

Synthesis and immunostimulatory activity of two α -S-galactosyl phenyl-capped ceramides

Niamh Murphy,^a Andreea Petrasca,^b Niamh M. Murphy,^b Vincent O'Reilly,^b
Paul Evans,^a Derek G. Doherty,^b and Xiangming Zhu^{a,*}

^a Centre for Synthesis and Chemical Biology, UCD School of Chemistry and Chemical Biology,
University College Dublin, Belfield, Dublin 4, Ireland

^b Institute of Molecular Medicine, Trinity College Dublin, St James's Hospital, Dublin, Ireland
E-mail: xiangming.zhu@ucd.ie

Dedicated to Prof. Richard Schmidt on the occasion of his 78th birthday

Abstract

Two α -S-linked galactosylceramides carrying ω -phenyl fatty acid chains were efficiently synthesized and were shown to have *in vitro* stimulatory activity for human natural killer T cells.

Keywords: α -Galactosylceramide, thioglycoside, glycosyl thiol, immunostimulant

Introduction

α -Galactosylceramides (α -GalCers) are a class of immunostimulatory agents that are specifically presented by the antigen-presenting molecule CD1d to T cell receptors (TCR) on the surface of natural killer T (NKT) cells.¹ NKT cells are thus stimulated by the CD1d-glycolipid complex and release a variety of T_{H1} and T_{H2} cytokines; an event associated with profound biological consequences.² Of all α -GalCers, KRN7000³ is the most thoroughly studied and has been tested as an immunotherapeutic agent against many diseases including cancer, diabetes, malaria and hepatitis B. Unfortunately, the efficacy of KRN7000 has been limited in many cases due to the reciprocal inhibition exhibited by T_{H1} and T_{H2} cytokines.⁴ As such, a great deal of effort has been devoted to the synthesis of KRN7000 analogues in the past decade in the hope of developing novel lead compounds with better cytokine-inducing selectivity and appropriate potency.^{5,6}

Time course cytokine release studies in mice have shown that once the NKT cell is activated by KRN7000, IFN- γ cytokine release levels (T_{H1} response) peak after 12 hours, while IL-4 levels (T_{H2} response) peak within 2 hours.⁷ Therefore, the stability of the α -GalCer/CD1d complex is currently a focus point in the design of potent KRN7000 analogues. Increasing the

binding interactions between the glycolipid and CD1d, and therefore increasing the stability of the complex, could result in greater levels of IFN- γ release and consequently promoting and prolonging the T_H1 response. Conversely, a less stable α -GalCer/CD1d complex could shorten NKT stimulation time resulting in a T_H2 biased response.⁴ Also, other factors, such as the mode of loading the glycolipid into CD1d and the rate of glycolipid dissociation from CD1d in lysosomes, could greatly influence the cytokine release profile as well.

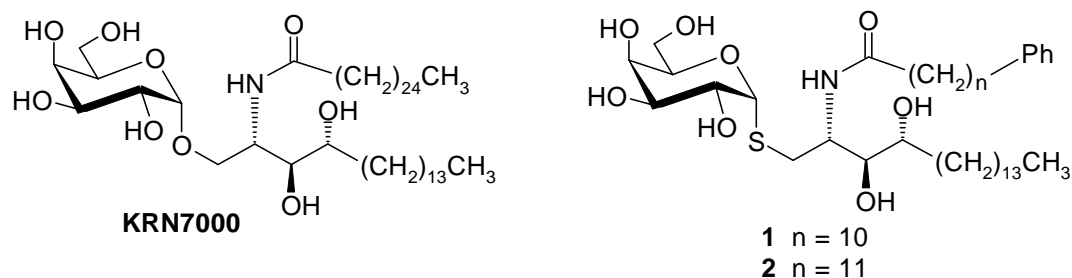


Figure 1. Structures of KRN7000 and target molecules **1** and **2**.

Recent crystallographic analysis has indicated that the lipid chains of α -GalCer are buried in a groove in CD1d containing two hydrophobic pockets (denoted as A' and C') and the galactose ring is exposed for the recognition by NKT TCR.⁸ The phytosphingosine chain sits in the hydrophobic C' pocket which ideally accommodates 18 carbons, while the longer acyl chain binds in the other hydrophobic A' pocket that can accommodate up to 26 carbons. In addition, several hydrogen bonds were identified and are assumed to anchor α -GalCer in a distinct orientation and position it in the lipid-binding groove. As such, the modification of the lipid chains of α -GalCer is a very important aspect for designing α -GalCer analogues seeing that the chains are strongly involved in the binding with CD1d. For instance, if the lipid chains could be rationally modified to enhance their hydrophobic interaction with CD1d, as mentioned above, the resultant complex may preferably induce the release of T_H1 cytokines, which are thought to be responsible for the antitumor, antiviral and antibacterial effects of α -GalCer. In this regard, a paper by Wong and co-workers attracted our attention, wherein several α -GalCer analogues with greater anticancer efficacy were reported.⁹ They introduced different aromatic groups into the fatty acyl chain with a view to designing tighter CD1d-binding glycolipids since X-ray crystal structures showed that the CD1d A' binding groove was lined with numerous aromatic side-chain residues. These analogues were expected to introduce extra aromatic interactions which could increase the stability of the CD1d/glycolipid complex, thereby favouring the induction of T_H1 cytokines. Indeed, some of these analogues with the appropriate fatty acid acyl chain length were four times more potent than α -GalCer itself and were biased towards IFN- γ secretion.^{9,10} Computational, docking of these analogues in the human CD1d hydrophobic groove was also conducted, and the results indicated that their binding mode did not vary much from the crystal structure of α -GalCer bound to hCD1d. In each case, the phytosphingosine chain was bound to

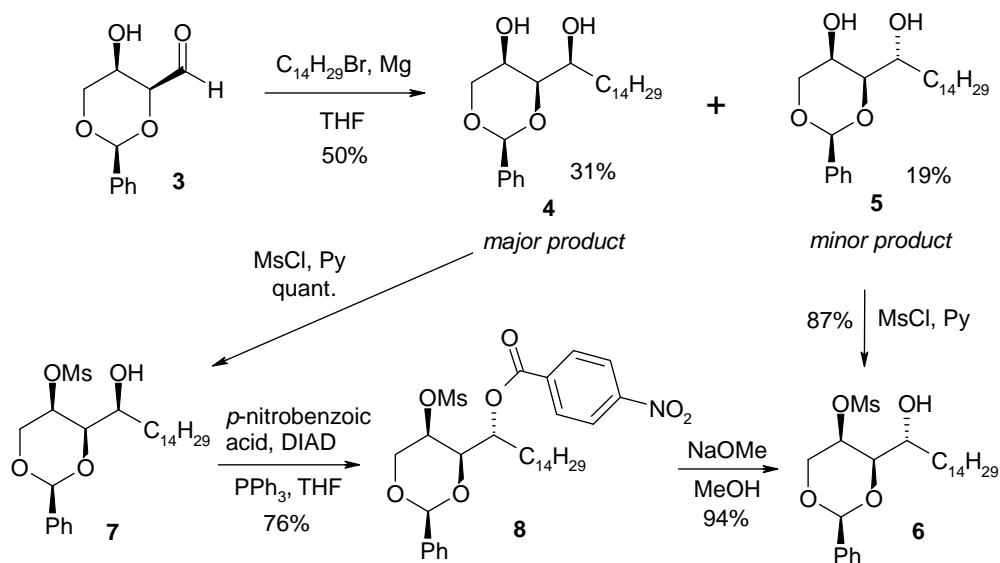
the C' pocket and the A' pocket was occupied by the modified fatty acid acyl chain with the galactose headgroup presented in nearly the same conformation as α -GalCer. Additional beneficial binding forces between the terminal phenyl group and Tyr73 or Trp40 were proposed.⁹

