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Synthesis of RCAI-172 (C6 epimer of RCAI-147) and its biological activity

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ABSTRACT

RCAI-147 is one of the hydroxylated analogues of KRN7000 which is known as a ligand for the activation of CD1d mediated invariant natural killer T cells (iNKT cells) and releases both T helper 1 (Th1) cytokines such as IFN-γ and T helper 2 (Th2) cytokines such as IL-4. KRN7000 has been anticipated as an antitumor drug or an adjuvant for viral infection such as influenza, because of its strong secretion of IFN-γ. In an interesting twist, it has been obvious in our previous paper that RCAI-147 induces much more Th2 cytokines (IL-4) than Th1 cytokines (IFN-γ) from iNKT cells compared to KRN7000, and shows fairly good result in the experimental autoimmune encephalomyelitis (EAE) test. Therefore, synthesis of RCAI-172 (C6-OH epimer of RCAI-147) was attempted to examine the biological activity. As a result, RCAI-172 was synthesized and its biological activity biased to Th2 response largely compared to that of KRN7000. However, this level decreased to approximately 61% compared to that of RCAI-147. And the clinical score of RCAI-172 for EAE suppression was disappointing. There exist seven chiral centers in the aglycon part of RCAI-172, and even though the change of configuration is just one position (C6-OH), the effect on both Th1/Th2 response and EAE test is fairly large.

1. Introduction[1]

Agelasphins are a series of the glycosphingolipids isolated from the marine sponge (*Agelas mauritianus*), and they show potent effects on the immune system of mice.[2] In 1995, KRN7000,[3] one of agelasphin related compounds having a structure of the α-galactosylceramides, was developed by Kirin Brewery Co. in the process of synthetic studies on agelasphins.

Invariant natural killer T (iNKT) cell is known as one of the lineages of immunocytes.[4] It produces immunoregulatory cytokines both T helper 1 (Th1)-type [such as interferon-γ (IFNγ)] and Th2-type [such as interleukin-4 (IL-4)] by the recognition of glycolipid antigens such as KRN7000 presented by CD1d,[5] which is an antigen presenting protein for the T cell receptor (TCR) of iNKT cells. The glycolipids stimulating the secretion of mainly Th1- or Th2-type cytokines are expected to be promising anticancer drugs or immunosuppressive agents and autoimmune disease treatment medicine, respectively.[6] It is thought that the balance of Th1 and Th2 cytokine production is important for therapeutic use. Because both types of Th1 and Th2 may reduce or countervail the beneficial effects of cytokines each other. Therefore, we need to develop more effective antigens that bias the production of Th1 and Th2 cytokines to one side or the other compared to KRN7000 which induce NKT cells to produce both Th1- and Th2-type cytokines in large quantities at the same time.

By the way, in our synthesis of the α-galactosylceramides, we found that two analogues [RCAI-147 and 160 in Fig. 1] of KRN7000 induced highly biased Th2 cytokine secretion.[7] As shown in Figure 1, they have several hydroxy groups on the octadecyl main chain and the C2 amide side chain, and showed good efficacy in the experimental autoimmune encephalomyelitis (EAE) for a mouse model of multiple sclerosis.[8] In addition, we synthesized both diastereomers of 4-deoxy-6-hydroxy analogues of KRN7000 (6*R*-isomer: RCAI-10, 6*S*-isomer: RCAI-45) in the early stage of our structure-activity relationship (SAR) study on KRN7000, and we found that the stereochemistry at C6-position highly influences the potency of the immunostimulatory activity.[9] Thereupon, we attempted to synthesize C6-epimer (RCAI-172, **15**) of RCAI-147. Firstly we tried to develop an effective short-step route for **15** (C6 epimer of RCAI-147), because the synthetic steps of RCAI-147 were long and its total yield was poor. And then we examined the biological activity of RCAI-172 in order to compare with that of RCAI-147 (Fig. 1).

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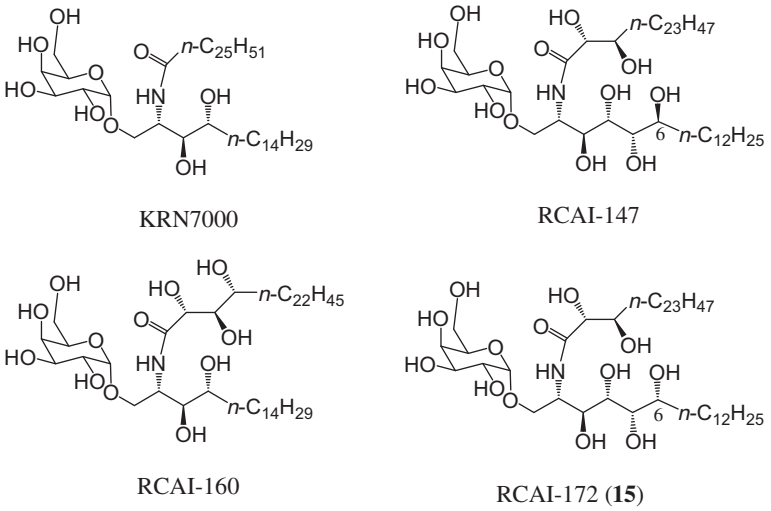
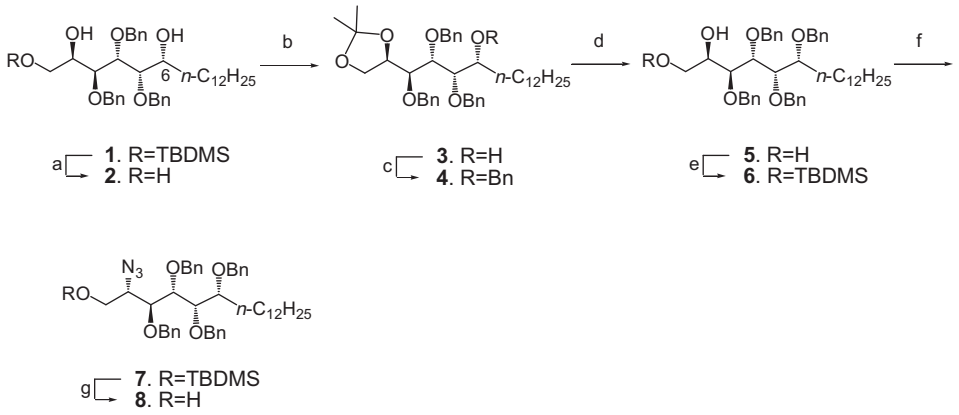
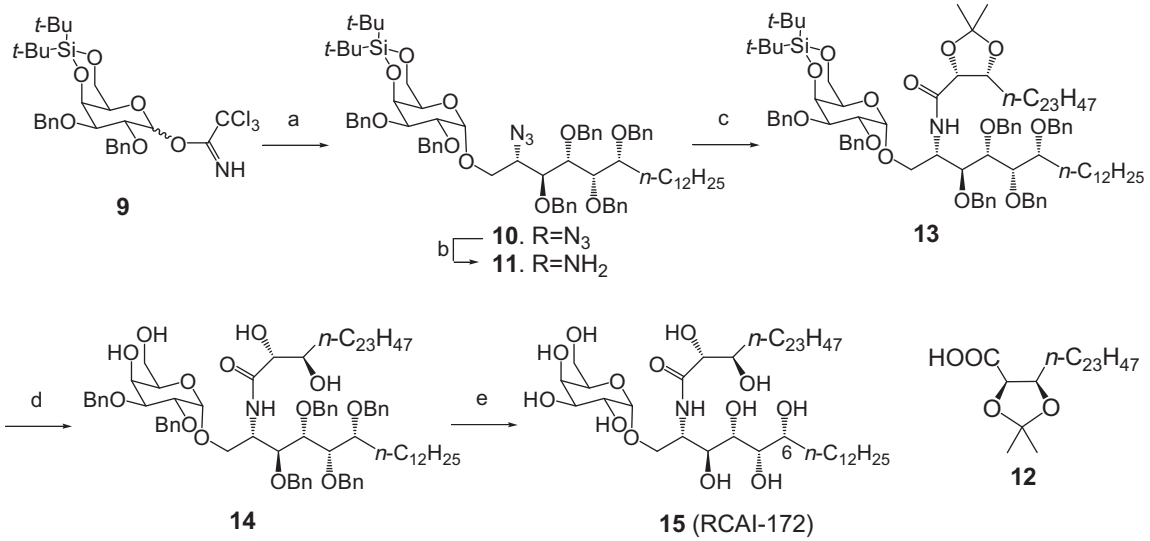


