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Synthesis of 6"-Triazole-Substituted α -GalCer Analogues as Potent iNKT Cell Stimulating Ligands

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Keywords: α -GalCer, CD1d, iNKT cell, triazole, IL-2, click chemistry

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Abstract

We report the synthesis of a small series of 6''-triazol-1-yl-substituted α -GalCer analogues by late-stage conversion of the 6''-OH to an azide group, copper-catalyzed azide-alkyne cycloaddition and final deprotection. When evaluated for their capacity to induce IL-2 secretion *in vitro*, all compounds proved equally potent or superior to α -GalCer. The S.A.R suggests that the improved antigenic activity is mainly triggered by the triazole functionalization in se. While the introduction of selected substituents at C-4 of this heterocyclic ring is tolerated, this generally fails to further improve antigenicity.

Introduction

Invariant natural killer T cells (iNKT cells) are a unique lymphocyte population characterized by markers associated with both NK and conventional T cells.¹ Their semi-invariant T cell receptor (TCR) is encoded by an invariant V α 14-J α 18 chain in mice (homologous V α 24J α 18 chain in humans), preferentially paired with V β 8.2, V β 7 or V β 2 (V β 11 in humans). Recognition of glycolipid antigens presented by the MHC class I-like protein CD1d and subsequent activation of these iNKT cells lead to a rapid release of copious amounts of proinflammatory and regulatory cytokines, including IFN- γ and IL-4. Hence they contribute to different immune responses against microorganisms,² tumors³ and self-antigens.⁴ Since iNKT cells bridge the innate and adaptive immune system, they represent an attractive target for immunotherapeutics.

The most extensively studied ligand for iNKT cells is KRN7000 (**1**, Figure 1), also referred to as α -galactosylceramide (α -GalCer), a structurally optimized compound derived from extracts of the marine sponge *Agelas Mauritanus*.⁵ Its potential in the treatment of several diseases such as certain tumors, infections and autoimmune diseases has been related to the activation of iNKT cells⁶ and downstream activation of other immune cells such as NK⁷ and B cells.⁸ However, the opposing activities of the simultaneous secreted Th1 and Th2 cytokines are considered a major limitation for the therapeutic applications of α -GalCer.⁹ For that reason, a lot of effort has been investigated in the search for potent α -GalCer analogues with a biased Th1 or Th2 profile.

Numerous modifications of all parts of KRN7000, i.e. the galactose, the phytosphingosine and the acyl moieties, have been investigated.¹⁰ This has led to the identification of OCH (**2**), a prototypical Th2 polarizer characterized by a reduced sphingosine chain and a slightly shortened acyl chain¹¹ and the Th1 polarizing α -C-GalCer (**3**), in which the O-anomeric link

is replaced by a CH₂ group.¹² S.A.R. studies revealed a wide variety of possible alterations in the ceramide part resulting in a shifted cytokine profile. By contrast, successful modifications of the galactose moiety are rather scarce and mostly limited to the 6''-position. 6''-modifications were first reported by the group of Savage, who showed that analogues containing different fluorophores¹³ or an acetamide group¹⁴ at this position were still able to activate iNKT cells similar to α -GalCer. This finding led different groups to explore alternative C-6''-modifications such as pegylation,¹⁵ *O*-methylation (RCAI-6) and 6''-deoxygenation.¹⁶ Previously, we demonstrated that a C-6''-naphthylurea derivative (NU- α -GalCer, **4**) exhibits marked anti-tumor effects in a mouse B16 melanoma model.¹⁷ A crystal structure of NU- α -GalCer complexed to CD1d and the TCR allowed relating this response to the induction of a third anchor in CD1d by the naphthylurea group, thereby probably enhancing the affinity for CD1d. Encouraged by these findings, we decided to investigate alternative C-6''-modifications. Here, we describe the synthesis and preliminary biological evaluation of a small series of α -GalCer derivatives containing different 4-substituted triazol-1-yl moieties at the C-6''-position.

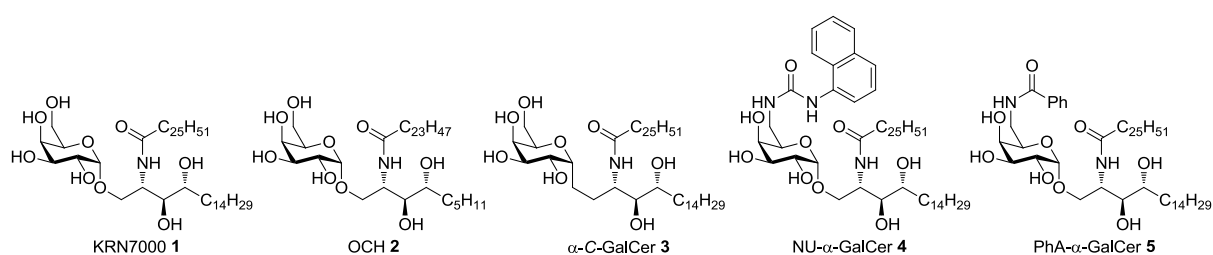


Figure 1. Structures of KRN7000, OCH, α -C-GalCer and NU- α -GalCer and PhA- α -GalCer.

The copper-catalyzed azide–alkyne cycloaddition (CuAAC) variant of Huisgen’s 1,3-dipolar cycloaddition provides an easy and reliable method to link two building blocks via a 1,2,3-triazole moiety. Besides being a rigid linking unit, the 1,4-disubstituted triazole also possesses desirable pharmacological properties.¹⁸ It is metabolically stable and, due to its high aromatic

stabilization, resistant towards acid and basic hydrolysis and towards reductive and oxidation conditions. It has a large dipole moment (about 5 D) and is able to participate in hydrogen bond formation, dipole-dipole and π -stacking interactions. Since the aforementioned crystal structure revealed an extra hydrogen bond between the urea carbonyl oxygen of NU- α -GalCer and Thr159 of CD1d, the capacity of a triazole moiety to act as H-bonding acceptor is most appealing. Moreover, a 1,4-disubstituted triazole group is suggested as a bioisostere of a trans-amide bond, which is interesting in view of the distinct Th1 profile of α -GalCer analogues with aromatic amides on C-6'' such as phenyl-substituted amide **5**.¹⁹ Hence, its corresponding triazole compound **9c** is synthesized and evaluated for comparative reasons. Finally, a docking experiment of C-6''-(1,2,3-triazol-1-yl)-substituted α -GalCer (**9a**) in the structure of the ternary complex indicates that introduction of aromatic substituents in position 4 of the triazole ring might favorably occupy the cleft that accommodates the naphthyl group of NU- α -GalCer (Figure 2). Hence, such aromatic substituents may induce a similar hydrophobic pocket in CD1d. This led us to select a homologous series of aromatic alkynes, allowing us to investigate the influence of the linker length between the triazole ring and the aromatic group on the iNKT cell activity.

