ORGANIC LETTERS

2002
Vol. 4, No. 8
1267−1270

Synthesis and NKT Cell Stimulating Properties of Fluorophore- and Biotin-Appended 6′′-Amino-6′′-deoxy-galactosylceramides

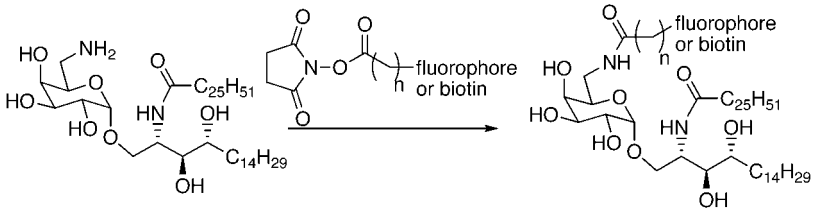
Xiao-Ti Zhou,[†] Claire Forestier,[‡] Randal D. Goff,[†] Chunhong Li,[†] Luc Teyton,[§] Albert Bendelac,[‡] and Paul B. Savage*,[†]

Department of Chemistry and Biochemistry, Brigham Young University, Provo, Utah 84602, Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, and Department of Immunology, Scripps Research Institute, La Jolla, California 92037

paul_savage@byu.edu

Received January 15, 2002

ABSTRACT



α-Galactosylceramides are potent stimulators of human T cells. Stimulation occurs through binding of the glycolipids by CD1d, presentation to T cells, and formation of a CD1d–glycolipid–T cell receptor complex. To facilitate the elucidation of the structural features of glycolipids necessary for T cell stimulation, α-galactosylceramides have been prepared with small molecules appended at the C6 position of the sugar. The appended molecules do not significantly influence the abilities of the glycolipids to stimulate T cells.

Peptide antigen presentation via major histocompatability complexes has long been recognized as a central element in adaptive immune responses. Recently, a parallel pathway that can elicit potent immune responses has begun to be elucidated. This pathway involves the presentation of glycolipids by CD1 proteins and is believed to be responsible for a portion of the innate immunity of mammals to bacteria.[1] There are five members of the CD1 gene family, CD1a−e, and these membrane-bound proteins have been characterized by their abilities to present classes of glycolipids, including those from bacterial membranes, to T cells. For example, CD1b has been shown to selectively present glycolipids found in the membranes of mycobacteria (e.g., *Mycobacterium tuberculosis*) to T cells and stimulate proliferation.[2] CD1d is another member of the gene family and has been characterized by its ability to bind and present α-galactosylceramides, including KRN7000[3] (**1**, in Figure 1), to natural killer T cells (NKT cells).[4] Glycosylceramides undergo

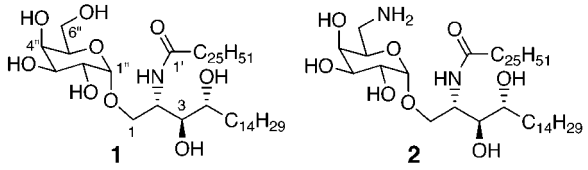


Figure 1. Structures of KRN7000 and 6-amino-6-deoxygalactosylceramide **2**.

[†] Brigham Young University.
[‡] Princeton University.
[§] Scripps Research Institute.

(1) For recent reviews, see: (a) Porcelli, S. A.; Modlin, R. L. *Annu. Rev. Immunol.* **1999**, *17*, 297. (b) Park, S.-H.; Bendelac, A. *Nature* **2000**, *406*, 788. (c) Moody, D. B.; Besra, G. S. *Immunology* **2001**, *104*, 243.

10.1021/ol025565+ CCC: $22.00
Published on Web 03/20/2002

endocytosis by antigen-presenting cells, processing in which oligosaccharides are truncated,[5] loading into CD1d in the endoplasmic reticulum, translocation to the cell surface, and presentation to NKT cells. Complexes between glycolipid-loaded CD1d proteins and T cell receptors form that can lead to stimulation of the T cell.

