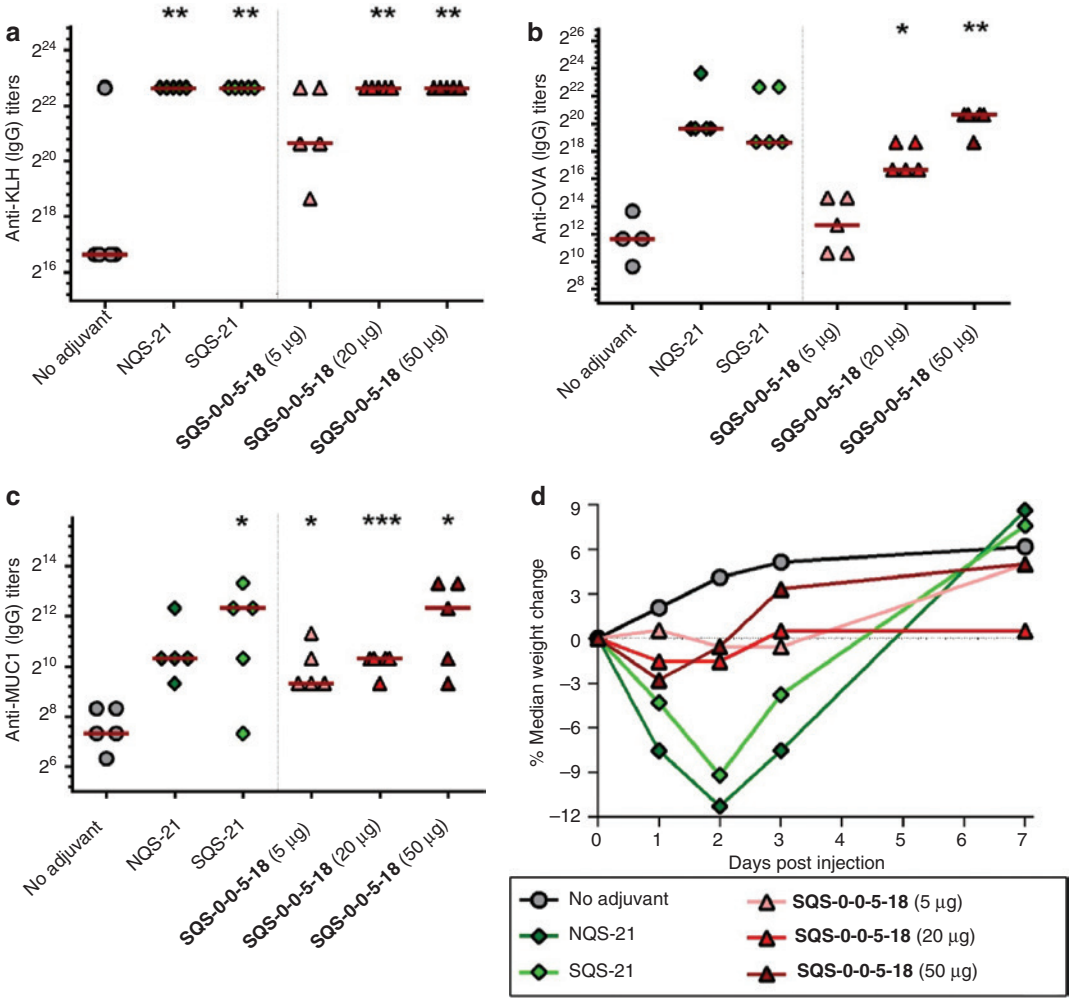


Fig. 10: Immunological evaluation of aryl iodide acyl chain variant **SQS-0-0-5-18** (5, 10, and 20 μg saponin, respectively) with OVA (20 μg) and MUC1–KLH (2.5 μg) vaccine. Antibody titers against (a) KLH (IgG), (b) OVA (IgG), and (c) MUC1 (IgG). (d) Toxicity assessment based on median mouse weight change over a week after first vaccine injection.

theless, this amine-terminated saponin allowed late-stage, chemoselective installation of different reporter groups for the synthesis of saponin mechanistic probes. Thus, Gin and co-workers introduced fluorescent or radiolabeled tags into the QS-saponin scaffold for subsequent imaging and in vivo biodistribution studies. Commencing with **SQS-0-0-5-11**, we synthesized FITC-labeled **SQS-0-0-5-12** [12] by reaction of the acyl chain domain amine with fluorescein isocyanate, and aryl iodide **SQS-0-0-5-18** [17] by 4-iodobenzoylation of the terminal amine in **SQS-0-0-5-11** (Scheme 5b).

In mouse vaccinations, these variants (10 μg **SQS-0-0-5-12**, 20 μg **SQS-0-0-5-18**) elicited comparable antibody responses to QS-21 and exhibited considerably lower weight loss (3.5 % and 1.5 % on day 1, 1 % and 1.5 % on day 2, respectively) (Figs. 9 and 10).

