Published in final edited form as:
Bioorg Med Chem Lett. 2009 August 1; 19(15): 4122–4125. doi:10.1016/j.bmcl.2009.06.005.

Synthesis and evaluation of 3”- and 4”-deoxy and -fluoro analogs of the immunostimulatory glycolipid, KRN7000

Ravinder Raju[a], **Bernard F. Castillo**[a], **Stewart K. Richardson**[a], **Meena Thakur**[a], **Ryan Severins**[b], **Mitchell Kronenberg**[b], and **Amy R. Howell**[a]

[a] Department of Chemistry, University of Connecticut, Storrs, CT 06269-3060

[b] Division of Developmental Immunology, La Jolla Institute for Allergy and Immunology, La Jolla, CA 92037

Abstract

Four 3”- and 4”-deoxy and -fluorogalactosyl ceramides were synthesized, and their ability to stimulate iNKT cells, based on levels of IL-2 production, was assessed in three NKT cell receptor hybridomas. In two of the hybridomas, 1.2 and 2H4, all of the analogs were immunostimulatory, while in the 1.4 hybridoma only the 4”-fluoro analog led to the production of significant levels of IL-2.

Cluster of differentiation 1 (CD1) proteins are cell surface glycoproteins distantly related to the class I and II antigen presenting molecules of the major histocompatibility complex (MHC).[1] Unlike the MHC-encoded molecules, which bind and present peptide antigens, CD1 proteins bind a variety of lipids and present them to subsets of lipid-specific T cells. CD1d, a member of the CD1 family, has been shown to present lipid antigens to a specialized subset of lymphocytes known as invariant natural killer T (iNKT) cells.[2–7] Over the past decade CD1d activated iNKT cells have been demonstrated to elicit a range of immune responses with potential implications for treating viral and bacterial infections, cancer and a variety of autoimmune conditions.[8–14] Most studies of the role of iNKT cells have utilized the α-galactosyl ceramide (GalCer), KRN7000 (Figure 1), identified by Kirin Brewery in SAR studies centered around agelasphin-9b, a potent immunostimulatory compound isolated from the *Agelus* genus of marine sponge.[15] The activity of KRN7000 was found to be mediated by its binding to CD1d and subsequent activation of iNKT cells.[16] Because of the therapeutic potential of iNKT cell stimulation, there has been a growing interest in understanding how the glycolipid structure impacts the immune response. Thus, numerous α-glycosyl ceramides (α-GlyCers) have been synthesized and evaluated for their ability to stimulate iNKT cells.

Using KRN7000 as a lead structure the majority of variations have been in the lipid portion.[17–23] Nevertheless, the carbohydrate moiety has been of interest[24–27] because it was assumed and later confirmed that it was this region of the antigen that protruded from the CD1d/glycolipid complex and interacted with the iNKT T cell receptor (TCR). SAR studies had shown that the C2”-OH (for numbering see Figure 1) was essential for iNKT cell activation and that there was wide tolerance for functional group and size variations on C6”.[24,27,28] In

Supplementary data

Supplementary data associated with this article (experimentals and characterization data) can be found, in the online version, at doi: .

contrast, much less was known about the influence of the C3" and C4"-OH's. The GluCer analog of KRN7000 was not as active but was still potent,[24] and substitution of the C3"-OH by OSO_3^- gave similar results (less than KRN7000, but still potent).[27] We synthesized 3"- and 4"-deoxy and -fluorogalactosyl ceramides **2–5** to probe the importance of the OH's in these positions in terms of iNKT cell stimulation. After this work was completed a ternary structure (KRN7000/CD1d/iNKT TCR) was reported.[29] It shows hydrogen bonding interactions between the C2"-OH and both CD1d and the iNKT TCR, while the 6"-OH is located in a large, open pocket. The structure also appears to show hydrogen bonding between the TCR and the C3"- and C4"-OH's. In this paper we describe the syntheses of compounds **2–5** and report their ability to stimulate iNKT cells, based on IL-2 production, in three hybridomas.

