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RCAI-56, a carbocyclic analogue of KRN7000: its synthesis and potent activity for natural killer (NK) T cells to preferentially produce interferon-γ

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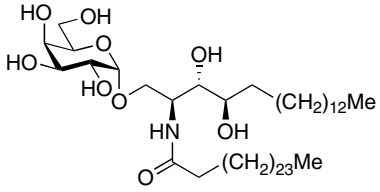
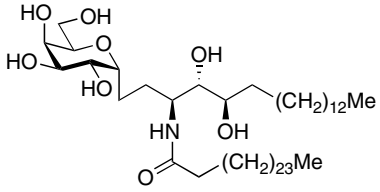
Abstract—RCAI-56 (**3**), a carbocyclic analogue of KRN7000 (**1**) was synthesized through an efficient coupling of a carba-α-D-galactose derivative **11** with cyclic sulfamidate derivative **13** of phytosphingosine to give **15**. Carbasugar derivative **11** was prepared by starting from methyl α-D-galactopyranoside (**4**), employing Pd(II)-catalyzed Ferrier rearrangement as the key step. RCAI-56 (**3**) is a potent stimulant of NKT cells in vivo to induce the production of Th1 biased cytokines such as interferon-γ in mice. According to the docking model of CD1d-**3** complex, its stabilization energy is approximately at the same level as that of the CD1d-**1** complex.

1. Introduction

In 1995, researchers at Kirin Brewery Co. developed an anticancer drug candidate KRN7000 (α-GalCer, **1**, Fig. 1)[1] through the modification of the structures of agelasphins, which had been isolated in 1993 as anticancer sphingolipids from the extract of an Okinawan marine sponge, *Agelas mauritianus*.[2,3] These sphingolipids exhibited antitumor activity in vivo in mice and human. It has been shown that **1** is a ligand to make a complex with CD1d protein, a glycolipid presentation protein on the surface of the antigen presenting cells of the immune system.[4] Natural killer (NK) T cells are activated by recognition of CD1d-**1** complex with their invariant Vα14 antigen receptors, and release both helper T(Th)1 and Th2 types of cytokines in large quantities at the same time.[5] Th1 type cytokines such as interferon(IFN)-γ mediate protective immune functions like tumor rejection, whereas Th2 type cytokines such as interleukin(IL)-4 mediate regulatory immune functions to ameliorate autoimmune diseases. Th1 and Th2 type cytokines can antagonize each other's biological actions.[6] Because of this antagonism, use of **1** for clinical therapy was unsuccessful. To circumvent this problem,



KRN7000 (α-GalCer, **1**)



α-*C*-GalCer (**2**)

Figure 1. Structure of KRN7000 (**1**) and α-*C*-GalCer (**2**).

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many groups are trying to develop new analogues of **1**, which induce NKT cells to produce only Th1 or Th2 type cytokines (see reviews[7–9]).

In 2003, Franck, Tsuji and their co-workers reported that their synthetic α-*C*-galactosylceramide (α-*C*-GalCer, **2**) caused an enhanced Th1 type response in vivo.[10] Derivative **2** was developed by replacing the glycosidic O atom at the galactose-ceramide linkage to a CH_2 group, and three different syntheses of **2** have already been reported.[11–13] Why analogue **2** induces NKT cells to release Th1 biased cytokines? It is proposed that **2** is stable against α-galactosidase in vivo, and therefore CD1d-**2** complex can stimulate NKT cells for a longer period than CD1d-**1** can do, causing Th1 biased response.[8]