Recently, several thioglycoside analogues of KRN7000 have been readily synthesized in a highly stereoselective manner in our laboratory,¹¹ in which an α -galactosyl thiol was used as the sugar building block. It is well known that thioglycosides often exhibit high stability against chemical and enzymatic hydrolysis,¹² so these analogues were expected to have relatively long lifetimes in a biological setting. Also, thioglycosides usually have more flexibility around the anomeric linkage compared with the corresponding *O*-glycosides owing to the longer C-S bond and weaker stereoelectronic effects. This increased flexibility may thus enable α -*S*-GalCer to sit differently than α -*O*-GalCer in the CD1d binding groove, resulting in an altered structure of the glycolipid/CD1d complex and a change in its affinity with the TCR of NKT cells. In addition, after binding with CD1d, the sugar head of α -*S*-GalCer may orientate in a different angle from that of α -GalCer as the C-S-C bond angle is significantly smaller than the C-O-C angle, which could result in differential recognition by TCR of NKT cells. The subsequent bioassay demonstrated that the α -*S*-GalCer possessed similar potency to KRN7000 in human NKT cell activation, and stimulated cytokine release in a similar CD1d-dependent manner as well.¹³ Therefore, the thioglycoside analogues may be superior to KRN7000 as a parent compound for developing immunostimulants for humans in view of its bioactivity, stability, ease of synthesis, and flexibility in terms of the preparation of other analogues. Based on this work, we wish to report here the synthesis of two new thioglycoside analogues of α -GalCer, **1** and **2** containing a terminal phenyl group in the fatty acid chain with the long-term aim of developing therapeutic analogues with greater anticancer efficacy.

Results and Discussion

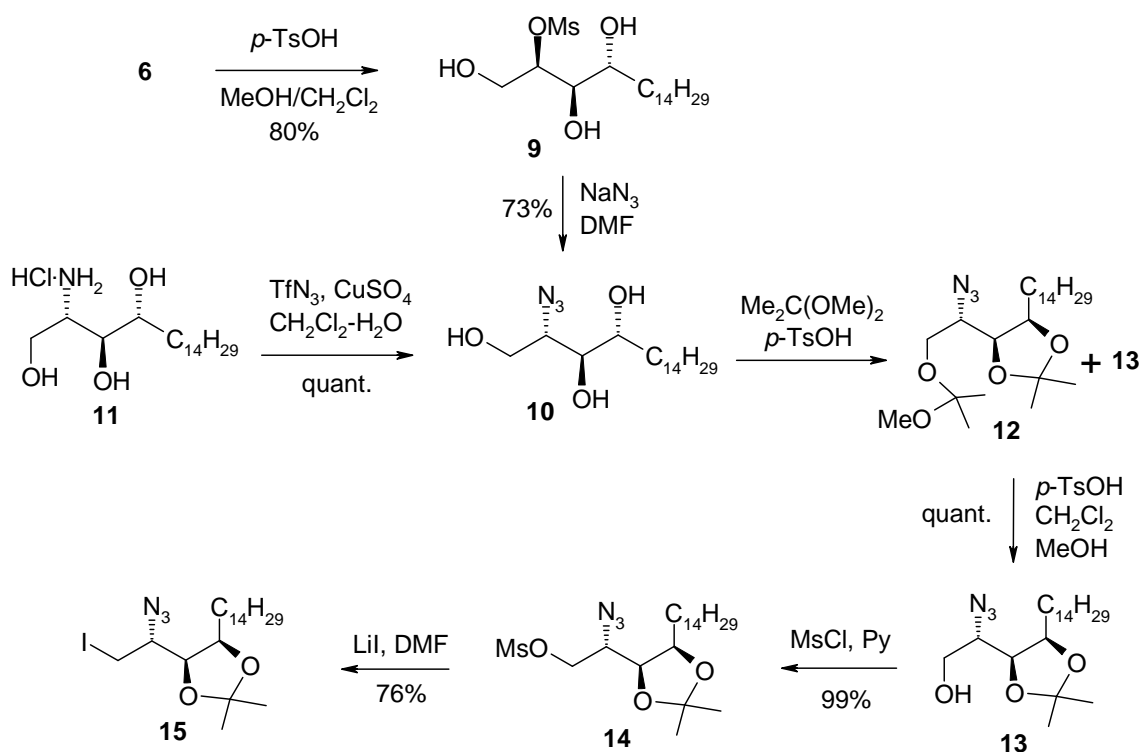
Our synthesis started with the preparation of phytosphingosine precursor **6**. The aldehyde **3** was first prepared by a known procedure¹⁴ and subjected to the Grignard reaction with tetradecylmagnesium bromide generated from 1-bromotetradecane and Mg to give the alcohols **4** and **5** in 47% overall yield (Scheme 1). As previously reported,¹⁵ the substrate-induced stereoselectivity favoured the formation of the unwanted isomer **4**, and the desired isomer **5** was produced as the minor product with a ratio of **4**:**5** = 1.6:1. Fortunately, **4** and **5** could be readily separated by flash column chromatography. Compound **5** was then treated with 0.9 equivalents of methanesulfonyl chloride in the presence of pyridine to provide the 2-*O*-mesylated intermediate **6**¹⁵ in 87% yield. As documented in the literature,¹⁵ the higher reactivity of the 2-OH group could be attributed to its greater nucleophilicity caused by intramolecular hydrogen bonding with the oxygen atoms of the dioxane ring. Meanwhile, in order to convert the major product **4** into the common intermediate **6**, we employed the Mitsunobu reaction to invert the configuration of the 4-OH group, as depicted in Scheme 1. As with **5**, selective mesylation of **4**

gave the 2-mesylyated intermediate **7**¹⁵ in a quantitative yield, which underwent Mitsunobu reaction with *p*-nitrobenzoic acid to produce the 4-OH-inverted *p*-nitrobenzoic ester **8** in high yield. Subsequently, **8** was treated with NaOMe in MeOH to provide the desired intermediate **6** in a 94% yield. Thus, by using the Mitsunobu reaction, both stereoisomers produced in the Grignard reaction could be capitalized upon for the synthesis of the target molecules.

