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RCAI-133, an *N*-methylated analogue of KRN7000, activates mouse natural killer T cells to produce Th2-biased cytokines†‡

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We synthesized seven new analogues of KRN7000: RCAI-133 and 154–159. RCAI-154, 156 and 158 are secondary-amide analogues having a hydroxy, a methyl or two methyl groups at the α-position of a linear C_{18}-acyl chain, respectively. RCAI-155, 157 and 159 are corresponding *N*-methylated tertiary amide analogues, and RCAI-133 is the *N*-methylated KRN7000. Among them, a PBS solution of RCAI-133 induced mouse lymphocytes to produce Th2-biasing cytokines *in vivo*, while RCAI-154–159 showed only weak or almost no immunostimulatory activity. Therefore, *N*-methylation led the glycolipid to produce predominantly Th2-type cytokines, while acyl α-substitution was detrimental to activity.

Introduction

KRN7000 (α-GalCer, **A**, Fig. 1) was developed by researchers at Kirin Brewery Co. as an anticancer drug candidate in 1995.[2] It was obtained through the structure–activity relationship studies on agelasphins, which are anticancer glycosphingolipids isolated from an Okinawan marine sponge, *Agelas mauritianus* (the main component is agelasphin-9b, **C**).[3,4] It has been known that these glycosphingolipids are ligands of CD1d protein, a glycolipid presentation protein of antigen presenting cells (APC).[5,6] The CD1d–glycosphingolipid complex is recognized by the invariant (mouse: Vα14, human: Vα24) T cell receptor (TCR) of natural killer T (NKT) cells, and activates them. In 2005, the crystal structures of the binary complexes of mouse and human CD1d-**A** were solved.[7,8] X-ray information of the ternary human and mouse CD1d-**A**–TCR complexes was also reported in 2007 and 2009, respectively.[9,10]

NKT cells activated with **A** simultaneously release both helper T type 1 (Th1) and Th2 cytokines in large quantities by a single injection. Th1 cytokines such as interferon (IFN)-γ mediate protective immune functions like tumor rejection, whereas Th2 cytokines such as interleukin (IL)-4 mediate regulatory immune functions to ameliorate autoimmune diseases. Additionally, Th1 and Th2 cytokines can antagonize each other in biological actions.[11] If there is a glycolipid which stimulates NKT cells to produce only (or highly biased) Th1 or Th2 cytokines, it can be a promising drug candidate.[12] Many research groups, therefore, are making efforts to develop such glycolipids.

To date, a world-wide effort has led to the synthesis and assay of numerous analogues of **A** (see reviews).[13] Some of them inducing Th2-type immune responses are shown in Fig. 1. OCH (**B**) is the pioneering Th2-biased cytokine inducer, and was derived by truncation of the two alkyl chains of **A**.[14,15] A 1,2,3-triazole (**D**)[16] and an ω-(dialkylamino)acyl (**E**)[17] analogues developed by Kim and co-workers in 2007 and 2010, respectively, induced enhanced IL-4 production compared to **A** *in vitro* (mouse). An α,α-difluoroacyl analogue (**F**) was developed by Linclau's group in 2009, and caused IL-4 biased cytokine production.[18] We reported that sulfonamido and ester analogues of **A** also induced Th2-pype immune responses in 2008 and 2010, respectively.[19,20] Modification of the sugar part or the sphingosine chain of **A** also generated some Th2-type analogues such as αGalCerMPEG (**G**) or **H**, respectively.[21,22]

In 2011, we reported that an *N*-methylated glycosphingolipid analogue of **A** (RCAI-127, **2**, Fig. 2) induced Th2-biased cytokine production *in vivo* when it was administered as liposome particles.[23] Because **2** showed weak Th2-type immunostimulatory activity when it was administered into mice as a phosphate buffered saline (PBS) solution, we employed the liposome-uptake technology to enhance the bioavailability of **2**.[24] We have continued our exploration to discover analogues of **2** that induce Th2-biased cytokine production that does not require a liposome formulation. In this regard we have prepared and evaluated derivatives possessing methyl groups, not limited to *N*-methyl, but also at the α-position of the acyl chain to study the influence of bulky groups near the amide linkage.

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† Electronic supplementary information (ESI) available: The synthetic procedure of α-galactosyl ceramides **3–9** and their ^{1}H- and ^{13}C-NMR spectra. See DOI: 10.1039/c3md00073g

‡ Synthesis of sphingosine relatives, Part XXXVII and Part XXXVI, see ref. 1.