Figure 1. Structures of KRN7000, RCAI-147, 160 and 172 (**15**).



Scheme 1. Reagents and conditions: (a) TBAF, THF, rt, 1 h, 98%; (b) 2,2-dimethoxypropane, *p*-TsOH·H_2O, rt, 1 h, 91%; (c) BnBr, NaH, DMF, rt, 1 h, 65%; (d) aq 46% HF, CH_2Cl_2–MeCN (1:1), rt, 30 min, 98%; (e) TBDMS-Cl, imidazole, catalytic DMAP, rt, 16 h, 99%; (f) Ph_3P, DEAD (in toluene), LDPPA, THF, 0 °C, 30 min, quant; (g) TBAF, THF, rt, 50 min, 96%.



Scheme 2. Reagents and conditions: (a) **8**, MS 4 Å, CH_2Cl_2, rt, 30 min, then AgOTf, rt, 16 h, 91%; (b) Me_3P, THF, rt, 2 h, then aq 1 M NaOH, rt, 2 h, 71%; (c) **12**, EDAC, DMAP, CH_2Cl_2–THF (1:1), rt, 3 h, 54%; (d) (1) aq HF, CH_2Cl_2–MeCN (1:1), rt, 3.5 h; (2) HF·pyridine (~70% HF, ~30% pyridine), pyridine, THF, rt, 2 h, two steps 44%; (e) H_2, $Pd(OH)_2$/C, rt, 16 h, 82%.

