Immunomodulatory α-Galactoglycosphingolipids: Synthesis of a 2′-*O*-Methyl-α-Gal-GSL and Evaluation of Its Immunostimulating Capacity

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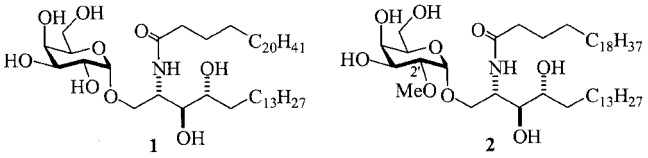
The total synthesis of 1-2-docosanoylamino-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-1,3,4-octadecanetriol (**2**), a 2′-methoxy analog of the immunostimulating α-galactoglycosphingolipid **1**, is reported. Stereoselective α-glycosylation of the azido precursor of sphingosine was successfully performed for the first time using an improved Mukaiyama reaction. When assayed in a 72 h splenocyte proliferation test, compound **2** was significantly less stimulatory than the non-methylated compound **1**, suggesting that the galactose 2-OH group is essential for the immunostimulatory activity of α-Gal-GSLs.

α-Galactoglycosphingolipids (α-Gal-GSLs) are glycosphingolipids, found in *Agelas* and *Axinella* sponges,[1−4] having an α-galactose as the first sugar attached to the ceramide headgroup. The simplest natural α-Gal-GSL, named agelasphin, shows a considerable immunostimulating activity, being the target of the T-cells response.[5] A synthetic analog, KRN7000 (**1**; Scheme 1), is now in clinical trials as an antitumor agent.[6] In the last few years, it has been demonstrated that the cell-mediated immune recognition of α-Gal-GSLs involves lipid-binding molecules known as CD1, a family of HLA-like, β2-microglobulin-associated transmembrane proteins.[7]



Scheme 1. KRN7000 (**1**) and its 2′-methoxy analog **2**

As part of our ongoing search for immunomodulating compounds, we discovered a number of more complex α-Gal-GSLs, which were tested on murine T-cell populations.[4] The obtained data suggested that the immunostimulating activity is reduced by glycosylation of the galactose 2-OH group, although some subsequent results obtained by other researchers seemed to be in contrast to these findings. This inconsistency was solved by a recent study,[8] demonstrating that α-Gal-GSLs glycosylated at the 2′- or 3′-positions are not active themselves, but are processed by the antigen presenting cells (APCs) with removal of the sugars linked to the galactose. A lysosomal enzyme, α-galactosidase A, was found to be responsible for the generation of the antigenic epitope recognized by T lymphocytes.

The lack of activity of glycosylated α-Gal-GSLs could be ascribed to either the steric hindrance of the additional sugar preventing binding to the T-cell receptor, or direct participation of the galactose 2-OH group in the binding to the receptor. In an attempt to clarify this point, we undertook a synthetic program directed to the preparation of a number of analogs modified at the key 2′-position in a way that cannot be affected by APC processing.

In this paper, we report the stereoselective total synthesis of the 2′-methoxy analog of the α-Gal-GSL, 2′-*O*-methyl-α-D-galactopyranosylceramide (**2**; Scheme 1), and a preliminary evaluation of its biological activity using lymphocyte proliferation tests.

Results and Discussion

The synthesis of glycosphingolipids involves the assembly of three fragments: a saccharide chain, a sphingoid base, and a fatty acid. A common strategy begins with the preparation of the protected saccharide moiety, followed by attachment of a sphingosine synthetic precursor, and *N*-acylation with the fatty acid to give the glycosyl ceramide. It is well established that the *O*-glycosylation reaction can be

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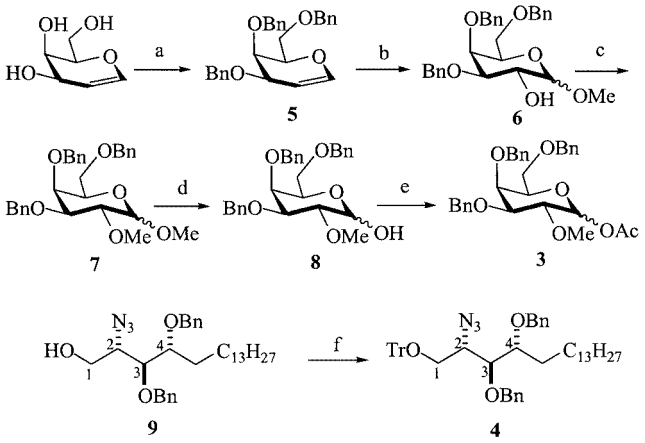
more suitably performed using the azido precursor of the sphingosine, rather than the sphingosine itself or the ceramide as glycosyl acceptor, because the former is a better nucleophile.[9]

In the synthesis of a GSL with an α-glycosidic bond, the need for a 1,2-*cis* glycosylation reaction with complete stereoselectivity is the most important aspect. While formation of 1,2-*trans* glycosides (β for glucose and galactose, α for mannose) can be easily achieved taking advantage of the participation of a neighboring group, such as *O*-acetyl or *O*-benzoyl, at C-2, no successful general method for 1,2-*cis* glycosylation has yet emerged. Each particular case thus requires a careful selection of the available techniques. Among them, the glycosylation reaction first proposed by Mukaiyama[10] in 1981 is worthy of note, as it produces α-glycosides with high selectivity but only modest yields. This reaction has previously been used in the synthesis of glycosphingolipids, giving the desired α-glycosyl ceramide with 36% yield.[11]

The original Mukayama glycosidation reaction involves a glycosyl fluoride as the glycosyl donor, and $AgClO_4$ and $SnCl_2$ as a Lewis acid catalytic system. An ion pair is postulated[12] to be produced by the cationic anomeric center and the perchlorate ion, with the perchlorate ion placed on the less-hindered β face of the sugar so as to cause nucleophilic attack at the α face. Over the years, several improved versions of the original Mukayama glycosidation reaction have appeared in an attempt to improve the yield of the reaction. One interesting modification has been proposed by Houdier and Vottéro,[13] involving the use of a glycosyl acetate as glycosyl donor, while the alcohol is activated with a trityl group; the catalytic system is similar, but $SnCl_4$ is used instead of $SnCl_2$.

