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

Adrianne Lee,[a] Kathryn J. Farrand,[b] Nina Dickgreber,[b] Colin M. Hayman,[a] Stefan Jürs,[a,c] Ian F. Hermans[b] and Gavin F. Painter[a,*]

[a]*Carbohydrate Chemistry, Industrial Research Limited, PO Box 31-310, Lower Hutt, New Zealand*
[b]*The Malaghan Institute of Medical Research, PO Box 7060, Wellington, New Zealand*
[c]*Institut für Organische Chemie der Universität Hamburg, Martin-Luther-King-Platz 6, 20146 Hamburg, Germany*

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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.

* Corresponding author. Tel.: +64 4 931 3103; fax: +64 4 931 3055; e-mail: g.painter@irl.cri.nz

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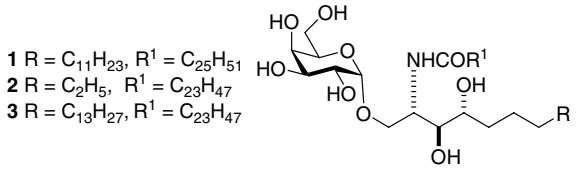
Stimulation of *i*NKT cells in vivo by injection of α-GalCer has been shown to provide a powerful stimulus to vaccine-induced immune responses.[4–6] However, α-GalCer may not be the best candidate for this adjuvant activity if multiple rounds of vaccination are required. It has been suggested that less 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

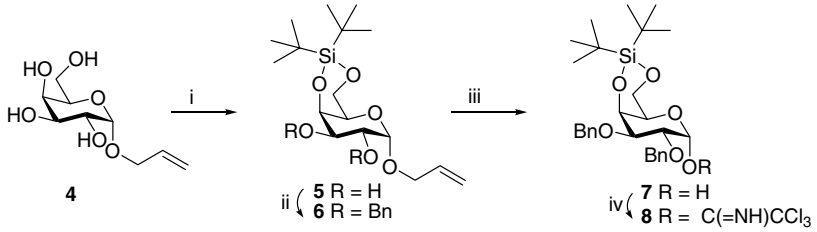
2.1. Synthesis of α-galactosyl-ceramides 1, 2 and 3

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.

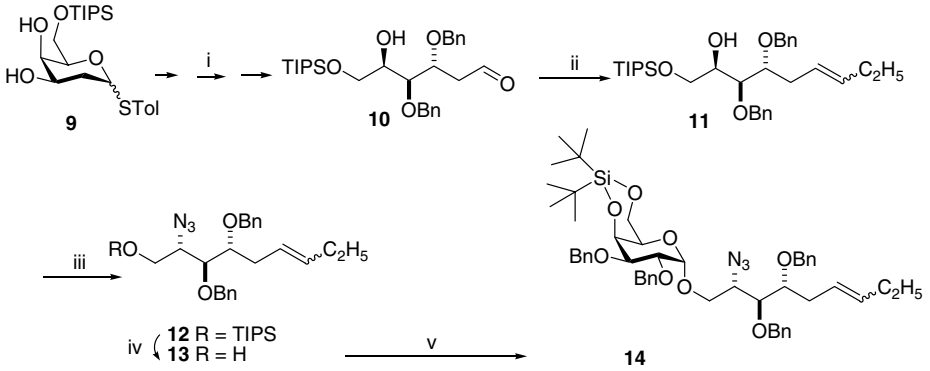
Figueroa-Pérez and Schmidt[27] have demonstrated that use of a 4,6-*O*-benzylidene acetal group within a glycosyl trichloroacetimidate donor can be used to afford α-galactosyl-ceramides in high yield and with good anomeric selectivity. Similarly, the 4,6-*O*-di-*tert*-butylsilylene (DTBS) group can function 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-

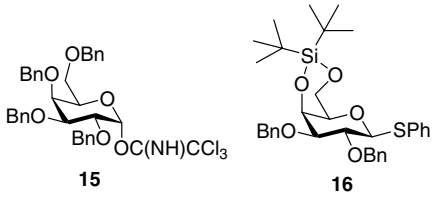


Scheme 1. Reagents, conditions and yields: (i) $^{t}Bu_2Si(OTf)_2$, DMAP, Py (65%); (ii) NaH, BnBr, DMF (66%); (iii) $Ph_2P_2(COD)Ir^{+}PF_6^{-}$, THF, then AcCl, MeOH–$CH_2Cl_2$; (iv) CCl_3CN, DBU, CH_2Cl_2 (steps iii and iv) 40% in total.



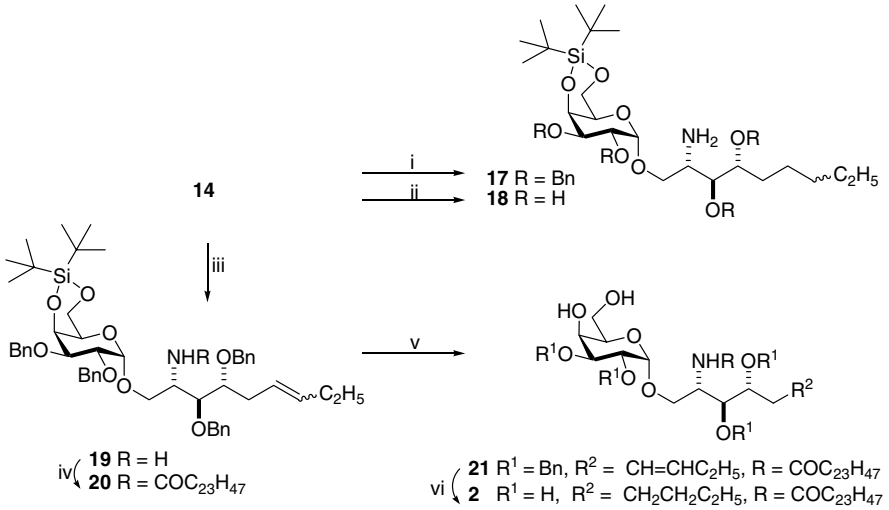
Scheme 2. Reagents, conditions and yields: (i) Ref. 19; (ii) $Ph_3P^+Pr{\cdot}Br^-$, *n*-BuLi, THF (88%); (iii) Ph_3P, DEAD, HN_3, THF (91%); (iv) TBAF, THF (85%); (v) **8**, TMSOTf, CH_2Cl_2 (66%).

ers,[19] glycosylation of the related acceptor **29** (vide infra) with donor **15** afforded the corresponding α-glycoside in 50% yield with a similar α:β ratio. Thioglycoside **16** was also prepared, but standard reaction conditions including activation with NIS/TFOH,[32] DMTSF[33] and MeOTf[34] only gave, at best, minor amounts of the desired galactoside **14**.



Reduction of the azide functional group and hydrogenation of the double bond of glycoside **14** were accomplished in one step by hydrogenation over Pd/C to give saturated amine **17** (Scheme 3). Somewhat surprisingly, the four benzyl ethers appeared to be completely resistant to hydrogenolysis under the conditions used. However, simultaneous hydrogenolysis of the benzyl groups, reduction of the azide and hydrogenation of the alkene to give compound **18** were achieved by hydrogen transfer reaction with ammonium formate.[35] This strategy should allow the introduction of unsaturated fatty acid moieties within the amido alkyl chain, which is currently under investigation. In an alternative procedure, as adopted by others,[19] the azido group of compound **14** was reduced with PMe_3 to afford amine **19** that was subsequently coupled with tetracosanoic acid to give amide **20** (Scheme 3). The silyl protecting group was removed with HF/Py to afford diol **21**, and the final deprotective-hydrogenation/hydrogenolysis with Pd-black afforded target compound **2**.

The above methodology, used to prepare the short chain α-galactosyl-ceramide derivative **2**, was then applied



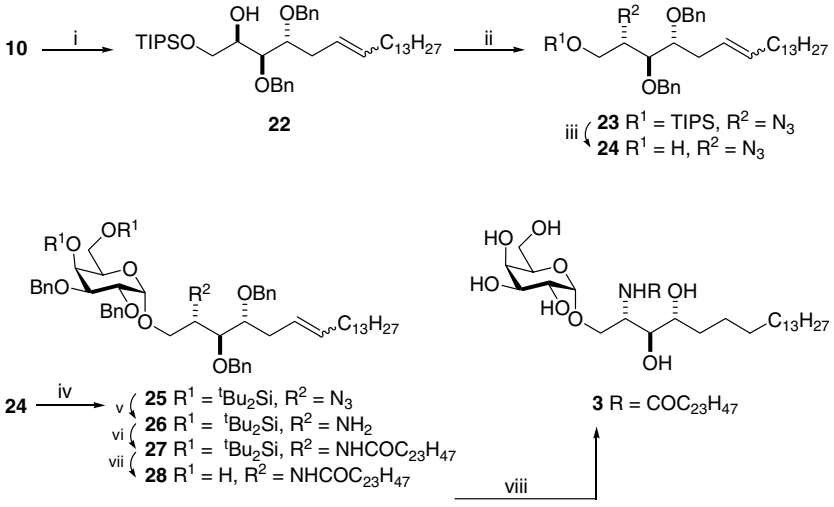
Scheme 3. Reagents, conditions and yields: (i) Pd–C, H_2 (96%); (ii) HCO_2NH_4, Pd–C, MeOH (40%); (iii) Me_3P, THF (99%); (iv) $C_{23}H_{47}CO_2H$, CH_2Cl_2, DMF, then DIPEA (70%); (v) HF·Py, THF (81%); (vi) Pd-black, H_2, $CHCl_3$/MeOH (50%).

with acceptor **24** and donor **8** to give α-galactosyl-ceramide **3** via intermediates **25**–**28**. Acceptor **24** was prepared from aldehyde **10**, by the method used for alcohol **13**, via intermediates **22** and **23** (Scheme 4).

