



Synthesis and biological activity of α -L-fucosyl ceramides, analogues of the potent agonist, α -D-galactosyl ceramide KRN7000

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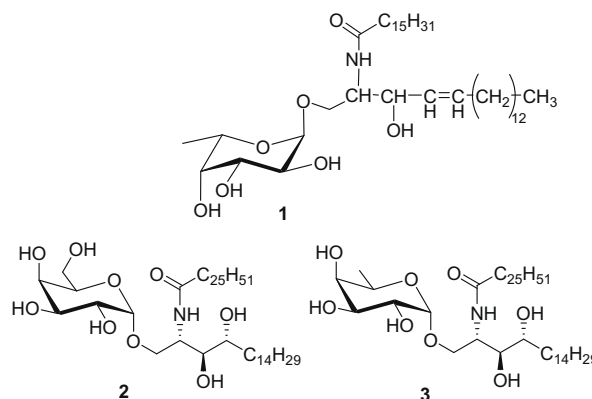
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ABSTRACT

Several L-fucoglycolipids are associated with diseases such as cancer, cystic fibrosis and rheumatoid arthritis. Activation of iNKT cells is known to lead to the production of cytokines that can help alleviate or exacerbate these conditions. α -Galactosyl ceramide (α -GalCer) is a known agonist of iNKT cells and it is believed that its fucosyl counterpart might have similar immunogenic properties. We herein report the synthesis of α -L-fucosyl ceramide derivatives and describe their biological evaluation. The key challenge in the synthesis of the target molecules involved the stereoselective synthesis of the α -glycosidic linkage. Of the methods examined, the per-TMS-protected glycosyl iodide donor was completely α -selective, and could be scaled up to provide gram quantities of the azide precursor **11**, from which a range of N-acylated α -L-fucosyl ceramides were readily obtained and evaluated for ex vivo expansion of human iNKT cells.

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Both D- and L-fucose (6-deoxy galactose) are widely found in nature.¹ Of interest, L-fucose is predominantly found in the α -configuration in the lipopolysaccharides (LPS) of Gram-negative bacteria and animal glycosphingolipids.^{1,2} With the exception of the monohexylceramide, α -L-fucosylceramide **1**, initially isolated from metastatic human adenocarcinoma,³ most fucosphingolipids are usually ceramide oligosaccharides.^{2,4} Many of these fucolipids are antigenic^{5–7} and play a role in tumour cell biology.^{8–10} Conversely, the synthetic glycolipid, α -galactosyl ceramide (α -GalCer)¹¹ (**2**), is a strong agonist of iNKT cells when bound to CD1d, triggering the release of diverse cytokines, including both Th1 and Th2 cytokines.^{12–14} It is believed that the release of Th1 cytokines may contribute to antitumour and antimicrobial functions while that of Th2 cytokines may help alleviate autoimmune diseases^{15–17} such as multiple sclerosis¹⁸ and arthritis.¹⁹



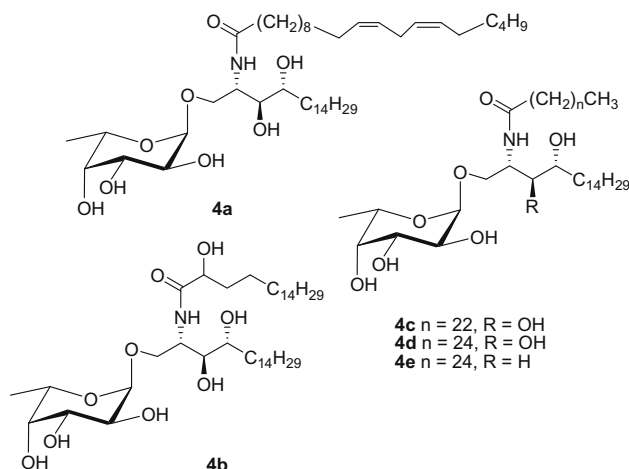
α -GalCer and its derivatives have proved to be and remain invaluable tools in understanding the functioning of CD1d and iNKT cells in a wide range of immune responses. Crystal structures of the bound α -GalCer-CD1d complex^{20–22} showed that the hydroxyl group at C-6 in the sugar head is not crucial for binding to the T-cell receptor (TCR) as opposed to C-2, C-3 and C-4. Hence, the α -D-fucosylceramide **3**²³ was shown to be a potent inducer of IFN- γ