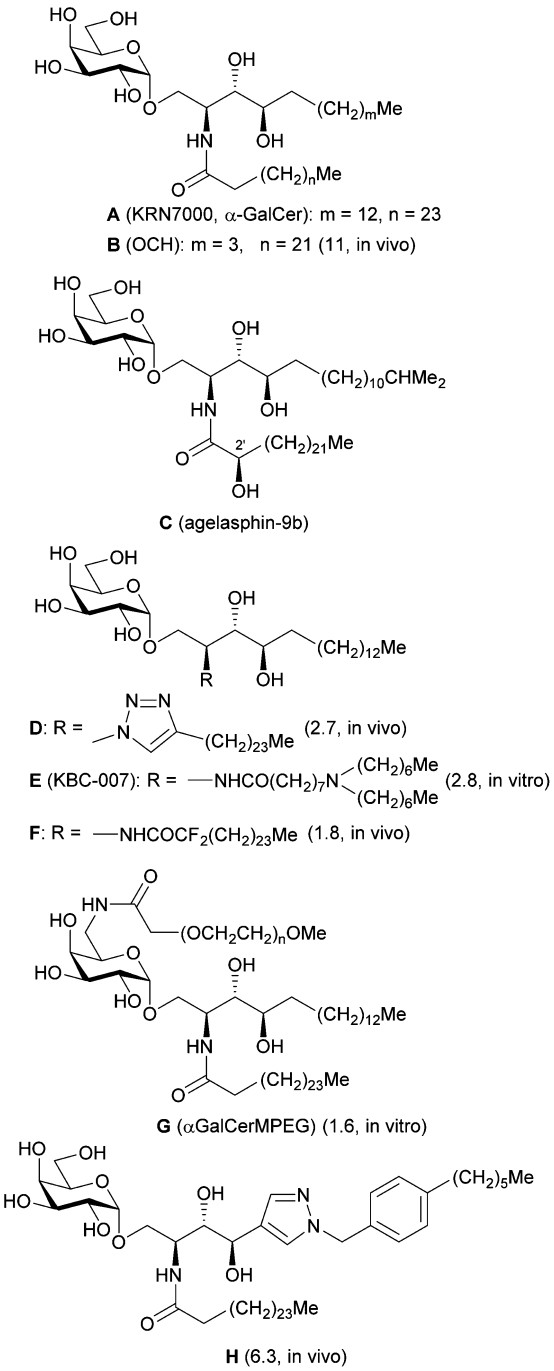


Fig. 1 Structures of KRN7000 (**A**), agelasphin-9b (**C**) and typical Th2-type glycolipids (**B** and **D–H**). IL-4/IFN-γ ratios of the relative intensities of cytokines produced by each compounds compared to those obtained by KRN7000 are shown in the parentheses.

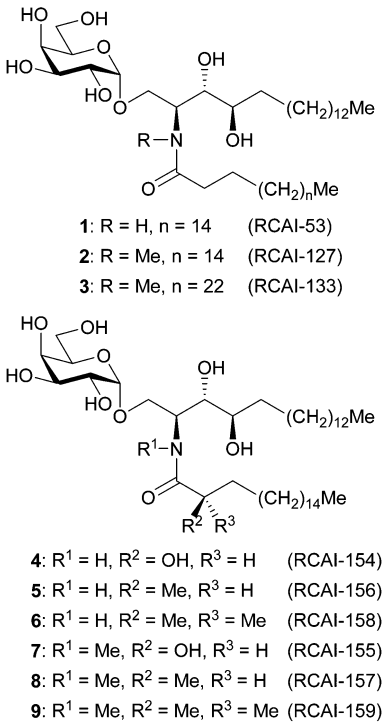


Fig. 2 Structures of synthesized analogues of **A**: RCAI-53, 127, 133 and 154–159.

Agelasphin-9b (**C**, Fig. 1) possesses a hydroxy group at the 2′-position with *R*-configuration.[3,4] In addition to **C**, many analogues possessing a 2*R*-hydroxy group were synthesized by Kirin's group,[2] and all of those showed a potent proliferative activity *in vitro* (mouse).[2] From these results, we thought that the 2′-position of **A** could be modified. We therefore planned to synthesize and evaluate analogues having a 2′*R*-hydroxy, a 2′*R*-methyl or 2′,2′-dimethyl groups (such as **4–6**, Fig. 2), along with the corresponding *N*-methylated analogues (such as **7–9**).

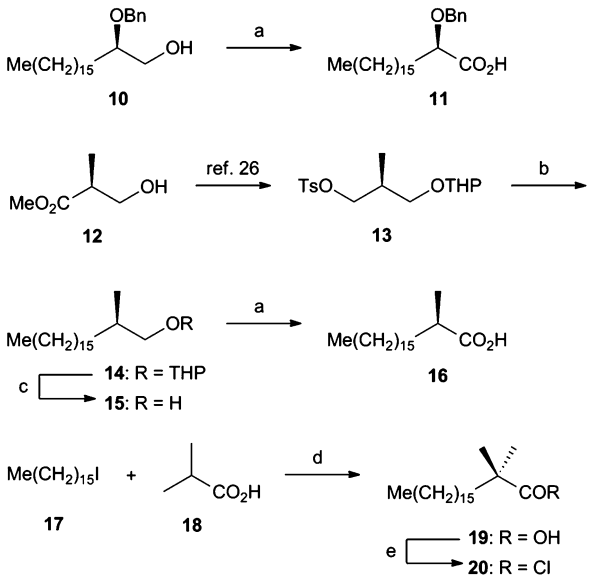
This communication describes the synthesis of RCAI-154–159 (**4–9**), whose immunostimulatory activity was compared with those caused by a C_{18}-acyl analogue (RCAI-53, **1**) and its *N*-methylated one (RCAI-127, **2**). It is found that **2** induces mouse lymphocytes to produce the largest amount of IL-4 among these analogues. Subsequent synthesis and evaluation of an *N*-methylated C_{26}-acyl analogue of **A** (= *N*-methylated KRN7000, RCAI-133, **3**) revealed **3** to be a highly Th2-biased cytokine inducer even when it was administered into mice as a PBS solution.