Due in part to the complexity of the glycolipid presentation pathway, little is known about the specific structural requirements for glycolipid binding by CD1d and T cell receptors. The limited structure−activity studies that have been conducted have used assays that do not distinguish between CD1d binding and NKT cell receptor binding.[3,4b,6,7] That is, in a typical procedure, glycolipids are added to antigen-presenting cells and T cells (of which NKT cells make up only a small percentage), and T cell stimulation is gauged. Nevertheless, it is well recognized that only the α anomers (and not the β) of galactosylceramides cause stimulation, that α-glucosylceramides are less active, and that the length of the lipid chains can influence how T cells respond to the compounds.[7]

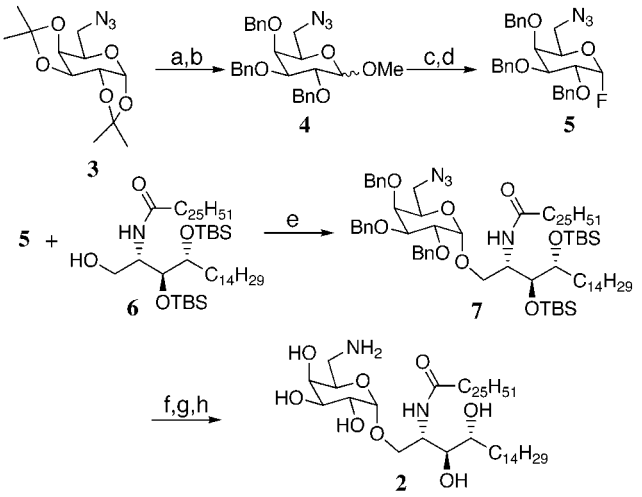
Challenges in understanding the roles of glycolipid structure in CD1d and NKT cell receptor binding have included the inability to effectively observe trafficking of glycolipids and difficulties in quantifying their association with CD1d and NKT cell receptors. An attractive means of overcoming these challenges is to label glycolipids with fluorophores or other small molecules (e.g., biotin) that allow observation of the compounds at low concentrations and/or provide a means of quantifying the association with CD1d and NKT cell receptors. α-Galactosylceramides have been prepared with labels (a fluorophore[8] or biotin[9]) at the end of one of the lipid chains; however, CD1d binds lipid chains within deep hydrophobic pockets.[10] Consequently, addition of the label on the lipid chains may interfere with the association with CD1d.[11]

Modeling of the CD1d−**1** complex suggested that the hydroxyl groups at C4″ and C6″ on galactose are not involved in complex formation.[12] Consequently, these positions may be the best locations for attachment of fluorescent tags or other small molecules. In addition, a derivative of **1** with a second sugar linked at the C6″ position was shown to not require processing for NKT cell stimulation,[5] suggesting that the CD1d−glycolipid−NKT cell receptor interaction tolerates small molecules appended at C6″.

By preparing 6-amino-6-deoxygalactosylceramide **2** (Figure 1), we have been able to attach a number of small molecules to the glycolipid.[13] The small molecules that have been attached include dansyl, a prodan derivative, and biotin. Examples of the labeled compounds have proven to have NKT cell stimulating properties similar to the parent glycolipid (**1**), suggesting that in a manner similar to **1**, the compounds go through endocytosis, CD1d loading, presentation on the cell surface, and binding to T cell receptors causing T cell stimulation.

The amine functionality was incorporated early onto the carbohydrate as the azide (**3**,[14] Scheme 1). The acetonides

Scheme 1. Preparation of α-6-Amino-6-deoxygalactosylceramide **2**[a]



[a] Reagents: (a) AcCl, MeOH (86% yield); (b) BnBr, 18-crown-6, NaH, THF (95% yield); (c) AcOH, H_2SO_4 (84% yield); (d) HF·pyridine, pyridine (78% yield); (e) MS 4Å, $AgClO_4$, $SnCl_2$, THF (44%); (f) TBAF, THF (81% yield); (g) PPh_3/H_2O, THF (quantitative yield); (h) NH_3/Na, −78 °C (53% yield).

were hydrolyzed with concomitant methylgalactoside formation,[15] and benzyl ethers at C2, C3, and C4 were formed

(2) For leading references, see: (a) Moody, B. D.; Reinhold, B. B.; Guy, M. R.; Beckman, E. M.; Frederique, D. E.; Furlong, S. T.; Ye, S.; Reinhold, V. N.; Sieling, P. A.; Modlin, R. L.; Besra, G. S.; Porcelli, S. A. *Science* **1997**, *278*, 283. (b) Jackman, R. M.; Stenger, S.; Lee, A.; Moody, D. B.; Rogers, R. A.; Niazi, K. R.; Sugita, M.; Modlin, R. L.; Peters, P. J.; Porcelli, S. A. *Immunity* **1998**, *8*, 341.

(3) Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sukai, T.; Sawa, E.; Yamaji, K.; Koezuka, Y.; Kobayashi, E.; Fukushima, H. *J. Med. Chem.* **1995**, *38*, 2176.