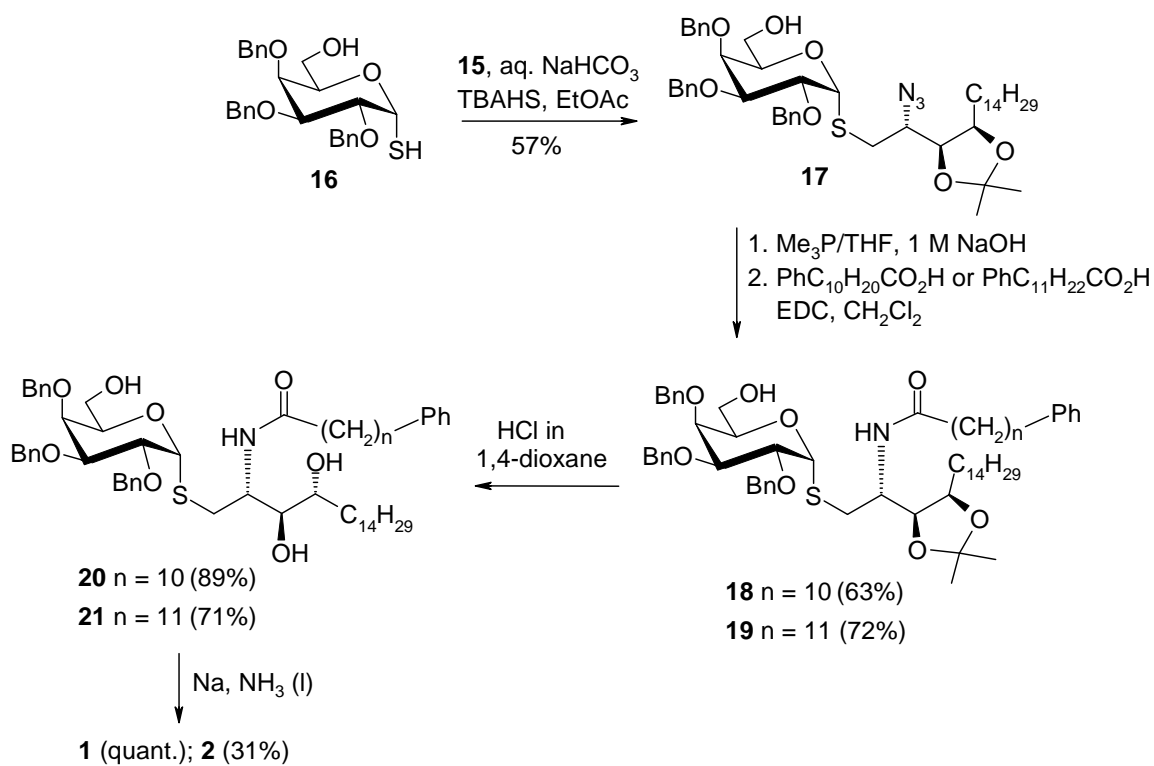


Scheme 1. Synthesis of intermediate **6**.

Removal of the benzylidene protecting group from **6** was then conducted by treatment with *p*-TsOH in MeOH- CH_2Cl_2 to afford **9**¹⁶ in 80% yield, which was treated subsequently with NaN_3 under heating to give the azide **10**¹⁶ in 74% yield. In this project, another goal was to optimize the previous synthetic route to the thioglycoside analogue of KRN7000 with a view to providing larger amounts of samples in a rapid way for more biological studies. Hence, the direct synthesis of the azide **10** from commercially available phytosphingosine **11** (as its hydrochloride salt) was investigated (Scheme 2). Following the literature procedures,¹⁷ the diazo transfer from triflic azide onto the amine was found to proceed extremely well, and **10** was produced in an excellent yield. A convenient and highly efficient route to the key intermediate **10** was thus established, which expedited the synthesis of α -S-GalCers. Compound **10** was then subjected to standard isopropylidene conditions (2,2-dimethoxypropane/*p*-TsOH).¹⁵ Perhaps not unexpectedly, the di-ketal intermediate **12** was formed in a mixture also containing **13** but this mixture could be converted directly into compound **13** without purification. Compound **13** was then mesylyated with methanesulfonyl chloride in pyridine to give compound **14** in almost quantitative yield, which was converted into the iodide **15** in a good yield by treatment with LiI in DMF.^{11a}



Scheme 2. Synthesis of sphingoid building block 15.

Scheme 3. Synthesis of α -S-galactosylceramides 1 and 2.

Subsequently, glycosylation of **15** with the pre-prepared α -galactosyl thiol **16**¹⁸ was carried out under phase transfer conditions, giving rise to thioglycoside **17**^{11a} in 57% yield. It is noteworthy that the reaction proceeded well regardless of the free hydroxy group present in the sugar **16**. Compound **17** was then exposed to Staudinger reduction conditions smoothly to produce the free amine intermediate, which was acylated with ω -phenylundecanoic acid or ω -phenyllauric acid in the presence of 1-ethyl-3-(dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) in CH₂Cl₂ to afford the amides **18** and **19** in good yields of 63% and 72%, respectively. To access the target molecules, **18** and **19** were then treated with 4 M HCl in dioxane which gave the corresponding partially protected *S*-glycolipids **20** and **21** in 89% and 71% yields. Subsequently, **20** and **21** were subjected to Birch reduction to furnish the target compounds **1** and **2** in quantitative and 31% yields, respectively. In the case of the lower yield starting material **21** was recovered (62%) due to the inclusion of an insufficient quantity of sodium. The synthetic samples were purified by flash column chromatography on silica gel (eluent: CHCl₃/MeOH 20:1→10:1), and characterized by NMR and HR-ESIMS.

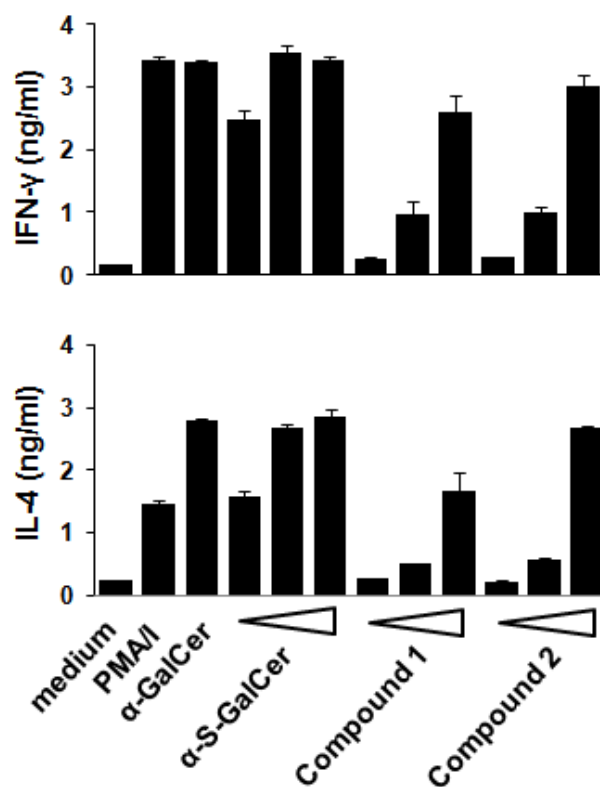


Figure 2. α -*S*-Linked galactosylceramides carrying ω -phenyl fatty acid chains display stimulatory activity for human NKT cells. CD1d-transfected HeLa cells were pulsed with medium alone, α -GalCer (0.1 μ g/mL), or the thioglycoside analogues α -*S*-GalCer, respectively, using ELISA. Figure 2 shows that comparable levels of IFN- γ and IL-4 were released by NKT cells stimulated with α -GalCer and α -*S*-GalCer. Compounds **1** and **2** also induced IFN- γ and IL-4

production but only when used at 100-fold higher concentrations (10 $\mu\text{g/mL}$) than those of the parent compound that were found to activate NKT cells. Compounds **1** and **2** did not appear to skew NKT cell cytokine production to $T_{\text{H}1}$ or $T_{\text{H}2}$. IL-10 was not detected in the supernatants of any of the co-cultures (not shown).

Compounds **1** and **2** were tested alongside KRN7000 (α -GalCer) and its thioglycoside analogue (α -S-GalCer) for their ability to stimulate cytokine production by human NKT cell lines *in vitro*. Mock-transfected or CD1d-transfected HeLa cells were pulsed for 18-24 hours with medium alone or with 0.1, 1 or 10 $\mu\text{g/mL}$ of glycolipid antigen.^{13,19} Equal numbers of expanded and rested NKT cells were added for a further 24 hours and cell supernatants were then harvested and assayed for the $T_{\text{H}1}$, $T_{\text{H}2}$ and regulatory cytokines IFN- γ , IL-4 and IL-10, Compound **1** or Compound **2** at 0.1, 1 and 10 $\mu\text{g/mL}$ (increasing concentrations denoted by triangles). After 24 hours equal numbers of expanded human NKT cells were added. As positive controls, NKT cells were stimulated with phorbol myristate acetate (50 ng/mL) and ionomycin (1 $\mu\text{g/mL}$) (PMA/I). After a further 24 hours, supernatants were harvested and analysed for IFN- γ (top), IL-4 (bottom) and IL-10 (not shown) production. Data are representative of experiments using 5 NKT cell lines.