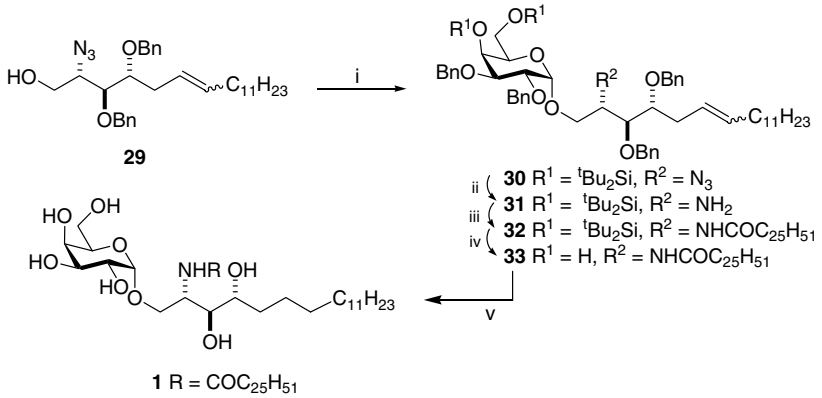
In a similar way, α-galactosyl-ceramide **1** was prepared from acceptor **29**[19] and donor **8**. As before, the glycosylation reaction afforded the α product **30** in good yield (82%) with no detectable amounts of the β anomer (Scheme 5). This result compares favourably with the literature. Figueroa-Pérez and Schmidt[27] observed glycosylation of a triol acceptor with the 4,6-benzylidene-protected trichloroacetimidate affording the α-glycoside in 73% yield. Later Wong[19] demonstrated that glycosylation of **29** with the same 4,6-benzylidene-protected donor afforded the corresponding α-glycoside in 68%. The spectroscopic data collected for **1** were in good agreement with that already reported.[15,23]

2.2. The stimulation effects of α-galactosyl-ceramides on *i*NKT cells in vivo

A hallmark of *i*NKT cell activation is the release of an array of different cytokines into the tissues. Included within this 'cytokine storm' are the cytokines IFN-γ, IL-4 and IL-12, which can be derived from the *i*NKT cells themselves, or from other immune cells that have been stimulated in turn by the activated *i*NKT cells. Thus, activated *i*NKT cells can interact with DC, driving 'maturation' characterized by the release of significant quantities of bioactive IL-12 (IL-12p70) into the serum. Maturation of DC is also associated with an increase in the expression of molecules involved in T cell stimulation on the DC surface.[4–6] We used these two attributes of *i*NKT cell-induced DC maturation to examine the biological activity of the synthetic glyco-



Scheme 4. Reagents, conditions and yields: (i) $Ph_3P^+C_{14}H_{29}\cdot Br^-$, *n*-BuLi, THF (84%); (ii) Ph_3P, DEAD, HN_3, THF (77%); (iii) TBAF, THF (94%); (iv) **8**, TMSOTf, CH_2Cl_2 (63%); (v) Me_3P, THF (94%); (vi) EDCl, HOBt, $C_{23}H_{47}CO_2H$, DIPEA, CH_2Cl_2/DMF (62%); (vii) HF·Py, THF (63%); (viii) Pd-black, H_2, THF/MeOH/H_2O (35:10:1) (79%).



Scheme 5. Reagents, conditions and yields: (i) **8**, TMSOTf, CH_2Cl_2 (82%); (ii) Me_3P, THF; (iii) EDCl, HOBt, $C_{25}H_{51}CO_2H$, DIPEA, CH_2Cl_2/DMF (86%); (iv) HF·Py, THF (79%); (v) Pd-black, H_2, THF/MeOH/H_2O (35:10:1) (85%).

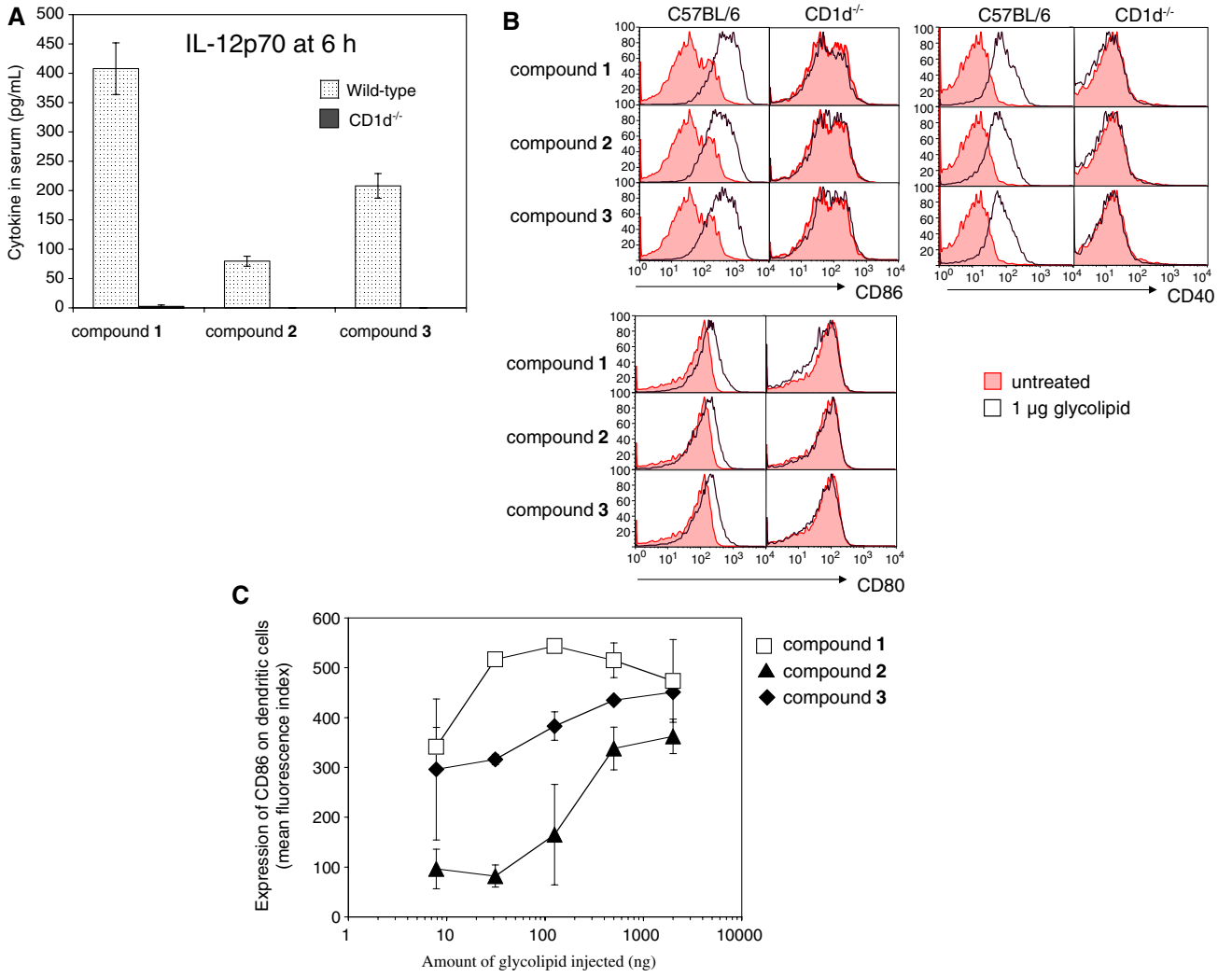


Figure 1. Injection of synthetic glycolipids induces activation of *i*NKT cells and subsequent maturation of dendritic cells. C57BL/6 (wild-type) and CD1d$^{-/-}$ mice ($n = 3$) were injected intravenously with 1 μg of glycolipid. (A) Serum was collected 6 h after the injection for the analysis of IL-12p70 levels. Mean cytokine levels ± SE are presented. (B) Spleens were removed 20 h after injection for antibody labelling and flow cytometry. The expression of CD40, CD80 and CD86 was assessed on CD11c^{+} dendritic cells. Representative FACS profiles are shown. (C) Titrated doses of glycolipids were injected intravenously into groups of C57BL/6 mice ($n = 3$) and spleens were removed 20 h later for the analysis of CD86 expression on CD11c^{+} dendritic cells. Mean fluorescence index ± SE are presented.

lipids. Injection of 1 μg of the synthetic compounds **1**, **2** or **3** into wild-type mice induced the release of IL-12p70 into the serum (Fig. 1A). In contrast, no IL-12p70 was released when the compounds were injected into CD1d-deficient mice, which are completely devoid of *i*NKT cells. This readout of *i*NKT cell activity showed compound **1** (α-GalCer) to have the most potent activity, and compound **2** (OCH) the least. The new derivative of α-GalCer, compound **3**, had intermediate activity. Using antibody labelling and flow cytometry, expression of the co-stimulatory molecules CD80 and CD86, and CD40 was shown to be increased on splenic DC (defined as CD11c^{+} cells) in wild-type animals following injection with 1 μg of **1**, **2** or **3** (Fig. 1B). No increase in the expression of these molecules was observed in CD1d-deficient animals. To assess the potency of the different compounds to induce *i*NKT cell-mediated maturation of DC, the mice were injected with titrated doses of the glycolipids. This analysis, shown in Figure 1C, again showed compound **1** (α-GalCer) to have the most potent activity, compound **2** (OCH) the least and compound **3** to have intermediate activity.