With both saponin variants established as potent adjuvants with low toxicity, the radiolabeled congener of **SQS-0-0-5-18** was generated by radioiodination of the corresponding aryl tributylstannane [17], and in vivo biodistribution as well as fluorescence imaging studies were performed in mice coadministered with OVA (20 μg). Initial efforts to utilize **[^{124}I]-SQS-0-0-5-18** in positron emission tomography (PET) imaging were complicated by high signal retention at the injection site, which made quantitation difficult at nearby sites such as the lymph nodes. Instead, "cut-and-count" studies were conducted with **[^{131}I]-SQS-0-0-5-18** and a structurally related inactive variant, **[^{131}I]-SQS-0-3-7-18**, devoid of the linear oligosaccharide. For the active saponin, preferential recovery of radioactivity compared to the inactive variant was observed at the injection site (17-fold higher) and the nearest lymph nodes (24-fold higher) at 24 h post-injection (Fig. 11). In mice treated with **[^{131}I]-SQS-0-0-5-18**, these two sites showed also high levels of radioactivity at 72 and 96 h post-injection, in contrast to other tissues and organs where large differences were initially observed. However, for the inactive variant **[^{131}I]-SQS-0-3-7-18** radioactivity cleared from all tissues at the later time points. These data indicate that the active saponin is retained at the injection site and accumulates in the lymph nodes, but the inactive variant does not. Similar trends were observed with the radiolabeled congeners of another pair of active/attenuated variants lacking the branched trisaccharide and incorporating quillaic acid and oleanolic acid triterpenes, respectively (vide infra), which overall provides a positive correlation between this biodistribution pattern and adjuvant activity.

As other QS-based adjuvant mixtures have been shown to affect antigen biodistribution [18, 19], the biodistribution of **[^{131}I]-OVA** in the presence and absence of active aryl iodide **SQS-0-0-5-18** was also explored. Unfortunately, elevated radioiodine uptake was observed in the thyroid tissue, suggesting rapid deiodination of **[^{131}I]-OVA**. Thus, in vivo mouse fluorescence imaging studies with FITC-labeled active variant **SQS-0-0-5-12** and Alexa-647-conjugated OVA (**OVA-A647**) were performed as an alternative strategy. At 24 h post-injection, **OVA-A647** colocalized at the lymph nodes and the injection site when coadministered with active **SQS-0-0-5-12** (Fig. 12a,b); however, in mice coinjected with the inactive variant **SQS-0-0-5-11**, **OVA-A647** was only observed at the injection site and did not accumulate in the lymph nodes. The active fluorescent saponin **SQS-0-0-5-12** was retained at both sites, consistent with the previous biodistribution results, and sublocalized to

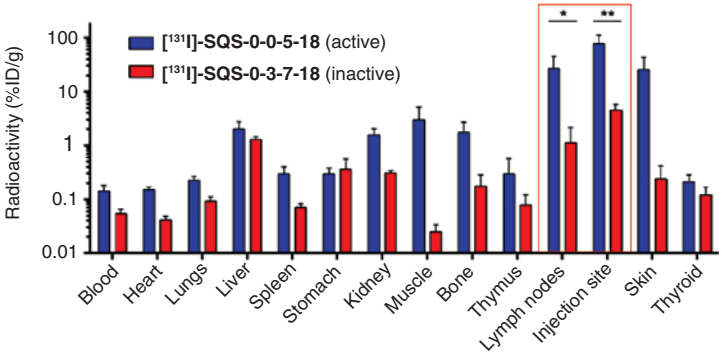


Fig. 11: Twenty four hours in vivo biodistribution of radiolabeled saponins (25 μCi) **SQS-0-0-5-18** (active) and **SQS-0-3-7-18** (inactive) in mice co-injected with 20 μg OVA and 20 μg of the corresponding cold saponin. Adapted from Ref. [4]. Statistical significance of the difference in recovery of radioactivity (%ID/g) only shown for lymph nodes and injection site.