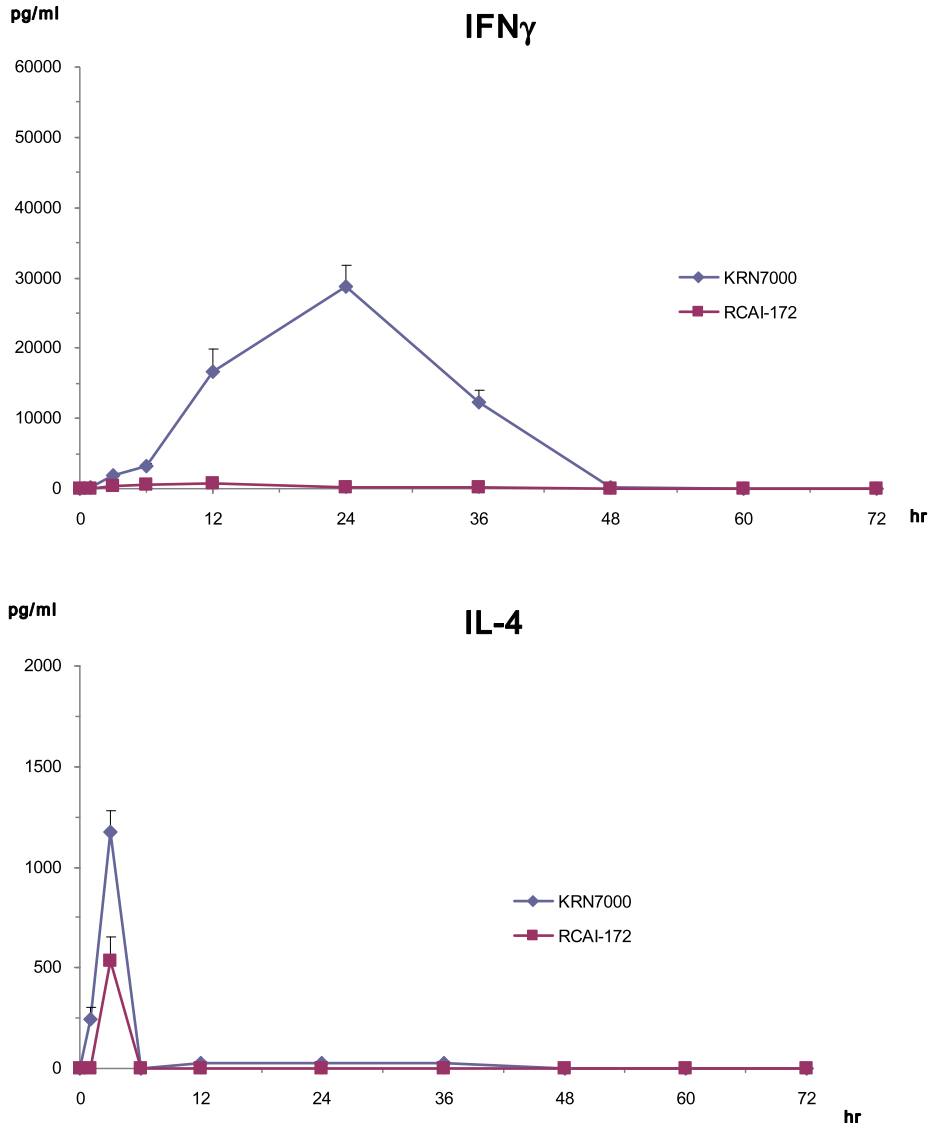


Figure 2. The concentrations of IFNγ and IL-4 upon administration of RCAI-172 using KRN7000 as a positive control.

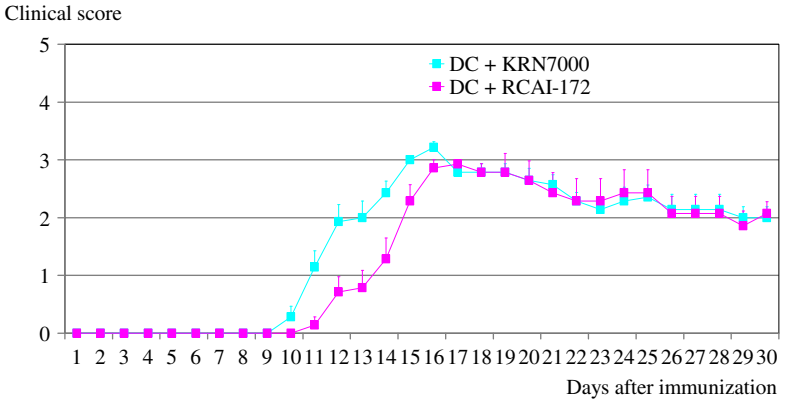


Figure 3. Clinical score of RCAI-172 compared to KRN7000 for EAE suppression.

2. Results and discussion

2.1. Synthesis

The *tert*-butyldimethylsilyl (TBDMS) group of the reported compound **1**[10] which was obtained by Grignard reaction of 2,3,4-tri-*O*-benzyl-6-*O*-*tert*-butyldimethylsilyl-D-galactose[10] and dodecylmagnesium bromide, was deprotected with tetra-*n*-butylammonium fluoride (TBAF) to yield a triol **2** (98% yield). Both C1- and C2-OH groups of compound **2** were protected as a 1,2-isopropylidenedioxy group of compound **3** (91% yield) with 2,2-dimethoxypropane using a catalytic amount of *p*-toluenesulfonic acid monohydrate ($TsOH{\cdot}H_2O$) in order to distinguish between two secondary alcohols, C2-OH and C6-OH. The remaining C6-OH of **3** was benzylated with benzyl bromide using sodium hydride as a base to give **4** (65% yield). The 1,2-isopropylidenedioxy group of **4** was deprotected with aqueous 46% hydrofluoric acid (HF) to afford diol **5** (98% yield), which was treated with TBDMS-Cl and imidazole as a base to give a silyl ether **6** (99% yield). Treatment of **6** with diethyl azodicarboxylate (DEAD in toluene), triphenylphosphine (Ph_3P), and then diphenylphosphoryl azide [DPPA, $(PhO)_2P(O)N_3$] in tetrahydrofuran (THF) at 0 °C for 30 min afforded azide **7** (quant) accompanying the inversion of configuration from *R* to *S* at C2-OH position.[7] Deprotection of TBDMS group from **7** with TBAF in THF afforded primary alcohol **8** as a glycosyl acceptor (Scheme 1).

Glycosylation of **8** with imidate **9**, obtained easily from 2,3-di-*O*-benzyl-4,6-*O*-(di-*tert*-butyl)silylene-D-galactopyranose[7] and CCl_3CN using Cs_2CO_3 as a base, was performed to afford α-anomer **10** (91% yield) by use of silver trifluoromethanesulfonate (AgOTf). The azide group of **10** was reduced to amine **11** (71% yield) by treatment with trimethylphosphine (Me_3P) and successively aqueous NaOH. Reaction of **11** with (2*R*,3*R*)-(isopropylidenedioxy)hexacosanoic acid **12**[7] in the presence of *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDAC) as a dehydrating agent yielded amide **13** (54% yield). Treatment of **13** with aqueous HF in CH_2Cl_2–CH_3CN, and then HF·pyridine (~70% HF, ~30% pyridine) yielded a tetraol **14**. The six benzyl groups of **14** were deprotected by using 20% palladium hydroxide on carbon [$Pd(OH)_2$/C] under hydrogen in THF to afford **15** (RCAI-172) (Scheme 2).

2.2. Biological activity

The cytokine-producing (IFNγ and IL-4) activity from iNKT cells of mice in vivo of RCAI-172 was investigated using KRN7000 as a positive control as shown in the Figure 2 (IFNγ and IL-4), and also RCAI-172 was tested for the suppression activity of the symptoms associated with experimental autoimmune encephalomyelitis (EAE)[8] as shown in Figure 3.

These glycolipid antigens were administered (2 μg/mouse) as phosphate buffered saline (PBS) solutions (10 μg/mL, 200 μL) into caudal vein. Serum concentrations of IFNγ and IL-4 were measured at the indicated time points. Data are means ±SD from three mice.[11]

Compound **15** (RCAI-172) has two successive (5*S*,6*R*)-5,6-dihydroxy groups next to C_4-hydroxy group on the phytosphingosine main-chain and also (2*R*,3*R*)-2,3-dihydroxyhexacosanamide on the amide side chain. As shown in Figure 2, the amount of IFNγ secretion mediated by RCAI-172 decreased to approximately 4.1% of KRN7000 (cf, approximately 5% of KRN7000 in the case of RCAI-147),[7] and the IL-4 production amount was down to approximately 35.3% of KRN7000 (cf, remaining approximately 70% of KRN7000 in the case of RCAI-147).[7] The cytokine secretion of RCAI-172 biased to Th2 response (almost 8.6 times; cf. approximately 14 times in the case of RCAI-147)[7] as compared to that of KRN7000. Therefore, the Th2 response of RCAI-172 was reduced to approximately 61% of RCAI-147, and RCAI-172 was tested for EAE[8,12] as shown in Figure 3.

