JOC The Journal of Organic Chemistry

Article

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Synthesis and Evaluation of QS-21-Based Immunoadjuvants with a Terminal-Functionalized Side Chain Incorporated in the West Wing Trisaccharide

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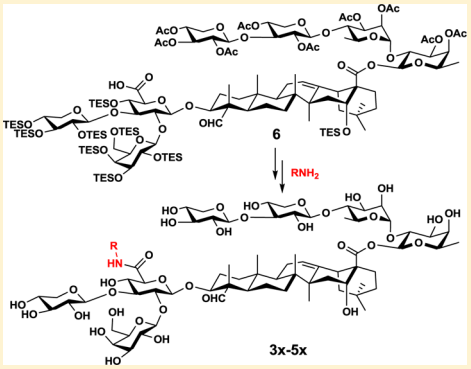
Supporting Information

ABSTRACT: Three QS-21-based vaccine adjuvant candidates with a terminal-functionalized side chain incorporated in the west wing trisaccharide have been synthesized. The terminal polar functional group serves to increase the solubility of these analogues in water. Two of the synthetic analogues have been shown to have adjuvant activity comparable to that of GPI-0100. The stand-alone adjuvant activity of the new synthetic analogues again confirmed that it is a feasible way to develop new saponin-based vaccine adjuvants through derivatizing at the west wing branched trisaccharide domain. Inclusion of an additional polar functional group such as a carboxyl group (as in **3x**) or a monosaccharide (as in **4x** and **5x**) is sufficient to increase the water solubility of the corresponding synthetic analogues to a level comparable to that of GPI-0100 and suitable for immunological studies and clinical application. The structure of the incorporated side chain has a significant impact on the adjuvant activity in terms of the magnitude and nature of the host's responses.



INTRODUCTION

Recent efforts in developing new vaccines to combat cancer and infectious diseases have relied heavily on subunit antigen constructs. However, the refined and homogeneous antigens are often less immunogenic, which necessitates the use of immune adjuvants to enhance the ability of vaccines to elicit strong and durable immune responses to specific antigens.[1−6] Despite the obvious benefits, the choice of adjuvant for human vaccines is severely limited. Alum (aluminum salts) was the sole adjuvant used in licensed human vaccines for over 80 years until the 1990s. But it is only efficient in eliciting a high antibody response with a Th2 profile instead of eliciting a protective Th1 response. A Th1 response is necessary for vaccines against cancers and intracellular pathogens such as HIV, TB, and malaria.[2,7−9] Since the 1990s, only a few other adjuvants have been approved for use in defined human vaccines, including oil in water emulsions (MF59 and AS03) used in influenza vaccines and a combination adjuvant (i.e., AS04, composed of monophosphoryl lipid A (MPL) adsorbed to alum) used in HBV and HPV vaccines in Europe and the United States. Despite the progress, developing subunit vaccines is still bottlenecked by the lack of safe and effective adjuvants. Because of their urgent need, the discovery and development of novel adjuvants has emerged as a critical frontline effort in vaccine research.

Having promising lead compounds is the critical first step toward successful development of synthetic vaccine adjuvants. Naturally occurring QS saponins can be promising leads in this regard. Among various vaccine adjuvants studied, QS-21 (**1**, Figure 1), a saponin adjuvant obtained from the bark of *Quillaja saponaria* (QS) Molina, stimulates mixed Th1 and Th2 responses. It significantly outperformed other classes of adjuvants (including glucan formulations, peptidoglycans, amphiphilic block copolymers, bacterial nucleosides, and bacterial lipopolysaccharide)[10,11] and has been evaluated in over 100 clinical trials of vaccines against cancer and infectious diseases.[10] Although it is the immunostimulant of choice in many clinical trials of vaccines, QS-21 has its own drawbacks. One of them is its chemical instability in stock solution, originated from the hydrolytically unstable ester moieties (i.e., the one that connects the side chain to the east wing oligosaccharide domain and the internal one in the side chain). SAR studies confirmed that removal of the east wing chain leads to QS-21's loss of the capability of boosting a lymphoproliferative response along with CTL production.[12−16] These inherent drawbacks prevent QS-21 from wider use. Despite recent progress in circumventing these limitations of QS-21,[17,18] (1) QS-based synthetic analogues appropriate for clinical use are still not available, (2) the molecular mechanisms by which QS saponins work are not understood, and (3) synthesis of the unnatural QS-21 analogues remains a challenging and time-consuming task.

To address these challenges, we have recently looked into a different type of synthetic QS-21 analogue. Different from the natural QS-21, which has a hydrolytically labile acyl side chain

Received: April 22, 2016
Published: October 6, 2016

DOI: 10.1021/acs.joc.6b00922
J. Org. Chem. 2016, 81, 9560−9566

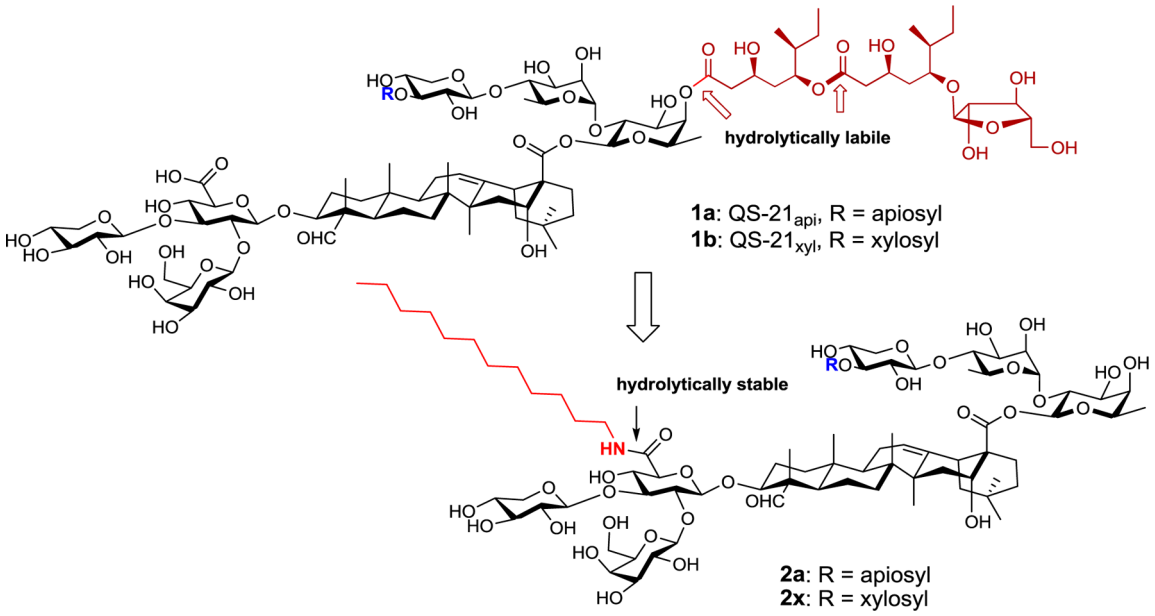


Figure 1. Natural QS-21 (**1**) and synthesized analogues (**2**).

