ORGANIC LETTERS

2006
Vol. 8, No. 5
911−914

Chemoenzymatic Syntheses of iGb3 and Gb3

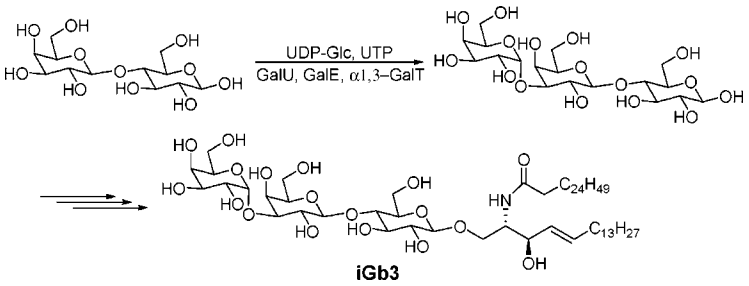
Qingjia Yao,[†,§] Jing Song,[‡,§] Chengfeng Xia,[†] Wenpeng Zhang,[†] and Peng George Wang*[,†]

Departments of Biochemistry and Chemistry, The Ohio State University, Columbus, Ohio 43210, and The State Key Laboratory of Microbial Technology, School of Life Science, Shandong University, Jinan, Shandong 250100, P.R. China

pgwang@chemistry.ohio-state.edu

Received December 19, 2005

ABSTRACT



Efficient chemoenzymatic syntheses of iGb3 and Gb3 have been developed. Isoglobotrihexose and globotrihexose were enzymatically synthesized by a three-enzyme system in both solid and solution phases. Then iGb3 and Gb3 were chemically synthesized by coupling of the corresponding trisaccharides with lipid.

CD1d-restricted invariant natural killer T (*i*NKT) cells (mouse Vα14 and human Vα24 NKT cells) regulate a number of critical biological conditions in vivo, including malignancy and infection, as well as autoimmune diseases, through the rapid secretion of cytokines and chemokines.[1,2] They act as a bridging system between innate and adaptive immunity.[3,4] Such cells specifically recognize microbial and endogenous glycolipid antigens in a CD1d-dependent way (Figure 1).[5,6]

α-Galactosyl ceramide (KRN7000) was the first discovered agonist ligand for *i*NKTs (Figure2).[7,8] However, α-galactosyl ceramide has not been found in mammals. Recently, Zhou et. al reported that isoglobotrihexosylceramide (iGb3)

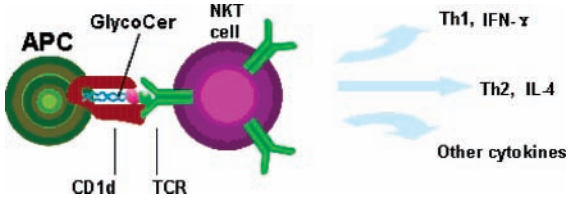


Figure 1. Acitvation of NKT cell.

[†] The Ohio State University.
[‡] Shandong University.
[§] Contributed equally to this work.

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10.1021/ol053070p CCC: $33.50
Published on Web 02/02/2006

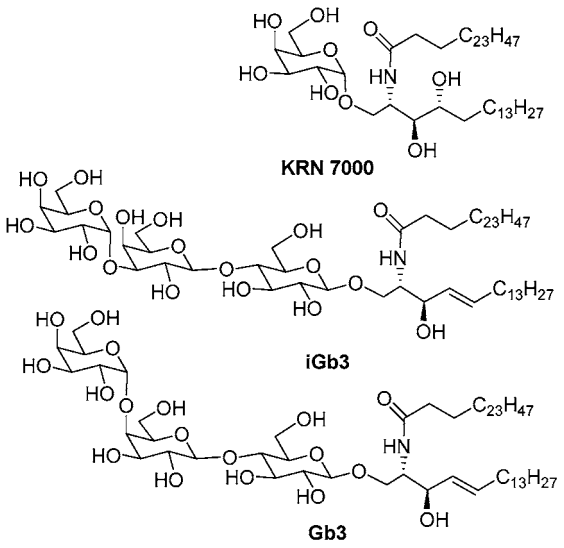

Figure 2. Structures of KRN7000, iGb3 and Gb3.

stimulates both human Vα24 NKT cell and mouse Vα14 NKT cells, and it was suggested that iGb3 acted as an endogenous ligand for *i*NKT cells.[9] In contrast, globotrihexosylceramide (Gb3) with a terminal Gal−α-(1→4)Gal sequence instead of Gal−α-(1→3)Gal in iGb3 has no stimulatory ability toward *i*NKT cells (Figure 2).

