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Synthesis and Biological Activities of 5-Thio-α-GalCers

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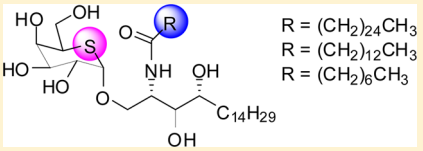
Supporting Information

ABSTRACT: NKT cells, a unique subset of T cells that recognizes glycolipid antigens presented by CD1d molecules, are believed to produce key cytokines of both Th1 and Th2 T cells and are thus involved in the control of several types of immune response. As an active glycolipid antigen having α-galactosyl ceramide core structure, KRN7000 showed promising immunostimulation activity and was selected as an anticancer drug candidate for further clinical application. In this report, three new KRN7000 structural analogues were designed and synthesized, in which the ring oxygen of the galactopyranose residue is replaced by a sulfur atom along with the variation on the lipid chain. Their abilities for stimulating mouse NKT cells to produce IFN-γ and IL-4 were evaluated both *in vivo* and *in vitro*.

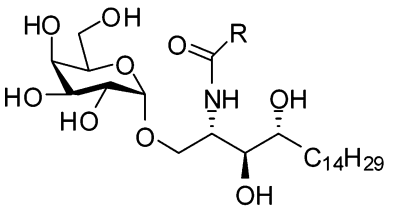


KEYWORDS: *KRN7000, 5-thio-α-GalCer, iNKT cells, antitumor activity*

Invariant natural killer T (iNKT) cells are a specialized subset of T cells that play an important role in tumor immunity and preventing autoimmunity.[1−4] The anti-infective activity of iNKT cells can be triggered by various viruses, bacteria, and parasites.[5,6] When stimulated by glycolipid antigens such as marine natural product agelasphin-9b,[7] the iNKT cells could rapidly release T helper 1 (Th1) cytokines (e.g., IFN-γ, TNF-α, IF-2) and Th2 cytokines (e.g., IL-4, IL-10).[8,9] Th1-biased cytokines are believed to be concerned with the antitumor, antibacterial, and antiviral activities, whereas the release of Th2-biased cytokines may relieve some autoimmune diseases.[10−12]

KRN7000 (**1**),[13] a synthetic α-GalCer (**1**, Figure 1) derived from the structural modification on the ceramide part of agelasphin-9b, presented convincing effects on the treatment of liver tumors,[14,15] metastatic cancers,[16,17] parasitic infections,[11,18] and autoimmune diseases.[19,20] The action mechanism of KRN7000 has been suggested in approximately three sequential steps. First, KRN7000 is combined with the CD1d protein to form a glycolipid−protein complex. Second, the KRN7000/CD1d complex is recognized by the T cell receptor (TCR) on the surface of NKT cells to form a three-molecule complex. Lastly, it stimulates NKT cells to release Th1 and Th2 cytokines rapidly.[21] Because of the mutual repulsion of Th1 and Th2 cytokines, the simultaneous high level production of both cytokines weaken the therapeutic effectiveness of KRN7000.[22] Therefore, some KRN7000 derivatives modifying on either sugar or ceramide moieties were synthesized and expected to selectively control the release of Th1 and Th2 cytokines.[23−27] For example, KRN7000 analogue with a longer acyl tail (**2**, Figure 1) exhibited a greater ability for activation of B cells and induced IL-2 from mouse NKT cells, and IL-4 and IFN-γ from human Vα24i NKT cells *in vitro*,[28,29] while the analogue with a relative shorter chain (**3**, Figure 1) influenced cytokine release toward Th2 bias with an immunomodulatory response.[30] In consideration of the intrinsic instability of *O*-glycosides *in vivo*, both *C*-bridged glycoside (α-*C*-GalCer)[31−33] and *S*-bridged glycoside (α-*S*-GalCer),[34−36] with the replacement of the anomeric oxygen by methylene or sulfur atom, respectively, were prepared to examine their biological activities. Comparing to the *O*-glycoside KRN7000, α-*C*-GalCer showed a stronger Th1 response *in vivo*, whereas the analogue α-*S*-GalCer did not activate the murine iNKT cells both *in vitro* and *in vivo*, but stimulated human iNKT cells *in vitro*.[36]

