ACS Medicinal Chemistry Letters

LETTER

pubs.acs.org/acsmedchemlett

The 3-Deoxy Analogue of α-GalCer: Disclosing the Role of the 4-Hydroxyl Group for CD1d-Mediated NKT Cell Activation

Dong Jae Baek,[†] Jeong-Hwan Seo,[†] Chaemin Lim,[†] Jae Hyun Kim,[†] Doo Hyun Chung,[‡] Won-Jea Cho,[§] Chang-Yuil Kang,[†] and Sanghee Kim*[,†]

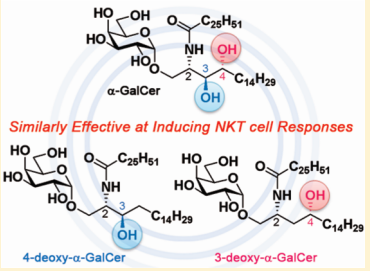
[†]College of Pharmacy, Seoul National University, San 56-1, Shilim, Kwanak, Seoul 151-742, Korea

[‡]College of Medicine, Seoul National University, 28 Yongon, Chongno, Seoul 110-799, Korea

[§]College of Pharmacy, Chonnam National University, Yongbong, Buk, Kwangju 500-757, Korea

Supporting Information

ABSTRACT: KRN7000, or α-GalCer, is a potent agonist for natural killer T (NKT) cells. The 3-hydroxyl group of its phytosphingosine moiety is important for activating NKT cells, whereas its 4-hydroxyl group is perceived to be less crucial. To experimentally determine the role of the 4-hydroxyl group, we synthesized the 3-deoxy analogue of α-GalCer. It was found that 3-deoxy-α-GalCer induced potent cytokine responses from NKT cells, comparable to those of both α-GalCer and 4-deoxy-α-GalCer. This result and our docking studies suggest that the effects of an absence of the 3-hydroxyl group are compensated by the presence of a hydroxyl group at the C-4 position. Thus, we conclude that the 4-hydroxyl group of α-GalCer is as important to the mechanism of action as the 3-hydroxyl group and that the two hydroxyl groups could play individual and cooperative roles in orienting the glycolipid into the proper position in CD1d to be recognized by the T cell receptor of NKT cells.



KEYWORDS: CD1d, natural killer T cells, T cell receptor, glycolipid, α-GalCer, deoxy analogues

KRN7000 (also commonly called α-GalCer, **1**, Figure 1) is a structurally modified analogue of the marine natural product agelasphins.[1] This glycolipid is the first-defined agonist for natural killer T (NKT) cells, and it remains the most extensively studied compound for the exploration of NKT cell biology and pharmacology.[2−6] α-GalCer activates NKT cells in a CD1d restricted manner; it first binds to the CD1d molecule of antigen-presenting cells, and the resulting α-GalCer/CD1d complex is then recognized by the conserved αβ T cell receptor (TCR) to form the ternary complex that leads to activation of the NKT cells. The activated NKT cells promptly secrete large amounts of T helper 1 and 2 (Th1 and Th2) cytokines, such as interferon-γ (IFN-γ) and interleukin-4 (IL-4), which play critical roles in the regulation of innate and adaptive immune responses.[7−9]

The recently achieved crystal structure of α-GalCer complexed with CD1d reveals that the acyl and phytosphingosine lipid chains of α-GalCer fit tightly into two hydrophobic pockets of the CD1d binding groove, whereas the galactose group protrudes from the CD1d cleft.[10−13] There are several hydrogen-bonding interactions between the surface residues of CD1d and the hydroxyl groups of the galactose and the phytosphingosine base that appear to be crucial for maintaining α-GalCer in the correct position for recognition by the TCR of NKT cells.

The hydrogen-bonding network in the crystal structure is well matched with the previous results from the structure−activity relationships (SAR) studies of α-GalCer analogues.[6,14−18] SAR studies on the phytosphingosine moiety of α-GalCer have shown that the analogue lacking the 4-hydroxyl group on the phytosphingosine (4-deoxy-α-GalCer, **2**, Figure 1) exhibits slightly reduced activity as compared to α-GalCer, whereas the analogue that lacks both 3- and 4-hydroxyl groups (3,4-dideoxy-α-GalCer, **3**, Figure 1) was inactive.[19−22] These SAR results, along with the observed hydrogen-bonding interactions of the 3-hydroxyl group of α-GalCer, with residues of both the CD1d (Asp-80) and the TCR (Arg-95), led to the general belief that the 3-hydroxyl group of phytosphingosine was crucial for activating NKT cells, whereas the 4-hydroxyl group was not crucial for activity.[16,20,23] However, the analogue lacking only the 3-hydroxyl group on the phytosphingosine (3-deoxy-α-GalCer, **4**, Figure 1) had never been prepared and evaluated to ascertain the individual impacts of the 3- and 4-hydroxyl groups on NKT cell activation. This lack of potentially important SAR data prompted us to synthesize and evaluate the 3-deoxy analogue **4**. Herein, we report our studies on this subject.

