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Synthesis of a novel α -galactosyl ceramide haptenated-lipid antigen, a useful tool in demonstrating the involvement of α NKT cells in the production of antilipid antibodies

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Abstract

A new haptenated derivative of α -galactosyl ceramide (α -GalCer) has been synthesized to assist in the study of the mechanism of T cell help for the production of B cell antibodies. Our synthetic route provides access to an amine intermediate which can easily be extended to generate an array of compounds, useful in various ongoing studies. Herein, we also describe the biological evaluation of the nitrophenyl (NP) haptenated α -GalCer and demonstrate its importance in such mechanistic studies. For instance, *in vivo* studies showed that NP- α -GalCer stimulates both T and B cell proliferation while *in vivo* studies in immunized mice showed the production of IgG anti-NP antibodies after exposure to NP- α -GalCer. The interpretation of these results helps towards a better understanding of T cell help for the production of antibodies.

INTRODUCTION

Antigen-presenting cells (APC) and lymphocytes are two major cell types that work together to ensure an effective immune response through the recognition and elimination or neutralization of pathogenic organisms and other foreign invaders in mammals (1-4). APC include macrophages, B lymphocytes and dendritic cells, while T cells, invariant natural killer (α NKT), and natural killer (NK) cells are three major types of lymphocytes important for immune defense. Specifically, B cells recognize their epitopes as part of either an intact protein, lipid molecule or polysaccharide, whereas T cells only recognize epitopes which are subcomponents of an intact molecule and are found in association with antigen-presenting molecules, such as MHC class II or CD1.

A distinctive class of T cells, known as invariant Natural Killer T (α NKT) cells display characteristics of both T cells and NK cells and play a crucial role in diverse immune responses and other pathologic conditions (5). The synthetic glycolipid α -galactosyl ceramide (α -GalCer) (6), known as KRN7000 (**1**), is a powerful agonist for these cells. When it is presented by CD1d it activates α NKT cells to release diverse cytokines, including both Th1 and Th2 cytokines (7). It is believed that the release of Th1 cytokines may contribute to antitumor and antimicrobial functions, while Th2 cytokines may help alleviate

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autoimmune diseases (8), such as multiple sclerosis (9) and arthritis (10) or helminth infections. Once stimulated, α NKT cells also activate other cells, such as dendritic cells, NK cells, peptide-reactive T cells, and B cells (2).

The detection of IgG antilipid antibodies, generated by lipid-specific B cells during infection and autoimmune disease, such as malaria (11) and systemic lupus erythematosus (12) respectively, suggests the existence of cooperation between α NKT cells and B cells (13). To better understand the mechanism of α NKT cell help for the production of antilipid antibodies, haptenated lipid antigens, containing both a B cell recognition component and an α NKT cell agonist are useful tools (Figure 1) (13). As part of ongoing studies on the emerging role for CD1d and α NKT cells/ B cell responses that are crucial in defense against infection, autoimmunity, and tumor immunity, easy access to hybrid haptenated lipids is therefore desired. Here, we propose that the modified α -GalCer derivative **2** (Figure 2) represents an ideal template which is easily amenable to such molecules. We believe that the amine functionality in compound **2** allows conjugation to different haptens, e.g. biotin or 4-hydroxy-3-nitrophenyl (NP) to yield substrates such as compound **3**, thereby making it an ideal scaffold. In this paper, we describe the syntheses of compounds **2** and **3** and describe the biological activities of compounds **3** and **4** (Figure 2). The hapten, 4-hydroxy-3-nitrophenyl (NP), was chosen as the B cell recognition component because the anti-NP B cell response has been extensively studied in mice and there are a number of B-cell specific immunologic tools available to investigate this response (14–16). Conversely, α -GalCer was selected as the α NKT cell component of this conjugated molecule because it is also a well studied antigen which stimulates robust activation of α NKT cells and for which there are a number of important immunologic tools available.

EXPERIMENTAL PROCEDURES

General Experimental Procedures

^1H NMR spectra were recorded at 400 MHz or 300 MHz, using Bruker AMX 400, Bruker AV 400, Bruker AV 300 and Bruker AC 300 spectrometers. ^{13}C NMR spectra were recorded at 100 MHz or 75 MHz, respectively, using Bruker AMX 400, Bruker AV 400, Bruker AV 300 and Bruker AC 300 spectrometers. Chemical shifts are reported as δ values (ppm) referenced to the following solvent signals: CHCl_3 , ^1H 7.26; CDCl_3 , ^{13}C 77.0; CH_3OH , ^1H 3.34; CD_3OD , ^{13}C 49.9. Quaternary carbons were reported only when observed. HRMS were recorded on a Micromass LCT spectrometer using a lock mass incorporated into the mobile phase. All reagents were obtained from commercial sources, and were used without further purification unless stated otherwise. Anhydrous solvents were purchased from Sigma–Aldrich, UK, stored over 4 Å molecular sieves and under an argon atmosphere. Analytical thin layer chromatography (TLC) was performed on aluminum plates pre-coated with Merck silica gel 60A F-254 as adsorbent. The developed plates were air dried, visualized by UV detection (at 254 nm) and/or stained with 5% phosphomolybdic acid in EtOH (MPA spray). Compounds were purified by flash column chromatography on Merck silica gel (particle size 40–63 μm mesh) or Fluka 60 (40–60 μm mesh) silica gel.

