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Synthesis and evaluation of human T cell stimulating activity of an α-sulfatide analogue

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Abstract—A concise synthesis of α-sulfatide **1**, an analogue of natural glycolipid antigens with potential anti-tumor activity, was performed. Two different approaches to the α-glycosidic bond were explored, resulting in a high yield and excellent stereoselectivity. Compound **1** combines the structural features of sulfated β-GalCer (sulfatide) and α-GalCer, which activate specific T cells. α-Sulfatide **1** was stimulatory for CD1d-restricted semi-invariant Natural Killer T (iNKT) cell clones, although less potent than α-GalCer, while it was not recognized by CD1a-restricted sulfatide-specific T cells.

1. Introduction

CD1 proteins constitute a family of highly conserved antigen presenting molecules (APM) which bind and present lipid and glycolipid molecules to T lymphocytes.[1] Diverse immunogenic lipids and glycolipids have been isolated and characterized from mammalian and bacterial sources.[2]

Many evidences confirm the immunological relevance of CD1-restricted T cells in bacterial infections, tumor immunosurveillance, and autoimmune response.[3] In particular T cells specific for self-glycosphingolipids are increased in the blood of individuals with multiple sclerosis; moreover, synthesis and presentation of self-glycosphingolipids is enhanced by bacterial infections.[4]

A highly stimulatory glycolipid is agelasphin, initially isolated from the marine sponge *Agelas mauritiana*.[5] Agelasphin is composed of a galactose bound to ceramide with an α-anomeric bond (α-GalCer) and when presented by CD1d it stimulates very efficiently T cells expressing a semi-invariant T cell receptor (TCR).[6] These T cells also express surface markers common to Natural Killer cells and therefore are indicated as invariant NKT (iNKT) cells.

The synthetic α-GalCer analogue KRN7000 is highly immunostimulatory for iNKT cells and has strong anti-tumor activities.[7] Its potential in the treatment of several diseases such as tumors, infectious diseases like malaria and hepatitis B, and autoimmune diseases like diabetes is currently being investigated.[8,9]

Upon activation by α-GalCer, iNKT cells release proinflammatory (Th1) and immunomodulatory (Th2) cytokines and participate in immunoregulation, in anti-tumor immune response, and in autoimmune diseases.[10] Many α-GalCer analogues have been designed and prepared with the aim to determine structure–activity relationship and to enhance bioactivity; in this context, sphingosine 3-OH and galactose 2-OH have been found essential for bioactivity,[7,11–13] whereas the length and structure of the lipid chains might influence the type of released cytokines.[14–16]

Keywords: Galactosylceramides; Glycosylation; NKT cell activation; Immunostimulation.
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Sulfatide, a β-D-galactosylceramide sulfated at position 3 of galactose, is a potent self-glycolipid antigen produced by mammalian cells.[17] Sulfatide is abundant in myelin and in serum and is synthesized in many different cell types. Moreover, it is highly immunogenic, as it stimulates both specific T and B cells. Sulfatide is the only known promiscuous ligand binding to all human CD1 proteins and activates specific T cells.[17] Like α-GalCer-reacting iNKT cells, also sulfatide-specific T cells react in different manner to sulfatides with variations in alkyl chain length.[18,19]

The mode of sulfatide binding to CD1 molecules has been elucidated by 3D structures of CD1a[20] and CD1d[19] in complex with sulfatide: two alkyl chains are inserted into hydrophobic pockets of CD1 projecting the 3-*O*-sulfogalactose moiety out of the binding groove on the CD1 surface which contacts the T cell receptor. Structural determinations of CD1d-sulfatide[19] and CD1d-α-GalCer[21] complexes allow to compare the binding mode of the two ligands on CD1d: the galactose moiety of sulfatide, due to β-anomeric linkage, is more exposed and projects out of the ligand binding groove compared to the α-linked galactose of α-GalCer.

With the aim to find more efficient bioactive compounds, the preparation of analogues of the natural glycolipid antigens, combining the structural features of sulfated β-GalCer (sulfatide) and α-GalCer, was planned. From a chemical point of view sulfatide and α-GalCer differ in several respects: in the anomeric linkage, in the presence of a E configured double bond between C4 and C5, instead of a hydroxyl at C4 of the sphingoid base (sphingosine in sulfatide versus phytosphingosine in α-GalCer), and the presence of sulfate at position 3 of galactose.

Considering the structural differences between sulfatide and α-GalCer, compound **1** (Fig. 1) containing 3-*O*-sulfogalactose α-linked to a sphingosine-derived ceramide is a hybrid glycolipid analogue, whose immunostimulatory activity was studied in the context of CD1a and CD1d mediated T cell activation.

Biological tests on this analogue should allow to evaluate the influence of these structural modifications on its immunostimulatory potential.

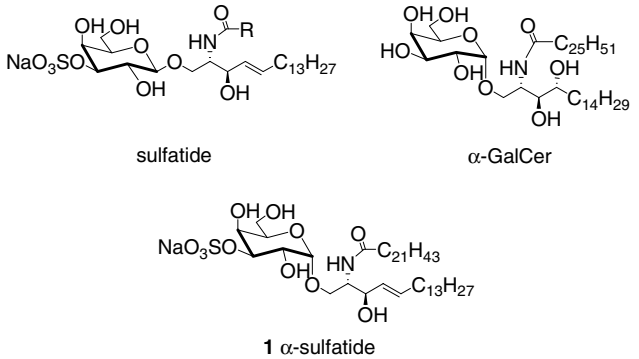


Figure 1. Structures of sulfatide, α-galactosylceramide, and α-sulfatide **1**.

In this paper, the synthesis of **1** starting from 3-*O*-benzoyl-D-*erythro* azidosphingosine[22] as well as the biological tests on this analogue are described.

