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Synthesis of α-Galactosyl Ceramide (KRN7000) and Analogues Thereof via a Common Precursor and Their Preliminary Biological Assessment

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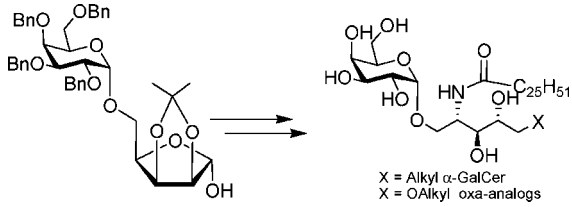

A new practical synthesis of α-GalCer and of its analogues is presented, opening the chance to easily modify the sphingosine chain. The common precursor is a disaccharide, obtained by coupling tetra-*O*-benzyl-D-galactose with allyl 2,3-*O*-isopropylidene-D-lyxofuranoside. Introduction of alkyl chains via Wittig reaction (for α-GalCer and OCH) or via Williamson reaction (for oxa analogues) followed by standard synthetic steps allows one to efficiently obtain such compounds. The analogues are able to activate iNKT cells when presented by CD1d expressing cells.

α-GalCer (KRN7000) is one of the most powerful activating glycolipids when presented by CD1d proteins expressed on antigen-presenting cells (e.g., monocytes, dendritic cells, and B cells). The stimulation of iNKT cells by engagement of the T-cell receptor initiates a cascade of events leading to the release of different Th1- or Th2-type cytokines.[1]

The crystal structure of CD1d alone or complexed with α-GalCer has been described,[2] highlighting the importance of the length of the acyl and the alkyl chains in modulating TCR binding affinity and cytokine bias.[3] Several syntheses of α-GalCer have been reported, based on the use of various galactosyl donors (e.g., phosphates, imidates, halides, thiosugars, etc.)[4] and a sphingosine or a ceramide acceptor, with variable efficiency.[5]

Here we describe a novel approach to the synthesis of α-GalCer (**11a**) and α-GalCer analogues starting from a disaccharide and building the lipidic part on the reducing end, thus avoiding the difficulties of glycosylation reactions on ceramide acceptors and opening an easy access to α-GalCer and various analogues modified on the sphingosine chain.[6]

We exploited D-lyxose as precursor, with the suitable stereochemistry, of the phytosphingosine moiety. However, differently from previous approaches[7] in which lyxose was converted into phytosphingosine before the glycosylation, we reversed the sequence by previously forming a 5-galactosyl lyxoside and introducing the lipid chain or mimics thereof on the obtained disaccharide.

Our synthetic route started from the bromide **1**, obtained from commercial tetra-*O*-benzyl-D-galactose by reaction with oxalyl bromide, which was coupled with the D-lyxose derivative **2** in presence of tri(1-pyrrolidine)phosphine oxide as activating agent[8] (Scheme 1), giving **3** together with traces of its β-anomer in 95% yield (α/β >95:5). Careful chromatography gave pure **3** in 84% yield. Acceptor **2** was easily obtained from D-mannofuranose following the procedure of Brimacombe et al.[9] The obtained disaccharide **3** was then deallylated to give the key intermediate **4** in 90% yield.

Disaccharide **4** is a versatile compound for the synthesis of either α-GalCer or its alkyl analogues (e.g., OCH) by Wittig olefination or for the synthesis of oxa analogues by Williamson alkylation.

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[§] Basel University Hospital.

