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Novel synthesis of α-galactosyl-ceramides and confirmation of their powerful NKT cell agonist activity

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Abstract—α-Galactosyl-ceramide (**1**) has been identified as a powerful modulator of immunological processes through its capacity to bind CD1d molecules and specifically activate invariant natural killer (NK)-like T cells (*i*NKT cells). This paper describes the synthesis of **1**, the analogous α-galactosyl-ceramide **3**, and its short chain analogue 'OCH' (**2**), by use of the 4,6-di-*O*-*tert*-butylsilylene (DTBS) protecting group to produce a powerful α-galactosylating agent. In vivo experiments confirmed these compounds to be potent and selective activators of *i*NKT cells in a CD1d-dependent manner, each inducing a unique profile of cytokine release. This synthesis strategy will permit the generation of novel derivatives for use in the study of the mechanism of *i*NKT cell activation.

Keywords: α-Galactosyl-ceramide; *i*NKT cells; Lipid antigen presentation; CD1d; Agelasphins; KRN 7000

1. Introduction

In contrast to most T cells that recognize specific peptide fragments bound to major histocompatibility complex (MHC) molecules, invariant natural killer (NK)-like T cells (*i*NKT cells) recognize glycolipids bound by the MHC-like molecule CD1d. After stimulation, *i*NKT cells can modulate the function of a number of immune cells including T cells,[1] B cells,[2] NK cells[3] and dendritic cells (DC),[4–6] primarily through the release of a spectrum of cytokines, including 'Th1' cytokines such as IFN-γ, and 'Th2' cytokines such as IL-4 and IL-13. Controlling the profile of cytokine release may be key to exploiting *i*NKT cell function for the treatment of various diseases. For example, the release of Th1 cytokines is likely to contribute to antitumour[7,8] and antimicrobial functions,[9] whereas the release of Th2 cytokines may attenuate autoimmune diseases such as multiple sclerosis[10] and arthritis.[11] The best known class of agonist ligands for *i*NKT cells[12] are the agelasphins, first isolated from a marine sponge for their antitumour attributes.[13,14] Later structure activity relationships identified the compound (2*S*,3*S*,4*R*)-1-(α-D-galactopyranosyloxy)-2-(*N*-hexacosanoylamino)octadecane-3,4-diol (**1**),[15] commonly referred to as α-galactosyl-ceramide (α-GalCer), as a candidate for clinical trials. α-GalCer specifically, and potently, stimulates *i*NKT cells to release Th1 and Th2 cytokines in vitro and in vivo. A short chain derivative of α-GalCer, known as 'OCH' (**2**), was shown to drive the release of a cytokine profile with a Th2 bias, thereby indicating a potential application in the treatment of autoimmune conditions.[10,12] While there has been considerable interest in using α-GalCer to stimulate *i*NKT cells in a therapeutic manner, it has recently been shown that repeated administration of α-GalCer induces long-term *i*NKT unresponsiveness in mice,[16] suggesting that there are limitations to the use of α-GalCer as a therapeutic option.

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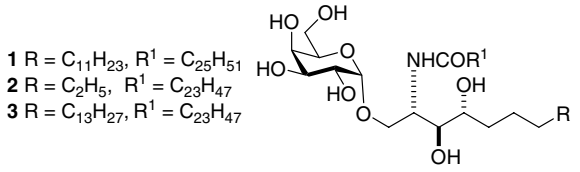
Stimulation of *i*NKT cells in vivo by injection of α-GalCer has been shown to provide a powerful stimulus to vaccine-induced immune responses.[4–6] However, α-GalCer may not be the best candidate for this adjuvant activity if multiple rounds of vaccination are required. It has been suggested that less potent stimulation of *i*NKT cells may avoid the induction of long-term unresponsiveness.[17] Here we report a novel method for the general synthesis of α-galactosyl-ceramides that can be used to generate different analogues of α-GalCer. We confirm that this synthesis strategy can be used to generate known structures α-GalCer (**1**) and OCH (**2**), and show that a third structure (**3**) has the capacity to stimulate *i*NKT cells in a CD1d-dependent manner with less potency than α-GalCer (**1**), but without the stronger Th2 bias of OCH (**2**).