incorporated to the east wing tetrasaccharide domain, the new analogues feature a plain aliphatic chain incorporated to the branched west wing trisaccharide domain through a hydrolytically stable amide bond. The design is inspired by the early work of developing the semisynthetic saponin analogue GPI-0100.[12−15,19] Marciani and co-workers prepared GPI-0100 from Quil A, a complex mixture of QS tree bark extracts (containing QS-21). They completely removed the east wing acyl side chain and replaced it with a plain dodecylamine chain on the other side of the saponins through the amide formation reaction. The obtained complex mixture retains adjuvanticity similar to that of Quil A, stimulating humoral and T-cell immunity along with antigen-specific CTL production. Although the immune stimulatory activity of GPI-0100 is lower than that of the natural saponins, toxicology studies indicated that GPI-0100 is 20 times less lethal in mice than QS-21. In mice, the ratio of acute toxic dose to effective dose is about 40−50, which allows the GPI-0100 dose to be significantly increased to achieve the desired immune response without early onset of toxicity. However, GPI-0100 is highly heterogeneous, with high variability in content and composition, which affects its efficacy and formulation and prevents its use in a clinical setting. Nevertheless, the early work of Marciani et al. on GPI-0100[12−15,19] and the structure−function studies of Soltysik et al.[20] provide us a valuable clue on the initial molecular design of QS analogues (as shown in Figure 1). We have recently synthesized structurally defined **2a** and **2x**,[21] the QS-21-derived components in GPI-0100.[12,16] The stand-alone adjuvant activity of **2x** confirmed that our new design is a feasible path to chemically stable QS-based saponin adjuvants. However, we noticed that **2x** has lower adjuvant activity and water solubility than GPI-0100. Herein, we report our efforts aimed at increasing the water solubility and improving the adjuvant activity of the synthetic analogues.

RESULTS AND DISCUSSION

We infer that inclusion of a polar functional group such as a carboxyl group (Figure 2, as in **3x**) or one extra sugar unit (Figure 2, as in **4x** and **5x**) will increase the water solubility. In

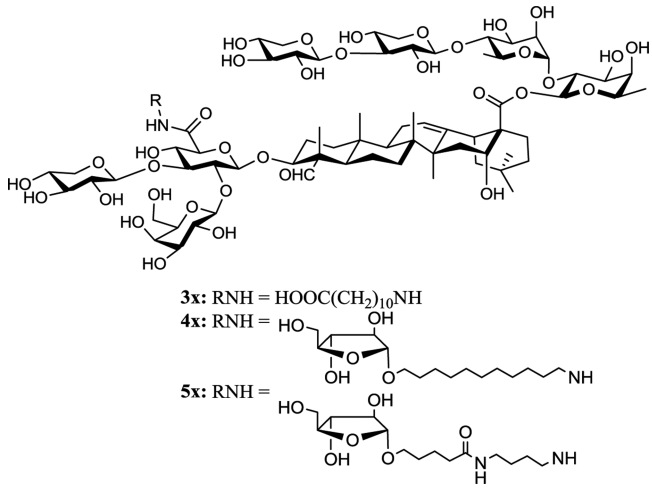


Figure 2. Design of new QS-21 analogues.

3x, the small polar terminal carboxyl group is expected to retain the adjuvant activity of GPI-0100 due to minimal structural variation. In **4x** and **5x**, we included a terminal L-arabinose unit to mimic the terminal structure of the natural acyl side chain in QS-21 (Figure 1). The analogue **5x** differs from **4x** in having an internal polar functional group, mimicking the internal ester moiety in the acyl side chain of the natural QS-21.

Retrosynthesis of the targets **3x**−**5x** leads to the common intermediate **6** and the different side chains **7**−**9** (Scheme 1). The intermediate **6** was also used in the synthesis of **2x**.[21] It can be obtained from the conjugate **10** (prepared in three steps from Quil A)[22,23] and a tetrasaccharide synthesized from the allyl glycoside building blocks **11**−**14** by using the two-stage activation of allyl glycosyl donor method recently developed in our laboratory.[21,24−26]

The side-chain 11-aminoundecanoic acid benzyl ester hydrochloride (**7**) for the synthesis of **3x** can be synthesized from the commercially available 11-aminoundecanoic acid (**15**) in a quantitative yield (Scheme 2).[27] From the compound **16**[28] and peracetyl L-arabinoside (**17**),[29] acid-promoted glyco-

DOI: 10.1021/acs.joc.6b00922
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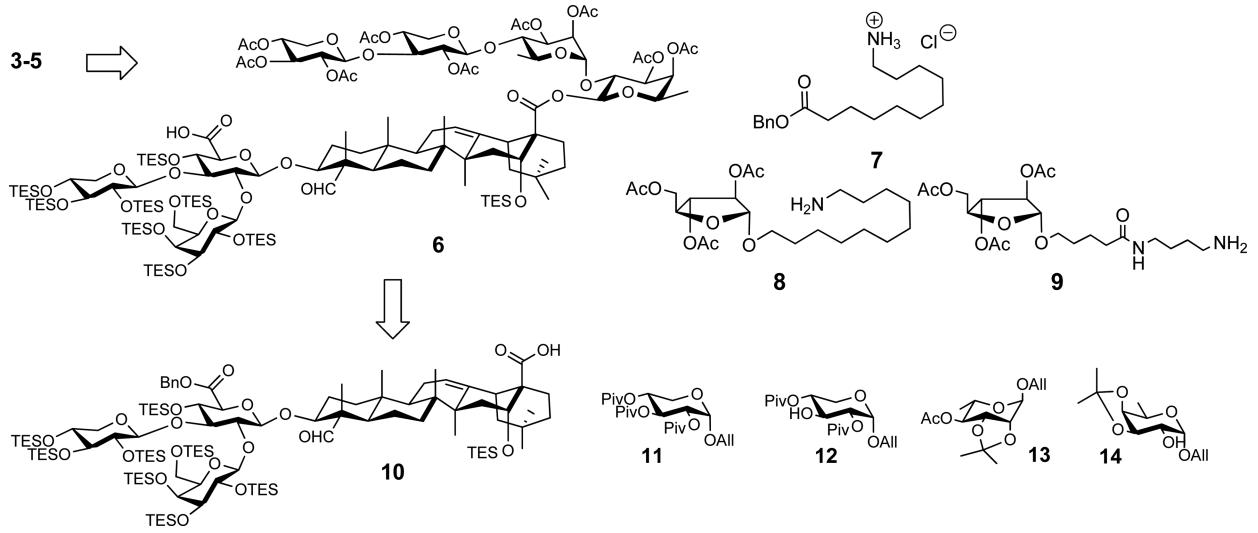
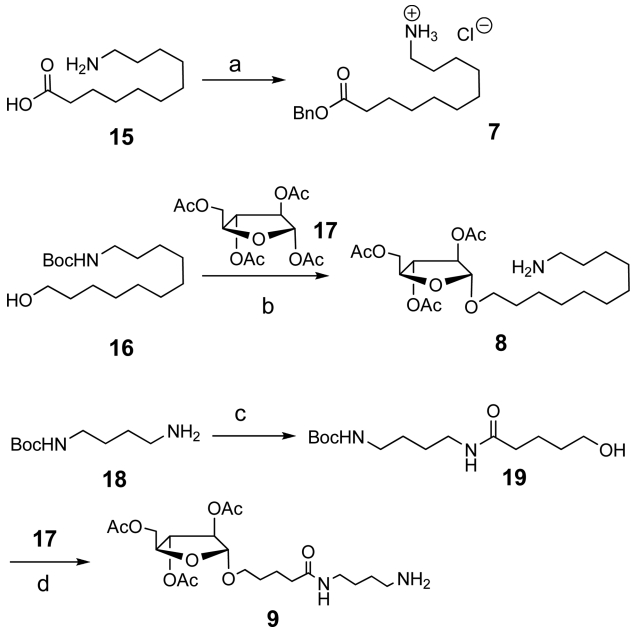
Scheme 1. Retrosynthesis of the Analogues 3−5