Results and discussions

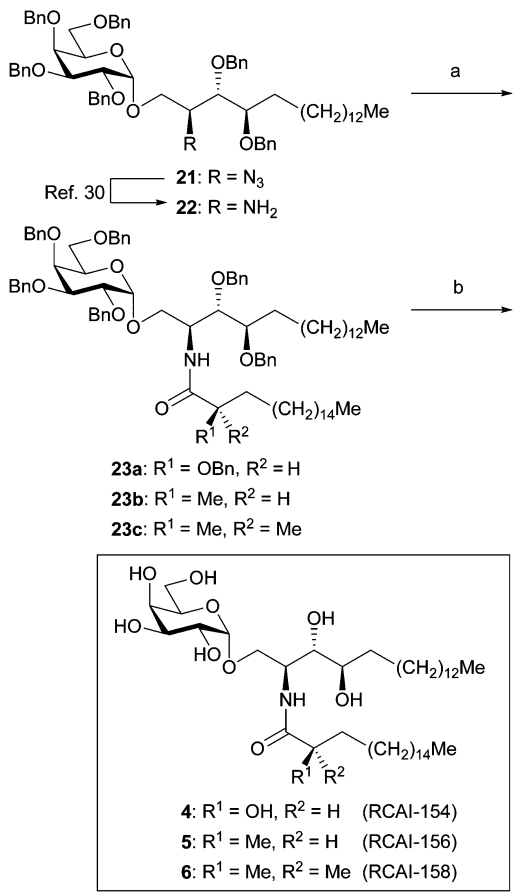
Synthesis of RCAI-154–159

As shown in Scheme 1, we prepared three kinds of acids, 2*R*-hydroxy-, 2*R*-methyl- and 2,2-dimethyloctadecanoic acids (**11**, **16** and **19**). Acid **11** was obtained by Jones oxidation of the known alcohol **10** (48%),[25] which could be prepared from commercially available octadecane-1,2-diol (see ESI†). Acid **16** was prepared from methyl (*S*)-3-hydroxy-2-methylpropanoate (**12**). According to our previous report,[26] **12** was converted to tosylate **13**, whose alkyl chain was then elongated by the Grignard reaction under Schlosser conditions[27] to give tetrahydropyranyl (THP) ether **14** in 58% yield. Deprotection of the THP group afforded alcohol **15** (89%),[28] which was oxidized with Jones reagent to yield **16** in 82% yield. Acid **19** was obtained by dianion alkylation of **18** with **17** in one step (81%).[29]

In Scheme 2, syntheses of analogues **4**, **5** and **6** (RCAI-154, 156 and 158) are summarized. The known amine **22** (ref. 30) was



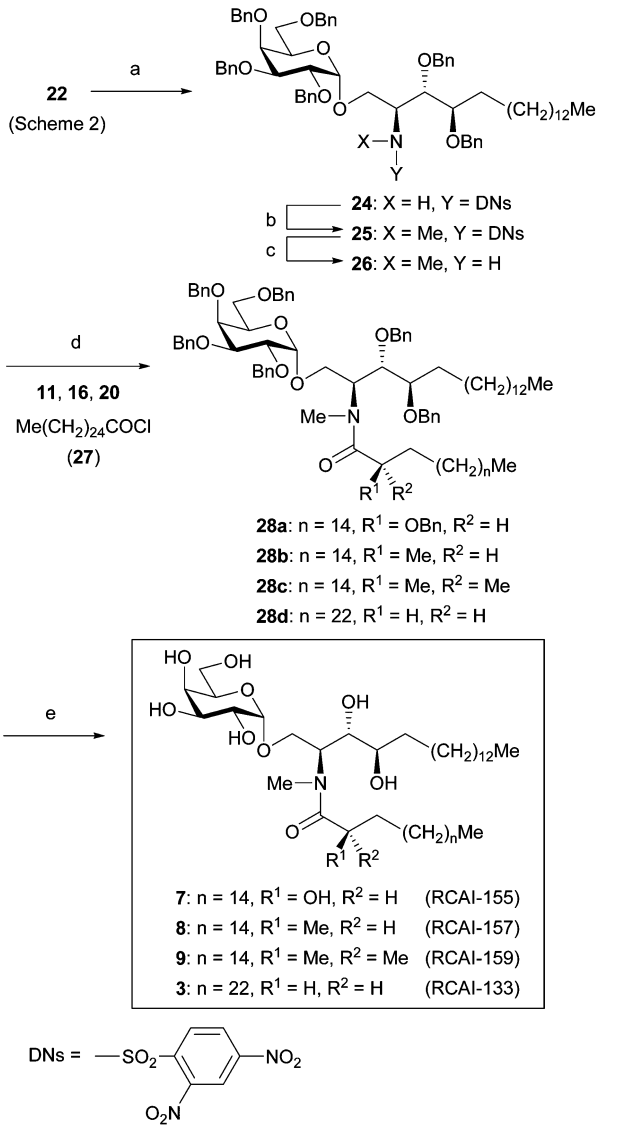
Scheme 1 Synthesis of three carboxylic acids **11**, **16** and **19**. *Reagents and conditions*: (a) Jones CrO_3, acetone, 0–25 °C, 48% for **11** and 82% for **16**. (b) $CH_3(CH_2)_{14}MgBr$, cat. Li_2CuCl_4, THF, −40 to 25 °C, 58% (c) *p*-TsOH, MeOH–CH_2Cl_2 (1 : 1), 25 °C, 89%. (d) NaH, LDA, THF, 0–25 °C, 81%. (e) $(COCl)_2$, benzene, 60 °C.