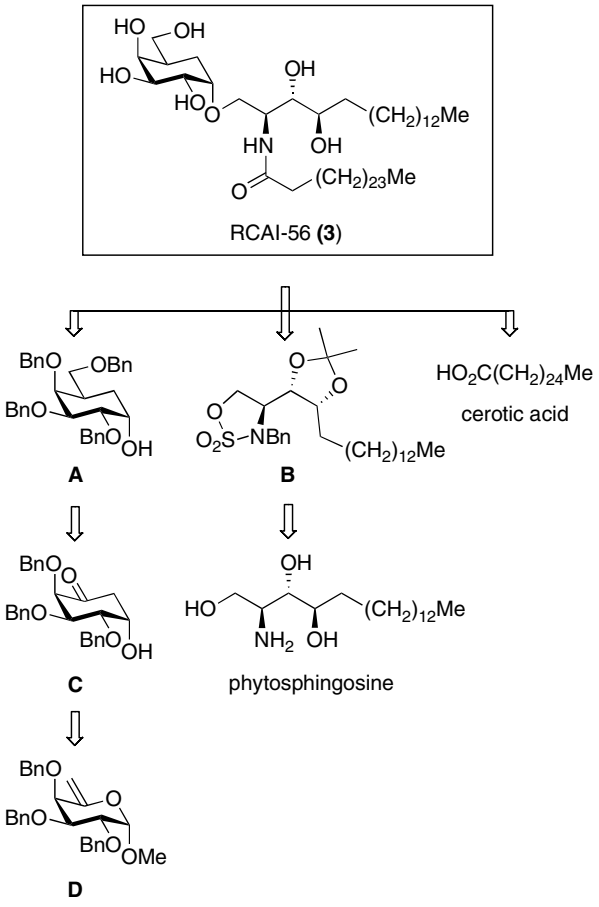
According to the X-ray crystallographic analysis of CD1d-**1** complex, the glycosidic O atom of **1** makes a hydrogen bonding with the α2 helix of CD1d (mouse: Thr156; human: Thr154).[14,15] Based on this result, we began our attempt to synthesize carbocyclic analogue **3** (Scheme 1), with the linking O atom and hence the stable ether structure. The new analogue **3** might make a more stable complex with CD1d than **2**, and might induce NKT cells to release Th1 biased cytokines. Whereas the O atom of the pyranose ring makes no hydrogen bonding with CD1d, the galactose part of **1** of the CD1d-**1** complex is placed out of the hydrophobic pocket of CD1d. Bioassay of analogue **3** would enable us to examine whether the pyranose O atom is important for the recognition by NKT cells or not.

As described below, we synthesized **3**, coined a code name RCAI-56 to it, and found it to be a potent activator of mouse NKT cells to produce interferon-γ. It must be added that, an independent and unsuccessful attempt of Chung and co-workers to provide the carbocyclic analogues of **1** was published very recently.[16]

2. Results and discussion

2.1. Retrosynthetic analysis

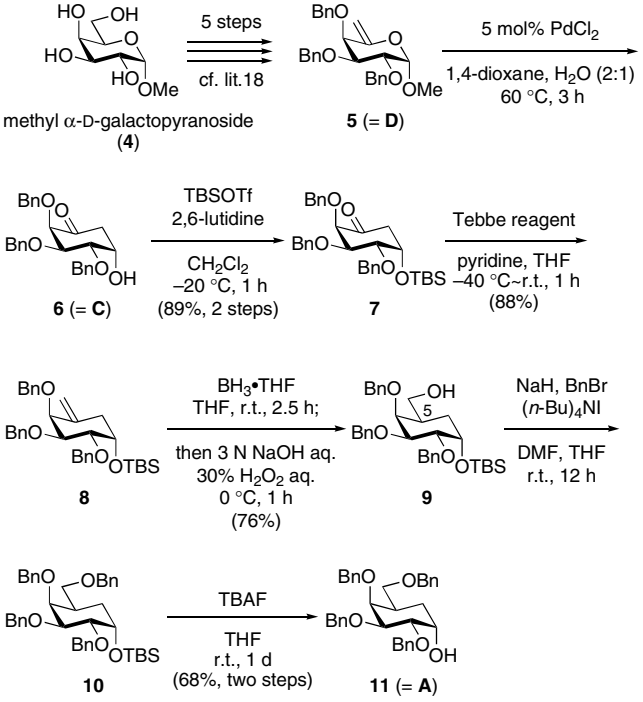
Scheme 1 shows the retrosynthetic analysis of RCAI-56 (**3**). It can be prepared from carba-α-D-galactose part **A**, phytosphingosine part **B**, and cerotic acid. We envisage that Bittman's cyclic sulfamidate **B**[17] is useful as an electrophile to attack the sodium alkoxide derived from **A**. Carba-α-D-galactose derivative **A** will be prepared from the known cyclohexanone **C**, which can be synthesized from *galacto*-hexenopyranoside **D**[18] via Pd(II)-catalyzed Ferrier rearrangement developed by Ikegami.[19] The cyclic sulfamidate **B** is to be prepared from commercially available phytosphingosine.