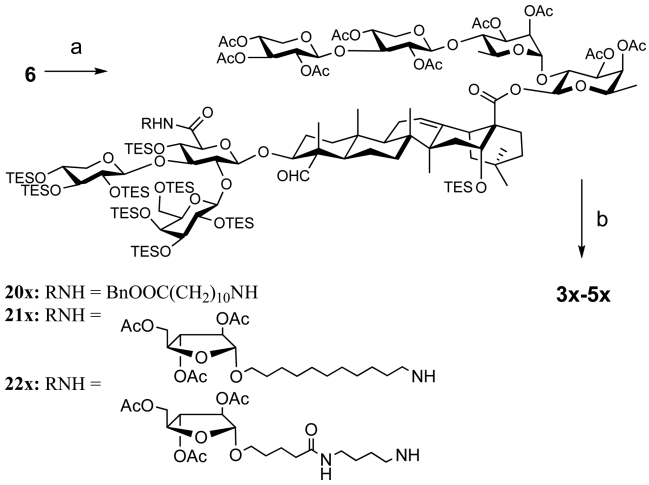


Scheme 2. Synthesis of the Side Chains[a]



[a]Reagents and conditions: (a) $SOCl_2$, BnOH, > 99%; (b) TESOTf (2 equiv), 0 to rt, 51%; (c) δ-lactone, THF, 75 °C, 94%; (d) TESOTf (2 equiv), 0 °C to rt, 79%.

Scheme 3. Synthesis of the Adjuvants 3x−5x[a]



[a]Reagents and conditions: (a) side chain **7**, **8**, or **9**, HATU, DIPEA, $CHCl_3$, 23 °C, 94% for **20x**, 82% for **21x**, 67% for **22x**; (b) TFA/H_2O (4:1), DCM, 0 °C; K_2CO_3, MeOH, 23 °C, 74% for **3x**, 70% for **4x**, 50% for **5x**.

sylation reaction led to the protected side chain **8** with an unoptimized yield of 51% for the synthesis of the analogue **4x**. For the synthesis of the side chain to be incorporated into **5x**, we started with the commercially available *N*-Boc-1,4-butanediamine (**18**). Thus, the reaction of **18** with δ-lactone resulted in the formation of **19** in a 94% yield.[30] In the presence of 2 equiv of TESOTf, the glycosyl acceptor **19** and the donor **17** provided the protected side chain **9** in a 79% yield.[29]

With the three side chains in hand, we attempted the divergent synthesis of the fully protected adjuvant analogues from the common intermediate **6** (Scheme 3). Thus, coupling of **6** with the side chains **7**−**9** by using a standard coupling reagent such as HATU[31] provided the conjugates **20x**, **21x**, and **22x** in 94%, 82%, and 67% yield, respectively. The subsequent deprotection consists of two steps, i.e., removal of the silyl protecting groups under acidic conditions (TFA/H_2O (4:1 v/v) at 0 °C) followed by removal of the acetyl groups under basic conditions (K_2CO_3 in MeOH). The ester moiety between the east wing linear tetrasaccharide and the quillaic acid remained intact under the reaction conditions.[32,33] After reversed-phase HPLC separation and lyophilization, the final products **3x**−**5x** were obtained as white powder in 74%, 70%, and 50% yield, respectively. Inclusion of a polar functional group such as a carboxyl group (as in **3x**) or one extra sugar unit (as in **4x** and **5x**) proved to be sufficient to increase their water-solubility to a level comparable to that of GPI-0100.

For immunological study, we first evaluated the effectiveness of **3x**−**5x** in augmenting immune responses to rHagB. The antigen rHagB is a recombinant, nonfimbrial adhesion hemagglutinin B from *Porphyromonas gingivalis*, a periodontal pathogen.[34−40] We used GPI-0100 and **2x** as the positive controls. BALB/c mice (female, 8−10 weeks of age, six per group) were injected with rHagB (20 μg) alone or rHagB (20 μg) with different adjuvants (i.e., GPI-0100, **2x** and **3x**−**5x**) via

DOI: 10.1021/acs.joc.6b00922
J. Org. Chem. 2016, 81, 9560−9566

a subcutaneous route (s.c.). Mice were immunized on days 0, 14, and 28. Prior to each immunization and at 42 days post the last immunization, mice were weighed and then serum was collected from each mouse and analyzed for anti-rHagB activity using an enzyme-linked immunosorbent assay (ELISA). GPI-0100 augments significantly higher ($P < 0.001$) IgG anti-rHagB antibody responses than that seen with rHagB alone at days 14, 28, and 42, and **2x** showed potentiation at day 42 ($P < 0.05$) (Figure 3).[21] The adjuvants **3x** and **4x** were also effective in

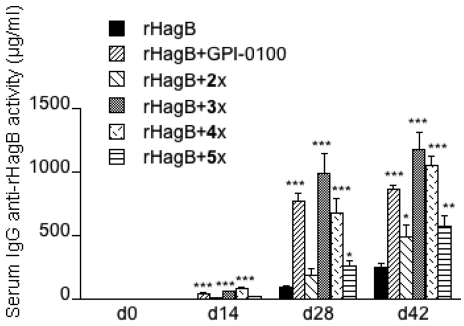


Figure 3. Serum IgG anti-rHagB in mice immunized (s.c.) with antigen ± adjuvant on days 0, 14, and 28. Values are expressed as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with mice immunized with rHagB alone.

enhancing the serum IgG anti-rHagB response after the initial immunization ($P < 0.001$) and subsequent immunizations, compared to that seen with antigen alone. Similarly, the adjuvant **5x** enhanced the anti-rHagB IgG responses at days 28 ($P < 0.05$) and 42 ($P < 0.01$). However, mice immunized with rHagB+**3x** or +**4x** had higher serum IgG anti-rHagB antibody responses compared to mice immunized with rHagB+**2x** or +**5x** at days 28 ($P < 0.001$) and 42 ($P < 0.01$). Interestingly, although the differences were not significant, the anti-rHagB responses potentiated by **3x** at days 28 and 42 were higher than that seen with GPI-0100. No difference was seen in the mean body weights of mice between groups (see Figure S1), indicating that the adjuvants lacked toxicity.