Literature search revealed that very few examples have been found for carbohydrates having the ring sulfur structure in natural products,[37] one interesting report is the 5-thio-D-



1 KRN7000 R = $(CH_2)_{24}CH_3$
2 α-GalCer 566 R = $(CH_2)_{12}CH_3$
3 PBS-25 R = $(CH_2)_6CH_3$

Figure 1. Structures of KRN7000 (**1**) and its active analogues **2** and **3**.

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mannopyranose isolated from metabolites of the marine sponge *Clathria pyramida* (Lendenfled) with antimicrobial activity.[38] In a molecular modeling study, the carbon−sulfur bond is proposed to be more soft with regard to absorb the deviations of bond lengths and bond angles between ethers and thioethers, thereby minimizing structural changes of carbohydrate moieties.[39] Some synthetic oligosaccharides, containing 5-thio-pyranosyl residue, show good inhibitory activities against exoglycosidases,[40−43] as represented by a synthetic 5-thio-L-fucopyranosyl disaccharide with inhibition activity on bovine epididymis α-L-fucosidase.[44] Therefore, it is possible to create bioactive oligosaccharides with remarkable alterations of physicochemical characteristics and biological activities[37] using ring-*S* instead of ring-*O* sugar unit. Accordingly, we designed 5-*S*-KRN7000 (**4**, Figure 2), in which the α-

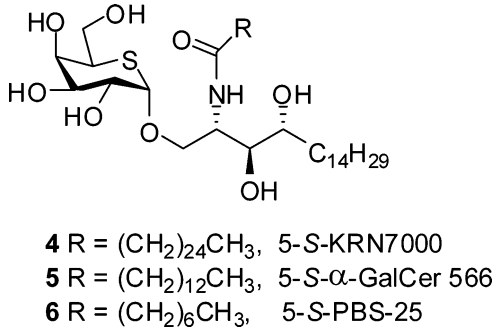


Figure 2. 5-*S*-Substituted KRN7000 analogues **4**, **5**, and **6**.

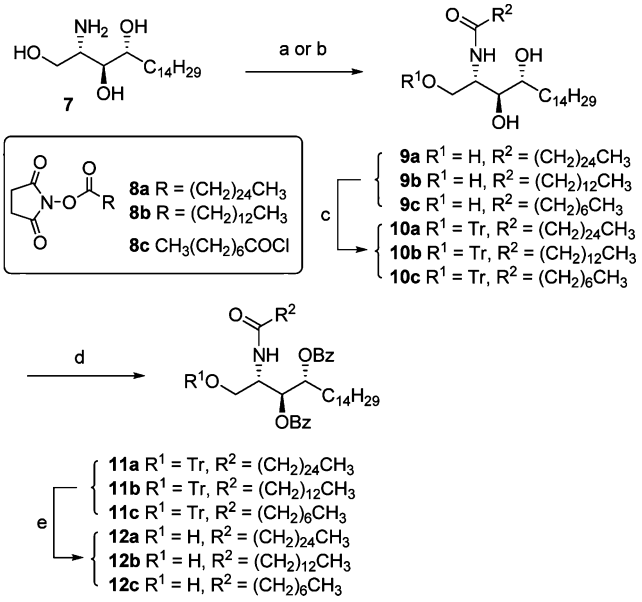
galactopyranosyl moiety of KRN7000 (**1**) is replaced by a 5-thio-galactopyranose residue, expecting to develop a new antitumor agent that would induce the release of cytokines selectively. We also synthesized compounds **5** and **6** (Figure 2) with varied lipid tails to explore the preliminary relationship between the efficacy of the cytokine release and the chemical structures.

