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The Total Synthesis of Immunostimulant α-Galactosylceramides from Naturally Configured α-Galactoside Raffinose

Zhenxing Zhang,[†] Wei Zhao,[†] Bin Wang,[†] Chengfeng Xia,[§] Wenpeng Zhang,*[,§] and Peng George Wang*[,†,‡,§]

College of Pharmacy, Nankai University, Tianjin 300071, China, State Key Laboratory of Elemento-organic Chemistry, Nankai University, Tianjin 300071, China, and Departments of Chemistry and Biochemistry, The Ohio State University, Columbus, Ohio 43210, United States

zhang.472@osu.edu; pwang@nankai.edu.cn

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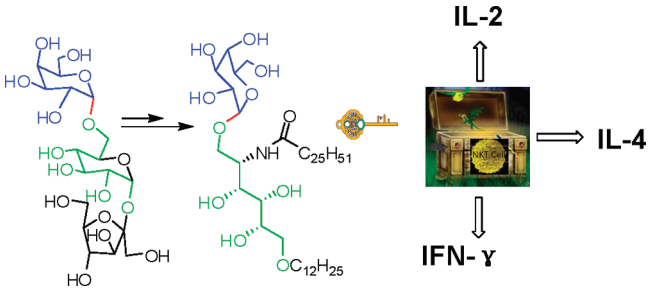
ABSTRACT



The total synthesis of absolute anomeric confirmation α-galactosylceramide analogues from raffinose is described. Using the naturally occurring α-galactoside raffinose as the starting material, the easily maneuverable protocol without glycosylation reactions ensured the critical α-linkage in the product and simplified the synthetic procedures. The immunostimulatory activities of the new α-galactosylceramides were validated by both *in vitro* and *in vivo* NKT cell stimulation assays.

α-Galactosylceramide is an agonist ligand for natural killer T cells (NKT cells). It has been proven that α-galactosylceramide is presented to the T cell receptor on the NKT cell surface by the CD1d molecules of antigen-presenting cells (APC). This stimulates the NKT immune response, which results in the rapid release of the Th1 and Th2 cytokines with significant influences.[1] KRN7000 is the most well-known molecule of the α-galactosylceramide class, and its therapeutic effects have been well studied in different disease models. Crystallographic analysis has indicated that the mouse CD1d (mCD1d) has a deep, narrow, and very hydrophobic ligand binding groove with two pockets denoted as A′ and F′.[2] Bioinformatic analysis has also suggested that the two long alkyl chains of the ligand are capable of being accommodated in the two hydrophobic pockets. Removal of the hydroxyl from the fatty acid chain has no significant effects on antigenicity. However, the biological activity is sensitive to the configuration between the sugar and the lipid, with the β-galactosylceramides only showing minimal or no activity.[3]

As the α-linkage is very critical for the biological functions of the glycosphingolipids, many different methodologies have been developed to chemically synthesize this critical structure. For example, the trichloroacetimidate donors have been successfully applied to the preparation of α-galactosylceramide.[4] Thioalkyl activated donors have also been used to prepare the α-galactosylceramide, although the selectivity was not ideal.[5] Glycosyl halides are other documented donors for the preparation of α-GalCer, with galactosyl fluoride, galactosyl bromide, and galactosyl iodide all being applied as donors in moderate yield and with good anomeric selectivity.[6] $AgClO_4$,[7] $BF_3 \cdot Et_2O$,[8] Bu_4NBr,[9] and phosphine oxide[10] are reported to serve as promoters for glycosidation. Phosphate[11] and

[†] College of Pharmacy, Nankai University.
[‡] State Key Laboratory of Elemento-organic Chemistry, Nankai University.
[§] The Ohio State University.

