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4,5-Cis Unsaturated α-GalCer Analogues Distinctly Lead to CD1d-Mediated Th1-Biased NKT Cell Responses

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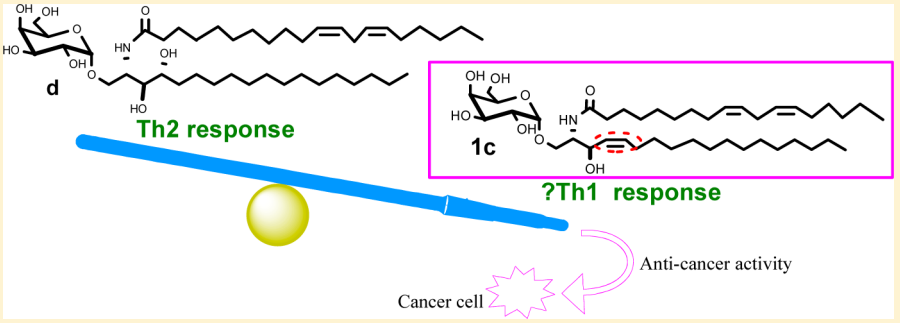
Supporting Information



ABSTRACT: The total synthesis of 4,5-cis unsaturated α-GalCer analogues was achieved, and their immune-response altering activity was assessed *in vitro* as well as *in vivo* in mice. Using glycosyl iodide as a glycosyl donor, construction of the sphingosine unit was shortened by four steps and single α-stereoselectivity was achieved in good yield (67%). With regard to the therapeutic use of α-GalCer, the novel analogues (**1b** and **1c**) distinctly induced a Th1-biased cytokine response, avoiding induction of a contradictory response and overstimulation.

INTRODUCTION

α-Galactosylceramides (α-GalCers) play an important role as immunoresponsive 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.[1,2] NKT cells are thus stimulated by the CD1d−glycolipid complex, eliciting a proinflammatory response through the secretion of T helper 1 (Th1) cytokines [interferon (IFN)-γ, tumor necrosis factor (TNF)-α] or an immunomodulatory response through the release of Th2 cytokines [interleukin (IL)-4, IL-10]. Of the α-GalCers, KRN7000 was found to be the most potent agonist and has been extensively evaluated in preclinical studies and clinical trials for treating cancers and other diseases.[3−6] However, even though Phase I clinical trials demonstrated that α-GalCer was safe and immunostimulatory, no optimistic responses in patients have been observed.[6,7] The efficacy of KRN7000 has been limited in many cases due to reciprocal inhibition exhibited by Th1 and Th2 cytokines[8] as well as, more importantly, overstimulation of iNKT cells, which can result in their entering a long-term anergic state, i.e., unresponsiveness to subsequent KRN7000 stimulation and preferential IL-4 production, which would be deleterious for long-term therapy.[9,10] As such, considerable efforts have been devoted to the synthesis of KRN7000 analogues in the hope of developing novel compounds with better cytokine-inducing selectivity and appropriate potency.[6] Since the binding site of CD1d is highly flexible, a wide variety of analogues of KRN7000 are acceptable.[6,11] This flexibility, at the same time, makes it difficult to determine the definitive partial structures of KRN7000 that result in analogues that are Th1- or Th2-type immune stimulants.

The altered stability of the CD1d−glycolipid complex influences the overall stability of the CD1d−glycolipid−TCR complex and is likely a factor that contributes to the resulting cytokine profile that is produced.[12] Therefore, the stability of the α-GalCer−CD1d complex is currently a focal 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 higher levels of IFN-γ release and consequently promote and prolong a Th1 response.

Recent crystallographic analysis has indicated that the lipid chains of α-GalCer are buried in a groove in CD1d containing

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two hydrophobic pockets and that the galactose ring is exposed for recognition by the NKT TCR.[13] In addition, several hydrogen bonds were identified and are assumed to anchor α-GalCer in a distinct orientation and to position it in the lipid-binding groove. As such, modification of the lipid chains of α-GalCer is a very important aspect of the design of α-GalCer analogues. However, surprisingly little attention has been devoted to 4,5-cis alkene sphingosine chain alterations. The crystal structure of the ternary complex of human NKT TCR with CD1d and KRN7000 highlighted the involvement of the 3- and 4-OH groups on the phytosphingosine scaffold through concomitant hydrogen bonding with CD1d's Asp80 residue and an additional H-bond between the donating Arg95 of the CDR3 R-loop and the 3-OH group of the glycolipidic ligand.[14] Nevertheless, Lacone et al. created a 4-deoxy analogue (**f**) that was nearly as active as KRN7000 in stimulating iNKT cells.[15] The 4,5-trans sphingosine chain analogue (**c**) showed immunoresponsive activity.[16]

During the exploration of immunostimulatory glycolipids, cis unsaturated lipids aroused our attention. For example, phospholipids with multiple double bonds were better recognized than those with a single double bond, and increasing the unsaturation of the acyl chain correlated with increased binding of self-phospholipid antigen to CD1d.[17] In studying immunostimulatory glycolipids of bacterial origin, the structures of the major fatty acids were elucidated as palmitate and oleate.[18] From Hénon's study,[19] four positions in the acyl chain emerged at which a Z double bond may help to supply a rigid bend relatively close to that found in the natural compound. The insertion of double bonds in a glycolipid also helps considerably to increase solubility, which is essential for biological testing.[20,21]

