Syntheses of Biotinylated α-Galactosylceramides and Their Effects on the Immune System and CD1 Molecules

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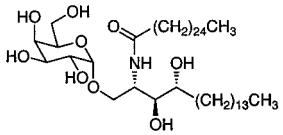
A representative α-galactosylceramide (α-GalCer), KRN7000, has strong immunostimulatory and antitumor activity. Recent studies demonstrated that KRN7000-pulsed antigen-presenting cells (APC) can activate natural killer T (NKT) cells, a novel T-cell lineage, and CD1d molecules on APC play an important role in the activation of NKT cells. However, it remains unclear whether α-GalCers actually bind to CD1d molecules. To address this question, we synthesized three kinds of biotinylated α-GalCer and a biotinylated *β*-GalCer and found that the biotinylated α-GalCers significantly stimulate the proliferation of murine spleen cells, but the biotinylated *β*-GalCer does not and that all biotinylated compounds bind to CD1d molecules.

Introduction

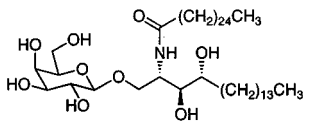
KRN7000, an α-galactosylceramide (α-GalCer, galactose bound to ceramide in an α-configuration; Chart 1), showed significant immunostimulatory[1] and antitumor activity in mice with liver metastasis of the Colon26 adenocarcinoma.[2] We previously suggested that KRN7000-pulsed dendritic cells (DC) activate mouse Vα14 natural killer T (NKT) cells, a novel lymphoid lineage. Furthermore, we suggested that the CD1 molecule on DC plays an important role in the activation of NKT cells by KRN7000,[3] and it was clearly demonstrated that CD1 molecules play a key role in the activation of Vα14/V*β*8 NKT cells by KRN7000-pulsed CD1-positive antigen-presenting cells (APC).[4] Furthermore, recent studies clearly demonstrated that KRN7000 can also activate human Vα24/V*β*11 NKT cells, a counterpart of mouse Vα14/V*β*8 NKT cells, through human CD1d molecules, the homologue of mouse CD1 molecules.[5−8] These findings suggest that the CD1d/KRN7000 complex was recognized by Vα14/V*β*8 or Vα24/V*β*11 T-cell receptors (TCR) on NKT cells. However, it remains unclear whether KRN7000 actually binds to CD1d molecules.

To address this question, we planned to conduct surface plasmon resonance experiments using immobilized α-GalCers and soluble CD1d molecules. To do this, we synthesized biotinylated α-GalCers which could bind to a streptavidin-coated sensor chip, thereby avoiding micelle or bilayer formation. In addition, it was demonstrated that CD1d-transfectant APC pulsed with *β*-GalCer (AGL-583),[9] which has the same ceramide moiety as KRN7000 (Chart 1), showed little or no activation of mouse and human NKT cells.[3−8] These findings lead to the question as to whether *β*-GalCers can bind to CD1d molecules or if these molecules are not antigenic for some other reason. To address this question, we also synthesized a biotinylated *β*-GalCer.

Chart 1. Chemical Structures of KRN7000 (α-GalCer) and AGL-583 (*β*-GalCer)



KRN7000



AGL-583

In this paper, we report the syntheses of three kinds of biotinylated α-GalCers, with different hydroxyl groups in the ceramide portion, and a biotinylated *β*-GalCer, and we report their effects on the proliferation of murine spleen cells. In addition, we describe that all the biotinylated compounds actually bind to mouse CD1 and to human CD1d molecules.

Chemistry

In the synthetic approach to the biotinylated α-GalCers and *β*-GalCer, we considered that the fatty acid moiety had appropriate length for binding the BIAcore sensor chips. Therefore, we first synthesized the long *ω*-biotinylated fatty acid (Scheme 1). The 12-aminododecanoic acid (**1**) was treated with 10% hydrochloric acid in methanol to give a methyl ester **2**. The methyl ester **2** was amidated with 6-(biotinylamino)hexanoic acid (**3**) using EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride) to give **4**. The methyl ester was hydrolyzed with sodium hydroxide in aqueous THF, MeOH to give biotinylated fatty acid **5**.

