Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 16 (2006) 2195–2199
Bioorganic & Medicinal Chemistry Letters

Synthesis and biological evaluation of α-galactosylceramide (KRN7000) and isoglobotrihexosylceramide (iGb3)

Chengfeng Xia,[a,b] Qingjia Yao,[a,b] Jens Schümann,[c] Emmanuel Rossy,[c] Wenlan Chen,[b] Lizhi Zhu,[a,b] Wenpeng Zhang,[a] Gennaro De Libero[c] and Peng George Wang[a,b,*]

[a]*Department of Biochemistry, The Ohio State University, Columbus, OH 43210, USA*
[b]*Department of Chemistry, The Ohio State University, Columbus, OH 43210, USA*
[c]*Experimental Immunology, Department of Research, University Hospital Basel, CH-4031 Basel, Switzerland*

Received 17 December 2005; revised 10 January 2006; accepted 11 January 2006
Available online 2 February2006

Abstract—Glycoceramides can activate NKT cells by binding with CD1d to produce IFN-γ, IL-4, and other cytokines. An efficient synthetic pathway for α-galactosylceramide (KRN7000) was established by coupling a protected galactose donor to a properly protected ceramide. During the investigation, it was discovered that when the ceramide was protected with benzyl groups, only β-galactosylceramide was produced from the glycosylation reaction. In contrast, the ceramide with benzoyl protecting groups produced α-galactosylceramide. Isoglobotrihexosylceramide (iGb3) was prepared by glycosylation of Galα1-3Galβ1-4Glc donor with 2-azidosphingosine in high yield. Biological assays on the synthetic KRN7000 and iGb3 were performed using human and murine *i*NKT cell clones or hybridomas.

α-Galactosylceramides (α-GalCers) were discovered from an extract of a marine sponge, *Agelas mauritianus*, by the Pharmaceutical Division of the Kirin Brewery Company in 1993 and were named agelasphins (AGLs).[1] Although a number of β-galactosylceramides have been reported, AGLs were the first reported galactosylceramides having an α-galactosyl linkage. All these compounds were active substances in prolonging the life span of mice when intraperitoneally inoculated with B16 mouse melanoma cells.[2] Therefore, various analogues were synthesized to explore for candidates with similar antitumor activity and that were readily available from large-scale chemical synthesis. Among these compounds, AGL-582 emerged as the most desirable candidate for clinical application and was designated as KRN7000 (Fig. 1).[3] Further studies revealed that α-GalCers could stimulate natural killer T (NKT) cells via presentation by CD1d. The NKT cells can regulate a variety of microbial, allergic, autoimmune, and tumor conditions through the rapid secretion of interleukin-4 (IL-4), interferon-γ (INF-γ), and other cytokines and chemokines.[4] Because α-GalCers were either separated from a marine sponge or chemically synthesized, they are not the natural products of mammalian cells. Evidences showed that during the development of NKT cells from the mainstream T cell precursor pool to mature NKT cells, some endogenous glycolipid antigens were essential for this process by presentation of CD1d.[5] Recently, Zhou et al. demonstrated that isoglobotrihexosylceramide (iGb3) was the primary endogenous agonist ligand for NKT cells.[6] Therefore, there has been high demand for both KRN7000 and iGb3 for immunology research and for preclinical investigation.

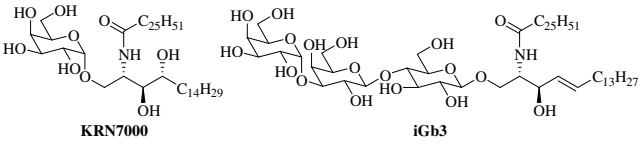
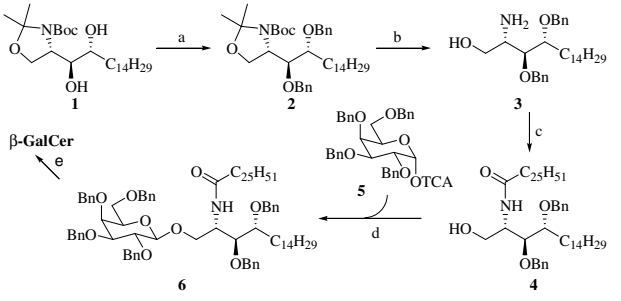


Figure 1. Structures of KRN7000 and iGb3.