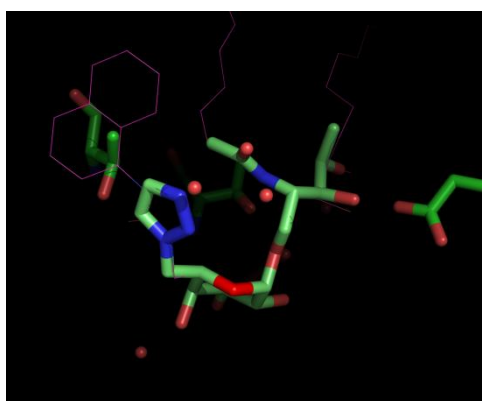


Figure 2. Docking of a 6''-triazole-substituted α -GalCer analogue (green: triazole compound; purple: NU- α -GalCer)

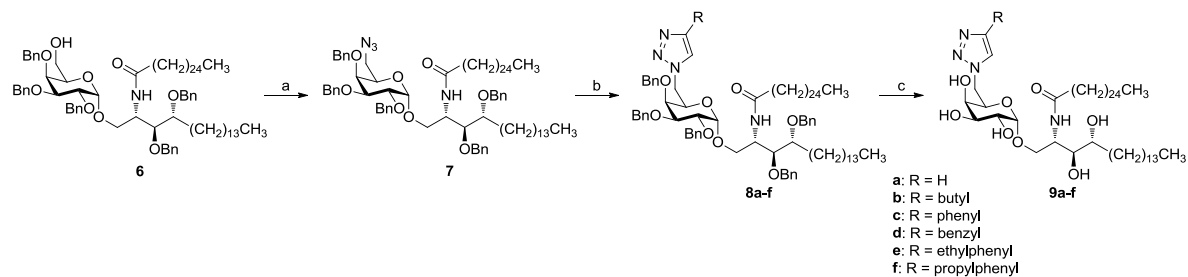
Incorporation of a triazole unit at the C-6'' position has very recently been described by the group of Besra for the synthesis of homodimeric α -GalCer analogues.²⁰ Pegylated as well as alkylene spacers of varying lengths were used to link the two α -GalCer units. Depending on the linker length, the pegylated dimers showed similar or lower iNKT cell stimulation as α -GalCer. By contrast, the alkylene derivatives were less active and proved to be insensitive to the linker length. Prompted by this report and the more recently published 6''-triazole α -GalCer analogues by the same group,²¹ we want to disclose the synthesis and initial biological results of a distinct series of 6''-triazole modified analogues.

Results and Discussion

Chemistry

The synthesis of the desired triazoles started from compound **6** (Scheme 1). We recently used this intermediate, which is obtained by regioselective opening of the 4,6-*O*-benzylidene precursor, for the synthesis of galacturonic acid and α -D-fucopyranosyl analogues of KRN7000.²² Conversion of the primary hydroxyl group to an azide group via a Mitsunobu reaction with diphenylphosphorazidate (DPPA) afforded compound **7** in excellent yield. Initial attempts to convert **7** to triazole **8b** using a CuAAC reaction at ambient temperature did not result in triazole formation, even after 48 hours. Based on a report by Carvalho and coworkers²³ and our own lab experiences, we then performed the CuAAC reaction under microwave conditions (70 °C) in the presence of the appropriate alkyne, successfully obtaining compounds **8b-f** in good yields. Due to solubility reasons, DMF was chosen as a solvent, rather than common mixtures such as H₂O/*t*BuOH or H₂O/THF. Aberrantly, the unsubstituted triazole **8a** was acquired by treatment with neat vinylacetate (120 °C). This procedure provides an improved and simple method compared to other reported alternatives such as the use of TMS-acetylene or acetylene gas.²⁴ Final debenzylation by a catalytic

hydrogenolysis afforded the desired analogues **9a-f** in moderate yields. In their recent publication, Besra and coworkers synthesized structurally related triazoles by CuAAC on the unprotected 6''-azido-6''-deoxy- α -GalCer applying conventional heating.



Scheme 1. Reagents and conditions: (a) Ph_3P , DEAD, DPPA, THF, $-20\text{ }^\circ\text{C}$, 92%; (b) appropriate alkyne, sodium ascorbate, CuSO_4 , DMF, μW ($70\text{-}120\text{ }^\circ\text{C}$, 250 W), 60%-97%; (c) Pd black, H_2 , EtOH/ CHCl_3 , 22%-53%.

Biological Evaluation

To assess the antigenic activity of the final compounds, bone marrow dendritic cells (BMDCs) were loaded during 20 hours with 100 ng/mL glycolipid. After 16 hours of coculture with 2C12 cells (iNKT cell hybridoma), IL-2 secretion was determined by ELISA. **9a-f** showed an efficacy to release IL-2 that was higher or comparable to α -GalCer (Figure 3). The analogues in which a butyl (**9b**) or phenyl (**9c**) substituent is directly attached to the triazole ring tend to induce the highest IL-2 secretion. Strikingly, the response of phenyl-substituted triazole **9c** contrasts with the *in vitro* results obtained by Besra and co-workers, where a remarkably reduced iNKT cell stimulatory activity is observed for this compound.²¹ A possible explanation for this discrepancy is the direct incubation of glycolipids, BMDCs and iNKT cell hybridomas in their assay, while we used glycolipid-loaded BMDCs. Introduction of varying linker lengths between the triazole and the phenyl moieties doesn't seem to significantly influence the antigenic activity. Hence we speculated that the mere presence of a triazol-1-yl ring at position 6'' suffices to cause a superior antigenic activity than

α -GalCer. Affirmatively, dendritic cells (DCs) loaded with the unsubstituted triazole analogue **9a** were even better in stimulating iNKT cells to release IL-2. This is consistent with the potent iNKT cell activity of the precursor azide reported in the publication of Besra and co-workers.²¹ The IL-2 secretion induced by butyl containing analogue **9b** is in line with the alkyl-substituted triazoles of Besra, where the one with an octyl chain is far more active than the one with a longer undecyl chain. Contradictory to the docking experiment, however, these data indicate that 1,4-substitution of the triazole moiety doesn't interfere in the interaction with CD1d and the TCR, provided that the attached group doesn't exceed a certain size.