We then assessed the subclass of the IgG antibody responses (Table 1). Immunization of mice with rHagB+GPI-0100 or

Table 1. Serum IgG Subclass Anti-rHagB Activity at Day 42[a]

entry	adjuvant	IgG1 (μg/mL)	IgG2a (μg/mL)	IgG2a/IgG1
1	none	264.6 ± 30.3	9.7 ± 2.8	0.036 ± 0.007
2	GPI-0100	756.4 ± 39.33[d]	327.2 ± 70.2[c]	0.5 ± 0.1[c]
3	**2x**	546.2 ± 41.7[b]	24.2 ± 6.3	0.04 ± 0.01
4	**3x**	950.5 ± 90.5[d]	380.0 ± 72.9[d]	0.40 ± 0.06[b]
5	**4x**	813.3 ± 88.8[d]	495.9 ± 65.2[d]	0.7 ± 0.1[d]
6	**5x**	460.7 ± 28.0	4.5 ± 0.8	0.010 ± 0.002

[a]Values are the mean ± SEM. [b]$P < 0.05$. [c]$P < 0.01$. [d]$P < 0.001$.

with rHagB+**2x**, +**3x**, or +**4x** resulted in significantly higher ($P < 0.05$ or $P < 0.001$) serum IgG1 anti-rHagB antibody levels by day 42 than seen in mice immunized with rHagB alone. A higher IgG1 response was also seen in mice receiving rHagB +**5x** than in mice immunized with antigen alone. Only a slight IgG2a response was induced in the mice receiving rHagB+**5x** or rHagB alone. Although the IgG2a response induced by rHagB +**2x** was higher than that seen with rHagB alone, GPI-0100, **3x**, and **4x** potentiated significantly higher ($P < 0.001$) serum IgG2a anti-rHagB responses. The IgG2a/IgG1 ratio of the anti-rHagB responses indicated that rHagB and rHagB+**2x** preferentially induced IgG1 antibody responses and that rHagB+**5x** further potentiated the response toward the IgG1 direction. However, both IgG1 and IgG2a responses to rHagB were potentiated by GPI-0100, **3x**, and **4x**. These findings suggest that following s.c. immunization, rHagB selectively induces a Th2-like response, whereas the adjuvants **3x** and **4x**, like GPI-0100, potentiate a mixed Th1- and Th2-like response to rHagB and that **5x** mainly enhanced a Th2-like response. Taken together, our results demonstrated that QS-21 derivatives **3x** and **4x** are comparable to GPI-0100 for potentiating systemic responses to rHagB.

In summary, three synthetic QS-21-based immune adjuvants have been derived. These new analogues are equipped with a terminal-functionalized side chain that connects to the west wing branched trisaccharide domain through a hydrolytically stable amide bond with the glucuronic acid moiety. The stand-alone adjuvant activity of the new synthetic analogues again confirmed that our molecular design is a feasible approach to new saponin adjuvants with different adjuvant properties. Addition of one polar functional group such as a carboxyl group (as in **3x**) or an extra sugar unit (as in **4x**) is sufficient to increase the water solubility of the corresponding synthetic analogues to a level comparable to that of GPI-0100 and suitable for immunological studies and potential clinical application. Thus, the structure of the incorporated side chain has a significant impact on adjuvant activity in terms of the magnitude and nature of the responses.

EXPERIMENTAL SECTION

General Methods. Organic solutions were concentrated by rotary evaporation at ca. 12 Torr. Flash column chromatography was performed employing 230−400 mesh silica gel. Thin-layer chromatography was performed using glass plates precoated to a depth of 0.25 mm with 230−400 mesh silica gel impregnated with a fluorescent indicator (254 nm). IR data are presented as frequency of absorption (cm^{-1}). ^1H NMR or ^{13}C NMR spectra were recorded on 300, 400, and 700 MHz NMR spectrometers; chemical shifts are expressed in parts per million (δ scale) downfield from tetramethylsilane. Data are presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet and/or multiple resonances), coupling constants in hertz (Hz), integration. HRMS was conducted with either an ESI or MALDI ionization method and with a TOF mass analyzer.

Materials. Tetrahydrofuran (THF), toluene, dichloromethane (DCM), and acetonitrile (MeCN) were distilled from appropriate drying reagents under a nitrogen atmosphere at 760 Torr. Other chemicals and solvents (such as methanol (MeOH), ethyl acetate (EtOAc), and petroleum ether (PE)) were obtained from commercial vendors and used without further purification.

Synthesis of side chain **8**: A solution of peracetyl L-arabinoside (83 mg, 0.26 mmol) and *N*-Boc-11-amino-1-undecanol (57 mg, 0.20 mmol) in 3.0 mL of DCM at 0 °C was treated with TESOTf (91 μL, 0.40 mmol). The reaction was stirred for 2 h at 0 °C and quenched with triethylamine. The reaction solution was concentrated for column purification on silica gel (eluted with DCM/MeOH 10:1) to afford **8** (70 mg, 79%) as a colorless oil: R_f = 0.5 (DCM/MeOH, 10:1); ^1H NMR (400 MHz, $CDCl_3$) δ 5.09 (s, 1 H), 5.03 (s, 1 H), 4.98 (d, J = 3.3 Hz, 1 H), 4.45 (m, 1 H), 4.26−4.23 (m, 2 H), 3.71 (m, 1 H), 3.46 (m, 1 H), 2.95 (s, 2 H), 2.11 (s, 9 H), 1.69−1.58 (m, 4 H), 1.35−1.28 (m, 14 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.2, 170.7, 170.2, 105.9, 81.7, 80.6, 68.0, 63.8, 40.5, 29.9, 29.8, 29.74, 29.69, 29.3, 27.9, 26.7, 26.4, 21.2; IR (neat) 2927, 28.56, 1744, 1679; HRMS (ESI-TOF) m/e $[M + H]^+$ calcd for $C_{22}H_{40}NO_8$ 446.2754, found 446.2751.

Synthesis of side chain **9**: *N*-Boc-1,4-butanediamine (4.50g, 24 mmol) and δ-lactone (2.87g, 28.7 mmol) were refluxed in THF for 2

DOI: 10.1021/acs.joc.6b00922
J. Org. Chem. 2016, 81, 9560−9566

days. The reaction solution was concentrated, and the residue was taken in EtOAc and washed with water. The organic layer was dried over anhydrous Na_2SO_4, concentrated, and recrystallized in EtOAc/DCM/PE to provide the intermediate **19** (6.5 g, 94%). A solution of peracetyl L-arabinoside (83 mg, 0.26 mmol) and **19** (58 mg, 0.20 mmol) in 3.0 mL of DCM at 0 °C was treated with TESOTf (120 μL, 0.52 mmol). The reaction was stirred for 1.5 h at 0 °C and then 12 h at room temperature before quenched with triethylamine. The reaction solution was concentrated for column purification on silica gel (eluted with DCM/MeOH 9:1) to afford **9** (77 mg, 84%) as a colorless oil: R_f = 0.6 (DCM/MeOH, 9:1); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.80 (t, *J* = 5.5 Hz, 1 H), 5.01 (s, 1 H), 5.00 (s, 2 H), 4.43 (m, 1 H), 4.43−4.20 (m, 2 H), 3.73 (m, 1 H), 3,44 (m, 1 H), 3.25 (m, 2 H), 3.01 (s, 2 H), 2.24 (m, 2 H), 2.14 (s, 3 H), 2.12 (s, 3 H), 2.10 (s, 3 H), 1.73−1.60 (m, 8 H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 175.0, 171.2, 170.7, 170.6, 106.0, 82.0, 80.4, 67.5, 63.6, 39.9, 38.9, 36.2, 31.3, 28.9, 26.6, 24.8, 22.9, 21.2, 21.0; IR (neat) 2938, 1733, 1673, 1638, 1547; HRMS (ESI-TOF) *m*/*e* $[M + H]^+$ calcd for $C_{20}H_{35}N_2O_9$ 447.2343, found 447.2339.

