Rapid Identification of Immunostimulatory α-Galactosylceramides Using Synthetic Combinatorial Libraries

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Two 60+-membered libraries of α-galactosylceramides have been prepared by reactions between activated ester resins and two core, fully deprotected galactosylated sphingoid bases. The libraries were evaluated for their ability to stimulate CD1d-restricted NKT cells, using in vitro stimulation of a murine NKT cell hybridoma line and for their ability to induce the expansion of NKT cells from peripheral blood mononuclear cells (PBMC) of a normal human subject. Our results showed that many compounds constructed on a C18-phytosphingosine base had significant stimulatory activity in both assays. Because no product purification was required, this approach is particularly attractive as a method for rapid synthesis of large libraries of potential immunomodulatory glycosylceramides.

Introduction

CD1 proteins are cell surface glycoproteins that are distantly related to the class I and class II antigen presenting molecules of the major histocompatibility complex (MHC). Unlike the MHC-encoded molecules which bind and present peptide antigens, CD1 proteins function by binding a variety of lipids and presenting these to various subsets of lipid-specific T cells.[1] CD1d is a member of the CD1 family that has been shown to present lipid antigens for recognition by the T cell receptors (TCRs) of a specialized subset of lymphocytes known as natural killer T cells (NKT cells).[2–4] CD1d-dependent NKT cells have been demonstrated to play a role in a variety of immune responses. A synthetic α-galactosylceramide (α-GalCer) known as KRN7000 has proved to be an invaluable tool for dissecting the function of CD1d and NKT cells in a wide range of immune responses. For example, the use of KRN7000 as a specific agonist for in vivo stimulation has provided evidence suggesting that NKT cell-mediated pathways may be used to inhibit hepatitis B virus replication[5] or protect against cancer,[6] diabetes,[7–10] malaria,[11,12] and tuberculosis.[13] An analogue of KRN7000 known in the literature as OCH, which is truncated in both the acyl chain and the sphingoid base, was found to selectively induce a T helper type 2 (T_H2)-biased cytokine response and to offer protection in mice against experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis.[14] More recently, OCH has been shown to protect against development of diabetes in NOD mice[15] and also to inhibit collagen-induced arthritis.[16] In spite of the growing interest in exploiting CD1d-mediated activation of NKT cells and the increased understanding of the role of the cytokines elicited via this route, extensive and systematic structure–activity relationship studies of glycolipid activators of CD1d are lacking. Earlier synthetic studies en route to the identification of KRN7000 focused on enhancing T-cell proliferation and included modest changes in acyl chain and sphingoid base lengths,[6,17,18] as well as a limited range of mono-, di-, and trisaccharides.[6,19–22] More recently, as the potential of certain structural analogues of KRN7000 to induce cytokine bias (either T_H2 or T_H1) has emerged, reports have appeared on a few individual compounds[23,24] or relatively small series' of compounds.[25–27] The largest series of α-GalCer analogues to be studied as NKT cells agonists that has been reported to date is a set of 34 acyl chain variants of KRN7000 recently described by Wong and co-workers.[27] These latter compounds were prepared by solution-phase methods, where template **1** was acylated and the resultant glycosphingolipids hydrolyzed. Each stage required chromatographic purification. We therefore carried out the current set of studies to demonstrate that a broader range of compounds, especially with extensive variation in the acyl chain, could be evaluated more systematically by a combinatorial approach. We used unprotected template **2**, containing the carbohydrate and sphingoid based moieties of KRN7000, in combination with activated ester resins. The resins were readily prepared from

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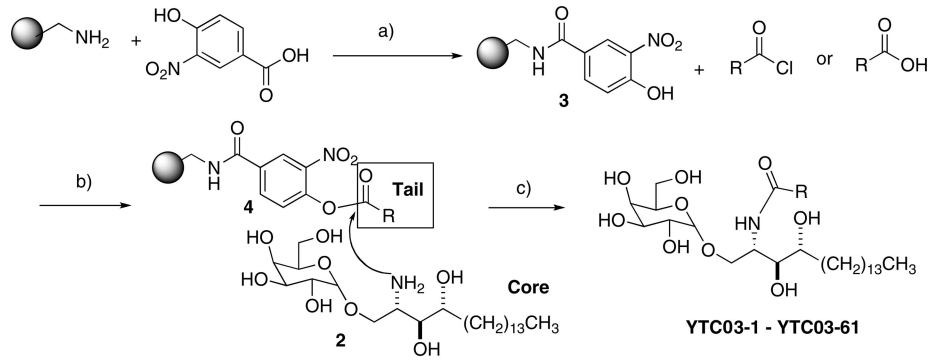
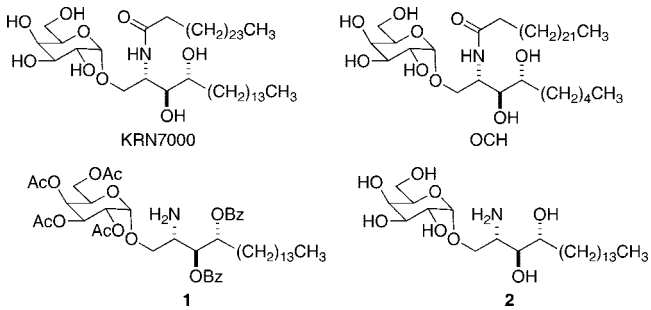


Figure 1. General strategy for solid-phase synthesis of α-GalCer library.

commercial acids or acyl chlorides. Product isolation was greatly facilitated because filtration and solvent removal provided the target α-GalCers. Product purity was readily evaluated by HPLC with confirmation of identity provided by MS analysis. Herein we report the results on the preparation and biological evaluation of two 60+-membered α-GalCer libraries.



Results and Discussion

Glycosylated sphingoid base template **2** was prepared from phytosphingosine using an intramolecular glycosidation strategy.[28] A library of α-GalCers was prepared by coupling **2** with activated ester resins using solid-phase synthesis (Figure 1). Phytosphingosine template **2** is represented as the "**Core**" structure, and the solid phase loaded components are represented as the "**Tail**". The amidation reaction between the activated ester on the resin and the free amine group of the α-galactosylphytosphingosine allowed us to rapidly and conveniently prepare the initial 61-membered library.

Polystyrene nitrophenol resin **3** was prepared from commercially available polystyrene AM amino resin by coupling with 4-hydroxy-3-nitrobenzoic acid in the presence of *N*,*N*′-diisopropylcarbodiimide (DIC) and *N*-hydroxybenzotriazole (HOBt). Activated ester resins **4** were then prepared using either the acyl halide in pyridine or the carboxylic acid (by DIC/HOBt coupling reaction). The "**Tails**" were chosen from commercial sources to provide structural diversity. Reactions between **2** (**Core**) and the activated esters **4** were then carried out to make the library of α-GalCers (designated as YTC03, Figure 2). DMSO was used as the solvent due to the poor solubility of α-galactosylphytosphingosine **2**. Completion of the reaction was confirmed by TLC with phosphomolybdic acid (PMA) or ninhydrin staining. The reactions were generally complete after gently shaking the mixtures overnight, and the products were isolated for biological study by concentration of the reaction filtrates. Sixty-one α-GalCers that met our standards of purity (>90% by HPLC) were synthesized.