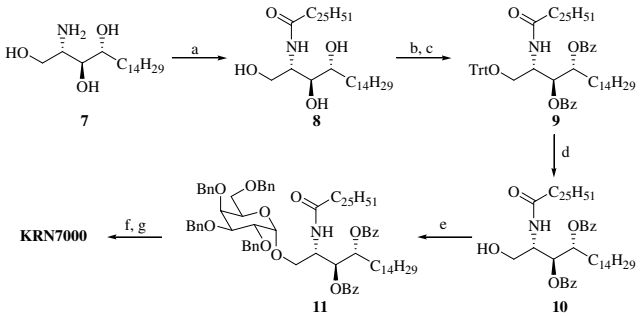
Several methods for synthesis of KRN7000 have been reported, which involved a crucial galactosylation reaction with different galactose donors and lipid acceptors.[7–10] Herein we used the active 'armed' perbenzyl-

Keywords: Immuno stimulator; Natural killer T cells; Glycoceramide; Glcosylation.
* Corresponding author. Tel.: +1 614 292 9884; fax: +1 614 688 3106; e-mail: wang.892@osu.edu

0960-894X/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2006.01.040



Scheme 1. Reagents and conditions: (a) NaH, BnCl, DMF, 87%; (b) TFA-H_2O (20:1), 85%; (c) $C_{25}H_{51}CO_2H$, EDCI, THF, 91%; (d) TMSOTf, Et_2O-THF (5:1), −23 °C, 57%; (e) H_2 (20 psi), 10% Pd/C, MeOH, 93%.



Scheme 2. Reagents and conditions: (a) $C_{25}H_{51}COSu$, THF, 94%; (b) TrtCl, DMAP, pyridine, 40 °C; (c) BzCl, DMAP, pyridine, 89% for two steps; (d) *p*TsOH, MeOH, 92%; (e) **5**, TMSOTf, Et_2O-THF (5:1), −23 °C, 59%; (f) NaOMe, MeOH, 94%; (g) H_2 (20 psi), 10% Pd/C, MeOH, 91%.

galactoside **5**[11] as a donor. We first used a ceramide **4** with 3,4-dihydroxyl groups protected by benzyl groups as an active 'armed' acceptor. As shown in Scheme 1, the partially protected phytosphingosine **1**[12] was subjected to NaH and then benzyl chloride in dry DMF to protect the two secondary hydroxy groups with benzyl group. Removal of the ketal and Boc with trifluoroacetic acid afforded the amine **3**. Then it was reacted with fatty acid under the condensation with 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (EDCI) to furnish the ceramide **4**. The ceramide **4** was subjected to glycosylation with donor **5** with TMSOTf as an activator under −23 °C. Surprisingly, the β-glycosylated product **6** was separated as a single glycosylation product. The anomeric configuration was further confirmed by NMR spectrums of β-GalCer which were consistent with reported data.[13]

We hypothesized that the formation of β-anomeric product was due to the high reactivity of the acceptor. While the donor was activated by TMSOTf, the armed acceptor attacked the donor in an SN2 fashion with a transition state **A** (Fig. 2), before the full development of oxacarbonium intermediate, resulting in β-configuration production. We then predicted that a less reactive acceptor would favor α-glycosylation through a fully developed oxacarbonium intermediate.