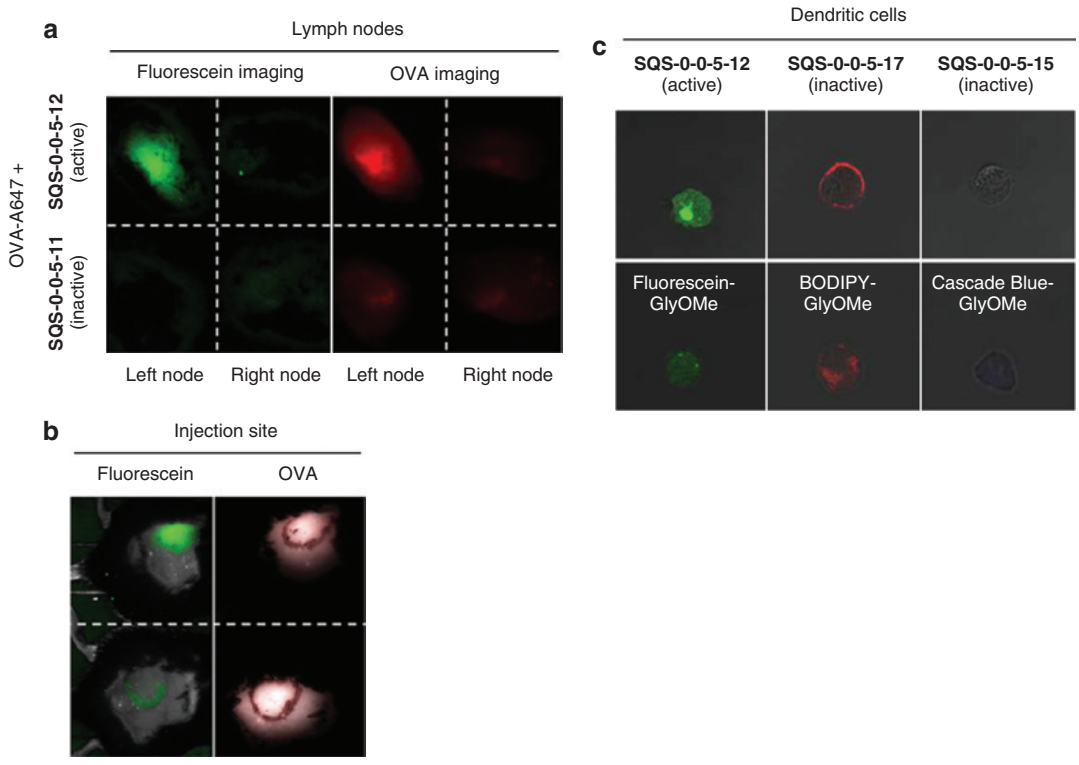
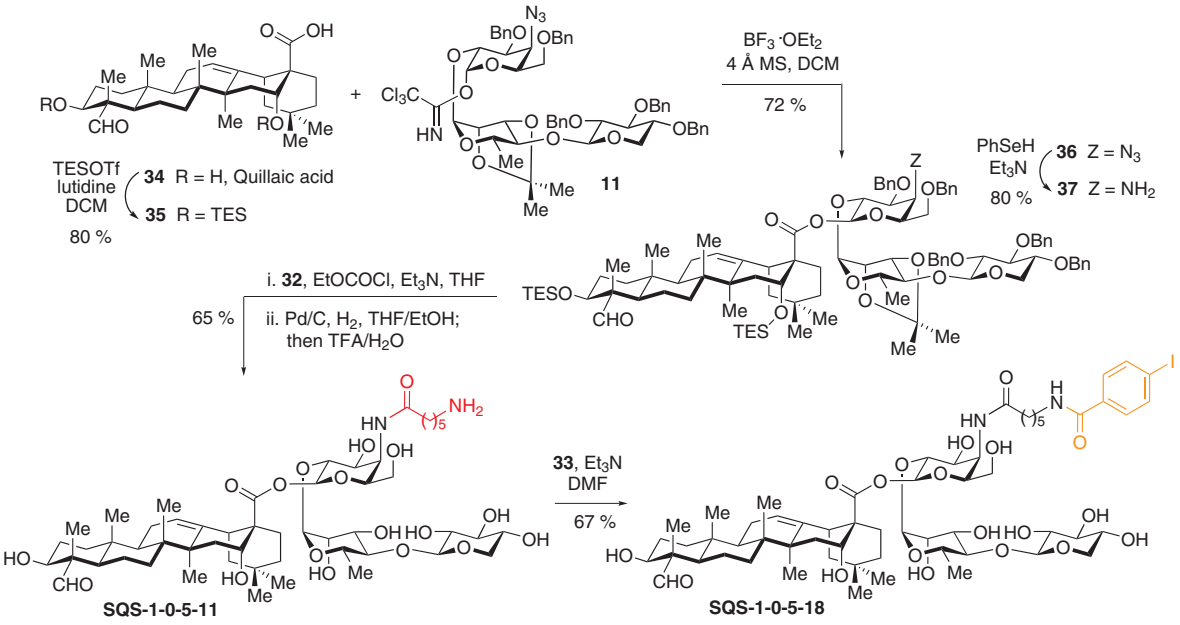


Fig. 12: Twenty four hours in vivo fluorescence imaging studies in mice injected with active fluorescein-conjugated saponin **SQS-0-0-5-12** or inactive non-fluorescent variant **SQS-0-0-5-11** (10 μg each), as well as OVA-Alexa647 (20 μg). Adapted from Ref. [4]. (a) Imaging of dissected lymph nodes (mice injected in left nodes, right nodes are negative controls). (b) Whole-body imaging at the injection site. (c) Subcellular localization of fluorescein-labeled active saponin **SQS-0-0-5-12** and inactive BODIPY (**SQS-0-0-5-17**) and Cascade Blue (**SQS-0-0-5-15**) saponins in immature dendritic cells imaged by confocal microscopy (upper panel); the corresponding glycine methyl esters (GlyOMe) labeled with each corresponding fluorophore were administered as negative controls (lower panel).

the cortex of the draining inguinal node, as assessed by immunohistochemistry of dissected lymph nodes. Flow cytometric analysis demonstrated that internalization of **SQS-0-0-5-12** was specific to dendritic cells within the lymph nodes, in agreement with subcellular localization studies in immature dendritic cells by confocal microscopy [12] that showed internalization of active **SQS-0-0-5-12** to a discrete cellular compartment, but not of related attenuated saponins bearing BODIPY **(SQS-0-0-5-17**) and Cascade Blue (**SQS-0-0-5-15**) fluorophores, or other fluorescent negative controls (Fig. 12c). Collectively, these studies suggest that active variants help direct antigen-presenting cells trafficking OVA to the lymph nodes for presentation of the antigen to the immune system, and provide preliminary mechanistic understanding of how these saponin adjuvants may potentiate the immune response.

Carbohydrate variants via deletion of the QS branched trisaccharide

In our next investigations, we prepared a truncated saponin variant, **SQS-1-0-5-18**, derived from **SQS-0-0-5-18** but devoid of the complete branched trisaccharide, to determine if this domain, as a whole, is required for adjuvant activity. Starting from quillaic acid, isolated and purified in gram quantities from commercial QS-extracts [7, 20], selective silylation of the hydroxyl groups followed by glycosylation of the triterpene C28-carboxylate with the aza-trisaccharide imidate **11** gave the triterpene–linear trisaccharide azide intermediate **36** (Scheme 6). The remaining steps towards **SQS-1-0-5-18** involved reduction of the azide, acyla-



Scheme 6: Synthesis of truncated aryl iodide acyl variant **SQS-1-0-5-18** devoid of the branched trisaccharide.

tion with the 6-aminohexanoic acyl chain, global deprotection via hydrogenolysis and acid hydrolysis, and late-stage chemoselective acylation of the side chain terminal amine with the aryl iodide succinimidyl ester **33**.