We carried out the initial analysis of NKT cell activation by the panel of α-GalCer analogues using the well-characterized, representative mouse NKT cell hybridoma, DN3A4-1.2, which provided a rapid and reproducible assay.[29] This hybridoma was originally established from $NK1.1^+$ thymocytes of a C57BL/6 mouse and expresses the canonical NKT T cell Vα14-α18 T cell receptor (TCR) α chain paired with Vβ8.2. When stimulated in vitro with relevant glycolipid ligands presented by CD1d, this hybridoma gives a dose-dependent increase in production of IL-2 that can be used to quantitatively assess the potency of TCR-dependent activation. Mouse CD1d-transfected RMA-S cells were pulsed with a range of concentrations of each glycolipid, washed, and then cultured with the DN3A4-1.2 hybridoma cells. As described in the Experimental Section, we modeled the dose response of IL-2 production by the hybridoma cells using a classic 4-parametric logistic equation and reported potency for NKT cell activation as the reciprocal of the glycolipid concentration giving 50% of the maximum achievable level of IL-2 production ($1/EC_{50}$).

As summarized in Figure 3, about half of the panel of α-galactosylceramides with *N*-acyl group substitutions showed activity above baseline, although these all showed reductions by at least one log unit relative to KRN7000. Analysis of the relative potencies of the compounds in this panel suggested a few clear structure–activity relationships. For example, the group of analogues which carried shortened acyl chains (from no carbons to 14) were recognized by hybridoma DN3A4-1.2 with $EC_{50} \sim 50$ nM, about 500 times less potent than that for DB04-1 (KRN7000). There was no clear correlation between potency and chain length for the range of chain lengths tested in this panel. One surprising result was the apparent lack of any detectable activity for the analogue with a 16-carbon *N*-acyl chain (YTC03-37). Goff et al. had seen modest cytokine stimulation with the same analogue,[25] and we suspect that our outcome may

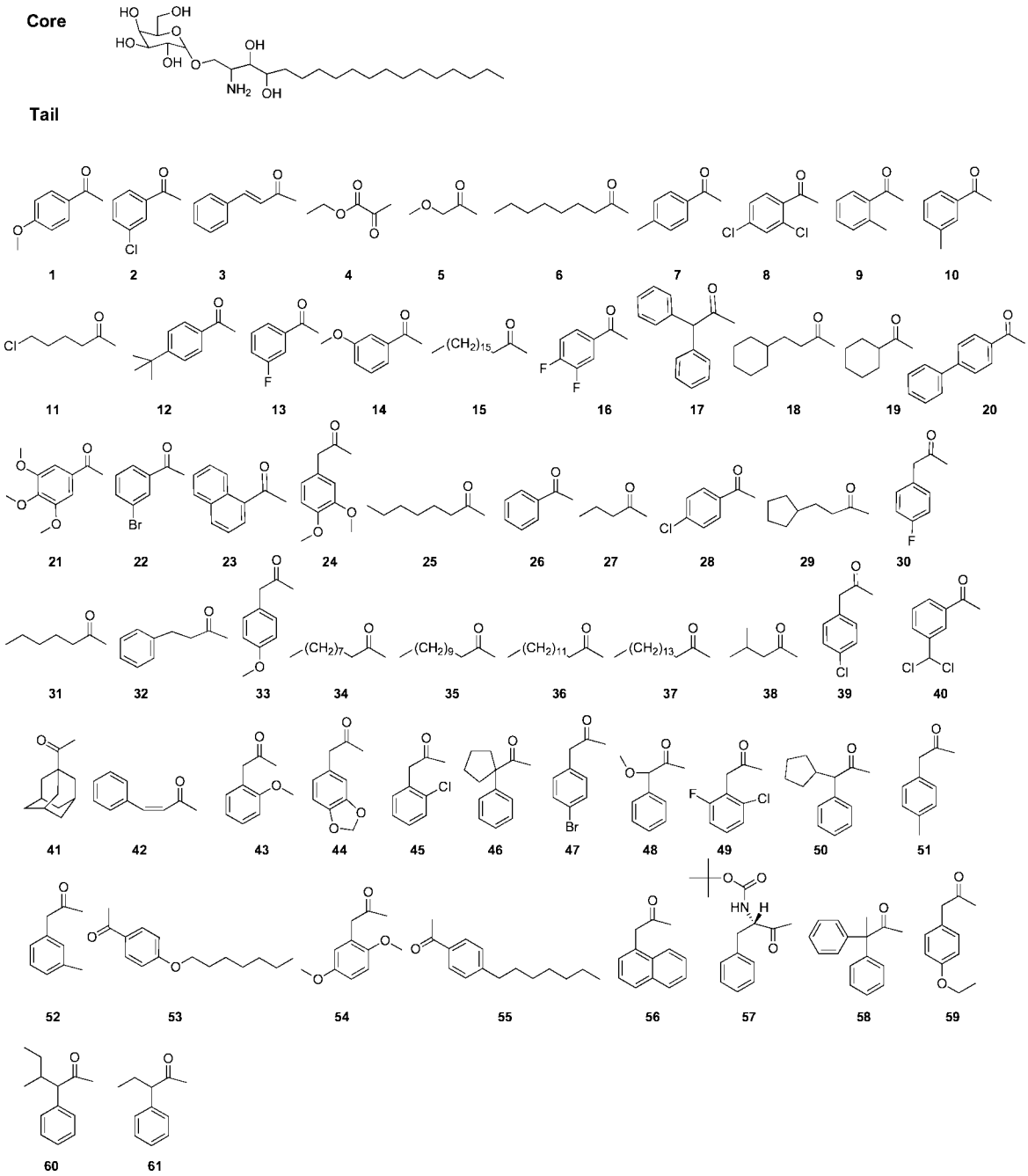


Figure 2. Core and tail components of α-GalCer library YTC03.

represent a technical limitation of our method for long-chain fatty acid tails (16 carbons or longer).[30] This may be due to the increased hydrophobicity of the product, leading to its retention in the resin matrix. TLC showed starting material consumption, and LC–MS confirmed the presence and purity of the product (but not the yield). However, precipitated material was observed in this case. An efficient solvent for recovery of the solid was not found, and such compounds may need to be prepared by conventional solution-phase chemistry. This problem was not seen with the shorter or aromatic acyl chains.

A striking correlation was observed for the NKT cell stimulating activity of the α-GalCers with aryl-substituted acyl chains. Most of the compounds that were variants of the benzoyl-*N*-substituted KRN7000 (Figure 3, benzoyl bracketed group) were not recognized by the hybridoma. However, with the aromatic ring moved one carbon atom away, a number of 2-phenylacetyl analogues were recognized with moderate potency. This was true even for the highly hindered compound with two phenyl groups attached to C2 of the acetyl substituent (YTC03-17), or for compounds where a phenyl group and a small acyl chain are attached to C2 (YTC03-60, -61). These findings were confirmed by a larger-scale independent synthesis of selected compounds (Figure 4) and point to substantial flexibility in the size limits of the lipid binding pockets of CD1d. That many aromatic substitutions could load, only if placed one carbon away from the amide bond, suggested that a limiting parameter would be the constriction of the narrow opening between the two alpha helices of CD1d.[31] A bulky moiety beyond the second carbon of the acyl chain would be past this constriction.