(4) For leading references, see: (a) Bendelac, A.; Rivera, M. N.; Park, S.-H.; Roark, J. H. *Annu. Rev. Immunol.* **1997**, *15*, 535. (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. *Science* **1997**, *278*, 1626. (c) Burdin, N.; Brossay, L.; Koezuka, Y.; Smiley, S. T.; Grusby, M. J.; Gui, M.; Taniguchi, M.; Hayakama, K.; Kronenberg, M. *J. Immunol.* **1998**, *161*, 3271. (d) Park, S.-H.; Weiss, A.; Benlagha, K.; Kyin, T.; Teyton, L.; Bendelac, A. *J. Exp. Med.* **2001**, *193*, 893.

(5) Prigozy, T. I.; Naidenko, O.; Qasba, P.; Elewaut, D.; Brossay, L.; Khurana, A.; Natori, T.; Koezuka, Y.; Kulkarni, A.; Kronenberg, M. *Science* **2001**, *291*, 664.

(6) (a) Kobayashi, E.; Motoki, K.; Yamaguchi, Y.; Uchida, T.; Fukushima, H.; Koezuka, Y. *Bioorg. Med. Chem.* **1996**, *4*, 615. (b) Uchimura, A.; Shimizu, T.; Nakajima, M.; Ueno, H.; Motoki, K.; Fukushima, H.; Natori, T.; Koezuka, Y. *Bioorg. Med. Chem.* **1997**, *5*, 1447. (c) Uchimura, A.; Shimizu, T.; Morita, M.; Ueno, H.; Motoki, K.; Fukushima, H.; Natori, T.; Koezuka, Y. *Bioorg. Med. Chem.* **1997**, *5*, 2245.

(7) Miyamoto, K.; Miyake, S.; Yamamura, T. *Nature* **2001**, *413*, 531.

(8) Sakai, T.; Ehara, H.; Koezuka, Y. *Org. Lett.* **1999**, *1*, 359.

(9) Sakai, T.; Naidenko, O. V.; Iijima, H.; Kronenberg, M.; Koezuka, Y. *J. Med. Chem.* **1999**, *42*, 1836.

(10) Zheng, Z.-H.; Castaño, A. R.; Segelke, B. W.; Stura, E. A.; Peterson, P. A.; Wilson, I. A. *Science* **1997**, *277*, 339.

(11) Naidenko, O. V.; Maher, J. K.; Ernst, W. A.; Sukai, T.; Modlin, R. L.; Kronenberg, M. *J. Exp. Med.* **1999**, *190*, 1069.

(12) Kamada, N.; Iijima, H.; Kimura, K.; Harada, M.; Shimizu, E.; Motohashi, S.; Kawano, T.; Shinkai, H.; Nakayama, T.; Sakai, T.; Brossay, L.; Kronenberg, M.; Taniguchi, M. *Internat. Immunol.* **2001**, *13*, 853.

(13) Fluorophores have been attached via amide bonds to C6 on other biologically active galactosides. For example, see: Ueda, M.; Wada, Y.; Yamamura, S. *Tetrahedron Lett.* **2001**, *42*, 3869.

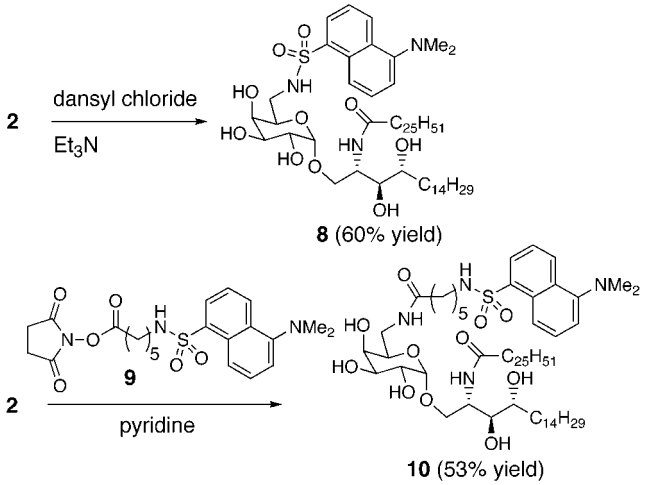
(14) Corey, E. J.; Sameulsson, B.; Luzzio, F. A. *J. Am. Chem. Soc.* **1984**, *106*, 3682.

