Diversity-Oriented Synthesis of a Molecular Library of Immunomodulatory α-Galactosylceramides with Fluorous-Tag-Assisted Purification and Evaluation of Their Bioactivities in Regard to IL-2 Secretion

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Abstract: Structural variants of α-galactosylceramide (α-GalCer) that stimulate invariant natural killer T (iNKT) cells constitute an emerging class of immunomodulatory agents in development for numerous biological applications. Variations in lipid chain length and/or fatty acids in these glycerolipids selectively trigger specific pro-inflammatory responses. Studies that would link a specific function to a structurally distinct α-GalCer rely heavily on the availability of homogeneous and pure materials. To address this need, we report herein a general route to the diversification of the ceramide portion of α-GalCer glycolipids. Our convergent synthesis commences from common building blocks and relies on the Julia–Kocienski olefination as a key step. A cleavable fluorous tag is introduced at the non-reducing end of the sugar that facilitates quick purification of products by standard fluorous solid-phase extraction. The strategy enabled the rapid generation of a focused library of 61 α-GalCer analogs by efficiently assembling various lipids and fatty acids. Furthermore, when compared against parent α-GalCer in murine cells, many of these glycolipid variants were found to have iNKT cell stimulatory activity similar to or greater than KRN7000. ELISA assaying indicated that glycolipids carrying short fatty N-acyl chains (1fc and 1ga), an unsubstituted (1fh and 1f) or CF3-substituted phenyl ring at the lipid tail, and a flexible, shorter fatty acyl chain with an aromatic ring (1ge, 1gf, and 1gg) strongly affected the activation of iNKT cells by the glycolipid-loaded antigen-presenting molecule, CD1d. This indicates that the method may benefit the design of structural modifications to potent iNKT cell-binding glycolipids.

Keywords: α-GalCer; ceramide; glycolipids; fluorous tag; olefination

1. Introduction

Natural killer T (NKT) cells are lymphocytes that show characteristics specific to T cells and classical NK cells and are considered critical to the innate and adaptive arms of the immune system [1,2]. In contrast to conventional CD4+ and CD8+ T cells that recognize peptide antigens bound to major histocompatibility complex (MHC) class I or II molecules, NKT cells recognize endogenous and exogenous glycolipid antigens that bind to the lipid-antigen-presenting glycoprotein molecule, CD1d [3,4]. Several synthetic and natural antigens of the invariant NKT (iNKT) cell have been defined, of which the most studied is KRN7000 (1a, Figure 1), an α-galactosylceramide (α-GalCer) discovered through structure–activity relationship (SAR) studies of marine-sponge-derived glycolipids [5]. The KRN7000-bound glycolipid CD1d binary complex engages in the activation of iNKT cells to
stimulate antigen-presenting cells (APCs), releasing cytokines resulting in the modulation of the immune response cascade [6]. These activated iNKT cells simultaneously secrete pro-inflammatory T helper type 1 (Th1) and T helper type 2 (Th2) cytokines [7]. α-GalCer exhibits potent anti-tumor activity by immunomodulation and has been shown to have various immunological stimulation activities in many immunotherapies [8]. However, the production of cytokines is related to the structures of the α-GalCer analogs, and some of them are mutually inhibitory. Moreover, serine-based synthetic α-GalCer glycolipid analogs are known as agonists, inducing a unique macrophage activation by providing costimulatory signals, for example, via lipopolysaccharide (LPS) receptor and toll-like receptor 4 (TLR4) [9,10]. Therefore, development of a glycolipid that can stimulate a specific immune response and produce a greater amount of specific cytokines is an attractive approach for immunotherapy [11].

Many structural analogs of α-GalCer based on the modification of KRN7000 were found to be potent immunomodulatory agents that act by CD1d-dependent iNKT cell stimulation [12]. SAR studies of α-GalCer analogs include two major classes of modification; variation in the hydrophilic polar carbohydrate head group that directly interacts with the T-cell antigen receptor (TCR) and variation in the acyl chain and the sphingoid base structures of the ceramide (Cer) moiety responsible for CD1d binding [13]. The long fatty acyl chain (C26:0) and phytosphingosine (Psp) chain of the Cer moiety in α-GalCer interact with many amino acid residues in the hydrophobic lipid-binding grooves of CD1d, A' pocket and F' pocket, respectively, resulting in the formation of a stable glycolipid–CD1d binary complex [6,14]. Thus, the fatty acyl chain of α-GalCer plays an important role in eliciting selective cytokine production. For example, an α-GalCer analog with a shorter length of Psp of Cer, commonly referred to as OCH (1b), switches the activity to a more selective Th2 response [15]. In addition, the introduction of double bonds, such as di-unsaturated C20 fatty N-acyl chain (C20:2) α-GalCer analog, leads to a bias toward Th2 responses [16]. Further introduction of a phenyl (such as 1c) [17] or para-fluoro phenyl ring to the fatty acyl chain strongly enhances the production of Th1 cytokines and has been demonstrated as a prototype for adjuvant development [18]. Longer hydrophobic lipid chains of Psp also selectively induce Th1 cytokines [19]. A polar amide group on the truncated fatty acyl chain of α-GalCer retains binding to CD1d and Th2-selective cytokine production [20]. Moreover, a Th1-selective CD1d-binding iNKT cell ligand, C34 (1d), bearing a nonpolar para-F substituted aromatic moiety in the lipid chain, was reported; it is also used as an adjuvant in breast cancer vaccines [21]. The stereochemistry of Psp was also reported to be important for the activity of α-GalCer. The change of chiral center of 2S, 3S, 4R-Psp resulted in loss or a significant decline in CD1d protein binding activity [22]. Further exploration of modifications of the pyranose ring, carba-analogs of α-GalCer [23], and 5-S-KRN7000 [24], and enzymatic hydrolysis-resistant glycolipids, such as α-C-GalCer [25], show potential in stimulating innate immune responses in mice that are biased toward induction of Th1 responses. A diether-containing α-GalCer analog was reported to show Th17 selectivity with improved IL-17 secretion [26].

![Figure 1. Structures of α-galactosylceramide (KRN7000, 1a) and its analogs (1b–1d).](image-url)
Overall, synthetic analogs that enhance CD1d protein–α-GalCer complex stability by noncovalent interaction, such as fatty acid tails containing aromatic rings, tend to stimulate a TH1-like immune response, while those that lower the stability of the CD1d-α-GalCer complex, such as truncation of the Psp chain of Cer of α-GalCer, induce a TH2 bias due to its short retention time on the surface of CD1d. Therefore, it may be possible to provide versatility to the generation of new α-GalCer analogs for SAR study by constructing an α-GalCer scaffold that allows post-glycosylation modifications both at the fatty acyl tail and Psp backbone derivatization of the natural C18 chain by olefination with virtually any olefination reagent [12].

Due to its wide range of biological activities, numerous strategies for the synthesis of α-GalCer and its derivatives have been developed. Most common methods involve the stereoselective union of a variety of glycosyl donors with a Psp acceptor [12,27]. Although iodides, phosphates, and other anomic leaving groups were employed for glycosylation with Psp, glycosyl trichloroacetimidates and aryl thioglycosides were widely used donors because of their popularity in preparative carbohydrate chemistry and the mild conditions required for their activation [28–33]. In addition to the use of amino acids [34] and the Chiron approach [35], the Psp moiety can also be obtained from cheap substrates, such as sugars and other chiral starting materials. For example, Garner’s aldehyde [36] and galactose-derived aldehyde [37] have been reacted with Wittig’s or Grignard’s reagents to give different lengths of Psp derivatives, which were then glycosylated with galactosyl donors and coupled with fatty acids. Unfortunately, the unavailability of Wittig and Grignard reagents in many cases limits structural modification of Psp chains. Olefin cross-metathesis has also been used as a method for producing Psp [38] and has been successfully applied on C- and O-allyl galactosides for direct construction of the Cer backbones; however, the method necessitates additional stereoselective epoxidation followed by epoxide opening steps to obtain the correct Psp configuration [39]. D-Lyxose, which has the requisite stereochemistry of all chiral centers required for Psp, is the most suitable precursor [40,41]. While such strategies have proven to be useful for a multitude of applications, these methods also have limitations; using them to synthesize structural analogs of α-GalCer leads to labor-intensive and time-consuming purification steps due to the linear synthetic scheme, and they do not allow easy access to a large library of α-GalCer analogs. Thus, the development of a more efficient and less time-consuming synthetic strategy is still necessary to speed up the purification steps and may facilitate the discovery of new immuno-adjuvants.