The synthesis of biotinylated α-GalCers is shown in Scheme 2. The α-galactosyl long chain base **7a** was synthesized from the approriate α-GalCer AGL-525 (**6a**)[1] by hydrolysis with *n*-tetrabutylammonium hydroxide in aqueous *n*-butanol. The resulting long chain base **7a** was amidated with biotinylated fatty acid **5** using EDC for coupling reagent to give biotinylated α-GalCer AGL-592 (**8a**). By the same method, we synthesized the

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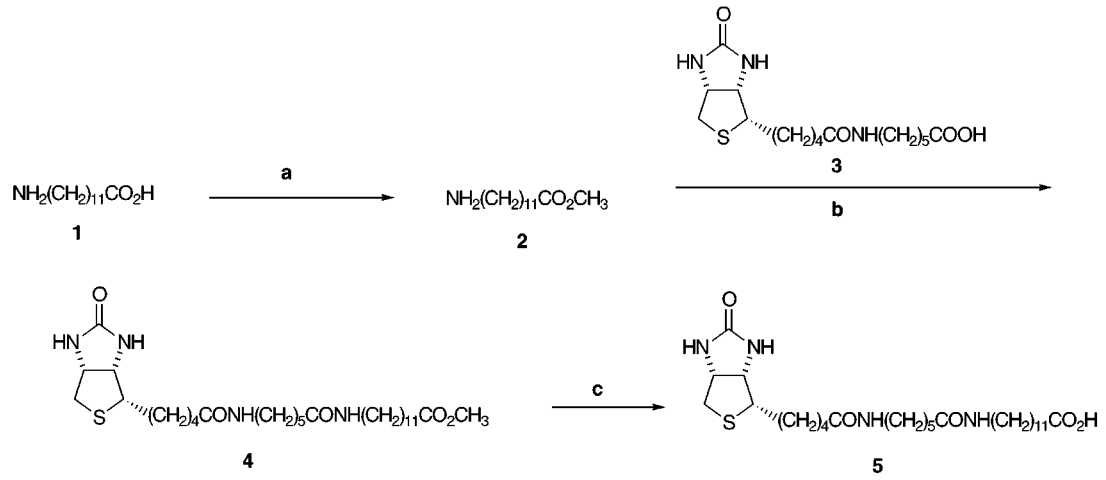
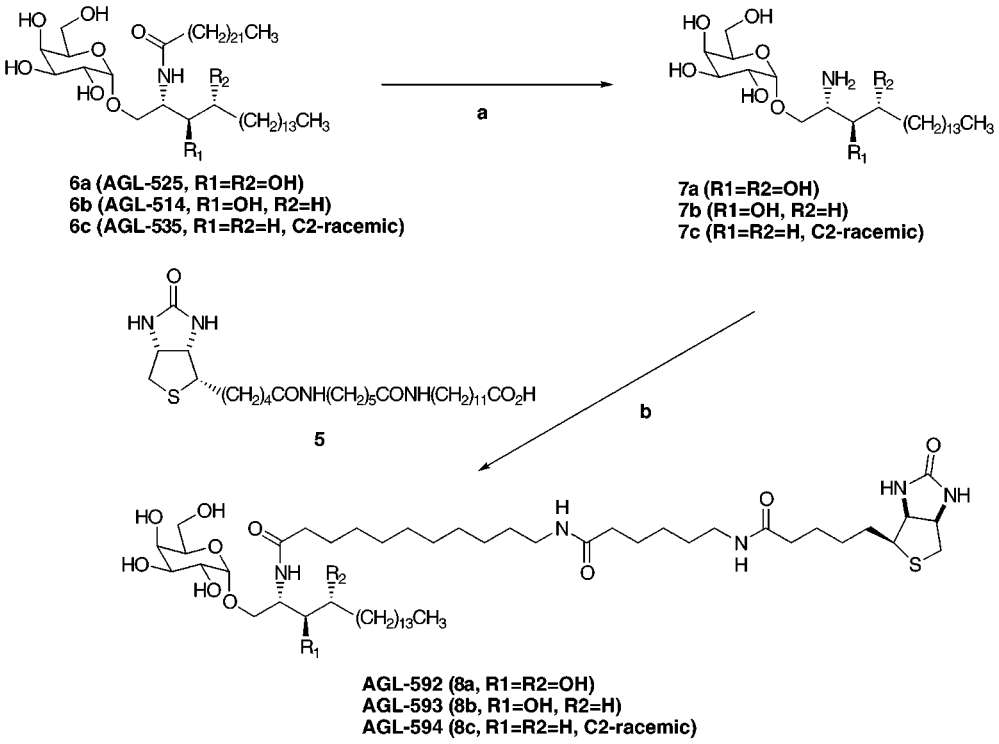
Scheme 1[a]



[a] (a) 10% HCl−MeOH, 60 °C, 2 h; (b) **3**, EDC, DMF, 30 °C, 18 h; (c) NaOH, THF, MeOH, H_2O.