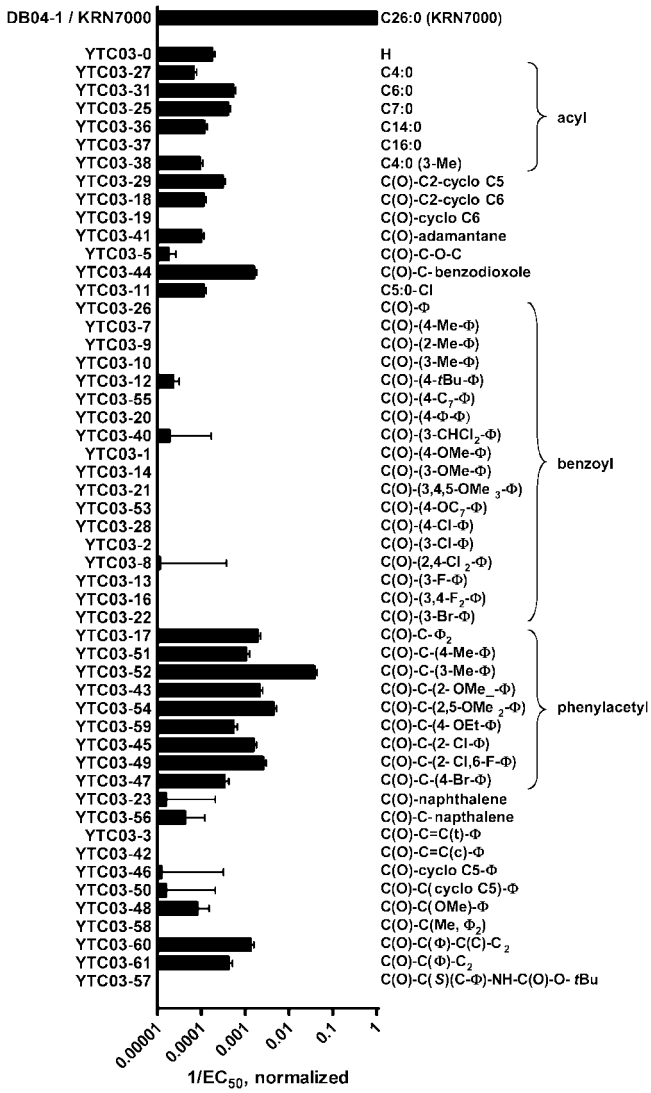


Figure 3. Potencies of 50 analogues in the YTC03 combinatorial panel for activation of the NKT hybridoma DN3A4-1.2 when presented by RMA-S.mCD1d cells.[29] Activities were determined using the DN3A4-1.2 hybridoma stimulation assay with measurement of IL-2 release. The relative potencies of the compounds are displayed as the reciprocal of the EC_{50} values, normalized to the $1/EC_{50}$ for the parent compound KRN7000. Median EC_{50} for KRN7000 in this experiment was 0.097 nM ($1/EC_{50} = 10.3$). Error bars represent standard errors of the log EC_{50} obtained by nonlinear regression of the dose–response curves for each analogue.

The utility of having access to a range of α-GalCers is further illustrated by results of an assay of the YTC03 library for its ability to induce the expansion of NKT cells from peripheral blood mononuclear cells (PBMC) of a normal human subject. As seen in Figure 5, the results were markedly different from those seen with the mouse hybridoma assay. The benzoyl compounds were generally more active than the phenylacetyl compounds, although it would be premature to make conclusions about substituent patterns. Interestingly, a significant number of compounds were more potent agonists than KRN7000 in this assay, including some, such as YTC03-7 and YTC03-40, that were at best minimally active in the mouse NKT hybridoma assay. This may reflect real differences between human and mouse CD1d and TCR structure or may be related to differences in the assays which measure proliferation in one case versus cytokine production

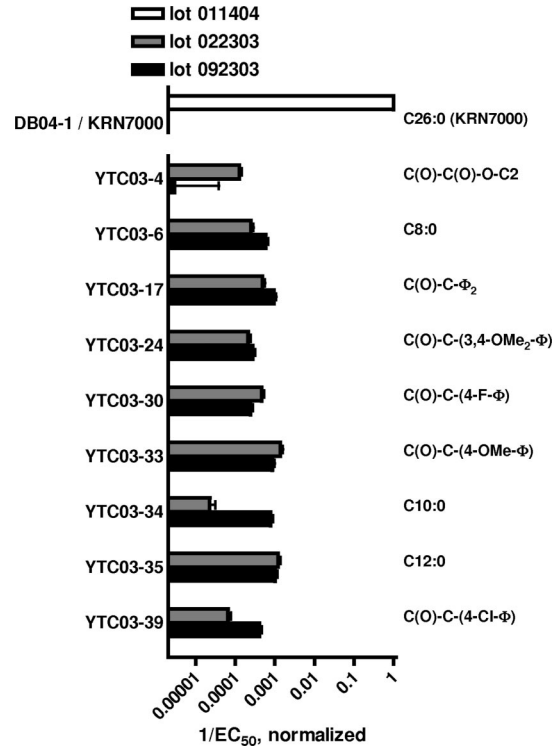


Figure 4. Analysis of biological potencies of selected analogues that were resynthesized on a larger scale. For each of the analogues indicated, a second synthesis was carried out to generate milligram amounts. The activities of these independently synthesized analogues (black bars) are shown together with the activities of the original samples produced by the combinatorial method (grey bars). Activities for all compounds were determined for direct comparison in one experiment using the DN3A4-1.2 hybridoma stimulation assay with measurement of IL-2 release and are normalized to the activity of KRN7000 (open bar). Median EC_{50} for KRN7000 in this experiment was 0.0136 nM ($1/EC_{50} = 73.4$). Error bars as described in Figure 3.

in the other. Considerably more studies will be needed to determine whether any of the compounds in this panel have properties that might make them superior to KRN7000 for particular applications. However, the analyses presented here confirm that some members of the combinatorial library have significant NKT cell stimulating activity in both the murine and human systems.

The number of active compounds in the α-GalCer library encouraged us to use the solid-phase approach to look at analogues of OCH, which had previously been reported as an activator of murine NKT cells that is able to selectively elicit the production of T_H2-type cytokines.[14] If these compounds stimulated IL-2 production, they would provide an excellent opportunity to look at cytokine biasing with previously unexplored acyl chain compounds. We chose glycosylated sphinganine template **5** rather than the corresponding phytosphingosine template because sphinganines are generally easier to prepare and are somewhat more soluble in typical organic solvents than the corresponding phytosphingosines. Moreover, we had previously demonstrated that the sphinganine analogues **6** and **7** of KRN7000 and OCH, respectively, elicited nearly the same cytokine levels as the parent phytosphingosine-containing α-GalCers when used to stimulate several mouse NKT cell hybridomas.[23]

