Modification of the Ceramide Moiety of Isoglobotrihexosylceramide on Its Agonist Activity in Stimulation of Invariant Natural Killer T Cells

Chengfeng Xia,[†] Jens Schümann,[‡] Rossy Emmanuel,[‡] Yalong Zhang,[†] Wenlan Chen,[†] Wenpeng Zhang,[†] Gennaro De Libero,[‡] and Peng George Wang*,[†]

Departments of Biochemistry and Chemistry, The Ohio State University, 876 Biological Sciences Building, 484 West 12th Avenue, Columbus, Ohio 43210, and Experimental Immunology, Department of Research, University Hospital Basel, CH-4031 Basel, Switzerland

Received January 26, 2007

Isoglobotrihexosylceramide (iGb3) is an endogenous antigen of mammalian cells and can stimulate invariant natural killer T (iNKT) cells to evoke autoimmune activities by the release of T helper 1 (Th1) and Th2 cytokines. Th1 cytokines are correlated with the antitumor and antiviral response, while Th2 cytokines are correlated with the amelioration of autoimmune diseases. iGb3 is a very weak agonist compared to the exogenous α-galactosylceramide; however, modification of the ceramide moiety has been advocated as one of the approaches to improve its stimulatory activity and to change the bias of release of Th1 and Th2 cytokines. Two analogues of iGb3, 2H-iGb3 and HO-iGb3 with different ceramide moieties, were synthesized. Bioassay results showed that HO-iGb3 was much more effective in stimulating iNKT cells than iGb3 at low concentration. The assay also showed that the CD1d/2H-iGb3 complexes are remarkably efficient in stimulating iNKT cells.

Introduction

Invariant natural killer T (iNKT[a]) cells are a subpopulation of T lymphocytes and express conserved, semi-invariant αβ (mouse Vα14-Jα18/Vβ8 or human Vα24-Jα18/Vβ11) T cell receptors (TCR) and natural killer (NK) receptors. They can be stimulated by the glycolipid antigens that are presented by major histocompatibility complex class-I-like protein CD1d for specific recognition. Upon stimulation, iNKT cells rapidly secrete copious cytokines (Figure 1). The interferon-γ (IFN-γ) and interleukin-2 (IL-2) belong to Th1-type cytokines, tend to produce the proinflammatory response, and are responsible for fighting bacterial, parasitic, and viral infections. However, the Th1 response also causes multiple sclerosis, lupus, and type 1 diabetes. Th2-type cytokines, including IL-4 and IL-10, can result in an immunomodulatory response. In excess, Th2 responses, however, will counteract the Th1 mediated autoimmune diseases.[1]

Agelasphins are a kind of α-galactosylceramide (α-GalCer) that were isolated from the marine sponge *Agelas mauritianus* in 1992. They are the first exogenous antigens that could be presented by CD1d to stimulate iNKT cells.[2] After decades of searching, isoglobotrihexosylceramide (iGb3) (Figure 1) was discovered as an endogenous mammalian glycosphingosine that can also stimulate iNKT cells via presentation by CD1d.[3] The most distinctive difference between agelasphins and iGb3 is that the agelasphins contain an α-linkage between the sugar and ceramide, while iGb3 has a β-linkage.

Since the discovery of α-GalCer, numerous researchers have struggled to demonstrate its structure−activity-relationship (SAR).[4] It was found that modification of the ceramide chains could polarize the secretion of Th1 and Th2 cytokines. The glycoceramide OCH, which is an α-GalCer analogue, is structurally distinct from other α-GalCer analogues in having a substantially shorter sphingosine chain.[5] OCH can stimulate iNKT cells to preferentially produce Th2 cytokines. Another glycoceramide C20:2, which contains two double bonds in the acyl chain, can induce strong Th1 production and induce cellular proliferation.[6]

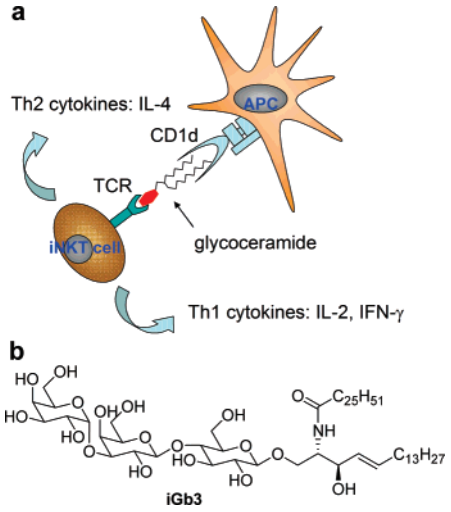


Figure 1. (a) Schematic illustration for the event of iNKT cells stimulation by glycoceramide. The glycoceramide is presented by the CD1d of APC to the receptor of iNKT cells. Upon glycoceramide stimulation, iNKT cells produce the Th1 and Th2 cytokines. (b) The structure of isoglobotrihexosylceramide (iGb3) consists of a trisaccharide and ceramide with a β-linkage and is the endogenous antigen of iNKT cells.

Crystallographic studies of the CD1d/α-GalCer complexes illustrated that the ceramide moiety of α-GalCer contributes to the binding with CD1d by anchoring α-GalCer in a distinct orientation.[7] Both alkyl chains are initially inserted perpendicularly to the β-sheet platform and then extend more laterally toward the ends of the A′ and F′ pockets (Figure 2). A specific

* To whom correspondence should be addressed. Phone: (+1) 614-292-9884. Fax: (+1) 614-688-3106. E-mail: wang.892@osu.edu.
[†] The Ohio State University.
[‡] University Hospital Basel.

[a] Abbreviations: iGb3, isoglobotrihexosylceramide; iNKT, invariant natural killer T; Th1, T helper 1; IFN-γ, interferon-γ; IL-2, interleukin-2; DDQ, 2,3-dichloro-5,6-dicyano-1,4-benzoquinone; DMAP, dimethylaminopyridine; EDCI, *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride; TMSOTf, trimethylsilyltrifluoromethane sulfonate; ELISA, enzyme linked immunosorbent serologic assay.

10.1021/jm0701066 CCC: $37.00
Published on Web 07/04/2007

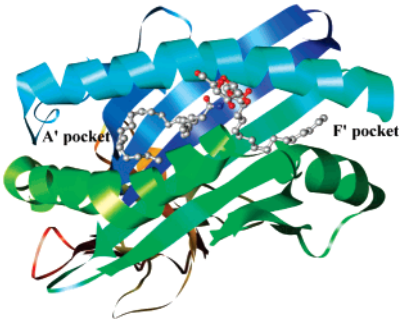


Figure 2. Crystal structure of α-GalCer with CD1d. There are two pockets in the CD1d molecule. The acyl chain is located in the A′ pocket, and the sphingosine chain is located in the F′ pocket. The sugar head stands outside for recognition by the TCR.

hydrogen-bond network was observed between CD1d and glycoceramide. Binding was assisted by these interactions, specifically between three conserved residues (mouse Asp153, Asp80, and Thr156; human Asp151, Asp80, and Thr154) in CD1d and the 2′-OH on the galactose ring, the 3-OH on the sphingosine chain, and the 1′-O glycosidic linkage. Aromatic residues Tyr73 (A′ pocket), Phe77, and Trp133 (F′ pocket) make extensive van der Waals contacts with the glycoceramide, stabilizing both alkyl chains after insertion into their individual binding pockets. The phytosphingosine moiety fully occupies the F′ pocket, which can accommodate linear alkyl chains of up to C_{18}. In pocket A′, a linear hydrophobic compound has been found, which acts as a "spacer lipid" similar to the detergent molecules initially noted in the CD1b phosphatidylinositol structure.[8] Such a factor could stabilize the hydrophobic binding pocket in the absence of an antigenic groove-filling ligand, such as a full-length α-GalCer for CD1d or a glucose monomycolate for CD1b.[9]

To the best of our knowledge, only a few analogues of iGb3 have been synthesized to explore its SAR.[10] Herein, we report the synthesis of two novel iGb3 analogues with different ceramides and their associated stimulatory activity of iNKT cells.

Results and Discussion

On the basis of the structure−activity relationship studies of α-GalCer, we assumed that varying the lipid portion of iGb3 would change its ability to bind with CD1d. This would further affect its activities in stimulating iNKT cells and could polarize iNKT cell release toward either the Th1 or the Th2 cytokines. The difference between the lipid moieties of endogenous iGb3 and exogenous α-GalCer is that the former is D-erythrosphingosine, while the latter is phytosphingosine. The D-erythrosphingosine has a double bond between C4 and C5, whereas the phytosphingosine has an extra hydroxy group on C4. The additional hydroxy group of phytosphingosine may form extra hydrogen bonds with CD1d and thereby increase the stability of the CD1d/glycoceramide complex. This additional hydroxyl group may also effect the orientation of the sugar head on the surface of CD1d for recognition by the T cell receptor (TCR) of iNKT cells. The trans double bond of D-erythrosphingosine has some function in fixing the conformation of lipid. Reduction of the double bond will increase the rotatable flexibility of the lipid, thus altering the rate of complex formation.