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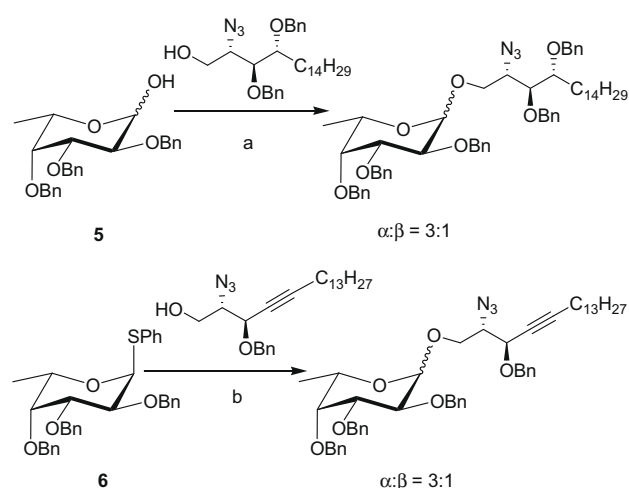
in mice *in vivo*, while a slightly different derivative, synthesised by Motoki et al.²⁴ showed strong lymphocytic proliferation stimulatory effects *in vitro* in mice.

While there are a few examples describing the synthesis of α -L-fucosylceramide **4b**²⁵ and other derivatives in the literature,^{26,27} their biological activities are yet to be fully explored. As part of various ongoing studies, and because of the aforementioned reasons, we have synthesised compounds **4a–4d** for biological evaluation and comparative studies.



Fan et al.²⁵ reported the synthesis of compound **4d**, while Okamoto et al.²⁷ reported that of compound **4e**. Both groups obtained their target compounds as an α , β mixture (3:1 ratio) from different glycosyl donors and acceptors (Scheme 1). On the other hand, in the original reported synthesis of the fucosylceramide **1**, only the α -anomer was isolated when another ceramide acceptor was used under different reaction conditions.

We recently reported the synthesis²⁸ of α -GalCer and other derivatives where we used *N*-iodosuccinimide (NIS) and triflic acid (TfOH) as a promoter, and benzoate protecting groups on the sphingosine base. Along with the nature of the protecting groups on both the glycosyl acceptor and donor, the choice of promoter, as well as temperature and reaction times can affect the stereoselectivity of the glycosylation reaction. In this study, we proposed to investigate whether our glycosyl acceptor **7** and reaction conditions could offer a better α -selectivity in the case of L-fucose.

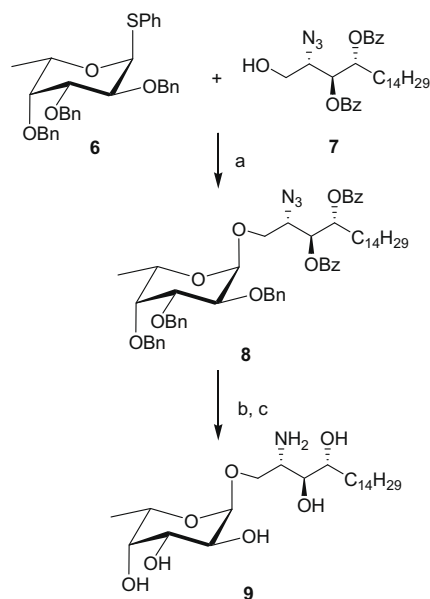


Scheme 1. (a) Me₂S, 2-Cl-pyridine, Tf₂O, CH₂Cl₂; (b) dimethyl(methylthio)sulfonium triflate (DMTST), CH₂Cl₂, 0 °C–rt.