Phenyl 3, 4-*O*-isopropylidene-6-*O*-*tert*-butyldiphenylsilyl-2-*O*-(5-azapentyl)-1-thio- β -D-galactopyranoside **13**

To a cold solution of phenyl 3, 4-*O*-isopropylidene-6-*O*-*tert*-butyldiphenylsilyl-1-thio- β -D-galactopyranoside **12** (2.00g, 3.64 mmol) in dry DMF (30 mL) was added sodium hydride (0.44 g, 18.2 mmol, 5 eq) in small amounts at 0 °C. The solution was allowed to stir for 20 minutes before the addition of 1, 5-dibromopentane (4 mL, 18.2 mmol, 5eq). The reaction mixture was stirred at room temperature for two hours, and then taken up in water (50 mL). The resulting mixture was then extracted with CH_2Cl_2 (3 $\times$ 20 mL). The combined organic

layers were washed with brine (30 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo. The crude bromide was further dissolved in DMSO (30 mL) and sodium azide (1.20 g, 18.2 mmol, 5 eq) was added. The reaction was stirred overnight at 60 °C, after which, it was taken up in water (50 mL) and extracted with CH_2Cl_2 (3×20 mL). The combined organic layers were then washed with brine (30 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was then purified by flash chromatography using Hexanes: EtOAc (4:1) to give the desired product as colorless oil (1.80 g, 2.72 mmol, 75%). ^1H NMR (CDCl_3): 7.65-7.75 (3H, m, Ar-*H*), 7.49-7.55 (2H, m, Ar-*H*), 7.32-7.44 (8H, m, Ar-*H*), 7.19-7.27 (2H, m, Ar-*H*), 4.55 (1H, d, $J_{1,2}=9.72$ Hz, H-1), 4.28 (1H, dd, $J_{4,3}=5.6$, $J_{4,5}=1.9$ Hz, H-4), 4.15 (1H, dd, $J_{3,2}=6.5$ Hz, H-3), 3.96-3.83 (3H, m, H-5, H-6a, H-6b), 3.72 (1H, ddd, $J_{1a,1b}=10.7$, $J_{1a,2a}=J_{1a,2b}=6.1$ Hz, $\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 3.59 (1H, ddd, $J_{1b,2a}=8.1$, $J_{1b,2b}=5.2$ Hz, $\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 3.34-3.39 (1H, m, H-2), 3.26-3.31 (2H, m, $\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 1.56-1.70 (4H, m, CH_2), 1.34, 1.47 (6H, 2s, $\text{C}(\text{CH}_3)_2$), 1.09 (2H, s, CH_2), 1.06 (9H, s, *t*-Bu); ^{13}C NMR (75 MHz, CDCl_3): 135.6, 134.8, 133.4, 131.4, 131.5, 129.7, 128.8, 127.7, 127.6, 127.2 (18 C_{Ar}), 109.8 ($\text{O}_2\text{C}(\text{CH}_3)_2$), 86.6 (C-1), 79.7, 79.2, 76.7, 73.4 (C-2, C-3, C-4, C-5), 71.7 ($\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 63.0 (C-6), 51.4 ($\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 29.5, 28.6 ($2 \times \text{CH}_2$), 28.0, 26.8, 26.6, 26.3 ($\text{C}(\text{CH}_3)_3$, $\text{C}(\text{CH}_3)_2$), 23.3 (CH_2), 19.2 ($\text{C}(\text{CH}_3)_3$); HRMS calcd for $\text{C}_{36}\text{H}_{47}\text{N}_3\text{O}_5\text{SiS}$ [$\text{M}+\text{Na}$] $^+$: 684.2904, found 684.2908.

Phenyl 2-O-(5-azapentyl)-1-thio- β -D-galactopyranoside 10

To a solution of **13** (1.50g, 2.27 mmol) in THF (30 mL) was added a 1M solution of tetrabutylammonium fluoride (4.50 mL, 2 eq). The reaction mixture was stirred at room temperature for 4 hours. Upon completion of the reaction, the reaction mixture was diluted with EtOAc (20 mL) and washed successively with water (20 mL) and brine (20 mL). The organic layer was dried over anhydrous Na_2SO_4 and evaporated. The residue was then dissolved in CH_2Cl_2 and TFA (5 mL) was added. The mixture was stirred at room temperature for 2 h, after which time TLC analysis indicated that the reaction was complete. The solvents were removed in vacuo and the residue was purified by flash chromatography using EtOAc to give the desired product as a colorless oil (0.68 g, 1.78 mmol, 78%). ^1H NMR (CDCl_3): 7.48-7.54 (2H, m, Ar-*H*), 7.26-7.35 (3H, m, Ar-*H*), 4.59 (1H, d, H-1, $J_{1,2}=9.7$ Hz), 4.06 (1H, d, $J_{4,3}=3.1$ Hz, H-4), 3.95 (1H, dd, $J_{6a,6b}=11.9$, $J_{6a,5}=5.9$ Hz, H-6a), 3.90 (1H, dd, H-6b), 3.80-3.69 (2H, m, $\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 3.63 (1H, dd, $J_{3,2}=8.9$ Hz, H-3), 3.58 (1H, m, H-5), 3.45 (1H, t, H-2) 3.28 (2H, t, $\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 1.56-1.71 (4H, m, CH_2), 1.07 (2H, s, CH_2); ^{13}C NMR (75 MHz, CDCl_3): 134.0, 131.1, 129.0, 127.4 (6 C_{Ar}), 87.7 (C-1), 78.5, 77.9, 75.1, 69.8 (C-2, C-3, C-4, C-5), 73.2 ($\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 62.6 (C-6), 51.4 ($\text{OCH}_2\text{C}_3\text{H}_6\text{CH}_2\text{N}_3$), 29.7, 28.6 ($2 \times \text{CH}_2$), 23.2 (CH_2), 19.2 ($\text{C}(\text{CH}_3)_3$); HRMS calcd for $\text{C}_{17}\text{H}_{25}\text{N}_3\text{O}_5\text{S}$ [$\text{M}+\text{Na}$] $^+$: 406.1413, found 406.1426.