Initially, the structure−activity relationship study of the ceramide of iGb3 focused on the functional groups on the lipid part. Two iGb3 analogues, 2H-iGb3 (**1**) and HO-iGb3 (**2**), were designed on the basis of the above rationale (Figure 3). Both of the two iGb3 analogues could be synthesized from the same

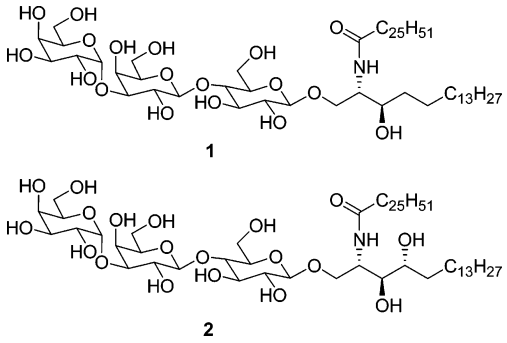


Figure 3. Two iGb3 analogues with different ceramides.

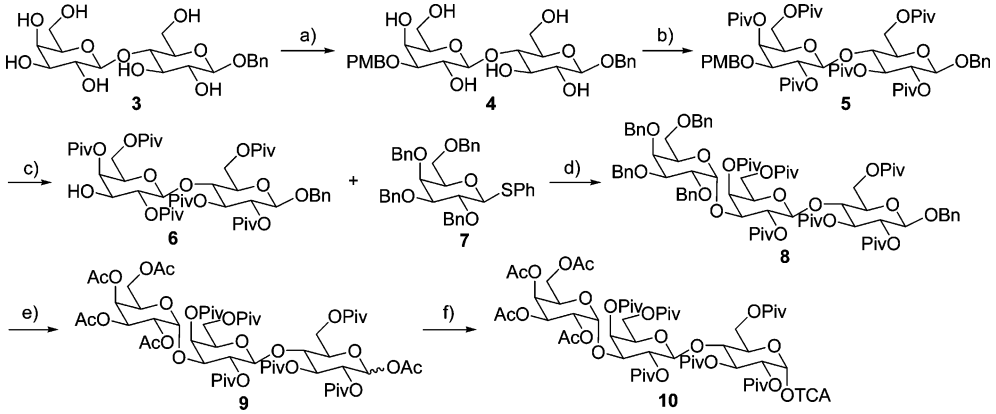
trisaccharide donor **10**, which was readily prepared from 1-benzyllactose **3** (Scheme 1). According to our published protocol, the 3″-OH of lactose **3** was activated by the formation of a dibutylstannylene acetal.[11] Regioselective monoalkylation, in a one-pot manner, by subjection to 4-methoxybenzyl chloride (PMBCl) afforded compound **4**. The remaining hydroxy groups were protected with a bulky pivaloyl group by treatment with pivaloyl chloride and DMAP in pyridine at 70 °C. The use of a bulky pivaloyl group is important for avoiding the formation of the orthoester in the glycosylation with the lipid. Selective deprotection of the PMB group was achieved by treatment with DDQ to release the 3″-OH to afford the disaccharide acceptor **6**.

The glycosylation between the perbenzylated phenylthioglycoside donor **7** and the lactosyl acceptor **6** was carried out at −30 °C under activation with *N*-iodosuccinimide (NIS)/triflic acid to produce the protected trisaccharide **8**. The structure of trisaccharide **8** was confirmed by the $J_{H1-H2} = 2.4$ Hz at δ 5.51 ppm, indicating an α linkage. The trisaccharide **8** was subjected to $Pd(OH)_2$ under hydrogen atmosphere to remove all the benzyl groups. The newly formed free hydroxy groups were then protected with acetyl groups by using acetic anhydride and pyridine to provide compound **9**. The anomeric acetyl group was selectively removed by treatment with benzylamine in THF. Finally, the donor trichloroacetimidate **10** was obtained by treatment with trichloroacetonitrile and 1,8-diazabicyclo[5.4.0]-under-7-ene (DBU).

Since the H-bonding of the amide group will reduce the reactivity of the ceramide acceptor, the azidosphingosine **11** was used as the acceptor.[12] After extensive experimentation with a variety of Lewis acids, we found that glycosylation catalyzed by TMSOTf at −20 °C in the presence of 4 Å molecular sieves worked extremely well with β-selective glycosylation to give compound **12** in excellent yield (Scheme 2). To incorporate the fatty acid on the lipid, the azide was reduced to an amine quantitatively with triphenylphosphine−H_2O in benzene at 60 °C. The crude amine was subjected to amide formation with cerotic acid in the presence of EDCI in THF to provide the protected glycoceramide **13**. The compound was then hydrogenated to remove the PMB protecting group and reduce the double bond on the lipid. Finally, the acetyl and pivaloyl protecting groups were removed under reflux conditions with a catalytic amount of NaOMe in anhydrous methanol to achieve **1** in high yield.

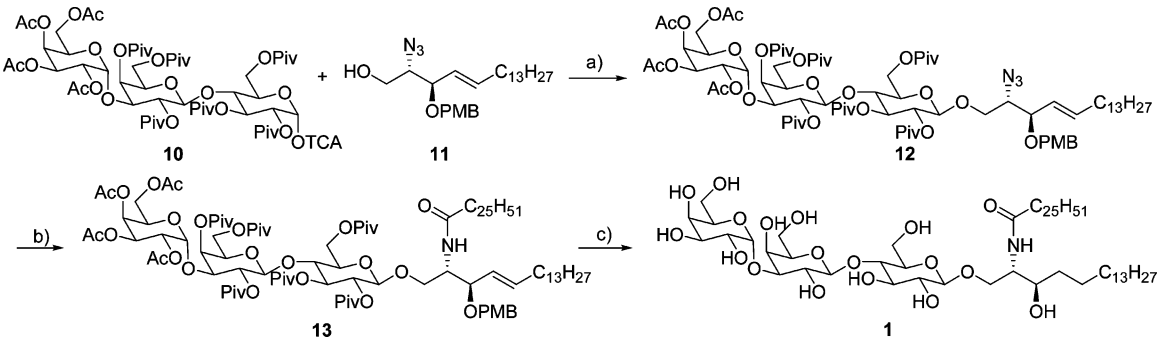
To prepare the iGb3 analogue that has the phytosphingosine type ceramide instead of the D-erythrosphingosine type ceramide, azidophytosphingosine **16** was used as an acceptor (Scheme 3). It was easily prepared from commercially available phytosphingosine **14** by conducting a diazotransfer reaction with $CuSO_4$ as the catalyst[13] to give the azido lipid **15**.[14] The primary hydroxyl group was selectively protected with trityl chloride,

Scheme 1. Synthesis of Trisaccharide Donor[a]



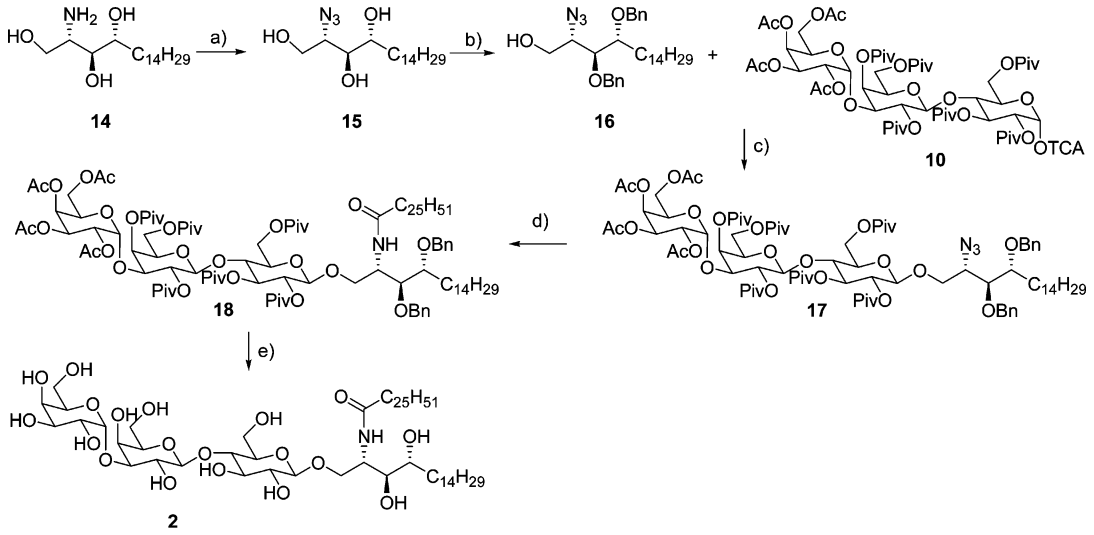
[a] Reagents and conditions: (a) (i) Bu_2SnO, MeOH, reflux; (ii) PMBCl, Bu_4NI, benzene, reflux; (b) PivCl, DMAP (cat.), pyridine, 52% for three steps; (c) DDQ, H_2O, DCM, 76%; (d) NIS, TfOH, DCM, −30 °C, 84%; (e) (i) H_2, Pd/C, MeOH; (ii) Ac_2O, pyridine, 97%; (f) (i) $BnNH_2$, THF; (ii) CCl_3CN, DBU, DCM, 78%.