Therefore, from these data, we anticipated that Z-unsaturations in the sphingosine chain would alter the balance in the proinflammatory and anti-inflammatory activities of NKT cells. Hence, to verify this assumption, we synthesized analogues with Z-unsaturations in the sphingosine chain. Then, preliminary assays of the synthetic analogues' immunoactivity were performed by measuring the production of IL-2 by mouse iNKT hybridoma 2H4 cells, and the selectivity and intensity of the responses generated by the screened agonists were evaluated using mouse primary splenocyte assays. Lastly, the synthesized analogues were tested *in vivo* in mice. We are committed to finding an appropriate and potent glycogen.

EXPERIMENTAL SECTION

Synthesis of α-GalCer Analogues. *General.* All reagents were obtained from commercial sources and used without further purification. Solvents were dried using standard methods. Reactions were monitored by TLC using a silica gel 60 F_{254} precoated plate (Merk, Darmstadt, Germany), and detection was performed by charring with sulfuric acid. Flash column chromatography was performed on silica gel 60 (100−400 mesh, Qingdao Marine Chemical Ltd., Qingdao, PRC). NMR spectra were recorded at ambient temperature (400 MHz for ^{1}H NMR and 101 MHz for ^{13}C NMR) on a Bruker DRX 400 (Karlsruhe, Germany). Tetramethylsilane was used as an internal standard. Chemical shifts are reported as δ values (ppm). Coupling constants are presented in hertz. Mass spectral data were determined by ESI (Micromass, UK).

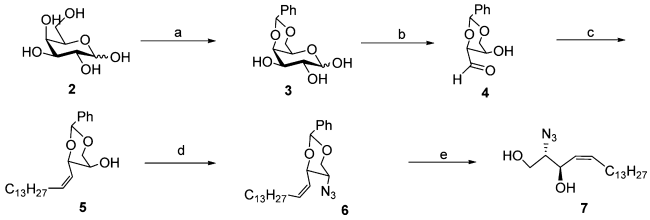
*(2S,3R,4Z)-2-Azido-octadec-4-ene-1,3-diol (**7**).* APTS (60 mg) was added to a solution of **6** (2.5 g, 6.0 mmol) in anhydride MeOH (30 mL). The solution was stirred at 40 °C for 4 days under an argon atmosphere. After the organic solvent was removed in vacuum, the residue was purified by column chromatography (petroleum ether/ethyl acetate 2:1) to afford **7** as a white solid (1.62 g, 82%) (Scheme 1). R_f = 0.49 (petroleum ether/ethyl acetate 2:1). ESI/MS (*m*/*z*) 326.3 $[M + H]^+$.

*(2,3,4,6-Tetra-O-trimethylsilyl-α-D-galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-azido-octadec-4-ene-1,3-diol (**10**).* TMSI (500 μL, 3.69 mmol) was added to a solution of **8** (2.0 g, 3.69 mmol) in CH_2Cl_2 (20 mL) at 0 °C. The reaction mixture was stirred under an argon atmosphere for 15 min. The solvent was removed under reduced pressure, and the glycosyl iodide intermediate obtained was dissolved in benzene (10 mL) and kept under an argon atmosphere. In a separate flask, a mixture of activated 4 Å molecular sieves (1000 mg), *n*-Bu_4NI (3.0 g, 8.10 mmol), *i*-Pr_2NEt (1000 μL, 5.89 mmol), and alcohol **7** (0.90 g, 2.77 mmol) in benzene (25 mL) was prepared and stirred under an argon atmosphere at 50 °C for 30 min. The solution of glycosyl iodide in benzene was then added dropwise over 20 min to this mixture, and the resulting mixture was stirred overnight. After removal of the solvent under reduced pressure, CH_2Cl_2 (50 mL) and H_2O (50 mL) were added and the phases were separated. The organic phase was concentrated under reduced pressure. The resulting solid was purified by flash column chromatography (petroleum ether/ethyl acetate 8.5:1.5) to afford glycoside **10** (single α-anomer) as a white solid (1.44 g, 67%). R_f = 0.35 (petroleum ether/ethyl acetate 3:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.62 (dd, *J* = 11.3, 7.4 Hz, 1H), 5.40 (t, *J* = 10.1 Hz, 1H), 4.75 (d, *J* = 3.1 Hz, 1H anomeric proton), 4.34 (t, *J* = 8.3 Hz, 1H), 3.98−3.90 (m, 1H), 3.80−3.65 (m, 4H), 3.64−3.26(m, 4H), 2.11 (dd, *J* = 14.3, 7.1 Hz, 2H), 1.65 (s, 1H), 1.43−1.32 (m, 2H), 1.34−1.18 (m, 20H), 0.87 (t, *J* = 6.4 Hz, 3H), 0.18 (s, 9H), 0.16 (s, 9H), 0.14 (s, 9H), 0.12 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 135.41, 127.92, 100.96 (C-1, anomeric carbon), 74.53, 73.98, 72.70, 71.90, 66.05, 63.32, 62.32, 61.77, 32.58, 30.11−29.81, 28.53, 23.14, 14.57, 1.64, 1.50, 1.33, 1.18, 0.60−0.00. ESI-HRMS (*m*/*z*) 798.4717 $[M + Na]^+$ (calcd: 798.4736).