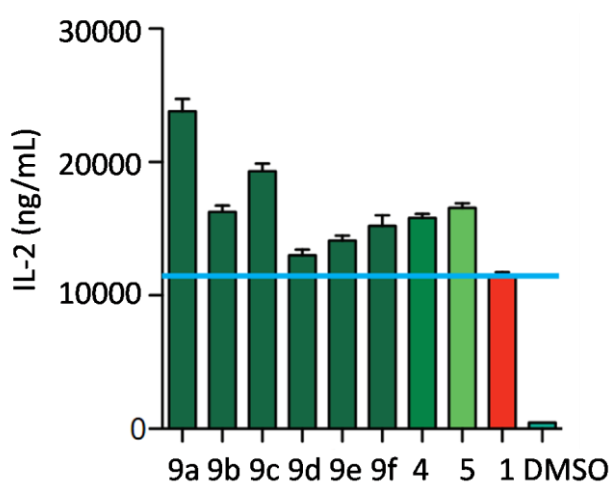


Figure 3. IL-2 secretion after coculture of 2C12 cells (iNKT cell hybridoma) with glycolipid (100 ng/mL) loaded BMDCs.

Conclusions

In summary, a series of phenyl-substituted C-6''-triazolyl α -GalCer analogues as well as a butyl- and non-substituted one have been synthesized using a copper-catalyzed azide-alkyne cycloaddition reaction. Evaluation of the *in vitro* IL-2 secretion induced by these compounds reveals them as potent iNKT cell stimulating ligands. We suggest that the observed increase in antigenic activity is mainly due to the triazole functionalization whereby further substitutions at C-4 of this heterocycle with small groups is tolerated.

Experimental Section

Chemical Synthesis

Precoated Macherey-Nagel SIL G/UV₂₅₄ plates were used for TLC, and spots were examined under UV light at 254 nm and further visualized by sulfuric acid-anisaldehyde spray. Column chromatography was performed on Biosolve silica gel (63-200 μm , 60 \AA). NMR spectra were obtained with a Varian Mercury 300 Spectrometer. Chemical shifts are given in ppm (δ) relative to the residual solvent signals, in the case of CDCl_3 : $\delta = 7.26$ ppm for ^1H and $\delta = 77.4$ ppm for ^{13}C and in the case of pyridine- d_5 : $\delta = 8.74$, 7.58 and 7.22 ppm for ^1H and $\delta = 149.9$, 135.5 and 123.5 ppm for ^{13}C . IR spectra were recorded on a Varian Scimitar 800 FT-IR spectrometer as KBr pellets. The absorption peaks are reported in cm^{-1} . Exact mass measurements were performed on a Waters LCT Premier XE TOF equipped with an electrospray ionization interface and coupled to a Waters Alliance HPLC system. Samples were infused in a in a $\text{CH}_3\text{CN}/\text{HCOOH}$ (1000:1) mixture at 10 mL/min. **(2S,3S,4R)-3,4-di-O-benzyl-1-O-(2,3,4-tri-O-benzyl- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (6).**

IR: 3485, 3319, 3036, 2922, 2851, 1647.

(2S,3S,4R)-3,4-di-O-benzyl-1-O-(6-azido-2,3,4-tri-O-benzyl-6-deoxy- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (7). To a solution of intermediate **6** (500 mg, 0.38 mmol) and triphenylphosphine (400 mg, 1.53 mmol) in THF (25 mL) at -20 $^\circ\text{C}$ were added DEAD (0.7 mL, 2.2 M) and DPPA (0.33 mL, 1.53 mmol). The reaction mixture was stirred overnight and after completion of the reaction, the solvent was removed under reduced pressure. Purification by column chromatography (hexanes/EtOAc: 9/1) resulted in azide **7** (471 mg, 92%) as a white solid.

^1H NMR (300 MHz, CDCl_3): δ 7.42-7.26 (m, 25H, arom. H), 5.87 (d, $J = 8,6$ Hz, 1H, NH), 4.98 (d, $J = 11.4$ Hz, 1H, $\text{CH}_2\text{-Ph}$), 4.84 (d, $J = 3.8$ Hz, 1H, H-1''), 4.82-4.72 (m, 4H, $\text{CH}_2\text{-Ph}$), 4.64 (d, $J = 11.9$ Hz, 1H, $\text{CH}_2\text{-Ph}$), 4.61-4.57 (m, 2H, $\text{CH}_2\text{-Ph}$), 4.51 (d, $J = 11.5$ Hz, 1H, $\text{CH}_2\text{-Ph}$), 4.49 (d, $J = 11.7$ Hz, 1H, $\text{CH}_2\text{-Ph}$), 4.32-4.24 (m, 1H, H-2), 4.02 (dd, $J = 3.5$ and 10.1 Hz, 1H, H-2''), 3.91-3.83 (m, 3H, H-3'', H-1, H-4''), 3.81-3.74 (m, 3H, H-3, H-1, H-5''), 3.55-3.51 (m, 1H, H-4), 3.47 (dd, $J = 8.1$ and 12.4 Hz, 1H, H-6''), 2.99 (dd, $J = 6.2$ and 12.4 Hz, 1H, H-6''), 2.01-1.85 (m, 2H, COCH_2), 1.64-0.98 (m, 72H, CH_2), 0.88 (t, $J = 6.6$ Hz, 6H, CH_3).

^{13}C NMR (75 MHz, CDCl_3): δ 173.01, 150.14, 150.03, 138.89, 138.78, 138.73, 138.60, 138.32, 130.30, 130.28, 128.68, 128.66, 128.63, 128.61, 128.57, 128.15, 128.06, 128.04, 127.97, 127.91, 127.84, 127.77, 127.69, 126.36, 126.34, 120.49, 120.43, 99.34, 80.09, 79.56, 79.04, 77.66, 77.44, 77.24, 76.81, 75.07, 74.87, 73.68, 73.51, 72.06, 70.25, 69.04, 51.61, 50.37, 36.98, 32.16, 30.06, 29.96, 29.94, 29.89, 29.84, 29.69, 29.64, 29.61, 29.59, 26.16, 25.95, 22.92, 14.35.

