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Stereoselective Synthesis and Immunogenic Activity of the *C*-Analogue of Sulfatide

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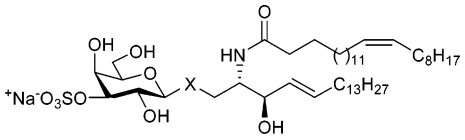
ABSTRACT



1a. X = O, **1b.** X = CH_2

The *C*-sulfatide 1b was synthesized through a [2,3]-Wittig sigmatropic rearrangement and a Horner–Wadsworth–Emmons olefination as the key steps. The *C*-analogue 1b is less immunogenic than natural sulfatide 1a, but induces a preferential secretion of the proinflammatory cytokine IFN-γ.

CD1 antigen-presenting molecules present different types of self and foreign lipid antigens to T lymphocytes which recognize the CD1-lipid complexes on the surface of antigen-presenting cells.[1] CD1-restricted T cells exert a wide range of effector functions. They secrete a large variety of cytokines upon T cell receptor (TCR) triggering, including IFN-γ and IL-4, and participate in both proinflammatory and anti-inflammatory immune responses.[2]

The role of T cells reacting against self-glycosphingolipids in autoimmune diseases, such as in Multiple Sclerosis, has been clarified by both in vitro and ex vivo data.[3] Recent studies have demonstrated the importance in their immunogenicity of both the lipid and the sugar structures. For example, modifications in the fatty acids of GM1 ganglioside or of phospholipids may profoundly hinder the response of specific T cells.[4] Also the structure of the sphingosine chain contributes to immunogenicity. These modifications have been associated with modifications in the structures of CD1-lipid complexes which interact with the TCR or with differences in the half-life of the immunogenic complexes.[5] Finally, the structure of the sugar moiety is also very important, mostly because the TCR establishes cognate interactions with parts of the hydrophilic moiety of glycolipids. Therefore, the position of the hydroxyl groups as well as the type of glycosidic bonds play critical roles in immunogenicity. Interestingly, modifications in the glyco-

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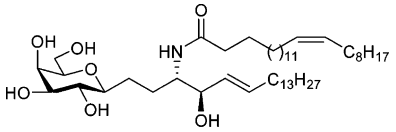
sidic bond may also confer upon the ligand the capacity to preferentially stimulate proinflammatory functions in the responding T cell population. Indeed, a *C*-glycoside analogue of α-galactosylceramide (α-GalCer) enhances proinflammatory T cell response as compared to natural α-GalCer.[6] Whether this is a general phenomenon occurring also with other types of glycolipid antigens and lipid-specific T cell populations is not known.

We have shown that sulfatide (**1a**), a β-D-galactosylceramide sulfated at position 3 of galactose, is one of the mammalian endogenously derived self-antigens presented by CD1 proteins.[7] Sulfatide **1a** is a natural ligand for all human CD1 family members and is presented by all CD1 molecules to specific T cells. Furthermore, the response to sulfatide is very frequent in Multiple Sclerosis patients (our unpublished results) and also occurs in mice during experimental allergic encephalomyelitis (EAE), a model of autoimmune brain disease.[8]

To investigate whether the substitution of the anomeric oxygen with a methylene affects the proinflammatory T cell responses also with sulfatide as the model antigen, we prepared **1b**, the *C*-glycoside isosteric analogue of sulfatide **1a**. This modification might confer novel binding conformations in the active site of CD1 molecules or might influence the binding capacity to CD1.[9,10]

Here we describe the synthesis of the *C*-sulfatide **1b** and show that this analogue is less stimulatory than sulfatide **1a**. The easy and highly stereoselective synthesis of the sulfatide analogue **1b** is based on the preparation of the β-*C*-galactosyl ceramide **2**, previously described by Dondoni et al. in an alternative synthetic approach,[11] followed by regioselective sulfation of galactose.

The key features of our approach to the skeleton of β-*C*-GalCer **2** are a [2,3]-Wittig sigmatropic rearrangement for the construction of the β-*C*-glycoside[12] and a Horner−Wadsworth−Emmons olefination for the installation of the carbon unsaturated chain.