Thus, we switched the protecting groups on the ceramide from benzyl to benzoyl (as ceramide **10**) to decrease the acceptor reactivity (Scheme 2). The commercially available phytosphingosine was condensed with hexacosanic acid *N*-hydroxysuccinimide ester to give the amide **8**.[10] Then the primary hydroxy group was selectively protected with trityl chloride in pyridine. When TLC showed that the starting material disappeared, benzoyl chloride was added to protect the remaining two hydroxyl groups without work-up to afford **9**. The trityl was removed in methanol with the presence of *p*-toluenesulfonic acid. Then the ceramide **10** was subjected to glycosylation with donor **5** under activator TMSOTf. The glycoceramide **11** was isolated as the sole product which had the desired α-configuration. Then the benzoyl groups on sphingosine and the benzyl groups on sugar were removed by saponification in anhydrous methanol with NaOMe and hydrogenation under catalysis of 10% Pd/C, respectively, to produce KRN7000.[14]

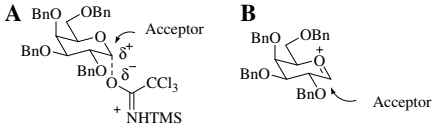
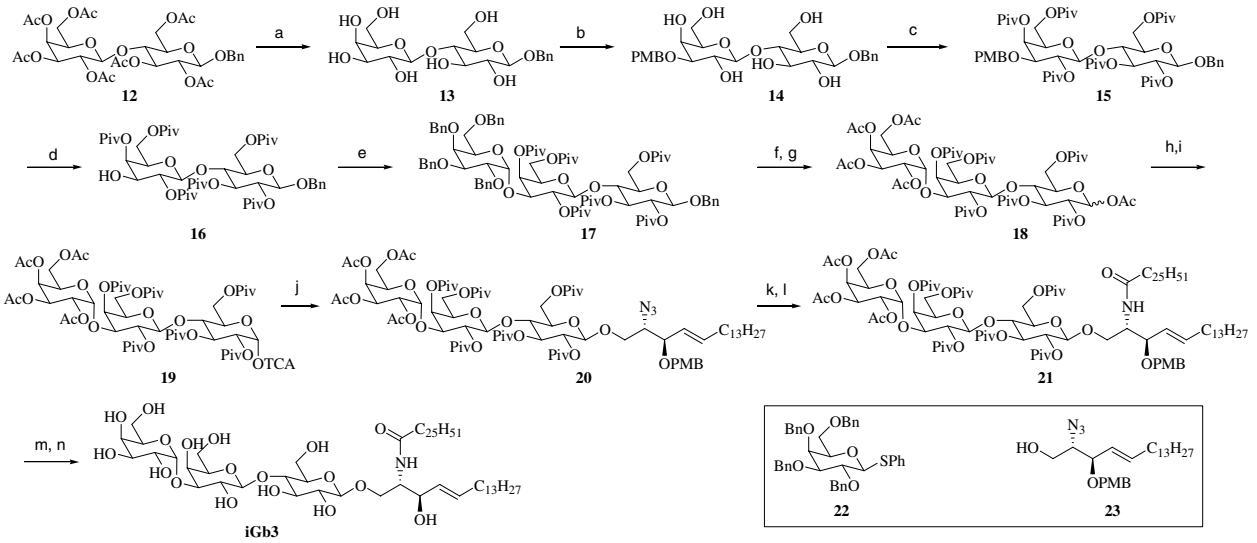


Figure 2. Transition state of glycosylation. (A) The armed acceptor attacked donor via SN2 pathway; (B) the disarmed acceptor reacted with fully formed oxacarbonium donor.

It is well known that the glycosylation outcome depends on the delicate balance of donor/acceptor pair reactivity. Herein we found out that the 'armed' sugar donor with 'armed' lipid acceptor produced a β-configuration and 'armed' sugar donor with 'disarmed' lipid acceptor produced an α-configuration. It is remarkable to see that the concept of 'armed' and 'disarmed' even works between a sugar donor and lipid acceptor.