Even though this method has never been used for glycosphingolipid preparation, we selected it for the crucial glycosylation step (Scheme 2) because the reported yields appeared very interesting. In addition, the stereochemical outcome of the reaction was not dependent on the anomeric configuration of the glycosyl acetate used as substrate, and this allowed an easier preparation of the glycosyl donor **3**.

The glycosyl donor 3,4,6-tri-*O*-benzyl-2-*O*-methylgalactopyranosyl acetate (**3**) was prepared as follows (Scheme 3). D-Galactal was first converted into 3,4,6-tri-*O*-benzylgalactal (**5**) and, after purification, converted into the 1,2-*trans*-methylglycoside **6** through a one-pot reaction involving stereoselective α-epoxidation, followed by in situ opening of the oxirane ring. The epoxidation was performed with Camp's reagent, i.e. *m*-chloroperoxybenzoic acid (MCPBA, 3.6 equiv.) and KF (7.2 equiv.)[14] in dichloromethane under anhydrous conditions. The reagent was added to a dichloromethane solution containing the galactal **5** and an excess of methanol. After SiO_2 chromatography, the desired methyl galactoside **6** (deriving from the α-oriented oxirane ring) was obtained in 56% yield as a mixture of anomers (β:α = 5:1), along with minor amounts of the α-*talo* stereoisomer from the β-oriented oxirane ring.

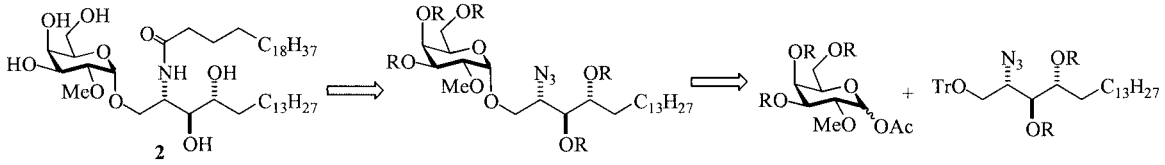


Scheme 3. Syntheses of 3,4,6-tri-*O*-benzyl-2-*O*-methyl-D-galactosyl acetate (**3**) and (2*S*,3*S*,4*R*)-2-azido-3,4-di-*O*-benzyl-1-*O*-trityl-1,3,4-octadecanetriol (**4**); reagents: (a) NaH, DMF, 0 °C, then BnBr (90%); (b) BzOOH/KF (1:2), CH_2Cl_2/MeOH, room temp. (56%); (c) NaH, THF, 0 °C, then MeI (82%); (d) 1 M aqueous TfOH/AcOH, 80 °C (82%); (e) Ac_2O, pyridine (quantitative); (f) TrCl, DMAP, pyridine, 60 °C (85%)

Conversion of **6** into the corresponding 2-*O*-methyl derivative **7** (82%) was accomplished by treatment with sodium hydride and methyl iodide under the usual conditions. Cleavage of the glycosidic bond to afford **8** (82% yield, mixture of anomers) was promoted by treatment with trifluoromethanesulfonic acid (1 M solution in water) and glacial acetic acid for two hours at 80 °C. Finally, the free 2-methoxysugar **8** was acetylated with acetic anhydride in pyridine to give the desired 3,4,6-tri-*O*-benzy-2-*O*-methylgalactopyranosyl acetate **3** as a mixture of anomers (α:β = 1:1) in quantitative yield.

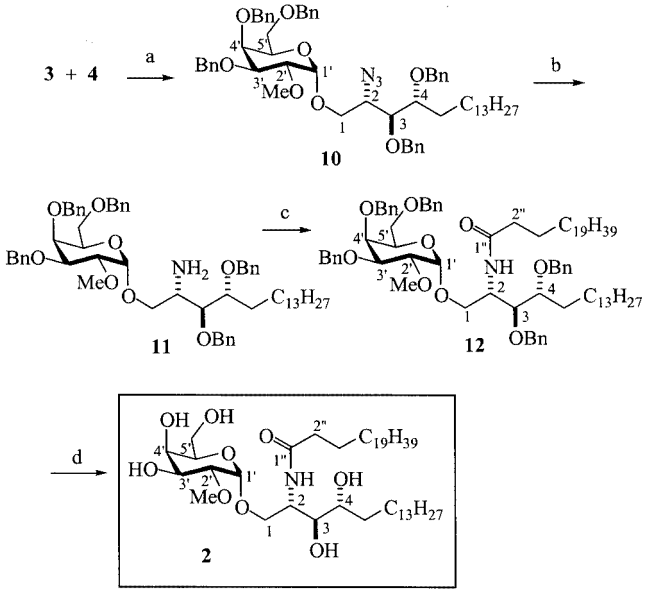
The glycosyl acceptor 2-azido-3,4-*O*-benzyl-1-*O*-trityl-1,3,4-octadecanetriol (**4**) was synthesized from the corresponding non-tritylated azide **9**, prepared as described previously.[15] Compound **9** was dissolved in pyridine and treated with a 10-fold molar excess of trityl bromide in the presence of *p*-(dimethylamino)pyridine at 60 °C giving, after chromatography, compound **4** in 85% yield.