The cytokines secretion of RCAI-172 biased to more Th2 (IL-4) than Th1 (IFNγ). This tendency was similar to that of RCAI-147,[7] but the intencity of RCAI-172 was reduced to 61% of RCAI-147. Therefore we anticipated that there might be a little difference between RCAI-147 and -172 in EAE test. But surprisingly the EAE score of RCAI-172 was far worse than that of RCAI-147, namely almost the same as that of KRN7000. The amount of Th2 (IL-4) cytokine secretion from iNKT cells mediated by RCAI-172 was reduced to about 35.3% of KRN7000. Whereas that of RCAI-147 was 70% of KRN7000. This difference in the amount of IL-4 should certainly affect EAE score of RCAI-172 negatively. It is not known exactly why.

However, it is important to find the fact, namely, 'the stereoisomer at the only one position has a possibility to make an unexpected large influence on the biological activity'.

Therefore to examine more reliable and deep discussion concerning the importance of the stereochemistry and functional groups on the side chain by comparison of the other related synthetic compounds, and also to examine more SAR studies, we need to synthesize many other stereoisomers at C3, C4, C5 and/or C6 positions on the lipid chain of RCAI-147.

In addition, the other mouse model tests for human autoimmune diseases such as type I diabetes, systemic lupus erythematosus (SLE) and/or rheumatoid arthritis may possibly find out the effectiveness of RCAI-172.

2.3. Discussion

Although it has not been clarified yet how the extra hydroxy groups of RCAI-172 (**15**) interact with the residues of CD1d within a **15**/CD1d complex, it was obvious that the stereochemistry of C6 hydroxy group influenced the immunostimulatory activity. When the relationship between the configuration of C4 hydroxy group and bioactivity of KRN7000 was investigated, its 4*R*-isomer induced larger amounts of cytokines than that of C4 epimer (4*S*-isomer).[13] According to the X-ray analysis, the C4 hydroxy group of KRN7000 makes no hydrogen-bonding interaction with any residues in human TCR/KRN7000/CD1d complex.[14] However, it is essential for the glycolipid to stimulate human iNKT cells.[15] It is thought that the inversion of the stereochemistry may change the binding conformation of glycolipid/CD1d complex, and also may cause to change the cytokine Th1/Th2 profiles secreted by iNKT cells, even if the hydroxyl group does not involved in the hydrogen-bonding network.

The iNKT cells secrete both Th1 and Th2 cytokines (such as IFNγ and IL-4, respectively) by stimulation of the α-galactosylceramides. In our previous report (cf. Ref. 7), for better EAE suppression effect, it is important to decrease the secretion amount of IFNγ less than 5–7% of KRN7000, and also to hold the secretion amount of IL-4 more than 60–70% of KRN7000 as shown in RCAI-147 and -160. In other words, not only the less secretion of IFNγ but also the more secretion of IL-4 gave the better clinical score of EAE suppression.

It is difficult to estimate better clinical score of EAE test from these few facts about the secretion amount of the cytokines IFNγ and IL-4. However we feel that there may be an upper limit of IFNγ and a lower limit of IL-4, for example, such as 'the amount of IFNγ has to be less than 5% of KRN7000, and the amount of IL-4 has to be at least more than 70% of KRN7000'.

In fact, a compound (RCAI-151)[7] increased IL-4 secretion amount to 138% of KRN7000. But the secretion amount of IFNγ was ~41% of KRN7000, and RCAI-151 biased to Th2 response (about 3.4 times compared to KRN7000). However this compound showed only weak suppression effect against EAE symptom. It may

be thought to be causally related to exceeding the upper limit of IFNγ.

Anyway, we may need to investigate the immunostimulatory activities of the other diastereomers of RCAI-147.

3. Conclusion

RCAI-172, C6 epimer of RCAI-147, was synthesized from a known compound (**1**)[10] in 11 steps. It is obvious that the cytokine secretion ability of RCAI-172 biases to the Th2 response as shown in Figure 2. However the IL-4 production was reduced to ~35% of KRN7000. This value was almost half compared to ~70% in the case of RCAI-147.[7] In addition, RCAI-172 did not show the effectiveness for the EAE suppression.[8,12] There exist seven chiral centers in the aglycon part of RCAI-172. Surprisingly, it is obvious that the change of configuration in just one position (C6-OH) has a fairly large effect both on the Th1/Th2 response and for the EAE suppression.

4. Experimental

4.1. General

IR spectra were measured with a Jasco FT/IR-460 plus spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian VNMRS-500 spectrometer using tetramethylsilane (TMS) as an internal standard. Mass spectra and high resolution MS were recorded with Jeol JMS-SX102A or Bruker BioAPEX II 70e FT-ICR mass spectrometers. Optical rotation value was measured with a Jasco P-1010 polarimeter. Column chromatography was carried out using silica gel 60 N (70–230 mesh ASTM, using ca 10–50 times weight of substrate if there is no indication) under a slightly elevated pressure for elution. Preparative TLC was carried out on a plate (Merck, Silica gel 60 F_{254}, 0.5 mm thick).