For the synthesis of the desired 3-deoxy analogue of α-GalCer **4**, the known D-*ribo*-phytosphingosine-derived compound **5**[24] was chosen as an ideal starting material given that its 2-amino and 4-hydroxyl groups were already suitably protected (Scheme 1). The primary hydroxyl group of **5** was selectively protected as its silyl

Received: March 30, 2011
Accepted: May 11, 2011
Published: May 16, 2011

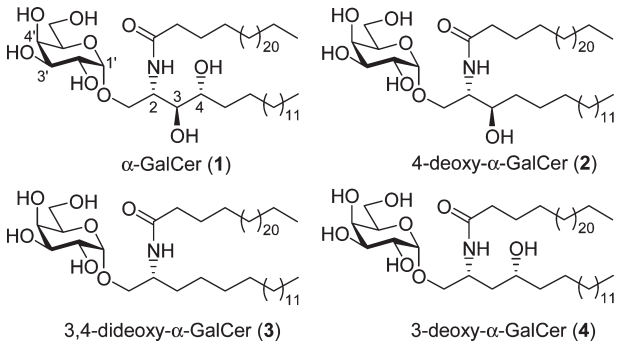
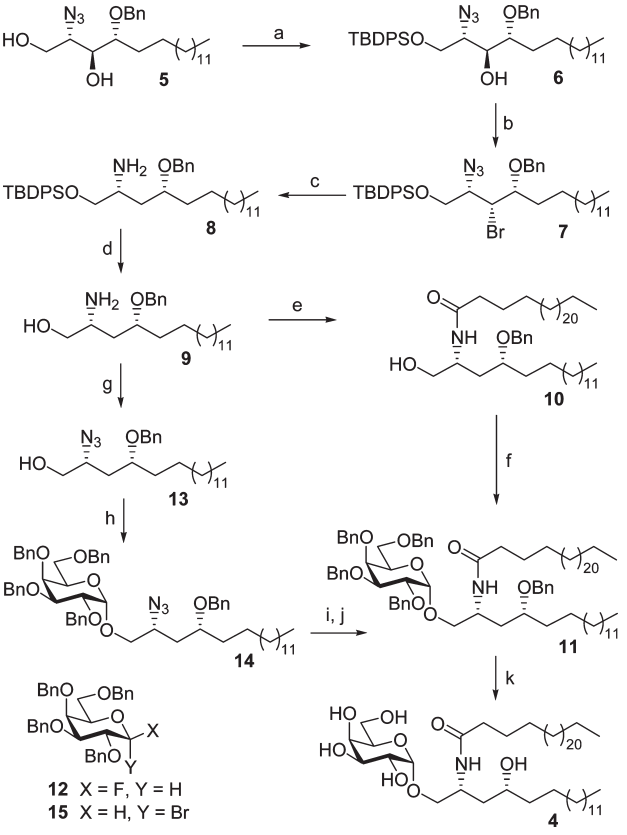


Figure 1. Structures of glycolipids **1**−**4**.

Scheme 1. Synthesis of 3-Deoxy-α-GalCer 4[a]



[a] Reagents and conditions: (a) TBDPSCl, Et_3N, CH_2Cl_2, room temperature, 99%. (b) CBr_4, PPh_3, CH_2Cl_2, 40 °C, 72%. (c) $NaBH_4$, $NiCl_2$, EtOH, room temperature, 85%. (d) Bu_4NF, THF, room temperature, 80%. (e) Hexacosanoic acid, EDCI, DMAP, CH_2Cl_2, room temperature, 78%. (f) Compound **12**, $AgClO_4$, $SnCl_2$, 4 Å molecular sieve, THF, −10 °C, ca. 10%. (g) TfN_3, K_2CO_3, $CuSO_4$, MeOH, CH_2Cl_2, H_2O, room temperature, 79%. (h) Compound **15**, Bu_4NBr, *N*,*N*-tetramethylurea, CH_2Cl_2, room temperature, 83%. (i) PPh_3, benzene/H_2O (10:1), 60 °C, 12 h. (j) Hexacosanoic acid, EDCI, DMAP, CH_2Cl_2, room temperature, 54% from **14**. (k) H_2, $Pd(OH)_2$, EtOH/CH_2Cl_2 (3:1), room temperature, 72%.