Having synthesized the two building blocks **3** and **4**, we performed the coupling reaction by dissolving the glycosyl



Scheme 2. Retrosynthetic analysis of (2*S*,3*S*,4*R*)-2-docosanoylamino-1-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-1,3,4-octadecanetriol (**2**)

acceptor **4** (130 mg, 0.17 mmol) and the glycosyl donor **3** (170 mg, 2.0 equiv.) in Et_2O and adding the obtained solution to the catalytic system, previously prepared by suspending $AgClO_4$ and $SnCl_4$ in dry Et_2O (Scheme 4). After workup, isolation of the reaction product was performed by preparative HPLC rather than column chromatography because all the reaction products showed similar retention times. The α-glycoside **10** was obtained in a satisfactory 69% yield, whereas only trace amounts of the β-isomer were obtained. This is a significant improvement on the yields reported so far for α-glycosylation reactions as applied to the synthesis of glycosphingolipids.



Scheme 4. Synthesis of compound **2**; reagents: (a) $AgClO_4$, $SnCl_4$, Et_2O (69%); (b) Ph_3SnH, AIBN, benzene, reflux (81%); (c) $C_{21}H_{43}COCl$, pyridine/CH_2Cl_2 (84%); (d) H_2, $Pd(OH)_2/C$, EtOH/AcOH, 40 °C (70%)

Completion of the synthesis[15] required reduction of the azide group with triphenyltin hydride, amidation with docosanoyl chloride, and removal of the benzyl protecting groups by hydrogenolysis over Pd/C, to afford the target compound **2** in 47% yield over the three steps, and 28% overall yield based on the azidosphingosine **9** used as starting material.

Preliminary evaluation of the biological activity of compound **2** compared to the non-methylated α-Gal-GSL KRN7000 (**1**) was performed using a splenocyte proliferation test. As expected, compound **1** stimulated spleen-cell proliferation from C57Bl/6 mice in a dose- and time-dependent manner (Figure 1). The activity peaked after 72 h of incubation at 37 °C. The proliferative response was significantly reduced when the 2′-methoxy analog **2** was used. These results indicate that a free galactose 2-OH group is essential for the activity of α-Gal-GSLs, and suggest that this hydroxyl group is probably directly involved in the binding to the TCR receptor. A more extensive examination of the biological activity of compound **2** and other modified α-Gal-GSLs is in progress and will be reported elsewhere.

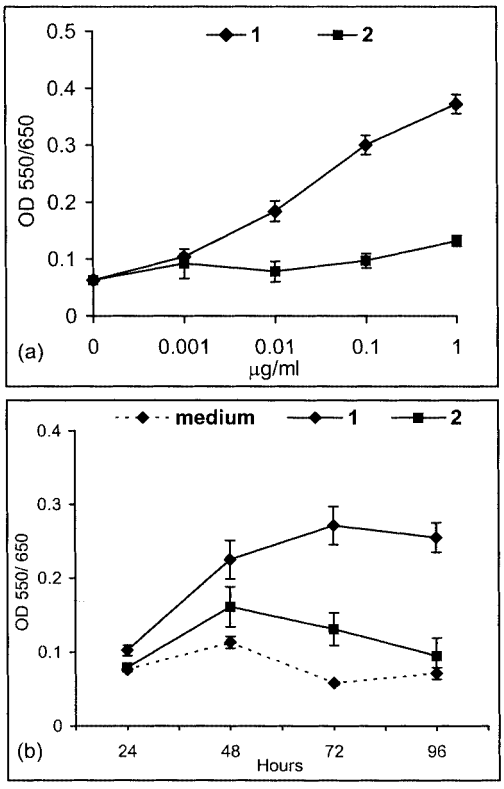


Figure 1. (a) Dose-dependent proliferation of C57Bl/6 spleen cells to the non-methylated α-Gal-GSL KRN7000 (**1**) or its 2′-methoxy analog (**2**) after 72 hours of culture; the results are representative of three different experiments conducted in quadruplicate; data are expressed as mean ± SD; (b) kinetics of the proliferative response of C57Bl/6 spleen cells to KRN7000 (**1**, 0.01 μg/mL) or its 2′-methoxy analog (**2**, 0.01 μg/mL); data are expressed as mean ± SD

Experimental Section

General Remarks: High resolution ESI-MS spectra were performed on a Micromass QTOF Micro mass spectrometer, dissolving the sample in MeCN/H_2O (1:1) with 0.1% TFA. ESI MS/MS experiments were performed on a Finnigan LCQ ion-trap mass spectrometer. The spectra were recorded by infusion into the ESI source using MeOH/$CHCl_3$ (4:1) as the solvent. Optical rotations were measured at 589 nm on a Perkin−Elmer 192 polarimeter using a 10-cm microcell. ^1H and ^{13}C NMR spectra were determined on a Bruker AMX-500 spectrometer at 500.13 and 125.77 MHz, respectively; chemical shifts are referenced to the residual solvent signal ($CDCl_3$: δ_H = 7.26 ppm; δ_C = 77.0 ppm; [D_5]pyridine: δ_H = 8.71, 7.56, and 7.19 ppm; δ_C = 149.8, 135.3, and 123.4 ppm). The spectra of new compounds were assigned with the aid of COSY and HMQC two-dimensional NMR experiments. The reverse multiple-quantum heteronuclear correlation (HMQC) spectra were recorded by using a pulse sequence with a BIRD pulse 0.5 s before each scan to suppress the signal originating from protons not directly bound to ^{13}C; the interpulse delays were adjusted for an average $^1J_{C,H}$ of 142 Hz.