Scheme 2. Synthesis of **1**[a]



[a] Reagents and conditions: (a) TMSOTf, DCM, −25 °C, 87%; (b) (i) PPh_3, H_2O, benzene, 50 °C; (ii) $C_{25}H_{51}CO_2H$, EDCl, DCM, 95%; (c) (i) 10% Pd/C, H_2, EtOH; (ii) NaOMe, MeOH, reflux, 82%.

Scheme 3. Synthesis of **2**[a]



[a] Reagents and conditions: (a) TfN_3, $CuSO_4$, DCM/MeOH/H_2O, 89%; (b) (i) TrtCl, DMAP, pyr, 50 °C; (ii) NaH, BnBr, DMF; (iii) *p*TsOH, MeOH, 68%; (c) TMSOTf, DCM, −25 °C, 81%; (d) (i) PPh_3, H_2O, benzene, 50 °C; (ii) $C_{25}H_{51}CO_2H$, EDCI, DCM, 84%; (e) (i) H_2, 10% Pd/C, MeOH; (ii) NaOMe, MeOH, reflux, 76%.

after which the two secondary hydroxy groups were protected with benzyl bromide. Finally, removal of the trityl group under acidic conditions afforded the lipid acceptor **16**.[15] Under the activation of TMSOTf, the azido acceptor **16** was glycosylated with donor **10** to produce glycolipid **17**. The azide was reduced and the cerotic acid was introduced, as in the preparation of **13**, to generate the protected glycoceramide **18**. Finally, the two benzyl groups were removed by hydrogenation with 10% Pd/C in methanol under hydrogen atmosphere and the remaining esters were hydrolyzed with NaOMe in refluxing MeOH to give **2**.

IFN-γ (Th1-type cytokine) and IL-4 (Th2-type cytokine) productions were measured to evaluate the abilities of the iGb3 analogues to stimulate iNKT cells. The relative potencies of iGb3, **1**, and **2** were measured with the use of human Vα24i NKT cell clone (JS7) and hCD1d-transfected human B lym-

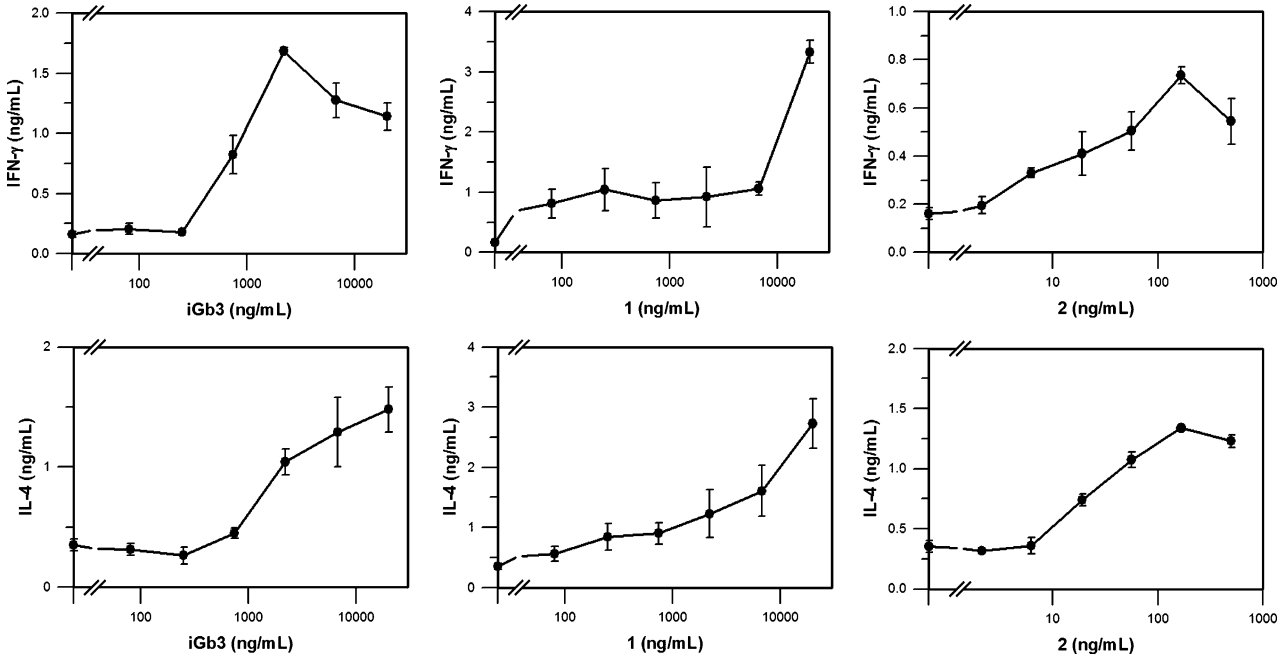


Figure 4. iNKT cells release IFN-γ and IL-4 cytokines when activated by iGb3 analogues.

phoblastoid (C1R). The release of cytokines was measured by enzyme linked immunosorbent serologic assay (ELISA) from cocultured supernatants of the iNKT cells and antigen presenting cells (APC) with different concentrations of glycoceramides (Figure 4). The results showed that **1** was slightly more active in stimulation of iNKT cells in low concentration relative to iGb3. At higher concentrations (e.g., 20 μg/mL), very efficient stimulation of iNKT cells was observed. These results indicated that reduction of the double bond on the lipid to the saturated bond increased the flexibility of the glycolipids, which made it easier for **1** to adopt the appropriate confirmation to enter the F′ pocket of CD1d. An impressive stimulation was observed when using **2** to activate iNKT cells. Both Th1 and Th2 cytokines were released when human iNKT cells were stimulated with **2** at 10 ng/mL (100-fold lower concentration than iGb3). This may result from the greater stability of the **2** and CD1d complex because the 4-OH of the phytosphingosine can form an additional hydrogen bond with the F′ pocket. The results are in agreement with the observation by Kronenberg et al.[16] They examined the interactions between the solubilized form of the TCR and CD1d-glycolipd complex by surface plasmon resonance. The equilibrium dissociation constants (K_D), based on TCR binding at equilibrium, are 0.35 μM for KRN7000 (same ceramide as **2**) and 1.12 μM for 4-deoxy-GalCer (same ceramide as **1**).

However, the modification of the lipid part of iGb3 by reducing the double bond or introducing an extra hydroxy group did not polarize the release of cytokines. Both of the Th1 and Th2 cytokines were produced when the iNKT cells were incubated with APC and iGb3 analogues. The reported modifications of α-GalCer to change the bias of Th1 and Th2 secretion were mostly successful from the lipids instead of the polar portion of ceramide. To obtain the selectivity toward either Th1 or Th2 cytokines response, more investigation is needed for the lipid part of the ceramide.

Conclusion

In summary, we described a SAR study guided by iGb3, an endogenous antigen for iNKT cells. Reduction of the double bond of the D-erythrosphingosine moiety gave **1**. Furthermore, replacement of the D-erythrosphingosine moiety with phytosphingosine led to the generation of **2**. Two iGb3 analogues with different ceramides were synthesized by first constructing the trisaccharide scaffold and then by introducing the lipid part. The release of cytokines by iNKT cells involves two stages. The glycoceramide is first loaded on CD1d, and then the CD1d/glycoceramide complex will bind to iNKT cells.[17] Any development of the glycoceramides that can improve one of the two stages will change the status of cytokine production by iNKT cells. The saturated lipid part of **1** increased its rotational flexibility, which was beneficial to its uploading on CD1d. Bioassay results showed that **2** is much more efficient in stimulating iNKT cells compared to iGb3 at very low concentrations. Introduction of the additional hydroxyl group on the ceramide part of iGb3 may improve the stability of the CD1d/glycoceramide complex.

Experimental Section

All solvents were dried with a solvent-purification system from Innovative Technology, Inc. All reagents were obtained from commercial sources and used without further purification. Analytical TLC was carried out on silica gel 60 F254 aluminum-backed plates (E. Merck). The 230−400 mesh size of the same absorbent was utilized for all chromatographic purifications. ^{1}H and ^{13}C NMR spectra were recorded at the indicated field strengths. The high-resolution mass spectra were collected at The Ohio State University Campus Chemical Instrumentation Center.

Benzyl 2,4,6-Tri-*O*-pivaloyl-3-(4-methoxybenzyl)-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-β-D-glucopyranoside (5). A suspension of benzyllactose **3** (13.0 g, 30.1 mmol) and Bu_2SnO (9.0 g, 36.1 mmol) in anhydrous MeOH (150 mL) was heated to reflux and stirred for 6 h. The solvent was removed in vacuo. Then the residue was dissolved in dry benzene (150 mL). *p*-Methoxybenzyl chloride (4.9 mL, 36.1 mmol), tetrabutylammonium iodide (4.43 g, 12.0 mmol), and 4 Å molecular sieves (5 g) were added. The resulting mixture was heated to reflux for another 6 h and then cooled to room temperature. The suspension was filtered through a Celite pad and the filtrate was concentrated and chromatographied (6:1 chloroform−methanol) to afford 10.6 g of crude product **4** (64% yield).