Synthesis of 20x. The QA−trisaccharide conjugate **6** (30 mg, 0.01 mmol)[21] and the side chain **7** (11 mg, 0.03 mmol) in 1.0 mL of chloroform were treated with HATU (14 mg, 0.04 mmol) and *N*,*N*-diisopropylethylamine (11 μL, 0.06 mmol) at room temperature overnight. The reaction mixture was then concentrated and purified directly with column chromatography on silica gel (eluted with PE/EtOAc gradient) to afford the amide **20x** (31 mg, 94%) as a white amorphous solid: R_f = 0.6 (PE/EtOAc, 1:1); $[\alpha]_D^{23}$ = −28.8 (*c* = 1.53, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) (characteristic protons) δ 9.68 (s, 1 H), 7.37−7.33 (m, 5 H), 6.11 (t, *J* = 5.5 Hz, 1 H), 5.44 (d, *J* = 7.8 Hz, 1 H), 5.28 (m, 1 H), 5.20 (d, *J* = 3.2 Hz, 1 H), 5.10−4.82 (m, 10 H), 4.79 (dd, *J* = 7.8, 5.8 Hz, 1 H), 4.58 (d, *J* = 6.0 Hz, 1 H), 4.55 (d, *J* = 7.3 Hz, 1 H), 4.52 (d, *J* = 7.0 Hz, 1 H), 4.48 (s, 1 H), 4.41 (d, *J* = 7.3 Hz, 1 H), 4.26 (d, *J* = 7.2 Hz, 1 H), 4.10 (dd, *J* = 12.0, 4.6 Hz, 1 H), 4.05 (dd, *J* = 11.9, 4.9 Hz, 1 H), 3.94 (s, 1 H), 3.90 (t, *J* = 8.1, 1 H), 3.84−3.65 (m, 7 H), 3.65−3.53 (m, 4 H), 3.50 (m, 1 H), 3.40−3.15 (m, 7 H), 3.12 (t, *J* = 10.0 Hz, 1 H), 2.83 (d, *J* = 11.6 Hz, 1 H), 2.35 (t, *J* = 7.6 Hz, 2 H), 2.25−2.20 (m, 1 H), 2.15 (s, 3 H), 2.10 (s, 3 H), 2.09−2.00 (m, 20 H), 1.20 (d, *J* = 6.2 Hz, 3 H), 1.07 (d, *J* = 6.2 Hz, 3 H), 1.02−0.81 (m, 91 H), 0.79 (s, 3 H), 0.78−0.55 (m, 50 H); ^{13}C NMR (176 MHz, $CDCl_3$) δ 212.2, 175.6, 173.7, 170.4, 170.2, 170.1, 169.9, 169.7, 169.4, 169.2, 169.1, 168.2, 143.4, 136.1, 128.5, 128.4, 128.3, 128.2, 121.6, 108.3, 107.3, 105.8, 104.7, 102.9, 102.4, 101.4, 101.3, 100.7, 100.6, 98.4, 93.9, 79.6, 78.8, 78.7, 77.5, 76.3, 76.2, 76.1, 75.8, 75.7, 75.4, 75.0, 72.6, 72.47, 72.45, 72.0, 71.6, 71.4, 71.36, 70.3, 70.27, 70.0, 69.8, 69.5, 69.2, 68.7, 68.2, 66.1, 65.4, 62.4, 61.5, 60.4, 57.7, 56.0, 53.9, 49.5, 49.3, 46.8, 46.1, 41.7, 40.9, 39.9, 39.3, 38.1, 36.1, 35.1, 34.5, 34.3, 32.8, 32.5, 31.9, 30.7, 30.5, 29.7, 29.6, 29.4, 29.3, 29.2, 29.1, 27.0, 26.4, 25.8, 24.9, 24.3, 23.4, 22.7, 21.0, 20.9, 20.8, 20.7, 20.6, 20.57, 20.53, 20.3, 17.7, 17.5, 15.92, 15.90, 14.1, 12.1, 7.6, 7.5, 7.3, 7.2, 7.1, 7.05, 6.9, 6.8, 5.9, 5.6, 5.4, 5.3, 5.26, 5.23, 5.22, 5.0, 4.4; IR (neat) 2952, 2875, 1750; MS (MALDI) *m*/*e* $[M + Na]^+$ (rel intens) calcd for $C_{159}H_{279}NNaO_{46}Si_9$ 3214.7378 (100.0), 3215.7411 (85.4), 3213.7344 (58.1), 3216.7445 (48.4), 3215.7374 (45.7), 3216.7407 (39.0), 3216.7346 (30.1), 3214.7340 (26.6), 3217.7380 (25.7), 3217.7441 (22.1), 3217.7479 (20.4), 3215.7313 (17.5), 3218.7413 (14.6), 3217.7342 (12.2), 3218.7376 (10.5), found 3213.420, 3214.436, 3215.444, 3216.446, 3217.446, 3218.446.

