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RCAI-61, the 6′-*O*-methylated analog of KRN7000: its synthesis and potent bioactivity for mouse lymphocytes to produce interferon-γ in vivo

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ABSTRACT

RCAI-61, the 6′-*O*-methylated analog of KRN7000, and six other analogs with modified 6′-position of the galactose moiety of KRN7000 were synthesized to examine their bioactivity for mouse lymphocytes. Methyl α-D-galactopyranoside was the starting material for RCAI-58, 61, 64, 83, 85, and 86, while RCAI-87 was prepared from methyl β-L-arabinopyranoside. Bioassay showed RCAI-61 to be a much more potent stimulant of mouse lymphocytes than KRN7000 and RCAI-56 to induce the production of a large amount of IFN-γ in vivo.

1. Introduction

In 1995, KRN7000 (**1**, α-GalCer, Fig. 1) was developed by researchers at Kirin Brewery Co. as an anticancer drug candidate.[1] It was obtained through the modification of the structure of agelasphins (main component is agelasphin-9b, **2**), which had been isolated in 1993 from the extract of an Okinawan marine sponge, *Agelas mauritianus*, as anticancer glycosphingolipids.[2,3] They are structurally characteristic α-galactosylceramides, and act as the immunostimulating agents to induce antitumor activity in vivo in mice and humans. In 1997, it was shown that α-glycosphingolipids can be a ligand to make a complex with CD1d protein, the major glycolipid presentation protein on the surface of the antigen presenting cells of immune system.[4] Natural killer (NK) T cells recognize this CD1d-glycosphingolipid complex with their invariant (mouse: Vα14, human: Vα24) T cell receptor (TCR), and then they are activated to release both helper T(Th)1- and Th2-types of cytokines at the same time in large quantities.[5]

Th1 cytokines [interferon(IFN)-γ, etc.] can induce protective immune response against pathogen infections or mediate tumor rejection, whereas Th2 cytokines [interleukin(IL)-4, etc.] mediate regulatory immune functions to ameliorate autoimmune disease or transplantation tolerance. These Th1 and Th2 cytokines can antagonize each other's biological functions.[6] Because of this problem, use of **1** for clinical therapy has been limited. To solve the problem, many research groups attempt to develop a novel

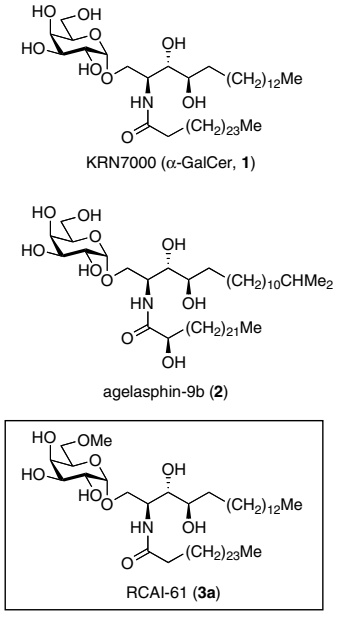

Figure 1. Structures of KRN7000 (α-GalCer, **1**), agelasphin-9b (**2**), and RCAI-61 (**3a**).

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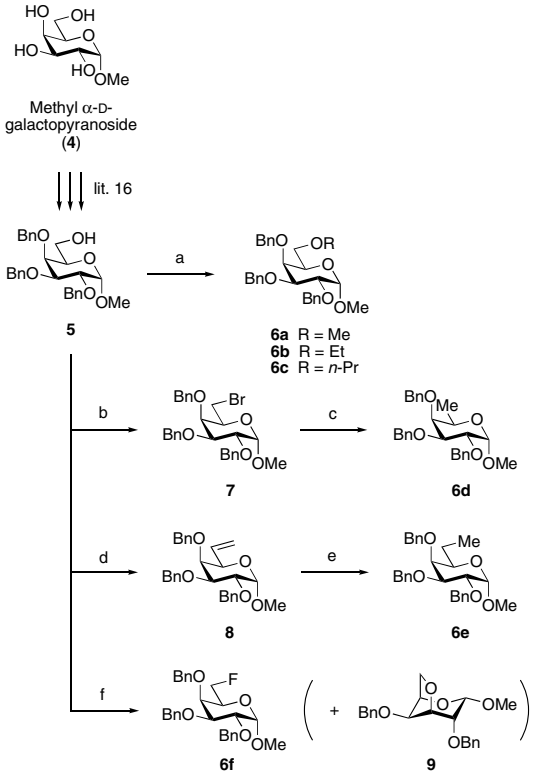