2. Results and discussion

2.1. Chemistry

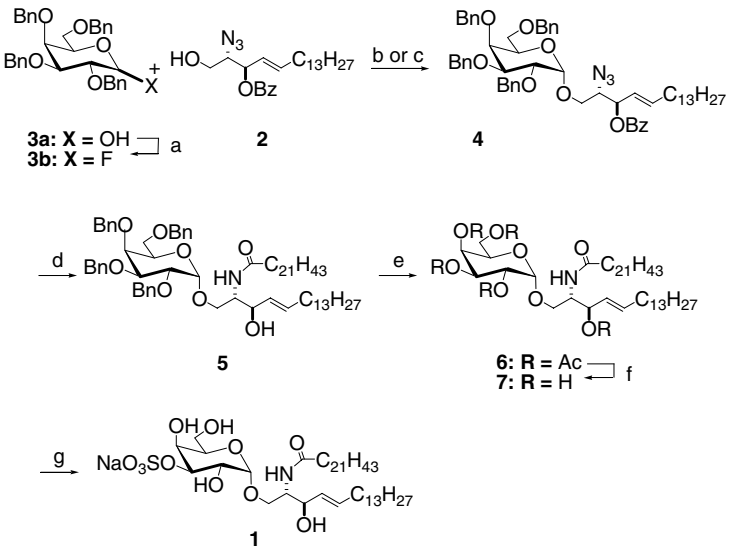
The synthetic strategy for the preparation of α-sulfatide **1** involves the assembly of a galactose unit and a protected azido precursor of sphingosine, followed by azide reduction, N-acylation with fatty acid, and final selective sulfation of 3-OH of galactose.

We successfully experienced (2*S*,3*R*,4*E*)-3-*O*-benzoyl azidosphingosine **2** as glycosyl acceptor for the preparation of β-galactosylceramides[22,23] and a very efficient protocol for the preparation of this synthon starting from D-glyceraldehyde was reported by us.[22]

So compound **2** was chosen as the lipid acceptor for the crucial glycosylation leading to α-sulfatide; due to the experience acquired in Mukaiyama glycosylation,[11–13] which has been applied for the preparation of different 2-*O*-analogues of α-GalCer, we first focused our attention on this procedure. The Mukaiyama glycosylation reaction involves a glycosyl fluoride as the glycosyl donor, and $AgClO_4$ and $SnCl_2$ as a Lewis acid catalytic system. Therefore, 2,3,4,6-tetra-*O*-benzyl-galactopyranosyl fluoride (**3b**) was prepared by reaction of tetrabenzylgalactose (**3a**) (Scheme 1) with DAST (Et_2NSF_3), a well-known reagent for mild and direct transformation of glycosyl hemiacetals into glycosyl fluorides.[24] The glycosyl fluoride was obtained in 80% yield. Following the protocol for the Mukaiyama glycosylation, the azidosphingosine acceptor **2** and the glycosyl fluoride donor (3 mol equiv) dissolved in dry THF were added to a flask containing the Lewis acid system, previously prepared suspending $AgClO_4$ (3 mol equiv) and $SnCl_4$ (3 mol equiv) in dry Et_2O. The mixture was allowed to react for 12 h at room temperature; after work-up, the isolation of the reaction product was performed by preparative HPLC. The α-glycoside **4** was obtained with complete α stereoselectivity, but only in a moderate 35% yield. In addition, unlike that in previous work, we were not able to recover any unreacted acceptor **2**.

With the aim to gain better yields, the Lemieux halide-ion catalytic α-glycosylation as modified by Kobayashi was explored, which potentially provides a straightforward and simple access to 1,2-*cis* glycosides in high yields and α-selectivity.[25,26] According to this procedure, the glycosyl bromide donor is generated in situ through the use of Appel agents (Ph_3P and CBr_4), and can then perform a halide-ion catalytic α-glycosylation with a large array of different acceptor alcohols, affording α-glycosides almost quantitatively.[25]

We applied this protocol to azidosphingosine acceptor **2**, conducting glycosylation as follows (Scheme 1): tetrabenzylgalactose **3a** was treated with Ph_3P (3 mol equiv) and CBr_4 (3 mol equiv) for 3 h at room tempera-



Scheme 1. Reagents and conditions: (a) **3a**, DAST, THF, rt, 20 min, 80%; (b) **2** and **3b**, $SnCl_2/AgClO_4$, Et_2O, −15 °C, 12 h, 35%; (c) **3a**, Ph_3P, CBr_4, then Bu_4NBr, **2** (CH_2Cl_2 solution), *N*,*N*-tetramethylurea, 4 Å ms, CH_2Cl_2, rt, 5 days, 92%; (d) (i) MeONa, MeOH, rt, 2 h; (ii) H_2S, pyridine, water, rt, 48 h; (iii) $C_{21}H_{43}COCl$, 50% aq AcONa, THF, rt, 3 h, 55% (three steps); (e) (i) Na, liquid ammonia, **5** (THF solution), −50 °C, 2 h; (ii) Ac_2O, Py, rt, 20 h, 78% (two steps); (f) MeONa, MeOH, 3 h, (quant); (g) Bu_2SnO, MeOH, reflux, 2 h; $Me_3N{\cdot}SO_3$, THF, rt, 2 h, 70%.

ture to produce the anomeric bromide, which was one-pot reacted with **2** (2 mol equiv) in the presence of tetrabutylammonium bromide (3 mol equiv), *N*,*N*-tetramethylurea, and molecular sieves. The reaction proceeded smoothly and the bromide donor (as monitored by TLC) was completely consumed after five days. In spite of an excess amount of Ph_3P in the reaction system, no azide reduction was observed. This result indicates that this α-glycosylation procedure can be successfully applied to 3-*O*-benzoylazidosphingosine **2** further than the model acceptor 6-azidohexanol,[24] so as to have access to α-linked glycosphingolipids.