The ability of the different compounds to induce different profiles of cytokine release was also assessed (Fig. 2). In agreement with the published data,[10,36] injection of 1 μg of α-GalCer (**1**) led to the release of considerable quantities of both IL-4 and IFN-γ into the serum, whereas injection of 1 μg of analogue OCH (**2**) induced a weaker response, with considerably less IFN-γ, indicating a Th2 bias. The injection of 1 μg of compound **3** induced a similar release of IL-4 to OCH (**2**), but the intermediate levels of IFN-γ indicated less Th2 bias.

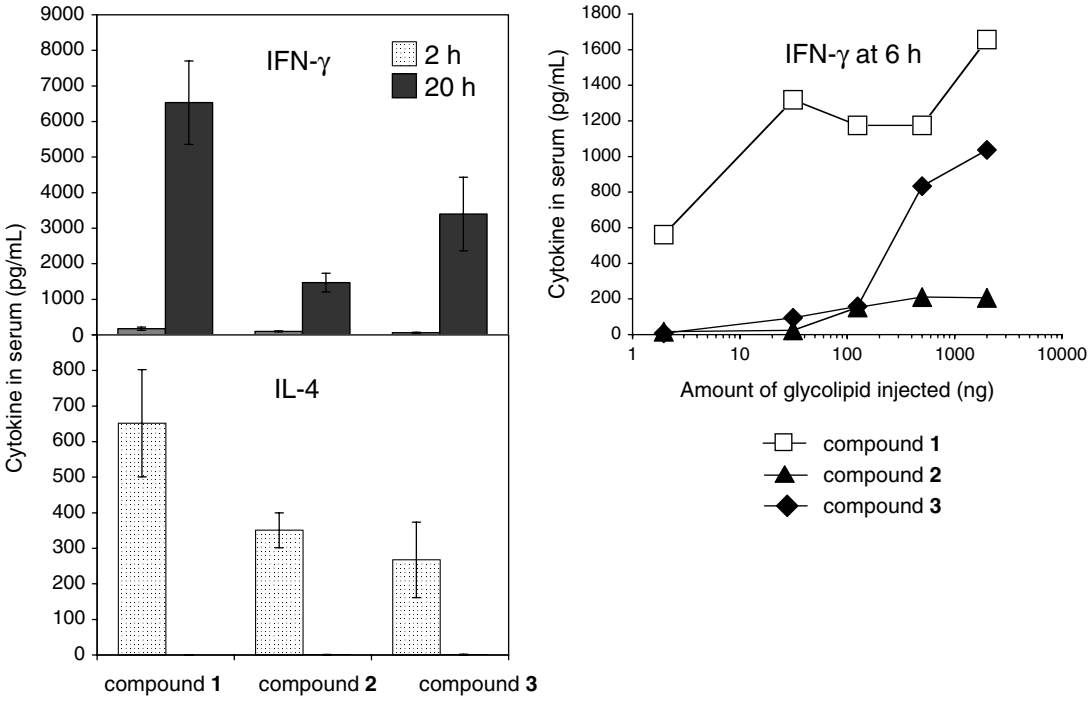


Figure 2. Kinetics of cytokine release into serum following the injection of glycolipids. C57BL/6 mice were injected intravenously with 1 μg of glycolipid (left panels, $n = 8$), or titrated doses of glycolipid (right panel) and then the serum was collected at the indicated times for analysis of IL-4 and IFN-γ levels.

This hierarchy of IFN-γ responses was shown again when titrated doses of glycolipids were injected (Fig. 2, right panel), with compound **3** inducing the release of significantly higher levels of IFN-γ than OCH (**2**) when more glycolipid was injected, but never exceeding the levels induced by α-GalCer (**1**).

3. Conclusions

We have reported a new synthetic protocol for the synthesis of α-galactosyl-ceramides that should have general applicability. The selectivity of the key glycosylation reaction was controlled by incorporation of the powerful α-directing 4,6-*O*-DTBS group into the galactosylating donor. The synthetic compounds prepared by this methodology were shown to be powerful *i*NKT cell agonists with the capacity to induce cytokine release and DC maturation in a CD1d-dependent manner. One of these structures (**3**), has the capacity to stimulate *i*NKT with less potency than α-GalCer (**1**), but without the stronger Th2 bias of OCH (**2**). Further study on the adjuvant activity of compound **3** is now being carried out, with specific emphasis on the ability of this compound to be utilized in repeated vaccination procedures. The new α-galactosyl-ceramide synthesis protocol described here may permit the investigation of new derivatives[37] in an attempt to further understand the mechanism of *i*NKT cell activation, and ways in which this cell type can be exploited therapeutically.

4. Experimental

4.1. General methods

Melting points were measured on a Gallenkamp capillary melting point apparatus and are uncorrected. Specific optical rotations, given in 10^{-1} deg cm^2 g^{-1}, were measured at ambient temperature using either a Jasco DIP-370 or DIP-1000 polarimeter with a cell of path length 1.0 dm. ^{1}H NMR spectra were obtained at either 300 or 500 MHz and referenced to the residual solvent peak. Chemical shifts are reported using the δ scale, and coupling constants (J) and separations are reported in hertz. ^{13}C NMR spectra were recorded at 75 or 125 MHz and chemical shifts are reported using the δ scale. FAB mass spectra were recorded on a Kratos MSORF or a VG70-250S double focusing, magnetic sector mass spectrometer under chemical ionization conditions using isobutene, ammonia or xenon as the ionizing gas, or under high-resolution FAB conditions in a glycerol or nitrobenzyl alcohol matrix. Electro-spray ionization (ESI) mass spectra were recorded on a PerSeptive Biosystems Mariner time of flight mass spectrometer. Elemental analyses were carried out by Dr. R. G. Cunninghame and associates at the Campbell Micro-

analytical Laboratory, University of Otago, Dunedin, New Zealand. Thin layer chromatography (TLC) was performed on Merck silica gel DC Alurolle Kieselgel $60F_{254}$ plates and were visualized under UV light and/or with a spray (5% w/v dodecamolybdophosphoric acid in EtOH) with subsequent heating. Flash column chromatography was carried out using Merck Kieselgel 60 (230–400 mesh). All chromatographic solvents were of reagent grade. THF was distilled from sodium-benzophenone ketyl under nitrogen and dichloromethane was distilled from phosphorus pentoxide. All other solvents and reagents were purified using the methods described by Perrin and Armarego.[38] Organic solutions were dried over $MgSO_4$.

4.2. Allyl 4,6-*O*-di-*tert*-butylsilylene-D-galactopyranoside (5)

Di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (3.40 mL, 10.6 mmol) was added drop-wise to a stirred solution of allyl α-D-galactopyranoside (1.94 g, 8.80 mmol) and DMAP (537 mg, 4.40 mmol) in pyridine (15 mL) cooled to −10 °C. After being stirred at 0 °C for 1 h and rt for 3 h, the reaction solution was diluted with H_2O (100 mL) and extracted with ether (2 × 100 mL). The combined organic extracts were washed with HCl (1 M, 150 mL) and H_2O (150 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (3:7 to 35:65) afforded the title compound **5** as an oil (1.85 g, 5.13 mmol, 58%); $[\alpha]_D^{20}$ +127 (*c* 0.9, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.97–5.83 (m, 1H), 5.35–5.25 (m, 1H), 5.23–5.16 (m, 1H), 4.99 (d, $J = 3.7$ Hz, 1H), 4.41 (d, $J = 3.2$ Hz, 1H), 4.28 (dd, $J = 12.5$, 2.2 Hz, 1H), 4.22–4.12 (m, 2H), 4.09–4.01 (m, 1H), 3.90–3.70 (m, 3H), 2.67 (d, $J = 9.6$ Hz, 1H), 2.37 (d, $J = 10.1$ Hz, 1H), 1.06 (s, 9H), 1.03 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 133.7, 117.6, 98.2, 73.3, 71.3, 69.8, 68.6, 67.5, 66.9, 27.6, 27.2, 23.4, 20.7; HRMS-ESI $[M+H]^+$ calcd for $C_{17}H_{33}O_6Si$: 361.2046. Found 361.2050.

4.3. Allyl 2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranoside (6)

Sodium hydride (60% dispersion in mineral oil, 439 mg, 11.0 mmol) was added to a stirred solution of diol **5** (1.80 g, 4.99 mmol) and benzyl bromide (1.44 mL, 12.5 mmol) in DMF (30 mL) cooled to −20 °C. After 3 h, the cold bath was removed and the reaction mixture was stirred at rt for a further 12 h when NH_4Cl solution (100 mL, satd aq soln) and H_2O (100 mL) were added. The mixture was extracted with ether (2 × 150 mL) and the combined ethereal extracts were washed with H_2O (2 × 100 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:4 to 3:7) afforded the title compound **6** as an oil (1.67 g, 3.09 mmol, 62%); $[\alpha]_D^{22}$ +66 (*c* 0.6, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.21 (m, 10H), 5.96–5.83 (m, 1H), 5.34–5.25 (m, 1H), 5.20–5.13 (m, 1H), 4.86 (d, $J = 12.0$ Hz, 1H), 4.78 (d, $J = 3.7$ Hz, 1H), 4.73 (s, 2H), 4.67 (d, $J = 12.0$ Hz, 1H), 4.49 (d, $J = 2.6$ Hz, 1H), 4.22–3.97 (m, 5H), 3.85 (dd, $J = 10.0$, 3.0 Hz, 1H), 3.62 (s, 1H), 1.06 (s, 9H), 0.99 (s, 9H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.1, 138.6, 134.1, 128.2, 127.5, 127.4, 117.5, 97.1, 77.7, 74.2, 73.6, 71.2, 71.1, 68.2, 67.3, 67.2, 27.6, 27.3, 23.4, 20.6; HRMS-ESI $[M+Na]^+$ calcd for $C_{31}H_{44}O_6NaSi$: 563.2805. Found 563.2813.