*(α-D-Galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-azido-octadec-4-ene-1,3-diol (**11**).* To the solution of **10** (1.44 g, 18.55 mmol) in MeOH (20 mL) was added *p*TSA (20.0 mg), and the reaction mixture was stirred for 5 h at rt. The reaction was quenched by the addition of $NaHCO_3$ (100 mg, 1.19 mmol), filtered, and then concentrated under reduced pressure. The resulting solid was purified by flash column chromatography (CH_2Cl_2/MeOH 9.4:0.6) to afford glycoside **11** (single α-anomer) as a white solid (860 mg, 95%). R_f = 0.41 (CH_2Cl_2/MeOH 7:1). ^{1}H NMR (400 MHz, MeOD) δ 5.75−5.58 (m, 1H), 5.53−5.40 (m, 1H), 4.89 (d, *J* = 3.4 Hz, 1H anomeric proton), 4.57 (dt, *J* = 14.6, 7.3 Hz, 1H), 3.97−3.90 (m, 2H), 3.88 (t, *J* = 6.0 Hz, 1H), 3.82−3.77 (m, 2H), 3.75−3.70 (m, 2H), 3.67−3.58 (m, 1H), 3.57−3.51 (m, 1H), 2.21−2.08 (m, 2H), 1.41 (d, *J* = 6.7 Hz, 2H), 1.30 (s, 20H), 0.91 (t, *J* = 6.4 Hz, 3H). ^{13}C NMR (101 MHz, MeOD) δ 134.10, 128.04, 99.76 (C-1, anomeric carbon), 71.20, 69.87, 69.54, 68.65, 67.53, 66.14, 65.94, 61.21, 31.62, 29.41−29.18, 29.03, 28.98, 27.44, 22.28, 13.05. ESI-HRMS (*m*/*z*) 510.3187 $[M + Na]^+$ (calcd: 510.3155).

*(α-D-Galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-amino-octadec-4-ene-1,3-diol (**12**).* PMe_3 (26.7 μL, 0.252 mmol) was added to a

Scheme 1. Synthesis Scheme for Sphingosine Backbone Building[a]



[a](a) $PhCH(OCH_3)_2$, APTS, DMF, 40 °C, 70%; (b) $NaIO_4$, buffer solution (pH 7.6), 53.9%; (c) $CH_3(CH_2)_{12}CH_2{}^+PPh_3Br^-$, *n*-BuLi, THF, 0 °C → rt, 51% (cis) + 15% (trans); (d) Tf_2O, pyridine, DCM, NaN_3, DMF, −15 °C → rt, overnight, 62%; (e) ATPS, MeOH, rt, 4 days, 82%.

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solution of azide **11** (35 mg, 0.072 mmol) in wet MeOH (2 mL) at rt, and the resulting solution was stirred for 2 h. Then, the solvent was concentrated under reduced pressure. The residue was then subjected to high vacuum at 30 °C for 24 h to remove the Me_3PO byproduct. Amine **12** was obtained as a white solid (32 mg, quant.) and used in the next step without further purification. R_f = 0.23 (CH_2Cl_2/MeOH 4:1).

*(α-D-Galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-octadecenamino-octadec-4-ene-1,3-diol (**1a**).* To a solution of ester **13a** (56.3 mg, 0.144 mmol) in THF (2 mL) were added amine **9** (32 mg, 0.069 mmol) and Et_3N (55 μL, 0.395 mmol). The reaction mixture was stirred at rt for 10 h. Then, EtOAc (5 mL) was added, and then the organic phases were evaporated under reduced pressure. Purification of the residue by column chromatography (CH_2Cl_2/MeOH 9.5:0.5) afforded glucosyl ceramide **1a** as a white solid (34.6 mg, 69%) (Scheme 2). R_f = 0.52 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, C_5D_5N) δ 8.40 (d, 1H, *J* = 9.8 Hz, −NH−), 5.95−5.88 (m, 1H), 5.59 (dd, 1H, *J* = 11.3, 7.3 Hz), 5.38 (d, 1H, *J* = 3.7 Hz, H-1 anomeric proton), 5.12−5.06 (m, 1H), 4.71−4.63 (m, 1H), 4.55−4.24 (m, 8H), 2.37 (t, 2H, *J* = 7.4 Hz), 2.24 (ddd, 2H, *J* = 22.1, 14.7, 7.0 Hz), 1.76 (dt, 2H, *J* = 14.6 Hz, 7.4 Hz), 1.36−1.18 (m, 52 H), 0.88 (dd, 6H, *J* = 6.8, 5.5 Hz). Some resonance overlaps in C_5D_5N. ^{13}C NMR (101 MHz, C_5D_5N) δ 173.60, 132.44, 131.79, 101.99 (C-1, anomeric carbon), 72.59, 71.43, 70.80, 70.31, 69.18, 68.31, 62.55, 55.10, 36.71, 32.06, 30.05, 29.91, 29.85, 29.78, 29.70, 29.63, 29.57, 29.53, 28.10, 26.20, 22.85, 14.23. ESI-HRMS (*m*/*z*) 728.6032 [M + H]$^+$ (calcd: 728.6040).