Scheme 1. Synthesis of the analogs of D-galactose modified at 6-position **6a**–**f**. Reagents and conditions: (a) NaH, MeI or EtBr or *n*-PrBr, $(n\text{-Bu})_4NI$ (when the alkyl bromide was used), DMF, THF, rt, 12–16 h (78–98%); (b) Ph_3P, CBr_4, pyridine, 65 °C, 13 h (87%); (c) $(n\text{-Bu})_3SnH$, AIBN, toluene, 95 °C, 17 h (94%); (d) (i) $(COCl)_2$, DMSO, CH_2Cl_2, −78 °C, 40 min; then Et_3N (quant.); (ii) Ph_3PMeBr, *n*-BuLi, THF, rt, 30 min (85%, two steps); (e) $H_2NNH_2{\cdot}H_2O$, 30% H_2O_2 aq, EtOH, rt, 18 h (87%); (f) DAST, Et_3N, CH_2Cl_2, reflux, 1 h (34%).

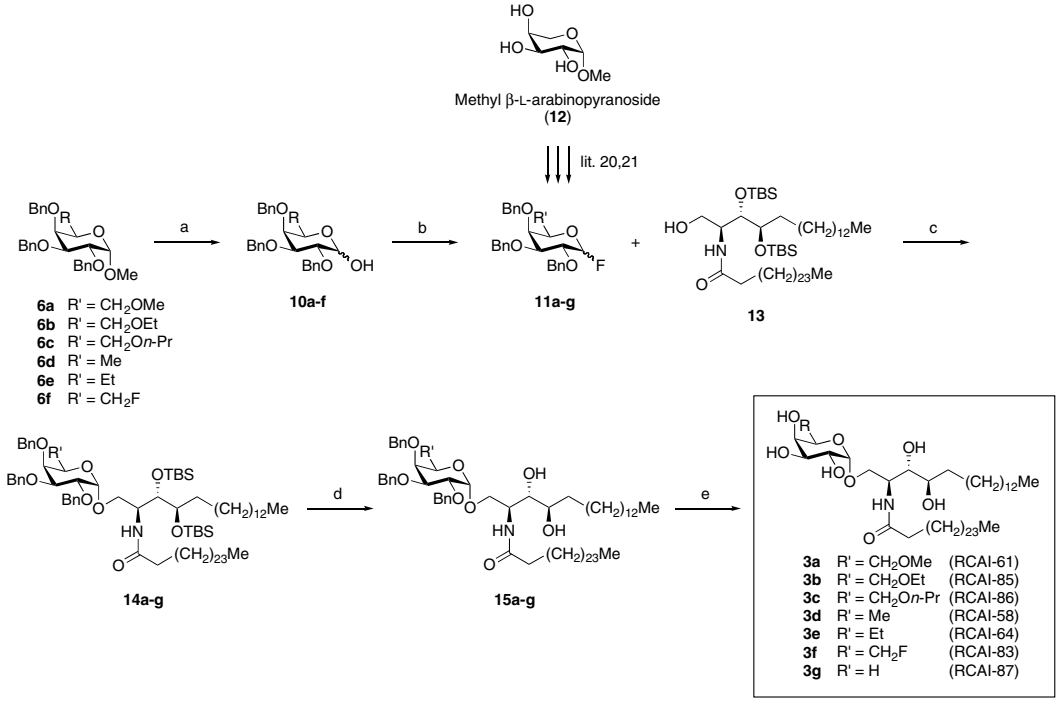
analog of **1**, which induces the production of either Th1 or Th2 cytokines.[7]