Phenyl 3-O-benzoyloxy-4, 6-O-di-*tert*-butylsilylene-2-O-(5-azapentyl)-1-thio- β -D-galactopyranoside 8

To a solution of **10** (0.52g, 1.36 mmol) in dry DMF (15 mL) was added di-*tert*-butylsilyl bis(trifluoromethanesulfonate) (0.90g, 2.04 mmol, 1.5 eq) at 0 °C, and the mixture was stirred for 30 minutes, then neutralized with Et_3N . The solution was taken up in water and extracted with EtOAc (3×20 mL). The organic layers were combined, washed with brine (20 mL), dried over anhydrous Na_2SO_4 and evaporated. The residue was then dissolved in pyridine (20 mL) and benzoyl chloride (0.27g, 1.92 mmol, 1.4 eq) was added. The mixture was stirred at room temperature for 2 h, after which time TLC analysis indicated that the reaction was complete. The reaction was quenched with methanol, diluted with CH_2Cl_2 (30 mL), washed successively with 1M HCl (3×20 mL), water (2×20 mL), brine (20 mL), dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was finally purified by flash chromatography using Hexanes: EtOAc (4:1) to give the desired product as a colorless oil (0.82 g, 1.31 mmol, 96%). ^1H NMR (CDCl_3): 8.06-8.13 (2H, m, Ar-*H*),

7.41-7.65 (6H, m, Ar-*H*), 7.27-7.34 (2H, m, Ar-*H*), 5.03 (1H, dd, $J_{3,2}=9.4$, $J_{3,2}=3.1$ Hz, H-3), 4.69-4.75 (2H, m, H-1, H-4), 4.22-4.28 (2H, m, H-6a, H-6b), 3.82-3.94 (2H, m, H-2, OCH₂C₃H₆CH₂N₃), 3.66 (1H, ddd, $J_{1a,1b}=9.0$, $J_{1a,2a}=J_{1a,2b}=6.2$ Hz, OCH₂C₃H₆CH₂N₃), 3.50 (1H, s, H-5), 3.05-3.18 (2H, m, OCH₂C₃H₆CH₂N₃) 1.26-1.57 (4H, m, CH₂), 1.09 (2H, s, CH₂), 1.16, 0.97 (18H, 2s, 2 × *t*-Bu); ¹³C NMR (75 MHz, CDCl₃): 133.2, 132.0, 131.9, 129.6, 128.8, 128.5, 127.4 (12 C_{Ar}), 88.5 (C-1), 77.3 (C-3), 76.7, 74.6, 70.6 (C-2, C-4, C-5), 73.3 (OCH₂C₃H₆CH₂N₃), 67.2 (C-6), 51.2 (OCH₂C₃H₆CH₂N₃), 29.7, 28.5 (2 × CH₂), 27.7, 27.4 (2 × C(CH₃)₃), 23.3, 20.7 (2 × C(CH₃)₃); HRMS calcd for C₃₂H₄₅N₃O₆SiS [M+Na]⁺: 650.2696, found 650.2693.

(2S, 3S, 4R)-2-*N*-tert-Butoxycarbonylamino-3,4-di(benzyloxy)-1-[3-*O*-benzyloxy-4,6-*O*-di-*tert*-butylsilylene-2-*O*-(5-azapentyl)-α-D-galactopyranosyloxy]-octadecane 14

A suspension of **10** (0.75g, 1.20 mmol), **9** (0.90g, 1.44 mmol, 1.2 eq), silver triflate (0.18g, 2.4mmol) and 4Å molecular sieves in anhydrous CH₂Cl₂ (10 mL) was stirred, with the exclusion of light for 10 minutes at room temperature under Ar before it was cooled to -78 °C. A solution of *p*-nitrobenzenesulfonyl chloride (0.24g, 1.20 mmol, 95% purity) in anhydrous CH₂Cl₂ (2 mL) was dropped into the above suspension at -78 °C. The reaction mixture was maintained at -78 °C for 30 minutes and allowed to warm up to 0 °C over the following 30 minutes, after which TLC analysis showed that the reaction was complete. The reaction was quenched with saturated NaHCO₃ (2 mL) and diluted with CH₂Cl₂ (5 mL) to give a suspension, which was filtered through Celite and the Celite pad was washed with CH₂Cl₂ (10 mL). The filtrate was separated and the organic phase was dried over anhydrous Na₂SO₄ and evaporated. The residue was purified by flash chromatography using Hexanes: EtOAc (5:1) to give the glycosylated compound **14** as a light yellow oil (1.02 g, 0.89 mmol, 75%). ¹H NMR (CDCl₃): 7.89-8.13 (6H, m, Ar-*H*), 7.32-7.64 (12H, m, Ar-*H*), 5.64 (1H, dd, $J_{3,2}=8.5$, $J_{3,4}=3.4$ Hz, H-3^{Cer}), 5.39-5.52 (2H, m, NHBOC, H-4^{Cer}), 5.24 (1H, dd, $J_{3,2}=10.3$, $J_{3,2}=3.1$ Hz, H-3), 5.00 (1H, d, $J_{1,2}=3.5$ Hz, H-1), 4.27 (1H, d, H-4), 4.06-4.32 (3H, m, H-6a, H-6b, H-2^{Cer}), 4.00 (1H, dd, H-2), 3.73-3.84 (2H, m, H-5, H-1a^{Cer}), 3.60-3.71 (2H, m, OCH₂C₃H₆CH₂N₃, H-1b^{Cer}), 3.44 (1H, ddd, $J_{1a,1b}=8.9$, $J_{1a,2a}=J_{1a,2b}=6.6$ Hz, OCH₂C₃H₆CH₂N₃), 2.84-3.06 (2H, m, OCH₂C₃H₆CH₂N₃), 1.80-2.02 (2H, m, H-5a^{Cer}, H-5b^{Cer}), 1.49 (9H, s, NHCO₂C(CH₃)₃), 1.15-1.13 (24H, m, CH₂), 1.06-1.14 (2H, m, CH₂), 0.94-0.98 (2H, m, CH₂), 1.03, 0.92 (18H, 2s, 2 × *t*-Bu), 0.87 (3H, t, CH₃); ¹³C NMR (75 MHz, CDCl₃): 133.3, 132.9, 133.2, 132.0, 131.9, 129.9, 129.7, 129.6, 129.4, 128.8, 128.5, 127.4 (18 C_{Ar}), 97.4 (C-1), 80.3 (NHCO₂C(CH₃)₃), 77.1 (C-3), 76.5, 74.8, 70.2 (C-2, C-4, C-5), 73.8 (C-3^{Cer}), 73.2 (OCH₂C₃H₆CH₂N₃), 71.8 (C-4^{Cer}), 67.0 (C-6), 61.7 (C-1^{Cer}), 51.2 (OCH₂C₃H₆CH₂N₃), 49.9 (C-2^{Cer}), 32.1-22.8 (13 × CH^{Cer}), 29.8, 28.4 (2 × CH₂), 27.6, 27.3 (2 × C(CH₃)₃), 23.1, 20.5 (2 × C(CH₃)₃), 14.8 (CH^{Cer}); HRMS calcd for C₆₃H₉₄N₄O₁₃Si[M+Na]⁺: 1165.6484, found 1165.6488.