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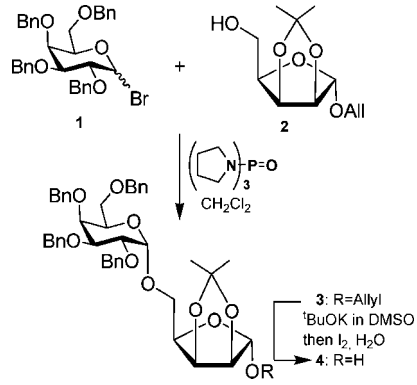
SCHEME 1. Key Intermediate Synthesis



To obtain α-GalCer and OCH, Wittig olefination was performed using the ylids derived from phosphonium salt obtained from *n*-tridecyl- or *n*-butylbromide. Opening of the furanosidic ring due to the Wittig reaction let the 2*R*-hydroxyl group ready for the introduction of the azido group via S_N2 after reduction of the resulting double bond via catalytic hydrogenation with Pt/C. Attempts to introduce the azido group before the double bond reduction in order to reduce both functional groups in a single step gave sluggish results during the substitution with NaN_3, possibly due to an intramolecular cycloaddition between the azido group and the double bond.

Activation of the hydroxyl group as chloromethanesulfonyl ester[10] allowed the subsequent substitution with NaN_3 to give the desired 2*S*-azide with inversion of configuration.

The azido group was then reduced by mild catalytic hydrogenation with Lindlar catalyst to the amines **9a**,**b**, which were condensed with hexacosanoic acid in presence of EDC, DIPEA, and HOBt[11] to give **10a**,**b**.

Hydrolysis of the isopropylidene and catalytic hydrogenolysis of the benzyl groups gave pure α-GalCer **11a** and OCH **11b** (Scheme 2), with an overall yield (from compound **4**) of 40% for α-GalCer and 34% for OCH.

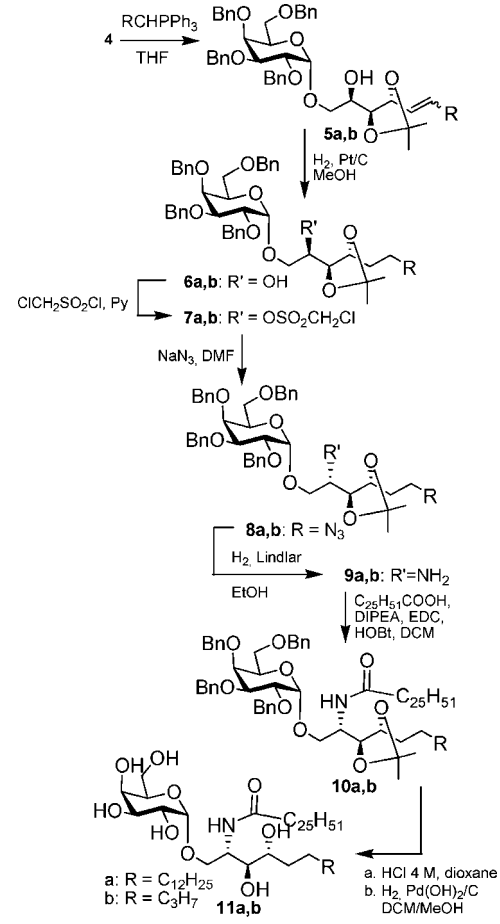
In a similar way, we have been able to perform the synthesis of a series of oxa analogues simply by alkylation of the alcohol **14**, derived from **4**, then following a similar strategy to that used for the alkyl derivatives.

Compound **14** is the starting point to obtain a new family of analogues of α-GalCer, with the presence of oxygen atom(s) on the alkyl chain.

Such isosteric substitution is expected not to change significantly the structure of the final compounds, but it could modify the electronic properties, allowing a possible modulation of their biological properties. Using different alkyl halides and an alkyl-*O*-mesylate (see Scheme 3), we built a small library of the above-mentioned compounds.

To explore different possibilities of substitution, we began synthesizing four analogues: one with a second atom of oxygen in the chain (**16a**), one with a saturated cycle (**16b**), one with an aromatic cycle (**16c**), and one with a simple alkylic chain (**16d**). To obtain such analogues, compound **4** was reduced with $NaBH_4$ to give the corresponding galactosyl lyxitol **12**, and the primary alcohol was then selectively protected as pivaloyl ester to give **13**. On compound **13**, the azido group in position 2 was

SCHEME 2. α-GalCer and OCH Synthesis



introduced using the same protocol shown for compound **7**. Removal of pivaloyl ester with tetrabutylammonium hydroxide in dioxane let the primary OH free for the subsequent alkylations (Scheme 3).