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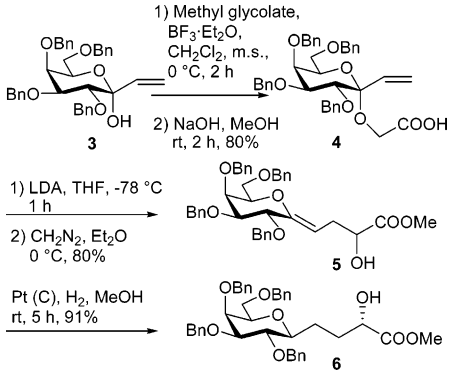
Starting from known 2,3,4,6-tetra-*O*-benzyl-1-*C*-vinyl-α-D-galactopyranose **3**,[13] galactoside **4** was obtained (Scheme 1) exclusively as the α-anomer in 90% yield by using a protocol reported in the literature for the corresponding

Scheme 1. Preparation of the β-*C*-Glycoside **6**



glucosidic compound.[12b] The dianionic [2,3]-Wittig rearrangement of **4** was carried out by treatment with excess LDA at −78 °C, followed by reaction of the crude with diazomethane to afford the α-hydroxy ester **5** as a single isomer, as clearly shown by the single set of peaks observed in the ^{1}H and ^{13}C NMR spectra. The *Z* geometry of the newly formed double bond was established by a NOE experiment, which showed an effect of 5% on the H-2 atom of galactose on irradiation of the vinylic hydrogen. Due to its acidic lability, compound **5** was promptly hydrogenated with Pt(C) in methanol to give compound **6** in high yield. The configuration of the new stereocenter at position 1 of galactose, generated after double bond reduction, was found to be β through the ^{1}H NMR coupling constant of 9 Hz between H-1 and H-2 of galactose. Moreover, compound **6** was used to establish the absolute configuration of the stereocenter at C-2 previously formed in the [2,3]-Wittig rearrangement. The analysis of the ^{1}H NMR spectra of its (*R*)- and (*S*)-MTPA esters[14] (see the Supporting Information) allowed us to unequivocally assign the absolute configuration at C-2 as *S* and confirmed the stereoisomeric purity and the complete transmission of stereochemical information to this new stereocenter during the rearrangement.

The stereochemistry at C-2 of compound **6** requires the introduction of a masked amino functionality at that position with retention of configuration. Therefore we decided to employ a Mitsunobu reaction with methanesulfonic acid as nucleophile on compound **6** (Scheme 2), followed by reaction with sodium azide. Mitsunobu mesylation was performed on compound **6** under the condition reported by Lawless et al.[15] followed by treatment with sodium azide in DMF, which

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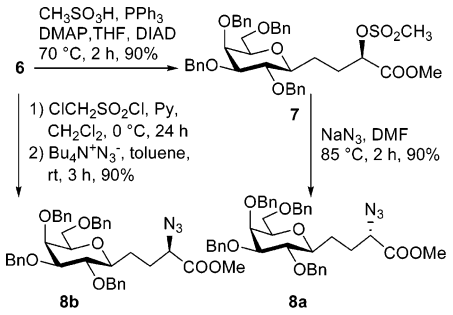
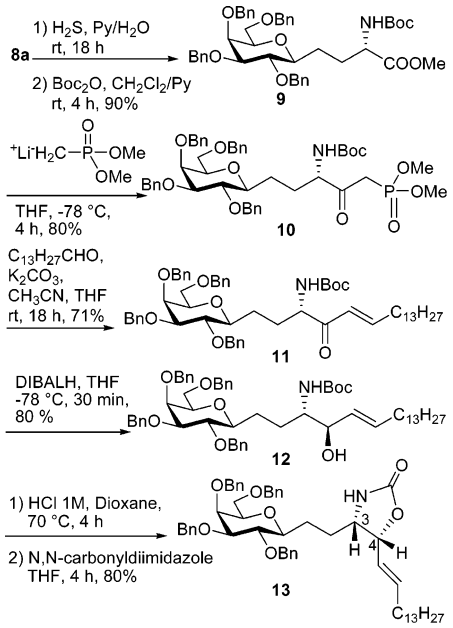
Scheme 2. Introduction of the Azido Moiety



afforded azide **8a** in high yield as a single diastereoisomer. The net retention of stereochemistry during this functional group interconversion was confirmed by comparing the physical data of compound **8a** with those of its C-2 epimer **8b**. Compound **8b** was derived from alcohol **6** by mesylation under standard conditions followed by displacement with tetrabutylammonium azide and showed a different set of NMR signals and the same ESI-MS molecular ion.