(2S,3S,4R)-2-*N*-tert-Butoxycarbonylamino-1-[2-*O*-(5-azapentyl)-α-D-galactopyranosyloxy]-octadecanediol 15

To a solution of **14** (0.93g, 0.814 mmol) in THF was added a 1M solution of tetrabutylammonium fluoride (2 mL, 2eq). The reaction mixture was stirred at room temperature for 6 hours. Upon completion of the reaction, the reaction mixture was diluted with EtOAc (20 mL) and washed successively with water (20 mL) and brine (20 mL). The organic layer was dried over anhydrous Na₂SO₄ and evaporated. The residue was then dissolved in MeOH and a 1M solution of NaOMe (5 mL) was added. The mixture was stirred at room temperature for 2 h, after which time TLC analysis indicated that the reaction was complete. The reaction was neutralized by the addition of Dowex 50WX8-200 resin. The latter was then filtered and the filtrate concentrated to give a residue which was purified by flash chromatography using EtOAc:MeOH (7:1) to give the title compound as a colorless syrup (0.49g, 0.710 mmol, 88%). ¹H NMR (CDCl₃): 4.90 (1H, d, $J_{1,2}=3.8$ Hz, H-1), 3.81

(1H, d, $J_{4,3}$ =3.1 Hz, H-4), 3.70 (1H, dd, $J_{1a,1b}$ =10.9, $J_{1a,2a}$ =4.4 Hz, H-1a^{Cer}), 3.65 (1H, dd, $J_{3,2}$ =9.8 Hz, H-3), 3.54-3.62 (5H, m, H-1b^{Cer}, H-2^{Cer}, H-3^{Cer}, H-4^{Cer}, H-5), 3.43 (1H, dd, H-2), 3.31-3.37 (2H, m, H-6a, H-6b), 3.20 (2H, ddd, $J_{1a,1b}$ =8.9, $J_{1a,2a}$ = $J_{1a,2b}$ =6.8 Hz, OCH₂C₃H₆CH₂N₃), 2.02-2.11 (2H, m, OCH₂C₃H₆CH₂N₃), 1.50 (9H, s, NHCO₂C(CH₃)₃) 1.35-1.60 (m, 8H, CH₂), 1.22-1.32 (2H, m, CH₂), 1.20-1.23 (68H, m, CH₂), 0.71 (6H, t, J =6.8 Hz, CH₃); ¹³C NMR (75 MHz, CDCl₃): 98.1 (C-1), 78.2, 76.1, 75.2, 71.4, 70.2, 69.2 (C-2, C-3, C-4, C-5, C-4^{Cer}, C-3^{Cer}), 71.2 (OCH₂C₃H₆CH₂N₃), 68.2 (C-1^{Cer}), 61.4 (C-6), 51.3 (OCH₂C₃H₆CH₂N₃), 50.1 (C-2^{Cer}), 36.5-22.0 (17 × CH₂), 27.5, 27.3 (2 × C(CH₃)₃), 14.0 (CH₃^{Cer}); HRMS calcd for C₃₄H₆₆N₄O₁₀[M+Na]⁺: 713.4677, found 713.4676.

(2S,3S,4R)-2-Amino-N-hexacosanoyl-1-[2-O-(5-azapentyl)-α-D-galactopyranosyloxy]]-3, 4-octadecanediol 16

Compound **15** (0.38g, 0.550 mmol) was dissolved in CH₂Cl₂: MeOH (2:1) (10 mL) and TFA (2 mL) was added. The reaction was stirred for 2 hours and concentrated in vacuo to give the crude amine which was used in the next step without purification. The crude amine was then dissolved in a 1:1 mixture of THF:NaOAc (saturated solution) (5 mL) and the freshly prepared acid chloride of hexacosanoic acid (0.25g, 0.603 mmol, 1.1eq) was added. The reaction was allowed to stir overnight at room temperature. The organic phase was then concentrated and the residue was purified by flash chromatography (gradient from CHCl₃ to 15% MeOH in CHCl₃) to give the acylated compound **16** as a white solid (0.38g, 0.39mmol, 71%). ¹H NMR (CDCl₃/ CD₃OD 2:1): 4.86 (1H, d, $J_{1,2}$ =3.6 Hz, H-1), 3.79 (1H, d, $J_{4,3}$ =3.2 Hz, H-4), 3.72 (1H, dd, $J_{1a,1b}$ =10.9, $J_{1a,2a}$ =4.6 Hz, H-1a^{Cer}), 3.64 (1H, dd, $J_{3,2}$ =9.8 Hz, H-3), 3.52-3.60 (5H, m, H-1b^{Cer}, H-2^{Cer}, H-3^{Cer}, H-4^{Cer}, H-5), 3.43 (1H, dd, H-2), 3.31-3.37 (2H, m, H-6a, H-6b), 3.20 (2H, ddd, $J_{1a,1b}$ =8.9, $J_{1a,2a}$ = $J_{1a,2b}$ =6.8 Hz, OCH₂C₃H₆CH₂N₃), 2.00-2.10 (2H, m, OCH₂C₃H₆CH₂N₃), 1.36-1.59 (m, 8H, CH₂), 1.25-1.35 (2H, m, CH₂), 1.20-1.24 (68H, m, CH₂), 0.73 (6H, t, J =6.8 Hz, CH₃); ¹³C NMR (75 MHz, CDCl₃): 97.7 (C-1), 77.2, 75.2, 72.1, 70.4, 69.6, 69.3 (C-2, C-3, C-4, C-5, C-4^{Cer}, C-3^{Cer}), 71.2 (OCH₂C₃H₆CH₂N₃), 67.4 (C-1^{Cer}), 61.5 (C-6), 51.1 (OCH₂C₃H₆CH₂N₃), 49.8 (C-2^{Cer}), 36.2, 32.9, 31.6, 29.4, 29.1, 28.3, 25.6, 22.8, 22.4 (CH₂), 13.7 (CH₃^{Cer}); HRMS calcd for C₅₅H₁₀₈N₄O₉ [M+Na]⁺: 991.8014, found 991.8018.