4.1.1. (2*R*,3*S*,4*R*,5*S*,6*R*)-3,4,5-(Tribenzyloxy)octadecane-1,2,6-triol (2)

To a solution of **1** (4.44 g, 6.04 mmol) in THF (80 mL) was added TBAF (1.0 M in THF, 22 mL). After stirring for 1 h at room temperature, the reaction mixture was diluted with EtOAc, washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a residue, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (3:1, then 1:1) gave **2** (3.67 g, 98%). IR ν_{max}(KBr) 3465, 2925, 2853, 1457, 1065 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* 7.0 Hz), 1.26 (19H, bs), 1.35–1.62 (3H, m), 3.54 (1H, dd, *J* 2.0, 7.4 Hz), 3.58 (1H, dd, *J* 4.9, 11.2 Hz), 3.64 (1H, m), 3.71 (1H, dd, *J* 5.7, 11.3 Hz), 3.76 (1H, t, *J* 3.2 Hz), 3.92 (1H, m), 4.10 (1H, dd, *J* 2.7, 7.4 Hz), 4.55, 4.73 (2H, AB-q, *J* 11.6 Hz), 4.58, 4.83 (2H, AB-q, *J* 11.0 Hz), 4.72, 4.78 (2H, AB-q, *J* 11.0 Hz), 7.27–7.36 (15H, m). ESI-MS: *m*/*z* 643.4 [M+Na]$^+$. HR ESI-MS: calcd for $C_{39}H_{56}O_6Na$: 643.3975; observed: 643.3952.

4.1.2. (2*R*,3*S*,4*R*,5*S*,6*R*)-1,2-(Isopropylidenedioxy)-3,4,5-(tribenzyloxy)octadecan-6-ol (3)

A solution of **2** (3.67 g, 5.15 mmol) in 2,2-dimethoxypropane (80 mL) containing *p*-toluenesulfonic acid monohydrate (70 mg) was stirred for 1.5 h at room temperature, and diluted with EtOAc, which was washed with satd aq $NaHCO_3$ and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a residue, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (9:1, then 6:1) gave **3** (3.55 g, 91%). IR ν_{max}(KBr) 3628, 2926, 2855, 1457, 1067 cm^{-1}. ^{1}H NMR (500 MHz, C_6D_6) δ 0.92 (3H, t, *J* 7.0 Hz), 1.31 (19H, bs), 1.36 (3H, s), 1.47 (3H, s), 1.52 (2H, m), 1.65 (1H, m), 3.69 (1H, dd, *J* 2.6, 5.8 Hz), 3.80–3.86 (3H, m), 3.93 (1H, m), 4.05 (1H, dd, *J* 6.3, 8.3 Hz), 4.52 (1H, m), 4.52–4.94 (6H, m), 7.10–7.44 (15H, m). ESI-MS: *m*/*z* 683.4 [M+Na]$^+$. HR ESI-MS: calcd for $C_{42}H_{60}O_6Na$: 683.4288; observed: 683.4289.

4.1.3. (2*R*,3*S*,4*R*,5*S*,6*R*)-1,2-(Isopropylidenedioxy)-3,4,5,6-(tetrabenzyloxy)octadecane (4)

To a solution of **3** (3.55 g, 5.37 mmol) in dry DMF (35 mL) were added NaH [60% oil dispersion, 656 mg (NaH: 393 mg, 16.4 mmol)] and BnBr (1.84 g, 10.7 mmol) at 0 °C, and the mixture was stirred for 1 h at room temperature. The reaction mixture was diluted with EtOAc, which was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a residue, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (19:1, then 9:1) gave **4** (2.61 g, 65%) as an oil. IR ν_{max}(KBr) 2924, 2853, 1516, 1496, 1455, 1067 cm^{-1}. ^{1}H NMR (500 MHz, C_6D_6) δ 0.92 (3H, t, *J* 6.8 Hz), 1.31 (19H, bs), 1.38 (3H, s), 1.48 (3H, s), 1.52 (1H, m), 1.70 (1H, m), 1.86 (1H, m), 3.81 (1H, m), 3.90–4.01 (5H, m), 4.49 (1H, m), 4.53, 4.58 (2H, AB-q, *J* 11.6 Hz), 4.68, 4.86 (2H, AB-q, *J* 11.8 Hz), 4.73 (2H, s), 4.74, 4.80 (2H, AB-q, *J* 11.5 Hz), 7.09–7.42 (20H, m). ESI-MS: *m*/*z* 773.5 [M+Na]$^+$. HR ESI-MS: calcd for $C_{49}H_{66}O_6Na$: 773.4757; observed: 773.4745.

4.1.4. (2*R*,3*S*,4*S*,5*S*,6*R*)-3,4,5,6-(Tetrabenzyloxy)octadecane-1,2-diol (5)

To a solution of **4** (2.61 g, 3.48 mmol) in CH_2Cl_2–MeCN (1:1, 140 mL) was added aq 46% HF (2.2 mL) at room temperature. The mixture was stirred for 15 min at room temperature, diluted with CH_2Cl_2, which was washed with satd aq $NaHCO_3$ and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give **5** (2.41 g, 98%) as an oil. IR ν_{max}(KBr) 3537, 2926, 2854, 1515, 1456 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* 7.0 Hz), 1.14–1.32 (19H, m), 1.48–1.68 (3H, m), 3.43 (1H, dd, *J* 5.0, 11.3 Hz), 3.48 (1H, m), 3.62 (1H, dd, *J* 6.4, 11.3 Hz), 3.68–3.71 (2H, m), 3.91 (1H, m), 4.09 (1H, dd, *J* 3.2, 7.4 Hz), 4.39, 4.50 (2H, AB-q, *J* 11.6 Hz), 4.43, 4.52 (2H, AB-q, *J* 11.4 Hz), 4.65, 4.78 (2H, AB-q, *J* 11.6 Hz), 4.77, 4.80 (2H, AB-q, *J* 10.8 Hz), 7.23–7.34 (20H, m). ESI-MS: *m*/*z* 733.4 [M+Na]$^+$. HR ESI-MS: calcd for $C_{46}H_{62}O_6Na$: 733.4444; observed: 733.4439.