*(α-D-Galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-((cis-9)-octadecenoicamino)-octadec-4-ene-1,3-diol (**1b**).* The procedure was the same as that for **1a**. **1b** was afforded as a white solid (33.5 mg, 67%) (Scheme 2). R_f = 0.52 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$ = 1:3) δ 5.64−5.49 (m, 1H), 5.45−5.29 (m, 3H), 4.90 (d, 1H, *J* = 3.5 Hz, H-1 anomeric proton), 4.45 (d, 1H), 4.02−3.91 (m, 2H), 3.88−3.67 (m, 7H), 2.20 (t, 2H, *J* = 7.5 Hz), 2.08−1.94 (m, 4H), 1.61 (s, 2H), 1.45−1.15 (m, 43H, CH_2), 0.89 (t, 6H, *J* = 6.5 Hz). CONH resonance not observed. ^{13}C NMR (101 MHz, MeOD/$CDCl_3$ = 1:3) δ 174.47 (CONH), 133.70, 129.57, 129.31, 128.55, 99.67 (C-1, anomeric carbon), 70.35, 69.92, 69.40, 68.69, 67.25, 66.99, 61.42, 53.66, 36.06, 31.51, 29.28, 29.23, 29.09, 28.99, 28.93, 28.89, 28.81, 27.36, 26.78, 25.45, 22.23, 13.46. ESI-HRMS (*m*/*z*) 748.5680 [M + Na]$^+$ (calcd: 748.5703).

*(α-D-Galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-(cis,cis-9,12-octadecadienoicamino)-octadec-4-ene-1,3-diol (**1c**).* The procedure was the same as that for **1a**. **1c** was afforded as a white solid (32.4 mg, 65%) (Scheme 2). R_f = 0.51 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, MeOD/$CDCl_3$ = 1:3) δ 5.58−5.49 (m, 1H), 5.43−5.27 (m, 5H,), 4.91 (d, 1H, *J* = 3.3 Hz, H-1 anomeric proton), 4.46 (t, 1H, *J* = 8.5 Hz), 4.01−3.94 (m, 1H), 3.89 (d, 1H, *J* = 2.2 Hz), 3.83−3.76 (m, 5H), 3.72−3.67 (m, 2H), 3.31 (t, 2H, *J* = 6.2 Hz), 2.17 (dd, 2H, *J* = 13.7, 6.1 Hz), 2.06 (dd, 4H, *J* = 13.5, 6.7 Hz), 1.59 (s, 2H), 1.43−1.21 (m, 38H), 0.94−0.85 (m, 6H). CONH resonance not observed. ^{13}C NMR (101 MHz, MeOD/$CDCl_3$ = 1:3) δ 174.01 (CONH), 132.73, 128.87, 128.80, 127.03, 126.96, 99.25 (C-1, anomeric carbon), 70.41, 69.36, 68.93, 68.23, 66.09, 65.47, 60.65, 53.40, 35.26, 31.00, 30.59, 28.90, 28.75, 28.72, 28.48, 28.40, 28.34, 28.27, 26.76, 26.15, 26.11, 24.98, 24.50, 21.67, 21.56, 12.43. ESI-HRMS (*m*/*z*) 746.5531 [M + Na]$^+$ (calcd: 746.5547).

*(α-D-Galactopyranosyl)-(1→1)-(2S,3R,4Z)-2-(boc-L-tryptophanamimo)-octadec-4-ene-1,3-diol (**1d**).* The procedure was the same as that for **1a**. **1d** was afforded as a white solid (30.9 mg, 60%) (Scheme 2). R_f = 0.50 (CH_2Cl_2/MeOH 9:1). ^{1}H NMR (400 MHz, DMSO-*d6*) δ 10.75 (s, 1H), 7.68 (d, *J* = 8.4 Hz, 1H), 7.53 (t, *J* = 13.0 Hz, 1H), 7.27 (d, *J* = 7.8 Hz, 1H), 7.12−7.03 (m, 1H), 7.00 (t, *J* = 7.3 Hz, 1H), 6.91 (t, *J* = 7.1 Hz, 1H), 6.74 (d, *J* = 8.0 Hz, 1H), 5.40−5.16 (m, 2H), 4.82 (s, 1H), 4.68 (s, 1H), 4.52 (s, 2H), 4.42 (s, 1H), 4.26 (d, *J* = 14.9 Hz, 2H), 4.14 (s, 1H), 3.80 (s, 1H), 3.73−3.35 (m, 7H), 3.00 (d, *J* = 12.4 Hz, 1H), 2.80 (t, *J* = 10.0 Hz, 1H), 2.12−1.83 (m, 2H), 1.40−1.01 (m, 31H), 0.80 (t, *J* = 6.6 Hz, 3H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 171.74, 155.16, 136.03, 131.63, 130.89, 127.42, 123.48, 120.72, 118.43, 118.02, 111.20, 110.37, 99.89 (C-1, anomeric carbon), 77.96, 71.22, 69.76, 68.90, 68.72, 66.62, 65.99, 60.70, 55.12, 53.68, 31.29, 29.25, 29.05, 28.89, 28.71, 28.13, 27.85, 27.18, 22.09, 13.94. ESI-HRMS (*m*/*z*) 770.4547 [M + Na]$^+$ (calcd: 770.4567).