Synthesis of 21x. The QA−trisaccharide conjugate **6** (30 mg, 0.01 mmol) and the side chain **8** (13 mg, 0.03 mmol) in 1.0 mL of chloroform were treated with HATU (11 mg, 0.03 mmol) and *N*,*N*-diisopropylethylamine (11 μL, 0.06 mmol) at room temperature overnight. The reaction mixture was then concentrated and purified directly with column chromatography on silica gel (eluted with PE/EtOAc gradient) to afford the amide **21x** (27 mg, 82%) as a white amorphous solid: R_f = 0.4 (PE/EtOAc, 1:1); $[\alpha]_D^{23}$ = −36.0 (*c* = 1.0, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) (characteristic protons) δ 9.68 (s, 1 H), 6.12 (t, *J* = 5.5 Hz, 1 H), 5.44 (d, *J* = 7.8 Hz, 1 H), 5.27 (s, 1 H), 5.2 (d, *J* = 4.5 Hz, 1 H), 5.1−4.95 (m, 6 H), 4.94−4.83 (m, 4 H), 4.79 (dd, *J* = 7.7, 6.0 Hz, 1 H), 4.58 (d, *J* = 5.9 Hz, 1 H), 4.56 (d, *J* = 7.3 Hz, 1 H), 4.51 (d, *J* = 7.3 Hz, 1 H), 4.48 (s, 1 H), 4.44 (t, *J* = 5.8 Hz, 1 H), 4.41 (d, *J* = 5.7 Hz, 1 H), 4.29−4.21 (m, 3 H), 4.11 (dd, *J* = 12.2, 4.3 Hz, 1 H), 4.05 (dd, *J* = 11.8, 5.2 Hz, 1 H), 3.93 (d, *J* = 1.7 Hz, 1 H), 3.90 (t, *J* = 8.0 Hz, 1 H), 3.85−3.20 (m, 25 H), 3.11 (t, *J* = 11.2 Hz, 1 H), 2.82 (d, *J* = 15.5 Hz, 1 H), 2.24 (t, *J* = 15.8 Hz, 1 H), 2.16 (s, 3 H); ^{13}C NMR (176 MHz, $CDCl_3$) δ 212.2, 175.6, 170.7, 170.4, 170.3, 170.1, 169.9, 169.72, 169.70, 169.4, 169.2, 169.1, 168.2, 143.4, 121.6, 105.5, 102.8, 101.4, 101.3, 100.7, 100.6, 98.4, 93.9, 86.0, 81.3, 80.1, 79.6, 78.75, 78.73, 77.5, 76.3, 76.2, 76.1, 75.8, 75.7, 75.4, 75.0, 72.6, 72.5, 72.4, 72.0, 71.6, 71.39, 71.36, 70.30, 70.27, 69.95, 69.8, 69.5, 69.3, 68.7, 68.2, 67.7, 65.4, 63.4, 62.4, 61.5, 60.4, 58.2, 53.9, 49.5, 49.3, 46.8, 46.1, 41.7, 40.9, 39.9, 39.3, 38.1, 36.1, 35.1, 34.5, 32.8, 32.5, 31.9, 30.6, 30.5, 29.71, 29.66, 29.62, 29.60, 29.58, 29.40, 29.37, 29.32, 27.0, 26.4, 26.0, 25.4, 24.3, 23.4, 22.7, 21.0, 20.9, 20.84, 20.82, 20.78, 20.71, 20.60, 20.57, 20.53, 20.3, 17.7, 17.5, 15.92, 15.90, 14.1, 12.1, 8.0, 7.6, 7.5, 7.4, 7.3, 7.15, 7.12, 7.09, 7.05, 7.0, 6.9, 6.8, 5.9, 5.6, 5.4, 5.3, 5.26, 5.23, 5.22, 4.9, 4.4; IR (neat) 2953, 2876, 1751; MS (MALDI) *m*/*e* $[M + Na]^+$ (rel intens) calcd for $C_{163}H_{289}NNaO_{52}Si_9$ 3368.7855 (100.0), 3369.7889 (87.6), 3367.7822 (56.7), 3370.7922 (50.8), 3369.7851 (45.7), 3370.7885 (40.0), 3370.7824 (30.1), 3371.7857 (26.4), 3368.7817 (25.9), 3371.7918 (23.2), 3371.7956 (21.4), 3369.7790 (17.1), 3372.7891 (15.3), 3371.7819 (12.2), 3370.7898 (10.7), 3372.7853 (10.6), found 3367.388, 3368.398, 3369.404, 3370.405, 3371.406.

Synthesis of 22x. The QA−trisaccharide conjugate **6** (61 mg, 0.02 mmol) and the side chain **9** (27 mg, 0.06 mmol) in 1.5 mL of chloroform were treated with HATU (24 mg, 0.06 mmol) and *N*,*N*-diisopropylethylamine (10 μL, 0.05 mmol) at room temperature overnight. The reaction mixture was then concentrated and purified directly with column chromatography on silica gel (eluted with PE/EtOAc gradient) to afford the amide **22x** (45 mg, 67%) as a white amorphous solid: R_f = 0.2 (PE/EtOAc, 1:2); $[\alpha]_D^{23}$ = −39.0 (*c* = 0.39, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) (characteristic protons) δ 9.66 (s, 1 H), 6.27 (t, *J* = 5.8 Hz, 1 H), 5.91 (t, *J* = 5.5 Hz, 1 H), 5.45 (d, *J* = 7.8 Hz, 1 H), 5.28 (s, 1 H), 5.20 (d, *J* = 3.3 Hz, 1 H), 5.12−5.09 (m, 2 H), 5.07−5.00 (m, 3 H), 4.90 (m, 1 H), 4.88−4.83 (m, 3 H), 4.78 (t, *J* = 6.8 Hz, 1 H), 4.59 (d, *J* = 6.0 Hz, 1 H), 4.56 (d, *J* = 7.2 Hz, 1 H), 4.51 (d, *J* = 7.4 Hz, 1 H), 4.48 (s, 1 H), 4.43−4.39 (m, 2 H), 4.31 (d, *J* = 7.5 Hz, 1 H), 4.26−4.21 (m, 2 H), 4.11 (dd, *J* = 12.2, 4.5 Hz, 1 H), 4.04 (dd, *J* = 11.8, 5.0 Hz, 1 H), 3.94 (s, 1 H), 3.90 (t, *J* = 7.9 Hz, 1 H), 3.82−3.71 (m, 9 H), 3.67−3.55 (m, 5 H), 3.51−3.43 (m, 2 H), 3.41−3.18 (m, 12 H), 3.12 (t, *J* = 10.7 Hz, 1 H), 2.84 (dd, *J* = 13.9, 3.4 Hz, 1 H), 2.24 (t, *J* = 13.8 Hz, 1 H), 2.20 (t, *J* = 7.6 Hz, 1 H), 2.16 (s, 3 H), 2.12−2.09 (m, 13 H), 2.08−2.00 (m, 23 H), 1.36 (s, 3 H), 1.30−1.25 (m, 8 H), 1.19 (d, *J* = 6.2 Hz, 3 H), 1.07 (d, *J* = 6.4 Hz, 3 H), 1.00−0.92 (m, 100 H), 0.88 (s, 3 H), 0.79 (s, 3 H), 0.77−0.57 (m, 61 H); ^{13}C NMR (176 MHz, $CDCl_3$) δ 211.5, 175.5, 172.8, 170.6, 170.3, 170.2, 170.0, 169.9, 169.8, 169.6, 169.3, 169.2, 169.0, 168.6, 143.4, 127.8, 121.6, 114.0, 105.7, 102.4, 101.5, 101.4, 100.7, 100.6, 98.4, 93.9, 85.3, 81.5, 80.1, 79.8, 78.8, 78.6, 76.3, 76.2, 75.8, 75.7, 75.5, 72.6, 72.4, 72.0, 71.7, 71.5, 71.4, 70.4, 70.3, 69.9, 69.6, 68.7, 68.2, 67.4, 65.4, 63.3, 62.4, 61.6, 60.5, 56.0, 54.0, 49.4, 49.3, 46.8, 46.3, 41.7, 40.9, 39.9, 39.1, 38.8, 38.1, 36.6, 36.2, 36.1, 35.1, 34.5, 32.8, 32.5, 31.9, 31.0, 30.6, 30.5, 29.7, 29.6, 29.4, 28.8, 28.5, 27.2, 26.6, 26.4, 25.2, 24.7, 24.4, 23.4, 23.3, 22.7, 22.5, 20.91, 20.86, 20.78, 20.76, 20.73, 20.68, 20.66, 20.58, 20.54, 20.5, 20.3, 17.7, 17.5, 15.91, 15.90, 14.09, 11.9, 7.9, 7.5, 7.42, 7.35, 7.22, 7.13, 7.12, 7.09, 6.99, 6.91, 6.82, 6.78, 5.8, 5.7, 5.5, 5.4, 5.31, 5.27, 5.24, 5.08, 5.0, 4.5; IR (neat) 2954, 2914, 2877, 1749, 1372, 1224, 1080; MS (MALDI) *m*/*e* $[M + Na]^+$ (rel intens) calcd for $C_{161}H_{284}N_2NaO_{53}Si_9$ 3369.7444 (100.0), 3370.7478 (86.5), 3368.7410 (57.4), 3371.7511 (49.5), 3370.7440 (45.7), 3371.7473 (39.5), 3371.7412 (30.1), 3369.7406 (26.2), 3372.7446 (26.1), 3372.7507 (22.6), 3372.7545 (20.6), 3370.7379 (17.3), 3373.7479 (14.9), 3372.7408 (12.2), 3371.7486 (10.9), 3373.7442 (10.5), found 3368.824, 3369.708, 3370.736, 3371.762, 3372.767, 3373.731.