The stereoselectivity of the glycosylation reaction (α/β ratio: 95:5) was established by integration of the H-1 doublets for the α-anomer (δ 4.87, $J = 3.5$ Hz) and for the β-anomer (δ 4.37, $J = 7.7$ Hz) in the ^{1}H NMR spectrum of the crude mixture. The α-glycoside **4** was recovered pure in 92% yield (based on **3a**) after work-up and purification by silica gel column chromatography; in addition, practically all the excess acceptor **2** was recovered from the reaction mixture.

Removal of benzyl ethers, benzoyl ester and azide reduction could have been performed simultaneously by treatment with sodium and liquid ammonia without affecting the olefinic moiety of sphingosine; but when glycoside **4** was subjected to these conditions a complex mixture of products was formed, from which it was not possible to isolate the deprotected amine.

Therefore, the procedure was conducted stepwise. To avoid acyl shift on amino group during azide reduction benzoyl ester was first removed by Zemplén transesterification. After that, azide reduction and N-acylation with docosanoyl chloride were carried out as previously described[18] to give α-GalCer **5** in 55% yield over three steps.

With amide **5** in hand, removal of benzyl protecting groups was easily achieved with sodium in liquid ammonia at −50 °C; the reaction was complete within 2 h and the crude was acetylated to facilitate chromatographic purification; the fully acetylated derivative **6** was obtained in satisfactory 78% yield.

Removal of acetyl groups on compound **6** afforded α-GalCer **7** and finally the sulfate was introduced at position 3 of galactose according to Flitsch et al.[27] to give the target compound **1** in 70% yield. Selective functionalization at position 3 of the 1,2-*cis* glycoside **7** was successfully accomplished through reaction of dibutylstannilene acetal with $SO_3{\cdot}Me_3N$, as indicated by the downfield shift of the H-3′ signal[18,22,23] in the ^{1}H NMR spectrum of α-sulfatide **1**. In this way, tedious manipulations of protecting groups on galactose donor[28] were avoided.

2.2. Biological evaluation

The biological activity of α-sulfatide **1** was tested by using CD1-restricted T cell antigen presentation assays in comparison with synthetic sulfatide or α-GalCer. We used different types of human T cells. The clone K34B9.1, which was obtained by stimulation with naturally occurring β-sulfatide,[3] is restricted by CD1a, and recognizes also synthetic analogues of β-sulfatide like a C-analogue[29] and a 4-*O*-sulfated analogue.[23] The iNKT cell clones BGA01 and BGA89 which recognize α-GalCer presented by CD1d, were recently obtained in our laboratories.

α-Sulfatide **1** is unable to stimulate CD1a-restricted and sulfatide-specific T cells when presented by CD1a (Fig. 2), probably due to the alpha configuration of the sulfated galactose moiety. Both interferon gamma (IFNγ) and interleukin-4 (IL-4) cytokines were not released after stimulation with this compound, whereas

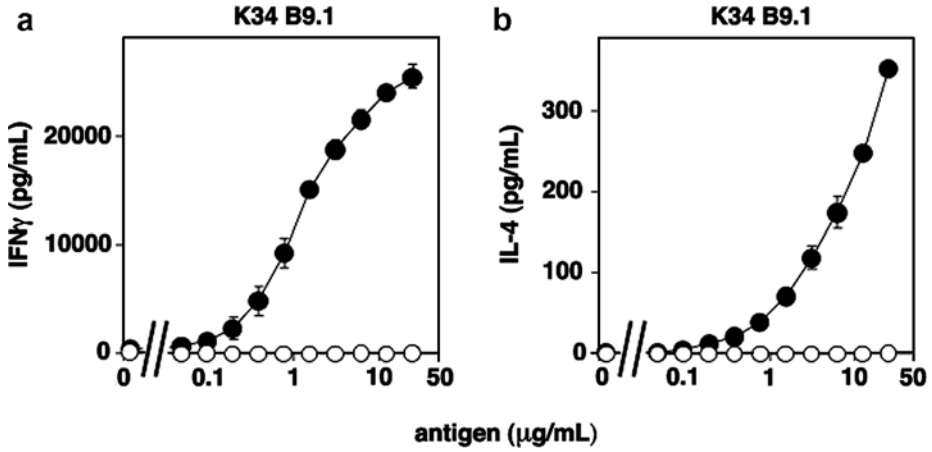


Figure 2. C1R cells expressing human CD1a were preincubated with *cis*-tetracosenoyl ($C_{24:1}$) sulfatide[18] (●) or with α-sulfatide **1** (○) at the doses indicated, before addition of the sulfatide-specific CD1a-restricted T cell clone K34B9.1. Released IFN-γ (a) and IL-4 (b) were measured by ELISA. Data are expressed as mean pg/ml ± SD of duplicates.

synthetic[18] but naturally occurring β-sulfatide was very active. Hence the β configuration is an important requisite of sulfatide to become recognized by CD1a-restricted T cells.

