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An improved synthesis of dansylated α-galactosylceramide and its use as a fluorescent probe for the monitoring of glycolipid uptake by cells

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ABSTRACT

A highly efficient synthesis of the biologically important fluorescent probe dansyl α-GalCer is presented. Key in our strategy is the incorporation of the fluorescent dansyl group at an early stage in the synthesis to facilitate in the monitoring and purification of intermediates via TLC and flash column chromatography, respectively, and the use of a high yielding α-selective glycosylation reaction between the phytosphingosine lipid and a galactosyl iodide donor. The ability of dansyl α-GalCer to activate *i*NKT cells and to serve as a fluorescent marker for the uptake of glycolipid by dendritic cells is also presented.

1. Introduction

In 1993, the pharmaceutical division of Kirin Breweries isolated a series of novel α-galactosyl ceramides from the marine sponge *Agelas mauritianus*.[1,2] Of the series, agelasphin-9b (AGL-9b, **1**, Fig. 1), consisting of a galactosyl moiety α-linked to a ceramide containing an N-acylated phytosphingosine backbone, exhibited potent anti-tumour activity against in vivo models of several murine tumour cells and later served as the parent compound for subsequent analogue syntheses and structure–activity studies.[3] During the course of this structure–activity work, KRN7000 (**2**) was found to have similar anti-tumour activity to **1** and, due to its easier synthesis, was deemed the more suitable candidate for clinical use. Since then, KRN7000, now widely known as α-galactosyl ceramide (α-GalCer), has been reported to have potential in the treatment of several diseases including cancer, malaria, type I diabetes, and multiple sclerosis, and equally importantly, has been shown to exert its therapeutic activity via its ability to bind to CD1d [a member of the CD1 family of proteins found on the surface of antigen presenting cells (APCs)] and activate a subset of T cells known as invariant natural killer T (*i*NKT) cells.[4–6] This discovery was remarkable and provided the first evidence that glycolipids, like their protein counterparts, can be presented by APCs and recognised by T cells to invoke an immune response.

Given the therapeutic potential of α-GalCer, much effort has been spent in developing robust routes for its synthesis.[3,7–10] In addition, a number of α-GalCer-derivatives containing a fluorophore[11–13] or a biotinylated probe[11,14,15] have been prepared with the objective of using these substrates to better understand the mechanism of *i*NKT cell activation by α-GalCer.[13,16,17] Though the ability of α-GalCer to bind to CD1d and activate *i*NKT cells has been robustly studied, only little information is known about lipid trafficking and how this influences presentation by CD1d and activation of *i*NKT cells.[18] Accordingly, many groups have focussed on derivatising the lipid portion of α-GalCer with a reporter probe in order to address such issues.[12–14] However, as CD1d binds lipid chains within deep hydrophobic pockets,[19] it has been proposed that the addition of a label on the lipid may interfere with α-GalCer-CD1d association,[17] and moreover, may influence intracellular trafficking.[18,20–22] Conversely, modelling of the CD1d-α-GalCer complex suggests that the hydroxyl groups at C4″ and C6″ on galactose are not involved in complex formation.[23] This theory is also supported by the preparation of a α-GalCer analogue containing an additional α-linked galactose at the C6″ position which was shown to stimulate *i*NKT cells without the need for processing.[24] Taken as a whole, it is generally accepted that the CD1d-glycolipid-NKT cell

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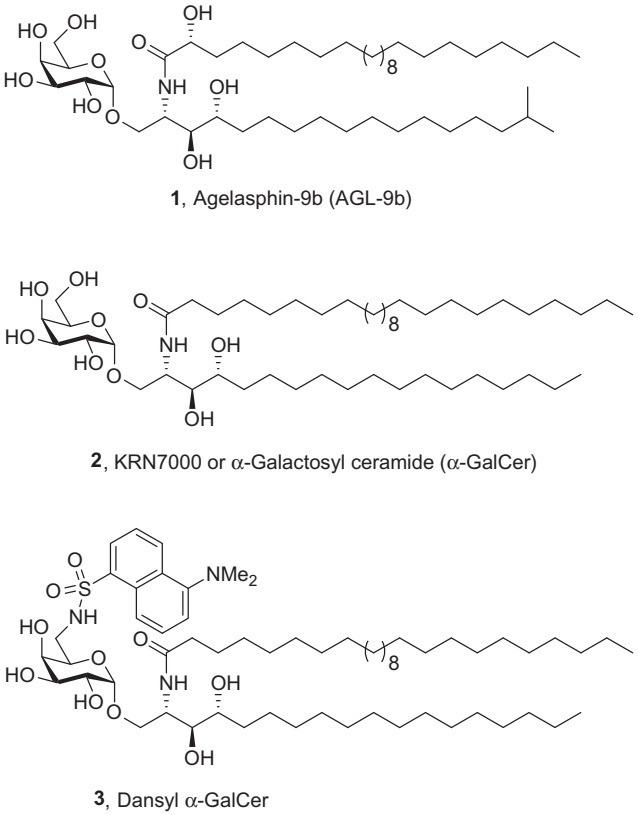


Figure 1. α-Galactosyl ceramide and derivatives.

