# *Brief Articles*

## Synthesis and Evaluation of 1,2,3-Triazole Containing Analogues of the Immunostimulant α-GalCer

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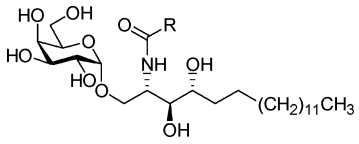
α-GalCer is the first defined and most potent agonistic antigen of the T cell receptor of natural killer T cells. We have prepared a series of 1,2,3-triazole-containing α-GalCer analogues in which the lipid chain lengths have been incrementally varied. We found that this isosteric replacement of α-GalCer's amide moiety with triazole increases the IL-4 versus IFN-$\gamma$ bias of released cytokines. The stimulatory effect was influenced by the length of the attached chain. In particular, the long-chained triazole analogues have a comparable stimulatory effect on cytokine production as α-GalCer and exhibit a stronger Th2 cytokine response.

### Introduction

Natural killer T (NKT[a]) cells are a unique subset of T cells that express both a conserved $\alpha\beta$ T cell receptor (TCR) and natural killer (NK) cell receptors. Unlike other T cells, NKT cells recognize glycolipid antigens presented by the major histocompatibility complex (MHC) class I-like molecule CD1d.[1] Upon recognition of the glycolipid/CD1d complex through the TCR, NKT cells rapidly produce large amounts of T helper 1 (Th1) and Th2 cytokines, such as interferon-$\gamma$ (IFN-$\gamma$) and interleukin 4 (IL-4), respectively. The released cytokines play critical roles in inducing a series of cellular activation events leading to the activation of innate and adaptive immune cells. In this cellular process, the Th1/Th2 balance is believed to be importantly related to the target of the immune response.[2]

The first defined and most potent agonistic antigen of the TCR is α-GalCer (also known as KRN7000, **1**, Figure 1), which is a structurally modified analogue of the marine natural product α-galactosylceramide, a member of agelasphins.[3] α-GalCer has been the most extensively studied valuable compound in exploration of NKT cell biology and pharmacology.[4,5] Since the introduction of α-GalCer, a number of analogues have been synthesized and evaluated to elucidate the structure−activity relationships (SAR).[5] The SAR studies on the sugar moiety have demonstrated that the galactose group is important for α-GalCer to bind CD1d and to activate NKT cells through their TCR.[6] The α-anomeric conformation is believed to be crucial for the ability of α-GalCer to act as a potent and efficient ligand for NKT cells.[6,7] It was also found that the equatorial configuration of the 2-hydroxyl group of the sugar moiety is essential for recognition by the TCR of NKT cells, while some flexibility can be accommodated at the 3- and 6-hydroxyl positions.[8−11]




**1** R = $-(CH_2)_{24}CH_3$ α-GalCer
**2** R = $-(CH_2)_6CH_3$

**Figure 1.** Chemical structures of **1** and **2**.

Modification of the fatty acid chain as well as that of the sphingosine base leads to changes in the cytokine release profile, presumably through alteration of glycolipid/CD1d complex stability. For example, the α-GalCer derivatives with a shorter fatty acid chain (typically 2 (PBS-25)) have an increased Th2 profile compared with that of α-GalCer.[12] Truncation of the phytosphingosine lipid chain by nine carbon atoms (known as OCH) biases toward the Th2 response.[13] Introduction of an aromatic group to the fatty acid chain enhances the Th1 cytokine profiles.[14] Interestingly, replacement of the anomeric oxygen of α-GalCer by $CH_2$ also alters the cytokine release profile. The *C*-glycoside analogue of α-GalCer stimulates strong Th1 responses in vivo from NKT cells.[15]

The SAR studies of α-GalCer were corroborated by two recent crystal structures of CD1d with α-GalCer.[16,17] The crystal structures showed that the lipid chains of α-GalCer fit tightly into the CD1d binding groove two hydrophobic pockets. There are several hydrogen-bonding interactions between the surface residues of CD1d and the hydroxyl groups of the galactose and sphingosine base, which can be considered crucial for maintaining α-GalCer in the correct position and orientation for recognition by the TCR. The crystal structure of human CD1d/α-GalCer revealed that the amide group of α-GalCer does not seem to be hydrogen-bonded to the surface residues of CD1d.[17] On the other hand, the crystal structure of mouse CD1 in