The phytosphingosine acceptor **7**²⁹ was synthesised from (2S,3S,4R)-2-azido-1,3,4-octadecanetriol³⁰ as described before. Given the previous success of a thiogalactoside in a similar glycosylation reaction with compound **7**²⁸ we chose to use the thiofucosyl donor **6**, rather than compound **5**. The thioglycoside **6** was thus obtained from commercially available L-fucose after standard procedures, as previously reported.³¹

With both the acceptor and donor in hand, we then proceeded to the critical glycosylation reaction. Interestingly, NIS/TfOH activation of **1** g of the thioglycoside **6** (Scheme 2) in anhydrous CH₂Cl₂ at –78 °C afforded the glycosylated compound **8**, almost exclusively as the α -anomer (α : β ratio = 9:1), in 68% yield after 2 h. Our results show a definite improvement in selectivity from the previously reported syntheses. Because the formation of the α -anomer is favoured by the anomeric effect, we rationalise that the latter is a governing factor under our reaction conditions. Both the lower temperature and the different reactivity of the acceptor **7** could potentially influence the stereoselectivity of the glycosylation reaction. These factors will have to be more thoroughly investigated in a later study. Subsequent Zemplen's deprotection of the benzoate protecting groups produced the azide intermediate in quantitative yields. Tandem hydrogenation of the azido group and hydrogenolysis of the benzyl ethers in methanol then produced the amine **9** as a white solid, which exhibited spectroscopic data consistent with the literature.²⁵

In an attempt to improve the stereoselectivity and circumvent the sometimes problematic removal of the benzyl ethers by hydrogenolysis, we embarked on a different glycosylation route. Recently, the use of glycosyl iodide donors has been revived by Gervay-Hague's group.^{32,33} They have demonstrated that glycosylation reactions employing glycosyl iodides and promoted by tetrabutyl ammonium iodide (TBAI) are generally quite stereoselective and fast. It has been hypothesized that TBAI catalyses the isomerization of the α -glycosyl iodide to the β -anomer,³⁴ thereby leading to the formation of an α -glycoside. They have successfully adapted this strategy to the synthesis of α -GalCer³⁵ and other α -fucosylglycosides.³⁶ Furthermore, the replacement of the benzyl ethers with trimethylsilyl ethers (TMS) made Gervay-Hague's synthetic strategy even more attractive. TMS protected sugars are easy to generate and their deprotection requires very mild acidic conditions, compatible with the glycosidic linkage.



Scheme 2. (a) NIS/TfOH, CH₂Cl₂, –78 °C to –20 °C, 68%; (b) NaOMe/MeOH, 92%; (c) H₂, Pd, 76%.

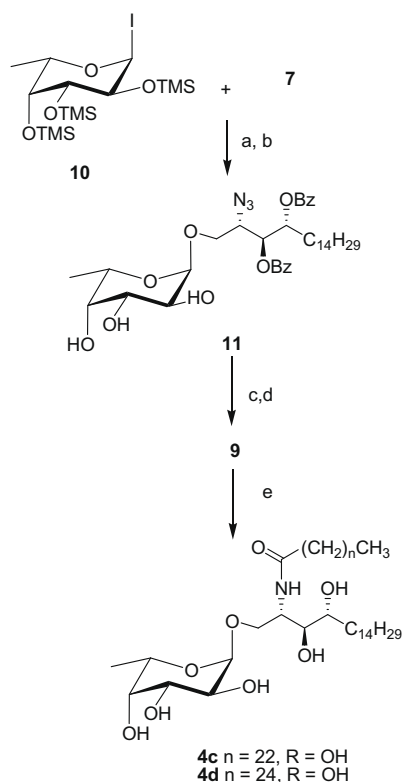
The per-*O*-trimethylsilyl- α -L-fucosylpyranosyl iodide **10** was generated by the reaction of the per-*O*-tetramethylsilyl- α -L-fucose with one equivalent of iodotrimethylsilane³⁵ and then added to the phytosphingosine acceptor **7** which was premixed with diisopropylethylamine (DIPEA) and TBAI (Scheme 3). After 2 days at room temperature, the solvent was evaporated and the TMS protecting groups were removed by treatment with an acidic resin in MeOH. Compound **11** was obtained as the α -anomer exclusively in an overall yield of 62%. The formation of the desired α -linkage was confirmed by the H-1 and C-1 signals in ¹H and ¹³C NMR. Methanolysis, followed by hydrogenation of the azide then afforded compound **9**.