Scheme 2[a]



[a] (a) *n*-Bu_4NOH, *n*-BuOH, H_2O, reflux, 72 h; (b) **5**, EDC, DMF, 70 °C, 5 h.

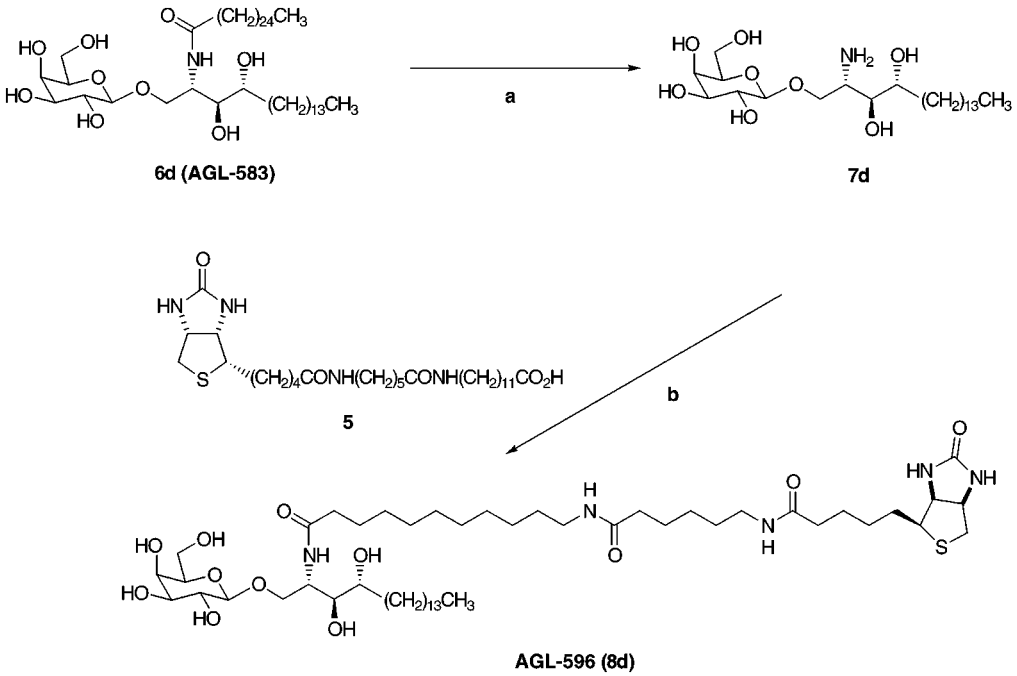
other two biotinylated α-GalCers, AGL-593 (**8b**) and AGL-594 (**8c**), from AGL-514 (**6b**)[1] and AGL-535 (**6c**),[1] respectively. In addition, the biotinylated *β*-GalCer AGL-596 (**8d**) was synthesized from AGL-583 (**6d**)[9] by the same reaction sequence shown in Scheme 3.

Results and Discussion

We previously reported that KRN7000 dose-dependently stimulates the proliferation of murine spleen cells from the relatively low concentration of 1 ng/mL. By contrast, little or no proliferative effect is induced by AGL-583, *β*-GalCer, having the same ceramide portion as KRN7000, even at a concentration of 100 ng/mL.[10] In addition, it was demonstrated that KRN7000-pulsed DC can enhance the antigen-presenting cell function of DC, but the DC pretreated with AGL-583 are not enhanced.[11,12] Furthermore, recent findings suggest that CD1d molecules on DC play a key role in the enhancement of the immune response via the KRN7000-induced activation of mouse and human NKT cells.[3−8] These data suggest that the CD1d/KRN7000 complex was recognized by NKT cells but that the CD1d/AGL-583 complex was not. To test this possibility, we conducted the following study.

We first undertook several surface plasmon resonance experiments using either soluble mouse CD1 or soluble human CD1d and KRN7000, but we could not clearly detect the binding of KRN7000 to these immobilized CD1 molecules, because the molecular weight of KRN7000 is much smaller than that of soluble CD1d molecules, and surface plasmon resonance detects the change in mass of material bound to the sensor chip

Scheme 3[a]



[a] (a) *n*-Bu_4NOH, *n*-BuOH, H_2O, reflux, 72 h; (b) **5**, EDC, DMF, 70 °C, 5 h.