Scheme 2 Synthesis of RCAI-154, 156 and 158 (**4–6**). *Reagents and conditions*: (a) **11** or **16**, EDC, DMAP, CH_2Cl_2, 25 °C, 82% for **23a**, 84% for **23b**. **20**, Et_3N, CH_2Cl_2, 25 °C, 93% for **23c**. (b) H_2, $Pd(OH)_2$–C, THF, 25 °C, 52–94%.

obtained by Staudinger reduction[31] from azide **21** (ref. 32) in 85% yield. Amine **22** was then condensed with **11** or **16** to afford amide **23a** or **23b** (82% and 84%, respectively). Amide **23c** was obtained by acylation of **22** with acyl chloride **20** (Scheme 1) in 93% yield.[33] Finally, deprotection of all of the benzyl groups of **23a–c** furnished **4**, **5** and **6** in 52–94% yield.[34]

Analogues **7**, **8** and **9** (RCAI-155, 157 and 159) were synthesized as shown in Scheme 3. Amine **22** was treated with 2,4-dinitrobenzenesulfonyl (DNs) chloride to give sulfonamide **24** (68%). After *N*-methylation, the DNs group of the resulting **25** was removed by treatment with thioglycolic acid to afford amine **26** in 68% yield (two steps).[35] Two-step transformation, acylation and hydrogenolysis of the benzyl groups of **28a–c**, similar to that employed in the synthesis of **4–6**, furnished **7**, **8** and **9** in 46–73% yield.[34]



Scheme 3 Synthesis of RCAI-155, 157 and 159 (**7–9**). *Reagents and conditions*: (a) DNsCl, pyridine, 25 °C, 68%. (b) MeI, K_2CO_3, DMF, 25 °C, 68%. (c) $HSCH_2CO_2H$, Et_3N, CH_2Cl_2, 25 °C, 99%. (d) **11** or **16** or **27**, EDC, DMAP, CH_2Cl_2, 25 °C, 88% for **28a**, 99% for **28b**. **20** or **27**, Et_3N, CH_2Cl_2, 25 °C, 88% for **28c**, 94% for **28d**. (e) H_2, $Pd(OH)_2$–C, THF, 25 °C, 32–88%.

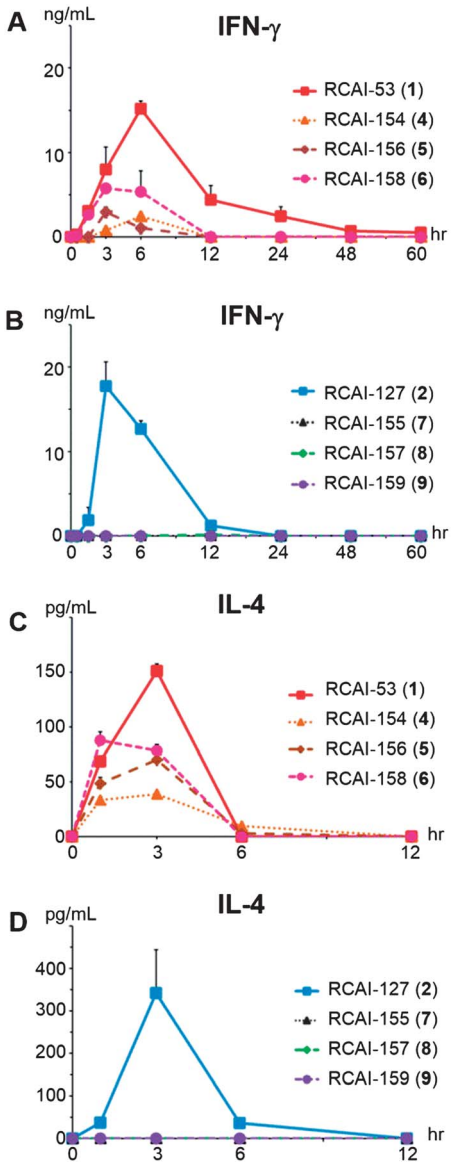


Fig. 3 Cytokine level in serum after intravenous injection into mice of PBS solutions of RCAI-154–159 (**4–9**) (2 μg per mouse). Serum concentrations of IFN-γ (A and B) and IL-4 (C and D) were measured at the indicated time points. Data are means ± SD from 3 mice and repeated two times with similar results.[36,37]