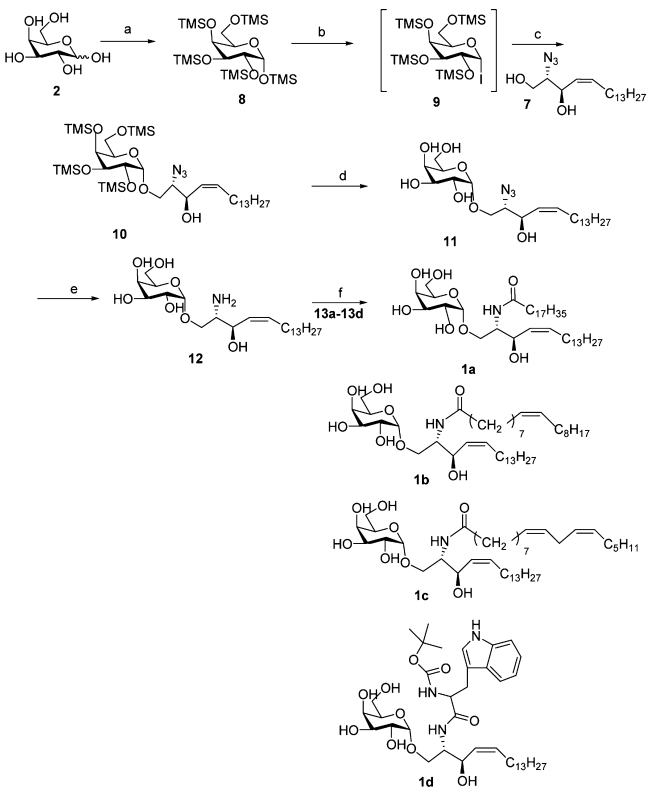
Immunological Assays. *Methods. Cell Culture.* The 2H4 mouse iNKT hybridoma and A20-CD1d cell lines were kindly provided by Mitchell Kronenberg (La Jolla Institute for Allergy and Immunology, La Jolla, CA). Cell lines were maintained in RPMI-1640 medium containing 10% fetal calf serum (FCS), 2 mM sodium pyruvate, 2 mM L-glutamine, and 50 μM 2-mercaptoethanol.

iNKT Hybridoma Cell Assay. The 2H4 cells secrete IL-2 upon recognition of glycolipids presented by A20-CD1d cells. α-GalCer and compounds dissolved at 1 mg/mL in DMSO were diluted in cell culture medium and pulsed to A20-CD1d cells (10^5 cells) for 16 h, after which the A20-CD1d cells were washed with culture medium and mixed with 2H4 hybridoma cells (5×10^4 cells). Then, the mixture (200 μL) was co-cultured for 24 h, and the released IL-2 was measured by ELISA (Shanghai ExCell Biology, Inc., Shanghai, China).

Mouse Primary Splenocyte Assay. Splenocytes (5×10^5) from C57BL/6 mice were cultured for 5 days with 0.1 μg/mL of α-GalCer, compounds, or DMSO (vehicle) in 96-well U-bottom plates containing RPMI-1640 supplemented with 10% FCS, L-glutamine, 2-mercaptoethanol, penicillin, and streptomycin in a humidified environment of 5% CO_2 at 37 °C in the presence of 25 IU/mL mouse IL-2 (PeproTech) added at day 2 of the culture. For cytokine determination, cell-free supernatants were collected at day 5, and IFN-γ and IL-4 levels were measured by ELISA.

In Vivo Assay. This study was approved by the local ethical committee. One microgram of the analogues and reference α-GalCer in 200 μL of PBS was injected intraperitoneally to C57BL/6 mice. Sera were collected at two time points (2 and 24 h), and both IFN-γ and IL-4 were measured by ELISA.

Scheme 2. Synthesis Scheme for α-GalCer Analogues[a]



[a](a) HMDS, TMSCl, pyridine, 75 °C, 2h, 91%; (b) TMSI, DCM, 0 °C, 15 min; (c) *n*-Bu_4NI, *i*-Pr_2Net, compound **7**, 4 Å molecular sieves, benzene, 50 °C, 30 min → overnight, 67%; (d) *p*TSA, MeOH, rt, 5 h, 95%; (e) PMe_3, MeOH, 30 °C, 24 h, quant; (f) **13a−13d**, Et_3N, THF, rt, 10 h, **1a−1d** (69, 67, 65, and 60%, respectively).