Synthesis of 3x. To the fully protected conjugate **20x** (30 mg, 9.4 μmol) in 2.0 mL of THF was added Pd/C (8 mg, 10%). After being shaken under hydrogen (55 psi) for 23 h, the reaction mixture was filtered through a Celite 545 plug and concentrated. The residue in 0.1 mL of DCM was treated with 0.6 mL of TFA/H_2O (4:1) at 0 °C for 40 min, and the liquid was then removed under vacuum at 0 °C. The

DOI: 10.1021/acs.joc.6b00922
J. Org. Chem. 2016, 81, 9560−9566

residue was dissolved in 1.2 mL of MeOH with five drops of chloroform and treated with K_2CO_3 (20 mg) overnight. The reaction solution was centrifuged, and half of the solution was concentrated and purified with RP-HPLC (MeCN/H_2O gradient). The product fraction was concentrated on a rotary evaporator at room temperature to remove MeCN, and the remaining water was then removed on a lyophilizer to afford **3x** (4.6 mg, 74%) as a white powder: ^{1}H NMR (400 MHz, CD_3OD) (characteristic protons) δ 9.35 (s, 1 H), 5.33 (d, J = 1.5 Hz, 1 H), 5.21 (s, 1 H), 5.19 (d, J = 8.3 Hz, 1 H), 4.70 (d, J = 7.6 Hz, 1 H), 4.52 (s, 1 H), 4.47 (d, J = 7.8 Hz, 1 H), 4.44−4.40 (m, 2 H), 4.38 (s, 1 H), 4.33 (d, J = 7.2 Hz, 1 H), 3.85−3.25 (m, 32 H), 3.20−3.05 (m, 7 H), 2.85 (dd, J = 13.9, 3.2 Hz, 1 H), 2.20 (t, J = 13.6 Hz, 1 H), 2.12 (t, J = 7.5 Hz, 1 H), 1.90−1.77 (m, 5 H), 1.72−1.58 (m, 4 H), 1.54−1.47 (m, 3 H), 1.45−1.40 (m, 5 H), 1.29 (s, 3 H), 1.27−1.20 (m 20 H), 1.11 (d, J = 6.4 Hz, 3 H), 1.07 (s, 3 H), 0.90 (s, 3 H), 0.84 (s, 3 H), 0.78 (s, 3 H), 0.65 (s, 3 H); ^{13}C NMR (176 MHz, CD_3OD) δ 212.2, 178.0, 171.6, 145.7, 124.0, 107.9, 106.5, 105.8, 105.4, 104.6, 102.1, 95.9, 88.2, 87.8, 87.2, 86.1, 79.1, 78.8, 78.6, 77.85, 77.76, 77.3, 76.3, 76.2, 76.1, 75.9, 75.3, 75.1, 74.5, 74.4, 73.5, 73.2, 72.7, 72.2, 71.8, 71.7, 71.5, 70.3, 69.4, 68.1, 68.0, 67.8, 62.8, 57.1, 50.9, 48.8, 43.7, 43.1, 41.9, 41.0, 40.2, 38.0, 37.5, 34.4, 34.2, 32.2, 31.7, 31.52, 31.47, 31.45, 31.36, 31.1, 28.7, 28.0, 27.8, 26.8, 25.6, 25.3, 22.3, 19.2, 18.5, 17.4, 17.3, 11.9; HRMS (ESI-TOF) m/e $[M - H]^-$ calcd for $C_{80}H_{128}NO_{37}$ 1694.8165, found 1694.8182.

Synthesis of 4x. To the fully protected conjugate **21x** (15 mg, 4.5 μmol) in 0.1 mL of DCM cooled in an ice−water bath was added 0.5 mL of TFA/H_2O (4:1). After being stirred at 0 °C for 40 min, the liquid was removed under vacuum at 0 °C. The residue was dissolved in 1.0 mL of MeOH with five drops of chloroform and treated with K_2CO_3 (10 mg) overnight. The reaction solution was centrifuged, and the solution was concentrated and purified with RP-HPLC (MeCN/H_2O gradient). The product fraction was concentrated on a rotary evaporator at room temperature to remove MeCN, and the remaining water was then removed on a lyophilizer to afford **4x** (5.6 mg, 70%) as a white powder: ^{1}H NMR (400 MHz, CD_3OD) (characteristic protons) δ 9.51 (s, 1 H), 5.47 (s, 1 H), 5.33 (s, 1 H), 5.32 (d, J = 8.2 Hz, 1 H), 4.83 (d, J = 7.6 Hz, 1 H), 4.60 (d, J = 7.7 Hz, 1 H), 4.55−4.50 (m, 3 H), 4.46 (d, J = 6.6 Hz, 1 H), 4.00−3.64 (m, 30 H), 3.62−3.37 (m, 19 H), 2.98 (d, J = 13.8 Hz, 1 H), 2.34 (t, J = 13.6 Hz, 1 H), 2.00−1.91 (m, 7 H), 1.85−1.70 (m, 5 H), 1.68−1.60 (m, 4 H), 1.58−1.45 (m, 7 H), 1.42−1.31 (m, 32 H), 1.24 (d, J = 6.3 Hz, 3 H), 1.21 (s, 3 H), 1.03 (s, 3 H), 0.98 (s, 6 H), 0.91 (s, 3 H), 0.78 (s, 3 H); ^{13}C NMR (176 MHz, CD_3OD) δ 209.9, 175.8, 169.3, 143.5, 121.7, 108.0, 105.6, 104.2, 103.6, 103.1, 102.3, 99.8, 93.6, 85.9, 84.9, 83.8, 83.7, 82.2, 77.3, 76.8, 76.6, 76.4, 75.6, 75.5, 75.0, 73.9, 73.8, 73.6, 72.8, 72.2, 71.2, 71.0, 70.4, 69.6, 69.5, 68.0, 67.5, 67.2, 65.8, 65.6, 61.6, 60.6, 56.3, 56.2, 56.1, 55.9, 55.8, 54.8, 41.4, 40.8, 39.6, 38.7, 37.9, 35.7, 35.2, 32.0, 30.8, 30.0, 29.5, 29.4, 29.3, 29.2, 29.1, 28.9, 26.5, 25.9, 25.8, 24.6, 23.3, 23.1, 20.7, 20.1, 16.9, 16.2, 16.1, 16.0, 15.9, 15.8, 15.7, 15.1, 15.0, 9.6; HRMS (ESI-TOF) m/e $[M - H]^-$ calcd for $C_{85}H_{138}NO_{40}$ 1812.8795, found 1812.8800.