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[a] Abbreviations: IL, interleukin; IFN-$\gamma$, interferon-$\gamma$; Th, T helper; NKT, natural killer T; TCR, T cell receptor; NK, natural killer; MHC, major histocompatibility complex; SAR, structure−activity relationships; CD, cluster of differentiation; RBL, rat basophilic leukemia; hCD1d, human CD1d; mCD1d, mouse CD1d; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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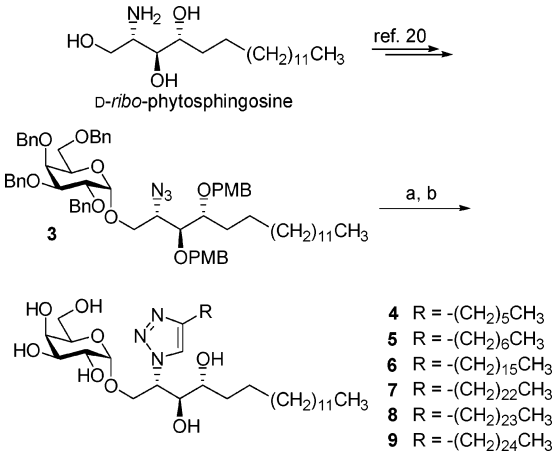
complex with **2** clearly showed that the NH of the amide group of **2** is involved in the hydrogen bonding to the α2 helix of CD1d, but the carbonyl of the amide does not seem to form a hydrogen bond.[16] These features may imply that the amide moiety of α-GalCer is left exposed for recognition by the TCR of NKT if the amide group does not have only the structural role of maintaining the fatty acid chain in a particular geometry.

The reported analogues of α-GalCer can be categorized in three groups according to their modified moieties of α-GalCer: sphingoid core modified, fatty acid modified, and sugar modified analogues. However, to the best of our knowledge, modification of the amide moiety of α-GalCer has not been reported. Because an amide group is often involved in hydrogen bond interactions with the binding site, isosteric replacement of the amide moiety of α-GalCer would be very interesting in gaining further insight into the NKT cell biology. The modification of the amide moiety could alter the stability of the glycolipid/CD1d complex and the position of the sugar head group in the binding groove as a result of the different electronic and steric character of the isosteres. As a consequence, the avidity and antigenic potency of galactosylceramides could be modulated. Moreover, the isosteric replacement could alter the metabolic transformation of α-GalCer, thereby influencing the subsequent immune responses.

Among the known isosteres for an amide group, 1,2,3-triazoles have gained increasing attention in drug discovery since the introduction of the concept of "click" chemistry by Sharpless.[18,19] 1,2,3-Triazoles serve as rigid linking units that can mimic the topological and electronic features of an amide bond. They can actively participate in hydrogen bonding and dipole−dipole interactions due to their strong dipole moment. However, unlike amides, triazoles are extremely stable to hydrolysis and oxidative/reductive conditions. To our knowledge, 1,2,3-triazoles have never been introduced into ceramide agents. Based on these considerations and the available crystal structure of CD1d/α-GalCer, we designed 1,2,3-triazole containing α-GalCer analogues. We present here the synthesis and preliminary evaluation of a series of 1,2,3-triazole-containing α-GalCer analogues in which the lipid chain lengths have been incrementally varied.

**Scheme 1.** Synthesis of α-GalCer Analogues **4**−**9**[a]




[a] Reagents and conditions: (a) R−C≡CH, 10 mol % $CuSO_4$, 40 mol % sodium ascorbate, *t*-BuOH/$H_2O$ (2:1), 50 °C; (b) $H_2$ gas, $Pd(OH)_2$, EtOH/$CH_2Cl_2$ (3:1), rt. Overall yields from **3**: **4**, 62%; **5**, 56%; **6**, 50%; **7**, 54%; **8**, 55%; **9**, 51%.

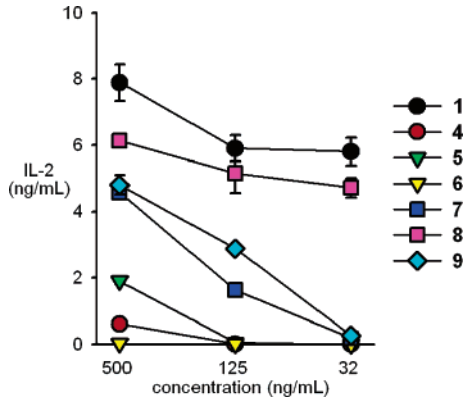



**Figure 2.** IL-2 secretion by DN32.D3 NKT hybridoma cells. IL-2 production was measured from co-cultured supernatants of NKT hybridoma DN32.D3 and mouse CD1d transfected RBL cells after 16 h of culture. Representative data of two individual experiments are expressed as the means ± SD of duplicates.