Pivaloyl chloride (5.4 mL, 43.47 mmol) was added to a solution of the above crude (3.00 g, 5.43 mmol) and 4-dimethylaminopy-

ridine (50 mg) in dry pyridine (20 mL). The reaction mixture was heated for 36 h at 70 °C and then allowed to cool to room temperature. The solvent was removed in vacuo. The residue was dissolved in EtOAc (100 mL), washed with 1 N HCl (60 mL), saturated aqueous $NaHCO_3$ (60 mL), and brine (50 mL) successively, dried (Na_2SO_4), and evaporated. The crude product was purified by silica gel flash chromatography (1:2 EtOAc−hexane) to give compound **5** (4.70 g 82%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32 (m, 5H), 7.13 (d, J = 8.6 Hz, 2H), 6.85 (d, J = 8.6 Hz, 2H), 5.51 (d, J = 2.5 Hz, 1H), 5.24 (t, J = 9.4 Hz, 1H), 5.06 (dd, J = 8.4, 9.4 Hz, 1H), 4.96 (dd, J = 8.4, 9.4 Hz, 1H), 4.84 (d, J = 11.4 Hz, 1H), 4.59 (m, 4H), 4.42 (d, J = 9.4 Hz, 1H), 4.27 (m, 2H), 4.13 (m, 2H), 3.88 (m, 2H), 3.80 (s, 3H), 3.69 (m, 1H), 3.54 (dd, J = 2.6, 9.4 Hz, 1H), 1.24 (s, 9H), 1.22 (s, 9H), 1.16 (s, 9H), 1.13 (s, 9H), 1.12 (s, 9H), 1.08 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 206.9, 177.9, 177.9, 177.5, 177.0, 176.7, 176.0, 159.2, 136.6, 129.3, 129.2, 128.4, 128.0, 100.3, 99.4. 78.0, 76.8, 73.9, 73.4, 71.8, 71.6, 71.5, 70.5, 70.1, 65.6, 62.0, 61.9, 55.2, 39.0, 38.95, 38.8, 38.7, 38.66, 30.9, 27.3, 27.29, 27.2, 27.1, 27.09, 27.06. HRMS calcd for $C_{57}H_{84}O_{18}Na$ ($[M + Na]^+$) 1079.5550, found 1079.5555.

Benzyl 2,4,6-Tri-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-β-D-glucopyranoside (6). To a solution of compound **5** (3.00 g, 2.83 mmol) in CH_2Cl_2 (27 mL) were added H_2O (3 mL) and DDQ (1.29 g, 5.55 mmol). The reaction mixture was stirred at room temperature for 12 h until compound **3** was no longer detectable in TLC. The mixture was diluted with CH_2Cl_2 (30 mL), washed with water (3 × 40 mL), dried (Na_2SO_4), and evaporated. Purified by silica gel flash chromatography (1:1 EtOAc−hexane) to give compound **6** (2.02 g, 76%) as a clear oil. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.32 (m, 5H), 5.34 (d, J = 2.5 Hz, 1H), 5.28 (t, J = 9.2 Hz, 1H), 4.95 (dd J = 8.4, 9.2 Hz, 1H), 4.84 (m, 2H), 4.60 (m, 2H), 4.49 (d J = 9.2 Hz, 1H), 4.33 (dd J = 4.6, 11.9 Hz, 1H), 4.12 (m, 2H), 3.88 (m, 3H), 3.57 (m, 1H), 2.61 (br, 1H), 1.24 (m, 54H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 178.7, 177.8, 177.6, 177.0, 176.5, 136.4, 128.3, 127.8, 99.7, 99.2, 73.8, 73.3, 72.6, 72.2, 71.7, 71.4, 71.2, 70.4, 69.2, 61.8, 61.6, 39.0, 38.8, 38.5, 30.7, 27.2, 26.9. HRMS calcd for $C_{49}H_{76}O_{17}Na$ ($[M + Na]^+$) 959.4975, found 959.4957.

Benzyl 2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-β-D-glucopyranoside (8). A suspension of acceptor **6** (1.04 g, 1.1 mmol), perbenzylgalactosyl donor **7** (1.05 g, 1.66 mmol), and 4 Å molecular sieves (2 g) in dry CH_2Cl_2 (20 mL) was stirred at room temperature for 30 min. After the mixture was cooled to −30 °C, *N*-iodosuccinimide (272 mg, 1.21 mmol) was added followed by TMSOTf (20 μL, 0.2 mmol). The resulting mixture was stirred at −30 °C for 2 h and then was allowed to warm slowly to 10 °C. The mixture was diluted with CH_2Cl_2 (20 mL), and the molecular sieves were filtered. The filtrate was washed with saturated aqueous $NaHCO_3$ (20 mL), 10% $Na_2S_2O_3$ (20 mL), and brine (20 mL), dried (Na_2SO_4), and concentrated. The residue was purified by silica gel flash chromatography (1:8 EtOAc−hexane) to furnish trisaccharide **8** (1.35 g, 84%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36−7.19 (m, 25H), 5.51 (d, J = 2.4 Hz, 1H), 5.16 (t, J = 9.6 Hz, 1H), 5.08 (dd, J = 10.0, 8.0 Hz, 1H), 4.90−4.77 (m, 5H), 4.67 (d, J = 11,6 Hz, 1H), 4.61 (d, J = 12.0 Hz, 1H), 4.56−4.50 (m, 3H), 4.47 (d, J = 10.8 Hz, 1H), 4.44 (d, J = 11.6 Hz, 1H), 4.35 (d, J = 11.6 Hz, 1H), 4.26 (d, J = 8.0 Hz, 1H), 4.24 (dd, J = 12.0, 5.6 Hz, 1H), 3.98−3.92 (m, 3H), 3.89−3.85 (m, 3H), 3.81 (t, J = 9.6 Hz, 1H), 3.65 (dd, J = 10.4, 3.2 Hz, 1H), 3.53−3.49 (m, 3H), 3.41 (dd, J = 6.8, 5.6 Hz, 1H), 1.23 (s, 9H), 1.21 (s, 9H), 1.18 (s, 9H), 1.16 (s, 9H), 1.14 (s, 9H), 1.11 (s, 9H), 1.08 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.0, 176.9, 176.7, 175.9, 138.9, 138.8, 138.7, 138.2, 136.6, 128.4, 128.3, 128.2, 128.1, 128.0, 127.95, 127.7, 127.6, 127.5, 127.4, 100.4, 99.4, 98.2, 78.9, 76.1, 75.2, 74.9, 73.9, 73.8, 73.5, 73.2, 73.1 72.1, 71.7 71.4, 70.7, 70.5, 70.3, 68.1, 67.5, 62.0, 61.97, 39.0, 38.9, 38.8, 38.7, 38.6, 30.9, 27.3, 27.3, 27.3, 27.2, 27.1. HRMS calcd for $C_{83}H_{110}O_{22}Na$ ($[M + Na]^+$) 1481.7381, found 1481.7327.

Acetyl 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-β-D-glucopyranoside (9). Palladium hydroxide (200 mg) was added to a solution of trisaccharide **8** (1.35 g, 0.93 mmol) in MeOH (15 mL), and the reaction mixture was stirred under hydrogen balloon pressure for 12 h. The palladium hydroxide was then filtered through a Celite pad and concentrated in vacuo. The crude residue was dissolved in anhydrous pyridine (15 mL). Acetic anhydride (0.7 mL) and DMAP (40 mg) were added, and the solution was stirred for 10 h. The solvent was removed in vacuo, and the residue was dissolved in EtOAc (40 mL). The organic layer was washed with 1 N HCl (30 mL), saturated aqueous $NaHCO_3$ (30 mL), and brine (30 mL) successively, dried over Na_2SO_4, concentrated in vacuo, and purified by silica gel flash chromatography (1:2 EtOAc−hexane) to afford compound **9** (1.1 g, 97%) as a white foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 5.68 (d, J = 8.4 Hz, 1H), 5.42−5.38 (m, 2H), 5.25−5.21 (m, 3H), 5.13 (m, 1H), 5.00 (m, 1H), 4.93 (t, J = 8.4 Hz, 1H), 4.46−4.40 (m, 2H), 4.31 (dd, J = 12.0, 4.0 Hz, 1H), 4.12−3.99 (m, 6H), 3.91 (m, 1H), 3.81 (dd, J = 6.4 Hz, 1H), 3.77 (m, 1H), 3.68 (m, 1H), 2.09 (s, 3H), 2.06 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.90 (s, 3H), 1.23−1.14 (m, 45H), 1.10 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 177.8, 177.6, 176.8, 176.7, 176.0, 170.3, 170.1, 170.0, 169.69, 168.8, 100.2, 94.3, 91.7, 73.0, 72.1, 71.2, 70.4, 69.8, 67.5, 67.47, 67.1, 66.9, 65.3, 61.7, 61.0, 60.4, 39.0, 38.97, 38.9, 38.8, 38.7, 38.68, 27.3, 27.31, 27.2, 27.1, 27.0, 26.9, 21.0, 20.6, 20.58, 20.5. HRMS calcd for $C_{58}H_{90}O_{27}Na$ ($[M + Na]^+$) 1241.5562, found 1241.5540.