Synthesis of 5x. To the fully protected conjugate **22x** (11 mg, 3.3 μmol) in 0.1 mL of DCM cooled in an ice−water bath was added 0.5 mL of TFA/H_2O (4:1). After stirred at 0 °C for 40 min, the liquid was removed under vacuum at 0 °C. The residue was dissolved in 1.0 mL of MeOH with five drops of chloroform and treated with K_2CO_3 (10 mg) overnight. The reaction solution was centrifuged, and the solution was concentrated and purified with RP-HPLC (MeCN/H_2O gradient). The product fraction was concentrated on a rotary evaporator at room temperature to remove MeCN, and the remaining water was then removed on a lyophilizer to afford **3x** (3.0 mg, 50%) as a white powder. ^{1}H NMR (400 MHz, CD_3OD) (characteristic protons) δ 9.51 (s, 1 H), 5.49 (s, 1 H), 5.36 (s, broad, 1 H), 5.34 (d, J = 8.3 Hz, 1 H), 4.00−3.70 (m, 26 H), 3.70−3.50 (m, 13 H), 3.00 (d, J = 13.8 Hz, 1 H), 2.40−2.23 (m, 3 H), 1.80−0.70 (m, 47 H), 0.99 (s, 3 H), 0.93 (s, 3 H); ^{13}C NMR (176 MHz, CD_3OD) δ 210.0, 175.8, 174.7, 169.4, 168.9, 143.4, 121.7, 108.1, 105.6, 104.2, 103.6, 103.1, 102.3, 99.9, 93.6, 85.9, 85.6, 85.0, 83.9, 83.8, 82.2, 77.3, 76.8, 76.5, 76.3, 76.0, 75.5, 75.0, 74.0, 73.9, 73.8, 73.6, 73.0, 72.8, 72.24, 72.15, 71.2, 71.0, 70.4, 70.0, 69.55, 69.47, 69.2, 68.0, 67.1, 67.0, 65.9, 65.8, 65.5, 61.7, 60.5, 56.32, 56.20, 56.07, 55.97, 55.8, 54.9, 48.1, 46.63, 46.56, 41.4, 40.8, 39.6, 38.6, 38.4, 37.9, 35.7, 35.4, 35.2, 32.1, 32.0, 30.9, 30.0, 28.6, 26.2, 25.7, 24.5, 23.3, 23.1, 22.6, 20.1, 16.9, 16.2, 16.1, 16.0, 15.9, 15.8, 15.7, 15.6, 15.1, 15.0, 9.6; HRMS (ESI-TOF) m/e $[M + H]^+$ calcd for $C_{83}H_{135}N_2O_{41}$ 1815.8540, found 1815.8558.

Immunological Evaluation of QS-21-Based Immune Adjuvants. *Mice and Immunization.* BALB/c mice were used in this study and were purchased from Frederick Cancer Research (Fredrick, MD). Mice were maintained within an environmentally controlled, pathogen-free animal facility at the University of Alabama at Birmingham. To assess the adjuvant activity of the QS-21-based immune adjuvant, groups of mice (8−10 weeks of age; six female mice per group) were immunized by a subcutaneous (s.c.) route with rHagB (20 μg) along or with GPI-0100 (100 μg) or with a synthetic adjuvant (100 μg) on days 0, 14, and 28. We used the s.c. route of immunization since we[21,36,39,40] and others[12−16,20] have used this route in previous studies to assess vaccines and since this route is used to vaccinate humans. Mice were weighed, and blood samples were collected prior to and at various time points following the initial immunization. Blood samples were obtained via the retro-orbital plexus by using heparinized capillary pipettes. The blood samples were centrifuged, and the serum was collected and stored at −20 °C until it was assayed. All studies were done in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols involving animal research were approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham (Protocol No. IACUC-20222 under Institutional Animal Assurance No. A-3255-01).

ELISA. The levels of specific serum IgG and IgG subclasses against rHagB in each group were determined by ELISA using Maxisorp microtiter plates (NUNC International, Roskilde, Denmark) coated with rHagB (1 μg/mL) or with optimal amounts of goat antimouse IgG, IgG1, or IgG2a (Southern Biotechnology Associates, Inc., Birmingham, AL) in borate buffer saline (BBS; 100 mM NaCl, 50 mM boric acid, 1.2 mM $Na_2B_4O_7$, pH 8.2) at 4 °C overnight. Plates were washed, and then BBS containing 1% bovine serum albumin (BSA) and 0.02% sodium azide was added to wells for 2 h at room temperature. Serial 2-fold dilutions of serum samples were added in duplicate to the plates. In addition, serial dilutions of a mouse immunoglobulin reference serum (MP Biomedicals, Solon, OH) were added to two rows of wells in each plate that had been coated with the appropriate antimouse IgG or IgG subclass reagent, in order to generate standard curves. After incubation (overnight at 4 °C) and washing of the plates, horseradish peroxidase-conjugated goat antimouse IgG or IgG subclass antibody (Southern Biotechnology Associates, Inc.) was added to the appropriate wells. After 4 h of incubation at room temperature, plates were washed and developed by *o*-phenylenediamine substrate with hydrogen peroxide. Color development was recorded at 490 nm. The concentrations of antibodies were determined by interpolation on standard curves generated by using the mouse immunoglobulin reference serum and constructed by a computer program based on four-parameter logistic algorithms (Softmax/Molecular Devices Corp., Menlo Park, CA).

Statistical Analysis. Statistical significance in antibody responses and body weights between groups was evaluated by ANOVA and the Tukey multiple-comparisons test using the InStat program (Graph Pad Software, San Diego, CA). Differences were considered significant at a P value < 0.05.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b00922.

^{1}H and ^{13}C NMR spectra of the new compounds (PDF)

DOI: 10.1021/acs.joc.6b00922
J. Org. Chem. 2016, 81, 9560−9566

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The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was supported by the NIH (AI099407) and the UAB CAS Interdisciplinary Innovation Team Award. We also thank Dr. Michael J. Jablonsky for assistance with NMR spectroscopy.

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DOI: 10.1021/acs.joc.6b00922
J. Org. Chem. 2016, 81, 9560−9566