Current research is hampered by the rather limited access to iGb3. Purification of iGb3 from natural sources requires unique biochemical and analytical apparatus and typically furnishes milligram quantities of the product in low yield.[10] Chemical syntheses of iGb3[9] and Gb3[11] have been reported; these have prepared the trisaccharide donors from lactose and galactose and coupled the donor with the lipid acceptor. The chemical synthesis of the lipid part has been well documented in recent years.[12,13] The complexity of the chemical synthesis mainly stems from the tedious preparation of the trisaccharide donors, which require selective protections, deprotections, and glycosylations. In the reported chemical syntheses of iGb3 and Gb3, the trisaccharide donor is prepared in 17−19 steps. However, chemical transformation of the free trisaccharide to the donor can be achieved in three steps. Therefore, taking advantage of the high efficiency of enzymatic syntheses of trisaccharides, a chemoenzymatic approach, including the enzymatic synthesis of the trisaccharide and subsequent chemical coupling of this sugar to ceramide, could dramatically shorten the current route to iGb3 and Gb3. Herein we report the efficient chemoenzymatic syntheses of iGb3 and Gb3.

First, we explored more efficient enzymatic syntheses of free trisaccharides. Generally, enzymatic glycosylation is one of the most practical methods for oligosaccharide synthesis.[14,15] The most effective approach to isoglobotrihexose and globotrihexose is the direct enzymatic synthesis using UDP-galactose (UDP-Gal) and lactose catalyzed by a single galactotransferase. However, the high cost of UDP-Gal limits application of the synthesis on a large scale. Multiple-enzyme sugar nucleotide regeneration systems have been extensively developed to avoid using costly stoichiometric amounts of sugar nucleotides.[16] Previously, we reported the enzymatic syntheses of globotrihexose and isoglobotrihexose using superbead or superbug techniques.[17,18] In these systems, UDP-Gal is generated through recycling of UTP by multiple enzymes as follows: UDP-Gal reacts with lactose to produce trisaccharide and the byproduct UDP. Then the UDP is recycled by the enzyme PykF to regenerate UTP. The UTP is further converted to UDP-Gal catalyzed by the enzymes GalU and GalPUT (Scheme 1). Nowadays, UTP is commercially available in large quantities at low price. Therefore, the direct use of UTP in stoichiometric amounts can dramatically simplify the multiple-enzyme UDP-Gal generation system. Herein we have developed a simpler two-enzyme UDP-Gal generation system to produce trisaccharides (Scheme 1). Briefly, glucosyl-1-phosphate reacts with UTP catalyzed by GalU to yield UDP-Glc. UDP-Glc is converted to UDP-Gal by epimerase, GalE.[19] A galactotransferase transfers the galactose from UDP-Gal to lactose and produces trisaccharide. For globotrihexose synthesis, a mixture of enzymes (GalE, GalU, and LgtC) was added to the solution of Glu-1-P, UTP, and lactose containing 0.01 M Tri-HCl and 0.01 M $MnCl_2$. The reaction mixture was stirred at room temperature for 2 days. The removal of proteins followed by Sephadex G-15 gel column separation provided globotrihexose in 82% yield. We also successively applied this system on solid phase to produce isoglobotrihexose in 78% yield. The His6-tagged enzymes GalE, GalU, and α-1,3-GalT were immobilized onto a Ni^{2+} resin column, and then a solution of Glu-1-P, UTP, and lactose containing 0.01 M Tri-HCl and 0.01 M $MnCl_2$ was circulated through the column driven by a pump at room temperature for 2 days. After the reaction was complete (monitored by TLC), the solution was pumped out for purification, and the column was ready for the next batch of reaction.

With the desired free trisaccharides in hand, we turned our attention to developing an efficient way of attaching the sugar to the ceramide to give a product with the β-configuration. It is generally recognized that an acyl protective group on C-2 in a glycosyl donor is required for β-selectivity in the glucosylation. Schmidt's glycosyl trichloroacetimidate