Remarkably, in mice vaccinated with MUC1–KLH and OVA, this truncated variant **SQS-1-0-5-18** elicited antibody responses comparable to QS-21 and the branched trisaccharide-containing **SQS-0-0-5-18**, although at a 50 µg dose (Fig. 13). Moreover, **SQS-1-0-5-18** was barely toxic, causing no mouse weight loss at 20 µg (+1.8 %, day 1) and only –1 % (day 1) at 50 µg [17]. These results revealed that deletion of the branched trisaccharide does not impair adjuvant activity and also lowers toxicity, yielding a greatly simplified and potent QS-21 variant that is more synthetically accessible and less toxic than the natural product.

Triterpene variants via specific modifications at C4-aldehyde and C16-hydroxyl group

The discovery that the branched trisaccharide is not necessary for activity allowed us to carry out detailed structure-activity relationship studies on the triterpene, particularly at its C4-aldehyde substituent and C16-hydroxyl group, by synthesizing several saponin variants with targeted modifications at these positions using other readily available triterpenes as starting materials (Fig. 14) [17]. The potential role of the aldehyde in the adjuvant's mode of action is only speculative, but it has been proposed to participate in Schiff base formation with a presumed T-cell surface receptor target [21, 22] based on the finding that QS-21 derivatives that compromise the C4-aldehyde substituent by reductive amination with exogenous amines exhibited significantly attenuated adjuvant activity [21]. However, these derivatives not only were devoid of the aldehyde (as intended), but also incorporated a positively-charged amino group that may be the origin of its decreased activity, analogously to the inactive acyl chain terminal amine variant **SQS-0-0-5-11** [12]. Thus, we introduced more subtle structural changes at the triterpene C4 position with the synthesis of caullophylogenin variant **SQS-1-11-5-18** and echinocystic acid variant **SQS-1-8-5-18**, in which the C4-aldehyde in quillaic acid variant **SQS-1-0-5-18** was replaced with a hydroxymethyl and a methyl group, respectively. Moreover, the related gypsogenin **SQS-1-9-5-18**, hederagenin **SQS-1-10-5-18**, and oleanolic acid **SQS-1-7-5-18** variants, in which the C16-hydroxyl group of the respective triterpene cores was orthogonally replaced by a proton, were also pursued (Fig. 14). The preparation of these new triterpene variants started

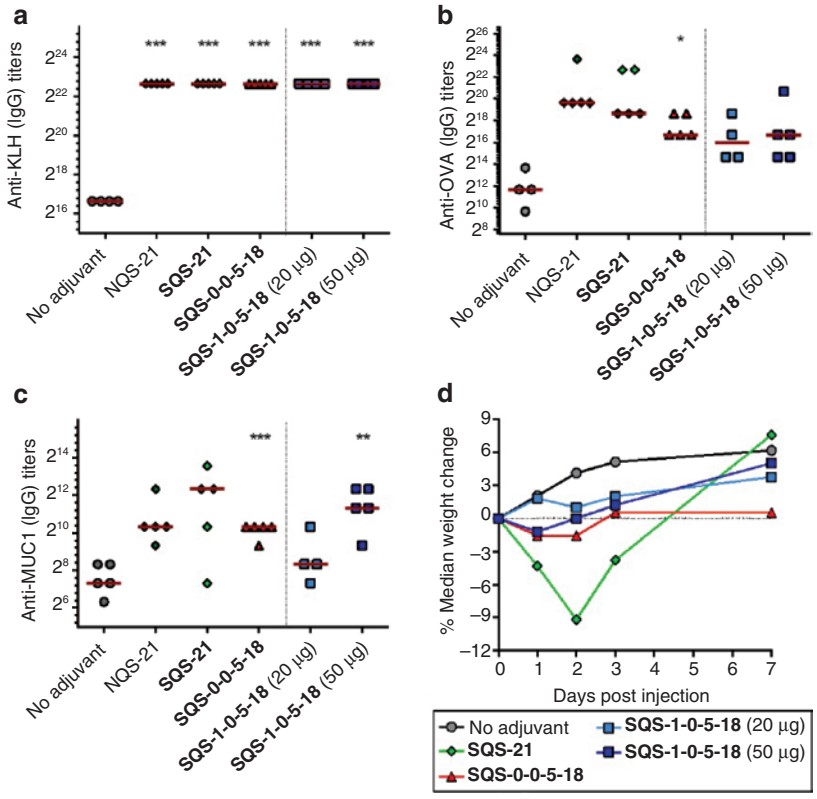
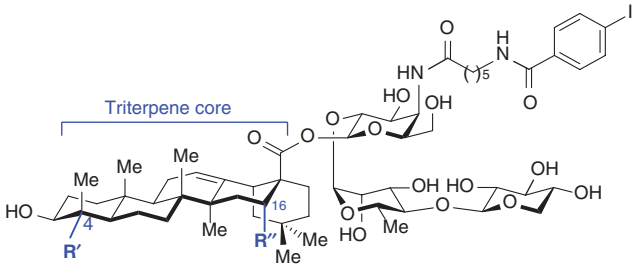


Fig. 13: Immunological evaluation of truncated aryl iodide variant **SQS-1-0-5-18** (10 and 20 μg saponin) with OVA (20 μg) and MUC1–KLH (2.5 μg) vaccine. Antibody titers against (a) KLH (IgG), (b) OVA (IgG), and (c) MUC1 (IgG). (d) Toxicity assessment based on median mouse weight change over a week after first vaccination.

from commercially available echinocystic acid, hederagenin, and oleanolic acid triterpenes, following a similar synthetic sequence to that of quillaic acid variant **SQS-1-0-5-18**, with the exception of caullophylogenin variant **SQS-1-11-5-18**, which was prepared from an advanced protected intermediate en route to **SQS-1-0-5-18**.

