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Synthesis and biological evaluation of α-galactosylceramide (KRN7000) and isoglobotrihexosylceramide (iGb3)

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Abstract—Glycoceramides can activate NKT cells by binding with CD1d to produce IFN-γ, IL-4, and other cytokines. An efficient synthetic pathway for α-galactosylceramide (KRN7000) was established by coupling a protected galactose donor to a properly protected ceramide. During the investigation, it was discovered that when the ceramide was protected with benzyl groups, only β-galactosylceramide was produced from the glycosylation reaction. In contrast, the ceramide with benzoyl protecting groups produced α-galactosylceramide. Isoglobotrihexosylceramide (iGb3) was prepared by glycosylation of Galα1-3Galβ1-4Glc donor with 2-azidosphingosine in high yield. Biological assays on the synthetic KRN7000 and iGb3 were performed using human and murine *i*NKT cell clones or hybridomas.

α-Galactosylceramides (α-GalCers) were discovered from an extract of a marine sponge, *Agelas mauritianus*, by the Pharmaceutical Division of the Kirin Brewery Company in 1993 and were named agelasphins (AGLs).[1] Although a number of β-galactosylceramides have been reported, AGLs were the first reported galactosylceramides having an α-galactosyl linkage. All these compounds were active substances in prolonging the life span of mice when intraperitoneally inoculated with B16 mouse melanoma cells.[2] Therefore, various analogues were synthesized to explore for candidates with similar antitumor activity and that were readily available from large-scale chemical synthesis. Among these compounds, AGL-582 emerged as the most desirable candidate for clinical application and was designated as KRN7000 (Fig. 1).[3] Further studies revealed that α-GalCers could stimulate natural killer T (NKT) cells via presentation by CD1d. The NKT cells can regulate a variety of microbial, allergic, autoimmune, and tumor conditions through the rapid secretion of interleukin-4 (IL-4), interferon-γ (INF-γ), and other cytokines and chemokines.[4] Because α-GalCers were either separated from a marine sponge or chemically synthesized, they are not the natural products of mammalian cells. Evidences showed that during the development of NKT cells from the mainstream T cell precursor pool to mature NKT cells, some endogenous glycolipid antigens were essential for this process by presentation of CD1d.[5] Recently, Zhou et al. demonstrated that isoglobotrihexosylceramide (iGb3) was the primary endogenous agonist ligand for NKT cells.[6] Therefore, there has been high demand for both KRN7000 and iGb3 for immunology research and for preclinical investigation.

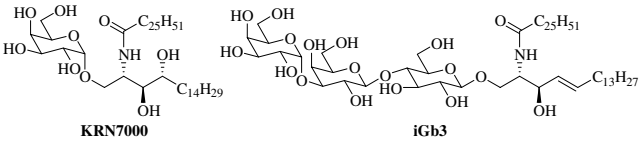

Figure 1. Structures of KRN7000 and iGb3.

Several methods for synthesis of KRN7000 have been reported, which involved a crucial galactosylation reaction with different galactose donors and lipid acceptors.[7–10] Herein we used the active 'armed' perbenzyl-

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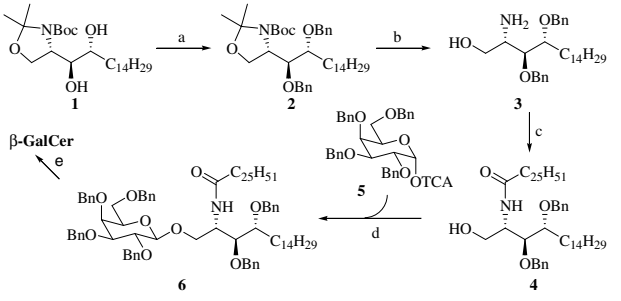
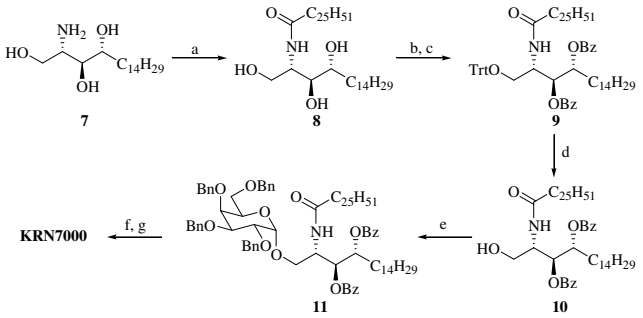


Scheme 1. Reagents and conditions: (a) NaH, BnCl, DMF, 87%; (b) TFA-H_2O (20:1), 85%; (c) $C_{25}H_{51}CO_2H$, EDCI, THF, 91%; (d) TMSOTf, Et_2O-THF (5:1), −23 °C, 57%; (e) H_2 (20 psi), 10% Pd/C, MeOH, 93%.