Conclusions

In conclusion, in this report two α -S-linked galactosylceramides (**1** and **2**) were efficiently synthesized based on our previously reported synthetic strategy. The Mitsunobu reaction was utilized to retrieve the unwanted diastereoisomer **4**, also formed in the original synthesis of the sphingoid building block, and in doing so the synthetic efficiency was greatly improved. The synthetic route was further optimized by directly converting the commercially available phytosphingosine **10** into the sphingoid building block. The synthetic strategy presented here could also be extended to the synthesis of other thioglycoside analogues of α -GalCer. The bioassays showed that compounds **1** and **2** bind to CD1d and stimulate human NKT cell lines *in vitro*. The addition of ω -phenyl fatty acid chains to α -S-GalCer rendered these compounds less potent as NKT cell stimulators with up to 100 times more of compound **1** or **2** required to elicit similar cytokine levels as seen for the parent compound. Addition of ω -phenyl fatty acid chains to α -S-GalCer did not result in skewing of $T_{\text{H}1}/T_{\text{H}2}$ cytokine profiles as demonstrated by measurement of IFN- γ and IL-4 production. This data shows that thioglycoside analogues of α -GalCer have bioactivity for human NKT cells. Because of their ease of synthesis and increased stability in biological systems, these compounds may be superior to their non-thioglycoside counterparts as adjuvants for immunotherapies.

Experimental Section

General. All chemicals used were reagent grade and used as supplied except where noted. Reactions were performed in oven-dried glassware under a nitrogen atmosphere using dry solvents. Solvents were evaporated under reduced pressure while maintaining the water bath temperature at 40 °C. All reactions were monitored by thin-layer chromatography (TLC) using silica gel 60 F₂₅₄ coated on aluminium sheets and the compounds visualized by UV, or by treatment with 8% H₂SO₄ in methanol followed by heating. Flash chromatography was performed with the appropriate solvent system using 40-60 μm silica gel. Optical rotations were measured at 20 °C with a Perkin-Elmer 343 polarimeter (1 dm cell) and are quoted with units of 10⁻¹degcm²g⁻¹. ¹H NMR spectra were obtained on a 300 and 400 MHz and reported in parts per million (δ) relative to the response of the solvent, or to TMS (0.00 ppm). Coupling constants (J) are reported in Hertz (Hz). ¹³C NMR spectra were recorded at 75 or 100 MHz by using CDCl₃ or C₅D₅N as solvent and are reported in δ relative to the response of the solvent and the signals were assigned with the aid of DEPT, HSQC spectra. Yields refer to chromatographically pure compounds and are calculated based on reagents consumed.

1,3-*O*-Benzylidene-2-*O*-methanesulfonyl-4-*O*-*p*-nitrobenzoyl-D-arabino-1,2,3,4-octadecane-tetrol (8). Triphenylphosphine (7.5 g, 29 mmol) and *p*-nitrobenzoic acid (4.7 g, 28 mmol) were mixed in anhydrous THF (66 mL) followed by the addition of DIAD (5.7 mL, 29 mmol). A solution of compound **7** (5.6 g, 12 mmol) in THF (40 mL) was added dropwise to the mixture and the reaction was left to stir overnight at room temperature. The mixture was concentrated *in vacuo*, and the residue was purified by column chromatography (*c*-Hex:EtOAc, 8:1→3:1), the collected compound was then flushed through a column of silica with CH₂Cl₂ to remove remaining impurities to afford **8** (5.5 g, 76%) as a white solid: R_f = 0.5 (*c*-Hex:EtOAc, 3:1); [α]_D = -17.8 (*c* 1.0 CHCl₃); ν_{max} (neat/cm⁻¹) 2923, 2853, 1643, 1343, 1276; ¹H NMR (400 MHz, CDCl₃) δ 8.30 (d, 2H, *J* 8.9 Hz, Ar), 8.18 (d, 2H, *J* 8.9 Hz, Ar), 7.53-7.51 (m, 2H, Ar), 7.43-7.39 (m, 3H, Ar), 5.66 (s, 1H, CH), 5.46 (ddd, 1H, *J* 3.4, 7.4, 9.1 Hz, H-4), 4.83-4.79 (m, 1H, H-2), 4.65 (dd, 1H, *J* 1.4, 13.3 Hz, H-1a), 4.25 (dd, 1H, *J* 1.4, 9.1 Hz, H-3), 4.16 (dd, 1H, *J* 0.9, 13.3 Hz, H-1b), 3.11 (s, 3H, CH₃), 2.06-1.98 (m, 1H, H-5a), 1.84-1.75 (m, 1H, H-5b), 1.24 (m, 24H, CH₂), 0.87 (t, 3H, *J* 6.8 Hz, CH₃) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 164.3, 150.8, 137.2, 135.9 (4C, 4° C), 130.9, 129.5, 128.6, 126.2, 123.8 (5C, Ar), 101.5 (CH), 78.3 (C-3), 77.0 (C-4), 69.8 (C-1), 68.9 (C-2), 38.9 (CH₃), 32.1 (CH₂), 31.2 (C-5), 29.89, 29.86, 29.82, 29.75, 29.73, 29.67, 29.57, 27.1, 24.7, 22.9 (10C, CH₂), 14.3 (CH₃) ppm [NB: 2 coincident C signals]; ESI-MS *m/z* 656.2 [M + Na]⁺; ESI-HRMS calcd for C₃₃H₄₇NO₉NaS [M + Na]⁺ 656.2869, found 656.2887.

3,4-*O*-Isopropylidene-2-(ω -phenylundecanoylamino)-1-*S*-(2',3',4'-tri-*O*-benzyl- α -D-galactopyranosyl)-D-ribo-3,4-octadecanediol (18). A 1M solution of triphenylphosphine in THF (1 mL, 1.0 mmol) was added dropwise to a solution of compound **17** (159 mg, 0.19 mmol) in THF (6 mL) at room temperature. After stirring for 2 h, 1M aqueous NaOH (3 mL) was added and the