3,4,6-Tri-*O*-benzyl-D-galactal (5): D-Galactal (250 mg, 1.71 mmol) was dissolved in dry DMF (10 mL), cooled to 0 °C, and NaH (170 mg, 6.7 mmol) was slowly added. After a few minutes, benzyl

bromide (0.70 mL, 5.9 mmol) was added dropwise. After stirring for 12 h at room temperature, water (100 mL) was added and the reaction mixture was extracted three times with EtOAc (150 mL). The combined organic layers were dried with Na_2SO_4 and concentrated under reduced pressure. The residue was purified by chromatography on silica gel to give 640 mg of **5** (1.54 mmol, 90%) as a colorless oil, identified by comparison of its ^1H and ^{13}C NMR spectra with those reported previously.[16]

Methyl 3,4,6-Tri-*O*-benzyl-D-galactoside (6): Anhydrous KF (450 mg, 7.7 mmol) was added to a DCM solution (25 mL) of freshly recrystallized *m*-chloroperoxybenzoic acid (640 mg, 3.7 mmol), previously dried over Na_2SO_4 and $CaSO_4$ (Sikkon, Fluka) for 20 min, and the suspension was stirred at room temperature for 30 min. After this time, a solution of compound **5** (640 mg, 1.54 mmol) in dichloromethane (DCM; 5 mL) and MeOH (5 mL) was added, and the reaction was allowed to proceed for 12 h at room temperature. Water (200 mL) was added to the reaction mixture, which was extracted with DCM (3 × 300 mL). The combined organic extracts were dried over Na_2SO_4, concentrated under reduced pressure, and purified by column chromatography on SiO_2 (*n*-hexane/EtOAc, 8:2) to give 400 mg (0.86 mmol, 56%) of **6** (mixture of anomers, α:β = 1:5) as a colorless oil. $[\alpha]_D = +2.9$ ($CHCl_3$, $c = 0.6$). ESIMS (positive ions): $m/z = 487$ $[M + Na]^+$. ^1H NMR (500 MHz, $CDCl_3$, β anomer): δ = 3.44 (dd, $J = 9.8$, 2.7 Hz, 1 H, 3-H), 3.54 (s, 3 H, OMe), 3.65−3.67 (overlapping signals, 3 H, 5-H and 6-H_2), 3.97−3.91 (overlapping signals, 2 H, 2-H and 4-H), 4.19 (d, $J = 7.6$ Hz, 1 H, 1-H), 4.48 and 4.44 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.73 and 4.64 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.88 and 4.61 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 7.38−7.23 (15 H, aromatic protons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, β anomer): δ = 56.9 (CH_3, OMe), 68.7 (CH_2, C-6), 71.2 (CH, C-2), 72.3 (CH_2, benzyl methylene group), 72.7 (CH, C-4), 73.6 (CH_2, benzyl methylene group), 73.7 (CH, C-5), 74.6 (CH_2, benzyl methylene group), 82.0 (CH, C-3), 104.0 (CH, C-1), 128.5−127.7 (CH, aromatic carbons) ppm.

Methyl 3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactoside (7): Compound **6** (400 mg, 0.86 mmol) was dissolved in dry THF (15 mL), cooled to 0 °C, and NaH (100 mg, 4.2 mmol) was added. After a few minutes methyl iodide (0.25 mL, 4.0 mmol) was added dropwise. After stirring for 12 h at room temperature, water (100 mL) was added and the reaction mixture was extracted three times with EtOAc (150 mL). The combined organic layers were dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by column chromatography on SiO_2 (*n*-hexane/EtOAc, 9:1) to give 340 mg (0.71 mmol, 82%) of **7** (mixture of anomers, α:β = 1:5) as a colorless oil. $[\alpha]_D = +6.5$ ($CHCl_3$, $c = 0.6$). ESIMS (positive ions): $m/z = 501$ $[M + Na]^+$. ^1H NMR (500 MHz, $CDCl_3$, β anomer): δ = 3.44 (dd, $J = 9.7$, 2.7 Hz, 1 H, 3-H), 3.53 (s, 3 H, OMe), 3.53−3.50 (overlapping signals, 2 H, 2-H and 5-H), 3.59 (m, 2 H, 6-H_2), 3.64 (s, 3 H, OMe), 3.87 (br. d, $J = 2.1$ Hz, 1 H, 4-H), 4.18 (d, $J = 7.6$ Hz, 1 H, 1-H), 4.45 and 4.42 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.76 and 4.72 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.94 and 4.62 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 7.41−7.25 (15 H, aromatic protons) ppm. ^1H NMR (500 MHz, $CDCl_3$, α anomer): δ = 3.42 (s, 3 H, OMe), 3.55 (m, 2 H, 6-H_2), 3.56 (s, 3 H, OMe), 3.84 (dd, $J = 9.7$, 3.1 Hz, 1 H, 2-H), 3.87 (dd, $J = 10.0$, 2.4 Hz, 1 H, 3-H), 3.90 (m, 1 H, 5-H), 3.93 (br. s, 1 H, 4-H), 4.50 and 4.42 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.82 and 4.71 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.91 (d, $J = 3.1$ Hz, 1 H, 1-H), 4.96 and 4.58 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 7.41−7.25 (15 H, aromatic protons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, β anomer): δ = 56.9 (CH_3, OMe), 60.9 (CH_3, OMe), 68.7 (CH_2, C-6), 72.8 (CH_2, benzyl methylene group), 73.2 (CH, C-5), 73.4 (CH, C-4), 73.5 (CH_2, benzyl methylene group), 74.3 (CH_2, benzyl methylene group), 81.5 (CH, C-3), 104.5 (CH, C-1), 128.7−127.7 (CH, aromatic carbons) ppm.