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RESULTS AND DISCUSSION

The biological activity of Z-unsaturated analogues (**1a**−**1d**) as iNKT agonists was preliminarily assayed *in vitro* by measuring the production of IL-2 by mouse iNKT hybridoma 2H4 cells. As depicted in Figure 1, **1a**, **1b**, and **1c** induced obvious IL-2 production, whereas **1d** failed to do so. The results indicated that **1a**, **1b**, and **1c** had the ability to activate NKT cells and thus caused the release of IL-2. Even though **1d** has a 4,5-cis alkene sphingosine chain and its acyl chain contains a short protected tryptophan, it had no activity. The acyl chain structure also determines whether an analogue has activity and influences the intensity of the response.

As a functional assay, we cultured whole spleen cells in the presence of the indicated compounds or vehicle and determined the production of Th1 and Th2 prototypic cytokines, IFN-γ and IL-4, by ELISA at day 5 of culture. Both **1b** and **1c** induced IFN-γ production of cultured splenocytes at concentrations of 2.5 and 5 μg/mL. More interestingly, we found that **1c** at 5 μg/mL induced the production of IFN-γ to a higher extent than did α-GalCer at 100 ng/mL (Figure 2). On the contrary, production of prototypic Th2 cytokine IL-4 was extremely weak after stimulation with **1b** and **1c**. **1b** and **1c** did not induce significant production of IL-4 above background levels (Figure 2). The data strongly suggest that **1b** and **1c** were recognized by iNKT cells, stimulating their activation in a way that seems to be more biased toward a Th1 response.

As is known, α-GalCer induces both Th1 and Th2 cytokines, and the two types of cytokines inhibit each other. There were no clinical responses in a Phase I study of α-GalCer in patients with solid tumors because the effects of Th1 cytokines were hindered by Th2 cytokines.[29] As a result, the efficacy of α-GalCer has been limited. Therefore, the selectivity toward either Th1 or Th2 cytokine responses is more critical. We found that both **1b** and **1c** induced a Th1-biased cytokine response, as deduced from the high level of IFN-γ in the splenocyte cultures compared with the very weak IL-4 induction. Thus, **1b** and **1c** have efficacy as potent Th1 response inducers but do not activate Th2 cytokines, thus avoiding one of the main problems in the therapeutical application of α-GalCer, i.e., the simultaneous and potent induction of contradictory responses.

To address the biological activity of the analogues and their physiological consequences more meaningfully, we performed an *in vivo* study. We administered 1 μg of the analogues intraperitoneally and measured the levels of IFN-γ and IL-4 in serum at 2 and 24 h. Similarly to the observations from the splenocyte cultures, **1b** and **1c** both induced obvious production of IFN-γ upon *in vivo* administration, as detected in the serum of treated mice 24 h later, whereas IL-4 induction *in vivo* was very barely over background levels at 2 h and essentially no IL-4 in serum was found at 24 h (Figure 3).

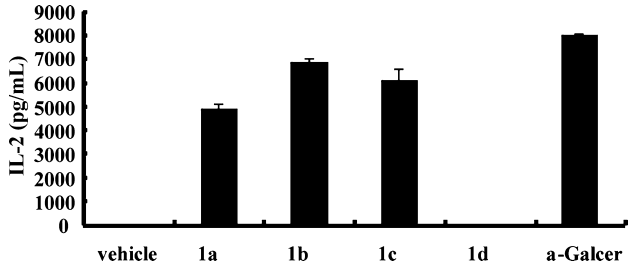
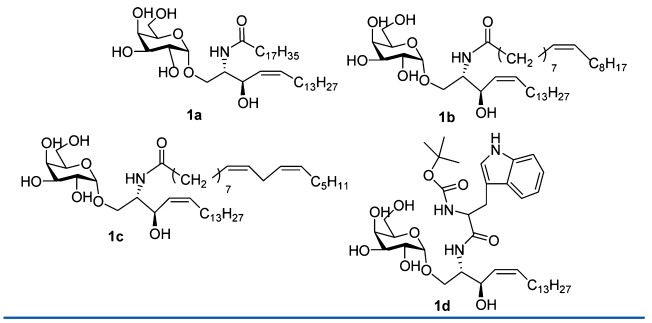


Figure 1. ELISA of IL-2 released by iNKT hybridoma 2H4 cells cultured with different Z-unsaturated analogues presented by A20-CD1d cells at a concentration of 1 μg/mL compared with α-GalCer at 100 ng/mL after 24 h of co-culture. Data are expressed as the mean ± SD from three independent experiments.



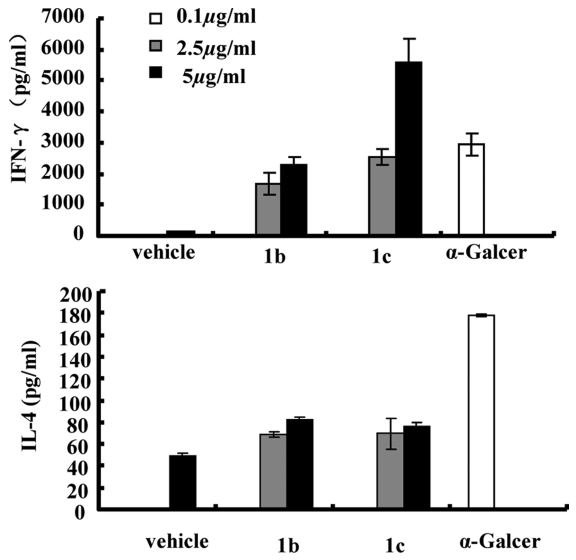


Figure 2. *In vitro* cytokine induction in cultured splenocytes. ELISA of IFN-γ and IL-4 in the culture supernatants at day 5 after compound incubation at the indicated concentrations. Data are expressed as the mean ± SD from three independent experiments.