In recent years, fluorous-based techniques have shown great promise in solution-phase chemical synthesis. Phase-tag-based separation are highly effective in facilitating product purifications [42]. In particular, substrates attached to a linear and light fluorous tag such as a perfluorooctyl (C₈F₁₇) or perfluorohexyl (C₆F₁₃) tail can easily be separated from nonfluorous materials by fluorous solid-phase extraction (F-SPE). Recently, the technique has been used for enzymatic synthesis of sialic acid containing glycans [43,44], human milk oligosaccharides [45], and chemical synthesis of oligosaccharides [46,47]. Furthermore, fluorous tags themselves are relatively unreactive and are invisible in ¹H NMR spectroscopy. The latter feature has an additional advantage in that reducing signals from the tag helps in facilitating compound characterization. Since F-SPE only differentiates between fluorous-tagged and non-tagged substances, it resembles filtration more than chromatography, and the compounds in an entire library can be purified by the same protocol.

We envisaged that a combination of Julia–Kocienski olefination and fluorous technique would rapidly assemble a small compound library based on the modification of Psp and fatty acid moieties of α-GalCer glycolipids for SAR studies. More specifically, it would be an attractive way to speed up purification of the synthetic intermediates after the glycosylation step. In this article, we report a diverse route to construct α-GalCer glycolipid analog 1 (Scheme 1) by modification of its Psp tail via Julia–Kocienski olefination and fatty acid chain addition by amide bond formation. The cleavable C₈F₁₇ fluorous tail at the non-reducing end of galactose allows for introducing rigidifying elements in the lipid
chains of Cer, thus providing ready access to various structurally related analogs. As a preliminary biological evaluation study, we examined only the functional reactivities of murine NK1.2 cells to iNKT cell antigen KRN7000 and an extended panel of α-GalCer glycolipid analogs to trigger interleukin-2 (IL-2) production. Finally, we demonstrated that some of these synthetic α-GalCer glycolipids possess greater activities for the stimulation of murine iNKT cells than their parent, KRN7000, highlighting the utility of this straightforward synthetic strategy in derivatizing substituents of the target compound to facilitate SAR study.

**2. Results and Discussion**

The retrosynthetic analysis for the synthesis of α-GalCer and its analogs (1) with the fluorous-tag-assisted methodology is illustrated in Scheme 1. The construction of the Cer portion was implemented in the late steps of the synthesis by introducing fatty acids through amide bond formation with the amine intermediate pre-masked as an azide (3), whose olefinic linkage was formed by Julia–Kocienski olefination using sulfone 4 with aldehydes. Compound 4 was obtained by a stereoselective glycosylation reaction of the relatively simple alcohol template 6 and 4,6-O-benzylidene-protected p-tolyl-1-thio-β-D-galactoside donor 5, whose cyclic acetal protection could be replaced by a fluorous tag later. Importantly, the presence of a cyclic 4,6-O-benzylidene acetal protecting group on galactosyl donors ensured α-stereoselectivity in the glycosylation [37]. On the other hand, the Psp chain precursor, benzothiazolyl sulfide (SB₇) derivative 6 was designed to be readily sourced from D-lyxose-derived alcohol using the Mitsunobu reaction. The strategy outlined in Scheme 1 is appealing for the generation of a library because the core structure 4 can be used to generate diverse analogs through the olefination reaction, and fatty acids can later be assembled in a modular fashion. Although the modified Julia–Kocienski olefination [48] has been reported for α-C-analogs of KRN7000 [49], to the best of our knowledge, a merger of the Julia–Kocienski olefination and fluorous-tag-assisted purification for creating a small α-GalCer glycolipid library with a variety of lengths in both the Psp and the acyl chain has not been examined.

The synthesis of SB₇ derivative 6a is shown in Scheme 2. Commercially available D-lyxose (7) was transformed to lactol 8, using our previously developed procedures [40]. NaBH₄-mediated reduction of lactol 8 produced 1,4-diol intermediate 9 (94%), which was readily converted to benzoate ester 10 by a temperature-controlled regioselective benzylation (benzoyl chloride, pyridine, 0 °C) at the primary hydroxyl group with high yield (89%). The secondary hydroxyl group in 10 was transformed to an azide by the
Mitsunobu reaction with diphenylphosphoryl azide (DPPA) with complete inversion at the reacting carbon center, and the basic removal of the benzoate ester produced the alcohol 11 (78% over two steps). The primary alcohol in 11 was then transformed to the tosylate 12; however, attempts to achieve the S_{n}2 substitution with 2-mercaptobenzothiazole (HSBr) under basic conditions were fruitless. Therefore, tosylate 12 was heated to reflux in neat triethyl phosphate to give 73% yield of the phosphonate derivative 13. Disappointingly, in a typical Horner–Wadsworth–Emmons olefination using undecanal and LiHMDS at −78 °C, the phosphonate 13 produced an intractable mixture of products. The Mitsunobu reaction of 11 with HSBr gave the corresponding sulfide 6a with 84% yield. Oxidation of 6a to the corresponding benzothiazol-2-yl sulfone 14 was readily achieved upon treatment with meta-chloroperoxybenzoic acid (m-CPBA) in dichloromethane in the presence of NaHCO_{3} to give 14 with 85% yield. While the strategy for synthesizing 6a was straightforward, in the initial examination into generating the Psp backbones, the sulfone 14 could not provide a satisfactory yield of Julia–Kocienski olefination (see Table 1 below). Therefore, we explored the less sterically hindered sulfone 15 for the synthesis of Psp.

Scheme 2. Synthesis of mercaptobenzothiazole (SBT) derivative 6a and its conversion to sulfone 14.

Table 1. Julia-Kocienski olefination reaction of sulfones (14 and 15) with undecanal.

| Entry | Substrate | Base | Temp (°C) | Product | Yield (%) |
|-------|-----------|------|-----------|---------|-----------|
| 1     | 14        | NaH  | 0         | 19      | 33        |
| 2     | 14        | NaH  | 78        | messy   | –         |
| 3     | 14        | LiHMDS | 0        | 19      | 20        |
| 4     | 14        | KHMD | 0         | 19      | 13        |
| 5     | 14        | KOH  | 0         | 19      | 30        |
| 6     | 14        | DBU  | 0         | 19      | 25        |
| 7     | 15        | NaH  | −78       | 20      | 53        |
| 8     | 15        | LiHMDS | 0       | 20      | 78        |

To reduce the steric hindrance, 6b, which bears a longer carbon chain, was prepared (Scheme 3). In our previous study [41], a Wittig reaction between 8 and a stabilized ylide Ph_{3}P = CHCO_{2}Me gave an undesired intramolecular cyclization adduct by Michael addi-
tion as the major product. Fortunately, after detailed investigation and careful optimization of the reaction conditions (Table S1), we found that an 85% yield of 16 (E/Z = 5/2) was obtained by changing the reaction solvent to CHCl₃ and performing the reaction at 50 °C. The alkene (16), without further purifications, was then routinely transformed to the saturated intermediate azide 17 by following established procedures [41]. Subsequent reduction of the methyl ester of 17 with DIBAL-H at 0 °C gave alcohol 18 (78% yield), which, upon reaction with HSB₆, afforded the sulfide 6b with a yield of 87% (Scheme 3). Sulfide 6b was then oxidized with m-CPBA to provide the corresponding sulfone 15 with a good yield (73%).

![Scheme 3. Synthesis of SBT derivative 6b and its conversion to sulfone 15.](image)

For the initial evaluation of the synthesis of Psp backbone by Julia–Kocienski olefination, as shown in Table 1, a relatively simple sulfone substrate 14 was selected as the target molecule for olefination. The bases and temperature were examined first using a carbonyl compound in a low-polarity solvent, THF (entries 1–6, Table 1). Accordingly, 14 was deprotonated with a base (NaH, LHMDS, KHMDS, KOH, or DBU) followed by addition of undecanal, but this resulted in a disappointing yield of the desired olefin 19 (13% to 33%) and some aldol condensation products. The low yield of the olefination in truncated sulfone 14 was likely due to the steric hindrance imparted by the structurally rigid protecting group with a cyclic acetal moiety (acetonide) adjacent to α-carbon of sulfone, which resulted in the difficulty of proton abstraction by a base. As a result, large amounts of starting material (14) remained unreacted, as determined by TLC.

Next, the Julia–Kocienski olefination reaction of a three-carbon spacer sulfone 15 was investigated. As with the investigation of truncated sulfone 14, the use of NaH as a base at −78 °C in the olefination of 15 with undecanal gave a moderate yield (53%) of the corresponding E-olefinic compound 20 (entry 7, Table 1). The reaction progressed smoothly with an improved outcome, and we obtained a 78% yield (trace amounts of Z-isomer formed, which was separated by simple column chromatography) with LiHMDS (entry 8, Table 1). This indicated that steric crowding was considerably eased in sulfone 15, and that sufficient reactivity for efficient coupling could be retained.