Although C18-*ribo*-phytosphingosine, the aminotriol found in KRN7000, is now readily available and inexpensive, this was not the case at the time of our initial studies, and we synthesized and evaluated the more easily accessible sphinganine analog **1**.[30] In assays using mouse cells it was shown to be nearly equipotent to KRN7000 and to elicit a similar cytokine profile. Therefore, we decided to prepare sphinganine-containing analogs **2–5**. Glycosylations to synthesize **2–5** were done with either ceramide **6**, prepared as previously described,[30] or sphingoid base **7**, derived by deprotection of known **8**,[30] as shown is Scheme 1.

The synthesis of 3"-deoxyGalCer **2** began with α-methyl galactopyranoside (Scheme 2). Acid catalyzed acetal formation using benzaldehyde diethyl acetal,[31] followed by non-regioselective acylation with benzoyl chloride under phase transfer catalyzed conditions, gave a mixture of mono- and diacylated sugars from which the desired 2-benzoyl derivative **9** was isolated by careful chromatography.[32] The undesired benzoate esters could be recycled by hydrolysis and reesterification. Deoxygenation at C3 was achieved via tin hydride reduction following acylation with phenyl chlorothionoformate. Cleavage of the benzoate provided **10**. Removal of the acetal was followed by perbenzylation. The anomeric methyl group was then hydrolyzed and the resulting mixture of anomers activated as the 2-mercaptopyridyl thioether according to a literature procedure.[33] β-Anomer **11** was the major product, and it was coupled with ceramide **6** in the presence of silver triflate to give an α-GalCer which was deprotected under standard conditions to give **2**.[33] Although the yield was low,[34] no β-anomer was detected, and significant amounts of both starting materials were recovered.

The synthesis of 4"-deoxyGalCer **3** began with commercially available glucose pentaacetate (Scheme 3). The derived β-thioglycoside was prepared by activation with boron trifluoride and addition of thiophenol. Global deacylation and selective acetalization gave **12**. Dibenzylation and regioselective, reductive acetal cleavage provided **13**.[35] A two stage removal of the 4"-OH similar to that used for **2**, glycosylation of sphingoid base **7** under standard conditions, and removal of the Bocprotecting group gave amine **14**. Acylation with the PNP ester of cerotic acid and subsequent hydrogenolysis provided 4"-deoxyGalCer **3**.

The synthesis of 3"-fluoroGalCer **4** began with 1,2:5,6-di-*O*-ispropylidene-α-D-glucofuranose (Scheme 4). Inversion of stereochemistry at the free OH and C4 was achieved by a three step process,[36–39] oxidation to the corresponding ketone,[40,41] conversion to the enol acetate and stereoselective reduction, presumably directed to the beta face by the bulky 1,2-α-acetonide. Selective cleavage of the acetate of **15** and fluorination with inversion gave **16**.[42] The bisacetonide was then hydrolyzed with simultaneous ring enlargement using an acidic ion exchange resin; subsequent acylation gave **17**. The anomeric acetate was converted to the thioglycoside, and the remaining acetates were cleaved. Tribenzylation gave **18**, which was coupled with ceramide **6** using NIS/AgOTf activation. Global hydrogenolysis gave **4**.

The synthesis of the 4"-fluoroGalCer **5**, like that of **3**, used β-thioglycoside **13** (Scheme 5). Inversion of the OH at C4 was realized by triflation, followed by treatment with tris

(dimethylamino)sulfonium difluorotrimethylsilicate (TASF), to give sugar donor **19**.[43] Glycosylation and debenzylation then followed standard conditions to give **5**.

The compounds were assessed in antigen presentation assays that have been described previously.[44] Three well-characterized iNKT cell hybridomas,[44] which are cells immortalized by cell-cell fusion, were used. These T cell hybridomas have TCRs containing the identical Vα14 chain characteristic of the iNKT cell population, paired with different TCR β chains. Hybridoma 2H4 has Vβ7, 1.2 has Vβ8.2, and 1.4 has Vβ10. While more than 50% of iNKT cells contain a Vβ8.2 TCR, and Vβ7 is also common, Vβ10 is only very rarely expressed in this population. To assay for antigenic potency, 1×10^5 A20 B lymphoma cells transfected to express high amounts of mouse CD1d were incubated overnight with the indicated amounts of the compounds; they were not toxic at any dose tested. These antigen-presenting cells were washed and cultured with 2×10^5 iNKT cell hybridoma cells. After a further overnight incubation, the supernatants of the cultures were assayed for the presence of the cytokine, interleukin-2 (IL-2), by enzyme-linked immunoassay (ELISA). Hybridoma cells only produce IL-2 when their TCR is engaged, and therefore the IL-2 measurement provides a bioassay for antigen recognition by the TCR.