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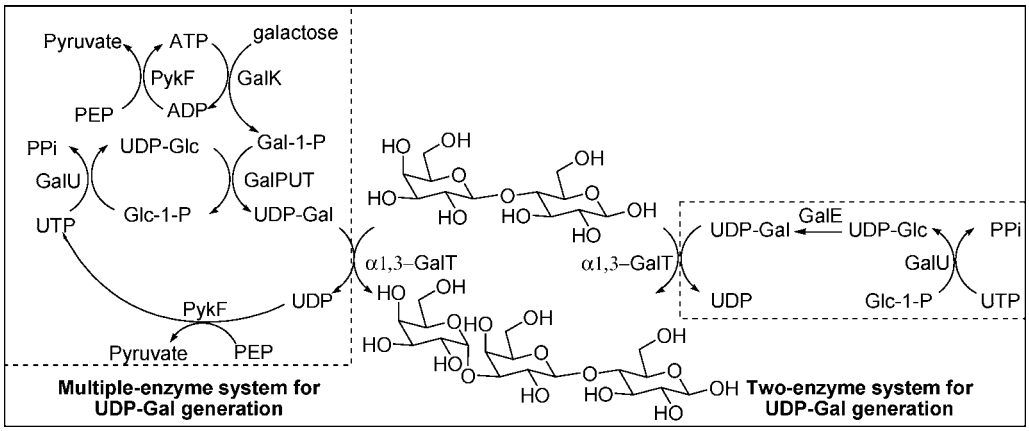
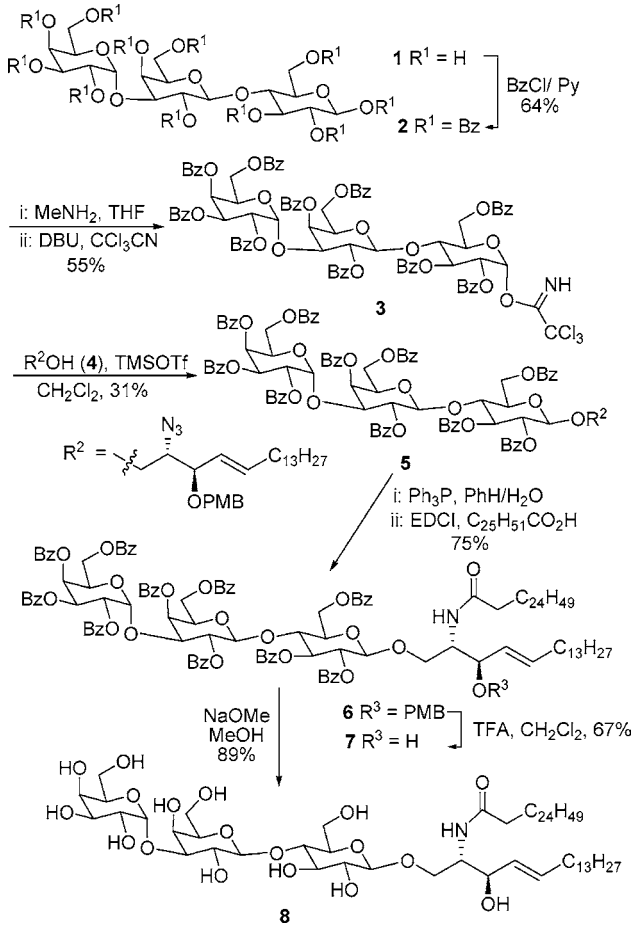
Scheme 1



glycosylation promoted by catalytic TMSOTf was our first choice, because of its relative preparative simplicity and the high reactivity of trichloroacetimidate glycosyl donors.[20] Regarding the lipid acceptor, we used 2-azido-sphingenine instead of the 2-*N*-hexacosanylsphingenine used by Savage in the chemical synthesis of iGb3, because the amide dramatically decreases the acceptor reactivity and leads to a low yield in glycosylation. It appears that the lipid acceptor with an acyl group at C-3 is not compatible with the azide reduction because of acyl migration. Therefore, the ether group, *p*-methoxybenzyl (PMB) was introduced to protect 3-OH, although this did entail an extra step later on for removal of the PMB group. Lipid acceptor **4** was synthesized by the reported procedure.[13]

First, we tried the perbenzoylated glycosyl trichloroacetimidate as glycosyl donor for the synthesis of iGb3 (Scheme 2). Isoglobotrihexose **1** was treated with benzoyl chloride in the presence of catalytic DMAP, and the reaction was stirred at 70 °C overnight to afford the perbenzoylated trisaccharide **2** in 64% yield. Selective deprotection of the anomeric benzoyl group with methylamine in THF was achieved at 50 °C.[21] Following the removal of solvent, the resulting alcohol was directly treated with trichloroacetonitrile in the presence of DBU to provide trichloroacetimidate donor **3** (55% yield over two steps).[22] The glycosylation of lipid acceptor **4** with donor **3** promoted by trimethylsilyl triflate gave an inseparable mixture of the desired *β*-glycosylation product **5** and the byproduct ortho ester, transformation of which to glycosyl lipid **5** was attempted by treatment of the mixture with stannic chloride.[23] Although the transformation did not afford more of the desired product **5** as we had expected, it was delightful in that it resulted in a separable mixture of product **5** and the ortho ester hydrolysis product, which facilitated column chromatographic purification. After purification, the *β*-glycosyl lipid **5** was obtained in 31% yield over two steps. The azido group was reduced with triphenylphosphine in a mixture of benzene and water at 70 °C.[24] After concentration, the resulting amine was directly coupled with cerotic acid in the presence of EDCI to provide protected iGb3 **6** with 75% yield over two steps.