The next planned key steps for the construction of the sphingoind skeleton were a Horner−Wadsworth−Emmons (HWE)[16] olefination to introduce the trans double bond and the aliphatic chain, followed by the stereoselective reduction of the keto group of the enone (Scheme 3).

Scheme 3. Preparation of the β-*C*-Galactosylsphingosine **12**



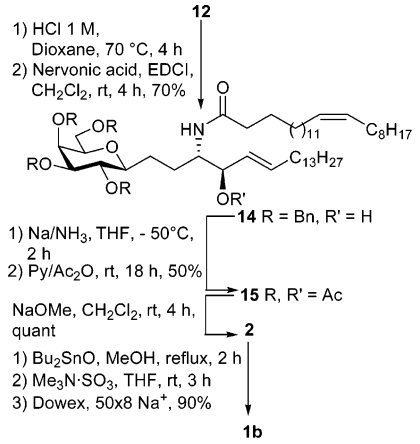
First, reduction of the azide and protection of the amino group as *N*-Boc derivative were necessary to avoid decomposition of the α-azido ester in basic conditions and considering that the steric hindrance of this bulky amino protecting group should enhance the selectivity of the reduction of the keto group on the HWE product.[17] Staudinger reduction of the azide did not proceed efficiently, instead reduction with hydrogen sulfide in pyridine/water followed by reaction of the crude amine with *tert*-butoxycarbonyl-anhydride (Boc_2O) in DCM/pyridine afforded the *N*-protected α-amino ester **9** in 90% yield. Compound **9** was then converted into the ketophosphonate **10** in 80% yield by treatment with 6 equiv of lithium dimethyl methylphosphonate in THF at −78 °C.

The HWE reaction of **10** with tetradecanal in the presence of anhydrous potassium carbonate[16,18] in acetonitrile exclusively afforded the *E*-enone **11** in 71% yield, as indicated by the coupling constant of 15.5 Hz between the vinylic protons. The carbonyl group of compound **11** was then subjected to hydride reduction: the optimal reagent was found to be DIBAL-H (3 equiv in THF at −78 °C),[19] which afforded a mixture of two diastereoisomers in a 95:5 ratio.[20]

The main isomer **12** was obtained pure after column chromatography and the absolute configuration of the new stereocenter at C-4 of the sphingoid base was unequivocally assigned as *R* by the preparation of the oxazolidinone **13**:[11,17,21] NOE analysis of **13** gave a 5% enhancement between H-3 and H-4 that is consistent with a cis relationship between the two protons, also confirmed by the 8 Hz coupling constant value. The assignment and the optical purity of the product were corroborated by preparation of the (*R*)- and (*S*)-MTPA esters of **12** and analysis of their ^{1}H NMR spectra (see the Supporting Information).

The last steps of the synthesis (Scheme 4) were the installation of the desired acyl chain,[22] the removal of the benzyl groups, and the introduction of the sulfate group at position 3 of galactose. Removal of the *tert*-butoxycarbonyl

Scheme 4. Synthesis of the *C*-Sulfatide **1b**



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(19) Reduction of **10** with L-Selectride gave a 1:1 mixture of the two diastereoisomers.
(20) The dr was deduced by integration of the C-5 signals at δ 5.40 (95%) for the major isomer and δ 5.45 (5%) for the minor isomer in the ^{1}H NMR spectrum of the crude mixture.

group of **12** with HCl in dioxane, followed by treatment of the crude amine with nervonic acid and EDCI in DCM gave the tetra-*O*-benzylgalactosyl ceramide analogue **14** in 70% yield. Debenzylation with sodium in liquid ammonia followed by exhaustive acetylation of the crude afforded **15** in 50% yield. Finally, compound **15** was submitted to Zemplén deacetylation to yield the β-*C*-galactosyl ceramide **2**, and the sulfate group at position 3 of galactose was introduced according to Flitsch et al.[23] affording the target compound **1b** in 90% yield over two steps.