Figure 2 shows the antigen dose-response curve of the three hybridomas. It contains representative data from one of three similar experiments. It is evident that the two hybridomas expressing the more commonly used Vβ8.2 (1.2) and Vβ7 (2H4) β chains responds nearly equivalently to all of the compounds, with the exception of compound 4”-deoxyGalCer **3**, which is a much weaker antigen. By contrast, the Vβ10 containing hybridoma (1.4) was much more sensitive to changes in the sugar, as it responded only toKRN7000, the sphinganine analog **1** of KRN7000 and the 4”-fluoroGalCer **5**.

The results of our studies provide a deeper understanding of the recognition of glycolipid antigens presented by CD1d to the invariant TCR expressed by iNKT cells. First, our data demonstrate that the TCR β chain can have a strong influence on antigen recognition. This must be an indirect influence, however, as the structural analysis shows that the β chain does not make direct contacts with the hexose sugar,[29] but instead it only makes a few contacts with CD1d.[45] It is possible, therefore, that the reduced affinity of the TCR α chain for the altered sugars, combined with the reduced affinity of Vβ10 for CD1d, causes the absence of reactivity of hybridoma 1.4 to compounds **2**, **3** and **4**. The complementarity determining regions (CDR) 3 of the β chain are highly diverse. Although NKT cells expressing Vβ10 are rare, the differences observed in this study may be important for understanding how β chain diversity influences a reactivity that is otherwise dominated by the TCR α chain. Second, our data demonstrate that the different H bonds the invariant TCR α chain makes with the 2”-, 3”- and 4”-positions of the galactose do not make equal contributions to the avidity of the TCR interaction, although this was not obvious from the TCR-αGalCer-CD1d tri-molecular structure. While the 2”-deoxy- and 2”-fluoroGalCers lost antigenic activity completely,[46] this was not true for the same modifications at the 3”- and 4”-positions. The antigen presenting cells we used express high amounts of CD1d, which might mask differences in TCR avidity for glycolipid antigens weaker than KRN7000. Furthermore, the results from testing compound **3** show the hydroxyl in the 4” position does have a clear influence on antigenic potency. The influences of the 3”- and 4”-position modifications are subtle, however, when compared to the absolute requirement for the hydroxyl at the 2”-position. In the trimolecular structure of the human TCR bound to an αGalCer human CD1d complex,[29] each of the three galactose hydroxyls makes multiple contacts with the TCR α chain that include van der Waals interactions and hydrogen bonds. However, the 2” position makes unique contacts with TCR α chain amino acids 95–96 in the critical CDR3, while the 3” and 4” hydroxyls both contact amino acid positions 28–30 in the TCR α chain CDR1. Therefore, we hypothesize that the 3”

OH can compensate partially for the absence of the 4” OH, and vice versa. Further studies of KRN7000 analogs may help in the design of more potent and selective agonists for iNKT cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors are grateful for financial support provided by NIH NIAID: RO1 AI057519, ARH and RO1 AI45053, MK.