4.1.5. (2*R*,3*S*,4*S*,5*S*,6*R*)-1-*tert*-Butyldimethylsilyloxy-3,4,5,6-(tetrabenzyloxy)octadecan-2-ol (6)

To a solution of **5** (2.41 g, 3.39 mmol) in dry CH_2Cl_2 (140 mL) was added TBDMS-Cl (1.02 g, 6.77 mmol), imidazole (690 mg, 10.1 mmol) and a catalytic amount of DMAP (7 mg). The mixture was stirred for 16 h at room temperature. The reaction mixture was diluted with CH_2Cl_2, which was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a residue, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (19:1, then 3:2) gave **6** (2.77 g, 99%). IR ν_{max}(KBr) 3538, 2925, 2854, 1518, 1508, 1456 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.03 (3H, s), 0.04 (3H, s), 0.88 (9H, s, and 3H, t, *J* 7.0 Hz), 1.10–1.32 (20H, m), 1.55–1.63 (2H, m), 3.49 (1H, dd, *J* 6.4, 10.8 Hz), 3.52 (1H, br, OH), 3.65–3.69 (3H, m), 3.84 (1H, dd, *J* 1.5, 4.2 Hz, C3-H), 3.92 (1H, t, *J* 6.6 Hz, changed to dt, *J* 1.5, 6.6 Hz, on addition of D_2O, C2-H), 4.14 (1H, dd, *J* 4.2, 6.4 Hz, C4-H), 4.41-4.83 (8H, m, benzylic protons), 7.22-7.33 (20H, m). ESI-MS: *m*/*z* 847.5 [M+Na]$^+$. HR ESI-MS: calcd for $C_{52}H_{76}O_6SiNa$: 847.5309; observed: 847.5299.

4.1.6. (2*S*,3*S*,4*S*,5*S*,6*R*)-2-Azido-1-*tert*-butyldimethylsilyloxy-3,4,5,6-(tetrabenzyloxy)octadecane (7)

Ph_3P (2.01 g, 7.66 mmol) and DEAD (2.2 M in toluene, 3.4 mL, 7.48 mmol) were added to a solution of **6** (1.40 g, 1.70 mmol) in dry THF (34 mL) under argon at room temperature. DPPA (2.11 g, 7.66 mmol) was added to this solution at 0 °C. After stirring for 30 min at 0 °C, the mixture was concentrated in vacuo to give a

mixture, which was chromatographed on a silica gel (85 g) column. Elution with hexane–EtOAc (19:1) gave **7** (1.44 g, quantitatively) as an oil. IR ν_{max}(KBr) 2926, 2855, 2098, 1455 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.04 (6H, s), 0.88 (3H, s), 0.88 (3H, t, *J* 7.1 Hz), 0.90 (9H, s), 1.19–1.30 (20H, m), 1.55–1.68 (2H, m), 3.53 (1H, m), 3.69 (1H, dd, *J* 3.2, 6.4 Hz), 3.76 (1H, m), 3.84 (1H, m), 4.01–4.05 (2H, m), 4.39–4.82 (8H, m), 7.17–7.32 (20H, m). ESI-MS: *m*/*z* 872.5 $[M+Na]^+$. HR ESI-MS: calcd for $C_{52}H_{75}N_3O_5SiNa$: 872.5374; observed: 872.5380.

4.1.7. (2*S*,3*S*,4*S*,5*S*,6*R*)-2-Azido-3,4,5,6-(tetrabenzyloxy)octadecan-1-ol (8)

To a solution of **7** (1.44 g, 1.70 mmol) in THF (30 mL) was added TBAF (1.0 M in THF, 5.1 mL). After stirring for 50 min at room temperature, the reaction mixture was concentrated in vacuo, and diluted with EtOAc, which was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a residue, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (9:1, then 4:1) gave **8** (1.20 g, 96%). IR ν_{max}(KBr) 3526 (br), 2925, 2853, 2097, 1455, 1065 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.89 (3H, t, *J* 7.0 Hz), 1.09–1.30 (20H, m), 1.53 (1H, m), 1.65 (1H, m), 2.77 (1H, t, *J* 5.6 Hz, OH), 3.46 (1H, m), 3.67–3.75 (3H, m), 3.83 (1H, t, *J* 3.8 Hz), 3.95 (1H, m), 4.06 (1H, dd, *J* 3.3, 7.7 Hz), 4.47 (2H, s), 4.48, 4.54 (2H, AB-q, *J* 11.4 Hz), 4.61, 4.78 (2H, AB-q, *J* 11.5 Hz), 4.77 (2H, s), 7.22–7.33 (20H, m). ESI-MS: *m*/*z* 758.5 $[M+Na]^+$. HR ESI-MS: calcd for $C_{46}H_{61}N_3O_5Na$: 758.4509; observed: 758.4492.

4.1.8. (2*S*,3*S*,4*S*,5*S*,6*R*)-2-Azido-3,4,5,6-(tetrabenzyloxy)octadecyl 2,3-di-*O*-benzyl-4,6-*O*-(di-*tert*-butyl)silylene-α-D-galactopyranoside (10)

(1) A solution of 2,3-di-*O*-benzyl-4,6-*O*-(di-*tert*-butyl)silylene-D-galactopyranose (1.47 g, 2.94 mmol) and CCl_3CN (3.0 mL, 29.7 mmol) in CH_2Cl_2 (30 mL) containing Cs_2CO_3 (1.11 g, 3.41 mmol) as a base was stirred for 16 h at room temperature, and the reaction mixture was diluted with CH_2Cl_2, which was washed with H_2O, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give crude trichloroacetimidoyl 2,3-di-*O*-benzyl-4,6-*O*-(di-*tert*-butyl)silylene-D-galactopyranoside (**9**), which was employed for the next reaction without further purification.

(2) To a solution of azido-alcohol **8** (1.27 g, 1.73 mmol) and the above obtained imidate (**9**) in dry CH_2Cl_2 (25 mL) was added MS 4 Å (dry powder, 3.0 g). After stirring for 30 min at room temperature, AgOTf (266 mg, 1.04 mmol) was added to this mixture. The mixture was stirred for 16 h at room temperature, and filtered. The filter cake was washed with CH_2Cl_2. The combined filtrate was washed with satd aq $NaHCO_3$, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a mixture which was chromatographed on a silica gel column. Elution with hexane–EtOAc (19:1, then 9:1) gave **10** (1.92 g, 91%) as an oil. IR ν_{max}(KBr) 2928, 2857, 2099, 1671, 1496, 1472, 1455 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* 6.9 Hz), 0.98 (9H, s), 1.04 (9H, s), 1.10–1.32 (20H, m), 1.50–1.65 (2H, m), 3.49–3.53 (2H, m), 3.67 (1H, dd, *J* 6.9, 9.8 Hz), 3.73 (1H, dd, *J* 3.9, 6.3 Hz), 3.78–3.83 (2H, m), 3.94–4.07 (6H, m), 4.39–4.55 (5H, m), 4.61–4.82 (9H, m), 7.15–7.41 (30H, m). ESI-MS: *m*/*z* 1240.7 $[M+Na]^+$. HR ESI-MS: calcd for $C_{74}H_{99}N_3O_{10}SiNa$: 1240.6997; observed: 1240.6995.

