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Synthesis and In Vivo Evaluation of 4-Deoxy-4,4-difluoro-KRN7000

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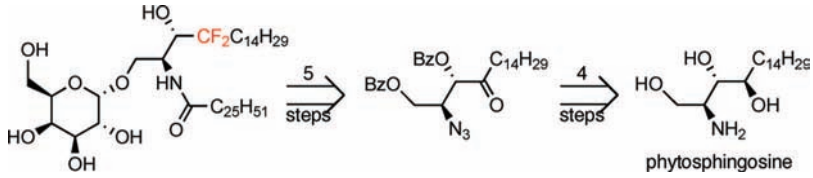
ABSTRACT



The synthesis of 4-deoxy-4,4-difluoro-KRN7000 starting from phytosphingosine is described. Key steps include a regioselective benzylation of azidophytosphingosine and a deoxofluor-mediated fluorination of the corresponding 4-ketone. This fluorination failed completely when the adjacent 3-OH was protected as benzyl ether but proceeded well when a benzoyl group was used. The biological evaluation reveals a bias toward Th1 cytokine induction upon Natural Killer T cell activation.

The discovery of KRN7000 **1** (Figure 1)[1] as potent stimulator of Natural Killer T-cells (NKT-cells) has stimulated intense research toward the immunomodulating properties of this class of exogenous (α-linked) glycolipids.

It has been shown that KRN7000 binds to the CD1d protein to form a complex that is subsequently recognized by NKT-cells, leading to a rapid release of cytokines. Unfortunately, both proinflammatory Th1 (*eg* IFN-γ and IL-2) and immunomodulatory Th2 (e.g., IL-4 and IL-10) cytokines are released, which antagonize each other's effects. The development of KRN7000 analogues that lead to polarization of cytokine release, and hence may have

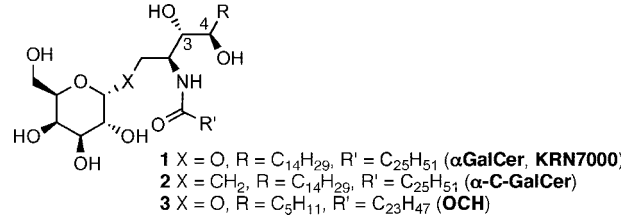


Figure 1. Structures of KRN7000 (**1**), α-*C*-GalCer (**2**), and OCH (**3**).

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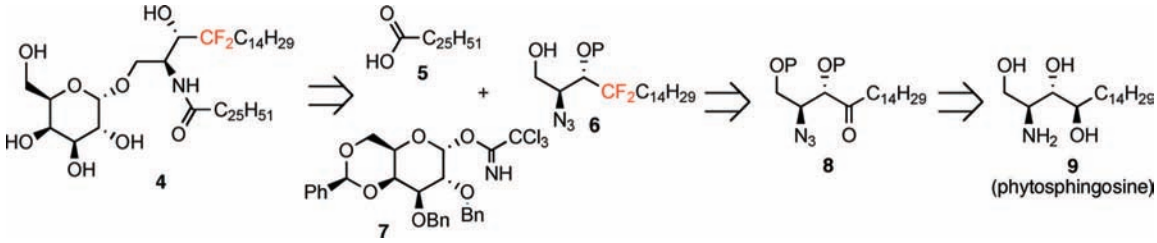
Scheme 1. Retrosynthetic Analysis



therapeutic potential, is currently an active area of research.[2] Examples include the *C*-glycoside α-*C*-GalCer **2**, which was found to induce a markedly Th1 skewed response,[3] while truncation of one of the lipid chains, such as in OCH **3**,[4] caused a Th2 bias in NKT cell response.

At present the relationship between glycolipid structure and cytokine polarization is not completely understood, but could relate to the stability of the glycolipid complex with CD1d,[5] or to a difference in glycolipid presenting cells.[6]

It has been established that the sphingosine 3-OH is much more important than the 4-OH for antigenic activity.[1,20] Following crystallographic studies of CD1d complexed with KRN7000[6a,7] and related GalCers,[8] it was shown that the 3-OH forms a H-bond with the CD1d Asp80. Furthermore, the crystal structure of the human NKT TCR in complex with CD1d bound to **1** reveals a H-bond between the 3-OH and Arg 95 of the CDR3α-loop.[9] Given the apparent pivotal role of the 3-OH in the interaction with both proteins, it was decided to synthesize the 4-deoxy-4,4-difluoro KRN7000 analogue **4**, in which the 3-OH would display a better hydrogen bond donating capacity, but a significantly reduced ability to accept a hydrogen bond,[10] and to investigate the effect of this modification on the immunostimulating properties.

An α-galactosylceramide construction strategy was envisioned in which the modified sphingosine **6** (Scheme 1) would be glycosylated with the galactosyl donor **7**,[11] followed by azide reduction, amide bond formation with **5**,[11b] and global deprotection. The sphingosine derivative **6** would be obtained by fluorination of ketone **8**, which would be accessible from the commercially available phytosphingosine **9**. Hence, the project started by investigating the conversion of **9** to **8** aiming for minimal protection/deprotection operations.