Table 1. Effects of KRN7000, Agl-592, Agl-596, Agl-593, and Agl-594 on the Proliferation of Murine Spleen Cells[a]

	[^{3}H]TdR incorporation (cpm)		
sample	1 ng/mL	10 ng/mL	100 ng/mL
vehicle	4628 ± 302	5054 ± 666	4975 ± 195
KRN7000	13499 ± 246*	17438 ± 967*	17636 ± 515*
AGL-592	5273 ± 306	10951 ± 244*	15292 ± 543*
AGL-596	4811 ± 245	4833 ± 241	5463 ± 462
AGL-593	4628 ± 302	8665 ± 1113*	14588 ± 138*
AGL-594	4803 ± 205	5330 ± 304	9427 ± 341*

[a] 2.5×10^5 cells/100 μL/well of spleen cells from C57BL/6 mice suspended in 10% FCS RPMI 1640 medium were plated on 96-well plates. At the same time, various concentrations of samples (10 μL/well) were added into each well, and the cell suspension was cultured at 37 °C, 5% CO_2, for 18 h. Then 0.5 μCi/well of tritium-thymidine ([^{3}H]TdR) was added into each well, and 8 h later, the [^{3}H]TdR uptake into the cells was measured by liquid scintillation counter. Each value shows the mean ± S.D.*$p < 0.05$ (vs vehicle-treated group).

(Naidenko et al., manuscript in preparation). We therefore planned to conduct surface plasmon resonance experiments using immobilized α-GalCers with the detection of the binding of soluble CD1d molecules (molecular weight of approximately 45 kDa).

On the basis of this plan, we synthesized biotinylated α-GalCer (AGL-592) and biotinylated β-GalCer (AGL-596) (Schemes 2 and 3) in order to conduct the surface plasmon resonance experiments using immobilized α-GalCer and β-GalCer. When we evaluated the effects of AGL-592 and AGL-596 on the proliferation of mouse spleen cells, it appeared that AGL-592 markedly stimulated the proliferative effect at concentrations of 10 and 100 ng/mL, although its potency was weaker than that of KRN7000, a positive control. Furthermore, AGL-596, with a β-linked galactose, showed little or no immunostimulatory activity even at a concentration of 100 ng/mL (Table 1). The surface plasmon resonance experiment using immobilized AGL-592 and AGL-596 and soluble mouse CD1 and human CD1d molecules demonstrated that both α-GalCer and β-GalCer actually bind to mouse CD1 and human CD1d molecules, and their binding affinities are almost equal (Naidenko et al., manuscript in preparation). These results demonstrate that the bindings of both α-GalCer and β-GalCer to CD1d molecules are independent of their spleen cell proliferation stimulatory effects, suggesting that the CD1d/GalCer complex exists on DC and that NKT cells could, interestingly, discriminate between the different glycosidic linkages between galactose and ceramide when the glycolipid is present on the cell surface as part of a CD1d/GalCer complex.

It was reported that the hydroxyl group in the 4 position in the ceramide moiety (4-OH) plays an essential role in the activation of human Vα24/Vβ11 NKT cells induced by α-GalCers[13] and that the hydroxyl group in the 3 position in the ceramide moiety (3-OH) plays a key role in the activation of mouse Vα14/Vβ8 NKT cells and human Vα24/Vβ11 NKT cells induced by α-GalCers.[1,3,5,7,13] On the basis of these findings, we then synthesized biotinylated α-GalCers with only the 3-OH (AGL-593) and without the 3- and 4-OH (AGL-594), to compare their binding affinities to mouse CD1 and human CD1d molecules with that of AGL-592 which has both 3- and 4-OH. As shown in Table 1, AGL-593 significantly stimulated the proliferation of spleen cells at concentrations of 10 and 100 ng/mL, and its potency was a little weaker than that of AGL-592. AGL-594 also enhanced the proliferative response, although the significant augmentation was observed only at the highest concentration of 100 ng/mL.

We then performed the surface plasmon resonance experiment using either soluble mouse CD1 or soluble human CD1d and immobilized compounds. AGL-593 and AGL-594 were immobilized on a streptavidin-coated BIAcore sensor chip. The presence of the galactose-containing lipid AGL-594 was confirmed by binding of terminal galactose-specific plant lectins *Ricinus communis* agglutinin (RCA I), which has a preference for

