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Article

Synthesis and Evaluation of Liposomal Anti-GM3 Cancer Vaccine Candidates Covalently and Noncovalently Adjuvanted by αGalCer

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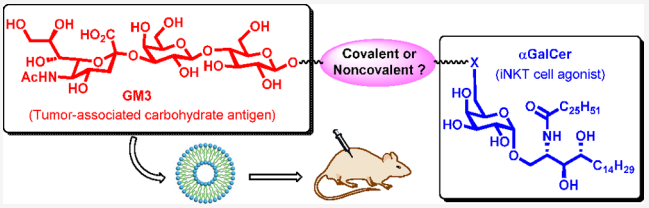
ABSTRACT: GM3, a typical tumor-associated carbohydrate antigen, is considered as an important target for cancer vaccine development, but its low immunogenicity limits its application. αGalCer, an iNKT cell agonist, has been employed as an adjuvant via a unique immune mode. Herein, we prepared and investigated two types of antitumor vaccine candidates: (a) self-adjuvanting vaccine **GM3-αGalCer** by conjugating GM3 with αGalCer and (b) noncovalent vaccine **GM3-lipid/αGalCer**, in which GM3 is linked with lipid anchor and coassembled with αGalCer. This demonstrated that βGalCer is an exceptionally optimized lipid anchor, which enables the noncovalent vaccine candidate **GM3-βGalCer/αGalCer** to evoke a comparable antibody level to **GM3-αGalCer**. However, the antibodies induced by **GM3-αGalCer** are better at recognition B16F10 cancer cells and more effectively activate the complement system. Our study highlights the importance of vaccine constructs utilizing covalent or noncovalent assembly between αGalCer with carbohydrate antigens and choosing an appropriate lipid anchor for use in noncovalent vaccine formulation.



INTRODUCTION

Many cancer cells are expressed with aberrant levels and types of carbohydrate structures on their surfaces, known as tumor-associated carbohydrate antigens (TACAs). Due to the structural conservatism and its important roles in tumorigenic processes, TACAs have been recognized as promising targets for the development of cancer vaccines.[1−6] GM3, a sialylated trisaccharide TACA, is highly expressed in a broad range of human malignancies; furthermore, the presence of this molecule in normal tissues is undetectable.[7] A variety of GM3-targeting cancer immunotherapies have been evaluated in the clinical trials, showing evidence of a clinical effect.[8,9] However, because of its weak immunogenicity and T cell independence, the GM3 antigen alone is unable to initiate the adaptive immune system to stimulate a robust immune response against tumor cells, thus the common strategy in preparing the anti-GM3 vaccine is to conjugate GM3 to immunogenic carriers, a modality known as semisynthetic vaccines. Unfortunately, semisynthetic cancer vaccines are suboptimal in some clinical trials, and the immune responses to the TACAs vary significantly from person to person. The possible reason might be the resulting anticarrier antibody responses.[10] To resolve this issue, fully synthetic vaccines with well-defined molecular structures are pursued, generally comprising an adjuvant, B cell epitopes, and Th cell epitopes.[11] These vaccines are able to eliminate inessential epitopes and elicit more specific immune responses.

Adjuvants play an essential role in the improvement of vaccine efficacy by providing appropriate "danger signals".[12−14] αGalCer, an iNKT cell glycolipid agonist, has been widely used as a vaccine adjuvant in the treatment of cancer, viruses, and bacteria.[15,16] iNKT cells are a unique population of T cells with immunomodulatory properties that link innate and adaptive immune responses. Unlike conventional T cells that see the peptides associated with MHC molecules, iNKT cells recognize glycolipid epitopes present by CD1d, an MHC class I-like molecule. The CD1d molecule is nonpolymorphic, therefore, αGalCer can act as a "universal helper".[17] Upon activation by glycolipids binding to CD1d, iNKT cells rapidly release potent cytokines leading to downstream activation of a broad spectrum of immune cells. Numerous strategies of αGalCer-based cancer therapy have been developed.[18−21] Clinical trials have demonstrated that iNKT cell agonists are immunologically active in humans and have good safety profiles.[22] Recently, iNKT cell agonists have been identified as potent immunological stimulants of a new class of carrier molecules for the construction of fully synthetic self-adjuvant conjugate vaccines.[23−32] In these vaccines, Th epitope peptides are not incorporated, and αGalCer plays a specific role: carrier molecular and adjuvant. The iNKT cells provide cognate or noncognate help to B-cells,[33,34] representing a novel mode of immunotherapy.

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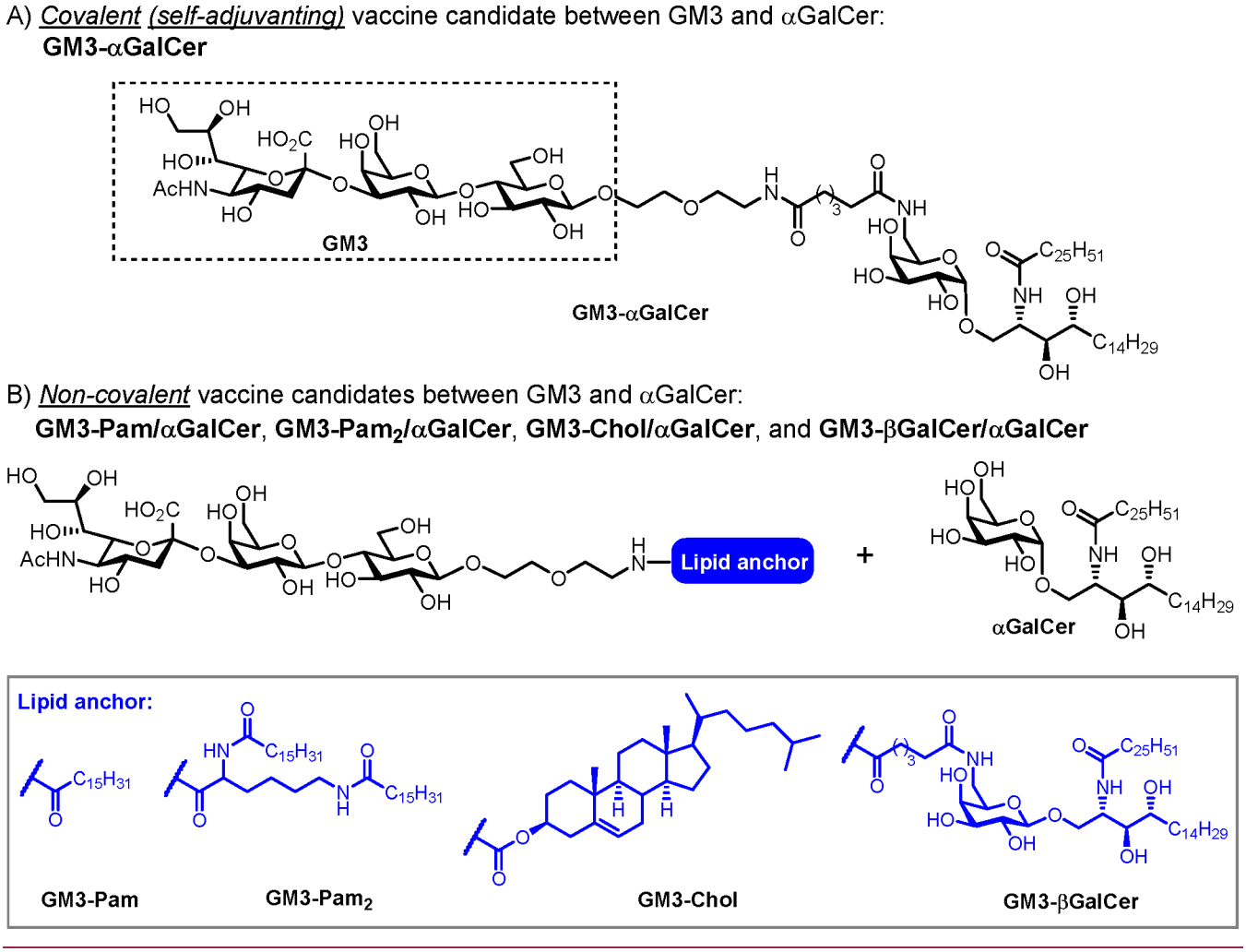
Scheme 1. (A) GM3 Covalently Conjugated with αGalCer: GM3-αGalCer and (B) Lipid-Modified GM3 Conjugates Non-Covalently Co-Formulated with αGalCer: GM3-Pam/αGalCer, GM3-Pam$_2$/αGalCer, GM3-Chol/αGalCer, and GM3-βGalCer/αGalCer



In the present study, we extended the self-adjuvanting approach to GM3, preparing **GM3-αGalCer** conjugate, of which the antibody response and anticancer properties were evaluated (Scheme 1A). Adjuvant and antigen codelivery to the same antigen-presenting cells can break tolerance to self-antigen and enhance antigen-specific response.[35−37] Covalent conjugation is an important strategy for codelivery. In addition to the self-adjuvanting (or covalent) strategy, an alternative method could be to form a liposome in which a lapidated antigen and lipophilic adjuvant are coassembled in a non-covalent manner, simplifying the synthesis and avoiding the modification of the adjuvant (Scheme 1B). A previous study demonstrated that an amphiphilic vaccine comprising of an antigen and adjuvant cargo linked to a lipophilic lipid tail is able to increase the potency of subunit vaccines.[38] Savage and co-workers successfully developed the noncovalent vaccine against *Streptococcus pneumoniae* serotype 14 polysaccharide, which outperformed the clinically used semisynthetic vaccine in potentiating the antibody response.[39,40] The antigenic polysaccharide was modified by lipid-tail, potentially able to match the amphiphilicity of the iNKT cell glycolipid agonist. However, it is noteworthy that the effects of lipidation are manifold,[41] and the hydrophobicity bought about by lipidation is amendable to the particular antigens, therefore, the lipid-anchor or hydrophobic foot appended needs to be optimized. For example, our recent study showed that the dipalmitoyl-tailed glycopeptide antigen MUC1 is more effective than the monopalmitoyl-tailed and free MUC1 in potentiating immunological responses.[42] In contrast, BenMohamed and co-workers reported that the antiviral T cell responses induced by the palmitoyl-tailed Th-CTL chimeric epitopes are irrespective of the number of lipid moieties.[43] Therefore, whether the lipid anchor attached to GM3 can trigger an optimal immune response remains to be investigated.

In our ongoing study of anti-TACA vaccines,[28,42,44] we prepared a covalent vaccine candidate, **GM3-αGalCer**, that can induce an effective antibody response. Furthermore, we also hypothesized that an alternative vaccine modality, in which GM3 antigen is modified with lipids of weak immunogenicity and coformulated noncovalently with αGalCer in a liposome, could elicit an antibody response as effective as that of **GM3-αGalCer**. To evaluate the impact of lipidation and to identify an optimal lipid anchor, we constructed four glycolipids: **GM3-Pam**, **GM3-Pam$_2$**, **GM3-Chol**, and **GM3-βGalCer**, which appended with monopalmitoyl (Pam), dipalmitoyl (Pam$_2$), cholesterol, and βGalCer (the anomer of αGalCer), respectively (Scheme 1B). The immune responses induced in mice by the vaccine candidates were characterized by the anti-GM3

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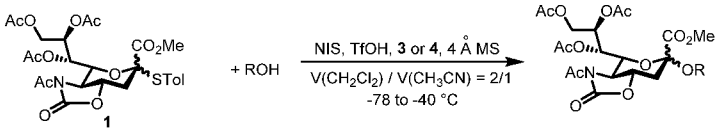
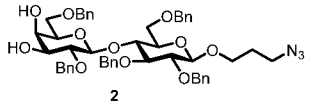
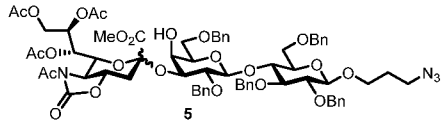
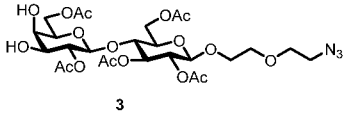
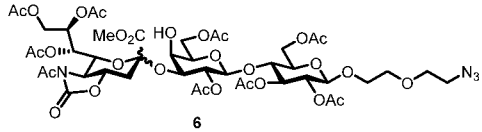
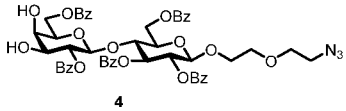
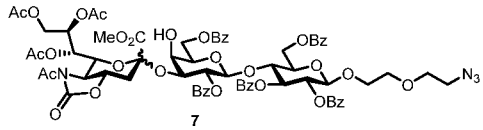
Table 1. Synthesis of GM3 by [1 + 2] Glycosylation



Entry	Acceptor [a]	Product	yield [b]	α/β ratio [c]
1[d]	 2	 5	71%	2:1
2	 3	 6	23%	1:2.5
3	 4	 7	31%	1:1.6

[a]Donor/acceptor = 1.2/1, −78 to −40 °C for 1 h. [b]Isolated yield. [c]Determined by ^{1}H NMR spectroscopic analysis of the unpurified reaction mixture. [d]Ref 54.

antibody titers and the concentrations of cytokines (IFN-γ and IL-4). The efficacies of the vaccine candidates were further evaluated by the binding of the induced sera to B16F10 tumor cells and the activation of complement-dependent cytotoxicity.