Finally, N-acylation with the fully saturated fatty acids, tetracosanoic acid (C24:0) and hexacosanoic acid (C26:0), was achieved via reaction of the corresponding acid chloride with the free amine **9** in a 1:1 mixture of THF and saturated sodium acetate solution (Scheme 3). Target compounds **4c** and **4d** were obtained as white solids after concentration of the organic phase and purification of the residue by flash chromatography. The spectroscopic data of the final compounds were consistent with the literature.²⁵ While compound **4a** was obtained by heating amine **9** with the *N*-hydroxysuccinimide activated ester of C20:2 fatty acid in a mixture of pyridine: water (9:1) at 50 °C overnight, compound **4b** was obtained via dicyclohexylcarbodiimide (DCC) activated coupling using procedures described previously.^{37,38}

To assess the biological activity of the α -L-fucosylceramides and compare these to α -GalCer (KRN7000, **2**), we assessed the ability of each compound to induce the expansion of iNKT cells in samples of human peripheral blood mononuclear cells (PBMC) during an eight-day in vitro culture.³⁹ The results showed that both the percentages and absolute numbers of iNKT cells in cultures were increased by stimulation with α -L-fucosylceramides with C26:0

(**4d**) > C18:0 (OH) (**4b**) > C24:0 (**4c**). The α -L-fucosylceramide containing a C26:0 fatty acid (**4d**) was the most active of the fucosyl series, and stimulated iNKT cell expansions in some donors that approached those seen with the prototype iNKT cell agonist KRN7000 (**2**). In contrast, the α -L-fucosylceramide containing the C20:2 fatty acid (**4a**) was found to lack detectable iNKT cell stimulating activity in any of the donors tested (Fig. 1B). Representative profiles obtained by flow cytometry of cultures from one normal blood donor are shown in Figure 1A. This analysis was carried out with PBMC from four separate donors (Fig. 1B). Although, differences were observed for the levels of iNKT cell expansion between different donors, all donors responded significantly to two of the α -L-fucosylceramide analogues (**4b** and **4d**).

In summary, in the current study we have developed an efficient method for the synthesis of a series of biologically active α -L-fucosylceramides.⁴⁰ The second method, employing the per-*O*-trimethylsilyl- α -L-fucosylpyranosyl iodide **10**, proved to be superior with a better α -selectivity and reasonably good yield in the glycosylation reaction. Given the marked difference in the stereochemistry of the α -fucosyl head group of these glycolipids compared to α -galactosyl group of strong iNKT cell activators like KRN7000, it is surprising that the compounds in the current series show such substantial activity. This would seem to further reinforce the notion that the TCR of iNKT cells, in spite of its relative limited variability, is nevertheless able to interact efficiently with a broad range of structurally diverse ligands. It is noteworthy that



Scheme 3. (a) TBAI, DIPEA, CH₂Cl₂, rt; (b) Dowex 50WX8-200, MeOH, rt, 62% over two steps; (c) NaOMe/MeOH, quantitative; (d) H₂, Pd, MeOH, 80%; (e) C₂₅H₅₁COCl or C₂₃H₄₇COCl, THF, NaOAc, 78–80%.