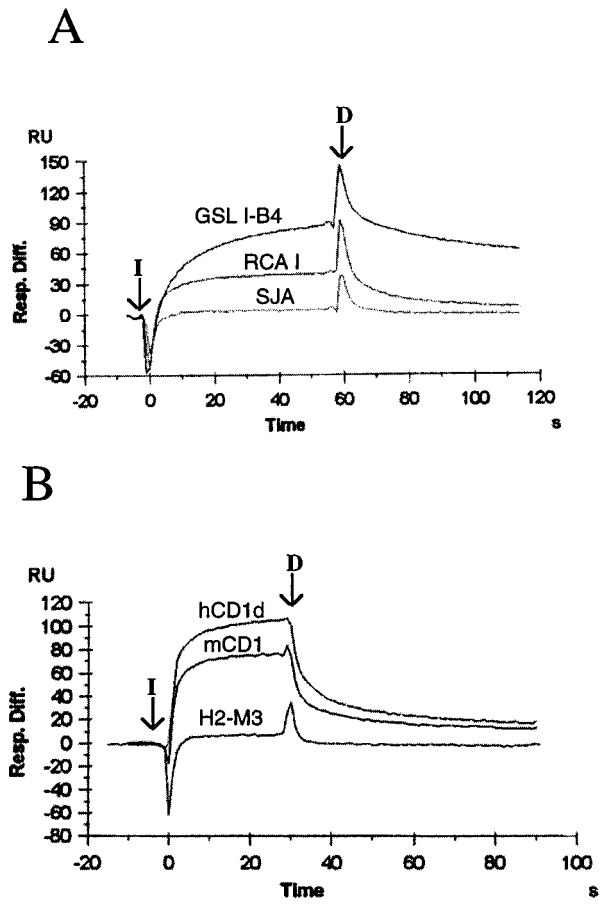


Figure 1. Binding of lectins and class I molecules to immobilized AGL-594. (A) Lectin binding. Galactose-specific lectins RCA I (8.7 mM) and GSL I-B4 (8.7 mM) specifically bind to immobilized AGL-594, while the GalNac-specific lectin SJA (8.7 mM) does not bind. Subtracted sensorgrams (Fc2 − Fc1) are shown. I indicates the start of the injection (association phase), and D indicates the end of the injection and the beginning of the dissociation phase. The dip in the sensorgram at the beginning of the association phase and the peak at the end of it are due to the time delay between Fc1 and Fc2. Binding was done in PBS with 0.5 mM $MgCl_2$ and 0.9 mM $CaCl_2$ at 10 mL/min flow rate. (B) Class I molecule binding. Binding of mouse CD1 (1.2 mM), human CD1d (2.6 mM), and M3 (0.8 mM) to immobilized AGL-594. Data are presented as in part A.

the *β*-linked anomer, and isolectin B4 of *Griffonia simplicifolia* lectin I (GSL I-B4) (Figure 1A), which has a preferance for the α-anomer. A negative control, *Sophora japonica* agglutin (SJA), which recognizes terminal *N*-acetylgalactosamine, did not bind to the immobilized glycolipid (Figure 1A). The same pattern of lectin binding was observed for the AGL-593-coupled biosensor chip (data not shown).

We have previously observed that soluble recombinant mouse CD1 and human CD1d are able to bind strongly to both biotinylated α-GalCer (AGL-592) and *β*-GalCer (AGL-596) (Naidenko et al., manuscript in preparation) when immobilized on a sensor chip. Similarly, both mouse CD1 and human CD1d can bind to immobilized AGL-594, while another nonclassical MHC class I molecule with a hydrophobic binding groove, M3,[14] did not bind (Figure 1B). Mouse CD1 and human CD1d also could bind to immobilized AGL-593 as well (data not shown). Analysis of the k_{on} and k_{off} in the BIAcore indicated that the affinity of the observed interaction of mouse CD1 and human CD1d proteins with immobilized AGL-593 and AGL-594 is in the 10−100 nM range. In light of the fact that mouse CD1 and human CD1d proteins bind equally well to AGL-593 and AGL-594, it is uncertain if the reported requirement for the hydroxyl groups in positions 3 and 4 of the long chain base reflects a specific feature of the interaction of the NKT cell T-cell receptor with these hydroxyl groups[3,5,13] or if it is simply due to the likely decreased solubility of these compounds. Furthermore, taking the report that the crystallographic structure of mouse CD1 shows a deep, narrow, and very hydrophobic ligand binding groove[15] into consideration, these results suggest that the long chain base and the binding groove play an important role in the binding of GalCer and CD1d molecules.