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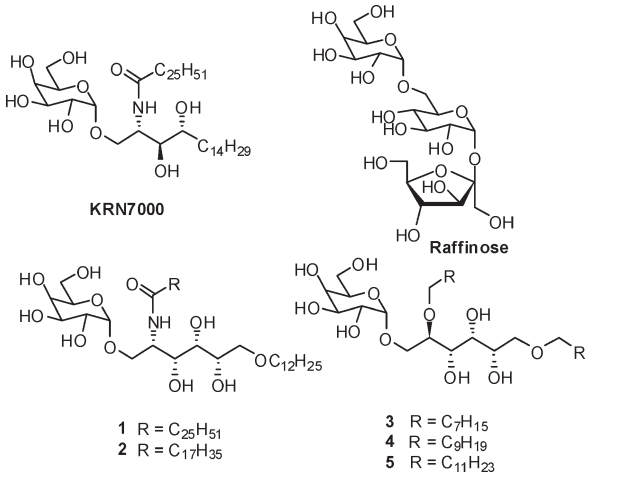
acetate[12] were also exploited for the preparation of α-GalCer. However, these glycosylation reactions produced mostly a mixture of α/β isomers which were very difficult to separate.

There is always suspicion about the anomeric purity of synthetic galactosylceramide as it is difficult to separate the α isomers from the β isomers, especially when they are present in trace amounts in the glycosylation reactions. Until now, there is no chemical glycosylation reaction in this area with 100% α/β selectivity. Time-consuming separation procedures are required to separate the isomers from the above synthetic methodologies, which lowers the reaction efficiency and prevents the methods from being extended to large scale synthesis. To overcome the difficulties and flaws of current synthetic methodologies, we chose to begin our synthesis of α-galactosyl lipids from the naturally configured α-galactoside, raffinose. By avoiding glycosylation reactions, a new and easily scaled synthesis of several KRN7000 analogues was developed via high yield reactions that could be easily adapted to industrial scale synthesis.

By comparing the KRN7000 structure and raffinose, we designed a series of new potential NKT cell ligands as shown in Scheme 1 (compounds **1**, **2**, **3**, **4**, **5**).

Scheme 1. Structures of KRN7000, Raffinose, and the Proposed α-Galactosylipids



From raffinose **6**, the fully benzylated derivative **7** was prepared in 80% yield by treatment with benzyl bromide and sodium hydride in DMF (Scheme 2). Partially benzylated sugar **8** was accessed, using a described procedure,[13] by selective cleavage of the β-fructofuranosidic linkage in **7** under acidic conditions in 90% yield. A subsequent reduction of hemiacetal **8** to diol **9** was conducted using $NaBH_4$ as the reductant which, among many other reductants screened, was found to be the best in terms of safety and reaction yield (Scheme 2).

Diol **9** was an ideal intermediate, having only two reactive sites for introducing the lipid chains. The easiest way of accomplishing this was through direct alkylation of

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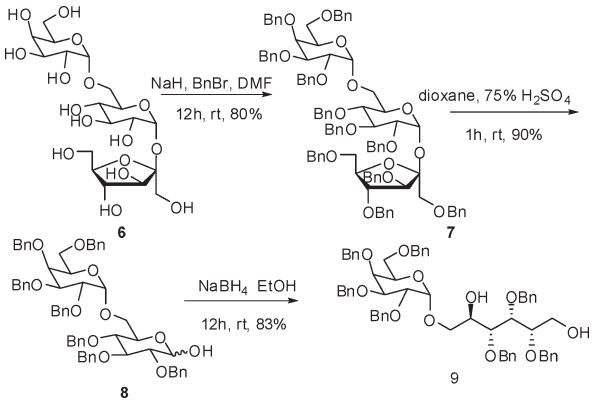
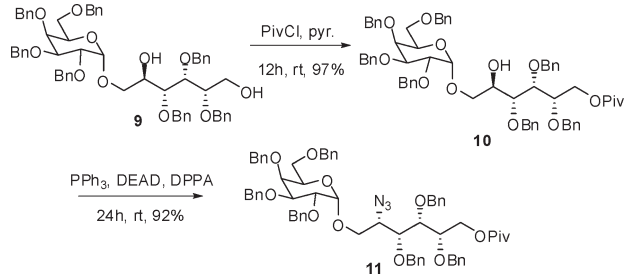
Scheme 2. Preparation of Intermediate Diol **9**



the two −OH groups with a 1-bromoalkane and NaH in DMF. Via this method, and following deprotection, a series of α-galactosyllipids **3**, **4**, **5** (Scheme 1) was obtained. Unfortunately, they had no stimulatory effect on our studied cells. So, we decided to produce a molecule that was more like the known KRN7000, and the amide moiety was needed.