In mice vaccinated with the four-antigen formulation (GD3–KLH, MUC1–KLH, and OVA), both variants retaining the C16-hydroxyl group, echinocystic acid variant **SQS-1-8-5-18** and caullophylogenin variant **SQS-1-11-5-18**, showed potent adjuvant activity, higher than or similar to QS-21, respectively (Fig. 15). Remarkably, while these variants contain the C16-hydroxyl group, neither of them bear the C-4 aldehyde substituent, which casts doubt on the Schiff-base hypothesis, at least within these truncated variants. On the other hand, gypsogenin **SQS-1-9-5-18**, hederagenin **SQS-1-10-5-18** and oleanolic acid **SQS-1-7-5-18** (not shown) variants, all lacking the C16-hydroxyl, exhibited attenuated adjuvant activities across all antigens, suggesting an important role for this functionality in adjuvant activity. Notably, none of these variants



QS saponin	Triterpene	R′	R″
SQS-1-0-5-18	Quillaic acid	CHO	OH
SQS-1-11-5-18	Caullophylogenin	CH_2OH	OH
SQS-1-8-5-18	Echinocystic acid	Me	OH
SQS-1-9-5-18	Gypsogenin	CHO	H
SQS-1-10-5-18	Hederagenin	CH_2OH	H
SQS-1-7-5-18	Oleanolic acid	Me	H

Fig. 14: Structures of triterpene variants via molecular editing at C4 (aldehyde) and C16 (hydroxyl group) positions of the triterpene.