Recently, the X-ray crystallographic analyses revealed the structures of mouse and human CD1d-**1**[8,9] and human CD1d-**1**-Vα24TCR complexes.[10] According to the X-ray analysis of human CD1d-**1**-TCR complex, the galactose head group of **1** on the surface of CD1d is presented to TCR. The TCR makes a rigid network of hydrogen bondings with the 2′-, 3′-, and 4′-hydroxy groups of the galactose part and with the 3-hydroxy group of the sphingosine chain of **1**, whereas 6′-hydroxy group does not make a hydrogen bonding with any residue of neither CD1d nor TCR. Modification of these hydroxy groups of the galactose moiety were examined in the past to some extent with no remarkable result.[7] The 2′-, 3′-, or 4′-hydroxy groups of KRN7000 (**1**) seem to play critical roles in TCR recognition, enabling the formation of hydrogen bondings which must be important for the expression of bioactivity. Any modification of these hydroxy groups weakened or abolished the bioactivities of KRN7000 (**1**) except for 3-*O*-sulfo-α-GalCer, which shows almost comparable activity.[11] We, therefore, decided to investigate the structure–activity relationships focused on the 6′-hydroxy group of the galactose part. As described below, we synthesized seven analogs of **1**, which have α-D-galactose moiety modified at 6′-position. Among them, especially the 6′-*O*-methylated galactose derivative **3a** (RCAI-61) was found to be a potent stimulant of NKT cells to induce a large amount of IFN-γ in mice in vivo. It should be added that D-galacturonic,[12,13] 6′-amino,[14] and 6′-acetamidogalactose analogs[15] show activity comparable to **1**.

2. Results and discussion

2.1. Syntheses of RCAI-58, 61, 64, 83, 85, and 86: the D-galactose analogs modified at 6-position (3a–f) and RCAI-87: the L-arabinose analog (3g) of KRN7000

To investigate the importance of the 6′-hydroxy group of **1**, we planned to synthesize five types of the analogs. Syntheses of the



Scheme 2. Synthesis of RCAI-58, 61, 64, 83, 85–87 (**3a**–**g**). Reagents and conditions: (a) concd H_2SO_4, Ac_2O, 0 °C, 30 min; NaOMe, MeOH, rt, 40 min (64–94%); (b) DAST, CH_2Cl_2, rt, 40 min (84–96%); (c) **13**, $SnCl_2$, $AgClO_4$, powdered MS 4 Å, THF, −18 to 5 °C, 1–2 h; (d) TBAF, THF, rt, 13–18 h (48–73%, two steps); (e) H_2, 20% $Pd(OH)_2$–C, EtOH–$CHCl_3$–THF (8:2:5), rt, 11–24 h (32–85%).

galactose derivatives (**6a**–**f**) modified at 6-position are summarized in Scheme 1. The 6-*O*-alkylated galactose derivatives **6a**–**c** were synthesized from alcohol **5**, which was prepared from methyl α-D-galactopyranoside (**4**) in three steps.[16] Williamson's etherification of the 6-hydroxy group of **5** gave **6a**–**c** in 78–98% yield. The 6-deoxy-D-galactose (D-fucose) derivative (**6d**) was also synthesized from alcohol **5**. The alcohol **5** was converted to bromide **7** (87%), which was then reduced with tri-*n*-butyltin hydride to give D-fucose derivative **6d** in 82% yield.[17] The 6-deoxy-6-methyl-D-galactose derivative **6e** was prepared from the known alkene **8**,[18] which could be derived from **5** via Swern oxidation followed by Wittig reaction (85%, two steps). The alkene **8** was reduced with diimide to afford the saturated derivative **6e** in 87% yield. Treatment of alcohol **5** with (diethylamino)sulfur trifluoride (DAST) in the presence of Et_3N afforded 6-deoxy-6-fluoro-D-galactose derivative **6f** (34%) together with a cyclized by-product **9**.[19] When this reaction was performed without Et_3N, only cyclized **9** was obtained.

Scheme 2 summarizes the completion of the synthesis of the desired analogs (**3a**–**g**) of KRN7000 (**1**). Acetolysis of **6a**–**f** with acetic anhydride in the presence of a catalytic amount of H_2SO_4 was followed by *O*-deacetylation with NaOMe in MeOH to afford hemiacetals **10a**–**f** (64–94%, two steps). These hemiacetals **10a**–**f** were treated with DAST to give fluorides **11a**–**f** in 48–73% yield. The L-arabinose derivative **11g** was prepared from methyl β-L-arabinopyranoside **12** according to the reported procedures.[20,21] The obtained fluorides **11a**–**g** were coupled with the ceramide **13**[22] under Mukaiyama conditions[23] to give protected α-galactosylceramides **14a**–**g**. Two *tert*-butyldimethylsilyl (TBS) groups of **14a**–**g** were removed with tetra-*n*-butylammonium fluoride (TBAF) to give alcohols **15a**–**g** (27–56%, two steps). Finally, hydrogenolysis of all of the benzyl groups of **15a**–**g** gave the desired analogs **3a**–**g** in 32–85% yield as colorless solids.[24]