(15) Singh, P. P.; Adams, G. A. *Carbohyd. Res.* **1970**, *12*, 261.

giving **4**. The methoxy group was then replaced by an acetoxy group,[16] followed by conversion to the anomeric fluoride (**5**).[17] Glycosyl bond formation[18] with **6**[19] gave **7**. The silyl protecting groups were removed[20] followed by reduction of the azide[21] and removal of the benzyl groups giving **2**.[22]

Reaction of **2** with a variety of acid chlorides and *N*-hydroxysuccinimidyl esters gave reasonable yields of the corresponding amides. Glycosylceramides are notoriously insoluble, and the influence of this insolubility in purification may have decreased reaction yields. Nevertheless, using dansyl chloride and a dansyl amide tethered to an *N*-hydroxysuccinimidyl ester[23] (**9**), we were able to prepare **8** and **10** (Scheme 2).

Scheme 2. Preparation of Dansyl-Appended Glycolipids **8** and **10**



[a] Reagents: (a) AcCl, MeOH (86% yield); (b) BnBr, 18-crown-6, NaH, THF (95% yield); (c) AcOH, H_2SO_4 (84% yield); (d) HF·pyridine, pyridine (78% yield); (e) MS 4 Å, $AgClO_4$, $SnCl_2$, THF (44% yield); (f) TBAF, THF (81% yield); (g) PPh_3/H_2O, THF (quantitative yield); (h) NH_3/Na, −78 °C (53% yield).

The constraints placed upon the carbohydrate portion of glycosylceramides by CD1d and T cell receptors are not fully understood, and it was not clear if the presence of a fluorophore or similarly sized appendage would be tolerated. Therefore, we used a tether between the fluorophore and the carbohydrate in **10** to determine how a flexible tether would influence CD1d and T cell receptor binding.

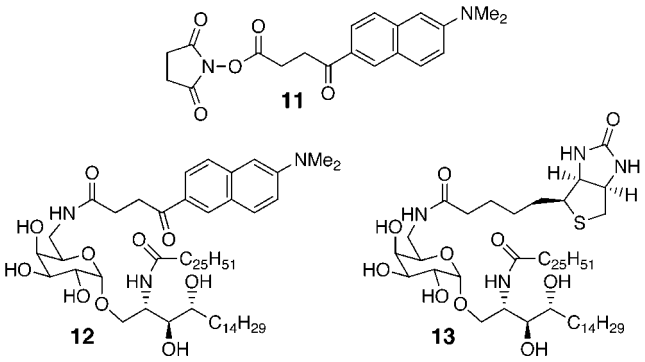


Figure 2. Structures of *N*-hydroxysuccinimidyl ester derivative of prodan (**11**) and prodan- and biotin-labeled α-galactosylceramides **12** and **13**, respectively.

Two other small molecules were appended onto **2**. Prodan is a fluorophore with a high quantum yield that responds to the polarity of its environment via large changes in its emission wavelength.[24] It is probable that the environment of a fluorophore appended to a galactosylceramide will change upon complex formation with a protein. Consequently, we anticipate being able to visualize the binding of the appended glycolipid via fluorescence modulation. In anticipation of attaching prodan to a glycolipid, compound **11** was prepared from 4-(6-methoxy-[2]naphthyl)-4-oxobutyric acid[25] by nucleophilic displacement of the methoxy group with lithium dimethylamide[26] followed by reaction with DCC and *N*-hydroxysuccinimide. Reaction of *N*-hydroxysuccinimidyl ester **11** with **2** gave **12** in 46% yield. Similarly, reaction of **2** with the *N*-hydroxysuccinimidyl ester of biotin gave **13** in 52% yield.

The abilities of compounds **8**, **10**, **12**, and **13** to stimulate NKT cells were gauged by measuring IL-2 production using

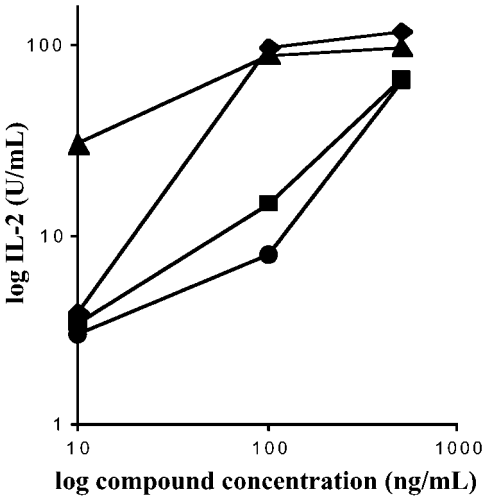


Figure 3. NKT cell stimulatory activity of fluorophore-appended 6-amino-6-deoxygalactosylceramides **8**, **10**, **12**. **1** (♦); **8** (●); **10** (▲); **12** (■).