In attempting to differentiate the 4-OH from the other alcohol groups, direct 1,3-protection, for example as silylene acetal,[12] proved not of use for our purposes. A high-yielding selective alcohol differentiation was developed from 1-*O*-trityl phytosphingosine **11** (Scheme 2). Masking the amino group of phytosphingosine **9** as an azide[13,14] was followed by regioselective tritylation of **10**.[15] Subsequent azide reduction led to **11**, upon which treatment with Boc_2O surprisingly led to the 3-*O* carbonate **12** as the only product in 80% yield. The origin for the selectivity is unclear. Given that there is no additional stabilizing group present on the nitrogen, prior amine protection followed by N→O migration of the Boc group under steric pressure from the trityl group is not expected under the employed reaction conditions.[16] The functionalization at the 3-position was easily proven by NMR spectroscopy. This remarkably high-yielding selective 3-OH protection step allowed us to easily obtain the desired ketone **13** by subsequent protection of the amino group with

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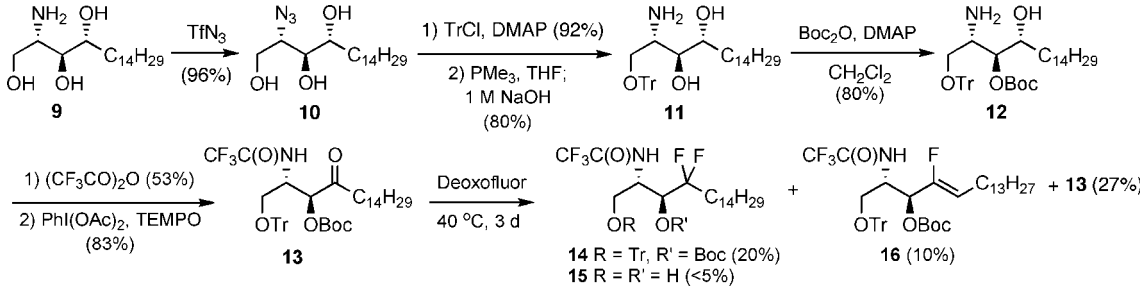
Scheme 2. First Synthesis of a 4-Deoxy-4,4-difluorophytosphingosine Derivative



trifluoroacetic anhydride, followed by oxidation of the 4-OH. Disappointingly, fluorination of **13** proceeded very slowly, with only 20% of the desired product **14** obtained after 3 days at 40 °C. In addition, the corresponding elimination product **16** was isolated in 10% yield, along with remaining starting material (27%) and deprotected **15** (<5%). The fluoroalkene **16** was obtained as a single geometric isomer as judged by ^{19}F NMR. The alkene was assigned as the *Z*-isomer by determination of the coupling constant between the vinylic fluorine and proton (^{1}H NMR, $^3J_{F-H} = 36.6$ Hz).

The desired derivative **14** was obtained in seven steps from **9**, and the removal of the three different protecting groups would need another two or three steps. Hence, it was decided to reconsider our approach by investigating another protecting strategy. Relying on the electron-withdrawing azido group to direct functionalization of the adjacent hydroxyl groups, a short, direct protection of the 1,3-diol moiety was achieved by benzylation of **10** under basic conditions (Scheme 3). By using an excess of base, but only 2 equiv of BnBr, an acceptable 49% yield was obtained of the desired 1,3-dibenzylated azidophytosphingosine **17**. Minor amounts of 1-*O*-benzylated (16%) and the tribenzylated product[17] (10%) were formed as well but were easily separated by chromatography, resulting in an attractive direct selective protection protocol. The position of the benzylation was assigned by NMR. Oxidation of **17** gave the ketone **18** in good yield. However, all attempts to effect fluorination of **18** completely failed.

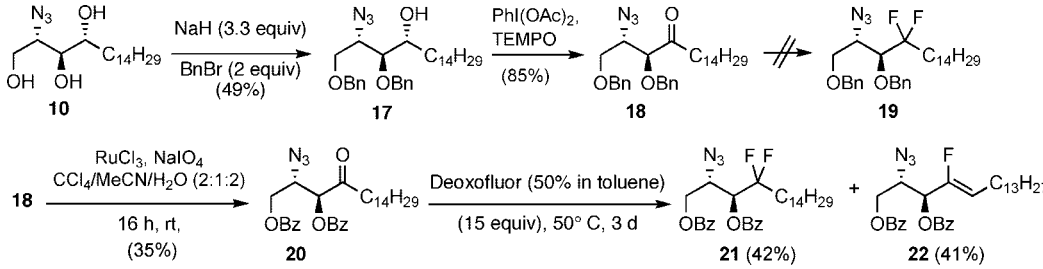
With the partially successful fluorination of **13** in mind, it was thought that an electron-withdrawing protecting group, such as a benzoate, would result in a more reactive ketone. Unfortunately, direct benzoylation of **10** under nucleophilic conditions (BzCl, pyridine) or basic conditions (NaH, BzCl) only led to 1,3-protection in low yield and selectivity, with the isomers not separable by chromatography (not shown), but a direct benzyl to benzoyl oxidation of **18** to **20** (Scheme 3) proved successful despite the moderate yield obtained.