References and notes

1. Brigl M, Brenner MB. Annu Rev Immunol 2004;22:817. [PubMed: 15032598]
2. Matsuda JL, Mallevaey T, Scott-Browne J, Gapin L. Curr Opin Immunol 2008;20:358. [PubMed: 18501573]
3. Wu D, Fujio M, Wong CH. Bioorg Med Chem 2008;16:1073. [PubMed: 18006319]
4. Sullivan BA, Kronenberg M. Curr Top Microbiol Immunol 2007;314:165. [PubMed: 17593661]
5. Bendelac A, Savage PA, Teyton L. Annu Rev Immunol 2007;25:297. [PubMed: 17150027]
6. Yu KOA, Porcelli SA. Immunol Lett 2005;100:42. [PubMed: 16083968]
7. Florence WC, Bhat RK, Joyce S. Expert Rev Mol Med 2008;10:e20.10.1017/S1462399408000732 [PubMed: 18601810]
8. Hammond JKL, Godfrey DI. Tissue Antigens 2002;59:353. [PubMed: 12144618]
9. Meyer EH, DeKruyff RH, Umetsu DT. Annu Rev Med 2008;59:281. [PubMed: 17937589]
10. Linsen L, Somers V, Stinissen P. Human Immunol 2005;66:1193. [PubMed: 16690406]
11. Cerundolo V, Salio M. Curr Top Microbiol Immunol 2007;314:325. [PubMed: 17593667]
12. Tupin E, Kinjo Y, Kronenberg M. Nature Rev Microbiol 2007;5:405. [PubMed: 17487145]
13. Swann JB, Coquet JMC, Smyth MJ, Godfrey DI. Curr Top Microbiol Immunol 2007;314:293. [PubMed: 17593666]
14. Biron CA, Brossay L. Curr Opin Immunol 2001;13:458. [PubMed: 11498302]
15. Natori T, Morita M, Akimoto K, Koezuka H. Tetrahedron 1994;50:2771.
16. Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. Science 1997;278:1626. [PubMed: 9374463]
17. Li Q, Ndonye RM, Illarionov PA, Yu KOA, Jerud ES, Diaz K, Bricard G, Porcelli SA, Besra GS, Chang YT, Howell AR. J Comb Chem 2007;9:1084. [PubMed: 17896821]
18. Morita M, Motoki K, Akimoto K, Natori T, Sakai T, Sawa E, Yamaji K, Koezuka Y, Kobayashi E, Fukushima H. J Med Chem 1995;38:2176. [PubMed: 7783149]
19. Miyamoto K, Miyake S, Yamamura T. Nature 2001;413:531. [PubMed: 11586362]
20. Goff RD, Gao Y, Mattner J, Zhou D, Yin N, Cantu C III, Teyton L, Bendelac A, Savage PA. J Am Chem Soc 2004;126:13602. [PubMed: 15493902]
21. Toba T, Murata K, Nakanishi K, Takahashi B, Takemoto N, Akabane M, Nakatsuka T, Imajo S, Yamamura T, Miyake S, Annoura H. Bioorg Med Chem Lett 2007;17:2781. [PubMed: 17419054]
22. Yu KOA, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, Fujiwara N, Arias I, Miyake S, Yamamura T, Chang YT, Besra GS, Porcelli SA. Proc Natl Acad Sci USA 2005;102:3383. [PubMed: 15722411]
23. Fujio M, Wu D, Garcia-Navarro R, Ho DD, Tsuji M, Wong CH. J Am Chem Soc 2006;128:9022. [PubMed: 16834361]
24. Motoki K, Morita M, Kobayashi E, Uchida T, Akimoto K, Fukushima H, Koezuka Y. Biol Pharm Bull 1995;18:1487. [PubMed: 8593464]
25. Uchimura A, Shimizu T, Morita M, Ueno H, Motoki K, Fukushima K, Natori T, Koezuka Y. Bioorg Med Chem 1997;5:2245. [PubMed: 9459022]