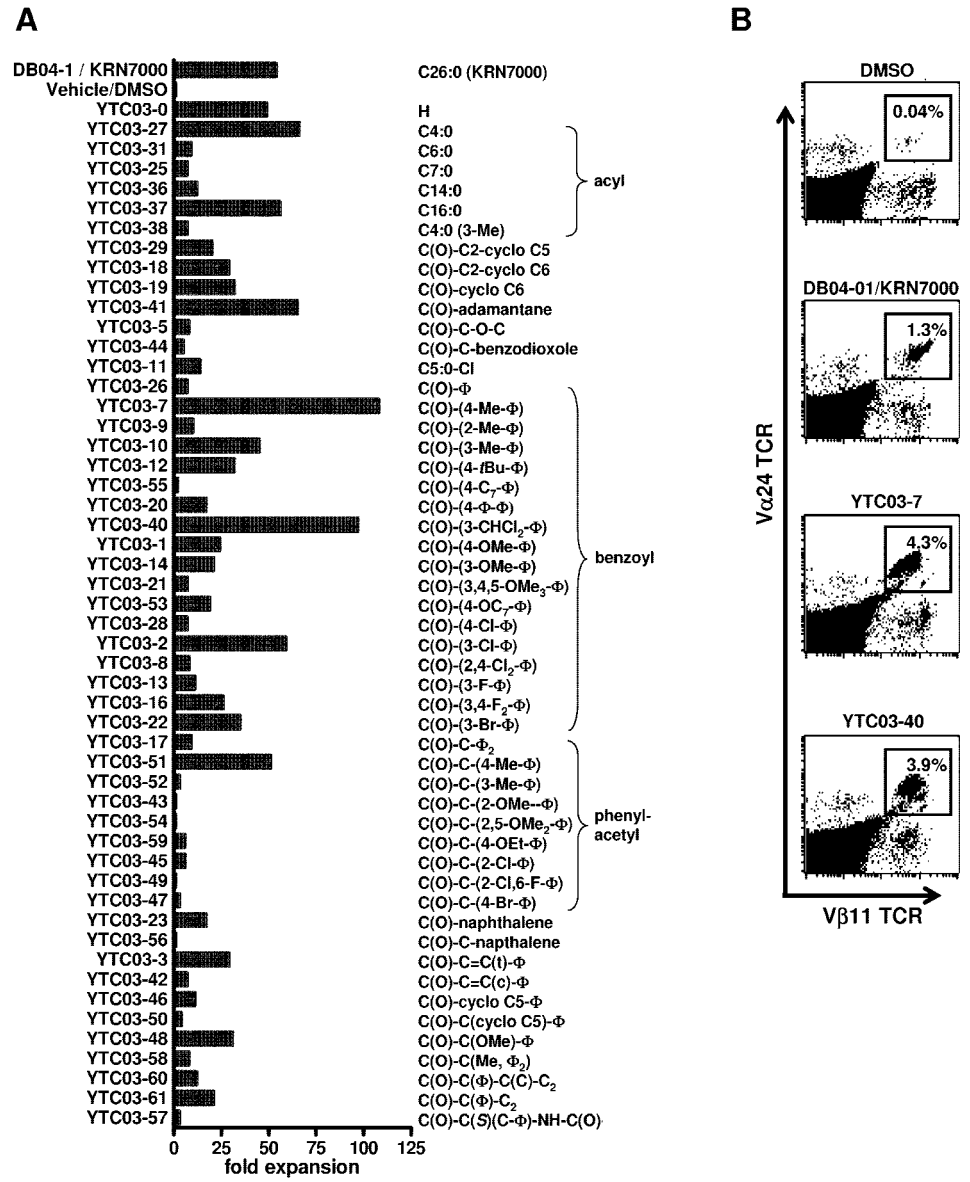
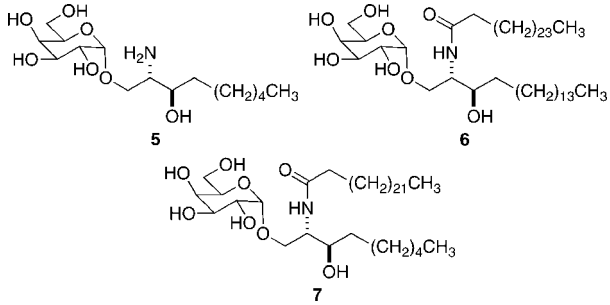


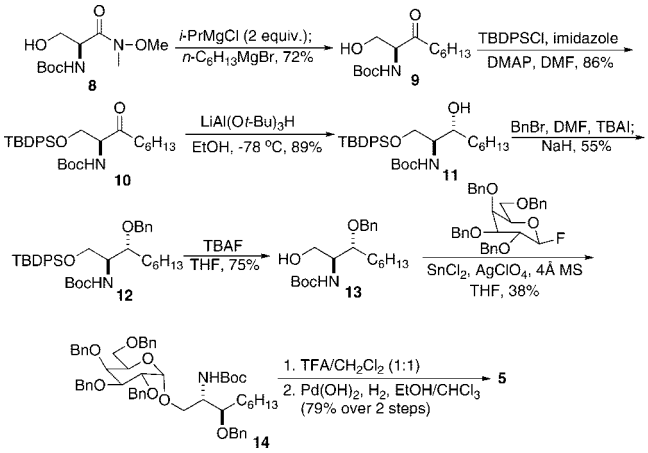
Figure 5. Potencies of 50 analogues from the YTC03 combinatorial panel for stimulating ex vivo expansion of human $V\alpha24^+/V\beta11^+$ NKT cells. Peripheral blood mononuclear cells (PBMC) from a normal healthy donor were incubated with each individual analogue at a final concentration of 100 nM. After 14 days of culture, NKT cell frequencies were determined by flow cytometry, and the fold expansion of NKT cells was calculated by comparison with vehicle control (A). NKT cell frequencies were defined as the percentage of $V\alpha24^+/V\beta11^+$ cells among gated lymphocytes, as shown in the representative panels on the right (B). Shown are staining of PBMC harvested after 14 days from cultures containing vehicle alone (DMSO), or 100 nM of KRN7000, YTC03-7, or YTC03-40, as indicated.



The sphinganine moiety of **5** was prepared (Scheme 1) from serine-derived Weinreb amide **8**, using methodology previously developed in our laboratory.[32] Double deprotonation of **8** followed by addition of *n*-hexylmagnesium bromide provided ketone **9**. Silyl protection of the alcohol, diastereoselective reduction of **10**, secondary alcohol benzylation, and cleavage of the silyl group provided glycosyl acceptor **13**. Glycosylation proceeded in modest yield, but with good selectivity. Template **5**, used for the construction of the second α-GalCer library, was secured by a two-stage deprotection.

The second α-GalCer library, YTC04, was prepared in the same manner as YTC03. Template **5** was more easily solubilized than glycosylated phytosphingosine **2**, and more α-GalCers (77) resulted. However, assessment of NKT cell stimulating activity using either the DN3A4-1.2 hybridoma assay (Supplementary Figure 1) or a normal mouse splenocyte culture system[26] (data not shown), revealed at most only trace levels of activity, indicating that they are either extremely weak or inactive as agonists for mouse NKT cells. NKT cells from peripheral blood mononuclear cells were not expanded by the YTC04 panel. Taken together, these

Scheme 1



results suggested that with the shortened C9 sphingoid base of the YTC04 library, there was substantially less tolerance for substitution of the long unsaturated acyl chain moiety with other shorter or more complex amide-linked tails. This may reflect the fact that with a C9 sphingoid chain, only part of the A' pocket of the lipid binding site in CD1d is occupied, and a relatively long and flexible fatty acyl group is therefore required to occupy the F' pocket of this site in order to impart sufficient binding of the α-GalCer to CD1d to lead to NKT cell activation.[31]

In conclusion, two 60+-membered α-GalCer libraries have been prepared by solid-phase protocols in a straightforward fashion, using two fully deprotected, glycosylated sphingoid bases with a range of acyl chains. The findings with respect to biological activities of these libraries were significant for several reasons, even though in our analysis using a mouse NKT cell hybridoma none of the compounds was as potent as KRN7000. For example, published studies of OCH have demonstrated that compounds of substantially lower potency than KRN7000 can have unique immunological properties of great potential significance in the mouse model, particularly if they cause a qualitative change in cytokine production or other effector activities of NKT cells. Like OCH, preliminary results in our laboratories indicate that compounds YTC03-04, -30, and -34 are T_H2-cytokine biasing compounds in the murine system,[33] and they are currently being investigated in greater depth. Moreover, the analogues in which galactosylphytosphingosine core **2** was coupled with derivatives of phenylacetic acid are also worthy of note, especially given that one of these compounds (YTC03-51) also showed strong expansion of human NKT cells. The tolerance of branching and aromatic structure one carbon removed from (but not directly adjacent to) the acyl carbonyl in the mouse system suggests further avenues for exploration. Along these lines, Wong and co-workers have reported that the attachment of a simple benzene to the end of chains with from 5 to 10 methylene units gave compounds that exhibited a T_H1 bias and greater potency than KRN7000 when used to activate human NKT cells.[27]