(2S,3S,4R)-2-Amino-N-hexacosanoyl-1-[2-O-(5-aminopentyl)-α-D-galactopyranosyloxy]]-3, 4-octadecanediol 3

Compound **16** (0.32g, 0.33 mmol) was dissolved in MeOH (10 mL) and stirred with Pd-C (5 mg) under H₂ overnight, after which time it was filtered through a pad of Celite, which was subsequently washed with MeOH. The combined filtrates were concentrated and the target compound **3** was obtained as a white solid (0.28g, 0.297 mmol, 90%). The crude amine was used without any further purification in the following acylation reactions.

Acylation to give compound 3

3-hydroxy-4-nitrophenyl acetic acid (17 mg, 0.09 mmol) was added to neat oxalyl chloride (1 mL) and stirred at 70 °C for 2 h, after which time the solution was cooled to rt and the unreacted oxalyl chloride was removed under a stream of argon. The residual volatiles were removed under reduced pressure. The resulting crude acyl chloride was dissolved in THF (1 mL) and added to a solution of amine **3** (50 mg, 0.05 mmol) in THF/NaOAc(sat) (1:1, 1 mL). The reaction was stirred vigorously overnight after which the organic phase was removed. The aqueous phase was further extracted with THF (2 × 1 mL), and the combined organic phases were concentrated. The residue was finally purified by flash chromatography (gradient from CHCl₃ to 15% MeOH in CHCl₃) to give the acylated compound **17** as a yellow solid (42 mg, 0.39 mmol, 71%). ¹H NMR (CDCl₃/ CD₃OD 2:1): 7.85 (1H, d, J =2.4

Hz), 7.36 (1H, dd, $J = 8.6, J = 2.4$ Hz), 6.90 (1H, d, $J = 8.6$ Hz), 4.81 (1H, d, $J_{1,2} = 3.6$ Hz, H-1), 3.74 (1H, d, $J_{4,3} = 3.1$ Hz, H-4), 3.25-3.69 (10H, m, H-1a^{Cer}, H-1b^{Cer}, H-2^{Cer}, H-3^{Cer}, H-4^{Cer}, H-2, H-3, H-5, H-6a, H-6b), 3.00 (2H, t, $J_{1a,2a} = J_{1a,2b} = 6.4$ Hz, OCH₂C₃H₆CH₂NHCO), 2.00-2.06 (2H, m, OCH₂C₃H₆CH₂NHCO), 1.32-1.50 (m, 8H, CH₂), 1.20-1.24 (68H, m, CH₂), 0.68 (6H, t, $J = 6.5$ Hz, CH₃); ¹³C NMR (75 MHz, CDCl₃): 173.2 (C=O), 138.8, 125.1, 120.4 (C_{Ar}), 98.1 (C-1), 77.3, 75.2, 72.9, 70.5, 69.8, 69.2 (C-2, C-3, C-4, C-5, C-4^{Cer}, C-3^{Cer}), 71.1 (OCH₂C₃H₆CH₂NHCO), 63.1 (C-1^{Cer}), 60.4 (C-6), 49.9 (C-2^{Cer}), 42.0 (COCH₂Ar), 39.5 (OCH₂C₃H₆CH₂NHCO), 31.9-21.1 (CH₂), 14.1 (CH^{Cer}₃); HRMS calcd for C₆₃H₁₁₅N₃O₁₃ [M+Na]⁺: 1144.8328, found 1144.8326.

Mice

All mice were maintained in Dana Farber Animal Facility according to IACUC guidelines. C57BL/6 WT were obtained from Jackson Laboratories. C57BL/6 V 14 Tg mice (iNK T TcR Tg) created by A. Bendelac (University of Chicago, Chicago) were provided by Mark Exley (Beth Israel Hospital, Boston). C57BL/6.SJL congenic B1-8^{hi} B cell receptor knock-in mice, previously created by insertion of a high-affinity NP-specific BcR transgene into the BCR coding region to maintain class switch components, were provided by M. Nussenzweig (Rockefeller University, New York). Mice were immunized as noted with 200 μ l intraperitoneally (IP) in PBS/0.1% BSA. Serum was collected by retro-orbital bleed and stored at -20°C until evaluation.

Murine Serum Antibody ELISA

NP-conjugated antigen challenge induces a heteroclitic response, where the resulting antibodies have higher affinity for NIP, so antibody is detected with an NIP-specific ELISA. Plates (Immulon 2 HB) were coated with 1 mg NIP (5)-OVAL in PBS and blocked with 10% soy milk/0.05% Tween/PBS. Serum was serially diluted in 0.1% soy milk/PBS and antibody detected with HRP-labeled goat anti-mouse IgG, IgM (Southern Biotech) developed with ABTS (Sigma A-1888). Concentration was extrapolated from IgG anti-NP (clone Pevch 1) or IgM anti-NP (clone J558) (provided by A. Ferguson, Boston University School of Medicine, Boston) standard curves.

