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## Synthesis of all stereoisomers of KRN7000, the CD1d-binding NKT cell ligand

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### ABSTRACT

KRN7000 is an important ligand identified for CD1d protein of APC, and KRN7000/CD1d complex can stimulate NKT cells to release Th1 and Th2 cytokines. In an effort to understand the structure–activity relationships, we have carried out the synthesis of a complete set of the eight KRN7000 stereoisomers, and their biological activities have been examined.

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In the early 1990s potent antitumor activity was located in the agelasphins extracted from Okinawan sponge *Agelas mauritianus* by Kirin Pharmaceuticals, and  $\alpha$ -galactosyl ceramide ( $\alpha$ -GalCer) named KRN7000 was found to have the best activity in the SAR (structure–activity relationship) studies of related synthetic glycolipids.<sup>1</sup> Further studies have revealed that T cell receptors (TCR) of NKT cells recognize KRN7000 presented by major histocompatibility complex (MHC) class I-like protein CD1d of antigen presenting cells (APC). Upon recognition of KRN7000/CD1d complex, NKT cells rapidly release large amounts of a broad range of cytokines including pro-inflammatory T helper 1 (Th1) cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$ , and anti-inflammatory Th2 cytokines such as interleukin-4, -10, and -13 (IL-4, -10, -13).<sup>2</sup> Th1 cytokines are thought to correlate with the antitumor, antiviral and antibacterial activities of KRN7000, whereas Th2 cytokine are believed to either delay or prevent the onset of autoimmune diseases such as type1 diabetes.<sup>3</sup>

Due to the biological significance, many  $\alpha$ -GalCer analogues have been synthesized and biologically evaluated to understand the structure–activity relationships (SAR). Studies focused on the sugar moiety of  $\alpha$ -GalCer indicated that the  $\alpha$ -anomeric galactose group is important for  $\alpha$ -GalCer to bind CD1d and to activate NKT cells through their TCR.<sup>4</sup> It was also found that the equatorial C2-OH group of the galactose is crucial for the activity of  $\alpha$ -GalCer; any modification at this position largely abolishes activity.<sup>5</sup> Modifications at the C3- and C6-OH groups of the galactose were found

to be tolerated.<sup>6</sup> The SAR studies involving the ceramide moiety of  $\alpha$ -GalCer can be separated into modifications of the acyl and phytosphingosine chain, and the polar portion of the ceramide. It was found that truncations of either the phytosphingosine or acyl chain resulted in  $\alpha$ -GalCer that biased NKT cells toward release of Th2 cytokines (IL-4).<sup>7</sup> Insertion of double bonds into the acyl chain of  $\alpha$ -GalCer (C20:2) seemed to bias toward Th2 responses.<sup>8</sup> Introduction of an aromatic residue to the acyl or phytosphingosine chain enhanced the Th1 cytokine profiles.<sup>9</sup> The  $\alpha$ -C-GalCer, in which the glycosidic oxygen was replaced by a methylene group stimulated strong Th1 responses in vivo from NKT cells.<sup>10</sup> The C3- and C4-OH groups of the phytosphingosine was reported essential for the activity of  $\alpha$ -GalCer, although C3-OH group being more important than C4-OH group.<sup>4</sup>

These results were supported by crystal structural studies. Two X-ray crystallography studies of the CD1d/ $\alpha$ -GalCer complexes have revealed that the lipid chains of  $\alpha$ -GalCer fit tightly into the CD1d binding groove (A' and F' pockets). The good hydrogen-bonding interactions were observed between CD1d and  $\alpha$ -GalCer [2-OH group of the galactose group and Asp151 (mouse Asp153) of the CD1d; 3-OH group of the phytosphingosine and Asp80 of CD1d]. These bonds serve to anchor  $\alpha$ -GalCer in a distinct orientation and position it in the lipid binding groove.<sup>11,12</sup> Recently, the crystal structure of human NKT TCR-CD1d- $\alpha$ -GalCer complex showed that  $\alpha$ -GalCer protrudes minimally from the CD1d cleft with only the galactose group exposed for recognition by the NKT TCR. There are several hydrogen-bonding interactions between the CDR1 $\alpha$  and CDR3 $\alpha$  loops of TCR and  $\alpha$ -GalCer [Phe29 $\alpha$  of the CDR1 $\alpha$  loops and C4-OH group of the galactose group; Ser30 $\alpha$  of the CDR1 $\alpha$