reaction was left to stir for a further 2 h. The solution was then diluted with EtOAc (15 mL) and washed with water (10 mL) and brine (10 mL). The organic layers were combined, dried over MgSO₄ and concentrated *in vacuo* to afford the amine as a colourless oil. A mixture of the crude amine, EDC (73 mg, 0.38 mmol), and ω-phenylundecanoic acid (60 mg, 0.23 mmol) in dry CH₂Cl₂ (15 mL) was stirred at room temperature overnight. The mixture was diluted with CH₂Cl₂ (15 mL) and washed with water (10 mL) and brine (10 mL), dried over MgSO₄ and concentrated *in vacuo* to give a residue which was purified by column chromatography (*c*-Hex/EtOAc, 10:1) to afford compound **18** (127 mg, 63%) as a white solid: *R*_f = 0.4 (*c*-Hex:EtOAc, 11:1); [α]_D = +28.1 (*c* 1.0 CHCl₃); ν_{max} (neat/cm⁻¹) 3429, 3293, 2920, 2852, 1644, 1544, 1461, 1130, 1074, 1031, 739, 699; ¹H NMR (400 MHz, CDCl₃) δ 7.39-7.24 (m, 18H, Ar), 7.17-7.15 (m, 2H, Ar), 5.89 (d, 1H, NH, *J* 9.4 Hz), 5.48 (d, 1H, *J* 5.4 Hz, H-1'), 4.93 (d, 1H, *J* 11.3 Hz, CH₂), 4.83 (d, 1H, *J* 11.7 Hz, CH₂), 4.76-4.76 (m, 3H, CH₂), 4.63 (d, 1H, *J* 11.5 Hz, CH₂), 4.29-4.25 (m, 2H, H-2', H-2), 4.17 (app t, 1H, *J* 5.0 Hz, H-5'), 4.10-4.05 (m, 2H, H-4, H-3), 3.87 (br s, 1H, H-4'), 3.77 (dd, 1H, *J* 7.1, 11.5 Hz, H-6a'), 3.57 (dd, 1H, *J* 2.5, 10.0 Hz, H-3'), 3.59 (dd, 1H, *J* 4.3, 11.6 Hz, H-6b'), 2.95 (dd, 1H, *J* 7.9, 14.9 Hz, H-1a), 2.82 (dd, 1H, *J* 2.2, 14.5 Hz, H-1b), 2.58 (t, 2H, *J* 7.8 Hz, H-11a'', H-11b'), 2.18-2.07 (m, 2H, H-2a'', H-2b''), 1.59-1.39 (m, 6H, H-5a, H-5b, H-3a'', H-3b'', H-10a'', H-10b''), 1.43 (s, 3H, CH₃), 1.32 (s, 3H, CH₃), 1.32-1.21 (m, 36H, CH₂), 0.88 (t, 3H, *J* 6.8 Hz, CH₃) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 172.9 (C=O), 142.9, 138.6, 138.15, 138.1 (4C, 4° C), 128.5, 128.4, 128.35, 128.30, 128.2, 128.15, 127.85, 127.80, 127.68, 127.55, 127.4, 126.9, 125.5 (12C, Ar), 108.0 (4° C), 87.5 (C-1'), 79.5 (C-3'), 78.6 (C-3/C-4), 77.7 (C-3/C-4), 76.4 (C-2'), 75.0 (C-4'), 74.7, 73.5, 72.5 (3C, CH₂), 72.0 (C-5'), 62.7 (C-6'), 49.5 (C-2), 39.9, 38.4 (2C, CH₂), 36.9 (C-2''), 36.0 (C-11''), 35.2 (C-1), 31.9, 31.5, 29.7, 29.65, 29.6, 29.55, 29.53, 29.50, 29.45, 29.36, 29.35, 29.34, 29.33, 29.28, 28.9 (15C, CH₂), 27.4 (CH₃), 26.9, 26.8, 25.7 (3C, CH₂), 25.5 (CH₃), 22.7 (CH₂), 14.1 (CH₃) ppm; ESI-HRMS calcd. For C₆₅H₉₄NO₈S [M - H]⁻ 1048.6700 found 1048.6688.

3,4-O-Isopropylidene-2-(ω-phenyldodecanoylamino)-1-S-(2',3',4'-tri-O-benzyl-α-D-galactopyranosyl)-D-ribo-octadecanediol (19). A 1M solution of triphenylphosphine in THF (0.21 mL, 0.21 mmol) was added dropwise to a solution of compound **17** (38 mg, 0.04 mmol) in THF (1.5 mL) at room temperature. After stirring for 2 h, 1M aqueous NaOH (0.6 mL) was added and the reaction was left to stir for a further 2 h. The solution was then diluted with EtOAc (10 mL) and washed with water (5 mL) and brine (5 mL). The organic layers were combined, dried over MgSO₄ and concentrated *in vacuo* to afford the amine as a colourless oil. A mixture of the crude amine, EDC (15 mg, 0.08 mmol) and ω-phenyldodecanoic acid (14 mg, 0.05 mmol) in dry CH₂Cl₂ (3 mL) was stirred at room temperature overnight. The mixture was diluted with CH₂Cl₂ (10 mL) and washed with water (5 mL) and brine (5 mL), the organic layer was dried over MgSO₄ and concentrated *in vacuo* to give a residue which was purified by column chromatography (*c*-Hex/EtOAc, 7:1) to afford compound **19** (32 mg, 72%) as a white solid: *R*_f = 0.3 (*c*-Hex:EtOAc, 5:1); ¹H NMR (400 MHz, CDCl₃) δ 7.40-7.25 (m, 18H, Ar), 7.18-7.16 (m, 2H, Ar), 5.88 (d, 1H, *J* 9.7 Hz, NH), 5.48 (d, 1H, *J* 5.4 Hz, H-1'), 4.93 (d, 1H, *J* 11.6 Hz, CH₂), 4.87 (d, 1H, *J* 11.8 Hz, CH₂), 4.76-4.66 (m, 3H, CH₂), 4.60 (d, 1H, *J* 11.5 Hz, CH₂), 4.29-4.25

(m, 2H, H-2', H-2), 4.17 (app t, 1H, J 5.7 Hz, H-5'), 4.11-4.05 (m, 2H, H-3, H-4), 3.87 (d, 1H, J 1.7 Hz, H-4'), 3.81-3.74 (m, 1H, H-6a'), 3.70 (dd, 1H, J 2.8, 10.0 Hz, H-3'), 3.59-3.54 (m, 1H, H-6b'), 2.92 (dd, 1H, J 7.9, 14.8 Hz, H-1a), 2.82-2.77 (m, 1H, H-1b), 2.59 (t, 2H, J 7.8 Hz, H-2a'', H2b''), 2.15 (dd, 1H, J 7.7, 14.7 Hz, H-12a''), 2.10 (dd, 1H, J 7.6, 14.5 Hz, H-12b''), 1.63-1.25 (m, 44H, CH₂), 1.32 (s, 3H, CH₃), 1.29 (s, 3H, CH₃), 0.88 (t, 3H, J 6.8 Hz, CH₃) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 173.2 (C=O), 143.1, 138.8, 138.40, 138.35 (4C, 4° C), 128.8, 128.61, 128.60, 128.56, 128.4, 128.11, 128.09, 128.05, 127.9, 127.8, 127.7, 125.8 (12C, Ar), 108.2 (4° C), 87.7 (C-1'), 79.8 (C-3'), 78.8 (C-3/4), 77.9 (C-3/4), 76.6 (C-2'), 75.3 (C-4'), 74.9, 73.8, 72.8 (3C, CH₂), 72.2 (C-5'), 63.0 (C-6'), 49.8 (C-2), 37.1 (C-12''), 36.2 (C-2''), 35.3 (C-1), 32.2, 31.8, 29.6 (3C, CH₂), 27.7 (CH₃), 27.1, 26.0 (2C, CH₂), 25.7 (CH₃), 22.9 (CH₂), 14.4 (CH₃) ppm [NB: 16 co-incident C signals]; ESI-MS m/z 1064.64 [M + H]⁺, 1086.75 [M + Na]⁺; ESI-HRMS calcd for C₆₆H₉₇NO₈NaS [M + Na]⁺ 1086.6833, found 1086.6825.

