ORGANIC LETTERS

2006
Vol. 8, No. 24
5493−5496

Thio-isoglobotrihexosylceramide, an Agonist for Activating Invariant Natural Killer T Cells

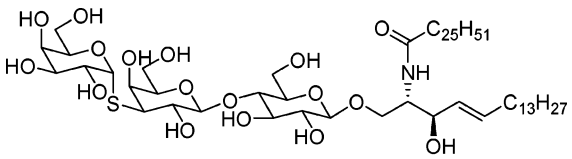
Chengfeng Xia,[†] Dapeng Zhou,*[,‡] Chengwen Liu,[‡] Yanyan Lou,[‡] Qingjia Yao,[†] Wenpeng Zhang,[†] and Peng George Wang*[,†]

Departments of Biochemistry and Chemistry, The Ohio State University, Columbus, Ohio 43210, and the Department of Melanoma Medical Oncology, University of Texas M.D. Anderson Cancer Center, 7455 Fannin Street #SCR2.3150, Houston, Texas 77054

pgwang@chemistry.ohio-state.edu; dzhou@mdanderson.org

Received September 5, 2006

ABSTRACT



S-iGb3

Thio-isoglobotrihexosylceramide (*S*-iGb3) might be resistant to α-galactosidases in antigen-presenting cells and have a longer retaining time in the lysosome before being loaded to CD1d. The biological assay showed that *S*-iGb3 demonstrates a much higher increase as a stimulatory ligand toward invariant natural killer T (*i*NKT) cells as compared to iGb3.

The well-known $CD4^+$ and $CD8^+$ T cells of the immune system typically recognize specific peptide antigens presented by major histocompatibility complex (MHC) class I or MHC class II molecules.[1] However, a unique subset of T cells called invariant natural killer T (*i*NKT) cells recognize glycolipid antigens presented by the MHC class I-like molecule called CD1d. *i*NKT cells represent a distinct population of T cells that express conserved $\alpha\beta$ T cell receptors (TCR) and natural killer (NK) receptors.[2,3] Mouse (m) Vα14 and human (h) Vα24 NKT cells regulate a number of critical biological conditions in vivo, including malignancy, infection, and autoimmune diseases, through the rapid secretion of T helper 1 (Th1) and Th2 cytokines and chemokines (Figure 1).[4,5]

It was found that α-galactosylceramides can stimulate *i*NKT cells to produce both interferon (IFN)-γ and interleukin (IL)-4 cytokines.[6] Recently, the first natural glycosphingolipid ligand, isoglobotrihexosylceramide (iGb3), was found to stimulate both human Vα24 NKT cells and mouse Vα14 *i*NKT cells.[7] In the lysosome, β-hexosaminidases remove the terminal GalNAc of iGb4 to produce iGb3 (Figure 2). Subsequently, iGb3 is degraded to β-lactosylceramide (β-LacCer) by the removal of the terminal Gal with α-galactosidase.

[†] The Ohio State University.

[‡] University of Texas M.D. Anderson Cancer Center.

(1) (a) Krummel, M. F.; Davis, M. M. *Curr. Opin. Immunol.* **2002**, *14*, 66. (b) Bromley, S. K.; Burack, W. R.; Johnson, K. G.; Somersalo, K.; Sims, T. N.; Sumen, C.; Davis, M. M.; Shaw, A. S.; Allen, P. M.; Dustin, M. L. *Annu. Rev. Immunol.* **2001**, *19*, 375. (c) Gao, G. F.; Jakobsen, B. K. *Immunol. Today* **2000**, *21*, 630.

(2) Parekh, V. V.; Wilson, M. T.; Van Kaer, L. *Crit. Rev. Immunol.* **2005**, *25*, 183.

(3) Bendelac, A.; Rivera, M. N.; Park, S.-H.; Roark, J. H. *Annu. Rev. Immunol.* **1997**, *15*, 535.

(4) Smyth, M. J.; Crowe, N. Y.; Hayakawa, Y.; Takeda, K.; Yagita, H.; Godfrey, D. I. *Curr. Opin. Immunol.* **2002**, *14*, 165.

(5) Serizawa, I.; Koezuka, Y.; Amao, H.; Saito, T. R.; Takahashi, K. W. *Exp. Anim.* **2000**, *49*, 171.