Without purification of the reduced diol **9**, monopivaloate **10** was synthesized in 97% yield by regioselective pivaloylation (PivCl/pyridine) at the primary −OH group.

Scheme 3. Synthesis of Azide **11**

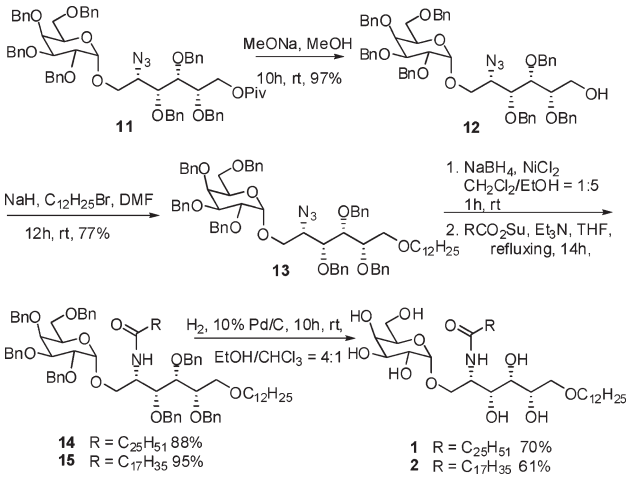


The synthesis of azide **11** was achieved in 92% yield by treating alcohol **10** with DPPA in the presence of Ph_3P and DEAD in toluene (Scheme 3). Deacylation via base-catalyzed methanolysis and alkylation with 1-bromododecane in the presence of NaH in DMF afforded ether **13** in 77% yield (Scheme 4).

Reduction of the azide **13** was achieved with sodium borohydride in the presence of a catalytic amount of nickel chloride, affording the corresponding amine. Treatment of this amine with active ester in the presence of Et_3N gave amide **14** and **15** both in 95% overall yield (2 steps). Finally, global debenzylation via catalytic hydrogenation over Pd/C gave the final α-galactosylceramide **1** and **2** in 70% and 63% yield respectively (Scheme 4).

The NKT stimulation activities of the new compounds were evaluated in both an *in vitro* hybridoma assay and *in vivo* mouse splenocytes stimulation assay.

Scheme 4. Synthesis of the New α-Galactosylceramides



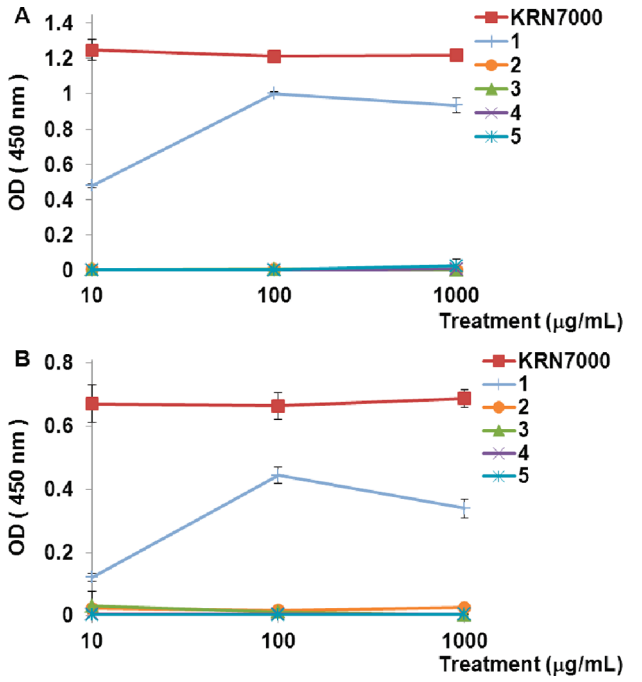


Figure 1. IL-2 cytokine releasing profile from stimulated NKT hybrodoma cells. (A) Hybridoma DN3A4-1.2. (B) Hybridoma N38-2H4.