On the basis of the above observation, the acetonide protection in 6b was converted to an acyclic ether derivative 6c to further reduce the steric effect, enhance the yield of olefination, simplify the post-glycosylation deprotection steps, and increase the ease of purification (Scheme 4). We anticipated that benzyl ethers would shorten one step of deprotection when compared with acetonide, as the former could be removed simultaneously with double bonds under hydrogenolytic debenzylation conditions. Thus, we accomplished cleavage of the acetonide protection in 6b with camphor sulfonic acid (CSA) in MeOH–CHCl₃ solution, generating the triol (21) with concomitant removal of the trityl protecting group with 83% isolated yield. The trityl ether at the primary hydroxyl group in 21 was reinstalled by reacting with trityl chloride for 8 h to provide 22 (85%), which was then treated with BnBr and NaH to produce 23 with 92% yield. Finally, the trityl ether was removed under acidic conditions to afford the target acceptor 6c (77% yield).
Before installing a fluorous tag, the α-galactosylation of the newly designed alcohol acceptor 6c was conducted (Scheme 5). Stereoselective formation of α-galactopyranosyl linkages such as those present in α-GalCer is considered challenging because of the absence of neighboring group participation [50]. The 1,2-cis-glycosides can be formed stereospecifically under thermodynamic control conditions and by using C2 nonparticipating groups, typically benzyl ether [51]. Galactosyl iodides are particularly attractive among commonly used glycosyl donors as they are known to undergo exclusive glycosylation with electron-rich lipid acceptors; they have been adopted in the synthesis of KRN7000 analogs [52]. Thus, the iodide donor (25), generated in situ from 24 (1.2 equiv.), was reacted with 6c at 90 °C in toluene, using TBAI as a promoter to provide 45% yield of the α-glycoside 26 (δH-1α = 4.90 ppm, J = 3.5 Hz) after column chromatography. However, only a slightly higher yield of the α-glycoside 29 (δH-1α = 4.93 ppm, J = 3.3 Hz) was obtained when a 4,6-O-benzylidene-protected iodide donor (28) was used in the glycosylation reaction (53%, two steps). It is noteworthy that a significant amount of hydrolyzed side product was observed in these galactosylation reactions, presumably because of the instability of iodide donors. Therefore, we decided to proceed with the galactosylation of 6c acceptor with the more stable 4,6-O-benzylidene-protected thioglycoside donor 5 (see below).

Thiogalactosyl 5, with its 4,6-O-benzylidene protection, is a thermally stable donor and is known to favor the generation of α-glycosidic bonds [37]. Thus, the glycosylation reaction between acceptor 6c and donor 5 was performed by employing N-iodosuccinimide (NIS)/silver triflate as the promoter/activating reagent system in CH2Cl2/THF/ether at −30 °C to give 29 with 72% yield of α-anomer and a selectivity of 5:1 (α:β, total yield of 87%), as shown in Scheme 6. It should be noted that the use of CH2Cl2 as solvent yields the product only with an α-anomer but with a slight decrease of yield to 60%. Hydrolysis of the 4,6-O-benzylidene acetel in 29 was readily achieved by using CSA to afford 4,6-diol 30 (with 81% yield). The direct reaction between 30 and heavy fluorous tag perfluorinated benzaldehyde 31 in the presence of acid only provided a low product yield. Fortunately, the use of 1-dimethoxymethyl-4-(1H,1H,2H,3H)-perfluoroundecyloxycarbene, which was generated in situ from 31 using trimethyl orthoformate in the presence of catalytic amounts of p-TsOH under conditions reported by the Takeuchi group [53], gave a 63% yield.
of $^1$benzylidene acetal 32. The reaction yield can be further improved to 85% by the addition of molecular sieves (AW-300) in CH$_3$CN/HFE-7100 (1:1, v/v) as co-solvent. Oxidation of sulfide 32 under conditions (m-CPBA, NaHCO$_3$) similar to those described above was straightforward in generating benzothiazolyl sulfone 4 with 94% yield. Notably, crude mixtures were quickly purified by an F-SPE cartridge with multiple washes using MeOH/H$_2$O (4:1) and 100% MeOH to elute out the desired fluorous-tagged glycosides (32 and 4). Typically, one cycle of fluorous-assisted purification takes less than 20 min to afford a relatively pure product.

![Scheme 6. Synthesis of the core structure, sulfone 4, for Julia–Kocienski olefination.](image-url)

As shown in Scheme 7, the Julia–Kocienski olefination was then applied to modify the Psp backbone on sulfone 4 by using various alkyl aldehydes to give olefins 33–41 as a mixture of two geometrical isomers (predominantly E-isomer) with yields of 67–93%, except for compound 41 (38%). Although the minor Z-isomer could not be separated at this stage, this was unnecessary since the ensuing steps, including azide reduction and installation of the fatty acyl chain, progressed smoothly. After a quick passage through F-SPE, the final decoration was accomplished by the chemoselective reduction of the azide in 33–41 to an amine via Staudinger reduction using PMe$_3$ in THF/H$_2$O followed by HBTU-mediated amide bond formation with nine different fatty acids (a–i) to produce $\alpha$-GalCer analogs 42–49 in the protected form with 82–97% yields (Table S2) [54]. Finally, acidic removal of 4,6-$^1$benzylidene acetal and catalytic hydrogenation with Pd(OH)$_2$ on carbon in MeOH/CH$_2$Cl$_2$ under an atmospheric pressure of H$_2$ was implemented to remove all the benzyl ethers and reduce the double bond in 42–49 to afford a total of 61 members of an $\alpha$-GalCer glycolipid library (ordinarily, the yield is higher than 70% over two steps for 42–49) including KRN7000 (1a) and its prototype 1c. The structures of all synthesized $\alpha$-GalCer analogs are listed in Table 2.

It is important to note that purification by F-SPE on FluroFlash$^\text{®}$ silica gel greatly facilitated the separation of fluorous tagged glycans from the non-fluorous mixture. The operational time for purification substantially decreased from hours to within 20 min when compared to traditional chromatographic purifications, resulting in a high purity of greater than 95% (as determined by $^1$H NMR analysis). However, products from the peptidic coupling require multiple purifications because of the presence of many reagents and other side products in the reaction mixture. Thus, the intermediates were not fully characterized, and only the final product spectra were collected and assigned. Importantly, after final cleavage, the fluorous tag was recycled with a recovery yield of 80% to 97%.
We noted in the case of 41 that the yield of olefination was low because of the rapid decomposition of 3-(thiophen-2-yl)-propanal. In the later deprotection step, it also proved difficult to achieve the selective removal of benzyl ether protecting groups under catalytic hydrogenolysis conditions in substrates containing the para-chloro phenyl moiety, 36, since the para-chloro phenyl moiety was reduced to an unsubstituted benzene ring. To circumvent this problem, an alternative strategy was applied by conducting global deprotection.

**Scheme 7.** Construction of an α-GalCer glycolipid library (1).

**Scheme 8.** A modified strategy for the synthesis of α-GalCer analogs (1bc.bd–1hc.hd).

We noted in the case of 41 that the yield of olefination was low because of the rapid decomposition of 3-(thiophen-2-yl)-propanal. In the later deprotection step, it also proved difficult to achieve the selective removal of benzyl ether protecting groups under catalytic hydrogenolysis conditions in substrates containing the para-chloro phenyl moiety, 36, since the para-chloro phenyl moiety was reduced to an unsubstituted benzene ring. To circumvent this problem, an alternative strategy was applied by conducting global deprotection first and then installing the fatty acyl chain, as shown in Scheme 8. Thus, a chlorophenyl or thiophene moiety could be incorporated into the fatty acid. Accordingly, compounds 33–35 and 37–40 were subjected to catalytic hydrogenolysis using Pearlman’s catalyst to simultaneously reduce azide, double bond, benzyl ether, and 4,6-O-benzylidene protecting groups in one step. The resulting amines were then coupled with 3-(thiophen-2-yl)-propanoic acid N-succinimidyl ester and 4-chlorobenzoic acid N-succinimidyl ester to provide corresponding α-GalCer analogs (1bc.bd–1hc.hd; Table 1) with overall yields of 65–79% (for two steps).
### Table 2. The structures and chemical yields of α-GalCer analogs (1) \(^{a}\).  