Scheme 1. Retrosynthetic analysis of RCAI-56 (**3**).

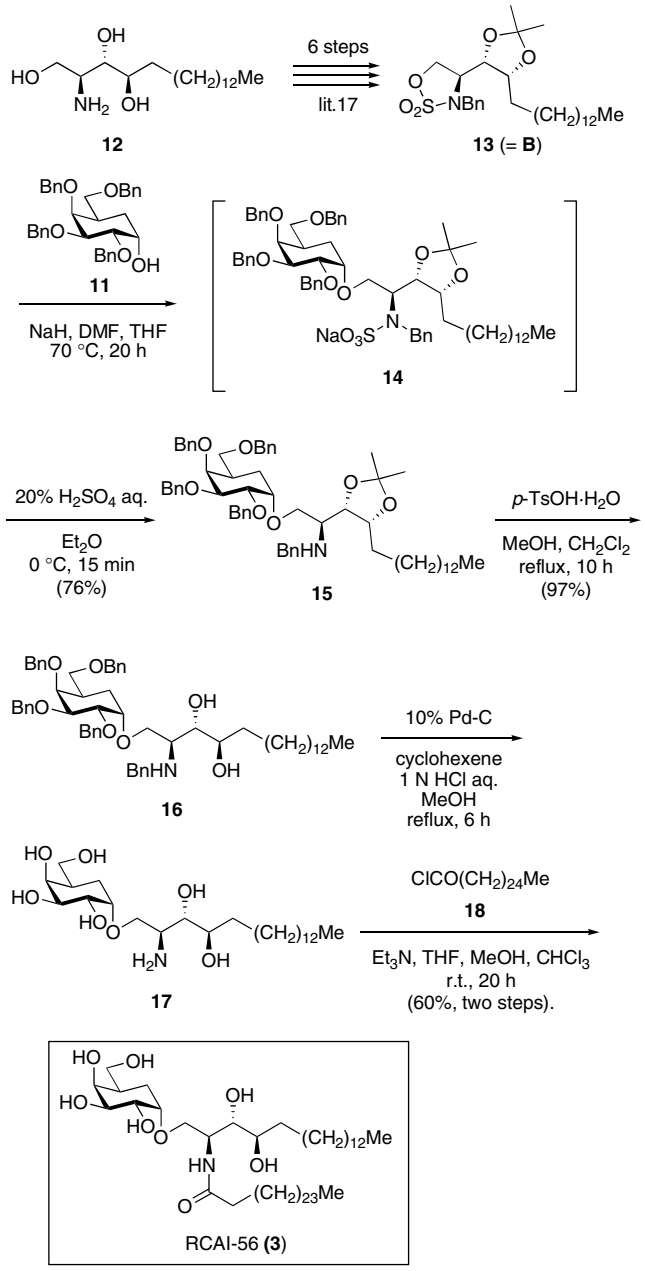
2.2. Synthesis of carba-α-D-galactose derivative 11

Scheme 2 summarizes the synthesis of carba-α-D-galactose derivative **11**. We adopted a method similar to the one developed for the synthesis of a carba-α-D-glucose derivative by Pohl and his co-workers.[20] The key reac-



Scheme 2. Synthesis of carba-α-D-galactose derivative **11**.

tion was Ikegami's modification of the Ferrier rearrangement (**5**→**6**).[19] The known building block **5** (=**D**), was prepared from commercially available methyl α-D-galactopyranoside (**4**) through five steps in a high overall yield.[18] The cyclohexane framework of ketone **6** (=**C**) was constructed by Pd(II)-catalyzed Ferrier rearrangement of **5**.[19] The free hydroxy group of the resulting **6** was protected as *tert*-butyldimethylsilyl(TBS) ether by treatment with *tert*-butyldimethylsilyl triflate (TBSOTf) at −20 °C to give TBS ether **7** (89%, two steps). This was then converted to alkene **8** by methylenation with Tebbe reagent (88%). Alkene **8** was subjected to hydroboration-oxidation by treatment with borane-THF and NaOH aq/H_2O_2 to furnish alcohol **9** with the desired β-configuration at C-5 (76%). Benzylation of the free hydroxy group of **9** yielded **10**. Removal of the TBS group of **10** with tetra-*n*-butylammonium fluoride (TBAF) furnished the desired tetrabenzylated carba-α-D-galactose derivative **11** (=**A**) in 68% yield (two steps).[21]



Scheme 3. Synthesis of RCAI-56 (**3**).