Scheme 2



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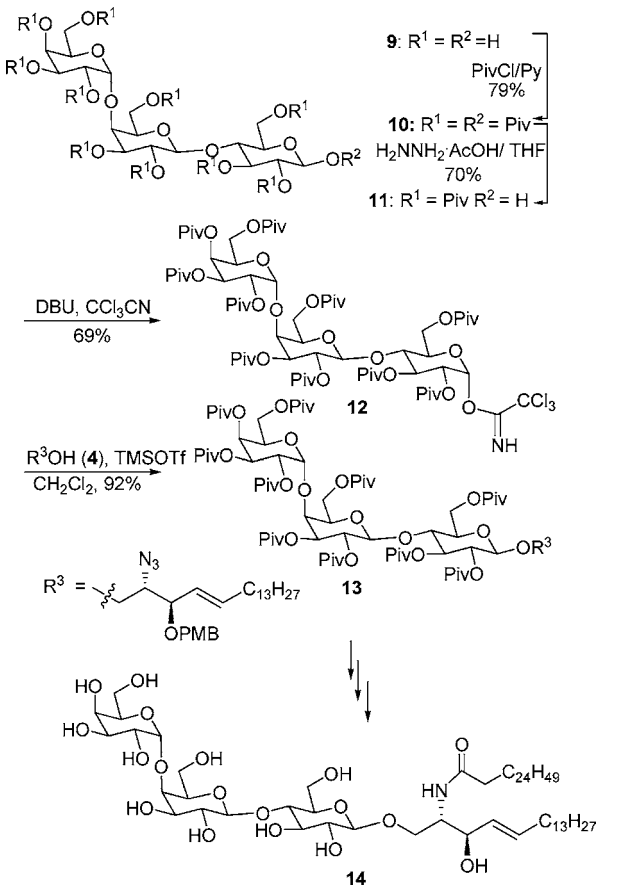
Scheme 3



To avoid incompatibility of a DDQ-mediated removal of the PMB group with the presence of double bond in compound **6**, PMB was removed by TFA in dichloromethane, resulting in compound **7** in 67% yield. Final global saponification of **7** with NaOMe provided iGb3 (**8**) in 89% yield.

Since use of the benzoyl group resulted in ortho ester formation in glycosylation, we next chose a more bulky pivaloyl group as a protecting group to prevent ortho ester formation. We turned our attention to the synthesis of Gb3 to optimize glycosylation using a perpivaloyl-protected donor. It was reasoned that the synthesis of Gb3 would also be suitable for iGb3 because of their highly structural similarity. iGb3 terminates with α-(1−3)-galactose instead of α-(1−4)-galactose in Gb3. This terminal slight difference does not affect glycosylation reactivity. As we expected, the glycosylation of lipid acceptor **4** with pivaloyl protected globotrihexosyl donor **12** produced a single glycosylation product with *β*-configuration in an excellent yield, 92% (Scheme 3).

Perpivalolyation of globotrihexose **9** with pivaloyl chloride in the presence of catalytic DMAP at 70 °C gave protected trisaccharide **10** with 79% yield. Selective deprotection of anomeric pivolyl group with the milder base, hydrazine acetate, at 50 °C provided compound **11** in 70% yield.[25] The alcohol **11** was converted to trichloroacetimidate **12** by treatment with trichloroacetonitrile in the presence of DBU with 69% yield. Trisaccharide **12** was activated with TMSOTf and coupled to lipid **4** to give the protected glycolipid **13** with 92% yield. Then compound **13** was converted to Gb3 (**14**) in the same way as in the synthesis of iGb3 with consistent yields.

In summary, we developed a three-enzyme system to synthesize the trisaccharide, isoglobotrihexose, and globotrihexose. iGb3 and Gb3 were synthesized from the corresponding enzymatically synthesized sugars. Two protecting groups, benzoyl and pivaloyl, on the donor part were tested for glycosylation. The pivaloyl group proved to be an ideal protecting group for the synthesis with excellent yield.

Acknowledgment. This project is supported by Ohio State University.

Supporting Information Available: Synthetic procedures and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

OL053070P

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