4.1.9. (2*S*,3*S*,4*S*,5*S*,6*R*)-2-Amino-3,4,5,6-(tetrabenzyloxy)octadecyl 2,3-di-*O*-benzyl-4,6-*O*-(di-*tert*-butyl)silylene-α-D-galactopyranoside (11)

To a solution of **10** (1.92 g, 1.58 mmol) in dry THF (30 mL) was added Me_3P (1.0 M solution in THF, 8.3 mL). After stirring for 2 h at room temperature, aq NaOH (1.0 M solution in H_2O, 30 mL) was added to this solution. The mixture was stirred for 2 h at room temperature, diluted with water, and extracted with $CHCl_3$, which was washed with H_2O and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (4:1, then 1:1) afforded **11** (1.33 g, 71%) as a gum. IR ν_{max}(KBr) 3031, 2926, 2856, 1496, 1455, 1362 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (3H, t, *J* 6.8 Hz) 1.00 (9H, s), 1.05 (9H, s), 1.17–1.30 (20H, m), 1.55–1.63 (2H, m), 3.25 (1H, m), 3.36 (1H, dd, *J* 8.3, 9.5 Hz), 3.54 (1H, s), 3.55 (1H, m), 3.66 (1H, dd, *J* 3.3, 6.0 Hz), 3.75 (1H, dd, *J* 4.7, 5.6 Hz), 3.81 (1H, dd, *J* 2.8, 9.9 Hz), 3.96–4.01 (4H, m), 4.08 (1H, dd, *J* 1.9, 12.5 Hz), 4.40–4.83 (10H, m), 7.19–7.42 (30H, m). ESI-MS: *m*/*z* 1192.7 $[M+H]^+$. HR ESI-MS: calcd for $C_{74}H_{102}NO_{10}Si$: 1192.7273; observed: 1192.7250.

4.1.10. (2*S*,3*S*,4*S*,5*S*,6*R*,2′*R*,3′*R*)-2-[2′,3′-(Isopropylidenedioxy)hexacosanoylamino]-3,4,5,6-(tetrabenzyloxy)octadecyl 2,3-di-*O*-benzyl-4,6-*O*-(di-*tert*-butyl)silylene-α-D-galactopyranoside (13)

To a solution of amine **11** (729 mg, 0.628 mmol) and (2*R*,3*R*)-(isopropylidenedioxy)hexacosanoic acid (**12**, 883 mg, 1.88 mmol) in dry CH_2Cl_2–THF (1:1, 70 mL) were added DMAP (921 mg, 7.54 mmol) and EDAC (1.45 g, 7.54 mmol). The mixture was stirred for 3 h at room temperature. The reaction mixture was diluted with CH_2Cl_2, which was washed with water and brine, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (19:1, then 7:1) gave **13** (547 mg, 54%) as an oil. IR ν_{max}(KBr) 2924, 2853, 1677, 1466, 1455 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.879 (3H, t, *J* 7.0 Hz), 0.883 (3H, t, *J* 7.0 Hz), 0.96 (9H, s), 1.02 (9H, s), 1.13–1.31 (65H, m, containing 3H singlet at 1.30 ppm), 1.38 (3H, s), 1.39–1.70 (4H, m), 3.50 (1H, s), 3.66 (1H, m), 3.70 (1H, m), 3.74 (1H, dd, *J* 3.0, 10.1 Hz), 3. 83 (1H, dd, *J* 2.4, 3.9 Hz), 3.90 (1H, dd, *J* 9.1, 11.5 Hz), 3.94 (1H, dd, *J* 4.0, 10.3 Hz), 3.99 (2H, bs), 4.09 (1H, dd, *J* 4.0, 6.5 Hz), 4.21 (1H, m), 4.31 (1H, m), 4.35–4.81 (16H, m), 7.00 (1H, d, *J* 8.3 Hz, NH), 7.18–7.41 (30H, m). ESI-MS: *m*/*z* 1665.1 $[M+Na]^+$. HR ESI-MS: calcd for $C_{103}H_{155}NO_{13}SiNa$: 1665.1165; observed: 1665.1166.

4.1.11. (2*S*,3*S*,4*S*,5*S*,6*R*,2′*R*,3′*R*)-2-[2′,3′-(Dihydroxy)hexacosanoylamino]-3,4,5,6-(tetrabenzyloxy)octadecyl 2,3-di-*O*-benzyl-α-D-galactopyranoside (14)

(1) To a solution of **13** (540 mg, 0.329 mmol) in CH_2Cl_2–CH_3CN (1:1, 200 mL) were added water (4 mL) and aq 46% HF (16 mL). After stirring for 3.5 h at room temperature, the reaction mixture was diluted with $CHCl_3$ (200 mL), and the whole was washed with satd aq $NaHCO_3$, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a mixture.

(2) The above obtained mixture was dissolved in dry THF (100 mL). Dry pyridine (1.80 g) and HF-pyridine complex (~70% HF, ~30% pyridine, 1.64 g) were added to this THF solution. After stirring for 2 h at room temperature, the reaction mixture was diluted with $CHCl_3$, which was washed with satd aq $NaHCO_3$, dried over $MgSO_4$, and filtered. The filtrate was concentrated in vacuo to give a mixture, which was chromatographed on a silica gel column. Elution with hexane–EtOAc (2:3, then 1:5) gave **14** (213 mg, two steps 44%) as a gum. IR ν_{max}(KBr) 3393 (br), 3063 (w), 3031 (w), 2924, 2852, 1648, 1523, 1497, 1466, 1455 cm^{-1}. ^1H NMR (500 MHz, $CDCl_3$) δ 0.879 (3H, t, *J* 7.0 Hz), 0.882 (3H, t, *J* 7.0 Hz), 1.01–1.30 (62H, m), 1.30–1.70 (4H, m), 2.11 (1H, dd, *J* 4.6, 8.0 Hz, OH), 2.49 (1H, s, OH), 3.42 (1H, dd, *J* 2.8, 3.9 Hz, on addition of D_2O), 3.44 (1H, d, *J* 5.6 Hz, OH), 3.47 (1H, dd, *J* 3.9, 11.7 Hz, on addition of D_2O), 3.53–3.66 (6H, m), 3.70 (1H, dd, *J* 3.1, 9.8 Hz), 3.77 (1H, dd, *J* 3.7, 9.8 Hz), 3.80 (1H, t, *J* 3.1 Hz), 3.85 (1H, d-like, *J* 5.8 Hz, OH), 3.91 (1H, d, *J* 2.5 Hz), 4.01 (1H, dd, *J* 3.4, 5.1 Hz), 4.22 (1H, dd, *J* 3.9, 10.2 Hz), 4.33 (1H, d, *J* 11.8 Hz), 4.48–4.78 (13H,