receptor interaction tolerates the appendage of small molecules at C6″ and that this is an ideal position to attach a fluorescent reporter group. Indeed, this was the conclusion made by Zhou et al. who developed a strategy for the preparation of dansylated α-GalCer derivative **3**.[11]

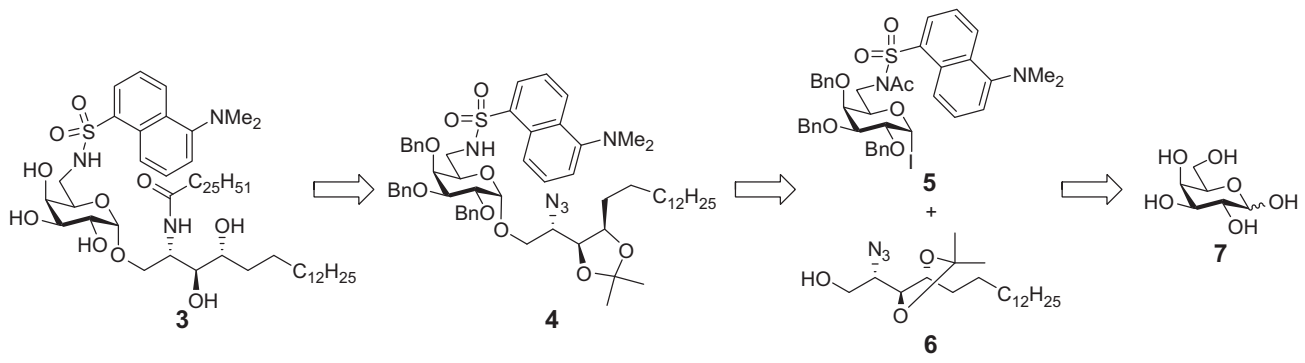
Despite there being much interest in α-GalCer, the need for a more efficient synthesis of an appropriately labelled fluorescent α-GalCer derivative remains. Indeed, our own desire to understand more about the mechanism by which α-GalCer is transferred to resident dendritic cells (DCs) during cancer immunotherapy,[25,26] drove us to devise an improved strategy for the synthesis of the dansylated α-GalCer derivative **3** (Fig. 1). Our objective was to achieve a robust synthesis of **3** with high reaction yields throughout, and to assess the ability of **3** to both activate *i*NKT cells and to serve as a fluorescent reporter group. The results of these studies are reported herein.

2. Results and discussion

2.1. Synthesis of dansylated α-GalCer

Our retrosynthetic analysis for the preparation of dansylated α-GalCer **3** is presented in Scheme 1. We chose to prepare **3** from the mono-lipidated derivative **4**, itself formed via the coupling of dansylated galactosyl iodide donor **5** to the phytosphingosine backbone **6**. Donor **5** and acceptor **6** are in turn both readily prepared from D-galactose (**7**). The reasoning behind these key disconnects are twofold. First, though it is possible to couple the complete ceramide lipid backbone to a suitably protected galactose donor, the ceramide is a particularly poor acceptor and yields for these glycosylations are typically modest (ca. 25–55%). This is a general phenomenon largely independent of the type of galactose donor used.[8,15,27–31] There are also advantages to be gained by incorporating the fluorescent dansyl group at an early stage in the synthesis for it has been well documented that molecules containing a chromophore are more easily monitored by TLC and purified by flash column chromatography.[32] Accordingly, in contrast to other syntheses of fluorescent α-GalCer probes,[11–13] we envisioned a glycosylation reaction involving the use of a fluorescent galactose donor, such as **5**.

With our synthetic strategy in place, our first goal was the preparation of the phytosphingosine backbone **6** (Scheme 2). Here, our lipid synthesis commenced with the selective tritylation of D-galactose (**7**) at the primary position,[33] followed by installation of an isopropylidene at the 3- and 4-positions to give the diol **8** in 85% (over two steps).[34] Diol **8** was then treated with a solution of $NaIO_4$ in THF/H_2O, which resulted in cleavage of the diol and formation of the formate ester **9** in quantitative yield. As a temporary protecting group, the formate at the 4-position was a pivotal step in our strategy as it prevented cyclisation to the corresponding D-lyxofuranose derivative, which in our hands, gave only modest yields (50–60%) when subject to the subsequent Wittig reaction. Treatment of ester **9** with excess ylide (BuLi, 2.4 equiv, phosphonium bromide, 2.5 equiv), however, gave the corresponding alkene **10** in good (78%) yield and in an *E*:*Z* ratio of approximately 1:10 based on ^1H NMR analysis. Subsequent conversion of alkene **10** to the required phytosphingosine backbone then followed via quantitative hydrogenation, using 1.5 wt % Pd/C, and treatment with triflic anhydride to give the intermediate triflate, which was converted to azide **11** in situ. During the installation of the azide functionality, it was found that the best yields were obtained when the reaction was preformed at −15 °C as higher temperatures resulted in the elimination of the triflate and the formation of the corresponding olefinic by-product. Selective removal of the trityl ether in the presence of the isopropylidene group was then attempted. This transformation has proven problematic in the past,[7]



Scheme 1. Retrosynthetic analysis for the preparation of dansylated α-GalCer **3**.