α-Sulfatide **1** is instead stimulatory for CD1d-restricted iNKT cell clones, although it is less potent than α-GalCer (Fig. 3). Using CD1d-restricted iNKT cell clones, a dose–response shift was observed. α-Sulfatide **1** induced the same half-maximal stimulation at doses about 1000 times larger than α-GalCer. This was observed with two iNKT cell clones, and was detected by measuring both IFNγ and IL-4 cytokines. These findings indicate that α-sulfatide **1** has the capacity to bind to CD1d and

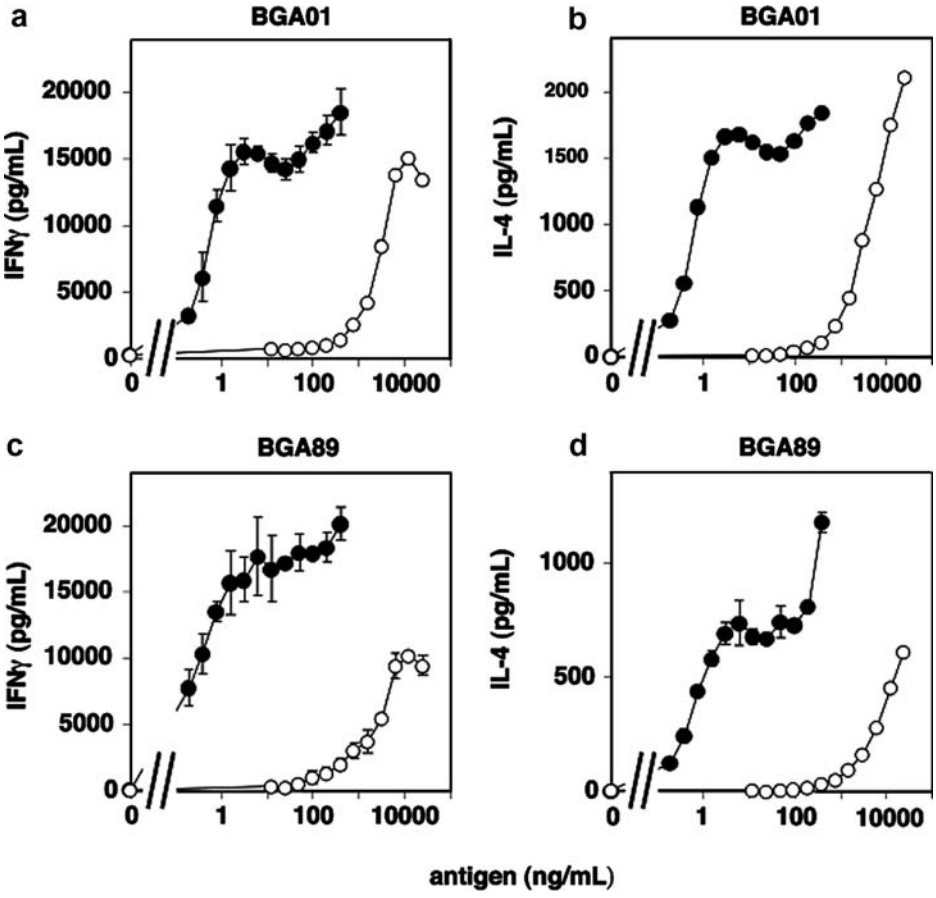


Figure 3. C1R cells expressing human CD1d were preincubated with α-GalCer (●) or with α-sulfatide **1** (○) at the doses indicated, before addition of the CD1d-restricted iNKT cell clone BGA01 (a and b) or BGA89 (c and d). Released IFN-γ (a and c) and IL-4 (b and d) were measured by ELISA. Data are expressed as mean pg/ml ± SD of duplicates.

stimulate iNKT cells. However, the presence of the sulfate group and the absence of phytosphingosine induce a striking reduction of the stimulatory capacity, although for the synthetic 3-*O*-sulfo-α-GalCer analogue[28] the presence of the sulfate itself does not seem to alter NKT cell activation. According to the recent crystal structure of the complex formed by CD1d and α-GalCer[21] the sulfate group most likely hinders the appropriate contact with the TCR. Moreover, an important role of the phytosphingosine in stabilizing CD1d binding or in ligand solubilization cannot be excluded.

3. Conclusions

In summary, this study has allowed to prepare a new interesting hybrid glycolipid analogue through a concise synthesis resulting in satisfactory yield and excellent stereoselectivity.

In the crucial α-galactosylation step performed through the Lemieux halide-ion catalytic procedure as modified by Kobayashi, the desired α-galactosylceramide derivative **4** was obtained in 92% yield.

The immunostimulatory activity of α-sulfatide **1** was studied in the context of CD1a and CD1d mediated T cell activation, indicating that T cells display a very specific recognition of the CD1-antigen complexes, when either CD1a or CD1d are the restriction elements. These findings show the fine discriminatory capacity of the TCR interacting with glycolipid antigens.

4. Experimental

4.1. General methods

Optical rotations were measured with a 241 Perkin-Elmer polarimeter at 20 °C. All NMR spectra were recorded at 303 K with a Bruker FT NMR Avance DRX500 spectrometer in $CDCl_3$ or $CDCl_3/CD_3OD$ solutions with TMS as internal standard; chemical shifts are reported as δ (ppm) relative to $CHCl_3$ fixed at 7.24 ppm for $CDCl_3$ and 7.60 ppm for $CDCl_3/CD_3OD$ solutions for ^1H NMR spectra and relative to $CDCl_3$ fixed at 77.00 ppm for ^{13}C NMR spectra.

MS spectra were recorded in the negative or positive ion-modes on a Thermo Quest Finnigan LCQ™DECA ion trap mass spectrometer; the mass spectrometer was equipped with a Finnigan ESI interface; data were processed by Finnigan Xcalibur software system. All reactions were monitored by TLC on Silica Gel 60 F-254 plates (Merck), spots being developed with 5% sulfuric acid in methanol/water (1:1), or with phosphomolybdate based reagent. Flash column chromatography was performed on Silica Gel 60 (230–400 mesh, Merck). Organic solutions were dried over sodium sulfate. All evaporations were carried out under reduced pressure at 40 °C. Dry solvents and liquid reagents were distilled prior to use: THF was distilled from sodium; dichloromethane and pyridine were distilled from calcium hydride; DMF and methanol were dried on 4 Å molecular sieves. All reagents were purchased from Aldrich. Docosanoyl chloride was prepared from docosanoic acid following a literature procedure.[30] The elemental analyses were consistent with the theoretical ones.