ether to afford compound **6**. To remove the C-3 hydroxyl group, alcohol **6** was first converted to bromide **7** with CBr_4 and PPh_3. The obtained bromide **7** was then reduced using $NaBH_4$ and $NiCl_2$ in EtOH. Under these reaction conditions, the azido group of **7** was concomitantly reduced to give amine **8** in good yield (85%).

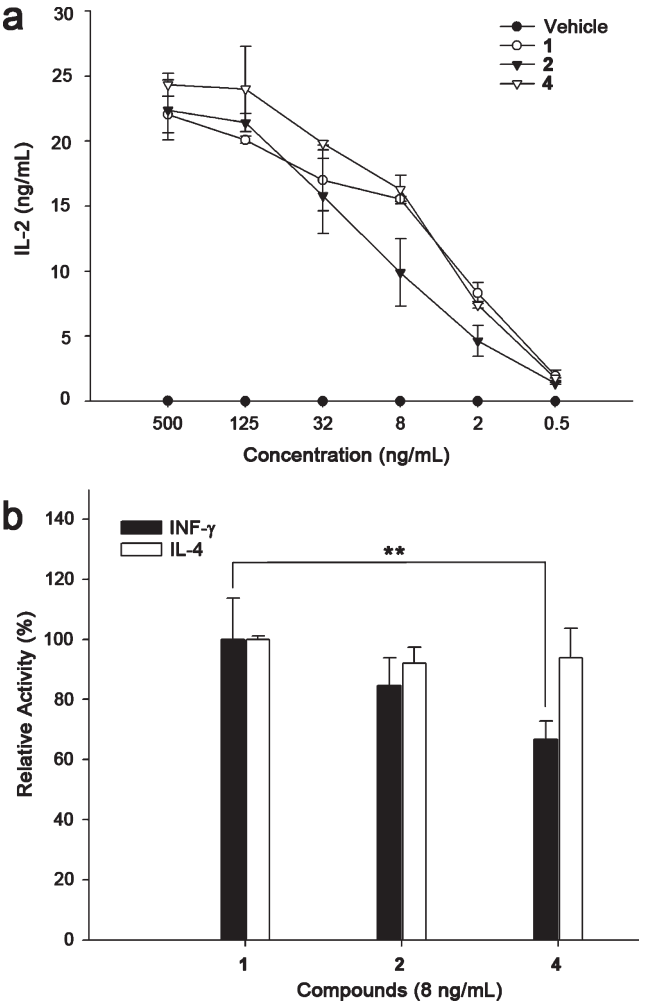


Figure 2. Biological evaluation of deoxy analogues. (a) IL-2 secretion by DN32.D3 NKT hybridoma cells. IL-2 production was measured from cocultured supernatants of NKT hybridoma DN32.D3 and mouse CD1d transfected RBL cells after 16 h of culture. Representative data of two individual experiments are expressed as the mean ± SD of duplicates. (b) IFN-γ and IL-4 secretion by mouse splenocytes. Cytokine production was measured after 72 h of culture. Results are expressed as relative activity. Representative data of two individual experiments are expressed as means ± SDs of triplicates. The statistical significance of the difference in secretion levels was determined by Student's *t* test. $^{**}p < 0.01$.

After successful removal of the C-3 hydroxyl group of *ribo*-phytosphingosine, the silyl protecting group in **8** was removed with Bu_4NF to furnish amino alcohol **9**. Acylation of **9** with hexacosanoic acid and EDCI gave ceramide **10**. For selective α-glycosyl bond formation, we employed the Mukaiyama glycosylation reaction[25] involving the reactive galactosyl fluoride **12** as the glycosyl donor. This reaction provided the desired α-galactoside **11**, but the yield was very low (ca. 10%). Attempts to increase the yield of **11** by varying the glycosyl donor and reaction conditions were not successful.