(16) Davis, N. J.; Flitsch, S. L. *J. Chem. Soc., Perkin Trans. 1* **1994**, 359.
(17) Hayashi, M.; Hashimoto, S.; Noyori, R. *Chem. Lett.* **1984**, 1747.
(18) Hashimoto, S.; Hayashi, M.; Noyori, R. *Tetrahedron Lett.* **1984**, *25*, 1379.
(19) (2*S*,3*S*,4*R*)-3,4-Bis-*tert*-butyldimethylsisyloxy-2-hexaosanoylamino-4-octadecanol was prepared by coupling of phytosphingosine (Avanti Polar Lipids) with hexacosanoic acid (DIC, HOBT) followed by the protection/deprotection scheme from ref 20.
(20) Takikawa, H.; Muto, S.; Mori, K. *Tetrahedron* **1998**, *54*, 3141.
(21) Vaultier, M.; Knouzi, N.; Carrié, R. *Tetrahedron Lett.* **1983**, *24*, 763.
(22) Sakai, T.; Ueno, H.; Natori, T.; Uchimura, A.; Motoki, K.; Koezuka, Y. *J. Med. Chem.* **1998**, *41*, 650.
(23) Wang, F.; Schwabacher, A. W. *J. Org. Chem.* **1999**, *64*, 8922.

an immobilized CD1d assay.[27] Briefly, soluble, biotinylated CD1d was loaded onto precoated avidin plates, and the plates were pulsed with incrementally varied concentrations of glycolipids. After being washed, the plates were treated with a CD1d-restricted Vα24 NKT cell hybridoma and IL-2 release was measured using ELISA.

Compounds **8**, **10**, **12**, and **13** were highly stimulatory. Although **8** and **12** appear to be slightly less efficient in the results from the experiment shown in Figure 3, no significant differences among the compounds were found in repeated experiments (at least three experiments for each compound). In a separate series of experiments (e.g., Figure 4), **13** was slightly but reproducibly more efficient in stimulating NKT cells than **1**. Similar results were observed using CD1d-transfected rat basophilic leukemia cells for antigen presentation to NKT cell hybridomas.

Interestingly, attachment of a dansyl group directly at C6″ (as in **8**) or through a tether did not cause a significant loss of stimulating properties. Similarly, alteration of the appended group (i.e., dansyl vs prodan vs biotin) did not greatly affect the abilities of glycolipids to stimulate NKT cells.

Because the glycolipids are appended in the carbohydrate portion of the molecules, it was anticipated that the labels would not interfere with association of the glycolipids with CD1d. Gratifyingly, we found that modification of the glycolipids at C6″ with small molecules allowed retention of NKT cell stimulating properties. Attachment of fluorophores and biotin to α-galactosylceramides is expected to facilitate elucidation of the structural features of glycolipids necessary for NKT cell stimulation. Fluorescence and surface plasmon resonance studies are underway to characterize the association of appended glycolipids with CD1d, and results will be reported in due course.

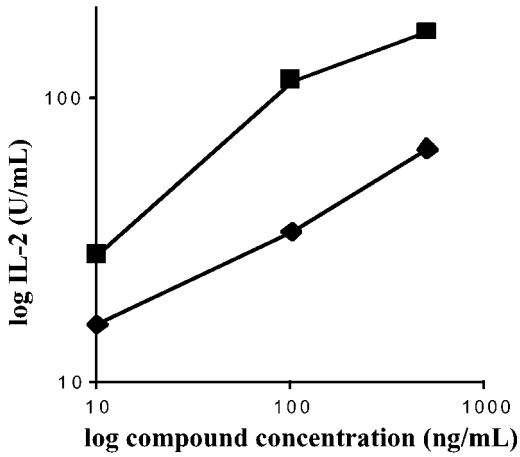


Figure 4. NKT cell stimulatory activity of biotin-appended 6-amino-6-deoxygalactosylceramide **13**. **1** (♦); **13** (■).

Acknowledgment. Generous financial support from the NSF (CAREER) (P.B.S.) and the Human Frontier Science Program (C.F.) is gratefully acknowledged.

Supporting Information Available: Experimental details for the syntheses of **8**, **10**, **12**, and **13**. This material is available free of charge via the Internet at http://pubs.acs.org.

OL025565+

(24) Weber, G.; Farris, F. J. *Biochemistry* **1979**, *18*, 3075.
(25) Khan, M. S. Y.; Siddiqui, A. A. *Indian J. Chem., Sect. B* **2000**, *39*, 614.
(26) Weber, G.; Farris, F. J. *Biochemistry* **1979**, *18*, 3075.
(27) Benlagha, K.; Weiss, A.; Beavis, A.; Teyton, L.; Bendelac, A. *J. Exp. Med.* **2000**, *191*, 1895.