2-(ω -Phenylundecanoylamino)-1-S-(2',3',4'-tri-*O*-benzyl- α -D-galactopyranosyl)-D-ribo-3,4-octadecanediol (20). 4M HCl in dioxane (0.2 mL) was added to a solution of compound **18** (100 mg, 96 μ mol) in CH₂Cl₂:MeOH (10 mL:2 mL) at 0 °C. The mixture was stirred for 2.5 h at room temperature, concentrated *in vacuo* and was purified by column chromatography (CH₂Cl₂:MeOH, 30:1) to afford compound **20** (86 mg, 89%) as a white solid: R_f = 0.7 (CH₂Cl₂:MeOH, 20:1); [α]_D = +29.0 (*c* 0.6 CHCl₃); ν_{\max} (neat/cm⁻¹) 3430, 2924, 2854, 1644, 1460, 1083; ¹H NMR (400 MHz, CDCl₃) δ 7.38-7.24 (m, 18H, Ar), 7.17-7.15 (m, 2H, Ar), 6.45 (d, 1H, J 8.5 Hz, NH), 5.46 (d, 1H, J 5.4 Hz, H-1'), 4.91 (d, 1H, J 11.4 Hz, CH₂), 4.83 (d, 1H, J 11.8 Hz, CH₂), 4.75-4.67 (m, 3H, CH₂), 4.59 (d, 1H, J 11.5 Hz, CH₂), 4.26 (dd, 1H, J 5.4, 9.7 Hz, H-2'), 4.18-4.16 (m, 2H, H-2, H-5'), 3.86-3.79 (m, 2H, H-6a', H-4'), 3.69 (dd, 1H, J 2.0, 9.9 Hz, H-3'), 3.54-3.47 (m, 3H, H-3, H-4, H-6b'), 3.06 (dd, 1H, J 9.3, 14.5 Hz, H-1a), 2.83 (dd, 1H, J 1.6, 14.2 Hz, H-1b), 2.58 (t, 2H, J 7.7 Hz, H-11a'', H-11b''), 2.19-2.12 (m, 2H, H-2a'', H-2b''), 1.67-1.42 (m, 6H, CH₂), 1.39-1.05 (m, 36H, CH₂), 0.88 (t, 3H, J 6.4 Hz, CH₃) ppm; ¹³C NMR (100 MHz, CDCl₃) δ 174.9 (C=O), 143.2, 138.9, 138.40, 138.38 (4C, 4° C), 128.8, 128.75, 128.70, 128.6, 128.5, 128.3, 128.2, 128.1, 128.0, 127.8, 127.3, 125.9 (12C, Ar), 87.7 (C-1'), 79.8 (C-3'), 77.2 (C-2'), 76.7 (C-3/4), 75.3 (C-4'), 74.9, 74.0 (2C, CH₂), 73.2 (C-3/4), 73.1 (CH₂), 72.8 (C-5'), 63.3 (C-6'), 53.1 (C-2), 37.1 (C-2''), 36.3 (C-11''), 33.4, 33.2 (2C, CH₂), 32.3 (C-1), 31.9, 30.07, 30.04, 30.02, 29.97, 29.92, 29.90, 29.87, 29.86, 29.77, 29.72, 29.70, 29.68, 29.61, 26.17, 26.09, 26.08, 26.06, 23.04 (19C, CH₂), 14.4 (CH₃) ppm; ESI-HRMS calcd for C₆₂H₉₁NO₈NaS [M + Na]⁺ 1032.6363, found 1032.6385.

2-(ω -Phenyl-dodecanoylamino)-1-S-(2',3',4'-tri-*O*-benzyl- α -D-galactopyranosyl)-D-ribo-octadecanediol (21). 4M HCl in dioxane (90 μ L) was added to a solution of compound **19** (48 mg, 45 μ mol) in CH₂Cl₂:MeOH (4.5 mL:0.9 mL) at 0 °C. The mixture was stirred for 2.5 h at room temperature, concentrated *in vacuo* and was purified by column chromatography (CH₂Cl₂:MeOH, 30:1) to afford compound **21** (33 mg, 71%): R_f = 0.7 (CH₂Cl₂:MeOH, 20:1); [α]_D = +12.1 (*c* 1.0 CHCl₃); ν_{\max} (neat/cm⁻¹) 3399, 3325, 2923, 2852, 1646, 1539, 1468, 1462, 1122, 1083, 1034, 736, 655; ¹H NMR (400 MHz, CDCl₃) δ 7.39-7.24 (m, 18H, Ar), 7.18-7.16 (m, 2H, Ar), 6.38 (d, 1H, J 8.8 Hz, NH), 5.47 (d, 1H, J 5.5 Hz, H-1'), 4.92 (d, 1H, J 11.8 Hz,

CH₂), 4.84 (d, 1H, *J* 11.8 Hz, CH₂), 4.72-4.68 (m, 3H, CH₂), 4.60 (d, 1H, *J* 11.6 Hz, CH₂), 4.27 (dd, 1H, *J* 5.4, 9.9 Hz, H-2'), 4.23-4.17 (m, 2H, H-5', H-2), 3.86-3.81 (m, 2H, H-6a', H-4'), 3.69 (dd, 1H, *J* 2.7, 10.0 Hz, H-3'), 3.59-3.54 (m, 2H, H-3, H-4), 3.50 (dd, 1H, *J* 3.1, 12.0 Hz, H-6b'), 3.06 (dd, 1H, *J* 9.4, 14.6 Hz, H-1a), 2.83 (dd, 1H, *J* 3.0, 14.7 Hz, H-1b), 2.59 (t, 2H, *J* 7.9 Hz, H-2a'', H-2b''), 2.17 (t, 2H, *J* 7.8 Hz, H-12a'', H-12b''), 1.66-1.25 (m, 44H, CH₂), 0.88 (t, 3H, *J* 6.5 Hz, CH₃) ppm; ¹³C NMR (400 MHz, CDCl₃) δ 174.8 (C=O), 143.1, 138.8, 138.31, 138.29 (4C, 4 °C), 128.7, 128.63, 128.60, 128.4, 128.2, 128.1, 128.0, 127.8, 127.7, 125.8 (10C, Ar), 87.5 (C-1'), 79.7 (C-3'), 76.8 (C-3/C-4), 76.6 (C-2'), 75.3 (C-4'), 74.8 (CH₂), 73.9 (CH₂), 73.1 (C-3/C-4), 73.0 (CH₂), 72.7 (C-5'), 63.2 (C-6'), 53.1 (C-2), 37.0 (C-2''), 36.2 (C-12''), 33.3 (CH₂), 33.1 (C-1), 32.2, 31.7, 29.93, 29.89, 29.81, 29.75, 29.6, 29.5, 26.03, 25.96, 22.9 (11C, CH₂), 14.3 (CH₃) ppm [NB: 12 co-incident C signals].

1-S-(α-D-Galactopyranosyl)-2-(ω-phenylundecanoylamino)-D-ribo-3,4-octadecanediol (1).