4.4. 2,3-Di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-D-galactopyranose (7)

1,5-Cyclooctadiene)bis(methyldiphenylphosphine)iridium(I) hexafluorophosphate (390 mg, 0.63 mmol) was added to a stirred solution of allyl glycoside **7** (1.75 g, 3.24 mmol) in THF (50 mL) under argon. This atmosphere was replaced with hydrogen for ca. 1 min and the hydrogen was replaced with argon. The mixture was stirred at 20 °C for 70 min, the solvent was removed in vacuo and the residue dissolved with stirring in CH_2Cl_2/MeOH (2:1, 30 mL). Acetyl chloride (1.0 mL, 12.9 mmol) was added to this solution and stirring continued for a further 90 min when solid $NaHCO_3$ (2.00 g, 23.8 mmol) was added. The mixture was stirred for an additional 5 min when H_2O (50 mL) was added. This mixture was extracted with ether (2 × 100 mL) and after drying and filtration the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:4 to 35:65) afforded the title compound **7** as an oil (843 mg, 1.68 mmol, 52%); ^{13}C NMR (75 MHz, $CDCl_3$), α-anomer only, δ 138.8, 138.2, 129.1, 128.3, 127.8, 127.4, 92.2, 77.5, 77.4, 74.8, 73.9, 70.9, 67.5, 67.4, 27.7, 26.9, 23.4, 20.7; HRMS-ESI $[M+Na]^+$ calcd for $C_{28}H_{40}SiNaO_6$: 523.2492. Found 523.2493.

4.5. *O*-(2,3-Di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyl) trichloroacetimidate (α:β, 9:1) (8)

DBU (24 μL, 0.17 mmol) was added drop-wise to a stirred solution of hemi-acetal **7** (825 mg, 1.65 mmol) and trichloroacetonitrile (0.830 mL, 8.25 mmol) in CH_2Cl_2 (20 mL) cooled to 0 °C. After 1 h, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (15:85) afforded the title α-compound **8** as an oil (840 mg, 1.30 mmol, 79%); ^{1}H NMR (300 MHz, $CDCl_3$), α anomer, δ 8.61 (s, 1H), 7.42–7.23 (m, 10H), 6.47 (d, $J = 3.5$ Hz, 1H), 4.89–4.67 (m, 4H), 4.59 (d, $J = 2.5$ Hz, 1H), 4.25–4.10 (m, 3H), 3.91 (dd, $J = 9.9$, 2.9 Hz, 1H), 3.83 (br s, 1H), 1.06 (s, 9H), 1.01 (s, 9H);

^{13}C NMR (75 MHz, $CDCl_3$), α anomer, δ 161.4, 138.7, 138.4, 128.3, 128.1, 127.8, 127.6, 95.8, 77.0, 73.8, 73.3, 71.1, 70.9, 70.1, 66.9, 27.7, 27.3, 23.4, 20.8.

4.6. (2*R*,3*S*,4*R*)-3,4-Di(benzyloxy)-1-triisopropylsilyloxy-non-6-en-2-ol (*E*:*Z*, 4:1) (11)

n-BuLi (1.44 M in hexanes, 5.60 mL, 8.10 mmol) was added drop-wise to a stirred solution of propyl triphenylphosphonium bromide (3.42 g, 8.88 mol) in THF (30 mL) cooled to −60 °C. After being stirred for 45 min, a solution of 3,4-di-*O*-benzyl-2-deoxy-6-*O*-triisopropylsilyl-D-galactopyranose (**10**) (1.69 g, 3.38 mmol) in THF (15 mL) was added drop-wise over 30 min to the reaction mixture. After being stirred for a further 12 h, the mixture was quenched with the addition of NH_4Cl solution (100 mL, satd aq soln) and H_2O (100 mL). The mixture was extracted with ether (2 × 150 mL) and the combined ethereal extracts were washed with brine (100 mL) and H_2O (100 mL). After drying and filtration the solvent, was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (5:95) afforded the title compound **11** as an oil (1.56 g, 2.96 mmol, 88%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32–7.21 (m, 10H), 5.55–5.37 (m, 2H), 4.70–4.59 (m, 4H), 3.93–3.85 (m, 1H), 3.79–3.67 (m, 4H), 3.05 (d, $J = 6.0$ Hz, 1H), 2.45–2.38 (m, 2H), 2.05–1.97 (m, 2H), 1.10–0.85 (m, 24H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 138.4, 133.9, 128.4, 128.3, 128.0, 127.8, 127.7, 127.6, 124.7, 80.2, 78.3, 73.8, 72.8, 71.4, 64.0, 29.0, 20.8, 18.0, 14.1, 12.0; HRMS-ESI $[M+H]^+$ calcd for $C_{32}H_{51}O_4Si$: 527.3557. Found 527.3550.

4.7. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-1-triisopropylsilyloxy-non-6-ene (*E*:*Z*, 4:1) (12)

Diethyl azodicarboxylate (1.80 mL, 11.4 mmol) and hydrazoic acid (0.7 M in toluene, 16.3 mL, 11.4 mmol) were added together to a stirred solution of triphenylphosphine (2.99 g, 11.4 mmol) in THF (25 mL) cooled to −78 °C. After 1 min, a solution of alcohol **11** (1.50 g, 2.85 mmol) was cannulated into the reaction mixture. After stirring for 10 h, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (5:95) afforded the title compound **12** as an oil (1.43 g, 2.58 mmol, 91%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.33–7.21 (m, 10H), 5.45–5.37 (m, 2H), 4.62–4.43 (m, 4H), 3.97–3.90 (m, 1H), 3.75–3.65 (m, 1H), 3.63–3.56 (m, 2H), 3.54–3.48 (m, 1H), 2.40–2.32 (m, 2H), 2.00–1.90 (m, 2H), 1.11–0.86 (m, 24H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 138.4, 138.2, 133.9, 128.4, 128.0, 127.7, 127.6, 124.8, 79.6, 79.1, 73.7, 72.2, 64.7, 64.6, 28.0, 20.8, 18.0, 14.1, 12.0; HRMS-ESI $[M+H]^+$ calcd for $C_{32}H_{50}N_3O_3Si$: 552.3616. Found 552.3590.

4.8. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-non-6-en-1-ol (*E*:*Z*, 4:1) (13)

A solution of TBAF in THF (1.0 M, 3.83 mL, 3.83 mmol) was added to a stirred solution of silyl ether **12** (1.41 g, 2.56 mmol) in THF (30 mL) cooled to 5 °C. After the addition the cold bath was removed and the reaction mixture stirred at ambient temperature for 90 min The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9) afforded the title compound **13** as an oil (860 mg, 2.17 mmol, 85%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.21 (m, 10H), 5.55–5.38 (m, 2H), 4.64–4.45 (m, 4H), 3.88–3.62 (m, 5H), 2.49–2.38 (m, 3H), 2.11–1.97 (m, 2H), 0.98–0.93 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 138.0, 137.7, 134.4, 128.5, 128.4, 128.1, 128.0, 127.9, 124.1, 80.5, 78.8, 73.7, 72.4, 63.3, 62.4, 28.2, 20.9, 14.1; HRMS-ESI $[M+H-N_2]^+$ calcd for $C_{23}H_{30}NO_3$: 368.2226. Found 368.2216.

4.9. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-non-6-ene (*E*:*Z*, 4:1) (14)

TMSOTf (46.0 μL, 0.251 mmol) was added drop-wise to a stirred mixture of donor (**8**) (809 mg, 1.25 mmol), acceptor (**13**) (378 mg, 0.956 mmol) and 4 Å molecular sieves (220 mg) in CH_2Cl_2 (30 mL) at −40 °C. The reaction mixture was allowed to warm to 0 °C over 2 h when Et_3N (2.5 mL) was added. The mixture was filtered through Celite and the filter cake washed with further CH_2Cl_2 (2 × 100 mL). The solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:19) afforded the title compound **14** (554 mg, 0.631 mmol, 66%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45–7.39 (m, 2H), 7.35–7.20 (m, 18H), 5.55–5.35 (m, 2H), 4.87–4.45 (m, 10H), 4.15–3.60 (m, 9H), 3.55 (br s, 1H), 2.47–2.34 (m, 2H), 2.09–1.95 (m, 2H), 1.05 (s, 9H), 0.99 (s, 9H), 0.92–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 139.1, 138.8, 138.3, 138.2, 134.1, 128.4, 128.3, 128.1, 127.8, 127.8, 127.7, 127.5, 127.5, 124.5, 99.1, 79.3, 79.3, 77.6, 74.3, 73.8, 73.6, 72.1, 71.2, 71.0, 68.2, 67.7, 67.1, 62.0, 28.1, 27.7, 27.4, 23.5, 20.9, 20.7, 14.2; Gated decoupled ^{13}C NMR (75 MHz, $CDCl_3$) selected data, δ 99.1, $^1J_{C1'-H1'}$ 168.6 Hz; HRMS-ESI $[M+H-N_2]^+$ calcd for $C_{51}H_{68}NO_8Si$: 850.4714. Found 850.4706.