Alkylations were performed following the classical Williamson's synthesis of ethers, where alcohol **14** was deprotonated and alkylated by an alkyl halide or mesylate (Scheme 3).

The last steps to each new compound followed the same strategy previously shown for α-GalCer: reduction of the azide by catalytic hydrogenation (using the Lindlar catalyst) to amine and subsequent condensation with hexacosanoic acid in presence of EDC, DIPEA, and HOBt.[11]

Final deprotection of the isopropylidene group using hydrochloric acid in dioxane and benzyl groups by catalytic hydrogenation with $Pd(OH)_2$ on charcoal afforded the target analogues **16a**−**d**.

The biological activities of the newly synthesized compounds were determined by a classical T-cell antigen presentation assay, using α-GalCer as positive control. Such preliminary screening allows one to evaluate how modifications on the sphingosine alkyl chain affect their activity with respect to α-GalCer. Figure 1 shows that when α-GalCer-specific iNKT hybridoma cells are stimulated with CD1d-transfected THP-1 cells, previously exposed to increasing concentrations (10^{-7}−10^{-5} g/mL) of either α-GalCer or compounds under evaluation for 2 h, a significant increase of IL-2 is detected in the medium 48 h later. The analysis of these curves demonstrates that all compounds were active in stimulating IL-2 release from iNKT cells (Figure 1).

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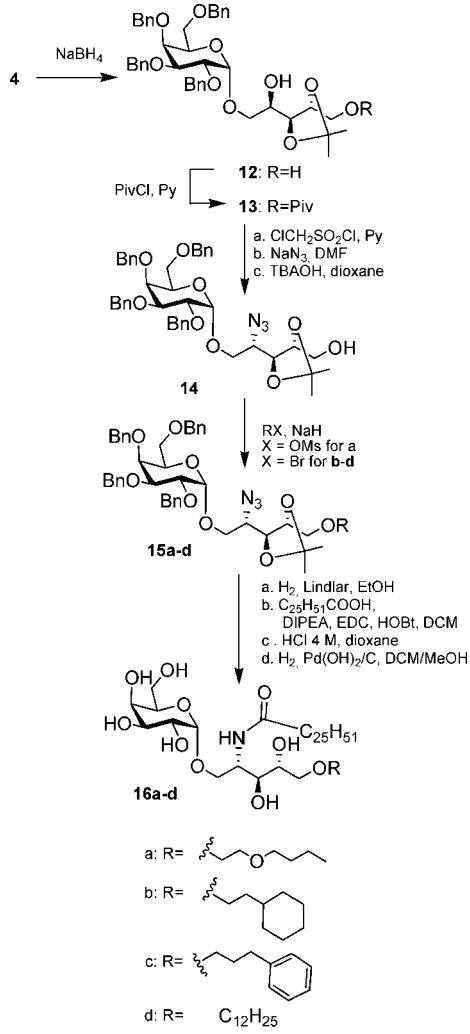
SCHEME 3. Synthesis of Oxa Analogues of α-GalCer



These results suggest that the performed structural modifications on the sphingoid chain led to derivatives that retain strong iNKT cell-stimulatory activity similar to that of α-GalCer. It is worthy to note that the presence of the oxygen seems not to have a major impact on the biological activity since compound **16a**, which contains two oxygen atoms, is as active as α-GalCer. Moreover, the activity is only slightly decreased when the chain ends with a ring, either aliphatic (as in **16b**) or aromatic (as in **16c**).