Recent studies have indicated major differences in the reactivity of human versus murine NKT cells to certain α-GalCer analogues such as OCH.[34] Indeed, our preliminary studies showed that several compounds in the YTC03 library may have strong activity for stimulating ex vivo expansion of human peripheral blood NKT cells, although most of these were actually extremely weak stimulators of the mouse NKT cell hybridoma that we studied. Our analysis of the mouse NKT cell response in the current study was limited to a single NKT cell hybridoma, so clearly it will be necessary to study the pattern of recognition of additional hybridomas or of primary mouse NKT cells to gain a full understanding of the biological activities of these combinatorial libraries. In addition, it will be of interest to examine responses obtained with other types of antigen presenting cells, which could potentially mediate processing events that are not revealed with CD1d transfected RMA-S cells that we used as APCs for mouse NKT cell assays in the current study. Further studies along these lines, and also potentially using murine splenocytes or intact mice for in vivo screening, will be essential to obtain a complete understanding of the biological activities of the α-GalCer libraries. The solid-phase approach demonstrated here would allow for rapid assembly of acyl chain varied α-GalCers that should further refine the understanding of how the acyl chain structure can influence the activation of NKT cells, the control of their profile of cytokine production, and other important effector functions.

Experimental Section

All chemicals were purchased from Sigma-Aldrich or Acros Organics and were used without further purification. The activated ester resins were prepared using anhydrous solvent and stored under anhydrous conditions to avoid possible hydrolysis. Completion of library reactions was monitored by staining with 10% PMA in ethanol solution or 10% ninhydrin in ethanol solution. Yield and purity were determined by LC–MS using a C18 column (20 × 4.0 mm) with 4 min elution using a gradient of 5–95% acetonitrile (containing 0.1% acetic acid)–water (containing 0.1% acetic acid), with UV detector at λ = 210, 230, 250 nm and an electrospray ionization source using negative mode. Melting points were determined in open Pyrex capillary tubes and are uncorrected. Infrared spectra were recorded on a 750 FT-IR spectrometer. ^{1}H NMR spectra were recorded at 400, 300, and 500 MHz with tetramethylsilane (TMS) as an internal standard. Chemical shifts (δ) are reported in ppm units, and coupling constants (J) are reported in Hertz. Abbreviations used are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. ^{13}C NMR spectra were recorded at 75, 100, or 125 MHz. Column chromatography was performed with 40 μm flash silica. For thin-layer chromatography for the intermediates involved in the preparation of template **5**, spots were visualized by UV, 10% molybdic acid in ethanol. or 0.5–1% potassium permanganate in water. Tetrahydrofuran was distilled from dark blue solutions of sodium/benzophenone. Methylene chloride was distilled from calcium hydride. Pyridine was distilled from KOH. Toluene was dried over MS (4 Å). Ethanol was dried over MS (4 Å).

Nitrophenol Polystyrene Resin 3. Commercially available polystyrene AM amino (1 g, 1.37 mmol/g) was suspended in DMF (10 mL) for 10 min before adding reagents. 4-Hydroxy-3-nitro-benzoic acid (1.6 g, 5 equiv),

DIC (1.26 mL, 5 equiv), and HOBt (1.3 g, 6 equiv) were added to the reaction solution. The reaction was complete after gentle shaking at room temperature overnight. The resin was filtered and washed with DMF (3 × 20 mL), methanol and DCM (5 × 20 mL) alternatively, and DCM (5 × 20 mL) and dried. It was then re-suspended in a 1:10 piperidine/DMF solution. The mixture was heated at 80 °C for 3 h before filtration, then washed and dried following the same procedure.

General Procedure of Preparing Activated Ester Resins Using Acyl Chlorides (1–43, 50). Activated ester resins **4** were prepared by a slight modification of literature procedures.[30,35] Nitrophenol resin **3** (1.0 g, 1.7 mmol) was suspended in NMP (10 mL). After 10 min, pyridine (1 mL, 12.4 mmol) and the appropriate acyl chloride (4.0 mmol) were added to the reaction mixture, and the mixture was shaken overnight at room temperature. The resin was filtered and washed with DMF (3 × 20 mL), methanol and DCM (5 × 20 mL) alternatively, and DCM (5 × 20 mL) and dried.

General Procedure for Activated Ester Synthesis Using Acid (44–49, 51–61). Diisopropylcarbodiimide (DIC, 1 mL, 6.4 mmol) was added to a mixture of nitrophenol resin **3** (1.0 g, 0.87 mmol), the appropriate acid (4.0 mmol), and HOBt (1.3 g, 6 equiv) in 1-methyl-2-pyrrolidinone (10 mL), and the mixtures were stirred overnight at room temperature. The resin was filtered, washed, and dried as before.

General Procedure for α-GalCer Synthesis. The activated ester resin (20 mg, 30 μmol) was added to a α-galactosyl phytosphingosine **2** (3 μmol) in DMSO (0.2 mL) and agitated on a shaker overnight at room temperature. Completion of the reaction was confirmed by TLC with negative PMA or ninhydrin staining. The reaction mixture was filtered and the solid was washed with DMSO (0.1 mL). The filtrate was then concentrated. The purity of product was tested by LC–MS before biological activity study. Only compounds of greater than 90% purity were assayed.