Our synthesis of iGb3 started with the chemical synthesis of Galα1-3Galβ1-4Glc reported by our laboratory (see Scheme 3).[15,16] The known 1-benzyl-peracetyl lactose **12** was deacetylated to generate 1-benzyl lactose **13**. The regioselective monoalkylation of the C3 hydroxy group of the galactose unit with *p*-methoxybenzyl chloride (PMBCl) was achieved through activating this hydroxy group with dibutylstannylene acetal. Originally, acetyl group was used to protect the remaining hydroxy groups on compound **13**. In the glycosylation of the trisaccharide donor with the lipid, however, only an orthoester was obtained. Efforts for transforming this orthoester to glycoside by treatment with tin chloride or other Lewis acids failed. The bulky pivaloyl group, which could significantly restrain the formation of the orthoester, was then employed to give **15**. Oxidative cleavage of the PMB group by DDQ released the 3′-OH to afford the disaccharide acceptor **16**. The glycosylation of acceptor **16** with perbenzyl phenyl thiogalactoside donor **22** was carried out under activation by NIS/TfOH to afford the protected trisaccharide **17** in a yield of 92%. At this juncture, the benzyl groups have to be switched to acetyl groups to avoid the reduction of the double bond on the lipid during hydrogenation.



Scheme 3. Reagents and conditions: (a) NaOMe, MeOH, quantitive; (b) Bu_2SnO, MeOH, reflux, then PMBCl, TBAI, toluene, 70 °C, 74%; (c) PivCl, DMAP, pyridine, 70 °C, 86%; (d) DDQ, DCM/H_2O, 93%; (e) **22**, NIS, TfOH, DCM, −20 °C, 91%; (f) H_2 (20 psi), 10% Pd/C, MeOH, 97%; (g) Ac_2O, pyridine, 93%; (h) $BnNH_2$, THF; (i) CCl_3CN, DBU, DCM, 75% for two steps; (j) **23**, TMSOTf, DCM, −20 °C, 82%; (k) PPh_3, PhH/H_2O, 50 °C; (l) $C_{25}H_{51}CO_2H$, EDCI, THF, 89% for two steps; (m) 10% TFA in DCM, 87%; (n) NaOMe, MeOH, reflux, 92%.

Selective exposure of the anomeric hydroxy group was achieved by treatment with benzylamine in THF. It was then converted to trichloroacetimidate **19**, which was ready for glycosylation with lipid **23**.

Although Savage used an amide compound as an acceptor for glycosylation, the yield is around 45%.[6] It is well known that the amide has remarkable effects on the glycosylation. So we used azide **23**[17] instead of amide for the acceptor. It was also found that if the 3-OH on sphingosine was protected with an ester, the acyl group would shift to the amine while in reduction of the azide.[18] PMB is an excellent protecting group which can be cleavaged by oxidation or in acidic condition. The glycosylation of Schmidt's type donor **19** and lipid **23** was performed at −20 °C for 2 h in 82% yield. The azide was reduced to an amine by triphenylphosphine with stoichiometric amount of water, then subjected to amide formation with cerotic acid and EDCI to afford protected iGb3 **21**. Cleavage of the PMB proceeded smoothly in 10% of trifluoroacetic acid in dichloromethane. Finally, all the ester groups were removed by reaction with sodium methoxide in refluxing methanol for 24 h to yield iGb3 in 92% yield.[19]

Liquid chromatography analysis coupled to mass spectrometry was performed to control the purity and confirm the mass of the synthetic compounds. After injection of large amounts of iGb3 (40 pmol), the base peak chromatogram and the ESI-MS spectra revealed highly pure compounds (Fig. 3A). The *m/z* 1186.7 and 1146.5 ions were attributed to the synthetic iGb3 by MS/MS spectra analysis. Low energy CID experiments performed on the sodiated adduct precursor ion allowed a partial structural analysis of iGb3 (Fig. 3B). The characteristic neutral loss of the sugar groups (Y ions) confirmed the loss of the 3 hexose groups. Fragmentation of the sodiated forms gave low intensity peaks and little structural information. Replacement of sodium by lithium was then performed