Bioassay of RCAI-154–159

The results of bioassay in mice *in vivo* are shown in Fig. 3.[36,37] The concentrations of cytokines in sera of mice were monitored after intravenous administration of synthesized analogues (**1**, **2** and **4–9**) as phosphate buffered saline (PBS) solutions into C57BL/6 mice.

From Fig. 3A and C, the cytokine concentrations of both IFN-γ and IL-4 induced by RCAI-154 (**4**), 156 (**5**) or 158 (**6**) were lower than those by RCAI-53 (**1**). The former three analogues have a hydroxy group or methyl group(s) at the α-position to the amide carbonyl group. So, the bulkiness around the amide group of these analogues is larger than that of **1**. According to the X-ray structure of CD1d-**A**,[7,8] the unoccupied volume of the binding site of CD1d around the amide group is not so large, so the increment of the bulkiness is thought to decrease the binding stability to the presentation protein, CD1d. Therefore, it was found that introduction of extra functional group(s) at the 2′-position diminished immunostimulatory activity. A similar tendency is observed in Fig. 3B and D. The most potent analogue was RCAI-127 (**2**), and the other analogues possessing extra substituent(s) at the 2′-position induced almost no cytokine production. From Fig. 3B and D, RCAI-127 (**2**) induced the largest amount of IL-4 (with the largest IL-4/IFN-γ ratio) among these analogues.

Synthesis and bioassay of RCAI-133

As described above, *N*-methylation was found to be an effective derivatization to obtain a Th2-type glycolipid. We then planned to investigate the immunostimulatory activity of an *N*-methylated C_{26}-acyl analogue, RCAI-133 (**3**, Fig. 2). As shown in Scheme 3, we synthesized **3** from **26** by acylation with cerotyl chloride[38] followed by deprotection (30%, two steps).[34] After administration of **3** as a PBS solution into mice, the cytokine concentrations in sera were monitored (Fig. 4). As can be seen, **3** induced a large amount of IL-4 and a small amount of IFN-γ. The IL-4/IFN-γ ratio of the cytokine concentrations at the peak time induced by **3** (1.7×10^{-2}) was most potent than those by **A** (0.43×10^{-2}) or **2** (0.95×10^{-2}). The IL-4/IFN-γ ratio of the relative intensities of cytokines produced by **3** compared to those obtained by **A** was 3.1. Therefore, RCAI-133 (**3**) was found to be the potent Th2-type immunostimulant.

According to the ^{1}H-NMR spectrum of **3**, the rotamer ratio was *ca.* 2 : 1 (ESI†).[34] These rotamers could not be separated, and are thought to be in equilibrium at the body temperature. Therefore, it is difficult to say which rotamer is more potent, or

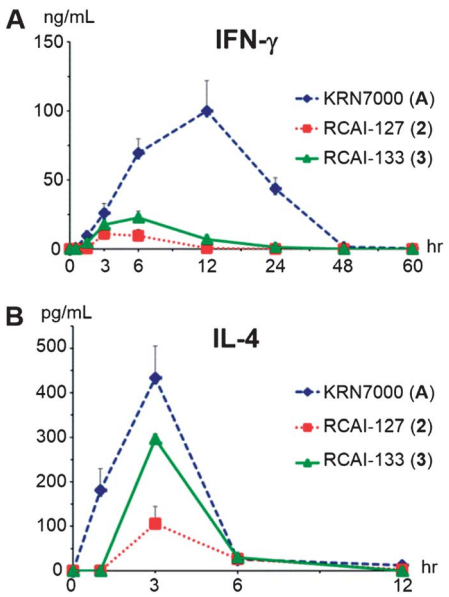


Fig. 4 Cytokine in serum after injection in mice of KRN7000 (**A**), RCAI-127 (**2**) or RCAI-133 (**3**) (2 μg per mouse). Serum concentrations of IFN-γ (A) and IL-4 (B) were measured at the indicated time points. Data are means ± SD from 3 mice and repeated two times with similar results.[36,37]

which rotamer makes a more stable complex with CD1d. At all events, the appended methyl group of RCAI-133 (**3**) seemed to weaken the binding affinity of **3** to CD1d. According to Oki *et al.*,[15] the analogues possessing low affinity to CD1d (like OCH, **B**) stimulate NKT cells to produce Th2-biased cytokines.[15] RCAI-133 (**3**) might be also one of such 'low affinity' ligands, and be the selective Th2-type immunostimulant, although further evidence is required to support this assumption.