2.2. Results of bioassay

Figure 2 shows the results of bioassay.[25] Concentrations of cytokines in sera of mice were measured after their intravenous injection of KRN7000 (**1**) or synthetic analogs.[26] As can be seen, all of the new analogs induced potent IFN-γ production in vivo in mice. These new analogs induced maximum production of IFN-γ at 24 h after injection, while in the case of KRN7000 (**1**), the maximum was observed at 12 h. These analogs lack their 6′-hydroxy group, which makes intramolecular hydrogen bonding with 4′-hydroxy groups. The enhanced activity of these analogs might be attributed to the increased stability of the CD1d-ligand-TCR complex by increasing the electron density at 4′-hydroxy group. Because of its ready availability and intensity of bioactivity, we chose RCAI-61 (**3a**) as the lead compound. As for RCAI-61 (**3a**), the dose dependence of its activity was also investigated. Indeed in comparison with KRN7000 (**1**), RCAI-61 (**3a**) brought about highly remarkable increase (×8.2 times, total amount of the produced IFN-γ at 2 μg/mouse in vivo) in the production of IFN-γ even at low concentration. In another experiment, it has also been found that RCAI-61 (**3a**) induces the production of a large amount of IL-12, which is one of the cytokines causing IFN-γ production, in mice in vivo. Accordingly, the production of a very large amount of IFN-γ upon injection of RCAI-61 (**3a**) might be partly due to its ability to induce the production of IL-12.

The overall yield of RCAI-61 (**3a**) was 23% based on methyl α-D-galactopyranoside (**4**) through 10 steps. In 2007, we reported the synthesis of RCAI-56 (**16**, Fig. 3) as the potent inducer of IFN-γ in mice and humans.[27] It was also synthesized from **4**, and its overall yield was only 6.7% (20 steps). Detailed bioassay results including

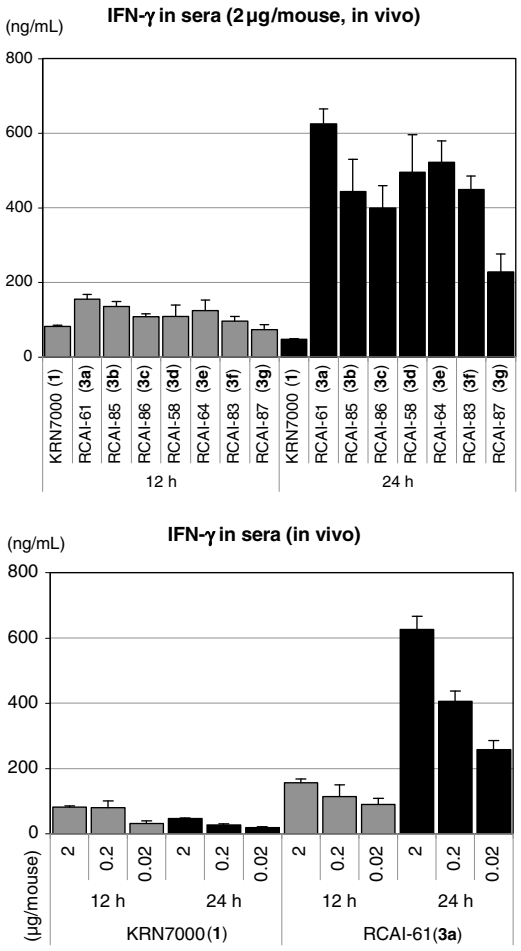


Figure 2. The concentrations of IFN-γ in sera in mice (i.v.).[26]

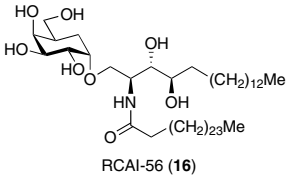


Figure 3. The structures of RCAI-56 (**16**).

the comparison of bioactivities in vivo of **3a** with **16** will be reported elsewhere in due course.[28] RCAI-61 (**3a**) seems to show stronger bioactivity than RCAI-56 (**16**).