The human CD1 family contains five members, designated CD1a, CD1b, CD1c, CD1d, and CD1e,[16,17] and they can be classified into two groups on the basis of the predicted amino acid sequence similarity: The CD1a, CD1b, and CD1c comprise group I, and mouse CD1 and human CD1d comprise the second group. Among the group I CD1 molecules, CD1b has been well-characterized by several research groups. They demonstrated that glycolipids such as glucomonomycolate (GMM) and lipoarabinomannan (LAM) from *Mycobacteria spp*. can work as antigens for a human T-cell subset[18] and that LAM, fragments derived from LAM and GMM, can bind to CD1b molecules.[19] In addition, it was also indicated that CD1b molecules broadly distribute in endosomal and lysosomal compartments of antigen-presnting cells, and the binding of glycolipid and CD1b molecules occurs in these compartments.[20−22] However, while CD1d molecules also distribute in these endosomal compartments,[23,24] it is controversial whether CD1d binds α-GalCer in these compartments. It is likely that biotinylated α-GalCers and *β*-GalCer will be useful tools to study if CD1d binds α-GalCers and *β*-GalCer in endosomal and lysosomal compartments.

In summary, the data in the present study demonstrate that α-GalCer and *β*-GalCer actually bind to mouse CD1 and human CD1d molecules, demonstrate that binding is independent of the presence of hydroxyl groups in the 3 and 4 positions of the long chain base, and suggest that biotinylated α-GalCers and *β*-GalCer will be useful tools to study the binding and distribution of CD1d antigens in APC.

Experimental Section

Chemical Methods. Column chromatography was performed on silica gel (Cica-Merck silica gel 60, particle size 0.063−0.200 mm). TLC analyses were done on silica gel plates (Merk, art. 5554). All melting points were measured on a Yanagimoto micromelting point apparatus and are uncorrected. Mass spectra were measured on a JEOL JMS SX/SX-102 mass spectrometer. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. Elemental analyses were recorded with a Perkin-Elmer 240C elemental analyzer. ^{1}H NMR spectra were obtained using a JEOL JNM-GX-500 FT NMR spectrometer; chemical shifts are expressed in δ units from tetramethylsilane (TMS) as an internal standard, and coupling constants (J) are reported in hertz (Hz).

Methyl 12-[6-(Biotinoylamino)hexanoylamino]dodecanoate (4). 12-Aminododecanoic acid (**1**) (1.00 g, 4.64 mmol) was dissolved in 10% HCl in methanol (40 mL) and stirred at 60 °C for 2 h. The mixture was concentrated. The residue was washed with Et_2O to give **2**. This amino ester hydro-

chloride **2** was used for the next step without further purification: 1.02 g (82%); FDMS *m/z* 230 $(M + 1)^+$. To a stirred solution of **2** (515 mg, 1.94 mmol) in DMF (20 mL) were added **3** (694 mg, 1.94 mmol) and WSC·HCl (504 mg, 2.62 mmol); after addition the mixture was stirred at 30 °C for 18 h. The mixture was concentrated, and the resulting residue was purified by chromatography on a silica gel column (50 g) using chloroform−methanol (10:1) as the eluent to give **4**: 420 mg (38%); $[\alpha]^{23}_D$ +14.4° (*c* 0.1, pyridine); mp 156−157 °C; FDMS *m/z* 569 $(M + 1)^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.68 (1H, t, J = 4.4 Hz), 7.70 (1H, t, J = 5.1 Hz), 6.40 (1H, brs), 6.34 (1H, brs), 4.28−4.32 (1H, m), 4.10−4.15 (1H, m), 3.57 (3H, s), 3.06−3.12 (1H, m), 2.95−3.03 (4H, m), 2.78−2.85(2H,m), 2.28 (2H, t, J = 7.3 Hz), 2.03 (2H, t, J = 7.1 Hz), 2.02 (2H, t, J = 7.3 Hz), 1.20−1.60 (30H, m). Anal. ($C_{29}H_{52}N_4O_5S$) C, H, N.

12-[6-(Biotinoylamino)hexanoylamino]dodecanoic Acid (5). To a stirred solution of **4** (260 mg, 0.46 mmol) in THF and methanol (1:1, 16 mL) was added a water solution of sodium hydroxide (500 mg, 4 mL). After the mixture was stirred at 40 °C for 2 h, the mixture was acidified with 2 M hydrochloric acid to pH 3. The resulting precipitates was collected by filtration and dried in vacuo to give **5**: 230 mg (91%); $[\alpha]^{23}_D$ +6.8° (*c* 0.1, pyridine); FDMS *m/z* 555 $(M + 1)^+$; ^{1}H NMR (500 MHz, DMSO-d_6) δ 7.6−7.74 (2H, m), 6.40 (1H, brs), 6.34 (1H, brs), 4.28−4.32 (1H, m), 4.10−4.15 (1H, m), 3.06−3.12 (1H, m), 2.95−3.03 (4H, m), 2.79−2.85 (2H, m), 2.18 (2H, t, J = 7.3 Hz), 2.03 (2H, t, J = 7.1 Hz), 2.02 (2H, t, J = 7.3 Hz), 1.20−1.52 (30H, m). Anal. ($C_{28}H_{50}N_4O_5S$) C, H, N.