2.3. Synthesis of RCAI-56 (3), the carbocyclic analogue of KRN7000

Completion of the synthesis of RCAI-56 (**3**) is illustrated in Scheme 3. The carba-α-D-galactose derivative **11** was coupled with cyclic sulfamidate **13** (=**B**), which was prepared from phytosphingosine (**12**) by Bittman's protocol,[17] to give amine **15** in 76% yield via acid hydrolysis of sodium sulfamidate intermediate **14**. Removal of the acetonide protective group of amine **15** afforded aminodiol **16** (97%). Hydrogenolysis of all of the benzyl groups of **16** was followed by acylation of the resulting **17** with cerotyl chloride (**18**)[13,22] to give RCAI-56 (**3**) as a colorless solid, mp 147–149 °C, in 60% yield (two steps).[23] The overall yield of **3** was 6.7% based on methyl α-D-galactopyranoside (**4**) through 20 steps.

2.4. Results of bioassay

Concentrations of IFN-γ and IL-4 in the sera of mice were measured after their treatment with either **1** or **3**. As shown in Figure 2, RCAI-56 (**3**) induced Th1 biased cytokine production in vivo. Indeed, in comparison with KRN7000 (**1**), RCAI-56 (**3**) brought about remarkable increase in the production of IFN-γ with concomitant decrease in IL-4 release. The structural differences between **3** and **1** are (i) the lack of the pyranose O atom and (ii) the replacement of the labile glycosidic linkage of **1** with a stronger ether linkage. Comparison of the

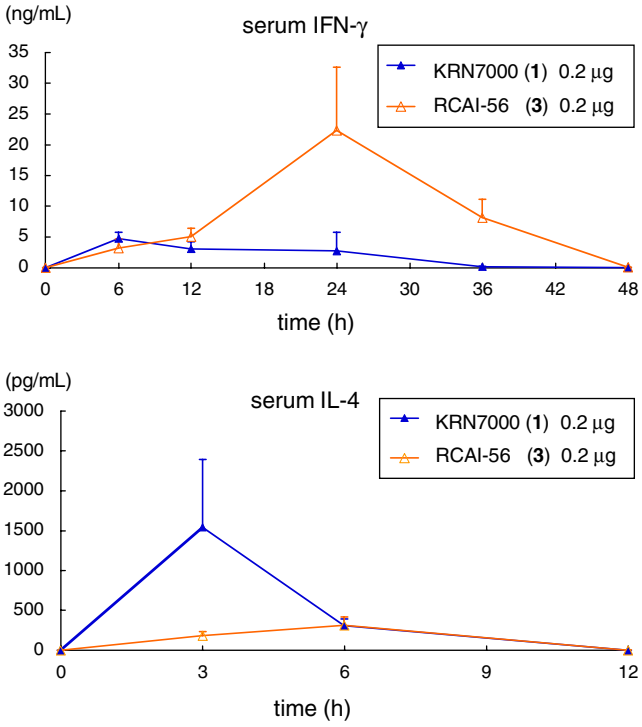


Figure 2. The concentrations of IFN-γ or IL-4 in serum in mice.[24]

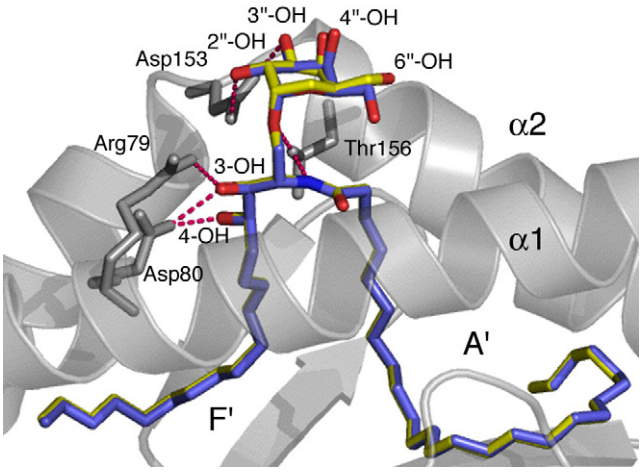


Figure 3. The docking model of mCD1d-KRN7000 (**1**, purple) and mCD1d-RCAI-56 (**3**, yellow).

bioactivities in vivo of α-*C*-GalCer (**2**) with **1** or **3** will be reported elsewhere.