Finally, it should be noted that we prepared liposome particles composed of **3**/DOPC/DC-Chol, which is the best liposome composition inducing Th2-type bioactivities in our previous examination.[23] Liposomal **3** also stimulated mouse lymphocytes to produce Th2-biased cytokines. The peak time concentration of IL-4 induced by liposomal **3** was, however, almost the same level as that induced by the PBS solution of **3**.

Conclusions

We synthesized six analogues of KRN7000 with a C_{18}-length acyl chain. The extra functional groups, an (*R*)-hydroxy group, an (*R*)-methyl group, or two methyl groups, were introduced at the 2-position to the amide carbonyl group of RCAI-57 (**1**) to yield RCAI-154 (**4**), 156 (**5**), and 158 (**6**), respectively, and of RCAI-127 (**2**) to afford 155 (**7**), 157 (**8**), and 159 (**9**), respectively. Their immunostimulatory activities were examined together with RCAI-57 (**1**) and 127 (**2**) in mice *in vivo*. Among them, RCAI-127 (**2**) induced a potent Th2-biased cytokine production.

RCAI-133 (**3**), an *N*-methylated KRN7000, was also synthesized and evaluated. Administration of its PBS solution induced mouse lymphocytes to produce a larger amount of Th2-biased cytokines compared to RCAI-127 (**2**), therefore, RCAI-133 (**3**) was found to be a potent stimulant of mouse lymphocytes to induce Th2-type immune responses.[39]