m), 6.81 (1H, d, *J* 8.8 Hz, NH), 7.20–7.40 (30H, m). ESI-MS: *m*/*z* 1484.9 $[M+Na]^+$. HR ESI-MS: calcd for $C_{92}H_{135}NO_{13}Na$: 1484.9831; observed: 1484.9845.

4.1.12. (2*S*,3*S*,4*S*,5*S*,6*R*,2′*R*,3′*R*)-2-[2′,3′-(Dihydroxy)hexacosanoylamino]-3,4,5,6-(tetrahydroxy)octadecyl α-D-galactopyranoside (15) (RCAI-172)

A solution of **14** (70 mg, 0.048 mmol) in THF (30 mL) containing $Pd(OH)_2$/C (20 wt.%, Degussa type, wet, ~50%, 140 mg) was stirred for 20 h under hydrogen atmosphere (balloon) at room temperature, and the reaction mixture was concentrated in vacuo to give a crude mixture, which was dissolved in pyridine. The pyridine solution was filtered, and the filter cake was washed with pyridine. The combined filtrate was concentrated in vacuo to give a crude product **15** to which dry Et_2O (10 mL) was added. The mixture was stirred for 16 h at room temperature to yield a powder, which was washed with Et_2O for several times to give **15** (36 mg, 82%) as a powder. $[\alpha]_D^{25}$ +53.1 (*c* 1.0, pyridine). IR ν_{max}(KBr) 3315 (br), 2920, 2850, 1638, 1562, 1468 cm^{-1}. ^1H NMR (500 MHz, pyridine-d_5): δ 0.87 (6H, t, *J* 7.0 Hz), 1.20–1.39 (58H, m), 1.50–1.62 (2H, m), 1.72–1.84 (2H, m), 1.95–2.02 (4H, m), 4.32–4.38 (3H, m), 4.39–4.43 (2H, m), 4.45–4.50 (3H, m), 4.52–4.55 (2H, m), 4.58 (1H, d, *J* 2.9 Hz), 4.62 (1H, d, *J* 9.3 Hz), 4.66 (1H, dd, *J* 3.9, 9.7 Hz), 4.71 (1H, d, *J* 4.9 Hz), 4.75 (1H, dd, *J* 6.1, 10.8 Hz), 4.86 (1H, dd, *J* 3.1, 9.0 Hz), 5.38 (1H, m), 5.64 (1H, d, *J* 3.9 Hz), 5.90 (1H, s, low and broad), 8.70 (1H, d, *J* 9.0 Hz, NH). ^{13}C NMR (125 MHz, pyridine-d_5): δ 14.31, 22.97, 26.49, 26.52, 29.64, 29.95, 30.02, 30.08, 30.12, 30.22, 30.25, 30.31, 32.15, 32.66, 34.58, 50.66, 62.65, 67.91, 70.30, 70.99, 71.74, 72.91, 73.15, 73.19, 73.50, 74.12, 74.40, 76.07, 101.22, 173.96. ESI-MS: *m*/*z* 944.7 $[M+Na]^+$. HR ESI-MS: calcd for $C_{50}H_{99}NO_{13}Na$: 944.7014; observed: 944.7031. Both compounds, RCAI-172 and -147[7] are stable at room temperature, and they are much more soluble compared to KRN7000 in the medium for bioassay.

4.2. Methods for measurement of biological activity

4.2.1. Bioassay (mouse in vivo)

The stock solutions (1.0 mg/mL in DMSO) of KRN7000 and RCAI-172 were diluted to 10 μg/mL in Dulbecco's phosphate buffered saline (Sigma, Product No. D8537) just before injection into mice. Each glycolipid solution (10 μg/mL, 200 μL) was administered intravenously. Peripheral blood was collected from the retro-orbital plexus of mice at indicated time points, using heparin-coated capillary tubes (Funakoshi Pharmaceutical, Japan), and plasma was prepared.

4.2.2. Cytokine measurement

The cytokine concentrations in plasma were quantified by cytometric bead array (CBA) system (BD Biosciences) for mouse IFN-γ and IL-4 according to the manufacturer's protocol.

4.2.3. EAE induction by active immunization in C57BL/6 (B6) mice, and Clinical Score[8,12]

EAE is induced in B6 female mice by immunization with an emulsion of MOG35-55 peptide in complete Freund's adjuvant (CFA). MOG in CFA (Hooke Lab) solution (200 μL) was subcutaneously injected at 2 sites on lower back (one injection over each hip/base of tail) with 100 μL of emulsion at each site on day 1. 5 ng/mL pertussis toxin (200 μL in PBS) was intraperitoneally injected on day 1 and day 3. Mice were observed daily until day 30 for clinical score as 0–5 graduations with 0.5 for intermediate scores. 0: No clinical signs, 1: flaccid tail, 2: hind limb weakness or abnormal gait, 3: complete hind limb paralysis, 4: complete hind limb paralysis + forelimb weakness or paralysis, 5: moribund or deceased. In order to analyze the effect of glycolipid on EAE, each glycolipd-pulsed GM-CSF induced DC (GM-DC) (5×10^5 cells/mouse) were intravenously injected on day 5 and day 3. GM-DC were induced by bone marrow by culturing with GM-CSF (10 ng/mL) for 5 days, and then enriched by CD11c MACS beads (Miltenyi Biotec). Each glycolipid (100 ng/mL) was pulsed by culturing GM-DC (5×10^6/mL) for 24 h.

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