4.10. (2*S*,3*S*,4*R*)-2-Amino-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-nonane (17)

A mixture of Pd on carbon (20%, 70 mg), and alkene **14** (355 mg, 0.404 mmol) in MeOH (30 mL) was stirred

under a hydrogen atmosphere for 12 h. The mixture was filtered through Celite and the cake washed with MeOH (100 mL). The solvent was removed in vacuo to afford the title compound **17** (332 mg, 0.389 mmol, 96%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.19 (m, 20H), 4.89–4.45 (m, 9H), 4.15–3.90 (m, 4H), 3.82 (dd, $J = 10.1$, 2.8 Hz, 1H), 3.75–3.65 (m, 2H), 3.59–3.50 (m, 2H), 3.40–3.30 (m, 1H), 3.20–3.10 (m, 1H), 1.95–1.75 (m, 2H), 1.38–1.15 (m, 6H), 1.05 (s, 9H), 0.97 (s, 9H), 0.92–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.0, 138.7, 138.6, 128.4, 128.1, 127.8, 127.6, 127.5, 127.0, 99.2, 82.1, 80.0, 77.9, 74.7, 73.7, 73.6, 72.1, 71.5, 71.0, 70.8, 67.4, 67.3, 52.9, 32.1, 30.6, 27.7, 27.4, 25.5, 23.5, 22.7, 20.7, 14.1; HRMS-ESI $[M+H]^+$ calcd for $C_{51}H_{72}NO_8Si$: 854.5027. Found 854.5019.

4.11. (2*S*,3*S*,4*R*)-2-Amino-1-(4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-nonane-3,4-diol (18)

A mixture of Pd on carbon (20%, 73 mg), ammonium formate (76 mg, 0.15 mmol) and tetrabenzyl ether **17** (30 mg, 0.03 mmol) in MeOH (5 mL) was heated under reflux for 3 h. The mixture was cooled, filtered through Celite and the cake washed with MeOH (3×30 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with MeOH/$CHCl_3$ (0:1 to 1:9) afforded the title compound **18** (6 mg, 0.012 mmol, 40%) as an oil; ^{1}H NMR (300 MHz, CD_3OD) δ 4.42–4.33 (m, 1H), 4.20 (dd, $J = 12.5$, 1.9 Hz, 1H), 4.01 (dd, $J = 12.5$, 1.4 Hz, 1H), 3.88–3.76 (m, 2H), 3.73–3.62 (m, 2H), 3.57–3.27 (m, 5H), 3.06–2.95 (m, 1H), 1.75–1.60 (m, 1H), 1.55–1.40 (m, 1H), 1.35–1.15 (m, 6H), 1.03 (s, 9H), 0.93 (s, 9H), 0.88–0.78 (m, 3H); ^{13}C NMR (75 MHz, CD_3OD) δ 101.5, 76.9, 75.4, 74.2, 71.8, 70.9, 70.1, 69.0, 68.2, 54.1, 35.0, 33.2, 28.2, 27.9, 26.2, 24.2, 23.7, 21.6, 14.4; HRMS-ESI $[M+H]^+$ calcd for $C_{23}H_{48}NO_8Si$: 494.3149. Found 494.3155.

4.12. (2*S*,3*S*,4*R*)-2-Amino-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-non-6-ene (*E*:*Z*, 4:1) (19)

Trimethylphosphine (1 M in THF, 0.820 mL, 0.880 mmol) was added to a stirred solution of azide **14** (145 mg, 0.165 mmol) in THF (15 mL) cooled to 0 °C and the mixture was stirred for 45 min. After stirring at ambient temperature for a further 1.5 h, NaOH (aq, 1 M, 50 mL, 1.50 mmol) was added. After a further 2 h, the mixture was diluted with EtOAc (150 mL) and washed with H_2O (2×100 mL), brine (100 mL) dried and filtered. The solvent was removed to give the title compound **19** (140 mg, 0.164 mmol, 99%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.30 (m, 2H), 7.28–7.14 (m, 18H), 5.52–5.30 (m, 2H), 4.78–4.40 (m, 11H), 4.07–3.60 (m, 7H), 3.50–3.42 (m, 2H), 3.32–3.23 (m 1H), 3.17–3.08 (m, 1H), 2.41–2.29 (m, 2H), 2.04–1.90 (m, 2H), 1.05 (s, 9H), 0.99 (s, 9H), 0.92–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 139.0, 138.6, 133.7, 128.4, 128.1, 127.8, 127.7, 127.6, 127.6, 127.5, 125.1, 99.2, 82.1, 79.9, 77.9, 74.7, 73.7, 72.0, 71.5, 71.1, 70.8, 67.4, 67.3, 52.9, 30.4, 28.4, 27.7, 27.4, 23.5, 20.9, 20.8, 14.2; HRMS-ESI $[M+H]^+$ calcd for $C_{51}H_{70}NO_8Si$: 852.4871. Found 852.4873.

4.13. (2*S*,3*S*,4*R*)-3,4-Di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-2-tetracosanoylamino-non-6-en (*E*:*Z*, 4:1) (20)

1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (38 mg, 0.197 mmol) and 1-hydroxybenzotriazole (27 mg, 0.197 mg) were added to a stirred suspension of tetracosanoic acid (60 mg, 0.164 mmol) in a CH_2Cl_2 (10 mL), DMF (4 mL) solvent mixture cooled to 0 °C. After 2 min, a solution of amine **19** (140 mg, 0.164 mmol) and DIPEA (70 μL, 0.394 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was stirred at ambient temperature for 12 h when it was diluted with the addition of EtOAc/Et_2O (4:1, 200 mL). The organic phase was washed with a satd aq soln of $NaHCO_3$ (100 mL), HCl (1 M, 100 mL) and brine (150 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (0:1 to 1:9) afforded the title compound **20** (139 mg, 0.115 mmol, 70%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.39 (m, 2H), 7.36–7.21 (m, 18H), 5.85 (d, $J = 8.5$ Hz, 1H), 5.55–5.37 (m, 2H), 4.86–4.45 (m, 10H), 4.36–4.25 (m, 1H), 4.15–3.93 (m, 3H), 3.82–3.70 (m, 4H), 3.59–3.51 (m, 2H), 2.54–2.35 (m, 2H), 2.10–1.97 (m, 2H), 1.92–1.83 (m, 2H), 1.53–1.40 (m, 2H), 1.28–1.20 (m, 40H), 1.06 (s, 9H), 0.99 (s, 9H), 0.91–0.84 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 172.9, 138.9, 138.5, 133.8, 128.4, 128.2, 127.9, 127.9, 127.8, 127.7, 127.6, 125.0, 99.5, 79.7, 79.4, 77.8, 74.6, 73.9, 73.4, 71.7, 70.9, 70.7, 68.1, 67.7, 67.3, 50.2, 36.8, 32.0, 29.8, 29.5, 29.4, 27.9, 27.7, 27.4, 25.8, 23.9, 22.7, 20.8, 20.8, 14.2; HRMS-ESI $[M+H]^+$ calcd for $C_{75}H_{115}NO_9Si$: 1202.8419. Found 1202.8389.

4.14. (2*S*,3*S*,4*R*)-3,4-Di(benzyloxy)-1-(2,3-di-*O*-benzyl-α-D-galactopyranosyloxy)-2-tetracosanoylamino-non-6-ene (*E*:*Z*, 4:1) (21)

A solution of HF·py (40 M, 0.350 mL, 14.0 mmol) was added to a stirred solution of amide **20** (135 mg, 0.112 mmol) in THF (10 mL) cooled to 0 °C. After 1 h, the mixture was diluted with EtOAc (100 mL) and washed with a satd aq soln of $NaHCO_3$ (50 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:1 to 3:1)

afforded the title compound **21** (96 mg, 0.09 mmol, 81%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36–7.22 (m, 20H), 5.78 (d, $J = 8.2$ Hz, 1H), 5.60–5.38 (m, 2H), 4.84 (d, $J = 2.9$ Hz, 1H), 4.79–4.39 (m, 9H), 4.02 (br s, 1H), 3.92–3.54 (m, 9H), 2.80–2.35 (m, 4H), 2.11–1.97 (m, 3H), 1.94–1.75 (m, 3H), 1.55–1.37 (m, 2H), 1.28–1.20 (m, 40H), 0.99–0.82 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 173.1, 138.5, 138.3, 138.1, 133.9, 128.6, 128.4, 128.0, 127.9, 127.8, 127.7, 124.7, 99.5, 80.3, 79.1, 77.6, 75.9, 73.5, 73.2, 72.6, 71.7, 69.9, 69.4, 68.5, 62.8, 50.5, 36.8, 32.0, 29.7, 29.6 29.5, 29.4, 28.0, 25.7, 22.7, 20.8, 14.2, 14.1; HRMS-ESI $[M+H]^+$ calcd for $C_{67}H_{100}NO_9$: 1062.7398. Found 1062.7408.