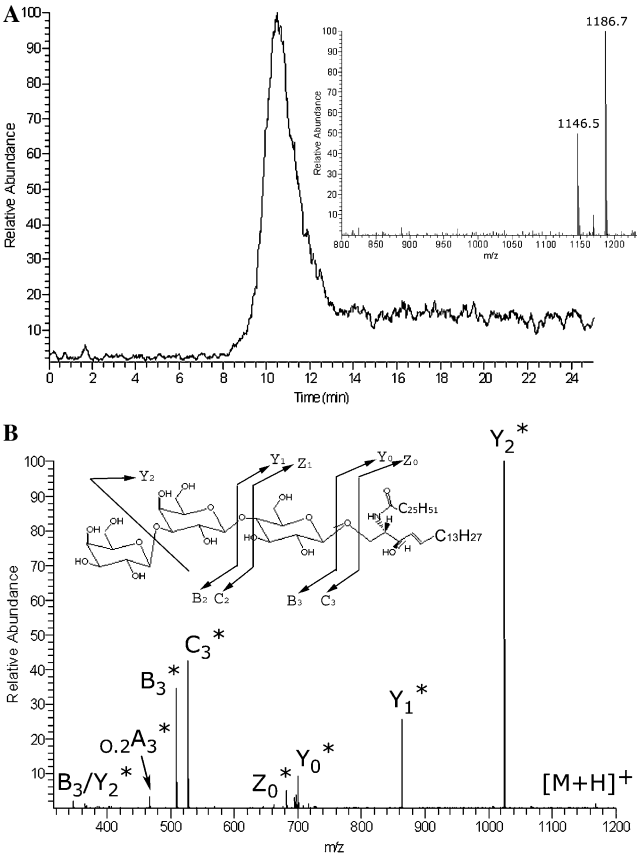


Figure 3. (A) Base peak chromatogram of iGb3 (40 pmol). The main chromatographic peak has been attributed to the sodiated form of iGb3 (m/z 1186.7). (Inset) Positive-ion mode spectra of the iGb3 standard. The *m/z* 1146.5 ion has been attributed to the dehydrated iGb3 by MS/MS analysis. (B) ESI-MS/MS spectra of the parent ion at *m/z* 1186.7 ($[M+Na]^+$). * represents the sodiated daughter ions. (Inset) Structure and fragmentation of iGb3 attributed from the ESI-MS/MS spectra. The nomenclature of Costello et al., expanded by Adams and Ann, has been used.

because it provides more informative structural data. Analysis in the presence of excess lithium was performed by direct infusion and generated similar fragmentation patterns (data not shown). These investigations confirmed the ceramide moiety as a d18:1/C26:0.

Since there has been some controversy to the reported activities of iGb3, here the bioassays on iGb3 were reported in detail. We performed T cell stimulation assays using human and murine *i*NKT cell clones or hybridomas, as previously described.[6] DMSO and methanol were used to solubilize iGb3 and stimulation experiments were performed at a final concentration of 1% DMSO or methanol, a concentration which is not toxic to cells. iGb3 stimulated human *i*NKT cells to release interleukin-4 (IL-4) and interferon-γ (IFN-γ) when presented by hCD1d-transfected human B lymphoblastoid cells (C1R) expressing high levels of hCD1d (Fig. 4A). The stimulatory capacity of iGb3 strongly depended on the solvent used. Only iGb3 dissolved in methanol was able to stimulate the *i*NKT clone. IL-4 and IFN-γ release could be observed already at iGb3 concentrations of 5 μg/mL and increased at higher doses in a dose-dependent manner. Control α-GalCer was active at doses as low as 10 pg/mL. Hence, iGb3 is less efficient than the very strong agonist α-GalCer in stimulating the used *i*NKT cell clone. The stimulatory capacity of iGb3 was then tested using C1R-hCD1d transfectants and peripheral blood mononuclear cell (PBMC)-derived human dendritic cells (DC), that is, professional antigen-presenting cells (APC) directly generated from fresh human blood. As shown in Figure 4B, iGb3 employed at 20 μg/mL in the presence of 1% methanol induced the release of both IL-4 and IFN-γ from human *i*NKT cells. The extent of stimulation was lower when DC was used as APC, likely because of the significantly lower expression levels of CD1d on DC as compared to C1R-hCD1d transfectants (not shown). Finally, we also employed iGb3, dissolved in methanol, in a murine *i*NKT stimulation system using bone marrow-derived mouse DC as APC and a murine *i*NKT hybridoma releasing interleukin-2 (IL-2) as responder cells. iGb3 clearly stimulated the murine *i*NKT hybridoma already at a concentration of 0.2 μg/mL (Fig. 4C).