26. Uchimura A, Shimizu T, Nakajima M, Ueno H, Motoki K, Fukushima H, Natori T, Koezuka Y. Bioorg Med Chem 1997;5:1447. [PubMed: 9377104]
27. Wu D, Xing GW, Poles MA, Horowitz A, Kinjo Y, Sullivan B, Bodmer-Narkevitch V, Plettenburg O, Kronenberg M, Tsuji M, Ho DD, Wong CH. Proc Natl Acad Sci USA 2005;102:1351. [PubMed: 15665086]
28. Zhou XT, Forestier C, Goff RD, Li C, Teyton L, Bendelac A, Savage PB. Org Lett 2002;4:1267. [PubMed: 11950339]
29. Borg NA, Wun KS, Kjer-Nielsen L, Wilce MCJ, Pellicci DG, Koh R, Besra GS, Bharadwaj M, Godfrey DI, McCluskey J, Rossjohn J. Nature 2007;448:44. [PubMed: 17581592]
30. Ndonye RM, Izmirian DP, Dunn MF, Yu KOA, Porcelli SA, Khurana A, Kronenberg M, Richardson SK, Howell AR. J Org Chem 2005;70:10260. [PubMed: 16323834]
31. Ferro V, Mocerino M, Stick RV, Tilbrook DMG. Aust J Chem 1988;41:813.
32. Hakamata W, Nishio T, Oku T. Carbohydr Res 2000;324:107. [PubMed: 10702877]
33. Zhiyuan Z, Magnusson G. Carbohydr Res 1994;262:79. [PubMed: 7954521]
34. The glycosylation reactions in this manuscript were not optimized. In general, we have found that glycosylations with thiophenyl glycosides proceed with higher yields and are more easily purified than those of the corresponding glycosylfluorides.
35. Janczuk AJ, Zhang W, Andreana PR, Warrick J, Wang PG. Carbohydr Res 2002;337:1247. [PubMed: 12151204]
36. Brimacombe JS, Foster AB, Hems R, Westwood JH, Hall LD. Can J Chem 1970;48:3946.
37. Reckendorf, WMz. Angew Chem 1967;79:151.
38. Slessor KN, Tracey AS. Can J Chem 1969;47:3989.
39. Elhalabi J, Rice KG. Nucleosides, Nucleotides, Nucleic Acids 2004;23:195. [PubMed: 15043147]
40. Watterson MP, Pickering L, Smith MD, Hudson SJ, Marsh PR, Mordaunt JE, Watkin DJ, Newman CJ, Fleet GWJ. Tetrahedron: Asymmetry 1999;10:1855.
41. Tadano, K-i; Idogaki, Y.; Yamada, H.; Suami, T. J Org Chem 1987;52:1201.
42. Kovac P, Glaudemans CPJ. Carbohydr Res 1983;123:326.
43. Doboszewski B, Hay GW, Szarek WA. Can J Chem 1987;65:412.
44. Burdin N, Brossay L, Degano M, Iijima H, Gui M, Wilson IA, Kronenberg M. Proc Natl Acad Sci USA 2000;97:10156. [PubMed: 10963678]
45. Scott-Browne JP, Matsuda JL, Mallevaey T, White J, Borg NA, McCluskey J, Rossjohn J, Kappler J, Marrack P, Gapin L. Nature Immunol 2007;8:1105. [PubMed: 17828267]
46. Wu D, Zajonc DM, Fujio M, Sullivan B, Kinjo Y, Kronenberg M, Wilson IA, Wong CH. Proc Natl Acad Sci USA 2006;103:3972. [PubMed: 16537470]