4.15. (2*S*,3*S*,4*R*)-1-(α-D-Galactopyranosyloxy)-2-tetracosanoylamino-nonane-3,4-diol (2)

A mixture of Pd-black (9 mg) and **21** (90 mg, 0.085 mmol) in $CHCl_3$/MeOH (15 mL, 1:2) was stirred under a hydrogen atmosphere for 12 h. The mixture was filtered through Celite and the cake washed with $CHCl_3$/MeOH/H_2O (70:20:2, 200 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with MeOH/chloroform (1:49 to 1:9) afforded the title compound **2** (30 mg, 0.043 mmol, 50%) as a white hygroscopic solid; ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD, 3:1) δ 4.83 (d, $J = 3.7$ Hz, 1H), 4.15–4.08 (m 1H), 3.88–3.76 (m, 2H), 3.75–3.55 (m, 7H), 3.49–3.42 (m, 2H), 2.16–2.07 (m, 2H), 1.65–1.41 (m, 2H), 1.27–1.10 (m, 51H), 0.85–0.76 (m, 6H); ^{13}C NMR (150 MHz, CD_3OD) δ 174.3, 99.6, 74.7, 71.8, 70.6, 70.1, 69.6, 68.8, 67.3, 61.7, 50.2, 36.3, 32.4, 31.7, 29.5, 29.2, 25.6, 25.2, 22.4, 13.7; HRMS-ESI $[M+H]^+$ calcd for $C_{39}H_{78}NO_9$: 704.5677. Found 704.5694; Anal. ($C_{39}H_{77}NO_9 \cdot 0.2CHCl_3$) C, H, N.

4.16. (2*R*,3*S*,4*R*)-3,4-Di(benzyloxy)-1-triisopropylsilyloxy-icos-6-en-2-ol (*E*:*Z*, 4:1) (22)

n-BuLi (1.44 M in hexanes, 6.75 mL, 9.74 mmol) was added drop-wise to a stirred solution of tetradecyl triphenylphosphonium bromide (5.60 g, 10.5 mmol) in THF (40 mL) cooled to −60 °C. After stirring for 1 h a solution of 3,4-di-*O*-benzyl-2-deoxy-6-*O*-triisopropylsilyl-D-galactopyranose (**10**) (1.74 g, 3.48 mmol) in THF (20 mL) was added drop-wise over 15 min to the reaction mixture. After being stirred for a further 12 h, the mixture was quenched with the addition of NH_4Cl solution (100 mL, satd aq soln) and H_2O (100 mL). The mixture was extracted with ether (2 × 200 mL) and the combined ethereal extracts were washed with brine (100 mL) and H_2O (100 mL). After drying and filtration, the solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9) afforded the title compound **22** as an oil (1.99 g, 2.92 mmol, 84%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.37–7.26 (m, 10H), 5.57–5.31 (m, 2H), 4.73–4.59 (m, 4H), 4.00–3.90 (m, 1H), 3.80–3.69 (m, 4H), 3.09–3.01 (m, 1H), 2.53–2.35 (m, 2H), 2.07–1.93 (m, 2H), 1.33 (m, 22H), 1.08–1.02 (m, 21H), 0.91–0.82 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 138.5, 133.9, 133.7, 132.4, 128.4, 128.4, 128.3, 128.2, 128.1, 127.9, 127.8, 127.6, 125.2, 80.2, 78.4, 73.8, 72.8, 71.5, 64.1, 32.0, 29.8, 29.7, 29.4, 29.1, 27.7, 22.8, 18.1, 14.2, 12.0; HRMS-ESI $[M+H]^+$ calcd for $C_{43}H_{73}O_4Si$: 681.5278. Found 681.5265.

4.17. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-1-triisopropylsilyloxy-icos-6-ene (*E*:*Z*, 4:1) (23)

Diethyl azodicarboxylate (1.80 mL, 11.4 mmol) and hydrazoic acid (0.7 M in toluene, 16.4 mL, 11.5 mmol) were added together to a stirred solution of triphenylphosphine (3.00 g, 11.5 mmol) in THF (40 mL) cooled to −78 °C. After 1 min a solution of alcohol **22** (1.95 g, 2.86 mmol) was cannulated onto the reaction mixture. After stirring for 10 h MeOH (1 mL) was added and the solvent was removed in vacuo. The residue was purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (0:1 to 5:95) afforded the title compound **23** as an oil (1.56 g, 2.21 mmol, 77%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35–7.26 (m, 10H), 5.55–5.39 (m, 2H), 4.73–4.50 (m, 4H), 4.08–4.00 (m, 1H), 3.87–3.78 (m, 1H), 3.75–3.64 (m, 2H), 3.63–3.58 (m, 1H), 2.50–2.37 (m, 2H), 2.08–1.95 (m, 2H), 1.35–1.20 (m, 22H), 1.08–1.02 (m, 21H), 0.91–0.83 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 138.5, 138.3, 132.4, 128.4, 128.0, 127.9, 127.8, 127.6, 125.3, 79.7, 79.1, 73.7, 72.2, 64.8, 64.7, 32.0, 29.8, 29.5, 29.4, 28.1, 27.7, 22.8, 18.0, 14.2, 12.0; HRMS-ESI $[M+H-N_2]^+$ calcd for $C_{43}H_{72}NO_3Si$: 678.5281. Found 678.5281.

4.18. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-icos-6-en-1-ol (*E*:*Z*, 4:1) (24)

A solution of TBAF in THF (1.0 M, 2.90 mL, 2.90 mmol) was added to a stirred solution of silyl ether **23** (1.40 g, 1.98 mmol) in THF (20 mL) cooled to 0 °C. After the addition, the cold bath was removed and the reaction mixture stirred at ambient temperature for 1 h. The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:9) afforded the title compound **24** as an oil (1.02 g, 1.86 mmol, 94%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38–7.27 (m, 10H), 5.59–5.35 (m, 2H), 4.68–4.52 (m, 4H), 3.90–3.62 (m, 5H), 2.50–2.38 (m, 3H), 2.07–1.95 (m, 2H), 1.33–1.21 (m, 22H), 0.91–0.82 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 138.0, 137.7, 132.8, 128.5, 128.4, 128.0, 128.0, 127.8, 124.6, 80.5, 78.8, 73.7, 72.4, 63.3,

62.3, 32.0, 29.7, 29.7, 29.6, 29.4, 28.3, 27.6, 22.7, 17.7, 14.1, 12.4; HRMS-ESI $[M+H-N_2]^+$ calcd for $C_{34}H_{52}NO_3$: 522.3947. Found 522.3947.

4.19. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-icos-6-ene (*E*:*Z*, 4:1) (25)

TMSOTf (19 μL, 0.108 mmol) was added drop-wise to a stirred mixture of donor **8** (350 mg, 0.542 mmol), acceptor **24** (265 mg, 0.482 mmol) and 4 Å molecular sieves (120 mg) in CH_2Cl_2 (20 mL) at −40 °C. The reaction mixture was allowed to warm to 0 °C over 2 h when Et_3N (2.5 mL) was added. The mixture was filtered through Celite and the filter cake was further washed with CH_2Cl_2 (2 × 100 mL). The solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (0:1 to 1:19) afforded the title compound **25** (312 mg, 0.302 mmol, 63%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.38 (m, 2H), 7.35–7.20 (m, 18H), 5.53–5.37 (m, 2H), 4.84 (d, $J = 11.8$ Hz, 1H), 4.79 (d, $J = 3.6$ Hz, 1H), 4.75–4.45 (m, 8H), 4.11–3.62 (m, 9H), 3.55 (s, 1H), 2.48–2.35 (m, 2H), 2.06–1.94 (m, 2H), 1.37–1.22 (m, 22H), 1.05 (s, 9H), 0.99 (s, 9H), 0.92–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 139.1, 138.8, 138.4, 138.2, 132.5, 128.4, 128.3, 128.1, 127.8, 127.8, 127.7, 127.5, 127.5, 125.0, 99.1, 79.3, 79.2, 77.6, 74.3, 73.9, 73.6, 72.1, 71.2, 71.0, 68.2, 67.7, 67.1, 62.0, 32.0, 29.8, 29.7, 29.5, 29.4, 28.1, 27.7, 27.4, 23.5, 22.7, 20.7, 14.2; Gated decoupled ^{13}C NMR (75 MHz, $CDCl_3$) selected data, δ 99.1, $^1J_{C1'-H1'}$ 168.3 Hz. HRMS-ESI $[M+H-N_2]^+$ calcd for $C_{62}H_{90}NO_8Si$: 1004.6436. Found 1004.6425.

4.20. (2*S*,3*S*,4*R*)-2-Amino-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-icos-6-ene (*E*:*Z*, 4:1) (26)

Trimethylphosphine (1 M in THF, 1.20 mL, 1.20 mmol) was added to a stirred solution of azide **25** (246 mg, 0.238 mmol) in THF (20 mL) cooled to 0 °C and stirred for 45 min. After stirring at ambient temperature for a further 1 h, NaOH (aq, 1 M, 2.00 mL, 2.00 mmol) was added. After a further 2 h, the mixture was diluted with EtOAc (150 mL) and washed with H_2O (2 × 100 mL), brine (100 mL) dried and filtered. The solvent was removed to give the title compound **26** (226 mg, 0.225 mmol, 94%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45–7.37 (m, 2H), 7.35–7.19 (m, 18H), 5.55–5.40 (m, 2H), 4.87–4.43 (m, 10H), 4.13–3.65 (m, 8H), 3.58–3.49 (m, 2H), 3.40–3.31 (m, 1H), 3.25–3.16 (m, 1H), 2.49–2.37 (m, 2H), 2.10–1.92 (m, 2H), 1.29–1.21 (m, 22H), 1.06 (s, 9H), 1.02 (s, 9H), 0.91–0.85 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 139.0, 138.7, 138.6, 132.2, 128.3, 128.1, 128.0, 127.8, 127.7, 127.6, 127.5, 125.6, 99.2, 82.1, 79.8, 77.9, 74.7, 73.7, 72.0, 71.5, 71.0, 70.8, 67.4, 67.3, 52.9, 32.0, 30.4, 29.7, 29.5, 29.4, 28.5, 27.7, 27.4, 23.5, 22.7, 20.7, 14.2; HRMS-ESI $[M+H]^+$ calcd for $C_{62}H_{92}NO_8Si$: 1006.6592. Found 1006.6567.