4.1.1. 2,3,4,6-Tetra-*O*-benzyl-D-galactopyranosyl fluoride (3b). Tetrabenzylgalactose **3a** (0.82 g, 1.52 mmol) was dissolved in dry THF (15 mL) and cooled at −30 °C. DAST (0.24 mL, 1.8 mmol) was rapidly added and the cooling bath removed immediately. After stirring for 30 min at room temperature, the mixture was cooled again at −30 °C and quenched with MeOH (2 mL). After evaporation of solvent under reduced pressure, water (200 mL) was added to the residue, that was extracted with CH_2Cl_2 (3× 300 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on SiO_2 (CH_2Cl_2) giving **3b** (0.58 g, 70% yield) as mixture of anomers, α/β about 1:1, colorless oil; ^1H NMR ($CDCl_3$), α anomer: δ 3.54 (d, 2H, J = 6.5 Hz, H_2-6), 3.94 (dd, 1H, $J_{3,2}$ = 10.0 Hz, $J_{3,4}$ = 2.5 Hz, H-3), 4.02 (br d, 1H, $J_{4,3}$ = 2.5 Hz, H-4), 4.03 (ddd, 1H, $J_{2,F}$ = 25.1 Hz, $J_{2,3}$ = 10.0 Hz, $J_{1,2}$ = 2.5 Hz, H-2), 4.10 (br t, 1H, J = 6.5 Hz, H-5), 4.41 (d, 1H, J = 11.9 Hz, CHPh), 4.47 (d, 1H, J = 11.9 Hz, CHPh), 4.57 (d, 1H, J = 11.7 Hz, CHPh), 4.72 (d, 1H, J = 11.8 Hz, CHPh), 4.75 (d, 1H, J = 11.4 Hz, CHPh), 4.81 (d, 1H, J = 11.4 Hz, CHPh), 4.84 (d, 1H, J = 11.8 Hz, CHPh), 4.93 (d, 1H, J = 11.7 Hz, CHPh), 5.59 (1H, dd, $J_{1,F}$ = 53.8 Hz, $J_{1,2}$ = 2.5 Hz, H-1), 7.39–7.24 (m, 20H, Ph); β anomer: δ 3.53 (1H, dd, $J_{3,2}$ = 9.7 Hz, $J_{3,4}$ = 2.3 Hz, H-3), 3.66–3.58 (3H, m, H-5, H_2-6), 3.90 (1H, br d, $J_{4,3}$ = 2.3 Hz, H-4), 3.93 (1H, ddd, $J_{2,F}$ = 13.3, $J_{2,3}$ = 9.7 Hz, $J_{2,1}$ = 7.1 Hz, H-2), 4.40 (d, 1H, J = 11.7 Hz, CHPh), 4.46 (d, 1H, J = 11.7 Hz, CHPh), 4.58 (d, 1H, J = 11.5 Hz, CHPh), 4.69 (d, 1H, J = 12.1 Hz, CHPh), 4.73 (d, 1H, J = 12.1 Hz, CHPh), 4.75 (d, 1H, J = 11.0 Hz, CHPh), 4.83 (d, 1H, J = 11.0 Hz, CHPh), 4.92 (d, 1H, J = 11.5 Hz, CHPh), 5.16 (dd, 1H, $J_{1,F}$ = 53.2 Hz, $J_{1,2}$ = 7.1 Hz, H-1), 7.37–7.23 (m, 20H, Ph). ^{13}C NMR ($CDCl_3$), α anomer: δ 68.2, 71.7, 73.1, 73.4, 73.6, 74.3, 74.8, 75.7 (CH, d, J_{C-F} = 24 Hz, C-2), 78.4, 106.0 (CH, d, J_{C-F} = 232 Hz, C-1), 127.5–128.5, 138.0–137.5; β anomer: δ 68.3, 73.1, 73.2, 73.5, 73.6, 74.6, 75.0, 79.1 (CH, d, J_{C-F} = 25 Hz, C-2), 81.0, 110.1 (CH, d, J_{C-F} = 227 Hz, C-1), 127.5–128.5, 138.0–137.5. ESI-MS (positive ion mode): m/z 565 $[M+Na]^+$.

The ^1H and ^{13}C NMR data of galactopyranosyl fluoride **3b** were in agreement with those reported in the literature by Nicolaou et al.[31] and Graziani et al.[32] who obtained the α- or β-fluoride through different synthetic approaches.

4.1.2. (2*S*,3*R*,4*E*)-2-Azido-3-benzoyloxy-1-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyloxy)-4-octadecene (4). (a) A solution of the azidosphingosine **2** (0.06 g, 0.15 mmol) and the fluoride **3b** (0.24 g, 0.45 mmol) in dry THF (4 mL) was added at −15 °C to a flask containing solid $AgClO_4$ (0.09 g, 0.45 mmol) and solid $SnCl_2$ (0.08 g, 0.45 mmol). After stirring for 12 h at room temperature,

the mixture was filtered through Celite and evaporated. The reaction mixture was diluted with CH_2Cl_2 (30 mL), washed with a saturated $NaHCO_3$ solution (20 mL), dried with Na_2SO_4, and taken to dryness. The residue was chromatographed by HPLC (*n*-hexane/EtOAc 9:1) giving **4** (0.05 g, 35% yield) as a colorless oil.