General Procedure for Scaled-Up α-GalCer Synthesis. The acyl chloride (2 eq) was added to a α-galactosyl phytosphingosine **2** (9 mg, 18.8 μmol) in THF/NaOAc (50% in H_2O) (3 mL, 1:1, v/v) and mixed 3 h at room temperature. The two layers were separated, and the aqueous layer was extracted with THF (3 × 1.5 mL). The organic extracts were combined and concentrated. Purification by flash chromatography on silica gel (chloroform/MeOH, 80:1 to 15:1) provided the target compounds as white solids (yield 67–78%).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-ethyloxalylamino)-1,3,4-octadecantriol (YTC03-4). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 4.93 (d, J = 3.7 Hz, 1H), 4.38–4.28 (m, 3H), 3.98–3.90 (m, 2H), 3.82–3.70 (m, 6H), 3.57–3.54 (m, 2H), 1.69 (m, 1H), 1.56 (m, 1H), 1.36 (t, J = 7.8 Hz, 3H), 1.27 (br s, 24H), 0.83 (t, J = 6.9 Hz, 3H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-octanoylamino)-1,3,4-octadecantriol (YTC03-6). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 4.88 (d, J = 3.7 Hz, 1H), 4.18 (m, 1H), 3.88–3.63 (m, 8H), 3.58–3.50 (m, 2H), 2.18 (t, J = 7.6 Hz, 2H), 1.66–1.49 (m, 4H), 1.24 (br s, 34H), 0.82 (m, 6H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-diphenylacetylamino)-1,3,4-octadecantriol (YTC03-17). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 7.30 (m, 10H), 4.95 (s, 1H), 4.82 (d, J = 3.7 Hz, 1H), 4.22 (m, 1H), 3.82 (dd, J = 10.6, 4.2 Hz, 1H), 3.75 (br d, J = 3.2 Hz, 1H), 3.73–3.58 (m, 4H), 3.54–3.40 (m, 4H), 1.58 (m, 1H), 1.47 (m, 1H), 1.22 (br s, 24H), 0.84 (t, J = 6.9 Hz, 3H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-[*N*-(3,4-dimethoxyphenyl) acetylamino]-1,3,4-octadecantriol (YTC03-24). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 6.87 (s, 1H), 6.83 (s, 2H), 4.82 (d, J = 3.7 Hz, 1H), 4.16 (m, 1H), 4.86–4.78 (m, 6H), 4.76–3.44 (m, 10H), 1.58 (m, 1H), 1.47 (m, 1H), 1.22 (br s, 24H), 0.85 (t, J = 6.9 Hz, 3H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-[*N*-(4-fluorophenyl)acetylamino]-1,3,4-octadecantriol (YTC03-30). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 7.20 (m, 2H), 6.96 (m, 2H), 4.82 (d, J = 3.7 Hz, 1H), 4.14 (m, 1H), 3.83–3.42 (m, 12H), 1.56 (m, 1H), 1.48 (m, 1H), 1.18 (br s, 24H), 0.82 (t, J = 6.9 Hz, 3H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-[*N*-(4-methoxyphenyl)acetylamino]-1,3,4-octadecantriol (YTC03-33). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 7.21 (d, J = 8.6 Hz, 2H), 6.84 (d, J = 8.6 Hz, 2H), 4.82 (d, J = 3.7 Hz, 1H), 4.18 (m, 1H), 3.83–3.40 (m, 15H), 1.61 (m, 1H), 1.51 (m, 1H), 1.22 (br s, 24H), 0.84 (t, J = 6.9 Hz, 3H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-decanoylamino)-1,3,4-octadecantriol (YTC03-34). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 4.87 (d, J = 3.7 Hz, 1H), 4.18 (m, 1H), 3.88–3.63 (m, 8H), 3.58–3.47 (m, 2H), 2.18 (t, J = 7.6 Hz, 2H), 1.66–1.49 (m, 4H), 1.24 (br s, 38H), 0.82 (m, 6H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-(*N*-dodecanoylamino)-1,3,4-octadecantriol (YTC03-35). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 4.86 (d, J = 3.7 Hz, 1H), 4.17 (m, 1H), 3.89–3.61 (m, 8H), 3.56–3.45 (m, 2H), 2.11 (t, J = 7.6 Hz, 2H), 1.68–1.50 (m, 4H), 1.24 (br s, 42H), 0.82 (m, 6H).

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Galactopyranosyl)-2-[*N*-(4-chlorophenyl)acetylamino]-1,3,4-octadecantriol (YTC03-39). ^{1}H NMR (300 MHz, $CDCl_3$–CD_3OD (2:1, v/v)) δ 7.28 (m, 4H), 4.826 (d, J = 3.7 Hz, 1H), 4.18 (m, 1H), 3.93–3.48 (m, 12H), 1.62 (m, 1H), 1.53 (m, 1H), 1.22 (br s, 24H), 0.88 (t, J = 6.9 Hz, 3H).

Preparation of Template 5. (2*S*)-Methyl[2-hydroxy-1-(methoxymethylcarbomyl)ethyl]carbamic acid *tert*-butyl ester (8). The protocol for the preparation of **8** was based on a procedure for the preparation of [2-hydroxy-1-(methoxymethylcarbomyl)ethyl]carbamic acid benzyl ester.[36] Boc-Serine (8.0 g, 39.0 mmol) was dissolved in dry CH_2Cl_2 (153 mL), and the solution was cooled to −15 °C under N_2. *N*,*O*-dimethylhydroxylamine hydrochloride (3.92 g, 40.0 mmol), followed by *N*-methylmorpholine (4.4 mL, 40.2 mmol), was added. After 5 min 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (7.7 g, 40.2 mmol) was added in five portions over 30 min. After stirring for 1 h at −15 °C, the reaction was quenched with HCl (1.0 M, 25 mL), and the layers were separated. The aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The combined organic extracts were washed with saturated $NaHCO_3$ (23 mL) and H_2O (23

mL), dried ($MgSO_4$), and concentrated to provide **8**[37] as a white solid (8.3 g, 86%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.71 (br s, 1H), 4.95 (br s, 1H), 3.82–3.78 (m, 5H), 3.23 (s, 3H), 2.90 (br s, 1H), 1.43 (s, 9H).

2*S*-2-(*N*-*tert*-Butoxycarbonyl)amino-1-hydroxynonan-3-one (9). (2S)-Methyl[2-hydroxy-1-(methoxymethylcarbomyl)-ethyl]carbamic acid *tert*-butyl ester (**8**) (4.2 g, 17 mmol) was dissolved in dry THF (33 mL) under N_2. The resulting solution was cooled to −15 °C, and *i*-propylMgCl (2.0 M in THF, 17 mL, 34 mmol) was added dropwise at −15 °C to afford a clear solution. After 5 min, hexylmagnesium bromide (0.63 M in THF, 35 mL, 22 mmol) was added at −15 °C. The resulting solution was stirred for 4 h at rt. The mixture was cooled to −20 °C, and HCl (1.0 M, 39 mL) was added, followed by EtOAc (30 mL). The two layers were separated, and the aqueous layer was extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were combined, washed with H_2O (50 mL), dried ($MgSO_4$), and concentrated. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 95:5 to 85:15) provided **9** as a pale yellow oil (3.4 g, 72%): $[\alpha]^{25}_D$ +36.3 (*c* 0.5, $CHCl_3$); IR (neat) 3422, 2931, 1709, 1500 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.67 (d, J = 5.6 Hz, 1H), 4.32 (br s, 1H), 3.94 (dd, J = 11.2, 3.2 Hz, 1H), 3.88 (dd, J =11.2, 3.6 Hz, 1H), 2.81 (br s, 1H), 2.63–2.48 (m, 2H), 1.62–1.55 (m, 2H), 1.44 (s, 9H), 1.34–1.21 (m, 6H), 0.87 (t, J = 6.4 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 208.3, 156.2, 80.4, 63.3, 61.8, 40.1, 31.7, 29.0, 28.5, 23.6, 22.6, 14.2; MS (EI) *m/z*: 200 ($M^+ - C_5H_{10}$), 160, 113, 104, 60, 57 (100); Anal. Calcd for $C_{14}H_{27}NO_4$: C, 61.51; H, 9.96; N, 5.12. Found: C, 61.70; H, 9.67; N, 5.01.

(2*S*)-2-(*N*-*tert*-Butoxycarbonyl)amino-1-*tert*-butyldiphenylsilanyloxynonan-3-one (10). A catalytic amount of DMAP was added to a solution of 2*S*-2-(*N*-*tert*-butoxycarbonyl)amino-1-hydroxynonan-3-one (**9**) (3.2 g, 11.7 mmol) and imidazole (2.39 g, 35.1 mmol) in dry DMF (5.8 mL) under N_2. After 10 min TBDPSCl (3.6 mL, 14.0 mmol) was added, and the mixture was stirred overnight at rt. The reaction mixture was diluted with saturated aqueous NH_4Cl (31 mL), and the aqueous layer was extracted with CH_2Cl_2 (3 × 30 mL). The organic extracts were combined, washed with H_2O (30 mL) and brine (30 mL), dried ($MgSO_4$), and concentrated. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 98:2 to 95:5) provided **10** as a colorless oil (5.1 g, 86%): $[\alpha]^{25}_D$ +38.2 (*c* 1.0, $CHCl_3$); IR (neat) 3433, 3072, 3049, 2956, 2931, 2858, 1710, 1491 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.63–7.37 (m, 10H), 5.54 (d, J = 8.0 Hz, 1H), 4.35 (t, J = 4.0 Hz, 1H), 4.05 (d, J = 4.0 Hz, 1H), 3.93 (d, J = 4.0 Hz, 1H), 2.53–2.47 (m, 2H), 1.61 (m, 2H), 1.46 (s, 9H), 1.31 (br s, 6H), 1.04 (s, 9H), 0.89 (t, J = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 207.7, 155.5, 135.7, 132.9, 130.1, 128.0, 79.8, 64.4, 61.3, 40.3, 31.7, 29.0, 28.5, 27.0, 26.8, 23.5, 22.6, 19.4, 14.2; Anal. Calcd for $C_{30}H_{45}NO_4Si$: C, 70.41; H, 8.86; N, 2.74. Found: C, 70.56; H, 8.84; N, 2.75.