The low yield of glycosylation product **11** could be attributed to the fact that the amide group of **10** diminishes the nucleophilicity of the primary hydroxyl group through hydrogen bonding.[26] An alternative approach was therefore devised in which an azido group was used in place of the amide during the glycosylation. The free amine group of **9** was first reconverted to

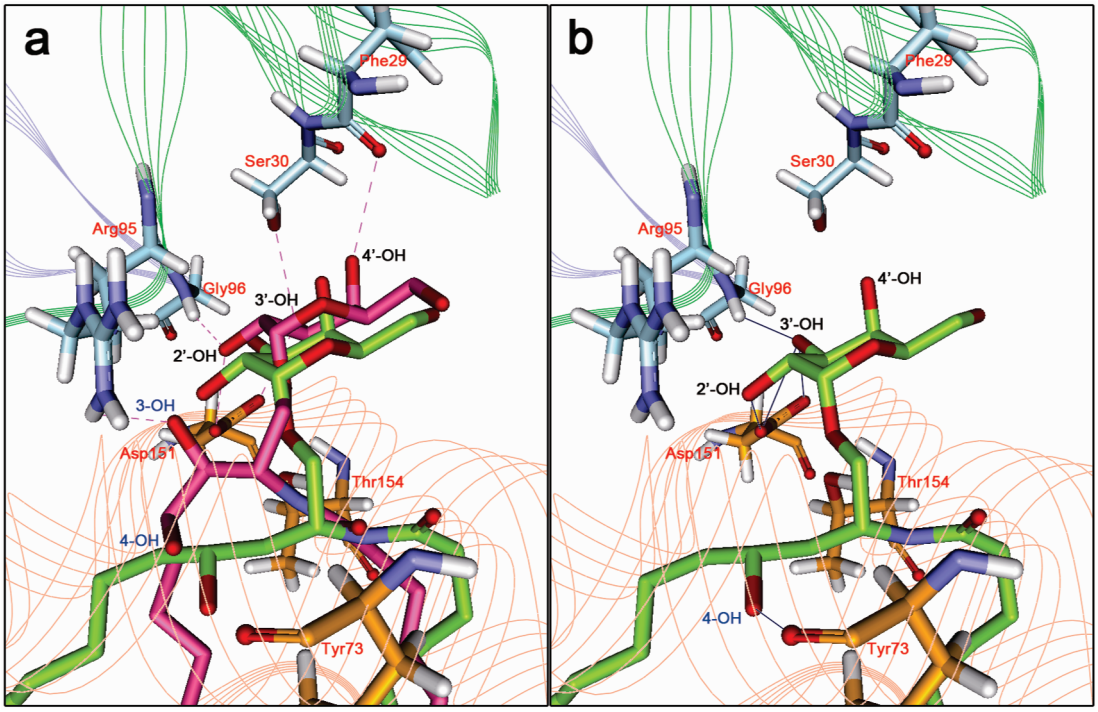


Figure 3. Comparison of the docking model of 3-deoxy-α-GalCer **4** (green backbone) with the crystal structure of α-GalCer **1** (pink backbone) within the hCD1d/TCR (Protein Data Bank code 2PO6). The key amino acid residues are shown in blue (TCR residues) and orange (CD1d residues). (a) α-GalCer **1** (shown in pink) and 3-deoxy-α-GalCer **4** (shown in green) are overlaid. The galactose headgroup of 3-deoxy-α-GalCer **4** is shifted down (ca. 1.3 Å) as compared to that of α-GalCer **1**. H-bonds between α-GalCer **1** and hCD1d/TCR are indicated by pink dashed lines. (b) Docking model of 3-deoxy-α-GalCer **4**. H-bonds are shown as dark blue solid lines.

an azide **13** via a modified diazo transfer reaction.[27] When the in situ-generated galactosyl bromide **15**[28] was employed as a galactosyl donor, the glycosylation of **13** was accomplished efficiently to give α-galactoside **14** as the only identifiable anomer in good yield (83%). Staudinger reduction of the azido group of **14** followed by condensation of the resulting amine with hexacosanoic acid led to the formation of **11**. This route required two more steps than the original but proved significantly more efficient and practical for the preparation of **11**. Finally, the global deprotection of the benzyl ethers by hydrogenolysis afforded the desired 3-deoxy analogue **4**.