(b) 2,3,4,6-Tetra-*O*-benzyl-D-galactose **3a** (0.30 g, 0.56 mmol) in dry CH_2Cl_2 (12 mL) was treated with Ph_3P (0.44 g, 1.67 mmol) and CBr_4 (0.55 g, 1.67 mmol) for 3 h at room temperature under argon. Then *N*,*N*-tetramethylurea (0.6 mL), Bu_4NBr (0.54 g, 1.67 mmol), molecular sieves (MS, 4 Å, 0.6 g), and 3-*O*-benzoyl azidosphingosine **2** (0.48 g, 1.11 mmol) in dry CH_2Cl_2 (6 mL) were added and stirred at room temperature for five days; at this time the bromide donor was completely consumed as evidenced by TLC analysis. Triethylamine (3 mL) was added and the mixture was diluted with CH_2Cl_2 (250 mL), filtered on Celite pad, washed with saturated aq $NaHCO_3$ (250 mL) and NaCl solution (250 mL), dried, and concentrated. The residue was purified by flash chromatography (petroleum ether/EtOAc 9:1) affording first compound **4** (0.49 g, 92% yield based on the amount of **3a**) as a colorless oil, then unreacted **2** (0.25 g) as a clear oil.

$[\alpha]_D^{20}$ + 11 ($CHCl_3$, *c* = 0.2); ^{1}H NMR ($CDCl_3$): δ 0.88 (t, 3H, *J* = 6.8 Hz, CH_3), 1.30–1.40 (m, 22H, 11 CH_2), 2.02 (q, 2H, *J* = 7.0 Hz, CH=CHCH_2), 3.55–3.45 (m, 3H, H-1a, H_2-6′), 3.74 (dd, 1H, $J_{1a,1b}$ = 10.9, $J_{1a,2}$ = 4.4 Hz, H-1a), 3.98–3.93 (m, 3H, H-5′, H-4′, H-3′), 4.00 (ddd, 1H, *J* = 7.7, 4.4, 4.4 Hz, H-2), 4.05 (dd, 1H, $J_{2',3'}$ = 9.5 Hz, $J_{2',1'}$ = 3.6 Hz, H-2′), 4.39 (d, 1H, *J* = 11.9 Hz, CHPh), 4.46 (d, 1H, *J* = 11.9 Hz, CHPh), 4.56 (d, 1H, *J* = 11.4 Hz, CHPh), 4.94 (d, 1H, *J* = 11.4 Hz, CHPh), 4.69 (d, 1H, *J* = 12.0 Hz, CHPh), 4.80 (d, 1H, *J* = 12.0 Hz, CHPh), 4.74 (d, 1H, *J* = 11.8 Hz, CHPh), 4.85 (d, 1H, *J* = 11.8 Hz, CHPh), 4.86 (d, 1H, $J_{1',2'}$ = 3.8 Hz, H-1′), 5.53 (ddt, 1H, $J_{4,5}$ = 15.2 Hz, $J_{4,3}$ = 7.9 Hz, $J_{4,6}$ = 1.4 Hz, H-4), 5.60 (dd, 1H, $J_{3,4}$ = 7.9 Hz, $J_{3,2}$ = 4.5 Hz, H-3), 5.88 (dt, 1H, $J_{5,4}$ = 15.2 Hz, $J_{5,6}$ = 6.8 Hz, H-5), 7.40–7.20 (m, 20 H, Ph), 7.45 (br t, 2H, J = 7.8 Hz, Ph), 7.57 (br t, 2H, J = 7.5 Hz, Ph), 8.06 (br d, 2H, J = 8.0 Hz, Ph). ^{13}C NMR ($CDCl_3$): δ 14.1, 22.7, 28.7, 29.2–29.7 (7C), 31.9, 32.3, 64.0, 67.9, 69.0, 69.9, 73.2, 73.3, 73.4, 74.7, 74.9, 75.1, 76.5, 78.6, 98.9, 122.9, 127.4–128.3 (Ph), 128.4 (Ph) 129.8 (Ph), 130.0 (Ph), 133.2, 137.9–138.8 (Ph), 138.6, 165.2. ESI-MS (positive-ion mode): *m*/*z* 974.5 $[M+Na]^+$.

4.1.3. (2*S*,3*R*,4*E*)-1-(2,3,4,6-Tetra-*O*-benzyl-α-D-galactopyranosyloxy)-2-(docosanoylamino)-3-hydroxy-4-octadecene (5). To a stirred solution of compound **4** (0.38 g, 0.40 mmol) in dry CH_2Cl_2 (10 mL) sodium methoxide in dry methanol (0.05 M solution, 4.8 mL) was added and the solution was stirred at room temperature for 2 h. The solution was neutralized with an ion exchange resin (Dowex 50 × 8, H^+ form), filtered, and concentrated. The crude was dissolved in pyridine/water (1:1, 12 mL). Hydrogen sulfide was bubbled into the solution for 20 min and the solution was stirred at room temperature for 48 h. The reaction mixture was concentrated and subjected to azeotropic distillation with toluene. The crude amine was dissolved in THF (20 mL) and treated with aqueous sodium acetate (50%, 18 mL) and freshly prepared docosanoyl chloride (0.16 g, 0.44 mmol) under vigorous stirring at room temperature for 4 h. The aqueous phase was extracted with THF (2× 40 mL). The combined organic layers were dried and concentrated. The residue was purified by flash chromatography (*n*-hexane/EtOAc 8:2 containing 0.1% Et_3N) affording compound **5** (0.25 g, 55% yield from **4**) as a foam.