3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactose (8): Compound **7** (340 mg, 0.71 mmol) was dissolved in glacial AcOH (2.5 mL), heated to 80 °C, and an aqueous 1 M solution of CF_3SO_3H (0.5 mL) was added. The reaction mixture was stirred at 80 °C for 2 h. After cooling, a saturated aqueous $NaHCO_3$ solution was added until effervescence disappeared, and the reaction mixture was partitioned between water (30 mL) and DCM (50 mL). The organic layer was washed with water (2 × 30 mL), and the combined aqueous layers were in turn washed with EtOAc (100 mL). All the organic layers were combined, dried over Na_2SO_4, and concentrated under vacuum, Column chromatography of the residue (*n*-hexane/EtOAc, 7:3) yielded 270 mg (0.58 mmol, 82%) of **8** (mixture of anomers, α:β = 3:1) as a colorless oil. $[\alpha]_D = +20$ ($CHCl_3$, $c = 0.2$). ESIMS (positive ions): $m/z = 508$ $[M - H+2Na]^+$, 487 $[M + Na]^+$. ^1H NMR (500 MHz, $CDCl_3$, α anomer): δ = 3.43 (dd, $J = 9.4$, 5.9 Hz, 1 H, 6-Hb), 3.55 (m, 1 H, 6-Ha), 3.57 (s, 3 H, OMe), 3.82 (dd, $J = 9.6$, 3.6 Hz, 1 H, 2-H), 3.88 (m, 1 H, 3-H), 3.90 (br. s, 1 H, 4-H), 4.17 (t, $J = 6.3$ Hz, 1 H, 5-H), 4.50 and 4.41 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.81 and 4.73 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.95 and 4.59 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 5.42 (d, $J = 3.1$ Hz, 1 H, 1-H), 7.43−7.26 (15 H, aromatic protons) ppm.

3,4,6-Tri-*O*-benzyl-2-*O*-methyl-D-galactosyl Acetate (3): Compound **8** (270 mg, 0.58 mmol) was dissolved in pyridine (2.0 mL) and Ac_2O (0.2 mL) was added. After 12 h, the reaction was quenched with MeOH, and after a further 30 min the mixture was dried under reduced pressure, to give 295 mg (0.58 mmol, quantitative) of **3** (mixture of anomers, α:β = 1:1) as a colorless oil. $[\alpha]_D = +22$ ($CHCl_3$, $c = 1.4$). ESIMS (positive ions): $m/z = 529$ ([M + Na]$^+$. ^1H NMR ($CDCl_3$, α anomer): δ = 2.12 (s, 3 H, Ac), 3.58 (s, 3 H, OMe), 3.63−3.50 (overlapping signals, 3 H, 3-H and 6-H_2), 3.69−3.65 (overlapping signals, 2 H, 2-H and 5-H), 3.94 (br. d, $J = 2.7$ Hz, 1 H, 4-H), 4.43 and 4.39 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.74 and 4.72 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.93 and 4.61 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 5.48 (d, $J = 8.1$ Hz, 1 H, 1-H), 7.41−7.24 (m, 15 H, aromatic protons) ppm. ^1H NMR ($CDCl_3$, β anomer): δ = 2.12 (s, 3 H, Ac), 3.50 (s, 3 H, OMe), 3.55 (m, 2 H, 6-H_2), 3.81 (dd, $J = 10.1$, 2.7 Hz, 1 H, 3-H), 3.92 (dd, $J = 10.1$, 3.7 Hz, 1 H, 2-H), 4.00 (m, 1 H, 5-H), 4.02 (br. d, $J = 2.7$ Hz, 1 H, 4-H), 4.46 and 4.40 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.82 and 4.72 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 4.95 and 4.58 (AB system, $J = 11.7$ Hz, 1 H each, geminal benzyl protons), 6.39 (d, $J = 3.7$ Hz, 1 H, 1-H), 7.41−7.24 (m, 15 H, aromatic protons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, α anomer): δ = 21.2 (CH_3, Ac), 60.9 (CH_3, OMe), 67.9 (CH_2, C-6), 72.8 (CH_2, benzyl methylene group), 73.1 (CH, C-4), 73.4 (CH_2, benzyl methylene group), 74.0 (CH, C-5), 74.6 (CH_2, benzyl methylene group), 79.8 (CH, C-2), 82.1 (CH, C-3), 94.3 (CH, C-1), 128.4−127.2 (CH, aromatic carbons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$, β anomer): δ = 21.2 (CH_3, Ac), 59.3 (CH_3, OMe), 68.3 (CH_2, C-6), 71.8 (CH, C-5), 73.0 (CH_2, benzyl methylene group), 73.5 (CH_2, benzyl methylene group), 74.5 (CH, C-4), 74.9 (CH_2, benzyl methylene group), 77.5

(CH, C-2), 78.5 (CH, C-3), 90.3 (CH, C-1), 128.4−127.2 (CH, aromatic carbons) ppm.