Sodium (161 mg, 7.0 mmol) was added to liquid NH₃ under N₂ at -78 °C after 1 h. Compound **20** (71 mg, 70 μmol) in THF (5 mL) was added to the solution and the mixture was allowed stir for 2 h at -78 °C. The reaction was quenched by the addition of NH₄Cl, and the NH₃ was left to evaporate overnight. The crude residue was purified by column chromatography (CHCl₃:MeOH, 10:1) to afford **1** (52.5 mg, quant.) as a white solid: *R_f* = 0.7 (CH₂Cl₂:MeOH, 11:1); [α]_D = +20.1 (*c* 0.2 C₅H₅N); *v*_{max} (neat/cm⁻¹) 3412, 2923, 2853, 1642, 1267, 746; ¹H NMR (400 MHz, C₅D₅N) δ 8.54 (d, 1H, *J* 8.7 Hz, NH), 7.37 (t, 2H, *J* 7.5 Hz, Ar), 7.29-7.22 (m, 3H, Ar), 6.11 (d, 1H, *J* 5.5 Hz, H-1'), 5.26-5.02 (m, 1H, H-2), 4.98 (dd, 1H, *J* 5.5, 9.9 Hz, H-2'), 4.91 (app t, 1H, *J* 5.6 Hz, H-5'), 4.60 (dd, 1H, *J* 7.2, 11.2 Hz, H-6a'), 4.52 (app d, 1H, *J* 2.6 Hz, H-4'), 4.42-4.38 (m, 3H, H-6b', H-3', H-3), 4.25 (app dt, 1H, *J* 2.0, 7.6 Hz, H-4), 3.72-3.62 (m, 2H, H-1a, H-1b), 2.59 (t, 2H, *J* 7.7 Hz, H-2a'', H-2b''), 2.55-2.49 (m, 2H, 11a'', 11b''), 2.31-2.24 (m, 1H, OH), 1.95-1.16 (m, 47H, OH, CH₂), 0.88 (t, 3H, *J* 6.7 Hz, CH₃) ppm; ¹³C NMR (100 MHz, C₅D₅N) δ 174.2 (C=O), 129.3, 129.2, 129.1, 126.5 (4C, Ar), 90.1 (C-1'), 78.0 (C-3'), 73.9 (C-5'), 73.0 (C-4), 72.8 (C-3), 71.4 (C-4'), 70.5 (C-2'), 63.4 (C-6'), 53.6 (C-2), 43.9, 37.3, 37.2, 36.6 (4C, CH₂), 32.5 (C-1), 32.3, 30.7, 30.6, 30.43, 30.41, 30.39, 30.3, 30.26, 30.21, 30.17, 30.15, 30.0, 29.95, 27.0, 26.8, 23.3 (16C, CH₂), 14.7 (CH₃) ppm [NB: 3 co-incident C signals]; ESI-HRMS calcd for C₄₁H₇₂NO₈S [M - H] 738.4979, found 738.4943.

1-S-(α-D-Galactopyranosyl)-2-(ω-phenyldodecanoylamino)-D-ribo-3,4-octadecanediol (2).

Sodium (23 mg, 0.98 mmol) was added to liquid NH₃ under N₂ at -78 °C after 1 h. Compound **21** (51 mg, 49 μmol) in THF (2.5 mL) was added to the solution and the mixture was allowed stir for 2 h at -78 °C. The reaction was quenched by the addition of NH₄Cl, and the NH₃ was left to evaporate overnight. The crude residue was purified by column chromatography (CHCl₃:MeOH, 20:1) to afford **2** (11 mg, 31%) as an off-white solid: *R_f* = 0.7 (CH₂Cl₂:MeOH, 11:1); [α]_D = +16.5 (*c* 0.2 C₅H₅N); *v*_{max} (neat/cm⁻¹) 3430, 2925, 2855, 1645, 1263, 749; ¹H NMR (400 MHz, C₅D₅N) δ 8.79 (d, 1H, *J* 7.43 Hz, NH), 7.37 (t, 2H, *J* 7.5 Hz, Ar), 7.30-7.24 (m, 3H, Ar), 6.11 (d, 1H, *J* 5.4 Hz, H-1'), 5.27-4.90 (m, 3H, H-2, H-2', H-5'), 4.59 (dd, 1H, *J* 7.2, 11.2 Hz, H-6a'), 4.52 (d, 1H, *J* 3.0 Hz, H-4'), 4.42-4.37 (m, 3H, H-6b', H-3', H-3), 4.27-4.22 (m, 1H, H-4), 3.68 (s, 1H, H-1a.), 3.67 (s, 1H, H-1b), 2.61 (t, 2H, *J* 7.7 Hz, H-2a'', H-2b''), 2.53 (dt, 2H, *J* 3.6 Hz, H-12a'',

H-12b"), 2.31-2.24 (m, 1H, OH), 1.99-1.83 (m, 4H, CH₂), 1.75-1.67 (m, 1H, OH), 1.61 (q, 2H, *J* 7.2, 14.3 Hz, H-3-a", H-3b"), 1.41-1.21 (m, 42H, CH₂), 0.89 (t, 3H, *J* 6.5 Hz, CH₃) ppm; ¹³C NMR (100 MHz, C₅D₅N) δ 174.3 (C=O), 129.3, 129.2, 126.5 (3C, Ar), 111.9 (4° C), 90.2 (C-1'), 78.1 (C-3'), 74.0 (C-5'), 72.9 (C-3, C-4), 71.5 (C-4'), 70.6 (C-2'), 63.5 (C-6'), 37.3 (C-12"), 36.6 (C-2"), 34.5, 32.8 (2C, CH₂), 32.6 (C-1), 32.3, 30.8, 30.6, 30.48, 30.46, 30.44, 30.38, 30.31, 30.30, 30.24, 30.20, 30.07, 30.01, 27.1, 26.9, 23.4 (16C, CH₂), 14.7 (CH₃) ppm [NB: C-2 not seen and 5 co-incident C signals]; ESI-HRMS calcd. For C₄₂H₇₅NNaO₈S [M + Na]⁺ 776.5111 found 776.5108.

NKT cell stimulation assays. NKT cells were enriched from human peripheral blood mononuclear cells using antibody-coated magnetic beads (Miltenyi Biotech, Gladbach Bergische, Germany) followed by sorting on a MoFlo™ XDP Cell Sorter (Beckman Coulter, High Wycombe, UK). Cells were then expanded for 2-3 weeks by mitogen stimulation in the presence of IL-2, as described¹⁹. This method typically leads to the generation of >10⁸ NKT cells starting with <10⁶ cells, with purities of >98% as determined by flow cytometry. Mock-transfected or CD1d-transfected HeLa cells were pulsed with medium alone or with KRN7000 (α-GalCer), its thioglycoside analogue (α-S-GalCer), or Compounds **1** and **2** in 96-well culture plates (10⁵ cells/well) as described previously^{13,19}. α-GalCer and α-S-GalCer were added at concentrations of 0.1 μg/ml, whereas Compounds **1** and **2** were tested at 0.1, 1 and 10 μg/ml. After 24 hours, equal numbers of expanded NKT cells, which were rested by culturing for at least 1 week in the absence of IL-2, were added to the glycolipid-pulsed HeLa transfectant cells. After a further 24 hours, cell supernatants were harvested and assayed for IFN-γ, IL-4 and IL-10 by ELISA (R&D Systems (Abingdon, UK)).

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References

1. Savage, P. B.; Teyton, L.; Bendelac, A. *Chem. Soc. Rev.* **2006**, *35*, 771. <http://dx.doi.org/10.1039/b510638a> PMID:16936925
2. Wu, L.; Gabriel, C. L.; Parekh, V. V.; Van Kaer, L. *Tiss. Antigen.* **2009**, *73*, 535. <http://dx.doi.org/10.1111/j.1399-0039.2009.01256.x> PMID:19392798