The above new discovery is beyond our expectation. In previous studies, aryl-containing acyl chains glycolipids or α-C-GalCers polarized toward a Th1 response.[26,27] The analogues containing cis-unsaturated C18−20 fatty acid with a pytos-phingosine chain (**1d, 2d**; Figure 4)[22] or 4,5-trans unsaturated chain (**e**, Figure 4)[23] potently induced a Th2-biased cytokine response. It is widely accepted that analogues containing a cis-unsaturated C18−20 fatty acid are Th2-biased agonists.[6,28] Interestingly, when the sphingosine chain was altered to Z-unsaturated analogues (**1b** and **1c**) with a cis-unsaturated C18 fatty acid, they distinctly induced a Th1-biased cytokine response. The diunsaturated C18 fatty acid analogue was better recognized than those with a single double bond, and the single double bond analogue was better recognized than those with saturated C18 fatty acid. Our studies also support the previous phenomena regarding the C20 fatty acid observed by Yu et al.[23] The diunsaturated C20 fatty acid analogue had similar immune activity as that of the diunsaturated C18 fatty acid analogue. In view of the results with analogues having more double bonds, e.g., compound **2d** (Figure 4), which did not show more promising immune activity, we did not produce more Z-unsaturated analogues. This implied that isomers

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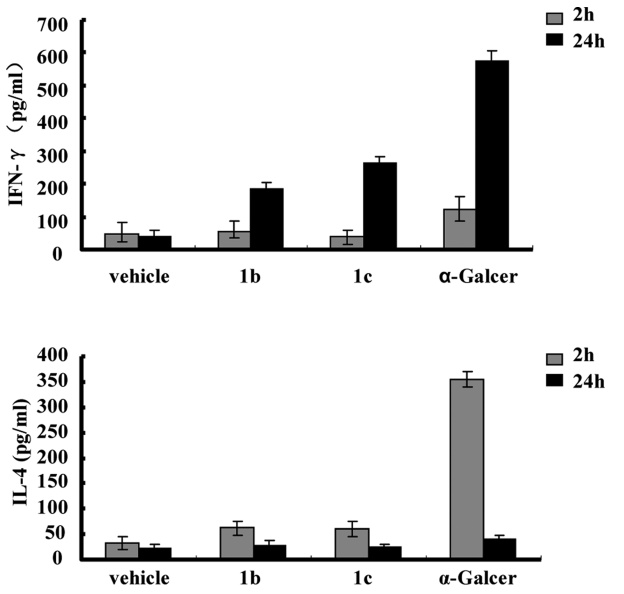


Figure 3. *In vivo* cytokine induction in mice. One microgram of the analogues and α-GalCer was i.p. injected into mice, and IFN-γ and IL-4 in serum were measured after 2 and 24 h.

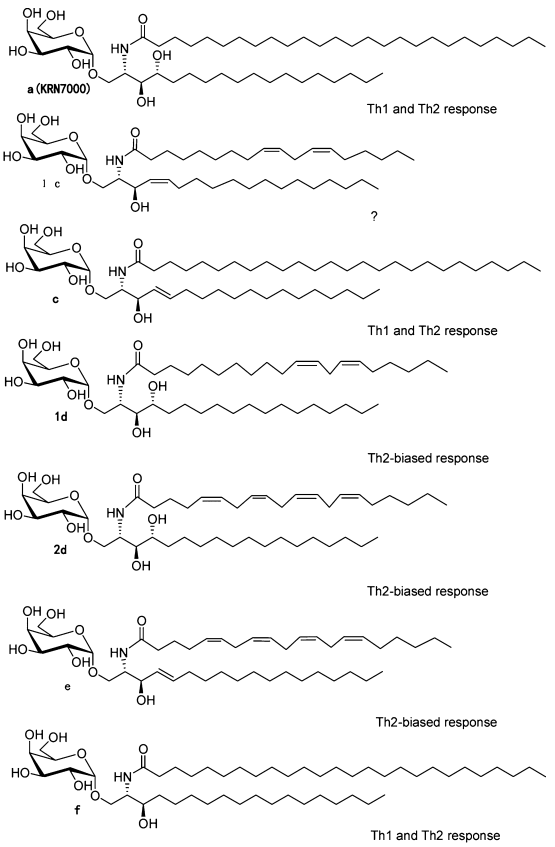


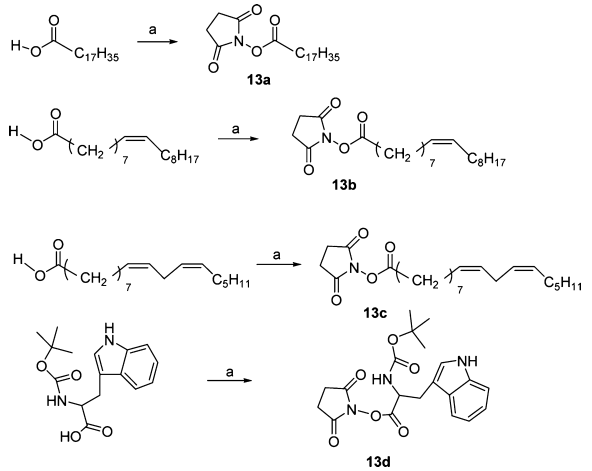
Figure 4. Comparison of different α-GalCer analogues.