2.5. Docking model of mouse(m)CD1d-RCAI-56 complex

Comparison between the computational docking models[25] of mCD1d-KRN7000(**1**) and mCD1d-RCAI-56(**3**) complexes are depicted in Figure 3. As can be seen in the figure, the binding conformations of **1** and **3** are not so different. The ligand binding score (Glide Score)[26] of the CD1d-ligand complexes were also calculated as −18.97 for **1** and −19.12 for **3**. Presence of the O atom between the carba-sugar and the ceramide of **3** therefore keeps the structure of mCD1d-**3** complex almost as same as that of the complex with **1**. This means that the stability of the connecting ether linkage together with the absence of the pyranose O atom must have caused the remarkable Th1 bias of RCAI-56 (**3**) in its ability to induce cytokine release.

3. Conclusion

In conclusion, we developed RCAI-56 (**3**), a new carba-α-D-galactose analogue of KRN7000. RCAI-56 (**3**) is a remarkably potent inducer of Th1 biased cytokine production in vivo. Further studies are in progress to clarify the structural requirements for a glycosphingolipid ligand in controlling the ratio of Th1/Th2 responses.

Acknowledgements

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23. Mp 147–149 °C; $[\alpha]_D^{23}$ +27.8 (*c* 0.32, pyridine); ^{1}H NMR of **3** (400 MHz, pyridine-d_5, 25 °C): $\delta = 8.43$ (d, $J = 8.4$ Hz, 1H, NH), 6.85–6.82 (m, 1H, OH), 6.37 (d, $J = 6.4$ Hz, 1H, OH), 6.31–6.28 (m, 1H, OH), 6.07 (d, $J = 5.2$ Hz, 1H, OH), 6.00–5.98 (m, 1H, OH), 5.97 (t, $J = 5.4$ Hz, 1H, OH), 5.21–5.18 (m, 1H, 2H), 4.69 (br s, 1H, 4″-H), 4.50 (dd, $J = 10$, 4.0 Hz, 1H, 1-H_a), 4.47–4.43 (m, 1H, 2″-H), 4.34–4.18 (m, 5H, 3-, 4-, 1″-, 3″-H, 6″-H_a), 4.26 (dd, $J = 10$, 5.2 Hz, 1H, 1-H_b), 4.00 (ddd-like, $J = 9.6$, 5.4, 4.8 Hz, 1H, 6″-H_b), 2.51–2.42 (m, 1H, 5″-H), 2.44 (t, $J = 7.6$ Hz, 2H, 2′-H_2), 2.33–2.24 (m, 1H, 5-H_a), 2.14–2.06 (m, 1H, 5a″-H_a), 2.00 (br t, $J = 13$ Hz, 1H, 5a″-H_b), 1.98–1.84 (m, 2H, 5-H_b, 6-H_a), 1.82 (quint.-like, $J = 7.6$ Hz, 2H, 3′-H_2), 1.76–1.67 (m, 1H, 6-H_b), 1.50–1.17 (m, 66H, 7-, 8-, 9-, 10-, 11-, 12-, 13-, 14-, 15-, 16-, 17-, 4′-, 5′-, 6′-, 7′-, 8′-, 9′-, 10′-, 11′-, 12′-, 13′-, 14′-, 15′-, 16′-,

17′-, 18′-, 19′-, 20′-, 21′-, 22′-, 23′-, 24′-, 25′-H_2), 0.85 (t, $J = 7.2$ Hz, 6H, 18-, 26′-H_3) ppm.

24. The serum samples were obtained at indicated times after the injection of KRN7000 (**1**) or RCAI-56 (**3**). Concentration was measured by ELISA (Cytometric Bead Array system; BD Bioscience).
25. KRN7000 (**1**) and RCAI-56 (**3**) molecules were docked into the X-ray structure of mCD1d (PDB code: 1Z5L)[14] using Glide 4.0 (Schrödinger, LLC). Initial structures of **1** and **3** were built based on co-crystallized ligands PBS and R16 in mCD1d. Hydrogen-bonding interaction between the ligands and the mCD1d and their visualization were generated by MacPyMOL (DeLano Scientific, LLC).
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