| R \(= \) R' = | (CH3)2CH2 | (CH3)2CH3 | (CH3)2CH2Ph | (CH3)2Ph | (CH3)2Ph | (CH3)2Ph | (CH3)2CH4CF3 | (CH3)2CH2CH2CH2Cl |
|---|---|---|---|---|---|---|---|---|
| C7H15 | 1ba (79) | 1ab (79) | 1aa (58) | 1ac (67) | 1ad (65) | 1ae (66) | 1af (79) | 1ag (81) |
| C15H31 | 1bb (72) | 1ab (89) | 1ac (77) | 1ad (72) | 1ae (72) | 1af (69) | 1ag (70) | 1ah (46) |
| C7H15 | 1bc (78) * | 1bb (78) | 1ba (73) | 1bc (76) | 1bd (75) * | 1be (73) | 1bf (67) * | 1bg (65) * |
| C9H19 | 1bd (77) * | 1bc (77) | 1be (77) | 1bd (77) | 1bc (77) | 1be (77) | 1bf (76) * | 1bd (77) * |
| Cl | 1be (74) | 1bc (74) | 1bd (74) | 1be (74) | 1bc (74) | 1bd (74) | 1be (74) | 1bc (74) |
| 3Cl | 1bf (70) | 1be (70) | 1bd (70) | 1be (70) | 1bc (70) | 1bd (70) | 1be (70) | 1bc (70) |
| Cl | 1bh (72) | 1be (72) | 1bd (72) | 1be (72) | 1bc (72) | 1bd (72) | 1be (72) | 1bc (72) |
| 5F | 1bi (71) | 1be (71) | 1bd (71) | 1be (71) | 1bc (71) | 1bd (71) | 1be (71) | 1bc (71) |

\(^{a}\) Isolated yields are shown in parentheses based on the transformation of 33–41 to 1a–1hi (for four steps).  
\(^{\ast}\) Obtained from the modified procedure (Scheme 8).  

We used V\(\alpha\)14-expressing murine NK1.2 (mNK1.2) cells and mCD1d-expressing A20 (CD1d-A20) cells to evaluate the bioactivities of the synthetic α-GalCer analogs, including glycolipids of the 1b, 1c, 1d, 1e, 1f, 1g, and 1h series (Table 2). As an initial biological study, ELISA assaying was used to determine the level of IL-2 secreted by murine NK1.2 cells upon stimulation by each glycolipid (1 \(\mu\)M). Fujimoto et al. also characterized IL-2 and INF-γ cytokine secretion level produced by the NKT cells upon stimulation by α-GalCer glycolipid ligands containing polar groups in the acyl chain [55]. As shown in Figure 2, glycolipids of the 1b, 1c, and 1d series were unable to stimulate NK1.2 cells. Each glycolipid in the 1e series (1ea: 496.7 ± 63.7, 1eb: 380 ± 131.4, 1ef: 363.3 ± 70.8, 1eg: 375 ± 65, 1eh: 526.7 ± 46.5, and 1ei: 575 ± 21.8) had activity comparable to 1a (α-GalCer) except 1ec (1ec: 161.7 ± 98.7 and 1ee (0, p < 0.0001)). Among the glycolipids in the 1f series, 1fa (770 ± 52.7, 1fh (805 ± 45), and 1fi (710 ± 57.7) were superior to 1a in stimulation of NKT cells. Five glycolipids [1fb (343.3 ± 20.2), 1fc (0, p < 0.0001), 1fd (0, p < 0.0001), 1ff (205 ± 65.4), and 1fg (0, p < 0.0001)] induced less IL-2 production than 1a. Meanwhile, glycolipid 1fe (531.7 ± 30.1) induced similar levels of IL-2 as 1a. Among the glycolipids in the 1g series, five [1ga (763.3 ± 171.8), 1ge (798.3 ± 24.7), 1gf (656.7 ± 47.3), 1gg (780 ± 86.8), and 1gh (645 ± 52.2)] exhibited greater heightened cell stimulatory activity than 1a, but three showed comparable activities [1gb (393.3 ± 38.8), 1gd (356.7 ± 83.3), and 1gi (470 ± 52.2)] and 1gc showed no activity (0, p < 0.0001). Most of the glycolipids in the 1h series displayed no (1ha, 1hb, 1hc, 1hd, and 1hf: 0, p < 0.0001) or lower IL-2-stimulating activities [1he (58.3 ± 20.8, p < 0.0001) and 1hh (320 ± 40.9)] when compared with 1a, except for 1hg (470 ± 18) and 1hi (398.3 ± 56.9).
These results suggest that possessing a shorter [1b series ((CH$_2$)$_3$CH$_3$)] or longer [1c series ((CH$_2$)$_9$(CH$_3$)] sphingosine chain than 1a [(CH$_2$)$_3$CH$_3$] might impair NKT-stimulatory activity, and that the Psp chain length in α-GalCer might be important for NKT cell activation. It has been reported that the lengths of the alkyl chain affect the stability of CD1d-glycolipid bound complexes, resulting in a modulated NKT cell TCR-binding affinity [56]. The non-stimulating activity of the synthetic 1b series glycolipids, containing shorter lipid chain length, is likely due to the formation of less stable ternary CD1d-glycolipid-iNKT cell TCR complex [57]. Notably, 1c series glycolipids possessing with 25 carbon in the sphingoid base chain abolished activity toward IL-2 cytokine production, possibly longer lipid chain cannot localize to the F’ pocket in the CD1d hydrophobic groove [6]. Moreover, in Psp chains with a terminal phenyl ring, the short carbon chain length [(CH$_2$)$_6$Ph, 1e series] appear to have less NKT-stimulating activity than the (CH$_2$)$_9$Ph containing 1f series glycolipids. Shorter chain [(CH$_2$)$_4$Ph] derivatives in the 1d series showed no activity, demonstrating spacer length was too short for binding interaction in the hydrophobic F’ pocket. However, further analysis by molecular dynamic simulations were essential to support the conclusion. Although the analog 1ea with a truncated acyl chain (C$_7$H$_{15}$) showed activity comparable to that of 1a, the corresponding glycolipid 1fa showed much higher levels of IL-2 production. Introduction of a terminal phenyl ring with a suitable spacer length can dramatically enhance overall cytokine secretion [17]. The results obtained from glycolipids 1eb, 1ei, 1fh and 1f are consistent with this model. The 4-fluorophenyl ring attached to acyl chain containing analog also demonstrated stronger cytokine response relative to α-GalCer [17]. Analog 1gf only induced a notable increase in IL-2 production among 1ef, 1ff, 1gf and 1hf α-GalCers. Notably, the addition of the CF$_3$ functional group (1g series) to the phenyl group (1e series) achieved increased bioactivity relative to the 1e series glycolipids. Furthermore, the IL-2 production levels of most of the 1g series glycolipids were equal or greater than that of α-GalCer (1a). In addition, the carbon chain length of the acyl chains with a phenyl group might slightly affect their ability to activate NKT cells.

![Figure 2](image)

Figure 2. Bioactivity of α-GalCer and its analogs. A20 cells overexpressing mCD1d were loaded with 1 μM of α-GalCer (1a) and indicated glycolipids (from the 1b, 1c, 1d, 1e, 1f, 1g, and 1h series), and then co-cultured with murine NK1.2 cells for three days. ELISA assaying was used to determine IL-2 in the supernatant produced by glycolipid-stimulated mNK1.2 cells. All values are expressed as mean ± SD. * p < 0.05, *** p < 0.001, and **** p < 0.0001 vs. 1a by one-way ANOVA with Dunnett’s multiple comparisons test. Ctrl: DMSO.

In this work, we primarily focused on the development of a robust synthetic route to lipid-modified α-GalCer glycolipids. Even though some of the synthetic glycolipids in the 1f and 1g series showed stronger IL-2 release relative to KRN7000 (1a), we are unable to identify T$_{H1}$/T$_{H2}$-selective cytokine responses with our α-GalCer analogs. Therefore, an in-depth study is required to determine the cytokine-biased glycolipid ligands. However,
these efforts may provide useful guidelines for rational ligand design strategy in the context of CD1d-binding glycolipid ligands.

3. Materials and Methods

Materials and reagents. All reactions were performed in oven-dried glassware (120 °C) under a nitrogen atmosphere unless indicated otherwise. All chemicals were purchased as reagent grade and used without further purification. Dichloromethane (CH₂Cl₂) was distilled over calcium hydride. Tetrahydrofuran (THF) and ether were distilled over sodium metal/benzophenone ketyl radical. Anhydrous N,N-dimethylformamide (DMF) and methanol (MeOH) were purchased from Merck. Molecular sieves (MS) for glycosylation were MS 4Å (Aldrich) and activated by flame. ¹H and ¹³C NMR spectra were recorded on either a Bruker AV-400 or AV-600 spectrometer operating at 400 or 600 MHz for ¹H and 100 or 150 MHz for ¹³C, respectively. Chemical shifts (δ) are reported in ppm and referenced to the deuterated solvent used (chloroform-d (CDCl₃), δ 7.24 and 77.0; methanol-d₄ (CD₂OD), δ 3.31 and 49.0; and acetone-d₆ (CD₃)₂CO, 2.05, and 29.84 and 206.26), with coupling constants (J) reported in Hz. Two-dimensional (COSY) experiments were used to assist in assignment of the products. High-resolution mass spectra were recorded under ESI-TOF mass spectroscopy conditions. Analytical thin-layer chromatography (TLC) was performed on pre-coated plates (silica gel 60). Silica gel 60 (E. Merck) was employed for all flash-chromatography experiments. All fluorous-assisted purification was performed using FluoroFlash® SPE cartridge (Fluorous Tech. Inc., USA). The reactions were monitored by examination under UV light (254 nm) and by staining with p-anisaldehyde, ninhydrin, cerium molybdate, or potassium permanganate solutions.