(6) (a) Xia, C.; Yao, Q.; Schuemann, J.; Rossy, E.; Chen, W.; Zhu, L.; Zhang, W.; De Libero, G.; Wang, P. G. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2195. (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) 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. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 1351.

(7) Zhou, D.; Mattner, J.; Cantu, C.; Schrantz, N., III; Yin, N.; Gao, Y.; Sagiv, Y.; Hudspeth, K.; Wu, Y.-P.; Yamashita, T.; Teneberg, S.; Wang, D.; Proia Richard, L.; Levery Steven, B.; Savage Paul, B.; Teyton, L.; Bendelac, A. *Science* **2004**, *306*, 1786.

10.1021/ol062199b CCC: $33.50
Published on Web 10/31/2006

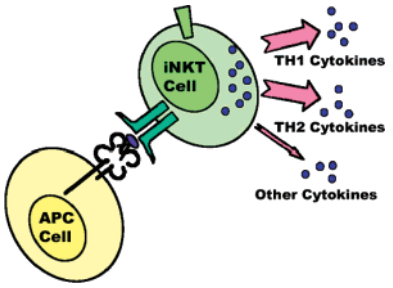


Figure 1. Activation of *i*NKT cells by α-GalCer/APC.

iGb3, but not iGb4 or *β*-LacCer, can be presented as an agonist ligand toward *i*NKT cell lines by plate-bound recombinant CD1d molecules. Hexb$^{-/-}$ cells can present iGb3 but fail to present iGb4, which directly indicates that processing of iGb4 into iGb3 is necessary for *i*NKT cell recognition.[7]

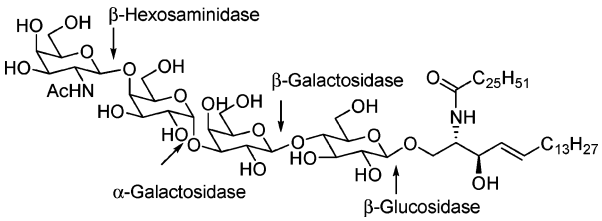


Figure 2. Metabolism of iGb4 in human cells.

Because the iGb3 is a natural "self" ligand, it is critical that the expression of this ligand should be tightly regulated. iGb3 can be hydrolyzed to *β*-LacCer to lose its agonist activity. The dynamics of the iGb3/CD1d complex that is presented to *i*NKT cells remains a puzzle.

We hypothesized that an α-galactosidase-resistant iGb3 analogue might be a valuable tool for studying the turnover of iGb3 in antigen-presenting cells. However, we first had to determine whether changes in the carbohydrate linkage preserve the agonist activity of iGb3. It is well-known that thioglycosides are resistant to glycosidase.[8] Conformational studies of *S*-linked oligosaccharides (thio-oligosaccharides) have suggested that the flexibility about the anomeric linkage is increased but that they still adopt a conformational space very similar to their *O*-linked counterparts.[9] Therefore, we hypothesize that the *i*NKT cell receptor should recognize the conformation of an iGb3 analogue with the distal galactose linked to LacCer through a thioglycoside. Herein, we report an efficient synthesis of *S*-iGb3 (Figure 3) and its bioassay, which showed that *S*-iGb3 can dramatically increase its stimulatory ability to *i*NKT cells compared to iGb3.

(8) Albrecht, B.; Puetz, U.; Schwarzmann, G. *Carbohydr. Res.* **1995**, *276*, 289.

(9) (a) Geyer, A.; Hummel, G.; Eisele, T.; Stefan, R.; Schjmidt, R. R. *Chem.−Eur. J.* **1996**, *2*, 981. (b) Aguilera, B.; Jimenez-Barbero, J.; Fernandez-Mayoralas, A. *Carbohydr. Res.* **1998**, *308*, 19. (c) Weimar, T.; Kreis, U. C.; Andrews, J. S.; Pinto, B. M. *Carbohydr. Res.* **1999**, *315*, 222.

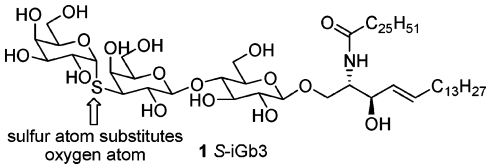


Figure 3. Structure of *S*-iGb3.