In vitro proliferation assay

B and T cells were purified by pan-B or pan-T MACS bead separation (Milteny-Biotec) according to the manufacturer's instructions. iNK T TcR Tg total splenic T cells were approx 40% iNK T cells (data not shown). Purified B and T cells were mixed at 1:1 ratio (1 $\times$ 10⁵ cells per well each) and labeled with 0.5 μ M CFSE (Sigma 21888) for 9 min in PBS, then quenched with FCS and washed extensively before culture. Proliferation was assessed by FACs as CFSE dilution on day 3. Murine-specific antibodies were anti-CD19 PerCP-Cy5.5 (1D3), anti-Thy1.2 APC(53-2.1), NA/LE anti-CD3 (145-2C11), and isotype controls (all BD Biosciences PharMingen). Cells were preblocked with unlabeled anti-Fc γ RIII, II (clone 2.4G2).

RESULTS AND DISCUSSIONS

Compound **4** was synthesized as reported *via* a pseudo-glycosylation reaction of compound **6** with a suitably protected β -GalCer derivative **5** (Scheme 1) (13). In this route, the six-carbon linker was first attached to the hapten (*p*-nitrophenol) before being added onto the sugar residue. While this remains an effective strategy for synthesizing derivatives of β -GalCer, we have observed that glycosylation reactions at this slightly hindered C-2 position can sometimes be tricky and dependent upon various factors, such as the reactivity of the donor; thus offering limited scope for hapten diversity. Therefore, we reason that the introduction of a linker into the sugar residue *via* a reaction other than glycosylation is worth

exploring. Also, in the interest of adaptability, the linker should facilitate the introduction of different haptens or other molecules *via* simple and diverse reactions.

Retrosynthetic analysis (Scheme 2) indicated that target compound **3** can be obtained by acylation of compound **7**, which in turn can be accessed from the coupling of sugar donor **8** with the sphingosine derivative **9**. This route is quite efficient as it entails the introduction of the linker at C-2 prior to glycosylation *via* an alkylation reaction, thereby eliminating the complications discussed above. It is noteworthy that compound **7** bears amine functionalities on both the sugar and the sphingosine base moieties. Judicious orthogonal protection of the two amino groups is therefore required as they are to be acylated with different carboxylic acids at distinct stages in the course of the synthetic route. Since the sugar moiety is to be subjected to a wider range of chemical reactions, we chose to protect the amino group as the corresponding azide. The latter is known to be very stable and compliant to diverse reaction conditions. As for the amino group on the sphingosine moiety, we opted for the acid sensitive BOC protecting group, which is also compatible with the benzoate protecting group. To ensure α -selectivity in the crucial glycosylation step, we relied on the directing effect of the bulky 4, 6-*O*-di-*tert*-butylsilylene (DTBS) group on the sugar moiety (**17**).

The phytosphingosine acceptor **9** (**18**) was synthesized from (2*S*, 3*S*, 4*R*)-2-amino-1, 3, 4-octadecanetriol using standard procedures summarized in Scheme 3. The spectroscopic data of compound **9** matched those previously reported (**18**). Regarding the synthesis of **10**, an appropriately protected galactosyl derivative had to be carefully chosen at the outset. Importantly, the protecting groups should withstand the basic reaction conditions for the formation of an alkoxide at C-2. In galactose, the *cis*-orientation of the hydroxyl groups at C-3 and C-4 facilitate their simultaneous protection as the acetonide, while the primary hydroxyl group at C-6 can be selectively blocked using diphenyltertiarybutylsilyl chloride, thereby leaving C-2 free for modification.

The crucial intermediate **12** was thus synthesized from β -D-galactopyranose pentaacetate according to published report (**19**). The alkoxide of **12**, which was generated *in situ* by treatment with NaH in DMF, was then reacted with commercially available 1, 5 dibromopentane to afford the bromide. The amine functional group was then introduced into the molecule by an S_N2 displacement of the bromide with sodium azide in DMSO. With the linker in place at C-2, we then proceeded to the preparation of the glycosyl donor **8**. The protecting groups were sequentially removed to give **10** in quantitative yields. Finally, introduction of the α -directing bulky DTBS group at C-4 and C-6, followed by benzylation at C-3 gave compound **8** as colorless syrup in 96% yield after purification.

With both the donor **8** and the acceptor **9** in hand, we next turned our attention to the glycosylation reaction. Because of the possible cleavage of the acid sensitive BOC group on the acceptor, we preferred avoiding the usual method of activation of the thioglycoside with NIS/TfOH. When using Crich's (**20**) relatively new method of activation with the commercially available *p*-nitrobenzenesulfonyl chloride (*p*-NO₂PhSCl) in conjunction with silver triflate in anhydrous CH₂Cl₂ at -78 °C over 2 h, we were pleased to observe that the desired glycosylated product **14** was obtained as the α -anomer exclusively in 75% yield after purification by flash chromatography. The formation of the α -linkage was confirmed by the H-1 and C-1 signals in ¹H and ¹³C NMR respectively.

Stepwise deprotection of compound **14** was then carried out. To avoid any probable base-induced migration of the benzoate to the free amine, we chose to remove the BOC protecting group on the aglycon last. Hence, treatment with TBAF and Zemplén's deprotection of the benzoate protecting groups produced the intermediate **15** as a thick syrup in quantitative yields. The BOC group was subsequently cleaved with TFA and the resulting

free amine was acylated with the fully saturated fatty acid, hexacosanoic acid. This was achieved by the reaction of the corresponding acid chloride with the free amine in a 1:1 mixture of THF and saturated sodium acetate solution. The azide intermediate **16** was obtained as a white solid after concentration of the organic phase and purification of the residue by flash chromatography. Ultimately, hydrogenation of the azide in methanol provided the desired scaffold **2**. Conjugation of the amine with the NP hapten was then accomplished by acylation with its corresponding activated carboxylic acid group. Compound **3** was obtained after stirring freshly prepared acid chloride of the (3-hydroxy-4-nitrophenyl) acetic acid with **2** in a 1:1 mixture of THF and saturated sodium acetate solution for 12 h (Scheme 6).