In conclusion, the synthetic route shown here allows an easy access to different carba (such as OCH) or oxa analogues of α-GalCer. The flexibility of our approach permits, in principle, introduction of any kind of side chain on the ceramide moiety and provides a powerful tool for analogue development and for elucidation of biological functions. The preliminary biological testing showed that the novel oxa analogues are able to activate efficiently iNKT cells, opening new possibilities for studying the role of these immunoregulatory cells.

Experimental Section

Key Intermediate (4) Synthesis. A quantity of 6.48 mmol (1.50 g) of allyl 2,3-*O*-isopropylidene-α-D-lyxofuranoside was coevaporated twice with anhydrous toluene and left under high vacuum for 2 h in the presence of activated molecular sieves (0.4 nm). Under Ar atmosphere, 8.43 mmol (5.09 g) of 2,3,4,6-tetra-*O*-benzyl-D-galactopyranosyl bromide,[12] dissolved in 65 mL of anhydrous dichloromethane, was added, giving a colorless solution; 19.45 mmol (5.00 g, 4.46 mL) of tri(1-pyrrolidine)phosphine oxide was added dropwise, and the reaction was stirred under Ar atmosphere for 24 h at room temperature. Solution was diluted with AcOEt and filtered on a plug of Celite. The crude obtained after evaporation of the solvent was purified by flash chromatography to give 4.12 g of pure allyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1→5)-2,3-*O*-isopropylidene-α-D-lyxofuranoside **3** in 84% yield.

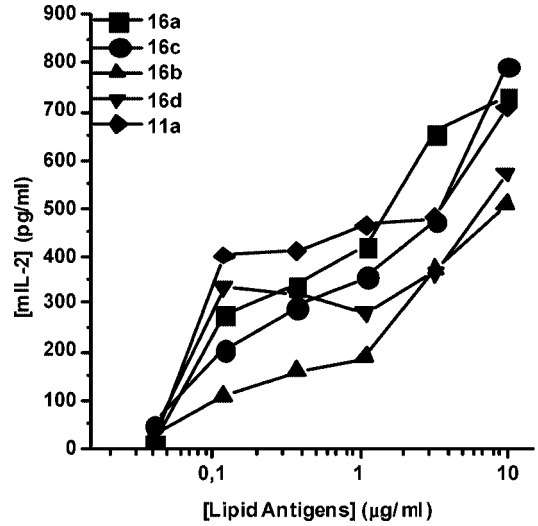


FIGURE 1. IL-2 release by iNKT hybridoma cells upon stimulation with the new glycolipid analogues.

R_f 0.68 (ETP/AcOEt = 8/2); $[\alpha]^{rt}_D = +7.0$ (c = 1.0, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40−7.24 (m, 20 H), 5.85 (ddd, 1 H, J = 5.2, 6.1, 10.4 Hz), 5.27−5.14 (m, 2 H), 5.02 (s, 1 H), 4.97−4.56 (m, 7 H), 4.47 and 4.42 (2d, 2 H, J = 11.9 Hz), 4.26 (m, 1 H), 4.14−3.88 (m, 7 H), 3.86 (dd, 1 H, J = 2.8, 6.1 Hz), 3.55 (m, 2 H), 1.41 (s, 3 H), 1.29 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.0, 138.8, 138.1, 134.0, 128.5−127.6, 117.6, 112.6, 105.4, 97.8, 85.2, 79.9, 79.0, 78.4, 76.5, 75.1, 74.9, 73.5, 73.2, 73.1, 69.2, 68.8, 26.2, 25.1. Anal. Calcd for $C_{45}H_{52}O_{10}$: C, 71.79; H, 6.96. Found: C, 71.90; H, 7.01.