(2*S*,3*S*,4*R*)-2-Azido-3,4-di-*O*-benzyl-1-*O*-trityl-1,3,4-octadecanetriol (4): Compound **9** (104 mg, 0.20 mmol), prepared as described previously,[15] was dissolved in 3.0 mL of dry pyridine. 4-Dimethylaminopyridine (DMAP, 2.0 mg, 0.016 mmol) and triphenylbromomethane (trityl bromide, 650 mg, 2.0 mmol) were added, and the reaction mixture was kept at 60 °C for 12 h. The reaction was quenched with water (30 mL), and after 15 min the reaction mixture was extracted with DCM (3 × 50 mL). The organic layers were dried over Na_2SO_4, concentrated under vacuum and chromatographed on SiO_2 (*n*-hexane/EtOAc, 99:1), to gave 130 mg (0.17 mmol, 85%) of **4** as a colorless oil. $[\alpha]_D = +12$ ($CHCl_3$, c = 1.1). ESIMS (positive ions): m/z = 788 $[M + Na]^+$, 760 $[M + Na - N_2]^+$, 243 $[trityl]^+$. ESI MS/MS (parent ion: m/z = 788): m/z = 760 $[M + Na - N_2]^+$; (parent ion: m/z = 760): m/z = 654 $[M + Na - N_2 - PhCHO]^+$, m/z = 654 $[M + Na - N_2 - TrH]^+$. ^1H NMR ($CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H, 18-H_3), 1.25 (alkyl chain CH_2 protons), 1.40 (m, 1 H, 6a-H), 1.50 (m, 1 H, 5b-H), 1.59 (m, 1 H, 5a-H), 3.36 (dd, J = 10.0, 8.4 Hz, 1 H, 1b-H), 3.49 (dd, J = 10.0, 2.8 Hz, 1 H, 1a-H), 3.56−3.51 (overlapping signals, 2 H, 3-H and 4-H), 3.76 (m, 1 H, 2-H), 4.41 (s, 2 H, benzyl protons), 4.57 and 4.47 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 7.46−7.06 (m, 25 H, aromatic protons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.0 (CH_3, C-18), 22.6 (CH_2, C-17), 25.4 (CH_2, C-6), 29.7−29.4 (CH_2, alkyl chain CH_2 groups), 30.0 (CH_2, C-5), 31.9 (CH_2, C-16), 63.2 (CH, C-2), 64.3 (CH_2, C-1), 72.0 (CH_2, benzylic CH_2 group), 73.5 (CH_2, benzyl methylene group), 79.1 (CH, C-3), 79.4 (CH, C-4), 128.7−126.9 (CH, aromatic carbons) ppm.

(2*S*,3*S*,4*R*)-2-Azido-3,4-di-*O*-benzyl-1-*O*-(2-*O*-methyl-3,4,6-tri-*O*-benzyl-α-D-galactopyranosyl)-1,3,4-octadecanetriol (10): $SnCl_4$ (50 μL from a 0.5 M toluene solution, 0.025 mmol) was added to a suspension of $AgClO_4$ (7.0 mg, 0.034 mmol) in anhydrous Et_2O (2 mL). The mixture was stirred for 4 h, and compound **3** (170 mg, 0.34 mmol) and compound **4** (130 mg, 0.17 mmol) were added simultaneously in an anhydrous Et_2O solution (2 mL). After 14 h, the reaction mixture was diluted with DCM (30 mL), washed with a saturated $NaHCO_3$ solution (20 mL), dried over Na_2SO_4, and taken to dryness. The residue was chromatographed by HPLC (*n*-hexane/EtOAc, 9:1) to gave 114 mg (0.12 mmol, 69%) of **10** as a colorless oil. $[\alpha]_D = +26$ ($CHCl_3$, c = 0.3). ESIMS (positive ions): m/z = 992 $[M + Na]^+$, 964 $[M + Na - N_2]^+$. ESI MS/MS (parent ion: m/z = 992): m/z = 964 $[M + Na - N_2]^+$; (parent ion: m/z = 964): m/z = 858 $[M + Na - N_2 - PhCHO]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H, H_3–18)), 1.25 (alkyl chain CH_2 protons), 1.40 (m, 1 H, 6a-H), 1.57 (m, 1 H, 5b-H), 1.67 (m, 1 H, 5a-H), 3.49 (m, 2 H, 6-H_2), 3.51 (s, 3 H, OMe), 3.60 (m, 1 H, 4-H), 3.76−3.69 (overlapping signals, 3 H, 1b-H, 2-H, and 3-H), 3.82 (dd, J = 9.6, 3.5 Hz, 1 H, 2′-H), 3.95−3.86 (overlapping signals, 3 H, 3′-H, 4′-H, and 5′-H), 4.03 (m, 1 H, 1a-H), 4.44 and 4.36 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.59 and 4.50 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.71 and 4.66 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.81 and 4.70 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.93 and 4.55 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 5.02 (d, J = 3.5 Hz, 1 H, 1′-H), 7.40−7.23 (25 H, aromatic protons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.0 (CH_3, C-18), 22.6 (CH_2, C-17), 25.4 (CH_2, C-6), 29.7−29.4 (CH_2, alkyl chain CH_2 groups), 30.0 (CH_2, C-5), 31.9 (CH_2, C-16), 58.9 (CH_3, OMe), 62.1 (CH, C-2), 68.6 (CH_2, C-1), 69.1 (CH_2, C-6′), 69.8 (CH, C-5′), 72.1 (CH_2, benzylic CH_2 group), 73.0 (CH_2, benzyl methylene group), 73.4 (CH_2, benzyl methylene group), 73.7 (CH_2, benzyl methylene group), 74.7 (CH_2, benzyl methylene group), 75.2 (CH, C-4′), 78.5 (CH, C-2′), 78.7 (CH, C-3′), 79.2 (CH, C-3), 79.5 (CH, C-4), 98.2 (CH, C-1′), 128.5−127.7 (CH, aromatic carbons), 138.8−138.1 (C, aromatic carbons) ppm.