For the *in vitro* hybridoma assay, as Figure 1 shows, among all of the new analogues synthesized, compound **1** caused significant stimulation of the two hybridoma cell lines, with comparable strength to KRN7000. All of the other four analogues did not show any stimulation in the hybridoma assay.

In the case of the *in vivo* mouse splenocytes culture assay, as Figure 2 shows, compound **1** showed significant stimulation of both cytokines, especially the Th2 cytokine. The activity of compound **1** is quite impressive among all of the

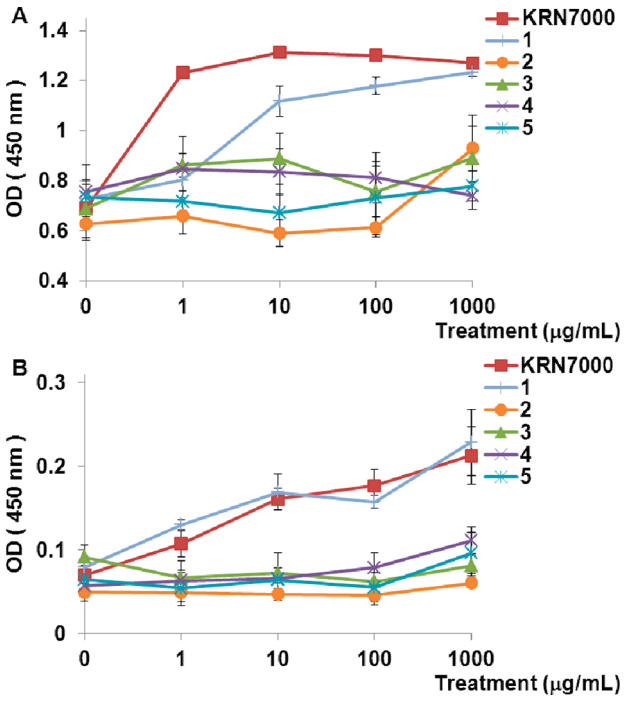


Figure 2. IFN-γ (A) and IL-4 (B) releasing profile from glycolipids stimulated mouse splenocytes.

known KRN7000 analogues, as it has comparable cytokine stimulation efficiency.

Based on these results, it can be concluded that the analogue from the newly developed synthetic methodology still maintains the immunogenicity against NKT cells. The synthetic strategy achieved its target of producing a functional KRN7000 analogue with the correct absolute configuration via a new and simplified procedure. The biological results also reveal that replacement of the acyl chain with a short carbon chain (compounds **3**, **4**, **5**) eliminates the analogues' immunogenicity, probably due to a lack of affinity or a lack of flexibility when the compounds bind to CD1d. At the same time, compound **1** maintained a high immunogenicity by keeping its long acyl chain for binding affinity (compound **2** does not have a long enough acyl chain). The introduction of an −O− group in the sphingosine chain does not significantly change the immunogenicity of the compound.

In summary, a new kind of α-galactosylceramide has been successfully synthesized by an easy method from raffinose. This group of novel α-galactosylceramides has been shown to be immunostimulatory for NKT cells by biological evaluation. In our synthesis, the naturally occurring α-galactoside, raffinose, was applied as the starting material to ensure the emergence of the α-linkage in the product and to simplify the synthetic route. This method also provides an efficient methodology for producing other galactosylceramides if the protocol is extended to other available saccharides and varied lipid chains. It will also prove useful for the facile generation of glycolipid libraries for novel NKT cell ligand discovery.

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Supporting Information Available. Experimental procedures for the preparation of all new compounds and the detail experimental procedures of the biological activity. This material is available free of charge via the Internet at http://pubs.acs.org.