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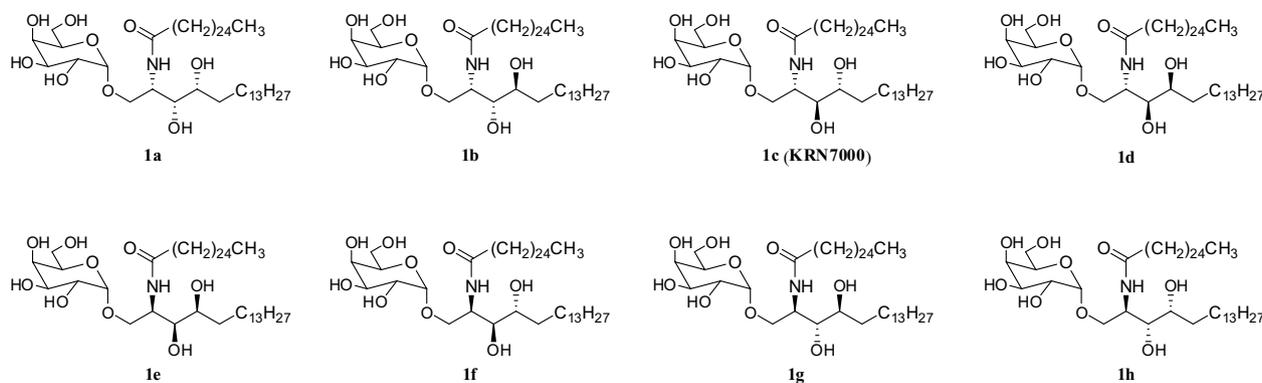


Figure 1. Structure of the eight KRN7000 stereoisomers.

loops and C3-OH group of the galactose group; Arg95 $\alpha$  of the CDR3 $\alpha$  loops and C3-OH group of the phytosphingosine; Gly96 $\alpha$  of the CDR3 $\alpha$  loops and C2-OH group of the galactose group.<sup>13</sup>

A variety of sugar and chain-modified analogues of KRN7000 have been prepared and their biological properties evaluated. However, a similar systematic study of phytosphingosine core-modified KRN7000 stereoisomers has not yet been carried out, probably because the stereoisomers of phytosphingosine are not readily available. Although phytosphingosine stereoisomers have been targets of much synthetic efforts, the complete set of all stereoisomers of phytosphingosine had not been available until recently. Previously, we developed practical synthetic methods for all four stereoisomers of sphingosine,<sup>14</sup> and extended them to gen-

erate all eight phytosphingosine stereoisomers in a divergent manner. These stereoisomers of sphingosine and phytosphingosine were also utilized in the construction of ceramide libraries, each library consisting of more than 500 compounds.<sup>15</sup> We wish to report herein the complete synthesis of all KRN7000 stereoisomers (Fig. 1) from the phytosphingosine stereoisomers and preliminary results on their biological activities in mouse and human iNKT cells.

Our syntheses of all eight KRN7000 stereoisomers entailed (1) preparation of appropriately protected derivatives of all phytosphingosines, (2) N-acylation of the backbone to ceramides, and (3)  $\alpha$ -glycosidation with protected galactose of the terminal hydroxyl group of the phytosphingosine residue after removal of the