(2*S*,3*S*,4*R*)-2-Amino-3,4-di-*O*-benzyl-1-*O*-(2-*O*-methyl-3,4,6-tri-*O*-benzyl-α-D-galactopyranosyl)-1,3,4-octadecanetriol (11): Ph_3SnH (300 μL, 412 mg, 1.2 mmol) and a small amount of AIBN were added to a solution of the azide **10** (114 mg, 0.12 mmol) in dry benzene (10 mL), and the resulting solution was allowed to react at room temperature for 24 h and subsequently for 1 h under reflux. The solution was cooled to room temperature and concentrated under reduced pressure. Column chromatography on SiO_2 (*n*-hexane/EtOAc, 6:4, with 0.1% pyridine) gave 90 mg (0.095 mmol, 81%) of **11** as a colorless oil. $[\alpha]_D = +20$ ($CHCl_3$, c = 0.2). HRESIMS (positive ions): m/z = 944.6071 ($[M + H]^+$, $C_{60}H_{82}NO_8$ gives 944.6040). ESIMS (positive ions): m/z = 966 $[M + Na]^+$, 944 $[M + H]^+$. ESI MS/MS (parent ion: m/z = 966): m/z = 874 $[M + Na - PhCH_3]^+$, 858 $[M + Na - PhCHOH]^+$, 487 $[sugar + Na]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 3 H, H_3–18), 1.25 (alkyl chain CH_2 protons), 1.45 (m, 1 H, 6a-H), 1.59 (m, 1 H, 5b-H), 1.69 (m, 1 H, 5a-H), 3.31 (m, 1 H, 2-H), 3.52 (s, 3 H, OMe), 3.55−3.45 (overlapping signals, 3 H, 1b-H, 6-H_2), 3.67 (m, 1 H, 3-H), 3.71 (m, 1 H, 4-H), 3.83 (dd, J = 10.1, 3.4 Hz, 1 H, 2′-H), 3.89 (dd, J = 10.1, 2.5 Hz, 1 H, 3′-H), 3.97−3.92 (overlapping signals, 2 H, 4′-H and 5′-H), 4.08 (dd, J = 9.9, 2.5 Hz, 1 H, 1a-H), 4.46 and 4.38 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.64 and 4.53 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.73 and 4.63 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.79 and 4.72 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.94 and 4.57 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 5.01 (d, J = 3.4 Hz, 1 H, 1′-H), 7.42−7.25 (25 H, aromatic protons) ppm.

(2*S*,3*S*,4*R*)-2-Docosanoylamino-3,4-di-*O*-benzyl-1-*O*-(2-*O*-methyl-3,4,6-tri-*O*-benzyl-α-D-galactopyranosyl)-1,3,4-octadecanetriol (12): Docosanoic acid (100 mg, 0.29 mmol) dissolved in $SOCl_2$ (0.63 mL, 1.0 g, 8.6 mmol) was refluxed for 90 min, and the excess of $SOCl_2$ removed under reduced pressure. A solution of the amine **11** (90 mg, 0.095 mmol) in dry pyridine (3 mL) and dry CH_2Cl_2 (3 mL) was added to the obtained docosanoyl chloride. After 12 h, the solvents were removed under vacuum, and the residue was partitioned between CH_2Cl_2 (100 mL) and a saturated $NaHCO_3$ aqueous solution (50 mL). The organic phase, dried over Na_2SO_4 and concentrated under reduced pressure, was subjected to column chromatography on SiO_2 (*n*-hexane/EtOAc, 8:2), giving 101 mg (0.080 mmol, 84%) of **12** as a colorless oil. $[\alpha]_D = +23$ ($CHCl_3$, c = 0.3). ESIMS (positive ions): m/z = 1288 $[M + Na]^+$. ESI MS/MS (parent ion: m/z = 1288): m/z = 824 $[M + Na - sugar]^+$, 487 $[sugar + Na]^+$. ^1H NMR (500 MHz, $CDCl_3$): δ = 0.88 (t, J = 6.9 Hz, 6 H, 18-H_3 and 22′′′-H_3), 1.25 (alkyl chain CH_2 protons), 1.63 (overlapping signals, 3 H, 3′′-H_2 and 5b-H), 1.69 (m, 1 H, 5a-H), 2.34 (t, J = 7.5 Hz, 2 H, 2′′-H_2), 3.44 (dd, J = 9.1, 6.8 Hz, 1 H, 6′b-H), 3.50 (s, 3 H, OMe), 3.53−3.48 (overlapping signals, 2 H, 4-H and 6′a-H), 3.77 (dd, J = 10.9, 3.5 Hz, 1 H, 1b-H), 3.83−3.79 (overlapping signals, 2 H, 2′-H and 3′-H), 3.87 (dd, J = 7.1, 2.0 Hz, 1 H, 3-H), 3.93−3.89 (overlapping signals, 2 H, 4′-H and 5′-H), 4.01 (dd, J = 10.9, 5.2 Hz, 1 H, 1a-H), 4.18 (m, 1 H, 2-H), 4.47 and 4.39 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.62 and 4.47 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.76 and 4.60 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.82 and 4.55 (AB system, J =

11.7 Hz, 1 H each, geminal benzyl protons), 4.91 and 4.55 (AB system, J = 11.7 Hz, 1 H each, geminal benzyl protons), 4.94 (br. s, 1 H, 1′-H), 6.22 (d, J = 8.7 Hz, 1 H, 2-NH), 7.41−7.22 (25 H, aromatic protons) ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ = 14.1 (CH_3, C-18 and C-22′′), 172.4 (C, C-1′′), 22.6 (CH_2, C-17 and C-21′′), 24.9 (CH_2, C-3′′), 30.0−29.1 (CH_2, alkyl chain CH_2 groups), 30.2 (CH_2, C-5), 31.9 (CH_2, C-16 and C-20′′), 34.1 (CH_2, C-2′′), 50.3 (CH, C-2), 59.2 (CH_3, OMe), 69.1 (CH_2, C-1), 69.2 (CH_2, C-6′), 69.8 (CH, C-5′), 71.7 (CH_2, benzylic CH_2 group), 72.7 (CH_2, benzylic CH_2 group), 73.5 (CH_2, benzylic CH_2 group), 74.7 (CH, C-4′), 74.7 (CH_2, benzylic CH_2 group), 78.4 (CH, C-3), 78.5 (CH, C-2′ or C-3′), 78.6 (CH, C-3′ or C-2′), 80.2 (CH, C-4), 98.8 (CH, C-1′), 128.4−127.3 (CH, aromatic carbons), 138.8−138.1 (C, aromatic carbons) ppm.