(2*S*,3*R*)-2-(*N*-*tert*-Butoxycarbonyl)amino-1-*tert*-butyldiphenylsilanyloxynonan-3-ol (11). LiAl(O-*tert*-Bu)$_3$H (16.2 g, 63.6 mmol) was added to dry EtOH (89 mL) at −78 °C under N_2. A solution of (2*S*)-2-(*N*-*tert*-butoxycarbonyl)amino-1-*tert*-butyldiphenylsilanyloxynonan-3-one (**10**) (5.4 g, 10.6 mmol) in dry EtOH (89 mL) was added dropwise. After stirring for 5 h at −78 °C, the mixture was diluted with aqueous citric acid (10%, 260 mL), followed by CH_2Cl_2 (30 mL). The cooling bath was removed, and the mixture was stirred for 1.5 h at rt. The reaction mixture was extracted with CH_2Cl_2 (3 × 100 mL), and the combined organic extracts were washed with H_2O (100 mL) and brine (100 mL), dried ($MgSO_4$), and concentrated. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 95:5 to 90:10) afforded **11** as a colorless oil (4.7 g, 89%): $[\alpha]^{25}_D$ +10.3 (*c* 1.0, $CHCl_3$); IR (neat) 3448, 3072, 3049, 2929, 2858, 1699, 1502 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64–7.38 (m, 10H), 5.29 (d, J = 8.0 Hz, 1H), 3.93 (dd, J = 12.0, 4.0 Hz, 1H), 3.83 (d, J = 8.0 Hz, 1H), 3.69 (m, 1H), 3.58 (br s, 1H), 2.85 (br s, 1H), 1.51 (br s, 4H), 1.46 (s, 9H), 1.29 (br s, 6H), 1.08 (s, 9H), 0.89 (t, J = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.5, 135.8, 132.9, 132.7, 130.3, 130.2, 128.1, 128.1, 79.6, 74.1, 64.4, 54.8, 34.7, 32.0, 29.5, 28.7, 27.1, 26.1, 22.8, 19.4, 14.3; Anal. Calcd for $C_{30}H_{47}NO_4Si$: C, 70.13; H, 9.22; N, 2.73. Found: C, 69.98; H, 9.41; N, 2.68.

(2*S*,3*R*)-2-(*N*-*tert*-Butoxycarbonyl)amino-1-*tert*-butyldiphenylsilanyloxy-3-benzyloxynonane (12). (2*S*,3*R*)-2-(*N*-*tert*-Butoxycarbonyl)amino-1-*tert*-butyldiphenylsilanyloxynonane-3-ol (**11**) (4.0 g, 7.8 mmol) was dissolved in dry DMF (26 mL) under N_2. Tetrabutylammonium iodide (4.6 g, 12.5 mmol) was added, followed by benzyl bromide (1.4 mL, 12.5 mmol). The mixture was cooled to 0 °C, and NaH (60% in mineral oil, 0.37 g, 15.6 mmol) was added. The cooling bath was removed, and the mixture was stirred for 2 h at rt. The reaction mixture was quenched with saturated aqueous NH_4Cl (67 mL) and extracted with Et_2O (5 × 50 mL). The combined organic extracts were washed with H_2O (100 mL) and brine (100 mL), dried ($MgSO_4$), and concentrated. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 99:1 to 97:3) provided **12** as a colorless oil (2.6 g, 55%): $[\alpha]^{25}_D$ +6.3 (*c* 1.0, $CHCl_3$); IR (neat) 3450, 3070, 2956, 2929, 2858, 1718, 1496 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.57–7.16 (m, 15H), 4.64 (s, 1H), 4.44 (m, 2H), 3.80 (m, 2H), 3.66 (dd, J = 12.0, 4.0 Hz, 1H), 3.52 (d, J = 4.0 Hz, 1H), 1.42 (br s, 2H), 1.37 (s, 9H), 1.18 (br s, 8H), 1.00 (s, 9H), 0.81 (t, J = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.7, 138.8, 135.8, 133.5, 129.9, 129.9, 128.5, 127.9, 127.7, 79.1, 72.2, 63.1, 60.6, 53.9, 32.0, 30.7, 29.9, 29.7, 28.6, 27.1, 25.5, 22.8, 21.2, 19.5, 14.4; Anal. Calcd for $C_{37}H_{53}NO_4Si$: C, 73.59; H, 8.85; N, 2.32. Found: C, 73.68; H, 8.66; N, 2.40.

(2*S*,3*R*)-2-(*N*-*tert*-Butoxycarbonyl)amino-3-benzyloxynonan-1-ol (13). Tetrabutylammonium fluoride (1.0 M in THF, 7.6 mL, 7.6 mmol) was added to a solution of (2*S*,3*R*)-2-(*N*-*tert*-butoxycarbonyl)amino-1-*tert*-butyldiphenylsilanyloxy-3-benzyloxynonane (**12**) (2.3 g, 3.8 mmol) in THF (18 mL) under N_2 at 0 °C. The cooling bath was removed, and the mixture was stirred for 4 h at rt. The reaction mixture was concentrated, and the residue was dissolved in CH_2Cl_2 (50 mL), washed with brine (20 mL), dried ($MgSO_4$), and concentrated. Purification by flash chromatography on silica gel (petroleum ether/EtOAc 95:5 to 85:15) provided **13** as a pale yellow oil (1.0 g, 75%): $[\alpha]^{25}_D$ −30.2 (*c* 1.0, $CHCl_3$);

IR (neat) 3442, 3064, 3032, 2929, 2858, 1695, 1498 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.33 (m, 5H), 5.28 (d, J = 4.0 Hz, 1H), 4.64 (d, J = 11.4 Hz, 1H), 4.50 (d, J = 11.4 Hz, 1H), 3.98 (d, J = 12.0 Hz, 1H), 3.65 (m, 3H), 2.88 (d, J = 8.0 Hz, 1H), 1.70 (m, 1H), 1.45 (s, 9H), 1.29 (br s, 9H), 0.90 (t, J = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 156.3, 138.2, 128.8, 128.2, 128.1, 82.3, 79.7, 73.1, 62.6, 53.5, 31.9, 31.5, 29.9, 29.7, 28.6, 25.8, 22.8, 14.3; Anal. Calcd for $C_{21}H_{35}NO_4$: C, 69.01; H, 9.65; N, 3.83. Found: C, 69.40; H, 9.27; N, 3.74.