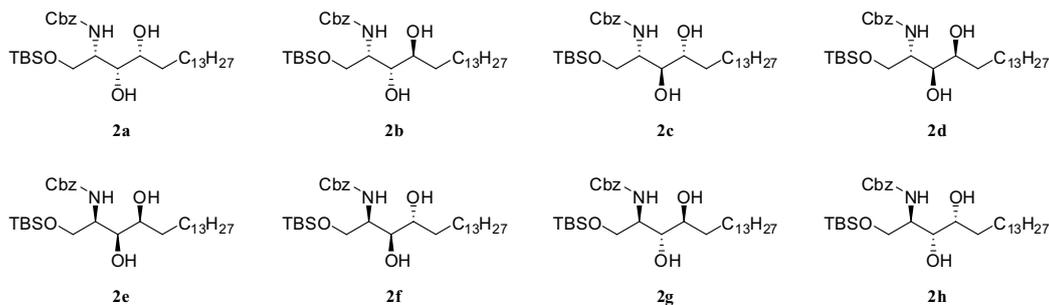
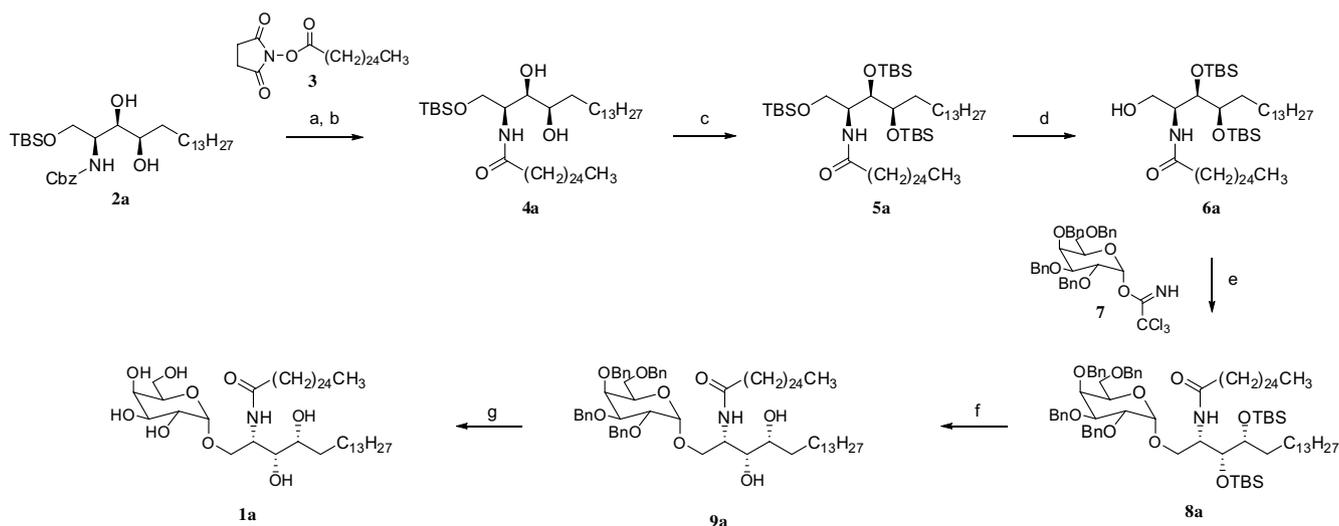


Figure 2. Structure of eight phytosphingosine stereoisomers derivatives.



Scheme 1. Reagents and conditions: (a) Pd/C, H<sub>2</sub>, THF, rt; (b) **3**, Et<sub>3</sub>N, THF, 50 °C, 85% (2 steps); (c) TBSOTf, 2,6-lutidine, CH<sub>2</sub>Cl<sub>2</sub>, rt, 90%; (d) HF-py, THF-pyridine, rt, 87% (e) BF<sub>3</sub>-OEt<sub>2</sub>, 4Å molecular sieves, THF-Et<sub>2</sub>O, -20 °C, 48%; (f) TBAF, THF, rt, 97%; (g) Pd(OH)<sub>2</sub>/C, H<sub>2</sub>, EtOH-CHCl<sub>3</sub>, rt, 88%.

**Table 1**  
Physical properties of KRN7000 stereoisomers

Compound	$[\alpha]_D^{25}$ (pyridine)	Mp (°C)
<b>1a</b>	+35.3 (c 0.30)	187
<b>1b</b>	+21.7 (c 0.51)	189
<b>1c</b>	+42.4 (c 0.50)	190
<b>1d</b>	+46.2 (c 0.51)	191
<b>1e</b>	+46.6 (c 0.43)	182
<b>1f</b>	+53.4 (c 0.35)	197
<b>1g</b>	+42.1 (c 0.49)	194
<b>1h</b>	+35.3 (c 0.36)	183