## Results and Discussion

For the divergent synthesis of the designed α-GalCer analogues, we employed the known galactosyl azido-phytosphingosine **3**[20] (Scheme 1) as the starting material in this synthesis. Compound **3**, which contains the galactose with an α-anomeric linkage and the proper protecting groups, was prepared in high overall yield from commercially available D-*ribo*-phytosphingosine according to the published five-step procedure.[20] The copper(I)-catalyzed Huisgen (click) reaction[21] between the obtained azido-phytosphingosine **3** and varied terminal alkynes provided 1,4-regioselective triazole products in high yield. The global deprotection of the benzyl-type protecting groups by hydrogenolysis afforded the desired triazole containing α-GalCer analogues **4**−**9**.

As a preliminary evaluation of triazoles **4**−**9**, IL-2 production was examined to assess their ability to stimulate immortalized NKT cells. The relative potencies were measured with the use of the Vα14$^+$ NKT cell hybridoma DN32.D3 and RBL cells transfected to express mouse CD1d.[22] As shown in Figure 2, the IL-2 production was highly influenced by the length of the attached chain. The short chain triazole analogues **4** and **5** turned out to be much less efficient than α-GalCer in IL-2 production. The medium-chained analogue **6** was virtually inactive. On the other hand, the long-chained triazoles **7**−**9** were slightly less effective at eliciting IL-2 production than the parent α-GalCer at a high concentration (500 ng/mL). At a lower concentration (32 ng/mL), only triazole **8** had a comparable stimulatory effect as α-GalCer, whereas the analogues **7** and **9**, of which the lipid chain lengths differ by only one carbon unit from that of **8**, did not exhibit notable activity.

While immortalized T cell hybridomas tend to produce IL-2 when stimulated, primary NKT cells tend to produce IFN-$\gamma$ (Th1) and IL-4 (Th2) along with a variety of other cytokines. Because Th1 and Th2 cytokines induce antagonistic biological effects and their balance is critically important,[2,23] we measured the levels of IFN-$\gamma$ and IL-4 in the supernatants of mouse splenocytes[23,24] cultured in the presence of triazoles **4**−**9**. Figure 3 shows the relative IFN-$\gamma$ and IL-4 production levels of triazoles when compared with those of α-GalCer. All the analogues tested seemed to induce a Th2 bias in the NKT cell cytokine responses. At a high concentration (125 ng/mL), all were less effective than α-GalCer in IFN-$\gamma$ production, while certain triazoles (**5**, **7**, and **8**) showed equivalent or greater IL-4 secretion than α-GalCer. At a lower concentration (32 ng/mL), relatively low levels of both IFN-$\gamma$ and IL-4 were produced by most analogues, except for **8**. Triazole **8**, which showed a good stimulatory effect on IL-2 production, induced less IFN-$\gamma$ but more IL-4 production compared with α-GalCer.

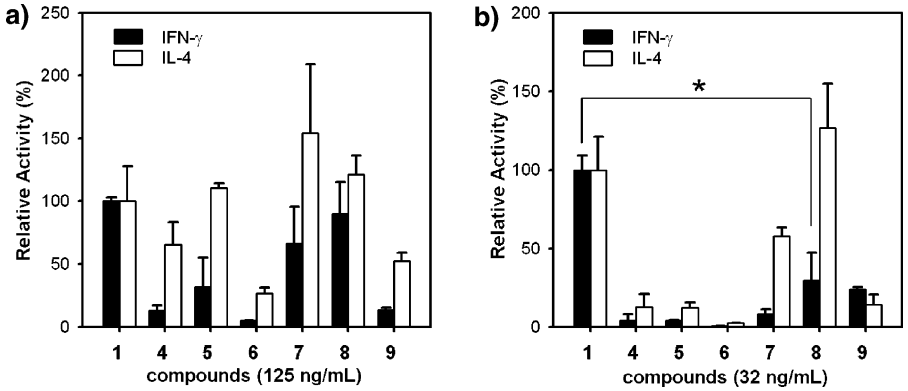



**Figure 3.** IFN-$\gamma$ and IL-4 secretion by mouse splenocytes when stimulated by α-GalCer (**1**) or triazoles **4**−**9**. IFN-$\gamma$ and IL-4 production was measured after 72 h of culture. Results are expressed as relative activities. Representative data of two individual experiments are expressed as the means ± SD of duplicates. (a) Relative activities at 125 ng/mL and (b) relative activities at 32 ng/mL. Statistical significance of the difference in secretion levels was determined by Student's *t* test. *$p < 0.05$.