(2*S*,3*R*)-3-Benzyloxy-2-(*N*-*tert*-butoxycarbonylamino)-1-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)nonane (14). (2*S*,3*R*)-2-(*N*-*tert*-Butoxycarbonyl)amino-3-benzyloxynonan-1-ol (**13**) (0.12 g, 0.36 mmol) was dissolved in THF (6.7 mL) under Ar at rt. Stannous chloride (0.20 g, 1.09 mmol), silver perchlorate (0.23 g, 1.1 mmol), and freshly ground 4 Å molecular sieves (1.7 g) were added successively. The mixture was stirred for 30 min and cooled to −10 °C. A solution of β-tetrabenzylgalactosyl fluoride (0.30 g, 0.57 mmol) in THF (6.7 mL) was added. The mixture was warmed gradually to rt and stirred overnight. The reaction mixture was filtered through Celite, and the filter cake was washed with CH_2Cl_2. The filtrate was washed with brine (30 mL), dried ($MgSO_4$), and concentrated. The residue was purified by flash chromatography on silica gel (petroleum ether/EtOAc 95:5 to 85:15) to provide **14** as a clear oil (0.12 g, 38%): $[\alpha]^{25}_D$ +27.8 (*c* 1.0, $CHCl_3$); IR (neat) 3444, 3062, 2925, 2857, 1714, 1496, 1454 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.38–7.23 (m, 25H), 5.03–4.37 (m, 12H), 4.04 (dd, J = 12.0, 4.0 Hz, 1H), 3.93 (m, 4H), 3.80 (dd, J = 12.0, 4.0 Hz, 1H), 3.71 (dd, J = 12.0, 4.0 Hz, 1H), 3.58 (m, 1H), 3.49 (m, 2H), 1.49 (m, 2H), 1.43 (s, 9H), 1.26 (br s, 8H), 0.86 (t, J = 8.0 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 155.9, 139.0, 138.9, 138.2, 128.6, 128.6, 128.6, 128.5, 128.5, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 99.2, 79.3, 79.2, 78.8, 75.2, 75.0, 73.7, 73.5, 73.2, 72.3, 69.9, 69.3, 68.6, 53.1, 32.0, 30.8, 29.9, 29.8, 28.7, 25.3, 22.9, 14.3; Anal. Calcd for $C_{55}H_{69}NO_9$: C, 74.38; H, 7.83; N, 1.58. Found: C, 73.99; H, 7.50; N, 1.46.

(2*S*,3*R*)-1-(α-D-Galactopyranosyl)-2-aminononan-3-ol (5). A solution of TFA:CH_2Cl_2 (1:1, 1.7 mL) was added to a solution of (2*S*,3*R*)-3-benzyloxy-2-(*N*-*tert*-butoxycarbonylamino)-1-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl) nonane (**14**) (0.21 g, 0.24 mmol) in CH_2Cl_2 (4.0 mL) under N_2 at rt. The mixture was stirred for 30 min then neutralized to pH approx. 8 with saturated aqueous $NaHCO_3$ (80 mL). The reaction mixture was stirred for 30 min and extracted with CH_2Cl_2 (50 mL) and $CHCl_3$ (50 mL). The combined organic extracts were concentrated to provide the amine as a pale yellow oil which was dissolved in EtOH (14 mL) and $CHCl_3$ (4.0 mL). $Pd(OH)_2$ (20% on carbon, 1.1 g) was added, and stirring was continued vigorously for 24 h under H_2 (1 atm). The mixture was filtered through Celite, and the filter cake was washed with $CHCl_3$ and MeOH. The filtrate was concentrated, and the residue was purified by flash chromatography on silica gel (CH_2Cl_2/MeOH/NH_4OH 85:10:5 to 75:20:5) to provide **5** as a white solid (56 mg, 79%): $[\alpha]^{25}_D$ +2.2 (*c* 1.0, CH_3OH:$CHCl_3$, 1:1); ^1H NMR (400 MHz, CD_3OD) δ 4.87 (br s, 6H), 4.04–3.69 (m, 8H), 3.54 (m, 1H), 3.30 (m, 1H), 1.49 (br s, 2H), 1.28 (br s, 8H), 0.90 (t, J = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, CD_3OD) δ 101.1, 73.2, 71.4, 71.2, 70.4, 70.3, 65.4, 63.1, 57.1, 34.4, 33.0, 30.4, 27.2, 23.8, 14.5; Hrms (FAB) calcd for $C_{15}H_{31}NO_7$ (M^+ + H) *m/z*: 337.2101. Found: 337.2085.

Materials and Methods for Biological Assays. Cell Lines. The RMA-S.mCD1d cell line, a C57BL/6 strain mouse thymoma line transfected to give high expression of mouse CD1d, was provided by S. Behar (Brigham and Women's Hospital, Harvard Medical School).[38] The NKT cell hybridoma DN3A4-1.2 was provided by M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA).[29] Both cell lines were maintained in RPMI-1640 medium containing the following supplements (complete RPMI): 10 mM HEPES, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 55 μM 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco-BRL, Grand Island, NY) and 10% heat-inactivated (55 °C, 30 min) fetal bovine serum (FBS, Gemini Biological Products, Calabasas, CA). Cells were cultured in a 37 °C humidified incubator with 5% CO_2.

NKT Cell Hybridoma Stimulation Assays. For in vitro NKT cell activation studies, glycolipids were dissolved to a concentration of 100 μM in 100% DMSO (Fisher Biotech, Fairlawn, NJ). RMA-S.mCD1d antigen-presenting cells were plated in 96-well flat-bottom tissue culture-treated plates at 50,000 cells/well with varying concentrations of glycolipids ranging from 100 nM to 5×10^{-2} nM, typically for 6 h at 37 °C in 100 μL of complete RPMI. Cells were washed three times by suspending them in PBS and pelleting by centrifugation (430 g for 5 min). DN3A4-1.2 hybridoma cells (50,000 cells/well) were then added in 100 μL of medium, and the cultures were incubated at 37 °C in a 5% CO_2 humidified incubator. Supernatants were collected after 20 h and assayed for IL-2 by capture ELISA. Prism 4.02 software (Graphpad Software, Inc., San Diego, CA) was used to analyze IL-2 ELISA data and to determine relative potencies of synthetic glycolipids, as previously described.[26] Standard curves for IL-2 ELISA, and dose response curves for IL-2 production in response to each glycolipid, were generated with nonlinear regression using the 4-parameter logistic equation. Potencies of glycolipids for activation of NKT cell hybridomas DN3A4-1.2 were compared using EC_{50} values (i.e., the concentration of glycolipid giving 50% of the maximum hybridoma IL-2 secretion response), and relative potencies were displayed as the reciprocal of the antilog of the log EC_{50} reported by the Prism program (i.e., $1/EC_{50}$).

In vitro Expansion of Human NKT Cells. Peripheral blood mononuclear cells (PBMC) were prepared from a randomly selected normal human donor blood by centrifugation on ficoll-hypaque. PBMC were distributed at 1 million cells per well in flat-bottom 96-well plates in 250 μL of RPMI-1640 medium with 10% FCS and individual glycolipids at 100 nM final concentration. Cells were harvested after 14 days of incubation and were stained with FITC-labeled anti-Vα24 and PE-labeled anti-Vβ11 antibodies (Clones C15 and C21 respectively, Immunotech, Marseille, France). Cells gating as lymphocytes based on forward and wide-angle light scatter were analyzed for fluorescence using a FACSCalibur flow cytometer (BD Biosciences), and the percentages of cells coexpressing both Vα24 and Vβ11 were determined

to quantitate NKT cells. The fold expansion of NKT cells was calculated by dividing percentage of NKT cells in PBMC cultures containing a particular glycolipid divided by the percentage of NKT cells in PBMC cultures containing vehicle only (DMSO, 0.02% final concentration).

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Supporting Information Available. Table summarizing the activities of all YTC03 compounds and acyl tail structures and biological evaluation of YTC04 library. This material is available free of charge via the Internet at http://pubs.acs.org.

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