RESULTS AND DISCUSSION

The self-adjuvanting vaccine candidate **GM3-αGalCer** was designed by following our previous research on an anti-STn vaccine.[28] As for the noncovalent candidates, Pam, Pam$_2$, Chol, and βGalCer were chosen as the lipid anchors. Notably, the anti-MUC1 vaccine using a lysine-backbone-based dipalmitoyl lipid anchor showed great potency in stimulating robust antibody response.[42] βGalCer, a weak iNKT cell agonist,[45] was chosen because of high structural similarity to αGalCer, which could render GM3 similar pharmacokinetics to αGalCer. In addition, GM3-βGalCer was used as a control in our study. In all lipid-modified GM3 conjugates, the linkers bear no immunogenic heterocycle in order to avoid immunological suppression.[46]

For the synthesis of GM3, highly regio- and stereoselective glycosylation is desirable. For the widely used donor, 4O,5N-acetyl oxazolidinone-protected *p*-toluenethiosialoside, the trans orientation of the oxazolidinone group always leads to excellent α selectivity of sialylation.[47−49] The [1 + 2] synthetic route for GM3 by using lactosyl as the acceptors has been reported due to the short steps.[50−55] On the basis of previous report, though the lactosyl diol **2** bearing multiple benzyl ether of protective groups as the acceptor only gave moderate yield of the trisaccharide product, it was very difficult to isolate the anomers (α/β ratio 2/1) by column chromatography.[54] Instead of benzyl ether, these acceptors with acetyl or benzoyl ester as the protective groups were applied. Due to the disarming effect of acetyl or benzoyl protective groups, both acceptors **3** and **4** afforded a mixture of trisaccharide compounds (α:β ratio 1:2.5 or 1:1.6, respectively) with a low yield (20−30%) (Table 1). This result was similar to a previous report.[56]

The synthesis of lipid-modified GM3 conjugates is outlined in Scheme 2. Instead of the [1 + 2] glycosylation reactions, an alternative [2 + 1] synthetic sequence was carried out, which followed the previously reported procedures with slight modifications (Scheme 2A).[57−59] Sialodisaccharide **13** was prepared by dibutyl phosphate sialoside **11** and **12** in a 83% yield (α/β ratio 9/1), whose α-sialyl linkage was verified by NMR. The chemical shifts of the H-3eq of sialic acid is 2.89 ppm (dd, J = 12.1, 3.2 Hz) for α-glycoside and 2.58 ppm (dd, J = 12.1, 3.6 Hz) for β-glycoside. Additionally, the chemical shift of C-2 of sialic acid is 97.3 ppm for α-glycoside and 99.4 ppm for β-glycoside.[59,60] Glycosylation of penta-*O*-acetylglucose (**8**) with 2-(2-azidoethoxy) ethan-1-ol and subsequent manipulation of the protecting groups offered glycosyl acceptor **10**.[61] The NIS/TfOH-promoted glycosylation between **13** and **10** provided the fully protected form of GM3 antigen **14** with high β-selectivity. The attempt to Staudinger reduction of azide **14** in the presence of PMe$_3$/wet MeOH led to an unidentified mixture probably due to the high electrophilicity of the carbonyl group in oxazolidinone. Therefore, our initial plan was to cleave the acetyl and oxazolidinone protective group at the same condition.[49,62] However, we found that treatment of **14** with a freshly prepared NaOMe/MeOH solution (the concentration of NaOMe was adjusted to be 2.0 mM) within 10 min at room temperature provided methyl carbonate **15** in high yield, allowing acetyl groups to be intact. The formation of methyl carbonate was confirmed by HRMS and NMR (a clearly observed upfield-shifted methyl group (δ = 1.86) of NHAc at the 5-position, and appearance of the methyl group (δ = 3.68) at carbonate). This result parallels Sugai and co-workers' report on the selective cleavage of oxazolidinone.[63] Staudinger reduction of **15** and amide-formation followed by global deprotection was uneventful, affording the desired lipid-anchored GM3 conjugates **GM3-αGalCer**, **GM3-βGalCer**, **GM3-Pam**, and **GM3-Pam$_2$**. Because the alkene in cholesterol may be saturated under the condition of catalytic hydrogenolysis, the above synthetic sequence is not suitable for the preparation of **GM3-Chol**. Therefore, an alternative route was carried out (Scheme 2B). After the removal of all the protecting groups, the GM3 antigen was conjugated with **22**, obtained from the reaction of *p*-nitrophenyl chloroformate with cholesterol,[64] to yield the

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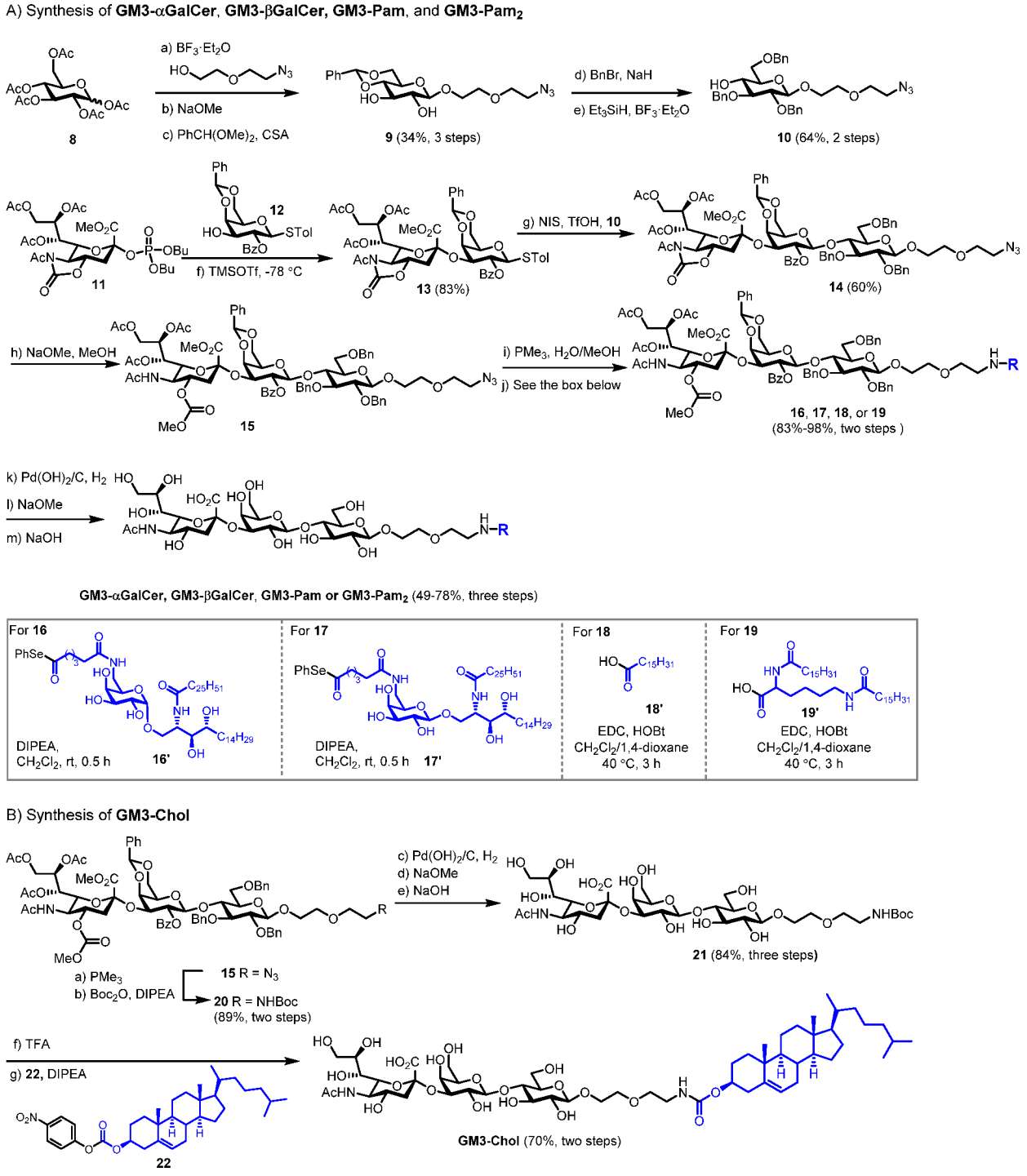
Scheme 2. Synthetic Routes to GM3-αGalCer, GM3-βGalCer, GM3-Pam, and GM3-Pam$_2$ (A) and GM3-Chol (B)



targeted conjugate **GM3-Chol**. All the lipid-modified GM3 conjugates were purified by size-exclusion chromatography, and their purities were confirmed by ^{1}H and ^{13}C NMR spectroscopy (see the Supporting Information).

To prepare the coating antigen for ELISA (Scheme 3), the terminal amino group derived from **21** was reacted with diethyl squarate in EtOH/H_2O at pH 8.0 resulting in squaric acid monoamide **23**,[65] which was dissolved together with BSA in a buffer solution at a pH of 9.5 to afford **GM3-BSA** conjugates (on average 7.4 molecules of GM3 per molecule of BSA). The loading of the GM3 antigen on BSA was determined by MALDI-TOF mass spectrometry (see the Supporting Information).

To investigate the multivalency of the GM3 antigen, all the GM3 conjugates were formulated into liposomes. In terms of covalent modality, **GM3-αGalCer** was incorporated into liposomes by hydration of a thin film of DSPC, cholesterol (Chol), and **GM3-αGalCer** (molar ratios 5:4:1) in a HEPES buffer (10 mm, pH 6.5) containing NaCl (145 mm), followed by sonication for 15 min. The above formulation replacing **GM3-αGalCer** with **GM3-βGalCer** was used as a control. For the noncovalent modality, liposomes of mixtures of lipid-anchoring

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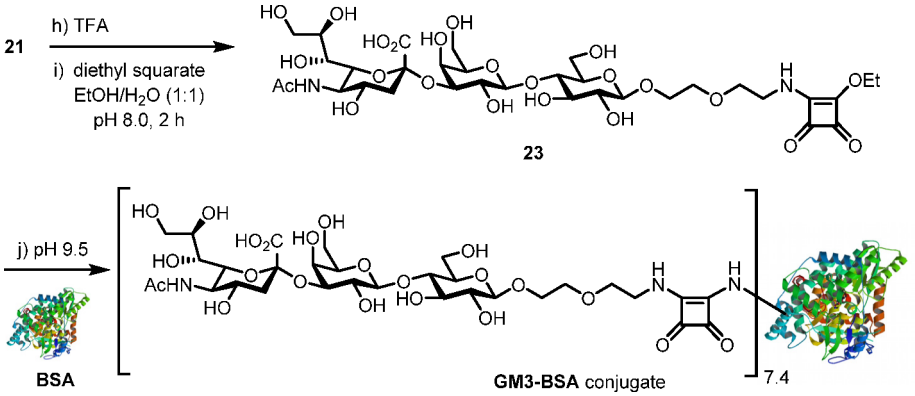
Scheme 3. Preparation of GM3-BSA Conjugate as Coating Antigen for ELISA



GM3 conjugates with ***α*GalCer** [molar ratios: DSPC/Chol/αGalCer/**GM3-Pam** (or **GM3-Pam$_2$**, **GM3-Chol**, **GM3-*β*GalCer**) 5:4:1:1] in the HEPES buffer were prepared via the same method as that of covalent modality. The contents of GM3 antigen (1.5 μg) and αGalCer (2 μg) were consistent in all vaccine candidates. The physicochemical properties of the vaccine candidate liposomes were evaluated (Figure S4). The dynamic light scattering (DLS) confirmed that the liposomes are sized with an approximate diameter range of 200−800 nm. Furthermore, the surface charges represented by zeta potential of liposomes were measured, all vaccine candidates had negative zeta potentials.