(2*S*,3*S*,4*R*)-2-Docosanoylamino-1-*O*-(2-*O*-methyl-α-D-galactopyranosyl)-1,3,4-octadecanetriol (2): The protected glycosphingolipid **12** (101 mg, 0.080 mmol) and $Pd(OH)_2$/C (50 mg, 20% w/w) were suspended in 95% EtOH (18 mL) and AcOH (2 mL). The obtained mixture was hydrogenated at a pressure of 3 bar in a Parr reactor for 48 h at 40 °C. The reaction mixture was filtered through Celite and the filtrate washed with 95% EtOH and $CHCl_3$. After removal of the solvents, the combined organic extracts were purified by reversed-phase column chromatography on RP-18 silica gel (MeOH/EtOAc, 95:5) to give 46 mg (0.056 mmol, 70%) of compound **2** as an amorphous solid. [α]D = +52 ($CHCl_3$/MeOH, 1:1, c = 0.3). HRESIMS (positive ions): m/z = 838.6777 ($[M + Na]^+$, $C_{47}H_{93}NNaO_9$ gives 838.6748). ESI MS/MS (parent ion: m/z = 838): m/z = 820 $[M + Na - H_2O]^+$, 662 $[M + Na - (sugar - H_2O)]^+$, 644 $[M + Na - sugar]^+$, 512 $[M + Na - docosanoyl]^+$. ^{1}H NMR (500 MHz, [D$_5$]pyridine): δ = 0.83 (t, J = 7.3 Hz, 6 H, 18-H_3 and 22′′-H_3), 1.31−1.20 (alkyl chain CH_2 protons), 1.47−1.33 (overlapping signals, 3 H, 7a-H and 4′′-H_2), 1.66 (m, 1 H, 6b-H), 1.81 (quintet, J = 7.3 Hz, 2 H, 3′′-H_2), 1.91 (m, 2 H, 5b-H and 6a-H), 2.21 (m, 1 H, 5a-H), 2.45 (t, J = 7.3 Hz, 2 H, 2′′-H_2), 3.58 (s, 3 H, OMe), 4.11 (dd, J = 9.7, 3.3 Hz, 1 H, 2′-H), 4.24 (m, 1 H, 4-H), 4.33 (m, 1 H, 3-H), 4.43−4.37 (overlapping signals, 4 H, 1b-H, 6-H_2, and 3′-H, and 6′a-H), 4.46 (m, 1 H, 5′-H), 4.49 (br. s, 1 H, 4′-H), 4.63 (dd, J = 10.5, 4.1 Hz, 1 H, 1a-H), 5.17 (m, 1 H, 2-H), 5.55 (d, J = 3.3 Hz, 1 H, 1′-H), 8.60 (d, J = 8.5 Hz, 1 H, 2-NH) ppm. ^{13}C NMR (125 MHz,[D$_5$]pyridine): δ = 14.2 (CH_3, C-18 and C-22′′), 23.0 (CH_2, C-17 and C-21′′), 26.5 (CH_2, C-3′′), 26.6 (CH_2, C-6), 30.3−29.5 (CH_2, alkyl chain CH_2 groups), 32.1 (CH_2, C-16 and C-20′′), 33.7 (CH_2, C-5), 36.8 (CH_2, C-2′′), 51.8 (CH, C-2), 58.5 (CH_3, OMe), 62.6 (CH_2, C-6′), 68.7 (CH_2, C-1), 70.5 (CH, C-3′), 71.0 (CH, C-4′), 72.7 (CH, C-4), 72.8 (CH, C-5′), 76.7 (CH, C-3), 80.0 (CH, C-2′), 98.6 (CH, C-1′), 173.5 (C, C-1′′) ppm.

Lymphocyte Proliferation Test: Spleens from C57Bl/6 mice (Charles River Italia, Calco, Como) were aseptically removed, minced and cell suspensions were incubated at 5×10^5/well in 96-well microtiter plates (3799 Costar Italia, Milan, Italy) using an RPMI 1640 medium supplemented with 100 U/mL of penicillin, 2 mM glutamine, 100 μg/mL streptomycin, 20 mM Hepes buffer (Euroclone) and 10% FCS (Euroclone). All reagents were free of endotoxin contamination by the LAL assay. Various doses of α-Gal-GSL, KRN7000 (**1**) or the 2′-methoxy analog of α-Gal-GSL, (2-*O*-methyl-α-D-galactopyranosyl)ceramide (**2**), were added. Concanavaline A (1 μg/mL) was used as positive control. Cell proliferation was measured using the MTT assay already described.[17] Plates were incubated for 72 h at 37 °C in 5% CO_2, then 20 μL of a 5 mg/mL solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide (MTT) (M-2128 Sigma) in PBS were added for an additional 3 hours at 37 °C. The plates were then centrifuged, the supernatants discarded and the dark blue formazan crystals dissolved using 100 μL of lysing buffer consisting of 20% (w/v) of a solution of SDS (Sigma), 40% of *N*,*N*-dimethyl formamide (Merck) in H_2O, at pH 4.7 adjusted with 80% acetic acid. The plates were then read on a microplate reader (Molecular Devices Co., Menlo Park, CA, USA) at a test wavelength of 550 nm and a reference wavelength of 690 nm. The results are expressed as OD 550/OD690 which mean that the values at OD 690 have been subtracted from the values at OD 550. All the tests were performed at least three times in quadruplicate and the statistical analysis was performed by one-way ANOVA with Scheffe F-test post hoc; p values less than 0.05 were considered to be significant.

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