IR: 3312, 2978, 2918, 2849, 2097, 1645. Exact mass (ESI-MS) for $\text{C}_{85}\text{H}_{128}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ found, 1333.9846; calcd, 1333.9805.

General procedure for CuAAC click reaction

To a solution of azide **7** (80 mg, 0.06 mmol) in DMF (2 mL) was added the appropriate alkyne, sodium ascorbate and copper(II)sulfate. The reaction mixture was stirred for 20 minutes at 70 °C in the microwave. After extraction with EtOAc, the organic phase was washed with brine and dried over Na_2SO_4 , followed by evaporation of the solvent. Purification by column chromatography (hexanes/EtOAc: 7.5/2.5) afforded the desired triazoles **8b** (74%), **8c** (60%), **8d** (95%), **8e** (80%) and **8f** (97%) .

(2*S*,3*S*,4*R*)-3,4-di-*O*-benzyl-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-(4-butyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (8b).

¹H NMR (300 MHz, CDCl₃): δ 7.27-7.03 (m, 26H, arom. H), 5.63 (d, $J = 8.5$ Hz, 1H, NH), 4.94 (d, $J = 11.1$ Hz, 1H, CH₂-Ph), 4.78 (d, $J = 3.5$ Hz, 1H, H-1''), 4.73 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.71 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.64 (d, $J = 11.7$ Hz, 2H, CH₂-Ph), 4.57 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.56 (d, $J = 11.1$ Hz, 1H, CH₂-Ph), 4.48 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.40 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.39 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.31 (dd, $J = 5.9$ and 13.7 Hz, 1H, H-6''), 4.21-4.15 (m, 1H, H-2), 4.12 (dd, $J = 5.9$ and 13.1 Hz, 1H, H-6''), 4.00-3.96 (m, 2H, H-2'', H-5''), 3.80 (dd, $J = 2.6$ and 10.2 Hz, 1H, H-3''), 3.68-3.59 (m, 3H, H-4'', H-3, H-1), 3.52-3.41 (m, 2H, H-1, H-4), 2.57 (app. t, $J = 7.7$ Hz, 2H, CH₂), 1.88-1.71 (m, 2H, COCH₂), 1.56-1.05 (m, 72H, CH₂), 0.84 (t, $J = 7.3$ Hz, 3H, CH₃), 0.81 (t, $J = 6.8$ Hz, 6H, CH₃).

¹³C NMR (75 MHz, CDCl₃): δ 173.01, 148.40, 138.82, 138.77, 138.56, 138.49, 138.44, 128.76, 128.68, 128.65, 128.62, 128.57, 128.16, 128.11, 128.09, 127.99, 127.92, 127.81, 127.66, 122.29, 99.41, 79.87, 79.78, 79.25, 77.67, 77.45, 77.25, 76.82, 76.47, 74.95, 74.76, 73.81, 73.51, 73.41, 72.08, 70.17, 50.28, 36.87, 32.16, 31.75, 30.43, 30.08, 29.96, 29.94, 29.91, 29.89, 29.85, 29.70, 29.61, 29.59, 26.06, 25.88, 25.52, 22.92, 22.55, 14.35, 14.05.

Exact mass (ESI-MS) for C₉₁H₁₃₈N₄O₈ [M+H]⁺ found, 1416.0526; calcd, 1416.0593.

(2*S*,3*S*,4*R*)-3,4-di-*O*-benzyl-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-(4-phenyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (8c).

¹H NMR (300 MHz, CDCl₃): δ 7.93-7.72 (m, 3H, arom. H), 7.31-7.18 (m, 28H), 5.55 (d, $J = 8.2$ Hz, 1H, NH), 4.95 (d, $J = 10.9$ Hz, 1H, CH₂-Ph), 4.78 (app. s, 1H, H-1''), 4.73-4.55 (m, 6H, CH₂-Ph), 4.47-4.34 (m, 4H, CH₂-Ph, H-6''), 4.25-4.18 (m, 2H, H-2, H-6''), 4.06-3.97 (m, 2H, H-5'', H-2''), 3.79 (app. d, $J = 10.0$ Hz, 1H, H-3''), 3.69-3.52 (m, 4H, H-4'', H-3, H-1),

3.42-3.41 (m, 1H, H-4), 1.82-1.66 (m, 2H, COCH₂), 1.49-0.99 (m, 72H, CH₂), 0.80 (t, *J* = 6.5 Hz, 6H, CH₃).

¹³C NMR (75 MHz, CDCl₃): δ173.19, 162.87, 138.76, 138.68, 138.58, 138.51, 138.48, 130.85, 129.03, 128.82, 128.69, 128.68, 128.63, 128.58, 128.28, 128.21, 128.13, 128.01, 127.98, 127.91, 127.81, 127.68, 125.88, 121.47, 99.69, 80.15, 79.45, 79.27, 77.70, 77.49, 77.28, 76.86, 76.45, 75.06, 74.75, 73.80, 73.60, 73.40, 72.00, 70.10, 68.90, 50.41, 36.86, 36.73, 32.17, 31.69, 30.50, 30.08, 29.95, 29.90, 29.84, 29.65, 29.60, 25.86, 22.93, 14.37.

Exact mass (ESI-MS) for C₉₃H₁₃₄N₄O₈ [M+H]⁺ found, 1436.0295; calcd, 1436.0274, [M+Na]⁺ found 1458.0104; calcd, 1458.0094, [M+K]⁺ found 1473.9843, calcd, 1473.9833.

(2*S*,3*S*,4*R*)-3,4-di-*O*-benzyl-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-(4-benzyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (8d)

¹H NMR (300 MHz, CDCl₃): δ7.30-7.09 (m, 31H, arom. H), 5.63 (d, *J* = 8.6 Hz, 1H, NH), 4.92 (d, *J* = 11.1 Hz, 1H, CH₂-Ph), 4.74 (d, *J* = 2.9 Hz, 1H, H-1''), 4.70 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.69 (d, *J* = 13.0 Hz, 1H, CH₂-Ph), 4.66 (d, *J* = 12.1 Hz, 1H, CH₂-Ph), 4.64 (d, *J* = 11.6 Hz, 1H, CH₂-Ph), 4.56 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.50 (d, *J* = 11.1 Hz, 1H, CH₂-Ph), 4.48 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.39 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.38 (d, *J* = 11.6 Hz, 1H, CH₂-Ph), 4.25 (dd, *J* = 5.6 and 13.8 Hz, 1H, H-6''), 4.16-4.09 (m, 2H, H-2, H-6''), 3.98-3.93 (m, 4H, CH₂-Ph, H-2'', H-5''), 3.79 (dd, *J* = 2.4 and 10.1 Hz, 1H, H-3''), 3.68 (app. s, 1H, H-4''), 3.64 (dd, *J* = 3.5 and 5.7 Hz, 1H, H-3), 3.55 (dd, *J* = 6.1 and 10.7 Hz, 1H, H-1), 3.45-3.38 (m, 2H, H-4, H-1), 1.89-1.70 (m, 2H, COCH₂), 1.54-1.18 (m, 72H, CH₂), 0.80 (t, *J* = 6.7 Hz, 6H, CH₃).