Synthesis of α-GalCer glycolipids library (1). The azides (33–41) were converted to amines by Staudinger reduction, and the resultant amines were subsequently reacted with 9-different fatty acids. In a typical procedure, an azide (1.0 eq.) was dissolved in THF:H₂O (1 mL, 1:1 v/v) to which a 1M solution of PME₃ in THF was added (2.5 eq.). After stirring at rt for 12 h or until the azide was completely consumed, the solution was evaporated under reduced pressure. The residue was subjected to a F-SPE cartridge to get the desired amine. A solution of amine (1 eq.) in anhydrous DMF (3 mL) was treated with acid (1.5 eq.), HBTU (1.2 eq.), and diisopropylethylamine (1.5 eq.) at 0 °C. The reaction mixture was stirred at rt for 16 h or until the azide was completely consumed, then concentrated under reduced pressure and extracted with EtOAc (3 × 10 mL). The combined organic extracts were washed with brine, dried over MgSO₄, and concentrated in vacuo. The residue was purified by a F-SPE cartridge (70% aqueous MeOH to 100% MeOH elution) to afford pure amides (42ai–49ai).

General procedure for the final deprotection of compounds 42ai–49ai. The fully unprotected glycolipid library was constructed by two deprotection steps: the hydrolysis of benzylidene acetal was performed using CSA following a similar method as described for 30, and subjected to reductive hydrogenolysis to remove benzyl ethers and reduce unsaturation in phytosphingosine chain. In a typical experiment, CSA (1.0 eq.) was added to a solution of corresponding 4,6-O-β-benzylidene derivative (1 eq.) in MeOH/CHCl₃ (1/1, 2 mL) at rt with vigorous stirring. After 8 h, the reaction mixture was quenched with Et₃N, concentrated under reduced pressure, and used without further purification. To a solution of above crude product in MeOH/CH₂Cl₂ (1/1, 2 mL), 10% Pd(OH)₂ (10 w/w %) was added. The resultant mixture was degassed and saturated with a balloon filled with H₂ gas and left stirring at rt for 8 h under a positive pressure of H₂. The catalyst was removed by filtration through celite, rinsed with MeOH. The combined filtrates were evaporated in vacuo, and subjected to flash column chromatography on silica to afford pure α-GalCer glycolipids (1).
J = 4.7, 10.4 Hz, 1H), 4.87 (d, J = 3.6 Hz, 1H). 13C NMR (150 MHz, CD3OD): δ 9.4, 14.5, 23.8, 23.9, 27.1, 27.2, 30.3, 30.5, 30.6, 33.0, 33.2, 33.3, 37.4, 48.1, 62.9, 68.4, 70.4, 71.2, 71.6, 72.7, 73.1, 75.7, 101.3, 176.0. HRMS (ESI-TOF) m/z calcd for C24H27NO4Na [M+Na]+ 516.3143, found 516.3140.

Compound 4. To a solution of 32 (170 mg, 0.21 mmol) in CH2Cl2 (20 mL) was added a solution of NaHCO3 (88 mg, 1.05 mmol) and followed by addition of MCPBA (36 mg, 0.63 mmol). The resulting solution was stirred at rt for 16 h. The solution was diluted with CH2Cl2, and quenched with saturated Na2S2O3 solution. The residue was filtered, washed with CHCl3, and concentrated in vacuo. The residue was dissolved in DMF (2 mL) and loaded onto an F-SPE cartridge. The cartridge was then successively washed with MeOH/H2O (4:1) to remove any non-fluorous materials and then 100% MeOH to elute out the desired pure sulfone 4 (163 mg, 94%) as a colorless oil. Rf 0.6 (n-hexanes/ EtOAc = 2/1). δ 11.65 = +25.76 (c = 1.5, CH2Cl2). 1H NMR (400 MHz, CDCl3): δ 1.61–1.98 (m, 4H), 2.04–2.11 (m, 2H), 2.23–2.36 (m, 2H), 3.39 (m, 2H), 3.53–3.57 (m, 2H), 3.61 (dt, J = 3.7, 7.5 Hz, 1H), 3.66 (dd, J = 6.2, 10.4 Hz, 1H), 3.73 (dd, J = 3.7, 6.8 Hz, 1H), 3.88 (dd, J = 1.5, 12.8 Hz, 1H), 3.94 (dd, J = 3.3, 10.4 Hz, 1H), 3.96 (dd, J = 3.1, 10.0 Hz, 1H), 4.01–4.06 (m, 1H), 4.02 (t, J = 6.1 Hz, 2H), F1a8CH2CH2CH2O, 4.07 (dd, J = 3.4, 10.0 Hz, 1H), 4.16 (d, J = 3.1 Hz, 1H), 4.36 (d, J = 11.4 Hz, 1H), 4.53 (d, J = 11.4 Hz, 1H), 4.53 (d, J = 11.2 Hz, 1H), 4.59 (d, J = 11.2 Hz, 1H), 4.63 (d, J = 11.8 Hz, 1H), 4.72 (d, J = 12.3 Hz, 1H), 4.78 (d, J = 12.3 Hz, 1H), 4.85 (d, J = 11.8 Hz, 1H), 4.93 (d, J = 3.4 Hz, 1H), 5.40 (s, 1H), 6.85 (m, 2H), 7.18–7.42 (m, 2H), 7.60 (doublet of quintet, J = 1.5, 7.7 Hz, 2H), 7.99 (m, 1H), 8.17 (m, 1H). 13C NMR (100 MHz, CDCl3): δ 18.8, 20.7, 28.1, 28.3, 54.7, 61.5, 63.2, 66.5, 68.3, 69.4, 72.1, 72.2, 73.8, 74.1, 74.6, 75.5, 76.0, 77.8, 78.5, 99.3, 101.1, 114.2 (2C), 122.5, 125.6, 127.7, 127.8 (2C), 128.4 (2C), 128.6 (4C), 131.0, 136.9, 137.9, 137.9, 138.8, 138.9, 152.9, 159.2, 165.9. HRMS (ESI-TOF) m/z calcd for C26H23F7N4O12S2Na [M+Na]+ 659.2121, found 659.2131.

Compound 6b. Disisopropyl azodicaboxylate (DIAD, 0.38 mL, 1.86 mmol) was added dropwise to a stirred solution of 18 (750 mg, 1.54 mmol), and PPh3 (470 mg, 1.86 mmol) in anhydrous THF (30 mL) at 0 °C under an atmosphere of nitrogen. Subsequently, 2-mercaptobenzothiazole (312 mg, 1.86 mmol) was added and the resulting solution was allowed to warm to rt over 5 h. EtOAc was then added, and the reaction mixture was sequentially washed with brine and water and then dried and concentrated in vacuo. Purification via flash silica gel column chromatography gave 6b as a yellow oil (850 mg, 87%). Rf 0.65 (n-hexanes/EtOAc = 4/1). δ 11.65 = +5.83 (c = 3.5, CH2Cl2). 1H NMR (400 MHz, CD2Cl2): δ 1.23 (s, 3H), 1.24 (s, 3H), 1.66–2.06 (m, 4H), 3.36 (dd, J = 7.3, 10.1 Hz, 1H), 3.49 (t, J = 7.2 Hz, 2H), 3.56 (dd, J = 2.5, 10.1 Hz, 1H), 3.66 (ddd, J = 2.5, 7.3, 9.1 Hz, 1H), 4.02 (dd, J = 7.5, 9.1 Hz, 1H), 4.24 (dd, J = 3.4, 5.7, 10.1 Hz, 1H), 7.26–7.54 (m, 54 (m, 15H); 13C NMR (100 MHz, CDCl3): δ 25.8, 26.6, 28.2, 28.6, 33.5, 50.6, 64.6, 73.9, 77.4, 87.5, 108.6, 121.1, 121.7, 124.4, 126.2, 127.3 (3C), 128.1 (6C), 128.9 (6C), 135.4, 143.9 (3C), 153.5, 167.1. HRMS (ESI-TOF) m/z calcd for C36H38N4O12S2Na [M+Na]+ 859.2121, found 859.2131.