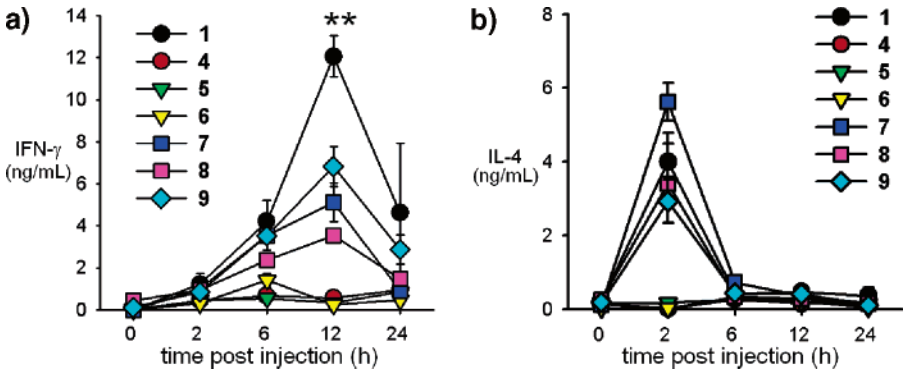



**Figure 4.** Serum IFN-$\gamma$ and IL-4 levels from mice injected (i.v.) with α-GalCer (**1**) or triazoles **4**−**9** (1 $\mu$g/mouse). Representative data of two individual experiments are shown as the means ± SD of three mice. (a) IFN-$\gamma$ secretion in vivo and (b) IL-4 secretion in vivo. The significance of the difference between α-GalCer and triazoles in IFN-$\gamma$ secretion was determined by Student's *t* test. *$p < 0.05$.

As an in vivo evaluation, we measured the serum cytokine levels after intravenous injection of compounds (1 $\mu$g/mouse) into naïve C57BL/6 mice.[24] Both α-GalCer and triazoles induced a rapid elevation of IL-4, with the peak value at 2 h and a delayed elevation of IFN-$\gamma$ with the peak value at 12 h after treatment (Figure 4). The medium- and short-chained triazoles **4**−**6** were nonstimulatory, as judged by this in vivo assay. The long-chained triazoles **7**−**9** provoked relatively small amounts of IFN-$\gamma$ compared to α-GalCer. However, they induced equal or greater IL-4 secretion than α-GalCer. This result, together with the in vitro results, shows that the long-chained triazole analogues have a comparable stimulatory effect on cytokine production as α-GalCer. However, their cytokine release profile is somewhat different from the parent α-GalCer. They seemed to bias cytokine secretion toward the Th2 response.

To understand the altered cytokine profiles and the interaction between the triazoles and CD1d, molecular modeling studies were performed. Figure 5 shows the binding mode of triazole **8** in the human CD1d binding grove. We observed that triazole analogue **8** in our docking model is oriented quite similarly to α-GalCer in its crystalline complex with hCD1d.[17] The galactose ring was presented in nearly the same position. The phytosphingosine chain fitted into the C′ pocket by adopting the same conformation (see Supporting Information). The lipid chain of triazole effectively filled the A′ pocket, suggesting that triazole might successfully serve as a linking unit that can mimic the atom placement of an amide bond of α-GalCer.

In terms of the hydrogen bond with the galactose ring, subtle differences are observed. The crystal structure of hCD1d/α-GalCer revealed the hydrogen bonding between Asp151 and

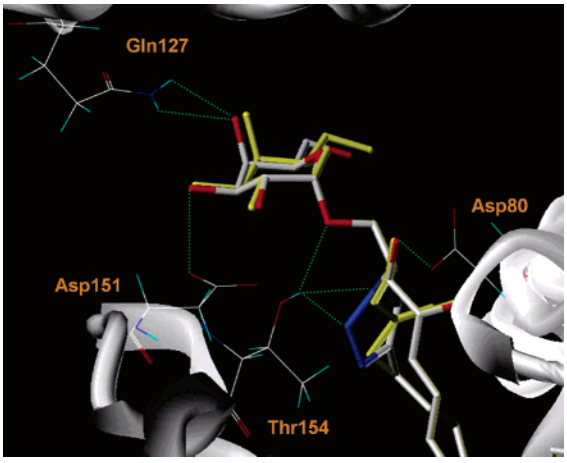



**Figure 5.** Docking model of triazole **8** (gray backbone) within the hCD1d binding groove. The FlexiDock docking procedure was utilized. The structure of hCD1d was obtained from the Protein Data Bank (1ZT4). The H-bonds are shown as a green dotted line. The superimposed α-GalCer (**1**) is shown in yellow.