4.21. (2*S*,3*S*,4*R*)-3,4-Di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-2-tetracosanoylamino-icos-6-ene (*E*:*Z*, 4:1) (27)

1-(3-Dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (52 mg, 0.269 mmol) and 1-hydroxybenzotriazole (36 mg, 0.269 mmol) were added to a stirred suspension of tetracosanoic acid (83 mg, 0.224 mmol) in a CH_2Cl_2 (10 mL), DMF (4 mL) solvent mixture cooled to 0 °C. After 30 min a solution of amine **26** (226 mg, 0.224 mmol) and DIPEA (90 μL, 0.539 mmol) in CH_2Cl_2 (10 mL) was added. The reaction mixture was stirred at ambient temperature for 12 h when it was diluted with the addition of EtOAc/Et_2O (4:1, 200 mL). The organic phase was washed with a satd aq soln of $NaHCO_3$ (100 mL), HCl (1 M, 100 mL) and brine (150 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:19 to 3:17) afforded the title compound **27** (188 mg, 0.138 mmol, 62%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.39 (m, 2H), 7.36–7.21 (m, 18H), 5.85 (d, $J = 8.5$ Hz, 1H), 5.55–5.39 (m, 2H), 4.86–4.45 (m, 10H), 4.36–4.25 (m, 1H), 4.15–3.94 (m, 3H), 3.82–3.70 (m, 4H), 3.60–3.50 (m, 2H), 2.54–2.35 (m, 2H), 2.08–1.95 (m, 2H), 1.91–1.83 (m, 2H), 1.52–1.40 (m, 2H), 1.27–1.21 (m, 62H), 1.04 (s, 9H), 1.00 (s, 9H), 0.91–0.84 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 172.8, 138.9, 138.5, 132.2, 128.4, 128.2, 127.9, 127.8, 127.7, 127.7, 127.6, 125.4, 99.5, 79.7, 79.3, 77.8, 74.6, 73.8, 73.4, 71.7, 70.9, 70.7, 68.0, 67.6, 67.2, 50.2, 36.8, 32.0, 29.7, 29.5, 29.4, 28.0, 27.7, 27.6, 27.4, 25.8, 23.4, 22.7, 20.7, 14.2; HRMS-ESI $[M+H]^+$ calcd for $C_{86}H_{138}NO_9Si$: 1357.0141. Found 1357.0155.

4.22. (2*S*,3*S*,4*R*)-1-(2,3-Di-*O*-benzyl-α-D-galactopyranosyloxy)-3,4-di(benzyloxy)-2-tetracosanoylamino-icos-6-ene (*E*:*Z*, 4:1) (28)

A solution of HF·py (40 M, 0.600 mL, 24.0 mmol) was added to a stirred solution of **27** (164 mg, 0.121 mmol) in THF (10 mL) cooled to 0 °C. After 1 h the mixture was diluted with EtOAc (100 mL) and washed with a satd aq soln of $NaHCO_3$ (50 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:1 to 4:1) afforded the title compound **28** (93 mg, 0.076 mmol, 63%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.36–7.24 (m, 20H), 5.75

(d, $J = 8.9$ Hz, 1H), 5.55–5.41 (m, 2H), 4.84 (d, $J = 3.2$ Hz, 1H), 4.79–4.40 (m, 9H), 4.02 (br s, 1H), 3.89–3.55 (m, 9H), 2.75–2.36 (m, 4H), 2.09–1.95 (m, 2H), 1.92–1.83 (m, 2H), 1.53–1.40 (m, 2H), 1.27–1.22 (m, 62H), 0.91–0.84 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 173.1, 138.5, 138.4, 138.1, 132.4, 128.6, 128.5, 128.0, 127.9, 127.8, 127.7, 125.1, 99.5, 80.4, 79.0, 77.6, 76.0, 73.5, 73.2, 72.7, 71.7, 69.9, 69.5, 68.6, 62.9, 50.5, 36.8, 32.0, 29.8, 29.5, 29.4, 28.1, 27.6, 25.8, 22.7, 14.2; HRMS-ESI $[M+H]^+$ calcd for $C_{78}H_{122}NO_9$: 1216.9120. Found 1216.9157.

4.23. (2*S*,3*S*,4*R*)-1-(α-D-Galactopyranosyloxy)-2-tetracosanoylamino-icosane-3,4-diol (3)

A mixture of Pd-black (9 mg) and **28** (90 mg, 0.074 mmol) in THF/MeOH/H_2O (7, 2 and 0.2 mL) was stirred under a hydrogen atmosphere for 5 h. The mixture was filtered through Celite and the cake washed with THF/MeOH/H_2O (70:20:2, 200 mL). The solvent was removed in vacuo and the residue purified by column chromatography on silica gel. Elution with MeOH/chloroform (1:9) afforded the title compound **3** (50 mg, 0.058 mmol, 79%) as a white hygroscopic solid; ^{1}H NMR (600 MHz, $CDCl_3/CD_3OD$, 3:1) δ 4.91 (d, $J = 3.8$ Hz, 1H), 4.19 (dd, $J = 9.5$ and 4.6 Hz, 1H), 4.07 (s, 1H), 3.93 (d, $J = 2.8$ Hz, 1H), 3.88 (dd, $J = 10.7$ and 4.7 Hz, 1H), 3.82–3.67 (m, 6H), 3.57–3.52 (m, 2H), 2.21 (t, $J = 7.5$ Hz, 2H), 1.70–1.52 (m, 4H), 1.35–1.24 (m, 68H), 0.91–0.87 (m, 6H); ^{13}C NMR (150 MHz, $CDCl_3/CD_3OD$, 3:1), referenced to CD_3OD, δ 172.9, 99.5, 74.4, 71.7, 70.5, 70.0, 69.5, 68.7, 67.1, 61.6, 50.2, 36.2, 32.2, 31.6, 29.4, 29.0, 25.5, 22.3, 13.6; HRMS-ESI $[M+H]^+$ calcd for $C_{50}H_{100}NO_9$: 858.7398. Found 858.7417.

4.24. (2*S*,3*S*,4*R*)-2-Azido-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-octadeca-6-ene (*E*:*Z*, 4:1) (30)

TMSOTf (19 μL, 0.105 mmol) was added drop-wise to a stirred mixture of donor **8** (884 mg, 1.37 mmol), acceptor **29** (550 mg, 1.054 mmol) and 4 Å molecular sieves (1.5 g) in CH_2Cl_2 (30 mL) at −20 °C. The reaction mixture was allowed to warm to 10 °C over 2.25 h when Et_3N (1 mL) was added. The mixture was filtered through Celite and the filter cake was further washed with CH_2Cl_2 (2 × 100 mL). The solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:39 to 1:19) afforded the title compound **30** (863 mg, 0.859 mmol, 82%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.45–7.38 (m, 2H), 7.38–7.12 (m, 18H), 5.56–5.33 (m, 2H), 4.84 (d, $J = 11.9$ Hz, 1H), 4.79 (d, $J = 3.5$ Hz, 1H), 4.75–4.42 (m, 8H), 4.12–3.58 (m, 9H), 3.55 (br s, 1H), 2.49–2.32 (m, 2H), 2.10–1.92 (m, 2H), 1.39–1.18 (m, 18H), 1.05 (s, 9H), 1.00 (s, 9H), 0.92–0.82 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 139.1, 138.8, 138.4, 138.2, 132.5, 128.40, 128.35, 128.3, 128.1, 127.81, 127.76, 127.7, 127.6, 127.54, 127.47, 125.0, 99.1, 79.33, 79.26, 77.6, 74.3, 73.9, 73.6, 72.1, 71.2, 71.0, 68.2, 67.7, 67.1, 62.0, 32.0, 29.74, 29.70, 29.65, 29.54, 29.47, 29.40, 28.1, 27.71, 27.65, 27.4, 23.4, 22.7, 20.7, 14.2; Gated decoupled ^{13}C NMR (75 MHz, $CDCl_3$) selected data, δ 99.1, $^1J_{C1'-H1'}$ 168.2 Hz. HRMS-ESI $[M+Na]^+$ calcd for $C_{60}H_{85}N_3O_8$-NaSi: 1026.6004. Found 1026.6007.