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-α-D-glucopyranosyl-(1→1)-trichloroacetimide (10). Benzylamine (0.2 mL, 1.8 mmol) was added to a solution of compound **9** (1.1 g, 0.90 mmol) in THF (20 mL). After being stirred overnight at room temperature, the reaction mixture was concentrated in vacuo. The residue was diluted with CH_2Cl_2 (20 mL) and then was washed with 1 N HCl (10 mL), saturated aqueous $NaHCO_3$ (10 mL), and brine (10 mL), dried over Na_2SO_4, and concentrated. The crude product was used for next step reaction without further purification.

The above crude residue was dissolved in dry CH_2Cl_2 (10 mL). Then CCl_3CN (0.9 mL, 9.0 mmol) and DBU (67 μL, 0.45 mmol) were added successively. The reaction mixture was stirred for 4 h. The solvent was removed in vacuo and the residue was purified by silica gel flash chromatography (1:2 EtOAc−hexane) to afford compound **10** (0.93 g, 78%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 8.62 (s, 1H), 6.45, (d, J = 3.5 Hz, 1H), 5.56 (t, J = 10.0 Hz, 1H), 5.39 (dd, J = 8.0, 2.5 Hz, 1H), 5.25−5.22 (m, 2H), 5.15 (dd, J = 10.0, 8.0 Hz, 1H), 5.01 (m, 1H), 4.94 (dd, J = 10.5, 4.0 Hz, 1H), 4.45 (m, 2H), 4.35 (dd, J = 10.0, 4.5 Hz, 1H), 4.14−4.03 (m, 7H), 3.96 (t, J = 9.5 Hz, 1H), 3.83 (t, J = 6.5 Hz, 1H), 3.79 (dd, J = 10.5, 3.0 Hz, 1H), 2.10 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 1.91 (s, 3H), 1.22 (s, 9H), 1.20 (s, 9H), 1.90 (s, 27H), 1.11 (s, 9H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.7, 177.63, 177.6, 177.5, 176.8, 176.6, 176.4, 176.0, 171.1, 170.3, 170.1, 170.0, 169.7, 160.9, 160.6, 100.3, 94.2, 92.6, 90.8, 74.1, 72.9, 72.0, 71.5, 70.1, 69.8, 68.7, 67.5, 67.0, 66.8, 65.3, 61.5, 61.46, 61.4, 61.0, 60.4, 39.0, 38.97, 38.9, 38.8, 38.75, 38.7, 27.3, 27.2, 27.16, 27.14, 27.1, 27.0, 21.0, 20.6, 20.4, 14.2. HRMS ($[M + Na]^+$) 1342.451 73, calcd. 1342.455 24.

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-β-D-glucopyranosyl(1→1)-(2*S*,3*R*,4*E*)-2-azido-3-*O*-(4-methoxybenzyl)-4-octadecen-1,3-diol. (12). A suspension of trisaccharide donor **10** (450 mg, 0.34 mmol), sphingosine acceptor **11** (193 mg, 0.41 mmol), and 4 Å molecular sieves (2 g) in dry CH_2Cl_2 (10 mL) was stirred at room temperature for 30 min. After cooling to −20 °C, TMSOTf (14 μL, 0.068 mmol) was added. The resulting mixture was stirred for 2 h and then diluted with CH_2Cl_2 (10 mL). Saturated aqueous $NaHCO_3$ (10 mL) was added to quench the reaction. The molecular sieves were filtered through a Celite pad. The filtrate was washed with brine (5 mL), dried over Na_2SO_4, and concentrated. The residue was purified by silica gel flash chromatography (1:3 EtOAc−hexane) to provide **12** (485 mg, 87%) as a clear oil.

^{1}H NMR (500 MHz, $CDCl_3$): δ 7.21 (d, J = 8.5 Hz, 1H), 6.87 (d, J = 8.5 Hz, 1H), 5.72 (dt, J = 15.5, 6.5 Hz, 1H), 5.42 (dd, J = 9.0, 2.0 Hz, 1H), 5.37 (dd, J = 15.5, 8.5 Hz, 1H), 5.28−5.25 (m, 3H), 5.22 (t, J = 9.8 Hz, 1H), 5.16 (dd, J = 10.0, 8.0 Hz, 1H), 5.05 (m, 1H), 4.87 (dd, J = 9.8, 7.5 Hz, 1H), 4.53 (d, J = 7.5 Hz, 1H), 4.52 (m, 1H), 4.50 (d, J = 11.5 Hz, 1H), 4.45 (d, J = 8.0 Hz, 1H), 4.30 (dd, J = 12.0, 5.5 Hz, 1H), 4.26 (d, J = 11.5 Hz, 1H), 4.16 (m, 1H), 4.11−4.05 (m, 4H), 3.91 (t, J = 9.5 Hz, 1H), 3.87−3.79 (m, 4H), 3.82 (s, 3H), 3.63 (dd, J = 10.5, 4.0 Hz, 1H), 3.59 (m, 1H), 3.53 (m, 1H), 2.17 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H), 1.94 (s, 3H), 1.38 (m, 2H), 1.32−1.26 (m, 22H), 1.25 (s, 9H), 1.24 (s, 9H), 1.23 (s, 9H), 1.22 (s, 9H), 1.20 (s, 9H), 1.16 (s, 9H), 0.89 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.7, 177.0, 176.8, 176.6, 176.0, 170.3, 170.1, 170.0, 169.7, 159.2, 137.9, 130.2, 129.2, 126.0, 113. 8, 100.7, 100.3, 94.4, 79.3, 76. 8, 74.5, 73.6, 73.5, 72.0, 71.6, 71.4, 70.0, 69.9, 68.6, 67.54, 67.5, 67.1, 66.9, 65.3, 64.2, 61.9, 61.6, 61.0, 55.2, 39.0, 38.95, 38.9, 38.7, 38.72, 38.67, 32.4, 31.9, 29.7, 29.65, 29.6, 29.5, 29.3, 29.2, 29.0, 28.0, 27.3, 27.2, 27.1, 27.09, 22.7, 21.0, 20.6, 20.5, 14.1. HRMS calcd for $C_{82}H_{129}N_3O_{28}Na$ ([M + Na]$^+$) 1626.8655, found 1626.8696.

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-*β*-D-glucopyranosyl-(1→1)-(2*S*,3*R*,4*E*)-2-hexacosanoylamino-3-*O*-(4-methoxybenzyl)-4-octadecen-1,3-diol (13). Triphenylphosphine (154 mg, 0.59 mmol) was added to a solution of azide **12** (480 mg, 0.29 mmol) in benzene (20 mL) and water (0.2 mL). The reaction mixture was stirred at 50 °C for 8 h. The solvent was evaporated under reduced pressure and azetroped with benzene (2 × 20 mL). Then it was dissolved in dry THF (10 mL) and treated with cerotic acid (167 mg, 0.42 mmol) and EDCI (81 mg, 0.42 mmol). After the mixture was stirred for 10 h at room temperature, the solvent was evaporated and the residue was partitioned between CH_2Cl_2 (20 mL) and water (10 mL). The organic layer was separated and dried over Na_2SO_4. After the layer was concentrated, the residue was purified by silica gel flash chromatography (1:3 EtOAc−hexane) to provide **13** (540 mg, 95%) as a foam. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.17 (d, J = 8.5 Hz, 1H), 6.82 (d, J = 8.5 Hz, 1H), 5.61 (dt, J = 15.0, 8.0 Hz, 1H), 5.50 (d, J = 8.5 Hz, 1H), 5.39 (dd, J = 6.0, 2.5 Hz, 2H), 5.30 (dd, J = 15.5, 8.5 Hz, 1H), 5.24−5.17 (m, 3H), 5.13 (dd, J = 10.0, 8.0 Hz, 1H), 5.02 (dd, J = 11.0, 3.5 Hz, 1H), 4.81 (dd, J = 9.5, 8.0 Hz, 1H), 4.46 (d, J = 9.0 Hz, 1H), 4.43 (d, J = 4.0 Hz, 1H), 4.41 (d, J = 8.0 Hz, 1H), 4.31 (dd, J = 12.5, 5.0 Hz, 1H), 4.18 (d, J = 11.0 Hz, 1H), 4.14−4.01 (m, 7H), 3.88 (t, J = 9.5 Hz, 1H), 3.82 (t, J = 6.5 Hz, 1H), 3.78 (s, 3H), 3.77−3.73 (m, 2H), 3.54 (m, 1H), 3.51 (dd, J = 9.0, 3.0 Hz, 1H), 2.08 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.95 (s, 3H), 1.62−1.48 (m, 4H), 1.30−1.21 (m, 68H), 1.22 (s, 9H), 1.21 (s, 9H), 1.19 (s, 9H), 1.17 (s, 9H), 1.16 (s, 9H), 1.14 (s, 9H), 0.86 (t, J = 6.5 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.6, 176.9, 176.8, 176.76, 176.0, 172.4, 170.3, 170.1, 170.0, 169.7, 159.1, 136.8, 130.6, 129.1, 127.5, 113.7, 100.8, 100.1, 94.4, 79.4, 74.4, 73.5, 73.4, 72.0, 71.3, 70.1, 69.9, 68.1, 67.5, 67.5, 67.1, 66.9, 65.3, 61.8, 61.5, 61.0, 55.2, 51.6, 39.0, 39.0, 38.9, 38.8, 38.7, 38.66, 36. 9, 32.3, 31.9, 29.7, 29.7, 29.65, 29.5, 29.4, 29.39, 29.35, 29.3, 29.28, 27.8, 27.3, 27.2, 27.21, 27.13, 27.11, 27.10, 25.7, 22.68, 21.0, 20.6, 20.5, 14.1. HRMS calcd for $C_{108}H_{181}NO_{29}Na$ ([M + Na]$^+$) 1980.2645, found 1980.2728.