Next, groups of five female BALB/c mice were immunized intraperitoneally with freshly prepared liposomes at biweekly intervals (days 1, 15, and 29), and sera were collected on days 14, 28, and 42. To determine whether the vaccine candidates can promote cytokine production, the secretion of IL-4 and IFN-γ after the first immunization was evaluated (Figure 1). All groups produced a high level of IL-4 and IFN-γ compared to the control **GM3-*β*GalCer**. This rapid production of cytokines indicated that the iNKT cells were activated for all the vaccine candidates. The cytokines induced between covalent and noncovalent candidates were not significantly different, confirming that 6-position conjugation exerts a marginal impact on the immunological activity of αGalCer. Interestingly, although the contents of αGalCer are consistent among the noncovalent vaccine candidates, **GM3-Chol/*α*GalCer** is more potent than **GM3-*β*GalCer/*α*GalCer** in promoting Th1-biasing cytokine response, indicating that the physicochemical properties of liposomes might affect the pharmacokinetics of αGalCer leading to different Th1/Th2 profiles.[66,67]

We then measured the antibody titers of IgM and IgG in serum by ELISA using GM3-BSA as the coating antigen (Figure 2). In terms of IgG, all noncovalent vaccine candidates but **GM3-*β*GalCer/*α*GalCer** failed to generate any notable amounts of anti-GM3 IgG responses in comparison to **GM3-*β*CalCer** on days 14, 28, and 42. Interestingly, although the dipalmitoyl lipid anchor exhibits high efficacy in cancer vaccines targeting MUC1,[42] the candidate **GM3-Pam$_2$/*α*GalCer** did not display significant potency. Consistent with our previous studies on vaccines targeting STn[28] and nicotine hapten,[30] the self-adjuvanting vaccine candidate **GM3-*α*GalCer** stimulated robust IgG antibody response, leading to 6, 11, and 26-fold higher IgG titers than **GM3-*β*GalCer** at days 14, 28, and 42, respectively. **GM3-*β*GalCer/*α*GalCer** elicited an effective IgG response similar to that of the covalent counterpart **GM3-*α*GalCer**. No significant difference was observed between them, suggesting

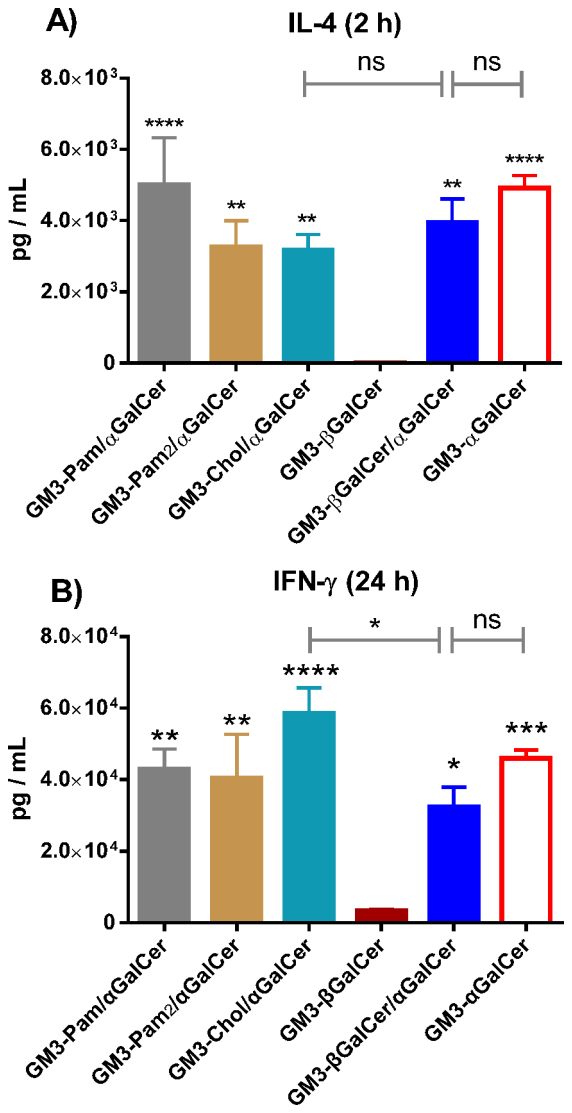


Figure 1. (A) IL-4 and (B) IFN-γ production induced by the vaccine candidates. The serum concentrations of cytokines were evaluated by ELISA at the indicated time points after the first injection. The data are indicated as the average value ± SEM. Asterisks without brackets indicate significant difference to the control **GM3-*β*GalCer** using one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks with brackets indicate significant difference using an unpaired two-tailed Student's *t* test. ns: not significant.

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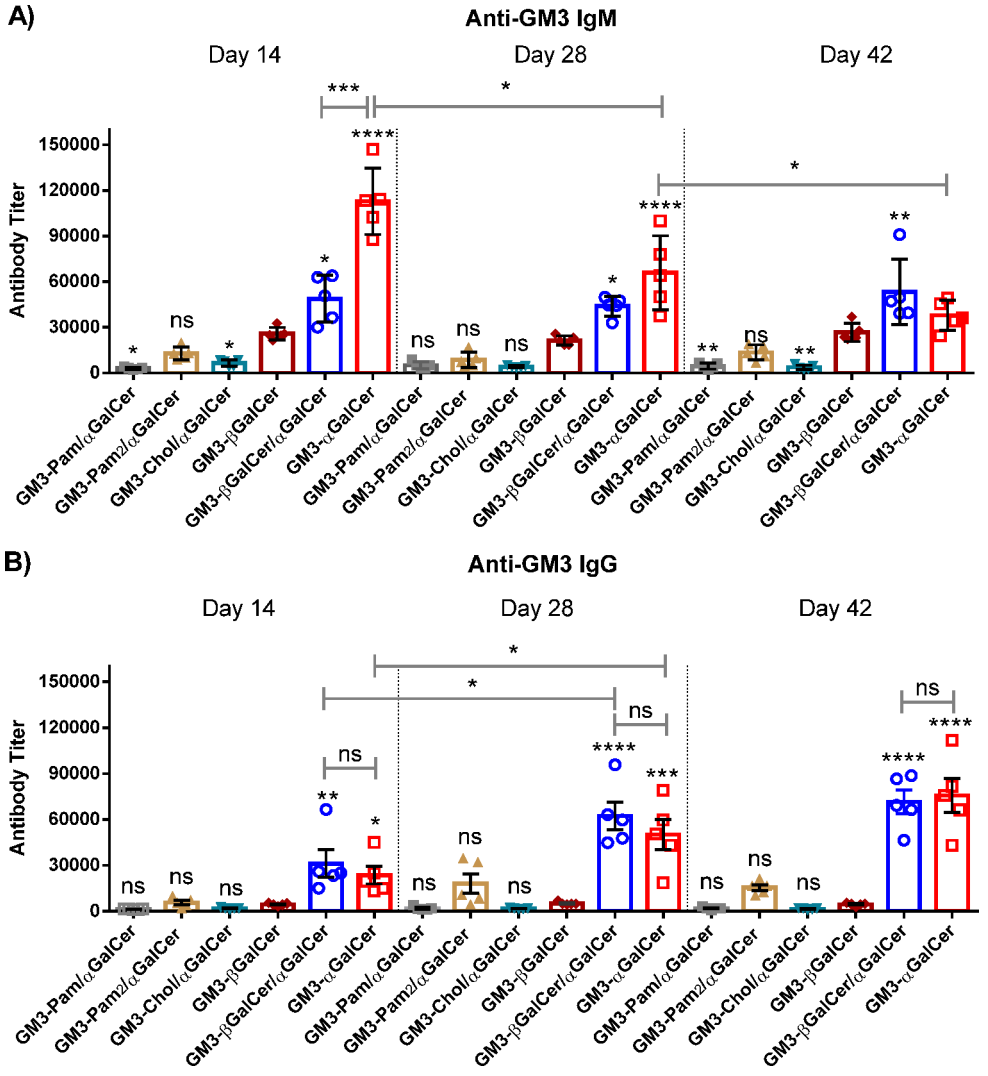

Figure 2. (A) Anti-GM3 IgM and (B) IgG response after intraperitoneal immunization of female BALB/c mice at days 1, 15, and 29 with the vaccine candidates. The data are indicated as the average ± SEM, and individual symbols represent individual mouse titers. Asterisks without brackets indicate significant difference to control **GM3-*β*GalCer** (days 14, 28, and 42, respectively) using one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks with brackets indicate significant difference using an unpaired two-tailed Student's *t* test. ns: not significant.

that *β*CalCer is the optimal lipid anchor. Notably, a significant increase was seen in the levels of IgG in mice receiving **GM3-*α*GalCer** and **GM3-*β*GalCer/*α*GalCer** following the second immunization, but the magnitude of the response only slightly increased following the third immunization, which might contribute to iNKT cell anergy.[68−70] In constrast, with respect to anti-GM3 IgM, the self-adjuvant vaccine candidate **GM3-*α*GalCer** stimulates high levels of IgM production following the first immunization, and the titers decrease gradually, indicating an effective antibody isotype switching from IgM to IgG. In contrast, **GM3-*β*GalCer/*α*GalCer** caused lower levels of IgM production, observed at day 14, this level of production was consistent until the end of the experiment. The pattern of IgG subclasses was also analyzed (Figure 3). Except for these groups immunized with **GM3-*α*GalCer** and **GM3-*β*GalCer/*α*GalCer**, other noncovalent vaccine candidates induced no significant levels of four IgG subtypes compared to **GM3-*β*GalCer**. No significant difference was observed between **GM3-*α*GalCer** and **GM3-*β*GalCer/*α*GalCer** for all IgG subtypes. Remarkably, unlike our study with STn antigen, higher levels of IgG3 over IgG1 were observed, which could be partly attributed to GM3 mediated suppression of T helper type 2-like NKT-cell responses.[71]

The optimized lipid anchor in the noncovalent modality leads to a potent vaccine candidate that induces comparable levels of anti-GM3 IgG to covalent candidate **GM3-*α*GalCer** and also demonstrated similar subtype profiles, which suggests that noncovalent formulation is feasible as an alternative to self-adjuvanting vaccines and is a potential approach for the facile preparation of the vaccine. Importantly, although all non-covalent vaccine candidates can provide appropriate danger signals and stimulate the rapid production of comparable levels of cytokines, the antibody response differs greatly, which could be partly attributed to the different amphiphilicity and resultant pharmacokinetics between lipid-modified GM3-lipid and *α*GalCer. Remarkably, we found that **GM3-Pam** and **GM3-Chol** are more soluble in MeOH than **GM3-Pam$_2$** and **GM3-*β*GalCer**, while *α*GalCer is almost insoluble in MeOH. Therefore, ***β*GalCer** might enable the amphiphilicity of lipid-anchoring GM3 to be more analogous to that of ***α*GalCer** than other lipid anchors, creating effective antigen/danger signal coformulations.[72]

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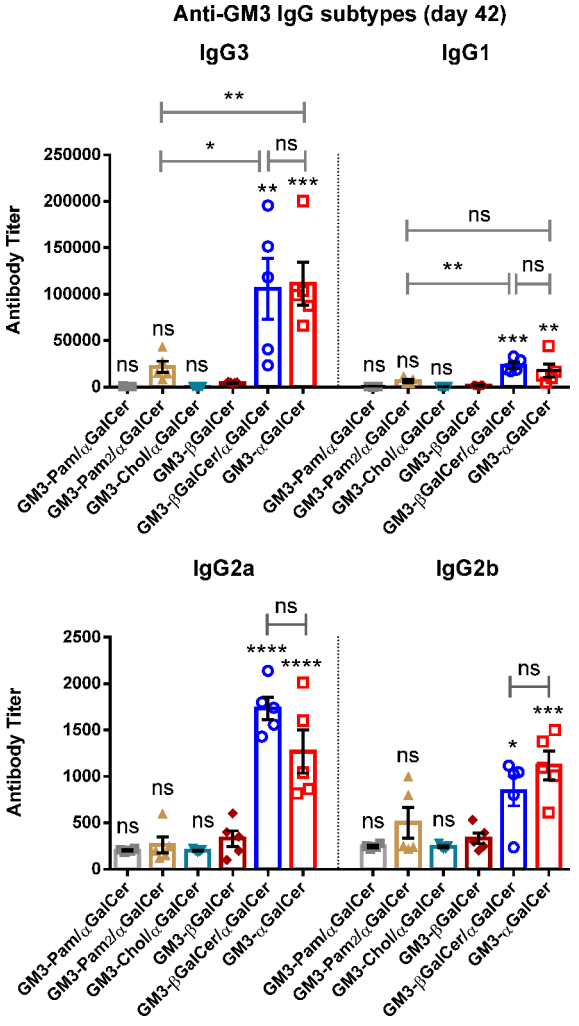

Figure 3. IgG subtype profiles at day 42. Data are represented as the mean ± SEM in each group (n = 5), individual symbols represent individual mouse titers. Asterisks without brackets indicate a significant difference to control **GM3-*β*GalCer** using one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks with brackets indicates significant difference using unpaired two-tailed Student's t test. ns: not significant.

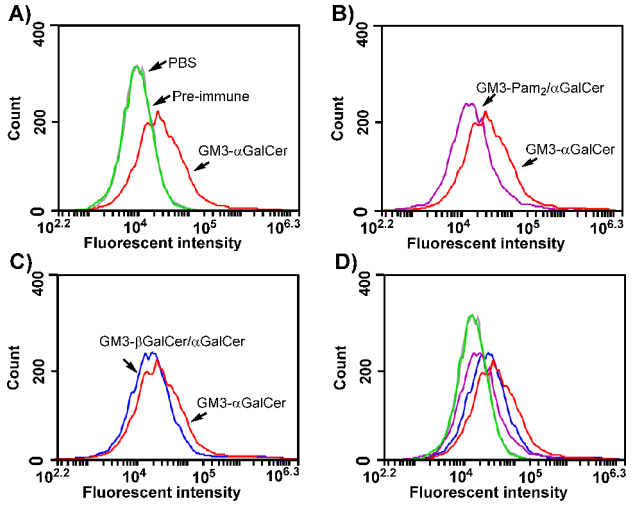

Figure 4. FACS of the induced binding of sera to B16F10 cells by the indicated vaccine candidates (negative control experiment was performed with the sera before immunization (green) and PBS (gray)). FITC-rabbit antimouse IgG was used for staining.