$[\alpha]_D^{20}$ + 24.8 (*c* 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 6H, *J* = 7.5 Hz, 2 CH_3), 1.20–1.40 (m, 58H, 29 CH_2), 1.57–1.65 (m, 2H, $COCH_2CH_2$), 2.02 (m, 2H, CH=CHCH_2), 2.15 (m, 2H, $COCH_2$), 3.48–3.57 (m, 2H, H_2-6′), 3.72 (dd, 1H, $J_{1a,1b}$ = 10.5 Hz, $J_{1a,2}$ = 4.0 Hz, H-1a), 3.83–3.92 (m, 3H, H-1b, H-3′, H-5′), 3.98–4.04 (m, 2H, H-4′, H-2), 4.06 (dd, 1H, $J_{2',3'}$ = 10.0 Hz, $J_{2',1'}$ = 3.5 Hz, H-2′), 4.16 (m, 1H, H-3), 4.40 (d, 1H, *J* = 12.0 Hz, CHPh), 4.50 (d, 1H, *J* = 12.0 Hz, CHPh), 4.58 (d, 1H, *J* = 11.5 Hz, CHPh), 4.73 (d, 1H, *J* = 11.5 Hz, CHPh), 4.75–4.82 (m, 2H, CHPh), 4.77 (d, 1H, $J_{1',2'}$ = 3.5 Hz, H-1′), 4.88 (d, 1H, *J* = 12.0 Hz, CHPh), 4.94 (d, 1H, *J* = 11.5 Hz, CHPh), 5.45 (ddt, 1H, $J_{4,5}$ = 15.5 Hz, $J_{4,3}$ = 5.5 Hz, $J_{4,6}$ = 1.7 Hz, H-4), 5.75 (dt, 1H, $J_{5,6}$ = 15.5 Hz, $J_{5,6}$ = 7.5 Hz), 6.43 (d, 1H, $J_{NH,2}$ = 8.0 Hz, NH), 7.20–7.60 (m, 20H, Ph). ^{13}C NMR ($CDCl_3$): δ 14.1 (2C), 22.7 (2C), 25.8, 29.3–30.0 (24C), 31.9 (3C), 32.4, 36.7, 52.8, 68.7, 69.0, 69.8, 72.7, 73.6, 74.0, 74.1, 74.4, 74.8, 75.8, 79.2, 99.1, 125.4–128.5 (Ph), 129.2, 132.9, 137.6, 138.0; 138.4, 138.5, 173.3. ESI-MS (negative-ion mode): *m*/*z* 1142.5 $[M-H]^-$.

4.1.4. (2*S*,3*R*,4*E*)-3-Acetoxy-1-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyloxy)-2-(docosanoylamino)-4-octadecene (6). Sodium (0.06 g) was added to cold (−50 °C), stirred liquid ammonia (12 mL) and after a few minutes deep blue solution was obtained, then compound **5** (0.16 g, 0.14 mmol) in anhydrous THF (2.4 mL) was added via cannula at such a rate that the solution was not discolored. The solution was stirred at −50 °C for 2 h while the blue color persisted; after that methanol (12 mL) was added and ammonia was allowed to evaporate. The mixture was concentrated and the residue was dissolved in pyridine–Ac_2O (6 mL/3 mL) and stirred for 20 h at room temperature. The reaction was quenched by addition of methanol, water was added (40 mL), and the aqueous layer was extracted with EtOAc (4× 60 mL). Combined organic layers were dried and concentrated. Flash chromatography of the crude (*n*-hexane/EtOAc 7:3) afforded compound **6** (0.10 g, 78%) as an amorphous solid.

$[\alpha]_D^{20}$ + 46.7 (*c* 1, $CHCl_3$). ^{1}H NMR ($CDCl_3$): δ 0.90 (t, 6H, *J* = 7.5 Hz, 2 CH_3), 1.18–1.45 (m, 58H, 29 CH_2), 1.57–1.68 (m, 2H, $COCH_2CH_2$), 2.02, 2.05, 2.06, 2.14, 2.16 (5s, 15H, 5 $COCH_3$), 2.04 (m, 2H, CH=CHCH_2), 2.15 (m, 2H, $COCH_2$), 3.57 (dd, 1H, $J_{1a,1b}$ = 10.5 Hz, $J_{1a,2}$ = 4.0 Hz, H-1a), 3.74 (dd, 1H, $J_{1b,2}$ = 3.5 Hz, H-1b), 4.03–4.20 (m, 3H, H-5′, H_2-6′), 4.38 (m, 1H, H-1b), 5.06 (d, 1H, $J_{4',3'}$ = 3.5 Hz, H-4′), 5.16 (dd, 1H,

$J_{3',2'} = 11.0$ Hz, $J_{3',4'} = 3.5$ Hz, H-3′), 5.30 (t, 1H, $J = 8.0$ Hz, H-3), 5.35 (dd, 1H, 1H, $J_{2',3'} = 11.0$ Hz, $J_{2',1'} = 3.5$ Hz, H-2′), 5.41 (dd, 1H, $J_{4,5} = 15.5$ Hz, $J_{4,5} = 5.5$ Hz, H-4), 5.48 (d, 1H, $J_{1',2'} = 3.5$ Hz, H-1′), 5.67 (d, 1H, $J_{NH,2} = 9.0$ Hz, NH), 5.82 (dt, 1H, $J_{4,5} = 15.5$ Hz, $J_{5,6} = 7.5$ Hz, H-5). ^{13}C NMR ($CDCl_3$): δ 14.1 (2C), 20.5, 20.6 (2C), 20.7, 21.1, 22.7 (2C), 25.8, 29.0–29.8 (24C), 31.9 (3C), 32.3, 36.9, 50.5, 61.8, 66.6, 67.4, 67.5, 67.8, 67.9, 72.9, 97.0, 124.8, 138.0, 169.6, 170.1, 170.2, 170.4, 170.5, 172.7. ESI-MS (positive-ion mode): m/z 1016.6 $[M+Na]^+$.

4.1.5. (2*S*,3*R*,4*E*)-1-(α-D-Galactopyranosyloxy)-2-(docosanoylamino)-3-hydroxy-4-octadecene (7). To a stirred solution of compound **6** (0.10 g, 0.10 mmol) in dry CH_2Cl_2 (4 mL) sodium methoxide in dry methanol (0.05 M solution, 0.8 mL) was added and the solution was stirred at room temperature for 3 h. The solution was neutralized with an ion exchange resin (Dowex 50×8, H^+ form), filtered, and concentrated affording **7** (0.08 g, quantitative) as a foam.