We started from the synthesis of lipid part of glycosyl acceptor **12** (Scheme 1). Commercially available phytosphingosine (**7**) was condensed[45] with *N*-hydroxysuccinimide ester (**8a** or **8b**) or octanoyl chloride (**8c**) in the presence of Et_3N in THF to afford the crude amide **9**, which was treated with triphenylchloromethane (TrCl) in pyridine to give tritylated derivative **10**. The remaining two hydroxyl groups of **10** were further blocked with benzoyl chloride in pyridine generating compound **11**, which was subjected to the detritylation with *p*-toluenesulfonic acid in methanol/CH_2Cl_2 and achieved acceptor **12** in good yield.

The synthetic route toward 5-thio-galactopyranosyl donor **15** is depicted in Scheme 2. Taking advantages of our recently reported method, 1,2,3,4,6-penta-*O*-acetyl-5-thio-D-galactopyranose (**13**)[46] was selected as the starting material. Removal of the anomeric acetate of **13** with benzylamine[47] in THF afforded the hemiacetal **14**, which was transformed into trichloroacetimidate **15** with CCl_3CN and DBU in a yield of 51% for two steps.

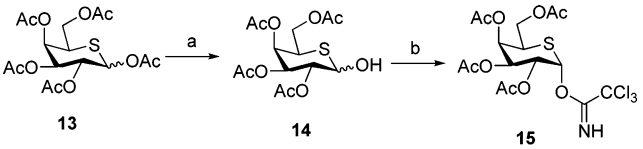
With donor **15** and acceptor **12** in hand, the coupling reaction was investigated as shown in Scheme 3. It was found that the glycosylation of 5-thio-galactopyranosyl donor **15** with lipid acceptors **12b** and **12c** furnished glycosides **16b** and **16c** in good yields in the presence of a catalytic amount of TMSOTf (0.05 equiv) in CH_2Cl_2 at −40 °C. However, the glycoside **16a** was afforded only in 35% isolated yield under

Scheme 1. Synthesis of the Acceptor 12[a]



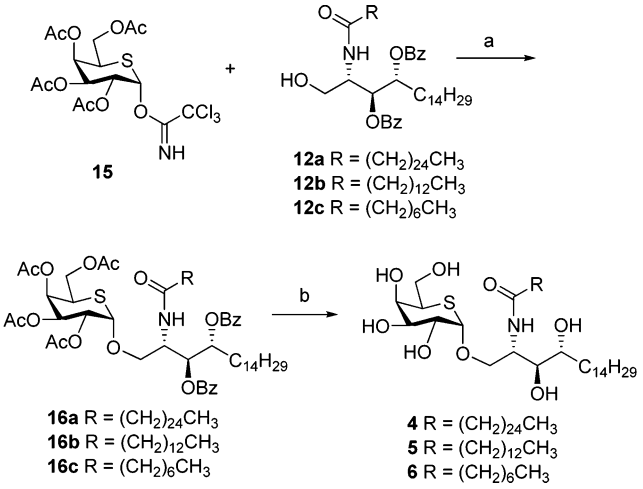
[a]Reagents and conditions: (a) compounds **8a** or **8b**, Et_3N, THF, 50 °C; (b) **8c**, Et_3N, THF, 0 °C; (c) TrCl, DMAP, Pyr, 80 °C, 80% for **10a**, 83% for **10b**, 84% for **10c** (over 2 steps); (d) BzCl, DMAP, Pyr, rt, 95% for **11a**, 89% for **11b**, 88% for **11c**; (e) TsOH, CH_2Cl_2/MeOH, rt, 88% for **12a**, 85% for **12b**, 85% for **12c**.

Scheme 2. Synthesis of the Donor 15[a]



[a]Reagents and conditions: (a) benzylamine, THF, rt, 62%; (b) CCl_3CN, DBU, CH_2Cl_2, rt, 82%.