3. Conclusion

In conclusion, we synthesized RCAI-58, 61, 64, 83, 85, and 86: the analogs with modified 6′-position (**3a**–**f**) and RCAI-87: L-arabinose analog (**3g**) of KRN7000 (**1**). Among them, the 6′-*O*-methylated analog of **1**, RCAI-61 (**3a**) is a remarkably potent inducer of IFN-γ in mice in vivo. From the pharmaceutical point of view, RCAI-61 (**3a**) may be the most hopeful analog due to its ready availability and strong bioactivity. So-called 'structure-based design of ligands' considering the X-ray results was successful in our case to make unusually potent ligands to induce IFN-γ production. Computational docking models are now being calculated, and the result will be reported shortly.

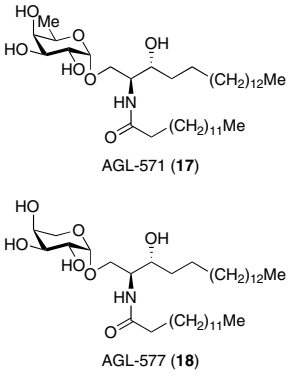


Figure 4. The structures of AGL-571 (**17**) and AGL-577 (**18**).

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24. All of the spectral data support each structures of synthesized analogs: for example, RCAI-61 (**3a**): $[\alpha]_D^{28}$ +46.7 (*c* 0.31, pyridine); ν_{max} (KBr): 3440 (br s, OH), 3280 (w, NH), 1640 (br s, C═O), 1540 (br m), 1080 (br s, C–O), 720 (w) cm^{-1}; δ_H (400 MHz, pyridine-d_5): 8.44 (1H, d, *J* = 8.8 Hz), 7.05 (1H, br s), 6.74 (1H, br s), 6.45 (1H, d, *J* = 6.4 Hz), 6.39 (1H, br s), 6.08 (1H, br s), 5.52 (1H, d, *J* = 4.0 Hz), 5.28–5.22 (1H, m), 4.64 (1H, dd, *J* = 10, 5.6 Hz), 4.65–4.58 (1H, m), 4.46 (1H, t, *J* = 6.4 Hz), 4.40–4.28 (4H, m), 4.14–4.08 (1H, m), 3.97 (1H, dd, *J* = 9.6, 5.6 Hz), 3.94 (1H, dd, *J* = 9.6, 6.4 Hz), 3.33 (3H, s), 2.42 (2H, t, *J* = 7.2 Hz), 2.33–2.20 (1H, m), 1.95–1.60 (5H, m), 1.48–1.16 (68H, m), 0.84 (6H, t, *J* = 6.8 Hz) ppm; δ_C (100 MHz, pyridine-d_5): 173.1, 101.5, 76.6, 73.0, 72.5, 71.4, 70.8, 70.7, 70.1, 68.9, 58.9, 51.2, 36.8, 34.3, 32.1, 30.4, 30.1, 30.04, 30.01, 29.93, 29.88, 29.83, 29.76, 29.6, 26.5, 26.4, 22.9, 14.3 ppm; HR-FABMS calcd for $C_{51}H_{102}NO_9$ $[M+H]^+$ 872.7555; found, 872.7553.
25. We reported the standard protocol for detection, isolation, and culture of mouse and human invariant NKT cells. See: Watarai, H.; Nakagawa, R.; Omori-Miyake, M.; Dashtsoodol, N.; Taniguchi, M. *Nat. Protocols* **2008**, *3*, 70–78.
26. The serum samples were obtained at indicated time points after injection (i.v.) of KRN7000 (**1**) or synthesized analogs (**3a**–**g**). Glycolipids: The stock solution of **1** [200 μg/mL in 0.5% polysorbate-20 (MP Biomedicals) or **3a**–**g** [1 mg/mL in dimethyl sulfoxide (DMSO)] was diluted to 10 μg/mL in Dulbecco's phosphate buffered saline (Sigma, Product No. D8537) just before injection into mice. Mice: 8–12-week-old female C57BL/6J mice (Charles River Laboratories, Inc.) Concentration of IFN-γ was measured by ELISA (Pierce Biotechnology, Inc.).
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28. It should be added that Kirin's research group reported their synthesis of similar analogs, α-D-fucopyranosyl (AGL-571, **17**, Fig. 4) and β-L-arabinopyranosyl (AGL-577, **18**) ceramides, which showed strong lymphocytic proliferation stimulatory effects in vitro in mice.[21]