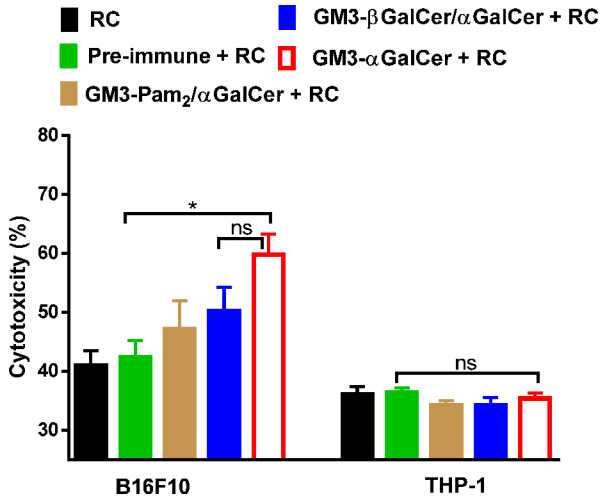

Figure 5. CDC for B16F10 and THP-1 cells that were activated by antisera that were induced in mice through vaccine candidates. **RC-inactive**: cell viability of inactivated rabbit complement, which was used for the negative control experiment; **RC**, rabbit complement. Data are represented as the mean ± SEM in each group (n = 4). Asterisks without brackets indicate significant differences to **RC** using one-way ANOVA followed by Dunnett's multiple comparison test. Asterisks with brackets indicate significant differences using an unpaired two-tailed Student's t test. ns: not significant.

Next, the ability of the antisera to recognize the GM3 antigen presented on highly metastatic B16F10 melanoma cells was evaluated by flow cytometry. As can be seen in Figure 4, antisera obtained from immunization with **GM3-*α*GalCer** displayed significant binding activity to GM3-expressing B16F10 cancer cells, and the percentage of positive cells was 47.9% (Figure S7) with enhanced mean fluorescent intensity (MFI: 45436). Although the sera obtained from mice immunized with **GM3-*β*GalCer/*α*GalCer** elicited IgG antibody titers equally as high as **GM3-*α*GalCer**, a slightly impaired recognition of B16F10 cancer cells was observed (positive cells 34.1%, MFI: 29275). This result indicated that the covalent linker between carbohydrate antigen and adjuvant was important for cancer cell recognition, consistent with a previous study by Boons et al.[73]

Finally, to evaluate the ability of activate the complement system, the complement-dependent cytotoxicity (CDC) assay was assessed and the percent of lysed cell was determined by applying a tetrazolium bromide (MTT) assay. As shown in Figure 5, antisera from **GM3-*α*GalCer** could effectively activate the complement system and stimulate significant cytotoxicity to B16F10 cells (cell lysis rate 60%), which was much higher than preimmune serum (cell lysis rate 41%). Under the same conditions, no significant cytotoxicity was observed for the antisera from **GM3-*β*GalCer/*α*GalCer**. Meanwhile, acute monocytic leukemia (THP-1) was used as the control due to the fact that it lacked the GM3 antigen on the cell surface.[74,75] There was no statistically significant cytotoxicity observed in THP-1 cells of each group. This result demonstrated that the antisera of GM3-*α*GalCer exhibited a moderate cytokine toxicity to B16F10 cells, but no cytotoxicity to other cells which lacked GM3 antigen.

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CONCLUSION

Given the unique immunology of iNKT cells and the valuable target, GM3, for diverse cancer immunotherapy, we evaluated two vaccine modalities to deliver antigen GM3 and adjuvant αGalCer to draining lymph nodes. The covalent vaccine candidate **GM3-αGalCer** is capable of promoting a high level of anti-GM3 IgG response, which exhibits strong binding activity to cancer cells and efficient activation of the complement system, underlining the importance of covalent linkers for the improvement of cancer vaccines. However, the limitation of covalent vaccine is that the ratio of antigen and adjuvant is fixed, and in some cases, the adjuvant's activity may be significantly influenced by the modification with linker. To facilitate the preparation of vaccines, an alternative modality was also explored. Although the induced antibodies have a slightly reduced recognition of B16F10 cancer cells, the noncovalent vaccine candidate **GM3-βGalCer/αGalCer** with the optimized lipid anchor could potentiate a comparable magnitude of IgG titer to **GM3-αGalCer**, suggesting that lipid anchors play an important role in altering antigen pharmacokinetics, and that lipidation of carbohydrate antigens has great potential in the preparation of simple and effective iNKT cell-based synthetic carbohydrate vaccines.

EXPERIMENTAL SECTION

Chemical Synthesis. *General Information.* All reactions were carried out under a dry Ar atmosphere using oven-dried glassware and magnetic stirring. The solvents were dried before use as follows: THF and Et_2O were heated at reflux over sodium benzophenone ketyl; toluene was heated at reflux over sodium; dichloromethane was dried over CaH_2. Anhydrous *N*,*N*-diisopropylethylamine and triethylamine were used directly as purchased. Commercially available reagents were used without further purification unless otherwise noted. Aluminum TLC sheets (silica gel 60 F_{254}) of 0.2 mm thickness were used to monitor the reactions. The spots were visualized with short wavelength UV light or by charring after spraying with a solution prepared from one of the following solutions: phosphomolybdic acid (5.0 g) in 95% EtOH (100 mL); *p*-anisaldehyde solution (2.5 mL of *p*-anisaldehyde, 2 mL of AcOH, and 3.5 mL of conc. H_2SO_4 in 100 mL of 95% EtOH); or ninhydrin solution (0.3 g ninhydrin in 100 mL of *n*-butanol; add 3 mL AcOH). Flash chromatography was carried out with silica gel 60 (230−400 ASTM mesh). NMR spectra were obtained on a 400-MHz or 600-MHz spectrometer. Proton chemical shifts are reported in ppm from an internal standard of residual chloroform (7.26 ppm) or methanol (3.31 ppm), and carbon chemical shifts are reported using an internal standard of residual chloroform (77.0 ppm) or methanol (49.1 ppm). Proton chemical data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad), coupling constant, and integration. Chemical shifts were referenced on residual solvent peaks: $CDCl_3$ (δ = 7.26 ppm for ^{1}H NMR and 77.00 ppm for ^{13}C NMR), CD_2Cl_2 (δ = 5.32 ppm for ^{1}H NMR and 53.84 ppm for ^{13}C NMR), CD_3OD (δ = 3.31 ppm for ^{1}H NMR and 49.00 ppm for ^{13}C NMR), D_2O (δ = 4.79 ppm for ^{1}H NMR). Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS was performed on an AB SCIEX 5800 spectrometer (Shimadzu AXIMA Assurance).

*2-(2-Azido-ethoxy)-ethyl 4,6-O-Benzylidene β-D-Glucopyranoside (**9**).* To a solution of penta-*O*-acetylglucose **8** (2 g, 5.12 mmol) and 2-(2-azidoethoxy) ethan-1-ol (0.88 g, 6.6 mmol) in anhydrous DCM (10 mL) was added $BF_3{\cdot}Et_2O$ (0.82 mL, 6.66 mmol). The reaction mixture was stirred at rt for 24 h and diluted with saturated aqueous $NaHCO_3$ (10 mL). The organic layer was separated, washed with aqueous $NaHCO_3$ (10 mL), dried over Na_2SO_4, and concentrated. The crude product was dissolved in 0.5 M NaOMe (5 mL) and stirred at rt for 0.5 h. After completion, the reaction was neutralized to pH 6−8 with 1 M HCl. After the solvent was evaporated under reduced pressure, the residue was purified by flash column chromatography (DCM/MeOH, 10:1, *v*/*v*) to provide the unprotected glucosyl derivative as intermediate (575 mg, 38% for two steps). To a stirred solution of the unprotected glucosyl derivative (440 mg, 1.5 mmol) in anhydrous CH_3CN (2 mL) was added benzaldehyde dimethyl acetal (2 mL, 1.32 mmol) and a catalytic amount of CSA (3.5 mg, 0.015 mmol) at rt. After stirring at rt for 2 h, the reaction mixture was quenched by Et_3N (4.2 μL, 0.03 mmol) and diluted with DCM (10 mL). After being washed with saturated $NaHCO_3$ (10 mL), the organic layer was dried ($MgSO_4$), concentrated, and purified by silica chromatography (DCM/MeOH, 30:1, *v*/*v*) to give **9** (520 mg, 90.6%) as a white solid; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.57−7.43 (m, 2H), 7.37 (d, *J* = 6.4 Hz, 3H), 5.53 (s, 1H), 4.45 (d, *J* = 7.8 Hz, 1H), 4.33 (dd, *J* = 10.6, 5.0 Hz, 1H), 4.04 (dt, *J* = 11.5, 3.7 Hz, 1H), 3.79 (dt, *J* = 26.6, 9.9 Hz, 3H), 3.74−3.62 (m, 3H), 3.54 (dt, *J* = 15.9, 8.8 Hz, 2H), 3.46 (dd, *J* = 9.8, 4.9 Hz, 1H), 3.39 (t, *J* = 5.0 Hz, 2H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 136.87, 129.23, 128.29, 126.25, 103.47, 101.83, 80.37, 74.41, 72.91, 70.15, 69.94, 69.14, 68.56, 66.35, 50.51. ESI-MS calcd. for $C_{17}H_{23}N_3O_7Na^+$ $[M + Na]^+$ 404.14, found 404.19.

*2-(2-Azido-ethoxy)-ethyl 2,3,6-Tri-O-benzyl-β-D-glucopyranoside (**10**).* Benzylidene acetal **9** (520 mg, 1.36 mmol) was dissolved in DMF (5 mL), and NaH (60% in mineral oil, 163.4 mg, 4.08 mmol) was added at 0 °C. After 5−10 min, benzyl bromide (648 μL, 5.4 mmol) was added dropwise at 0 °C and the reaction mixture was allowed to stir for 0.5 h at rt. After completion, the reaction was quenched with MeOH (5 mL). The solvent was evaporated under reduced pressure, and the residue was purified by silica chromatography (PE/EA, 2:1, *v*/*v*) to give the fully protected glucosyl derivative **9′** (564 mg, 80.2%) as a white solid; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.48 (d, *J* = 6.4 Hz, 2H), 7.40−7.31 (m, 9H), 7.28 (dd, *J* = 13.1, 5.5 Hz, 4H), 7.20 (s, 1H), 5.55 (s, 1H), 4.92 (dd, *J* = 11.2, 8.5 Hz, 2H), 4.79 (dd, *J* = 11.2, 8.6 Hz, 2H), 4.56 (d, *J* = 7.7 Hz, 1H), 4.34 (dd, *J* = 10.5, 5.0 Hz, 1H), 4.02 (dt, *J* = 9.8, 4.3 Hz, 1H), 3.84−3.71 (m, 3H), 3.69 (d, *J* = 7.2 Hz, 3H), 3.61 (t, *J* = 5.2 Hz, 2H), 3.48 (t, *J* = 8.1 Hz, 1H), 3.40 (td, *J* = 9.6, 5.0 Hz, 1H), 3.28 (q, *J* = 4.1 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.37, 137.19, 128.82, 128.20, 128.17, 128.12, 127.90, 127.55, 127.50, 125.88, 104.13, 100.96, 81.87, 81.27, 80.66, 75.03, 74.97, 70.29, 69.86, 69.32, 68.62, 65.87, 50.50. ESI-MS calcd. for $C_{31}H_{35}N_3O_7Na^+$ $[M + Na]^+$ 584.24, found 584.38. Then, compound **9′** (50 mg, 0.1 mmol) was dissolved in dry DCM (5 mL), followed by addition of Et_3SiH (189 μL, 1.2 mmol). The mixture was stirred at rt for 10 min and then cooled to −5 °C, and $BF_3{\cdot}OEt_2$ (25 μL, 0.2 mmol) was added and stirred at −5 °C for another 6 h. The reaction was quenched by Et_3N (50 μL) and washed with saturated $NaHCO_3$. The organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by silica chromatography (PE/EA, 2:1, *v*/*v*) to give **10** as a colorless oil (42 mg, 79.7%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.46−7.24 (m, 15H), 4.95 (dd, *J* = 17.3, 11.2 Hz, 2H), 4.72 (d, *J* = 11.2 Hz, 2H), 4.63−4.52 (m, 2H), 4.47 (d, *J* = 6.7 Hz, 1H), 4.04 (dt, *J* = 10.9, 4.2 Hz, 1H), 3.83−3.72 (m, 2H), 3.69 (dd, *J* = 10.3, 5.3 Hz, 2H), 3.63−3.57 (m, 2H), 3.45 (q, *J* = 4.8, 4.3 Hz, 3H), 3.29 (dd, *J* = 6.3, 4.1 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 138.47, 138.43, 137.79, 128.48, 128.35, 128.30, 127.99, 127.92, 127.79, 127.66, 127.61, 103.77, 83.81, 81.48, 75.20, 74.47, 73.94, 73.56, 71.25, 70.37, 70.08, 69.85, 69.05, 50.57. ESI-MS calcd. for $C_{31}H_{37}N_3O_7Na^+$ $[M + Na]^+$ 586.25, found 586.21.