¹³C NMR (75 MHz, CDCl₃): δ172.98, 147.54, 139.32, 138.82, 138.53, 138.46, 138.34, 128.85, 128.83, 128.71, 128.69, 128.64, 128.62, 128.58, 128.17, 128.11, 128.06, 128.04, 128.00, 127.95, 127.88, 127.81, 127.68, 126.97, 123.19, 99.27, 79.97, 79.58, 79.21, 77.67,

77.45, 77.25, 76.83, 76.42, 74.90, 74.71, 73.82, 73.49, 73.39, 72.04, 70.06, 68.41, 50.52, 50.17, 36.89, 32.38, 32.16, 30.34, 30.10, 29.97, 29.94, 29.89, 29.86, 29.71, 29.61, 29.60, 26.11, 25.90, 22.93, 14.36.

Exact mass (ESI-MS) for C₉₄H₁₃₆N₄O₈ [M+H]⁺ found, 1450.0453; calcd, 1450.0431, [M+Na]⁺ found 1472.0304; calcd, 1472.0250.

(2*S*,3*S*,4*R*)-3,4-di-*O*-benzyl-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-(4-ethylphenyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (8e).

¹H NMR (300 MHz, CDCl₃): δ 7.30-7.06 (m, 31H, arom. H), 5.64 (d, *J* = 8.5 Hz, 1H, NH), 4.93 (d, *J* = 11.3 Hz, 1H, CH₂-Ph), 4.77 (d, *J* = 3.7 Hz, 1H, H-1''), 4.73-4.62 (m, 3H, CH₂-Ph), 4.57 (d, *J* = 11.7 Hz, 1H, CH₂-Ph), 4.52 (d, *J* = 9.2 Hz, 1H, CH₂-Ph), 4.48 (d, *J* = 9.6 Hz, 1H, CH₂-Ph), 4.40 (d, *J* = 11.5 Hz, 1H, CH₂-Ph), 4.39 (d, *J* = 11.5 Hz, 1H, CH₂-Ph), 4.29 (dd, *J* = 5.92 and 13.7 Hz, 1H, H-6''), 4.22-4.15 (1H, H-2), 4.14 (dd, *J* = 6.7 and 13.9 Hz, 1H, H-6''), 4.01-3.95 (m, 2H, H-2'', H-5''), 3.79 (dd, *J* = 2.6 and 10.2 Hz, 1H, H-3''), 3.65-3.57 (m, 3H, H-4'', H-3, H-1), 3.51-3.41 (m, 2H, H-1, H-4), 2.96-2.82 (m, 4H, CH₂-CH₂-Ph), 1.88-1.70 (m, 2H, COCH₂), 1.53-1.04 (m, 72H, CH₂), 0.81 (t, *J* = 6.7 Hz, 6H, CH₃).

¹³C NMR (75 MHz, CDCl₃): δ 173.04, 147.29, 141.38, 138.80, 138.76, 138.56, 138.48, 138.41, 128.76, 128.69, 128.66, 128.61, 128.58, 128.17, 128.11, 128.09, 128.01, 127.93, 127.82, 127.65, 126.31, 122.61, 99.37, 79.84, 79.25, 77.67, 77.45, 77.25, 76.83, 76.48, 74.93, 74.71, 73.82, 73.54, 73.42, 72.08, 70.09, 68.50, 50.36, 50.28, 36.88, 35.71, 32.16, 30.45, 30.08, 29.96, 29.89, 29.86, 29.70, 29.61, 29.59, 27.61, 26.05, 25.89, 22.93, 21.28, 14.43, 14.36.

Exact mass (ESI-MS) for C₉₅H₁₃₈N₄O₈ [M+H]⁺ found, 1464.0540; calcd, 1464.0593.

(2*S*,3*S*,4*R*)-3,4-di-*O*-benzyl-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-(4-propylphenyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (8f).

¹H NMR (300 MHz, CDCl₃): δ 7.36-7.17 (m, 31H, arom. H), 5.73 (d, *J* = 8.5 Hz, 1H, NH), 5.03 (d, *J* = 11.9 Hz, 1H, CH₂-Ph), 4.87 (d, *J* = 3.5 Hz, 1H, H-1''), 4.82 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.80 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.73 (d, *J* = 12.0 Hz, 2H, CH₂-Ph), 4.66 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.65 (d, *J* = 11.27 Hz, 1H, CH₂-Ph), 4.57 (d, *J* = 11.6 Hz, 1H, CH₂-Ph), 4.49 (d, *J* = 11.8 Hz, 1H, CH₂-Ph), 4.47 (d, *J* = 11.6 Hz, 1H, CH₂-Ph), 4.40 (dd, *J* = 6.0 and 13.8 Hz, 1H, H-6''), 4.32-4.20 (m, 2H, H-2, H-6''), 4.11-4.05 (m, 2H, H-5'', H-2''), 3.89 (dd, *J* = 2.6 and 10.0 Hz, 1H, H-3''), 3.76 (app. d, *J* = 1.5 Hz, 1H, H-4''), 3.74-3.66 (m, 2H, H-3, H-1), 3.59 (dd, *J* = 4.6 and 10.7 Hz, 1H, H-1), 3.54-3.50 (m, 1H, H-4), 2.77-2.64 (m, 4H, CH₂-CH₂-Ph), 2.05-1.81 (m, 4H, CH₂, CH₂, COCH₂), 1.61-1.28 (m, 72H, CH₂), 0.90 (t, *J* = 6.7 Hz, 6H, CH₃).