Following successful synthesis, the NP-derivatives (**3** and **4**) were tested *in vivo* and *in vitro* for recognition by *α*NKT and B cells. Mice expressing an increased frequency of B cells with an NP-specific B cell receptor (B1-8 BcR Tg) were found to develop high titers of both IgM and IgG anti-NP antibodies in their serum (Figure 3) when immunized with **4**, but not

GalCer or PBS/0.1% BSA. Because T cell help is required for class switch to IgG, the presence of NP-specific IgG antibodies following immunization with **4** suggests T cell help is being provided, most likely by *α*NKT cells recognizing the α -GalCer component of the derivative (Figure 1). In depth investigations of this immunologic cooperation have been published elsewhere (13). Furthermore, the requirement of *α*NKT activation and response for both the specific antibody production and class-switch was demonstrated by Batista *et al.* (21) in their *in vivo* studies using α -GalCer conjugated with the antigen CGG (chicken gamma globulin). Therefore it can be inferred that *α*NKT cells helps antilip antibody production.

The biological function of **3** and **4** haptenated with NP (3-hydroxy-4-nitrophenyl) using the two different approaches described above were then compared *in vitro*. To compare functional recognition of **3** and **4** by *α*NKT cells and B cells, isolated cell populations were mixed, labeled with CFSE, incubated *in vitro*, then assessed for proliferation after three days of culture. Proliferation in response to control stimuli (Figure 4, lower panel) confirm the specificity of the assay, since only B cells respond to the B cell stimulus, LPS (*Salmonella enterica*, Sigma); *α*NKT cells respond more strongly to the T cell stimulus, anti-CD3; and neither cell type responds to the media alone. *α*NKT cells respond to α -GalCer, and while there is no selective advantage for NP-specific B cells, there are likely some B cells specific for α -GalCer in any population which contribute to the low level of proliferation observed in the α -GalCer stimulated group. Our data show that proliferative responses by *α*NKT cells and B cells were predominantly comparable following stimulation with **3** and **4** despite their slight structural differences. (Figure 4, upper panel). Importantly, previous *in vitro* experiments (13) showed that NP- α -GalCer **4** stimulated 3.2 μ g/ml anti-NP IgG by day 7, whereas α -GalCer stimulated <0.05 μ g/ml IgG anti NP, clearly indicating that both **3** and **4** induce substantial proliferation of both B and T cells.

CONCLUSION

We have described a simple synthesis of a highly biologically attractive glycolipid template **2**, which can be conjugated to multiple molecules, exemplified by the synthesis of compound **3**. Furthermore, the biological evaluation of the haptenated derivatives of α -GalCer showed that the biologic recognition of **3** and **4** is virtually the same and that they are both important tools in studying the mechanism of *α*NKT cell help for the production of antilipid antibodies.

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Abbreviations

APC	antigen-presenting cell
IgG	immunoglobulin G
IgM	immunoglobulin M
DMF	dimethylformamide
CFSE	carboxyfluorescein succinimidyl ester

LITERATURE CITED

- (1). Bendelac A, Bonneville M, Kearney JF. Autoreactivity by design: innate B and T lymphocytes. *Nat. Rev. Immunol.* 2001; 1:177–186. [PubMed: 11905826]
- (2). Kronenberg M. Toward an understanding of NKT cell biology: progress and paradoxes. *Annu. Rev. Immunol.* 2005; 23:877–900.
- (3). Brutkiewicz RR. CD1d ligands: the good, the bad, and the ugly. *J. Immunol.* 2006; 177:769–775. [PubMed: 16818729]
- (4). Bendelac A, Rivera MN, Park SH, Roark JH. Mouse CD1-specific NK1 T cells: development, specificity, and function. *Annu. Rev. Immunol.* 1997; 15:535, 562. [PubMed: 9143699]
- (5). Hong S, Scherer DC, Mendiratta SK, Serizawa I, Koezuka Y, Van Kaer L. Lipid antigen presentation in the immune system: lessons learned from CD1d knockout mice. *Immunol. Rev.* 1999; 169:31–44. [PubMed: 10450506]
- (6). Kawano T, Cui J, Koezuka Y, Taura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H, Taniguchi M. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science.* 1997; 278:1626–1629. [PubMed: 9374463]
- (7). (a) Crowe NY, Uldrich AP, Kyparissoudis K, Hammond KJL, Hayakawa Y, Sidobre S, Keating R, Kronenberg M, Smyth MJ, Godfrey DI. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J. Immunol.* 2003; 171:4020–4027. [PubMed: 14530322] (b) Burdin N, Brossay L, Kronenberg M. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur. J. Immunol.* 1999; 29:2014–2025. [PubMed: 10382765] (c) Carnaud C, Lee D, Donnars O, Park SH, Beavis A, Koezuka Y, Bendelac A. Cutting edge: Cross-talk between cells of the innate immune system: NKT cells rapidly activate NK cells. *J. Immunol.* 1999; 163:4647–4650. [PubMed: 10528160]
- (8). (a) Taniguchi M, Harada M, Kojo S, Nakayama T, Wakao H. The regulatory role of Valpha14 NKT cells in innate and acquired immune response. *Annu. Rev. Immunol.* 2003; 21:483–513. (b) Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L. NKT cells: what's in a name? *Nat. Rev. Immunol.* 2004; 4:231–237. [PubMed: 15039760] (c) Gonzalez-Aseguinolaza G, Van Kaer L, Bergmann CC, Wilson JM, Schmiege J, Kronenberg M, Nakayama T, Taniguchi M, Koezuka Y, Tsuji M. Natural killer T cell ligand alpha-galactosylceramide enhances protective immunity induced by malaria vaccines. *J. Exp. Med.* 2002; 195:617–624. [PubMed: 11877484]
- (9). Miyamoto K, Miyake S, Yamamura T. A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells. *Nature.* 2001; 413:531–534. [PubMed: 11586362]