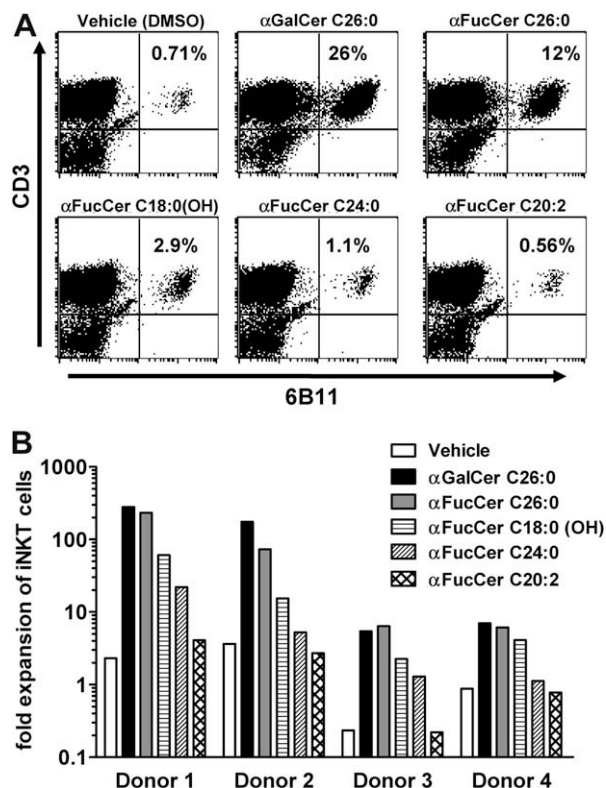


Figure 1. Ex vivo expansion of human iNKT cells by α -L-fucosylceramides. PBMC from four different donors were stimulated with the indicated glycolipids at a concentration of 250 nM in the presence of low levels of exogenous IL-2 and IL-7. At day 8, cultures were harvested and analysed by flow cytometry using monoclonal antibodies specific for CD3 and for the invariant TCR α chain expressed by iNKT cells (6B11). (A) Dot plots showing relative levels of CD3⁺ 6B11⁺ iNKT cells are shown for one representative donor. Numbers in upper right quadrant indicate percentages of total lymphocytes that are iNKT cells. (B) Absolute numbers of iNKT cells in the cultures were determined by flow cytometry using fluorescent counting beads, and the values of iNKT cell fold expansion were determined by dividing by the input number of iNKT cells.

the C20:2 *N*-acyl variant of α -fucosylceramide (**4a**) showed no detectable iNKT cell stimulating activity, given that the α -galactosyl version of this compound is an extremely potent iNKT cell agonist.⁴¹ This suggests that modification of the carbohydrate moiety can significantly alter the influence of the lipid moiety of the ligand on CD1d presentation and iNKT cell responses. Although the mechanism for this remains unclear, it is an important consideration for synthetic strategies that seek to combine biologically active alterations of the carbohydrate and lipid moieties of iNKT cell ligands.