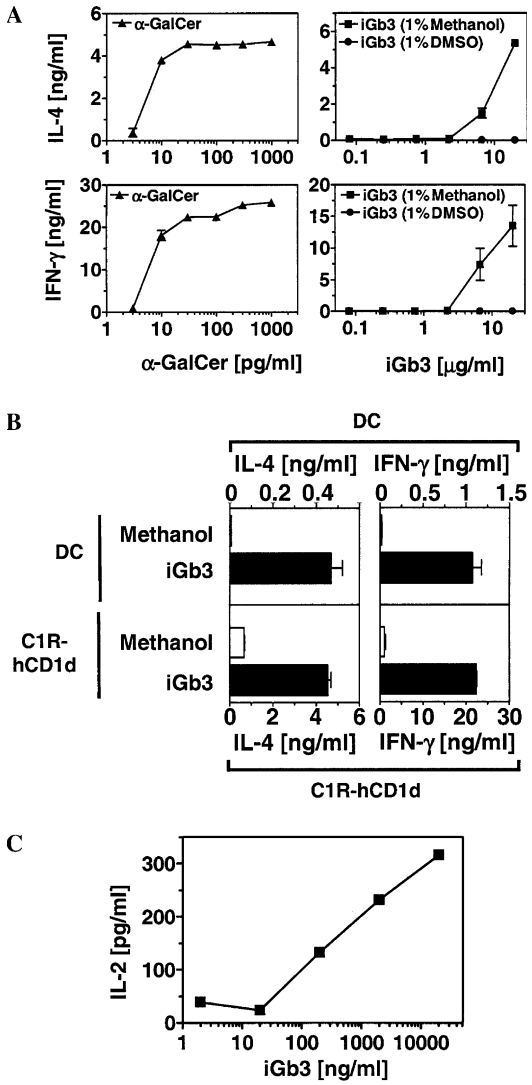


Figure 4. (A) Stimulation of a human Vα24*i* NKT cell clone (JS7) by human CD1d-transfected C1R cells (C1R-hCD1d) in the presence of different concentrations of α-GalCer or iGb3. iGb3 was solubilized using methanol or DMSO, both at final concentration of 1% (v/v) in the stimulation assay. Diagrams show the mean release of IL-4 (upper panel) or IFN-γ (lower panel) to cell culture supernatant after 40 h, determined by ELISA, as pg/mL ± SD. (B) iGb3 (solubilized in methanol) was used to stimulate the human Vα24*i* NKT cell clone JS63. C1R-hCD1d cells or human monocyte-derived DC were used as APC. iGb3 was solubilized in methanol. The bar charts show the mean release of IL-4 (left diagram) or IFN-γ (right diagram) in culture supernatants (ng/mL ± SD) in the presence of iGb3 (20 μg/mL) or methanol alone. Final methanol concentration was 1% (v/v). (C) Stimulation of the mouse Vα14*i* NKT hybridoma FF1 by murine bone marrow-derived DC in the presence of different concentrations of iGb3. iGb3 was solubilized in methanol. The diagram shows the mean release of IL-2 (pg/mL ± SD) in cell culture supernatants determined by ELISA.