the 2′-hydroxyl group of the galactose,[17] and the crystal structure of mCD1 in complex with **2** showed two hydrogen bondings between the 2′-and 3′-hydroxyl group and the corresponding Asp.[16] In our simulations, Asp151 engaged in only one hydrogen bond with the 3′-hydroxyl group of the galactose ring of **8**. We speculated that the absence of a hydrogen bond at the 2′-hydroxyl group seemed to cause the slightly different positions of the hydroxyl groups from those found in α-GalCer. The

amide group of α-GalCer is not involved in hydrogen bonding in the reported crystal structure,[17] whereas the triazole ring of **8** seems to form two hydrogen bonds with Thr154 in our docking model. Thus, it can be inferred that the slightly altered topological features and hydrogen-bonding patterns might influence the cytokine release profile, possibly through alteration of the interaction between TCR and CD1d.

In conclusion, we have found that the bioisosteric replacement of α-GalCer's amide moiety with triazole increases the IL-4 versus IFN-$\gamma$ bias of released cytokines. The stimulatory effect was influenced by the length of the attached chain. In particular, the long-chained triazole analogues have a comparable stimulatory effect on cytokine production as α-GalCer and exhibit a stronger Th2 cytokine response. Because IL-4 is a key cytokine for the control of autoimmune diseases such as type 1 diabetes and multiple sclerosis, the triazole analogues might be more useful than α-GalCer for the treatment of these diseases. This study also provides a novel direction for further investigation into the modification of the amide moiety of glycosylceramide and its influence on immune responses, as well as application to other ceramides. However, because the shift of the cytokine profile could result from numerous factors, the actual origin of the enhanced Th2 selectivity will need to be clarified by further studies.