Scheme 2. Reagents and conditions: (a) $C_{25}H_{51}COSu$, THF, 94%; (b) TrtCl, DMAP, pyridine, 40 °C; (c) BzCl, DMAP, pyridine, 89% for two steps; (d) *p*TsOH, MeOH, 92%; (e) **5**, TMSOTf, Et_2O-THF (5:1), −23 °C, 59%; (f) NaOMe, MeOH, 94%; (g) H_2 (20 psi), 10% Pd/C, MeOH, 91%.

galactoside **5**[11] as a donor. We first used a ceramide **4** with 3,4-dihydroxyl groups protected by benzyl groups as an active 'armed' acceptor. As shown in Scheme 1, the partially protected phytosphingosine **1**[12] was subjected to NaH and then benzyl chloride in dry DMF to protect the two secondary hydroxy groups with benzyl group. Removal of the ketal and Boc with trifluoroacetic acid afforded the amine **3**. Then it was reacted with fatty acid under the condensation with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDCI) to furnish the ceramide **4**. The ceramide **4** was subjected to glycosylation with donor **5** with TMSOTf as an activator under −23 °C. Surprisingly, the β-glycosylated product **6** was separated as a single glycosylation product. The anomeric configuration was further confirmed by NMR spectrums of β-GalCer which were consistent with reported data.[13]

We hypothesized that the formation of β-anomeric product was due to the high reactivity of the acceptor. While the donor was activated by TMSOTf, the armed acceptor attacked the donor in an SN2 fashion with a transition state **A** (Fig. 2), before the full development of oxacarbonium intermediate, resulting in β-configuration production. We then predicted that a less reactive acceptor would favor α-glycosylation through a fully developed oxacarbonium intermediate.

Thus, we switched the protecting groups on the ceramide from benzyl to benzoyl (as ceramide **10**) to decrease the acceptor reactivity (Scheme 2). The commercially available phytosphingosine was condensed with hexacosanic acid *N*-hydroxysuccinimide ester to give the amide **8**.[10] Then the primary hydroxy group was selectively protected with trityl chloride in pyridine. When TLC showed that the starting material disappeared, benzoyl chloride was added to protect the remaining two hydroxyl groups without work-up to afford **9**. The trityl was removed in methanol with the presence of *p*-toluenesulfonic acid. Then the ceramide **10** was subjected to glycosylation with donor **5** under activator TMSOTf. The glycoceramide **11** was isolated as the sole product which had the desired α-configuration. Then the benzoyl groups on sphingosine and the benzyl groups on sugar were removed by saponification in anhydrous methanol with NaOMe and hydrogenation under catalysis of 10% Pd/C, respectively, to produce KRN7000.[14]

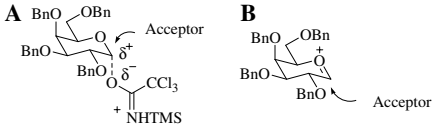


Figure 2. Transition state of glycosylation. (A) The armed acceptor attacked donor via SN2 pathway; (B) the disarmed acceptor reacted with fully formed oxacarbonium donor.

It is well known that the glycosylation outcome depends on the delicate balance of donor/acceptor pair reactivity. Herein we found out that the 'armed' sugar donor with 'armed' lipid acceptor produced a β-configuration and 'armed' sugar donor with 'disarmed' lipid acceptor produced an α-configuration. It is remarkable to see that the concept of 'armed' and 'disarmed' even works between a sugar donor and lipid acceptor.

Our synthesis of iGb3 started with the chemical synthesis of Galα1-3Galβ1-4Glc reported by our laboratory (see Scheme 3).[15,16] The known 1-benzyl-peracetyl lactose **12** was deacetylated to generate 1-benzyl lactose **13**. The regioselective monoalkylation of the C3 hydroxy group of the galactose unit with *p*-methoxybenzyl chloride (PMBCl) was achieved through activating this hydroxy group with dibutylstannylene acetal. Originally, acetyl group was used to protect the remaining hydroxy groups on compound **13**. In the glycosylation of the trisaccharide donor with the lipid, however, only an orthoester was obtained. Efforts for transforming this orthoester to glycoside by treatment with tin chloride or other Lewis acids failed. The bulky pivaloyl group, which could significantly restrain the formation of the orthoester, was then employed to give **15**. Oxidative cleavage of the PMB group by DDQ released the 3′-OH to afford the disaccharide acceptor **16**. The glycosylation of acceptor **16** with perbenzyl phenyl thiogalactoside donor **22** was carried out under activation by NIS/TfOH to afford the protected trisaccharide **17** in a yield of 92%. At this juncture, the benzyl groups have to be switched to acetyl groups to avoid the reduction of the double bond on the lipid during hydrogenation.