*p-Tolyl (Methyl 5-Acetamido-7,8,9-tri-O-acetyl-5-N,4-O-carbonyl-3,5-dideoxy-D-glycero-α,β-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-1-thio-β-D-galactopyranoside (**13**).* The donor **13** was prepared as previously described.[59] **13** was purified by silica flash chromatography with toluene and EA (6/1 to 3/1) as the eluent to give **13** as a white solid (α-glycoside 347 mg, 75%; β-glycoside 37 mg, 8%). **13α**: ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.20−8.11 (m, 2H), 7.59 (t, *J* = 7.4 Hz, 1H), 7.48 (dd, *J* = 7.9, 5.4 Hz, 4H), 7.44−7.30 (m, 6H), 7.09−6.99 (m, 2H), 5.53 (d, *J* = 5.6 Hz, 2H), 5.34 (s, 2H), 4.92 (d, *J* = 9.7 Hz, 1H), 4.58 (dd, *J* = 9.6, 3.5 Hz, 1H), 4.51−4.42 (m, 2H), 4.36 (d, *J* = 12.3 Hz, 1H), 4.12 (dd, *J* = 14.5, 8.3 Hz, 2H), 4.08 (d, *J* = 3.5 Hz, 1H), 3.99 (dd, *J* = 12.2, 6.3 Hz, 1H), 3.70 (d, *J* = 21.8 Hz, 2H), 3.53−3.47 (m, 1H), 3.44 (s, 3H), 2.89 (dd, *J* = 12.1, 3.2 Hz, 1H), 2.43 (s, 3H), 2.34 (d, *J* = 13.5 Hz, 4H), 2.19 (s, 3H), 2.08−2.00 (m, 5H). ^{13}C NMR (150 MHz, CD_2Cl_2): δ 172.01, 171.08, 170.76, 170.20, 168.91, 165.35, 153.79, 138.57, 138.26, 133.92, 133.60, 130.66,

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130.41, 129.87, 129.46, 128.84, 128.55, 126.81, 101.47, 97.35, 86.13, 75.29, 73.90, 71.75, 69.83, 69.54, 68.46, 68.32, 63.95, 59.02, 36.91, 24.82, 21.55, 21.30, 20.97, 20.65. **13*β***: ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.08−7.95 (m, 2H), 7.64−7.56 (m, 1H), 7.53−7.43 (m, 4H), 7.43−7.31 (m, 5H), 7.16 (t, J = 7.3 Hz, 1H), 7.07 (d, J = 7.9 Hz, 2H), 5.84 (t, J = 1.9 Hz, 1H), 5.64 (s, 1H), 5.59−5.51 (m, 1H), 5.32 (t, J = 9.7 Hz, 1H), 5.09 (dd, J = 12.1, 2.6 Hz, 1H), 4.81 (d, J = 10.0 Hz, 1H), 4.49 (dd, J = 12.4, 3.8 Hz, 3H), 4.41−4.35 (m, 1H), 4.33−4.27 (m, 1H), 4.19−4.10 (m, 1H), 3.90 (dd, J = 12.1, 9.3 Hz, 1H), 3.72 (s, 1H), 3.63 (s, 1H), 3.50 (dd, J = 11.3, 9.5 Hz, 1H), 3.19 (s, 3H), 2.58 (dd, J = 12.1, 3.6 Hz, 1H), 2.35 (d, J = 2.6 Hz, 4H), 2.32 (s, 3H), 2.14 (s, 3H), 2.08 (s, 4H), 2.03 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 171.39, 171.07, 170.93, 169.96, 169.31, 165.70, 164.79, 153.35, 138.42, 137.69, 134.56, 133.02, 130.34, 129.71, 129.46, 129.14, 128.36, 128.09, 126.67, 126.43, 100.95, 99.39, 84.77, 75.89, 75.55, 75.10, 74.17, 74.09, 71.88, 69.67, 69.05, 68.25, 63.13, 58.96, 52.62, 36.64, 29.63, 24.32, 21.27, 21.02, 20.86, 20.60. ESI-MS calcd. for $C_{46}H_{49}NO_{18}SNa^+$ $[M + Na]^+$ 958.25, found 958.27.

*2-(2-Azido-ethoxy)-ethyl (Methyl 5-Acetamido-7,8,9-tri-O-acetyl-5-N,4-O-carbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside (**14**).* Glycosyl donor **13** (80 mg, 0.086 mmol) and acceptor **10** (57 mg, 0.10 mmol) were dissolved in anhydrous DCM (3 mL) containing activated 4 Å powdered molecular sieves (100 mg) under argon atmosphere and then cooled to −20 °C, stirred 1 h, and followed by addition of NIS (38 mg, 0.172 mmol) and TfOH (2.5 μL, 0.025 mmol). The reaction mixture was stirred at −20 °C for 20 min and gradually raised to 0 °C for a period of 2 h, then quenched with triethylamine (20 μL) and warmed to rt. The mixture was diluted with DCM (5 mL) and filtered through a pad of Celite. The filtrate was washed with 20% aqueous $Na_2S_2O_3$ solution, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by flash column chromatography (6:1, DCM/EA, *v*/*v*) using silica gel to give **14** (72 mg, 60%) as a light-yellow solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.14 (d, J = 7.6 Hz, 2H), 7.57 (t, J = 7.4 Hz, 1H), 7.52 (dd, J = 6.7, 3.0 Hz, 2H), 7.44 (t, J = 7.6 Hz, 2H), 7.37−7.27 (m, 8H), 7.27−7.14 (m, 10H), 5.56 (dt, J = 13.5, 9.6 Hz, 2H), 5.48 (dd, J = 10.1, 7.9 Hz, 1H), 5.34 (s, 1H), 5.09 (d, J = 11.3 Hz, 1H), 5.02 (d, J = 8.0 Hz, 1H), 4.89 (dd, J = 15.1, 11.2 Hz, 2H), 4.67 (d, J = 11.2 Hz, 1H), 4.50−4.39 (m, 3H), 4.35 (d, J = 7.9 Hz, 1H), 4.29 (d, J = 11.9 Hz, 1H), 4.20 (d, J = 12.0 Hz, 1H), 4.06 (d, J = 12.2 Hz, 1H), 3.99 (dd, J = 12.4, 7.1 Hz, 1H), 3.95 (dd, J = 11.6, 4.1 Hz, 1H), 3.88 (dd, J = 11.1, 6.7 Hz, 2H), 3.77−3.72 (m, 1H), 3.72−3.62 (m, 3H), 3.60 (d, J = 10.7 Hz, 1H), 3.56 (t, J = 5.2 Hz, 1H), 3.52 (d, J = 10.2 Hz, 1H), 3.49 (s, 3H), 3.45 (dd, J = 10.9, 5.9 Hz, 1H), 3.40 (t, J = 8.5 Hz, 1H), 3.36 (d, J = 5.7 Hz, 2H), 3.23 (q, J = 5.2 Hz, 2H), 2.91 (dd, J = 12.1, 3.3 Hz, 1H), 2.44 (s, 3H), 2.19 (s, 3H), 2.00 (s, 3H), 1.78 (d, J = 12.9 Hz, 1H), 1.74 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 171.94, 170.82, 170.20, 168.40, 164.98, 153.40, 139.08, 138.39, 137.75, 133.27, 129.94, 128.85, 128.54, 128.18, 128.02, 127.92, 127.43, 127.38, 126.92, 126.44, 103.49, 101.06, 100.74, 97.04, 83.11, 81.73, 77.77, 77.21, 77.00, 76.79, 75.18, 75.01, 74.73, 74.45, 74.30, 73.02, 72.92, 72.81, 71.41, 71.15, 70.32, 69.82, 69.15, 69.00, 68.62, 67.83, 66.15, 63.67, 58.81, 52.91, 50.56, 36.81, 24.64, 21.36, 20.61. ESI-MS calcd. for $C_{70}H_{78}N_4O_{25}Na^+$ $[M + Na]^+$ 1397.49, found 1397.75.

*2-(2-Azido-ethoxy)-ethyl (Methyl 5-Acetamido-7,8,9-tri-O-acetyl-4-O-methoxycarbonyl-3,5- dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside (**15**).* To a solution of **14** (20 mg, 0.015 mmol) in anhydrous MeOH (3 mL) was added freshly prepared solution of 1 M NaOMe (6 μL), and the mixture was stirred at rt for 5−10 min, then neutralized with Amberlite 15 (H^+) resin. The resin was filtered off and washed with DCM/MeOH (1/1, *v*/*v*), the filtrate was concentrated to provide a residue **15** that was used directly in the next step without further operation. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.06 (d, J = 7.7 Hz, 2H), 7.56 (t, J = 7.4 Hz, 1H), 7.52−7.40 (m, 5H), 7.30 (ddt, J = 24.2, 13.0, 7.4 Hz, 14H), 7.16 (t, J = 3.3 Hz, 3H), 5.59−5.50 (m, 1H), 5.44 (dd, J = 10.1, 7.9 Hz, 1H), 5.35 (s, 1H), 5.27−5.18 (m, 2H), 5.11 (d, J = 11.2 Hz, 1H), 4.94 (d, J = 8.0 Hz, 1H), 4.86 (dd, J = 11.2, 7.3 Hz, 2H), 4.68 (d, J = 11.2 Hz, 2H), 4.43 (dd, J = 10.9, 4.5 Hz, 2H), 4.37−4.32 (m, 1H), 4.31−4.23 (m, 2H), 4.11 (d, J = 12.5 Hz, 1H), 4.01 (ddd, J = 15.0, 7.6, 3.5 Hz, 3H), 3.97−3.85 (m, 3H), 3.81−3.71 (m, 1H), 3.66 (d, J = 18.6 Hz, 5H), 3.60−3.49 (m, 6H), 3.47 (s, 1H), 3.44−3.36 (m, 1H), 3.30 (d, J = 8.0 Hz, 2H), 3.23 (q, J = 3.5, 2.3 Hz, 2H), 2.68 (dd, J = 12.7, 4.5 Hz, 1H), 2.20 (s, 3H), 2.02 (s, 3H), 1.86 (d, J = 4.6 Hz, 6H), 1.63 (d, J = 12.4 Hz, 1H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.69, 170.27, 170.05, 168.33, 164.88, 155.24, 139.07, 138.60, 138.34, 137.82, 133.10, 129.86, 128.78, 128.48, 128.25, 128.17, 128.02, 127.97, 127.86, 127.67, 127.63, 127.39, 126.92, 126.44, 103.48, 100.82, 100.79, 96.78, 83.05, 81.68, 75.29, 74.50, 74.26, 73.36, 73.02, 72.47, 72.34, 71.65, 70.96, 70.31, 69.82, 68.99, 68.67, 67.27, 67.04, 66.21, 62.52, 55.10, 52.78, 50.56, 49.41, 38.20, 29.53, 23.23, 21.35, 20.75, 20.65. ESI-MS calcd. for $C_{71}H_{82}N_4O_{26}Na^+$ $[M + Na]^+$ 1429.51, found 1429.39.

*(2S,3S,4R)-3,4-Dibenzyloxy-2-hexacosanoylamino-octadecane-(2,3-di-O-benzyl-6-deoxy-6-N-(2-(2-(methyl 5-Acetamido-7,8,9-tri-O-acetyl-4-O-methoxycarbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyloxy)-ethoxy)-ethylamino)-oxohexanamido-α-D-galactopyranoside (**16**).* To a solution of **15** (20 mg, 0.014 mmol) in 5 mL of wet MeOH was added PMe_3 (140 μL, 0.14 mmol) at rt, and the resulting solution was stirred for 3 h. Then, the solvent was concentrated under reduced pressure. The residue was then subjected to high vacuum at 30 °C for 24 h to remove the Me_3PO byproduct. The residue was dissolved in DCM (2 mL) and followed by addition of **16′** (α-GalCer-selenoester, 10 mg, 0.0067 mmol) and DIPEA (2.4 μL, 0.014 mmol). The reaction mixture was set to stir for 0.5 h at rt, then, the mixture was evaporated and purified by column chromatography (DCM/MeOH 20:1, *v*/*v*) to give **16** (18 mg, 98%) as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.09 (d, J = 7.8 Hz, 2H), 7.61 (t, J = 7.5 Hz, 1H), 7.57−7.45 (m, 3H), 7.44−7.31 (m, 32H), 7.28 (dd, J = 4.8, 2.2 Hz, 5H), 7.24−7.16 (m, 3H), 6.44 (s, 1H), 6.01 (d, J = 9.4 Hz, 2H), 5.58 (s, 1H), 5.48 (t, J = 9.0 Hz, 1H), 5.40 (s, 1H), 5.34−5.27 (m, 1H), 5.23 (d, J = 9.4 Hz, 1H), 5.17 (d, J = 11.1 Hz, 1H), 4.97 (d, J = 8.0 Hz, 1H), 4.90 (d, J = 11.5 Hz, 1H), 4.85 (d, J = 3.6 Hz, 1H), 4.81 (d, J = 7.6 Hz, 1H), 4.78−4.72 (m, 2H), 4.68 (d, J = 13.1 Hz, 1H), 4.62 (d, J = 12.3 Hz, 1H), 4.56−4.51 (m, 1H), 4.47 (dd, J = 12.8, 5.3 Hz, 1H), 4.38 (d, J = 7.7 Hz, 1H), 4.32 (t, J = 11.7 Hz, 1H), 4.16 (d, J = 12.2 Hz, 1H), 4.10−4.01 (m, 2H), 4.01−3.87 (m, 3H), 3.82 (t, J = 9.9 Hz, 1H), 3.73 (s, 3H), 3.71−3.61 (m, 2H), 3.58 (d, J = 12.0 Hz, 3H), 3.52−3.40 (m, 2H), 3.16 (s, 1H), 2.73 (dd, J = 12.5, 3.6 Hz, 1H), 2.25 (s, 3H), 2.13 (s, 2H), 2.05 (d, J = 8.4 Hz, 4H), 1.99−1.93 (m, 1H), 1.90 (d, J = 7.9 Hz, 4H), 1.79 (s, 6H), 1.70−1.61 (m, 2H), 1.57 (d, J = 5.9 Hz, 3H), 1.28 (d, J = 8.6 Hz, 63H), 0.92 (t, J = 6.7 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.69, 173.07, 172.69, 170.65, 170.24, 170.06, 168.37, 164.90, 155.27, 139.07, 138.49, 137.89, 133.12, 132.04, 130.04, 129.88, 128.79, 128.52, 128.42, 128.36, 128.30, 128.25, 128.05, 127.98, 127.89, 127.83, 127.78, 127.70, 127.64, 126.98, 126.45, 103.55, 100.83, 99.38, 96.86, 83.16, 81.79, 79.90, 75.80, 75.34, 74.64, 74.36, 73.65, 73.40, 73.07, 72.47, 72.37, 72.10, 71.70, 71.04, 70.12, 69.71, 68.84, 68.72, 68.59, 68.42, 67.31, 67.14, 66.28, 62.57, 55.08, 52.77, 50.45, 49.57, 39.64, 39.08, 38.27, 36.71, 35.91, 35.72, 31.91, 29.72, 29.69, 29.45, 29.35, 25.72, 24.96, 24.75, 23.23, 22.67, 21.34, 20.74, 20.65, 14.10.