α-D-Galactopyranosyl-(1→3)-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→1)-(2*S*,3*R*,4*E*)-2-hexacosanoylamino-4-octadecen-1,3-diol (1). A suspension of protected glycolipid **13** (42 mg, 0.021 mmol) and 10% Pd/C (10 mg) in ethanol (2 mL) was stirred under H_2 atmosphere (1 atm) for 2 h. The suspension was filtered through a Celite pad, and the filtrate was concentrated. The residue was dissolved in dry MeOH (2 mL), and freshly prepared NaOMe (5 mg, 0.09 mmol) was added. The resulting mixture was heated to reflux for 24 h. After the mixture was cooled to room temperature, the precipitate was collected by centrifuge. The precipitate was washed with MeOH (2 × 2 mL) and dissolved in pyridine. The insoluble impurities were removed by centrifuge and the clear solution was concentrated to give **1** (20 mg, 82%) as a white powder. ^{1}H NMR (500 MHz, pyridine-d_5): δ 8.39 (d, J = 7.9 Hz, 1H), 5.67 (d, J = 3.5 Hz, 1H), 5.07 (d, J = 4.9 Hz, 1H), 5.03 (t, J = 6.1 Hz, 1H), 4.89 (d, J = 7.8 Hz, 1H), 4.77 (dd, J = 10.3, 4.6 Hz, 1H), 4.73 (dd, J = 9.9, 3.7 Hz, 1H), 4.70 (m, 1H), 4.68 (d, J = 2.7 Hz, 1H), 4.59 (d, J = 2.7 Hz, 1H), 4.56−4.52 (m, 3H), 4.48−4.43 (m, 4H), 4.33 (dd, J = 11.0, 5.1 Hz, 1H), 4.27−4.21 (m, 4H), 4.18 (dd, J = 10.5, 3.3 Hz, 1H), 4.06−4.03 (m, 2H), 3.88 (m, 1H), 2.48 (t, J = 7.4 Hz, 2H), 1.93−1.83 (m, 5H), 1.58 (m, 1H), 1.42−1.21 (m, 68H), 0.90 (t, J = 6.6 Hz, 3H), 0.89 (t, J = 7.1 Hz, 3H). ^{13}C NMR (125 MHz, pyridine-d_5): δ 173.1, 105.3, 105.2, 97.5, 81.9, 79.9, 76.5, 76.3, 74.5, 72.6, 71.4, 71.1, 70.6, 70.3, 70.2, 65.8, 62.0, 61.8, 61.6, 54.7, 36.7, 34.7, 31.9, 31.89, 30.0, 29.9, 29.8, 29.77, 29.7, 29.68, 29.6, 29.5, 29.4, 29.37, 26.3, 26.2, 22.7, 14.0. HRMS calcd for $C_{62}H_{119}NO_{18}Na$ ([M + Na]$^+$) 1188.8325, found 1188.8307.

(2*S*,3*S*,4*S*)-2-Azidooctadecan-1,3,4-triol (15). To a mixture solution of CH_2Cl_2 (5 mL) and H_2O (5 mL) containing NaN_3 (2.05 g, 31.5 mmol) cooled at 0 °C was added dropwise Tf_2O (1.1 mL, 6.3 mmol) in 20 min. After addition, the resulting mixture was stirred for 3 h. The organic layer was separated, and the aqueous portion was extracted with CH_2Cl_2 (2 × 2 mL). The combined organic layer was washed with saturated aqueous Na_2CO_3.

To a suspension of phytosphingosine **14** (1.0 g, 3.1 mmol), K_2CO_3 (2.18 g, 15.8 mmol), and Cu_2SO_4 (20 mg) in a mixture of MeOH (4 mL) and H_2O (3 mL) was added the above organic layer, which contained TfN_3. More MeOH was added to make the mixture a homogeneous solution. The reaction mixture was stirred overnight at room temperature. The organic solvent was removed in vacuo. The aqueous portion was extracted with ethyl acetate. After being dried over anhydrous Na_2SO_4, it was purified by silica gel flash chromatography (1:1 EtOAc−hexane) to give 0.96 g of product **15** in 89% yield. ^{1}H NMR (400 MHz, $CDCl_3$): δ 4.02 (dd, J = 11.6, 5.2 Hz, 1H), 3.92 (dd, J = 11.6, 4.4 Hz, 1H), 3.82 (m, 1H), 3.78 (m, 1H), 3.69 (m, 1H), 1.64−1.54 (m, 3H), 1.46−1.28 (m, 23H), 0.91 (t, J = 6.8 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 74.8, 72.6, 63.4, 62.0, 32.0, 31.9, 29.6, 29.5, 29.3, 25.7, 22.6, 14.0.

(2*S*,3*S*,4*S*)-2-Azido-3,4-di-*O*-benzyloctadecan-1,3,4-triol (16). A solution of azidosphingosine **15** (0.40 g, 1.2 mmol), trityl chloride (1.3 g, 4.7 mmol), and a catalytic amount of DMAP in dry pyridine (5 mL) was stirred at 50 °C for 10 h. The reaction mixture was diluted with CH_2Cl_2 and washed with cooled 1 N HCl, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over anhydrous Na_2SO_4 and concentrated. The residue was then dissolved in dry DMF (6 mL) and then treated with 60% NaH (106 mg, 2.6 mmol) for 10 min. BnCl (0.30 mL, 2.6 mmol) was added by syringe, and the mixture was stirred overnight. After the solvent was removed in vacuo, the residue was dissolved in water and extracted with ethyl ether. The organic extraction was concentrated and then dissolved in a mixture of CH_2Cl_2 and MeOH (2:1, 9 mL). *p*TsOH·H_2O (95 mg, 0.5 mmol) was added, and the mixture was stirred for 1 h at room temperature. After the mixture was concentrated, the residue was purified by silica gel flash chromatography (1:6 EtOAc−hexane) to give 0.43 g of acceptor **16** in 68% yield for three steps. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37−7.28 (m, 10H), 4.73 (d, J = 11.3 Hz, 1H), 4.69 (d, J = 11.3 Hz, 1H), 4.65 (d, J = 11.4 Hz, 1H), 4.59 (d, J = 11.4 Hz, 1H), 3.92 (dd, J = 11.7, 5.1 Hz, 1H), 3.82 (dd, J = 11.7, 5.1 Hz, 1H), 3.73 (m, 1H), 3.70 (m, 1H), 3.66 (m, 1H), 1.71 (m, 1H), 1.59 (m, 1H), 1.45 (m, 1H), 1.37−1.27 (m, 23H), 0.91 (t, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 138.0, 137.7, 128.6, 128.5, 128.1, 128.03, 128.0, 127.9, 80.5, 79.0, 73.7, 72.6, 63.1, 62.3, 32.0, 30.3, 29.7, 29.68, 29.6, 29.59, 29.4, 25.5, 22.7, 14.1.

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-*β*-D-glucopyranosyl-(1→1)-(2*S*,3*S*,4*S*)-2-azido-3,4-di-*O*-benzyloctadecan-1,3,4-triol (17). A suspension of trisaccharide donor **10** (82 mg, 0.06 mmol), phytosphingosine acceptor **16** (37 mg, 0.071 mmol), and 4 Å molecular sieves (0.5 g) in dry CH_2Cl_2 (2 mL) was stirred at room temperature for 30 min. After the mixture was cooled to −20 °C, TMSOTf (3 μL, 0.012 mmol) was added. The resulting mixture was stirred for 2 h and then diluted with CH_2Cl_2 (10 mL). Saturated aqueous $NaHCO_3$ (10 mL) was added to quench