Compound 6c. CSA (500 mg, 2.15 mmol) was added to the solution of 23 (1.67 g, 2.15 mmol) in MeOH/CHCl3 (1/1, 40 mL). After being stirred at rt for 8 h, the reaction mixture was quenched with Et3N and then extracted with EtOAc (3 × 20 mL). The combined extracts were washed with brine, dried over MgSO4, and concentrated in vacuo. The residue was purified by flash silica gel column chromatography to yield 6c (1.2 g, 77%). Rf 0.25 (n-hexanes/EtOAc = 4/1). δ 11.65 = +11.33 (c = 2.67, CH2Cl2). 1H NMR (400 MHz, CDCl3): δ 1.76–2.00 (m, 4H), 2.13 (brs, 1H), 2.38 (t, J = 6.3 Hz, 2H), 2.65 (dd, J = 4.8, 9.6 Hz, 1H), 3.71 (m, 2H), 3.78 (dd, J = 5.3, 11.6 Hz, 1H), 3.85 (dd, J = 4.6, 11.6 Hz, 1H), 4.54 (d, J = 11.3 Hz, 1H), 4.60 (d, J = 11.3 Hz, 1H), 4.66 (d, J = 11.3 Hz, 1H), 4.70 (d, J = 11.3 Hz, 1H), 7.25–7.41 (m, 12H), 7.73 (d, J = 8.1 Hz, 1H), 7.83 (d, J = 8.1 Hz, 1H). 13C NMR (100 MHz, CDCl3): δ 25.0, 28.9, 33.4, 62.3, 63.2, 72.3, 73.7, 78.3, 79.7, 120.9, 121.4, 124.2, 126.0, 127.9, 128.0 (3C), 128.2 (2C), 128.5 (2C), 128.6 (2C), 135.2, 137.5, 137.6, 153.3, 168.8. HRMS (ESI-TOF) m/z calcd for C25H31N4O12S2 [M+H]+ 535.1832, found 535.1839.
Compound 15. To a solution of 6b (850 mg 1.27 mmol) in CH₂Cl₂ (20 mL) at rt was added solid NaHCO₃ (534 mg, 6.36 mmol) and then MCPBA (660 mg, 3.82 mmol). The reaction mixture was stirred at rt for 16 h. The solution was diluted with CH₂Cl₂, and then washed with saturated aq. Na₂SO₃ and saturated aqueous NaHCO₃. The aqueous layer was extracted with CH₂Cl₂, and the combined organic layers were dried over MgSO₄, filtered, and concentrated in vacuo. The residue was purified by flash chromatography to yield sulfone 15 (652 mg, 73%) as a colorless oil: Rf 0.3 (n-hexanes/EtOAc = 4/1); [α]D¹⁰ = +8.13 (c = 7, CH₂Cl₂): ¹H NMR (400 MHz, CDCl₃): δ 1.19 (s, 3H), 1.21 (s, 3H), 1.61–1.71 (m, 1H), 1.80–2.02 (m, 3H), 3.34 (dd, J = 7.2, 10.0 Hz, 1H), 3.52 (dd, J = 2.3, 10.0 Hz, 1H), 3.57 (dd, J = 2.3, 7.2, 9.2 Hz, 1H), 3.74 (t, J = 7.8 Hz, 2H), 3.99 (dd, J = 5.7, 9.2 Hz, 1H), 4.19 (dd, J = 3.1, 5.7, 10.6 Hz, 1H), 7.25–7.37 (m, 9H), 7.48–7.52 (m, 6H), 7.68–7.76 (m, 2H), 8.23–8.32 (m, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 19.9, 24.9, 27.3, 27.7, 53.9, 60.2, 64.4, 75.5, 76.9, 87.1, 108.2, 123.0, 125.0, 127.1 (3C), 127.8 (7C), 128.1, 128.6 (6C), 136.3, 143.9 (3C), 152.8, 166.7; HRMS (ESI-TOF) m/z calc for C₂₆H₂₆O₃S₂ Na [M+Na⁺] 691.2019, found 691.2018.

Compound 18. DIBAL-H (1 M in THF, 4.84 mL, 4.84 mmol) was added to a solution of 17 (1 g, 1.94 mmol) in anhydrous THF (10 mL) at 0 °C dropwise. The resulting mixture was stirred at 0 °C for 2 h under an atmosphere of nitrogen or until TLC indicated complete disappearance of starting materials. The reaction mixture was quenched with satd NH₄Cl(aq) and then diluted with EtOAc and aq. HCl (1.0 M) until a clear solution was obtained. The aqueous layer was extracted with EtOAc (3 × 20 mL) and the combined organic layers were washed with satd NaHCO₃(aq) and brine, dried over MgSO₄, and concentrated under reduced pressure. The product (18, 738 mg, 78%) was isolated as a colorless oil after flash column chromatography on silica gel. Rf 0.2 (n-hexanes/EtOAc = 2/1); [α]D¹⁰ = +3.20 (c = 6, CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃): δ 1.22 (s, 3H), 1.24 (s, 3H), 1.51–1.64 (m, 2H), 1.72–1.77 (m, 2H), 3.35 (dd, J = 7.6, 10.0 Hz, 1H), 3.56 (dd, J = 2.4, 10.0 Hz, 1H), 3.58–3.64 (m, 3H), 3.66 (dd, J = 2.4, 7.4, 9.0 Hz, 1H), 4.04 (dd, J = 5.5, 9.0 Hz, 1H), 4.19 (dd, J = 2.7, 5.5, 9.4 Hz, 1H), 7.26–7.54 (m, 15H); ¹³C NMR (100 MHz, CDCl₃): δ 25.0, 25.8, 27.5, 29.8, 60.5, 61.4, 64.5, 75.8, 77.5, 87.0, 107.8, 127.1 (3C), 127.8 (6C), 128.6 (6C), 143.9 (3C). HRMS (ESI-TOF) m/z calc for C₂₉H₃₅N₃O₄S₂ Na [M+Na⁺] 510.2365, found 510.2365.

Compound 21. A solution of 6b (2 g, 3.14 mmol) in MeOH/CHCl₃ (1/1, 50 mL) was treated with CSA (728 mg, 3.14 mmol), and the resulting mixture was stirred at rt for 8 h. The reaction mixture was quenched with Et₃N and concentrated in vacuo. The residue was purified by flash silica gel column chromatography to yield compound 21 (934 mg, 83%). Rf 0.2 (n-hexanes/EtOAc = 1/1); [α]D²⁰ = +16.83 (c = 3.0, CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃): δ 1.55–2.14 (m, 4H), 3.38 (m, 2H), 3.54 (dd, J = 4.8, 6.8 Hz, 1H), 3.61 (m, 2H), 3.76 (dd, J = 7.9, 11.6 Hz, 1H), 3.92 (dd, J = 3.3, 11.6 Hz, 1H), 7.28–7.32 (dt, J = 1.0, 7.6 Hz, 1H), 7.40–7.44 (dt, J = 1.0, 7.6 Hz, 1H), 7.80 (t, J = 7.6 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 26.9, 33.0, 34.6, 62.6, 66.8, 72.4, 75.9, 122.1, 122.4, 125.6, 127.5, 136.2, 154.4, 169.7; HRMS (ESI-TOF) m/z calc for C₁₄H₁₈N₄O₄S₂ Na [M+Na⁺] 377.0713, found 377.0718.