Scheme 3. Synthesis of KRN7000 Analogues[a]



[a]Reagents and conditions: (a) **12**, TMSOTf, CH_2Cl_2; for **16a**, 0 °C, 35%; for **16b**, −40 °C, 59%; for **16c**, −40 °C, 47%; (b) NaOMe, MeOH, rt, quantitative.

optimized reaction conditions, ascribed to the poor solubility of **12a** in CH_2Cl_2 at low temperature (0−40 °C). Several

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researchers have reported extensive efforts directed at glycosylation reactions with 5-thiopyranosyl donors. It seemed that, from these examples, both the yields and the stereoselectivity of the reactions were dependent on the glycosyl acceptor.[40−43] We were very glad that the glycosyl ceramides **16a−c** were prepared as the unique α-linked products stereoselectively, as well as the recovered acceptors. No β-isomers were isolated in any attempted efforts in our case. Global deacylation of **16a−c** was conducted smoothly with methanolic NaOMe to obtain KRN7000 analogues **4**, **5**, and **6** all in quantitative yields.

The abilities of KRN7000 and its 5-thio analogues **4**, **5**, and **6** in stimulating the cytokine production of NKT cells were investigated under designed *in vitro* and *in vivo* conditions. First, we measured the serum levels of IFN-γ and IL-4 after intraperitoneal (i.p.) injection of the glycolipids into B6 mice at several time points (Figure 3a,b). Consistent with previous studies,[12,48] KRN7000 (**1**) injection resulted in a rapid production of IL-4 peaked at 3 h and a delayed but extended elevation of IFN-γ (continuously released at high concentrations from 6 to 24 h) in B6 mice. Compared with KRN7000 (**1**), 5-thio-KRN7000 (**4**) presented a similar tendency and ability to induce the release of key immunomediators IFN-γ and IL-4 in mice. Analogue 5-thio-α-GalCer 566 (**5**) induced a slightly decreased production of IFN-γ compared to **1** and **4**, while 5-Thio-PBS-25 (**6**) had a low stimulation as shown in Figure 3a. One thing to note is that injection of **5** greatly reduced the production of IL-4; however, the production of IFN-γ still remained relatively high. Therefore, *S*-analogue **5** may be used specifically for inducing iNKT cells to produce Th1 cytokines. As shown in Figure 3b, all three 5-thio analogues of KRN7000 could stimulate IFN-γ and IL-4 production *in vivo*, and the productivities were increased with the length of the acyl tail. After 24 h, the production of IFN-γ induced by 5-thio-KRN7000 (**4**) remained high (1722 ± pg/mL), as compared to the level of IFN-γ induced by KRN7000 (2127 ± pg/mL). The induced production of IL-4 by the three analogues was rapidly ceased by 12 h.

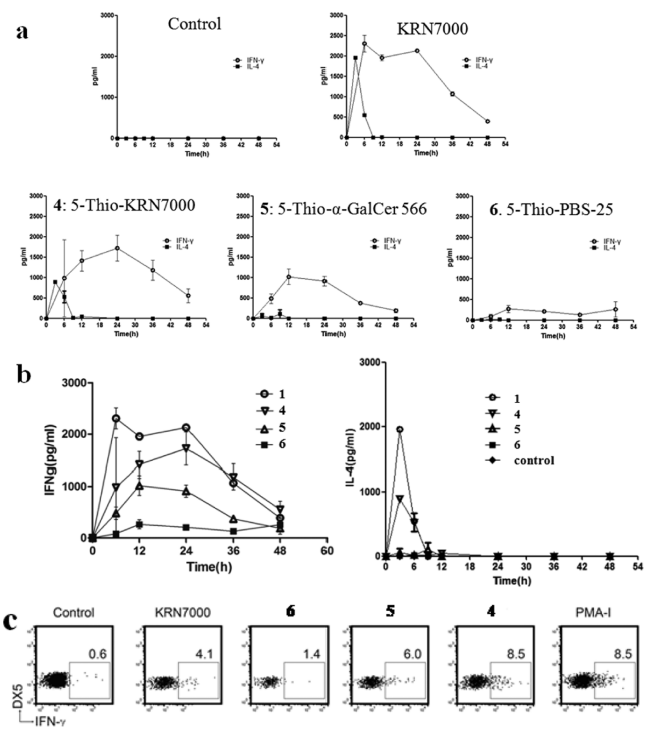

Figure 3. Bioassay of KRN7000 and its analogues *in vivo* and *in vitro*. (a,b) Cytokine secretion *in vivo* after injection of KRN7000 or its analogues (100 μg/kg). Every group contains two mice and their serum of indicated times were detected individually. (c) IFN-γ secretion by mouse splenocytes *in vitro*. For control, splenocytes were only incubated in complete 1640 medium. PMA (Phorbol 12-myristate 13-acetate)-I (ionomycin) were added to the cells as a positive control. The cells were gated on $CD3^+DX5^+CD1d$ $tetramer^+$ NKT cells.