*(2S,3S,4R)-3,4-Dibenzyloxy-2-hexacosanoylamino-octadecane-(2,3-di-O-benzyl-6-deoxy-6-N-(2-(2-(methyl 5-Acetamido-7,8,9-tri-O-acetyl-4-O-methoxycarbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyloxy)-ethoxy)-ethylamino)-oxohexanamido-β-D-galactopyranoside (**17**).* The preparation of **17** is similar to that of **16**. White solid, yield (45 mg, 95%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.07 (d, J = 7.7 Hz, 2H), 7.58 (t, J = 7.6 Hz, 1H), 7.48 (dd, J = 19.0, 11.5 Hz, 4H), 7.32 (ddq, J = 33.6, 24.3, 7.3 Hz, 40H), 7.18 (d, J = 5.0 Hz, 3H), 6.78 (d, J = 7.4 Hz, 1H), 6.07 (d, J = 5.8 Hz, 1H), 6.00 (d, J = 8.8 Hz, 1H), 5.55 (d, J = 7.7 Hz, 1H), 5.46 (t, J = 9.1 Hz, 1H), 5.38 (s, 1H), 5.32 (d, J = 9.7 Hz, 1H), 5.28 (d, J = 9.6 Hz, 1H), 5.15 (d, J = 11.2 Hz, 1H), 4.94 (d, J = 8.0 Hz, 1H), 4.86 (dd, J = 18.2, 11.1 Hz, 2H), 4.80 (s, 2H), 4.77 (s, 1H), 4.73−4.62 (m, 5H), 4.59 (d, J = 11.5 Hz, 1H), 4.51 (d, J = 11.5 Hz, 1H), 4.49−4.22 (m, 7H), 4.13 (d, J = 12.2 Hz, 1H), 4.03 (dt, J = 17.4, 9.4 Hz, 4H), 3.94 (t, J = 8.9 Hz, 3H), 3.88 (d, J = 3.4 Hz, 1H), 3.79 (d, J = 7.5

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Hz, 2H), 3.71 (d, J = 7.8 Hz, 4H), 3.63 (d, J = 8.5 Hz, 1H), 3.57 (d, J = 15.2 Hz, 5H), 3.50−3.36 (m, 3H), 3.36−3.13 (m, 6H), 2.70 (dd, J = 12.7, 4.5 Hz, 1H), 2.22 (s, 3H), 2.03 (s, 4H), 1.87 (d, J = 10.3 Hz, 6H), 1.74−1.58 (m, 5H), 1.56−1.41 (m, 9H), 1.26 (d, J = 19.3 Hz, 84H), 0.90 (t, J = 6.9 Hz, 7H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.90, 172.92, 172.83, 170.73, 170.35, 170.28, 170.13, 168.37, 164.95, 155.28, 139.04, 138.50, 138.25, 137.86, 133.20, 129.91, 128.84, 128.39, 128.03, 127.86, 127.73, 127.05, 126.47, 104.19, 103.55, 100.82, 96.88, 83.19, 81.77, 80.75, 80.46, 78.83, 78.11, 77.28, 75.40, 75.18, 74.72, 74.31, 73.45, 73.11, 73.00, 72.89, 72.55, 72.35, 72.20, 71.90, 71.72, 71.03, 70.17, 69.70, 69.01, 68.87, 68.72, 67.30, 67.13, 66.77, 66.30, 65.95, 62.62, 55.14, 54.57, 52.84, 50.13, 49.44, 42.73, 39.84, 39.11, 38.26, 36.61, 35.66, 31.95, 29.39, 29.32, 26.01, 25.65, 24.96, 24.67, 23.27, 22.72, 21.40, 20.80, 20.71, 18.66, 17.36, 14.16.

*2-(2-N-Hexadecanamide-ethoxy)-ethyl-(methyl 5-Acetamido-7,8,9-tri-O-acetyl-4-O-methoxycarbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside (**18**).* To the DCM/1,4-dioxane (2 mL, 3/1, v/v) solution of terminal amine prepared by Staudinger reduction of azide **15** (30 mg, 0.021 mmol) was added palmitic acid **18′** (6.7 mg, 0.026 mmol), EDC (6.2 mg, 0.032 mmol), and HOBt (4.4 mg, 0.032 mmol) at rt. The reaction mixture was raised to 40 °C and stirred at 40 °C for 2−3 h, and concentrated under reduced pressure. The residue was purified by flash column chromatography (DCM/MeOH 20:1, v/v) using silica gel to give **18** as a white solid. (32 mg, 89% over 2 steps). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23−7.88 (m, 2H), 7.57 (t, J = 7.4 Hz, 1H), 7.47 (dt, J = 15.3, 6.7 Hz, 4H), 7.40−7.21 (m, 18H), 7.20−7.12 (m, 3H), 5.83 (s, 1H), 5.54 (t, J = 6.7 Hz, 1H), 5.44 (dd, J = 10.1, 8.0 Hz, 1H), 5.35 (s, 1H), 5.25 (dd, J = 9.7, 2.2 Hz, 1H), 5.15 (dd, J = 20.1, 10.4 Hz, 2H), 4.93 (d, J = 8.0 Hz, 1H), 4.85 (dd, J = 14.6, 11.0 Hz, 2H), 4.68 (d, J = 11.1 Hz, 1H), 4.43 (dd, J = 10.8, 3.7 Hz, 2H), 4.37−4.32 (m, 1H), 4.31−4.21 (m, 2H), 4.11 (d, J = 12.3 Hz, 1H), 4.01 (ddd, J = 12.8, 8.3, 3.3 Hz, 3H), 3.96−3.87 (m, 3H), 3.81−3.73 (m, 1H), 3.73−3.23 (m, 15H), 2.69 (dd, J = 12.7, 4.6 Hz, 1H), 2.20 (s, 3H), 2.02 (s, 3H), 1.98 (td, J = 7.4, 2.1 Hz, 2H), 1.87 (s, 3H), 1.85 (s, 3H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 173.21, 170.69, 170.27, 170.07, 168.34, 164.87, 155.26, 139.02, 138.40, 138.27, 137.83, 133.13, 129.89, 128.79, 128.51, 128.29, 128.26, 128.04, 127.98, 127.67, 127.63, 126.98, 126.45, 103.54, 100.84, 100.80, 96.79, 83.13, 81.76, 77.35, 75.38, 74.70, 74.29, 73.34, 73.05, 72.47, 72.35, 71.66, 70.97, 70.14, 69.86, 68.81, 68.68, 67.23, 67.06, 66.25, 62.55, 55.12, 52.79, 49.45, 38.97, 38.26, 36.55, 31.91, 29.67, 29.50, 29.35, 25.63, 23.25, 22.69, 21.37, 20.77, 20.67, 14.13. ESI-MS calcd. for $C_{87}H_{114}N_2O_{27}Na^+$ $[M + Na]^+$ 1641.75, found 1641.77.

*2-(2-N-(N-α, ε-Bis-hexadecanamide)-L-lysyl)-ethoxy)-ethyl-(methyl 5-Acetamido-7,8,9-tri-O-acetyl-4-O-methoxycarbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-O-benzyl-β-D-glucopyranoside (**19**).* The preparation of **19** is similar to that of **18**. Yield (48 mg, 84%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.20−7.94 (m, 2H), 7.61−7.53 (m, 1H), 7.52−7.41 (m, 4H), 7.40−7.27 (m, 9H), 7.26 (d, J = 4.7 Hz, 6H), 7.19−7.12 (m, 3H), 6.51 (t, J = 5.6 Hz, 1H), 6.36 (d, J = 7.8 Hz, 1H), 5.76 (t, J = 5.8 Hz, 1H), 5.53 (ddd, J = 9.1, 6.1, 2.7 Hz, 1H), 5.44 (dd, J = 10.1, 7.9 Hz, 1H), 5.36 (s, 1H), 5.32 (d, J = 9.5 Hz, 1H), 5.26 (dd, J = 9.5, 2.3 Hz, 1H), 5.12 (d, J = 11.3 Hz, 1H), 4.93 (d, J = 8.0 Hz, 1H), 4.84 (dd, J = 11.2, 4.2 Hz, 2H), 4.68 (d, J = 11.1 Hz, 1H), 4.43 (dd, J = 10.7, 3.5 Hz, 2H), 4.38−4.23 (m, 4H), 4.16−4.08 (m, 1H), 4.07−3.97 (m, 3H), 3.96−3.85 (m, 3H), 3.74 (dd, J = 16.3, 10.4 Hz, 1H), 3.69 (s, 3H), 3.68−3.62 (m, 1H), 3.62−3.55 (m, 2H), 3.53 (s, 3H), 3.49−3.36 (m, 2H), 3.31 (dd, J = 9.4, 4.2 Hz, 3H), 3.22−3.10 (m, 2H), 2.68 (dd, J = 12.7, 4.6 Hz, 1H), 2.19 (s, 3H), 2.16 (dd, J = 8.5, 6.9 Hz, 2H), 2.10 (dd, J = 8.6, 6.8 Hz, 3H), 2.02 (s, 3H), 1.86 (d, J = 2.8 Hz, 6H), 1.63−1.53 (m, 4H), 1.25 (d, J = 3.6 Hz, 48H), 0.88 (t, J = 6.8 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 173.48, 173.37, 171.82, 170.66, 170.29, 170.23, 170.05, 168.36, 164.88, 155.24, 139.09, 138.53, 138.26, 137.85, 133.10, 129.99, 129.86, 128.76, 128.49, 128.28, 128.24, 128.20, 128.12, 128.02, 127.96, 127.89, 127.66, 127.49, 126.93, 126.42, 103.43, 100.79, 96.86, 83.06, 81.75, 75.26, 74.59, 74.26, 73.43, 73.04, 72.51, 72.33, 71.71, 71.00, 70.06, 69.49, 68.95, 68.70, 67.38, 67.12, 66.24, 62.55, 55.06, 52.74, 52.57, 49.44, 39.18, 38.63, 38.20, 36.74, 36.51, 32.11, 31.88, 29.66, 29.64, 29.62, 29.55, 29.50, 29.37, 29.32, 29.29, 28.86, 25.78, 25.63, 23.18, 22.64, 22.29, 21.31, 20.71, 20.63, 14.08. ESI-MS calcd. for $C_{109}H_{156}N_4O_{29}Na^+$ $[M + Na]^+$ 2008.08, found 2008.09.