For a preliminary biological evaluation of 3-deoxy analogue **4**, we used CD1d-specific NKT cell hybridoma cells (DN32.D3) that are immortalized NKT cells and that tend to produce IL-2 upon stimulation. The parent α-GalCer **1** and its 4-deoxy analogue **2** were also tested for comparison.[29,30] As shown in Figure 2a, 4-deoxy analogue **2** displayed comparable stimulatory effects to α-GalCer **1**, confirming previous reports that the activity of 4-deoxy analogue **2** does not differ significantly from that of α-GalCer.[19] Contrary to the expectation, however, the 3-deoxy analogue **4** was slightly more effective than α-GalCer at promoting IL-2 production. Because IL-2 secretion is dependent on antigen-loaded CD1d,[31] these results indicate that the 3-deoxy analogue **4** efficiently binds to CD1d and triggers TCR signaling in a manner similar to α-GalCer.

To determine whether the 3-deoxy analogue **4** can stimulate cytokine release from intact NKT cells, we measured the levels of IFN-γ and IL-4 in in vitro culture supernatants of mouse splenocytes stimulated with 8 ng/mL of compounds **1**, **2**, and **4**. Figure 2b shows the relative IFN-γ and IL-4 production levels of **2** and **4** when compared with those of **1**. As expected, the NKT stimulation activity of 4-deoxy analogue **2** did not significantly differ from that of α-GalCer **1**. Our evaluation showed that 3-deoxy analogue **4** could induce NKT cell cytokine responses similarly to **1**. However, compound **4** seemed to bias cytokine secretion toward the Th2 response; 3-deoxy analogue **4** showed a comparable stimulatory effect on IL-4 production as α-GalCer **1**, whereas it promoted smaller amounts of IFN-γ production as compared to **1**.

The above biological results suggest that the 3-deoxy analogue **4** can be favorably accommodated within the CD1d binding groove. To understand the binding mode of **4**, we performed molecular modeling studies on the interaction of analogues **2** and **4** with CD1d and the NKT TCR.[32,33] The X-ray crystallographic structure of ternary complex α-GalCer/hCD1d/TCR (PDB code 2PO6)[12] was used for this docking analysis. The best possible geometry of the analogue within the CD1d/TCR complex was searched using Surflex-Dock. The docking scores of compounds were in partial agreement with the biological data. The 3-deoxy analogue **4** gave a better docking score than either α-GalCer **1** or its 4-deoxy analogue **2** (see Figure S1 in the Supporting Information), although analogue **4** forms fewer hydrogen bonds with the CD1d/TCR complex than **1** or **2** (see Figure S2 in the Supporting Information). In our docking model, the 4-deoxy analogue **2** occupies virtually the same position and forms the same set of hydrogen bonds as α-GalCer in its crystalline complex with hCD1d/TCR (see Figure S3 in the Supporting Information). However, 3-deoxy analogue **4** sits considerably deeper in the groove than α-GalCer **1** as shown in Figure 3a. The galactose headgroup is laterally shifted approximately 1.3 Å toward the center of the binding groove.[34] The absence of a hydroxyl group in the 3-position seems to cause the

dx.doi.org/10.1021/ml2000802 |ACS Med. Chem. Lett. 2011, 2, 544–548

sphingoid base to shift itself lower in the pocket to establish new hydrogen bonds with CD1d/TCR complex (Figure 3b).

Although the computational modeling studies do not provide conclusive proof, our model offers a view of how 3-deoxy analogue **4** may bind CD1d and present its galactose epitope to the TCR of NKT cells. Throughout these molecular modeling and biological studies, the effect of the absence of the 3-hydroxyl group appears to be compensated by the presence of a hydroxyl group at the C-4 position. Thus, the 3- and 4-hydroxyl groups of phytosphingosine are both individually effective at orienting the glycolipid in CD1d and inducing NKT cells responses, although the position of the hydroxyl group affects how the glycolipid binds to CD1d.

In summary, we have prepared the α-GalCer analogue lacking the 3-hydroxyl group on the phytosphingosine (3-deoxy-α-GalCer, **4**) to ascertain the individual impacts of the 3- and 4-hydroxyl groups on NKT cell activation. Contrary to the previous perception, 3-deoxy-α-GalCer was found to be similarly effective at inducing NKT cells responses as both α-GalCer and 4-deoxy-α-GalCer. Together with our docking studies, this result suggests that the 4-hydroxyl group of α-GalCer is as important as the 3-hydroxyl group and that the two hydroxyl groups could play both individual and cooperative roles in orienting the glycolipid into the proper position in CD1d to be recognized by the TCR of NKT cells. We believe that 3-deoxy-α-GalCer represents an important new tool for improving the understanding of glycolipid−CD1d interactions and the NKT response. Additionally, it could provide guidance regarding the development of nonstereotypical immunostimulating agents that are structurally distinct from the typical phytosphingosine-containing galactosylceramide.