Under Ar atmosphere, 1.39 mmol (1.05 g) of **3** was dissolved in 14 mL of anhydrous DMSO, and then 2.08 mmol (0.23 g) of ^{t}BuOK was added, giving a brown solution. The reaction was stirred at 80 °C for 1 h, and then it was allowed to cool to ambient temperature. A drop of water was added to destroy the excess of ^{t}BuOK, and the reaction was diluted with AcOEt. Organic phase was washed three times with water and once with brine. After anhydrification with Na_2SO_4, solvent was evaporated on vacuum. The crude was then dissolved in 28 mL of THF and 2.78 mmol (0.71 g) of I_2, 5.56 mmol (0.44 g, 0.45 mL) of pyridine, and 5 mL of water was added. After 1 h stirring at room temperature, the volume of solvent was reduced, and solution was diluted with AcOEt. Organic phase was washed three times with a 5% solution of $Na_2S_2O_3$, with 1 N HCl, with a saturated solution of $NaHCO_3$, and with water and brine.

After anhydrification with Na_2SO_4, solvent was evaporated under vacuum, and the crude was purified by flash chromatography, giving 0.891 g of pure **4** in 90% yield.

R_f 0.09 (ETP/AcOEt = 8/2); $[\alpha]^{rt}_D = +30.9$ (c = 2.5, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40−7.23 (m, 20 H), 5.36 (s, 1 H), 4.98−4.67 (m, 4 H), 4.88 (d, 1 H, J = 3.0 Hz), 4.55 (d, 1 H, J = 11.5 Hz), 4.54 (d, 1 H, J = 5.8 Hz), 4.45 and 4.40 (2d, 2 H, J = 11.9 Hz), 4.44−4.42 (m, 1 H), 4.06−3.98 (m, 4 H), 3.90−3.77 (m, 1 H), 3.53 (d, 2 H, J = 6.3 Hz), 3.29 (br s, 1 H), 1.41 (s, 3 H),

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1.29 (s, 3 H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 139.0, 138.8, 138.7, 128.5−128.4, 112.6, 101.1, 98.4, 98.0, 96.8, 85.5, 79.1, 78.8, 73.5, 73.3, 73.1, 69.3, 68.8, 66.5, 66.1, 60.6, 26.2, 25.2. Anal. Calcd for $C_{42}H_{48}O_{10}$: C, 70.77; H, 6.79. Found: C, 70.68; H, 6.65.

General Procedure for Wittig Reaction. Under Ar atmosphere, 7.00 mmol of the appropriate phosphonium bromide was dissolved in 30 mL of anhydrous THF in the presence of activated molecular sieves (0.4 nm) and cooled to 0 °C; 7.00 mmol (4.38 mL) of *n*BuLi (solution 1.6 M in hexane) was added. After 15 min, 2.80 mmol (2.00 g) of compound **4**, dissolved in 10 mL of anhydrous THF, was added dropwise, and the solution was allowed to warm to ambient temperature.

The solution was stirred at room temperature for 2 h and quenched by addition of solid NH_4Cl, diluted with AcOEt, washed four times with a saturated solution of NH_4Cl, and with water and brine. After anhydrification with Na_2SO_4, solvent was removed under vacuum, and the crude was purified by flash chromatography to give the product.

The ^{13}C NMR analysis showed a 95:5 ratio of the *E*/*Z* isomers. Since the double bond would be reduced in the next step, the single isomers were not separated.

General Procedure for Alkylation of Compound 14. Under Ar atmosphere, 0.135 mmol (100 mg) of **14** was dissolved in 1.5 mL of anhydrous DMF and cooled to 0 °C; 0.20 mmol of NaH was added, and after 15 min, 0.160 mmol of alkyl bromide (or alkyl methanesulfonate) was added dropwise. Solution was allowed to warm to rt and stirred overnight. The reaction was quenched with 1 mL of an aqueous saturated solution of NH_4Cl. Solution was diluted with AcOEt and washed three times with water and brine. After anhydrification with Na_2SO_4, the solvent was removed under vacuum, and the product was purified by flash chromatography (62−75% yield).

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Supporting Information Available: General experimental methods, additional experimental procedures, analytical data, and copies of ^{1}H and ^{13}C NMR of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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