Compound 22. Et₃N (1.1 mL, 8.0 mmol) was added dropwise to a solution containing triol 21 (2.3 g, 7.05 mmol) and trityl chloride (2.16 g, 7.75 mmol) in anhydrous CH₂Cl₂ (60 mL) at rt under an atmosphere of N₂. After being stirred at rt for 8 h, the solution was concentrated in vacuo, and extracted with EtOAc (3 × 50 mL). The combined organic extracts were washed with brine, dried over MgSO₄, and evaporated under reduced pressure. The residue was purified by flash silica gel column chromatography to get mononitrilated ether 22 (3.57 g, 85%). Rf 0.25 (n-hexanes/EtOAc = 2/1); [α]D²⁰ = +5.83 (c = 6.2, CH₂Cl₂): ¹H NMR (400 MHz, CDCl₃): δ 1.52–2.0 (m, 4H), 2.93 (brs, 1H), 3.20 (dt, J = 7.7, 13.8 Hz, 1H), 3.38 (dt, J = 7.5, 13.8 Hz, 1H), 3.44 (dd, J = 5.3, 9.9 Hz, 1H), 3.67 (m, 4H, H-4, H-6, H7), 3.79 (brs, 1H), 7.19–7.50 (m, 17H), 7.71 (d, J = 8.0 Hz, 1H), 7.78 (d, J = 8.0 Hz, 1H); ¹³C NMR (100 MHz, CDCl₃): δ 25.6, 29.9, 32.3, 62.3, 63.5, 70.8, 74.1, 87.5, 120.8, 120.9, 124.2, 126.0, 127.1 (3C), 127.8 (6C), 128.4 (6C), 134.7, 143.2 (3C), 152.5, 167.7; HRMS (ESI-TOF) m/z calc for C₃₃H₃₂N₄O₃S₂ Na [M+Na⁺] 619.1808, found 619.1809.
Compound 23. The diol 22 (1.25 g, 2.09 mmol) and TBAI (232 mg, 0.63 mmol) were dissolved in DMF (20 mL) and cooled to 0 °C. A 60% dispersion of NaH in mineral oil (251 mg, 10.47 mmol) was added followed by addition of benzyl bromide (0.75 mL, 6.28 mmol). The reaction was warmed to rt with stirring over 8 h, quenched with MeOH and then concentrated in vacuo. The residue was extracted with EtOAc (3 × 20 mL), and the combined extracts were washed with water, brine, dried over MgSO₄, and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel to obtain 23 (1.5 g, 92%) as colorless oil. Rf 0.2 (n-hexanes/EtOAc = 9:1).

\[ \alpha_{D}^{25} = +3.52 \] (c = 2.53, CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃): δ 1.68–1.94 (m, 4H), 3.25 (t, J = 7.0 Hz, 2H), 3.38 (dd, J = 8.1, 10.0 Hz, 1H), 3.45 (dd, J = 2.8, 10.0 Hz, 1H), 3.54–3.60 (m, 2H), 3.78 (dd, J = 2.8, 5.4, 8.1 Hz, 1H), 4.04 (s, 2H), 4.46 (d, J = 11.2 Hz, 1H), 4.57 (d, J = 11.2 Hz, 1H), 7.08–7.45 (m, 27H), 7.73 (d, J = 7.9 Hz, 1H), 7.83 (d, J = 7.9 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 24.9, 28.6, 33.5, 64.1, 64.1, 71.9, 73.5, 73.8, 75.8, 78.6, 82.7, 120.8, 121.4, 125.0, 127.0 (3C), 127.6, 127.6, 127.8 (8C), 127.9 (2C), 128.2 (2C), 128.3 (2C), 128.6 (6C), 131.5, 137.6, 143.9, 143.6 (3C), 153.2, 166.9. HRMS (ESI-TOF) m/z calcd for C_{47}H_{64}N_{2}O_{2}S_{2}Na [M+Na]^+ 799.2747, found 799.2750.

Compound 29. A stirred suspension of p-tolylthiogalactoside donor 5 (1.0 g, 1.6 mmol), acceptor 6c (1.4 g, 2.4 mmol), and activated MS 4Å (21 mg, 0.081 mmol) was added. The solution was stirred for an additional 1 h and warmed to −10 °C. The reaction mixture was quenched with Et₃N (2 mL), and then filtered through a pad of celite. The filtrate was poured into satd NaHCO₃ (5 mL) and extracted with EtOAc (3 × 20 mL). The combined extracts were washed with brine, dried over MgSO₄, and evaporated under reduced pressure. The residue was purified by flash column chromatography on silica gel to give product 29 (1.3 g, 72%, α-anomer) as a colorless oil. Rf 0.25 (n-hexanes/EtOAc = 4:1).

\[ \alpha_{D}^{31} = +18.93 \] (c = 5.5, CH₂Cl₂). ¹H NMR (400 MHz, CDCl₃): δ 1.69–1.90 (m, 4H), 3.24 (t, J = 6.7 Hz, 2H), 3.52 (m, 1H), 3.64–3.70 (m, 3H), 3.72 (dd, J = 4.0, 5.7 Hz, 1H), 3.80 (d, J = 12.5 Hz, 1H), 3.92–4.02 (m, 3H), 4.06 (dd, J = 3.4, 10.0 Hz, 1H), 4.11 (d, J = 3.2 Hz, 1H), 4.44 (d, J = 11.4 Hz, 1H), 4.54 (d, J = 11.4 Hz, 1H), 4.61 (m, 2H), 4.63 (d, J = 11.8 Hz, 1H), 4.71 (d, J = 12.3 Hz, 1H), 4.77 (d, J = 12.3 Hz, 1H), 4.83 (d, J = 11.8 Hz, 1H), 4.93 (d, J = 3.3 Hz, 1H), 5.38 (s, 1H), 7.18–7.48 (m, 27H), 7.71 (d, J = 7.8 Hz, 1H), 7.81 (d, J = 8.1 Hz, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 25.2, 28.9, 33.6, 61.8, 63.1, 68.3, 69.4, 72.0, 72.2, 73.7, 73.9, 74.7, 75.5, 75.9, 78.4, 78.7, 99.2, 101.2, 121.1, 121.6, 124.3, 126.2, 126.5, 127.7, 127.8 (2C), 127.92 (2C), 127.95 (2C), 128.0, 128.1 (2C), 128.2 (2C), 128.3 (2C), 128.4 (2C), 128.5 (2C), 128.6 (4C), 129.0, 130.5, 138.0 (1C), 138.9 (2C), 153.4, 167.1. HRMS (ESI-TOF) m/z calcd for C_{55}H_{69}N_{2}O_{2}S_{2}Na [M+Na]^+ 987.3437, found 987.3439.

Compound 30. Compound 30 was prepared by following the similar method as described in the synthesis of 21 in a mixture of MeOH and CH₂Cl₂. Starting from 29 (500 mg, 0.52 mmol), pure compound 30 (318 mg) was isolated as a yellow oil in 81% yield. Rf 0.2 (n-hexanes/EtOAc = 1/1). ¹H NMR (400 MHz, CDCl₃): δ 1.74–1.99 (m, 4H), 2.71 (brs, 2H, OH), 3.26 (t, J = 6.70 Hz, 2H), 3.66–3.77 (m, 6H), 3.82 (dd, J = 5.1, 11.0 Hz, 1H), 3.87 (dd, J = 2.9, 10.0 Hz, 1H), 3.90 (dd, J = 2.8, 10.0 Hz, 1H), 3.98 (dd, J = 2.4, 10.2 Hz, 1H), 4.03–4.04 (m, 1H), 4.49 (d, J = 11.4 Hz, 1H), 4.57 (d, J = 11.4 Hz, 1H), 4.62 (d, J = 11.3 Hz, 1H), 4.66 (d, J = 10.4 Hz, 1H), 4.66 (d, J = 11.7 Hz, 1H), 4.68 (d, J = 10.3 Hz, 1H), 4.77 (d, J = 11.7 Hz, 1H), 4.78 (d, J = 11.3 Hz, 1H), 4.89 (d, J = 2.8 Hz, 1H), 7.22–7.41 (m, 22H), 7.71–7.74 (m, 1H), 7.82–7.84 (m, 1H). ¹³C NMR (100 MHz, CDCl₃): δ 25.1, 28.9, 33.6, 62.0, 63.0, 68.4, 69.1, 69.6, 72.1, 72.8, 73.3, 73.9, 75.7, 77.4, 78.7, 78.8, 98.5, 121.1, 121.6, 124.3, 126.2, 127.8, 127.9 (2C), 128.0, 128.1 (2C), 128.2 (2C), 128.3 (2C), 128.4 (2C), 128.5 (2C), 128.6 (2C), 132.7 (2C), 135.3, 138.0, 138.1 (2C), 138.5, 153.4, 167.1. HRMS (ESI-TOF) m/z calcd for C_{48}H_{63}N_{2}O_{2}S_{2} [M+H]^+ 877.3305, found 877.3301.