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- Peripheral blood mononuclear cells (PBMC) were obtained by Ficoll-hypaque centrifugation of leucocyte concentrates obtained from healthy volunteer blood donors. For ex vivo expansion of human iNKT cells, 4 million PBMC were cultured in wells of 24-well tissue culture plates in RPMI-1640 medium with 10% foetal calf serum and recombinant IL-2 (60 IU/mL) and IL-7 (5 ng/mL) (Peprotech). Glycolipids were solubilised in 100% DMSO and added directly to culture media to achieve a final concentration of 250 nM. Control wells received an amount of DMSO vehicle identical to that added with the glycolipids (0.00125%). Cultures were incubated for 8 days at 37 °C in a 5% CO₂ humidified incubator. Cultures were harvested and the cells stained with fluorochrome labelled monoclonal antibodies specific for CD3 and the iNKT cell TCR (6B11, BD Biosciences). Samples were also stained with propidium iodide to exclude dead cells, and a known number of Caltag fluorescent counting beads (Invitrogen) were added to the samples to allow quantitation of absolute cell numbers. The total number of iNKT cells (CD3⁺, 6B11⁺, PI negative lymphocytes) was calculated by normalising according to counting beads and the fold expansion values were calculated based on the initial number of iNKT cells. Data were collected on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences).
- Selected data for new compounds.** Compound **8**: ¹H NMR (CDCl₃): δ 7.92–8.04 (10H, m, Ar-H), 7.10–7.64 (15H, m, Ar-H), 5.42–5.50 (2H, m, H-3^{cer}, H-4^{cer}), 5.02 (1H, d, $J_{1,2}$ = 3.6 Hz, H-1), 4.72–4.84 (6H, m, 3 \times CH₂Ph), 3.98 (1H, dd, $J_{2,3}$ = 11.5 Hz, H-2), 3.75–3.90 (3H, m, H-4, H-3, H-1a^{cer}), 3.55 (1H, m, H-5), 3.68 (1H, dd, $J_{1a,1b}$ = 10.4, $J_{1b,2}$ = 8.6 Hz, H-1b^{cer}), 1.88 (2H, m, H-5a^{cer}, H-5b^{cer}), 1.27 (3H, d, $J_{5,6}$ = 6.4 Hz, H-6), 1.24 (24H, m, CH₂), 0.89 (3H, t, CH₃); ¹³C NMR (75 MHz, CDCl₃): δ 99.5 (C-1); HRMS calcd for C₅₉H₇₃N₃O₉ [M+Na]⁺: 990.5245, found 990.5248. Compound **9**: ¹H NMR (CD₃OD): δ 4.73 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1), 3.91–3.96 (2H, m, H-3, H-4), 3.68–3.72 (1H, dd, $J_{1a,1b}$ = 9.9, $J_{1b,2}$ = 3.9 Hz, H-1a^{cer}), 3.61–3.68 (2H, m, H-4, H-3^{cer}, H-4^{cer}), 3.52–3.55 (1H, dd, $J_{2,3}$ = 6.0 Hz, H-2), 3.43–3.49 (1H, m, H-5), 3.39–3.42 (1H, dd, $J_{1b,2}$ = 8.6 Hz, H-1b^{cer}), 3.35 (1H, m, H-2^{cer}), 1.89 (2H, m, H-5a^{cer}, H-5b^{cer}), 1.28 (3H, d, $J_{5,6}$ = 6.4 Hz, H-6), 1.24 (24H, m, CH₂), 0.89 (3H, t, CH₃); ¹³C NMR (75 MHz, CD₃OD): δ 102.5 (C-1); HRMS calcd for C₂₄H₄₉NO₇ [M+Na]⁺: 486.3403, found 486.3309. Compound **11**: ¹H NMR (CDCl₃): δ 7.36–7.86 (10H, m, Ar-H), 5.60 (1H, dd, $J_{3,4}$ = 4.8, $J_{3,2}$ = 6.8 Hz, H-3^{cer}), 5.51 (1H, ddd, $J_{4,5a}$ = 4.2, $J_{4,5b}$ = 8.5 Hz, H-4^{cer}), 4.82 (1H, d, $J_{1,2}$ = 2.8 Hz, H-1), 3.88–4.00 (3H, m, H-2, H-4, H-5), 3.62–3.80 (3H, m, H-3, H-2^{cer}, H-1a^{cer}), 3.71 (1H, dd, $J_{1a,1b}$ = 10.4, $J_{1b,2}$ = 6.5 Hz, H-1b^{cer}), 1.76–1.91 (2H, m, H-5a^{cer}, H-5b^{cer}), 1.10–1.43 (24 H, m, CH₂), 1.07 (3H, d, $J_{5,6}$ = 6.6 Hz, H-6), 0.85 (3H, t, CH₃); ¹³C NMR (75 MHz, CDCl₃): δ 100.9 (C-1); HRMS calcd for C₃₈H₅₅N₃O₁₀ [M+Na]⁺: 720.3836, found 720.3811. Compound **4a**: HRMS calcd for C₄₄H₈₃NO₈ [M+Na]⁺: 776.6013, found 776.6011. Compound **4b**: HRMS calcd for C₄₂H₈₃NO₈ [M+Na]⁺: 768.5962, found 768.5941. Compound **4c**: HRMS calcd for C₄₈H₉₅NO₈ [M+Na]⁺: 836.6956, found 836.6953. Compound **4d**: HRMS calcd for C₅₀H₉₉NO₈ [M+Na]⁺: 864.7268, found 864.7240.
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