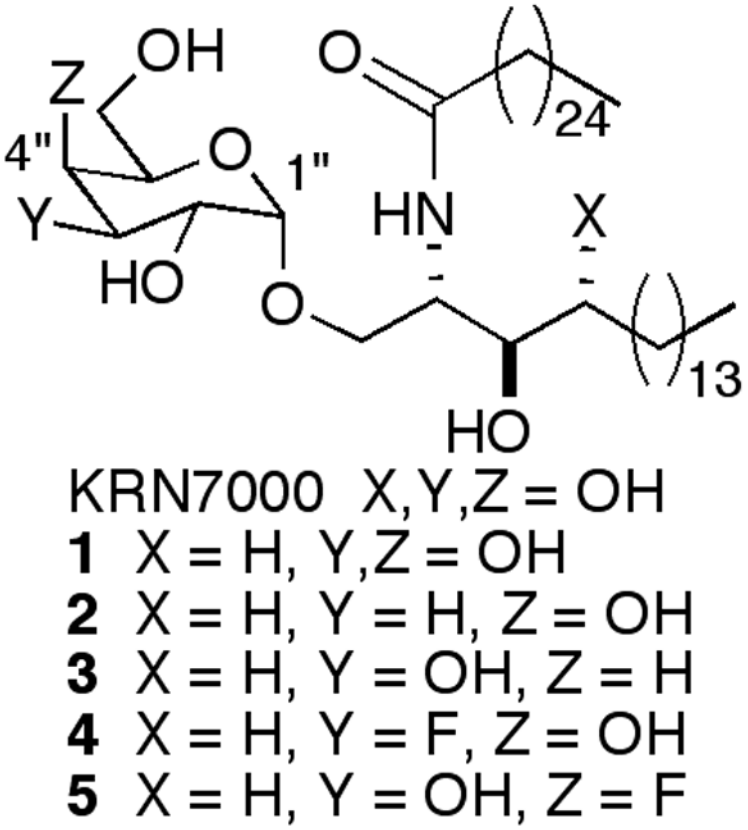


Figure 1.
Structures of KRN7000, its related sphinganine analog **1** and the targets **2–5** of this study.

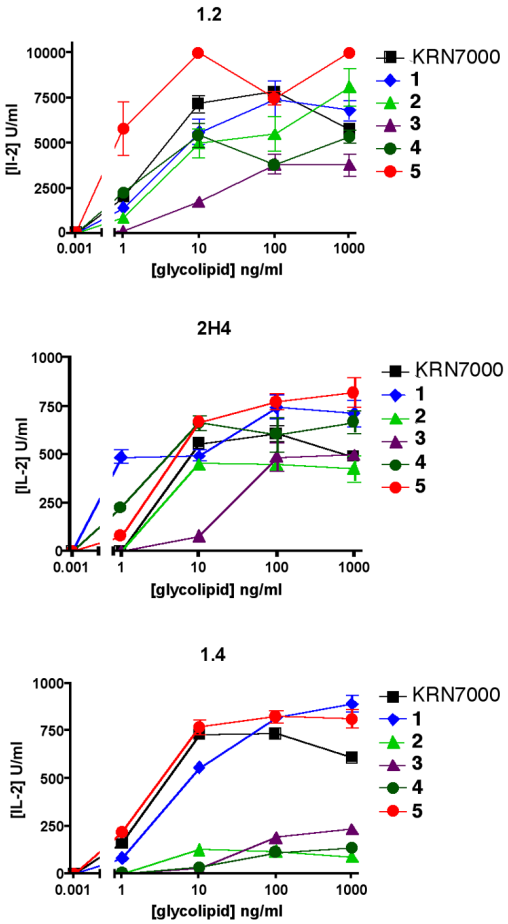
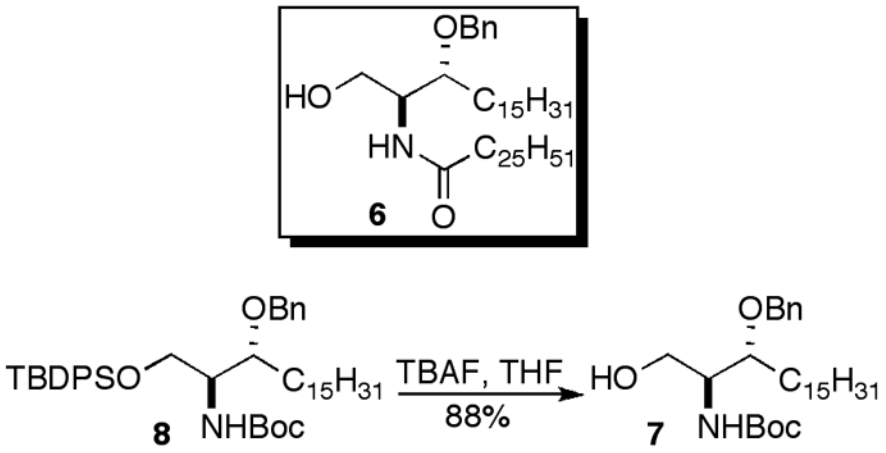
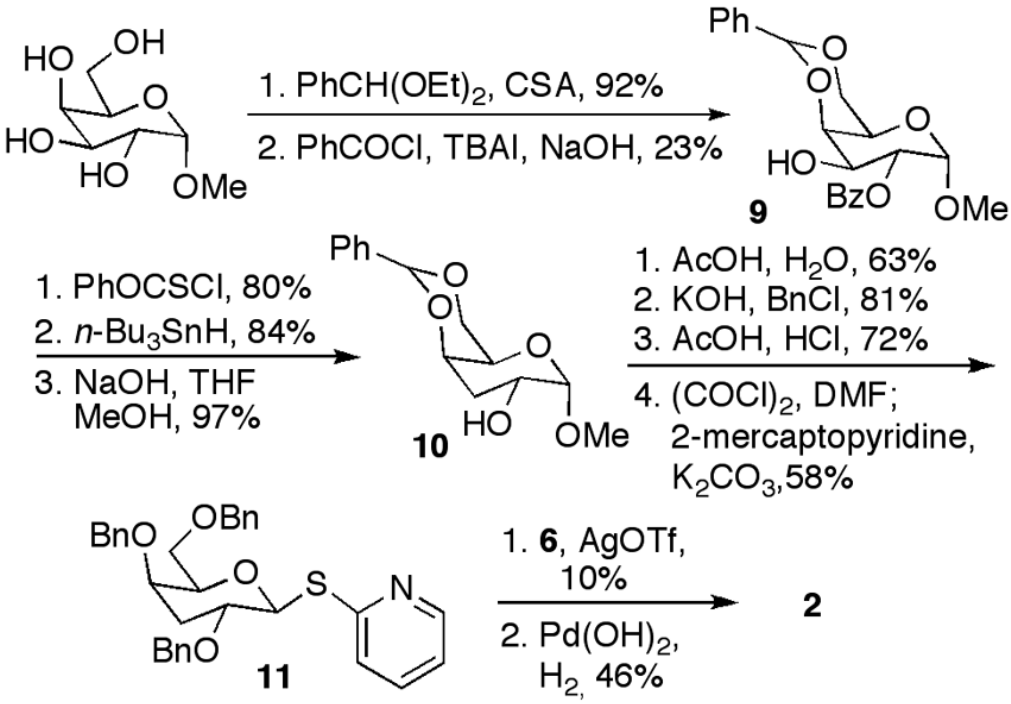