(2*S*,3*S*,4*R*)-1-*O*-(α-D-Glactopyranosyl)-2-[*N*-12-(6-(biotinoylamino)hexanoylamino)dodecanoylamino]-1,3,4-octadecanetriol (8a, AGL-592). To a solution of AGL-525 (**6a**) (100 mg, 0.12 mmol) in *n*-butanol (15 mL) was added 10% *n*-tetrabutylammonium hydroxide (5 mL), and the mixture was stirred under reflux for 72 h. The mixture was concentrated. The residue was dissolved in methanol (5 mL), the resulting precipitate (tetracosanoic acid main) was removed by filtration. The filtrate was concentrated and purified by chromatography on a silica gel column (10 g) using chloroform−methanol−10% NH_3 (100:100:1) to give amine **7a** [FDMS *m/z* 480 $(M + 1)^+$]. This amine **7a** was used for the next step without further purification. To a stirred solution of amine **7a** in DMF (5 mL) were added **5** (66 mg, 0.12 mmol) and EDC (46 mg, 0.24 mmol), and the mixture was stirred at 70 °C for 5 h. The mixture was concentrated and purified by chromatography on a silica gel column (20 g) using chloroform−methanol (4:1) as the eluent to give AGL-592 (**8a**): 10.0 mg (8.4%); $[\alpha]^{23}_D$ +103.6° (*c* 0.1, pyridine); mp 145−146 °C; FDMS *m/z* 1038 $(M + Na)^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ 10.71 (1H, d, J = 15.9 Hz), 8.51 (1H, d, J = 9.3 Hz), 8.47 (1H, d, J = 8.3 Hz), 8.34 (1H, t, J = 5.5 Hz), 8.27 (1H, t, J = 5.0 Hz), 5.57 (1H, d, J = 3.2 Hz), 5.26 (1H, m), 4.62−4.70 (3H, m), 4.56 (1H, m), 4.51 (1H, m), 4.36−4.48 (5H, m), 4.32 (2H, brs), 3.40−3.50 (4H, m), 2.32−2.48 (6H, m), 1.55−2.00(12H, m), 1.20−1.50 (40H, m), 0.87 (3H, t, J = 5.1 Hz). Anal. ($C_{52}H_{97}N_5O_{12}S$) C, H, N.

Using the same method, AGL-593 (**8b**), AGL-594 (**8c**), and AGL-596 (**8d**) were prepared from AGL-514 (**6b**), AGL-535 (**6c**), and AGL-583 (**6d**), respectively.

(2*S*,3*R*)-1-*O*-(α-D-Glactopyranosyl)-2-[*N*-12-(6-(biotinoylamino)hexanoylamino)dodecanoylamino]-1,3-octadecanediol (8b, AGL-593): 12 mg from AGL-514 (**6b**) (10%); $[\alpha]^{23}_D$ +26.0° (*c* 0.1, pyridine); mp 142−143 °C; FDMS *m/z* 1022 $(M + Na)^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ 8.52 (1H, d, J = 8.5 Hz), 7.89−7.92 (2H, m), 7.54 (1H, d, J = 3.4 Hz), 7.52 (1H, d, J = 3.2 Hz), 5.45 (1H, d, J = 3.7 Hz), 4.72 (1H, m), 4.64 (1H, dd, J = 3.6 Hz, 10.0 Hz), 4.40−4.60 (8H, m), 4.37 (1H, dd, J = 5.6 Hz, 10.7 Hz), 4.27 (1H, m), 3.45 (4H, m), 3.32 (1H, m), 2.84−2.95 (2H, m), 2.48 (2H, t, J = 6.8 Hz), 2.37 (2H, t, J = 7.6 Hz), 2.34 (2H, t, J = 7.1 Hz), 1.55−1.90 (12H, m), 1.20−1.55 (42H, m), 0.91 (3H, t, J = 5.1 Hz). Anal. ($C_{52}H_{97}N_5O_{11}S$) C, H, N.