4.25. (2*S*,3*S*,4*R*)-2-Amino-3,4-di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-octadeca-6-ene (*E*:*Z*, 4:1) (31)

Trimethylphosphine (1 M in THF, 4.0 mL, 4.0 mmol) was added to a stirred solution of azide **30** (779 mg, 0.776 mmol) in THF (40 mL) cooled to 0 °C. After 45 min the cooling bath was removed and the reaction mixture was stirred at ambient temperature for a further 2 h. Sodium hydroxide (aq, 1 M, 7.5 mL, 7.50 mmol) was added and after a further 2 h the mixture was diluted with EtOAc (200 mL) and washed with H_2O (3 × 100 mL), brine (50 mL), dried ($MgSO_4$) and filtered. The solvent was removed to give the title compound **31** (836 mg), which was used for the next step without further purification; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.46–7.38 (m, 2H), 7.38–7.13 (m, 18H), 5.58–5.32 (m, 2H), 4.87–4.41 (m, 10H), 4.16–3.67 (m, 6H), 3.59–3.48 (m, 2H), 3.41–3.30 (m, 1H), 3.25–3.15 (m, 1H), 2.53–2.34 (m, 2H), 2.09–1.91 (m, 2H), 1.39–1.16 (m, 18H), 1.05 (s, 9H), 1.00 (s, 9H), 0.92–0.82 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 139.0, 138.7, 138.6, 132.2, 128.4, 128.1, 128.0, 127.8, 127.7, 127.6, 127.54, 127.50, 125.6, 99.2, 82.1, 79.9, 77.9, 74.7, 73.7, 71.9, 71.5, 71.0, 70.8, 67.4, 67.3, 52.9, 32.0, 30.4, 29.7, 29.6, 29.5, 28.5, 27.7, 27.4, 23.5, 22.7, 20.7, 14.2; HRMS-ESI $[M+H]^+$ calcd for $C_{60}H_{88}NO_8Si$: 978.6279. Found 978.6264.

4.26. (2*S*,3*S*,4*R*)-3,4-Di(benzyloxy)-1-(2,3-di-*O*-benzyl-4,6-*O*-di-*tert*-butylsilylene-α-D-galactopyranosyloxy)-2-hexacosanoylamino-octadeca-6-ene (*E*:*Z*, 4:1) (32)

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (80 mg, 0.418 mmol) and 1-hydroxybenzotriazole (57 mg, 0.418 mmol) were added to a stirred suspension of hexacosanoic acid (138 mg, 0.349 mmol) in a CH_2Cl_2 (20 mL), DMF (8 mL) solvent mixture cooled to 0 °C. After 30 min a solution of amine **31** (341 mg, 0.349 mmol) and DIPEA (146 μL, 0.836 mmol) in CH_2Cl_2 (20 mL) was added. The reaction mixture was stirred at ambient temperature for 18 h when it was diluted with the addition of CH_2Cl_2 (150 mL) and washed with water (3 × 100 mL). The organic phase was dried

($MgSO_4$), concentrated and taken up again in EtOAc (200 mL) and washed further with water (3×100 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:19 to 3:17) afforded the title compound **32** (406 mg, 0.299 mmol, 86%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.44–7.39 (m, 2H), 7.45–7.16 (m, 18H), 5.80 (d, $J = 8.5$ Hz, 1H), 5.56–5.32 (m, 2H), 4.83 (d, $J = 11.7$ Hz, 1H), 4.78–4.42 (m, 9H), 4.36–4.25 (m, 1H), 4.16–3.93 (m, 3H), 3.84–3.68 (m, 4H), 3.61–3.46 (m, 2H), 2.56–2.30 (m, 2H), 2.11–1.91 (m, 2H), 1.91–1.80 (m, 2H), 1.56–1.40 (m, 2H), 1.39–1.12 (m, 62H), 1.05 (s, 9H), 0.99 (s, 9H), 0.93–0.81 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 172.8, 139.0, 138.6, 138.5, 132.2, 128.5, 128.4, 128.2, 127.9, 127.75, 127.72, 127.63, 127.57, 127.5, 125.4, 99.5, 79.8, 79.4, 77.8, 74.6, 73.8, 73.5, 71.7, 71.0, 70.8, 68.1, 67.7, 67.2, 50.3, 36.8, 32.0, 29.8, 29.7, 29.53, 29.50, 29.46, 28.0, 27.7, 27.6, 27.4, 25.8, 23.5, 22.8, 20.8, 14.2; HRMS-ESI $[M+Na]^+$ calcd for $C_{86}H_{137}NO_9NaSi$: 1378.9960. Found 1379.0016.

4.27. (2*S*,3*S*,4*R*)-1-(2,3-Di-*O*-benzyl-α-D-galactopyranosyloxy)-3,4-di(benzyloxy)-2-hexacosanoylamino-octadeca-6-ene (*E*:*Z*, 4:1) (33)

A solution of HF·py (approx 38 M, 0.85 mL, 32 mmol) was added to a stirred solution of **32** (350 mg, 0.258 mmol) in THF (10 mL) cooled in an ice/water bath. After 1 h the cold mixture was diluted with EtOAc (100 mL) and washed with a satd aq soln of $NaHCO_3$ (50 mL). After drying and filtration, the solvent was removed and the residue purified by column chromatography on silica gel. Elution with EtOAc/light petroleum (1:1) afforded the title compound **33** (248 mg, 0.204 mmol, 79%) as an oil; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.38–7.18 (m, 20H), 5.74 (d, $J = 8.9$ Hz, 1H), 5.59–5.35 (m, 2H), 4.84 (d, $J = 3.1$ Hz, 1H), 4.80–4.37 (m, 9H), 4.02 (br s, 1H), 3.94–3.50 (m, 9H), 2.75–2.33 (m, 4H), 2.10–1.95 (m, 2H), 1.93–1.82 (m, 2H), 1.55–1.40 (m, 2H), 1.39–0.97 (m, 62H), 0.96–0.75 (m, 6H); ^{13}C NMR (75 MHz, $CDCl_3$), major isomer, δ 173.1, 138.5, 138.4, 138.1, 132.5, 128.6, 128.5, 128.0, 127.9, 127.8, 127.7, 125.1, 99.6, 80.4, 79.0, 77.6, 76.0, 73.5, 73.2, 72.7, 71.7, 69.9, 69.5, 68.6, 62.9, 50.5, 36.9, 32.0, 29.8, 29.5, 29.4, 28.1, 27.6, 25.8, 22.7, 14.2; HRMS-ESI $[M+Na]^+$ calcd for $C_{78}H_{121}NO_9Na$: 1238.8939. Found 1238.8947.

4.28. (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-hexacosanoylamino-3,4-octadecandiol (1)

To a solution of **33** in $MeOH/CH_2Cl_2$ (4:1) was added $Pd(OH)_2$ on carbon (20%, 120 mg). The suspension was stirred under a hydrogen atmosphere for 3.5 h after which time the atmosphere was swapped for Ar. The reaction mixture was diluted with THF/MeOH/H_2O (35:10:1, 30 mL) and stirred for 30 min before filtering through Celite®. The filter cake was rinsed with further THF/MeOH/H_2O (35:10:1, approx 100 mL). The solvent was removed in vacuo and the residue purified by flash chromatography on reversed phase (C18) silica (eluant neat MeOH to 1:1 MeOH/$CHCl_3$) to provide the title compound **1** (67 mg, 85%) as a white powder. $[\alpha]_D$ +42.4 (*c* 0.4, pyridine); lit.[23] +42.2 (*c* 0.54, pyridine), lit.[15] +43.6 (*c* 1.0, pyridine); HRMS-ESI $[M+Na]^+$ calcd for $C_{50}H_{99}NO_9Na$: 880.7218. Found 880.7186. The ^{1}H and ^{13}C NMR spectra of this material were in good agreement to that reported.[15,23]

4.29. Materials and methods for DC maturation assays

4.29.1. Mice. C57BL/6 were from breeding pairs originally obtained from Jackson Laboratories, Bar Harbor, Maine. Also used were $CD1d^{-/-}$ mice,[39] which are devoid of CD1d-restricted *i*NKT cells.

4.29.2. Administration of glycolipids. Each glycolipid (5 mg) was solubilized in 500 μL of chloroform/MeOH/water at a ratio of 10:10:3, and then diluted to 200 μg/mL in 0.5% Tween/phosphate-buffered saline (PBS). For injections, each glycolipid was diluted in PBS and injected into the lateral tail vein.

4.29.3. Phenotyping DC from spleen. Antibody staining and flow cytometry were used to examine the expression of maturation markers on dendritic cells in the spleen following injection of glycolipids. Splenocyte preparations were prepared by gentle teasing of splenic tissue through gauze in Iscove's Modified Dulbecco's Medium with 2 mM glutamine, 1% penicillin–streptomycin, 5×10^{-5} M 2-mercapto-ethanol and 5% foetal bovine serum (all Invitrogen, Auckland, New Zealand), followed by lysis of red blood cells with RBC lysis buffer (Puregene, Gentra Systems, Minneapolis, MN, USA). Antibody staining was performed in PBS, 2% foetal bovine serum and 0.01% sodium azide. The anti-FcγRII monoclonal antibody 2.4G2 was used at 10 mg/mL to inhibit non-specific staining. Monoclonal antibodies (all BD Biosciences Pharmingen, San Jose, CA, USA) were used to examine expression of the maturation markers CD40, CD80 and CD86 on $CD11c^+$ dendritic cells.

4.29.4. Analysis of cytokine release into serum. Blood was collected from the lateral vein at different time intervals after glycolipid administration. Serum was collected after blood had clotted, and the levels of cytokines IL-12p70, IL-4 and IFN-γ were assessed by cytokine bead array technology (BD Biosciences Pharmingen), according to the manufacturer's instructions.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2006.09.006.

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