We reasoned that *S*-iGb3 could be synthesized from thiotrisaccharide donor **2** and a ceramide acceptor. The thiotrisaccharide would be prepared by the direct glycosylation of 3′-thio-lactoside derivative **3** with a galactosyl donor (Figure 4).

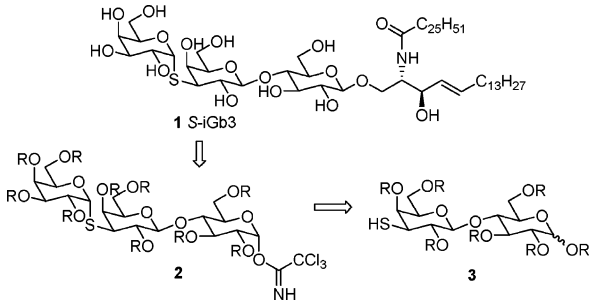
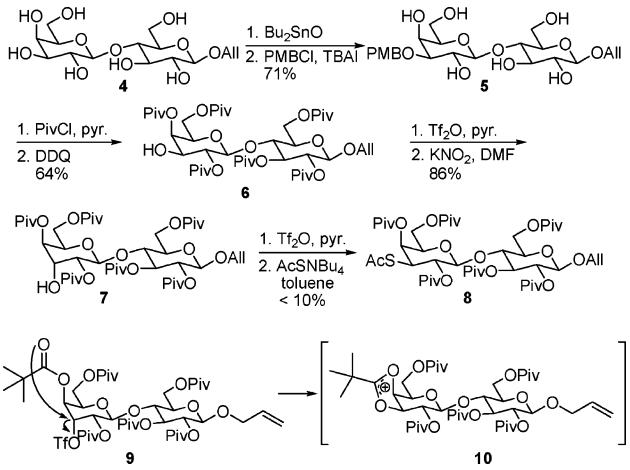


Figure 4. Retrosynthetic analysis.

First, we explored an efficient way to replace 3′-OH by a thio group, leaving the stereochemistry intact (Scheme 1).

Scheme 1. Synthesis of a Thio-lactose Moiety

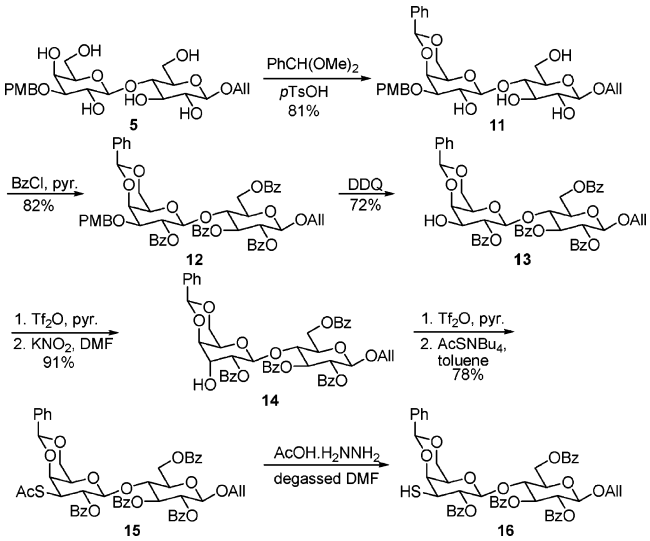


Compound **6** was prepared from the known compound **5**,[10] which was generated from allyl *β*-D-lactose **4**[10] by selectively dibutyltinoxide-promoted alkylation with *p*-methoxylbenzyl

(PMB) chloride,[11] in two steps including pivaloylation and PMB removal using DDQ. To get the original stereochemistry of the thio group, the inversion of 3′-OH stereochemistry was required before the second reversal in the introduction of thio. Therefore, the equatorial 3′-OH of **6** was converted to an axial configuration by treatment with triflic anhydride followed by KNO_2. Compound **7** was triflated again, and 3′-*O*-triflate was substituted with $AcSNBu_4$ to give *S*-acetate **8** in a S_N2 manner reforming the original stereochemistry.[12] However, the yield was very low (<10%) and varied in every trial. We assumed that the C-4′ hydroxyl derivative was formed because of the neighboring participation of 4′-*O*-ester in compound **9** via the cyclic intermediate **10** (Scheme 1).[13]