The ketone **20** is now easily accessible in four steps from phytosphingosine **9**. As predicted, the fluorination now did proceed, if still very slowly, with a yield of 42% for the desired **21**. The corresponding elimination product **22** was formed in an approximate 1:1 ratio with **21**, and the products were separable by preparative HPLC. The *Z*-fluoroalkene **22** was again obtained as a single geometric isomer ($^3J_{F-H} =$ 37.0 Hz).

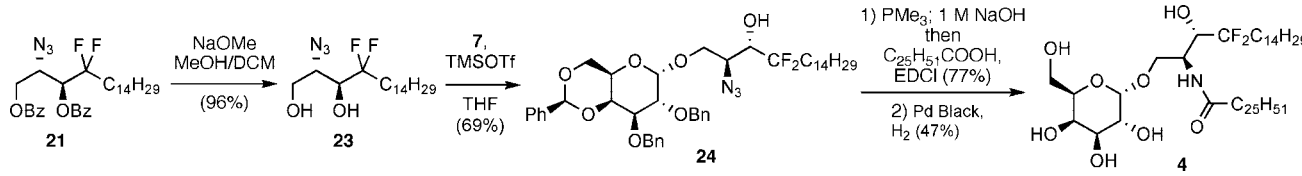
The final stages for the synthesis of **4** are shown in Scheme 4. Deprotection of **21** proceeded in excellent yield, and TMSOTf-induced galactosidation with galactosyl donor **7**[18] occurred selectively at the primary alcohol of **23**. No β-isomer was detected. Finally, azide reduction allowed introduction of the amide chain[11b] which was followed by global deprotection.

To investigate whether **4** could elicit CD1d-dependent NKT cell activation in vivo, wild-type C57Bl/6 mice, CD1d$^{-/-}$ as well as Jα18$^{-/-}$ mice were injected with 5 μg of **4**. Injection into wild-type C57B1/6 mice resulted in significantly reduced serum levels of IL-4 (Figure 2), while similar levels of IFN-γ production were detected compared to KRN7000. No cytokine production was observed when compound **4** was injected in either Jα18$^{-/-}$ or CD1d$^{-/-}$ mice, indicating that this glycolipid induces CD1d-dependent TCR activation of NKT cells.

Scheme 3. Direct Benzylation Approach



Scheme 4. Final Assembly to 4-Deoxy-4,4-difluoro KRN7000



This result confirms that the 4-OH is not required for activity and that replacement with a *gem*-difluoro group at that position is allowed. The fluorination will result in an increased hydrogen bond donating ability for the 3-OH,[10] which is expected to strengthen binding between **4** and CD1d through the interaction with Asp-80. Conversely, the fluorination will drastically reduce the hydrogen bond acceptor ability of the 3-OH group,[10] which would significantly weaken the binding to Arg 95 of the NKT TCR. Hence, a possible interpretation of the biological data is that a tighter binding between the glycolipid with CD1d is beneficial for cytokine polarization,[5] and given the minimal loss in activity compared to KRN7000 **1**, the interaction involving Arg 95 of the NKT TCR is of less importance.

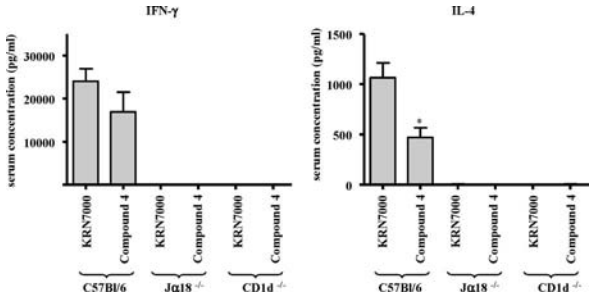


Figure 2. In vivo cytokine release by KRN7000 and 4-deoxy-4,4-difluoro-KRN7000 **4**.

In summary, the synthesis of a 4-deoxy-4,4-difluorosphingosine derivative **23** was achieved in five steps from phytosphingosine,[19] featuring a selective protection reaction of the azidosphingosine 1- and 3-OH. Fluorination of the C4-ketone was promoted by an electron-withdrawing protecting group at the 3-OH. α-Galactosylation of **23**, followed by introduction of the ceramide side chain, yielded 4-deoxy-4,4-difluoro-KRN7000 **4** in a further four steps. Biological evaluation showed that **4** induced CD1d-dependent TCR activation of NKT cells and that the modification modulates NKT cell responses resulting in the induction of a different cytokine pattern compared to KRN7000, with a bias toward Th1 cytokine release.

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Supporting Information Available: Experimental details, characterization, and copies of ^{1}H, ^{13}C, and (when relevant) ^{19}F spectra for **17**, **18**, **20**−**24**, and **4**.This material is available free of charge via the Internet at http://pubs.acs.org.

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