## Experimental Section

**A. Preparation of 8:** To a solution of **3** (195 mg, 0.18 mmol) and 1-hexacosyne (95 mg, 0.26 mmol) in *t*-BuOH/$H_2O$ (6 mL, 1:1) were added 0.5 M $CuSO_4$ (52 $\mu$L, 26 $\mu$mol) and 1 M sodium ascorbate (105 $\mu$L, 105 $\mu$mol) at rt. The reaction mixture was heated for 1 day at 50 °C and then diluted with EtOAc. It was washed with brine, and the aqueous layer was extracted with EtOAc (×2). The combined organic layers were dried over $MgSO_4$ and concentrated in vacuo. Purification of the residue by column chromatography on silica gel (hexane/EtOAc, 6:1) afforded 1,2,3-triazole (187 mg, 72%) as a colorless syrup: $[\alpha]^{24}_D$ +17.9 (*c* 1.2, $CHCl_3$); $^1$H NMR (300 MHz, $CDCl_3$) $\delta$ 0.89 (t, $J$ = 6.9 Hz, 6H), 1.27−1.57 (m, 76H), 2.60 (m, 2H), 3.11 (m, 1H), 3.46 (m, 2H), 3.70 (t, $J$ = 6.3 Hz, 1H), 3.77 (s, 3H), 3.79 (s, 3H), 3.80 (m, 1H), 3.90 (d, $J$ = 1.8 Hz, 1H), 3.99 (dd, $J$ = 3.6, 9.9 Hz, 1H), 4.09 (dd, $J$ = 3.6, 6.3 Hz, 1H), 4.18 (m, 2H), 4.29 (d, $J$ = 11.4 Hz, 1H), 4.35 (d, $J$ = 11.4 Hz, 2H), 4.45 (d, $J$ = 11.7 Hz, 2H), 4.51 (d, $J$ = 11.7 Hz, 1H), 4.53 (d, $J$ = 11.7 Hz, 1H), 4.57 (d, $J$ = 11.7 Hz, 1H), 4.66 (d, $J$ = 12.0 Hz, 1H), 4.69 (d, $J$ = 11.4 Hz, 1H), 4.76 (d, $J$ = 11.7 Hz, 1H), 4.82 (d, $J$ = 3.6 Hz, 1H), 4.89 (d, $J$ = 11.4 Hz, 1H), 4.90 (m, 1H), 6.82−6.86 (m, 4H), 7.15−7.37 (m, 24 H), 7.42 (s, 1H); $^{13}$C NMR (75 MHz, $CDCl_3$) $\delta$ 14.1, 22.6, 25.4, 25.6, 29.30, 29.31, 29.36, 29.43, 29.5, 29.59, 29.64, 29.7, 31.9, 55.11, 55.14, 61.8, 67.3, 68.8, 69.6, 71.4, 72.7, 72.9, 73.4, 74.7, 76.2, 78.2, 78.8, 79.0, 98.7, 113.6, 113.7, 121.6, 127.3, 127.4, 127.49, 127.54, 127.6, 127.7, 128.11, 128.13, 128.2, 128.26, 128.29, 129.48, 129.52, 129.9, 130.2, 137.9, 138.4, 138.5, 138.6, 147.8, 159.1, 159.2; HRMS (FAB) calcd for $C_{94}H_{137}O_{10}N_3$, 1468.0304 ($M^+$); found, 1468.0286. The obtained compound (70 mg, 47 $\mu$mol) was dissolved in EtOH/$CH_2Cl_2$ (4 mL, 3:1), and $Pd(OH)_2$ (350 mg, 500 wt %) was added. The reaction mixture was hydrogenated (1 atm) at rt for 5 h. The catalyst was removed by filtration through a pad of Celite and rinsed with EtOH/$CH_2Cl_2$ (3:1). The filtrate was centrifuged (×1000 rpm) twice for 10 min, and the supernatant was concentrated in vacuo. The residual solid was triturated with hexane/EtOAc (1:1, ×3) to give pure triazole **8** (31 mg, 77%) as a white solid; mp 167−169 °C; $[\alpha]^{25}_D$ +41.9 (*c* 1.5, pyridine); $^1$H NMR (300 MHz, $C_5D_5N$) $\delta$ 0.86 (t, $J$ = 6.9 Hz, 6H), 1.24−1.38 (m, 62H), 1.56−1.83 (m, 6H), 2.10−2.21 (m, 2H), 2.76 (t, $J$ = 7.5 Hz, 2H), 4.13 (m, 1H), 4.32−4.51 (m, 6H), 4.61 (dd, $J$ = 3.9, 9.9 Hz, 1H), 4.69 (dd, $J$ = 6.9, 11.1 Hz, 1H), 4.95 (dd, $J$ = 4.5, 10.8 Hz, 1H), 5.48 (d, $J$ = 3.6 Hz, 1H), 5.98 (td, $J$ = 3.9, 6.9 Hz, 1H), 8.27 (s, 1H); $^{13}$C NMR (75 MHz, $C_5D_5N$) $\delta$ 14.3, 22.9, 26.25, 26.29, 29.61, 29.64, 29.8, 29.9, 30.0, 30.2, 32.1, 34.3, 62.7, 62.8, 67.4, 70.2, 71.0, 71.5, 72.2, 73.2, 76.7, 101.7, 122.1, 147.9; HRMS (FAB) calcd for $C_{50}H_{98}O_8N_3$, 868.7354 ($[M + H]^+$); found, 868.7361; Anal. ($C_{50}H_{97}O_8N_3$) C, H, N.

**B. Biological Assay Protocol.** Immunological evaluations were performed as previously described. In brief, the procedure was as follows.

**Determination of the Stimulating Activity for NKT Hybridoma Cells:**[22,25] Mouse CD1d transfected rat basophilic leukemia (RBL) cells were loaded with α-GalCer or triazoles at various concentrations for 4 h. After removing the free glycolipids by washing with PBS 3 times, the RBL cells were incubated with DN32.D3 NKT hybridoma cells for 16 h. IL-2 secretion levels in the supernatant were determined by ELISA.

**Evaluation of the Cytokine Levels Produced by Primary Splenocytes:**[23] Splenocytes from naïve C57BL/6 mice were cultured in the presence of α-GalCer or triazoles at various concentrations for 72 h. IFN-$\gamma$ and IL-4 secretion levels in the supernatant were determined by ELISA, respectively. The absolute values of IFN-$\gamma$ and IL-4 release after treatment with compounds **1** and **8** were given in Supporting Information.

**Examination of the IFN-$\gamma$ and IL-4 Production Levels In Vivo:**[24] α-GalCer or triazoles (1 $\mu$g/mouse) were injected into naïve C57BL/6 mice. The serum concentrations of IFN-$\gamma$ and IL-4 at each time point were determined by ELISA, respectively.

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**Supporting Information Available:** Experimental details corresponding to the synthesis of the compounds described in this paper, spectral data for all relevant compounds, and molecular modeling methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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