To prevent this neighboring group participation, an acetal group was used to protect the 4′-OH instead of an ester. The benzylidene was selected as the protecting group to protect the C-4′ and C-6′ hydroxyl groups.[13] Compound **11** was prepared by 4,6-benzylidene protection of lactoside **5** and subsequent benzoylation (Scheme 2). Following the same

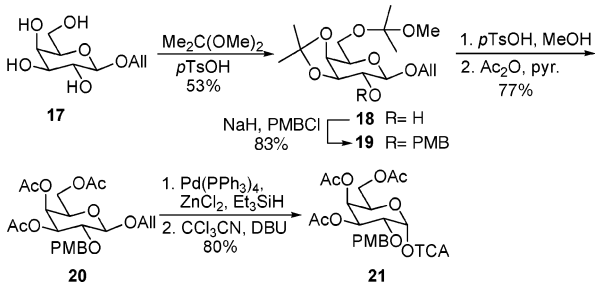
Scheme 2. Improved Synthetic Protocol of a Thio Acceptor



procedure as that in the synthesis of compound **8**, the thioester **15** was obtained in high yield. The acetyl was selectively removed by treatment with hydrazine acetate in degassed DMF to afford the thio-lactose derivative **16**.

The preparation of the galactosyl donor was outlined in Scheme 3. The benzyl group could not be used as a protecting group because of the incompatibility with the double bond in the lipid part at hydrogenation in the

Scheme 3. Synthesis of a Galactosyl Donor



deprotection stage. Our initial attempts toward the synthesis of the *per*-PMB protected galactoside donor gave a poor yield in the allyl group removal stage because some of the PMB groups were not stable. So, we switched and employed acetyl groups to protect the C-3, C-4, and C-6 hydroxyl groups, and only the C-2 hydroxyl group was protected with a PMB group for α-selectivity in glycosylation. The commercially available allyl *β*-D-galactose **17** was treated with dimethoxypropane (DMP) and a catalytic amount of *p*-TsOH to give **18**.[14] Then, the C-2 hydroxyl group was protected with PMB. Removal of the ketal in acidic conditions and direct acetylation of the resulting triol afforded compound **20**. The allyl group was removed by $Pd(PPh_3)_4$ in the presence of $ZnCl_2$ and Et_3SiH[15] followed by treatment with CCl_3CN and DBU to afford the donor **21**.[16]

The glycosylation of thio **16** with donor **21** using TMSOTf as a catalyst gave *S*-trisaccharide **22** in good yield (Scheme 4). The allyl group was removed and converted to Schmidt's-type donor **23** following procedures similar to those in the preparation of donor **21**. The protected glycolipid **25** was generated by glycosylation of donor **23** with azido-sphingosine **24**.[6a,17] The employment of an azido instead of a ceramide was to avoid the low reactivity resulting from H-bonding of the amido group. The azide was reduced with PPh_3 in the presence of trace amounts of water. The resulting amine was directly amidated with cerotic acid without further purification to give the protected *S*-iGb3 **27**. The benzylidene and two PMB groups were removed by 10% TFA in DCM. Finally, all the acyl groups were removed with NaOMe in methanol to afford the *S*-iGb3.

The bioassay using mouse (C57BL6) bone marrow derived dendritic cells showed that the *S*-iGb3, similar to iGb3, stimulates *i*NKT cell lines at a concentration above 10 ng/mL. A 10-fold increase was observed for *S*-iGb3 compared to iGb3 (Figure 5). The results indicated that the conformation change of *S*-iGb3 does not abolish the *i*NKT cell recognition. We cannot conclusively explain our observation that *S*-iGb3 is a stronger agonist ligand by using bone marrow derived dendritic cells as antigen-presenting cells. The most