2. Results and discussion

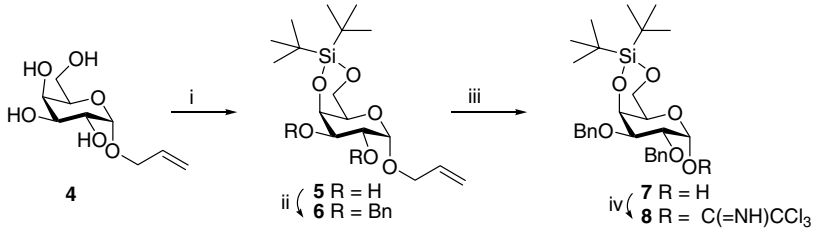
2.1. Synthesis of α-galactosyl-ceramides 1, 2 and 3

A number of the reported syntheses of α-galactosyl-ceramides depend on chemical glycosylation of a sphingosine derivative in the key step.[18–26] Because the glycosidic bond forming reactions are not normally stereospecific, most of these methods require tedious separations of anomeric products. Furthermore, those reactions that are highly α-selective are often not high yielding.[25] A method for facilitating the isolation of the α-anomers of the mixtures is based on preparing them by use of 3,4,6-tri-*O*-acetyl-2-*O*-benzyl-α-D-galactosyl bromide. Release of the C-2 hydroxyl group of the products aids in the chromatographic removal of the β-anomers.[21] The application of 2,3,4,6-tetra-*O*-benzyl-α-D-galactosyl bromide in the presence of tetrabutylammonium bromide, which induces its in situ anomerization, has also been used successfully in the synthesis of α-galactosyl-ceramides in a selective manner. However, these reactions generally require extended reaction times[22] and are often not high yielding.

Figueroa-Pérez and Schmidt[27] have demonstrated that use of a 4,6-*O*-benzylidene acetal group within a glycosyl trichloroacetimidate donor can be used to afford α-galactosyl-ceramides in high yield and with good anomeric selectivity. Similarly, the 4,6-*O*-di-*tert*-butylsilylene (DTBS) group can function as a powerful α-directing group in galactosylation donors and, significantly, under circumstances in which the corresponding 4,6-*O*-benzylidene donors can give only β-products.[28] We therefore reasoned that it should be useful within donors in the synthesis of α-galactosyl-ceramides. With this in mind, we prepared the galactosyl donor **8** from allyl α-D-galactopyranoside (**4**)[29] via compounds **5**–**7** (Scheme 1). The 4,6-*O*-DTBS group was installed by reaction with di-*tert*-butylsilyl bis(trifluoromethanesulfonate) in the presence of DMAP. Benzylation of diol **5** with NaH/BnBr needed to be monitored carefully as excess reagent and/or extended reaction times led to cleavage of the DTBS group. Nevertheless, the fully protected **6** was made in 66% yield. Deprotection of the anomeric hemi-acetal and installation of the α-imidate function proceeded without incident.

There are a number of elegant syntheses of the ceramide moiety or precursors thereof. In this study, we chose as a convenient glycosyl acceptor the sphingosine derivative **13**, which was prepared via compounds **10**–**12** from the 2-deoxy-D-*lyxo*-hexose derivative **9** by application of the existing protocols (Scheme 2).[19]

The glycosylation with imidate **8** of acceptor **13** proceeded smoothly to afford the desired α-glycoside **14** in good yield with no detectable β-anomer (Scheme 2). The stereochemistry of the newly formed glycosidic linkage was established as α from the C-1 heteronuclear one-bond ^{13}C–^{1}H coupling constant of 168.6 Hz.[30,31] In a comparative experiment, to confirm the stereo-directing effect of the DTBS group, glycosylation of **13** with donor **15** afforded the expected glycoside together with the β-anomer (70% combined yield, α:β ratio 2.5:1, estimated from ^{1}H NMR) that proved difficult to separate. In a similar experiment reported by Wong and co-work-



Scheme 1. Reagents, conditions and yields: (i) $^{t}Bu_2Si(OTf)_2$, DMAP, Py (65%); (ii) NaH, BnBr, DMF (66%); (iii) $Ph_2P_2(COD)Ir^{+}PF_6^{-}$, THF, then AcCl, MeOH–$CH_2Cl_2$; (iv) CCl_3CN, DBU, CH_2Cl_2 (steps iii and iv) 40% in total.