■ ASSOCIATED CONTENT

Supporting Information. Figures showing the docking scores of compounds **1**, **2**, and **4** (Figure S1), the hydrogen-bonding modes of compounds **2** and **4** (Figure S2), and the docking conformation of compound **2** superimposed with α-GalCer (**1**) (Figure S3), detailed information on the experimental procedures, analytical data for all new compounds, and copies of ^{1}H and ^{13}C NMR spectra for selected compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: 82-2-880-2487. Fax: 82-2-888-0649. E-mail: pennkim@snu.ac.kr.

Funding Sources

This work was supported by a National Research Foundation of Korea grant funded by the Korea government (MEST) (Grant 20100027763).

■ REFERENCES

(1) 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.

(2) Tupin, E.; Kinjo, Y.; Kronenberg, M. The unique role of natural killer T cells in the response to microorganisms. *Nat. Rev. Microbiol.* **2007**, *5*, 405–417.

(3) Balato, A.; Unutmaz, D.; Gaspari, A. A. Natural killer T cells: an unconventional T-cell subset with diverse effector and regulatory functions. *J. Invest. Dermatol.* **2009**, *129*, 1628–1642.

(4) Cerundolo, V.; Silk, J. D.; Masri, S. H.; Salio, M. Harnessing invariant NKT cells in vaccination strategies. *Nat. Rev. Immunol.* **2009**, *9*, 28–38.

(5) Banchet-Cadeddu, A.; Hénon, E.; Dauchez, M.; Renault, J.-H.; Monneaux, F.; Haudrechy, A. The stimulating adventure of KRN 7000. *Org. Biomol. Chem.* **2011**, *9*, 3080–3104.

(6) Mori, K.; Tashiro, T. Sphingolipids and glycosphingolipids—Their syntheses and bioactivities. *Heterocycles* **2011**, *83*, 951–1003.

(7) Kronenberg, M. Toward an understanding of NKT cell biology: Progress and Paradoxes. *Annu. Rev. Immunol.* **2005**, *23*, 877–900.

(8) Kaer, L. V. α-Galactosylceramide therapy for autoimmune diseases: Prospects and obstacles. *Nat. Rev. Immunol.* **2005**, *5*, 31–42.

(9) Murphy, N.; Zhu, X.; Schmidt, R. R. α-Galactosylceramides and analogues—Important immunomodulators for use as vaccine adjuvants. *Carbohydr. Chem.* **2010**, *36*, 64–100.

(10) Zajonc, D. M.; Cantu, C., III; 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.

(11) 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 α-galactosylceramide. *Nat. Immunol.* **2005**, *6*, 819–826.

(12) Borg, N. A.; Wun, K. S.; Kjer-Nielsen, L.; Wilce, M. C. J.; Pellicci, D. G.; Koh, R.; Besra, G. S.; Bharadwaj, M.; Godfrey, D. I.; McCluskey, J.; Rossjohn, J. CD1d-lipid-antigen recognition by the semi-invariant NKT T-cell receptor. *Nature* **2007**, *448*, 44–49.

(13) Pellicci, D. G.; Patel, O.; Kjer-Nielsen, L.; Pang, S. S.; Sullivan, L. C.; Kyparissoudis, K.; Brooks, A. G.; Reid, H. H.; Gras, S.; Lucet, I. S.; Koh, R.; Smyth, M. J.; Mallevaey, T.; Matsuda, J. L.; Gapin, L.; McCluskey, J.; Godfrey, D. I.; Rossjohn, J. Differential Recognition of CD1d-α-GalactogylCeramide by the Vβ8.2 and Vβ7 semi-invariant NKT T cell receptors. *Immunity* **2009**, *31*, 47–59.

(14) Goff, R. D.; Gao, Y.; Mattner, J.; Zhou, D.; Yin, N.; Cantu, C., III; Teyton, L.; Bendelac, A.; Savage, P. B. Effects of lipid chain lengths in α-galactosylceramides on cytokine release by natural killer T cells. *J. Am. Chem. Soc.* **2004**, *126*, 13602–13603.