3. Banchet-Cadeddu, A.; Hénon, E.; Dauchez, M.; Renault, J. H.; Monneaux, F.; Haudrechy, A. *Org. Biomol. Chem.* **2011**, *9*, 3080.
<http://dx.doi.org/10.1039/c0ob00975j> PMID:21394364
4. Berkers, C. R.; Ovaa, H. *Trends Pharmacol. Sci.* **2005**, *26*, 252.
<http://dx.doi.org/10.1016/j.tips.2005.03.005> PMID:15860372
5. (a) Wu, D.; Fujio, M.; Wong, C. H. *Bioorg. Med. Chem.* **2008**, *16*, 1073.
<http://dx.doi.org/10.1016/j.bmc.2007.10.026> PMID:18006319 PMCID:2279229
(b) Murphy, N.; Zhu, X.; Schmidt, R. R. In *Specialist Periodical Reports: Carbohydrate Chemistry – Chemical and Biological Approaches*, Royal Society of Chemistry, London 2010, Vol. 36, p 64.
6. (a) Trappeniers, M.; Chofor, R.; Aspeslagh, S.; Li, Y.; Linclau, B.; Zajonc, D. M.; Elewaut, D.; Van Calenbergh, S. *Org. Lett.* **2010**, *12*, 2928-2931.
<http://dx.doi.org/10.1021/ol100934z> PMID:20518554 PMCID:2903208
(b) Schombs, M.; Park, F. E.; Du, W.; Kulkarni, S. S.; Gervay-Hague, J. *J. Org. Chem.* **2010**, *75*, 4891-4898.
<http://dx.doi.org/10.1021/jo100366v> PMID:20387787 PMCID:2912955
(c) Shiozaki, M.; Tashiro, T.; Koshino, H.; Nakagawa, R.; Inoue, S.; Shigeura, T.; Watarai, H.; Taniguchi, M.; Mori, K. *Carbohydr. Res.* **2010**, *345*, 1663.
<http://dx.doi.org/10.1016/j.carres.2010.05.003> PMID:20591421
(d) Harrak, Y.; Barra, C. M.; Delgado, A.; Castano, A. R.; Llebaria, A. *J. Am. Chem. Soc.* **2011**, *133*, 12079.
<http://dx.doi.org/10.1021/ja202610x> PMID:21728320
(e) Jervis, P. J.; Cox, L. R.; Besra, G. S. *J. Org. Chem.* **2011**, *76*, 320.
<http://dx.doi.org/10.1021/jo102064p> PMID:21155575 PMCID:3018865
(f) Zhang, Z.; Zhao, W.; Wang, B.; Xia, C.; Zhang, W.; Wang, P. G. *Org. Lett.* **2011**, *13*, 4530.
<http://dx.doi.org/10.1021/ol201695n> PMID:21815670 PMCID:3398385
(g) O'Reilly, C.; Murphy, P. V. *Org. Lett.* **2011**, *13*, 5168.
PMid:21875123
(h) Deng, S.; Mattner, J.; Zang, Z.; Bai, L.; Teyton, L.; Bendelac, A.; Savage, P. B. *Org. Biomol. Chem.* **2011**, *9*, 7659.
<http://dx.doi.org/10.1039/c1ob06276j> PMID:21927724 PMCID:3308336
(i) Pauwels, N.; Aspeslagh, S.; Vanhoenacker, G.; Sandra, K.; Yu, E. D.; Zajonc, D. M.; Elewaut, D.; Linclau, B.; Van Calenbergh, S. *Org. Biomol. Chem.* **2011**, *9*, 8413.
<http://dx.doi.org/10.1039/c1ob06235b> PMID:22042483 PMCID:3246390

7. (a) Miyamoto, K.; Miyake, S.; Yamamura, T. *Nature* **2001**, *413*, 531.
<http://dx.doi.org/10.1038/35097097> PMID:11586362
(b) Pal, E.; Tabira, T.; Kawano, T.; Taniguchi, M.; Miyake, S.; Yamamura, T. *J. Immunol.* **2001**, *166*, 662.
8. Koch, M.; Stronge, V. S.; Shepherd, D.; Gadola, S. D.; Mathew, B.; Ritter, G.; Fersht, A. R.; Besra, G. S.; Schmidt, R. R.; Jones, E. Y.; Cerundolo, V. *Nat. Immunol.* **2005**, *6*, 819.
<http://dx.doi.org/10.1038/ni1225> PMID:16007090
9. Fujio, M.; Wu, D. G.; Garcia-Navarro, R.; Ho, D. D.; Tsuji, M.; Wong, C. H. *J. Am. Chem. Soc.* **2006**, *128*, 9022.
<http://dx.doi.org/10.1021/ja062740z> PMID:16834361
10. Chang, Y. J.; Huang, J. R.; Tsai, Y. C.; Hung, J. T.; Wu, D.; Fujio, M.; Wong, C. H.; Yu, A. L. *Proc. Natl. Acad. Sci. U.S.A* **2007**, *104*, 10299.
<http://dx.doi.org/10.1073/pnas.0703824104> PMID:17566107 PMCID:1965507
11. (a) Dere, R. T.; Zhu, X. *Org. Lett.* **2008**, *10*, 4641
<http://dx.doi.org/10.1021/ol8019555> PMID:18808135
(b) Zhu, X.; Dere, R. T.; Jiang, J. *Tetrahedron Lett.* **2011**, *52*, 4971.
<http://dx.doi.org/10.1016/j.tetlet.2011.07.103>
12. Driguez, H. *ChemBioChem.* **2001**, *2*, 311.
[http://dx.doi.org/10.1002/1439-7633\(20010504\)2:5<311::AID-CBIC311>3.0.CO;2-L](http://dx.doi.org/10.1002/1439-7633(20010504)2:5<311::AID-CBIC311>3.0.CO;2-L)
13. Hogan, A. E.; O'Reilly, V.; Dunne, M. R.; Dere, R. T.; Zeng, S. G.; O'Brien, C.; Amu, S.; Fallon, P. G.; Exley, M. A.; O'Farrelly, C.; Zhu, X.; Doherty, D. G. *Clin. Immunol.* **2011**, *140*, 196.
<http://dx.doi.org/10.1016/j.clim.2011.03.016> PMID:21493160
14. Stephens, B. E.; Liu, F. *Tetrahedron Lett.* **2007**, *48*, 9116.
<http://dx.doi.org/10.1016/j.tetlet.2007.10.141>
15. Schmidt, R. R.; Maier, T. *Carbohydr. Res.* **1988**, *174*, 169.
[http://dx.doi.org/10.1016/0008-6215\(88\)85090-0](http://dx.doi.org/10.1016/0008-6215(88)85090-0)
16. Figueroa-Pérez, S.; Schmidt, R. R. *Carbohydr. Res.* **2000**, *328*, 95.
[http://dx.doi.org/10.1016/S0008-6215\(00\)00092-6](http://dx.doi.org/10.1016/S0008-6215(00)00092-6)

17. (a) Du, W.; Gervay-Hague, J. *Org. Lett.* **2005**, 7, 2063
<http://dx.doi.org/10.1021/ol050659f> PMID:15876055
(b) Diaz, Y. R. G.; Wojno, J.; Cox, L. R.; Besra, G. S. *Tetrahedron: Asymmetry* 2009, 20, 747
<http://dx.doi.org/10.1016/j.tetasy.2009.02.020>
(c) P. B. Alper, S.-C. Hung and C.-H. Wong, *Tetrahedron Lett.* **1996**, 37, 6029.
[http://dx.doi.org/10.1016/0040-4039\(96\)01307-X](http://dx.doi.org/10.1016/0040-4039(96)01307-X)
18. Zhu, X.; Dere, R. T.; Jiang, J.; Zhang, L.; Wang, X. *J. Org. Chem.* **2011**, 76, 10187.
<http://dx.doi.org/10.1021/jo202069y> PMID:22059806
19. O'Reilly, V.; Zeng, S. G.; Bricard, G.; Atzberger, A.; Hogan, A. E.; Jackson, J.; Feighery, C.; Porcelli, S. A.; Doherty, D. G. *PLoS One* **2011**, 6, e28648.
<http://dx.doi.org/10.1371/journal.pone.0028648> PMID:22174854 PMCID:3236218