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33 Partial racemization took place when **11** or **16** was treated with $(COCl)_2$ (in benzene, 60 °C, 1 h) to prepare the corresponding acyl chlorides.
34 Due to the existence of the *N*-methyl group, ^{1}H- and ^{13}C-NMR spectra of RCAI-133 (**3**), 155 (**7**) and 157 (**8**) were recorded as the mixture of two rotamers, while the NMR spectra of RCAI-159 (**9**) were broadened.
Physical data of RCAI-133 (3): Mp 174–176 °C; $[\alpha]_D^{20}$ + 41.6 (*c* 0.31, pyridine); ν_{max} (KBr): 3440 (br s, OH), 3300 (s, OH), 1610 (br s, CO), 1270 (m), 1070 (s, C–O), 1055 (s, C–O), 720 (m) cm^{-1}; δ_H (500 MHz, pyridine-d_5, rotamer ratio = *ca.* 2 : 1): the typical peaks of the major rotamer: 5.41 (0.7H, d, J = 3.5 Hz, 1″-H), 3.25 (2H, s, NMe), 2.35 (0.7H, dt, J = 15, 7.5 Hz, 2′-H$_a$), 2.32 (0.7H, dt, J = 15, 7.5 Hz, 2′-H$_b$), 0.84 (4H, d, J = 7.0 Hz, 18-H$_3$, 18′-H$_3$) ppm. The typical peaks of the minor rotamer: 5.44 (0.3H, d, J = 3.5 Hz, 1″-H), 3.39 (1H, s, NMe), 2.89 (0.3H, dt, J = 15, 7.5 Hz, 2′-H$_a$), 2.85 (0.3H, dt, J = 15, 7.5 Hz, 2′-H$_b$), 0.85 (2H, t, J = 7.0 Hz, 18-H$_3$, 18′-H$_3$) ppm; δ_C (126 MHz, pyridine-d_5): the typical peaks of the major rotamer: 173.8 (1′-C), 101.7 (1″-C) ppm. The typical peaks of the minor rotamer: 174.2 (1′-C), 101.3 (1″-C) ppm; HRMS (ESI+): calcd for $C_{51}H_{102}NO_9$ [M + H]$^+$ 872.7549; found 872.7547.
RCAI-154 (4): Mp 218–220 °C; $[\alpha]_D^{25}$ + 49.7 (*c* 0.31, pyridine); ν_{max} (KBr): 3350 (br s, OH, NH), 1645 (br s, CO), 1620 (br s, CO), 1535 (br m), 1075 (br s, C–O) cm^{-1}; δ_H (500 MHz, pyridine-d_5): 8.51 (1H, d, J = 9.5 Hz), 6.73 (1H, br s), 6.65–6.22 (5H, m), 6.12 (1H, br s), 5.60 (1H, d, J = 4.0 Hz), 5.31–5.26 (1H, m), 4.67–4.62 (2H, m), 4.59 (1H, dd, J = 7.0, 3.0 Hz), 4.53 (1H, br d, J = 2.5 Hz), 4.50–4.47 (1H, m), 4.47 (1H, dd, J = 11, 4.0 Hz), 4.42–4.30 (5H, m), 4.29–4.25 (1H, m), 2.32–2.24 (1H, m), 2.23–2.15 (1H, m), 2.03–1.94 (1H, m), 1.94–1.83 (2H, m), 1.79–1.61 (4H, m), 1.46–1.16 (47H, m), 0.84 (6H, t, J = 7.0 Hz) ppm; δ_C (126 MHz, pyridine-d_5): 175.0, 101.3, 76.6, 73.1, 72.4, 72.3, 71.6, 71.0, 70.2, 68.2, 62.7, 50.5, 35.6, 34.5, 32.1, 30.4, 30.2, 30.04, 30.03, 30.00, 29.99, 29.98, 29.9, 29.6, 26.5, 25.8, 22.9, 14.3 ppm; HRMS (ESI+): calcd for $C_{42}H_{83}NO_{10}Na$ [M + Na]$^+$ 784.5915; found 784.5912.
RCAI-155 (7): Mp 118–122 °C; $[\alpha]_D^{21}$ + 46.0 (*c* 0.30, pyridine); ν_{max} (KBr): 3300 (br s, OH), 1640 (br s, CO), 1610 (br s, CO), 1075 (br s, C–O) cm^{-1}; δ_H (500 MHz, $CDCl_3$, rotamer ratio = *ca.* 1 : 1): the typical peaks: 5.42 (1H, d, J = 3.5 Hz, 1″-H), 5.30–5.26 (1H, m, 2-H), 3.45 (1.5H, s, NMe), 3.44 (1.5H, s, NMe), 0.85 (3H, t, J = 7.0 Hz), 0.84 (3H, t, J = 7.0 Hz) ppm; δ_C (126 MHz, pyridine-d_5): the typical peaks of the major rotamer: 176.4 (1′-C), 101.9 (1″-C) ppm. The typical peaks of the minor rotamer: 176.2 (1′-C), 101.5 (1″-C) ppm; HRMS (ESI+): calcd for $C_{43}H_{85}NO_{10}Na$ [M + Na]$^+$ 798.6071; found 798.6074.
RCAI-156 (5): Mp 143–145 °C; $[\alpha]_D^{21}$ + 46.4 (*c* 0.30, pyridine); ν_{max} (KBr): 3280 (br s, OH, NH), 1640 (br s, CO), 1540 (br s), 1145 (br m, C–O), 1075 (br s, C–O) cm^{-1}; δ_H (500 MHz, pyridine-d_5): 8.35 (1H, d, J = 8.5 Hz), 6.93 (1H, br s), 6.70–6.24 (4H, m), 6.06 (1H, br s), 5.57 (1H, d, J = 3.5 Hz), 5.22–5.18 (1H, m), 4.65 (1H, dd, J = 11, 5.0 Hz), 4.63 (1H, d, J = 9.5, 3.5 Hz), 4.55 (1H, br d, J = 2.5 Hz), 4.50 (1H, dd, J = 6.0, 5.5 Hz), 4.46–4.39 (3H, m), 4.37 (1H, dd, J = 11, 6.0 Hz), 4.33–4.27 (2H, m), 2.62–2.55 (1H, m), 2.29–2.22 (1H, m), 1.94–1.84 (3H, m), 1.69–1.61 (1H, m), 1.49–1.17 (54H, m), 0.84 (6H, t, J = 7.0 Hz) ppm; δ_C (126 MHz, pyridine-d_5): 176.7, 101.7, 76.7, 73.0, 72.5, 71.7, 71.0, 70.3, 68.8, 62.7, 51.5, 41.4, 34.9, 34.2, 32.1, 30.3, 30.12, 30.11, 30.04, 30.01, 29.99, 29.98, 29.91, 29.89, 29.6, 28.0, 26.5, 22.9, 18.5, 14.3 ppm; HRMS (ESI+): calcd for $C_{43}H_{85}NO_9Na$ [M + Na]$^+$ 782.6122; found 782.6113.
RCAI-157 (8): Mp 131–133 °C; $[\alpha]_D^{21}$ + 30.9 (*c* 0.29, pyridine); ν_{max} (KBr): 3320 (br s, OH), 1610 (br s, CO), 1150 (m), 1080 (br s, C–O) cm^{-1}; δ_H (500 MHz, pyridine-d_5, rotamer ratio = *ca.* 7 : 3): the typical peaks of the major rotamer: 5.40 (0.7H, d, J = 4.0 Hz, 1″-H), 3.34 (2.1H, s, NMe), 0.84 (4.2H, d, J = 7.0 Hz, 18-H$_3$, 18′-H$_3$) ppm. The typical peaks of the minor rotamer: 5.47 (0.3H, d, J = 4.0 Hz, 1″-H), 3.45 (0.9H, s, NMe), 0.85 (1.8H, t, J = 7.0 Hz, 18-H$_3$, 18′-H$_3$) ppm; δ_C (126 MHz, pyridine-d_5): the typical peaks of the major rotamer: 177.3 (1′-C), 101.8 (1″-C), 17.8 (2′-Me) ppm. The typical peaks of the minor rotamer: 177.9 (1′-C), 101.1 (1″-C), 18.8 (2′-Me) ppm; HRMS (ESI+): calcd for $C_{44}H_{87}NO_9Na$ [M + Na]$^+$ 796.6279; found 796.6274.
RCAI-158 (6): Mp 132–134 °C; $[\alpha]_D^{23}$ + 56.3 (*c* 0.31, pyridine); ν_{max} (KBr): 3380 (br s, OH, NH), 1640 (br s, CO), 1535 (br m), 1155 (m), 1070 (br s, C–O) cm^{-1}; δ_H (500 MHz, pyridine-d_5): 7.46 (1H, d, J = 8.5 Hz), 6.46 (1H, br s), 5.57 (1H, d, J = 4.0 Hz), 5.31 (5H, br s), 5.15–5.10 (1H, m), 4.65 (1H, dd, J = 10, 4.0 Hz), 4.62–4.58 (2H, m), 4.51–4.47 (2H, m), 4.43–4.37 (3H, m), 4.28–4.25 (2H, m), 2.28–2.20 (1H, m), 1.94–1.82 (2H, m), 1.69–1.59 (3H, m), 1.45–1.17 (53H, m), 1.33 (3H, s), 0.84 (6H, t, J = 7.0 Hz) ppm; δ_C (126 MHz, pyridine-d_5): 177.5, 101.5, 76.5, 73.1, 72.4, 71.7, 71.0, 70.2, 68.5, 62.6, 51.5, 42.4, 41.7, 34.2, 32.1, 30.7, 30.4, 30.14, 30.08, 30.02, 30.01, 30.00, 29.98, 29.97, 29.93, 29.92, 29.6, 26.5, 25.8, 25.7, 25.3, 22.9, 14.3 ppm; HRMS (ESI+): calcd for $C_{44}H_{87}NO_9Na$ [M + Na]$^+$ 796.6279; found 796.6264.