the reaction. The molecular sieves were filtered through a Celite pad. The organic layer was separated, washed with brine (5 mL), dried over Na_2SO_4, and concentrated. The residue was purified by silica gel flash chromatography (1:3 EtOAc−hexane) to provide **17** (83 mg, 81%) as a clear oil. ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.37−7.30 (m, 10H), 5.43 (dd, J = 5.5, 2.0 Hz, 2H), 5.32−5.16 (m, 4H), 5.06 (dd, J = 10.0, 3.0 Hz, 1H), 4.89 (dd, J = 9.5, 8.0 Hz, 1H), 4.67 (d, J = 11.5 Hz, 1H), 4.62 (d, J = 11.5 Hz, 1H), 4.58 (d, J = 11.5 Hz, 1H), 4.52 (d, J = 11.5 Hz, 1H), 4.51 (d, J = 10.0 Hz, 1H), 4.45 (dd, J = 7.5, 1.5 Hz, 2H), 4.30 (dd, J = 12.0, 5.0 Hz, 1H), 4.17 (t, J = 6.5 Hz, 1H), 4.14−4.07 (m, 4H), 4.03 (dd, J = 15.5, 8.0 Hz, 1H), 3.92 (t, J = 9.5 Hz, 1H), 3.86 (t, J = 6.8 Hz, 1H), 3.81 (dt, J = 10.0, 3.0 Hz, 1H), 3.72 (m, 1H), 3.60 (m, 2H), 3.54 (m, 4H), 2.14 (s, 3H), 2.10 (s, 3H), 2.06 (s, 3H), 1.95 (s, 3H), 1.61 (m, 2H), 1.32−1.18 (m, 24H), 1.24 (s, 9H), 1.23 (s, 9H), 1.22 (s, 9H), 1.21 (s, 9H), 1.21 (s, 9H), 1.17 (s, 9H), 0.90 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.7, 177.1, 176.9, 176.6, 176.1, 170.3, 170.2, 170.1, 169.8, 163.5, 138.4, 138.0, 128.4, 128.0, 127.8, 127.78, 127.7, 100.8, 100.2, 94.5, 91.9, 79.3, 79.2, 74.5, 73.8, 73.5, 73.4, 72.0, 71.97, 71.6, 71.4, 70.0, 69.5, 67.54, 67.5, 67.1, 66.9, 65.4, 61.9, 61.5, 61.0, 39.1, 39.0, 38.9, 38.73, 38.72, 38.7, 31.9, 30.0, 29.73, 29.7, 29.66, 29.6, 29.57, 29.4, 27.4, 27.23, 27.2, 27.12, 27.11, 27.1, 25.4, 22.7, 21.0, 20.6, 20.5, 14.1. HRMS calcd for $C_{88}H_{135}N_3O_{28}Na$ ([M + Na]$^+$) 1704.9124, found 1704.9106.

2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl-(1→3)-2,4,6-tri-*O*-pivaloyl-*β*-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-pivaloyl-*β*-D-glucopyranosyl-(1→1)-(2*S*,3*S*,4*S*)-2-hexacosanoylamino-3,4-di-*O*-benzyl-octadecan-1,3,4-triol (18). Triphenylphosphine (25 mg, 0.049 mmol) was added to a solution of azide **17** (83 mg, 0.049 mmol) in benzene (5 mL) and water (0.1 mL). The reaction mixture was stirred at 50 °C for 10 h. The solvent was evaporated under reduced pressure and azetroped with benzene (2 × 10 mL). Then it was dissolved in dry THF (2 mL) and treated with cerotic acid (31 mg, 0.078 mmol) and EDCI (15 mg, 0.078 mmol). After the mixture was stirred for 10 h at room temperature, the solvent was evaporated and the residue was partitioned between CH_2Cl_2 (10 mL) and water (10 mL). The organic layer was separated and dried over Na_2SO_4. After the layer was concentrated, the residue was purified by silica gel flash chromatography (1:3 EtOAc−hexane) to provide **18** (84 mg, 84%) as a foam. ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.36−7.27 (m, 10H), 5.67 (d, J = 8.2 Hz, 1H), 5.43 (br, 2H), 5.29−5.21 (m, 3H), 5.16 (dd, J = 10.0, 8.0 Hz, 1H), 5.06 (dd, J = 10.6, 2.9 Hz, 1H), 4.87 (dd, J = 9.3, 7.9 Hz, 1H), 4.80 (d, J = 11.3 Hz, 1H), 4.62 (d, J = 11.7 Hz, 1H), 4.57 (d, J = 11.2 Hz, 1H), 4.51 (d, J = 11.7 Hz, 1H), 4.45 (d, J = 9.8, 7.9 Hz, 2H), 4.39−4.30 (m, 2H), 4.16−4.04 (m, 8H), 3.90 (t, J = 9.5 Hz, 1H), 3.86−3.78 (m, 3H), 3.63 (m, 1H), 3.54 (m, 1H), 3.46 (m, 1H), 2.14 (s, 3H), 2.09 (s, 3H), 2.05 (s, 3H), 1.95 (s, 3H), 1.64 (m, 2H), 1.53 (m, 2H), 1.31−1.23 (m, 70H), 1.26 (s, 9H), 1.25 (s, 9H), 1.22 (s, 9H), 1.21 (s, 9H), 1.18 (s, 9H), 1.16 (s, 9H), 0.90 (t, J = 6.4 Hz, 6H). ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.8, 177.6, 176.9, 176.85, 176.8, 176.0, 172.4, 170.3, 170.2, 170.0, 169.7, 138.8, 138.6, 128.4, 128.3, 127.8, 127.7, 127.6, 127.5, 101.1, 100.1, 94.4, 80.0, 78.5, 74.4, 73.7, 73.5, 73.3, 72.0, 71.6, 71.3, 69.9, 68.9, 67.5, 67.49, 67.1, 66.9, 65.8, 65.3, 61.8, 61.5, 61.0, 49.8, 39.1, 39.0, 38.83, 38.8, 38.7, 38.68, 36.7, 31.9, 29.8, 29.7, 29.65, 29.6, 29.4, 29.37, 29.36, 29.3, 29.1, 27.3, 27.2, 27.18, 27.1, 27.09, 26.1, 25.6, 22.7, 21.0, 20.6, 20.5, 14.1. HRMS calcd for $C_{114}H_{185}NO_{29}$-Na ([M + Na]$^+$) 2058.3115, found 2058.3051.

α-D-Galactopyranosyl-(1→3)-*β*-D-galactopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→1)-(2*S*,3*S*,4*S*)-2-hexacosanoylamino-octadecan-1,3,4-triol (2). A suspension of glycoceramide **18** (34 mg, 0.017 mmol) and 10% Pd/C in MeOH (3 mL) was shaken for 4 h under H_2 atmosphere (40 psi). The catalytic Pd/C was filtered off through a Celite pad, and the filtrate was concentrated. Then it was dissolved in dry MeOH (5 mL), and freshly prepared NaOMe (4 mg, 0.07 mmol) was added. The resulting mixture was heated to reflux for 24 h. After the mixture was cooled to room temperature, the precipitate was collected by centrifuge. The precipitate was washed with MeOH (2 × 2 mL) and dissolved in pyridine. The insoluble impurities were removed by centrifuge and the clear solution was concentrated to give **2** (15 mg, 76%) as a white powder. ^{1}H NMR (500 MHz, pyridine-d_5): δ 8.51 (d, J = 8.8 Hz, 1H), 7.72 (br, 1H), 7.38 (d, J = 5.1 Hz, 1H), 7.07 (br, 1H), 6.71 (br, 1H), 6.64 (br, 1H), 6.50 (br, 2H), 6.43 (br, 2H), 6.12 (br, 1H), 5.86 (d, J = 7.1 Hz, 1H), 5.81 (br, 1H), 5.65 (br, 1H), 5.12 (m, 1H), 5.03 (d, J = 7.7 Hz, 1H), 5.00 (m, 1H), 4.87 (d, J = 7.8 Hz, 1H), 4.76 (dd, J = 10.5, 5.4 Hz, 1H), 4.73 (m, 1H), 4.66 (m, 1H), 4.58−4.49 (m, 4H), 4.47−4.40 (m, 4H), 4.37−4.34 (m, 2H), 4.31 (m, 1H), 4.26−4.18 (m, 4H), 4.01 (m, 2H), 3.81 (m, 1H), 2.42 (t, J = 7.3 Hz, 2H), 2.21 (m, 1H), 1.93 (m, 2H), 1.81 (m, 2H), 1.67 (m, 1H), 1.43−1.19 (m, 66H), 0.86 (t, J = 6.7 Hz, 3H), 0.85 (t, J = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, pyridine-d_5): δ 173.2, 105.2, 105.0, 97.5, 81.9, 90.0, 76.4, 76.3, 76.26, 75.6, 74.5, 72.6, 72.5, 71.4, 70.6, 70.5, 70.3, 70.2, 65.8, 62.0, 61.8, 61.6, 51.9, 36.7, 33.3, 31.9, 31.89, 30.1, 29.9, 29.8, 29.77, 29.7, 29.68, 29.6, 29.5, 29.4, 29.37, 26.4, 26.1, 22.7, 14.0. HRMS calcd for $C_{62}H_{119}NO_{19}Na$ ([M + Na]$^+$) 1204.8274, found 1204.8356.

Cell Stimulation and Cytokines Measurements. Methanol was used to solubilize iGb3 and its analogues. The stimulation experiments were performed at a final concentration of 1% methanol, a concentration that is not toxic to cells. Glycoceramides at indicated concentrations were incubated with hCD1d-transfected human B lymphoblastoid (C1R). After 2 h, CD1d-restricted human Vα*24i* NKT cell clones (JS7) were added, and they were cocultured for 36 h. IL-4 and IFN-γ were quantified in cell culture supernatants by Sandwich ELISA. First, capture antibody against the cytokine was coated to the ELISA plate. After the antigen binding, a biotinylated second antibody was used. Streptavidin−HRP conjugate was applied to finally quantitate the antigen amount.