¹³C NMR (75 MHz, CDCl₃): δ 173.04, 147.93, 142.09, 138.84, 138.79, 138.57, 138.51, 138.44, 128.79, 128.76, 128.72, 128.70, 128.66, 128.64, 128.61, 128.58, 128.18, 128.13, 128.10, 128.02, 127.94, 127.83, 127.68, 126.17, 126.07, 122.41, 99.43, 79.80, 79.26, 77.73, 77.30, 76.88, 76.50, 74.96, 74.75, 73.84, 73.54, 73.44, 72.08, 70.15, 68.94, 50.31, 36.88, 35.65, 34.87, 32.18, 31.29, 30.42, 30.30, 30.09, 29.98, 29.96, 29.94, 29.91, 29.87, 29.72, 29.63, 26.06, 25.91, 25.39, 22.95, 18.06, 14.38.

Exact mass (ESI-MS) for C₉₆H₁₄₀N₄O₈ [M+H]⁺ found, 1478.0772; calcd, 1478.0749.

(2*S*,3*S*,4*R*)-3,4-di-*O*-benzyl-1-*O*-(2,3,4-tri-*O*-benzyl-6-deoxy-6-(triazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (8a).

Azide **7** was dissolved in vinyl acetate and stirred at 120 °C in the microwave. After 6 h, the reaction mixture was evaporated to dryness. Purification by column chromatography (hexanes/EtOAc: 7.5/2.5) afforded the desired triazole **8a** (93 mg, 71%).

¹H NMR (300 MHz, CDCl₃): δ 7.53 (dd, *J* = 0.9 and 7.3 Hz, 2H, arom. H), 7.36-7.22 (m, 25H, arom. H), 5.68 (d, *J* = 8.5 Hz, 1H, NH), 5.01 (d, *J* = 11.2 Hz, 1H, CH₂-Ph), 4.84 (d, *J* =

3.6 Hz, 1H, H-1''), 4.80 (d, $J = 11.8$ Hz, 1H, CH₂-Ph), 4.78 (d, $J = 11.8$ Hz, 1H, CH₂-Ph), 4.71 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.70 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.64 (d, $J = 11.7$ Hz, 1H, CH₂-Ph), 4.63 (d, $J = 11.2$ Hz, 1H, CH₂-Ph), 4.55 (d, $J = 11.6$ Hz, 1H, CH₂-Ph), 4.47 (d, $J = 11.8$ Hz, 1H, CH₂-Ph), 4.45 (d, $J = 11.8$ Hz, 1H, CH₂-Ph), 4.39 (dd, $J = 5.2$ and 13.9 Hz, 1H, H-6''), 4.29 (d, $J = 7.7$ and 13.9 Hz, 1H, H-6''), 4.23-4.17 (m, 1H, H-2), 4.07-4.01 (m, 2H, H-2'', H-5''), 3.87 (dd, $J = 2.6$ and 10.1 Hz, 1H, H-3''), 3.77 (app. d, $J = 1.3$ Hz, 1H, H-4''), 3.68 (dd, $J = 3.6$ and 5.2 Hz, 1H, H-3), 3.62 (dd, $J = 6.9$ and 10.6 Hz, 1H, H-1), 3.55-3.47 (m, 2H, H-1, H-4), 1.95-1.77 (m, 2H, COCH₂), 1.61-1.13 (m, 72H, CH₂), 0.88 (t, $J = 6.7$ Hz, 6H, CH₃).

¹³C NMR (75 MHz, CDCl₃): δ 172.95, 149.61, 138.59, 138.49, 138.33, 138.27, 138.08, 133.65, 128.57, 128.51, 128.48, 128.45, 128.41, 128.37, 128.03, 127.89, 127.88, 127.85, 127.79, 127.73, 127.62, 127.47, 124.77, 116.19, 99.01, 79.69, 79.61, 78.96, 76.25, 74.73, 74.63, 73.58, 73.38, 73.11, 71.96, 69.94, 50.40, 50.04, 36.69, 31.94, 30.28, 29.83, 29.74, 29.69, 29.67, 29.62, 29.46, 29.39, 29.37, 25.86, 25.67, 22.70, 14.13.

Exact mass (ESI-MS) for C₈₇H₁₃₀N₄O₈ [M+H]⁺ found, 1359.9907; calcd, 1359.9967, [M+Na]⁺ found, 1381.9797; calcd, 1381.9786.

General procedure for debenzylation

A solution of the protected triazole (0.06 mmol) in CHCl₃ (0.4 mL) and EtOH (1.2 mL) was hydrogenated under atmospheric pressure in the presence of palladium black (10 mg). Upon reaction completion, the mixture was diluted with pyridine and filtered through celite. The filter cake was rinsed with CHCl₃ and EtOH and the filtrate was evaporated to dryness. After purification by column chromatography (CH₂Cl₂/MeOH: 8/2), final compounds **9a** (31%), **9b** (50%), **9c** (40%), **9d** (22%), **9e** (31%) and **9f** (53%) were obtained as white powders.

(2*S*,3*S*,4*R*)-1-*O*-(6-deoxy-6-(triazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (9a).

¹H NMR (300 MHz, pyridine-*d*₅): δ 8.42 (d, *J* = 8.9 Hz, 1H, NH), 8.21 (d, *J* = 0.9 Hz, 1H, arom. H), 7.92 (d, *J* = 0.9 Hz, 1H, arom. H), 5.5 (d, *J* = 3.7 Hz, 1H, H-1''), 5.26-5.19 (m, 1H, H-2), 5.14-4.99 (m, 2H, H-6''), 4.67-4.61 (m, 2H, H-2'', H-5''), 4.38-4.30 (3H, H-1, H-3'', H-3), 4.26-4.21 (m, 2H, H-4, H-4''), 4.09 (dd, *J* = 4.9 Hz and 10.6 Hz, 1H, H-1), 2.50-2.36 (m, 2H, COCH₂), 2.01-1.09 (m, 72H, CH₂), 0.88 (t, *J* = 6.7 Hz, 6H, CH₃).

¹³C NMR (75 MHz, pyridine-*d*₅): δ 171.92, 149.07, 148.72, 148.36, 147.99, 135.00, 134.66, 134.33, 134.00, 133.64, 132.60, 124.24, 122.98, 122.65, 122.32, 121.99, 121.61, 99.98, 75.54, 71.23, 69.78, 69.67, 69.47, 68.57, 67.06, 50.30, 49.77, 35.57, 33.34, 30.94, 29.21, 28.97, 28.86, 28.83, 28.75, 28.69, 28.63, 28.58, 28.43, 25.27, 25.20, 21.76, 13.10.