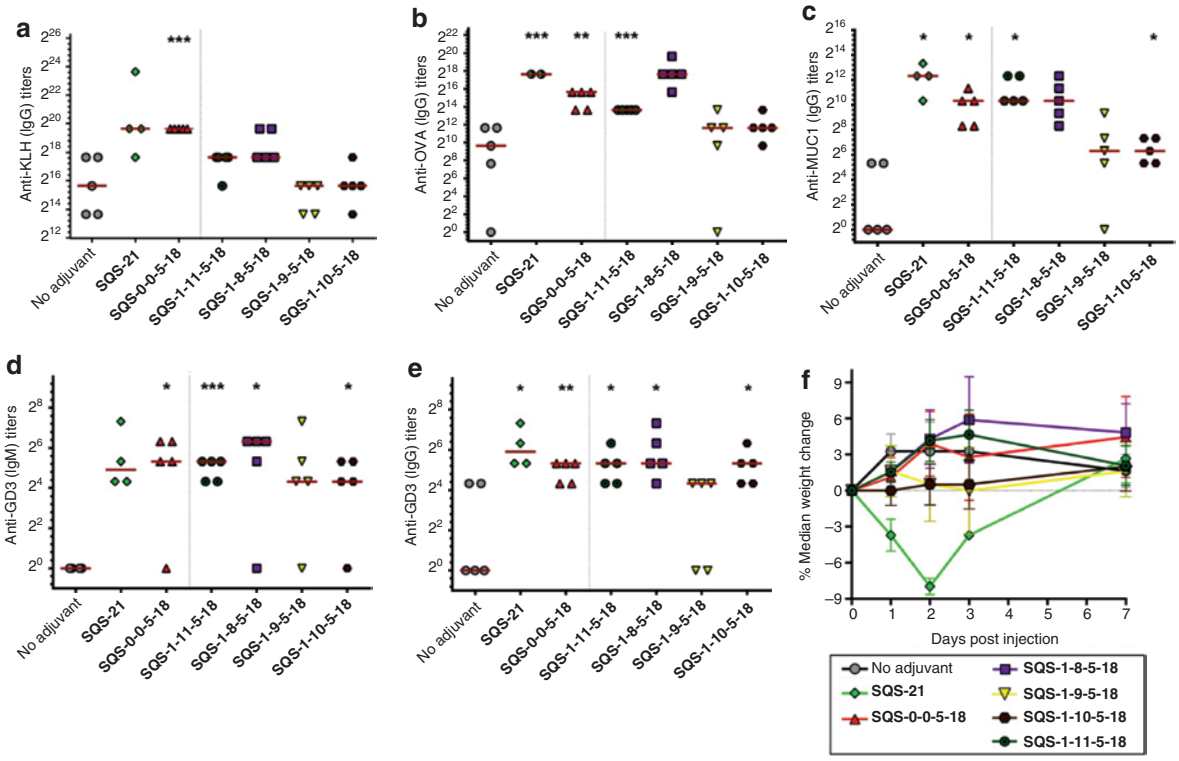


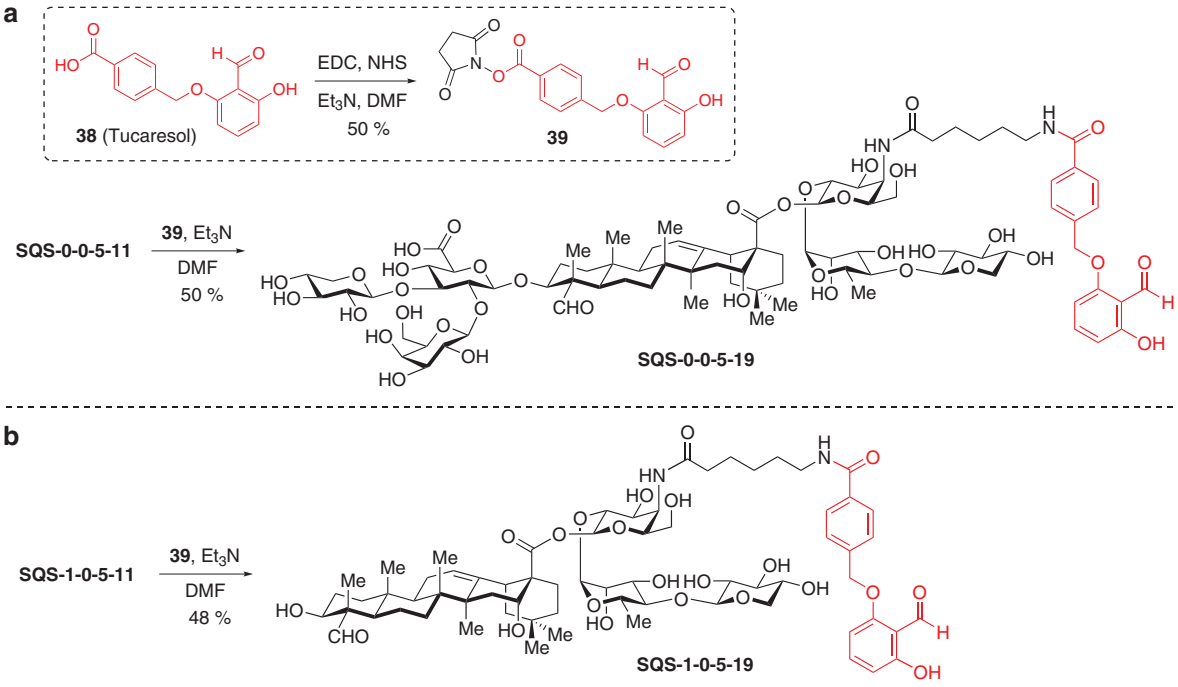
Fig. 15: Immunological evaluation of triterpene variants **SQS-1-8-5-18**, **SQS-1-9-5-18**, **SQS-1-10-5-18**, and **SQS-1-11-5-18** (50 μg saponin) with OVA (20 μg), MUC1–KLH (2.5 μg) and GD3–KLH (5 μg) vaccine. Antibody titers against (a) KLH (IgG), (b) OVA (IgG), (c) MUC1 (IgG), (d) GD3 (IgM), and (e) GD3 (IgG). (f) Toxicity assessment based on median mouse weight change over a week after first vaccination.

showed any appreciable toxicity as assessed by mouse weight loss (Fig. 15f). These results indicate that potent adjuvant activity requires the C16-hydroxyl group but not the C4-aldehyde substituent, which is consistent with the identification of other saponin adjuvants lacking the C4-aldehyde and bearing the C16-hydroxyl [23–25]. The discovery of this potent, non-toxic and synthetically accessible (23 steps total) echinocystic acid variant **SQS-1-8-5-18** provides an attractive lead for further advancement and mechanistic studies in the future.

Saponin–tucaresol conjugates to explore the effect of aldehyde-based adjuvant combinations on adjuvant activity

The previous hypothesis that QS-21 may interact with putative T-cell surface receptors via Schiff base formation through its C4-aldehyde substituent [21, 22] had its origin on the identification of aldehyde-containing adjuvants, e.g. tucaresol, that react forming imines with amino groups on the surface of T cells, leading to T-cell costimulation and activation, and Th1 immune responses [26–28]. On this basis, and given the increasing interest in using combinations of adjuvants to achieve optimal vaccine efficacy [29], we next designed and synthesized novel saponin–tucaresol conjugates, i.e. branched-trisaccharide containing **SQS-0-0-5-19** and its truncated congener **SQS-1-0-5-19**, to investigate if the aldehyde-based activity of tucaresol might further enhance the adjuvant activity of QS saponin variants [30].

These new variants were synthesized by chemoselective acylation with tucaresol succinimidyl ester at the acyl chain terminal amine of the corresponding saponin precursors (Scheme 7), and were evaluated in mice together with equimolar combinations of related saponin variants (**SQS-0-0-5-18** and **SQS-1-0-5-18**)



Scheme 7: (a) Synthesis of saponin–tucaresol conjugate **SQS-0-0-5-19** incorporating the branched trisaccharide. (b) Synthesis of truncated saponin–tucaresol conjugate **SQS-1-0-5-19** devoid of the left-hand carbohydrate.

and tucaresol. While the saponin–tucaresol conjugates retained potent adjuvant activity, similar to their aryl iodide saponin congeners, the presence of tucaresol, either in combination (with **SQS-0-0-5-18** or **SQS-1-0-5-18**), or covalently incorporated into the acyl chain, as in **SQS-0-0-5-19** and **SQS-1-0-5-19**, did not significantly enhance the adjuvant activity of our QS saponins [30].

Linear trisaccharide variants via versatile divergent synthesis

In considering further specific molecular modifications of the QS saponin scaffold, our goal was to provide access to saponin variants that can be prepared efficiently in a streamlined and versatile synthetic route. Thus, we developed a novel divergent strategy and synthesized three saponin variants with variation of individual sugars and connectivities within the linear trisaccharide (Fig. 16) that were prepared in less steps than the previous lead compound **SQS-1-0-5-18** [31]. The first analog, rhamnose–rhamnose variant **SQS-1-0-10-18** (22 total steps), incorporated a rather conservative modification with a single sugar substitution at the third, terminal residue, and was synthesized following the same linear, convergent approach used for the parent saponin **SQS-1-0-5-18** bearing the original linear trisaccharide. More profound structural changes took the form of lactose variant **SQS-1-0-11-18** (16 total steps), in which the entire terminal disaccharide was replaced by commercially available lactose, and regioisomeric variant **SQS-1-0-12-18** (19 total steps), in which the original terminal disaccharide and the acyl chain exchanged positions within a more readily accessible 2-azidogalactose bridging sugar. For the synthesis of the latter two variants, a new synthetic approach was designed consisting of stepwise glycosylation of the triterpene with a bridging monosaccharide residue followed by installation of the desired terminal disaccharides, enabling expedited preparation of the lactose variant **SQS-1-0-11-18** in only 16 steps. Moreover, through its versatile triterpene–monosaccharide late-stage diversification point, this divergent strategy sets the stage for more efficient access to a range of linear trisaccharide variants for the rapid identification of new, potentially improved saponin adjuvants that are synthetically accessible and easily scalable for further clinical development in vaccines.