In summary, we developed an efficient synthetic route for the synthesis of KRN7000 by controlling the galactosidic configuration using different protecting groups of the ceramide. We also synthesized iGb3 from Galα1-3Galβ1-4Glc and 2-azido-sphingosine in high yield. Biological assays showed KRN7000 and iGb3 could stimulate both human and murine *i*NKT cells to produce cytokines.

Acknowledgments

This project was supported by Ohio State University to Peng George Wang and by SNF (Grant 3100A0-109918) to Gennaro De Libero.

References and notes

1. Natori, T.; Koezuka, Y.; Higa, T. *Tetrahedron Lett.* **1993**, *34*, 5591.
2. Notari, T.; Morita, M.; Akimoto, K.; Koezuka, Y. *Tetrahedron* **1994**, *50*, 2771.
3. Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Koezuka, Y.; Kobayashi, E.; Fukushima, H. *J. Med. Chem.* **1995**, *38*, 2176.

4. Brigl, M.; Brenner, M. *Annu. Rev. Immunol.* **2004**, *22*, 817.
5. Kronenberg, M. *Annu. Rev. Immunol.* **2005**, *23*, 877.
6. Zhou, D.; Mattner, J.; Cantu, C., III; Schrantz, N.; Yin, N.; Gao, Y.; Sagiv, Y.; Hudspeth, K.; Wu, Y.; Yamashita, T.; Teneberg, S.; Wang, D.; Proia, R. L.; Levery, S. B.; Savage, P. B.; Teyton, L.; Bendelac, A. *Science* **2004**, *306*, 1786.
7. Morita, M.; Natori, T.; Akimoto, K.; Osawa, T.; Fukusima, H.; Koezuka, Y. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 699.
8. Figueroa-Perez, S.; Schmidr, R. R. *Carbohydr. Res.* **2000**, *328*, 95.
9. Plettenberg, O.; Bodmer-Narkevitch, V.; Wong, C. H. *J. Org. Chem.* **2002**, *67*, 4559.
10. Song, S.; Lee, T.; Jung, S.; Kim, D. *Synthesis* **2004**, *6*, 847.
11. Alessandro, D.; Alberto, M.; Alessandro, M. *J. Org. Chem.* **1999**, *64*, 933.
12. Shirota, O.; Nakanishi, K.; Berova, N. *Tetrahedron* **1999**, *55*, 13643.
13. β-GalCer: ^{1}H NMR (500 MHz, pyridine-d_5) δ 8.49 (d, J = 8.5 Hz, 1H), 5.14 (m, 1H), 4.89 (d, J = 7.5 Hz, 1H), 4.18 (dd, J = 10.5, 5.5 Hz, 1H), 4.53 (d, J = 2.0 Hz, 1H), 4.49–4.43 (m, 2H), 4.41 (d, J = 6.0 Hz, 1H), 4.36 (t, J = 6.0 Hz, 1H), 4.20 (t, J = 6.4 Hz, 1H), 4.12 (dd, J = 9.4, 2.7 Hz, 1H), 4.02 (t, J = 5.8 Hz, 1H), 2.40 (t, J = 7.3 Hz, 2H), 2.20 (m, 1H), 1.93-1.89 (m, 2H), 1.81–1.76 (m, 2H), 1.67 (m, 1H), 1.43-1.14 (m, 66H), 0.88–0.83 (m, 6H); ^{13}C NMR (125 MHz, pyridine-d_5) δ 174.44, 107.22, 77.98, 76.65, 76.28, 73.62, 73.54, 71.67, 71.10, 63.23, 53.19, 37.82, 34.42, 33.05, 33.04, 31.25, 31.10, 30.94, 30.86, 30.83, 30.76, 30.68, 30.55, 27.59, 27.30, 23.86, 15.21. Data can be found in: Morita, M.; Natori, T.; Akimoto, K.; Osawa, T.; Fukusima, H.; Koezuka, Y. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 699.