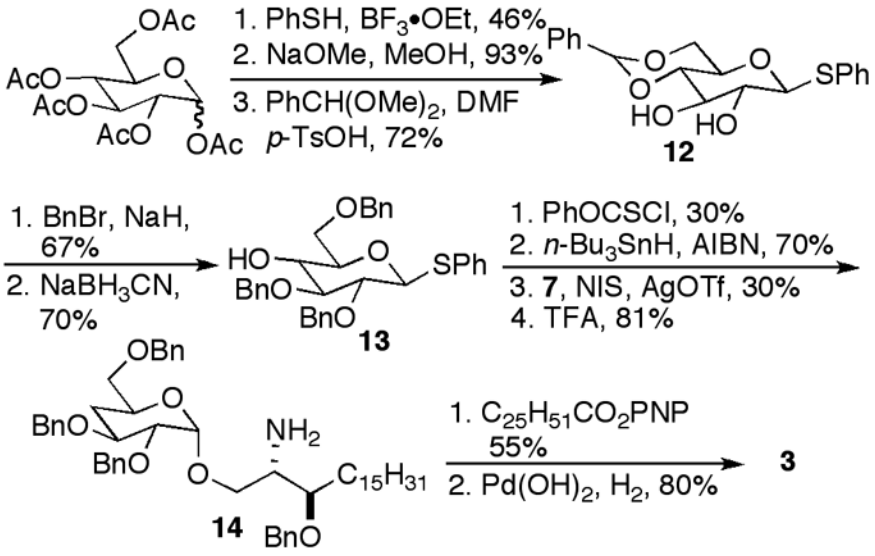
Figure 2.
NKT cell responses to glycolipid analogs of KRN7000 with alterations in the galactose moiety.