- (10). Chiba A, Oki S, Miyamoto K, Hashimoto H, Yamamura T, Miyake S. Suppression of collagen-induced arthritis by natural killer T cell activation with OCH, a sphingosine-truncated analog of alpha-galactosylceramide. *Arthritis Rheum.* 2004; 50:305–313. [PubMed: 14730629]
- (11). Naik RS, Branch OLH, Woods AS, Vijaykumar M, Perkins DJ, Nahlen BL, Lal AA, Cotter RJ, Costello CE, Ockenhouse CF, Davidson EA, Gowda DC. Glycosylphosphatidylinositol anchors of *Plasmodium falciparum*: molecular characterization and naturally elicited antibody response that may provide immunity to malaria pathogenesis. *J Exp Med.* 2000; 192:1563–1575. [PubMed: 11104799]
- (12). Gourley IS, McMillan SA, Bell AL. Clinical features associated with a positive anticardiolipin antibody in Irish patients with systemic lupus erythematosus. *Clin Rheumatol.* 1996; 15:457–460. [PubMed: 8894358]
- (13). Leadbetter EA, Brigl M, Illarionov P, Cohen N, Luteran MC, Pillai S, Besra GS, Brenner MB. NK T cells provide lipid antigen-specific cognate help for B cells. *PNAS.* 2008; 105:8339–8344. [PubMed: 18550809]
- (14). Boscardin SB, Hafalla JC, Masilamani RF, Kamphorst AO, Zebroski HA, Rai U, Morrot A, Zavala F, Steinman RM, Nussenzweig RS, Nussenzweig MC. Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *J Exp Med.* 2006; 203:599–606. [PubMed: 16505139]
- (15). Wolniak KL, Noelle RJ, Waldschmidt TJ. Characterization of (4-hydroxy-3-nitrophenyl) acetyl (NP)-specific germinal center B cells and antigen-binding B220-cells after primary NP challenge in mice. *J. Immunol.* 2006; 177:2072–2079. [PubMed: 16887965]
- (16). Furukawa K, Shimizu T, Murakami A, Kono R, Nakagawa M, Sagawa T, Yamato I, Azuma T. Strategy for affinity maturation of an antibody with high evolvability to (4-hydroxy-3-nitrophenyl) acetyl hapten. *Mol. Immunol.* 2007; 44:2436–2445. [PubMed: 17118452]
- (17). Kimura A, Imamura A, Ando H, Ishida H, Kiso M. A novel synthetic route to -Galactosyl Ceramides and iGb3 using DTBS-directed -selective galactosylation. *Synlett.* 2006; 15:2379–2382.
- (18). Fujio M, Wu D, Garcia-Navarro R, Ho DD, Tsuji M, Wong CH. Structure-based discovery of glycolipids for CD1d-mediated NKT cell activation: tuning the adjuvant versus immunosuppression activity. *J. Am. Chem. Soc.* 2006; 128:9022–9023. [PubMed: 16834361]
- (19). Broddefalk J, Bergquist KE, Kihlberg J. Use of acid-labile protective groups for carbohydrate moieties in synthesis of glycopeptides related to type II collagen. *Tetrahedron.* 1998; 54:12047–12070.
- (20). Crich D, Cai F, Yang F. A stable, commercially available sulfonyl chloride for the activation of thioglycosides in conjunction with silver trifluoromethanesulfonate. *Carbohydr. Res.* 2008; 343:1858–1862. [PubMed: 18374318]
- (21). Barral P, Eckl-Dorna J, Harwood NE, De Santo C, Salio M, Illarionov P, Besra GS, Cerundolo V, Batista FD. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help *in vivo*. *PNAS.* 2008; 105:8345–8350. [PubMed: 18550831]

Figure 1. *i*NKT cells provide cognate help for lipid-specific B cells.

Figure 2. CD1d agonist KRN7000 and analogues.

Figure 3. NP-haptenated derivative (4) of GalCer, but not GalCer, stimulates *in vivo* production of NP-specific IgM (left panel) and IgG (right panel) by B1-8 BcR Tg mice. (n = 2-4 mice/group, representative example from more than 2 experiments).

Figure 4. Alternatively synthesized NP-haptenated derivatives 3 and 4 stimulate similar levels of iNKT and B cell proliferation.

Mixtures of T cells from V 14 Tg mouse and B cells from B1-8 BcR Tg mouse were stimulated with 10-0.01ng/ml NP- GalCer (top panel), or media, 1μg/mlLPS, 10μg/ml anti-CD3, 10ng/ml GalCer as controls(bottom panel). Proliferation is measured as % B and T cells diluting CFSE. Statistics performed by Prism software ANOVA analysis (*=p<0.05 for 2 vs 17). Each bar represents the mean of pools of triplicate wells from two experiments.

Scheme 1.
Synthesis of compound **4**.

Scheme 2.
Retrosynthetic analysis of target compound 3.

Scheme 3.

(a) $\text{C}_4\text{H}_9\text{Si}(\text{C}_6\text{H}_5)_2\text{Cl}$, Pyridine, quant; (b) $(\text{BOC})_2\text{O}$, CH_3CN , Et_3N , 72%; (c) BzCl , Pyridine, 84%; (d) TBAF, THF, 82%.

Scheme 4.

(a) DMF, NaH, $(\text{CH}_2)_5\text{Br}_2$, quant; (b) NaN, DMSO, 60°C , 75%; (c) TBAF, THF, quant; (d) TFA, CH_2Cl_2 , 78%; (e) $\text{Si}(\text{OTf})_2(\text{C}_4\text{H}_9)_2$, DMF, Et_3N , quant; (f) BzCl , Pyridine, 96%.

Scheme 5.

(a) *p*-NO₂PhSCl, AgOTf, CH₂Cl₂, -78 °C, 75%; (b) TBAF, THF, quant; (c) NaOMe/MeOH, 88%; (d) TFA, CH₂Cl₂, quant; (e) C₂₅H₅₁COCl, THF:NaOAc(1:1), 71%; (f) H₂, Pd, MeOH, 90%.

Scheme 6.

(a) THF:NaOAc (1:1), 71%.