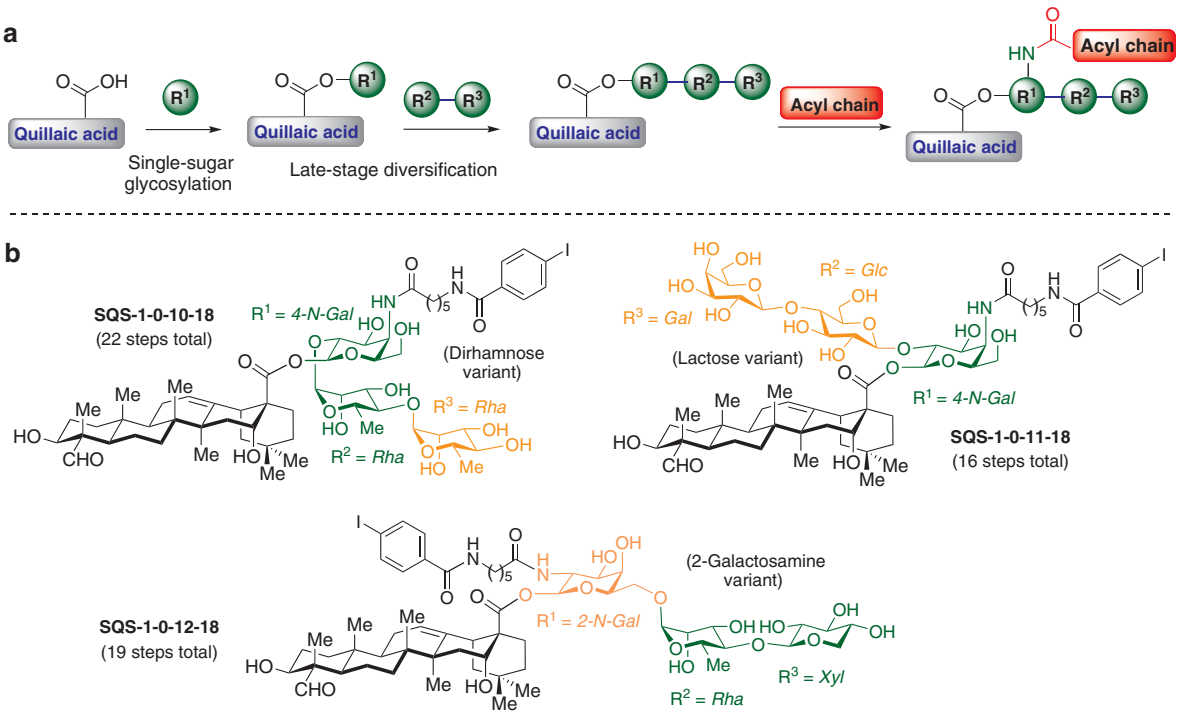


Fig. 16: (a) Versatile divergent strategy towards linear oligosaccharide saponin variants. (b) Structures of synthetically streamlined linear oligosaccharide variants with modified, readily accessible individual sugars.

Conclusions

QS-21 is a highly promising adjuvant investigated in numerous vaccine clinical trials but suffers several drawbacks: limited supply, difficulty in purification to homogeneity, toxic side effects, and hydrolytic instability, as well as a poorly understood mechanism of action. It is, therefore, necessary to discover and gain access to better saponin adjuvants that can potentiate the activity of molecular vaccines. The design, synthesis and biological evaluation of QS saponins was a subject of long-standing interest in the Gin group for more than ten years. The synthetic technology first applied to the total synthesis of **QS-21-api** and **QS-21-xyl** was subsequently exploited to prepare novel saponin variants with increased chemical stability, potent adjuvant activity and low toxicity. These studies have offered detailed information of saponin structure–activity relationships, while also providing streamlined access to optimized variants and saponin mechanistic probes. As a result, initial investigations into the enigmatic mechanism of action of these QS saponins have been possible by performing early biodistribution and fluorescence imaging studies. Preliminary results on this front pointed to a potential role for active saponin adjuvants in the trafficking of antigen by antigen-presenting cells to the lymph nodes, a known site for immune cell maturation. Moreover, the pronounced SAR established above together with the observed correlation between saponin conformation and adjuvant activity, suggest a mechanism of action involving interaction with discrete molecular target, setting the stage for additional studies in this context. Further work in this area is currently being pursued in my laboratories at CIC bioGUNE (Spain) with the goal of providing further mechanistic understanding and streamlined synthetic access to novel saponin constructs that will enable more rapid and rational discovery of new adjuvants and optimized adjuvant–antigen combinations for future vaccines.