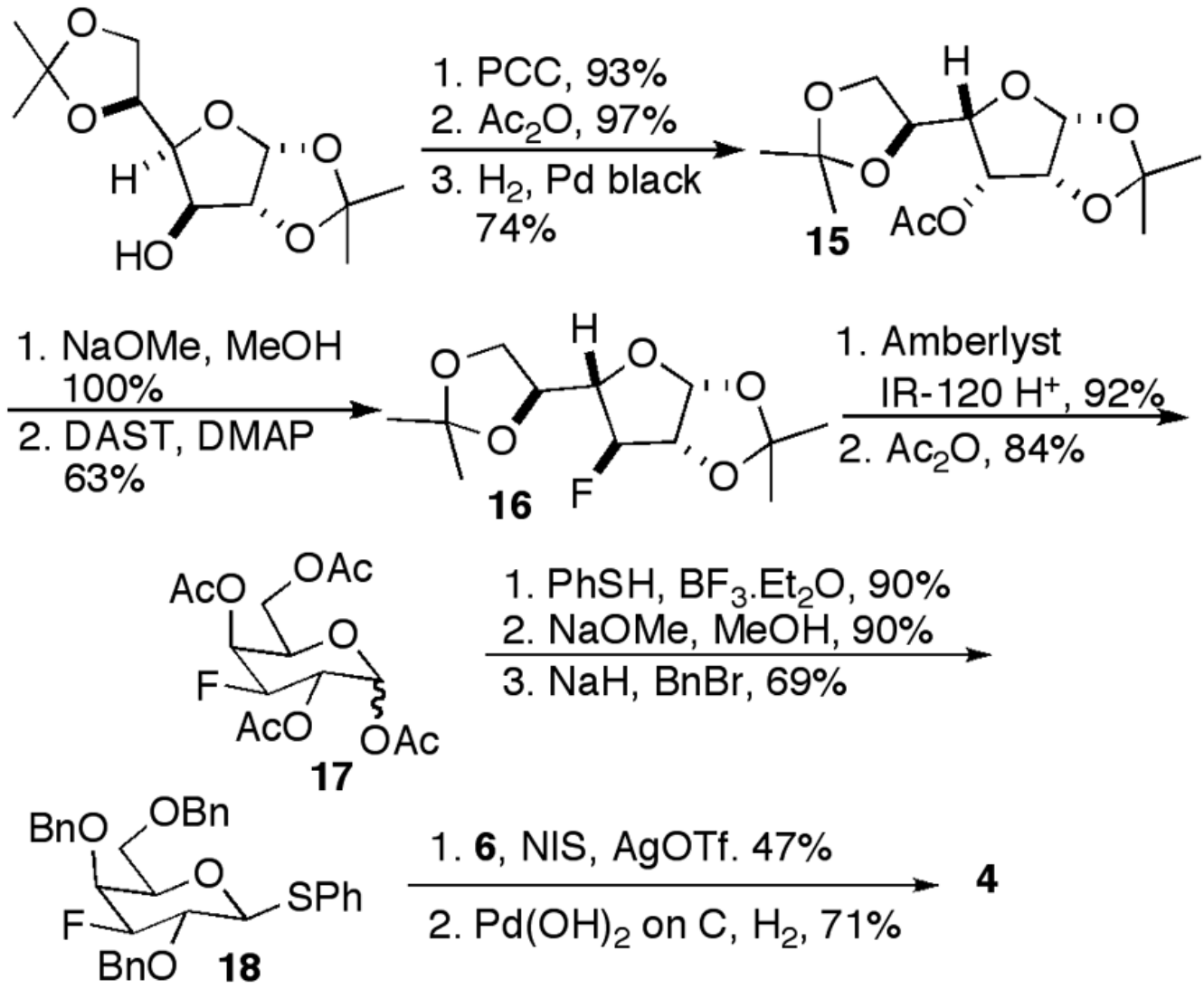
Scheme 1.



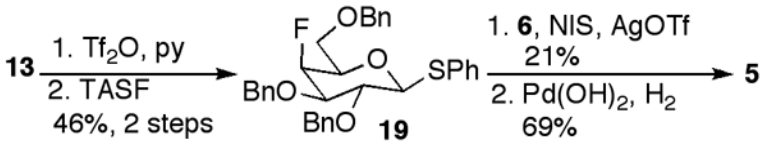
Scheme 2.



Scheme 3.



Scheme 4.



Scheme 5.