(10) Kononov, L. O.; Kornilov, A. V.; Sherman, A. A.; Zyryanov, E. V.; Zatonsky, G. V.; Shashkov, A. S.; Nifant'ev, N. E. *Bioorg. Khim.* **1998**, *24*, 608.
(11) Zhang, W.; Wang, J.; Li, J.; Yu, L.; Wang, P. G. *J. Carbohydr. Chem.* **1999**, *18*, 1009.
(12) Rich, J. R.; Szpacenko, A.; Palcic, M. M.; Bundle, D. R. *Angew. Chem., Int. Ed.* **2004**, *43*, 613.
(13) Liakatos, A.; Kiefel, M. J.; Von Itzstein, M. *Org. Lett.* **2003**, *5*, 4365.
(14) Pozsgay, V.; Jennings, H. J. *Carbohydr. Res.* **1988**, *179*, 61.
(15) Eisele, T.; Windmuller, R.; Schmidt, R. R. *Carbohydr. Res.* **1998**, *306*, 81.
(16) Maruyama, M.; Takeda, T.; Shimizu, N.; Hada, N.; Yamada, H. *Carbohydr. Res.* **2000**, *325*, 83.
(17) Yao, Q.; Song, J.; Xia, C.; Zhang, W.; Wang, P. G. *Org. Lett.* **2006**, *8*, 911.

Scheme 4. Synthesis of *S*-iGb3

likely explanation is that the thioglycoside is resistant to α-galactosidases in the antigen-presenting cells, thus *S*-iGb3 has a longer retaining time in the lysosome before being loaded to CD1d. We have assumed that the degradation of iGb3 requires the physical interaction of glycolipids with saposins, and iGb3/CD1d will be inaccessible to glycosidase cleavage.[18]

However, we cannot exclude the possibility that the conformational change of *S*-iGb3 creates a conformation that binds to TCR differentially from iGb3. It is also possible that *S*-iGb3 and iGb3 prefer a different binding orientation to apo-E, which was recently identified as a necessary component for lipid antigen uptake by antigen-presenting cells.[19]

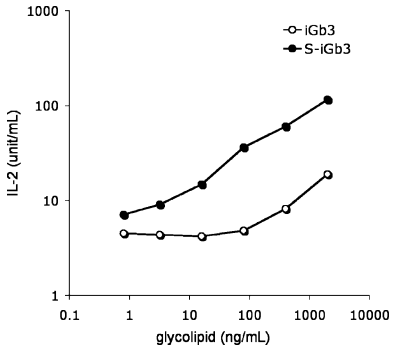


Figure 5. *S*-iGb3 and iGb3 dissolved in DMSO at 1.0 mg/mL. They were further diluted in a cell culture medium at indicated concentrations and pulsed to 100 000 mouse (C57BL6) bone marrow derived dendritic cells overnight, after the dendritic cells were washed with a culture medium and mixed with 50 000 DN32.D3 hybridoma cells. They were cocultured for 24 h, and the released IL2 was measured by CTLL assay. Data are representative of three independent experiments.

In summary, we successfully constructed 3′-thio-lactose via a repeated triflation−stereochemistry inversion sequence by using benzylidene as a protecting group on the C-4′ and C-6′ hydroxyl groups of lactose to eliminate neighboring group participation. Glycosylation of the 3′-thiolactose with a suitable galactosyl donor afforded the thio-trisaccharide. After introduction of the ceramide moiety, *S*-iGb3 was synthesized in 18 steps with 6.5% overall yield. The bioassay proved that *S*-iGb3 had significantly higher activity in stimulation of *i*NKT cells. *S*-iGb3 is a valuable tool for mechanistic studies on iGb3 processing in antigen-presenting cells. Further bioassays are ongoing in vivo and in vitro to ascertain the detailed mechanism.

Acknowledgment. This project was supported by The Ohio State University to Peng George Wang and the M.D. Anderson Cancer Center to Dapeng Zhou.

Supporting Information Available: Full experimental details as well as spectral data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

OL062199B

(18) (a) Zhou, D.; Cantu, C.; Sagiv, Y., III; Schrantz, N.; Kulkarni, A. B.; Qi, X.; Mahuran, D. J.; Morales, C. R.; Grabowski, G. A.; Benlagha, K.; Savage, P. B.; Bendelac, A.; Teyton, L. *Science* **2004**, *303*, 523. (b) Zhou, D. *Curr. Protein Pept. Sci.* **2006**, *7*, 325.

(19) Van den Elzen, P.; Garg, S.; Leon, L.; Brigl, M.; Leadbetter, E. A.; Gumperz, J. E.; Dascher, C. C.; Cheng, T.-Y.; Sacks, F. M.; Illarionov, P. A.; Besra, G. S.; Kent, S. C.; Moody, D. B.; Brenner, M. B. *Nature* **2005**, *437*, 906.