Acknowledgment. This project was supported by The Ohio State University (support to Peng George Wang) and by Swiss National Foundation (Grant 3100A0-109918 to Gennaro De Libero).

Supporting Information Available: Proton and carbon NMR spectra of compounds **3**−**8**, **10**−**12**, **14**−**18**; elemental analysis results. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) (a) Kronenberg, M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* **2005**, *26*, 877−900. (b) Van Kaer, L. α-Galactosylceramide therapy for autoimmune diseases: prospects and obstacles. *Nat. Rev. Immunol.* **2004**, *5*, 31−42.

(2) (a) Natori, T.; Koezuka, Y.; Higa, T. Agelasphins, novel α-galactosylceramides from the marine sponge *Agelas mauritianus*. *Tetrahedron Lett.* **1993**, *34*, 5591−5592. (b) Kawano, T.; Cui, J.; Koezuka, Y.; Toura, I.; Kaneko, Y.; Motoki, K.; Ueno, H.; Nakagawa, R.; Sato, H.; Kondo, E.; Koseki, H.; Taniguchi, M. CD1d-restricted and TCR-mediated activation of Vα14i NKT cells by glycosylceramides. *Science* **1997**, *278*, 1626−1629. (c) Van Der Vliet, H. J. J.; Nishi, N.; Koezuka, Y.; Peyrat, M. A.; Von Blomberg, B. M. E.; Van Den Eertwegh, A. J. M.; Pinedo, H. M.; Giaccone, G.; Scheper, R. J. Effects of α-galactosylceramide (KRN7000), interleukin-12 and interleukin-7 on phenotype and cytokine profile of human Va24$^+$ Vβ11$^+$ T cells. *Immunology* **1999**, *98*, 557−563.

(3) Zhou, D.; Mattner, J.; Cantu, C., 3rd; Schrantz, N.; Yin, N.; Gao, Y.; Sagiv, Y.; Hudspeth, K.; Wu, Y.-P.; Yamashita, T.; Teneberg, S.; Wang, D.; Proia, R. L.; Levery, S. B.; Savage, P. B.; Teyton, L.; Bendelac, A. Lysosomal glycosphingolipid recognition by NKT cells. *Science* **2004**, *306*, 1786−1789.

(4) (a) Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Koezuka, Y.; Kobayashi, E.; Fukushima, H. Structure−activity relationship of α-galactosylceramides against B16-bearing mice. *J. Med. Chem.* **1995**, *38*, 2176−2187. (b) Morita, M.; Natori, T.; Akimoto, K.; Osawa, T.; Fukushima, H.; Koezuka, Y. Syntheses of α-, *β*-monoglycosylceramides and four diastereomers of an α-galactosylceramide. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 699−704. (c) Kobayashi, E.; Motoki, K.; Uchida, T.; Fukushima, H.; Koezuka, Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol. Res.* **1995**, *7*, 529−534. (d) Wu, D.; Xing, G.-W.;

Poles, M. A.; Horowitz, A.; Kinjo, Y.; Sullivan, B.; Bodmer-Narkevitch, V.; Plettenburg, O.; Kronenberg, M.; Tsuji, M.; Ho, D. D.; Wong, C.-H. Bacterial glycolipids and analogs as antigens for CD1d-restricted NKT cells. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1351−1356.

(5) Miyamoto, K.; Miyake, S.; Yamamura, T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature* **2001**, *413*, 531−534.

(6) Yu, K. O. A.; Im, J. S.; Molano, A.; Dutronc, Y.; Illarionov, P. A.; Forestier, C.; Fujiwara, N.; Arias, I.; Miyake, S.; Yamamura, T.; Chang, Y.-T.; Besra, G. S.; Porcelli, S. A. Modulation of CD1d-restricted NKT cell responses by using *N*-acyl variants of α-galactosylceramides. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 3383−3388.

(7) (a) Koch, M.; Stronge, V. S.; Shepherd, D.; Gadola, S. D.; Mathew, B.; Ritter, G.; Fersht, A. R.; Besra, G. S.; Schmidt, R. R.; Jones, E. Y.; Cerundolo, V. The crystal structure of human CD1d with and without a-galactosylceramide. *Nat. Immunol.* **2005**, *6*, 819−826. (b) Zajonc, D. M.; Cantu, C.; Mattner, J.; Zhou, D.; Savage, P. B.; Bendelac, A.; Wilson, I. A.; Teyton, L. Structure and function of a potent agonist for the semi-invariant natural killer T cell receptor. *Nat. Immunol.* **2005**, *6*, 810−818. (c) Wu, D.; Zajonc, D. M.; Fujio, M.; Sullivan, B. A.; Kinjo, Y.; Kronenberg, M.; Wilson, I. A.; Wong, C.-H. Design of natural killer T cell activators: structure and function of a microbial glycosphingolipid bound to mouse CD1d. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 3972−3977.

(8) Gadola, S. D.; Zaccai, N. R.; Harlos, K.; Shepherd, D.; Castro-Palomino, J. C.; Ritter, G.; Schmidt, R. R.; Jones, E. Y.; Cerundolo, V. Structure of human CD1b with bound ligands at 2.3 Å, a maze for alkyl chains. *Nat. Immunol.* **2002**, *3*, 721−726.

(9) Batuwangala, T.; Shepherd, D.; Gadola, S. D.; Gibson, K. J. C.; Zaccai, N. R.; Fersht, A. R.; Besra, G. S.; Cerundolo, V.; Jones, E. Y. The crystal structure of human CD1b with a bound bacterial glycolipid. *J. Immunol.* **2004**, *172*, 2382−2388.

(10) Xia, C.; Zhou, D.; Liu, C.; Lou, Y.; Yao, Q.; Zhang, W.; Wang, P. G. Thio-isoglobotrihexosylceramide, an agonist for activating invariant natural killer T cells. *Org. Lett.* **2006**, *8*, 5493−5496.

(11) (a) Xia, C.; Yao, Q.; Schuemann, J.; Rossy, E.; Chen, W.; Zhu, L.; Zhang, W.; De Libero, G.; Wang, P. G. Synthesis and biological evaluation of α-galactosylceramide (KRN7000) and isoglobotrihexosylceramide (iGb3). *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2195−2199. (b) Zhang, W.; Wang, J.; Li, J.; Yu, L.; Wang, P. G. Large scale synthesis of a derivative of an α-galactosyl trisaccharide epitope involved in the hyperacute rejection of xenotransplantation. *J. Carbohydr. Chem.* **1999**, *18*, 1009−1017.

(12) Nicolaou, K. C.; Caulfield, T. J.; Katoaka, H. Total synthesis of globotriaosylceramide (Gb3) and lysoglobotriaosylceramide (lysoGb3). *Carbohydr. Res.* **1990**, *202*, 177−191.

(13) Alper, P. B.; Hung, S.-C.; Wong, C.-H. Meta catalyzed diazo transfer for synthesis of azides from amines. *Tetrahedron Lett.* **1996**, *37*, 6029−6032.

(14) (a) Chiu, H.-Y.; Tzou, D.-L. M.; Patkar, L. N.; Lin, C.-C. A facile synthesis of phytosphingosine from diisopropylidene-D-mannofuranose. *J. Org. Chem.* **2003**, *68*, 5788−5791. (b) van den Berg, R. J. B. H. N.; Boltje, T. J.; Verhagen, C. P.; Litjens, R. E. J. N.; Van der Marel, G. A.; Overkleeft, H. S. An efficient synthesis of the natural tetrahydrofuran pachastrissamine starting from D-ribo-phytosphingosine. *J. Org. Chem.* **2006**, *71*, 836−839. (c) Murata, K.; Toba, T.; Nakanishi, K.; Takahashi, B.; Yamamura, T.; Miyake, S.; Annoura, H. Total synthesis of an immunosuppressive glycolipid, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactosyl)-2-tetracosanoylamino-1,3,4-nonanetriol. *J. Org. Chem.* **2005**, *70*, 2398−2401.

(15) Fan, G.-T.; Pan, Y.-s.; Lu, K.-C.; Cheng, Y.-P.; Lin, W.-C.; Lin, S.; Lin, C.-H.; Wong, C.-H.; Fang, J.-M.; Lin, C.-C. Synthesis of α-galactosylceramide and the related glycolipids for evaluation of their activities on mouse splenocytes. *Tetrahedron* **2005**, *61*, 1855−1862.

(16) Sidobre, S.; Hammond, K. J. L.; Benazet-Sidobre, L.; Maltsev, S. D.; Richardson, S. K.; Ndonye, R. M.; Howell, A. R.; Sakai, T.; Besra, G. S.; Porcelli, S. A.; Kronenberg, M. The T cell antigen receptor expressed by Vα14i NKT cells has a unique mode of glycosphingolipid antigen recognition. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12254−12259.

(17) Zhou, D. The immunological function of iGb3. *Curr. Protein Pept. Sci.* **2006**, *7*, 325−333.

JM0701066