The biological activity of the synthetic sulfatide analogue **1b** was compared to the synthetic[22] but naturally occurring sulfatide **1a** by using a T-cell antigen presentation assay. The CD1a-restricted human T cell clones K34B9.1 and K34A6.2 were selected for these studies because both react to natural sulfatide and release the proinflammatory cytokine IFN-γ.[7] Results (Figure 1) show that *C*-sulfatide **1b** has lower

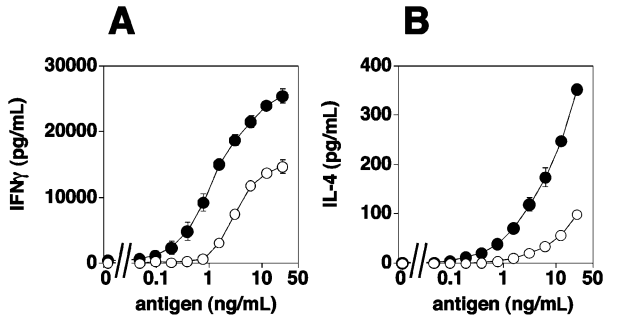

Figure 1. Glycolipid immunogenicity is influenced by the *C*-glycosidic bond. C1R cells expressing human CD1a were preincubated with sulfatide **1a** (filled circles) or with *C*-sulfatide **1b** (open circles) at the doses indicated, before addition of sulfatide-specific CD1a-restricted T cell clone K34B9.1. IFN-γ (A) or IL-4 (B) released were measured by ELISA. Data are expressed as mean pg/mL $\pm$ SD of duplicates. Similar results were obtained with the K34A6.2 T cell clone (see the Supporting Information).

potency and efficacy than sulfatide **1a** in inducing release of both IFN-γ and IL-4. Moreover, the *C*-sulfatide **1b** doses necessary to induce the same cytokines amounts as those induced by sulfatide **1a** were 5 times larger for IFN-γ and almost 10 times larger for IL-4. This indicates that *C*-sulfatide **1b** may induce a modest but preferential secretion of the proinflammatory IFN-γ.

In conclusion, we have developed an efficient and facile synthesis of the sulfatide analogue **1b**. Worthy of note is the very high stereoselectivity observed in each synthetic step, which allowed us to obtain the proper β-stereochemistry at the *C*-anomeric position and to construct the sphingosine skeleton with the correct stereochemistries at positions 3 and 4.

C-Sulfatide **1b** is less stimulatory than sulfatide **1a**. These findings are opposite to those obtained with α-*C*-galactosylceramide which stimulates natural killer T cells more effciently than α-*O*-galactosylceramide,[6,24] thus indicating that modifications of the glycosidic bond do not always result to the same functional effect.

The reduced potency of *C*-sulfatide **1b** might be the consequence of reduced CD1a loading in early endosomes or on the plasma membrane. This effect might also be ascribed to different populations of conformations around the *C*-glycosidic bond as compared to those around the *O*-glycosidic one. Therefore, a sufficient number of stimulatory CD1a complexes might be generated only with large doses of *C*-sulfatide **1b**.

The reduced efficacy indicates that sulfatide **1b** forms a complex with CD1a that is slightly different from the complex formed by sulfatide with an *O*-glycosidic bond. This difference might derive from altered hydrogen bond interactions between 3-*O*-sulfogalactose and Arg73, Arg76, Glu154, and Ser77 of CD1a, which fix the position of the hydrophilic part of sulfatide.[25] Moreover, although not explicitely indicated in ref 25 it cannot be excluded that the anomeric oxygen is involved in hydrogen bonds with Arg73 and Thr158 of CD1a. Such modifications might change the strength of interaction with the TCR of the two cell clones tested in this study and thus lead to reduced stimulatory capacity of sulfatide **1b**. Whether this modified interaction also leads to different signaling in the TCR, which in turn induces preferential IFN-γ release, will be the basis of further investigations.

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Supporting Information Available: Experimental procedures and full spectroscopic data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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