IR: 3462, 3451, 3335, 2922, 2850, 1653.

Exact mass (ESI-MS) for C₅₂H₁₀₀N₄O₈[M+H]⁺ found, 909.7681; calcd, 909.7619, [M+Na]⁺ found, 931.7450; calcd, 931.7439.

(2*S*,3*S*,4*R*)-1-*O*-(6-deoxy-6-(4-butyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (9b).

¹H NMR (300 MHz, pyridine-*d*₅): δ 8.42 (d, *J* = 8.7 Hz, 1H, NH), 7.93 (s, 1H, arom. H), 6.83 (br. s, 1H, OH), 6.37 (br. s, 1H, OH), 6.05 (br. s, 1H, OH), 5.52 (d, *J* = 3.9 Hz, 1H, H-1''), 5.26-5.19 (m, 1H, H-2), 5.03 (app. d, *J* = 6.5 Hz, 2H, H-6''), 4.70-4.62 (m, 2H, H-5'', H-2''), 4.41 (dd, *J* = 5.4 and 10.7 Hz, 1H, H-1), 4.32 (dd, *J* = 3.2 and 9.9 Hz, 1H, H-3''), 4.27-4.22 (m, 3H, H-3, H-4'', H-4), 4.16 (dd, *J* = 4.9 and 10.6 Hz, 1H, H-1), 2.83 (t, *J* = 7.6 Hz, 2H, CH₂), 2.48-2.39 (m, 2H, COCH₂), 1.94-1.11 (m, 76H, CH₂), 0.93-0.86 (m, 9H, CH₃).

^{13}C NMR (75 MHz, pyridine- d_5): δ 171.94, 100.11, 75.56, 71.24, 69.83, 69.72, 69.41, 68.62, 67.25, 50.21, 49.90, 35.58, 33.32, 30.94, 30.81, 29.24, 28.98, 28.86, 28.83, 28.75, 28.74, 28.71, 28.64, 28.61, 28.44, 28.43, 25.29, 25.21, 24.59, 21.76, 21.39, 13.10, 12.82.

Exact mass (ESI-MS) for $\text{C}_{56}\text{H}_{108}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ found, 965.8295; calcd, 965.8240, $[\text{M}+\text{Na}]^+$ found, 987.8087; calcd, 987.8059, $[\text{M}+\text{K}]^+$ found, 1003.7825; calcd, 1003.7799.

(2*S*,3*S*,4*R*)-1-*O*-(6-deoxy-6-(4-phenyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (9c).

^1H NMR (300 MHz, pyridine- d_5): δ 8.66 (s, 1H, arom. H), 8.40 (d, $J = 8.7$ Hz, 1H, NH), 8.21-8.18 (m, 2H, arom. H), 7.50-7.45 (m, 2H, arom. H), 7.38-7.33 (m, 1H, arom. H), 5.55 (d, $J = 3.7$ Hz, 1H, H-1''), 5.28-5.19 (m, 1H, H-2), 5.10 (app. d, $J = 5.5$ Hz, 2H, H-6''), 4.75 (t, $J = 6.6$ Hz, 1H, H-5''), 4.66 (dd, $J = 3.7$ and 9.8 Hz, 1H, H-2''), 4.45 (dd, $J = 5.6$ and 10.6 Hz, 1H, H-1), 4.35 (dd, $J = 3.2$ and 9.9 Hz, 1H, H-3''), 4.27-4.22 (m, 3H, H-3, H-4, H-4''), 4.15 (dd, $J = 4.9$ and 10.8 Hz, 1H, H-1), 2.45-2.22 (m, 2H, COCH_2), 1.90-1.17 (m, 72H, CH_2), 0.88 (t, $J = 6.7$ Hz, 6H, CH_3).

^{13}C NMR (75 MHz, pyridine- d_5): δ 171.95, 149.40, 149.07, 148.71, 148.35, 146.54, 134.66, 134.33, 134.00, 130.91, 128.07, 126.94, 124.95, 122.99, 122.66, 122.33, 122.00, 121.60, 121.01, 100.05, 75.56, 71.22, 69.79, 69.54, 69.25, 68.60, 67.07, 50.34, 49.83, 35.54, 33.32, 30.94, 29.23, 28.97, 28.87, 28.83, 28.75, 28.74, 28.70, 28.61, 28.58, 28.44, 28.43, 25.26, 25.18, 21.76, 13.10.

Exact mass (ESI-MS) for $\text{C}_{58}\text{H}_{104}\text{N}_4\text{O}_8$ $[\text{M}+\text{H}]^+$ found, 985.7953; calcd, 985.7927, $[\text{M}+\text{Na}]^+$ found, 1007.7729; calcd, 1007.7746.

(2*S*,3*S*,4*R*)-1-*O*-(6-deoxy-6-(4-benzyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (9d)

¹H NMR (300 MHz, pyridine-d₅): δ 8.41 (d, *J* = 8.7 Hz, 1H, NH), 7.91 (s, 1H, arom. H), 7.47-7.45 (m, 2H, arom. H), 7.41-7.36 (m, 2H, arom. H), 7.28-7.26 (m, 1H, arom. H), 5.48 (d, *J* = 3.7 Hz, 1H, H-1''), 5.23-5.19 (m, 1H, H-2), 5.01-4.99 (m, 2H, H-6''), 4.64-4.59 (m, 2H, H-2'', H-5''), 4.36 (dd, *J* = 5.2 and 10.7 Hz, 1H, H-1), 4.32-4.25 (m, 3H, H-4, H-4'', H-3, CH₂-Ph), 4.21 (app. d, *J* = 2.4 Hz, 1H, H-3''), 4.14 (dd, *J* = 4.6 and 10.7 Hz, 1H, H-1), 2.48-2.41 (m, 2H, COCH₂), 1.93-1.26 (m, 72H, CH₂), 0.88 (t, *J* = 6.6 Hz, 6H, CH₃).

¹³C NMR (75 MHz, pyridine-d₅): δ 171.92, 149.06, 148.70, 148.35, 147.98, 146.06, 139.26, 135.00, 134.66, 134.33, 133.99, 133.64, 128.02, 127.78, 125.45, 122.98, 122.65, 122.32, 122.20, 121.99, 100.13, 75.55, 71.30, 69.79, 69.71, 69.47, 68.56, 67.44, 50.37, 49.83, 35.58, 33.29, 31.33, 30.95, 29.24, 28.98, 28.87, 28.83, 28.75, 28.74, 28.71, 28.64, 28.61, 28.55, 28.44, 28.43, 28.37, 25.31, 25.22, 21.76, 13.10.