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References

[1] D. Christensen. *Hum. Vaccin. Immunother*. **12**, 2709 (2016).
[2] A. D. Pasquale, S. Preiss, F. T. Da Silva, N. Garçon. *Vaccines* **3**, 320 (2015).
[3] A. Fernández-Tejada, W. E. Walkowicz, D. S. Tan, D. Y. Gin. *Methods Mol. Biol.* **1494**, 45 (2017).
[4] A. Fernández-Tejada, D. S. Tan, D. Y. Gin. *Acc. Chem. Res*. **49**, 1741 (2016).
[5] S. Soltysik, J.-Y. Wu, J. Recchia, D. A. Wheeler, M. J. Newman, R. T. Coughlin, C. R. Kensil. *Vaccine* **13**, 1403 (1995).
[6] D. J. Marciani, J. B. Press, R. C. Reynolds, A. K. Pathak, V. Pathak, L. E. Gundy, J. T. Farmer, M. S. Koratich, R. D. May. *Vaccine* **18**, 3141 (2000).
[7] P. Wang, Y.-J. Kim, M. Navarro-Villalobos, B. D. Rohde, D. Y. Gin. *J. Am. Chem. Soc.* **127**, 3256 (2005).
[8] K. Deng, M. M. Adams, P. Damani, P. O. Livingston, G. Ragupathi, D. Y. Gin. *Angew. Chem. Int. Ed*. **47**, 6395 (2008).
[9] K. Deng, M. M. Adams, D. Y. Gin. *J. Am. Chem. Soc*. **130**, 5860 (2008).
[10] SQS nomenclature: Except for the first three analogues SQS-0101, -0102, and -0103, the QS saponin variants are designated by a four-number code corresponding to the four domains of QS-21, left to right (Figure 1), with '0' indicating the natural product structure at each position and variants numbered sequentially from 1 in order of design.
[11] M. M. Adams, P. Damani, N. R. Perl, A. Won, F. Hong, P. O. Livingston, G. Ragupathi, D. Y. Gin. *J. Am. Chem. Soc*. **132**, 1939 (2010).
[12] E. K. Chea, A. Fernández-Tejada, P. Damani, M. M. Adams, J. R. Gardner, P. O. Livingston, G. Ragupathi, D. Y. Gin. *J. Am. Chem. Soc*. **134**, 13448 (2012).
[13] P. Wang, Q. Dai, P. Thogaripally, P. Zhang, S. M. Michalek. *J. Org. Chem*. **78**, 11525 (2013).
[14] P. Wang, D. A. Devalankar, Q. Dai, P. Zhang, S. M. Michalek. *J. Org. Chem*. **81**, 9560 (2016).
[15] B. A. Garcia, J. L. Poole, D. Y. Gin. *J. Am. Chem. Soc*. **119**, 7597 (1997).
[16] W. E. Walkowicz, A. Fernández-Tejada, C. George, F. Corzana, J. Jiménez-Barbero, G. Ragupathi, D. S. Tan, D. Y. Gin. *Chem. Sci.* **7**, 2371 (2016).
[17] A. Fernández-Tejada, E. K. Chea, C. George, N. Pillarsetty, J. R. Gardner, P. O. Livingston, G. Ragupathi, J. S. Lewis, D. S. Tan, D. Y. Gin. *Nature Chem*. **6**, 635 (2014).
[18] P. Duewell, U. Kisser, K. Heckelsmiller, S. Hoves, P. Stoitzner, S. Koernig, A. B. Morelli, B. E. Clausen, M. Dauer, A. Eigler, D. Anz, C. Bourquin, E. Maraskovsky, S. Endres, M. Schnurr. *J. Immunol*. **187**, 55 (2011).
[19] M. T. Scott, M. Goss-Sampson, R. Bomford. *Int. Arch. Allergy Appl. Immunol*. **77**, 409 (1985).
[20] D. F. Elliott, G. A. R. Kon. *J. Chem. Soc*. 1130 (1939).
[21] S. Soltysik, J.-Y. Wu, J. Recchia, D. A. Wheeler, M. J. Newman, R. T. Coughlin, C. R. Kensil. *Vaccine* **13**, 1403 (1995).
[22] D. J. Marciani. *Drug Discov. Today* **8**, 934 (2003).
[23] N. Castro-Diaz, B. Salaun, R. Perret, S. Sierro, J. F. Romero, J.-A. Fernandez, A. Rubio-Moraga, P. Romero. *Vaccine* **30**, 388 (2012).
[24] D. Nico, F. N. Santos, G. P. Borja-Cabrera, M. Palatnik, C. B. Palatnik de Sousa. *Vaccine* **25**, 649 (2007).
[25] K. Oda, H. Matsuda, T. Murakami, S. Katayama, T. Ohgitani, M. Yoshikawa. *Biol. Chem.* **381**, 67 (2000).
[26] J. Rhodes, H. Chen, S. R. Hall, J. E. Beesley, D. C. Jenkins, P. Collins, B. Zheng. *Nature* **377**, 71 (1995).
[27] H. Chen, J. Rhodes. *J. Mol. Med*. **74**, 497 (1996).
[28] J. Rhodes. *Clin. Exp. Immunol*. **130**, 363 (2002).
[29] G. Mutwiri, V. Gerdts, S. van Drunen Littel-van den Hurk, G. Auray, N. Eng, S. Garlapati, L. A. Babiuk, A. Potter. *Expert Rev. Vaccines* **10**, 95 (2011).
[30] A. Fernández-Tejada, E. K. Chea, C. George, J. R. Gardner, P. O. Livingston, G. Ragupathi, D. S. Tan, D. Y. Gin. *Bioorg. Med. Chem.* **22**, 5917 (2014).
[31] A. Fernández-Tejada, D. S. Tan, D. Y. Gin. *Chem. Commun.* **51**, 13949 (2015).