(15) Tsuji, M. Glycolipids and phospholipids as natural CD1d-binding NKT cell ligands. *Cell. Mol. Life Sci.* **2006**, *63*, 1889–1898.

(16) Savage, P. B.; Teyton, L.; Bendelac, A. Glycolipids for natural killer T cells. *Chem. Soc. Rev.* **2006**, *35*, 771–779.

(17) Wu, D.; Fujio, M.; Wong, C.-H. Glycolipids as immunostimulating agents. *Bioorg. Med. Chem.* **2008**, *16*, 1073–1083.

(18) Nadas, J.; Li, C.; Wang, P. G. Computaional structure activity relationship studies on the CD1d/glycolipid/TCR complex using AMBER and AUTODOCK. *J. Chem. Inf. Model.* **2009**, *49*, 410–423.

(19) Lacône, V.; Hunault, J.; Pipelier, M.; Blot, V.; Lecourt, T.; Rocher, J.; Turcot-Dubois, A.-L.; Marionneau, S.; Douillard, J.-Y.; Clément, M.; Le Pendu, J.; Bonneville, M.; Micouin, L.; Dubreuil, D. Focus on the controversial activation of human iNKT cells by 4-deoxy analogue of KRN7000. *J. Med. Chem.* **2009**, *52*, 4960–4963.

(20) Ndonye, R. M.; Izmirian, D. P.; Dunn, M. F.; Yu, K. O. A.; Porcelli, S. A.; Khurana, A.; Kronenberg, M.; Richardson, S. K.; Howell, A. R. Synthesis and evaluation of sphinganine analogues of KRN7000 and OCH. *J. Org. Chem.* **2005**, *70*, 10260–10270.

(21) Sidobre, S.; Hammond, K. J. L.; Bénazet-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 $V_{\alpha}14i$ NKT cells has a unique mode of glycosphingolipid antigen recognition. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 12254–12259.

(22) Miyamoto, K.; Miyake, S.; Yamamura, T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing T_H2 bias of natural killer T cells. *Nature* **2001**, *413*, 531–534.

(23) 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.

(24) Trappeniers, M.; Goormans, S.; Beneden, K. V.; Decruy, T.; Linclau, B.; Al-Shamkhani, A.; Elliott, T.; Ottensmeier, C.; Werner, J. M.; Elewaut, D.; Van Calenbergh, S. Synthesis and in vitro evaluation of α-GalCer epimers. *ChemMedChem* **2008**, *3*, 1061–1070.

(25) Mukaiyama, T.; Murai, Y.; Shoda, S. An efficient method for glucosylation of hydroxy compounds using glucopyranosyl fluoride. *Chem. Lett.* **1981**, 431–432.

(26) Du, W.; Kulkarni, S. S.; Gervay-Hague, J. Efficient, one-pot syntheses of biologically active α-linked glycolipids. *Chem. Commun.* **2007**, 2336–2338.

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

(28) Nishida, Y.; Shingu, Y.; Dohi, H.; Kobayashi, K. One-pot α-glycosylation method using appel agents in *N*,*N*-dimethylformamide. *Org. Lett.* **2003**, *5*, 2377–2380.

(29) Compounds **1** and **2** were prepared according to the procedure in the literature; see refs 30 and 14.

(30) Kim, S.; Song, S.; Lee, T.; Jung, S.; Kim, D. Practical Synthesis of KRN7000 from phytosphinghosine. *Synthesis* **2004**, 847–850.

(31) Zhou, D.; Mattner, J.; Cantu, C., III; 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.

(32) For selected examples for other computational studies in the α-GalCer analogues, see Zhang, W.; Xia, C.; Nadas, J.; Chen, W.; Gu, L.; Wang, P. G. Introduction of aromatic group on 4′-OH of α-GalCer manipulated NKT cell cytokine production. *Bioorg. Med. Chem.* **2011**, *19*, 2767–2776. Also see refs 33 and 18.

(33) Hénon, E.; Dauchez, M.; Haudrechy, A.; Banchet, A. Molecular dynamics simulation study on the interaction of KRN 7000 and three analogues with human CD1d. *Tetrahedron* **2008**, *64*, 9480–9489.

(34) The lateral shift of the sugar headgroup at the CD1d surface has also been observed in the crystallographic structure of an mCD1d/GalA-GSL complex. For more information, see ref 23.