*2-(2-N-tert-Butyloxycarbonylamido-ethoxy)-ethyl-(methyl 5-Acetamido-7,8,9-tri-O-acetyl-4-O-methoxycarbonyl-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonate)-(2 → 3)-(2-O-benzoyl-4,6-O-benzylidene-β-D-galactopyranosyl)-(1 → 4)- 2,3,6-tri-O-benzyl-β-D-glucopyranoside (**20**).* To the solution of azide **15** (50 mg, 0.036 mmol) in 2 mL DCM/MeOH/H_2O (1/1/0.01, $v/v/v$) was added PMe_3 (360 μL, 0.36 mmol) under the argon atmosphere. The reaction mixture was stirred at rt for 2 h, and then the solvent was removed under reduced pressure. Then the residue was dissolved in 2 mL of DCM, followed by addition of Boc_2O (15.8 mg, 0.072 mmol) and DIPEA (12 μL, 0.072 mmol). The reaction mixture was stirred at rt for 2 h. The solvents were evaporated under reduced pressure, and the resulting residue was purified by flash column chromatography (DCM/MeOH, 40:1, v/v) using silica gel to give **20** (48 mg, 89%) a colorless solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 5.53 (s, 1H), 5.44 (t, J = 9.1 Hz, 1H), 5.35 (s, 1H), 5.28−5.23 (m, 1H), 5.17 (d, J = 9.9 Hz, 1H), 5.11 (d, J = 11.1 Hz, 1H), 4.93 (d, J = 7.9 Hz, 2H), 4.86 (dd, J = 11.2, 3.7 Hz, 2H), 4.68 (d, J = 11.0 Hz, 1H), 4.46−4.38 (m, 2H), 4.38−4.29 (m, 2H), 4.26 (d, J = 12.0 Hz, 1H), 4.10 (d, J = 12.1 Hz, 1H), 4.05−3.98 (m, 1H), 3.97 (d, J = 3.7 Hz, 1H), 3.90 (dd, J = 18.6, 10.2 Hz, 3H), 3.75 (q, J = 10.0 Hz, 1H), 3.69 (s, 2H), 3.68−3.55 (m, 1H), 3.53 (s, 2H), 3.52−3.35 (m, 2H), 3.18 (d, J = 20.3 Hz, 2H), 2.69 (dd, J = 12.8, 4.6 Hz, 1H), 2.20 (s, 3H), 2.02 (s, 3H), 1.85 (d, J = 11.2 Hz, 6H), 1.65 (s, 4H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.28, 169.86, 169.65, 167.95, 164.47, 154.86, 138.71, 138.09, 137.46, 132.72, 129.49, 128.39, 128.11, 127.87, 127.81, 127.64, 127.59, 127.25, 126.54, 126.06, 103.14, 100.47, 100.40, 96.38, 82.72, 81.29, 74.92, 74.18, 73.91, 72.96, 72.64, 72.08, 71.97, 71.25, 70.59, 69.66, 68.48, 68.34, 68.30, 66.85, 66.66, 65.84, 62.14, 54.72, 52.39, 49.08, 39.84, 37.86, 27.99, 22.87, 20.98, 20.28. ESI-MS calcd. for $C_{76}H_{92}N_2O_{28}Na^+$ $[M + Na]^+$ 1503.57, found 1503.39.

*(2S,3S,4R)-3,4-Dihydroxy-2-hexacosanoylamino-octadecane-(2,3-di-O-benzyl-6-deoxy-6-N-(2-(2-((5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranosyloxy)-ethoxy)-ethyl-amino)-oxohexanamido-α-D-galactopyranoside (**GM3-αGalCer**).* Glycolipid conjugate **16** (16 mg, 0.0067 mmol) was dissolved in DCM/MeOH (1/1, v/v) and carefully degassed. Pearlman's catalyst (17 mg, 20% Pd on carbon, normally contain 50% water) was added. The suspension was stirred under H_2 atmosphere for 16 h. After restoring Ar atmosphere, the reaction was filtered through a pad of Celite which was prewashed with DCM/MeOH (1:1, v/v). The residue obtained was dissolved in NaOMe (4 mL, 1:1, v/v) and stirred at 45 °C for 3−4 h. A freshly prepared solution of NaOMe [prepared by addition of 11.5 mg (0.5 mmol) sodium metal to 1 mL of MeOH, and cooled to rt], it was neutralized with Amberlite 15 (H^+) resin. The resin was filtered off and washed with DCM/MeOH (1:1, v/v). The filtrate and washings were combined and concentrated *in vacuo* to provide the residue, to which was added 0.4 mL of THF/H_2O (1:1, v/v) followed by 0.1 mL of 2.0 M aqueous solution of NaOH at rt. After the solution was stirred at rt overnight, it was neutralized with 1.0 M aqueous solution of HCl to pH 7. Then, the reaction mixture was concentrated *in vacuo*, then purified on a LH-20 column using DCM/MeOH (1:1, v/v) as the eluent to give **GM3-αGalCer** after lyophilization as a white solid (7 mg, 62% over 3 steps). ^{1}H NMR (400 MHz, $CDCl_3$/CD_3OD 1/1): δ 4.90 (t, J = 4.2 Hz, 1H), 4.45 (d, J = 7.9 Hz, 1H), 4.38 (d, J = 7.6 Hz, 1H), 4.19 (s, 1H), 4.01 (q, J = 6.5, 6.1 Hz, 3H), 3.95−3.46 (m, 21H), 3.42 (d, J = 4.9 Hz, 2H), 2.79 (d, J = 12.7 Hz, 1H), 2.06 (d, J = 7.2 Hz, 5H), 1.94 (d, J = 12.4 Hz, 1H), 1.65 (s, 14H), 1.27 (s, 99H), 0.96 (t, J = 7.5 Hz, 2H), 0.89 (t, J = 6.7 Hz, 10H). ^{13}C NMR (100 MHz, $CDCl_3$/CD_3OD 1/1): δ 103.79, 102.91, 99.75, 79.95, 77.71, 76.36, 75.22, 74.93, 74.83, 74.15, 74.01, 73.32, 72.83, 72.09, 71.79, 70.85, 69.88, 69.50, 69.31, 69.21, 68.69, 68.56, 68.15, 66.86, 64.29, 63.95, 63.81, 63.18, 63.10, 61.17, 60.72, 52.70, 52.35, 52.05, 50.49, 39.75, 39.63, 39.07, 36.17, 35.33, 34.29, 31.80, 31.79, 31.11, 29.70, 29.58, 29.52, 29.49, 29.46, 29.35, 29.24, 29.20, 29.07, 25.87, 25.79, 25.05, 22.50, 21.91, 18.51. HR ESI-MS calcd. for $C_{83}H_{154}N_4O_{30}Na^+$ $[M + Na]^+$ 1710.0540, found 1710.0514.

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*(2S,3S,4R)-3,4-Dihydroxy-2-hexacosanoylamino-octadecane-(2,3-di-O-benzyl-6-deoxy-6-N-(2-(2-((5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranosyloxy)-ethoxy)-ethyl-amino)-oxohexanamido-β-D-galactopyranoside (**GM3-βGalCer**).* The preparation of **GM3-βGalCer** is similar to that of **GM3-αGalCer.** White solid, yield (21 mg, 78% over 3 steps). ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$ 1/1): δ 4.41 (d, *J* = 7.8 Hz, 1H), 4.34 (d, *J* = 7.5 Hz, 2H), 4.18 (d, *J* = 6.8 Hz, 3H), 4.08 (s, 1H), 3.97 (s, 3H), 3.90−3.80 (m, 3H), 3.74 (d, *J* = 10.1 Hz, 4H), 3.69−3.45 (m, 21H), 3.39 (s, 5H), 2.75 (d, *J* = 12.8 Hz, 1H), 2.24 (d, *J* = 27.6 Hz, 11H), 2.02 (d, *J* = 3.6 Hz, 4H), 1.89 (d, *J* = 12.0 Hz, 1H), 1.61 (s, 10H), 1.49 (s, 1H), 1.23 (s, 121H), 0.84 (t, *J* = 6.8 Hz, 13H). ^{13}C NMR (150 MHz, $CDCl_3/CD_3OD$ 1/1, *v/v*): δ 176.06, 175.49, 174.97, 171.53, 104.44, 104.40, 103.53, 99.05, 80.42, 77.13, 76.02, 75.68, 75.47, 74.77, 74.53, 73.98, 73.84, 73.73, 72.67, 71.92, 71.81, 70.59, 70.15, 69.94, 69.81, 69.55, 69.28, 68.77, 67.78, 64.35, 61.96, 61.30, 53.13, 50.99, 40.35, 39.74, 36.95, 36.11, 32.54, 32.10, 30.33, 30.26, 29.98, 29.95, 26.61, 25.78, 23.25, 22.67, 14.36. HR ESI-MS calcd. for $C_{83}H_{154}N_4O_{30}Na^+$[M + Na]$^+$ 1710.0540, found 1710.0516.

*2-(2-N-Hexadecanamide-ethoxy)-ethyl-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**GM3-Pam**).* The preparation of **GM3-Pam** is similar to that of **GM3-αGalCer**. White solid, yield (12 mg, 67% over 3 steps). ^{1}H NMR (600 MHz, CD_3OD): δ 4.62 (s, 2H), 4.42 (d, *J* = 7.8 Hz, 1H), 4.34 (d, *J* = 7.9 Hz, 1H), 4.04 (d, *J* = 9.7 Hz, 1H), 3.98 (dd, *J* = 10.0, 5.6 Hz, 1H), 3.94−3.80 (m, 6H), 3.79−3.66 (m, 9H), 3.56 (ddt, *J* = 18.5, 13.0, 7.5 Hz, 10H), 3.48 (d, *J* = 8.9 Hz, 1H), 3.41 (d, *J* = 8.9 Hz, 2H), 3.35 (q, *J* = 5.4 Hz, 2H), 3.26 (t, *J* = 8.7 Hz, 2H), 2.84 (d, *J* = 12.8 Hz, 1H), 2.19 (t, *J* = 7.5 Hz, 2H), 2.00 (s, 3H), 1.72 (s, 1H), 1.58 (s, 2H), 1.28 (s, 26H), 0.89 (t, *J* = 6.9 Hz, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 104.80, 104.02, 80.69, 77.35, 76.76, 76.24, 76.01, 74.65, 74.44, 72.67, 70.90, 70.55, 70.33, 69.81, 69.53, 69.07, 68.71, 64.28, 62.44, 61.70, 53.68, 41.81, 39.96, 36.79, 32.77, 30.48, 30.35, 30.17, 30.02, 26.74, 23.43, 22.31, 14.14. HR ESI-MS calcd. for $C_{43}H_{78}N_2O_{21}Na^+$ [M + Na]$^+$ 981.4989, found 981.4968.

*2-(2-N-(N-α, ε-Bis-hexadecanamide)-L-lysyl)-ethoxy)-ethyl-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**GM3-Pam$_2$**).* The preparation of **GM3-Pam$_2$** is similar to that of **GM3-αGalCer**. White solid, yield (8 mg, 49% over 3 steps). ^{1}H NMR (400 MHz, $CDCl_3/CD_3OD$ 1/1, *v/v*): δ 4.44−4.24 (m, 5H), 4.04−3.49 (m, 25H), 3.45 (d, *J* = 8.9 Hz, 1H), 3.41−3.34 (m, 2H), 3.13 (t, *J* = 6.9 Hz, 3H), 2.77 (d, *J* = 12.9 Hz, 1H), 2.35 (t, *J* = 7.4 Hz, 1H), 2.22 (q, *J* = 9.7, 7.7 Hz, 3H), 2.13 (t, *J* = 7.6 Hz, 2H), 2.05−1.95 (m, 5H), 1.89−1.73 (m, 4H), 1.58 (p, *J* = 7.5 Hz, 8H), 1.48 (t, *J* = 7.3 Hz, 2H), 1.25 (d, *J* = 14.1 Hz, 76H), 0.95−0.76 (m, 14H). ^{13}C NMR (100 MHz, $CDCl_3/CD_3OD$): δ 104.32, 103.29, 80.41, 78.20, 76.67, 76.05, 75.47, 75.17, 74.04, 73.77, 71.95, 70.36, 69.81, 68.98, 63.91, 61.98, 61.24, 41.21, 39.47, 39.24, 36.68, 36.43, 32.25, 32.08, 30.00, 29.98, 29.96, 29.94, 29.89, 29.87, 29.81, 29.73, 29.67, 29.57, 29.15, 27.44, 26.34, 26.17, 23.28, 22.96, 14.04. HR ESI-MS calcd. for $C_{65}H_{120}N_4O_{23}Na^+$ [M + Na]$^+$ 1347.8236, found 1347.8279.

*2-(2-N-tert-Butyloxycarbonylamido-ethoxy)-ethyl-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**21**).* The preparation of **21** from **20** is similar to that of **GM3-αGalCer**. White solid, yield (14 mg, 84%, over 3 steps). ^{1}H NMR (600 MHz, CD_3OD): δ 4.43 (d, *J* = 7.8 Hz, 1H), 4.35 (t, *J* = 7.2 Hz, 1H), 4.09. − 3.96 (m, 3H), 3.89 (dt, *J* = 7.8, 4.6 Hz, 4H), 3.85−3.80 (m, 3H), 3.79−3.71 (m, 7H), 3.65 (tt, *J* = 18.6, 10.0 Hz, 9H), 3.60−3.45 (m, 10H), 3.41 (d, *J* = 9.3 Hz, 2H), 3.29−3.17 (m, 4H), 2.77 (dd, *J* = 12.9, 4.3 Hz, 1H), 2.00 (s, 3H), 1.88 (t, *J* = 11.8 Hz, 1H), 1.43 (s, 9H).^{13}C NMR (150 MHz, CD_3OD): δ 175.26, 172.51, 105.02, 104.26, 100.02, 80.72, 77.74, 76.79, 76.44, 76.22, 75.30, 74.69, 72.83, 72.62, 71.16, 71.03, 70.68, 70.02, 69.81, 69.42, 68.74, 64.71, 62.42, 61.85, 53.69, 41.18, 28.76, 22.67. ESI-MS calcd. for $C_{32}H_{55}N_2O_{22}^-$ [M − H]$^-$ 819.33, found 819.80.