1-*O*-(α-D-Glactopyranosyl)-2-[*N*-12-(6-(biotinoylamino)-hexanoylamino)dodecanoylamino]octadecanol (8c, AGL-594): 10 mg from AGL-535 (**6c**) (9%); $[\alpha]^{23}_D$ +25.2° (*c* 0.1, pyridine); mp 140−141 °C; FDMS *m/z* 1006 $(M + Na)^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ 8.28−8.30 (1H, m), 7.88−7.91 (2H, m), 7.53−7.55 (2H, m), 5.38−5.40 [1H (5.39, d, J = 3.7 Hz) and (5.37, d, J = 3.7 Hz)], 4.35−4.70 (9H, m), 3.40−3.50 (5H, m), 2.84−2.95 (2H, m), 1.24−2.00 (56H, m), 0.80−085 (3H, m). Anal. ($C_{52}H_{97}N_5O_{10}S$) C, H, N.

(2*S*,3*S*,4*R*)-1-*O*-(β-D-Glactopyranosyl)-2-[*N*-12-(6-(biotinoylamino)hexanoylamino)dodecanoylamino]-1,3,4-octadecanetriol (8d, AGL-596): 10 mg from AGL-583 **(6d)** (9%); $[\alpha]^{23}_D$ −4.8° (*c* 0.1, pyridine); mp 138−139 °C; FDMS *m/z* 1038 $(M + Na)^+$; ^{1}H NMR (500 MHz, C_5D_5N) δ 10.71 (1H, d, J = 15.1 Hz), 8.48 (1H, d, J = 9.3 Hz), 8.44 (1H, d, J = 9.0 Hz), 8.35 (1H, m), 8.28 (1H, m), 5.15 (1H, m), 4.91 (1H, d, J = 7.8 Hz), 4.79−4.84 (1H, m), 4.32−4.57 (8H, m), 4.21 (1H, m), 4.13 (1H, m), 4.04 (1H, m), 3.40−3.50 (4H, m), 2.30−2.45 (6H, m), 1.50−2.00 (12H, m), 1.20−1.50 (40H, m), 0.87 (3H, t, J = 4.6 Hz). Anal. ($C_{52}H_{97}N_5O_{12}S$) C, H, N.

Biological Methods. Animals: Female C57BL/6 (B6) and BDF_1 mice purchased from Nippon SLC (Shizuoka, Japan) were used in this study. Mice were maintained under our standard laboratory conditions.[11]

KRN7000: The KRN7000, (2*S*,3*S*,4*R*)-1-*O*-(α-D-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol, used for the present study was synthesized in our laboratory[1] and was used as a positive control.

Spleen cell proliferation assay: The spleens from C57BL/6 mice were dissociated in RPMI 1640, and the red cells were lysed with Tris NH_4Cl. The cells were washed three times with PBS (Nissui Pharmaceutical, Tokyo, Japan). The spleen cells (2.5×10^5 cells/well) and graded doses (1, 10, and 100 ng/mL) of samples or control vehicle (0.001, 0.01, and 0.1% DMSO) were plated into 96-well plates (Nunc, Napervilla, IL), and the plates were then cultured at 37 °C in 95% air, 5% CO_2 for 18 h. Tritium-thymidine ([^{3}H]TdR, 0.5 μCi/well; Du Pont/NEN Research Products, Boston, MA) was added into each well, and 8 h later, [^{3}H]TdR uptake into cells was measured by a liquid scintillation counter.

Surface plasmon resonance: A BIAcore X biosensor system (Biacore, Uppsala, Sweden) was used for the binding experiments. Biotinylated GalCer compounds were diluted to 50 mg/mL into Biacore flow buffer, HEPES-buffered saline (HBSt; 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% (v/v) surfactant P20), and captured onto a flow cell 2 (Fc2) of a streptavidin sensor chip (BIAcore) at a flow rate of 5 mL/min, which yielded 200−400 response units (RU) of immobilized ligand. Flow cell 1 (Fc1) of the sensor chip did not have any ligand immobilized and was used as a negative control surface. Binding data (in RU) is represented as signal from Fc2 minus nonspecific signal in Fc1. Presence of immobilized biotinylated α-GalCer was confirmed by adding lectins RCA I, GSL I-B4, and SJA (Vector Laboratories, Burlingame, CA). Binding studies were performed at 20 mL/min flow rate by injecting mouse CD1, human CD1d, or M3 soluble recombinant proteins diluted in HBSt. Soluble M3 protein was a gift from the laboratory of Dr. J. Deisenhofer (University of Texas Southwestern Medical Center, Dallas, TX). All soluble class I molecules were made in insect tissue culture cells, as described previously.[25] Sensor chip surfaces were regenerated after binding with HBSt containing 0.1% Triton (Sigma, St. Louis, MO).

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