having one Z double bond may change the extension direction of the sphingosine chain and help to supply a stable bend relatively close to CD1d. These findings help to explain the structural reason for the effect on the Th1 response and should promote further research to control the appropriate proinflammatory activities of NKT cells. The results demonstrate that even relatively minor changes in the key structure of a glycolipid ligand can result in a surprising degree of influence on the selectivity of cytokine induction.

Due to their intriguing bioactivities, great advances have been made in the synthesis and structure−function studies of KRN7000 analogues in order to rectify some of the deficiencies of KRN7000. Concerning the full synthesis, two key steps are difficult and challenging, termed α-glycosidic bond specific formation and diastereo- and enantiomeric sphingosine backbone building. As a suitable chiral source, D-galactose was employed for constructing a functionalized sphingosine unit according to previous work.[24] Intermediates **5**, **6**, and **7** were synthesized through modified methods. To control the configuration of the glycoside in the α-orientation, several elegant strategies have been developed. To retain unsaturation in the lipid chain under hydrogenation conditions, glycosyl iodide was chosen as a glycosyl donor.[25] Because glycosyl iodide is quite reactive, the hydroxyl of sphingosine **7** did not need to be protected, as was done for other donors, and the procedures for constructing the sphingosine unit and deprotection were shortened by at least four steps. With glycosyl iodide formation, glycosylation and detrimethylsilylation led to α-stereoselectivity in good yield (67%). The α configuration of the newly introduced glycosidic linkage (**1a**−**1d**) was confirmed by ^{1}H NMR. The azide group of compound **11** was reduced by trimethylphosphine in MeOH at room temperature for 2 h to produce amino derivative **12**, which was not characterized at this stage and condensed directly with fatty acid *N*-hydroxysuccinimide esters. To simplify the amide coupling, the fatty acids were activated by turning the fatty acid into fatty acid *N*-hydroxysuccinimide esters in advance (Scheme 3). The proton of the NH−CO group of **1b** and **1c** was not observed in deuterated solvent (MeOD/$CDCl_3$ = 1:3). ^{13}C NMR spectrum of **1b** and **1c** showed resonances at 174.47 and 174.01 ppm corresponding to the carbons of the NH−CO groups. In short, our constringent and rapid pathway increased the efficiency of full synthesis of α-GalCer compounds for the further assessment of their structure−activity relationships.

In summary, the synthesis of compounds related to α-GalCer with Z-unsaturation in the sphingosine chain resulted in the identification of **1a**, **1b**, and **1c** as new iNKT agonists. In

Scheme 3. Synthesis Scheme for Fatty Acid *N*-Hydroxysuccinimide Esters[a]



[a](a) NHS, DCCI, dry ethyl acetate, 35 °C, 15 h, **13a**−**13d** (85, 87, 89, and 83%, respectively).

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addition, **1b** and **1c** were potent Th1-biased activators of iNKT cells. Our new findings may overturn long-held notions about immune-responsive agonists and inspire people to consider extending the sphingosine chain, an approach that used to be ignored. A more complete assessment of the biological properties of **1b** and **1c** is warranted.

■ ASSOCIATED CONTENT

Supporting Information

Analytical data for compounds **10**, **11**, and **1a−d**. The synthetic procedures for compounds **5** and **6**. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00047.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

α-GalCer, α-galactosylceramide; APTS, *p*-toluenesulfonic acid; *n*-Bu4NI, tetrabutylammonium iodide; δ, chemical shift; dd, doublet of doublets; CD1d, antigen-presenting molecule; DCCI, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; FCS, fetal calf serum; Hz, hertz; HRMS, high-resolution mass spectrometry; IFN, interferon; iNKT, invariant natural killer; *i*-Pr_2Net, *N*,*N*-diisopropylethylamine; MeOH, methanol; NKT, natural killer T; *J*, coupling constant; m, multiple; *m*/*z*, mass-to-charge ratio; mmol, millimole; MS, mass spectrometry; NHS, *N*-hydroxysuccinimide; NMR, nuclear magnetic resonance; ppm, parts per million; R_f, rate of flow; t, triple; TCR, T cell receptors; Th1, proinflammatory cytokines; Th2, immunomodulatory cytokines; THF, tetrahydrofuran; TLC, thin-layer chromatography; TMSCl, trimethylsilyl chloride; TMSI, trimethylsilyl iodide; pTSA, *p*-toluenesulfonamide

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