*2-(2-N-Cholesterylcarbamate-ethoxy)-ethyl-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**GM3-Chol**).* Trisaccharide **21** (12 mg, 0.014 mmol) was added 35% aqueous trifluoroacetic acid solution (500 μL, TFA/H_2O, 35/65, *v/v*) and kept 10−15 min at rt, then equal volume toluene was added, concentrated via rotary evaporator. The residue was further dried at rt for 5 h under high vacuum. Then, the residue was dissolved in DCM/DMF (1/1, 2 mL, *v/v*), followed by cholesteryl nitrophenyl carbonate **22**[64] (24 mg, 0.044 mmol) and DIPEA (3.8 μL, 0.022 mmol). The mixture was kept stirring until TLC analysis ($CHCl_3$/MeOH/0.2% $CaCl_2$ 50:50:1) showed the complete consumption of amine. The residue was coevaporated with toluene three times to remove the DMF and washed with Et_2O to remove the redundant cholesterol. The desired product **GM3-Chol** was obtained from further purification by size exclusion column chromatography (LH-20, eluted by DCM/MeOH 1:1), white solid (11.5 mg, 70% over 2 steps). ^{1}H NMR (600 MHz, $CDCl_3$ /CD_3OD 1/1): δ 5.33 (s, 1H), 4.34 (d, *J* = 45.7 Hz, 3H), 3.97 (s, 2H), 3.90−3.33 (m, 22H), 3.14 (d, *J* = 7.8 Hz, 1H), 2.76 (d, *J* = 29.6 Hz, 1H), 2.29 (s, 2H), 1.99 (d, *J* = 9.4 Hz, 5H), 1.83 (s, 3H), 1.66−0.73 (m, 30H), 0.66 (s, 3H). ^{13}C NMR (100 MHz, CD_3OD): δ 123.14, 104.84, 104.04, 77.41, 76.75, 76.42, 76.23, 76.05, 75.43, 75.09, 72.12, 70.93, 70.68, 70.59, 70.41, 69.56, 69.11, 64.64, 64.29, 62.46, 62.44, 57.86, 57.29, 55.58, 53.70, 53.40, 51.36, 49.43, 49.21, 49.00, 48.79, 48.57, 43.51, 40.85, 40.41, 39.41, 38.00, 37.09, 36.83, 32.95, 32.74, 28.99, 28.86, 25.03, 24.65, 22.89, 22.65, 22.42, 22.30, 21.88, 19.51, 18.95, 18.08, 14.11, 12.88, 12.03. HR ESI-MS calcd. for $C_{55}H_{92}N_2O_{22}Na^+$ [M + Na]$^+$ 1155.6034, found 1155.6053.

*2-(2-N-(2-Ethoxy-3,4-dioxocyclobut-1-en-1-yl)-aminoethoxy)-ethyl-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-non-2-ulopyranosylonic acid)-(2 → 3)-(β-D-galactopyranosyl)-(1 → 4)-β-D-glucopyranoside (**23**).* The crude amine prepared by deprotection of Boc group in trisaccharide **21** (20 mg, 2.43 μmol) was dissolved in EtOH/H_2O (*v/v* = 1/1, 1 mL). A solution of aqueous $NaHCO_3$ (saturated) was added until reaching pH = 8.0. Then the mixture was treated with 3,4-diethoxy-3-cyclobutene-1,2-dione (12.4 mg, 7.32 μmol). After stirring for 1.5 h at rt, the mixture was neutralized by AcOH. The solvent was evaporated under reduced pressure, and the residue was triturated with EA to removed nonpolar impurities. The crude product was dissolved in minimal MeOH, and the pure product was precipitated after acetone was gradually added. The precipitate was dried to provide **23** (18 mg, 87%) as a colorless solid. ^{1}H NMR (600 MHz, CD_3OD): δ 4.74 (dd, *J* = 11.0, 6.7 Hz, 2H), 4.43 (d, *J* = 8.0 Hz, 1H), 4.35 (d, *J* = 7.8 Hz, 1H), 4.10−4.02 (m, 1H), 4.01−3.45 (m, 21H), 3.42 (s, 1H), 3.26 (s, 1H), 2.86 (d, *J* = 12.4 Hz, 1H), 2.01 (s, 3H), 1.72 (d, *J* = 11.4 Hz, 1H), 1.46 (t, *J* = 7.1 Hz, 3H). ESI-MS calcd. for $C_{33}H_{51}N_2O_{23}^-$ [M − H]$^-$ 843.30, found 842.73.

GM3-BSA. To the solution of squaric acid monoamide **23** (8 mg, 0.0095 mmol) in 5 mL of 70 mM $Na_2B_4O_7$ and 35 mM $KHCO_3$ aqueous solution was added BSA (21 mg, 0.31 μmol) at rt. After being incubated at rt for 48 h, the mixture was centrifuged using the Microcon (Millipore) under 6000*g* for 15 min. And the resulting aqueous solution was lyophilized to give **GM3-BSA** conjugate. The average loading of GM3 on BSA was 7.4, the value was estimated by MALDI-TOF-MS.

Immunological Test. *Materials and Reagents.* DSPC was purchased from TCI. Cholesterol was purchased from Energy Chemical. Peroxidase-conjugated AffiniPure goat antimouse kappa, IgA, IgE, IgG1, IgG2a, IgG2b, and IgG3 antibodies were purchased from Southern Biotechnology, and peroxidase-conjugated AffiniPure goat antimouse kappa antibody IgG and IgM were purchased from Jackson ImmunoResearch. Mouse IL-4 and IFN-γ ELISA kits were obtained from BD Pharmingen. B16F10 cells was purchased from China Infrastructure of Cell Line.

Liposomal Formulation of Vaccine Candidates. Liposomal formulations of vaccine candidates were prepared by following a previously reported protocol.[27,29,41] As for covalent vaccine candidate **GM3-αGalCer** and control **GM3-βGalCer**, the liposomes consist of DSPC, cholesterol, and **GM3-αGalCer** (or **GM3-βGalCer**) in a molar ratio of 5:4:1. In terms of noncovalent vaccine candidates, the liposomes consist of DSPC, cholesterol, αGalCer, and **GM3-Pam** (**GM3-Pam$_2$**, **GM3-Chol**, or **GM3-βGalCer**) in a molar ratio of 5:4:1:1. After the mixture of corresponding ingredients was dissolved in

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a mixture of DCM/MeOH (1:1, *v*/*v*, 2 mL), the solvents were removed under reduced pressure through rotary evaporation, which generated a thin lipid film on the flask wall. This film was hydrated and subjected to freeze/thaw cycles to produce multilamellar vesicles; the film was hydrated with HEPES buffer (20 mM, pH 7.5) containing NaCl (150 mM), and the mixture was sonicated 15 min at rt before use.

Immunization of Mice. Female BALB/c mice of 6−8 weeks age used for immunological studies were purchased from Laboratory Animal Centre of Huazhong Agriculture University. All mice were used according to the animal ethics guidelines (Regulations of Hubei Province on the Administration of Experimental Animals and Measures of Hubei Province on Administration of Laboratory Animal Licenses). Groups of five female BALB/c mice (age 6−8 weeks) were bred in the Laboratory Animal Centre of Huazhong Agriculture University. Mice were immunized by intraperitoneal injection on days 1, 15, and 29. The mice were bled on day 0 before initial immunization and on days 14, 28, and 42 after boost immunizations. In addition, sera collected at 2 and 24 h after the first injection were analyzed for the secretion of cytokines IFN-γ and IL-4. Mouse blood samples were clotted to obtain antisera that were stored at −80 °C before use.

In vivo Cytokine Assay. The cytokine levels in sera were evaluated using ELISA kits (IFN-γ and IL-4, BD Pharmingen) according to the manufacturer's protocol. Briefly, 96-well plates were coated with capture antibodies dissolved in the coating buffer per well and incubated overnight at 4 °C. The wells were then blocked with BSA for 1 h at rt. After blocking, 100 μL/well of standard, sera, or control were added and incubated for 2 h at 28 °C. After washing, the working detector (detection antibody and Sav-HRP reagent) was added to each well. The plates were incubated for 1 h at 28 °C. Then, the plates were washed, and the tetramethyl benzidine (TMB) substrate solution was added. The reactions were stopped after 30 min at 28 °C with a stopping solution. The absorbance was measured at 450 nm by the plate reader.

ELISA Procedure. A 96-well microtiter plate (Costar type 3590, Corning Inc.) was first coated with **GM3-BSA** (2 μg/plate), which had been dissolved in a 0.1 M bicarbonate buffer (pH = 9.6). The plate was then incubated at 4 °C overnight. The plate was then washed three times with PBST (0.05% Tween-20 in PBS), followed by the addition of 3% (*w*/*v*) BSA in PBS to each well and incubation at 37 °C for 1 h. After the plate was washed again with PBST, serially diluted sera were added to microwells (100 μL/well) and incubated for 1 h at 37 °C and washed (three times). A 1:5000 diluted solution of horseradish peroxidase (HRP)-conjugated goat antimouse IgG (γ-chain specific) or IgM (μ-chain specific), IgG1, IgG2a, IgG2b, IgG3, IgA, or IgE in 0.1% BSA/PBS (100 μL per well) was added to each well, respectively. The plate was incubated for 1 h at 37 °C. After a final wash (three times), substrate solutions that were freshly prepared with a 9.5 mL critic buffer at pH 5.0, 0.5 mL (1.6 mg/mL) TMB, and 32 μL 3% (*w*/*v*) urea hydrogen peroxide were added to the wells (100 μL per well). Color was allowed to develop in the dark for 8 min and then a solution of 2 M H_2SO_4 was added to quench the reaction. The optical density was measured at 450 nm. The antibody titer was defined as the highest dilution showing an absorbance of 0.1, after subtracting the background. Preimmunization sera have been used as the negative control.

FACS Assay Protocol. B16F10 cells were cultured in RPMI-1640 containing 10% FBS and 1% antibiotic. After, B16F10 cells were harvested and washed with FACS buffer (PBS containing 5% FBS). The cells were incubated with normal or anti-GM3 serum induced by the vaccine candidates (1:10 dilution in FACS buffer, contained PBS (1×), 10% FBS, and 0.1% sodium azide) for 1 h at 4 °C. Then cells were washed three times with 1% BSA/PBS and incubated with FITC-linked goat antimouse kappa antibody (Alexa Fluor 488-conjugated Goat Anti-Mouse IgG (H+L), Jackson ImmunoResearch, diluted 1:50) for 30 min at 4 °C. After washing three times with 1% BSA in PBS, the cells were added to the FACS buffer and analyzed by flow cytometry (BD Calibur).

Complement Dependent Cytotoxicity Assay (CDC). B16F10 cells or THP-1 cells[76] (1×10^4 cell/well) were seeded in the wells of the 96-well plate. After incubation at 37 °C overnight, the plate was washed with medium without FBS, then incubated with these antisera (diluted 1:25, 50 μL per well) from vaccinated mice for 1 h. After washing with PBS solution, the rabbit complement (at a rabbit sera dilution of 1:50) in 1% BSA/PBS was added. The rabbit complement inactivated by treatment at 65 °C for 30 min was used as the control. After incubation for 2 h, 0.5% MTT solution in PBS was added (20 μL/well) and incubated for 2 h. After removing the medium, DMSO was added (150 μL/well) and the absorption was analyzed at the wavelength of 490 nm. The survival rate of cells was measured with the following formula. All assays were conducted in four independent experiments. Cytotoxicity (%) = [1-(experimental OD/control OD)] × 100.

Statistical Analysis. Data reported in the figures were analyzed, and charts were generated using Prism 6 (GraphPad Software). All values and error bars are mean ± SEM. Significance between two groups was determined by unpaired two-tailed Student's *t* test. Significance of multiple groups in comparison to the control group was evaluated using one-way ANOVA followed by a Dunnett's multiple comparison test. Asterisks (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; and ****, $P \leq 0.001$) indicate significant differences.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.0c01186.

MALDI-TOF mass spectrometry determination of the average level of GM3 incorporated on BSA protein, ELISA evaluation of anti-GM3 antibody levels of IgA and IgE on Day 42, and ^{1}H and ^{13}C NMR spectra of the new compounds (PDF)

Molecular formula strings data (CSV)

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

αGalCer, α-galactosylceramide; APCs, antigen-presenting cells; BSA, bovine serum albumin; CDC, complement-dependent cytotoxicity; CTL, cytotoxic T lymphocyte; DCs, dendritic cells; DCM, dichloromethane; DIPEA, diisopropylethylamine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; EA, ethyl acetate; ELISA, enzyme-linked immunosorbent assay; EDC, 1-ethyl-3-(3-(dimethylamino)propyl)-carbodiimide; FACS, fluorescence activated cell sorter; FBS, fetal serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HOBt, 1-hydroxy-1H-benzotriazole; IFN-γ, interferon-γ; IL-4, interleukin-4; iNKT, invariant natural killer T; MHC, major histocompatibility complex; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide; NIS, *N*-iodosuccinimide; PE, petroleum ether; PBS, phosphate-buffered saline; SEM, standard error of measurement; TACAs, tumor-associated carbohydrate antigens; TfOH, trifluoromethanesulfonic acid; Th, T helper; TLR, toll-like receptor

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