Compound 32. To a solution of ³⁷-benzaldehyde 31 (305 mg, 0.52 mmol) in MeOH (5 mL) was added trimethyl orthoformate (0.12 mL, 1.05 mmol), IR-120 (150 mg). The reaction was stirred at rt until TLC indicated completed disappearance of starting material.
The reaction mixture was quenched with Et₃N and then filtered. The filtrate was evaporated under reduced pressure and purified by flash column chromatography on silica gel to give F-benzaldehyde dimethylacetal. To the above F-benzaldehyde dimethylacetal in acetonitrile (5 mL) and HFE-7100 (5 mL) was added compound 30 (460 mg, 0.52 mmol), CSA (36 mg, 0.16 mmol), and AW-300 (500 mg). The reaction was stirred at rt until TLC indicated complete disappearance of starting material. The reaction was quenched with Et₃N and concentrated in vacuo. The residue was subjected to a F-SPE cartridge (FluoroFlash® Slica Gel, 40 μm) and eluted by 80% MeOH. MeOH and then 100% MeOH to afford pure F-benzylidene derivative 32 (643 mg, 85% yield). R f 0.4 (n-hexanes/EtOAc = 2:1). [α] D [31] = +23.53 (c = 2.33, CH₂Cl₂).1H NMR (600 MHz, CDCl₃): δ 1.84–2.04 (m, 4H), 2.18–2.46 (m, 4H), 3.38 (t, ʃ = 6.9 Hz, 2H), 3.65 (m, 1H), 3.78–3.83 (m, 3H), 3.86 (dd, ʃ = 4.5, 5.6 Hz, 1H), 3.93 (dd, ʃ = 1.4, 12.6 Hz, 1H), 4.07–4.13 (m, 3H), 4.15 (t, ʃ = 6.00 Hz, 2H, F₆-OCH₂CH₂OH), 4.18 (dd, ʃ = 3.4, 10.1 Hz, 1H), 4.24 (d, ʃ = 2.8 Hz, 1H), 4.58 (d, ʃ = 11.5 Hz, 1H), 4.68 (d, ʃ = 11.5 Hz, 1H), 4.71 (d, ʃ = 10.6 Hz, 1H), 4.75 (d, ʃ = 10.6 Hz, 1H), 4.77 (d, ʃ = 11.8 Hz, 1H), 4.84 (d, ʃ = 12.3 Hz, 1H), 4.90 (d, ʃ = 12.3 Hz, 1H), 4.97 (d, ʃ = 11.8 Hz, 1H), 5.07 (d, ʃ = 3.4 Hz, 1H), 5.47 (s, 1H), 6.98 (d, ʃ = 8.7 Hz, 2H), 7.31–7.54 (m, 24H), 7.85 (d, ʃ = 7.5 Hz, 1H), 7.95 (d, ʃ = 8.0 Hz, 1H). 13C NMR (150 MHz, CDCl₃): δ 20.7, 25.3, 28.1, 30.0, 33.7, 61.8, 63.1, 66.5, 68.4, 69.4, 72.1, 72.2, 73.7, 74.0, 74.7, 75.6, 76.0, 78.5, 78.8, 99.3, 101.1, 114.2, 121.1, 121.6, 124.4, 126.2, 127.6, 127.7, 127.8 (2C), 127.9 (3C), 128.0 (4C), 128.1, 128.2 (2C), 128.3 (2C), 128.4 (2C), 128.5 (2C), 128.6 (2C), 128.7 (2C), 131.1, 135.3, 138.0, 138.2, 138.9 (2C), 135.3, 159.2, 167.1. HRMS (ESI-TOF) m/z calcd for C₆₆H₄₁F₂₁Ng₂Na [M+Na]+ 1463.3506, found 1463.3511.

General procedure for the synthesis of alkenes (33–41) via Julia–Kocienski olefination reaction. In a typical procedure, a solution of sulfone (4 (820 mg, 0.56 mmol) in anhydrous THF (10 mL) was treated at 0 °C with LiHMDS (1.2 mL, 1.2 mmol, 1 M in THF) with vigorous stirring. After 15 min, a solution of aldehyde (80 mg, 0.83 mmol) in anhydrous THF (2 mL) was added dropwise to 0 °C. The reaction mixture was gradually warmed to rt and allowed to stir for 16 h under an atmosphere of nitrogen. The reaction mixture was quenched with satd NH₄Cl(aq), extracted with EtOAc (3 × 20 mL), filtered, and concentrated under reduced pressure. The residue was subjected to a FluoroFlash® cartridge to afford pure alkene in 80% MeOH elution to afford 33 (540 mg, 73%). R f 0.55 (n-hexanes/EtOAc = 2:1); [α] D [33] = +27.93 (c = 2.5, CH₂Cl₂).1H NMR (400 MHz, CDCl₃): δ 0.97 (ddt, ʃ = 0.6, 3.4, 7.5 Hz, 3H), 1.59 (m, 1H), 1.76 (m, 1H), 1.96–2.16 (m, 6H), 2.32 (m, 2H), 3.58 (m, 1H), 3.66–3.77 (m, 4H), 3.89 (dt, ʃ = 1.9, 12.6 Hz, 1H), 4.00–4.11 (m, 4H), 4.05 (t, ʃ = 5.8 Hz, 2H, F₆-OCH₂CH₂OH), 4.16 (d, ʃ = 3.1 Hz, 1H), 4.50 (dd, ʃ = 2.3, 11.5 Hz, 1H), 4.59–4.63 (m, 2H), 4.68 (d, ʃ = 11.9 Hz, 1H), 4.69 (d, ʃ = 11.3 Hz, 1H), 4.75 (d, ʃ = 12.3 Hz, 1H), 4.82 (d, ʃ = 12.3 Hz, 1H), 4.87 (d, ʃ = 11.9 Hz, 1H), 4.98 (d, ʃ = 3.2 Hz, 1H), 5.27–5.47 (m, 2H), 5.42 (s, 1H, benzylidene), 6.88 (d, ʃ = 8.4 Hz, 2H), 7.22–7.34 (m, 18H) 7.40 (d, ʃ = 7.3 Hz, 2H), 7.45 (d, ʃ = 8.5 Hz, 2H).13C NMR (100 MHz, CDCl₃): δ 14.1, 20.7, 23.2, 25.7, 28.6, 30.3, 61.9, 63.1, 66.5, 68.6, 69.4, 72.2, 72.3, 73.7, 73.9, 74.8, 75.6, 75.9, 78.9, 79.2, 99.3, 101.1, 114.2 (2C), 126.7, 127.7, 127.8, 127.9 (4C), 128.0 (2C), 128.1 (4C), 128.4 (2C), 128.5 (2C), 128.6 (4C), 128.7 (2C), 131.1, 135.3, 138.0, 138.2, 138.9 (2C), 135.3, 159.2. HRMS (ESI-TOF) m/z calcd for [M+Na]+ 1338.4107, found 1338.4102.

Bioactivity assay
A20 cells (2 × 10⁴) overexpressing mCD1d were loaded with chemically synthesized glycolipids (1 μM) and then cultured with mNK1.2 cells (2 × 10⁶). Upon stimulation by glycolipids, mNK1.2 cells secreted IL-2 into culture medium. Three days later, supernatants were collected to determine the level of IL-2 by enzyme-linked immunosorbent assay (ELISA).

ELISA
Mouse IL-2 in cell culture supernatants was determined by DuoSet ELISA Development System according to the manufacturer’s procedures (R&D System, Minneapolis, MN, USA). Briefly, supernatant and standards were serially diluted, added into each well, and incubated for 2 h at room temperature (RT). After washing with the wash buffer, 100 μL of the detection antibody was added to each well and incubated for 2 h at RT. After washing,
each well was incubated with 100 μL of diluted streptavidin-HRP for 20 min at RT, followed by incubating with 100 μL of substrate solution for 20 min at RT. The reaction was stopped by adding stop solution and read at 450 nm with SpectraMax M2 (Molecular Device, San Jose, CA, USA).

4. Conclusions

In summary, a diversity-oriented strategy was developed for constructing an α-GalCer library with different lengths of phytosphingosines by using Julia–Kocienski olefination and fluorous-tag-assisted purification. This strategy offers two branching points for easy structural diversification on the ceramide portion: the lipid chain lengths of the phytosphingosine tail and acyl amide, both of which can be modulated using various aldehydes and fatty acids with different lengths in the olefination and peptidic coupling steps, respectively. In addition, from a green-chemistry perspective, the fluous tag is recyclable, and organic solvent waste was reduced (through elution with 80% MeOH and water during F-SPE). In conclusion, we obtained the core building block 4 in 10-steps with a total yield of 15.8% from known compound 17, followed by another five-step manipulation, and we successfully obtained 61 α-GalCer glycolipids including the immunomodulatory glycolipid KRN7000 (1a) and its prototype (1c) by using this approach. We have demonstrated that structurally distinct forms of synthetic α-GalCer with alterations in their ceramide portions can be designed to generate immunomodulators that stimulate murine NK1.2 cells characterized by their induction of IL-2 secretion. We anticipate that this straightforward route will be very attractive for the modular synthesis of various glycosceramides for biological studies.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms232113403/s1; Tables S1 and S2: synthesis of compounds 1bb–Ihi, 6a, 9–14, 26, 34–41, and 42a–49i, characterization and NMR spectra of all new compounds. References [58–60] are cited in the supplementary materials.

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54. The structures of fully protected compounds 42–49, and their corresponding isolated yields are provided in Table S1 of the Supplementary materials. A total of 65 compounds were prepared and are characterized by mass spectra (HRESI-MS).

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