$[\alpha]_D^{20} + 30.6$ (c 0.5, $CHCl_3/CH_3OH$, 1:1). ^{1}H NMR ($CDCl_3/CD_3OD$, 1:1): δ 0.88 (t, 6H, $J = 7.5$ Hz, 2 CH_3), 1.20–1.40 (m, 58H, 29 CH_2), 1.55–1.64 (m, 2H, $COCH_2CH_2$), 2.02 (m, 2H, CH=CHCH_2), 2.18 (t, 2H, $J = 7.5$ Hz, $COCH_2$), 3.68–3.83 (m, 7H, H-1a, H-1b, H-2′, H-3′, H-5′, H_2-6′), 3.92 (br d, 1H, $J_{4',3'} = 2.8$ Hz, H-4′), 3.95 (m, 1H, H-2), 4.07 (br t, 1H, $J = 7.0$ Hz, H-3), 4.87 (d, 1H, $J_{1',2'} = 3.5$ Hz, H-1′), 5.44 (ddt, 1H, $J_{4,5} = 15.5$ Hz, $J_{4,3} = 7.5$ Hz, $J_{4,6} = 1.0$ Hz, H-4), 5.71 (dt, 1H, $J_{5,4} = 15.5$ Hz, $J_{5,6} = 6.5$ Hz, H-5). ^{13}C NMR ($CDCl_3/CD_3OD$, 1:1): δ 13.7 (2C), 22.5 (2C), 25.9, 29.2–29.5 (24C), 31.8 (3C), 32.3, 36.3, 53.7, 61.6, 67.3, 69.0, 69.6, 70.1, 70.7, 72.0, 99.9, 129.2, 134.0, 174.7. ESI-MS (negative-ion mode): m/z 782.6 $[M-H]^-$.

4.1.6. (2*S*,3*R*,4*E*)-1-[3-*O*-(Sodium oxysulfonyl)-α-D-galactopyranosyloxy]-2-(docosanoylamino)-3-hydroxy-4-octadecene (1). Compound **7** (0.08 g, 0.06 mmol) and Bu_2SnO (0.02 g, 0.1 mmol) were stirred in MeOH (4 mL) at reflux under argon for 2 h. The solvent was evaporated off under reduced pressure, and the dibutylstannylene complex was treated with $Me_3N{\cdot}SO_3$ (0.02 g, 0.14 mmol) in THF (4 mL) for 2 h. The solvent was removed under reduced pressure, then the residue was dissolved in $CHCl_3$/MeOH, 1:1 (2 mL), loaded onto a cation exchange resin column (Dowex 50×8 Na^+ form, 0.5×6 cm), eluted with $CHCl_3$/MeOH (1:1), concentrated under reduced pressure and subjected to flash chromatography ($CHCl_3$/MeOH, 9:1) to give the target compound **1** (70%, 0.06 g) as a foam.

$[\alpha]_D^{20} + 42.0$ (c 0.5, CH_3OH). ^{1}H NMR ($CDCl_3/CD_3OD$, 1:1): δ 0.88 (t, 6H, $J = 7.5$ Hz, 2 CH_3), 1.20–1.40 (m, 58H, 29 CH_2), 1.55–1.64 (m, 2H, $COCH_2CH_2$), 2.03 (m, 2H, CH=CHCH_2), 2.20 (t, 2H, $J = 7.5$ Hz, $COCH_2$), 3.69–3.82 (m, 4H, H-1a, H-1b, H_2-6′), 3.85 (br t, 1H, $J = 5.5$ Hz, H-5′), 3.96 (m, 1H, H-2), 4.05 (dd, 1H, $J_{2',3'} = 10.5$ Hz, $J_{2',1'} = 4.0$ Hz H-2′), 4.12 (t, 1H, $J = 7.5$ Hz, H-3), 4.34 (br d, 1H, $J_{4',3'} = 2.5$ Hz, H-4′), 4.50 (dd, 1H, $J_{3',2'} = 10.5$ Hz, $J_{3',4'} = 2.5$ Hz, H-3′), 4.87 (d, 1H, $J_{1',2'} = 3.5$ Hz, H-1′), 5.44 (ddt, 1H, $J_{4,5} = 15.5$ Hz, $J_{4,3} = 7.5$ Hz, $J_{4,6} = 1.0$ Hz, H-4), 5.71 (dt, $J_{5,4} = 15.5$ Hz, $J_{5,6} = 6.5$ Hz, H-5). ^{13}C NMR ($CDCl_3/CD_3OD$, 1:1): δ 13.6 (2C), 22.5 (2C), 25.9, 29.2–29.5 (24C), 31.8 (3C), 32.3, 36.2, 53.6, 61.5, 66.9, 67.1, 68.1, 70.7, 71.2, 78.1, 99.6, 129.5, 134.0, 174.8. ESI-MS (negative-ion mode): m/z 862.8 $[M-Na]^-$.

4.2. Generation of T cell clones and activation assays

The K34B9.1 T cell clone specific for sulfatide was generated as previously described.[17] The iNKT cell clones BGA01 and BGA82 were obtained by α-GalCer stimulation of peripheral blood mononuclear cells of healthy donors are type I iNKT. To investigate their capability to react to α-sulfatide **1**, T cells (10^5/well) were incubated for 48 h together with C1R-CD1d-transfected cells (2.5×10^4/well) and increasing doses of glycolipids. T cell activation was detected by measuring the amounts of IFNγ and IL-4 released in the supernatant, using ELISA as previously described.[17]

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