protecting group. Thus, we first prepared substantial quantities of protected derivatives of the eight stereoisomers of phytosphingosine (Fig. 2: **2a–2h**) from L- and D-serine via four sphingosine stereoisomers as previously reported.<sup>14,15</sup> Briefly, both the L- and D-serine were converted to the four stereoisomers of N,O-diprotected sphingosine. The double bond of each sphingosine isomer was epoxidized to provide the 'up' and 'down' epoxides, which after separation was regioselectively reduced to give the appropriately protected phytosphingosine isomers, respectively. In this way each sphingosine stereoisomer was converted to two phytosphingosine isomers shown in Figure 2. Transformation of each phytosphingosine stereoisomer to  $\alpha$ -galactosylceramide is illustrated for the case of isomer **2a**, and all the other isomers were similarly transformed to the corresponding stereoisomers of KRN7000. Hydrogenolysis of N-Cbz-protected **2a** over Pd/C generated the free amine, which was conveniently acylated with the N-hydroxysuccinimide activated ester **3** in the presence of triethylamine<sup>16</sup> to give ceramide **4a**. The two hydroxyl groups of **4a** were protected with TBSOTf and 2,6-lutidine in CH<sub>2</sub>Cl<sub>2</sub> to yield the O-TBS-protected ceramide **5a**. Now the regioselective removal of the TBS group from the primary hydroxyl in **5a** was needed, and it was accomplished by treatment with HF-py in THF-pyridine<sup>17</sup> to afford the intermediate **6a**. The glycosylation of **6a** with the perbenzylated galactosyl trichloroacetimidate **7**<sup>18</sup> in the presence of BF<sub>3</sub>·OEt<sub>2</sub> gave  $\alpha$ -galactoside **8a** in modest yield. All protecting groups in **8a** were sequentially removed by treatment with TBAF in THF, and the resulting diol **9a** with Pd(OH)<sub>2</sub>/C in EtOH-CHCl<sub>3</sub> to provide the target compound, KRN7000 stereoisomer **1a** (Scheme 1). By employing the identical procedures on the other stereoisomers of phytosphingosine (**2b–2h**), the corresponding KRN7000 stereoisomers (**1b–1h**) were uneventfully prepared. The melting point and specific rotation data for all stereoisomers of KRN7000 are listed in Table 1.

The biological activities of these isomers have been examined in both mouse and human iNKT cells in terms of in vitro proliferation and induction of IFN- $\gamma$ , IL-4 and IL-13. The preliminary data indicate the following trends for the stereoisomers of KRN7000; (1) differential activity was observed between mouse and human iNKT cells with mouse cells being more sensitive, and (2) differential activity was also observed for different isomers. More specifically, with mouse iNKT cells the following observations have been made: (1) good in vitro proliferation with **1c** (KRN7000) > **1a**, **1d**, **1g**, and much weaker proliferation with **1b**, **1f**, and **1h**, and (2) good induction of IFN- $\gamma$ , IL-4/IL-13 with **1c** and **1d**, and weak induction of IFN- $\gamma$  and IL-13 at a high dose of **1a**, and induction of IL-4 at high dosage of **1b**, **1a**, **1h**. With human iNKT cells, the following trends have been observed; (1) the stereoisomers (2S) derived from L-serine, namely **1a**, **1b**, **1c**, and **1d** show high potency (IFN- $\gamma$ , IL-4, and IL-13 production), while the isomers (2R) derived from D-serine, that is, **1e**, **1g**, and **1h** exhibit weak potency (IFN- $\gamma$  and IL-4 production), but decent IL-13 production, and (2) isomer **1f** shows virtually

no activity except IL-13 production at high dose. The isomer **1d**, having the inverted C4-OH group stereochemistry when compared with **1c**, displays comparable potency (proliferation and cytokine secretion) with those for **1c** (KRN7000). Thus, the 3-D spatial orientations of C2-NH<sub>2</sub> group and C3-OH group of phytosphingosine appear crucial for the activity, and the configuration of C2-NH<sub>2</sub> group is much more important than that of C3-OH group; the stereochemical variation of C4-OH group seems not so significant. The more extensive studies of the biological activity of KRN7000 stereoisomers are in progress.<sup>19</sup>

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19. Details of the biological studies will be published in due course.