14. KRN7000: ^{1}H NMR (500 MHz, pyridine-d_5) δ 8.43 (d, J = 8.6 Hz, 1H), 5.55 (d, J = 3.5 Hz, 1H), 5.25–5.21 (m, 1H), 4.66-4.61 (m, 2H), 4.53 (m, 1H), 4.49 (t, J = 5.8 Hz, 1H), 4.42–4.36 (m, 4H), 4.31–4.29 (m, 1H), 2.43 (t, J = 7.4 Hz, 2H), 2.23 (m, 1H), 1.92–1.86 (m, 2H), 1.82-1.77 (m, 2H), 1.63 (m, 1H), 1.44–1.18 (m, 66H), 0.88–0.83 (m, 6H); ^{13}C NMR (125 MHz, pyridine-d_5) δ 174.56, 102.81, 77.96, 74.29, 72.88, 72.26, 71.58, 69.95, 63.94, 52.78, 38.08, 35.60, 33.40, 31.64, 31.42, 31.31, 31.27, 31.20, 31.14, 31.08, 30.89, 27.78, 27.66, 24.20, 15.54; HRMS ($[M+Na]^+$) 880.7240 calcd. 880.7212.
15. Janczuk, A. J.; Zhang, W.; Andreana, P. R.; Warrick, J.; Wang, P. G. *Carbohydr. Res.* **2002**, *337*, 1247.
16. Zhang, W.; Wang, J.-Q.; Li, J.; Yu, L.-B.; Wang, P. G. *J. Carbohydr. Chem.* **1999**, *18*, 1009.
17. Taniguchi, M.; Seino, K-i.; Nakayama, T. *Nat. Immunol.* **2003**, *4*, 1164.
18. Rai, A. N.; Basu, A. *Org. Lett.* **2004**, *6*, 2861.
19. iGb3: ^{1}H NMR (500 MHz, pyridine-d_5): δ 8.31 (d, J = 8.0 Hz, 1H), 7.53 (br, 1H), 7.32 (br, 1H), 6.98 (br, 1H), 6.66 (br, 1H), 6.58 (br, 1H), 6.52 (br, 1H), 6.42 (br, 1H), 6.38 (br, 1H), 6.36 (br, 1H), 6.09 (br, 1H), 6.01 (dd, J = 15.5, 6.0 Hz), 5.91 (dt, J = 15.3, 6.5 Hz, 1H), 5.76 (br, 1H), 5.64 (d, J = 3.8 Hz, 1H), 5.05–5.00 (m, 2H), 4.87 (d, J = 12 Hz, 1H), 4.81–4.75 (m, 3H), 4.72–4.69 (m, 1H), 4.65 (m, 1H), 4.56 (m, 1H), 4.54–4.49 (m, 3H), 4.47–4.39 (m, 4H), 4.32–4.29 (m, 1H), 4.24–4.20 (m, 3H), 4.15 (dd, J = 9.5, 2.5 Hz, 1H), 4.05–4.00 (m, 2H), 3.86–3.84 (m, 1H), 2.42 (t, J = 7.0 Hz, 2H), 2.06 (q, J = 6.5 Hz, 2H), 1.82 (m, 2H), 1.39–1.21 (m, 66H), 0.87 (t, J = 6.5 Hz, 3H), 0.86 (t, J = 7.0 Hz, 3H); ^{13}C NMR (100 MHz, pyridine-d_5): δ 174.20, 133.54, 133.16, 106.37, 98.59, 83.00, 81.06, 77.58, 77.47, 77.40, 75.68, 73.74, 73.60, 72.52, 71.73, 71.40, 71.30, 66.88, 63.13, 62.92, 62.73, 55.81, 50.56, 37.85, 33.65, 33.04, 33.02, 31.40, 30.94, 30.92, 30.89, 30.81, 30.70, 30.63, 30.54, 30.51, 27.31, 23.83, 15.18; HRMS ($[M+Na]^+$) 1186.8136, calcd. 1186.8168.