Exact mass (ESI-MS) for C₅₉H₁₀₆N₄O₈ [M+H]⁺ found, 999.8134; calcd, 999.8089, [M+Na]⁺ found, 1021.7906; calcd, 1021.7908, [M+K]⁺ found, 1037.7676; calcd, 1037.7648.

(2*S*,3*S*,4*R*)-1-*O*-(6-deoxy-6-(4-ethylphenyltriazol-1-yl)-α-D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (9e).

¹H NMR (300 MHz, pyridine-d₅): δ 8.43 (d, *J* = 8.7 Hz, 1H, NH), 7.88 (s, 1H, arom. H), 7.36-7.31 (m, 3H, arom. H), 7.27-7.25 (m, 2H, arom. H), 5.50 (d, *J* = 3.9 Hz, 1H, H-1''), 5.26-5.19 (m, 1H, H-2), 5.01-4.96 (m, 2H, H-2'', H-5''), 4.67-4.61 (m, 2H, H-6''), 4.39 (dd, *J* = 5.3 and 10.6 Hz, 1H, H-1), 4.61 (dd, *J* = 3.2 and 9.9 Hz, 1H, H-3'' or H-4'') 4.56 (m, 2H, H-3, H-4), 4.49 (app. d, *J* = 2.4 Hz, H-3'' or H-4''), 4.15 (dd, *J* = 5.0 and 10.7 Hz, 1H, H-1), 3.22-3.11 (m, 4H, CH₂), 2.47-2.40 (m, 2H, COCH₂), 1.94-1.24 (m, 72H, CH₂), 0.89 (t, *J* = 6.7 Hz, 6H, CH₃).

¹³C NMR (75 MHz, pyridine-d₅): δ 173.49, 150.58, 150.21, 149.86, 147.64, 142.41, 136.17, 135.84, 135.51, 129.24, 129.12, 126.72, 124.49, 124.16, 123.83, 123.50, 123.18, 123.12,

101.57, 77.11, 72.76, 71.34, 71.22, 70.91, 70.14, 51.76, 51.42, 37.10, 36.35, 34.85, 32.46, 32.45, 30.75, 30.49, 30.38, 30.34, 30.27, 30.25, 30.21, 30.14, 30.11, 29.96, 29.94, 28.44, 26.80, 26.72, 23.27, 14.61.

Exact mass (ESI-MS) for C₆₀H₁₀₈N₄O₈ [M+H]⁺ found, 1013.8259; calcd, 1013.8240.

(2*S*,3*S*,4*R*)-1-*O*-(6-deoxy-6-(4-propylphenyltriazol-1-yl)- α -D-galactopyranosyl)-2-hexacosylamino-octadecane-1,3,4-triol (9f).

¹H NMR (300 MHz, pyridine-d₅): δ 8.38 (d, *J* = 8.7 Hz, 1H, NH), 7.93 (s, 1H, arom. H), 7.37-7.24 (m, 5H, arom. H), 7.11 (br. s, 1H, OH), 6.94 (br. s, 1H, OH), 6.82 (d, *J* = 2.9 Hz, 1H, OH), 6.36 (d, *J* = 5.0 Hz, 1H, OH), 6.04 (d, *J* = 3.9 Hz, 1H, OH), 5.51 (d, *J* = 3.7 Hz, 1H, H-1''), 5.26-5.18 (m, 1H, H-2''), 5.03 (d, *J* = 5.03 Hz, 2H, H-6''), 4.71-4.62 (m, H-5'', H-2''), 4.41 (dd, *J* = 5.4 and 10.4 Hz, 1H, H-1), 4.33-4.20 (m, 5H, H-3'', H-3, H-4, H-4''), 4.15 (dd, *J* = 5.0 and 10.7 Hz, 1H, H-1), 2.86 (t, *J* = 7.6 Hz, 2H, CH₂), 2.72 (t, *J* = 7.6 Hz, 2H, CH₂), 2.41 (ddd, *J* = 3.0 and 7.5 Hz, 2H, COCH₂), 2.10 (quintet, *J* = 7.6 Hz, 2H, CH₂), 1.90-1.18 (m, 72H, CH₂), 0.87 (t, *J* = 6.6 Hz, 6H, CH₃).

¹³C NMR (75 MHz, pyridine-d₅): δ 173.43, 150.58, 150.22, 149.87, 149.49, 148.06, 142.91, 136.51, 136.17, 135.84, 135.51, 135.14, 129.33, 129.10, 126.51, 124.49, 124.16, 123.83, 123.50, 123.10, 101.63, 77.07, 72.75, 71.34, 71.21, 70.88, 70.13, 68.79, 51.70, 51.40, 37.08, 35.92, 34.83, 32.46, 32.45, 31.95, 30.75, 30.50, 30.38, 30.34, 30.27, 30.25, 30.22, 30.15, 30.12, 29.96, 29.94, 26.79, 26.72, 25.92, 23.28, 14.62.

Exact mass (ESI-MS) for C₆₁H₁₁₀N₄O₈ [M+H]⁺ found, 1027.8478; calcd, 1027.8402.

Computational modeling

The starting conformation of the triazole was generated using OpenBabel version 2.3.1²⁵ and graphical visualisation of the complex was done with PyMol.²⁶ The structure was

subsequently positioned into the binding pocket of Nu- α -GalCer (crystal structure was taken from the PDB, code 3QUZ) by superimposing the sugar ring of the triazole compound onto the corresponding ring of Nu- α -GalCer. Torsion angles were rotated to adopt a low energy conformation.

Biological evaluation

Lyophilized glycolipids were dissolved in pure DMSO (Sigma) at 10 mg/mL concentration and stored at -20°. Glycolipids were solubilized at 100 ng/mL concentration by adding vehicle (96mg/mL sucrose, 10mg/mL sodium deoxycholate, 0,05% Tween 20), warming to 80°C for 20 minutes and sonication for 10 minutes.

For *in vitro* stimulation, murine iNKT hybridoma N38-2C12 (V α 14V β 8.1/8.2*b*) cells at 5.10E4 cells/well in 96-well plates were stimulated with the 10E5 cells/well glycolipid pulsed BMDCs in cDMEM for 16 hours at 37°C, and levels of murine IL-2 secretion were determined by ELISA.

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