*RCAI-159 (**9**)*: Mp 137–139 °C; $[\alpha]_D^{20}$ + 49.7 (*c* 0.30, pyridine); ν_{max} (KBr): 3450 (br s, OH), 1590 (br s, CO), 1080 (br s, C–O) cm^{-1}; δ_H (500 MHz, pyridine-d_5): 6.85–5.80 (6H, m), 4.76 (1H, br d, J = 8.5 Hz), 4.67 (1H, dd, J = 10, 4.0 Hz), 4.62 (1H, d, J = 2.5 Hz), 4.48–4.37 (5H, m), 4.46 (1H, dd, J = 10, 3.5 Hz), 4.37–4.32 (1H, m), 4.13–4.07 (1H, m), 3.43 (3H, br s), 2.23–2.14 (1H, m), 1.97–1.85 (2H, m), 1.74–1.59 (3H, m), 1.32–1.16 (56H, m), 0.85 (6H, t, J = 7.0 Hz) ppm; δ_C (126 MHz, pyridine-d_5): 177.9, 101.6, 73.0, 72.7, 71.7, 71.0, 70.4, 62.6, 43.3, 41.1, 32.1, 30.7, 30.4, 30.2, 30.11, 30.05, 30.03, 30.01, 30.00, 29.96, 29.9, 29.6, 27.4, 26.6, 25.6, 22.9, 14.3 ppm; HRMS (ESI+): calcd for $C_{45}H_{89}NO_9Na$ $[M + Na]^+$ 810.6435; found 810.6433.The ^{1}H- and ^{13}C-NMR spectra of the newly synthesized analogues are shown in the ESI.†

35 T. Fukuyama, M. Cheung, C.-K. Jow, Y. Hidai and T. Kan, *Tetrahedron Lett.*, 1997, **38**, 5831.

36 The sera samples were collected at 1, 3, 6, 12, 24, 36, 48, and 60 h after intravenous injection of glycolipids (2 μg per mouse). The measurement of cytokine concentration was performed using the ELISA system (Thermo Fisher Scientific K.K.) for IFN-γ, and the cytometric bead array (CBA) system (BD Bioscience) for IL-2, 4, 5, 6, 10, 13, and 12p70. Only the results of IFN-γ and IL-4 are shown in Fig. 3 for the simplification of the discussion.

37 All experiments were in accordance with protocols approved by RIKEN Animal Care and Use Committee.

38 Cerotyl chloride (**27**) was prepared under modified conditions of Martin's: cerotic acid (1 equiv.) was treated with $(COCl)_2$ (10 equiv.) in dry benzene at 60 °C for 1–1.5 h (see ESI†)T. Heidelberg and O. R. Martin, *J. Org. Chem.*, 2004, **69**, 2290.

39 Very recently two new findings on the modifications of the acyl chain of KRN7000 were disclosed, one reporting two Th-2 biasing glycolipids with aromatic or heterocyclic rings on the acyl chain,[39a] while the other indicating the pro-*S* hydrogen at the α-position of the acyl group of KRN7000 can be substituted with bulkier groups without loosing its immunostimulative activity.[39b] (*a*) M. Groettrup, P. Wipf, M. Müller and J. Pierce, WO/2013/007792 A1; (*b*) P. J. Jervis, P. Polzella, J. Wojno, J.-P. Jukes, H. Ghadbane, Y. R. Garcia Diaz, G. S. Besra, V. Cerundolo and L. R. Cox, *Bioconjugate Chem.*, 2013, **24**, 586.