As shown in Figure 3a,b, IFN-γ was measured in the serum of glycolipid antigen-treated mice; however, the level of IFN-γ secretion might be affected by other types of cells interacting with iNKT cells after iNKT cells were activated *in vivo*. To directly compare the ability of KRN7000 and its 5-thio analogues in stimulating the cytokine production of NKT cells, KRN7000 and compounds **4**, **5**, and **6** were examined to stimulate NKT cells *in vitro*. As shown in Figure 3c, all synthetic samples could activate iNKT cells to produce IFN-γ *in vitro*. In particular, 5-thio-KRN7000 (**4**), as well as 5-thio-α-GalCer 566 (**5**), stimulated higher IFN-γ production of iNKT cells than that of KRN7000. About 8.5% iNKT cells produced IFN-γ after the stimulation with **4**, which was comparable to that of PMA-I stimulation.[49] Only 5-Thio-PBS-25 (**6**) stimulated iNKT cell activation at a lower level. Thus, all three KRN7000 analogues induced iNKT cells *in vitro* to release Th1 cytokines, but the activating abilities of 5-thio-KRN7000 (**4**) and 5-thio-α-GalCer 566 (**5**) were more potent.

In summary, we have successfully prepared three KRN7000 *S*-sugar analogues with acceptable total yields applying stereoselective α-glycosylation of 5-*S*-galactopyranosyl trichloroacetimidate and phytosphingosine derivatives. The abilities of KRN7000 and its 5-thio analogues **4**, **5**, and **6** to stimulate the cytokine production of NKT cells were investigated both *in vitro* and *in vivo*. *In vitro* testing of 5-thio-KRN7000 (**4**) and 5-thio-α-GalCer 566 (**5**) stimulated higher IFN-γ production of iNKT cells than that of KRN7000. Compared to KRN7000 (**1**), *S*-analogue **4** presented a similar tendency and ability to induce the release of key immunomediators IFN-γ and IL-4 in mice. It is quite interesting to note that the *S*-analogue **5** could compress the production of IL-4 in the presence of relatively high IFN-γ production. Therefore, **5** may be useful in specific inducing iNKT cells to produce Th1 cytokines. This biological data paves the way for developing new potent immunostimulating agents. Studies on deeper exploration of the three 5-thio analogues and the synthesis of other 5-thio-α-GalCers containing modified ceramide moiety are under investigation in our laboratory.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details for the synthesis of key compounds and procedure of bioassays. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

KRN7000, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol; iNKT, invariant natural killer T cells; Th1, T helper 1; Th2, T helper 1; IFN-γ, interferon γ; TNF-α, tumor necrosis factor-α; IF-2, interleukin-2; IL-4, interleukin-4; IL-10, interleukin-10; α-GalCer, α-galactosylceramide; TCR, T cell receptor; MS, mass spectrum; PBS-25, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galacto pyranosyl)-2-(*N*-octanoylamino)-1,3,4-octadecanetriol; α-GalCer 566, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-tetradecanoylamino)-1,3,4-octadecanetriol; NEt_3, triethylamine; THF, tetrahydrofuran; CCl_3CN, trichloroacetonitrile; DBU, 1,8-diazabicyclo(5.4.0)undec-7-ene; TMSOTf, trimethylsilyl trifluoromethanesulfonate; ELISA, enzyme linked immunosorbent assay; BFA, brefeldin A; APC, adenomatous polyposis coli; PMA-I, phorbol 12-myristate 13-acetate ionomycin

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